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Cómo intervenir mediante la dieta en el incremento del consumo de drogas inducido por el estrés social

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CERTIFICAN:

Que la Tesis Doctoral presentada por Don Francisco Ródenas González, con el título "Cómo intervenir mediante la dieta en el incremento del consumo de drogas inducido por el estrés social" ha sido realizada bajo su dirección. Tras haberla examinado hacen constar su autorización para que se realicen los trámites conducentes a su defensa.

Y para que conste a los efectos oportunos, firman el presente certificado en Valencia a 27 de octubre de 2022.

Fdo.: Dra. Marta Rodríguez Arias Fdo.: Dra. María del Carmen Blanco Gandía

A mi familia, Francisco, Daría y Rocío

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Abreviaturas

A1r y A2r	Receptores de adenosina 1 y 2
AA	Autoadministración operante
ACTH	Hormona Adenocorticotropa
AgRP	Proteína relacionada con el Agutí
ATP	Nucleótido Adenosina Trifosfato
ATV	Área Tegmental Ventral
CART	Péptido relacionado a la Cocaína y Anfetamina
CB1r y CB2r	Receptores Endocannabinoides 1 y 2
CPF	Corteza Pre-Frontal
CPL	Condicionamiento de Preferencia de Lugar
CRF	Factor Liberador de Corticotropina
CRHR1	Receptor de Corticotropina 1
D1r D2r	Receptores de Dopamina 1 y 2
DA	Dopamina
DC	Dieta Cetogénica
DPN	Día Postnatal
DRG	Dieta rica en grasas, carbohidratos y azúcares
DS	Derrota Social
FR1	Ratio Fijo 1
FR3	Ratio Fijo 3
HHA	Eje Hipotálamo-Hipofisio-Adrenal
HIP	Hipocampo
IL-6	Interleucina 6
NAcc	Núcleo Accumbens
NPY	Neuropéptido Y
Oprμ	Receptor Opioide μ
POMC	Pro-opiomelanocortina
Post-C	Post-Condicionamiento
PR	Ratio Progresivo
Pre-C	Pre-Condicionamiento
TUS	Trastornos por uso de sustancias
βOHB	β-hidroxibutirato

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Resumen

La respuesta a una situación de estrés es un proceso adaptativo al medio que favorece la supervivencia de los individuos (Ulrich-Lai & Herman, 2009). Sin embargo, una prolongada exposición al estrés puede provocar alteraciones en nuestro estado físico y mental. En la sociedad actual, el estrés al que nos exponemos de forma más habitual es de carácter social (Hartsell & Neupert, 2019), y sus efectos pueden afectar a numerosos procesos neuropsicológicos. En el desarrollo de un proceso adictivo, la exposición a estresores sociales puede favorecer el inicio, el mantenimiento, la escalada y la recaída en el consumo de drogas (Koob & Schulkin, 2019; Montagud-Romero et al., 2018). Esta potente influencia del estrés en el desarrollo de la adicción se explica por la estrecha relación existente entre los sistemas neurales que controlan la respuesta al estrés y la respuesta a las drogas.

Sin embargo, aunque los resultados indican que la exposición a estrés social incrementa los efectos reforzantes de las drogas, se observa que una parte de la población manifiesta conductas de afrontamiento resilientes y que se muestran protegidos de estas consecuencias negativas provocadas por el estrés (Ballestín et al., 2021; Brodnik et al., 2017). Actualmente comienzan a estudiarse los distintos factores que pueden promover una respuesta adaptativa al estrés y por lo tanto potenciar una respuesta resiliente. Algunas intervenciones ambientales en investigación preclínica han demostrado su eficacia reduciendo el incremento en el consumo de drogas provocado por el estrés social, como el ejercicio físico (Reguilón et al., 2020; Ferrer-Pérez et al., 2022), las condiciones de estabulación (Ferrer-Pérez et al., 2019b) o el enriquecimiento ambiental (Reguilón et al., 2021). Más recientemente, se han comenzado a desarrollar intervenciones nutricionales que pueden ser efectivas para potenciar esta respuesta resiliente. Por ejemplo, se ha observado que el incremento en los efectos reforzantes de la cocaína inducidos por

estrés social puede reducirse en ratones alimentados con una dieta rica en grasas (DRG), carbohidratos y azúcares (Blanco-Gandía et al., 2018).

El hecho de que estas intervenciones nutricionales puedan modular la respuesta de estrés y a las drogas se explica porque algunos tipos de alimentos, como la grasa o los azúcares, ejercen potentes efectos sobre el sistema de recompensa cerebral, induciendo cambios en el sistema dopaminérgico, opiáceo y endocannabinoide (Johnson & Kenny, 2010). Esta estimulación del sistema de refuerzo sugiere que este tipo de dietas también podrían actuar como un reforzador alternativo. Sabemos que las DRG pueden disminuir los efectos reforzantes condicionados de la cocaína (Morales et al., 2012) y reducir su búsqueda durante la extinción de las memorias asociadas al refuerzo condicionado (Blanco-Gandía et al., 2017b). Estos resultados apoyan la teoría de que la exposición continua a una DRG podría tener un papel como reforzador alternativo. Sin embargo, la exposición continua a grasas y azúcares provoca trastornos metabólicos, como un aumento significativo de peso y de los niveles de leptina (Blanco-Gandía et al., 2017b). Por lo tanto, se hace necesario evaluar la eficacia de otro tipo de administración de estas DRG, así como el empleo de otras dietas altas en grasa que no contienen excesivos niveles de carbohidratos, como la dieta cetogénica (DC).

La DC es una dieta rica en grasas, en la que obtiene un aporte de 80% de calorías en forma de grasas, un 15% de proteínas y solo un 5% de calorías en forma de carbohidratos. Por lo tanto, cuando se ingiere esta dieta se reducen drásticamente los niveles de glucosa disponible en el organismo y se generan cuerpos cetónicos como el β-hidroxibutirato (βOHB) (Masino et al., 2012). Esta reducción de glucosa y aumento de βOHB modifica el estado metabólico del organismo, entrando en lo que se conoce como estado de cetosis (Politi et al., 2011). Estos cambios de metabolismo provocados por la DC se han utilizado como intervención terapéutica en diferentes trastornos como algunos tipos de epilepsia, aunque se han realizado muy pocos

estudios sobre su efecto en el consumo de drogas. En estudios preclínicos con modelos animales se ha observado como la DC reduce la respuesta de sensibilización del síndrome de abstinencia a la cocaína (Martínez et al., 2019), los síntomas de abstinencia de alcohol (Dencker et al., 2018), y la hiperlocomoción y ansiedad provocada por la retirada del alcohol (Bornebusch et al., 2021).

Todos los estudios que se han mencionado sugieren que, tanto la DRG como la DC podrían reducir el consumo de drogas, así como potenciar el desarrollo de una respuesta resiliente que reduzca las consecuencias provocadas por la exposición al estrés social.

OBJETIVOS E HIPÓTESIS

La comunidad científica comienza cada vez más a interesarse por identificar aquellos factores que puedan fomentar el desarrollo de la resiliencia y reducir las consecuencias que la exposición al estrés social tiene sobre la adicción. Por ello, el **primer objetivo general** de la presente Tesis Doctoral (TD) ha sido confirmar la existencia de esta población resiliente a los efectos reforzantes de la cocaína incrementados por la exposición a estrés social. Para desarrollar este objetivo, los animales empleados en esta TD fueron expuestos a un procedimiento de Derrota Social (DS) previo a un Condicionamiento de preferencia de lugar (CPL) de cocaína, y se analizaron las conductas de afrontamiento durante la exposición al estrés social. Nuestra hipótesis principal plantea que, de todos los animales expuestos a estrés social, algunos sujetos manifestarán resistencia al incremento de los efectos reforzantes de la cocaína inducidos por el estrés, y esos mismos sujetos resilientes manifestarían conductas de afrontamiento particulares durante la DS.

Sabemos que el consumo de una DRG induce neuroadaptaciones en el sistema de recompensa, modificando el funcionamiento de la neurotransmisión dopaminérgica,

del sistema opioide endógeno y del sistema endocannabinoide, lo que va a modificar la respuesta de los animales a los efectos gratificantes de las drogas de abuso. También tenemos en cuenta la DC, que podría influir en el proceso adictivo a través de sus interacciones con el sistema de la adenosina y la dopamina. Por ello, el **segundo objetivo general** de esta TD ha sido identificar cómo la exposición a estas dietas interactúa con los efectos reforzantes de la cocaína y el alcohol. Para desarrollar este objetivo, utilizamos la administración intermitente de una DRG evaluando sus efectos sobre el proceso de extinción de un CPL inducido por la cocaína, así como la administración continua de una DC para evaluar su influencia sobre los efectos reforzantes condicionados de la cocaína empleando un CPL. Así mismo, estudiamos el consumo de alcohol empleando un procedimiento de Autoadministración oral de etanol (AA). Nuestra hipótesis principal plantea que la DRG es capaz de acelerar la extinción de los recuerdos asociados a la cocaína y evita la reinstauración de cocaína inducida por una dosis de recuerdo o "*priming*". También hipotetizamos que la DC aceleraría la extinción de los recuerdos asociados a la cocaína en comparación con los animales alimentados con una dieta estándar, bloquearía la reinstauración de la conducta de búsqueda de cocaína y reduciría tanto el consumo como la motivación por obtener alcohol.

Sin embargo, considerando que estas dietas no solo pueden modular la sensibilidad a los efectos reforzantes de las drogas, sino que también son capaces de disminuir los efectos que induce el estrés social, nuestro **tercer objetivo general** fue evaluar los efectos de estas dietas sobre el desarrollo de la resiliencia al estrés social. Para desarrollar este objetivo, administramos de forma intermitente y limitada una DRG evaluando sus efectos sobre un CPL inducido por la cocaína tras la exposición a un procedimiento de DS. Nuestra hipótesis planteó que el propio valor reforzante de la DRG actuaría como un reforzados alternativo al estrés social, evitando el incremento de los efectos reforzantes de la cocaína inducidos por dicho estrés.

MATERIAL Y MÉTODOS

En la presente TD se han empleado 2 cepas distintas de ratones: ratones albinos de la cepa OF1 y ratones de la cepa C57BL/6J. El número total de ratones se especifica en cada uno de los estudios presentados. A lo largo de los seis estudios, los animales recibieron distintos tratamientos farmacológicos en función del procedimiento experimental. Durante el procedimiento de CPL se les inyectó por vía intraperitoneal 1mg/kg o 10mg/kg de cocaína (según el diseño del estudio). Durante el procedimiento de AA, se utilizaron distintas concentraciones de una solución de sacarina y etanol (según el diseño del estudio). Por otro lado, se han administrado 3 dietas distintas a los animales. Una dieta estándar (13 Kcal % de grasa, 67 Kcal % de carbohidratos y 20 Kcal % de proteína; 2,9 kcal/g), una DRG (45 Kcal % de grasa, 36 Kcal % de carbohidratos y 19% Kcal de proteína; 4,6 kcal/g) y una DC (90,5% kcal de grasa, 0,3% kcal de carbohidratos y 9,1% kcal de proteína; 6,7 kcal/g).

Para estudiar las consecuencias conductuales y fisiológicas del estrés social, se ha empleado el protocolo de DS validado y descrito en detalle en trabajos previos (Ferrer-Pérez et al., 2019a; Rodríguez-Arias et al., 2017) y que se fundamenta en el modelo intruso-residente. Este procedimiento consta de 4 sesiones de 25 minutos que se repiten a intervalos de 72h. Cada sesión está compuesta por 3 fases. En la primera y tercera fase se introduce al intruso en la jaula del residente durante 10 minutos, en los que está protegido de los ataques, pero no de las amenazas del residente por medio de una rejilla. En la segunda fase se retira la rejilla y se permite la confrontación durante 5 minutos. Éstas sesiones son registradas con una videocámara y se analizan los encuentros mediante un programa informático que permite registrar el tiempo dedicado a realizar las diferentes conductas durante la DS.

Para evaluar los efectos de la respuesta condicionada de la cocaína se ha empleado el CPL. En el desarrollo del CPL empleamos cajas idénticas de plexiglás con dos

compartimentos de diferente color (blancas vs negras) y distinta textura de suelo (liso en el compartimento negro y rugoso en el blanco). El procedimiento completo de CPL consta de tres fases: Adquisición (Pre-Condicionamiento, Condicionamiento y Post-Condicionamiento), Extinción y Reinstauración. En la fase de adquisición, durante el **pre-condicionamiento** (Pre-C), los ratones tienen libre acceso a ambos compartimentos de la caja de condicionamiento. Al tercer día, el tiempo que cada animal pasa en cada compartimento es registrado y se eligen los compartimentos asociados a la cocaína y al salino. En la fase de **condicionamiento**, los animales son condicionados con 1mg/kg o 10mg/kg de cocaína (según el diseño del estudio) al compartimento asignado tras el Pre-C. Durante la tercera fase o **post-condicionamiento** (Post-C), se registra el tiempo que los ratones (sin ningún tipo de tratamiento) pasan en cada compartimento. La diferencia en segundos entre el tiempo que los animales permanecen en el compartimento asociado con la droga durante la prueba de Post-C, y el tiempo que pasan durante la prueba de Pre-C es una medida del grado de condicionamiento inducido por la droga. Una vez establecida la preferencia por el compartimento asociado a la droga, los animales se exponen dos veces por semana a una sesión de extinción que consiste en colocarlos en el compartimento central permitiéndoles la exploración de ambos compartimentos. Una vez que ya no muestran preferencia por el compartimento asociado a la cocaína, y tras 24h de confirmarse esta extinción, se evalúan los efectos de una dosis de cocaína de recuerdo o *priming* con una dosis del 50% de la dosis administrada durante el condicionamiento.

Para evaluar los efectos gratificantes del alcohol, empleamos el procedimiento de AA de etanol. Las cajas de AA están equipadas con una luz, dos orificios para el hocico de los animales, un receptáculo central para dejar caer la solución líquida, y una bomba de infusiones compuesta por una jeringa y una luz de estímulo. De los dos orificios para el hocico de los animales, cuando el animal introduce el hocico en el orificio activo, se administran 37 µl de solución liquida. Cuando el animal

introduce el hocico en el orificio inactivo no administra ningún compuesto ni se expone ningún estímulo. Este protocolo consta de tres fases. En la fase de entrenamiento, los animales deben introducir el hocico en los orificios activos para obtener 37 µl de sacarina. En la fase de sustitución el porcentaje de sacarina se reduce progresivamente a medida que aumenta la concentración de etanol. En la última fase de consumo de etanol (al 6%) se evalúa el número de respuestas proporcionadas en los orificios activos, el consumo de etanol y la motivación para obtenerlo.

Con el fin de evaluar las conductas de ansiedad empleamos la prueba del Laberinto Elevado en Cruz. El laberinto está compuesto por dos brazos abiertos, dos brazos cerrados y una plataforma central elevada 45 cm sobre el nivel del suelo. Al principio de cada ensayo, los ratones experimentales se colocan en la plataforma central frente a un brazo abierto y se les permite explorar la superficie del laberinto durante 5 minutos. El comportamiento mostrado por los ratones durante la prueba se registra mediante un sistema de seguimiento automatizado que registra el número de entradas y el tiempo pasado en cada sección del laberinto.

También empleamos la prueba de Campo abierto para analizar el comportamiento locomotor espontáneo de los ratones. En este caso, se coloca a los animales en cajas de campo abierto y se registra y analiza su actividad mediante un software de seguimiento.

Para evaluar la retención en la memoria empleamos el test de evitación pasiva. Esta prueba se realiza en dos días. El primero es el *Training* donde se coloca al ratón en un compartimento iluminado y blanco, estando cerrada la puerta a otro compartimento negro. A partir del momento en el que se abre la puerta se registra la latencia de paso al compartimento oscuro. Cuando el animal pasa a este compartimento, la puerta se cierra automáticamente y se le administra una leve descarga eléctrica. A las 24 horas, el día del *Test*, se realiza el mismo procedimiento, con el registro de la latencia de paso al compartimento negro.

Para evaluar la memoria espacial, empleamos el laberinto de Hebb-Williams que permite medir el proceso de adquisición del aprendizaje a través de la motivación para escapar del agua fría. En este procedimiento, los ratones deben nadar desde el compartimento de salida de los diferentes laberintos configurados hasta el compartimento de meta que está seco, con el fin de escapar del agua fría. Las pruebas se estructuran en 8 días de procedimiento compuestas por 3 días de prueba y 5 días de laberintos fáciles y difíciles donde se analiza el tiempo que tardan los animales en salir de dichos laberintos. Se realizan 8 ensayos en cada laberinto.

Por último, se emplearon distintos tipos de medidas fisiológicas y biológicas a lo largo de los seis estudios. El estado de cetosis se evaluó analizando los niveles plasmáticos de βOHB de los animales a partir de la extracción de sangre de la vena de la cola, empleando un monitor On Call GK Dual y tiras reactivas de cetonas. Para determinar la concentración de IL-6, leptina y la grelina en plasma, se emplearon los respectivos kit ELISA siguiendo las instrucciones del fabricante. Para el análisis de expresión génica, se emplearon RT-PCR cuantitativas evaluando la expresión génica de CB1r, el receptor opioide μ (Oprμ), los receptores dopaminérgicos D1r y D2r, los receptores de adenosina A1r y A2r y el receptor de corticotropina 1 (CRHR1).

RESULTADOS Y CONCLUSIONES

Utilizando los modelos de DS y CPL hemos demostrado que algunos individuos son capaces de desarrollar un perfil resiliente que les protege de las consecuencias psicofisiológicas derivadas del estrés social. Pero además de caracterizar el perfil conductual y neurobioquímico de estos animales resilientes, en esta TD nos hemos centrado en estudiar el papel que ejerce la dieta en la respuesta al estrés. Como resultado más importante debemos destacar que ambas dietas, DRG y DC, son capaces de modular los efectos reforzantes de la cocaína y del etanol. Además, podemos confirmar que la DRG puede actuar como un reforzador alternativo

acelerando la extinción de las memorias asociadas a la cocaína, evitando además la reinstauración de la conducta condicionada, y favoreciendo el desarrollo de un perfil resiliente. A continuación, se presentan las principales conclusiones de la presente TD.

Existe una población resiliente a los efectos del estrés social que no muestra un incremento de los efectos reforzantes condicionados de la cocaína

Nuestros resultados confirman que la experiencia de la DS induce un incremento a largo plazo de los efectos reforzantes condicionados de una dosis subumbral de cocaína. Además, se ha confirmado la existencia de una población de ratones derrotados que no han manifestado dicha preferencia, siendo caracterizados como resilientes al estrés social. El análisis etológico de la conducta de estos animales resilientes durante los encuentros agonísticos muestra que pasan más tiempo exhibiendo conductas de ataque, así como menor tiempo en las conductas de sumisión y huida. Por lo tanto, estos resultados permiten la identificación de ciertas características conductuales que aparecen en animales resilientes al estrés social, que pueden actuar como factor de protección frente al desarrollo de adicción a drogas.

La administración de dietas ricas en grasa (DRG y DC) modifica los efectos reforzantes de la cocaína y el alcohol

Nuestros resultados indican que la administración de una DRG controlada actúa como un reforzador alternativo, acelerando el proceso de extinción y bloqueando también la reinstauración de la búsqueda de cocaína en ratones macho. Sin embargo, los efectos reforzantes de la cocaína y el potencial de la DRG como reforzador alternativo difiere en ratones hembra. Los ratones hembra muestran una mayor sensibilidad a los efectos reforzantes de la cocaína, pero extinguen los recuerdos

asociados a la cocaína más rápido que los ratones macho. Estas diferencias de sexo también se observan en la respuesta que tienen los animales a los efectos moduladores de la DRG, donde no todas sus administraciones actúan como reforzador alternativo en hembras. Basándonos en nuestros resultados, confirmamos que la administración de una recompensa natural, como la DRG, puede actuar como reforzador alternativo ante el potente efecto de la cocaína en los ratones macho, pero de manera menos clara en los ratones hembra.

También hemos evaluado el efecto de la DC sobre los efectos reforzantes y el consumo de cocaína y alcohol. En primer lugar, se ha demostrado que el aumento de cetonas como el βOHB no provoca cambios significativos en la actividad locomotora, la memoria o el aprendizaje dependiente del hipocampo, evitando así la afectación en la ejecución de otras pruebas conductuales. Sin embargo, sí que se ha observado que los animales sometidos a la DC muestran un incremento en la ansiedad tras 7 días de consumo de esta dieta que podría reducirse tras un periodo de adaptación (Ciarlone et al., 2016; Huang et al., 2019).

La DC no evita la adquisición de un CPL inducido por una dosis efectiva de cocaína. Sin embargo, la DC administrada tanto a lo largo de todo el proceso experimental (antes, durante y después del CPL) como únicamente en el proceso de extinción, es capaz de acelerar el proceso de extinción de la conducta condicionada y evitar la reinstauración de la preferencia. Con estos resultados, confirmamos que la DC puede considerarse un enfoque nutricional prometedor en el tratamiento de la adicción a la cocaína y, aunque no debe considerarse como un tratamiento exclusivo, puede contribuir a atenuar los recuerdos relacionados con el consumo de cocaína, así como el riesgo de recaída. Similares resultados se han observado tras la administración de DC antes de comenzar la exposición al alcohol, ya que disminuye su ingesta, aunque no interfiere en la motivación para obtenerlo.

La administración intermitente de una DRG potencia la resiliencia a los efectos del estrés social

Sabemos que el estrés social sensibiliza el sistema de refuerzo y que la DRG ejerce una influencia directa sobre este sistema, por lo que hemos evaluado el efecto que tiene esta dieta sobre el incremento de los efectos reforzantes de la cocaína provocado por la exposición a estrés social. Nuestros resultados indican que la administración de pequeñas cantidades de DRG antes de cada DS bloquea el desarrollo de CPL inducido por una dosis subumbral de cocaína. Este resultado sugiere que el consumo de comida palatable podría estar actuando como refuerzo alternativo o *comfort food*. Además, cuando la ingesta de este tipo de dietas se realiza con posterioridad a la exposición del estrés social, durante la adquisición del condicionamiento con la cocaína, también actúa como reforzador alternativo, bloqueando igualmente el desarrollo del CPL. Sin embargo, hemos observado que, si la administración de una DRG se prolonga en el tiempo, no ejerce ningún efecto protector e incluso puede sensibilizar el sistema de recompensa, como sugieren estudios previos (Blanco-Gandía, et al., 2017b). Con estos resultados planteamos que la administración controlada de una DRG podría ser una estrategia útil para mitigar los efectos del estrés social sobre los efectos reforzantes de la cocaína, potenciando así un perfil resiliente.

Modificación en la expresión de genes inducido por la DRG y la DC

La DRG administrada de forma intermitente y limitada, tanto antes como durante el CPL de cocaína, no induce cambios en la expresión del gen Oprμ. Sin embargo, independientemente del tipo de dieta administrada, sí que observamos diferencias de sexo en la expresión de este gen, ya que los ratones hembra presentan una menor expresión en comparación con los ratones macho. Con estos datos hipotetizamos que, teniendo en cuenta que la activación del sistema opioide influye en el

aprendizaje o la realización de conductas basadas en la recompensa de las drogas (Koob & Le Moal, 2005), las diferencias de sexo en la expresión del gen Oprμ podrían explicar que los ratones machos modifiquen sus procesos de aprendizaje durante las extinciones de cocaína de forma distinta a las hembras.

También se ha observado que, en ausencia de estrés, la DRG no induce ningún cambio en la expresión del gen CB1r en ratones hembra, pero si provoca un aumento de expresión cuando esta dieta se administra previa a las sesiones de extinción de cocaína en ratones macho, coincidiendo con una reducción del número de sesiones necesarias para extinguir la conducta condicionada. Varios estudios asocian el receptor CB1 a los procesos de aprendizaje de la extinción y la recaída (Khaleghzadeh-Ahangar & Haghparast, 2015; Yu et al., 2011), por lo que la sobreexpresión del gen CB1r en este grupo de animales podría explicar que aprendan más rápidamente a identificar la ausencia de la cocaína en el compartimento previamente asociado a ella.

Finalmente, también podemos confirmar que la DS induce un aumento en la expresión del gen CRHR1. En la mayoría de los grupos experimentales que se exponen a una DRG, no se observa cambios en la sobreexpresión del gen CRHR1. Sin embargo, cuando se presenta un aumento de las Kcal ingeridas derivadas del consumo de una DRG sí que observamos una reducción en su expresión. Nuestros resultados sugieren que, al ingerir una cantidad elevada de Kcal derivadas de la DRG, se produciría un efecto similar al de los antagonistas de CRHR1, reduciendo la expresión de este gen y por tanto la actividad del eje HHA (Ulrich-Lai et al., 2011).

Estudiamos igualmente los efectos de la DC en la expresión génica de diferentes genes y observamos que esta dieta solo produce un incremento en la expresión de A1r sin provocar alteraciones en la expresión del gen Oprμ, el gen A2r o en los genes de los receptores dopaminérgicos. Paralelamente, al administrar la DC y exponer a los ratones al alcohol observamos cambios en la expresión génica con un incremento

en los genes D1r, D2r y A2r. A partir de estos resultados, y teniendo en cuenta que se ha demostrado que la DC aumenta los niveles de adenosina y activa sus receptores (Lusardi et al., 2015), nuestra hipótesis inicial plantea que la administración de una DC disminuye los efectos gratificantes de las drogas actuando a través del binomio adenosina-dopamina. Teniendo en cuenta estos resultados, sugerimos que el efecto de la DC sobre estos receptores provoca la reducción en la ingesta de alcohol, previniendo así el desarrollo de la conducta adictiva.

Valor traslacional del estudio y futuras investigaciones

Los modelos animales son una herramienta muy potente en investigación básica, pero debemos ser prudentes a la hora de trasladar los resultados obtenidos a la conducta humana. Nuestros resultados muestran que la comida palatable puede actuar como una recompensa alternativa a la cocaína y el alcohol, pero también observamos que estos efectos están condicionados por el sexo. Es por ello que, teniendo en cuenta que la administración de DRG es menos eficaz en hembras y que las consecuencias cognitivas y conductuales de algunos trastornos psiquiátricos como los trastornos por uso de sustancias, la ansiedad y la depresión difieren entre hombres y mujeres (Bangasser & Cuarenta, 2021), remarcamos la necesidad de continuar estudiando las diferentes pautas de administración y dietas con alto contenido en grasa en ambos sexos, ya que no podemos asumir que las intervenciones ambientales o nutricionales sean igualmente efectivas en ambos. Paralelamente, también hemos demostrado que la DRG puede facilitar el desarrollo de un perfil resiliente en roedores, ya que esta intervención nutricional es capaz de reducir los efectos reforzantes de la cocaína incrementados por el estrés social. Teniendo en cuenta estos resultados, y tras confirmar que la DC también influye en el sistema de recompensa provocando una disminución del consumo de alcohol, consideramos necesario continuar estudiando el efecto de la DC sobre las consecuencias derivadas del estrés social.

1. INTRODUCCIÓN

1. Introducción General

1. Introducción general

El estrés actúa como un proceso de adaptación de los individuos al medio que, afrontado correctamente, favorece nuestra supervivencia (Ulrich-Lai & Herman, 2009). Sin embargo, una prolongada percepción del estrés puede provocar alteraciones en nuestro estado físico y mental. En la sociedad actual, el estrés más frecuente al que nos exponemos es de carácter social (Hartsell & Neupert, 2019), y su influencia puede afectar a otros procesos neuropsicológicos. En el desarrollo de un proceso adictivo, por ejemplo, la exposición a estresores sociales puede favorecer el inicio, el mantenimiento, la escalada y la recaída en el consumo de drogas (Koob, 2008; Koob & Schulkin, 2019; Montagud-Romero et al., 2018; Sinha et al., 2011). La influencia del estrés en el desarrollo de adicción se explica por la estrecha relación existente entre los sistemas neurales que controlan la respuesta al estrés y la respuesta a las drogas. Ante una situación de estrés, el eje Hipotálamo-Hipofisio-Adrenal (HHA), provoca la liberación de hormonas como el factor liberador de corticotropina (CRF) (Covington & Miczek, 2001; Goeders, 2002). En el área tegmental ventral (ATV), una de las regiones principales que participan en el sistema de recompensa implicado en la adicción, hay neuronas dopaminérgicas que expresan receptores sensibles al CRF (Boyson et al., 2014; Haass-Koffler & Bartlett, 2012). Al activarse estos receptores, se produce un aumento de la actividad dopaminérgica en regiones específicas del sistema de recompensa como en el Núcleo Accumbens (NAcc), la amígdala y la corteza pre-frontal (CPF) (Steketee & Kalivas, 2011). Este aumento de actividad dopaminérgica provocada por la exposición a estrés resulta en un incremento de la sensibilidad en las propiedades reforzantes de diferentes sustancias de abuso (Han et al., 2017; Sinha et al., 2011).

Para conocer los mecanismos neurobiológicos que subyacen a los efectos del estrés en la adicción a las drogas, se han desarrollado modelos animales como la derrota social (DS), que permiten estudiar las consecuencias conductuales y fisiológicas a

corto y largo plazo del estrés social (Shimamoto, 2018). Con este modelo, se ha confirmado como la exposición a DS en ratones es capaz de aumentar los efectos reforzantes de la cocaína evaluados en un paradigma de preferencia de lugar condicionada (CPL) (Ferrer-Pérez et al., 2018; Hymel et al., 2014; McLaughlin et al., 2006; Montagud-Romero et al., 2018; Rodríguez-Arias et al., 2017) y en un procedimiento de autoadministración operante (AA) (Boyson et al., 2014; Holly et al., 2016; Newman et al., 2018).

Paralelamente, aunque los resultados indican que la exposición a estrés social incrementa los efectos reforzantes de las drogas, se observa que una parte de la población manifiesta conductas de afrontamiento resilientes que les protege de estas consecuencias provocadas por el estrés (Ballestín et al., 2021; Brodnik et al., 2017; Krishnan et al., 2007). Por ello, comienzan a estudiarse distintos factores que pueden inducir una respuesta satisfactoria al estrés y potenciar las respuestas resilientes. Algunas intervenciones farmacológicas como la administración de oxitocina exógena, administrada antes de cada DS, ha conseguido bloquear el aumento de los efectos reforzantes de la cocaína inducidos por el estrés social (Ferrer-Pérez, et al., 2019a). Diferentes manipulaciones ambientales también han demostrado su eficacia reduciendo las consecuencias provocadas por la DS en la adicción, como el ejercicio físico (Reguilón et al., 2020; Ferrer-Pérez et al., 2022; Calpe-López et al., 2022), las condiciones de estabulación (Ferrer-Pérez et al., 2019b) o el enriquecimiento ambiental (Reguilón et al., 2021; Aujnarain et al., 2018). Más recientemente, se han comenzado a desarrollar intervenciones nutricionales que pueden ser efectivas para potenciar la resiliencia y de hecho se ha observado que los efectos reforzantes de la cocaína inducidos por estrés social pueden reducirse en ratones alimentados con una dieta rica en grasas, carbohidratos y azúcares (DRG) (Blanco-Gandía et al., 2018).

El hecho de que estas intervenciones nutricionales puedan modular la respuesta de estrés y la respuesta a drogas se explica porque, a pesar de que la alimentación está

controlada por mecanismos homeostáticos (Seeley et al., 2003), también está influida por mecanismos hedónicos orquestados por el sistema de recompensa (Saper et al., 2002; Zheng & Berthoud, 2007). Algunos tipos de alimentos, como los encontrados en las DRG, tienen efectos considerables en este sistema de recompensa del cerebro, produciendo cambios en el sistema dopaminérgico, opiáceo y endocannabinoide (Davis et al., 2008; Johnson & Kenny, 2010). Estos alimentos que contienen un alto porcentaje de grasa y azúcar provocan la estimulación de regiones cerebrales como el ATV, la CPF, la amígdala y el NAcc (de Macedo et al., 2016), estructuras implicadas en todo el proceso adictivo (Volkow et al., 2013). Al compartir el mismo sistema, se ha comprobado en modelos animales como la DRG aumenta la AA de cocaína en ratas (Puhl et al., 2011) y ratones (Blanco-Gandía et al., 2017a), y también provoca un aumento de los efectos reforzantes de los psicoestimulantes evaluados con un CPL, como la anfetamina (Kuhn et al., 2013) o la cocaína (Blanco-Gandía et al., 2017a; Peleg-Raibstein et al., 2016).

La estimulación del sistema de refuerzo sugiere que este tipo de dietas también podrían actuar como un reforzador alternativo, y de hecho se ha confirmado que su consumo puede disminuir los efectos reforzantes condicionados de la cocaína (Morales et al., 2012) y las anfetaminas (Davis et al., 2008), atenuar la AA de cocaína en ratas (Wellman et al., 2007) y reducir la búsqueda de cocaína durante la extinción de las memorias asociadas al refuerzo condicionado (Blanco-Gandía et al., 2017b). Estos resultados refuerzan la teoría de que la exposición continua a una DRG podría tener un papel como reforzador alternativo, atenuando las propiedades reforzantes y alterando los aspectos gratificantes de los psicoestimulantes (Morales et al., 2012). Sin embargo, esta exposición continua a grasas y azúcares provoca trastornos metabólicos como un aumento significativo de peso y del tejido adiposo o un aumento drástico de los niveles de leptina (Blanco-Gandía et al., 2017b; Morales et al., 2012; Wellman et al., 2007). Por lo tanto, resulta necesario evaluar la eficacia de otro tipo de administración de estas DRG, así como el empleo de otras dietas altas

en grasa que no contienen excesivos niveles de carbohidratos derivados del azúcar, como la dieta cetogénica (DC).

La DC es una dieta rica en grasas, con una proporción aproximada de 80% de calorías de grasa, 15% de calorías de proteínas y 5% de calorías de carbohidratos, que reduce drásticamente los niveles de glucosa disponible en el organismo y genera cuerpos cetónicos como el β-hidroxibutirato (βOHB) (Masino et al., 2012). Esta reducción de glucosa y aumento de βOHB modifica el estado metabólico, entrando en lo que se conoce como estado de cetosis (Politi et al., 2011). Estos cambios de metabolismo provocados por la DC se han empleado como intervención terapéutica en diferentes trastornos como la epilepsia (Kessler et al., 2011; Masino et al., 2011; Neal et al., 2008), la enfermedad de Alzheimer o el Parkinson (Henderson et al., 2009; Kashiwaya et al., 2000; Tieu et al., 2003; Yang & Cheng, 2010; Yao et al., 2011) así como el autismo (Evangeliou et al., 2003).

Entre los principales mecanismos neuronales que podrían explicar sus efectos, cabe destacar su influencia en la modulación de los canales de potasio sensibles al nucleótido adenosina trifosfato (ATP) y el aumento de la neurotransmisión GABAérgica y de purinas como la adenosina, que han sido los más estudiados (Lusardi et al., 2015; Masino et al., 2012). En concreto, se ha observado cómo la DC, aumenta los niveles de adenosina y activa sus receptores (Lusardi et al., 2015), inhibiendo la excitabilidad de las neuronas, lo que explica sus efectos terapéuticos en enfermedades como la epilepsia (Kawamura et al., 2010; Masino et al., 2011). Por otro lado, la adenosina también está estrechamente vinculada a la acción dopaminérgica, ya que sus receptores se colocalizan en las neuronas GABAérgicas, lo que sugiere que la modificación de uno de los sistemas puede conducir a la regulación del otro (Fuxe et al., 2010). Teniendo en cuenta el efecto de la DC sobre la adenosina y el sistema dopaminérgico, y la implicación de estos sistemas en la

adicción, se plantea que esta dieta podría modular de alguna forma el desarrollo del proceso adictivo.

Sin embargo, hay muy pocos estudios que han demostrado el efecto que una DC puede tener en la adicción a las drogas. En estudios preclínicos con modelos animales se ha observado cómo esta dieta puede reducir las respuestas habituales de sensibilización que se manifiestan durante el síndrome de abstinencia a la cocaína (Martínez et al., 2019), reducir los síntomas de abstinencia de alcohol (Dencker et al., 2018), y también reducir la hiperlocomoción y la ansiedad provocada por la retirada del alcohol (Bornebusch et al., 2021). Por otro lado, en investigación clínica también se ha observado cómo en personas con trastorno por consumo de alcohol, la DC es capaz de reducir los síntomas de abstinencia, disminuir el deseo de consumo y además reducir la cantidad de benzodiacepinas necesarias para mitigar el malestar del síndrome de abstinencia (Wiers et al., 2021).

Con todos estos datos, se evidencia que la exposición a estrés social es capaz de aumentar los efectos reforzantes de las drogas de abuso, aunque algunos individuos son capaces de desarrollar un perfil resiliente que les protege de las consecuencias psicofisiológicas del estrés. Además, es posible potenciar esa respuesta resiliente con algunos tipos de intervenciones farmacológicas y ambientales, como las intervenciones nutricionales. En resumen, los estudios realizados hasta la fecha demuestran que la estrecha relación entre las DRG y el sistema de recompensa cerebral (Hajnal et al., 2004; Rada et al., 2010), su capacidad para disminuir de las consecuencias provocadas por el estrés (Pecoraro et al., 2004; Ulrich-Lai et al., 2011), y su interacción con los efectos gratificantes de las drogas, (Blanco-Gandía et al., 2018). Todo ello sugiere que estas dietas podrían emplearse como un reforzador alternativo potenciando el desarrollo de la resiliencia, y reduciendo las consecuencias provocadas por la exposición al estrés social.

2. Estrés social y consumo de drogas

2.1. Estrés social

La respuesta al estrés es una estrategia saludable y adaptativa que nos ayuda a afrontar las situaciones diarias necesarias para nuestra supervivencia (Ulrich-Lai & Herman, 2009) pero en algunas ocasiones, la experiencia del estrés se prolonga en el tiempo convirtiéndose realmente en un estímulo nocivo que provoca cambios en nuestro estado físico y mental. En nuestra sociedad moderna, el estrés se ha convertido en un problema importante, donde los principales factores de estrés que sufren las personas son de carácter social como la ausencia de apoyo social, aspectos socioeconómicos, vida escolar, estado civil, rol laboral, género o discriminación (Hartsell & Neupert, 2019). Inevitablemente, los acontecimientos socialmente estresantes forman parte de la vida cotidiana de los seres humanos. A veces se trata de eventos puntuales que generan una respuesta de estrés elevada pero breve que implican una rápida recuperación de la homeostasis, pero en otras ocasiones, el evento se prolonga más de lo que el individuo desearía (Bains et al., 2015; Neumann, 2008). Con la alteración de la homeostasis se produce un desequilibrio en el correcto funcionamiento de diversos sistemas cerebrales y hormonales que puede promover efectos adversos en nuestra salud, tanto en las dimensiones fisiológicas como psicológicas (Hartsell & Neupert, 2019; Lupien et al., 2009; Mcewen, 2004). De hecho, existe una amplia literatura que relaciona el estrés social con algunas enfermedades epidémicas en nuestra sociedad, como el cáncer (Krizanova et al., 2016), la obesidad (Ouakinin et al., 2018) e incluso en los trastornos por uso de sustancias (TUS) (Ruisoto & Contador, 2019). Con respecto a las adicciones, muchos estudios han puesto de manifiesto el papel del estrés social en todas las etapas del proceso adictivo, favoreciendo el inicio, el mantenimiento, la escalada y la recaída en el consumo de drogas (Koob, 2008; Koob & Schulkin, 2019; Montagud-Romero et al., 2018; Sinha et al., 2011), lo que sugiere que los circuitos cerebrales que regulan la recompensa y el refuerzo mantienen una estrecha relación con el sistema de estrés.

2.1.1. Estrés y sistema de refuerzo

En los últimos años se han detallado cuáles son los sustratos neurobiológicos que intervienen en la respuesta al estrés social y su influencia en la adicción a las drogas (Koob & Schulkin, 2019). En la respuesta al estrés participan diferentes mecanismos que implican la activación del eje HHA, provocando la liberación de hormonas como el CRF (Covington & Miczek, 2005; Goeders, 2002). Cuando el CRF se une a sus receptores localizados en la adenohipófisis, promueve la síntesis de pro-opiomelanocortina (POMC), hormona α-estimulante de melanocitos, β-endorfina y hormona adenocorticotropa (ACTH) (Arnett et al., 2016). Tras ser sintetizada, la ACTH se libera en el torrente sanguíneo hasta llegar a la corteza suprarrenal, induciendo la síntesis de glucocorticoides, como el cortisol en humanos y la corticosterona en roedores (Tsigos et al., 2016). Por un lado, los receptores de glucocorticoides promueven reacciones catabólicas con el objetivo de obtener energía para superar la situación de estrés, y por otro, suprimen funciones que no son inmediatamente necesarias para la supervivencia con el objetivo de optimizar recursos (Goeders & Clampitt, 2002). Para conseguir una regulación óptima de este sistema de estrés, cuando se manifiesta una liberación excesiva de éstos glucocorticoides, se produce una retroalimentación negativa sobre el hipotálamo y la hipófisis anterior, disminuyendo la producción de CRF a nivel hipotalámico y una disminución de la síntesis de POMC y ACTH en la adenohipófisis (de Kloet et al., 2005).

El CRF modula muchas respuestas fisiológicas y de comportamiento relacionadas con el estrés, pero, además, tiene receptores localizados en estructuras clave del sistema de recompensa, lo que explica su influencia en la adicción a las drogas (Holly & Miczek, 2016; Koob & Schulkin, 2019; Zorrilla et al., 2014). En el ATV, por ejemplo, hay neuronas dopaminérgicas que expresan estos receptores sensibles al CRF (Boyson et al., 2014; Haass-Koffler & Bartlett, 2012). Al activarse estos

receptores, se produce un aumento de la actividad dopaminérgica en regiones específicas del sistema de recompensa como en el NAcc, la amígdala y la CPF (Steketee & Kalivas, 2011) (Figura 1). Esta hiperactividad del sistema dopaminérgico causada por el CRF se traduce en una mayor sensibilidad a las propiedades reforzantes de diferentes sustancias de abuso, como por ejemplo la cocaína (Han et al., 2017; Sinha et al., 2011), confirmando el papel crítico que tiene el estrés en la modulación de la motivación, la búsqueda y el consumo de drogas (Holly & Miczek, 2016; Koob & Volkow, 2010). Así, numerosos estudios muestran que esta activación de los receptores CRF en el ATV incrementa el consumo de cocaína y también puede inducir su reinstauración (Blacktop et al., 2011; Boyson et al., 2014; Wang, 2005). También se ha comprobado que la administración de antagonistas de estos receptores previene la sensibilización dopaminérgica y evitan la escalada del consumo de cocaína provocados por el estrés (Boyson et al., 2014; Ferrer-Pérez et al., 2018; Lodge & Grace, 2005; Specio et al., 2008). El aumento de los niveles de DA inducido por el CRF en estructuras como la amígdala es especialmente relevante durante la abstinencia, ya que la sobreactivación de esta estructura tiene un papel clave en el estado emocional negativo que se experimenta durante la misma, lo que podría explicar los procesos de refuerzo negativo que impulsan la compulsividad de la adicción en momentos de estrés (Zorrilla et al., 2014).

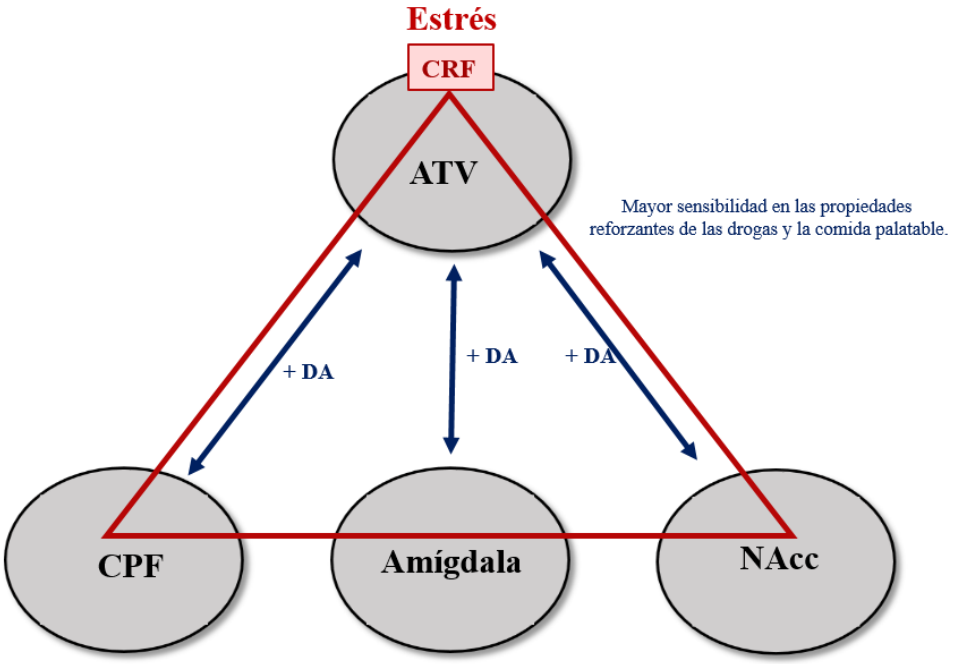


Figura 1. Efecto del estrés y el factor liberador de corticotropina (CRF) en el sistema de recompensa.

2.1.2. Modelos animales de estrés social y adicción

La investigación preclínica empleando modelos animales es esencial para aumentar la comprensión de los mecanismos neurobiológicos que subyacen a los efectos del estrés en la adicción a las drogas. En este sentido, entre todos los paradigmas que inducen estrés social en roedores como el aislamiento social, la inestabilidad social o la separación materna, la DS se considera el más representativo para estudiar sus consecuencias fisiológicas y conductuales (Hammels et al., 2015; Neisewander et al., 2012). La DS es una herramienta útil para recrear experiencias como el acoso, el abuso físico o la subordinación (Miczek et al., 2008), ya que este paradigma imita

estrechamente la realidad de las relaciones entre subordinados y agresores en los seres humanos (Björkqvist, 2001). También denominado paradigma intruso-residente, se basa en el ataque territorial de un macho residente a un intruso coespecífico, permitiendo estudiar las consecuencias conductuales y fisiológicas a corto y largo plazo del estrés social (Shimamoto, 2018). Por ejemplo, se ha podido observar cómo los roedores expuestos a la DS muestran síntomas similares a la depresión, anhedonia, aumento de la ansiedad, evitación social e incluso deterioro de las funciones cognitivas (Higashida et al., 2018; Riga et al., 2015; Zhang et al., 2016).

Por otro lado, para evaluar la vulnerabilidad a los efectos gratificantes de las drogas de abuso en modelos animales, dos de los protocolos más empleados gracias a su viabilidad y validez son el paradigma de CPL y el procedimiento de AA. En el CPL se utiliza un estímulo motivacional primario (la droga) como estímulo incondicionado, que, tras una exposición repetida, convierte las claves ambientales que inicialmente eran neutras en propiedades motivacionales secundarias, convirtiéndose en un estímulo condicionado (Aguilar et al., 2009; Tzschentke, 2007). Cuando el animal se expone al contexto en el que se administró la sustancia, pasa más tiempo en el compartimento emparejado con la droga (si el estímulo es apetitivo). Por otro lado, la AA evalúa los efectos reforzadores primarios de las drogas en función del esfuerzo que realiza el animal para obtener la droga, pudiendo también evaluar así la motivación por el consumo. En este modelo, el animal realiza una respuesta operante, como pulsar una palanca para obtener el refuerzo de la droga (Moeller & Stoops, 2015).

Tomando como referencia estos modelos animales de estrés y adicción, la literatura científica ha demostrado que la DS puede interferir en distintas fases del proceso adictivo de las drogas de abuso. Por ejemplo, la exposición a DS en ratones es capaz de aumentar los efectos reforzantes de la cocaína evaluados en un CPL (Ferrer-Pérez

et al., 2018; Hymel et al., 2014; McLaughlin et al., 2006; Montagud-Romero et al., 2018; Rodríguez-Arias et al., 2017). En estos estudios, los animales derrotados, es decir, expuestos a un estresor social, muestran una mayor sensibilidad a los efectos reforzantes de la cocaína, donde se manifiesta un aumento de la conducta de la búsqueda de la droga. Uno de los mecanismos que explica este efecto de sensibilización causado por el estrés social es el aumento de la transmisión dopaminérgica en las proyecciones del ATV a la CPF y al NAcc (Han et al., 2017; Holly et al., 2016; Steketee & Kalivas, 2011), que son las principales estructuras implicadas en los efectos gratificantes de los psicoestimulantes (Koob, 2009; Sinha, 2008). Además, este incremento de los efectos reforzantes de la cocaína causado por la DS también se ha observado empleando la AA de cocaína (Boyson et al., 2014; Holly et al., 2016; Newman et al., 2018), donde se observa tanto un aumento del consumo como de la motivación por conseguir la droga. Sin embargo, el efecto del estrés social no solo se ha confirmado en el proceso de adquisición de la conducta adictiva. También se ha observado que, una vez extinguida la conducta de búsqueda de la cocaína, la exposición a las DS puede restablecer el CPL en roedores (Land et al., 2009; Titomanlio et al., 2013), aumentando la susceptibilidad de la reinstauración inducida por una dosis de recuerdo o "*priming*" de cocaína (Montagud-Romero et al., 2016).

2.2. Resiliencia y adicción

En los últimos años se ha producido un gran aumento del estudio del fenómeno de la resiliencia al estrés. La resiliencia se define como la capacidad de los individuos para mantener un correcto funcionamiento psicológico y físico cuando experimentan un estrés crónico o de alta intensidad, lo que les permite adaptarse a estas situaciones, aprender de ellas e incluso evitar la aparición de las consecuencias psicofisiológicas provocadas por estos estresores (Charney, 2004; Chmitorz et al., 2018; Rutter, 2012).

Debido al creciente interés por este fenómeno y su gran relevancia en el desarrollo del bienestar personal, muchas de las investigaciones actuales tienen como objetivo identificar cuáles son las características psicológicas y biológicas que favorecen una adecuada gestión y adaptación al estrés, especialmente ante el estrés social (Pfau & Russo, 2015). Por ejemplo, algunos investigadores han propuesto que existe una serie de comportamientos y rasgos psicológicos específicos, como la flexibilidad cognitiva, el afrontamiento activo, el optimismo o el sentimiento de pertenencia a un grupo de referencia, que pueden favorecer una respuesta resiliente en los seres humanos (Laird et al., 2019; Wood & Bhatnagar, 2015). Hasta la fecha, la mayoría de los estudios se han centrado en los efectos positivos que tiene el desarrollo de la resiliencia ante consecuencias derivadas del estrés como la depresión, la ansiedad o el trastorno de estrés postraumático (Finnell & Wood, 2016; Krishnan, 2014; Russo et al., 2012), sin embargo, en los últimos años comienza a estudiarse cada vez más los efectos que tiene la resiliencia sobre otras patologías como la adicción.

Hasta el año 2010, la literatura relacionada con la resiliencia y la adicción era escasa, pero en la última década se está experimentando un aumento en el número de estudios que abordan esta interacción (Ballestín et al., 2021; Brodnik et al., 2017; Ferrer-Pérez et al., 2019b). Los estudios epidemiológicos actuales, por ejemplo, confirman que existe una relación entre la baja resiliencia y el incremento de las conductas adictivas (Kennedy et al., 2019; Yen et al., 2019), lo que evidencia la necesidad de identificar los rasgos conductuales y fisiológicos que puedan reducir los efectos del estrés sobre las propiedades gratificantes de las drogas de abuso, así como los sustratos neurobiológicos que favorezcan el desarrollo de la resiliencia. Por este motivo, la investigación preclínica resulta esencial para estudiar el impacto del estrés y el desarrollo de la resiliencia en la adicción.

Muchos de los estudios preclínicos sobre la resiliencia al estrés social utilizan el modelo de DS, donde los animales son categorizados como susceptibles cuando

manifiestan las consecuencias psicofisiológicas provocadas por la exposición al estrés, como síntomas similares a la depresión, anhedonia, ansiedad o evitación social (Higashida et al., 2018; Riga et al., 2015; Zhang et al., 2016), mientras que son considerados resilientes cuando no desarrollan esas consecuencias. Por ejemplo, la caracterización de resiliencia más habitual que podemos encontrar en la literatura científica se basa en el comportamiento social, donde los animales que mantienen un mayor tiempo de contacto social después de la exposición a la DS son considerados resilientes, mientras que los considerados susceptibles muestran evitación social (Golden et al., 2011; Henriques-Alves & Queiroz, 2016; Russo et al., 2012; Zhang et al., 2016). También encontramos estudios donde los ratones son considerados resilientes cuando no presentan anhedonia (Delgado et al., 2011) o evitación ante el olor de un depredador (Brodnik et al., 2017), sin embargo, hay muy pocos estudios que tienen en cuenta los efectos reforzantes de las drogas inducidos por el estrés a la hora de caracterizar a los animales como resilientes o susceptibles.

En un estudio llevado a cabo por Krishnan et al. (2007) se demostró por primera vez que, tras la exposición a la DS, los ratones susceptibles mostraban anhedonia, evitación social y comportamientos similares a la ansiedad mientras que los ratones resilientes no mostraban estos síntomas. Sin embargo, este estudio también fue pionero a la hora de demostrar las diferencias entre ratones susceptibles y resilientes en cuanto a la sensibilidad a los efectos gratificantes de la cocaína, donde sólo los ratones susceptibles desarrollaron una conducta de búsqueda a la cocaína evaluado en un CPL, mientras que los ratones resilientes y los no estresados no manifestaron dicha conducta. En el estudio nombrado previamente de Brodnik et al. (2017), también se observó que los animales resilientes que no manifestaron conductas de ansiedad ni evitación del olor del depredador, tampoco mostraron un aumento de los efectos reforzantes de la cocaína ni aumento de motivación para autoadministrarse esta droga, efecto que si se observó en los animales susceptibles. En esta línea, se han obtenido resultados similares en nuestro laboratorio al observar que la DS induce

un aumento de los efectos gratificantes condicionados de la cocaína sólo en aquellos animales caracterizados como susceptibles en función de su interacción social (Ballestín et al., 2021). En este último estudio, además se observó que los ratones resilientes mostraron una conducta de afrontamiento activo durante la exposición a estrés diferente a los susceptibles. Estos datos coinciden con los estudios que confirman que durante la exposición estrés, existen estrategias conductuales que limitan la experiencia del estrés y pueden promover la resiliencia (Russo et al., 2012). Por ejemplo, se ha identificado que los animales que adoptan posturas menos sumisas cuando son amenazados y atacados por el adversario durante la DS, muestran menos evitación social, lo que sugiere que esta estrategia conductual de afrontamiento podría reducir los efectos del estrés (Wood et al., 2010).

Actualmente, comienzan a estudiarse distintos factores que pueden inducir una respuesta satisfactoria al estrés y potenciar las respuestas resilientes, utilizado manipulaciones conductuales y ambientales como la exposición al ejercicio físico (Holmes, 2014; Sciolino et al., 2015) o el enriquecimiento ambiental (Hutchinson et al., 2012), sin embargo, como se ha mencionado anteriormente, hay pocos trabajos que estudien la potenciación de resiliencia para evitar el incremento de los efectos reforzantes de las drogas de abuso.

2.2.1 Intervenciones farmacológicas, ambientales y nutricionales.

Utilizando intervenciones farmacológicas como la administración de oxitocina exógena, administrada antes de cada DS se ha conseguido bloquear el aumento de los efectos reforzantes de la cocaína inducidos por el estrés social, potenciando así la resiliencia (Ferrer-Pérez et al., 2019a). Además, en estos estudios, los ratones que manifiestan menor sensibilidad a los efectos reforzantes de la cocaína, también muestran resiliencia a las conductas de evitación social. Estos efectos beneficiosos de la oxitocina también se han observado en los síntomas depresivos o ansiógenos

derivados de la exposición al estrés social (Dodhia et al., 2014; Luo et al., 2017; Schwaiger et al., 2019).

Las manipulaciones ambientales también se han utilizado como herramienta para fomentar el desarrollo de la resiliencia y reducir las consecuencias provocadas por la DS, entre las que encontramos el enriquecimiento ambiental, el ejercicio físico o las condiciones de estabulación. Se ha observado por ejemplo que los ratones a los que se les permite realizar ejercicio físico voluntario antes, durante o después de la exposición a la DS se vuelven resilientes al incremento en los efectos reforzantes de la cocaína (Ferrer-Pérez et al., 2022; Calpe-López et al., 2022) y el alcohol (Reguilón et al., 2020). Las condiciones de estabulación que simulan relaciones sociales positivas, como el alojamiento en pareja, también aumentan la resiliencia al estrés y revierten la potenciación de la recompensa de la cocaína inducida por la DS, efectos que están mediados por la oxitocina (Ferrer-Pérez et al., 2019b). En esta misma línea, también se ha informado de que el enriquecimiento ambiental incrementa la resiliencia al estrés (Aujnarain et al., 2018; Reguilón et al., 2021), evitando así el desarrollo de los efectos fisiológicos y conductuales desadaptativos provocados por el estrés crónico y agudo (Bahi, 2017). Por ejemplo, los ratones realojados en condiciones de enriquecimiento ambiental después de estrés físico o social parecen estar protegidos contra los efectos de estas experiencias de estrés (Ashokan et al., 2016; Bahi, 2017), reduciendo la búsqueda y la reinstauración de cocaína inducida por el estrés (Chauvet et al., 2009).

Todos estos resultados confirman que es posible desarrollar intervenciones con un relevante potencial terapéutico capaces de fomentar un perfil resiliente, por lo que se evidencia la necesidad de seguir buscando factores ambientales protectores que puedan amortiguar los efectos del estrés social. Por ejemplo, la literatura actual sugiere que las intervenciones nutricionales, como aquellas que emplean alimentos palatables con un alto componente hedónico, como las DRG, pueden ser

consideradas como otro factor protector a tener en cuenta. Estas dietas también parecen influir en el estrés, actuando como "alimento reconfortante" debido al efecto de alivio que presentan en condiciones de malestar psicológico (Bhatnagar et al., 2001; Dallman et al., 2003). De hecho, muchas personas informan de un incremento en el consumo de alimentos palatables cuando están expuestos al estrés (Groesz et al., 2012; Kim et al., 2013; Laugero et al., 2011), lo que provoca una reducción de los niveles de cortisol en plasma y una disminución del estrés percibido (Fernández et al., 2003). En estudios preclínicos también se ha observado esta compensación hedónica del estrés. Los roedores expuestos a estrés crónico muestran una mayor preferencia por este tipo de alimentos (Coccurello et al., 2018; Hassan et al., 2019; Packard et al., 2014; Pecoraro et al., 2004), reduciendo la respuesta a los estresores agudos como la hiperactividad del eje HHA (Foster et al., 2009; la Fleur et al., 2005; Pecoraro et al., 2004; Ulrich-Lai et al., 2011). En esta línea, algunos investigadores ya han confirmado que los efectos negativos causados por la DS, como la conducta de ansiedad o la anhedonia, disminuyen con la administración de una DRG (MacKay et al., 2017; Sial et al., 2021) sugiriendo que esta dieta modula la activación del eje HHA (Pecoraro et al., 2004; Ulrich-Lai et al., 2007) y reduce los niveles elevados de corticosterona en modelos animales (Francolinsilva et al., 2006; la Fleur et al., 2005; Suchecki et al., 2003). Este efecto también podría extenderse a los efectos gratificantes de drogas de abuso, de hecho, en un estudio previo de nuestro equipo de investigación se ha confirmado cómo los efectos gratificantes de la cocaína inducidos por el aislamiento social, otro paradigma animal para inducir estrés, disminuyen en ratones alimentados con DRG (Blanco-Gandía et al., 2018).

Todos estos resultados destacan que la DRG presenta una clara relación con el sistema de recompensa cerebral (Hajnal et al., 2004; Rada et al., 2010), siendo capaz de reducir las consecuencias provocadas por el estrés (Pecoraro et al., 2004; Ulrich-Lai et al., 2011) y que además interactúa con los efectos gratificantes de las drogas (Blanco-Gandía et al., 2018). En conclusión, las DRG podrían actuar como un

reforzador alternativo potenciando el desarrollo de la resiliencia, y reduciendo las consecuencias provocadas por la exposición al estrés social en los efectos reforzantes de la cocaína.

3. La dieta rica en grasa como moduladora del sistema de refuerzo

3. La dieta rica en grasa como moduladora del sistema de refuerzo

La alimentación es crucial para mantener las reservas de energía necesarias para la supervivencia en todas las especies del reino animal. Por esta razón los cerebros de los mamíferos han evolucionado desarrollando sistemas neuronales que impulsan el comportamiento alimentario, presentando además ciertos alimentos un importante valor reforzante (Saper et al., 2002). Muchas investigaciones hasta la fecha han tenido como objetivo comprender los principales mecanismos homeostáticos que regulan la alimentación (Kobeissy et al., 2008), pero también sus mecanismos hedónicos, ya que, en muchos casos, la ingesta de comida viene guiada por el placer y no por una necesidad energética nutricional (Gold, 2011).

En esta sección, examinaremos la regulación homeostática y hedónica de la alimentación y examinaremos los mecanismos de recompensa que confieren a la comida sus propiedades reforzantes. Así, para comprender mejor la naturaleza gratificante de la comida, es necesario considerar primero los diferentes mecanismos sensoriales homeostáticos y hedónicos que sustentan el impulso de la alimentación. Por un lado, la homeostasis energética está controlada principalmente por los circuitos neuronales del hipotálamo y el tronco cerebral, mientras que los aspectos de recompensa y motivación de la conducta alimentaria se rigen por las neuronas de las regiones límbicas y la corteza cerebral (Grill & Hayes, 2012; Waterson & Horvath, 2015).

3.1. Mecanismos homeostáticos de la alimentación

El hipotálamo es la estructura cerebral implicada en el control del estado homeostático del organismo y es el principal centro regulador de la ingesta de alimentos y agua (Seeley et al., 2003). Cuando se detectan alteraciones en las reservas de energía, el cerebro activa una serie de respuestas metabólicas y

conductuales dirigidas a mantener el equilibrio energético, regulando así el estado nutricional (Saper et al., 2002). En 1951 se demostró que las lesiones producidas en determinadas regiones del hipotálamo de las ratas producían una disminución de la ingesta de comida y agua (Anand & Brobeck, 1951), mientras que la estimulación de estas regiones provocaba a un aumento de la ingesta (Coons & Cruce, 1968; Delgado & Anand, 1952). Son varios núcleos los que constituyen el hipotálamo: el núcleo arqueado, el núcleo paraventricular, el hipotálamo lateral, el hipotálamo dorsomedial y el hipotálamo ventromedial (Schneeberger et al., 2014). De todos ellos, el núcleo arqueado destaca como centro regulador de la homeostasis energética y contiene neuronas sensibles a la señalización de hormonas que regulan la alimentación, como la leptina y la grelina. La leptina es una hormona descubierta en 1994 (Zhang et al., 2016) que aumenta sus niveles en sangre cuando se ingieren alimentos y disminuyen cuando hay privación de comida (Frederich et al., 1995; Maffei et al., 1995). Particularmente en el núcleo arqueado, encontramos neuronas que producen neuropéptidos orexigénicos que inducen el hambre, como el neuropéptido Y (NPY) y la proteína relacionada con Agouti (AgRP) (Graham et al., 1997; Spanswick et al., 2000). Estas neuronas son inhibidas por la leptina, reduciendo así la sensación de hambre. Por otro lado, la leptina también estimula otro tipo de neuronas del núcleo arqueado, que sintetizan neuropéptidos anorexígenos que inducen la saciedad, como el péptido relacionado a la cocaína y anfetamina (CART) y la POMC (Cowley et al., 2001; Mercer et al., 2013), provocando así la sensación de saciedad para reducir la ingesta de alimentos.

La grelina, descubierta más recientemente, es la otra hormona que participa conjuntamente con la leptina en este binomio apetito-saciedad (Horvath et al., 2001). Esta hormona se sintetiza en el estómago cuando los animales y seres humanos están privados de comida, como señal de hambre (Kojima et al., 1999), lo que provoca el aumento de la ingesta de alimentos (Cummings et al., 2001; Wren et al., 2000). Al igual que ocurre con la leptina, existen receptores de grelina en el hipotálamo

ventromedial y en el núcleo arqueado (Horvath et al., 2001). Al activarse estos receptores, se estimulan las neuronas AgRP/NPY, lo que induce el hambre y motiva el comportamiento de búsqueda de alimentos (Shintani et al., 2001; Wu et al., 2017). La interacción entre estas hormonas es crucial en la regulación nutricional (Cummings et al., 2001, 2002).

3.2 Mecanismos hedónicos y sistema de recompensa

A pesar de que la regulación nutricional entre hambre y saciedad es fundamental para favorecer la supervivencia, la alimentación no está controlada únicamente por mecanismos homeostáticos, también está influida por el placer de la ingesta y la motivación para conseguir la recompensa alimentaria (Saper et al., 2002; Zheng & Berthoud, 2007).

Desde el punto de vista del sistema de recompensa, se distinguen dos efectos principales que rigen los mecanismos hedónicos, *Liking* (gustar) y *Wanting* (querer, necesitar) (Hoebel et al., 2009). Por un lado, el '*liking'* se relaciona con el componente hedónico que nos suscita un estímulo, es decir, el efecto placentero propio de la comida u otras recompensas como las drogas de abuso. Por otro lado, el '*wanting'* se relacionaría con el componente que promueve nuestra motivación hacia el consumo de ese estímulo placentero. Es decir, en un estado inicial ingerimos alimentos placenteros porque nos gustan y por su propio valor reforzante, pero este comportamiento prolongado en el tiempo puede sensibilizar el circuito de recompensa y hacer que ciertos alimentos palatables se vuelvan más una necesidad (*want*) que un gusto (*like*) (Robinson & Berridge, 2008). En cuanto a los mecanismos neurobiológicos que sustentan este comportamiento (Figura 2), se encontrarían por un lado la dopamina (DA), y por otro lado, los sistemas opioide y cannabinoide (Morales & Berridge, 2020). Los sistemas opioide y cannabinoide se ocupan principalmente (pero no exclusivamente) del "*liking*" por la comida, es decir, de

provocar efectos placenteros cuando se ingiere. Particularmente, se ha observado cómo la activación de los receptores opioides o cannabinoides endógenos en el NAcc parece estimular el apetito, al aumentar la palatabilidad de la comida (Di Marzo et al., 2001; Onaivi et al., 2002). Por otro lado, el sistema dopaminérgico está implicado principalmente (pero no exclusivamente) en el "*wanting*", es decir, en el proceso motivacional de la ingesta.

Esta implicación de la DA en el '*wanting'* explica que la obtención o anticipación de la recompensa (el alimento) tenga más valor para el individuo que el efecto placentero de la comida en sí. La DA es un neurotransmisor ya conocido por sus diversas funciones fisiológicas, como la actividad locomotora, la emoción, los procesos cognitivos, la recompensa, el sueño y la ingesta de alimentos (Baladi et al., 2012). La DA ha sido objeto de una amplia investigación desde su descubrimiento (Carlsson et al., 1957) y su regulación también está implicada en varios trastornos como la enfermedad de Parkinson, el trastorno por déficit de atención con hiperactividad, el síndrome de Tourette y el abuso de drogas (Koob & Volkow, 2010; Swanson et al., 2007; Mink, 2006).

Se han identificado cuatro vías dopaminérgicas principales: los sistemas nigroestriatal, mesolímbico, mesocortical y tuberoinfundibular (Andén et al., 1964; Dahlström & Fuxe, 1964). En relación a la recompensa, la DA actúa sobre el sistema mesocorticolímbico, proyectándose desde el ATV al NAcc, a la CPF y al hipocampo (HIP) (Bassareo & Di Chiara, 1997; Di Chiara, 1998). La activación de este sistema está directamente relacionada con la respuesta hedónica y las recompensas obtenidas de distintas sustancias, como algunos tipos de alimentos y las drogas de abuso (Kelley et al., 2005; Volkow et al., 2013). En este sentido, las investigaciones demuestran que la alimentación hedónica modula los mecanismos neuronales relacionados con el procesamiento de la recompensa, lo que provoca el mantenimiento del deseo por este tipo de alimentos (Avena et al., 2008; Volkow et

al., 2013). De hecho, los estudios clínicos de imágenes cerebrales en humanos han demostrado que la liberación de DA generada cuando los seres humanos se exponen a estímulos relacionados con comida correlaciona con sus calificaciones subjetivas de deseo de la comida (Volkow et al., 2002). Además, en modelos animales se ha observado que la ingesta de comida aumenta la DA extracelular en el NAcc de ratas (Hernández & Hoebel, 1988). Incluso cuando se agotan los niveles de DA, los animales pueden dejar de alimentarse hasta llegar a morir de hambre, pero si se restauran los niveles de DA con L-DOPA, un precursor de la DA, el comportamiento alimentario vuelve a la normalidad (Sotak et al., 2005; Szczypka et al., 2001).

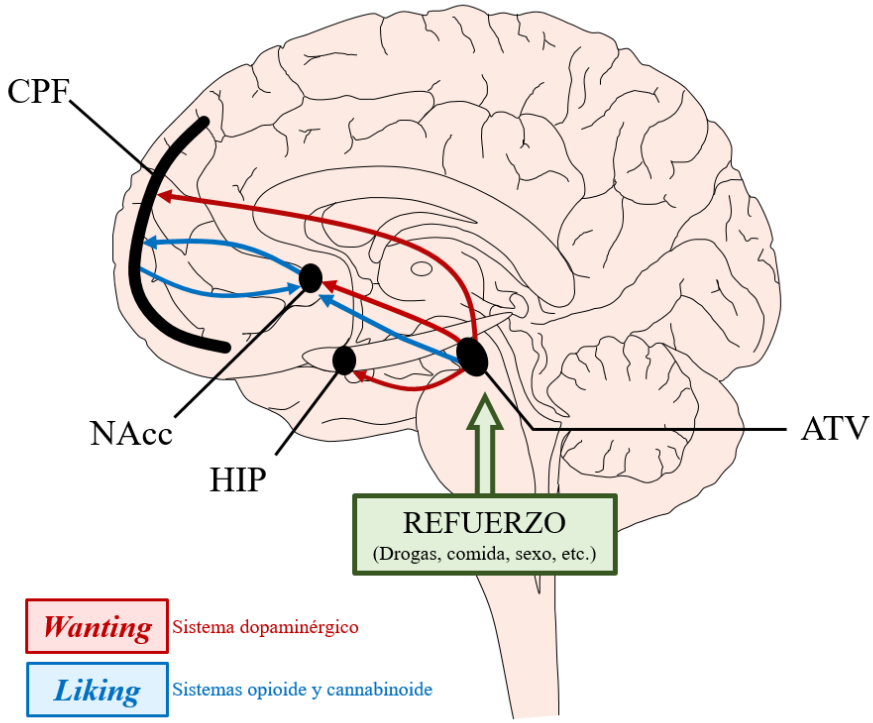


Figura 2. Sistema dopaminérgico implicado en *Wanting* (querer, necesitar) y sistemas opioide y cannabinoide implicado en *Liking* (gustar). Área tegmental ventral (ATV), Núcleo Accumbens (NAcc), hipocampo (HIP) y corteza prefrontal (CPF).

3.3. Tipos de dieta rica en grasa y su relación con la adicción

3.3.1. Dieta rica en grasa, carbohidratos y azúcares

La alimentación es una conducta esencial para la supervivencia, y por ello, nuestro cerebro ha desarrollado mecanismos que generan placer cuando ingerimos alimentos, aumentando las probabilidades de repetir la conducta. Sin embargo, no todos los alimentos provocan la misma intensidad a la hora de experimentar los efectos placenteros. Durante la evolución, nuestro organismo se ha desarrollado en un contexto de escasez nutricional, lo que explica nuestra preferencia biológica innata por alimentos con alto contenido en grasa, carbohidratos y azúcares, alimentos que inducen una potente liberación de DA en nuestro sistema de recompensa cerebral, produciendo una gran sensación de placer (de Macedo et al., 2016). De hecho, los estudios preclínicos realizados hasta la fecha han aportado pruebas sólidas que confirman que el acceso a las DRG tiene efectos considerables en el sistema de recompensa del cerebro, produciendo cambios en el sistema dopaminérgico, opiáceo y endocannabinoide (Davis et al., 2008; Johnson & Kenny, 2010). Sin embargo, aunque este mecanismo hedónico favorece la búsqueda innata de estos alimentos, en nuestro contexto actual el consumo de estas DRG ya no es imprescindible para favorecer la supervivencia, por lo que el exceso de su consumo puede llegar a ser perjudicial.

Como se ha comentado previamente, el sistema dopaminérgico está implicado en la respuesta hedónica de distintos estímulos, como las drogas de abuso y los alimentos (Kelley et al., 2005; Volkow et al., 2013). La relación entre alimentación y refuerzo es más evidente cuando se habla de alimentos que evolutivamente favorecían nuestra supervivencia al incrementar las reservas calóricas, como los alimentos que contienen un alto porcentaje de grasa y azúcar, cuyo consumo provoca la estimulación de regiones cerebrales como el ATV, la CPF, la amígdala y el NAcc (de Macedo et al., 2016). De hecho, también se ha observado cómo el consumo de estas DRG reduce la densidad del transportador activo de DA (Huang et al., 2006) y

produce un aumento más pronunciado de los niveles endógenos de DA en el NAcc comparado con otro tipo de alimentos (Bello & Hajnal, 2010; Rada et al., 2010). Además, se ha sugerido que sujetos con bajos niveles endógenos de DA en el NAcc pueden compensar este déficit consumiendo estas DRG para elevar sus niveles de DA y obtener así efectos gratificantes (Barson et al., 2012).

La DA se libera cuando se presenta un alimento apetecible, especialmente si es palatable, pero como se ha descrito anteriormente, aunque el NAcc y la señalización de DA son los principales agentes relacionados en la recompensa producida por el consumo de estos alimentos (Kelley et al., 2005; Smith et al., 2009), los receptores opioides y los cannabinoides también modulan la alimentación y la recompensa.

El sistema opioide endógeno destaca por su papel en la analgesia y la modulación de las funciones gastrointestinales, endocrinas, de aprendizaje y de memoria (Bodnar, 2022), estando también implicado en la modulación de la respuesta a una amplia gama de estímulos gratificantes, como la recompensa de las drogas, la música/el arte, el sexo, el humor y la obtención de dinero (Carelli, 2002; De Vries & Shippenberg, 2002; Everitt & Robbins, 2005). Entre otras regiones, sus receptores (μ, δ y κ) se expresan particularmente en estructuras implicadas en el sistema de recompensa, el control de la ingesta de alimentos y el apetito impulsado por la recompensa (Ding et al., 1996; George et al., 1994; Will et al., 2003). En el ATV, por ejemplo, la activación de los receptores μ localizados en sus neuronas GABAérgicas provoca un aumento de DA en el NAcc (Chefer et al., 2009; Shalev, 2002). A través de este mecanismo, la señalización opioide se asocia con la percepción de palatabilidad de los alimentos y sus propiedades hedónicas (Cota et al., 2006), es decir, el efecto placentero que se percibe al ingerir el alimento. Por ejemplo, Kawahara et al. (2013) demostraron que la ingesta de una DRG aumenta la liberación de DA en el NAcc a través de la activación de receptores μ opioide en el ATV, lo que provoca un aumento de efectos gratificantes y el valor hedónico de este tipo de dieta. Apoyando todos

estos resultados, varios estudios también han observado que la administración de agonistas de los receptores μ opioides directamente en el NAcc y el ATV aumentan la ingesta de alimentos palatables (Figlewicz & Sipols, 2010; Peciña, 2008; Zhang et al., 2003), mientras que la administración de antagonistas la disminuye (Bodnar, 2022).

Por otro lado, el sistema endocannabinoide es un sistema de transmisión química que también desempeña un papel modulador en los circuitos de recompensa/refuerzo del sistema mesolímbico y regula una gran variedad de procesos, incluyendo el sistema inmunológico, cardiovascular, endocrino y nervioso, pero también está implicado en el desarrollo neuronal, la motivación, el control emocional y el metabolismo energético (Cristino et al., 2014; Lu & Mackie, 2016; Mechoulam & Parker, 2013). Los receptores endocannabinoides específicos son CB1r y CB2r. Los CB1r se encuentran principalmente en el sistema nervioso central y son abundantes en regiones relacionadas con el sistema de recompensa como el ATV, NAcc y la CPF (Freund et al., 2003). Estos receptores tienen un importante papel en la regulación de la conducta alimentaria, especialmente en el NAcc, provocando un aumento en la motivación para comer al incrementar el valor hedónico de la comida (Kirkham, 2003). Se ha demostrado, por ejemplo, que la administración de agonistas de cannabinoides en el NAcc, como la anandamida, estimulan la ingesta de alimentos a través de los receptores CB1r (Mahler et al., 2007; Verty et al., 2005). También se ha revelado que la densidad de estos receptores en el NAcc está inversamente relacionada con el consumo de alimentos palatables, lo que indica que el aumento de la ingesta de una DRG puede aumentar la activación de los CB1r en esta región y provocar a largo plazo su regulación a la baja como mecanismo compensatorio (Harrold et al., 2002). De hecho, la administración de antagonistas de los receptores CB1 pueden reducir la ingesta compulsiva de una DRG (Parylak et al., 2012). Todos estos resultados sugieren que los endocannabinoides modulan la ingesta de alimentos ricos en grasa y carbohidratos a través del CB1r. Los estudios realizados en seres

humanos apoyan estos hallazgos y muestran que determinados alelos específicos para el CB1r son más comunes en personas con un mayor índice de grasa corporal (Jaeger et al., 2008; Russo et al., 2007).

- **Papel modulador de las DRG en la adicción**

Las drogas de abuso y la comida palatable afectan a mecanismos cerebrales comunes, concretamente al sistema de recompensa (Volkow et al., 2013). Ambas estimulan regiones cerebrales comunes como el hipotálamo lateral, el ATV, la CPF o la amígdala (de Macedo et al., 2016), reducen la densidad del transportador activo de DA (Huang et al., 2006) y activan las neuronas dopaminérgicas del NAcc (Kelley et al., 2005; Rada et al., 2005). Esta activación dopaminérgica provocada por la DRG afecta a las vías neuronales implicadas en la motivación y la recompensa, al igual que se observa con las drogas de abuso (Grigson, 2002; Hajnal et al., 2008; Pelchat, 2006). De hecho, la regulación a la baja de los receptores dopaminérgicos en el NAcc que se produce como mecanismo compensatorio en personas que presentan una adicción, también se observa en sujetos con obesidad (Volkow et al., 2013). Al estimular el mismo sistema, también se ha observado en modelos animales que el uso excesivo de estas DRG puede provocar efectos similares a los que encontramos en la adicción a las sustancias de abuso, como la tolerancia, el síndrome de abstinencia e incluso la manifestación de búsqueda compulsiva de este tipo de alimentos palatables (Adams et al., 2015), efectos provocados por la liberación sostenida de DA en el NAcc (Murray et al., 2015). Estas similitudes han llevado a investigar cómo los componentes nutricionales pueden influir en el desarrollo de la adicción a las drogas (Volkow et al., 2017) y se ha demostrado que estas dietas pueden funcionar como una puerta de entrada para el desarrollo de la adicción (Blanco-Gandía et al., 2017a, 2017c; Levy et al., 2013; Peleg-Raibstein et al., 2016; Puhl et al., 2011).

Hasta la fecha, muchos estudios preclínicos confirman esta relación entre la ingesta de DRG y los efectos reforzantes de las drogas de abuso. Por ejemplo, el consumo de esta dieta aumenta la AA de cocaína en ratas (Puhl et al., 2011) y ratones (Blanco-Gandía et al., 2017a), demostrando su influencia en las propiedades gratificantes de la cocaína. Pero también se han observado cómo la exposición de esta dieta aumenta los efectos reforzantes de los psicoestimulantes evaluados con un CPL, como la anfetamina (Kuhn et al., 2013) o la cocaína (Blanco-Gandía et al., 2017a; Peleg-Raibstein et al., 2016) e incluso, incrementa aún más la actividad locomotora que producen estos psicoestimulantes (Baladi et al., 2015; Collins et al., 2015; Serafine et al., 2015).

Sin embargo, a pesar de las evidencias que afirman que el consumo de esta dieta puede sensibilizar a los efectos reforzantes de las drogas, en un estudio previo de nuestro equipo de investigación también observamos que la DRG podría actuar como un reforzador alternativo que reduce la búsqueda de cocaína durante la extinción de las memorias asociadas al refuerzo condicionado (Blanco-Gandía et al., 2017b). En este estudio, los animales expuestos a una DRG de forma continua, tras haber sido condicionados en un CPL inducido por cocaína, extinguieron más rápidamente los recuerdos asociados a esta droga y mostraron una menor sensibilidad para reinstaurar la preferencia. Dicho de otro modo, la exposición a esta dieta palatable durante la fase de extinción pudo actuar como un reforzador alternativo al reducir la búsqueda compulsiva de la droga. Con estos resultados, podemos confirmar que, aunque las DRG pueden modificar el metabolismo y el sistema de recompensa provocando un aumento en la vulnerabilidad a los efectos gratificantes como la cocaína y el alcohol (Blanco-Gandía et al., 2017b; Puhl et al., 2011), también pueden presentar efectos protectores en la adicción (Blanco-Gandía et al., 2017b). De hecho, también se ha confirmado que la exposición continua a una DRG atenúa la AA de cocaína en ratas (Wellman et al., 2007) y disminuye los efectos reforzantes condicionados de la cocaína (Morales et al., 2012) y la anfetamina (Davis et al., 2008), confirmando que

las propiedades gratificantes de los psicoestimulantes se ven alteradas de forma significativa por el consumo de una dieta alta en grasas. Todos estos resultados refuerzan la teoría de que la exposición continua a una DRG podría tener un papel como reforzador alternativo. Se sugiere que esta dieta provoca una liberación constante de DA en el NAcc que atenúa las propiedades reforzantes y altera los aspectos gratificantes de los psicoestimulantes (Morales et al., 2012). Sin embargo, a pesar de los aparentes efectos beneficiosos que podría tener el consumo de estas DRG sobre la adicción, esta exposición continua a grasas y azúcares provoca trastornos metabólicos como un aumento significativo de peso y de tejido adiposo, un aumento drástico de los niveles de leptina e incluso, el propio cese de la administración de esta comida palatable puede generar un síndrome de abstinencia que induce una mayor sensibilidad a los efectos gratificantes y motores de la cocaína (Blanco-Gandía et al., 2017b; Morales et al., 2012; Wellman et al., 2007).

3.3.2. Dieta cetogénica

Hoy en día podemos encontrar otras dietas altas en grasa que no contienen elevados niveles de carbohidratos, como la DC. Esta dieta tiene una larga historia de uso clínico y ha ganado un considerable interés en los últimos años debido a sus efectos potencialmente beneficiosos en un amplio espectro de enfermedades (Zhu et al., 2022). Comenzó a utilizarse con objetivo terapéutico ya en 1920 cuando se observó que producía efectos similares en el organismo a los que se producen con el ayuno, el cual ya comenzaba a demostrar efectos beneficiosos en algunos trastornos como la epilepsia (Wheless, 2008; Yuen & Sander, 2014). La DC es una dieta rica en grasas, con una proporción aproximada de 80% de calorías de grasa, 15% de calorías de proteínas y 5% de calorías de carbohidratos, con la cual, al disminuir a menos del 5-10% los hidratos de carbono, se reduce la cantidad de glucosa disponible en el organismo, dejando de ser la principal fuente de energía (Masino & Rho, 2010; Politi et al., 2011). Es entonces cuando el cuerpo comienza a utilizar las reservas de grasa,

descomponiendo los ácidos grasos y creando cuerpos cetónicos en el hígado como la acetona, el acetoacetato y el βOHB (Hartman et al., 2007; Masino & Rho, 2010). Esta reducción de los niveles de glucosa y aumento de los cuerpos cetónicos modifica el estado metabólico, entrando en lo que se conoce como estado de cetosis (Figura 3). La cetosis puede alcanzarse de dos maneras: a través de la dieta (Politi et al., 2011) o a través del ayuno (Newman & Verdin, 2014; Owen et al., 1967).

El βOHB, compuesto no volátil y estable que se libera en el torrente sanguíneo (Achanta & Rae, 2017) y constituye hasta el 70% de los cuerpos cetónicos sintetizados en las mitocondrias hepáticas (Dedkova & Blatter, 2014), es utilizado como principal indicador del estado de cetosis tanto en humanos (Wiers et al., 2021) como en modelos animales (Hernández et al., 2018; Huang et al., 2019; Politi et al., 2011). Una vez que se ha sintetizado el βOHB en el hígado, se libera y distribuye a otros tipos de células cerebrales para convertirse en su principal fuente de energía, compensando así la reducción de glucosa (Achanta & Rae, 2017). Además, aunque se conoce la contribución de βOHB al metabolismo energético en el cerebro, a medida que avanzan las investigaciones se van descubriendo otros efectos que pueden convertir este cuerpo cetónico en un potencial agente terapéutico, como su implicación en el proceso de estrés oxidativo, la neuroinflamación o el mecanismo mitocondrial (Wang et al., 2022).

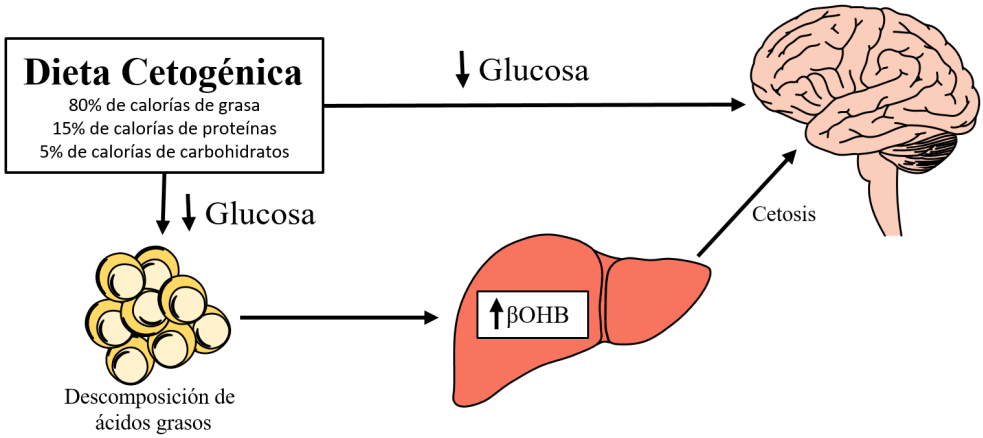


Figura 3. Efectos de la Dieta Cetogénica en el metabolismo. La disminución de carbohidratos reduce la cantidad de glucosa disponible en el organismo, provocando la descomposición de ácidos grasos lo que incrementa los niveles de cuerpos cetónicos en el hígado como la acetona, el acetoacetato y el β-hidroxibutirato (βOHB). Este proceso produce un cambio del estado metabólico, entrando en lo que se conoce como estado de cetosis.

Evolutivamente, el ser humano ha pasado buena parte de su existencia en estado cetogénico, sobre todo en invierno, cuando los hidratos de carbono eran limitados. Los cuerpos cetónicos desempeñan un papel muy importante en el desarrollo normal del cerebro del feto (Cotter et al., 2011; Cunnane et al., 2016), ya que la leche materna, al tener un alto contenido en grasas y ácidos grasos de cadena media, induce un estado de cetosis en el recién nacido que favorece su desarrollo cerebral (Breckenridge & Kuksis, 1967; Nehlig, 2004). Las investigaciones realizadas hasta la fecha parecen indicar que la DC podría tener una importante influencia neuroprotectora (Gasior et al., 2006), sin embargo, el mecanismo a través del cual es beneficiosa sigue siendo objeto de estudio. La DC se ha utilizado con éxito en diferentes trastornos como la epilepsia (Kessler et al., 2011; Masino et al., 2011; Neal et al., 2008), la enfermedad de Alzheimer y Parkinson (Henderson et al., 2009; Kashiwaya et al., 2000; Tieu et al., 2003; Yang & Cheng, 2010; Yao et al., 2011), el

cáncer cerebral (Schmidt et al., 2011; Seyfried et al., 2012), el autismo (Evangeliou et al., 2003), e incluso en la esclerosis lateral amiotrófica (Zhao et al., 2006). Los resultados de todos estos estudios sugieren que la DC induce un proceso de normalización cuando el funcionamiento del metabolismo está alterado (Zhu et al., 2022), lo que puede explicar sus efectos beneficiosos en diversos trastornos neurológicos.

La DC provoca distintos cambios en nuestro organismo, pero uno de los principales mecanismos neuronales que podrían explicar sus efectos fisiológicos es su influencia en la modulación de los canales de potasio sensibles al ATP y el aumento de la neurotransmisión GABAérgica y de purinas como la adenosina (Lusardi et al., 2015; Masino et al., 2012). El tratamiento con DC provoca un aumento de moléculas de ATP en el tejido cerebral, más de los que genera la glucosa extracelular (Abdelmalik et al., 2007; Deng-Bryant et al., 2011; Hartman et al., 2007). Este aumento en ATP extracelular se desfosforila rápidamente en adenosina (Dunwiddie et al., 1997), manteniendo una estrecha relación con los sistemas adenosinérgicos, sugiriendo que realmente la DC provoca efectos moduladores regulando la actividad de la adenosina a través de éste nucleótido (Masino et al., 2012; Masino & Geiger, 2008) (Figura 4a). De hecho, se ha observado cómo la DC aumenta los niveles de adenosina y activa sus receptores (Lusardi et al., 2015), inhibiendo la excitabilidad de las neuronas, lo que explica sus efectos terapéuticos en enfermedades como la epilepsia (Kawamura et al., 2010; Masino et al., 2011). Además, se ha observado que en ratones transgénicos que presentan una disfunción del receptor A1 de la adenosina (A1r), presentan algunos síntomas fisiológicos como convulsiones espontáneas, que son revertidas cuando se administra una DC (Masino et al., 2011), concluyendo que las convulsiones propias de la epilepsia causadas por la deficiencia de adenosina se reducen con esta dieta al aumentar la actividad de A1r.

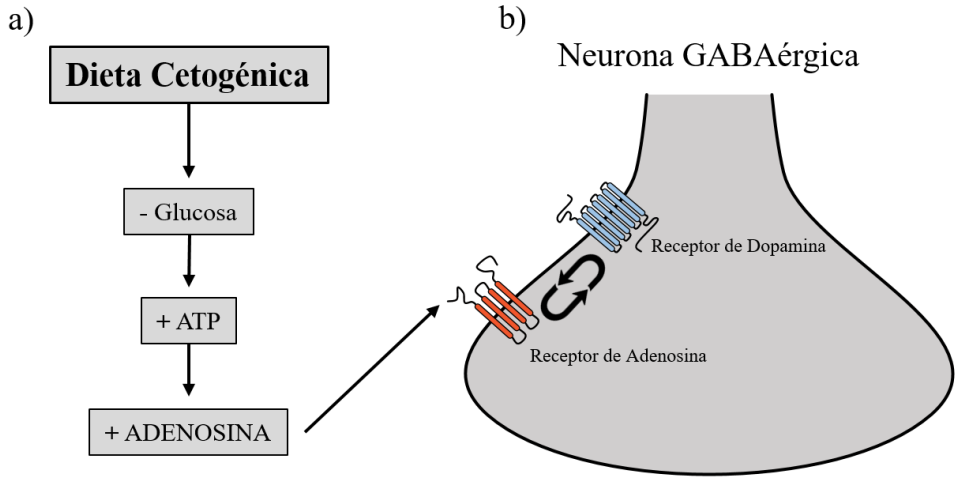


Figura 4. a) Efecto de la Dieta Cetogénica en la neurotransmisión GABAérgica y la adenosina. b) Interacción antagónica entre los heterodímeros de Adenosina y Dopamina.

La regulación de la adenosina también está estrechamente vinculada a la acción dopaminérgica, ya que sus receptores se colocalizan en las neuronas GABAérgicas, lo que sugiere que la modificación de uno de los sistemas puede conducir a la regulación del otro (Ferré et al., 1997) (Figura 4b). De hecho, existe una interacción antagónica entre los heterodímeros de A1r-D1r frente a los A2r-D2r (Franco, 2000). Por ejemplo, se ha descubierto que los agonistas de A1r disminuyen significativamente la afinidad de unión de D1, lo que indica que la función A1r en el heterodímero A1r-D1r es inhibir la señalización de la DA (Fuxe et al., 2010).

- **Papel modulador de la DC en la adicción**

Hasta la fecha, hay muy pocos estudios que han demostrado el efecto que una DC puede tener en la adicción a las drogas. Uno de estos estudios ha sido el realizado por el equipo de Martínez et al. (2019) que evaluaron el potencial de una DC como tratamiento para el síndrome de abstinencia de cocaína. Para ello, administraron una

DC a ratas durante tres semanas y después recibieron inyecciones diarias de cocaína (15 mg/kg) durante una semana. Observaron que las respuestas habituales de sensibilización que se manifiestan durante el síndrome de abstinencia de cocaína en ratas, como las respuestas locomotoras estereotipadas, eran mucho menores que en las ratas alimentadas con dieta estándar. Una de las explicaciones propuestas por este estudio es que los cambios en la adenosina provocados por la DC pueden tener un potencial terapéutico a través de acciones en los heterodímeros de los receptores de adenosina-dopamina. De hecho, se ha propuesto que la respuesta de algunas drogas como los psicoestimulantes también está mediada por la adenosina (Berrendero et al., 2003), al observar que ratones modificados genéticamente que no expresan receptores de adenosina, manifiestan un consumo más elevado de cocaína durante la AA. En esta misma línea, se ha observado también que la administración de antagonistas de los A2r produce un aumento de conductas de búsqueda de la droga, potenciándose la recaída en la AA de cocaína (O'Neill et al., 2012; Weerts & Griffiths, 2003) mientras que los agonistas A2r provocan el efecto contrario al reducir la sensibilización locomotora a la cocaína y a la morfina (Filip et al., 2006; Listos et al., 2008), e incluso disminuyen la AA de cocaína (Knapp et al., 2001; Wydra et al., 2015). Así pues, Martínez et al. (2019) plantean que la DC podría estar atenuando la transmisión dopaminérgica a través de la activación de los receptores de adenosina (Ferré et al., 1997; Filip et al., 2006).

Por otro lado, los estudios que han evaluado la interacción de una DC y el alcohol también son muy escasos. Con respecto al efecto de esta dieta en los síntomas del síndrome de abstinencia, se ha observado que personas con trastorno por consumo de alcohol presentan menos síntomas de abstinencia, manifiestan un menor deseo de consumo y requieren menos benzodiacepinas para mitigar el malestar después de tres semanas de la administración de una DC (Wiers et al., 2021). En investigación preclínica, también se ha visto que los síntomas de abstinencia de alcohol en ratas, como las conductas de rigidez e irritabilidad, se reducen cuando se implementa la

DC (Dencker et al., 2018), coincidiendo con los resultados obtenidos en ratones, donde ésta dieta también reduce la hiperlocomoción y la ansiedad provocada por la retirada del alcohol (Bornebusch et al., 2021). En lo que respecta al efecto de la DC en el consumo, Wiers et al. (2021) también desarrollaron una parte preclínica en su estudio centrada en la reducción de la ingesta de alcohol en ratas. Tras la administración durante 8 semanas de esta dieta, observaron que estas ratas no mostraban una escalada en la ingesta de alcohol, efecto que si se manifestó en ratas alimentadas con una dieta estándar. Estos estudios sugieren que el efecto que tiene la DC sobre la adenosina, relacionada con el consumo y la abstinencia al alcohol, puede explicar sus efectos moduladores. De hecho, se ha visto tanto en ratas como ratones que la estimulación A2r con agonistas reduce la ingesta de alcohol, mientras que sus antagonistas la aumentan (Bonaventura et al., 2012; Nam et al., 2013).

Con la información expuesta en los apartados anteriores, podemos confirmar que la exposición a estrés social es capaz de aumentar los efectos reforzantes de las drogas de abuso, sin embargo, algunos individuos son capaces de desarrollar un perfil resiliente que les protege de las consecuencias psicofisiológicas de ese estrés. También observamos cómo algunos tipos de intervenciones farmacológicas y ambientales son capaces de provocar un aumento de la conducta resiliente y, teniendo en cuenta el papel que tienen los distintos tipos de dieta con alto contenido en grasas en el sistema de refuerzo y sus efectos sobre la psicofisiológica del estrés, nos planteamos si estas dietas podrían potenciar la resiliencia y disminuir o bloquear los efectos reforzantes de las drogas como el alcohol y la cocaína que son incrementados por la exposición a estrés social.

2. OBJETIVOS E HIPÓTESIS

2. Objetivos e hipótesis

El estrés social es un factor de riesgo muy importante que influye directamente sobre las distintas fases del proceso adictivo, sin embargo, no todo el mundo desarrolla vulnerabilidad al incremento de los efectos reforzantes de las drogas inducidos por el estrés. Existe una población de sujetos que presenta una respuesta resiliente a los efectos adversos del estrés social. La comunidad científica comienza cada vez más a interesarse por identificar aquellos factores que puedan fomentar el desarrollo de la resiliencia y reducir las consecuencias que la exposición al estrés social tiene sobre la adicción. Así, el **primer objetivo general** de la presente TD es confirmar la existencia de esta población resiliente a los efectos reforzantes de la cocaína incrementados por la exposición a estrés social. Para desarrollar este objetivo, se expondrán a los animales a un procedimiento de DS previo a un CPL de cocaína, y se analizarán las conductas manifestadas durante la exposición al estrés social. Nuestra hipótesis principal es que, de todos los animales expuestos a estrés social, y posteriormente a una dosis subumbral en un CPL de cocaína, algunos sujetos manifestarán resistencia a los efectos reforzantes de la cocaína inducidos por el estrés, y esos mismos sujetos resilientes manifestarán diferentes conductas de afrontamiento durante la DS.

Por otro lado, los hallazgos actuales de la literatura muestran que el consumo de algunas DRG induce neuroadaptaciones en el sistema de recompensa, influyendo sobre las vías dopaminérgicas, opioides y endocannabinoides, lo que modifica la respuesta de los animales a los efectos gratificantes de las drogas de abuso. También tenemos en cuenta otras dietas altas en grasa, pero bajas en carbohidratos y azúcares, como la DC, que también podría influir en el proceso adictivo a través de sus interacciones con el sistema de adenosina y dopamina. Por ello, el **segundo objetivo general** de esta TD es identificar cómo la exposición a estas dietas interactúa con los efectos reforzantes de la cocaína y el alcohol. Para desarrollar este objetivo, hemos

utilizado como metodología principal la administración intermitente de una DRG evaluando sus efectos sobre el proceso de extinción de un CPL inducido por la cocaína, así como la administración continua de una DC para evaluar su influencia sobre los efectos reforzantes de la cocaína empleando un CPL, y sobre los efectos reforzantes del alcohol empleando un procedimiento de AA. Nuestra hipótesis principal es que la DRG será capaz de acelerar la extinción de los recuerdos asociados a la cocaína y evitará la reinstauración de cocaína inducida por una dosis de recuerdo o "*priming*". También hipotetizamos que la DC acelerará la extinción de los recuerdos asociados a la cocaína en comparación con los animales alimentados con una dieta estándar, bloqueará la reinstauración de la conducta de búsqueda de cocaína y reducirá tanto el consumo como la motivación por obtener alcohol.

Sin embargo, considerando que estas dietas no solo pueden modular la sensibilidad a los efectos reforzantes de las drogas, sino que también son capaces de disminuir los efectos que induce el estrés social, nuestro **tercer objetivo general** es evaluar los efectos de estas dietas sobre el desarrollo de la resiliencia al estrés social. Para desarrollar este objetivo, hemos administrado una DRG intermitente evaluando sus efectos sobre un CPL inducido por la cocaína tras la exposición a un procedimiento de DS. Nuestra hipótesis es que el propio valor reforzante de la DRG actuará como un reforzador alternativo al estrés social, evitando el incremento de los efectos reforzantes de la cocaína inducidos por dicho estrés.

Los conocimientos adquiridos contribuirán sin duda a tener en cuenta estos factores nutricionales para la prevención y el tratamiento de los trastornos por abuso de sustancias.

Estudio 1. El primer objetivo de este estudio fue demostrar que existían dos poblaciones de animales expuestos a DS que se comportaban como Resilientes o Susceptibles en función de su respuesta al incremento de los efectos reforzantes de la cocaína. Como segundo objetivo, quisimos evaluar las diferencias en el comportamiento de estos animales durante la exposición a la DS. Como tercer objetivo, caracterizamos la respuesta neuroinflamatoria de los animales Resilientes o Susceptibles midiendo los niveles estriatales de interleucina 6 (IL6).

Hipótesis

- El conjunto total de animales expuestos a DS manifestarán un incremento a largo plazo de los efectos gratificantes de la cocaína en un CPL.
- Una parte de los animales expuestos a DS serán Resilientes y no mostrarán un incremento a largo plazo de los efectos reforzantes de la cocaína.
- Los animales caracterizados como Resilientes y Susceptibles mostrarán diferencias de comportamiento durante el afrontamiento del estrés en las DS.
- La exposición a la DS influirá en los niveles estriatales de IL6.

Estudio 2. El objetivo principal de este estudio fue analizar el efecto de una DRG sobre el proceso de extinción y reinstauración a la cocaína condicionada en un CPL. Como segundo objetivo nos planteamos si estos efectos mostrarán diferencias entre machos y hembras. Como tercer objetivo evaluamos los efectos de la administración de la DRG sobre la grelina, la leptina, el sistema opioide y cannabinoide.

Hipótesis

- La administración limitada e intermitente de la DRG no influirá sobre el peso corporal de los animales.
- Los animales alimentados con una DRG limitada e intermitente no manifestarán cambios en los niveles de grelina y leptina.
- Las hembras serán más sensibles a los efectos reforzantes de la cocaína inducidos por un CPL.
- Los animales alimentados con una DRG limitada e intermitente acelerarán la extinción de los recuerdos asociados a la cocaína y evitarán la reinstauración de cocaína inducida por una dosis *priming*.
- Los animales alimentados con una DRG limitada e intermitente manifestarán cambios en el sistema opioide y cannabinoide.

Estudio 3. El objetivo de este estudio fue evaluar la influencia de una DRG, administrada de forma intermitente, sobre el incremento de los efectos reforzantes de la cocaína inducido por la DS en ratones adolescentes. También caracterizamos los cambios que se produjeron en el sistema cannabinoide, opioide y en CRF.

Hipótesis

- La administración limitada e intermitente de la DRG no influirá sobre el peso corporal de los animales.
- Los animales alimentados con una DRG limitada e intermitente durante la exposición a DS disminuirán el incremento de los efectos reforzantes de la cocaína inducidos por el estrés social evaluados en un CPL.
- Los animales alimentados con una DRG limitada e intermitente durante el desarrollo del CPL, tras la exposición a DS, disminuirán el incremento de los efectos reforzantes de la cocaína inducidos por el estrés social.
- Los animales alimentados con una DRG limitada e intermitente y expuestos a DS manifestarán cambios en el sistema cannabinoide, opioide y en CRF.

Estudio 4. El objetivo principal de este estudio fue evaluar los efectos de una DC sobre las funciones cognitivas y conductuales en ratones para descartar las posibles interferencias que pueda provocar esta dieta en otras pruebas conductuales.

Hipótesis

- Los animales alimentados con DC mostrarán un incremento en los niveles de β-hidroxibutirato.
- La administración de DC no influirá sobre el peso corporal de los animales.
- Los animales alimentados con una DC no manifestarán alteraciones en la actividad locomotora espontánea.
- Los animales alimentados con una DC no manifestarán alteraciones en la memoria.
- Los animales alimentados con una DC no manifestarán alteraciones en el aprendizaje hipocampal.
- Los animales alimentados con una DC no manifestarán un incremento en los niveles de ansiedad.

Estudio 5. El principal objetivo de este estudio fue analizar la influencia de una DC sobre los efectos reforzantes de la cocaína evaluados en un CPL. Como segundo objetivo evaluamos el efecto de la DC sobre el proceso de extinción de los recuerdos asociados a la droga y en la reinstauración de la conducta de búsqueda de cocaína tras una dosis de recuerdo o "*priming*".

Hipótesis

- Los animales alimentados con una DC mostrarán un incremento en los niveles de β-hidroxibutirato.
- La administración de DC no influirá sobre el peso corporal de los animales.
- Los animales alimentados con una DC adquirirán un CPL inducido por la cocaína.
- Los animales alimentados con una DC presentarán un proceso de extinción de los recuerdos asociados a la cocaína más rápido en comparación con los animales alimentados con una dieta estándar.
- Los animales alimentados con una DC bloquearán la reinstauración de la conducta de búsqueda de cocaína.

Estudio 6. El objetivo principal del presente estudio fue evaluar si una DC podría modular los efectos gratificantes del alcohol utilizando el paradigma de AA. También evaluamos los efectos de la DC y el alcohol en el sistema dopaminérgico, opioide, cannabinoide y de la adenosina.

Hipótesis

- Los animales alimentados con una DC mostrarán un incremento en los niveles de β-hidroxibutirato.
- La administración de DC no influirá sobre el peso corporal de los animales.
- Los animales alimentados con una DC mostrarán una reducción en la motivación por obtener alcohol.
- Los animales alimentados con una DC mostrarán una reducción del consumo de alcohol.
- Los animales alimentados con una DC y expuestos al procedimiento de AA de alcohol manifestarán cambios en el sistema dopaminérgico, opioide, cannabinoide y adenosina.

3. MATERIAL Y MÉTODO

3. Material y método

3.1. Animales

En la presente TD se han empleado 2 cepas distintas de ratones: ratones albinos de la cepa OF1 (en los estudios 2, 3, 4, 5 y 6; Figura 5a) y ratones de la cepa C57BL/6J (en el estudio 1; Figura 5b), ambos adquiridos por los Laboratorios Charles River (Barcelona, España). El número total de ratones se especifica en cada uno de los estudios presentados. Los ratones experimentales llegaron a nuestro laboratorio en el día postnatal (DPN) 21 (en los estudios 1, 3, 4 y 5) y en el DPN 42 (en los estudios 2 y 6). Todos los ratones (excepto los utilizados como oponentes agresivos) se alojaron en grupos de cuatro en jaulas de plástico (25 × 25 × 14,5 cm). Los ratones OF1 utilizados como oponentes agresivos fueron alojados individualmente en jaulas de plástico (23 × 13,5 × 13 cm) durante un mes previo a los experimentos para inducir una mayor agresividad (Rodríguez-Arias et al., 1998).

a) b)

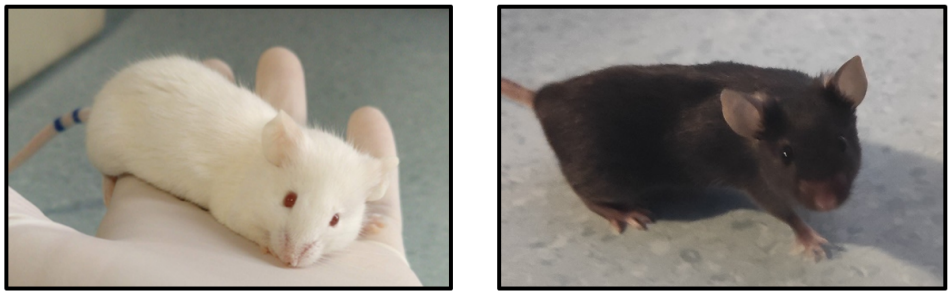


Figura 5. a) Ratones albinos de la cepa OF1 empleados en estudios 2, 3, 4, 5 y 6. b) Ratones de la cepa C57BL/6J empleados en el estudio 1.

Las condiciones ambientales constaron de una temperatura de 21 ± 2 ºC y una humedad relativa del 55%. Los ratones fueron mantenidos durante todo el procedimiento en un ciclo de luz/oscuridad de 12h/12h (8:00h – 20:00h) con agua y *pellets ad libitum*, excepto durante las pruebas comportamentales o durante la

administración de dietas altas en grasa. Todos los procedimientos de tratamiento y cuidado de los ratones obedecieron a las leyes y regulaciones nacionales, regionales y locales de acuerdo con las directrices que se ajustan a la Directiva 2010/63/UE del Parlamento Europeo y del Consejo, de 22 de septiembre de 2010, relativa a la protección de los animales utilizados para fines científicos. Además, los experimentos fueron aprobados por el Comité Ético de experimentación y bienestar animal de la Universidad de Valencia.

3.2. Tratamiento farmacológico

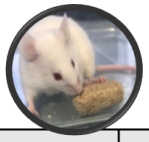
Los animales recibieron distintos tratamientos farmacológicos en los estudios expuestos en la presente TD. Durante el procedimiento de CPL se les inyectó por vía intraperitoneal 1mg/kg (en los estudios 1, 3 y 4) o 10mg/kg (en los estudios 2 y 5) de cocaína (Laboratorios Alcaliber S. A. Madrid, Spain) disuelta en una solución de NaCl 0,9% en un volumen de 0,1mL/10g de peso corporal. Se ha demostrado que la dosis de 1mg/kg es considerada una dosis subumbral, que no induce preferencia de lugar en la prueba CPL en ratones estándar no estresados (Maldonado et al., 2006; Vidal-Infer et al., 2012), mientras que los ratones expuestos a estrés social sí desarrollan preferencia (Rodríguez-Arias et al., 2017). Por otro lado, la dosis de 10mg/kg de cocaína es una dosis efectiva que induce la reinstauración de la búsqueda de cocaína con la mitad de la dosis recibida previamente (5mg/Kg de cocaína) (Duart-Castells et al., 2020).

En el estudio 6, durante la fase de entrenamiento de AA de etanol, se utilizó una solución de sacarina al 0,2% (p/v) disuelta en agua (Sigma-Aldrich, Madrid, España). Durante las fases de sustitución de AA, se utilizó una mezcla de concentración de sacarina al 0,15% disuelta en agua y 2% de etanol (Merck, Madrid, España) para la primera subfase; en la segunda subfase se utilizó una mezcla de solución de sacarina al 0,10% en agua y 4% de etanol; y, en la tercera subfase, se

utilizó una mezcla de solución de sacarina al 0,05% en agua y 6% de etanol. Durante los ratios fijos 1 y 3, y el ratio progresivo de AA, se diluyó el etanol en agua utilizando una solución de etanol al 6%.

3.3. Condiciones de alimentación

A lo largo de los 6 estudios expuestos en la presente TD se han administrado 3 dietas distintas a los animales (Figura 6). Una dieta estándar (Teklad Global Diet 2014, 13 Kcal % de grasa, 67 Kcal % de carbohidratos y 20 Kcal % de proteína; 2,9 kcal/g), una dieta rica en grasas y carbohidratos (DRG) (TD.06415, 45 Kcal % de grasa, 36 Kcal % de carbohidratos y 19% Kcal de proteína; 4,6 kcal/g) y una dieta cetogénica (DC) (TD.96355, 90,5% kcal de grasa, 0,3% kcal de carbohidratos y 9,1% kcal de proteína; 6,7 kcal/g). Todas las dietas fueron suministradas por Envigo Teklad Diets (Barcelona, España). Todos los animales fueron alimentados con la dieta estándar *ad libitum* excepto cuando se administraron las dietas con alto contenido en grasas en función de los requerimientos de los experimentos, administrando la DRG en los estudios 2 y 3, y la DC en los estudios 4, 5 y 6. La administración de DRG se basó en el modelo de acceso limitado e intermitente descrito por Corwin et al. (1998), en el que los animales no privados de alimento tienen un acceso esporádico y limitado a la DRG. La administración de DC consta de un acceso *ad libitum* para inducir el estado de cetosis.



Macronutrientes	Dieta estándar Teklad Global Diet 2014 %Kcal	Dieta rica en grasas (DRG) TD.06415 %Kcal	Dieta Cetogénica (DC) TD.96355 %Kcal
Proteínas	20	19	9,2
Carbohidratos	67	36,2	0,3
Grasas	13	44,8	90,5
Composición de grasas		Manteca de cerdo Aceite de soja	Manteca vegetal Aceite de maíz

Figura 6. Componentes de las dietas administradas en la presente TD. Dieta estándar *ad libitum* empleada en todos los estudios excepto cuando se administraron las dietas con alto contenido en grasas (Teklad Global Diet 2014, 13 Kcal % de grasa, 67 Kcal % de carbohidratos y 20 Kcal % de proteína; 2,9 kcal/g), DRG empleada en los estudios 2 y 3 (TD.06415, 45 Kcal % de grasa, 36 Kcal % de carbohidratos y 19% Kcal de proteína; 4,6 kcal/g) y DC empleada en los estudios 4, 5 y 6 (TD.96355, 90,5% kcal de grasa, 0,3% kcal de carbohidratos y 9,1% kcal de proteína; 6,7 kcal/g).

3.4. Pruebas Conductuales

3.4.1. Derrota Social

El protocolo de DS se ha validado y descrito en detalle en trabajos previos (Ferrer-Pérez et al., 2019a; Montagud-Romero et al., 2016; Rodríguez-Arias et al., 2017) y está basado en el ataque territorial de un macho residente (oponente agresivo) a un intruso coespecífico (sujeto experimental), permitiendo estudiar las consecuencias conductuales y fisiológicas a corto y largo plazo del estrés social (Shimamoto, 2018). En este procedimiento (aplicado en estudios 1 y 3), se emplearon ratones macho de 42 días de edad de la cepa OF1 como residentes que fueron aislados en cajas de 21 x 32 x 20 cm. 10 días antes de comenzar las sesiones de DS se dejó de limpiar la viruta de sus cajas con el fin de potenciar su territorialidad. Previo a la realización de las sesiones, se confirmó que los ratones residentes presentaban características agresivas antes de comenzar estas sesiones. La DS consta de 4 sesiones de 25

minutos a intervalos de 72h, donde cada sesión está compuesta por 3 fases (Figura 7). En la primera fase se introduce al intruso en la jaula del residente durante 10 minutos, en los que está protegido de los ataques, pero no de las amenazas del residente por medio de una rejilla de 27 x 18 cm colocada siempre en el mismo lado de la caja (Covington & Miczek, 2001). En la segunda fase se retira la rejilla y se permite la confrontación durante 5 minutos. En la tercera y última fase se coloca nuevamente la rejilla durante 10 minutos más, volviendo a permitir las amenazas del residente, pero sin posibilidad de realizar ataque sobre el intruso. Se empleó un procedimiento similar al descrito anteriormente para los ratones controles, pero sin la presencia del ratón residente. Todos los enfrentamientos se realizaron en la sala experimental, distinta del animalario, con luz blanca y únicamente estaban presentes los ratones que iban a participar en cada enfrentamiento.

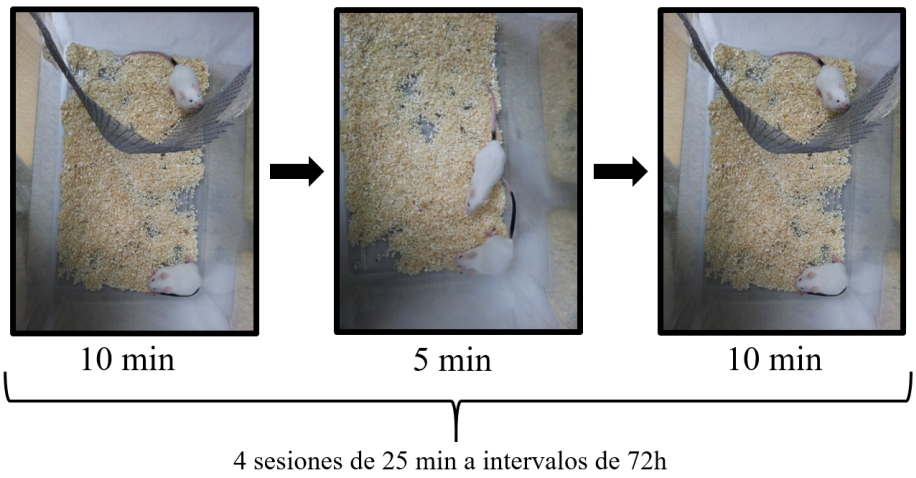


Figura 7. Protocolo de DS aplicado en estudios 1 y 3. Consta de 4 sesiones de 25 minutos a intervalos de 72h donde cada sesión está compuesta por 3 fases: primera fase de 10min donde el ratón experimental está protegido de los ataques, pero no de las amenazas del residente por medio de una rejilla, segunda fase de 5min donde se permite la confrontación y tercera fase de 10min donde nuevamente el ratón experimental está protegido de los ataques, pero no de las amenazas del residente.

Éstas sesiones fueron registradas con una videocámara y se evaluaron las conductas de huida, sumisión y ataque de los sujetos experimentales, mientras que en los animales residentes se midieron las conductas de amenaza y ataque para confirmar la existencia de confrontación. Una vez finalizado el procedimiento, se analizaron los encuentros mediante el programa informático *Raton Time* que permite registrar el tiempo dedicado a realizar las diferentes conductas durante la DS (Martínez et al., 1991).

3.4.2. Condicionamiento de la Preferencia de Lugar

El CPL es un modelo basado en el aprendizaje clásico o pavloviano, que evalúa la recompensa condicionada inducida por diferentes estímulos (Bardo & Bevins, 2000; Tzschentke, 2007). Ha sido ampliamente utilizado para estudiar los efectos de recompensa de las drogas adictivas condicionadas (Blanco-Gandía et al., 2017b; Han et al., 2017; Rodríguez-Arias et al., 2017), ya que los estímulos contextuales pueden adquirir propiedades apetitivas secundarias (efectos gratificantes condicionados) cuando se combinan con un reforzador primario (Tzschentke, 2007). Se ha empleado el procedimiento de CPL para evaluar la respuesta condicionada de la cocaína en los estudios 1, 2, 3 y 5.

Para el desarrollo del CPL se emplearon 12 cajas idénticas de plexiglás (Figura 8) con dos compartimentos de igual tamaño (30,7 cm de largo x 31,5 cm de ancho x 34,5 cm de alto), separadas por un área central de color gris (13,8 cm de largo x 31,5 cm de ancho x 34,5 cm de alto). Los compartimentos tienen paredes de diferente color (blancas vs negras) y distinta textura de suelo (liso en el compartimento negro y rugoso en el blanco). Los animales son entrenados para asociar un ambiente específico con el efecto de la droga administrada y el otro compartimento diferente con solución salina (Bardo & Bevins, 2000; Sanchis-Segura & Spanagel, 2006).

Ambos compartimentos presentan una puerta tipo guillotina que los separa del pasillo central. Cada uno de los compartimentos de condicionamiento cuenta con 4 células fotoeléctricas, mientras que la zona central tiene 6, lo que permite el registro de la posición del animal y los cruces de un compartimento al otro. El equipo es controlado por dos ordenadores IBM PC mediante el uso de un software MONPRE 2z (CIBERTEC, SA, España).

Figura 8. Caja de CPL compuesta por compartimentos con paredes de diferente color (blancas vs negras) y distinta textura de suelo (liso en el compartimento negro y rugoso para el blanco). Estas cajas han sido empleadas para evaluar la respuesta condicionada de la cocaína en los estudios 1, 2, 3 y 5. El procedimiento completo de CPL consta de tres fases: Adquisición (Pre-Condicionamiento, Condicionamiento y Post-Condicionamiento), Extinción y Reinstauración.

Adquisición de CPL

La fase de adquisición de la conducta condicionada durante el CPL consta de 3 fases, las cuales se llevaron a cabo durante el ciclo de oscuridad y siguiendo un procedimiento "no sesgado" en términos de la preferencia inicial espontánea (Manzanedo et al., 2001).

Durante la primera fase o **pre-condicionamiento** (Pre-C), los ratones tuvieron libre acceso a ambos compartimentos de la caja de condicionamiento durante 15 minutos (900s) cada día durante 3 días. Al tercer día, el tiempo que cada animal pasa en cada compartimento fue registrado durante 900s. Los animales que muestran una fuerte aversión (menos del 33% del tiempo de la sesión, es decir menos de 300s) o una fuerte preferencia (más del 67%, es decir más de 600s) por alguno de los compartimentos fueron descartados del procedimiento. Uno de los compartimentos se elige para ser asociado con la cocaína de tal manera que, dentro de cada grupo, la mitad de los animales reciben la cocaína en el compartimento donde han manifestado menos preferencia y la otra mitad en el compartimento donde han manifestado mayor preferencia, balanceándose también el color del compartimento. No deben existir diferencias significativas en el tiempo que los animales han pasado en el compartimento asociado a la cocaína o al salino en la fase de pre-condicionamiento. Esta medida es de gran importancia para el procedimiento experimental, ya que ayuda a evitar que exista algún tipo de sesgo en la preferencia antes de comenzar el experimento.

En la segunda fase (**condicionamiento**), los animales fueron condicionados con 1mg/kg o 10mg/kg de cocaína (según el diseño del estudio) mediante 4 asociaciones con el compartimento asignado tras el Pre-C. Los animales recibieron 2 inyecciones cada día: una administración de solución salina antes de ser introducidos al compartimento no asociado durante 30 minutos, y después de un intervalo de 4 horas, recibieron cocaína antes de ser introducidos al compartimento asociado con la droga durante 30 min. El área central no fue utilizada durante el condicionamiento y su acceso fue bloqueado mediante las puertas tipo guillotina.

Durante la tercera fase o **post-condicionamiento** (Post-C), en el 8º día del procedimiento, las guillotinas que separaban ambos compartimentos fueron retiradas y el tiempo que los ratones (sin ningún tipo de tratamiento) pasaban en cada

compartimento fue registrado durante 900s. La diferencia en segundos entre el tiempo que los animales permanecen en el compartimento asociado con la droga durante la prueba de Post-C, y el tiempo que pasan durante la prueba de Pre-C es una medida del grado de condicionamiento inducido por la droga. Si esta diferencia es significativamente positiva, entonces la droga ha inducido una preferencia por el compartimento asociado con la misma, mientras que lo opuesto indica la inducción de una aversión.

Extinción del CPL

Una vez establecida la preferencia por el compartimento emparejado con la droga, los ratones se sometieron dos veces por semana a una sesión de extinción que consistió en colocar a los animales en el compartimento central (sin las puertas de guillotina que separan los compartimentos) durante 900s. La condición de extinción se cumple cuando hay una diferencia significativa entre las puntuaciones registradas en esta fase y las puntuaciones de la Post-C en dos sesiones consecutivas y una falta de diferencia significativa entre los valores de esta fase y la prueba Pre-C.

Reinstauración del CPL

Tras 24h de confirmarse la extinción, se evaluaron los efectos de una dosis de cocaína de recuerdo o *priming*. En este proceso de reinstauración se administró una dosis de recuerdo con dosis decrecientes (la mitad de la dosis anterior) hasta que se confirmó que las dosis administradas fueron inefectivas, lo que significa que no hay reinstauración de la conducta de búsqueda (5 mg/kg tras la extinción del CPL inducido por 10 mg/kg; y 2,5 mg/kg tras la extinción si los animales manifiestan reinstauración con 5 mg/kg). El desarrollo de la prueba de reinstauración fue el mismo que el realizado en el Post-C (deambulación libre durante 900s), pero tras la administración de la dosis de recuerdo de cocaína.

3.4.3. Autoadministración oral de etanol.

Este procedimiento se basa en el empleado por Navarrete et al. (2014) y fue utilizado en el estudio 6. La administración oral de etanol se llevó a cabo en 8 cajas operantes (MED Associated Inc., Georgia, VT, USA). Un paquete de software (Cibertec, SA, España) controla la exposición de estímulos, la administración de solución liquida (compuesto variable según la fase del procedimiento) y registra las respuestas operantes proporcionadas por los animales. Las cámaras de AA se colocaron dentro de cajas de aislamiento acústico (Figura 9) equipadas con una luz, dos orificios para el hocico de los animales, un receptáculo central para dejar caer la solución líquida, una bomba de infusiones compuesta por una jeringa y una luz de estímulo. De los dos orificios para el hocico de los animales, cuando el animal introduce el hocico en el activo, se administra 37 µl de solución liquida, junto con un estímulo luminoso de 0,5s y un estímulo auditivo de 0,5s, a lo que sigue un periodo de espera de 6s. Cuando el animal introduce el hocico en el orificio inactivo, no administra ningún compuesto ni se expone ningún estímulo. Este protocolo consta de tres fases (Figura 10): fase de entrenamiento, fase de sustitución por sacarina y fase de consumo de etanol al 6%.

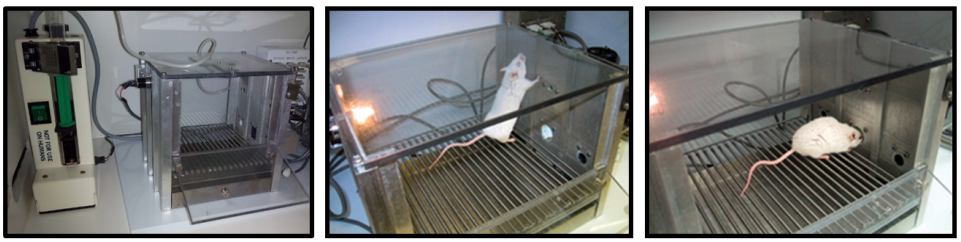


Figura 9. Caja operante de AA de etanol empleada en el estudio 6, equipada con una luz, dos orificios para el hocico de los animales, un receptáculo para dejar caer la solución líquida, una bomba de infusiones compuesta por una jeringa y una luz de estímulo.

Fase de entrenamiento (8 días)

En la fase de entrenamiento, los animales debían introducir el hocico en los orificios activos para obtener 37 µl de sacarina (0,2% (p/v)). Para facilitar la adquisición del aprendizaje, antes de la primera sesión de entrenamiento se retiró el agua durante 24 horas, y se proporcionó comida 1h antes de la sesión, facilitando el aumento de la motivación por introducir el hocico en el orificio activo. Sólo durante los 3 días siguientes, 1h antes de iniciar la sesión operante, los animales tuvieron acceso a la comida, pero no al agua (postprandial). En los cuatro días siguientes y durante el transcurso del experimento, para evitar la ingesta de etanol debido a la sed, el agua estaba disponible en cualquier momento y la comida estaba disponible durante 1h después de cada sesión de entrenamiento (preprandial).

Fase de sustitución de la sacarina (9 días)

En esta fase, el porcentaje de sacarina se redujo progresivamente a medida que aumentaba la concentración de etanol (Roberts et al., 2001; Samson et al., 1987). Los animales tuvieron acceso a cada combinación de soluciones durante tres sesiones consecutivas (0,15% Sac -2% EtOH; 0,10% Sac -4% EtOH; 0,05% Sac -6% EtOH).

Fase de consumo de etanol al 6% (11 días)

En esta fase se evalúa el número de respuestas proporcionadas en los orificios activos, el consumo de etanol al 6% (p/v) y la motivación para obtenerlo. En primer lugar, los animales fueron expuestos a 5 días de sesiones de ratio fijo 1 (FR1) que requiere que el animal active 1 vez el orificio activo para obtener el refuerzo de etanol. Después de cada sesión, se recogió el líquido restante en el receptáculo y se cuantificó con una micropipeta. Después de las sesiones de FR1, los animales fueron expuestos al programa de ratio fijo 3 (FR3) durante 5 días, en el que tenían que introducir tres veces el hocico en el orificio activo para obtener un refuerzo de etanol. Por último, para establecer el punto de ruptura de cada animal, que es el número

máximo de veces que el animal introduce el hocico en el orificio activo para obtener el refuerzo, se llevó a cabo una sesión de ratio progresivo (PR). En esta fase, para conseguir el refuerzo se incrementa el número de veces necesario que el animal debe introducir el hocico en el orificio activo, en función de la serie: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. A partir de esta escala se calculó el punto de ruptura o *breaking point* que el animal proporcionaba y que define la motivación del animal hacia el consumo de etanol. Por ejemplo, si un animal introduce el hocico en el orificio activo un total de 108 veces, esto significaba que era capaz de responder un máximo de 40 veces consecutivas para conseguir un refuerzo. Por lo tanto, el valor del punto de ruptura para este animal sería 40. Todas las sesiones duraron 1 h, excepto la sesión de PR, que duró 2 h (Navarrete et al., 2014).

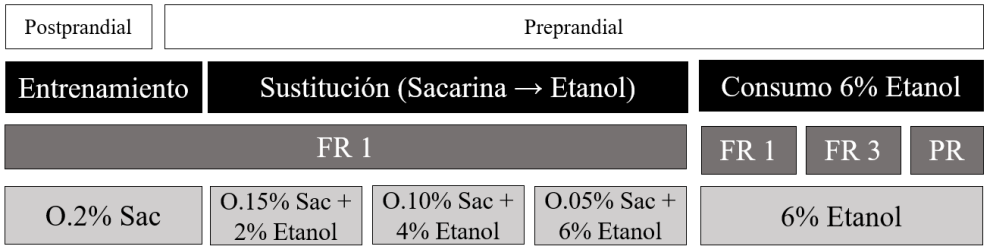


Figura 10: Protocolo de AA de etanol compuesto por tres fases: fase de entrenamiento, fase de sustitución por sacarina y fase de consumo de etanol al 6%.

3.4.4. Laberinto Elevado en Cruz

La prueba del Laberinto Elevado en Cruz se llevó a cabo en el estudio 4 siguiendo esencialmente el procedimiento descrito por Daza-Losada et al. (2009). El laberinto está compuesto por dos brazos abiertos (30 × 5 × 0,25 cm) y dos brazos cerrados (30 × 5 × 15 cm), y una plataforma central (5 × 5 cm) elevada 45 cm sobre el nivel del

suelo (Figura 11). El material del suelo del laberinto es de plexiglás negro mientras que las paredes de los brazos cerrados son de plexiglás transparente. Los brazos abiertos presentan un borde pequeño (0,25 cm) para proporcionar al animal un soporte adicional. Para disminuir el estrés experimental, los ratones se habituaron a la sala de experimentación durante 1 hora antes de la prueba. Al principio de cada ensayo, los ratones experimentales se colocaron en la plataforma central frente a un brazo abierto y se les permitió explorar la superficie del laberinto durante 5 minutos. Tras cada ensayo, la plataforma fue limpiada con una solución de agua al 7% de alcohol y se esperó a que se secase antes de proceder al siguiente ensayo.

Figura 11. Laberinto Elevado en Cruz empleado en el estudio 4 compuesto por dos brazos abiertos, dos brazos cerrados con paredes de plexiglás transparente y una plataforma central.

El comportamiento mostrado por los ratones durante la prueba se registró mediante un sistema de seguimiento automatizado (EthoVision XT 11, Noldus; Figura 12) que registra el número de entradas y el tiempo empleado en cada sección del laberinto (brazos abiertos, brazos cerrados, plataforma central). Tanto el tiempo transcurrido en los brazos como el número de entradas se consideran medidas de los efectos ansiolíticos y/o ansiógenos de sustancias u otras manipulaciones.

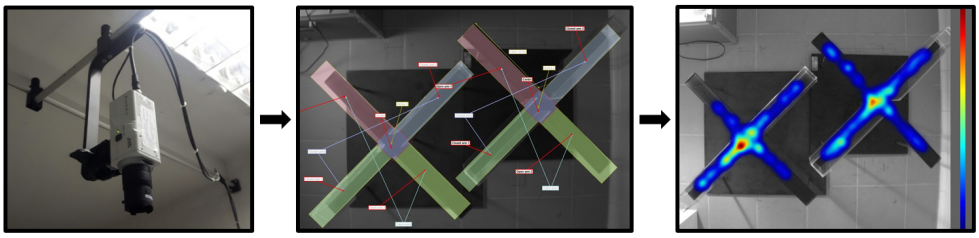


Figura 12. Sistema de seguimiento automatizado EthoVision XT 11 Noldus, empleado para evaluar el comportamiento de los animales en el Laberinto Elevado en Cruz que registra el número de entradas y el tiempo empleado en cada sección del laberinto (brazos abiertos, brazos cerrados, plataforma central).

3.4.5. Campo abierto

El comportamiento locomotor espontáneo de los ratones se cuantificó en un Campo Abierto durante un periodo de 1 hora. La prueba de Campo Abierto se realizó en una caja de plástico opaco (30 x 30 x 15 cm) que se dejó abierta en la parte superior. El animal se colocó en la caja y su actividad se registró automáticamente mediante un software de seguimiento (EthoVision XT 11, Noldus; Figura 13). Esta prueba fue empleada en el estudio 6 y el parámetro estudiado fue la distancia total recorrida (cm) y el tiempo cerca de la pared y del centro (s).

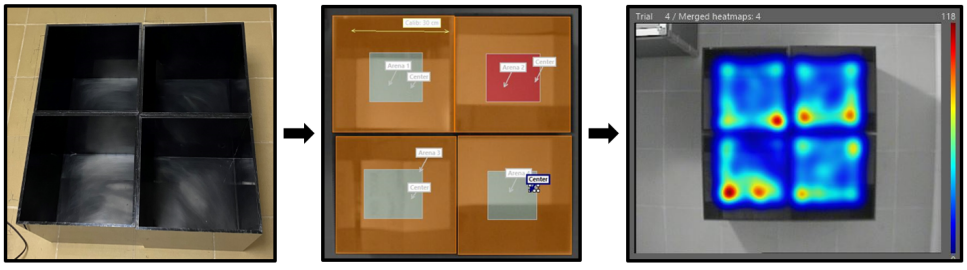


Figura 13. Sistema de seguimiento automatizado EthoVision XT 11 Noldus, empleado en el estudio 6 para evaluar el comportamiento locomotor espontáneo de los animales en un Campo Abierto que registra la distancia total recorrida (cm) y el tiempo cerca de la pared y del centro (s).

3.4.6. Test de Evitación Pasiva

El test de evitación pasiva es una prueba que evalúa la retención en la memoria de una leve descarga eléctrica recibida el día previo y fue empleada en el estudio 6. La caja de evitación pasiva (Ugo Basile, Comerio-Varese, Italia) se divide en dos compartimentos de metacrilato, uno blanco y uno negro, de 15 x 9,5 x 16,5 cm cada uno (Figura 14). El compartimento blanco está iluminado con una bombilla de luz blanca de 10W ubicada en la tapa de la caja, en contraste con el compartimento negro, que es oscuro y es donde se administra el shock eléctrico. Los compartimentos están divididos por una puerta automática corrediza, situada en el centro, que se abre pasado el tiempo a determinar por el experimentador, en este caso 60s. El suelo de la caja se compone de 48 barras de acero inoxidable separadas por 8mm de distancia y con un diámetro de 0,7mm.

Esta prueba se realiza en dos días. El primero es el *Training* o fase de adquisición y el segundo el *Test* o fase de retención. El día de *Training* se coloca al ratón en el compartimento blanco, estando la puerta automática cerrada. Tras un período de 60 segundos de habituación en el compartimento iluminado, la puerta corrediza se abre y el animal tiene la oportunidad de cruzar al compartimento negro. A partir del momento en el que se abre la puerta se registra la latencia de paso al compartimento negro, siendo el tiempo máximo de 300 segundos. Cuando el animal pasa a este compartimento, la puerta se cierra automáticamente y se le administra una leve descarga eléctrica de 0,5mA durante 3 segundos. Transcurrido este tiempo, se retira al animal del aparato y se le devuelve a su caja. A las 24 horas, el día del *Test*, se evalúa la memoria. Se realiza el mismo procedimiento, con el registro de la latencia de paso al compartimento negro, sin embargo, si el animal cruza no se le administrará la descarga. Si el animal recuerda la descarga del día anterior no pasará al compartimento negro y la latencia será de 300 segundos, que es el tiempo máximo registrado en el Test, ya que, aunque prefiera los espacios oscuros recordará la situación aversiva del día anterior.

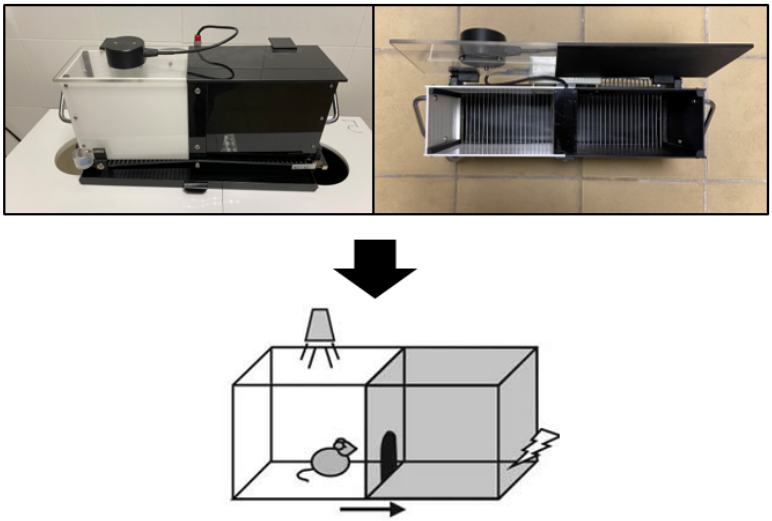


Figura 14. Caja empleada en el test de evitación pasiva que evalúa la retención en la memoria de una leve descarga eléctrica recibida el día previo, aplicada en el estudio 6. Consta de un compartimento blanco iluminado con una bombilla de luz blanca de 10W ubicada en la tapa de la caja, en contraste con el compartimento negro, que es oscuro y es donde se administra el shock eléctrico.

3.4.7. Laberinto de Hebb-Williams

Esta prueba fue originalmente diseñada para medir la inteligencia en ratas (Hebb & Williams, 1946), en la que a través de 12 configuraciones distintas podía obtenerse una gran cantidad de información acerca del curso del aprendizaje. Los laberintos fueron diseñados con diferentes niveles de dificultad y se ha empleado previamente para conocer los efectos de lesiones en el hipocampo, implicado en la memoria espacial (Kimble, 1963), para diferenciar el tipo de aprendizaje según la especie animal (Livesey, 1966) o evaluar el efecto de distintas sustancias sobre el aprendizaje y conducta en ratones (Vidal-Infer et al., 2012). El laberinto Hebb-Williams permite medir el proceso de adquisición del aprendizaje a través de la motivación para escapar del agua fría que hay en él y ha sido empleado en el estudio 6.

El suelo y paredes del laberinto están compuestos de plexiglás negro (60cm de ancho x 60cm de largo x 10cm de alto) y está cubierto por una tapa de plexiglás transparente de las mismas medidas (Figura 15). El suelo tiene líneas blancas que marcan la división del laberinto en 36 cuadrantes de 10cm x 10cm. Contiene un compartimento rectangular de salida y uno de meta, ambos de 14cm de largo por 9cm de ancho, ubicados de forma diagonal en esquinas opuestas. Los diferentes laberintos se configuran con unas barreras negras de 10cm de alto y 2,5cm de base de plexiglás negro de diferentes medidas que se fijan fácilmente a la tapa transparente con tornillos, de forma que posibilita cambiar la estructura de cada laberinto.

El laberinto Hebb-Williams contiene agua mantenida a 12 ± 2º C que se controla mediante un termómetro tras cada ensayo realizado. El nivel de profundidad del agua es de 3,5cm, por lo que el ratón no tiene que nadar, ya que el agua sólo le llega hasta el abdomen. En el compartimento de meta se coloca un lecho de papel seco para que salir del laberinto resulte reforzante para el animal. El procedimiento empleado en esta TD se basa en el utilizado por Galsworthy et al. (2005), en el que los ratones deben nadar desde el compartimento de salida hasta el compartimento de meta que está seco, con el fin de escapar del agua fría. Las pruebas se estructuran en 8 días de procedimiento compuestas por 3 días de prueba y 5 días de laberintos fáciles y difíciles (Figura 16). Al finalizar cada ensayo el animal pasa por una caja individual con un lecho de viruta e iluminada por una luz roja, de forma que se facilite el secado de cada uno de ellos. Todo el procedimiento se ejecuta bajo condiciones de luz tenue, simulando el período de actividad de los ratones.

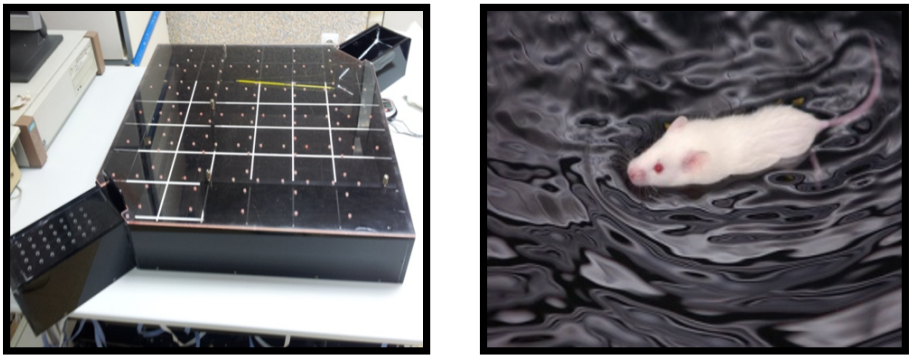


Figura 15. Laberinto Hebb-Williams utilizado para evaluar el proceso de adquisición de aprendizaje empleado en el estudio 6. El laberinto está compuesto por un suelo y paredes de plexiglás negro, una tapa de plexiglás transparente, un compartimento rectangular de salida, otro compartimento de meta y barreras de plexiglás negro de diferentes medidas empeladas para cambiar la estructura de cada laberinto.

El primer día se realiza un período de habituación al laberinto o prueba de campo abierto, permitiendo al animal explorarlo sin agua y sin barreras. En la prueba de campo abierto sólo se realiza un ensayo, que finaliza con la salida del animal por el compartimento de meta o transcurridos 5 minutos. Los dos siguientes días se realizan dos laberintos de prueba con agua y sin zonas de error. En estos laberintos se realizan 4 ensayos por día con un máximo de 5 minutos por ensayo. El objetivo de estos 3 días de prueba es que el animal se adapte y habitúe a esta rutina diaria, y establezca el hábito de ir hacia la meta sin entretenerse explorando. Tras realizar la habituación en campo abierto y los dos días de prueba, se realizan 5 días con laberintos de test, compuestos por 8 ensayos cada día con un máximo de 5 minutos por ensayo. Si un ratón realiza 3 ensayos seguidos sin salir del laberinto finaliza la prueba y no participa más en el laberinto de ese día. A lo largo de los 5 días se alternan laberintos fáciles y difíciles según la clasificación de Stanford & Brown (2003). Los laberintos 1, 3 y 4 son considerados como fáciles y los 5 y 8 como difíciles. El tiempo se

controla con un cronómetro, que comienza la cuenta cuando el animal pone las 4 patas fuera del compartimento de salida y cerrándose tras él la puerta de la salida para impedir que vuelva a entrar. La puerta del compartimento de meta se mantiene abierta durante todo el ensayo. Se considera que el animal entra en una zona de error cuando pone las 4 patas sobre ella y sólo se contabiliza el número de entradas en las zonas de error. La zona 1 se encuentra aproximada al camino principal; sin embargo, la zona 2 es la más alejada y el ratón debe pasar previamente por la zona 1 para acceder a la zona 2. Existen dos zonas de error en todos los laberintos salvo en el 5, en el que hay 3 zonas de error. En el transcurso de todos los ensayos se registraron tanto la latencia de llegada a la meta como el número de entradas en cada zona de error.

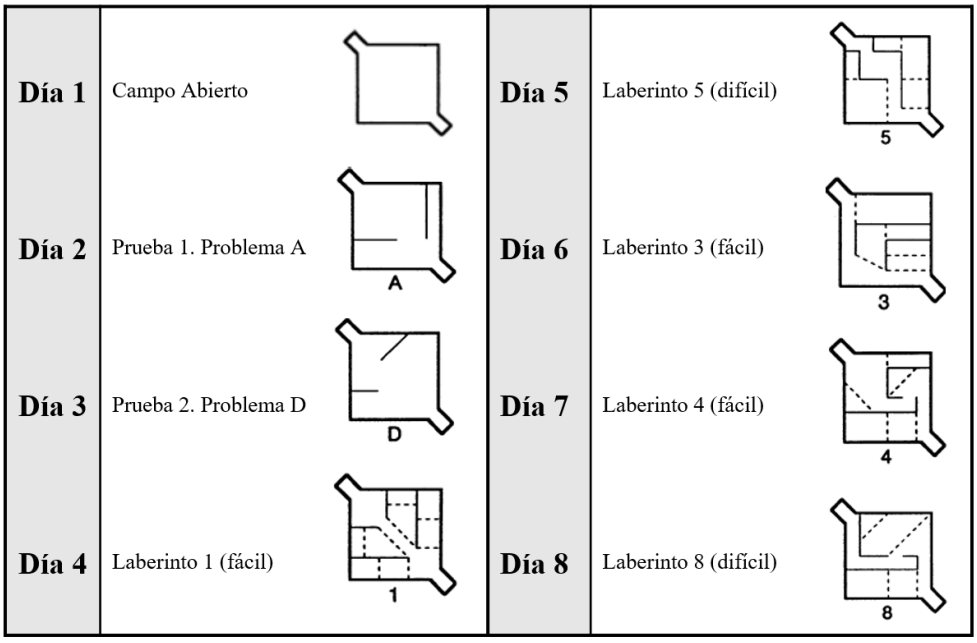


Figura 16. Composición de los laberintos específicos empleados a lo largo de 8 días en el procedimiento del laberinto Hebb-Williams (3 días de prueba y 5 días de laberintos fáciles y difíciles).

3.5. Medidas fisiológicas/biológicas

3.5.1. Recogida de muestras

Al finalizar los experimentos, se practicó la eutanasia a los animales mediante dislocación cervical y se extrajeron inmediatamente los cerebros del cráneo y se colocaron en una placa fría. Se eliminaron el cerebelo y los bulbos olfatorios y se diseccionó el estriado. Las muestras de tejido cerebral se almacenaron inmediatamente a -80ºC hasta su posterior análisis.

3.5.2. Estado de cetosis: niveles plasmáticos de β-hidroxibutirato

Los niveles de βOHB de los animales se midieron a partir de la extracción de sangre de la vena de la cola, empleando un monitor On Call GK Dual y tiras reactivas de cetonas (ACON Laboratories, Inc., San Diego, CA, USA). Los niveles de βOHB se analizaron en los estudios 4, 5 y 6.

3.5.3. Determinación de los niveles estriatales de interleucina 6 (IL-6)

Para determinar la concentración de IL-6 en el cuerpo estriado de los animales (estudio 1), utilizamos un kit ELISA de IL-6 de ratón (Figura 17) de la casa comercial Abcam (Ref: ab100712) y seguimos las instrucciones del fabricante. Para determinar la absorbancia, empleamos un lector de microplacas iMark (Bio-RAD) controlado por el software Microplate Manager 6.2. La densidad óptica se leyó a 450 nm y los resultados finales se calcularon utilizando una curva estándar, expresando como pg/mg para muestras de tejido. La sensibilidad del test es de <2 pg/mg. Todas las muestras fueron analizadas por duplicado.

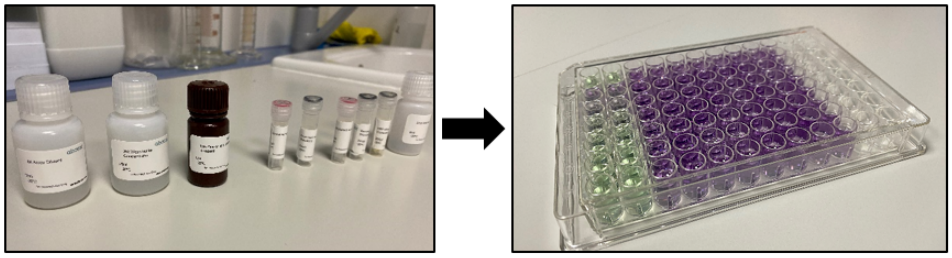


Figura 17. Kit ELISA de IL-6 de ratón de la casa comercial Abcam empleado en el estudio 1.

3.5.4. Determinación plasmática de los niveles de leptina y grelina

Para la cuantificación de la leptina y la grelina en plasma (estudio 2), se empleó un kit ELISA (Merck-Sigma Aldrich, Saint Louis, EE.UU.) siguiendo las instrucciones del fabricante. La sensibilidad de la prueba es de 0,2. Todas las muestras se realizaron por duplicado.

3.5.5. Análisis de expresión génica: Aislamiento del ARN y RT-PCR cuantitativa

Para el análisis de expresión génica de los animales, se emplearon RT-PCR cuantitativas siguiendo el protocolo del fabricante, utilizando el método del reactivo Tri (Sigma-Aldrich, St. Louis, MO, USA) para aislar el ARN total del cuerpo estriado. La transcripción inversa de 1 mg de ARN total se realizó utilizando el kit de síntesis de ADNc Transcriptor First Strand (Thermo Fisher Scientific, Madrid, España). La amplificación de los genes diana y de los genes de mantenimiento (b-glucuronidasa y Gusb) se realizó utilizando la mezcla maestra de expresión génica Taqman (Thermo Fisher Scientific, Madrid, España) en un sistema LightCycler 480 (Roche Diagnostics) siguiendo las instrucciones del fabricante. Los códigos de ensayo de los cebadores utilizados son Mm01212171_s1 para CB1r analizado en los estudios 2, 3 y 6, Mm01188089_m1 para el receptor opioide μ (Oprμ) analizado en los estudios 2, 3 y 6, Mm02620146 y Mm00438545 para los receptores

dopaminérgicos D1r y D2r analizados en el estudio 6, Mm01308023 y Mm00802075 para los receptores de adenosina A1r y A2r analizados en el estudio 6 y Mm00432670 para el receptor de corticotropina 1 (CRHR1) analizado en el estudio 3. Los datos se analizaron con el software de cuantificación relativa LightCycler 480 y se normalizaron con respecto al producto de amplificación de la b-glucuronidasa o Gusb (Mm00446953).

3.6. Estadística

Para analizar los niveles estriatales de IL-6, la determinación plasmática de los niveles de leptina y grelina, la expresión génica, el incremento (%) de peso corporal, el laberinto Elevado en Cruz y el campo abierto se realizaron ANOVAs univariantes. Para analizar las conductas manifestadas durante la DS, el peso corporal, el consumo de Kcal intermitente durante las sesiones de DRG, el consumo de dieta estándar, los niveles plasmáticos de βOHB, el test de evitación pasiva, laberinto de Hebb-Williams y la AA de etanol se realizaron ANOVAs mixtos. Además, para analizar el proceso de adquisición del CPL, se realizaron ANOVAs de medidas repetidas. En todos los estudios, tras el ANOVA, se calcularon pruebas post-hoc de Bonferroni siempre que fue necesario. Para evaluar la relación entre las conductas manifestadas durante la DS, y el grado de condicionamiento durante el CPL (*Conditioning Score),* se realizó el coeficiente de correlación de Pearson. Por último, los datos relacionados con la extinción y la reinstauración del CPL se analizaron mediante la prueba t de Student y la prueba de Kaplan-Meier, con comparaciones de Breslow (Wilcoxon generalizado). Los análisis estadísticos se realizaron con SPSS Statistics (v.26; IBM, NY, EE.UU.). Los datos se expresaron como media ± SEM y se consideró un valor de $p<0,05$ estadísticamente significativo. Los datos específicos de las pruebas estadísticas se encuentran descritos en cada estudio, en la sección de resultados.

4. RESULTADOS

Estudio 1

Caracterización conductual y neuroinmune de la resiliencia al estrés social: efectos reforzantes de la cocaína.

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(Anexo 1)

Caracterización conductual y neuroinmune de la resiliencia al estrés social: efectos reforzantes de la cocaína

Behavioral and neuroimmune characterization of resilience to social stress: rewarding effects of cocaine

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Resumen

Numerosos estudios preclínicos han demostrado que el estrés social incrementa la vulnerabilidad a los efectos reforzantes de la cocaína. Sin embargo, los resultados obtenidos no son homogéneos, observándose siempre una subpoblación que no muestra dicho incremento. Utilizando el modelo de derrota social (DS) repetida en ratones, en este trabajo hemos querido caracterizar conductualmente a los ratones resilientes al incremento de los efectos reforzantes de la cocaína inducido por el estrés social. Utilizamos ratones adultos macho de la cepa C57/BL6 a los que sometimos al protocolo de DS repetida y tres semanas más tarde, realizamos el Condicionamiento de Preferencia de Lugar (CPL) inducido por una dosis no efectiva de cocaína (1mg/kg). Una vez finalizado este procedimiento se midieron los niveles estriatales de interleucina 6, ya que el estrés social produce una respuesta de neuroinflamación. No se observó CPL en los ratones controles, pero los animales derrotados tomados en conjunto desarrollaron preferencia. Sin embargo, esta muestra se pudo dividir en ratones resilientes (no desarrollaron preferencia) y susceptibles (presentaron CPL). Durante las derrotas sociales, los animales resilientes pasaron menos tiempo en las conductas de huida y sumisión que los catalogados como susceptible y presentaron conductas de ataque hacia el ratón residente, manifestando por tanto resistencia a ser derrotados. No se observaron diferencias en la respuesta de neuroinflamación, probablemente debido al largo periodo de tiempo trascurrido desde la última derrota social. Nuestros resultados sugieren que un estilo de afrontamiento activo al estrés social va a ser determinante en la protección del sujeto a desarrollar un trastorno por uso de drogas.

Palabras clave: resiliencia, cocaína, estrés social, afrontamiento, interleucina 6

Abstract

Preclinical studies have shown that social stress increases vulnerability to the reinforcing effects of cocaine. However, the results are not always homogeneous, revealing a subpopulation that does not show a preference for cocaine. Thus, the main aim of the present study was to characterize the behavioral profile of resilient mice to the stress-induced rewarding effects of cocaine using an animal model of repeated social defeat stress (SD). To this end, male adult mice of the C57/BL6 strain were exposed to SD and, three weeks later, assessed using the Conditioned Place Preference paradigm induced by an ineffective dose of cocaine (1mg/kg). Afterwards, the striatal levels of interleukin 6 were measured, as social stress usually induces a neuroinflammatory response. Control mice did not develop CPP, while defeated mice did overall develop a preference for the drug-paired compartment. Based on the conditioning score that they exhibited, the SD sample was subdivided into resilient (did not develop preference) and susceptible mice (developed preference). During the SD sessions, resilient animals showed less flight and submission behaviors than susceptible mice and they presented attack behaviors towards the residents, thereby showing their resistance to being defeated. There were no differences in the neuroinflammatory response, probably due to the long time elapsed after the last SD session. These results suggest that an active coping style to social stress may be decisive in protecting the individual from developing an addiction.

Keywords: resilience, cocaine, social stress, coping, interleukin 6

1. INTRODUCCIÓN

La exposición al estrés es un factor ambiental que se ha relacionado de forma muy directa con la aparición de trastornos psiquiátricos, como la depresión, la ansiedad o los trastornos por abuso de sustancias. Sin embargo, no todos los sujetos son igual de vulnerables a las consecuencias del estrés (Krishnan et al., 2007; Lutter et al., 2008). En los últimos años se ha producido un gran incremento en el estudio del fenómeno de la resiliencia al estrés. Se define como resiliencia a la capacidad que presentan los individuos de mantener un funcionamiento psicológico y físico adaptativo, y evitar la aparición de enfermedades mentales cuando se exponen a un estrés crónico o de elevada intensidad (Charney, 2004). Los mecanismos responsables de la resiliencia van a promover una respuesta apropiada y no patológica al estrés (Chmitorz et al., 2018). En los últimos años se han comenzado a identificar las características psicológicas y biológicas de los individuos resilientes al estrés social (Pfau & Russo, 2015). Por ejemplo, existen una serie de conductas y rasgos psicológicos, como la flexibilidad cognitiva, el afrontamiento activo, el optimismo o la sensación de pertenencia a un grupo, que pueden favorecer una respuesta de resiliencia en seres humanos (Wood & Bhatnagar, 2015; Laird et al., 2019). Sin embargo, la mayoría de estos estudios se han centrado en la resiliencia al desarrollo de depresión, ansiedad o trastorno de estrés postraumático (Russo et al., 2012; Krishnan, 2014; Finnell & Wood, 2016), siendo muy escasos los estudios que evalúan la resiliencia al incremento en el consumo de drogas.

La mayoría de los estudios preclínicos sobre resiliencia al estrés utilizan el modelo de derrota social (DS) repetida o crónica. Este modelo presenta una gran relevancia etológica y traslacional, ya que la forma más común de estrés experimentada por los seres humanos proviene de su ambiente social. Este modelo está basado en el paradigma intruso/residente, que consiste en introducir a un animal macho (intruso) en el territorio de otro (residente), que confrontará y dominará al primero (Miczek et

al., 2008; Chaouloff, 2013). Numerosos estudios han demostrado que la DS repetida incrementa el consumo de cocaína y de alcohol (Miczek et al., 2008; Burke & Miczek, 2014; Rodríguez-Arias et al., 2016, 2017; Montagud-Romero et al., 2016a; Ferrer-Pérez et al., 2018a). Este incremento se ha asociado a una respuesta de neuroinflamación, ya que en los animales derrotados se ha constatado un incremento de marcadores de inflamación como las citoquinas o las quemoquinas, un incremento en la permeabilidad de la barrera hematoencefálica, así como una activación de la microglía (Rodríguez-Arias et al., 2017, 2018; Ferrer-Pérez et al., 2018a).

Al igual que ocurre en los estudios realizados en seres humanos, en la mayoría de los estudios preclínicos se ha evaluado el desarrollo de resiliencia al desarrollo de depresión o ansiedad en ratones expuestos a DS repetida. En estos estudios, a las 24h de finalizar la última DS se categoriza a los animales en resilientes o susceptibles en función de su conducta en una prueba de interacción social. Serán resilientes aquellos que presentan un elevado tiempo de contacto social, mientras que los susceptibles mostraran evitación social (Krishnan et al., 2007; Russo et al., 2012; Golden et al., 2011; Henriques-Alves & Queiroz, 2015; Zhan et al., 2018). Algunos estudios han confirmado que entre los factores que median la resiliencia se encuentra una menor respuesta neuroinflamatoria en los animales resilientes (Wang et al., 2018).

Los resultados anteriormente mencionados nos han llevado a plantear como objetivo principal del presente trabajo la caracterización de los ratones expuestos a DS repetida que sean resilientes al incremento a largo plazo de los efectos reforzantes de la cocaína. Para ello, tres semanas después de la última DS, realizamos un condicionamiento de la preferencia de lugar (CPL) con una dosis subumbral de cocaína que no es efectiva en animales controles pero que sí induce preferencia en aquellos derrotados socialmente (Montagud-Romero et al., 2016a, 2016b). La caracterización conductual se realizará evaluando el comportamiento que

presentaron aquellos animales resilientes durante las DS. Finalmente, una vez finalizado el procedimiento conductual, estudiaremos la respuesta neuroinflamatoria midiendo los niveles estriatales de interleucina 6 (IL6).

2. MATERIAL Y MÉTODOS

2.1. Animales

En el presente estudio se utilizaron 43 ratones adultos macho de la cepa C57/BL6. 28 de ellos se emplearon como sujetos experimentales (derrota social) y 15 se utilizaron como grupo control (expuestos solo a exploración). También se emplearon otros 10 ratones albinos macho de la cepa OF1 como ratones residentes en la DS repetida. Todos los ratones fueron adquiridos en los Laboratorios Charles River (Barcelona, Spain.). Los ratones experimentales llegaron en el día postnatal (DPN) 21 y fueron estabulados en grupos de 4 en cajas de plástico de 26x20x13cm. Los 10 ratones de la cepa OF1 fueron alojados de forma aislada para su uso como residentes durante la DS repetida. Las condiciones ambientales fueron de una temperatura de 21±2ºC y una humedad relativa del 55%. Los ratones fueron mantenidos durante todo el procedimiento en un ciclo de luz/oscuridad de 12h/12h (8:00h – 20:00h) y con agua y pellets *ad libitum*, excepto durante las pruebas comportamentales. Todos los procedimientos de tratamiento y cuidado de los ratones obedecieron a las leyes y regulaciones nacionales, regionales y locales de acuerdo con las directrices marcadas por la comunidad internacional recogidas en *European Community Council Directives* (86/609/EEC, 24 *November* 1986). Este estudio se realizó en la Unidad de Investigación de Psicobiología de las Drogodependencias del departamento de Psicobiología, Facultad de Psicología, Universitat de València. Fue aprobado por el Comité Ético de experimentación y bienestar animal de la Universidad de Valencia 2017/VSC/PEA/00224-A1507028485045.

2.2. Tratamiento farmacológico

Los animales recibieron tratamiento farmacológico únicamente durante el procedimiento de CPL. Tanto a los ratones del grupo control como a los del grupo experimental se les inyectó por vía intraperitoneal 1mg/kg de cocaína disuelta en una solución de NaCl 0,9%. Esta dosis es considerada una dosis subumbral, no mostrando preferencia de lugar en la prueba CPL en ratones estándar (Maldonado et al., 2006; Vidal-Infer et al., 2012), mientras que sí desarrollan preferencia los ratones expuestos a DS repetida (Rodríguez-Arias et al., 2017).

2.3. Recogida de muestras

Para la obtención de muestras seguimos el procedimiento realizado en estudios previos (Ferrer-Pérez et al., 2018b). Se sacrificaron ratones por dislocación cervical y posteriormente se decapitaron. Los cerebros se extrajeron rápidamente y el estriado se diseccionó siguiendo el procedimiento descrito por Heffner y colaboradores (Heffner et al., 1980), manteniéndose en hielo seco hasta que se almacenaron a -80 ° C.

Antes de realizar la determinación de IL-6, los cerebros se homogeneizaron y se prepararon siguiendo el procedimiento descrito por Alfonso-Loeches y colaboradores (2010). Los estriados se homogeneizaron en 250 mg de tejido / 0,5ml de tampón de lisis frío (NP-40 al 1%, Tris-HCl 20mM, pH 8, NaCl 130mM, NaF 10mM, 10µg/ml de aprotinina, 10µg/ml leupeptina, DTT 40mM, Na3VO4 1mM y PMSF 10mM). Los homogeneizados de cerebro se mantuvieron en hielo durante 30 minutos y se centrifugaron a la velocidad máxima durante 15 minutos, después se recogió el sobrenadante y se determinaron los niveles de proteína mediante el ensayo de Bradford de ThermoFisher (Ref: 23227).

2.4. Diseño experimental

En la tabla 1 se puede encontrar en detalle el diseño experimental del presente estudio. Todos los ratones llegaron con 21 días de edad al laboratorio. Después de 3 semanas de adaptación en el animalario, en el DPN 47, comenzaron las 4 sesiones de DS. Posteriormente, a las 3 semanas de la última DS realizamos el CPL (3 días de pre-condicionamiento, 4 días de condicionamiento y 1 día de post-condicionamiento). Por último, tras la finalización de todo el procedimiento experimental, procedimos al sacrificio de los animales para la recogida de muestras biológicas.

Tabla 1

Diseño Experimental

	Derrota social / Exploración					**CPL (1mg/kg cocaína)**			**Recogida de muestras**
	1.ª	2.ª	3.ª	4.ª	3 semanas	Pre-C test	Condicionamiento	Post-C test	
DPN	47	50	53	56		76 - 78	79 - 82	83	84

2.5. Aparatos y procedimiento

2.5.1. Derrota Social

El protocolo de DS realizado en este estudio se ha validado y descrito en detalle en trabajos previos (Montagud-Romero et al., 2016a; Rodríguez-Arias et al., 2017; Ferrer-Pérez et al., 2019). La DS repetida consta de 4 sesiones de 25 minutos a intervalos de 72h, realizándose en los días postnatales 47, 50, 53 y 56. La sesión de DS repetida está compuesta por 3 fases. En la primera fase se introduce al intruso en la jaula del residente durante 10 minutos, en los que está protegido de los ataques, pero no de las amenazas del residente por medio de una rejilla. En la segunda fase

se retira la rejilla y se permite la confrontación durante 5 minutos. En la tercera y última fase se coloca nuevamente la rejilla durante 10 minutos más.

Las sesiones de DS repetida fueron registradas con una videocámara y durante el procedimiento se evaluaron en los animales intrusos las conductas de huida, sumisión y ataque, mientras que en los animales residentes se midieron las conductas de amenaza y ataque. En la DS repetida realizada con los 15 ratones controles se empleó un procedimiento similar al descrito anteriormente, pero sin la presencia del ratón residente. Una vez finalizado el paradigma, se procedió al análisis de los encuentros mediante el programa informático que permite registrar el tiempo dedicado a realizar diferentes conductas (Martínez et al., 1991).

2.5.2. Condicionamiento de la Preferencia de Lugar

El CPL es un modelo basado en el aprendizaje clásico o pavloviano, para evaluar la recompensa condicionada inducida por diferentes estímulos (Bardo & Bevin, 2000; Tzschentke, 2007). Ha sido ampliamente utilizado para estudiar los efectos de recompensa de las drogas adictivas condicionadas (Aguilar et al., 2009; Yap et al., 2015; Rodríguez-Arias et al., 2016; Blanco-Gandía et al., 2017), ya que los estímulos contextuales pueden adquirir propiedades apetitivas secundarias cuando se combinan con un reforzador primario (Tzschentke, 2007).

Para el CPL se emplearon 12 cajas idénticas de plexiglas con dos compartimentos de igual tamaño (30.7 cm de largo X 31.5 cm de ancho X 34.5 cm de alto), separadas por un área central de color gris (13.8 cm de largo X 31.5 cm de ancho X 34.5 cm de alto). Los compartimentos tienen paredes de diferente color (blancas vs negras) y distinta textura de suelo (liso en el compartimento negro y rugoso para el blanco). Los animales son entrenados para asociar un ambiente específico con el efecto de la

droga administrada, y en el otro compartimento diferente con solución salina (García-Pardo et al., 2017). Ambos compartimentos presentan una puerta tipo guillotina, que los separa del compartimento central. Cada uno de los compartimentos de condicionamiento cuenta con 4 células fotoeléctricas, mientras que la zona central tiene 6, lo que permite el registro de la posición del animal y los cruces de un compartimento al otro. El equipo es controlado por dos computadoras IBM PC mediante el uso de un software MONPRE 2z (CIBERTEC, SA, España).

El CPL consiste en 3 fases, las cuales se llevaron a cabo durante el ciclo de oscuridad y siguiendo un procedimiento "no sesgado" en términos de la preferencia inicial espontánea (Manzanedo et al., 2001). Durante la primera fase o pre-condicionamiento (Pre-C), los ratones tuvieron libre acceso a ambos compartimentos del aparato durante 15 minutos (900s) cada día durante 3 días. Al tercer día, el tiempo que cada animal pasa en cada compartimento fue registrado durante 900s. Los animales que muestran una fuerte aversión (menos del 33% del tiempo de la sesión) o una fuerte preferencia (más del 67%) por alguno de los compartimentos son descartados del procedimiento. De acuerdo con lo anterior, en el presente experimento se descartaron un total de 2 animales que no cumplían con los criterios establecidos. La asignación de los compartimentos se realiza con un contrabalanceado. Uno de los compartimentos se elige para ser asociado con la cocaína de tal manera que, dentro de cada grupo, la mitad de los animales reciben la cocaína en el lugar menos preferido y la otra mitad en el más preferido, balanceándose también el color del compartimento. No deben existir diferencias significativas en el tiempo que los animales han pasado en el compartimento asociado al fármaco o al vehículo en la fase de pre-condicionamiento. Esta medida es de gran importancia para el procedimiento experimental, ya que ayuda a evitar que exista algún tipo de sesgo en la preferencia antes de comenzar el experimento.

En la segunda fase (condicionamiento), los animales fueron condicionados con 1mg/kg de cocaína mediante 4 asociaciones con el compartimento asignado tras el Pre-C. Se ha observado que 1mg/kg es una dosis subumbral, es decir, una dosis que no conlleva a la adquisición del CPL, a menos que se manipulen otras variables como el estrés o rasgos del comportamiento (Vidal-Infer et al., 2012; Arenas et al., 2014; Montagud-Romero et al., 2014; Rodríguez-Arias et al., 2016; Blanco-Gandía et al., 2018). Los animales recibieron 2 inyecciones (cocaína y vehículo) cada día: una administración de solución salina antes de ser confinados al compartimento no asociado durante 30 minutos, y después de un intervalo de 4 horas, recibieron cocaína antes de ser confinados al compartimento asociado con la droga durante 30 min. El área central no fue utilizada durante el condicionamiento y su acceso fue bloqueado mediante puertas tipo guillotina.

Durante la tercera fase o post-condicionamiento (Post-C), en el 8º día del procedimiento, las guillotinas que separaban ambos compartimentos fueron retiradas y el tiempo que los ratones, sin ningún tipo de tratamiento, pasaban en cada compartimento fue registrado durante 900s. La diferencia en segundos entre el tiempo que los animales permanecen en el compartimento asociado con la droga durante la prueba de Post-C, y el tiempo que pasan durante la prueba de Pre-C es una medida del grado de condicionamiento inducido por la droga (*Conditioning Score*). Si esta diferencia es positiva, entonces la droga ha inducido una preferencia por el compartimento asociado con la misma, mientras que lo opuesto indica la inducción de una aversión. Una vez finalizado el CPL, se dividió a los animales derrotados en resilientes o susceptibles. Se consideraron resilientes aquellos que no presentaron incremento en la preferencia por el compartimento asociado a la cocaína y susceptibles a los que si incrementaron la preferencia.

2.5.3. Ensayo ELISA de IL-6

Para determinar la concentración de IL-6 en el estriado, utilizamos un kit ELISA de IL-6 de ratón de la casa comercial Abcam (Ref: ab100712) y seguimos las instrucciones del fabricante. Para determinar la absorbancia, empleamos un lector de microplacas iMark (Bio-RAD) controlado por el software Microplate Manager 6.2. La densidad óptica se leyó a 450 nm y los resultados finales se calcularon utilizando una curva estándar, expresando como pg/mg para muestras de tejido. La sensibilidad del test es de <2 pg/mg. Todas las muestras fueron analizadas por duplicado.

2.5.4. Análisis de los datos

Para confirmar el efecto de la DS repetida en el CPL se realizó un ANOVA univariante con los datos del *Conditioning Score* con una variable entre-sujetos "Estrés" con dos niveles: Exploración y Derrota Social. Una vez hecha la división entre Resilientes y Susceptibles, realizamos un nuevo análisis univariante con la variable entre-sujetos "Grupo" con tres niveles: Exploración, DS Susceptibles y DS Resilientes. Este mismo análisis se aplicó a los datos de los niveles estriatales de IL6. Los resultados obtenidos en el análisis etológico de la DS se analizaron utilizando un ANOVA de dos vías con una variable entre-sujetos "Grupo" con dos niveles: Resilientes y Susceptibles y una variable intra-sujetos "Derrota" de 2 niveles: DS1 (primera sesión de derrota social) y DS4 (cuarta sesión de derrota social). Los análisis post-hoc se realizaron utilizando la prueba de ajuste de Bonferroni, considerando los intervalos de significación de $p<0.05$, $p<0.01$ y $p<0.001$. También se realizó el coeficiente de correlación de Pearson para determinar si existe relación entre la variable Huida y *Conditioning Score* de todos los animales que realizaron DS repetida.

3. RESULTADOS

3.1. Solo los animales susceptibles desarrollan CPL

Respecto al *Conditioning Score* del CPL (Figura 1a), el ANOVA mostró un efecto significativo en la variable Estrés [$F(1.36)=7.147$; $p<0.05$], indicando que los animales derrotados pasaron significativamente más tiempo en el compartimento asociado a la droga que los animales no estresados ($p<0.05$).

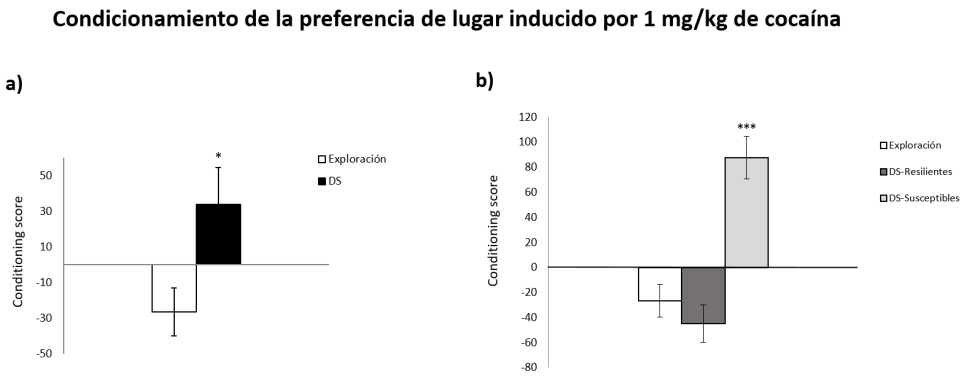

Figura 1. Efecto de la DS repetida en la adquisición del CPL inducido por 1 mg/kg de cocaína en ratones macho C57/BL6. Las barras representan la diferencia del tiempo (s) pasado en el compartimento asociado a la droga antes y después de las sesiones de condicionamiento (*conditioning score*). (a) Grupos de tratamiento: Exploración y DS repetida. (b) Tras el Post-C, los animales derrotados se dividieron en Resilientes y Susceptibles en función de su nivel de condicionamiento. *$p<0.05$, diferencia significativa respecto al grupo Exploración. ***$p<0.001$, diferencia significativa respecto al grupo exploración y DS-resilientes.

Cuando se dividió al grupo de animales derrotados en Resilientes y Susceptibles (Figura 1b), el ANOVA mostró un efecto significativo en la variable Grupo [$F(2.38)=21.287$; $p<0.001$]. Los animales susceptibles pasaron significativamente más tiempo en el compartimento asociado a la droga comparados con los otros dos grupos ($p<0.001$ en ambos casos).

3.2. Los ratones resilientes muestran una respuesta de afrontamiento al estrés durante las DS

En la Tabla 2 se muestran los datos relativos a la conducta de los ratones derrotados durante la primera y la cuarta DS. Respecto a la conducta de Huida, el ANOVA mostró un efecto significativo en la variable Grupo [$F(1,24) = 9.962$; $p<0.01$], ya que los animales del grupo DS-resilientes mostraron significativamente menos tiempo realizando conductas de este tipo ($p<0.01$). Con respecto a la conducta de Sumisión, el ANOVA mostró un efecto significativo en la interacción de las variables Derrota x Estrés [$F(1,24) = 5.171$; $p<0.05$], ya que los animales resilientes pasaron menos tiempo realizando conductas de sumisión durante la primera sesión de DS repetida con respecto a los animales susceptibles ($p<0.05$).

Tabla 2.
Resultados de DS repetida en intrusos.

Resilientes	Huida	Lat. Huida	Sumisión	Lat. sumisión	Ataque	Lat. ataque
DS1	34 ± 3**	14 ± 10	23 ± 5*	49 ± 22	3 ± 1	217 ± 37
DS4	34 ± 4**	4 ± 1	25 ± 7	101 ± 37	0 ± 0	300 ± 0
Susceptibles	Huida	Lat. Huida	Sumisión	Lat. sumisión	Ataque	Lat. ataque
DS1	42 ± 7	9 ± 3	35 ± 9+	60 ± 28	1 ± 0	276 ± 24
DS4	49 ± 6	4 ± 1	15 ± 4	83 ± 27	0 ± 0	300 ± 0

Nota. Conductas evaluadas durante la DS en intrusos. Datos presentados como valores medios ± SEM. *p<0.05, **p<0.01 diferencias con respecto a Susceptibles. + p<0.05 diferencias con respecto a DS4 (efecto grupo-derrota).

También se evaluó la presencia de conducta de ataque por parte de los animales intrusos frente a los residentes. Solo observamos una tendencia en la variable Derrota [$F(1,24)=3.865$; $p= 0,061$] que nos indica que los animales intrusos atacaron más en la primera sesión de DS. Observamos que un 29% de los animales catalogados como

Resilientes, frente a un 8% de los Susceptibles, atacó a su residente en la primera DS repetida. Ningún animal intruso atacó en la cuarta DS repetida.

Finalmente se evaluó la relación entre la conducta de Huida mostrada por los todos los intrusos en el primer y cuarto encuentro de la DS repetida y su conditioning score en el CPL, para comprobar si el tiempo pasado de alguna de estas conductas podría ser un indicador de condicionamiento que se produciría posteriormente (Figura 2). Solo se obtuvo un coeficiente de correlación de Pearson significativo entre el tiempo pasado en Huida y el Conditioning Score ($r = 0.241$, $p<0.05$). Es decir, a mayor tiempo en la conducta de huida durante los encuentros de DS, mayor es la preferencia por la droga en el CPL.

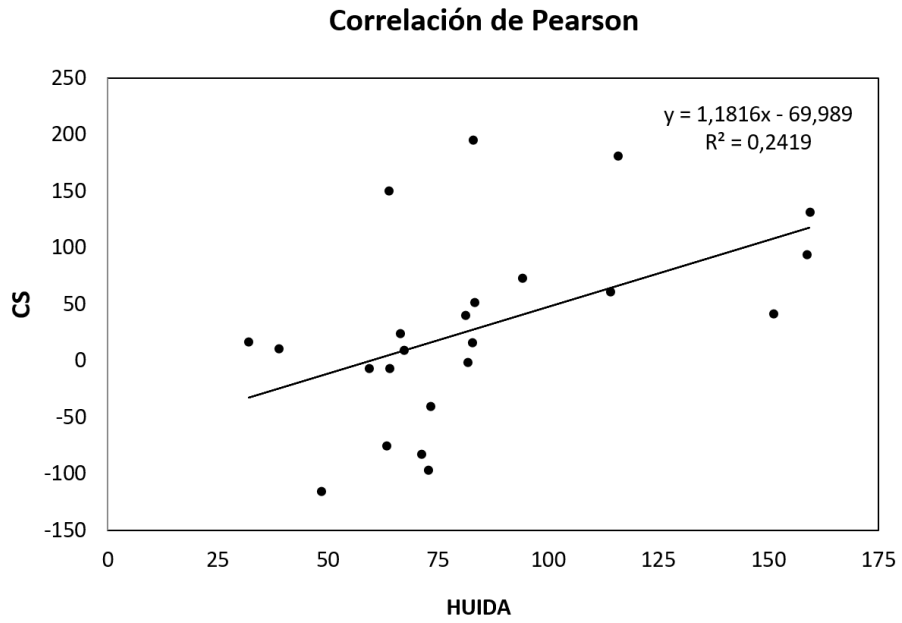

Figura 2. Gráfica de regresión para la Correlación de Pearson entre la conducta de Huida en la DS repetida y el *Conditioning Score* (CS). La línea de tendencia representa la regresión lineal de datos (y = 1,1816x – 58,989; r2 = 0.2419).

Respecto a la conducta de amenaza por parte de los residentes (Tabla 3) el ANOVA muestra un efecto en la variable derrota [F (1,24) = 6.070; p<0.05] indicando que los residentes amenazaron más en la DS1 que en la DS4 (p<0.05). Sin embargo, no hay diferencias significativas en la variable grupo, indicando que tanto Resilientes como Susceptibles han sido expuestos al mismo estrés.

Tabla 3
Resultados de DS repetida en residentes

Residentes de los animales Resilientes	Amenaza	Lat. Amenaza	Ataque	Lat. ataque
DS1	38 ± 6#	8 ± 3	26 ± 5	26 ± 17
DS4	28 ± 5	4 ± 1	20 ± 3	4 ± 1
Residentes de los animales Susceptibles	Amenaza	Lat. Amenaza	Ataque	Lat. ataque
DS1	35 ± 6#	9 ± 4	28 ± 6	35 ± 25
DS4	21 ± 3	8 ± 2	34 ± 5	3 ± 1

Nota. Interacción social de Residentes durante el paradigma intruso-residente para inducir la DS. Datos presentados como valores medios ± SEM. Diferenciamos entre los residentes que han atacado a aquellos posteriormente catalogados como resilientes y susceptibles. # p<0.05 respecto a DS4 (efecto derrota).

3.3. Niveles estriatales de Il6

El ANOVA de los niveles estriatales de IL-6 (Figura 3) no mostró diferencias significativas.

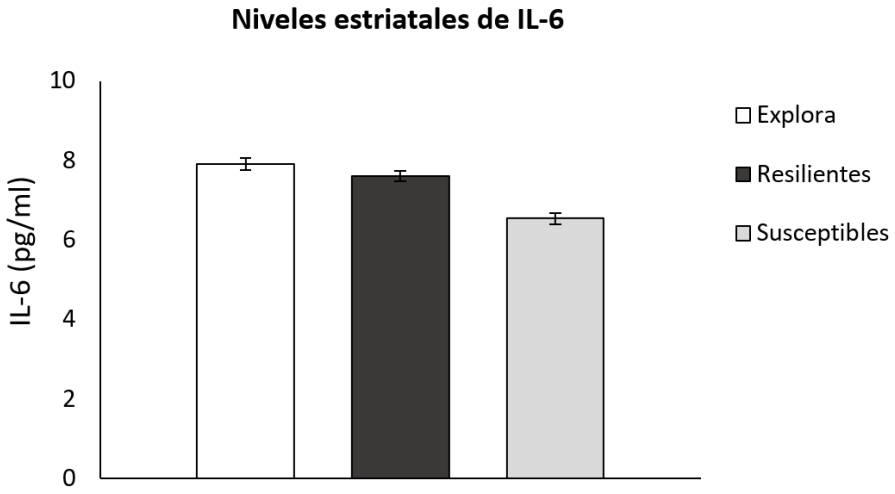

Figura 3. Niveles estriatales de IL-6. Efecto de la DS repetida en los niveles de IL-6 en ratones macho C57/BL6, teniendo en cuenta la subdivisión entre resilientes y susceptibles. Los datos se presentan como Media ± S.E.M. (pg/ml).

4. DISCUSIÓN

Los resultados del presente trabajo confirman que la DS repetida incrementa los efectos reforzantes de la cocaína en el CPL, pero además hemos demostrado por primera vez que los resultados obtenidos en los animales estresados no son homogéneos. Podemos distinguir entre los animales derrotados una población susceptible que ha desarrollado CPL con una dosis no efectiva de cocaína. Pero también hay una parte de los animales derrotados que se comportan como los no estresados, es decir, son resilientes no desarrollando CPL. Pero quizá el resultado más interesante es que el afrontamiento de la DS es diferente en ambos tipos de animales. Los resilientes muestran una menor conducta de huida y de sumisión frente al agresor durante la DS. La conducta de huida correlaciona positivamente con los resultados analizados de CPL, es decir, a más conducta de huida, más preferencia desarrollará el animal por la cocaína. Por lo tanto, una respuesta de afrontamiento activo, mostrando menos huida y sumisión durante un estresor social, reduce la sensibilización a los efectos reforzantes de la cocaína. Estos ratones resilientes, también presentan conductas de ataque al residente, manifestando resistencia a la derrota, algo que no se observa en ninguno de los animales susceptibles. Los cambios en los niveles de IL6 no difieren entre animales estresados o controles, no observándose tampoco diferencia entre aquellos resilientes o susceptibles. Esto puede ser debido a que nuestro estudio se realiza tres semanas después de la última DS.

4.1. Resiliencia y susceptibilidad al incremento de los efectos reforzantes de la cocaína

Nuestros resultados confirman que la experiencia de la DS repetida durante la edad adulta induce un incremento a largo plazo de los efectos reforzantes condicionados de una dosis subumbral de cocaína (1mg/kg), ya que lo hemos evaluado tres semanas

después de la última DS repetida. El paradigma de CPL es ampliamente utilizado para evaluar los efectos condicionados de las drogas (Aguilar et al., 2009) y refleja las propiedades motivacionales secundarias de las drogas, así como su potencial del abuso (Tzschentke, 2007). Por lo tanto, la exposición a una DS repetida induciría un incremento prolongado en el tiempo del valor motivacional de la cocaína incrementándose por tanto su potencial de abuso en los sujetos estresados. Nuestros resultados confirman numerosos estudios que han demostrado que la DS en ratones adolescentes y adultos, aumenta los efectos reforzantes de la cocaína utilizando el CPL (Arenas et al., 2016; Montagud-Romero et al., 2016a; Rodríguez-Arias et al., 2015, 2017; Ferrer-Pérez et al., 2018a), o la AA de cocaína (Boyson et al., 2011; Holly et al., 2016; Newman et al., 2018; Arena et al., 2019).

Pero este estudio va más allá y demuestra que en nuestra población de ratones derrotados, aunque en conjunto todos desarrollan preferencia con una dosis subumbral de cocaína, podemos diferenciar dos tipos de sujetos. Los ratones resilientes a pesar de haber sido estresados no presentan una respuesta a los efectos reforzantes condicionados de la cocaína (CPL). Por el contrario, los animales susceptibles si que desarrollan un incremento por el compartimento asociado a la cocaína. Aunque numerosas evidencias relacionan el estrés con el desarrollo de conductas adictivas (Lüthi & Lüscher, 2014; Polter & Kauer, 2014; Gold et al., 2015), también se ha demostrado que hay sujetos que desarrollan una buena competencia psicosocial en condiciones de elevado riesgo como el maltrato infantil o un estatus socioeconómico adverso (McGloin & Widom, 2001; Hjemdal et al., 2012; Brody et al., 2013). No existen prácticamente estudios en modelos animales que evalúen el fenómeno de la resiliencia al desarrollo de vulnerabilidad al consumo de drogas tras la exposición a un estresor social. Un reciente estudio utilizando como modelo de estrés la exposición al olor de un depredador, clasificó a sus ratones en resilientes y susceptibles basándose en la presencia de ansiedad en el laberinto

elevado en cruz y en la evitación del contexto asociado al olor (Brodnik et al., 2017). En este estudio, observaron que los ratones susceptibles presentaban un incremento a los efectos motores y dopaminérgicos de la cocaína, así como una mayor motivación para autoadministrarse esta droga. Estos efectos no se observaron en los animales resilientes, aunque en ambos tipos de ratones se observó un incremento en la liberación de DA inducida por la cocaína.

4.2. Diferente afrontamiento del estrés social en animales resilientes y susceptibles

La DS repetida es un modelo naturalista de estrés social que imita las situaciones de la vida real y por lo tanto presenta una gran validez ecológica y etológica (Tornatzky & Miczek, 1993). Algunas investigaciones recientes, empleando modelos animales de estrés social, han observado que las estrategias de afrontamiento se asocian con la resiliencia o vulnerabilidad al estrés (Wood et al., 2015; Chen et al. 2015, Finnell et al., 2017; Pearson-Leary et al., 2017). Sin embargo, estos estudios clasifican a los animales en resilientes o susceptibles basándose en la conducta social y la ansiedad mostrada por los animales al día siguiente de la última DS (Russo et al., 2012; Krishnan, 2014; Finnell & Wood, 2016). En estos estudios, los ratones resilientes no presentan anhedonia (Delgado et al., 2011), evitación social (Krishnan et al., 2007; Golden et al., 2011; Henriques-Alves & Queiroz, 2015) o evitación ante el olor de un depredador (Brodnik et al., 2017). Hasta le fecha ningún estudio ha caracterizado a los animales resilientes al incremento de los efectos reforzantes de las drogas de abuso y, por lo tanto, se desconoce si las diferentes estrategias de afrontamiento al estrés influyen en la sensibilidad a dichos efectos reforzantes. Lo que sí sabemos es que los ratones que no mostraron conductas de ansiedad ni evitación al olor de un depredador presentan adaptaciones neuroquímicas que afectan específicamente a la función del sistema DA y por lo tanto podrían modificar la eficacia reforzadora de la cocaína (Brodnik et al., 2017).

El estudio etológico de la conducta durante el desarrollo de las derrotas sociales demostró en primer lugar que no había diferencias en la conducta que los animales residentes mostraron a los intrusos, ya fueran resilientes o susceptibles. Es decir, todos fueron expuestos a un mismo nivel de estrés. Sin embargo, sí que observamos que los ratones que posteriormente se clasificarían como resilientes mostraron una menor conducta de huida en comparación con los susceptibles. Además, hemos observado una correlación positiva entre conducta de huida y el incremento de los efectos reforzantes condicionados de la cocaína en el CPL. Cuanto menos huyen los animales, menor es el efecto reforzante que les produce la cocaína. Igualmente, los animales resilientes también mostraron durante la primera DS una menor conducta de sumisión, aunque ya no observamos diferencias entre resilientes y susceptibles en la cuarta DS. Los ratones resilientes, al experimentar que sus conductas de afrontamiento no reducen la intensidad del ataque, presentan una adaptación conductual. La flexibilidad de las estrategias de afrontamiento se ha asociado con indicadores de resiliencia emocional, como una menor reactividad del eje HHA y un aumento de la neuroplasticidad (Hawley et al., 2010, Lambert et al., 2014). Por lo tanto, nuestros resultados indican que un afrontamiento activo y una adecuada adaptación del mismo reducen los efectos reforzantes de la cocaína. En apoyo a nuestros resultados, otros estudios también han confirmado que los ratones que no presentan estrategias de afrontamiento pasivo como la huida, muestran menor anhedonia (Wood et al., 2015), menor ansiedad y mayor interacción social (Duclot et al., 2011; Hollis et al., 2011; Kumar et al., 2014). Los animales resilientes también presentaron conductas de ataque contra los residentes durante el primer enfrentamiento, habiéndose asociado esta estrategia de afrontamiento activo con la resistencia a la derrota (Finnell & Wood, 2016).

En resumen, los animales resilientes desarrollan una estrategia de afrontamiento del estrés activa, ya que atacan al residente y tardan más en asumir que han sido

derrotados. Esta resistencia puede hacer que los resilientes no experimenten con tanta intensidad la DS como sí lo hacen los animales susceptibles. Se ha observado que los ratones que emplean conductas de afrontamiento activo durante la DS muestran niveles de corticosterona plasmática más baja, mayor capacidad de respuesta noradrenérgica durante el estrés y una mayor actividad simpática en respuesta a la derrota (Wood et al., 2010; Gómez-Lázaro et al., 2011; Pérez-Tejada et al., 2013). Este tipo de respuesta es muy adaptativa, ya que permite limitar la respuesta al estrés (Koolhaas et al., 2011). Otro factor que puede explicar el desarrollo de resiliencia es la sensación de control durante la DS, ya que los ratones resilientes no huyen del agresor e incluso muestran conductas de ataque. Curiosamente, el consumo de cocaína solo se observa incrementado en los ratones intrusos, pero no en los residentes que inician el ataque, aunque en ambos tipos de animales se produce una respuesta hormonal al estrés (Covington & Miczek, 2001, 2005; Covington et al., 2005; Boyson et al., 2014). El ratón residente mantiene el control del encuentro, lo que puede estar ejerciendo un papel protector sobre la respuesta al estrés del eje hipotálamo hipofiso adrenal (Boyson et al., 2014). Por lo tanto, nuestros animales resilientes pueden experimentar un cierto control de la situación de estrés.

Contrariamente, los animales susceptibles mostraron un afrontamiento pasivo, aceptando la derrota con mayor tiempo en huida y sumisión, sin presentar ninguna conducta agresiva hacia el residente. Este afrontamiento pasivo durante la DS se ha asociado previamente con la aparición de ansiedad y depresión (Wood et al., 2010, Chen et al., 2015, Pearson-Leary et al., 2017).

4.3. Respuesta de neuroinflamación tras las DS repetida

En los años 90 se esbozó la llamada teoría neuroinflamatoria de la depresión (por ejemplo, Maes et al., 2009), basándose en el incremento de mediadores inflamatorios en pacientes con depresión. En la actualidad existen numerosos estudios que demuestran el papel del sistema inmune en la vulnerabilidad al desarrollo de enfermedad mental (Réus et al., 2015: Menard et al., 2017). Igualmente se cree que el trastorno por uso de drogas se relaciona con cambios en la actividad del sistema inmunitario (Clark et al., 2013; Cui et al., 2014). Estudios tanto clínicos como preclínicos han demostrado que los psicoestimulantes como la cocaína activan componentes centrales y periféricos del sistema inmune (Clark et al., 2013; Araos et al., 2015; Moreira et al., 2016). Más recientemente también se ha demostrado que el estrés social induce una activación del sistema inmune, incrementando los niveles periféricos de citoquinas, activando la microglía o incluso incrementando la permeabilidad de la barrera hematoencefálica (Pfau & Russo, 2016; Rodríguez-Arias et al., 2017, 2018; Ferrer-Pérez et al., 2018a).

Se ha descrito que, tras la DS, los ratones susceptibles que desarrollan aislamiento social y ansiedad muestran niveles más altos de IL-6 que aquellos animales resilientes (Hodes et al., 2016). Sin embargo, nuestros resultados no confirman esta menor respuesta inflamatoria en animales resilientes. Los niveles de Il-6 no fueron más altos en los animales derrotados comparados con los controles, y tampoco se observaron diferencias entre resilientes y susceptibles. La discrepancia en los resultados puede deberse fundamentalmente a que en el estudio de Hodes et al. (2016), la medición de la IL-6 se realizó 24h después de la última DS, sin embargo, en nuestro estudio se realizó al final de todo el procedimiento, cuando ya había pasado más de un mes desde la última DS repetida. En esta misma línea ya habíamos demostrado previamente que tras el CPL ya no se observaban incrementos en los niveles estriatales de IL-6 en los animales derrotados (Ferrer-Pérez et al., 2018a).

Dado que la caracterización de los animales en resilientes o susceptibles requiere del desarrollo del CPL, nuestro diseño experimental implica que las mediciones se realicen al menos 4 semanas tras la última DS. Por lo tanto, nuestros resultados indican que un mes después de la última DS no existen deferencias en la respuesta neuroinflamatoria.

Los modelos animales son una herramienta muy potente, pero debemos ser prudentes a la hora de trasladar los resultados obtenidos a la conducta humana. El modelo de DS puede extrapolarse como situaciones de estrés psicológico o social, a las que estamos expuestos durante gran parte de nuestra vida. Nuestros resultados permiten la identificación de algunas características conductuales que aparecen en animales resilientes a esta DS, que pueden actuar como factor de protección frente al desarrollo de adicción a drogas. Un afrontamiento activo, pero al mismo tiempo flexible se destaca como la característica conductual más relevante de los sujetos resilientes. El estudio de las estrategias conductuales o farmacológicas que subyacen a la resiliencia nos permitirá disminuir la vulnerabilidad al TUS inducido por el estrés social.

5. Reconocimientos:

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Instituto de Salud Carlos III, Red de Trastornos Adictivos (RD16/0017/0007) y Unión Europea, Fondos FEDER “una manera de hacer Europa”.

6. Conflicto de intereses: Los autores declaran no tener ningún conflicto de intereses.

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Estudio 2

A limited and intermittent access to a high-fat diet modulates the effects of cocaine-induced reinstatement in the Conditioned Place Preference in male and female mice.

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(Anexo 2)

A limited and intermittent access to a high-fat diet modulates the effects of cocaine-induced reinstatement in the Conditioned Place Preference in male and female mice

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Abstract

Rationale: Palatable food and drugs of abuse activate common neurobiological pathways and numerous studies suggest that fat consumption increases vulnerability to drug abuse. In addition, preclinical reports show that palatable food may relieve craving for drugs, showing that an ad libitum access to a high-fat diet (HFD) can reduce cocaine-induced reinstatement.

Objective: The main aim of the present study was to evaluate the effect of a limited and intermittent exposure to HFD administered during the extinction and reinstatement processes of a cocaine-induced Conditioned Place Preference (CPP).

Methods: Male and female mice underwent the 10mg/kg cocaine CPP. From post-conditioning onwards, animals were divided into four groups: SD (Standard Diet), HFD-MWF with 2h access to the HFD on Mondays, Wednesdays and Fridays; HFD-24h, with 1h access every day and HFD-Ext with 1h access to the HFD before each extinction session.

Results: Our results showed that all HFD administrations blocked reinstatement in males, while only the HFD-MWF was able to inhibit reinstatement in females. In addition, HFD-Ext males needed fewer sessions to extinguish the preference, which suggests that administration of fat before being exposed to the environmental cues is effective to extinguish drug-related memories. HFD did not affect Oprμ gene expression but increased CB1r gene expression in the striatum in HFD-Ext males.

Conclusions: These results support that palatable food could act as an alternative reward to cocaine, accelerating extinction and blocking reinstatement, these effects being sex specific.

Keywords

Extinction; cocaine; reinstatement; high-fat diet; conditioned place preference

1. Introduction

Drug addiction is defined as a chronic disorder characterized by relapse accompanied by the compulsion to seek and take the drug and the loss of control in limiting intake (Koob and Le Moal 1997). When access to the drug is prevented, a negative emotional state emerges, reflecting a motivational withdrawal syndrome (Koob and Volkow 2010). Due to this negative emotional state, drugs of abuse become negative reinforcement during withdrawal (Koob and Le Moal 2001), leading to relapse due to the impelling need to consume (Koob and Volkow 2010). Therefore, it is not surprising that patients who undergo an addiction treatment tend to seek alternative reinforcements, including natural rewards, such as palatable food (high-fat and/or sugar-rich food), in order to stimulate brain circuits of reward (Salamone et al. 2005). In fact, clinical evidence emphasizes the frequent use of palatable food to decrease drug craving during withdrawal (Cowan and Devine 2008).

Preclinical studies have also pointed to this relation. Orsini and coworkers (2014) reported that rats with a history of chronic amphetamine exposure increased their food consumption. In addition, Loebens and Barros (2003) observed that animals fed with a high-fat diet (HFD) are more prone to depression during cocaine withdrawal in the forced swimming test (Loebens and Barros 2003). Moreover, other studies suggest that fat intake may represent a competitive reward for drugs. The conditioned place preference (CPP) procedure evaluates the role of environmental cues associated with the rewarding effects of drugs of abuse, such as cocaine, which were decreased by previous exposure to HFD (Morales et al. 2012). In this line, the present work is a follow-up of our previous study reporting that continuous HFD administration during the extinction of cocaine-induced CPP reduced the sessions required to extinguish the preference and decreased the sensitivity to drug priming-induced reinstatement (Blanco-Gandía et al. 2017a).

Epidemiological studies have shown high levels of comorbidity between eating disorders (bulimia and binge eating disorder) and substance abuse (Becker and Grilo 2016; Conason et al. 2006; Holderness et al. 1994; Nøkleby 2012; Flores-Fresco et al. 2018). We speculate that this high comorbidity could in part be due to shared reinforcing properties between palatable foods and drugs of abuse. For example, certain foods, particularly those rich in sugar and fat, are potent rewards that promote eating even in the absence of energetic requirements (Lenoir et al. 2007), being able to trigger learned associations between environmental stimulus and reward (Volkow et al. 2011). In fact, several studies have pointed out that a continuous access to fat diminishes the rewarding effects of cocaine (Morales et al. 2012; Thanos et al. 2010; Blanco-Gandía et al. 2017a). Palatable food activates the reward system (DiLeone et al. 2012; Narayanaswami et al. 2013) through the activation of the mu-opioid receptor pathway in the VTA (Pitman and Borgland 2015) and the cannabinoid system (Parylak et al. 2012).

The present work relates to the idea of palatable food as an alternative reward. In previous studies, a continuous high-fat diet produced harmful metabolic effects (Blanco-Gandía et al. 2017b). Here we aim to study if an intermittent and limited access to a HFD could also diminish cocaine-associated memories without causing changes on bodyweight or metabolism, proving that a sporadic exposure could be sufficient to extinguish the preference and block reinstatement. Our study will be the first to evaluate the possible counteracting effects of intermittent and limited access to a HFD on the extinction and reinstatement of cocaine-induced CPP. Our general hypothesis is that the limited access to a HFD will accelerate the extinction of cocaine-associated memories and will reduce cocaine-priming reinstatement. To assess metabolic disturbances, we will measure bodyweight and leptin and ghrelin changes after HFD exposure. Because the opioid and cannabinoid systems play a crucial role in food and drug reward (de Macedo et al. 2016), we also evaluated the

effects of HFD administrations on the opioid mu receptor (Oprμ) and CB1 receptor gene expression (CB1r) in the striatum. Oprμ and CB1r are implicated in food reward processes and palatability (Kessler et al., 2016; Bello et al, 2014), as well as in various forms of learning and memory, including acquisition and reinstatement of cocaine-associated memory (Sticht et al., 2015; Hu et al., 2015). Given that sex differences in the vulnerability to drug seeking have been scarcely studied (Carroll et al. 2002), we will conduct our study in both male and female mice. For example, several data suggest a more intense response to cocaine in female rodents, with more psychomotor sensitization (Holly et al. 2012), faster acquisition of cocaine self-administration (Martini et al. 2014), and higher a breaking point of the progressive ratio and cocaine reinstatement (Lynch 2006). Sex-specific differences have also been described in response to HFD, with male rodents being more susceptible to physiological changes (Grove et al. 2010; Mela et al. 2012; Wang et al. 2017; Gelineau 2017).

2. Material and Methods

2.1. Subjects

A total of 60 female and 47 male mice of the OF1 outbred strain were acquired commercially from Charles River (Barcelona, Spain). Animals were 42 days old when they arrived at the laboratory and were all housed under standard conditions in groups of 4-5 (cage size 28 x 28 x 14.5 cm). Mice were exposed to a reverse light cycle (white lights on from 19:30-7:30), and the vivarium was controlled for constant temperature (21 ± 2ºC). Food (standard diet) and water were available *ab libitum* except during the behavioral tests. All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The

Animal Use and Care Committee of the University of Valencia approved the present study.

2.2. Drug treatment

For CPP, animals were injected intraperitoneally (IP) with 10 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S.A., Madrid, Spain) diluted in 0.9% NaCl (saline) in a volume of 0.1mL/10 g bodyweight.

2.3. Experimental design

Animals first underwent the 10 mg/kg cocaine-induced CPP procedure from post-natal day (PND) 43. From PND 53 onwards, all mice were, from this moment on, exposed twice a week to extinction sessions (Table 1). Female and male mice were randomly divided into four groups: Standard Diet (SD), Daily high-fat diet (HFD-24h), Monday, Wednesday and Friday high-fat diet (HFD-MWF) and high-fat diet 1h before Extinction (HFD-Ext).

Table 1

Experimental Design. Abbreviations: PND, postnatal days; HFD, high-fat diet

			PND 53-Onwards				
	Male	Female	Monday	Tuesday	Wednesday	Thursday	Friday
SD	n=12	n=15	Standard diet	Standard diet	Standard diet	Standard diet	Standard diet
HFD-24h	n=11	n=15	HFD 1h	HFD 1h	HFD 1h	HFD 1h	HFD 1h
HFD-MWF	n=12	n=15	HFD 2h		HFD 2h		HFD 2h
HFD-Ext	n=12	n=15	HFD 1h before EXT			HFD 1h before EXT	

2.4. Apparatus and Procedure

2.4.1. Conditioned Place Preference

For Place Conditioning, we employed sixteen identical Plexiglas boxes with two equally sized compartments (30.7 cm length x 31.5 cm width x 34.5 cm height) separated by a grey central area (13.8 cm length x 31.5 cm width x 34.5 cm height). The compartments have different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossing from one compartment to the other. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

Acquisition of CPP

The procedure of Place Conditioning, unbiased in terms of initial spontaneous preference, was performed as described previously (Maldonado et al. 2006) and consisted in three phases. To summarize the main aspects, in the first phase, known as Pre-C, mice were allowed access to both compartments of the apparatus for 15 min (900 s) per day for 3 days. On day 3, the time spent in each compartment over a 900-s period was recorded, and animals showing a strong unconditioned aversion (less than 33% of the session time) or preference (more than 67%) for any compartment were excluded for the rest of the experiment (total excluded: 4). Half of the animals in each group received the drug or vehicle in one compartment, and the other half in the other compartment. After assigning the compartments, no significant differences were detected between the time spent in the drug-paired and vehicle-paired compartments during the pre-conditioning phase. In the second phase (conditioning), which lasted 4 days, animals received an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30

min. After an interval of 4 hours, they received an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. Confinement was carried out in both cases by closing the guillotine door that separated the two compartments, making the central area inaccessible. During the third phase, known as Post-C, the guillotine door separating the two compartments was removed (day 8) and the time spent by the untreated mice in each compartment during a 900-s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates that an aversion has been developed.

Extinction of CPP

When the preference for the drug-paired compartment was established, mice underwent twice a week (Monday and Thursday) an extinction session that consisted of placing the animals in the apparatus (without the guillotine doors separating the compartments) for 15 min. The extinction condition was fulfilled when there was a significant difference between CPP scores and Post-C scores in two consecutive sessions and a lack of significant difference between CPP and Pre-C test values.

Reinstatement of CPP

Twenty-four hours after extinction had been confirmed, the effects of a priming dose of cocaine were evaluated. Reinstatement tests were the same as those carried out in Post-C (free ambulation for 15 min), except that animals were tested 15 min after administration of the respective dose of cocaine. When reinstatement of the preference was achieved, after a subsequent weekly extinction process, a new reinstatement test was conducted with progressively lower doses of the drug until

the CPP was completely extinguished. This procedure of extinction-reinstatement was repeated with decreasing doses (half the previous dose) until a priming dose was confirmed to be ineffective (5 mg/kg after extinction of CPP induced by 10 mg/kg; and 2.5 mg/kg after extinction if animals showed reinstatement with 5 mg/kg). HFD conditions were maintained until the end of the experiment. Priming injections were administered in the vivarium, which constituted a non-contingent place to that of the previous conditioning procedure.

2.4.2. Feeding conditions

After the Post-c test in the CPP, each group began their intermittent access to the different HFD patterns. Our feeding procedure is based on the limited access model described by Corwin et al. (1998), in which non-food-deprived animals have sporadic and limited access to a HFD. Two different types of diet were used in this study. A standard diet (Teklad Global Diet 2014, 13 Kcal % fat, 67 Kcal % carbohydrates, and 20 Kcal % protein; 2.9 kcal/g), and the HFD (TD.06415, 45 Kcal % fat, 36 Kcal % carbohydrates and 19% Kcal protein; 4.6 kcal/g). Both diets were supplied by Harlan Laboratories Models, S. L. (Barcelona, Spain) and will be referred to from now on as the standard diet and the high-fat diet (HFD).

As we described on Table 1, on postnatal day (PND) 53, mice were randomly divided into 4 groups of males and 4 groups of females with similar average bodyweights and assigned to one of the following conditions: Standard Diet (SD), Daily high-fat diet (HFD-24h), Monday, Wednesday and Friday high-fat diet (HFD-MWF) and high-fat diet before Extinction (HFD-Ext). All the groups had standard diet access during the whole procedure, and the HFD-24h group had a one-hour access to HFD from Monday to Friday; the HFD-MWF had a two-hour access to HFD on Monday, Wednesday and Friday and lastly, animals in the HFD-Ext had a one-hour HFD

access prior to each extinction session (twice a week). The SD group remained undisturbed in their home cage.

All groups were fed ad libitum with the standard diet (Teklad Global Diet 2014) in their own cages and water was freely available. After acquiring cocaine preference, during the extinction process animals continued with SD in their home cages, but only the animals in the HFD groups were taken for a limited time into a separated plastic cage with access to the HFD. Exposure to the HFD took place in different individual plastic cages 2-3 hours after the beginning of the dark phase. HFD intake was measured after each session. Animals were weighed every week throughout the study, at which point their intake of the standard diet in their home cage was also measured in grams.

2.4.3. Determination of plasma leptin and ghrelin levels

For leptin and ghrelin plasma quantification, an ELISA kit was employed for leptin and ghrelin (Merck-Sigma Aldrich, Saint Louis, USA) following the manufacturer's instructions. The sensitivity of the test is 0.2. All samples were run in duplicate.

2.4.4. Gene expression analyses: RNA isolation and quantitative RT-PCR

At the end of the experiments, animals were euthanized by cervical dislocation and the brains were immediately removed from the skull and placed on a cold plaque. Cerebellum and olfactory bulbs were eliminated and the striatum was dissected. Brain tissue samples were immediately stored at -80ºC until the rt-PCR assay was performed. The whole striatum was employed for this analysis.

Total RNA from the striatum was isolated using the Tri Reagent Method (Sigma-Aldrich, St. Louis, MO, USA), as described in the manufacturer’s protocol. Reverse

transcription of 1 mg of total RNA was performed using the Transcriptor First Strand cDNA synthesis kit (Thermo Fisher Scientific, Madrid, Spain). Amplification of the target and housekeeping (b-glucuronidase) genes was performed using the Taqman Gene Expression Master Mix (Thermo Fisher Scientific, Madrid, Spain) in a LightCycler 480 System (Roche Diagnostics) following the manufacturer's instructions. The assay codes of the primers used are Mm01212171_s1, Mm01188089_m1 and Mm00446953_m1 corresponding to CB1r, Oprμ and Gusb, respectively. Data were analyzed using the LightCycler 480 relative quantification software and were normalized to the amplification product of b-glucuronidase.

2.5. Statistics

To test for the acquisition of CPP, the time spent in the drug-paired compartment was analyzed with a (2)x2x4 mixed ANOVA. The two between factors were Sex (male and female) and Diet (SD, HFD-24h, HFD-MWF, and HFD-Ext). The within subjects factors tested the measurement differences between the pre- and post-CPP phases (Pre-C and Post-C). Since at this point of measurement all the animals had been maintained with the standard diet, the Diet factor simply functions as a control check for the effectiveness of the random assignment to produce equivalent groups prior to exposure to the extinction-reinstatement manipulation (i.e., the expectation is that there will not be an effect for Diet). To compare whether extinction/reinstatement is achieved within the same group, data related to extinction, 2.5 mg/kg reinstatement and 5 mg/kg reinstatement were analyzed by means of Student's t-test. The time required for the preference to be extinguished was analyzed by means of the Kaplan-Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate. The extinction analyses were performed within every group; therefore, no comparisons among groups were made. Ghrelin and leptin plasma levels and CB1r and Oprμ gene expression values were analyzed

by a one-way ANOVA, considering the between variable –Sex–, with two levels (male and female) and the between variable –Diet–, with 4 levels (SD, HFD-24h, HFD-MWF, HFD-Ext). All data are presented as mean ± standard error of mean (SEM). A p-value <0.05 was considered statistically significant. Analyses were performed using SPSS v26.

3. Results

Information relating to bodyweight and food intake can be found in the supplementary material S1. No bodyweight and food intake differences were found between the four diet groups within each sex group.

3.1. Conditioned place preference

Results of the 10 mg/kg cocaine-induced CPP are presented in Fig 1a (males) and 1b (females). Regarding the main effects, the ANOVA for the time spent in the drug-paired compartment revealed an effect of the variable Days [$F(1,99)=138,410$; $p<0.001$], which indicates that all animals developed conditioned place preference (Post-C vs Pre-C). Bonferroni's post-hoc comparisons showed that in all cases, males and females spent significantly more time in the drug-paired compartment in Post-C than in Pre-C ($p<0.001$ in all cases). No effect was found in the variables Sex nor Diet separately. Regarding the interactions, there was no significant effect in the three-way interaction Days x Sex x Diet, but the ANOVA did reveal a significant effect of the interaction Days x Sex [$F(1,99)=3,833$; $p<0.05$]. Bonferroni's post-hoc comparisons showed that female mice spent more time in the drug-paired compartment in Post-C than male mice ($p<0.05$), showing a stronger CPP but only in two groups: those that will received the HFD tree days a week (HFD-MWF) or previously to each extinction sessions (HFD-Ext).

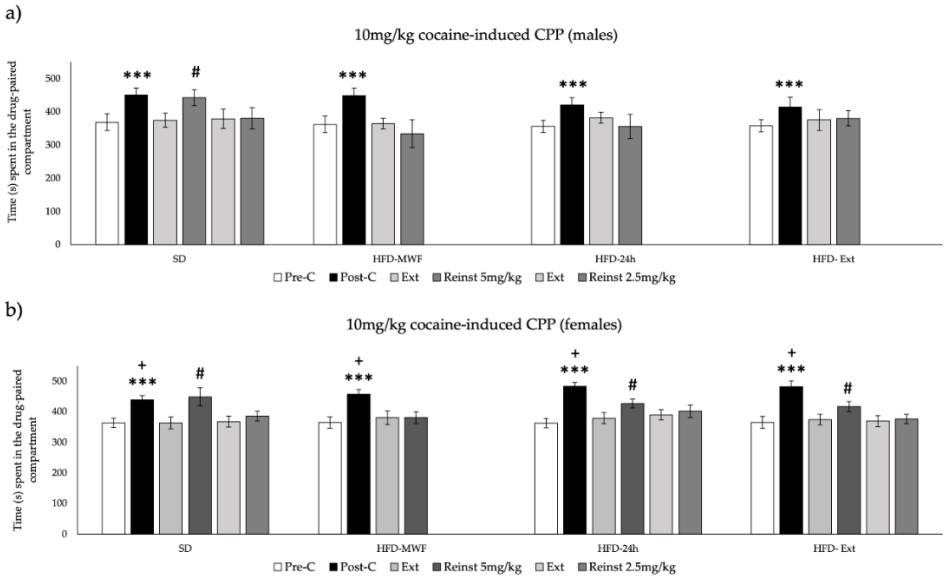

Fig 1 Effects of HFD during the extinction-reinstatement process in Conditioned place preference (CPP) in males **(a)** and females **(b)**. CPP was induced by 10 mg/kg of cocaine in male and female mice fed with a standard diet and exposed after Post-C to different dietary conditions: SD group (standard diet throughout the procedure), HFD-24h group (one hour access to HFD from Monday to Friday), in HFD-MWF group (two hours access to high fat diet on Monday, Wednesday and Friday) and in the HFD-Ext group (one hour high fat diet access twice a week, prior to extinction sessions). Bars represent the time (± SEM) in seconds spent in the drug-paired compartment in the pre-conditioning test (white bars), the post-conditioning test (black bars), in the last extinction session (light gray bars) and in the reinstatement test (dark gray bars). The first reinstatement test was evaluated 15 min after a priming dose of 5 mg/kg of cocaine, while the second reinstatement test was evaluated 15 min after a priming dose of 2.5 mg/kg of cocaine. ***$p<0.001$ significant difference with respect to the Pre-C (ANOVA); +$p<0.05$ significant difference in female Post-C vs male Post-C. #$p<0.05$ significant difference with respect to extinction (Student's t test).

To compare if there was a reinstatement into drug seeking after achieving extinction within the same group, Student's t-tests were performed. Reinstatement with a priming dose of 5 mg/kg cocaine (Fig 1a) was achieved in the male SD group (t=-2.772; d.f. 11; p=0.018) but after subsequent extinction sessions, the Student's t-test

showed that no reinstatement with a priming dose of 2.5 mg/kg cocaine was achieved (t=-0.078; d.f. 11; p=0.939). In females (Figure 1b), the Student's t-test showed reinstatement with a priming dose of 5 mg/kg cocaine in SD (t=-2.297; d.f. 14; p=0.038), HFD-24h (t=-2.529; d.f. 14; p=0.024) and HFD-Ext (t=-2.282; d.f. 14; p=0.039). Extinction was confirmed with the Student's t-test, where no group of males and females showed any differences with respect to the Pre-C test (Data not shown). After two subsequent extinction sessions, the Student's t-test showed that no reinstatement with a priming dose of 2.5 mg/kg cocaine was achieved in any group (SD: t=-1.241; d.f. 14; p=0.235; HFD-24h: t=-0.684; d.f. 14; p=0.505 and HFD-Ext: t=-0.303; d.f. 14; p=0.767).

With regards to the time required to extinguish the preference, we found that in males (Figure 2), the SD, HFD-MWF and HFD-24h groups required a total of 16, 21 and 17 sessions respectively to achieve extinction, while HFD-Ext required only 8 sessions to extinguish the preference. The Kaplan-Meier analysis showed that the HFD-Ext male group required significantly less time to achieve extinction than the SD group (χ^2=4.336; $p<0.05$), with no significant differences with respect to the other groups.

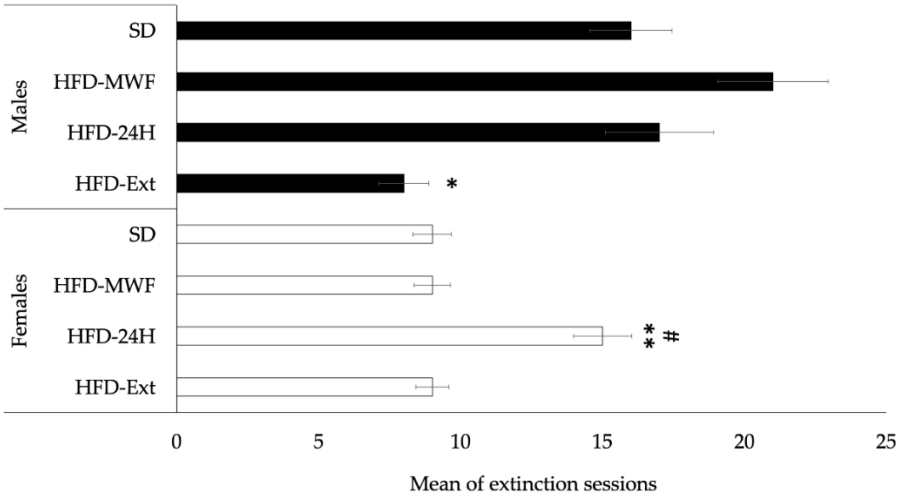

Fig 2 Mean of extinction sessions. The bars represent the mean value (± SEM) of the number of sessions required for the preference to be extinguished after the Post-C test. Preference was considered to be extinguished when an animal spent 370s or less in the drug-paired compartment on two consecutive days. When the preference was not extinguished in a mouse, the number of days needed to achieve extinction in the whole group was assigned to that animal. *p<0.05, **p<0.01 with respect to the respective Standard diet group (SD); #p<0.05 with respect to HFD-MWF and HFD-Ext.

In female groups, the SD, HFD-MWF and HFD-Ext groups required 9 sessions to achieve extinction, while the HFD-24h group required a total of 15 sessions to achieve extinction (Fig 2). The Kaplan-Meier analysis revealed that the HFD-24h group required significantly more time to achieve extinction than the SD group (χ^2=8.431; p<0.01), HFD-MWF (χ^2=.4.250; p<0.05) and HFD-Ext (χ^2=4.289; p<0.05).

3.2. Effects of different HFD eating patterns and sex on plasma leptin and ghrelin levels, CB1r and Oprμ gene expression

There were no significant effects of the different intermittent eating patterns or sex on circulating leptin [$F(3,56)=0.975$; $p=0.411$] and ghrelin levels [$F(3,56)=0.440$; $p=0.725$) (Table 2). Regarding the real-time PCR analyses, the ANOVA indicated in the CB1r gene expression (Fig 3a) an effect of the interaction Diet*Sex [$F(3,56)=3.340$; $p<0.05$]. There was an increased CB1r gene expression only in males of the HFD-Ext group with significant differences with respect to the SD group ($p<0.01$), HFD-MWF group ($p<0.01$) and HFD-24H group ($p<0.001$). Moreover, the HFD-Ext male group showed a significant increase against their corresponding HFD-Ext female group ($p<0.05$).

Table 2

Plasma Leptin (ng/ml) and Ghrelin (pg/ml) levels.

	Plasma Leptin (ng/ml)		**Plasma Ghrelin (pg/ml)**	
	Males	Females	Males	Females
SD	3.87 ± 0.49	3.64 ± 0.46	487 ± 51	356 ± 53
HFD-MWF	2.46 ± 0.77	4.03 ± 0.53	477 ± 78	471 ± 51
HFD-24h	3.07 ± 0.61	3.03 ± 0.70	475 ± 76	437 ± 60
HFD-Ext	3.01 ± 0.49	3.18 ± 0.79	482 ± 69	398 ± 50

Note. Data are presented as mean values ± SEM (n=8/condition)

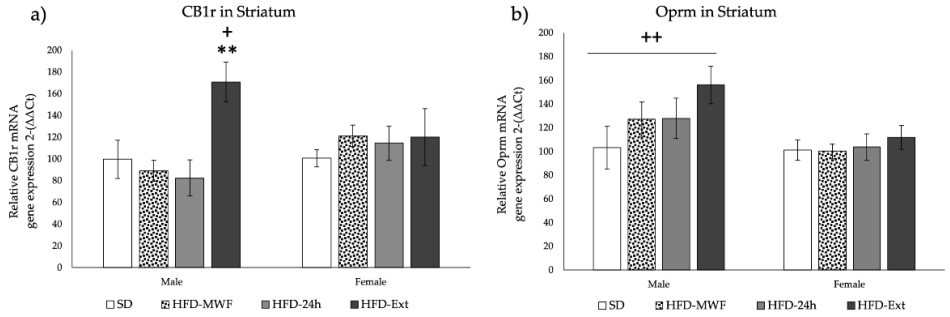

Fig 3 Real-time PCR Gene expression in the striatum. (a) CB1r relative gene expression evaluation in the striatum region (n = 8/condition). (b) Oprμ relative gene expression evaluation in the striatum region (n=8/condition). The columns represent means and the vertical lines ± SEM of relative (2-ΔΔCt method) gene expression in the striatum of OF1 mice. **p<0.01 significant differences with respect to the rest of the groups. +p<0.05; ++p<0.01 significant differences with respect to their corresponding female group.

Regarding Oprμ gene expression (Figure 3b), the ANOVA revealed a significant effect in the variable Sex [F(1,55)=7,604; p<0.01], as overall, HFD male mice overexpressed Oprμ with respect to females (p<0.01).

4. Discussion

The present study noticed that the intermittent and limited access to a HFD may prevent reinstatement of the conditioned rewarding effects of cocaine in males and specific administration schedules can facilitate or disrupt the extinction of the cocaine associated memories in males and females. Related to this, this study highlights the important role of sex in these effects, since there were great differences between the results obtained in male and female mice. Once 10mg/kg cocaine induced CPP was acquired, all the mice were exposed to a different intermittent administration of HFD. The main results of the present work indicate that in males, exposure to the HFD in any of the three patterns employed blocked the reinstatement of the preference with a priming dose of 5 mg/kg of cocaine. However, in female mice only one HFD administration (HFD-MWF) was effective in blocking the

reinstatement of cocaine-induced preference. Extinction of the memories associated with cocaine was faster when access to the HFD was prior to each extinction session, but this phenomenon occurred only in males.

As we mentioned in the Introduction, this is a follow-up of a previous study where we confirmed that a continuous HFD administration during cocaine withdrawal undermined reinstatement of cocaine-induced CPP, thereby acting as an alternative reward. However, that administration pattern induced several metabolic consequences (Blanco-Gandía et al. 2017a). Therefore, the aim of the present study was to evaluate a reduced administration pattern of HFD that could have effects on cocaine reward without inducing metabolic disturbances, such as increased bodyweight or changes in circulating leptin or ghrelin levels. We have shown that the metabolic harm induced by HFD is not necessary to disrupt the extinction and reinstatement of the CPP induced by cocaine, given that a limited and intermittent exposure was sufficient to block cocaine-related effects in a sex-specific manner. Although the HFD did not modify these parameters or affect the Oprμ gene expression, we found changes in the CB1r gene expression in the striatum of male animals. Surprisingly, we observed that only males that showed a faster extinction of cocaine-related memories (HFD-Ext) exhibited an overexpression in CB1r.

Conditioned rewarding effects of cocaine

Most of the studies performed to date focus on the role of palatable food on the acquisition of the self-administration/CPP of drugs (Davis et al. 2008; Morales et al. 2012, Blanco-Gandía et al. 2017a, 2017b). Prevention of the reinstatement of cocaine self-administration in rats has been obtained by pairing cocaine-related stimuli with food (Kearns and Weiss 2007). In that study, one of the groups was submitted to extinction by pairing a tone with the presentation of food in a different context. When this group was exposed to the tone in the original context, their renewal was

significantly lower than that observed in the group exposed to the tone but without alternative reward to cocaine. Kearns and Weiss (2007) suggested that pairing a drug-related stimuli such as a tone, to another reward different from the drug enhances the reduction of the reinstatement into drug seeking and prevents relapse. To date, no data regarding food administration before being exposed to a drug-related context are available. In our study, we evaluated for the first time how intermittent access to HFD may modulate the extinction/reinstatement process.

We have previously shown that ad libitum access to a HFD accelerated the extinction process and blocked the reinstatement of cocaine seeking in adult mice (Blanco-Gandía et al. 2017a). However, an important weakness of that study was that continuous access to HFD induced bodyweight gain and dysregulation of hormone levels, with an increase in leptin and a decrease in ghrelin signaling, pointing to a metabolic syndrome and discarding the possibility of employing palatable food as an alternative reward to cocaine. However, in the present study, none of the intermittent and limited access to HFD schedules employed affected bodyweight, confirming that limited access to a HFD does not promote obesity (Corwin et al. 1998; Hudson et al. 2007; Blanco-Gandía et al. 2017b). In addition, there were no changes in leptin or ghrelin signaling in any of the groups, independently of HFD administration. These results confirmed previous studies (Blanco-Gandía et al. 2017b), showing that intermittent access to palatable food does not induce the negative consequences that were observed after ad libitum access (Davis et al. 2008; Morales et al. 2012; Blanco-Gandía et al. 2017a).

In agreement with previous reports indicating sex differences in the response to cocaine, we have observed a marked sex difference in the establishment of cocaine-induced CPP. Female mice spent more time in the drug-paired compartment during Post-C than males, suggesting a stronger sensitivity to the rewarding effects of

cocaine. In line with our results, female rodents exhibit an increased sensitization to the locomotor effects of cocaine (Holly et al. 2012), faster acquisition of cocaine self-administration with persistence in the progressive ratio schedule (Martini et al. 2014; Lynch 2006), and develop cocaine-induced CPP with less pairing sessions than males (Russo et al. 2010).

However, after Post-C, female mice took less time to extinguish the preference than males, which suggests that cocaine-related memories are stronger in males. These results are in the line with previous studies showing that female mice require fewer extinction sessions than males to extinguish the CPP (Hilderbrand and Lasek 2014), and that estradiol administration facilitates extinction of cocaine CPP in female rats (Twining et al 2013). However, no positive effects of the diet on the extinction process were observed in females, conversely to the results in males. Male mice exposed to HFD prior to each extinction session (HFD-Ext) exhibited a faster extinction of the drug-related memories. The HFD-Ext male group required significantly fewer sessions than the control group as well as fewer sessions than the HFD-MWF and HFD-24h groups. This effect could be related to the administration of HFD before every exposure to the context associated with cocaine reward. This is supported by previous data, where it has been suggested that pairing food with the old contextual cues related to the drug can prevent relapse (Kearns and Weiss, 2007). An additional explanation is that the more rapid extinction in the HFD-Ext male group could be due to satiety and not be specific to HFD, as some studies have found that caloric restriction lowers the threshold dose for cocaine CPP and increases the persistence of extinction (Zheng et al., 2012; Jung et al., 2016). Thus, we cannot rule out that satiation in this group modulates the extinction process.

Hence, it can be argued that in order to diminish drug seeking in male mice, the moment in which HFD is administered plays a key role. Extinction recruits a new

learning process (Lattal et al. 2006; Nic Dhonnchadha et al. 2013), especially in the hippocampus, which is particularly involved in the extinction of drug-associated memories (Szalay et al. 2013). Exposure to fat before each extinction session became a stimulus that predicted a non-reinforced context without cocaine and therefore accelerated the extinction of the preference. This effect is clear in males (HFD-Ext), but not in females, maybe due to their faster extinction of the preference. It is important to note that several studies have shown that HFDs lead to impairments in cognitive function such as memory or learning (Hwang et al., 2010; Valladolid-Acebes et al., 2013). In a previous study from our laboratory, we evaluated if a limited and intermittent access (exposed to the same conditions as the HFD-MWF group in this study) produced comparable impairments as the continuous access to a HFD (Blanco-Gandía et al., 2019). Our results showed that animals exposed to a limited and intermittent access to HFD showed no differences with respect to animals fed with a standard diet, which supports that the data obtained in the male HFD-Ext group are not due to a recall impairment.

As expected, 5mg/kg of cocaine induced reinstatement in male and female mice fed with the standard diet. All three of the HFD administrations were effective in males, which did not reinstate their preference after a priming dose of cocaine. With a similar or lower number of extinction sessions than the control group, none of the male groups exposed to HFD reinstated the preference, suggesting that intermittent and limited HFD administration is a good alternative reinforcer. Therefore, the fact that the HFD blocked the reinstatement of preference in males, in all the schedules employed, independently of its administration schedule, suggests that fat could also act as an alternative reinforcer competing with cocaine.

Different results were observed in female mice, with only one group not reinstating the preference with 5mg/kg cocaine, the female HFD-MWF group. The sex

differences found in the response to HFD could be explained through the fact that female rats exhibited a higher cocaine priming-induced reinstatement response than males (Lynch 2006) with a greater magnitude of reinstatement to cocaine-induced CPP (Bobzean et al. 2010). Several studies have suggested that the neural systems mediating cocaine reinforcement could also show sex differences, with dopamine response induced by cocaine in several brain areas being greater in female (Becker 1999; Becker and Ramirez 1981; Walker et al. 2001). Results even showed that this higher dopaminergic sensitivity in females could be independent of gonadal hormones (Bazzett and Becker 1994, Castner et al. 1993, McDermott et al. 1994). However, the remaining question is why only the HFD-MWF pattern was a protective pattern to reinstate cocaine preference in females. Firstly, it was only in this group where females ate significantly more fatty food. Secondly, we have previously shown that administration for several weeks is a risky pattern that induces neurobiological changes similar to those produced by chronic drug administration and could interfere in the reward system (Blanco-García et al. 2017a; Corwin et al. 1998; Puhl et al. 2011). We can hypothesize that HFD-MWF females were protected from reinstatement into cocaine seeking because they may have developed another preference for HFD. Some studies have reported that, after drug withdrawal, there is increased overeating, and it is even recommended to counteract craving (Bane et al. 1993; Orsini et al. 2014). In this context, authors propose the concept of 'addiction transfer', where one addiction is replaced by another, and could explain the behavioral outcomes exhibited by the HFD-MWF female group (Chechlacz et al. 2009).

Based on our results, we hypothesize that the administration of a natural reward, such as food, was not enough to block the potent effect of cocaine on female mice. However, in the case of males, who acquired the preference with less intensity than females, HFD became a good alternative reinforcer. Although preclinical studies are

limited, several reports show that drug withdrawal induces an increase in food consumption. Orsini and coworkers (2014) showed that chronic exposure to amphetamine (9 injections) increased food consumption in male rats after cessation, discarding the possibility of a rebound from amphetamine-induced anorexia, as all the animals, control and amphetamine-treated, weighted the same when withdrawal began. In the same line, there is a reduction in the rewarding properties of drugs when a HFD is administered. For example, Wellman and coworkers (2007) demonstrated that a free access to HFD for 45 days diminished the acquisition of cocaine self-administration in male rats. Other findings on female rats showed that, after a 14-day exposure to cocaine, a specific increase in fat and carbohydrate consumption occurred, which was not seen in protein consumption (Bane et al. 1993). Most of these studies have been performed in male rodents, and our results highlighted the necessity of studying the response to palatable food in females as well as the limitation of the abovementioned results.

The different schedules of intermittent accesses to the HFD caused animals to receive a different number of rewarding experiences, which can be considered a limitation of this study. Those groups needing more sessions to extinguish the preference consequently were exposed to a higher number of fat administrations. However, a lack of reinstatement did not correlate to the number of HFD sessions in male or female mice. In addition, another important limitation to take into account in future studies is the variety of manipulations per week during the extinction process, given that, during this period, the SD groups were only moved to perform the extinction sessions, while the HFD groups were also moved for HFD access.

Neurobiology changes induced by intermittent and limited access to HFD

The endocannabinoid and the endogenous opioid systems are crucial in the addiction process and regulate feeding behaviors (Kessler et al. 2016; Bello et al. 2014).

Opioid signaling is closely related to the rewarding properties of food, regulating palatability (Esch and Stefano 2004). For example, a continuous access to a HFD induces a significant reduction in Oprμ gene expression in the VTA (Blendy et al. 2005; Vucetic et al. 2011). Moreover, some studies point that CB1 activation in the NAcc and VTA modulates both dopaminergic and opioidergic pathways (Mellis et al. 2007). CB1 receptor antagonists are capable of reducing binge-eating and mediate the extracellular dopamine release produced by a HFD (Parylak et al. 2012; Mellis et al. 2007). All these results support the idea that intermittent fat ingestion can modify reward pathways through different systems such as the opioid and cannabinoid systems. While the endogenous opioid system is related to the hedonic properties of food and modulates the release of DA-anticipating food, the endocannabinoid system is related to the homeostatic control of intake and positive feedback on the specific intake of fatty food (Koch 2001). In contrast with the numerous changes in gene expression induced by ad libitum administration of HFD (Blanco-Gandia et al. 2017a), the intermittent and the limited access to HFD during the extinction process practically did not induce variations. HFD administration did not induce any changes in the Oprμ gene expression, with the exception of a higher expression in males with respect to females. Results from previous studies are still controversial and depend on many factors, as some studies have reported that intermittent access to HFD for several weeks decreases mRNA expression of the Oprμ receptor in the NAcc in male mice (Blanco-Gandía et al. 2017b), while others reported the same reduction after a continuous access to a HFD or cafeteria diet in male mice (Ong et al. 2013; Vucetic et al. 2011) with no changes in females (Ong et al. 2013). The present results do not confirm that HFD administration changes Oprm gene expression, since we only found differences between sexes but not due to HFD intake. A previous study by Kawahara and co-workers (2013) showed that consumption of palatable food increased DA release in the NAcc via activation of the Oprm pathway in the VTA, and it is well known that this projection has an

influence on learning or performance of behaviors based on drug reward (Koob and LeMoal 2005). Regarding CPP studies, the Oprm antagonist naloxone was effective in blocking the reacquisition of a cocaine-induced CPP (Sticht et al., 2010). Although there are very few studies that focus on this relationship, this could account for the difference between males and females, indicating that male mice are modifying their learning processes during extinctions more efficiently than females.

Although HFD did not induce any changes in CB1r gene expression in females, an increase was detected in the HFD-Ext male group. Some studies have reported that HFD upregulates endocannabinoid levels (Massa et al. 2010; Higuchi et al. 2012) and that CB1 antagonists reduce binge eating (Parylak et al. 2012). We have only observed this upregulation in the HFD-Ext group, which could be related to the time required to extinguish the preference and thus, males in this group could have quickly learned to notice the absence of the conditioned drug in the CPP. This would support that CB1 receptors are modulating the extinction and reinstatement processes, as previous studies show that CB1 antagonists such as Rimonabant or AM251 are able to block cocaine and morphine extinction and CPP reinstatement (Yu et al., 2011; Khaleghzadeh-Ahangar and Haghparast, 2015). A limitation of the present work is in the analysis of the complete striatum without differentiating the ventral from the dorsal part, which could have offered more specific changes.

5. Conclusion

The results of the present work support our initial hypothesis, showing that palatable food could act as an alternative reward to cocaine by decreasing its conditioned rewarding memories and blocking cocaine priming-induced reinstatement. These effects are conditioned by sex, as females were less sensitive to the protective action of HFD. Taken together, the present results point out that a sporadic access to HFD

stimulates the same pathways activated by drugs, as well as decreasing craving and drug-related memories.

Currently, no controlled studies in humans have been performed to test the role of palatable food in attenuating drug withdrawal. It is well known that palatable food is generally used as self-medication to escape from a negative mood state or stressful situations (Groesz et al. 2012; Ifland et al. 2009; Kim et al. 2013). In 1989, Hatcher reported that substances such as sugar, aspartame, chocolate and nutritional supplements were compulsively used by patients in rehabilitation centers (Hatcher 1989). Several reports highlighted that during drug withdrawal and abstinence, overeating is a well-known problem in rehabilitation centers (Edge and Gold 2011). For example, Cowan and Devine (2008) found that patients at different stages of recovery from substance addictions experienced an increase in food intake, weight changes, and used food in recovery. Overeating and post-addiction obesity is so common that most abstinence-oriented drug treatment programs schedule diet counseling and mandatory exercise programs (Cowan and Devine 2008). Our results support these clinical reports and suggest that, acting as an alternative reward, palatable food may attenuate drug craving and the vulnerability to relapse. In particular, a more effective reduction in the extinction of drug-related memories occurs when a HFD is consumed temporally close to a drug-related cue exposure.

Contrary to an ad libitum access, our feeding conditions do not have significant consequences on bodyweight or hormonal levels, with minimal changes in opioid and cannabinoid receptors gene expression. These results indicate that it would be beneficial to introduce the intermittent use of palatable food as a “rehabilitation-tool” in drug treatment programs, allowing patients to eat it when drug craving is happening. The translational value of this study relies on the lack of metabolic adverse effects of any of the three HFD administrations. However, and no less

important, this treatment was significantly less effective in females, which indicates the necessity of conducting more studies in females, since we cannot assume that environmental or nutritional interventions are equally effective in both sexes.

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Conflicts of interest

All authors declare that they have no competing interests.

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Supplementary material.

Journal: Psychopharmacology

A limited and intermittent access to a high-fat diet modulates the effects of cocaine-induced reinstatement in the Conditioned Place Preference in male and female mice

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2. Material and Methods

2.5. Statistics

Statistical comparisons between all the groups were only calculated in the weeks where they matched to the corresponding extinction-reinstatement sessions, as there were groups that extinguished earlier than others and did not participate in the analyses of subsequent weeks. A previous ANOVA of bodyweight and intermittent access showed a significant effect of the variable Sex, but not in home cage food intake. Consequently, two separate ANOVAs were performed for male and female mice. Data related to bodyweight were analyzed by a mixed ANOVA, with the between variable -Diet-, with 4 levels (SD, HFD-24h, HFD-MWF, HFD-Ext)- and a within variable -Week-, with 6 levels for males (weeks 1 to 6) and 4 levels for females (weeks 1 to 4). Data related to the intermittent access of fat were analyzed identical to the bodyweight's data, except that the SD group was not considered in the between variable Diet, as that group was not exposed to the intermittent access to fat. As the initial analysis revealed no significant influence of Sex, data related to home cage food intake were analyzed by a three-way repeated measures ANOVA, considering the between variable –Sex–, with two levels (male and female), the between variable –Diet–, with 4 levels (SD, HFD-24h, HFD-MWF, HFD-Ext) and a within variable -Week-, with 4 levels (weeks 1 to 4).

3. Results

3.1. Bodyweight, food intake and intermittent access to HFD

An initial three-way ANOVA (Sex, Diet and Weeks) for the bodyweight in all mice revealed an effect of the variable Sex [$F(1,99)=555.928$; $p<0.001$] as male mice exhibited greater bodyweight than female mice ($p<0.001$). When comparing

bodyweights separated by sex, and considering only the two variables Diet and Weeks (Fig S1a and S1b), the ANOVA revealed an effect of the variable Week in both males [$F(5,215)=218.049$; $p<0.001$] and females [$F(5,280)=160.647$; $p<0.001$] showing that all mice increased their bodyweight throughout the experiment with no difference between groups. No significant differences were found on the variable Diet.

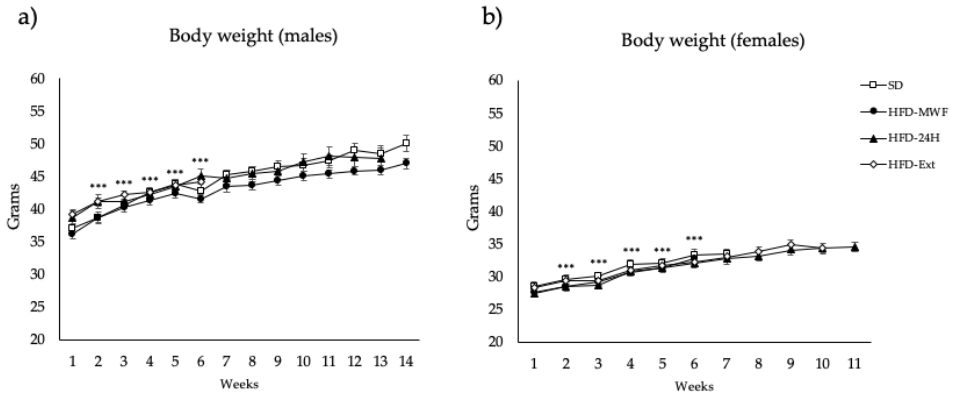

Fig S1 Increase of bodyweight of male (a) and female (b) animals in the SD group (standard diet during all the procedure), HFD-24h group (one hour access to HFD from Monday to Friday), in HFD-MWF group (two hours access to HFD on Monday, Wednesday and Friday) and in the HFD-Ext group (one hour HFD access twice a week, prior to extinction sessions). Data are represented as the mean (±SEM) amount of bodyweight measured weekly. Only data included in the ANOVA comparisons are represented with symbols of significance. No difference was found between groups. ***$p<0.001$ significant difference with respect to week 1.

With respect to the daily standard diet food intake (Fig S2a and S2b), the repeated measures ANOVA confirmed that no significant effect was found on the variable Sex [$F(1,16)=1.570$; $p=0.228$], Diet [$F(3,16)=3.172$; $p=0.053$] and Week

[F(3,48)=0.828; p=0.485]. There was also a significant interaction between the three factors (Sex x Diet x Week) [F(9,48)=2.345; p=0.028). Bonferroni post-hoc comparisons revealed that within the HFD-Ext groups, males showed an increased intake in comparison to females on week 1 (p=0.039). In addition, males in the SD group showed a more important diminution of daily standard diet intake in week 1 than in week 2(p=0.033), and a higher intake in week 2 than in week 3 (p=0.044).

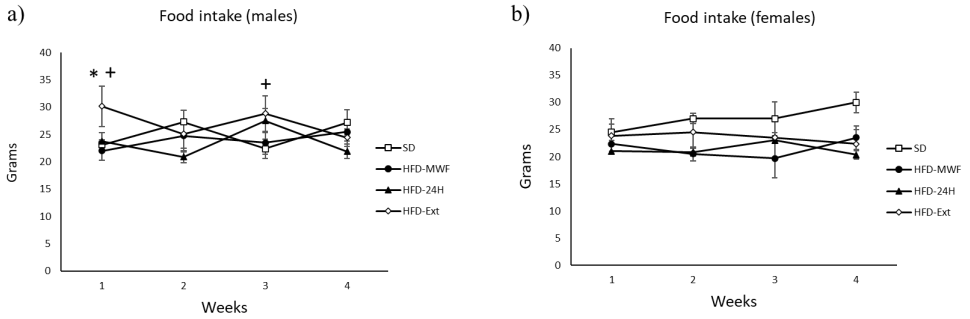

Fig S2 Daily standard food intake of male (a) and female (b) animals in the SD group (standard diet throughout the procedure), HFD-24h group (one hour access to HFD from Monday to Friday), in HFD-MWF group (two hours access to HFD on Monday, Wednesday and Friday) and in the HFD-Ext group (one hour HFD access twice a week, prior to extinction sessions). Data are represented as the mean (±SEM) amount of grams of standard diet measured weekly. *p<0.05 HFD-Ext males vs HFD-Ext females; + p<0.05 SD male group vs week 2.

With regards to fat intake during the intermittent access, the ANOVA revealed an effect of the variable Week only in males [F(3,96)=7.050; p<0.001], as there was a general escalation in intake throughout the 4 weeks (Fig S3a). However, there was also a significant difference on the variable Diet [F(2,32)=5.341; p<0.01], as the

HFD-Ext group showed a general decreased intake with respect to the HFD-MWF group and HFD-24h group ($p<0.05$). There was no interaction effect between factors.

Regarding females (Fig S3b), an ANOVA of the intermittent access to HFD revealed an effect of the interaction Week*Diet [$F(6,123)=3.516$; $p<0.01$] as HFD-MWF and HFD-24h exhibited a significant escalation through weeks ($p<0.01$) with respect to HFD-Ext, which showed a stable intake throughout the process. Moreover, HFD-MWF females showed a significant increase with respect to HFD-24h females ($p<0.01$).

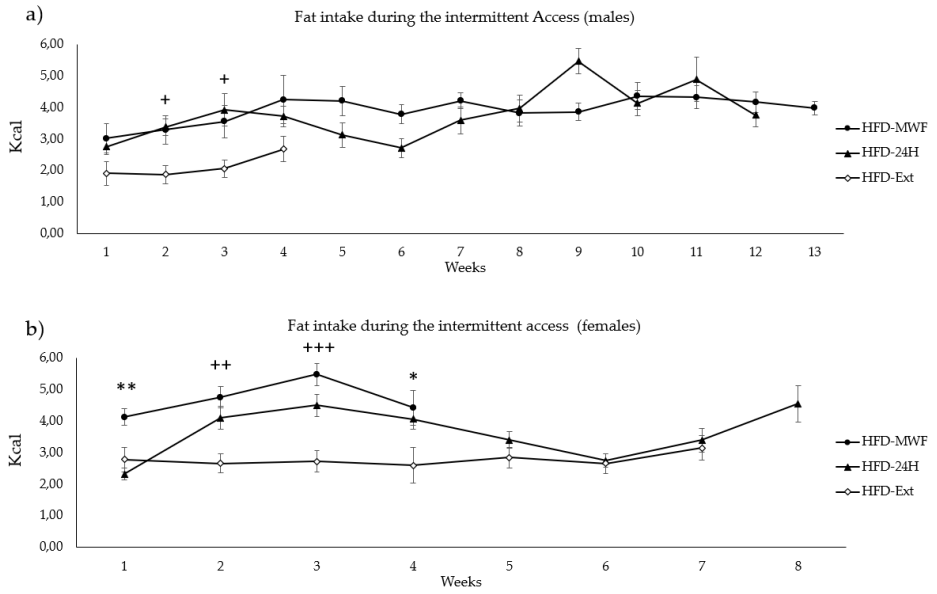

Fig S3 Escalation of the HFD intake in males (a) and females (b). Intake during HFD sessions in the HFD-24h group (one hour access to HFD from Monday to Friday), in HFD-MWF group (two hours access to high fat diet on Monday, Wednesday and Friday) and in the HFD-Ext group (one hour high fat diet access twice a week, prior to extinction sessions). Only data included in the ANOVA comparisons are represented with symbols of significance. **$p<0.01$ significant differences with respect to HFD-Ext and HFD-24H; +$p<0.05$, ++$p<0.01$, +++$p<0.001$ significant difference with respect to HFD-Ext

Estudio 3

Blocking the increased reinforcing effects of cocaine induced by social defeat: effects of palatable food.

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En preparación

Blocking the increased reinforcing effects of cocaine induced by social defeat: effects of palatable food

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Abstract

Rationale: It is known that stress is closely involved in the addiction process, playing an important role in the initiation, escalation, and maintenance of addiction. On the other hand, some types of food with high sugar and carbohydrate content, known as palatable food, have considerable effects on the brain's reward system by stimulating structures involved throughout the addictive process. Moreover, preclinical studies suggest that this stimulation of the brain's reward system by high-fat diets (HFD) could act as an alternative reinforcer, attenuating the reinforcing properties and altering the rewarding aspects of psychostimulants induced by stress.

Objective: The main aim of the present study was to evaluate the effect of a limited and an intermittent exposure to a HFD administered during an exposure to social stress performed by Social Defeat (SD) and after an exposure to SD during a 1mg/kg cocaine Conditioned Place Preference (CPP) acquisition.

Methods: a total of 105 male mice were divided in two experiments. Experiment 1 consisted in modulating SD episodes with different patterns of HFD access: EXP (animals in the standard diet without performing SD), SD (animals in the standard diet during SD and CPP sessions), SD-Pre (1h access to the HFD before each session of SD), SD-MWF (2h access to the HFD on Monday, Wednesday and Friday during the two weeks of SD) and SD-Post (2h access to the HFD after 4h of each SD episode). Three weeks after the last SD, all groups performed the CPP. Experiment 2 consisted in modulating the effects of stress on CPP acquisition with different patterns of HFD access: EXP, SD, CPP-Pre (1h access to the HFD before each conditioning session), CPP-MWF (2h access to the HFD on Monday, Wednesday and Friday during the two weeks of CPP) and Extended-CPP (2h access to the HFD on Monday, Wednesday and Friday from the last SD episode to the end of CPP).

Results: Our results showed that access to a HFD administered during the period of SD episodes counteracted the increased sensitivity that SD produces on the

reinforcing effects of cocaine. We also observed that access to a HFD before the conditioning session (CPP-Pre) or three days a week (CPP-MWF) during the acquisition of CPP blocked this increased sensitivity. With respect to gene expression, all mice exposed to SD, regardless of diet, showed a decrease in the CB1r and an increase of CRHR1 gene expression, except for the SD-Post group. In neither of both experiments were differences in the Oprm gene expression observed.

Conclusions: These results support that palatable food could be a good alternative reinforcer that blocks acquisition of cocaine preference in stressed animals.

1. Introduction

Stress plays a fundamental role in the initiation, escalation, and maintenance of addiction (Burke & Miczek, 2015; Koob, 2010; Logrip et al., 2012; Sinha et al., 2011), being a key element in the negative emotional state produced by dependence, leading to substance withdrawal (Koob, 2009) and relapse into drug use (Koob, 2010; Koob & Volkow, 2010). The main stressor in human beings is social stress (Dickerson & Kemeny, 2004), which arises from social relations and the environment in which individuals develop. Due to the importance of the deleterious physical and psychological consequences that social stress has on human beings, animal models of stress like Social Defeat (SD) have been developed to explore its consequences (Lu et al., 2003; Miczek et al., 2008). Employing this model, many preclinical studies have shown that social stress induces long-term consequences such as increased anxiety (Weathington & Cooke, 2012), decreased exploration behavior and social interaction (Burke et al., 2011), and increases in the conditioned rewarding effects of psychostimulants like cocaine, in adult (Ferrer-Pérez et al., 2018; Montagud-Romero et al., 2015, 2016; Rodríguez-Arias et al., 2017) and adolescent rodents (Burke et al., 2016; Burke & Miczek, 2015; Rodríguez-Arias et al., 2018).

Stress can also have an influence on nutritional habits. Clinical studies have reported that consumption of palatable foods increases with exposure to stressful situations (Groesz et al., 2012; Kim et al., 2013; Laugero et al., 2011; Tryon et al., 2013), due to the effects of palatable food on relieving psychological distress conditions, acting as comfort food (Bhatnagar et al., 2001; Dallman et al., 2003). For example, eating palatable food in humans lead to reduced plasma cortisol levels and decreased perceived stress (Fernandez et al., 2003; Leigh Gibson, 2006). In preclinical studies, rodents exposed to chronic stress show an increased preference for high-fat diets (HFD) compared to chow intake (Packard et al., 2014; Pecoraro et al., 2004),

reducing the response to acute stressors such as hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis (Foster et al., 2009; la Fleur et al., 2005; Pecoraro et al., 2004; Ulrich-Lai et al., 2011). In this line, a recent study from our group showed that mice isolated since adolescence and exposed to a palatable food intermittently showed reduced corticosterone levels with respect to their isolated counterparts fed only with the standard diet (Blanco-Gandía et al., 2018), which suggests that palatable food intake reduces the physiological stress response.

Therefore, social stress is capable of increasing the reinforcing effects of psychostimulants (Montagud-Romero et al., 2015) and HFD (Kim et al., 2013), that could be explained by common mechanisms. As with drugs of abuse, foods including a high proportion of calories, fat and/or sugar, as HFDs do, cause a pleasurable effect by increasing dopamine release in the nucleus accumbens (NAcc) (DiLeone et al., 2012; Rada et al., 2010) activating the ventral tegmental area (VTA), the prefrontal cortex and the amygdala (de Macedo et al., 2016), all of them critical structures of the reward system (Volkow et al., 2013). In fact, several studies have suggested that nutritional interventions can impact the response to drugs of abuse or even the development of addiction. For example, exposure to HFDs can modulate the development of alcohol and cocaine addiction, whether by increasing the sensitivity to reward (Avena et al., 2008; Blanco-Gandía, et al., 2017a, 2017b; Ledesma, et al., 2017; Puhl et al., 2011) or by decreasing it while acting as an alternative reinforcement in negative affective states induced by drug cessation (Blanco-Gandía et al., 2017c). In this line, studies in animal models have pointed out that consumption of HFD during cocaine withdrawal accelerates the extinction of cocaine seeking and blocks reinstatement, both when administered continuously (Blanco-Gandía et al., 2017c) or intermittently (Ródenas-González et al., 2021) and also decreases the behavioral withdrawal symptoms of cocaine (Loebens & Barros, 2003). These results suggest that HFD may act as an alternative reinforcer. Indeed, the interaction between HFD, stress and drug use could be explained by a common

neurobiological system, which is composed not only by the dopaminergic system, but also by the HPA axis, the cannabinoid, and the opioid systems, all of which are involved in stress, addiction, palatable food consumption (Parylak et al., 2012; Sakamoto et al., 2015) and reward (Cristino et al., 2014).

These three variables (stress, drugs of abuse intake and nutritional habits) require special attention in adolescents. Adolescence is a critical period in which structural changes in many limbic and cortical regions can be disrupted by several factors, such as social, hormonal, neurochemical, stress or dietary conditions (Baladi et al., 2012; Daws et al., 2011; Spear, 2000). For example, during adolescence, the reinforcing effects of drugs are enhanced, as sensitivity to reward is greater during this age (Steinberg, 2010). Nevertheless, the effects of palatable food on the cocaine-reinforcing effects increased by social stress in adolescent mice has not been evaluated.

Given the common neurobiological pathways that stimulate fat intake and drugs of abuse, and their interactions with the stress system, we hypothesize that palatable food intake could modulate the development of cocaine-induced conditioned place preference in adolescent mice that occurs with subthreshold doses of cocaine. To study this, we aimed to explore their interaction from two different approaches: a) Preventing the effects of stress on cocaine preference by modulating the SD episodes with a HFD administration; and b) Once the animals have been exposed to stress, blocking the effects of stress on cocaine acquisition by modulating the CPP procedure with HFD administration. In addition, considering its relevance in stress, addiction and palatable food reward, we also explored the gene expression of the opioid receptor μ (Oprm), the cannabinoid receptor 1 (CB1r) and the Corticotropin-releasing hormone receptor 1 (CRHR1) in brain samples of animals (striatum) at the end of the experiments.

2. Material and methods

2.1. Subjects

A total of 105 male mice of the OF1 outbred strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4 (cage size 28×28×14.5 cm) for 5 days prior to initiating the experimental procedure, at a constant temperature (21 ± 2 °C), with a reversed light schedule (white lights on 19:30–7:30) and food and water available ad libitum (except during the behavioral tests). All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The Animal Use and Care Committee of the University of Valencia approved the present study.

2.2. Drugs

For CPP, animals were injected i.p. with 1 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S. A. Madrid, Spain) diluted in physiological saline. The 1 mg/kg dose of cocaine used to induce CPP was based on previous studies (Maldonado et al., 2006; Vidal-Infer et al., 2012) in which it was shown to be a subthreshold dose that is not effective in standard animals.

2.3. Apparatus and procedure

2.3.1. Experimental design

All mice arrived at the laboratory on PND 21. After 5 days of adaptation in the vivarium, at PND 26, they were exposed to the procedure of Social Defeat (SD),

except for the exploration group. Following the last SD encounter, animals were kept in the vivarium for three weeks housed in their respective home cages, and then they performed the Conditioned Place Preference (CPP) (PND 55) with 1mg/kg cocaine. After completing the entire experimental procedure, the animals were euthanized to enable the collection of biological samples.

In this study, two different experiments were performed: Experiment 1 consisted in modulating SD episodes with different patterns of high-fat diets (HFD) access and Experiment 2 consisted in modulating the effects of stress on CPP acquisition with different patterns of HFD access. An overall and more detailed description of the sets of animals and experimental procedure of each experiment is provided in Figure 1.

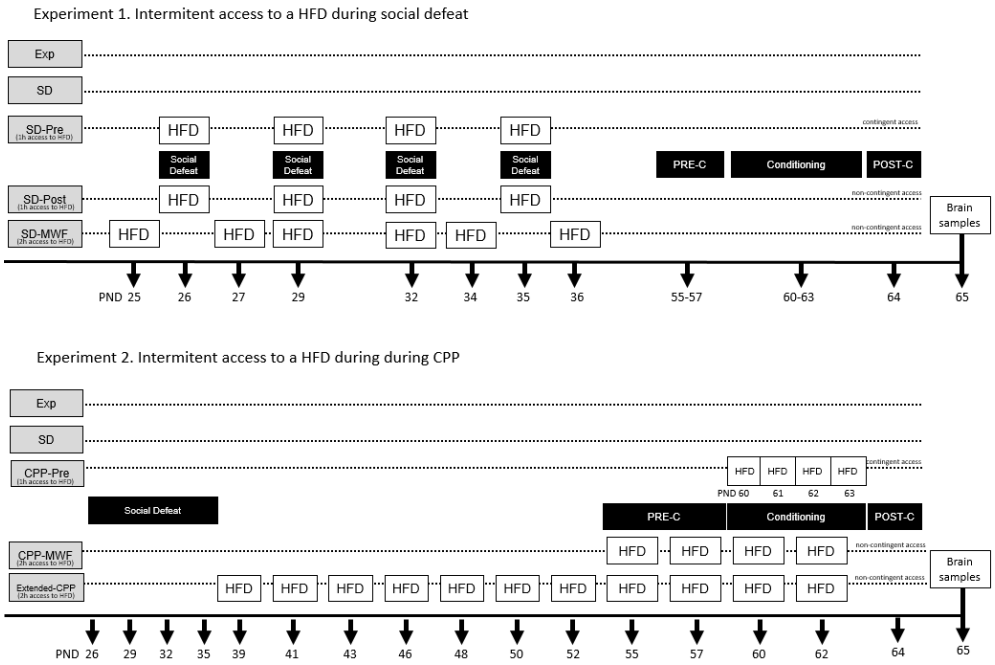

Fig. 2. Experimental design

Control groups (n=27) were randomly divided into two groups: Exploration (EXP) and Social Defeat (SD). Mice in these groups performed all procedures (Exploration-SD and CPP) to confirm that SD increases sensitivity to cocaine subthreshold doses (Montagud-Romero et al., 2015). Both groups were fed throughout the experiment with the standard diet.

In Experiment 1 (n=64), all the experimental animals were exposed to 4 episodes of SD with different HFD administrations during the two weeks of stress exposure. They were randomly divided into 5 groups with similar average body weights (25–26 g) and assigned either to EXP (animals in the standard diet without performing SD), SD (animals in the standard diet during SD and CPP sessions), SD-Pre (1h access to the HFD before each session of SD), SD-MWF (2h access to the HFD on Monday, Wednesday and Friday during the two weeks of SD, accessing the HFD after SD sessions on days that overlapped) and SD-Post (2h access to the HFD after 4h of each SD episode). Three weeks after the last SD, all groups performed the CPP.

In Experiment 2 (n=68), mice were randomly divided into 5 groups with similar average body weights (25–26 g) and assigned either to EXP (animals in the standard diet without performing SD), SD (animals in the standard diet during SD and CPP), CPP-Pre (1h access to the HFD before each conditioning session), CPP-MWF (2h access to the HFD on Monday, Wednesday and Friday during the two weeks of CPP) and Extended-CPP (2h access to the HFD on Monday, Wednesday and Friday from the last SD episode to the end of CPP, i.e. for a total of 5 weeks).

2.3.2 Feeding conditions

Two different types of diet were administered in the study. A standard diet (Teklad Global Diet 2014, 13 Kcal % fat, 67 Kcal % carbohydrates and 20% Kcal protein; 2,9 kcal/g; no sugars added) was given to the control groups, and a high-fat diet

(TD.06415, 45 Kcal % fat, 36 Kcal % carbohydrates and 19% Kcal protein; 4,6 Kcal/g; 20% of carbohydrates are sucrose) was administered in a limited way to the high-fat diet groups. Both diets were supplied by Harlan Laboratories Models, S. L. (Barcelona, Spain) and will be referred to from now on as the standard diet, while the sporadic limited access to the high-fat food will be referred to as the HFD. Ad libitum standard diet and water were always freely available in their home cages. Animals were weighed every week throughout the study, and their daily intake of standard diet in their home cage was also measured.

2.3.3. Repeated social defeat encounters

Animals in the corresponding group were exposed to four episodes of SD lasting 25 min each. Each episode consisted of three phases, which began by placing the experimental animal or intruder in the home cage of the aggressive opponent or resident for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the male-aggressive resident (Covington & Miczek, 2001). In the second phase, the wire mesh was removed from the cage and a 5-min period of confrontation began. In the third phase, the wire mesh was put back for 10 min to allow social threats from the resident. Mice were exposed to SD on postnatal days (PNDs) 26, 29, 32, and 35. The exploration group (EXP) underwent the same protocol, but without the presence of a Resident mouse in the cage. Following this last phase, animals were kept in the vivarium for 3 weeks and housed in their respective groups.

2.3.4. Conditioning place preference

For Place Conditioning, we employed sixteen identical Plexiglas boxes with two equally sized compartments (30.7 cm length x 31.5 cm width x 34.5 cm height) separated by a gray central area (13.8 cm, length x 31.5 cm, width x 34.5 cm height).

The compartments have different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

Acquisition of CPP

The procedure of Place Conditioning, unbiased in terms of initial spontaneous preference, was performed as described previously (Maldonado et al., 2006) and consisted of three phases. To summarize the main aspects, in the first phase, known as Pre-Conditioning (Pre-C), mice at 55 PND were allowed access to both compartments of the apparatus for 15 min (900s) per day on 3 days. On day 3, the time spent in each compartment over a 900s period was recorded, and animals showing a strong unconditioned aversion (less than 33% of the session time) or preference (more than 67%) for any compartment were excluded from the rest of the experiment. Half the animals in each group received the drug or vehicle in one compartment, and the other half in the other compartment. After assigning the compartments, no significant differences were detected between the time spent in the drug paired and vehicle-paired compartments during the preconditioning phase. In the second phase (conditioning), which lasted 4 days, animals received an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30 min. After an interval of 4 h, they received an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. Confinement was carried out in both cases by closing the guillotine door that separated the two compartments, making the central area inaccessible. During the third phase, known as post-conditioning (Post-C), the guillotine door separating the two compartments was removed (day 8) and the time spent by the untreated mice in

each compartment during a 900s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates that an aversion has developed.

2.3.5. Gene expression analyses: RNA isolation and quantitative RT-PCR

At the end of the experiments, animals were euthanized by cervical dislocation and the brains were immediately removed from the skull and placed on a cold plaque. Cerebellum and olfactory bulbs were eliminated, and the striatum was dissected. Brain tissue samples were immediately stored at -80ºC until the rt-PCR assay was performed.

Total RNA from the striatum was isolated using the Tri Reagent Method (Sigma-Aldrich, St. Louis, MO, USA), as described in the manufacturer's protocol. Reverse transcription of 1 mg of total RNA was performed using the Transcriptor First Strand cDNA synthesis kit (Thermo Fisher Scientific, Madrid, Spain). Amplification of the target and housekeeping (b-glucuronidase) genes was performed using the Taqman Gene Expression Master Mix (Thermo Fisher Scientific, Madrid, Spain) in a LightCycler 480 System (Roche Diagnostics) following the manufacturer's instructions. The assay codes of the primers used are Mm01212171, Mm01188089 and Mm00432670 for cannabinoid receptor 1 (CB1r), opioid receptor μ (Oprm) and CRHR1, respectively. Data were analyzed using the LightCycler 480 relative quantification software and were normalized to the amplification product of b-glucuronidase or Gusb (Mm00446953).

2.4. Statistics

Data related to body weight were analyzed by a mixed ANOVA, with the between variable -Diet-, with 5 levels (EXP, SD, SD-Pre, SD-MWF, SD-Post for experiment 1 and EXP, SD, CPP-Pre, CPP-MWF, Extended-CPP for experiment 2) and a within variable -Week-, with 6 levels (weeks 1 to 6). Data for the mean of total Kcal intake were analyzed by a one-way ANOVA with the between variable Diet (SD-Pre, SD-MWF, SD-Post for Experiment 1 and CPP-Pre, CPP-MWF, Extended-CPP for Experiment 2).

For the CPP procedure, the time spent in the drug-paired compartment was analyzed by two repeated measures ANOVA, with the between variable –Diet–, with 5 levels (EXP, SD, SD-Pre, SD-MWF, SD-Post for Experiment 1 and EXP, SD, CPP-Pre, CPP-MWF, Extended-CPP for Experiment 2), and a within variable –Days–, with two levels (Pre-C and Post-C). The gene expression data were analyzed by a one-way ANOVA with the between variable –Group– (EXP, SD, SD-Pre, SD-MWF, SD-Post for Experiment 1 and EXP, SD, CPP-Pre, CPP-MWF, Extended-CPP for Experiment 2). All data are presented as mean ± standard error of mean (SEM). A p-value <0.05 was considered statistically significant. Analyses were performed using SPSS v26.

3. Results

3.1. Experiment 1. Modulating SD episodes with palatable food

3.1.1. Body weight and mean of total Kcal intake in HFD sessions.

Results obtained in an initial ANOVA for the body weight (Figure 2a) revealed no significant differences of the variable *Diet* as all mice exhibited similar body weights. The ANOVA revealed an effect of the variable *Week* [$F(5,295)=1064.030$;

p<0,001] as all mice increased their body weight in Weeks 2, 3, 4, 5 and 6 with respect to Week 1 (p<0.001 in all cases).

The ANOVA of the of mean Kcal intake per session (Figure 2b) revealed significant differences on the variable *Diet*: [F(2,34)=62.104; p<0,001] as SD-Post consumed more Kcal during HFD sessions with respect to SD-MWF and SD-Pre (p<0.001).

Information relating to percentage of increase of body weight, standard food intake and HFD intake of both experiments can be found in the supplementary material S1.

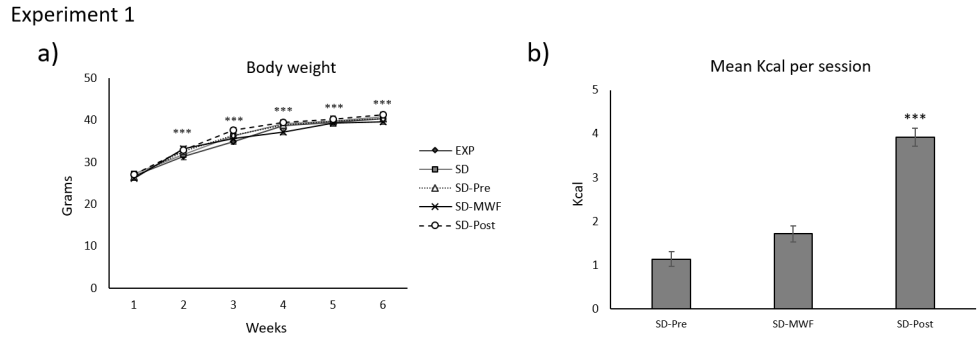

Fig. 2. (a) Body weight of mice over the 6 weeks in Experiment 1. EXP (n=12) and SD (n=15) mice received a standard diet during the HFD sessions and animals in the HFD condition underwent three additional temporary conditions: SD-Pre (n=14) had 1h access to the HFD before each session of social defeat, SD-MWF (n=13) had 2h access to the HFD on Monday, Wednesday and Friday during the two weeks of social defeat and SD-Post (n=10) had 2h access to the HFD after 4h of each session of social defeat. Data present mean (± SEM) amount of body weight. ***p<0.001 significant difference with respect to week 1. **(b) Mean Kcal per session.** Data are represented as the mean Kcal intake (±SEM) amount of HFD per session. ***p<0.001 significant difference with respect SD-Pre and SD-MWF.

3.1.2. Cocaine induced CPP

Results of the 1 mg/kg cocaine-induced CPP from Experiment 1 are presented in Figure 3. The ANOVA for the time spent in the drug-paired compartment revealed

an effect of the interaction *Days x Diet* [F(4,59)=6.836; p<0.001], showing that only animals exposed to Social Defeat and fed with the Standard Diet spent more time in the drug-paired compartment in Post-C (p<0.001).

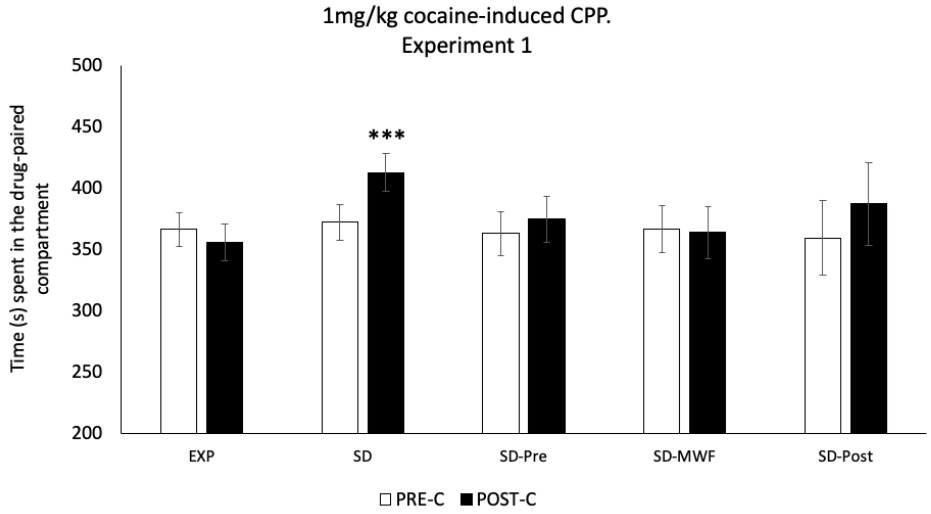

Fig. 3. Effects of modulating SD episodes with palatable food on cocaine induced CPP. CPP induced by 1 mg/kg of cocaine in mice exposed to exploration and standard diet (EXP)(n=12), exposed to Social Defeat and standard diet (SD)(n=15) or high fat diet (HFD) with three additional temporary conditions: SD-Pre (n=14) had 1h access to the HFD before each session of social defeat, SD-MWF (n=13) had 2h access to the HFD on Monday, Wednesday and Friday during the two weeks of social defeat and SD-Post (n=10) had 2h access to the HFD after 4h of each session of social defeat). Bars represent the mean (±SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (white) and post-conditioning (black). ***p < 0.001 significant difference with respect to Pre-C.

3.1.3. Gene expression analyses

For CB1 gene expression (Figure 4a), the ANOVA revealed a significant effect of the variable *Group* [F(4,32=9.739; p<0.001)]. All mice exposed to SD, regardless of diet, exhibited a significant decrease in CB1r gene expression in comparison with their corresponding EXP group (p<0.001). Regarding the expression of the CRHR1

(Fig 4b) the ANOVA revealed a significant effect of the variable *Group* [F(4,33=10.076; p<0.001)]. Mice in the SD, SD-Pre and SD-MWF groups exhibited a significant increase in CRHR1 gene expression in comparison with the Exp and SD-Post groups (p<0.01 in both cases). No significant differences were obtained in the gene expression of Oprm (Fig 4c).

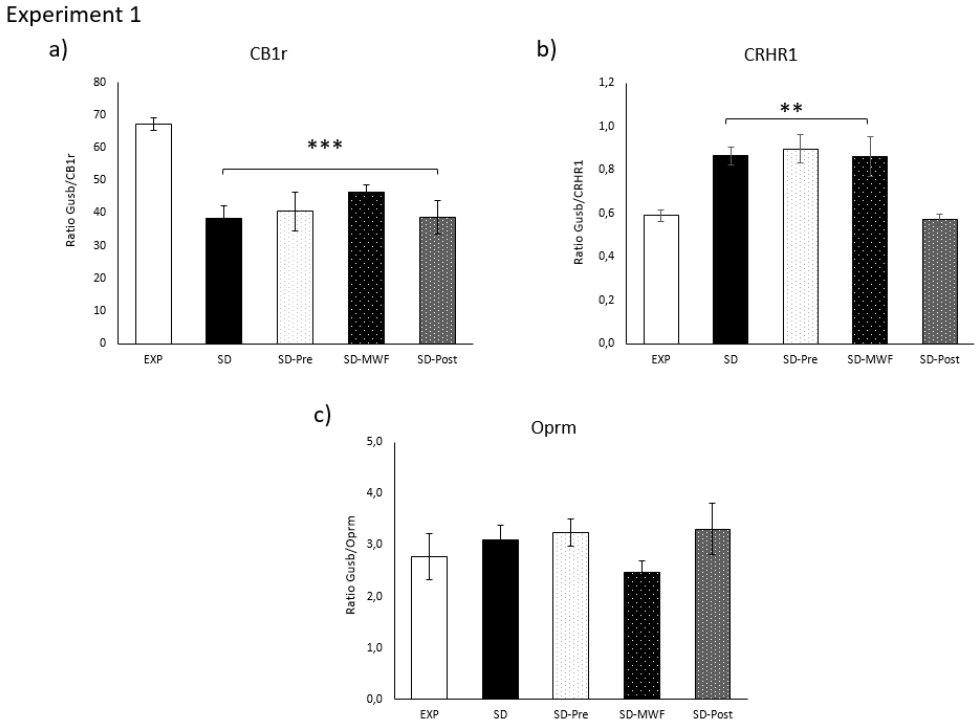

Fig. 4. Real-time PCR Gene expression in the striatum (n=8/condition). (a) Cannabinoid receptor 1 - CB1r (b) Corticotropin-releasing hormone receptor 1 - CRHR1 (c) Opioid receptor μ - Oprm. The columns represent means and the vertical lines ± SEM of gene expression in the striatum of OF1 mice. **p<0.01, ***p<0.001 significant differences with respect to the EXP group.

3.2. Experiment 2. Modulating the increase of cocaine-induce CPP with palatable food

3.2.1. Body weight and mean of total Kcal intake in HFD sessions.

Results obtained in an initial ANOVA for the body weight (Figure 5a) revealed no significant differences of the variable *Diet* as all mice exhibited similar body weights. The ANOVA revealed an effect of the variable *Week* [$F(5,315)=1549.255$; $p<0.001$] as all mice increased their body weight in Weeks 2, 3, 4, 5 and 6 with respect to Week 1 ($p<0.001$ in all cases).

The ANOVA of the Kcal intake per session (Figure 5b) revealed no significant differences of the variable *Diet*, as all mice consumed similar Kcal amounts during HFD sessions.

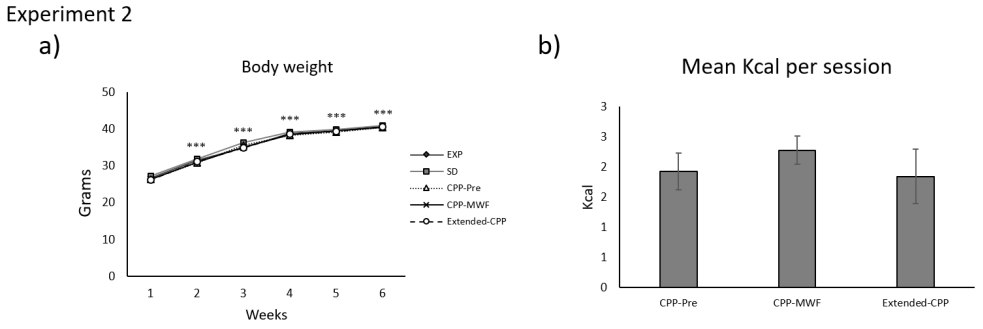

Fig. 5. (a) Body weight of mice over the procedure in Experiment 2. EXP (n=12) and SD (n=15) mice received a standard diet during the HFD sessions and animals in the HFD condition underwent three additional temporary conditions: CPP-Pre (n=11) had 1h access to the HFD before each conditioning session, CPP-MWF (n=15) had 2h access to the high-fat diet on Monday, Wednesday and Friday during the two weeks of CPP and Extended-CPP (n=15) had 2h access to the high-fat diet on Monday, Wednesday and Friday from the end of social defeat to the end of CPP. Data present mean (± SEM) amount of body weight. ***p<0.001 significant difference with respect to Week 1. **(b) Mean Kcal per session.** Data are represented as the mean Kcal intake (±SEM) amount of HFD per session.

3.2.2. Cocaine induced CPP

Results of the 1 mg/kg cocaine-induced CPP from Experiment 2 are presented in Figure 6. The ANOVA for the time spent in the drug-paired compartment revealed a significant effect of the interaction *Days x Diet* [$F(4,64)=4.909$; $p<0,01$], showing that SD and Extended-CPP spent more time in the drug-paired compartment in Post-C ($p<0.001$ and $p<0.05$ respectively).

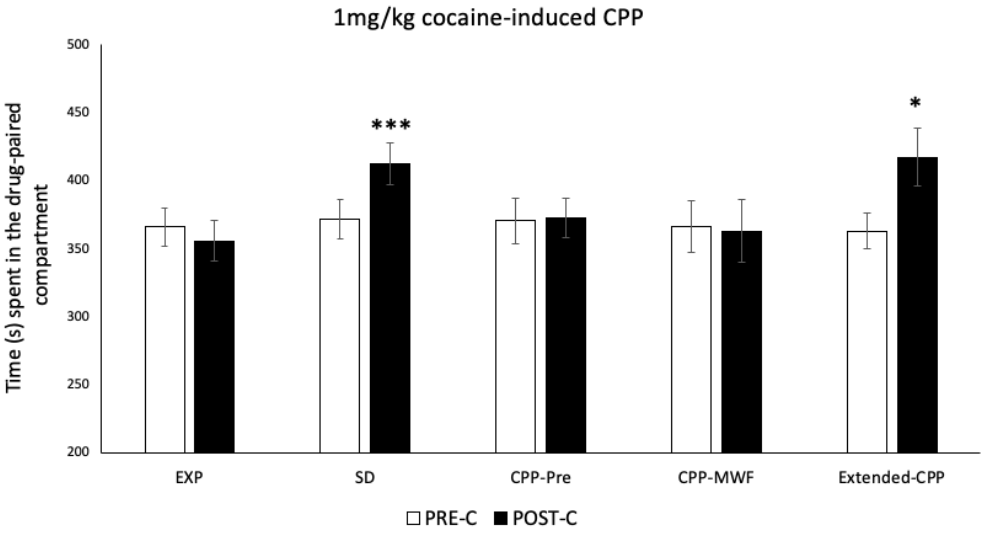

Fig. 6. Effects of exposure to a HFD during CPP on cocaine induced CPP. CPP induced by 1 mg/kg of cocaine in mice exposed to exploration and standard diet (EXP) (n=12), exposed to Social Defeat and standard diet (SD) (n=15) or HFD with three additional temporary conditions: CPP-Pre (n=11) had 1h access to the HFD before each conditioning session, CPP-MWF (n=15) had 2h access to the HFD on Monday, Wednesday and Friday during the two weeks of CPP and Extended-CPP (n=15) had 2h access to the HFD on Monday, Wednesday and Friday from the end of social defeat to the end of CPP. Bars represent the mean (±SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (white) and post-conditioning (black). *$p < 0.05$; ***$p < 0.001$ significant difference with respect to Pre-C.

3.2.3. Gene expression analyses

For CB1 gene expression (Fig 7a), the ANOVA revealed a significant effect of the variable *Group* [F(4,35=12.651; p<0.001)]. All groups exposed to a SD, regardless of diet, exhibited a significant decrease in CB1r gene expression in comparison with their corresponding Exp group (p<0.001). Regarding the expression of the CRHR1 (Fig 7b), the ANOVA revealed a significant effect of the variable *Group* [F(4,35=2.766; p<0.05)]. Mice in the SD group exhibited a significant increase in CRHR1 gene expression in comparison with Exp (p<0.05). No significant differences were obtained in the gene expression of Oprm (Fig 7c).

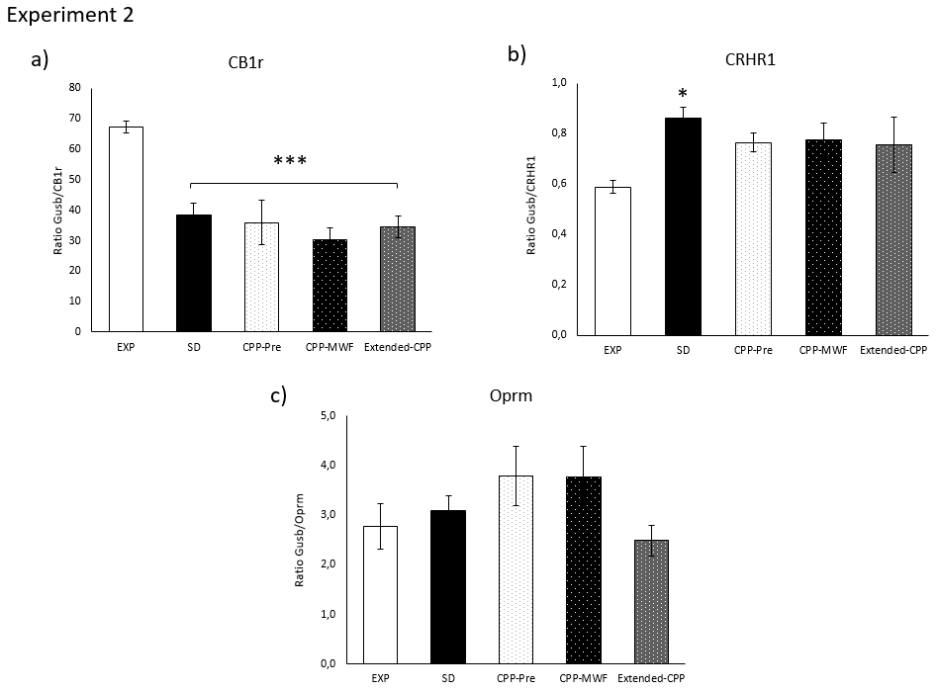

Fig. 7. Real-time PCR Gene expression in the striatum (n=8/condition). (a) Cannabinoid receptor 1 - CB1r (b) Corticotropin-releasing hormone receptor 1 - CRHR1 (c) Opioid receptor μ - Oprm. The columns represent means and the vertical lines ± SEM of gene expression in the striatum of OF1 mice. *p<0.05, ***p<0.001 significant differences with respect to the EXP group.

4. Discussion

The present work evaluates the effects of modulating the increased reinforcing effects of cocaine induced by social stress with a HFD in two critical moments: a) during exposure to SD stress and b) after exposure to SD stress and during the period of CPP acquisition.

Our results in Experiment 1 confirmed that access to a HFD administered during the period of the SD episodes counteracted the increased sensitivity that SD produces on the reinforcing effects of cocaine. None of the defeated groups fed with any of the three patterns of HFD administration developed CPP with a subthreshold dose of cocaine (1 mg/kg). With respect to gene expression, all mice exposed to SD, regardless of diet, showed a decrease in the CB1r and an increase of CRHR1 gene expression, except for the SD-Post group, which did not exhibit this increase.

In Experiment 2, we observed that access to a HFD prior to conditioning (CPP-Pre) or access three days a week (CPP-MWF) during the acquisition of CPP blocked the increased sensitivity to the conditioned reinforcing effects of cocaine induced by SD. The results obtained with the gene expression analyses were similar to that of Experiment 1, as all mice exposed to SD decreased CB1r gene expression. Furthermore, the CRHR1 gene expression increased in animals exposed to SD and normalized in all mice exposed to HFD during the CPP procedure. In none of the experiments were differences in the Oprm gene expression observed.

- ***Effects of HFD in body weight and Kcal intake.***

In both experiments, the intermittent access to a HFD did not produce any significant increase in body weight, which is in line with results obtained in previous studies with this administration pattern (Blanco-Gandía et al., 2017b; Hudson et al., 2007; Ródenas-González et al., 2021). Some preclinical studies have observed that ad

libitum access to a HFD leads to a metabolic syndrome, increasing adiposity and leptin levels, and interfering ghrelin and insulin signaling (Blanco-Gandía et al., 2017c; Davis et al., 2008; Morales et al., 2012). However, with our results, we can suggest that some of these alterations can be avoided with intermittent access.

Regarding HFD Kcal intake, in Experiment 1 the SD-Post group presented a significant increase in the mean of Kcal taken with respect to the other HFD treatment groups. This group had access to HFD after the SD episode, suggesting that this increased intake may be due to a compensatory response to stress, acting as comfort food. Several studies have reported the same outcome, where mice exposed to social stress subsequently increase their intake of HFD (Coccurello et al., 2018; Hassan et al., 2019; Sinha & Jastreboff, 2013).

- ***Modulating the increase in cocaine conditioned reward induced by social stress with palatable food***

As expected and in line with previous studies, in the present work SD mice fed with the standard diet exhibited an increased sensitivity to a subthreshold dose of cocaine, developing CPP for the drug-paired compartment. This result has been reported in several studies, where socially stressed animals show an increased vulnerability to the rewarding effects of cocaine in the self-administration (SA) and CPP paradigms (Han et al., 2017; Neisewander et al., 2012; Reguilón et al., 2017; Shimamoto, 2018), including a subthreshold dose of cocaine (Ferrer-Pérez et al., 2019; Giménez-Gómez et al., 2021; Montagud-Romero et al., 2021).

In Experiment 1, we observed that the socially defeated groups that were exposed to the different patterns of HFD administration during the two weeks of social encounters did not develop CPP for cocaine. This result suggests that palatable food consumption might be acting as an alternative reward (comfort food), as previous

studies corroborate. For example, administering a HFD in socially stressed animals due to isolation buffers the effects of cocaine, presenting an attenuated response in cocaine-induced motor hyperactivity (Erhardt et al., 2006), decreasing the corticosterone response and blocking the acquisition of cocaine CPP (Blanco-Gandía et al., 2018). HFD could reduce the HPA activity (Auvinen et al., 2012; Pecoraro et al., 2004), acting as an alternative reinforcer, which leads to a reduction in the reinforcing effects of cocaine caused by that stress, especially in adolescence when sensitivity to reward is enhanced (Blanco-Gandía et al., 2018; Steinberg, 2010).

The results obtained in Experiment 2, in which animals were exposed to different administration patterns of HFD during the CPP procedure, show that all SD groups exposed to different patterns of HFD during CPP acquisition did not develop cocaine preference, except for the Extended-CPP group. This group began to take HFD intermittently just after finishing the last episode of SD and until the CPP ended. This group showed an increased sensitivity to the rewarding effects of the subthreshold dose of cocaine, just as the SD animals fed with the standard diet. This suggests that when HFD exposure is prolonged over time, it has no effect or it can even sensitize the reward system, as previous studies suggest (Blanco-Gandía et al., 2017a, 2017b; Puhl et al., 2011). Supporting this hypothesis, we have noticed that 6 weeks of intermittent HFD administration increases the sensitivity of adolescent mice to a subthreshold dose of cocaine, with the mice also needing more time to extinguish the preference (Blanco-Gandía et al., 2017b). On the other hand, the present results indicate that when the intermittent administration of HFD is shorter and/or provided before each CPP session, the reinforcing effects of cocaine increased by social stress are blocked. Based on our previous and present results, we hypothesized that controlled administration of HFD might be a useful strategy to mitigate the effects of social stress on the reinforcing effects of cocaine, especially when this administration is prior to cocaine exposure. However, taking into account

the influence of this diet in reward circuits and its effects when administered for a prolonged period of time, it is crucial to determine the optimal pattern.

- ***Changes in the striatal gene expression after HFD administration of socially defeated mice***

In order to give a neurobiological explanation to the possible positive effects that HFD exerts on the consequences of SD, and taking into account the relevance of the cannabinoid and opioid system in addiction and HFD (Barson et al., 2012; Kawahara et al., 2013) and corticotrophin-releasing factor in stress (Puhl et al., 2011), we also explored striatal changes in gene expression of Oprm, CB1r and CRHR1. Our results showed that SD induces a reduction in CB1r and an increase in CRHR1 gene expression, while not producing any change in Oprm expression. Only when administered after suffering from an episode of SD (SD-Post) was HFD capable of decreasing CRHR1 gene expression to similar levels to those observed in the exploration group. HFD interventions during acquisition of CPP in Experiment 2 did not induce any change.

Regarding CB1r expression, all groups exposed to SD, regardless of diet, showed a decrease of CB1r gene expression, suggesting that SD may have long-term effects on the cannabinoid system. This marked reduction in SD animals was not reverted with any HFD administration pattern in either of the experiments. This is in line with previous studies that have demonstrated that CB1 signaling modulates regulation of the stress response (Valverde & Torrens, 2012). For example, chronic stress is associated with a reduction of CB1r gene expression in the hippocampus (Hill et al., 2005; Hu et al., 2011; Reich et al., 2009), and in the striatum (Rossi et al., 2008; Wang et al., 2010). Furthermore, stimulation of CB1 receptors reduces stress-induced effects as anhedonia (Rademacher & Hillard, 2007), or even depressive behaviors (Gobbi et al., 2005) and passive stress-coping behavior (Steiner et al.,

2008). In this line, previous studies have also suggested that HFD decreases CB1r gene expression in the N Acc of adults (Bello et al., 2012; Martire et al., 2014) and adolescent rodents (Blanco-Gandía et al., 2017b). As HFD and SD induce the same effect on CB1r gene expression by reducing it, this could explain why HFD administration did not reverse the reduction that SD produced in these animals.

On the other hand, mice exposed to HFD after each SD (SD-Post) was the group with the highest Kcal intake per session and the only one that decreased CRHR1 gene expression, suggesting that the rise in fat Kcal intake may play a role in this effect. In addition, although a tendency of increasing CRHR1 gene expression was observed in the HFD groups in Experiment 2, this was not strong enough to achieve significant differences with respect to the non-stressed animals. This result confirms what other studies have reported, namely that CRHR1 gene expression is usually increased as a response to stress (Logrip et al., 2012). This expression can lead to an increase in the vulnerability to the rewarding effects of cocaine in CPP and SA, where some studies have demonstrated that CRHR1 antagonists can block this effect (Boyson et al., 2014; Ferrer-Pérez et al., 2018). The outcome obtained in the present work with the SD-Post group suggests that HFD appears to have a similar effect to that of CRHR1 antagonists, reducing CRHR1 expression and HPA activity (Foster et al., 2009; la Fleur et al., 2005; Pecoraro et al., 2004; Ulrich-Lai et al., 2011). Moreover, other studies have demonstrated that a HFD can reduce corticosterone levels in isolated mice (Blanco-Gandía et al., 2018) and induced by restraint (Zeeni et al., 2013), while also reducing other consequences of social stress, such as social avoidance, anxiety and depression behavior (MacKay et al., 2017; Maniam & Morris, 2010; Otsuka et al., 2019). As previously mentioned, there is a tendency to increase palatable food consumption in rodents exposed to stress (Coccurello et al., 2018; Pecoraro et al., 2004; Zellner et al., 2006); thus, one possible explanation would lie in the increased Kcal intake of palatable food that the SD-Post group shows, which may return CRHR1 gene expression to normal.

Finally, our results throughout the study, both in Experiment 1 and Experiment 2, suggest that neither social stress nor intermittent HFD administration for two weeks causes alterations in Oprm1 gene expression, although the implication of the endogenous opioid system in fat consumption has been described (Sakamoto et al. 2015). This could be explained by the short exposure times to the HFD compared to other studies, suggesting that the amount of kcal ingested by mice in our study was not enough to induce any change.

Conclusion

The results obtained in this study confirm that palatable food could be a good alternative reinforcer that blocks acquisition of cocaine preference in stressed animals. However, although this palatable diet appears to be effective in reducing the consequences of social stress, the duration of exposure to HFD and the specific period of administration appears to be an important factor to be considered. Our results suggest that long exposures to the HFD may not be effective. Brief administration of HFD after SD or during the acquisition of the preference for cocaine can reduce the conditioned rewarding effects of this drug by reducing the increased expression of CRF1r. Future studies should address other systems related to stress and reward to provide a broader explanation to the positive effect of HFD on the consequences of social stress.

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Supplementary material

Blocking the increased reinforcing effects of cocaine induced by social defeat: effects of palatable food

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2. Material and Methods

2.4. Statistics

Data related to standard diet intake were analyzed by a mixed ANOVA, with the between variable -Diet-, with 5 levels (EXP, SD, SD-Pre, SD-MWF, SD-Post for experiment 1 and EXP, SD, CPP-Pre, CPP-MWF, Extended-CPP for experiment 2) and a within variable -Week-, with 6 levels (weeks 1 to 6). Data related to the percentage of increase in bodyweight were analyzed by a one-way ANOVA with the between variable Diet (EXP, SD, SD-Pre, SD-MWF, SD-Post for experiment 1 and EXP, SD, CPP-Pre, CPP-MWF, Extended-CPP for experiment 2).

Data related to limited and intermittent access to the HFD were analyzed by a mixed ANOVA, with the between variable -Diet-, with 3 levels (SD-Pre, SD-MWF, SD-Post for experiment 1 and CPP-Pre, CPP-MWF, Extended-CPP for experiment 2)- and a within variable -Days-, with 4 levels (day 1 to 4). Statistical comparisons to HFD of SD-MWF in experiment 1 were only calculated in the sessions where this group coincided with social defeat (sessions 2,3,4 and 6). Statistical comparisons to HFD of experiment 2 were only calculated in the first 4 sessions.

3. Results

3.1. Experiment 1. Modulating SD episodes with palatable food

3.1.1. Percentage of increase of body weight, standard food intake and intermittent access to HFD

The ANOVA for the percentage of increase of body weight from week 1 to 6 (Fig S1a) revealed no significant differences of the variable *Diet*, as all mice exhibited similar increase of weight gain in all weeks. With respect to the daily standard diet

food intake (Fig S1b) the ANOVA did not reveal significant differences in intake between groups.

The ANOVA of the HFD intake (Fig S1c) revealed an effect of the variable *Diet* [F(2,34)=12.591; p<0,001] as SD-Post group showed increase intake with respect to SD-Pre and SD-MWF (p<0.001 and p<0.01, respectively). No significant differences were found in the variable *Days*, as HFD intake was stable throughout the four fat intake sessions.

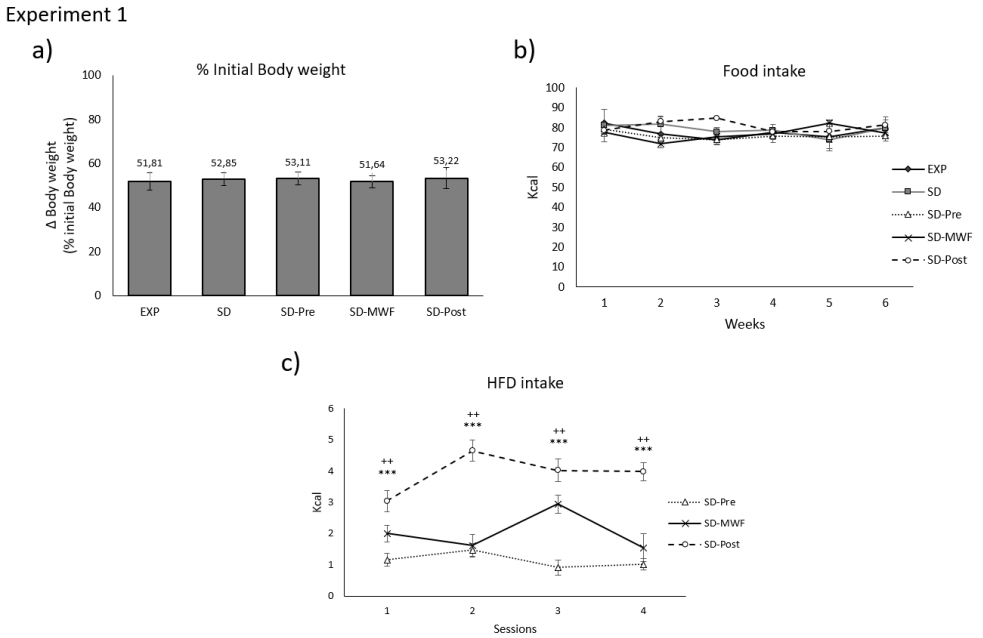

Fig. 1S. (a) Percentage of increase in bodyweight (% Δ BW) in week 6 respect initial bodyweight. EXP (n=12) and SD (n=15) mice received a standard diet during the HFD sessions and animals in the HFD condition underwent three additional temporary conditions: SD-Pre (n=14) had 1h access to the HFD before each session of social defeat, SD-MWF (n=13) had 2h access to the HFD on Monday, Wednesday and Friday during the two weeks of social defeat and SD-Post (n=10) had 2h access to the HFD after 4h of each session of social defeat. **(b) Standard food intake**. Daily intake (Kcal) of standard food per cage of 4

mice (mean ± SEM). **(c) Fat intake during HFD sessions**. HFD intake (Kcal) during fat intake sessions in the SD-Pre, SD-MWF and SD-Post. Data are represented as the mean (±SEM) amount of HFD measured by session. ***p<0.001 significant difference with respect SD-Post; ++p<0.01 significant difference with respect SD-Pre

3.2. Experiment 2. Modulating acquisition of CPP with palatable food

3.2.1. Percentage of increase of body weight, standard food intake and intermittent access to HFD

The ANOVA for the percentage of increase of body weight from week 1 to 6 (Fig S2a) revealed no significant differences of the variable *Diet*, as all mice exhibited similar increase of weight gain in all weeks. With respect to the daily standard diet food intake (Fig S2b) the ANOVA did not reveal significant differences in intake between groups as all animals ate a similar amount every week.

The ANOVA of the HFD intake (Fig S2c) revealed an effect of the variable *Days* [F(1,38)=12.998; p<0,001] as all mice showed a general increase intake in session 4 with respect to the session 1 (p<0.01). No significant differences were found of the variable *Diet*, showing that all animals ate a similar amount of HFD in the first 4 sessions.

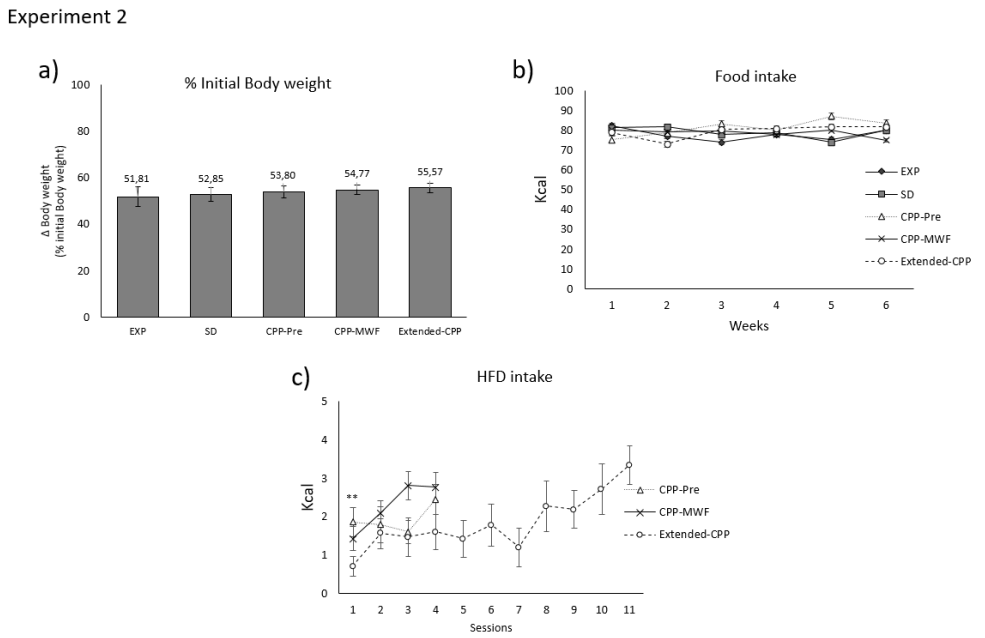

Fig. 2S. (a) Percentage of increase in bodyweight (% Δ BW) in week 6 respect initial bodyweight. EXP (n=12) and SD (n=15) mice received a standard diet during the HFD sessions and animals in the HFD condition underwent three additional temporary conditions: CPP-Pre (n=11) had 1h access to the HFD before each conditioning session, CPP-MWF (n=15) had 2h access to the high-fat diet on Monday, Wednesday and Friday during the two weeks of CPP and Extended-CPP (n=15) had 2h access to the high-fat diet on Monday, Wednesday and Friday from the end of social defeat to the end of CPP. **(b) Standard food intake**. Daily intake (Kcal) of standard food per cage of 4 mice (mean ± SEM). **(c) Fat intake during HFD sessions**. HFD intake (Kcal) during fat intake sessions in the CPP-Pre, CPP-MWF and Extended-CPP. Data are represented as the mean (±SEM) amount of HFD measured by session. **p<0.01 significant difference with respect to session 4.

Estudio 4

Cognitive profile of male mice exposed to a Ketogenic Diet.

Ródenas-González, F., Blanco-Gandía, M.C., Miñarro, J., y Rodríguez-Arias, M.

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(Anexo 3)

Cognitive profile of male mice exposed to a Ketogenic Diet

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Abstract

In recent years, nutritional interventions for different psychiatric diseases have gained increasing attention, such as the ketogenic diet (KD). This has led to positive effects in neurological disorders such as Parkinson's disease, addiction, autism or epilepsy. The neurobiological mechanisms through which these effects are induced and the effects in cognition still warrant investigation, and considering that other high-fat diets (HFD) can lead to cognitive disturbances that may affect the results achieved, the main aim of the present work was to evaluate the effects of a KD to determine whether it can induce such cognitive effects. A total of 30 OF1 male mice were employed to establish the behavioral profile of mice fed a KD by testing anxiety behavior (Elevated Plus Maze), locomotor activity (Open Field), learning (Hebb Williams Maze), and memory (Passive Avoidance Test). The results revealed that the KD did not affect locomotor activity, memory or hippocampal-dependent learning, as similar results were obtained with mice on a standard diet, albeit with increased anxiety behavior. We conclude that a KD is a promising nutritional approach to apply in research studies, given that it does not cause cognitive alterations.

Keywords: Ketosis, Ketogenic Diet, High-Fat Diet, Anxiety, Locomotor activity, Learning, Memory.

1. Introduction

In recent years, it has been widely discussed whether dietary modifications may be an important factor in several diseases [1,2]. Many of these new interventions modify one or more macronutrients, such as high-fat diets, low-carbohydrate or low-sugar diets [3,4]. An example that has been recently explored as a therapeutic target is the ketogenic diet (KD), which leads to changes in the body's own metabolism [5,6]. The KD has traditionally been used in epilepsy [7,8], but lately, it has also been used as a nutritional intervention to investigate its effects in other neurological disorders, such as Alzheimer's disease [9], Parkinson's disease [10], autism [11] and, most recently, addiction [12,13]. However, more research with randomized control trials is required to provide conclusive results.

The KD is a diet high in fat, low in carbohydrates and moderated in proteins. One of the main features of the KD is the reduction in carbohydrate intake, which reduces the production of glucose and induces the body to use fat stores, breaking down fatty acids and creating ketone bodies in the liver, like β-hydroxybutyrate (βOHB) [7,14]. This metabolic process is known as ketosis, which can be achieved by strict adherence to a KD [15], or by prolonged fasting [16,17]. βOHB is a non-volatile and stable compound released into the bloodstream [18] and constitutes up to 70% of the ketone bodies synthesized in liver mitochondria [19], being the main indicator of ketosis both in humans [13] and in animal models such as rats [20] and mice [12,21].

To date, numerous studies with high-fat diets have reported negative effects on behavior, such as locomotor activity [22,23], or cognition, such as learning deficits [24–27], impaired hippocampus-dependent memory [23,25,28,29] and even anxiety-like behavior [30]. These effects are crucial in animal model research, as a subtle deficit in these capabilities could be interfering with the results obtained on many levels. Sometimes, certain behavioral procedures in preclinical research require the animal to learn a task or remember an object. If dietary treatments like HFDs affect

behavior and cognition, we could be attributing benefits, harms, or no significant results to other causes and not to the diet itself, leading us to contaminated conclusions.

Therefore, as the KD is increasing its popularity in research in several neurological diseases, such as anxiety, depression, bipolar disorder or attention deficit hyperactivity disorder [31], it is still necessary to establish a baseline of the behavioral and cognitive profile of the chronic administration of this type of diet in order to confirm if it is safe or, on the contrary, if it has similar effects to those of traditional high-fat diets [21].

As mentioned above, the main difference between the KD and HFDs is that traditional high-fat diets not only contain an important percentage of carbohydrates, with a significant proportion of sugar, but also contain fats obtained from lard and soybean oil. In previous behavioral studies, it has been reported that some of the main factors contributing to cognitive alterations are the metabolic effects of HFD exposure, such as increased fat intake and body weight gain and the general metabolic dysfunction, with alterations in insulin, ghrelin and leptin levels [24,32–34].

Therefore, the aim of the present work was to evaluate the cognitive and behavioral differences observed in young and cognitively healthy mice exposed to a KD to explore the effects of this diet in standard animals. For this purpose, we provided the KD to OF1 mice and evaluated the differences in their anxiety behavior with the Elevated Plus Maze, their spontaneous locomotor activity using the Open Field test, their memory with the Passive Avoidance test and their hippocampal learning with the Hebb-Williams maze.

2. Material and Methods

2.1. Subjects

A total of 30 male mice of the OF1 outbred strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4 (cage size 28 x 28 x1 4.5cm) for three days prior to initiating the experimental feeding condition (PND 25), at a constant temperature (21±2°C), lights on from 8:00 to 20:00, and food and water available ad libitum (except during the behavioral tests). All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The Committee for the Use and Care of Animals of the University of Valencia approved the study (2019/VSC/PEA/0065). The size of the sample was determined with the G*Power program [35], estimating the need to include 13 mice per experimental group. An expected effect size of d = 1.5 (α = .05 and statistical power = .95) was taken, based on the results of the previous study of Blanco-Gandía et al. [24], which employed a similar experimental design, strain of mice, age of the animals and behavioral tests.

2.2. Apparatus and procedure

2.2.1 Feeding conditions and experimental design

Two different types of diet were used in this study: the standard diet (SD) (Teklad Global Diet 2014, 13 kcal % fat, 67 kcal % carbohydrates and 20% kcal protein; 2,9kcal/g) and the ketogenic diet (KD) (TD.96355, 90.5 % kcal from fat [vegetable shortening and corn oil], 0.3% kcal from carbohydrates and 9.1% kcal from protein; 6.7 kcal/g). The different diets were supplied by Envigo Teklad Diets (Barcelona,

Spain). In this experiment (Fig.1a), OF1 male mice (n=30) arrived in the laboratory on PND 21 and were randomly divided into 2 groups (n=15/condition) with similar average body weights (25-26 g) and assigned either SD or KD feeding conditions. Tests were performed one week after the diet had been initiated in order to evaluate if it had induced alterations in anxiety behavior (Elevated Plus Maze on PND32), motor activity (Open Field on PND 33), memory (Passive Avoidance Test on PND 34) or learning (Hebb Williams Maze on PND 36). All animals were under their specific feeding conditions from one week before the behavioral tests began (PND 25) until the end of the experiment (PND 44). On PND 44, the KD group was switched back to the SD until the end of the experiment to reevaluate anxiety behavior with the standard diet (PND 51). Body weight and Beta-hydroxybutyrate (βOHB) plasma levels were measured every week throughout the study.

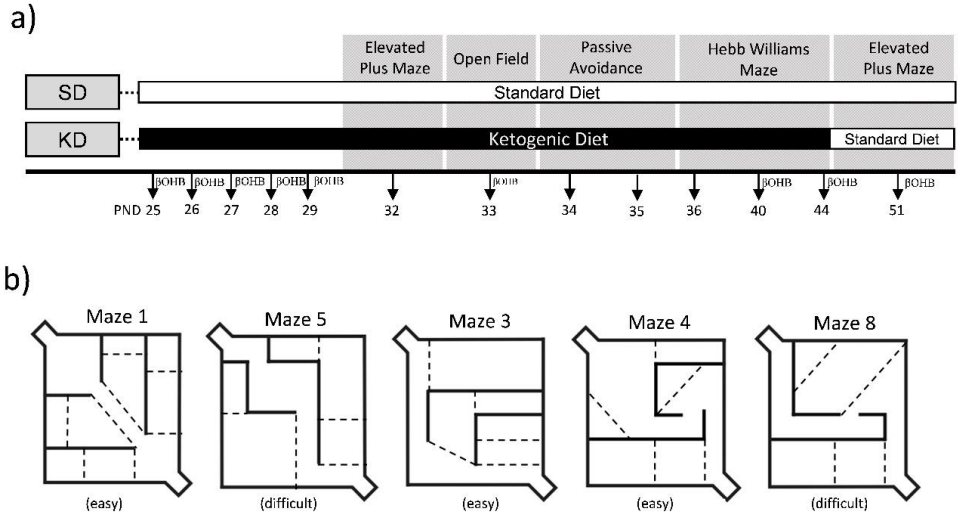

Fig.1. (a) Experimental design. (b) Hebb-Williams Maze configuration and difficulty.

2.2.2. Ketosis status: β-hydroxybutyrate plasma levels

Plasma β-hydroxybutyrate was measured weekly from the tail vein with a On Call GK Dual monitor and ketone test strips (ACON Laboratories, Inc., San Diego, CA).

2.2.3. Elevated Plus Maze

The EPM consisted of two open arms (30x5x0.25cm) and two enclosed arms (30x5x15cm). The junction of the four arms formed a central platform (5x5cm). The floor of the maze was made of black Plexiglas, and the walls of the enclosed arms of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide additional grip for the animals. The entire apparatus was elevated 45 cm above floor level. In order to facilitate adaptation, mice were transported to the dimly illuminated laboratory 1h prior to testing. At the beginning of each trial, subjects were placed on the central platform facing an open arm, and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The behavior displayed by the mice was recorded automatically by an automated tracking control software (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The measurements recorded during the test period were frequency of entries, time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. Number of open-arm entries, time spent in open arms and percentage of open-arm entries are generally used to characterize the anxiolytic effects of drugs [36,37].

2.2.4. Open Field

The spontaneous locomotor behavior of the mice was quantified in an Open Field for a period of 1 hour. The Open Field test was performed in an opaque plastic box (30x30x15cm) left open at the top. The animal was placed in the box and its activity was recorded automatically by tracking software (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The parameter studied was the total distance traveled (cm) and time near the wall and center (s).

2.2.5 Passive Avoidance test

For the Passive Avoidance test, a step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy) was employed. This cage is made of Perspex sheets and divided into two compartments (15 cm × 9.5 cm × 16.5 cm each one). The safe compartment is white and illuminated by a light fixture (10 W) fastened to the cage lid, whereas the "shock" compartment is dark and made of black Perspex panels. The two compartments are divided by an automatically operated sliding door at floor level. The floor is made of 48 stainless steel bars with a diameter of 0.7 mm and situated 8 mm apart.

Passive Avoidance tests were carried out following the procedure described in Aguilar et al. [38]. On the day of training, each mouse was placed in the illuminated compartment facing away from the dark compartment. After a 60 s period of habituation, the door leading to the dark compartment was opened. When the animal had placed all four paws in the dark compartment, a footshock (0.5 mA, 3 s) was delivered and the animal was immediately removed from the apparatus and returned to its home cage. The time taken to enter the dark compartment (step-through latency) was recorded. Retention was tested 24h later, following the same procedure but without the shock. The maximum step-through latency was 300 s.

2.2.6 Hebb-Williams Maze

The maze used in our experiment is made of black plastic and measures 60 cm wide x 60 cm long x 10 cm high. It contains a start box and a goal box (both 14 cm wide x 9 cm long), which are positioned at diagonally opposite corners. The maze contains cold water at a wading depth (15°C, 3,5cm high), while the goal box is stocked with fresh dry tissue. Several maze designs are produced by fixing different arrangements of barriers to a clear plastic ceiling. This apparatus allows the cognitive process of routed learning and the motivation of water escape to be measured.

The procedure followed was based on that employed by Galsworthy et al. [39], in which mice must navigate the maze and cross from the wet starting box to the dry goal box in order to escape the cold water. Animals underwent a 5-min habituation period (dry sand, no barriers) on day 1, and undertook problem A on day 2 and problem D on day 3 (4 trials/day) (practice mazes). Mice were subsequently placed in mazes 1, 5, 3, 4 and 8 on separate days (Fig. 1b), on which 8 trials took place (see Rabinovitch & Rosvold [40] for all maze designs). The time limit for reaching the goal box was 5 min, after which the mouse was guided to the box if necessary. The total latency score (sum of the latencies in all the problem trials in each maze) was registered.

2.3. Statistical analysis

Data relating to βOHB and body weight were analyzed by a mixed ANOVA with one between-subjects variable – "Diet", with 2 levels, (SD and KD) - and a within variable – "Days", with 9 (Baseline, Days 1-4, 7, 15, 19 and 25) or 4 levels (Baseline and weeks 1-3).

Data relating to the Elevated Plus Maze and Open Field test were analyzed by a one-way ANOVA with a between variable - "Diet", with 2 levels for Open Field (SD and

KD) and three levels for the Elevated Plus Maze test (SD, KD and 7 days post-KD). The Passive Avoidance test was analyzed by a two-way ANOVA, with the same between variable and one within variable - "Days", with 2 levels (training day and test 24h). The data of the Hebb-Williams maze were analyzed by a two-way ANOVA with one between subject variable – "Diet" - and one within subject variable – "Maze", with five levels. The Bonferroni adjustment was employed for post hoc comparisons. All results are expressed as mean ± S.E.M. Analyses were performed using SPSS v26.

3. Results

3.1. β-hydroxybutyrate (βOHB) and body weight.

The ANOVA of the βOHB plasma levels (Fig. 2a) revealed a significant effect of the interaction "Days x Diet" [$F(8,224) = 25,594$; $p < 0.001$], as the KD group showed increased levels of βOHB in comparison with the SD group at 24h, 48h, 72h, 96h, 7, 15 and 19 days ($p < 0.001$ in all cases), but showed no differences at 25 days ($p = 0.195$). There were also significant increases within the KD group on Days 1, 2, 3, 4, 7, 15 and 19 with respect to baseline ($p < 0.001$ in all cases), but no differences with respect to 25 days.

The ANOVA of body weight (Fig. 2b) revealed a significant effect of the interaction "Week x Diet" [$F(3,84) = 14,364$; $p < 0.001$] as mice on the KD displayed lower body weight at baseline and during week 1 compared to weeks 2 and 3 ($p < 0.001$, in all cases). Mice fed a SD showed higher body weight in Week 3 compared to Baseline, and Weeks 1 and 2 ($p < 0.001$, in all cases). In addition, the SD group exhibited higher body weight than the KD group at Baseline ($p < 0.05$) and in Week 1 ($p < 0.01$).

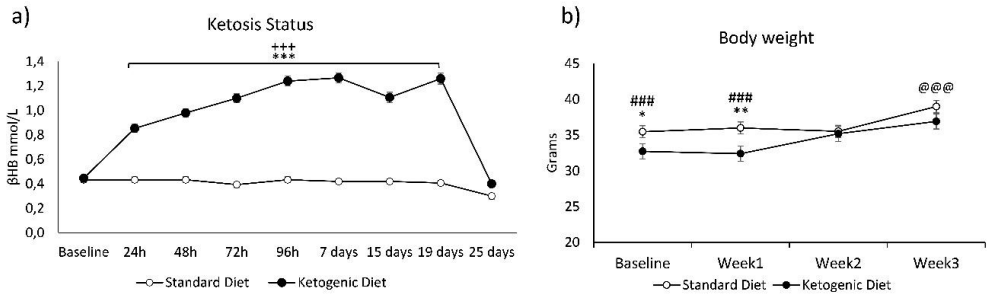

Fig. 2. β-hydroxybutyrate plasma levels and weekly body weight. (a) Ketosis status. Data are represented as the mean (±SEM) amount of βOHB. **(b) Weekly body weight**. Data are represented as the mean (±SEM) body weight measured weekly. ***p<0.001, **p<0.01, *p<0.05 significant difference with respect to the SD group. +++p<0.001 significant difference with respect to baseline. ### p<0.001 significant difference with respect to week 2 and 3 of KD. @@@p<0.001 significant difference with respect to the rest of the weeks of SD.

3.2 Elevated Plus Maze

The ANOVA (see Table 1) revealed an effect of the variable "Diet" for the time [F (2,42) =4.838; p=0.013] and percentage of time [F (2,42) =4.944; p=0.012] spent in the open arms of the maze. Animals belonging to the KD group spent less time and percentage of time in the open arms than the SD group (p<0.01 in both cases).

There was also a significant effect of the variable "Diet" for the percentage of open entries [F (2,42) = 3.317 p=0.046]. The KD group showed a lower percentage of open entries than the SD group (p<0.05). There was no effect of the variable Diet on the total number of entries [F(2,42)= 0.428; p=0.655]. There were no significant differences between the SD group and 7 days post-KD.

	Standard Diet	Ketogenic Diet	7 days post-Ketosis
Time OA	67 ± 15	20 ± 3 **	50 ± 11
% Time OA	33 ± 6	12 ± 2**	24 ± 5
% Open Entries	50 ± 4	31 ± 6*	43 ± 5
Total Entries	37 ± 4	37 ± 3	33 ± 4

Table 1. Effects of a KD on male mice in the Elevated Plus Maze (n=15/group). Data are presented as mean values ± S.E.M. *p<0.05; **p<0.01 significant difference with respect to SD.

3.3 Open Field

The ANOVA of the Open Field (Fig. 3 and Table 2) revealed no significant differences in the variable "Diet" for the distance traveled [F(1,18)= 0.175; p=0.681], time near the wall [F(1,18)= 0.731; p=0.404] and time in the center [F(1,18)= 0.038; p=0.848].

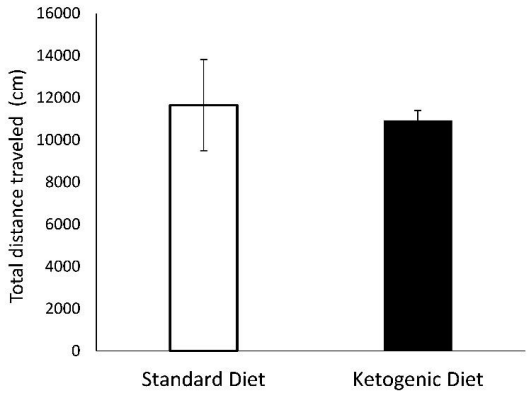

Fig. 3. Effects of a KD on the total distance covered in 1h in the Open Field by mice (n=10/group). Data are presented as mean values ± S.E.M.

	Near the Wall	Center	Rest of the field	Total time
Standard Diet	1283 ± 42	519 ± 41	1798 ± 11	3600
Ketogenic Diet	1334 ± 47	510 ± 29	1756 ± 28	3600

Table 2. Effects of a KD in the time (s) near the wall, center, rest of the field and total time in the Open Field by mice (n=10/group). Data are presented as mean values ± S.E.M.

3.4 Passive Avoidance test

The results of the Passive Avoidance test are presented in Figure 4. The ANOVA revealed an effect of the variable "Days" [$F(1,28)=146.006$; $p<0.001$], with all the groups presenting longer step-through latencies in the 24h test with respect to the training session ($p<0.001$). The ANOVA did not show significant differences for the variable "Diet" [$F(1,28)=0.335$; $p=0.567$] or the interaction "Days x Diet" [$F(1,28)=0.017$; $p=0.896$]. All animals remembered the footshock of the training session.

Fig. 4. Effects of a KD on the time taken for male mice to enter the dark compartment in the Passive Avoidance test during training and 24h after training (n=15/group). Data are presented as mean values ± S.E.M. *** $p<0.001$ significant differences with respect to training.

3.5 Hebb-Williams Maze

The ANOVA for the total latency score (Fig. 5.) revealed an effect of the variable "Maze" [$F(4,96)=10.456$; $p<0.001$]. Maze 1 was significantly easier for all the groups than mazes 3, 4 and 5 ($p<0.001$ in all cases) and 8 ($p<0.01$), as the animals took less time to reach the goal. There were no significant differences in the variable "Diet" [$F(1,24)=0.040$; $p=0.843$] or the interaction "Maze x Diet" [$F(4,96)=0.406$; $p=0.804$].

Fig. 5. Effects of a KD on the total latency score to reach the goal in the 8 trials of male mice in the Hebb-Williams maze. The mazes were classified as easy (1, 3 and 4) or difficult (5 and 8). (n=15/group) ***$p<0.001$ significant difference with respect to Maze 3, 4 and 5; ++ $p<0.01$ significant difference with respect to Maze 8. Data are presented as mean values ± S.E.M.

4. Discussion.

The ketogenic diet has been assessed to determine if it can be used as a nutritional treatment for numerous pathologies, such as epilepsy, Parkinson's disease, Alzheimer's disease, cancer, and more recently, drug addiction [7,9,13]. Although it has been observed that the KD could be an effective treatment option in certain pathologies, such as in children with drug-resistant epilepsy [41], further research is needed to achieve conclusive results in neurological disorders. Preclinical research in these fields studies the possible effects of nutritional interventions, and to draw conclusions, it is necessary to combine physiological results with behavioral outcomes. Experimental treatments, context or researchers can interfere with animal behavior, sometimes leading to inaccurate research conclusions.

As stated in the introduction, the main aim of the present work was to address whether the KD per se had any effects on anxiety, locomotor activity, memory, and learning. Our results confirmed that, even when the KD altered the animal's metabolism, with the increase in ketones, this diet did not affect the cognitive profile of mice, as no significant changes were observed in locomotor activity, memory, or hippocampal-dependent learning with respect the group fed a standard diet. Interestingly, animals on a KD showed an increase in anxiety behavior 7 days after beginning the KD regimen.

Results related to metabolism confirmed that animals exposed to a KD rapidly displayed a ketotic state, as ketone body levels of βOHB significantly increased from the first 24h onwards. When the individual is in a ketotic state, the reduction of carbohydrates in the diet leads to a drastic reduction in the levels of glucose, which ceases to be available as the main source of energy, inducing the body to generate ketone bodies in the liver [42,43]. This rise in blood ketone bodies induces a different metabolism [15] in which ketones, rather than sugar, become the main source of energy. In our study, these values remained stable while the animals were fed with

the KD, but returned to normal 24h after they were switched back to the SD. βOHB levels are widely used as a biomarker of ketosis in mice and humans [15], and our results are consistent with other studies that indicate that KD increases βOHB levels in both mice [21] and rats [20]. With respect to body weight and the KD, preclinical studies have revealed some disagreement, as different studies suggest that a KD can increase or decrease body weight in rats [12,44–46]. This may be due to methodological issues, as it is necessary to match eucaloric diets. In our study, even when animals rapidly entered a ketotic state, their body weight did not differ from that of animals exposed to the SD, which also corresponds with previous results [21,47].

Our results showed that animals fed with the KD spent less time and percentage of time in the open arms of the EPM and made a lower percentage of open entries than those fed with the SD. This result is in contrast with the only other study that evaluated anxiety in animals fed on a KD, which reported no changes with respect to animals fed with a SD [21]. Another study reported that 8 weeks of ketone supplementation also did not induce any changes in anxiety [48]. A plausible explanation for the result obtained in our study is that, in every previous study, anxiety was evaluated 3 months after being on the KD and 8 weeks after supplementation, while we measured anxiety only 7 days after the beginning of KD administration. Adenosine receptors in GABAergic neurons play an essential role in anxiety regulation [49,50] and KD induces modifications in the adenosinergic systems [51], which could explain these initial alterations. The increase in anxiety observed in our study could be due to the short time of habituation to this type of diet. We confirmed that this increase in anxiety was due to the KD, as anxiety levels returned to normal when animals were switched back to the SD. To confirm the results obtained in the EPM, we assessed the time spent near the wall in the Open Field test and found no significant differences between both groups. This may be due to the fact that the EPM is much more sensitive [52], and that the EPM and the

Open Field tests can measure different aspects of anxiety [53]. Indeed, several studies have reported symptoms of anxiety in mice using the EPM, but not with the Open field test [54]. Combining our results with those of previous studies, we could hypothesize that anxiety symptoms produced by the KD would be reduced over time. This result is an important issue to consider in future studies, as it indicates that it is prudent to lengthen the diet adaptation phase before the beginning of any behavioral test. For example, anxiety levels may be interfering if the animal is unable to complete the task of exiting a maze, and the researcher may misattribute this behavior to a lack of learning ability.

Focusing on the cognitive profile and the possible effects of a KD, no changes were observed in locomotion, learning or memory, with the exception of anxiety. In this line, the results of the present work showed that mice on the KD displayed similar locomotion abilities to animals fed the SD when evaluated in the Open Field test, which suggests that the KD does not alter locomotion behavior. The Open Field is a commonly used test, and several studies have confirmed that a KD does not affect general locomotor activity in rats [55,56] and mice [57]. Similar results have been reported by studies evaluating locomotor activity in mice receiving a ketone supplementation [48]. This result could be novel and promising, as studies employing common HFDs have reported alterations in locomotion, such as hyperlocomotion [24,58], or a decrease in activity [59,60]. This outcome would be crucial in studies on pathologies like epilepsy or Parkinson's disease, where a locomotor alteration may mask the real effects of nutritional interventions [10,61].

Regarding implicit memory, independently of the dietary treatment, all animals presented longer step-through latencies in the 24h test with respect to the training session, confirming that they remembered the footshock received earlier. These results confirm those of a previous study in which a KD did not affect a contextual fear-conditioning task in rats [62].

In the same line are the results obtained in hippocampal-dependent learning, which showed that the KD group required the same time as the SD group to reach the goal in all the mazes, confirming that a KD does not alter acquisition of learning. Mice fed with the SD and the KD spent a similar amount of time in the easy or difficult (5) mazes. In general, difficult mazes can discriminate between groups when there is a cognitive deficit in any of them. In the present study, after 8 trials, regardless of diet, all mice always learned the task. A possible explanation for the reduction of time needed to complete Maze 8 despite being considered a difficult maze is the experience that had been previously acquired. The day before performing Maze 8, all mice completed Maze 4, which presents a comparable configuration (Fig 1b) and thus were familiarized with the spatial configuration and learned the task comparatively faster. The Hebb-Williams maze is a very sensitive test which is employed to detect spatial learning deficiencies, but there are no studies to date employing this maze to test KD effects. However, results from other studies have shown no deficits in the Y-maze or Water Maze in male mice kept on a KD for 3 months [21], or in the Novel Object Recognition test [63]. This result is in contrast with previous studies on common HFDs, where it has been shown that continuous exposure to a HFD induces marked memory and spatial learning deficits [24,29,64–67]. This affection might be triggered by leptin levels, which are significantly increased with HFDs [24,68]. It has been shown that when leptin levels increase, memory and learning can be affected [29,69]. In fact, recent studies have suggested that not only does the KD not produce learning and memory impairments but it rescues hippocampal memory deficiencies in mice presenting impairments caused by age [70] or rats exposed to chronic stress environments [71]. Although the KD has different characteristics and metabolic properties from the common HFDs employed to date, it is still a high-fat diet. Thus, it is necessary to confirm the lack of detrimental effects induced by it. Conventional animal HFDs contain approximately 30-40% of carbohydrates, of which approximately 20% are sugars;

while in the KD, the main component is fat (90%), followed by protein and less than 5% carbohydrates. These differences in sugar and carbohydrate composition are the main explanation for the physiological effects of KDs on several diseases. Although both diets are high in fat, the changes that they produce in metabolic status are significantly different.

In preclinical studies, anxiety and locomotor behavior are both commonly used as behavioral complements in several areas of knowledge. In addition, learning and memory are most employed in areas of neurodegenerative disorders and drug addiction, areas in which the KD is increasingly becoming a focus of interest [9,13]. A pharmacological or nutritional treatment can have a significant physiological effect (cells, metabolism), but this effect might not be reflected in a behavioral improvement. For example, a specific treatment could clear amyloid beta protein without an improvement in the learning or memory performance of the animals. Another example could be found in models of addiction, where animals have to learn an operant task to obtain the drug (models of self-administration) [72] or make a contextual association with the drug (models of conditioned place preference), which requires memory and learning abilities for the acquisition process [73]. If the diet per se is affecting learning and memory, the researcher might draw wrong conclusions about the drug, as food, rather than the drug itself, could be influencing the animals' cognitive ability. Therefore, preclinical models of behavior help us to confirm whether the different pharmacological or nutritional treatments are effective. To achieve this end, we must ensure that the treatment per se does not affect behavior or cognition.

5. Conclusion

The present study shows that the KD is a nutritional intervention that does not affect behavior or cognitive performance in male mice. However, one important limitation of this study has been not exploring the effects of this diet in female mice. It should be noted that understanding sex differences in the clinical application of a KD is a very relevant factor to take into account, especially given that the cognitive and behavioral consequences of some psychiatric disorders differ between males and females, as in anxiety and depressive disorders [74]. Therefore, further research is needed to know the effects of a KD in females. In addition, in this study we have evaluated the short-term cognitive effects of a KD, but a longer exposure to this diet before the behavioral testing would verify the lack of cognitive alterations.

Future studies in the addiction and neurodegenerative disease fields that are exploring nutritional interventions with the KD can safely employ different tests of memory, learning and locomotor activity, as well as anxiety, always providing a prudent habituation time to the diet, as anxiety could otherwise alter the results of other tests.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author Contributions:

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Estudio 5

Effects of ketosis on cocaine-induced reinstatement in male mice.

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(Anexo 4)

Effects of ketosis on cocaine-induced reinstatement in male mice

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Abstract.

In recent years, the benefits of the ketogenic diet (KD) on different psychiatric disorders have been gaining attention, but the substance abuse field is still unexplored. Some studies have reported that palatable food can modulate the rewarding effects of cocaine, but the negative metabolic consequences rule out the recommendation of using it as a complementary treatment. Thus, the main aim of this study was to evaluate the effects of the KD on cocaine conditioned place preference (CPP) during acquisition, extinction, and reinstatement. 41 OF1 male mice were employed to assess the effects of the KD on a 10 mg/kg cocaine-induced CPP. Animals were divided into three groups: SD, KD, and KD after the Post-Conditioning test. The results revealed that, while access to the KD did not block CPP acquisition, it did significantly reduce the number of sessions required to extinguish the drug-associated memories and it blocked the priming-induced reinstatement.

Keywords: Ketosis, Cocaine, Ketogenic Diet, High-Fat Diet, Reward, Conditioned Place Preference

1. Introduction

The ketogenic diet (KD) is a high-fat, low-carbohydrate, and protein-balanced diet [1] that induces a specific metabolic status named ketosis. A ketosis status involves a significant change in the main source of energy used by the body and the brain, in which the reduction in carbohydrate intake reduces glucose production, leading the body to use up fat stores [2]. When carbohydrates are reduced to less than 5-10%, fatty acids break down and create ketone bodies in the liver, such as β-hydroxybutyrate (βOHB), which are indicators of nutritional ketosis [3]. Due to this special metabolic status that KD induces, in the last years this diet has been employed as complementary treatment in several neurological disorders, such as epilepsy or neurodegenerative diseases [4–6]. However, there are other diseases like drug addiction, in which the role of diet is just beginning to be studied, but the role of a KD is hardly explored.

Drugs of abuse and palatable diets affect common brain mechanisms, namely the reward system [7]. Both stimulate common brain regions like the lateral hypothalamus, ventral tegmental area, prefrontal cortex or amygdala [8], reduce dopamine active transporter density [9] and activate dopaminergic neurons of the nucleus accumbens [10,11]. This dopaminergic activation caused by palatable food affects neural pathways involved in motivation and reward, such as drugs of abuse [12–14]. For example, the downregulation of dopaminergic receptors in the nucleus accumbens, which is characteristic of the addictive process, is also found in obesity [7].

In recent years, some nutritional interventions have proved to be a modulating factor in the addiction process. For example, in a series of studies, it was observed that a high-fat diet (HFD) can be an important modulating factor of the rewarding properties of cocaine. This effect seems to be dependent on the access pattern of

palatable diets, such as intermittently or continuously. While intermittent access in a vulnerable period, such as adolescence, increases sensitivity to cocaine in the conditioned place preference paradigm (CPP) [15,16], continuous HFD access seems to reduce it [17]. Likewise, HFD administration after acquisition of CPP reduces the number of sessions needed to achieve extinction, suggesting that the diet had a role as an alternative reinforcer and diminished the drug-related memories [17]. Recently, it was demonstrated that a HFD administered in an intermittent schedule, which does not affect metabolic indicators like ghrelin, leptin or bodyweight, also reduced the time required to achieve extinction and blocked reinstatement of cocaine preference in adult male and female mice [18]. To date, studies regarding a possible beneficial interaction of the KD with substance use disorders are scarce. Thus, with all these results regarding HFDs, in the present study we asked ourselves whether other types of diet, such as the KD, could exert a modulation on the conditioned rewarding effects of cocaine.

For example, regarding alcohol, a recently published preclinical - clinical study [19] confirmed that people with an alcohol use disorder maintained on a KD manifested fewer withdrawal symptoms than those on a standard (American) diet. The preclinical data showed that access to a KD reduced ethanol consumption in rats [19] and, more recently, it has also been demonstrated in mice [20]. It seems that the KD could also be advantageous in decreasing ethanol withdrawal symptoms in rats and mice [20,21]. Regarding cocaine, to date only one study has reported decreased cocaine-induced stereotypies and sensitization in male and female rats maintained on a KD, suggesting that this nutritional intervention may act on the dopaminergic system [22].

The present work employed the CPP procedure, which evaluates the contextual cues related to the rewarding effects of a drug. Considering the previous results obtained

with a HFD, we hypothesized that a KD, which changes the metabolic status in the individual, would block the cocaine-induced CPP acquisition and accelerate the extinction of cocaine-related memories in the mice that acquired CPP. Finally, KD may be able to block reinstatement of cocaine-seeking behaviour.

2. Material and Methods

2.1. Subjects

A total of 45 male mice of the OF1 strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4-5 (cage size 28 x 28 x1 4.5cm) at a constant temperature (21±2°C), lights on from 8:00 to 20:00, and food and water available *ad libitum*. All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The Committee for the Use and Care of Animals of the University of Valencia approved the study (2019/VSC/PEA/0065).

2.2. Apparatus and procedure:

2.2.1. Experimental design

To avoid stressful social conditions in their home cages, animals arrived on PND 21 at the laboratory, but the experiment began during their young adulthood, on PND 42. Animals were randomly divided into 3 groups (Fig.1) with similar average body weights (37–40 g): mice fed the standard diet throughout the whole procedure (SD, n=12), mice fed the ketogenic diet throughout the procedure, from PND 42 (KD, n=14), and mice fed the SD until the end of the CPP procedure and a KD after the

Post-C test and until the end of the extinction sessions (PostCPP-KD, n=15). Animals underwent a 10 mg/kg cocaine induced CPP procedure on PND 52, and then underwent an extinction session once a week in order to evaluate the effects of the KD on the extinction of the preference. Body weight and Beta-hydroxybutyrate (βOHB) plasma levels were measured before the Pre-C test, after the Post-C test, and 7 days after the Post-C.

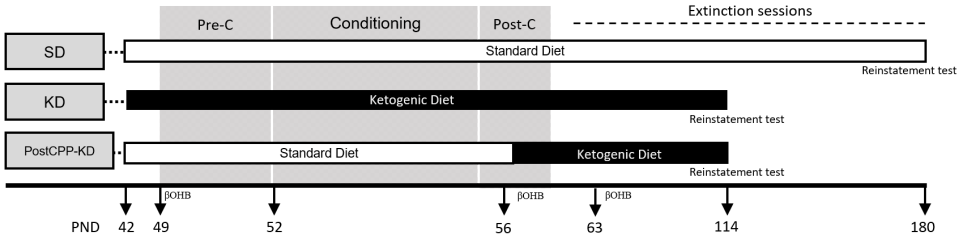

Fig.1. Experimental design.

2.2.2. Feeding conditions and ketosis

Two types of diet were administered in this study: the standard diet (SD) (Teklad Global Diet 2014, 13 kcal % fat, 67 kcal % carbohydrates and 20% kcal protein; 2,9kcal/g) and the ketogenic diet (KD) (TD.96355, 90.5 % kcal from fat, 0.3% kcal from carbohydrates and 9.1% kcal from protein; 6.7 kcal/g). Both diets were supplied by Envigo Teklad Diets (Barcelona, Spain).

To evaluate if animals were on a ketosis status, plasma β-hydroxybutyrate from the tail vein was measured weekly with an On Call GK Dual monitor and ketone test strips (ACON Laboratories, Inc., San Diego, CA).

2.2.3. Drug treatment

For CPP, animals were injected intraperitoneally (IP) with 10 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S.A., Madrid, Spain) diluted in 0.9% NaCl (saline) in a volume of 0.001mL/kg body weight. The dose of 10 mg/kg cocaine has been demonstrated to be an effective dose that induces reinstatement with half the previous received dose in standard mice [18,23].

2.2.4. Conditioned Place Preference

For Place Conditioning, we employed sixteen identical Plexiglas boxes with two equally sized compartments (30.7 cm length x 31.5 cm width x 34.5 cm height) separated by a grey central area (13.8 cm length x 31.5 cm width x 34.5 cm height). The compartments have different coloured walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

Acquisition of CPP

The procedure of Place Conditioning, unbiased in terms of initial spontaneous preference, was performed as described previously [24] and consisted of three phases. To summarize the main aspects, in the first phase, known as Pre-C, mice were allowed access to both compartments of the apparatus for 15 min (900 s) per day for 3 days. On day 3, the time spent in each compartment over a 900-s period was recorded, and animals showing a strong unconditioned aversion (less than 33% of the session time) or preference (more than 67%) for any compartment were excluded from the rest of the experiment (number of mice excluded: 4). The

procedure of assignment is unbiased, assigning half of the animals in each group to the drug or vehicle in one compartment (e.g. white), and the other half in the other compartment (e.g. black). Additionally, half of the animals are assigned to the initially preferred compartment and the other half to their non-preferred compartment.

After assigning the compartments, no significant differences were detected between the time spent in the drug-paired and vehicle-paired compartments during the pre-conditioning phase. In the second phase (conditioning), which lasted 4 days, animals received an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30 min. After an interval of 4 hours, they received an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. Confinement was made possible in both cases by closing the guillotine door that separated the two compartments, rendering the central area inaccessible. During the third phase, known as Post-C, the guillotine door separating the two compartments was removed (day 8) and the time spent by the untreated mice in each compartment during a 900-s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates that an aversion has developed.

Extinction of CPP

When preference for the drug-paired compartment had been established, all groups underwent a weekly extinction session in which they were placed in the apparatus (without the guillotine doors separating the compartments) for 15 min. Results were checked every week for each group to confirm if criteria had been satisfied. The

extinction condition was fulfilled when there was a lack of a significant difference between CPP scores and Pre-C test values in two consecutive sessions.

Reinstatement of CPP

Twenty-four hours after extinction had been confirmed, the effects of a priming dose of cocaine were evaluated. The reinstatement test was the same as those carried out in Post-C (free ambulation for 15 min), except that animals were tested 15 min after administration of the respective dose of cocaine (5 mg/kg). Priming injections were administered in the vivarium, which constituted a non-contingent place to that of the previous conditioning procedure. If animals reinstated the preference, the extinction sessions continued in time and when the criteria were met again, the next half-dose (2.5mg/kg) was administered. If they did not reinstate the preference, then the experiment finished. Therefore, each group can finish the procedure at different times.

2.3. Statistical analysis

Data relating to βOHB were analysed by a mixed ANOVA with one between-subjects variable – "Diet", with 3 levels (SD, KD and PostCPP-KD) - and a within variable – "Days", with 3 levels (Pre-C, Post-C and DAY7Post-C). Data relating to bodyweight were analysed by a mixed ANOVA with one between-subjects variable – "Diet", with 3 levels (SD, KD and PostCPP-KD) - and a within variable – "Weeks", with 8 levels (Baseline and Weeks 1-7). Body weight was compared until week 7 due to different extinction-reinstatement timings.

For the CPP procedure, the time spent in the drug-paired compartment was analysed by a repeated measures ANOVA, with the between-subjects variable - "Diet", with 3 levels (SD, KD and PostCPP-KD) - and a within variable – "Days", with two levels (Pre-C and Post-C). To compare whether extinction/reinstatement had been achieved

within the same group, data relating to extinction and 5 mg/kg reinstatement were analysed by means of Student's t-test. The time required for the preference to be extinguished was analysed by means of the Kaplan-Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate. All results are expressed as mean ± S.E.M. Analyses were performed using SPSS v26.

3. Results

3.1 Increased β-hydroxybutyrate (βOHB) and body weight.

With respect to βOHB plasma levels (Fig. 2a), the ANOVA revealed a significant effect of the interaction "Days x Diet" [$F(4,76) = 34{,}714$; $p < 0.001$], as the KD group showed increased levels of βOHB with respect to SD and PostCPP-KD when measurements were taken in Pre-C ($p < 0.001$) and Post-C ($p < 0.001$). The KD and PostCPP-KD groups exhibited higher levels than the SD group 7 days after Post-C, ($p < 0{,}001$ in both cases). Moreover, the PostCPP-KD group's levels were higher 7 days after Post-C when compared to pre-C and post-C measures ($p < 0.001$ in both cases).

Regarding changes in body weight (Fig. 2b), the ANOVA revealed no significant differences in the variable "Diet" [$F(2,38) = .019$; $p = 0.981$], as all groups presented similar weight throughout the procedure. There was a significant effect of the variable "Week" [$F(7,266) = 254{,}571$; $p < 0.001$], since mice showed higher body weight in weeks 1 to 7 than at baseline ($p < 0.001$, in all cases).

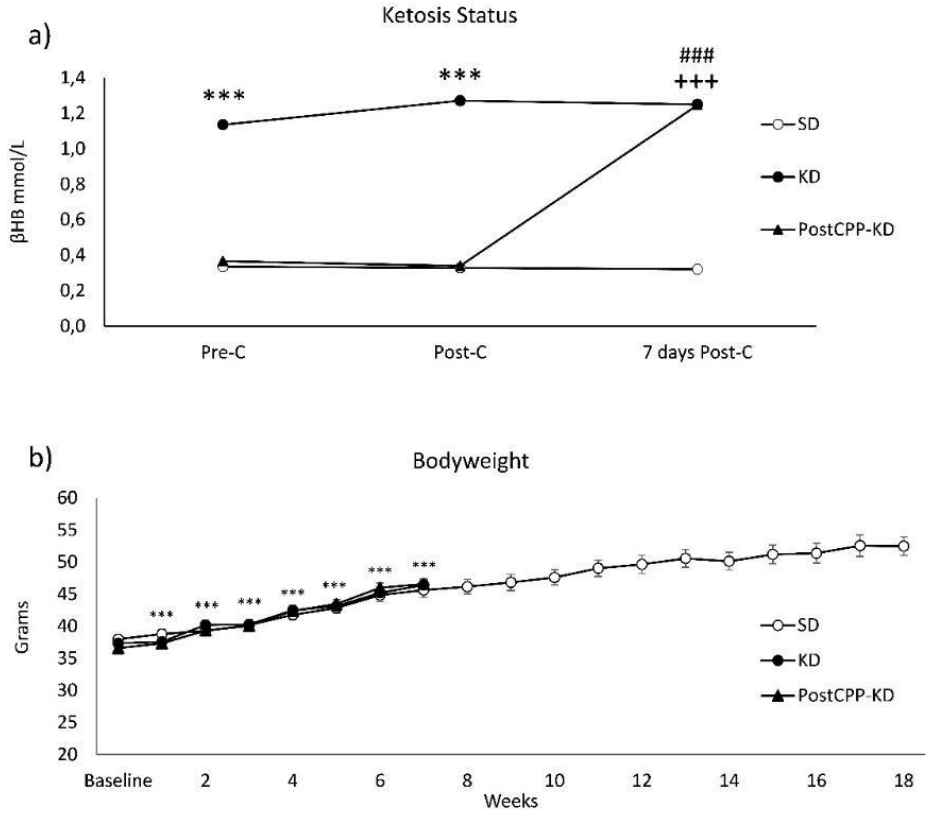

Fig.2. β-hydroxybutyrate plasma levels and weekly body weight. (a) Ketosis status. Data are represented as the mean (±SEM) amount of βOHB. ***p<0.001 significant difference with respect to the rest of the groups. +++p<0.001 significant difference with respect to SD. ###p<0.001 significant difference with respect to Pre-C and Post-C. **(b) Weekly body weight**. Data are represented as the mean (±SEM) body weight measured weekly. ***p<0.001 significant difference with respect to Baseline.

3.2. Conditioned place preference

The ANOVA for the time spent in the drug-paired compartment (Fig. 3) revealed an effect of the variable "Days" [F(1,38)=111,919; p<0.001]. Bonferroni's post-hoc comparisons showed that the mice spent significantly more time in the drug-paired

compartment in Post-C than in Pre-C ($p<0.001$ in all cases). These results indicate that the three groups developed CPP.

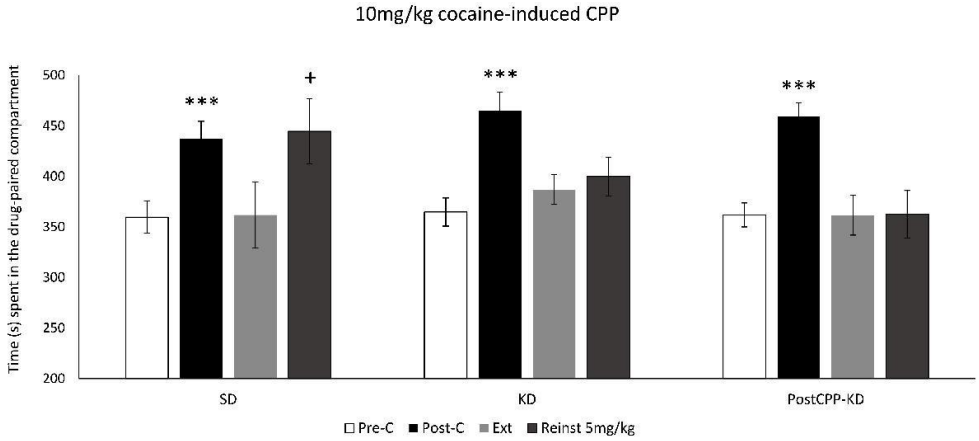

Fig. 3. Effects of KD during the extinction-reinstatement process in the Conditioned Place Preference (CPP) paradigm. Bars represent the time (± SEM) in seconds spent in the drug-paired compartment in the Pre-Conditioning test (white bars), the Post-conditioning test (black bars), the last extinction session (light gray bars) and the reinstatement test (dark gray bars). The reinstatement test was evaluated 15 min after a priming dose of 5 mg/kg of cocaine. ***$p<0.001$ significant difference with respect to the Pre-C. + $p<0.05$ significant difference with respect to Ext.

With regards to the time required to extinguish the preference (Fig. 4), the SD group required a total of 19 sessions, while the KD and the PostCPP-KD groups required only 8 sessions. The Kaplan-Meier analysis revealed that the SD group required significantly more sessions than the other two groups to extinguish the preference ($p< 0,05$ in both cases).

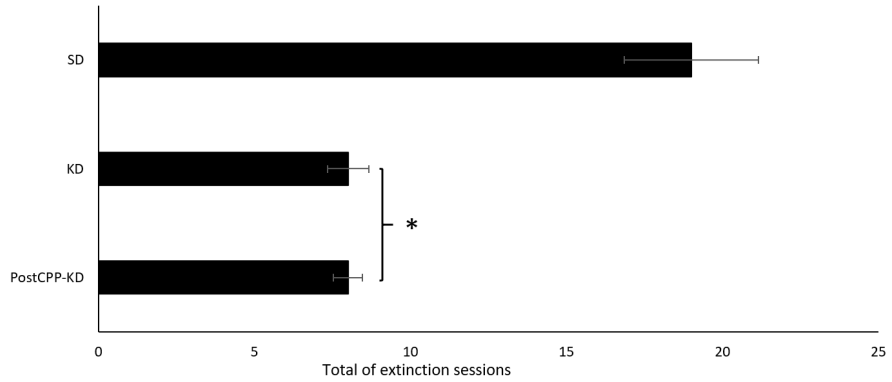

Fig. 4. Extinction. The bars represent the total value (± SEM) of the number of sessions required for the preference to be extinguished after the Post-C test. The Kaplan-Meyer analyses showed * $p<0.05$ significant difference with respect to SD.

Reinstatement of drug-seeking after achievement of extinction was evaluated with Student's t-tests, which showed that reinstatement with a priming dose of 5 mg/kg cocaine was achieved only in the SD group ($t=-2.943$; d.f. 9; $p=0.016$).

4. Discussion.

The aim of the present study was to evaluate whether a KD can modulate the conditioned rewarding effects of cocaine in two critical moments: during acquisition and/or during extinction/reinstatement of the preference. The results showed that all animals, regardless of being fed a KD or SD, developed a place preference for the cocaine-paired compartment after administration of 10 mg/kg of cocaine. However, during the extinction-reinstatement process both groups fed with the KD needed fewer sessions for the preference to be extinguished than the SD group. In the reinstatement test, induced by a priming dose of 5mg/kg of cocaine (half the previously received dose), only the SD group exhibited preference for the drug-

paired compartment, confirming that being on a KD blocked reinstatement with 5mg/kg cocaine. To date, only one study has evaluated how access to a KD mediates the effects of cocaine. Martinez et al., [22] reported that access to a KD over three weeks reduced cocaine withdrawal symptoms in rats. In that study, rats received daily cocaine injections and after cessation, withdrawal symptoms such as stereotyped locomotor responses appeared. Their results showed that the animals fed a KD showed weaker cocaine-induced stereotyped response than those fed a SD.

Studies with other drugs of abuse, such as ethanol, have reported similar results, with rats or mice on KD displaying milder ethanol withdrawal symptoms [20,21]. As mentioned in the introduction, the KD reduces ethanol self-administration during acute withdrawal in rats and withdrawal symptoms during ethanol detoxification in humans [19]. In addition, previous studies by our research group have shown that access to a KD for 7 days prior to an ethanol self-administration test, and maintaining it for 4 weeks, reduces ethanol consumption compared to animals on a SD [25].

One of the main therapeutic effects of the KD is the increase that it produces in adenosine transmission, and one of the possible explanations for the effects of a KD on drug addiction is the relationship between adenosine and dopamine [22]. Several studies have demonstrated that there is an antagonistic interaction between the adenosine A1 - Dopamine D1 and adenosine A2A - dopamine D2 receptors [26], especially in GABAergic neurons. For example, D1 binding affinity is decreased by A1 agonists, suggesting that the A1 receptor modulates dopaminergic transmission [27]. It has been proposed that the response to drugs, such as psychostimulants, is also mediated by adenosine [28–30]. On the other hand, there are preclinical studies that have demonstrated that A2 agonists reduce cocaine and morphine locomotor sensitization [30,31] and decrease cocaine self-administration [32]. However, antagonist administration causes comparable effects to psychostimulants and enhances relapse into cocaine self-administration [33]. Thus, the main hypothesis of

this work is that KD could attenuate dopaminergic transmission through activation of adenosine receptors [31,34]. In fact, in a previous study, we observed that a 4-5-week KD access led to alterations in the adenosine, dopamine and cannabinoid gene expression of mice [25]. Although the adenosine-dopamine modulation would not be strong enough to block the acquisition of cocaine-induced CPP, it could diminish the strength of the conditioning and therefore reduce the number of sessions needed to extinguish the preference, as well as the power of a cocaine-priming dose to reinstate drug-seeking behaviour. However, more extensive studies are needed to confirm the neurobiological mechanisms underlying these effects, especially ones considering female mice in them.

Our results, in line with those of Martínez et al., [22], also suggest another possible explanation for the KD modulation of addiction, which could be the role of differences in βOHB blood levels. Even when the KD contains more than double the calories as the SD, animals in the KD group did not gain more body weight than mice in the SD group. Previous results suggest differences in energy expenditure or lower food intake in KD-fed animals, with studies showing increases or decreases in body weight with respect to the control groups [21,25,35]. Nevertheless, some studies have reported that butyrate, which is a histone deacetylase inhibitor, keeps mice metabolically normal when maintained on a high-fat diet, with low glucose and insulin levels and normal body weight [36]. Butyrate is a product of bacterial anaerobic fermentation [37] and closely related to βOHB, the main source of energy for mammals during ketosis [38]. There are some studies that have reported that the overexpression of HDAC increases effects caused by cocaine [39]. Therefore, if βOHB could be acting as an endogenous HDAC inhibitor [40, 41], it would contribute to the final effects of ketosis on cocaine extinction.

The KD may be considered as a promising nutritional approach in the treatment of cocaine addiction. Although the diet cannot be an exclusive treatment, it can contribute to the attenuation of the memories related to cocaine consumption, as well as the risk of relapse. This study supports what previous studies with other types of high-fat diets have suggested, and it is that nutritional interventions can modulate the conditioned effects of drugs like cocaine, which today does not yet have a definitive treatment.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author Contributions

Francisco Ródenas-González: Methodology, Software, Validation, Formal Analysis, Investigation, Data Curation, Writing-Review and Editing, Visualization. **M. Carmen Blanco-Gandía**: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Data Curation, Writing-Review and Editing, Visualization. **José Miñarro:** Conceptualization, Resources, Supervision, Project, Funding Acquisition. **Marta Rodríguez-Arias:** Conceptualization, Resources, Data Curation, Writing-Review and Editing, Visualization, Supervision, Project Administration, Funding Acquisition.

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Estudio 6

Ketogenic Diet Decreases Alcohol Intake in Adult Male Mice.

Blanco-Gandía, M.C., **Ródenas-González, F.,** Pascual, M., Reguilón, M.D., Guerri, C., Miñarro, J., y Rodríguez-Arias, M.

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(Anexo 5)

Ketogenic Diet Decreases Alcohol Intake in Adult Male Mice

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Abstract

The classic ketogenic diet is a diet high in fat, low in carbohydrates, and well-adjusted proteins. The reduction in glucose levels induces changes in the body's metabolism, since the main energy source happens to be ketone bodies. Recent studies have suggested that nutritional interventions may modulate drug addiction. The present work aimed to study the potential effects of a classic ketogenic diet in modulating alcohol consumption and its rewarding effects. Two groups of adult male mice were employed in this study, one exposed to a standard diet (SD, n = 15) and the other to a ketogenic diet (KD, n = 16). When a ketotic state was stable for 7 days, animals were exposed to the oral self-administration paradigm to evaluate the reinforcing and motivating effects of ethanol. Rt-PCR analyses were performed evaluating dopamine, adenosine, CB1, and Oprm gene expression. Our results showed that animals in a ketotic state displayed an overall decrease in ethanol consumption without changes in their motivation to drink. Gene expression analyses point to several alterations in the dopamine, adenosine, and cannabinoid systems. Our results suggest that nutritional interventions may be a useful complementary tool in treating alcohol-use disorders.

Keywords: Ketosis, alcohol, ketogenic, ketone, adenosine, dopamine

1. Introduction

Motivation to seek drugs of abuse and highly palatable foods is regulated by the reward system [1]. Previous studies have shown psychological and biological commonalities between palatable food intake and drug addiction [2,3] and recent studies have indicated that nutritional habits are an important modulating factor in the development of cocaine [4,5] and alcohol addiction [6,7]. Palatable diets change metabolism and the reward system by increasing vulnerability to the rewarding effects of psychostimulants and depressants, such as cocaine and alcohol [4,6,8], but little is known about the protective effects that nutrition could have in the development of drug addiction. In a recent study, a high-fat diet was shown to reduce relapse into cocaine seeking in adult male mice [5]. However, the diet employed in those studies was rich in saturated fats and sugar, producing metabolic syndrome and obesity when the HFD administration was continuous and therefore, it cannot be recommended as a drug-addiction combined therapeutic. This metabolic syndrome is avoided when the administration of HFD is intermittent, although the accelerated extinction and the blockade of reinstatement is maintained [9]. Nevertheless, this contribution opened a new gateway to focus on nutrition as a possible complementary treatment in the field of drug addiction. For this reason, we considered the classic ketogenic diet (KD), a diet still high in fats, to prevent the development of escalation in alcohol consumption.

Today we can find several eucaloric protocols like the medium-chain triglyceride diet (MCT), the modified Atkins diet (MAD), the low glycemic index treatment (LGIT) or the classic ketogenic diet (KD). The KD is a high-fat, low-carbohydrate, and protein-balanced diet that induces a different metabolic state, in which ketone bodies are used as the main energy source [10,11]. The decrease in carbohydrates reduces the amount of glucose, which ceases to be available as the main source of energy [11]. This is where the body begins to use fat storages, breaking down fatty acids and creating ketone bodies in the liver (e.g., acetoacetate, β-hydroxybutyrate or βOHB to produce ATP, adenosine triphosphate) [11,12]. The rise in blood ketone

bodies appears as a response to low glucose levels, known as metabolic state ketosis. Ketosis can be achieved in two ways: through diet (nutritional ketosis) or through fasting. In the present work we will focus on nutritional ketosis.

Ketosis is not new. Evolutionarily, humans have spent a good part of their existence in ketotic state (especially in winter, where carbohydrates such as fruits and vegetables were limited). Moreover, ketone bodies play a very important role in the normal fetal brain development [13,14], as breast milk is high in fat and medium-chain fatty acids, inducing a ketotic state in the new-born [15,16]. The KD is neuroprotective [17], since ketone bodies cross the blood-brain barrier without difficulty and increase metabolic efficiency by improving mitochondrial function [18,19]. It reduces oxidative stress, with antioxidant and anti-inflammatory effects, inhibiting inflammatory markers such as interleukins and tumour necrosis factor alpha [20]. However, the mechanism through which a KD is beneficial is still under study. The KD has been used successfully for different disorders such as epilepsy [21,22], Alzheimer's and Parkinson disease [23,24,25,26,27], brain cancer [28,29], autism [30,31], and amyotrophic lateral sclerosis [32]. Based on all of these studies, we can hypothesize that ketones induce a normalization process when metabolism functioning is dysregulated, which can account for its beneficial effects on all of these neurological disorders.

Diet and nutrients can exert great changes in neural plasticity, modifying circuits and normalizing their function [33,34,35]. One of the most important KD neural mechanisms is its modulation of ATP-sensitive potassium channels and increase of GABAergic and purine neurotransmission such as adenosine [36,37]. The KD activates adenosine receptors, which are the basis of its therapeutic effects on diseases such as epilepsy, as it inhibits the excitability of neurons [38,39,40]. Adenosine regulation is closely linked to the dopaminergic action, as its receptors are colocalized on GABAergic neurons together with dopamine D2 receptors, suggesting that modifying one may lead to the regulation of the other [41]. In fact,

there is an antagonistic interaction between the heterodimers of the adenosine A1—Dopamine D1 vs. adenosine A2A—dopamine D2 receptors [42]. Although some of the results are controversial, there is evidence indicating that adenosine mediates the response of drugs such as opiates, cannabinoids, and psychostimulants [43,44,45,46]. For example, cocaine self-administration (SA) induces an upregulation of A2 receptors and a downregulation in D2 receptors as a compensation, a situation that is reversed with abstinence [47].

To date, only a few studies have shown the protective effect that a KD can have on drug addiction. Martínez and co-workers [48] showed that animals on a KD showed decreased cocaine-induced stereotypies and sensitization in male and female rats, suggesting that the KD modulates the dopaminergic system. Regarding alcohol, KD has been reported to decrease alcohol withdrawal symptoms in rats [49] and mice [50], as well as reduce alcohol consumption in rats [51]. The recently published work by Wiers and co-workers [51] combines a preclinical and a clinical study. In relation to the clinical study, Wiers and co-workers [51] also found that people with alcohol-use disorder who were on a KD had fewer withdrawal symptoms and required fewer medication the first week of detoxification than those following a standard American diet [51].

Therefore, the main aim of the present study is to evaluate whether a KD could modulate the rewarding effects of alcohol by acting through the adenosine-dopamine binomial. To achieve this objective, the behavioral effect of a KD on the oral ethanol SA was studied. Additionally, we evaluated the main changes in dopamine and adenosine receptor gene expression in the striatum, taking into account that diets and nutrition can disrupt the normal plasticity in the dorsal striatum [52]. Moreover, given the implication of the cannabinoid and opioid systems in the rewarding effects of not only alcohol but also high-fat foods [53,54], we included the analysis of the CB1r and Oprm expression.

2. Materials and Methods

2.1. Subjects

This study was performed with 47 OF1 male mice (Charles River, Écully France), which were housed under standard conditions (21 ± 2 °C) in groups of 4 (cage size 28 × 28 × 14.5 cm). Animals were 21-days old on arrival at the laboratory but initiated the experimental feeding condition on PND 42. Lights were turned on from 8:00–20:00, and food and water were available ad libitum. All procedures involving mice and their care complied with the national, regional, and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes. The Animal Use and Care Committee of the University of Valencia approved the study with the code 2019/VSC/PEA/0065 on 23 March 2019.

2.2. Drugs treatment

Absolute ethanol (EtOH) (Merck, Madrid, Spain) was dissolved in water using a w/v percentage, i.e., a 6% (w/v) EtOH solution equivalent to a 7.6% (v/v) EtOH solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water.

2.3. Apparatus and Procedure

2.3.1. Experimental Design

In the present study we employed two animal sets. In the first set, animals in the standard diet (SD, n = 15) group received the standard diet throughout the procedure, while animals in the ketogenic diet (KD, n = 16) group received the ketogenic diet from young adulthood, PND 42, until the end of the experiment. All the animals began the training phase on PND 49 and the 6% EtOH consumption phase on PND 66, when mice are already considered adults. Bodyweight and beta-hydroxybutyrate (βOHB) blood levels were measured every week throughout the study. During the

SA procedure, animals had 1 h access to food per day. About 15% weight loss was produced by the food restriction schedule [55]. Brains were collected at the end of the SA for gene expression analysis.

In the second set, a total of 16 animals were used (n = 8 per diet). They had the same feeding conditions and initiated the diet conditions on the same PND as the first set, but without any behavioral manipulations. In order to test only the effects of KD, animals in this second set were not exposed to alcohol. They were euthanized for brain gene expression analysis on PND 77, as with the first set. A detailed description of the experimental procedure is shown in Figure 1.

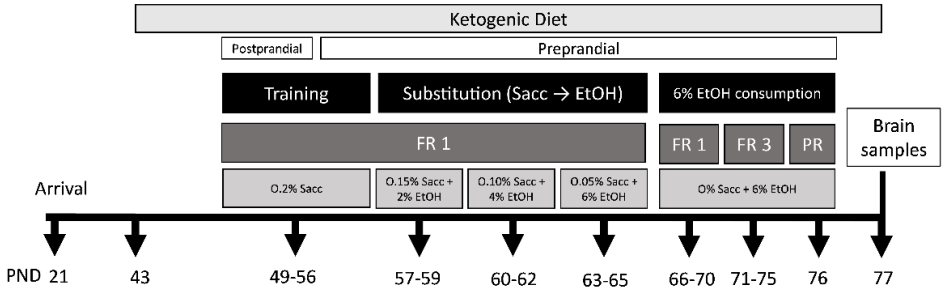

Figure 1. Experimental design for the 1st set of mice.

2.3.2. Feeding Conditions

Two different types of diets were used in this study. The SD group was fed with the standard diet (Teklad Global Diet 2014, 13 kcal % fat, 67 kcal % carbohydrates and 20% kcal protein; 2,9 kcal/g) and the KD group with a ketogenic diet (TD.96355, 90.5% kcal from fat, 0.3% kcal from carbohydrates and 9.1% kcal from protein; 6.7 kcal/g). The different diets were supplied by Envoi Teklad Diets (Barcelona, Spain).

2.3.3. Ketosis status: β-hydroxybutyrate blood levels

Blood β-hydroxybutyrate was measured weekly from the tail vein with an On Call GK Dual monitor and ketone test strips (ACON Laboratories, Inc., San Diego, CA, USA).

2.3.4. Oral Ethanol self-administration

Following the previously published protocol [55], eight modular operant chambers (Med Associates Inc., Georgia, VT, USA) and Med-PC IV were employed to carry out the oral EtOH SA. These cages contain two small holes with photocells that register nose-poke responses. Active nose-pokes activated a 0.5 s stimulus light and buzzer beep, which were followed by the delivery of 37 μL of EtOH, followed by a time-out of 6 s. Inactive nose pokes did not have any effect. This protocol consists of three phases: training phase, saccharin substitution phase, and 6% EtOH consumption phase.

Training phase (8 days)

In the training phase, animals had to nose-poke into the active wholes to obtain 37 μL of saccharin (0.2% (w/v)). To facilitate learning acquisition, two days before beginning the training, chow was restricted to 1 h/day and water was suspended for 24 h before the first session. Only during the subsequent 3 days, 1 h before initiating the operant session animals had access to food but not to water (postprandial). On the subsequent four days and during the course of the experiment, to avoid EtOH intake due to thirst, water was available anytime and food was available for 1 h after each training session (preprandial).

Saccharin substitution (9 days)

In this phase, saccharin percentage was progressively reduced as the EtOH concentration was gradually augmented [55,56]. Animals had access to each combination for three consecutive sessions (0.15% Sac −2% EtOH; 0.10% Sac −4% EtOH; 0.05% Sac −6% EtOH).

6% Ethanol consumption (11 days)

This phase evaluates the number of active nose-poke responses, the 6% EtOH (w/v) intake and motivation to obtain it. First, animals were exposed to 5 days of fixed ratio 1 (FR1) sessions, in which the number of effective responses on the active nose-poke and EtOH consumption (all) was measured. After each session, the remaining fluid in the receptacle was collected and quantified with a micropipette. Following the FR1 sessions, animals were exposed to the fixed ratio 3 (FR3) schedule for 5 days, where they had to respond three times with an active nose poke to obtain one EtOH reinforcement. To set the breaking point for each animal, which is the maximum number of active nose-pokes the animal is capable of accomplishing to obtain one reinforcement, a progressive ratio (PR) session with a 2 h duration was carried out. The response requirement to achieve reinforcements increased corresponding to the series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The breaking point that the animal achieved was calculated based on this scale, which defines the animal's motivation toward EtOH consumption.

2.3.5. RNA isolation, reverse transcription, and quantitative RT-PCR

After the PR session, mice were euthanized by cervical dislocation, and their brains were extracted and striata dissected. Brain tissue samples were immediately stored at −80 °C until the qRT-PCR assay was performed.

Following the manufacturer's protocol, the Tri Reagent Method (Sigma-Aldrich, St. Louis, MO, USA) was employed to isolate the total RNA from the striatum. Reverse transcription of 1 mg of total RNA was performed via the Transcriptor First Strand cDNA synthesis kit (Thermo Fisher Scientific, Madrid, Spain). Amplification of the target and housekeeping (b-glucuronidase) genes was completed employing the Taqman Gene Expression Master Mix (Thermo Fisher Scientific, Madrid, Spain) in a LightCycler 480 System (Roche Diagnostics, Madrid, Spain). The assay codes of the primers used were Mm02620146 and Mm00438545 for dopamine receptors 1 and 2 (DrD1, DrD2), Mm01308023 and Mm00802075 for adenosine receptors A1 and A2 (ADORA1, ADORA2a), and Mm01188089 and Mm00446953 for cannabinoid receptor 1 (CB1r) and opioid receptor μ (Oprm), respectively. Data were analyzed using the LightCycler 480 relative quantification software and normalized to the amplification product of b-glucuronidase.

2.4. Statistics

Data relating to body weight and βOHB were analyzed by a mixed ANOVA with one between-subjects variable diet (with 2 levels, standard diet (SD) and ketogenic diet (KD)) and a within variable PND with 6 levels: Baseline and Weeks 1–5.
Regarding EtOH SA, a two-way ANOVA was performed with the variable diet (standard or ketogenic) as a between variable and days (5 levels for FR1 or FR3) as a within variable. To analyze breaking point values in the progressive ratio, a Student's test was performed. The gene expression data were analyzed by a two-way ANOVA with two between variables, diet (with 2 levels, standard diet (SD) and ketogenic diet (KD)) and EtOH (no-alcohol self-administration (NO-SA) and alcohol self-administration (SA). Bonferroni post-hoc tests were also analyzed. Data are presented as mean ± SEM. Analyses were performed using SPSS v26 (IBM SPSS Statistics for Windows, Version 26.0. IBM Corp, Armonk, NY, USA).

3. Results

3.1. Increased β-hydroxybutyrate (βOHB) and bodyweight in mice fed on KD.

Results regarding βOHB blood levels (Figure 2) revealed a significant effect of the interaction week x diet [$F(5145) = 12{,}899$; $p < 0.001$]. The KD group showed increased levels of βOHB in comparison with the SD group in Weeks 1, 2, 3, 4, 5 ($p < 0.001$ in all cases). There were also significant increases within the KD group on weeks 1, 2, 3, 4, 5 with respect to the baseline ($p < 0.001$), as well as increases in the SD group in Weeks 3, 4, and 5 with respect to the baseline ($p < 0.05$), probably due to coincidence of food deprivation during the SA procedure.

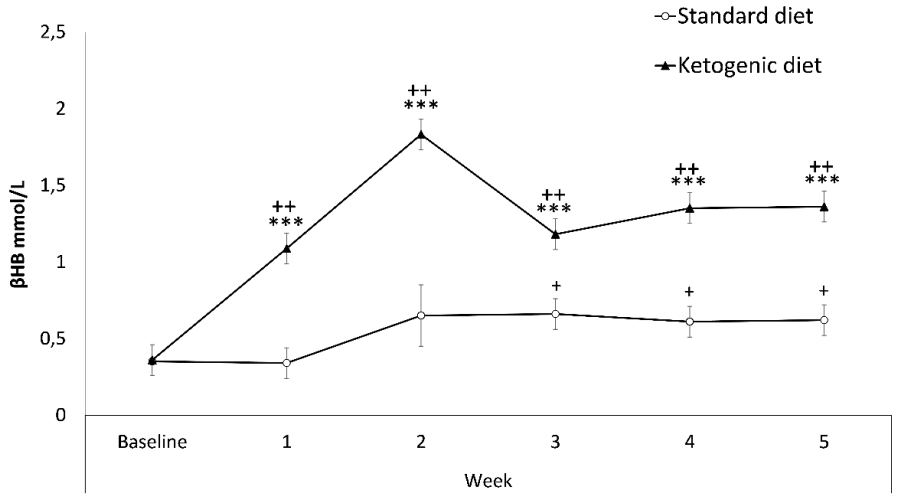

Figure 2. Weekly β-hydroxybutyrate blood levels. Data are represented as the mean (±SEM) amount of βOHB measured weekly. ***p<0.001 significant difference with respect to SD group. ++p<0.001; +p<0.05 significant difference with respect to the baseline.

Regarding changes in bodyweight (Figure 3), the ANOVA revealed a significant effect of the variable diet [$F(1,29) = 6.731$; $p < 0.05$], as the KD group presented a higher bodyweight than the SD group ($p < 0.05$, SD = 38 gr vs. KD = 40 gr). There

was also a significant effect of the variable week [$F(5145) = 46.893$; $p < 0.001$], as all weeks showed lower bodyweight than baseline ($p < 0.001$). In addition, during Week 2 (when the food deprivation for oral SA started) bodyweight was significantly lower than the rest of the weeks ($p < 0.001$). Finally, there was an effect of the interaction week x diet [$F(5145) = 3.705$; $p < 0.01$]. The KD group exhibited higher body weight with respect to the SD group on weeks 2, 3, and 4 ($p < 0.001$; $p < 0.01$, and $p < 0.05$ respectively).

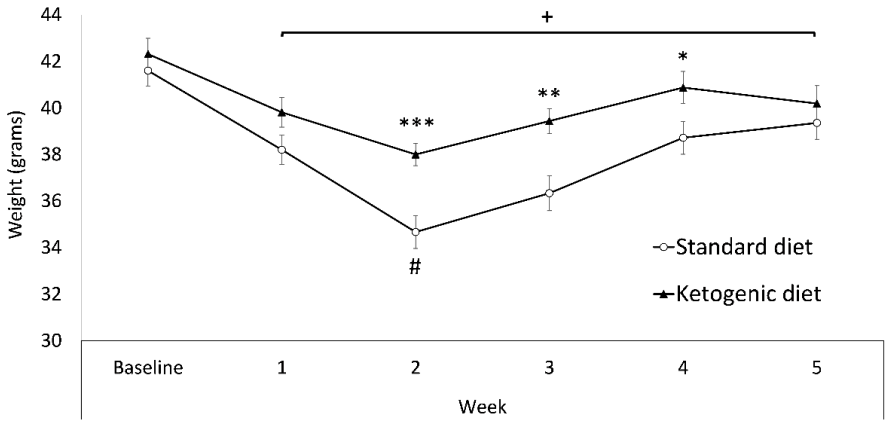

Figure 3. Weekly bodyweight. Data are represented as the mean (±SEM) bodyweight measured weekly. ***p<0.001; **p<0.01; *p<0.05 significant difference with respect to the SD group. +p<0.05 significant difference with respect to the baseline. #p<0.001 significant difference with respect the rest of the weeks.

3.2. Ketogenic diet decreased oral ethanol self-administration

Regarding the number of active responses during the FR1 schedule of EtOH SA (Figure 4a), the ANOVA did not show any significant differences between SD and KD. With respect to EtOH consumption (g/kg) during the FR1 schedule (Figure 4b), the ANOVA reported a significant effect of the variable diet [$F(1,29) = 10.554$; $p <$

0.01], as the KD group exhibited a decreased oral SA of EtOH with respect to the SD group ($p < 0.01$; KD = 0.51 ± 0.04 g/kg vs. SD = 1.04 ± 0.08 g/kg).

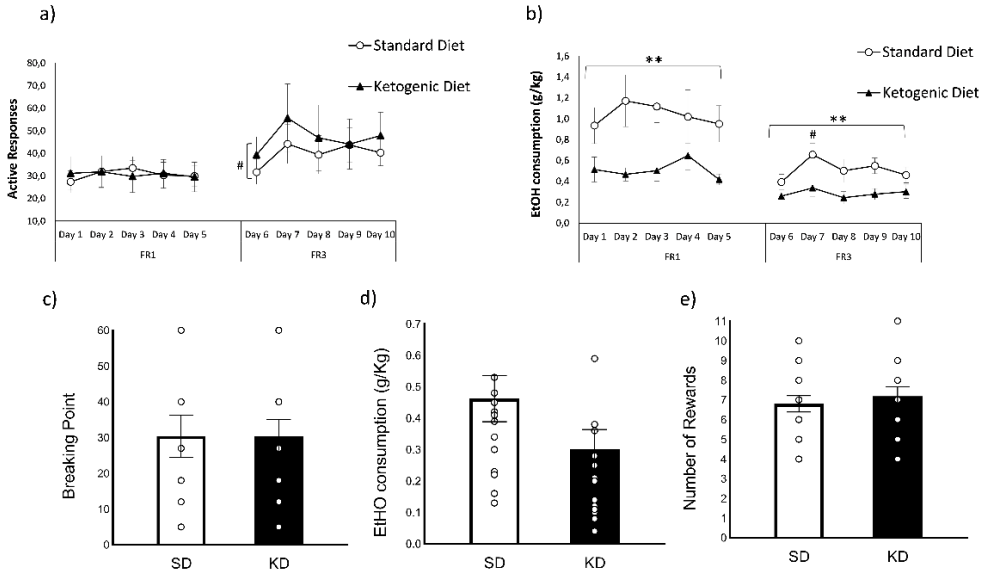

Figure 4. Oral EtOH self-administration (SD n=15; KD n=16). (a) The dots represent means and the vertical lines ± SEM of the amount of the number of active responses and (b) the volume of 6% EtOH consumption during FR1 and FR3 (in g/kg). (c) The columns represent means and the vertical lines ± SEM of breaking point values (d) the volume of 6% EtOH consumption (in g/kg) and (e) the number of rewards obtained during PR. **p<0.01, significant difference with respect to the SD group. #p<0.05, significant differences with respect to Day 7.

During the FR3 schedule, the ANOVA for the number of active responses (Figure 4a) showed a significant effect of the variable day [$F(4116) = 2942$; $p < 0.05$], as all mice exhibited lower number of active responses on Day 6 with respect to Day 7 ($p = 0.076$). With regards to EtOH consumption (g/kg) during the FR3 schedule (Figure 4b), significant differences were reported in the variable diet [$F(1,29) = 8142$; $p < 0.01$], since the KD group showed a decreased oral SA of EtOH ($p < 0.01$; KD = 0.28 ± 0.03 g/Kg vs. SD = 0.51 ± 0.04 g/Kg). There was also an effect of the variable

day [$F(4116) = 2638$; $p < 0.05$], as all mice showed higher EtOH intake in day 7 with respect to day 6 ($p < 0.05$).

Regarding the progressive ratio (Figure 4c,d,e), there were no significant differences in the breaking point ($t = -0.005$ d.f. 29; $p = 0.99$), EtOH consumption ($t = 1674$ d.f. 29; $p = 0.105$), and the number of rewards ($t = -0.616$ d.f. 29; $p = 0.54$).

3.3. Gene expression analyses.

3.3.1. Ketogenic diet induced increased expression of DrD1 and DrD2 gene expression af-ter ethanol self-administration.

For DrD1 gene expression (Figure 5a), the ANOVA revealed a significant effect of the variable diet [$F(1,28 = 14.652$; $p < 0.001$], EtOH [$F(1,28) = 8.378$; $p < 0.01$] and the interaction diet x EtOH [$F(1,28) = 8.142$; $p < 0.01$]. With regards to DrD2 expression (Figure 5b), the ANOVA revealed an effect of the variable EtOH [$F(1,28) = 8.625$; $p < 0.01$] and the interaction diet x EtOH [$F(1,28) = 8.639$; $p < 0.01$]. Exposure to a KD induced a significant increase in DrD1 and DrD2 expression in KD animals after the EtOH SA with respect to the rest of the groups ($p < 0.001$ in all cases).

3.3.2. Opposite changes in ADORA1 and ADORA2 gene expression in response to keto-genic diet and ethanol self-administration.

For the adenosine receptor A1 gene expression (ADORA1, Figure 5c), the ANOVA revealed a significant effect of the interaction diet x EtOH [$F(1,28) = 6.099$; $p < 0.05$]. Mice in the KD group that did not perform SA (KD-NO SA) showed an overexpression of ADORA1 with respect to the corresponding SD group ($p < 0.01$) as well as with respect to the KD-SA group ($p < 0.01$).

Regarding the expression of the ADORA2 gen (Figure 5d), the ANOVA revealed a significant effect of the variable EtOH [$F(1,28) = 10.587$; $p < 0.01$] and the interaction diet x EtOH [$F(1,28) = 10.615$; $p < 0.01$]. After the EtOH SA, mice exposed to the KD showed a significant overexpression in ADORA2 with respect to their corresponding SD-SA group ($p < 0.01$) and KD-NO SA group ($p < 0.001$).

3.3.3. Ketogenic diet decreases CB1r gene expression.

With regards to CB1r gene expression (Figure 5e), the ANOVA revealed an effect of the variable EtOH [$F(1,28) = 5.851$; $p < 0.05$] and the interaction diet x EtOH [$F(1,28) = 5.862$; $p < 0.05$]. Bonferroni post-hoc analyses showed that KD-NO SA mice exhibited a significant decrease in CB1r gene expression in comparison with their corresponding SD group ($p < 0.05$) and the KD-SA group ($p < 0.01$). No significant differences were obtained in the gene expression of the opioid receptor mu (Figure 5f).

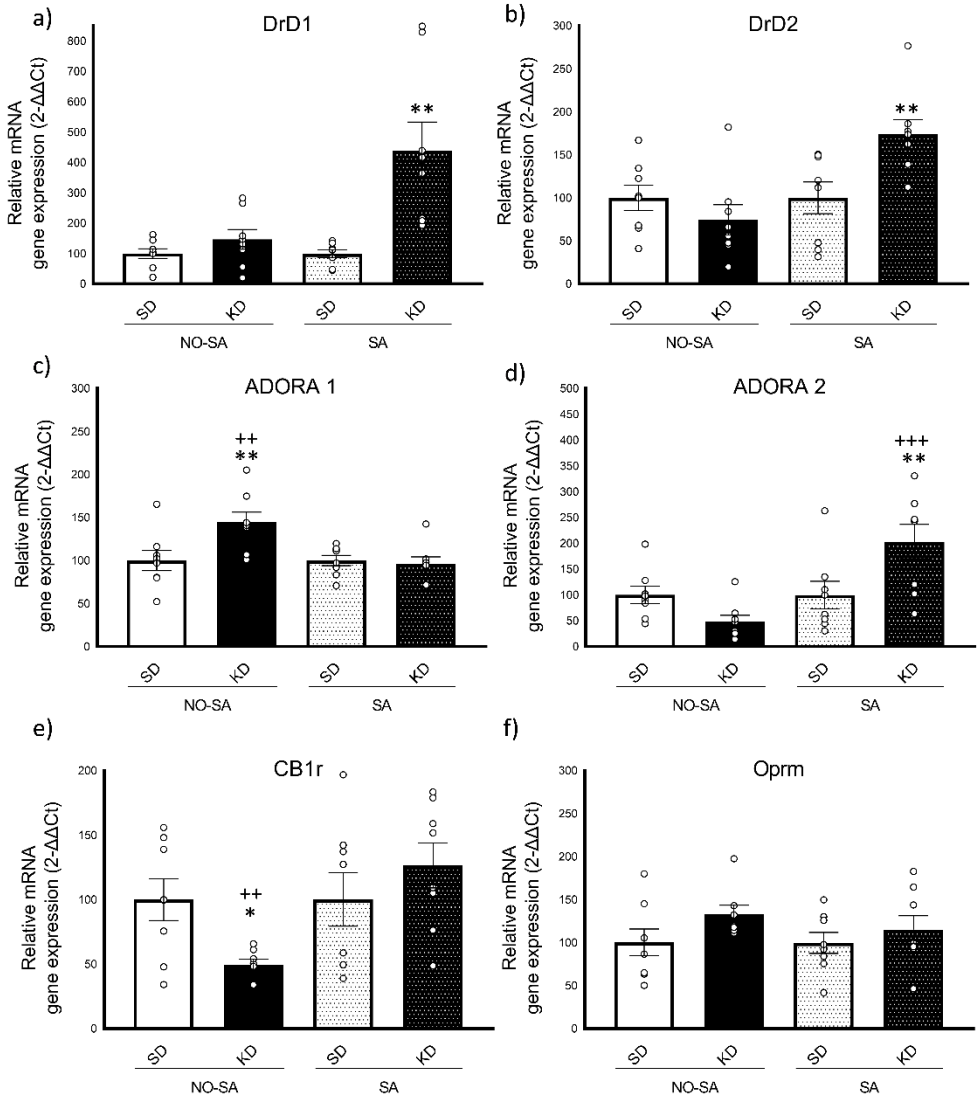

Figure 5. Real-time PCR Gene expression in the striatum (n=8/condition). (a) Dopamine receptor D1 gene - DrD1 (b) Dopamine receptor D2 gene - DrD2 (c) Adenosin receptor A1 gene - ADORA1 (d) Adenosin receptor A2 gene - ADORA2 (e) Cannabinoid receptor 1 - CB1r (f) Opioid receptor μ - Oprm. The columns represent means and the vertical lines ± SEM of relative (2-ΔΔCt method) gene expression in the striatum of OF1 mice. *p<0.05; **p<0.01 significant differences with respect to the corresponding SD group. ++p<0.01; +++p<0.001 significant differences with respect to their corresponding KD group.

4. Discussion

The main aim of the present work was to evaluate whether a KD could modulate the rewarding and motivational effects of alcohol. In addition, the role in these diet-induced changes of receptors related to the reward process, such as adenosine, dopaminergic, cannabinoid, and opioid systems, were also considered through gene expression studies. The possible beneficial effects of nutrition as a complement to substance-use disorder treatments have been scarcely studied. Therefore, this work opens a new line of research in drug addiction, given that to date only unhealthy diets such as cafeteria, high-sugar, and high-fat diets have been investigated in this field (for review see [57,58]).

The results found in the present work revealed that the KD induces an overall decrease in alcohol SA but does not affect the motivation to get the drug. Furthermore, the KD by itself induces opposite changes in the gene expression of ADORA1 and CB1r, which disappeared after alcohol consumption. Moreover, KD after EtOH SA induced striatal increases in the gene expression of ADORA2, DrD1, and DrD2 genes. No changes in Oprm gene expression were observed.

4.1. Ketogenic diet modulates bodyweight and increases β-hydroxybutyrate blood levels

Animals in the KD group rapidly turned into a ketotic state and their βOHB levels stabilized after 7 days. The ketotic state was maintained throughout the experiment, even during EtOH SA, where a slight decrease in βOHB levels was observed. These could be due to a decrease of βOHB in the liver induced by alcohol, as it has been recently shown [59]. These authors showed that βOHB administration had an anti-inflammatory and hepatoprotective effect in mice with ethanol-induced liver disease, suggesting its possible therapeutic role for alcohol-use disorders. On the other hand, we have also observed a slight increase in βOHB levels in the control group as a

result of food deprivation during EtOH SA, confirming that fasting also increases βOHB levels [60].

With respect to the bodyweight, our KD-exposed mice significantly increased their bodyweight in comparison to animals in the standard diet. Even though all groups experienced a decrease in bodyweight when SA food deprivation began (1 h/day access), the group exposed to KD maintained its weight above the SD group. To date, studies have shown conflicting results regarding the effects of KD on bodyweight. While some studies have found that KD for 2 weeks induces weight loss in rats [49], other studies show that bodyweight is increased by KD in rats when administered for 6 to 8 weeks, although the weight gain was slower than that observed in SD-fed animals [61,62]. Moreover, other studies reveal that male mice fed with a KD for a month showed an increase in brown adipose tissue (up to a 40% greater than controls) in agreement with our results [63].

4.2. Ketogenic diet diminishes ethanol self-administration

Regarding EtOH SA, our results showed a general decrease in alcohol consumption in the KD group, both in FR1 and FR3 schedules. There were no differences in the number of effective responses between groups, meaning that animals on KD did not drink all the EtOH they had access to. This decrease in EtOH consumption was maintained across the ten days of self-administration, even during the FR3, where animals were demanded more work to obtain the drug.

Nevertheless, this decrease in alcohol intake did not modify the motivation to obtain the reward, as it was observed in the active responses and PR schedule, where no differences were observed between KD and SD. The PR indicates the breaking point or limit of active responses by the animals, that is, to what extent a mouse is willing to introduce its nose into the hole repeatedly to obtain one reinforcement (in this

case, 38 µL 6% EtOH). Discarding an incorrect learning of the task (no differences in training or substitution), this result confirms that the mechanisms involved in FR1 and FR3 schedules (rates of operant responding) are not the same as those involved in PR (motivation) [64]. In fact, the PR schedule is complementary to the FR schedules and is indicative of the motivation for seeking the drug [65,66], while FR1 assesses the potential liability of a drug and the consumption based on its unconditioned psychopharmacological effects [67]. Moreover, these results support the fact that mice fed on KD do not show a motivational alteration towards reward.

To date, few studies have explored the relationship between alcohol consumption and KD, most of them focusing on ketoacidosis, a state in which the body's insulin is so low that the liver produces excess levels of ketone bodies that would have neurotoxic properties [68]. Nevertheless, only a few studies have evaluated the interaction between KD and EtOH, one of them focused on the reduction of alcohol intake in rodents [51]. In this study, animals were first trained to lever press for alcohol in the vapor self-administration paradigm. Next, the rats were divided into two groups and exposed to a KD or SD for 8 weeks, after which both groups were returned to SD. Then, to induce alcohol dependence, half of the rats were exposed to chronic alcohol vapor for 7 weeks, and the other half, as a control group, were exposed to air. After this, animals were subjected again to a vapor self-administration paradigm. Their results showed that animals on KD-alcohol vapor did not exhibit an escalation in EtOH intake, as they self-administered EtOH to the same degree as non-dependent rats, suggesting that the KD exerted a protective effect. Regarding alcohol withdrawal symptoms, another study implemented a KD in rats before alcohol administration (intragastric administration) until the end of the procedure, although they did not verify whether the KD had increased ketone bodies [49]. Their results indicate that the KD was effective in reducing rigidity and irritability behaviors but the mechanism through which the KD had a positive effect on alcohol

withdrawal symptoms remains unclear. Similar results have been obtained with administration of ketone monoester in mice [50], which reduces handling-induced convulsions and anxiety-like behaviors in early alcohol withdrawal, as with the KD.

Although the study focused on a psychostimulant drug, Martínez and co-workers [48] evaluated the potential of a KD as a therapy for cocaine addiction. The administration of a KD for three weeks to female and male rats was able to block cocaine sensitization, hyperlocomotion, and stereotypies, confirming a robust action of the KD on dopamine-mediated behaviors [48]. One of the explanations proposed by this study is that KD-mediated changes in adenosine may have therapeutic potential via actions at adenosine-dopamine receptor heterodimers.

Taking into account the different bodyweight of both groups, the results of EtOH consumption were calculated based on the individual weight of each subject (g/kg) in order for these differences to be avoided. A limitation of the present study could be the lack of control on the kcal intake between groups. Based on their bodyweight, we hypothesized that mice on KD ate a greater amount of kcal in an hour compared to the SD group during the SA procedure, indicating that the hypothesis of satiation as a contributor to the decrease in EtOH consumption should be taken into consideration. However, this possibility should be ruled out, as food intake occurred postprandially to SA, i.e., animals were introduced into the SA box 23 h after their last food intake. In addition, a recent study reported that food-deprived mice decreased their food intake after EtOH SA [69]. These results may explain why the SD group, which showed higher alcohol consumption, consumed fewer calories from food after consuming EtOH. In addition, food intake or satiation did not affect their EtOH motivation, as seen in the breaking point results. However, future studies should employ a eucaloric protocol to rule out this possible impact.

Nevertheless, even though studies report that a KD has beneficial effects, for example in weight loss in obese patients [70], the possible long-term effects of this diet remains unknown, probably due to the difficulty of adherence to a strict KD over time. Some studies have already reported that there can be adverse effects such as lipid abnormalities [71,72], hypoglycemia and dehydration [73,74], dysregulation of glucose levels [75,76] or nephrolithiasis [77]. Although some of these consequences have been seen in patients on the KD [77], the effects of a ketotic state in patients with an alcohol-use disorder may be pronounced, as they already present a poor nutritional status [78]. Thus, future studies should address this issue, considering the basic blood (cholesterol, low and high density lipoprotein, triglycerides, glucose) and liver parameters (transaminases) and investigate the possibility of using ketone esters as a supplement to a balanced diet, which also increase blood BHB [79,80].

4.3. Ketogenic diet diminishes ethanol intake through the adenosine-dopamine binomial

Our initial hypothesis was that the administration of a KD would diminish the EtOH rewarding effects by acting through the adenosine-dopamine binomial. Our results showed that KD increased ADORA1 gene expression without affecting ADORA2 or dopaminergic genes. Therefore, KD induced an overexpression of the ADORA1 gene with regards to the DrD1 gene. When animals on KD were exposed to EtOH, the changes in gene expression were completely different. KD and EtOH exposure increased the expression of the DrD1, DrD2, and ADORA2 genes. Under these circumstances, there was an overexpression of the DrD1 gene with respect to the ADORA1 gene and no change of balance was observed with regard to ADORA2 and DrD2, as both genes increased their expressions.

The binomial refers to the mutual antagonistic interactions between the heterodimer's A1-D1 and A2-D2 receptors [42]. Adenosine receptors are

particularly expressed in GABAergic neurons in the striatum and are colocalized post-synaptically with dopamine receptors [41]. A1 agonists have been found to significantly decrease the binding affinity of D1, indicating that the function of the A1 receptor in the A1-D1 heterodimer is to inhibit dopamine signaling via the D1 receptor [81].

Although the activation of the A1 receptor has been demonstrated to be one of the main anti-seizure mechanisms of the KD [36], it seems that the A2 receptor collects more evidence in the addiction field, and this supports the results obtained in this study. For example, a mixed antagonism of both the A1 and A2 receptors, but with higher potency on the latter, produces similar effects for those generated by psychostimulants and enhances relapse into cocaine SA in baboons [82]. Likewise, A2 agonists decrease cocaine SA [83] and cocaine and morphine locomotor sensitization in rodents [46,84]. Finally, A2 knockout animals exhibit a significant decrease in cocaine SA, although conditioned place preference and sensitization were not affected [45,85].

There are also studies suggesting that adenosine mediates EtOH intake as well. Confirming our results, Feltmann and co-workers [86] reported an increase in the density of the A2-D2 heteroreceptor in the striatum of rats that voluntarily drank alcohol for 12 weeks, although a reduction of the striatal density of D2-D2 homoreceptor complexes was also observed. In addition, A2 receptor stimulation with A2 agonists attenuated alcohol intake, while the A2 antagonist increased EtOH intake in alcohol-preferring rats [87] and mice [88].

Moreover, studies about KDs and dopaminergic activity are scarce. However, it has been reported that 3 weeks of KD does not change DA in the Nucleus Accumbens [89]. A possible explanation for the decreased EtOH SA observed in mice on KD

could be the relative increase of the ADORA1 gene, which in turn inhibits DrD1. With respect to the heterodimers A2-D2, KD did not induce any changes but both genes were overexpressed after oral EtOH SA, which indicates that there is no change in the balance of these genes.

As we mentioned, the GABAergic system is one of the most likely targets of EtOH [90] in addition to ketone bodies [91]. KD induces an increase in GABA-mediated inhibition and activates the GABA-B receptors [11,92]. Results showing the efficacy of GABA-B agonists decreasing EtOH SA [93,94,95] sustain the hypothesis that a KD modulates the GABAergic system through a change in the adenosine-dopamine heterodimer. Taking into account that both EtOH and KD interact with the GABAergic system and produce alterations in the adenosine-dopamine heterodimer, we can hypothesize that ketone bodies could induce a decrease in EtOH consumption through this mechanism.

The cannabinoid and opioid systems are both involved in fatty food intake and reward [4,5]. In this study we observed that the KD induced a significant underexpression of the CB1 gene, but after EtOH consumption, this underexpression was normalized. Calorically dense foods increase CB1r density in the Nucleus Accumbens, leading to their downregulation [96], which could be the case for KD. Moreover, it is important to note that most GABAergic inhibitory interneurons express presynaptic CB1r in abundance, which modulate the release of GABA at the synapses [97,98]. Regarding Oprm, this study reported no significant changes except for a trend to be overexpressed in the KD groups. Some studies suggest that Oprm presents an increase of expression in the brain areas processing reward associated with palatable foods [99,100], but there are no studies about the effects of KD on the opioid system.

5. Conclusions

KD may be a useful nutritional complement to the existing pharmacological therapies in alcohol addiction, especially considering the undernourishment that alcohol produces. In addition, we propose the adenosine-dopamine binomial as an interesting target that deserves to be explored in alcohol addiction. One important limitation of the KD is the difficulty to maintain adherence over time, and a permanent ketotic state is not considered realistic as a way of life. Stemming from this, there is a wide range of research that can be done, including testing other ketogenic-related therapies such as ketone esters, which promote an increase in blood βOHB levels. Furthermore, even when our results confirm that a KD could have a positive effect on reducing alcohol intake in mice, further research is needed to know the long-term effects of KD on metabolic health, such as basic blood and liver parameters. To date, although there is a scarce number of published studies highlighting the protective effects of KD on drug effects, the results obtained have been reported in preclinical studies, therefore more studies assessing the effect of different ketogenic diets on addiction in humans are needed. Many studies remain to be carried out with KD, and it is necessary to elucidate the exact neurobiological mechanisms through which KD modulates addiction. Even so, these results highlight once again the great relevance of nutritional interventions in mental and substance-use disorders.

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5. CONCLUSIÓN GENERAL

5. Conclusión General

La presente TD se ha centrado en estudiar las consecuencias que la exposición al estrés social induce sobre los efectos de las drogas de abuso, específicamente sobre el refuerzo condicionado de la cocaína y el consumo de etanol. Numerosos estudios realizados en laboratorios de investigación preclínica, incluido el nuestro, han demostrado que la exposición a un modelo de estrés social, la DS, es capaz de modificar la respuesta a diferentes drogas. El modelo de DS permite estudiar los mecanismos neurobiológicos que subyacen a los efectos conductuales y fisiológicos a corto y largo plazo del estrés social (Shimamoto, 2018). Este modelo ha sido de gran utilidad para confirmar cómo la exposición a DS en ratones es capaz de aumentar los efectos reforzantes de la cocaína (Ferrer-Pérez et al., 2018; Hymel et al., 2014; McLaughlin et al., 2006; Montagud-Romero et al., 2018; Rodríguez-Arias et al., 2017) y el alcohol (Boyson et al., 2014; Holly et al., 2016; Newman et al., 2018). En concreto, cabe destacar el incremento a largo plazo en los efectos reforzantes condicionados de la cocaína utilizando el modelo de CPL. En esta TD hemos empleado el CPL, donde la cocaína es el estímulo incondicionado, que, tras una exposición repetida, confiere a las claves ambientales que inicialmente eran neutras propiedades motivacionales secundarias, convirtiéndolas en un estímulo condicionado (Aguilar et al., 2009; Tzschentke, 2007). También hemos empleado el procedimiento de AA, con el que hemos evaluado los efectos reforzantes primarios de alcohol en función del esfuerzo que realiza el animal para obtenerlo (Moeller & Stoops, 2015), evaluando también la motivación por el consumo.

Utilizando estos modelos, hemos demostrado que algunos individuos son capaces de desarrollar un perfil resiliente que les protege de las consecuencias psicofisiológicas derivadas de dicho estrés, no mostrando este incremento en los efectos reforzantes de la cocaína. Pero además de caracterizar el perfil conductual y neurobioquímico de estos animales resilientes, el objetivo de esta TD ha sido el potenciar este tipo de

respuesta. Estudios recientes confirman que algunos tipos de intervenciones farmacológicas y ambientales son capaces de provocar un aumento de esta respuesta resiliente, como el enriquecimiento ambiental, el ejercicio físico o la administración de oxitocina (Ashokan et al., 2016; Aujnarain et al., 2018; Bahi, 2017; Calpe-López et al., 2022; Ferrer-Pérez et al., 2019a). En esta TD nos hemos centrado en estudiar el papel que ejerce la dieta en la respuesta al estrés, una variable que está presente continuamente en nuestra vida y que solo recientemente se ha comenzado a tener en consideración. Nos hemos centrado en el papel de la dieta con un alto contenido en grasas debido a su conocido efecto sobre el sistema de refuerzo y sus efectos sobre la respuesta al estrés. Para estudiar la influencia que la dieta alta en grasa ejerce sobre el incremento de los efectos reforzantes de las drogas por el estrés social, hemos empleado dos tipos distintos de dieta. Por un lado, una DRG con alto componente en grasas, carbohidratos y azúcares, administrada de forma intermitente y limitada (Corwin et al., 1998) y en segundo lugar una DC con alto componente en grasas, pero baja en carbohidratos y azúcares, administrada de forma continua para inducir un estado de cetosis (Masino & Rho, 2010).

Como resultado más importante de la presente TD debemos destacar que ambas dietas evaluadas son capaces de modular los efectos reforzantes de la cocaína y del etanol. Además, podemos confirmar que la DRG puede actuar como un reforzador alternativo acelerando la extinción de las memorias asociadas a la cocaína, evitando además la reinstauración de la conducta condicionada, favoreciendo el desarrollo de un perfil resiliente. A continuación, se presentan las principales conclusiones de la presente TD.

1º- Existe una población resiliente a los efectos del estrés social que no muestra un incremento de los efectos reforzantes condicionados de la cocaína

Nuestros resultados confirman que la experiencia de la DS induce un incremento a largo plazo de los efectos reforzantes condicionados de una dosis subumbral de cocaína (**Estudios 1 y 3**), coincidiendo con resultados obtenidos en numerosos estudios previos de nuestro laboratorio (Ferrer-Pérez et al., 2018; Montagud-Romero et al., 2016; Rodríguez-Arias et al., 2017). Además, se ha confirmado la existencia de una población de ratones derrotados que no han manifestado dicha preferencia, siendo caracterizados como resilientes al estrés social (**Estudio 1**). El análisis etológico de la conducta de estos animales resilientes durante los encuentros agonísticos muestra cómo estos ratones pasan más tiempo exhibiendo conductas de ataque, así como menor tiempo en las conductas de sumisión y huida, en comparación con los ratones caracterizados como susceptibles. Hemos observado una correlación positiva entre el tiempo empleado en la conducta de huida y el tiempo pasado en el compartimento asociado a la cocaína. Es decir, cuanto menos huyen los animales, menor es el efecto reforzante inducido por la cocaína. Por lo tanto, nuestros resultados indican que un afrontamiento activo y una adecuada adaptación del mismo reducen el incremento den los efectos reforzantes de la cocaína que induce la DS. En apoyo a nuestros resultados, otros estudios también han confirmado que los ratones que no presentan estrategias de afrontamiento como la huida, muestran menor anhedonia (Wood & Bhatnagar, 2015), menor ansiedad y mayor interacción social (Duclot et al., 2011; Hollis et al., 2011; Kumar et al., 2014). Contrariamente, los animales susceptibles muestran un afrontamiento pasivo, aceptando la derrota con mayor tiempo en huida y sumisión, sin presentar ninguna conducta agresiva hacia el residente. Este afrontamiento pasivo durante la DS se ha asociado previamente con la aparición de ansiedad y depresión (Chen et al., 2015; Pearson-Leary et al., 2017; Wood et al., 2010), lo que explicaría el incremento de los efectos reforzante de la cocaína en nuestros animales susceptibles.

En resumen, estos resultados permiten la identificación de ciertas características conductuales que aparecen en animales resilientes al estrés social, que pueden actuar como factor de protección frente al desarrollo de adicción a drogas. Un afrontamiento activo, pero al mismo tiempo flexible, se destaca como la característica conductual más relevante de los sujetos resilientes.

2º La administración de dietas ricas en grasa (DRG y DC) modifica los efectos reforzantes de la cocaína y el alcohol (1er Objetivo)

Influencia de la administración intermitente de la DRG sobre los efectos reforzantes de la cocaína

Con el objetivo de comprobar si algunas intervenciones nutricionales son capaces de modular el sistema de refuerzo para fomentar un perfil resiliente, se ha analizado el efecto de distintas dietas altas en grasa en el proceso adictivo. En estudios previos de nuestro equipo de investigación, observamos cómo la administración de una DRG previa a un CPL de cocaína provoca un aumento en la sensibilización de sus efectos reforzantes, confirmando su influencia sobre el sistema de refuerzo (Blanco-Gandía et al., 2017b). Sin embargo, cuando la DRG se administra con posterioridad a la adquisición de la preferencia en el CPL, se observa una reducción en el número de sesiones necesarias para extinguir la preferencia y además se disminuye la sensibilidad a la reinstauración inducida por la cocaína (Blanco-Gandía et al., 2017b), demostrando el potencial de esta dieta como reforzador alternativo. El problema con la administración continuada de la DRG son las consecuencias metabólicas, como un incremento de peso corporal y alteraciones en los niveles de grelina y leptina. Estos efectos metabólicos se pueden evitar administrando este tipo de dietas de forma intermitente y limitada, y hemos demostrado en esta TD que esta administración controlada sigue actuando como un reforzador alternativo, acelerando el proceso de extinción y bloqueando también la reinstauración de la

búsqueda de cocaína en ratones macho (**Estudio 2**). Sin embargo, los efectos reforzantes de la cocaína y el potencial de la DRG como reforzador alternativo difiere en ratones hembra. Los ratones hembra muestran una mayor sensibilidad a los efectos reforzantes de la cocaína, lo que es concordante con previos estudios donde muestran una mayor sensibilización a sus efectos locomotores (Holly et al., 2016), una adquisición más rápida de la AA de cocaína (Lynch, 2006; Martini et al., 2015) y el desarrollo de un CPL inducido por la cocaína con menos sesiones de emparejamiento que los ratones macho (Russo et al., 2003). Sin embargo, sí que hemos observado que extinguen los recuerdos asociados a la cocaína más rápido que los ratones macho. Estas diferencias de sexo también se observan en la respuesta que tienen los animales a los efectos moduladores de la DRG, donde no todas sus administraciones actúan como reforzador alternativo en hembras, probablemente porque los sistemas neurales que median el refuerzo podrían mostrar diferencias de sexo. De hecho, se ha comprobado que la respuesta dopaminérgica inducida por distintos reforzadores, como la cocaína, es mayor en hembras que en machos (Becker, 1999; Becker & Ramírez, 1981). Basándonos en nuestros resultados, confirmamos que la administración de una recompensa natural, como la DRG, puede actuar como reforzador alternativo ante el potente efecto de la cocaína en los ratones macho, pero no en ratones hembra.

3º Influencia de la DC sobre los efectos reforzantes de la cocaína y el alcohol (2º Objetivo)

En la presente TD, también hemos evaluado el efecto de otra dieta alta en grasa, pero en este caso baja en carbohidratos y azúcares, como es la DC, sobre los efectos reforzantes y el consumo de cocaína y alcohol. En primer lugar, se ha demostrado que el aumento de cetonas como el βOHB no provoca cambios significativos en la actividad locomotora, la memoria o el aprendizaje dependiente del hipocampo,

evitando así la afectación en la ejecución de otras pruebas conductuales (**Estudio 4**). Sin embargo, sí que se ha observado que los animales sometidos a la DC mostraban un incremento en la ansiedad tras 7 días de consumo de esta dieta. Hasta la fecha, los pocos estudios que evalúan el efecto de la DC en la ansiedad afirman que esta dieta no induce ningún cambio en esta conducta (Ciarlone et al., 2016; Huang et al., 2019), aunque se evaluó tras 2 o 3 meses de mantener un estado de cetosis. Por todo ello, hipotetizamos que los síntomas de ansiedad provocados por la DC se reducirán con el tiempo. Este resultado es importante, ya que refleja la necesidad de prolongar la fase de adaptación a la DC antes de desarrollar cualquier prueba conductual.

Con respecto al efecto modulador de la DC en los efectos reforzantes condicionados de la cocaína (**Estudio 5**), esta dieta no evita la adquisición de un CPL con una dosis efectiva de cocaína (10 mg/kg). Sin embargo, la DC administrada tanto a lo largo de todo el proceso experimental (antes, durante y después del CPL) como únicamente en el proceso de extinción, es capaz de acelerar el proceso de extinción de la conducta condicionada y evitar la reinstauración de la preferencia. Actualmente, sólo un estudio ha evaluado cómo el acceso a una DC modula los efectos de la cocaína (Martínez et al., 2019), en el que se muestra que la administración de esta dieta durante tres semanas reduce los síntomas de abstinencia de cocaína en ratas. Con estos resultados, confirmamos que la DC puede considerarse un enfoque nutricional prometedor en el tratamiento de la adicción a la cocaína y, aunque no debe considerarse como un tratamiento exclusivo, puede contribuir a atenuar los recuerdos relacionados con el consumo de cocaína, así como el riesgo de recaída.

Similares resultados se han observado tras la administración de DC antes de comenzar la exposición al alcohol, ya que disminuye su ingesta, aunque no interfiere en la motivación para obtenerlo (**Estudio 6**). Hasta la fecha, los pocos estudios que han evaluado el efecto de la DC sobre el consumo de alcohol afirman que esta dieta evita la escalada en la ingesta (Wiers et al., 2021) y reduce los síntomas de

abstinencia en ratas (Dencker et al., 2018) y en seres humanos (Wiers et al., 2021), sugiriendo también que la DC puede emplearse como intervención nutricional para reducir las consecuencias derivadas del trastorno por uso de alcohol.

4º La administración intermitente de una DRG potencia la resiliencia a los efectos del estrés social (3er Objetivo)

Sabemos que el estrés social sensibiliza el sistema de refuerzo y que la DRG ejerce una influencia directa sobre este sistema, por lo que hemos evaluado el efecto que tiene esta dieta sobre el incremento de los efectos reforzantes de la cocaína provocado por la exposición a estrés social. Nuestros resultados indican que la ingesta de una DRG es capaz de bloquear esta sensibilización (**Estudio 3**). La administración de pequeñas cantidades de DRG antes de cada DS bloquea el desarrollo de CPL inducido por una dosis subumbral de cocaína. Este resultado sugiere que el consumo de comida palatable podría estar actuando como refuerzo alternativo o *comfort food*. Varios estudios apoyan estos resultados al observar que la administración de una DRG en animales socialmente estresados disminuye la actividad motora inducida por la cocaína (Erhardt et al., 2006), disminuyendo la respuesta de la corticosterona o bloqueando la adquisición de CPL por cocaína (Blanco-Gandía et al., 2018). Además, cuando la ingesta de este tipo de dietas se realiza con posterioridad a la exposición del estrés social, durante la adquisición del condicionamiento con la cocaína, también actúa como reforzador alternativo, bloqueando igualmente el desarrollo de CPL. Sin embargo, hemos observado que, si la administración de una DRG se prolonga en el tiempo, no ejerce ningún efecto protector e incluso puede sensibilizar el sistema de recompensa, como sugieren estudios previos (Blanco-Gandía, et al., 2017b). Con estos resultados planteamos que la administración controlada de una DRG podría ser una estrategia útil para mitigar los efectos del estrés social sobre los efectos reforzantes de la cocaína, potenciando

así un perfil resiliente. Sin embargo, teniendo en cuenta la influencia de esta dieta en los circuitos de recompensa y sus efectos cuando se administra durante un periodo de tiempo prolongado, es esencial identificar un patrón de ingesta adecuado.

5º Modificación en la expresión de genes inducido por la DRG y la DC

Aunque la pauta de administración intermitente de DRG que se ha empleado en esta TD no induce cambios ni en el peso corporal de los animales, ni provoca cambios en los niveles de leptina y grelina, sí que se producen cambios a nivel cerebral en la expresión de diversos genes implicados en el proceso adictivo.

La DRG administrada de forma intermitente y limitada, tanto previa a la exposición a CPL de cocaína (**Estudio 3**), como a posteriori (**Estudio 2**), no induce cambios en la expresión del gen Oprμ. Sin embargo, algunos estudios si han reportado que cuando la DRG se administra de forma continua, pueden presentarse ciertas alteraciones como una reducción de la expresión de este gen (Ong et al., 2013; Vucetic et al., 2011). Por lo tanto, estos resultados confirman que la administración limitada de DRG no es suficiente para alterar la expresión del gen del receptor opiáceo mu. Independientemente del tipo de dieta administrada, sí que observamos diferencias con respecto al sexo en la expresión de este gen, ya que los ratones hembra presentan una menor expresión en comparación con los ratones macho. En resumen, no podemos confirmar que el efecto modulador de la DRG sobre los efectos reforzantes de la cocaína se deba directamente al efecto de esta dieta sobre el sistema opioide, pero si hipotetizamos que, teniendo en cuenta que la activación del sistema opioide influye en el aprendizaje o la realización de conductas basadas en la recompensa de la drogas (Koob & Le Moal, 2005), las diferencias de sexo en la expresión del gen Oprμ podrían explicar que los ratones machos modifiquen sus procesos de aprendizaje durante las extinciones de cocaína de forma distinta a las hembras.

También se ha observado que, en ausencia de estrés, la DRG no induce ningún cambio en la expresión del gen CB1r en ratones hembra, pero si provoca un aumento de expresión cuando esta dieta se administra previa a las sesiones de extinción de cocaína en ratones macho, coincidiendo con una reducción del número de sesiones necesarias para extinguir la conducta condicionada. Varios estudios asocian el receptor CB1 a los procesos de aprendizaje de la extinción y la recaída (Khaleghzadeh-Ahangar & Haghparast, 2015; Yu et al., 2011), por lo que la sobreexpresión del gen CB1r en este grupo de animales podría explicar que aprendan más rápidamente a identificar la ausencia de la cocaína en el compartimento previamente asociado a ella. Nuestros resultados también han puesto de manifiesto que el sistema cannabinoide se relaciona estrechamente con la experiencia de estrés social, ya que todos los grupos expuestos a dicho estrés, independientemente del tipo de administración de DRG, muestran una disminución en la expresión del gen CB1r (**Estudio 3**). Estos resultados coinciden con estudios previos que han demostrado que la señalización CB1 modula la regulación de la respuesta al estrés (Valverde & Torrens, 2012) ya que se ha asociado la exposición a un estrés crónico con una reducción de la expresión génica de CB1r en el hipocampo (Hill et al., 2005; Hu et al., 2011; Reich et al., 2009) y en el estriado (Rossi et al., 2008; Wang et al., 2010). En resumen, la DS induce una disminución en la expresión del gen CB1r y la exposición limitada e intermitente a una DRG no es capaz de modificar este efecto del estrés social.

Finalmente, también podemos confirmar que la DS induce un aumento en la expresión del gen CRHR1 **(Estudio 3)**. En la mayoría de los grupos experimentales que se exponen a una DRG, no se observan cambios en la sobreexpresión del gen CRHR1. Sin embargo, cuando se presenta un aumento de las Kcal ingeridas derivadas del consumo de una DRG sí que observamos una reducción en su expresión. Se conoce que la sobreexpresión de CRHR1 puede conducir a un aumento de la vulnerabilidad a los efectos gratificantes de la cocaína, e incluso se ha

demostrado que los antagonistas de CRHR1 pueden bloquear esta vulnerabilidad (Boyson et al., 2014; Ferrer-Pérez et al., 2018). Otros estudios han demostrado que una DRG puede reducir los niveles de corticosterona en ratones aislados (Blanco-Gandía et al., 2018), y además reducir otras consecuencias del estrés social, como la conducta de evitación social, la ansiedad y la depresión (MacKay et al., 2017; Maniam & Morris, 2010; Otsuka et al., 2019). Nuestros resultados sugieren que, al ingerir una cantidad elevada de Kcal derivadas de la DRG, se produciría un efecto similar al de los antagonistas de CRHR1, reduciendo la expresión de este gen y por tanto la actividad del eje HHA (Foster et al., 2009; la Fleur et al., 2005; Pecoraro et al., 2004; Ulrich-Lai et al., 2011).

En resumen, la DS produce cambios en la expresión de genes como son el CB1r y el CRHR1 que solo pueden ser revertidos parcialmente por la administración de una DRG.

Estudiamos igualmente los efectos de la DC en la expresión génica de diferentes genes y observamos que esta dieta solo produce un incremento en la expresión del gen A1r sin provocar alteraciones en la expresión del gen Oprμ, el gen A2r o en los genes de los receptores dopaminérgicos. Paralelamente, al administrar la DC y exponer a los ratones al alcohol observamos cambios en la expresión génica con un incremento en los genes D1r, D2r y A2r. A partir de estos resultados, y teniendo en cuenta que se ha demostrado que la DC aumenta los niveles de adenosina y activa sus receptores (Lusardi et al., 2015), nuestra hipótesis inicial plantea que la administración de una DC disminuye los efectos gratificantes de las drogas actuando a través del binomio adenosina-dopamina. Se ha propuesto que el desarrollo de la conducta adictiva estaría mediado por la interacción antagónica entre los receptores de adenosina A1 - dopamina D1 y adenosina A2 - dopamina D2 (Berrendero et al., 2003; Franco, 2000; Listos et al., 2008; Soria et al., 2006). Aunque en nuestros resultados la modulación adenosina-dopamina provocada por la DC no sería lo

suficientemente fuerte como para bloquear la adquisición del CPL inducido por la cocaína, si puede haber disminuido la fuerza del condicionamiento y requerir un menor número de sesiones para extinguir la preferencia. Con respecto al papel del binomio adenosina-dopamina y el consumo de alcohol, sabemos que la estimulación del receptor A2 atenúa la ingesta de alcohol, mientras que su antagonismo lo reduce en modelos de roedores (Bonaventura et al., 2012; Nam et al., 2013). Teniendo en cuenta estos resultados, y observando que nuestros animales manifiestan un aumento en la expresión del gen A1r tras la administración de la DC, y un aumento de la expresión de los genes D1r, D2r y A2r cuando son expuestos a dicha dieta y al alcohol, sugerimos que el efecto de la DC sobre estos receptores provoca la reducción en la ingesta de alcohol, previniendo así el desarrollo de la conducta adictiva.

Valor traslacional del estudio y futuras investigaciones

Los modelos animales son una herramienta muy potente en investigación básica, pero debemos ser prudentes a la hora de trasladar los resultados obtenidos a la conducta humana. Nuestros resultados confirman que no todas las poblaciones expuestas a estrés social manifiestan las mismas consecuencias derivadas de dicha exposición, como el incremento de los efectos reforzantes de las drogas. Además, estas diferencias vienen determinadas por distintos patrones de comportamiento, siendo el afrontamiento activo del estrés un perfil conductual que permite adaptarse al estresor y desarrollar una respuesta resiliente. Con los resultados obtenidos en esta TD de carácter preclínico pretendemos fomentar el estudio de las estrategias conductuales o farmacológicas que potencian a la resiliencia, lo que permitirá disminuir las devastadoras consecuencias que produce la exposición continuada al estrés social.

Hasta la fecha, se han desarrollado muy pocos estudios en humanos que evalúen el papel que tiene la comida palatable en el desarrollo de la adicción. Sin embargo, es muy habitual que este tipo de alimentos se utilicen como automedicación para reducir un estado de ánimo negativo (como el síndrome de abstinencia) o afrontar situaciones estresantes (Groesz et al., 2012; Ifland et al., 2009; Kim et al., 2013). Nuestros resultados muestran que la comida palatable puede actuar como una recompensa alternativa a la cocaína y el alcohol, pero también observamos que estos efectos están condicionados por el sexo. Es por ello que, teniendo en cuenta que la administración de DRG es menos eficaz en hembras y que las consecuencias cognitivas y conductuales de algunos trastornos psiquiátricos como los trastornos por usos de sustancias, la ansiedad y la depresión difieren entre hombres y mujeres (Bangasser & Cuarenta, 2021), remarcamos la necesidad de continuar estudiando las diferentes pautas de administración y dietas con alto contenido en grasa en ambos sexos, ya que no podemos asumir que las intervenciones ambientales o nutricionales sean igualmente efectivas en ambos sexos. Paralelamente, también hemos demostrado que la DRG puede facilitar el desarrollo de un perfil resiliente en roedores, ya que esta intervención nutricional es capaz de reducir los efectos reforzantes de la cocaína incrementados por el estrés social. Teniendo en cuenta estos resultados, y tras confirmar que la DC también modula en el sistema de recompensa provocando una disminución del consumo de alcohol, consideramos necesario continuar estudiando el efecto de la DC sobre las consecuencias derivadas del estrés social.

Aunque la presente TD pone de manifiesto la gran relevancia que tienen las intervenciones nutricionales en los trastornos mentales y en el consumo de sustancias, es necesario realizar muchos más estudios para explorar los mecanismos neurobiológicos exactos a través de los cuales estas dietas modulan la adicción y la resiliencia.

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6. ANEXOS

ANEXO 1: Estudio 1.

Caracterización conductual y neuroinmune de la resiliencia al estrés social: Efectos reforzantes de la cocaína.

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Behavioral and neuroimmune characterization of resilience to social stress: Rewarding effects of cocaine

Caracterización conductual y neuroinmune de la resiliencia al estrés social: Efectos reforzantes de la cocaína

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Abstract

Preclinical studies have shown that social stress increases vulnerability to the reinforcing effects of cocaine. However, the results are not always homogeneous, revealing a subpopulation that does not show a preference for cocaine. Thus, the main aim of the present study was to characterize the behavioral profile of resilient mice to the stress-induced rewarding effects of cocaine using an animal model of repeated social defeat stress (SD). To this end, male adult mice of the C57/BL6 strain were exposed to SD and, three weeks later, assessed using the Conditioned Place Preference paradigm induced by an ineffective dose of cocaine (1mg/kg). Afterwards, the striatal levels of interleukin 6 were measured, as social stress usually induces a neuroinflammatory response. Control mice did not develop CPP, while defeated mice did overall develop a preference for the drug-paired compartment. Based on the conditioning score that they exhibited, the SD sample was subdivided into resilient (did not develop preference) and susceptible mice (developed preference). During the SD sessions, resilient animals showed less flight and submission behaviors than susceptible mice and they presented attack behaviors towards the residents, thereby showing their resistance to being defeated. There were no differences in the neuroinflammatory response, probably due to the long time elapsed after the last SD session. These results suggest that an active coping style to social stress may be decisive in protecting the individual from developing an addiction.

Keywords: Resilience; cocaine; social stress; coping; interleukin 6.

Resumen

Numerosos estudios preclínicos han demostrado que el estrés social incrementa la vulnerabilidad a los efectos reforzantes de la cocaína. Sin embargo, los resultados obtenidos no son homogéneos, observándose siempre una subpoblación que no muestra dicho incremento. Utilizando el modelo de derrota social (DS) repetida en ratones, en este trabajo hemos querido caracterizar conductualmente a los ratones resilientes al incremento de los efectos reforzantes de la cocaína inducido por el estrés social. Utilizamos ratones adultos macho de la cepa C57/BL6 a los que sometimos al protocolo de DS repetida y tres semanas más tarde, realizamos el Condicionamiento de Preferencia de Lugar (CPL) inducido por una dosis no efectiva de cocaína (1mg/kg). Una vez finalizado este procedimiento se midieron los niveles estriatales de interleucina 6, ya que el estrés social produce una respuesta de neuroinflamación. No se observó CPL en los ratones controles, pero los animales derrotados tomados en conjunto desarrollaron preferencia. Sin embargo, esta muestra se pudo dividir en ratones resilientes (no desarrollaron preferencia) y susceptibles (presentaron CPL). Durante las derrotas sociales, los animales resilientes pasaron menos tiempo en las conductas de huida y sumisión que los catalogados como susceptible y presentaron conductas de ataque hacia el ratón residente, manifestando por tanto resistencia a ser derrotados. No se observaron diferencias en la respuesta de neuroinflamación, probablemente debido al largo periodo de tiempo trascurrido desde la última derrota social. Nuestros resultados sugieren que un estilo de afrontamiento activo al estrés social va a ser determinante en la protección del sujeto a desarrollar un trastorno por uso de drogas.

Palabras clave: Resiliencia; cocaína; estrés social; afrontamiento; interleucina 6.

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Exposure to stress is an environmental factor which has been directly related to the onset of psychiatric disorders such as depression, anxiety or substance abuse disorders. However, not all subjects are equally vulnerable to the consequences of stress (Krishnan et al., 2007; Lutter et al., 2008). Recent years have seen a great increase in the study of the phenomenon of stress resistance. Resilience is defined as the ability of individuals to maintain adaptive psychological and physical functioning, and to avoid the occurrence of mental illness when exposed to chronic or high intensity stress (Charney, 2004), with the mechanisms responsible for resilience promoting an appropriate and non-pathological response to stress (Chmitorz et al., 2018). In recent years, researchers have begun to identify the psychological and biological characteristics of individuals resistant to social stress (Pfau & Russo, 2015). For example, there are a number of behaviors and psychological traits, such as cognitive flexibility, active coping, optimism, or the feeling of belonging to a group, which can favor a resilient response in humans (Wood & Bhatnagar, 2015; Laird , Krause, Funes & Lavretsky, 2019). However, most of these studies have focused on resilience to the development of depression, anxiety or post-traumatic stress disorder (Russo, Murrough, Han, Charney & Nestler, 2012; Krishnan, 2014; Finnell & Wood, 2016), with very few studies assessing resilience to escalating drug use.

Most preclinical studies on stress resilience use the repeated or chronic model of social defeat (SD). This model has great ethological and translational relevance since the most common form of stress experienced by humans originates in their social environment. This model is based on the resident-intruder paradigm, in which a male (intruder) animal is introduced into the territory of another (resident), who will confront and dominate the first (Miczek, Yap & Covington, 2008; Chaouloff, 2013). Numerous studies have shown that repeated SD increases the use of cocaine and alcohol (Miczek et al., 2008; Burke & Miczek, 2014; Rodríguez-Arias et al., 2016, 2017; Montagud-Romero et al., 2016a; Ferrer-Pérez et al., 2018a). This increase has been associated with a neuroinflammatory response since defeated animals have shown an increase in inflammation markers such as cytokines or chemokines, greater blood-brain barrier permeability as well as activation of the microglia (Rodríguez-Arias et al., 2017, 2018; Ferrer-Pérez et al., 2018a).

As with studies on humans, in most preclinical studies the development of resilience to the development of depression or anxiety has been assessed in mice exposed to repeated SD. In these studies, 24 hours after finishing the final SD, animals are categorized as resilient or susceptible depending on their behavior in a social interaction test. Those maintaining higher social contact time are resilient, while the susceptible show social avoidance (Krishnan et al., 2007; Russo et al., 2012; Golden, Covington, Berton & Russo, 2011; Henriques-Alves & Queiroz, 2015; Zhan et al., 2018). Some studies have confirmed that among the factors that mediate resilience is a lower neuroinflammatory response in resilient animals (Wang et al., 2018).

These results have led us to propose as a main objective of the present study the characterization of those mice exposed to repeated SD which are resilient to the long-term increase of the rewarding effects of cocaine. To this end, three weeks after the final SD, we carried out the Conditioned Place Preference paradigm (CPP) with a sub-threshold dose of cocaine, a dose which is not effective in control animals but which does induce preference in those socially defeated (Montagud-Romero et al., 2016a, 2016b). Behavioral characterization was carried out by assessing the behavior of animals showing resilience during SD. Finally, once the behavioral procedure was completed, we studied the neuroinflammatory response by measuring the striatal levels of interleukin 6 (IL6).

Material and Methods

Animals

We used 43 adult male mice of the C57BL6 strain, with 28 as experimental subjects (social defeat) and 15 as a control group (exposed only to exploration). Another 10 male albino mice of the OF1 strain were also used as resident mice in the repeated SD. All mice were purchased from Charles River Laboratories (Barcelona, Spain.). The experimental mice arrived on postnatal day (PND) 21 and were housed in groups of 4 in 26x20x13 cm plastic cages. The 10 OF1 strain mice were housed in isolation for use as residents during repeated SD. The environmental conditions were a temperature of 21 ± 2ºC and a relative humidity of 55%. The mice were kept throughout the procedure in a 12-hour light/dark cycle (8:00-20:00) and with water and pellets *ad libitum,* except during behavioral tests. All procedures for the treatment and care of mice complied with national, regional and local laws and regulations in accordance with international community guidelines, as set out in *European Community Council Directives* (86/609/EEC, 24 *November* 1986). The study was carried out in the Drug Addiction Psychobiology Research Unit of the Department of Psychobiology, Faculty of Psychology, University of Valencia. It was approved by the Animal Experimentation and Welfare Ethics Committee of the University of Valencia 2017/VSC/PEA/00224-A1507028485045.

Pharmacological treatment

The animals were subjected to drug treatment only during the CPP procedure. Mice in both the control and the experimental group were injected intraperitoneally with a 1 mg/kg dose of cocaine dissolved in 0.9% NaCl

solution. This is considered a sub-threshold dose showing no preference of place in the CPP test with standard mice (Maldonado, Rodríguez-Arias, Castillo, Aguilar & Miñarro, 2006; Vidal-Infer, Aguilar, Miñarro & Rodríguez-Arias, 2012), while mice exposed to repeated SD do develop preference (Rodríguez-Arias et al., 2017).

Sample collection

To obtain samples we followed the procedure of previous studies (Ferrer-Pérez et al., 2018b). Mice were killed by cervical dislocation and subsequently decapitated. Brains were quickly removed and the striatum dissected after the procedure described by Heffner et al. (Heffner, Hartman & Seiden, 1980) and kept on dry ice until stored at -80° C.

Before determining IL-6 levels, the brains were homogenized and prepared following the procedure described by Alfonso-Loeches et al. (2010). The striata were homogenized as 250 mg of tissue/0.5 ml of cold lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 8, 130 mM NaCl, 10 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 40 mM DTT, 1 mM Na3VO4 and 10 mM PMSF). Brain homogenates were kept on ice for 30 minutes and centrifuged at a speed of 11.519 x g for 15 minutes, after which the supernatant was collected and protein levels were determined by the Bradford assay (Thermo Fisher, ref: 23227).

Experimental design

Table 1 shows the experimental design of the present study in detail. All mice arrived at the laboratory aged 21 days. After three weeks of adaptation in the animal facility, at PND 47, the four SD sessions began. Three weeks after the final SD, we performed the CPP (three days of pre-conditioning, four days of conditioning and one day of post-conditioning). Finally, after completing the entire experimental procedure, the animals were killed to enable the collection of biological samples.

Apparatus and procedure

Social Defeat

The SD protocol carried out in this study has been previously validated and described in detail (Montagud-Romero et al., 2016a; Rodríguez-Arias et al., 2017; Ferrer-Pérez et al., 2019). Repeated SD consists of four 25-minute sessions at 72-hour intervals, on postnatal days 47, 50, 53 and 56. The repeated SD session consists of three phases. In the first phase, the intruder is introduced into the resident's cage for ten minutes, where it is protected from the attacks, but not threat, of the resident by means of a wire partitition. In the second phase, the partition is removed and confrontation is allowed for five minutes. In the third and last phase, the partition is replaced for a further ten minutes.

The repeated SD sessions were recorded with a video camera to enable assessment of the intruder animal's flight, submission and attack behaviors, and the resident's threat and attack behaviors. In the repeated SD with the 15 control mice, a procedure similar to that described above was used, but without the presence of the resident mouse. After completing the paradigm, the analysis of the encounters was carried out using a computer program with which the time spent performing different behaviors can be recorded (Martínez, Miñarro & Simón, 1991).

Conditioned Place Preference (CPP)

CPP is a model based on classical or Pavlovian learning to assess the conditioned reward induced by different stimuli (Bardo & Bevin, 2000; Tzschentke, 2007). It has been widely used to study the reward effects of conditioned addictive drugs (Aguilar, Rodríguez-Arias & Miñarro, 2009; Yap et al., 2015; Rodríguez-Arias et al., 2016; Blanco-Gandía et al. 2017) since contextual stimuli can acquire secondary appetitive properties when combined with a primary enhancer (Tzschentke, 2007).

For CPP, we used 12 identical plexiglas cages with two compartments of equal size (30.7 cm long by 31.5 cm wide by 34.5 cm high), separated by a central gray area (13.8 cm long by 31.5 cm wide by 34.5 cm high). The compartments have different color walls (white vs black) and different floor texture (smooth in the black compartment and rough for the white). Animals are trained to associate one specific environment with the effect of the drug administered, and the other compartment with saline solution (García-Pardo, Rodríguez-Arias, Miñarro & Aguilar, 2017). A guillotine door separates each compartment from the central compartment. Each of the conditioning compartments has four photoelectric cells, while the central zone has six, to allow the position of the animal and the crossings from one compartment to the other to be recorded. The equipment is controlled by two IBM PC computers running MONPRE 2z software (CIBERTEC, SA, España).

CPP comprises three phases, carried out during the dark cycle and following an 'unbiased' procedure in terms of the spontaneous initial preference (Manzanedo, Aguilar,

Table 1. *Experimental Design.*

	Social Defeat/Exploration					CPP (1mg/kg cocaine)			Sample collection
	1th	2th	3th	4th	3 weeks	Pre-C test	Conditioning	Post-C test	
PND	47	50	53	56		76 - 78	79 - 82	83	84

Rodríguez-Arias & Miñarro, 2001). During the first phase or pre-conditioning (Pre-C), the mice had free access to both compartments of the apparatus for 15 minutes (900s) each day for 3 days. On the third day, the time each animal spent in each compartment was recorded for 900s. Animals showing strong aversion (less than 33% of the session time) or strong preference (more than 67%) for any compartment were excluded from the procedure. In the present experiment, a total of two animals were excluded for not meeting the established criteria. Compartment allocation was counterbalanced. One of the compartments was chosen for association with cocaine in such a way that, within each group, half the animals received the cocaine in the least preferred place and the other half in the most preferred, and compartment color was also balanced. There should be no significant differences in the time that animals spend in the compartment associated with the drug or vehicle in the pre-conditioning phase. This measure is of great importance for the experimental procedure as it helps to avoid any preference bias before starting the experiment.

In the second phase (conditioning), the animals were conditioned with 1 mg/kg of cocaine through four associations with the compartment allocated after Pre-C. It has been observed that 1 mg/kg is a sub-threshold dose, i.e., a dose that does not lead to the acquisition of CPP, unless other variables such as stress or behavioral traits are manipulated (Vidal-Infer et al., 2012; Arenas et al., 2014; Montagud-Romero et al., 2014; Rodríguez-Arias et al., 2016; Blanco-Gandía, Montagud-Romero, Aguilar, Rodríguez-Arias & Miñarro, 2018). The animals received two injections (cocaine and vehicle) each day: a saline administration before being confined to the non-associated compartment for 30 minutes, and after an interval of four hours they received cocaine before being confined to the compartment associated with the drug for 30 min. The central area was not used during conditioning and access to it was blocked by guillotine doors.

During the third phase, post-conditioning (Post-C), on the 8th day of the procedure, the guillotine doors separating both compartments were removed and the time that the mice spent in each compartment, without any treatment, was recorded for 900s. The difference in seconds between the time that the animals remained in the compartment associated with the drug during the Post-C test and the time they spent during the Pre-C test is a measure of the degree of conditioning induced by the drug (*Conditioning Score*). If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates the induction of an aversion. Once CPP was completed, the defeated animals were divided into resilient or susceptible. Those who exhibited no increase in preference for the cocaine-associated compartment were considered resilient and those whose preference did increase were susceptible.

ELISA IL-6 assay

To determine the concentration of IL-6 in the striatum, we use a mouse IL-6 ELISA kit from Abcam (Ref: ab100712) and followed the manufacturer's instructions. To determine absorbance, we use an iMark microplate reader (Bio-RAD) controlled by Microplate Manager 6.2 software. The optical density was read at 450 nm and the final results were calculated using a standard curve, expressed as pg/mg for tissue samples. The sensitivity of the test is <2 pg/mg. All samples were analyzed in duplicate.

Data analysis

To confirm the effect of repeated SD on CPP, univariate ANOVA was performed on the *Conditioning Score* data with the inter-subject variable 'Stress' with two levels: Exploration and Social Defeat. A K-means cluster analysis was performed using the *Conditioning Score* values to separate the animals into Resilient and Susceptible subgroups. After this division, we performed a new univariate analysis with the three level inter-subject variable 'Group' (Exploration, SD Susceptible and SD Resilient). The same analysis was applied to the striatal levels of IL-6 data. The results obtained in the ethological analysis of SD were analyzed using a two-way ANOVA with the two level inter-subject variable 'Group' (Resilient and Susceptible), and the intra-subject variable 'Defeat' of 2 levels: SD1 (first session) and SD4 (fourth session of social defeat). Post-hoc analyses were performed using the Bonferroni fit test, taking $p < 0.05$, $p < 0.01$ and $p < 0.001$ as significance intervals. The Pearson correlation coefficient was also calculated to determine possible relationships between the Flight variable and *Conditioning Score* of all animals performing repeated SD.

Results

Only susceptible animals develop CPP

Regarding the CPP *Conditioning* Score (Figure 1a), the ANOVA showed a significant effect for the Stress variable [$F(1,36) = 7.147$; $p < 0.05$], indicating that defeated animals spent significantly more time in the drug-associated compartment than non-stressed animals ($p < 0.05$).

Animals were classified as Resilient and Susceptible using K-means cluster analysis [$F(1,24) = 37.748$; $p < 0.001$]. When the group of defeated animals was divided into Resilient and Susceptible subgroups (Figure 1b), ANOVA showed a significant effect for the Group variable [$F(2,38) = 23.289$; $p < 0.001$]. Susceptible animals spent significantly more time in the drug-associated compartment compared to the other two groups ($p < 0.001$ in both cases). Figure 1c shows the individual scores of the three experimental

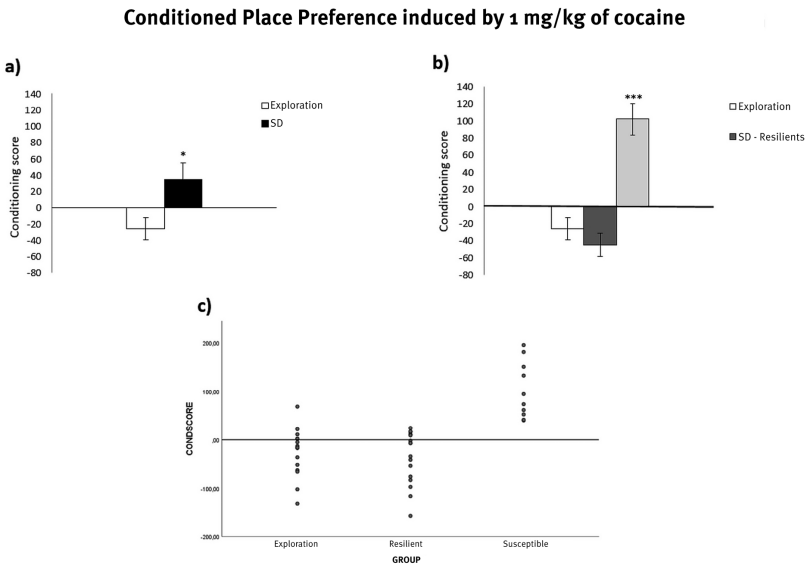

Figure 1. Effect of repeated SD on CPP acquisition induced by 1 mg / kg of cocaine in male C57/BL6 mice. The bars represent the difference in time (s) spent in the compartment associated with the drug before and after the conditioning sessions *(conditioning score)*. (a) Treatment groups: Exploration and repeated SD. (b) After Post-C, the defeated animals were divided into Resilient and Susceptible subgroups according to their level of conditioning. * p<0.05, significant difference compared to Exploration group. *** p <0.001, significant difference compared to Exploration and SD-Resilient group. (c) Individual values of the *Conditioning Score* of the Exploration, Resilient and Susceptible groups.

groups performed by a simple distribution of the *Conditioning Score* data.

Resilient mice show a coping response to stress during SD

Table 2 shows the data relating to the behavior of the defeated mice during the first and fourth SD. In terms of Flight, ANOVA showed a significant effect for the Group variable [$F(1,24) = 16.578$; $p < 0.001$] given the significantly less time SD-Resilient group animals spent behaving in this way ($p < 0.001$). With respect to Submission, ANOVA showed a significant effect for the interaction of Defeat x Stress variables [$F(1,24) = 4.163$; $p < 0.05$], with resilient animals spending less time behaving submissively during the first repeated SD session in comparison to susceptible animals ($p<0.05$).

The presence of attack behavior by intruders against residents was also assessed. We only observed a trend in

Table 2. *Results of repeated SD on intruders.*

Resilient	Flight	Lat. Flight	Submission	Lat. submission	Attack	Lat. attack
SD1	32 ± 3***	15 ± 10	22 ± 6*	56 ± 6	3 ± 2	228 ± 33
SD4	32 ± 3***	3 ± 3	26 ± 7	89 ± 7	0 ± 0	300 ± 0
Susceptible	**Flight**	**Lat. Flight**	**Submission**	**Lat. submission**	**Attack**	**Lat. attack**
SD1	47 ± 7	6 ± 3	34 ± 8+	51 ± 8	1 ± 1	271 ± 30
SD4	51 ± 8	4 ± 8	12 ± 4	99 ± 4	0 ± 0	300 ± 0

Note. Behavior assessed during SD. Data presented as mean values in seconds ± SEM. *p<0.05, ***p<0.001 differences with respect to Susceptibles. + p<0.05 differences with respect to SD4 (group-defeat effect).

the variable Defeat [F(1,24) = 3.023; p = 0.095], which tells us that intruders attacked more in the first SD session. We observed that, compared to 10% of Susceptibles, 25% of the animals classified as Resilient attacked their resident in the first repeated SD. No intruder animals attacked in the fourth repeated SD.

Finally, we assessed the relationship between the Flight behavior shown by all intruders by adding the first and fourth meeting of the repeated SD and their *Conditioning Score* in the CPP to see if the time spent behaving in these ways could be an indicator of conditioning which would occur later (Figure 2). A significant Pearson correlation coefficient was only obtained between the time in Flight mode and the *Conditioning Score* (r = 0.241, p <0.05). That is to say, the longer flight behavior continued during the

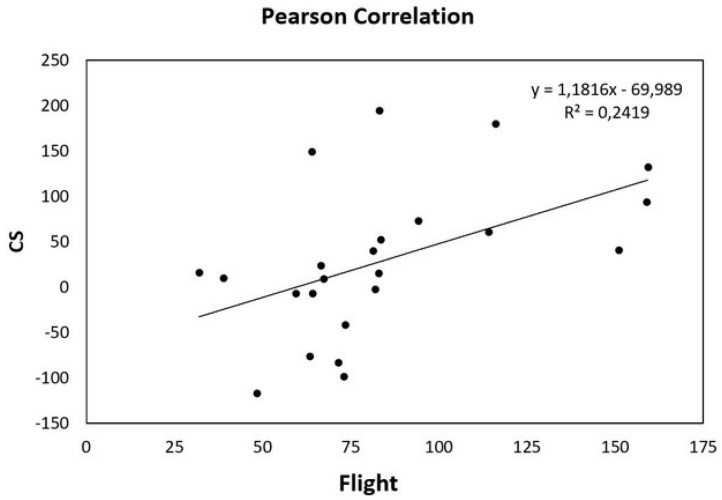

Figure 2. Regression plot for the Pearson correlation between flight during repeated SD and *Conditioning Score* (CS). The trend line represents the linear regression of data (y = 1.1816x - 58.989; r2 = 0.2419).

SD encounters, the greater the preference for the drug in CPP.

Regarding Threat by residents (Table 3), the ANOVA yields an effect for Defeat [F(1,24) = 6.535; p <0.05], indicating that residents threatened more in SD1 than in

Table 3. *Results of repeated SD on residents.*

Resident vs Resilient animals	Threat	Lat. threat	Attack	Lat. attack
SD1	36 ± 5#	10 ± 4	26 ± 5	4 ± 15
SD4	28 ± 5	4 ± 1	22 ± 3	4 ± 1
Resident vs Susceptible animals	**Threat**	**Lat. threat**	**Attack**	**Lat. attack**
SD1	36 ± 7#	6 ± 2	29 ± 9	37 ± 29
SD4	21 ± 4	8 ± 3	33 ± 4	3 ± 1

Note. Social interaction of Residents during the intruder-resident paradigm to induce SD. Data presented as mean values in seconds ± SEM. Differentiation between Residents attacking what were later categorized as Resilients and Susceptibles. # p<0.05 with respect to SD4 (defeat effect).

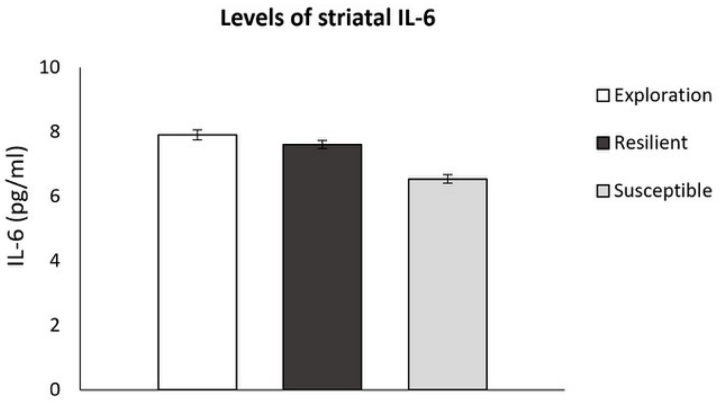

Figure 3. Striatal levels of IL-6. Effect of repeated SD on IL-6 levels in male C57/BL6 mice, taking into account the subdivision into resilient and susceptible. Data are shown as means ± S.E.M. (pg/ml).

SD4 ($p < 0.05$). However, there are no significant differences in the Group variable, implying that both Resilient and Susceptible were exposed to the same stress.

Striatal levels of Il6

The ANOVA for striatal levels of IL-6 (Figure 3) yielded no significant differences.

Discussion

The results of the present study confirm that repeated SD increases the rewarding effects of cocaine in CPP, but we have also demonstrated for the first time that the results obtained in stressed animals are not homogeneous. In defeated animals we can distinguish a susceptible population which has developed CPP with a non-effective dose of cocaine. However, there are also some defeated animals which behave like unstressed animals, that is, they are resilient without not developing CPP, although perhaps the most interesting result is that coping with SD is different in both types of animals. Resilients exhibit lower levels of flight and submissive behavior when facing the aggressor during SD. Flight behavior correlates positively with the analyzed results of CPP, i.e., the stronger the flight behavior, the more the animal will develop a preference for cocaine. Therefore, an active coping response, with less flight and submission during a social stressor, reduces sensitization to the rewarding effects of cocaine. These resilient mice also show attack behaviors against the resident, manifesting resistance to defeat, something that is not observed in any of the susceptible animals. The changes in IL-6 levels do not differ between stressed or control animals, and no difference is observed between those that are resilient or susceptible. This may be because our study was carried out three weeks after the final SD.

Resilience and susceptibility to increased cocaine reinforcing effects

Our results confirm that the experience of repeated SD during adulthood induces a long-term increase in the conditioned rewarding effects of a sub-threshold dose of cocaine (1 mg/kg) since we assessed this three weeks after the final repeated SD. The CPP paradigm is widely used to assess the conditioned effects of drugs (Aguilar et al., 2009) and reflects their secondary motivational properties, as well as their potential for abuse (Tzschentke, 2007). Exposure to repeated SD may thus induce a long-term increase in the motivational value of cocaine, thereby increasing its potential for abuse in stressed subjects. Our results confirm numerous studies showing that SD in adolescent and adult mice increases the rewarding effects of cocaine using CPP (Arenas et al., 2016; Montagud-Romero et al., 2016a; Rodríguez-Arias et al., 2015, 2017; Ferrer-Pérez et al., 2018a), or self-administration of cocaine (Boyson, Miguel, Quadros, DeBold & Miczek, 2011; Holly et al., 2016; Newman, Leonard, Arena, Almeida & Miczek, 2018; Arena, Covington, Herbert, DeBold & Miczek, 2019).

This study actually goes further and shows that although as a whole in our population of defeated mice they all develop preference with a sub-threshold dose of cocaine, we can distinguish two types of subjects. Resilient mice, despite being stressed, do not respond to the conditioned

rewarding effects of cocaine (CPP). Conversely, susceptible animals do develop increased preference for the cocaine-associated compartment. Although there is a great deal of evidence linking stress to the development of addictive behaviors (Lüthi & Lüscher, 2014; Polter & Kauer, 2014; Gold, Machado-Vieira & Pavlatou, 2015), it has also been shown that there are subjects who develop good psychosocial competence in high-risk conditions such as child abuse or adverse socioeconomic status (McGloin & Widom, 2001; Hjemdal, Friborg & Stiles, 2012; Brody et al., 2013). However, there are practically no studies with animal models assessing the phenomenon of resilience to the development of vulnerability to drug use after exposure to a social stressor. A recent study using exposure to the smell of a predator as a stress model classified its mice as resilient and susceptible based on the presence of anxiety in the cruciform raised labyrinth and avoidance of the context associated with the smell (Brodnik, Double, España & Jaskiw, 2017). This study observed that susceptible mice showed increased motor and dopaminergic effects of cocaine as well as a greater motivation to self-administer this drug. These effects were not seen in resilient animals, although in both types of mice an increase in cocaine-induced DA release was observed.

Different coping with social stress in resilient and susceptible animals

Repeated SD is a naturalistic model of social stress which mimics real-life situations and therefore has great ecological and ethological validity (Tornatzky & Miczek, 1993). Some recent research, using animal models of social stress, have observed that coping strategies are associated with resilience or vulnerability to stress (Wood et al., 2015; Chen et al. 2015, Finnell et al., 2017; Pearson-Leary et al., 2017). However, these studies classify animals as resilient or susceptible based on social behavior and anxiety shown by animals on the day after the final SD (Russo et al., 2012; Krishnan, 2014; Finnell & Wood, 2016). In these studies, resilient mice do not present anhedonia (Delgado et al., 2011), social avoidance (Krishnan et al., 2007; Golden et al., 2011; Henriques-Alves & Queiroz, 2015) or avoidance at the smell of a predator (Brodnik et al., 2017). To date, no studies have characterized animals resilient to the increased rewarding effects of drugs of abuse and, therefore, it is not known whether different stress coping strategies influence the sensitivity to such rewarding effects. What we do know is that mice showing no anxiety behavior or avoidance at the smell of a predator have neurochemical adaptations that specifically affect the function of the DA system and could therefore modify the rewarding efficacy of cocaine (Brodnik et al., 2017).

The ethological study of behavior during the social defeats showed firstly that there were no differences in the behavior of the resident animals towards intruders, whether resilient or susceptible. That is, all were exposed to the same level of stress. However, we did observe that the mice which would later be classified as resilient exhibited less flight behavior compared to the susceptible mice. In addition, we observed a positive correlation between flight behavior and the increase in the conditioned rewarding effects of cocaine in the CPP. The less the animals flee, the lower the rewarding effect produced by cocaine. Likewise, resilient animals also showed less submissive behavior during the first SD, although we no longer observed differences between resilient and susceptible animals in the fourth SD. Resilient mice, experiencing that their coping behaviors do not reduce the intensity of the attack, exhibit a behavioral adaptation. The flexibility of coping strategies has been associated with indicators of emotional resilience, such as reduced HHA axis reactivity and increased neuroplasticity (Hawley et al., 2010, Lambert et al., 2014). Our results therefore indicate that active coping and adequate adaptation reduce the rewarding effects of cocaine. Supporting our results, other studies have also confirmed that mice which do not have passive coping strategies such as flight show less anhedonia (Wood et al., 2015), less anxiety and greater social interaction (Duclot, Hollis, Darcy & Kabbaj, 2011; Hollis, Duclot, Gunjan & Kabbaj, 2011; Kumar et al., 2014). Resilient animals also exhibited attack behaviors against residents during the first confrontation, this active coping strategy having been associated with defeat resistance (Finnell & Wood, 2016).

In short, resilient animals develop an active stress coping strategy, since they attack the resident and take longer to accept they have been defeated. This resistance can cause the resilient to experience SD less intensely than the susceptible animals do. It has been observed that mice employing active coping behaviors during SD evidence lower plasma corticosterone levels, greater capacity for noradrenergic response during stress and greater sympathetic activity in response to defeat (Wood, Walker, Valentino & Bhatnagar, 2010; Gómez-Lázaro et al., 2011; Pérez-Tejada et al., 2013). This type of response is very adaptive, since it allows the response to stress to be limited (Koolhaas et al., 2011). Another factor which can explain the development of resilience is the feeling of control during SD, since the resilient mice do not flee from the aggressor and even exhibit attack behaviors. Interestingly, cocaine use is only increased in intruder mice, but not in residents which initiate the attack, although in both types of animals there is a hormonal response to stress (Covington & Miczek, 2001, 2005; Covington et al., 2005; Boyson et al., 2014). The resident mouse maintains control of the encounter, which may exert a protective effect on the stress response of the hypothalamus-pituitary-adrenal axis (Boyson et al., 2014). Therefore, our resilient animals may experience a certain level of control of the stress situation.

Conversely, the susceptible animals showed passive confrontation, accepting defeat with more time in flight and submission and without presenting any aggressive behavior towards the resident. This passive coping during SD has previously been associated with the onset of anxiety and depression (Wood et al., 2010, Chen et al., 2015, Pearson-Leary et al., 2017).

Neuroinflammation response after repeated SD

In the 1990s, the so-called neuroinflammatory theory of depression was proposed (for example, Maes et al., 2009), based on the increase in inflammatory mediators in patients with depression. There are currently numerous studies demonstrating the role of the immune system in the vulnerability to the development of mental illness (Réus et al., 2015; Menard, Pfau, Hodes & Russo, 2017). It is also believed that substance use disorder is related to changes in the activity of the immune system (Clark, Wiley & Bradberry, 2013; Cui, Shurtleff & Harris, 2014). Both clinical and preclinical studies have shown that psychostimulants such as cocaine activate central and peripheral components of the immune system (Clark et al., 2013; Araos et al., 2015; Moreira et al., 2016). More recently it has also been shown that social stress triggers an activation of the immune system, increasing peripheral levels of cytokines, activating microglia or even increasing the permeability of the blood brain barrier (Pfau & Russo, 2016; Rodríguez-Arias et al., 2017, 2018; Ferrer-Pérez et al., 2018a).

It has described that after SD, susceptible mice which develop social isolation and anxiety show higher levels of IL-6 than resilient animals (Hodes, Ménard & Russo, 2016). However, our results do not confirm this lower inflammatory response in resilient animals. Il-6 levels were not higher in defeated animals compared to controls, and no differences were observed between resilient and susceptible mice. The discrepancy in the results may be mainly due to the fact that in the study by Hodes et al. (2016), the measurement of IL-6 was carried out 24 hours after the final SD, while in our study it was done at the end of the entire procedure, when more than a month had passed since the final repeated SD. Similarly, we had previously demonstrated that after CPP, increases in striatal levels of IL-6 in defeated animals were no longer observed (Ferrer-Pérez et al., 2018a). Since the characterization of animals as resilient or susceptible requires the development of CPP, our experimental design involves making measurements at least four weeks after the final SD. Our results therefore indicate that one month after the final SD there are no differences in the neuroinflammatory response.

Animal models are a very powerful tool, but we must be cautious when transferring the results to human behavior. We can extrapolate from the SD model to situations of psychological or social stress to which we are exposed for much of our lives. Our results allow the identification of some behavioral characteristics which appear in animals resistant to this SD and which can act as a protective factor against the development of drug addiction. Active but flexible coping stands out as the most relevant behavioral characteristic of resilient subjects. The study of behavioral or pharmacological strategies underlying resilience will allow us to reduce vulnerability to SUD induced by social stress.

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Conflict of interests

The authors declare no conflict of interest.

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ANEXO 2: Estudio 2.

A limited and intermittent access to a high-fat diet modulates the effects of cocaine-induced reinstatement in the conditioned place preference in male and female mice.

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ORIGINAL INVESTIGATION

A limited and intermittent access to a high-fat diet modulates the effects of cocaine-induced reinstatement in the conditioned place preference in male and female mice

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Abstract
Rationale Palatable food and drugs of abuse activate common neurobiological pathways and numerous studies suggest that fat consumption increases vulnerability to drug abuse. In addition, preclinical reports show that palatable food may relieve craving for drugs, showing that an ad libitum access to a high-fat diet (HFD) can reduce cocaine-induced reinstatement.
Objective The main aim of the present study was to evaluate the effect of a limited and intermittent exposure to HFD administered during the extinction and reinstatement processes of a cocaine-induced conditioned place preference (CPP).
Methods Male and female mice underwent the 10 mg/kg cocaine CPP. From post-conditioning onwards, animals were divided into four groups: SD (standard diet); HFD-MWF with 2-h access to the HFD on Mondays, Wednesdays, and Fridays; HFD-24h, with 1-h access every day; and HFD-Ext with 1-h access to the HFD before each extinction session.
Results Our results showed that all HFD administrations blocked reinstatement in males, while only the HFD-MWF was able to inhibit reinstatement in females. In addition, HFD-Ext males needed fewer sessions to extinguish the preference, which suggests that administration of fat before being exposed to the environmental cues is effective to extinguish drug-related memories. HFD did not affect Oprμ gene expression but increased CB1r gene expression in the striatum in HFD-Ext males.
Conclusions These results support that palatable food could act as an alternative reward to cocaine, accelerating extinction and blocking reinstatement, these effects being sex specific.

Keywords Extinction · Cocaine · Reinstatement · High-fat diet · Conditioned place preference

Introduction

Drug addiction is defined as a chronic disorder characterized by relapse accompanied by the compulsion to seek and take the drug and the loss of control in limiting intake (Koob and Le Moal 1997). When access to the drug is prevented, a negative emotional state emerges, reflecting a motivational withdrawal syndrome (Koob and Volkow 2010). Due to this

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negative emotional state, drugs of abuse become negative reinforcement during withdrawal (Koob and Le Moal 2001), leading to relapse due to the impelling need to consume (Koob and Volkow 2010). Therefore, it is not surprising that patients who undergo an addiction treatment tend to seek alternative reinforcements, including natural rewards, such as palatable food (high-fat and/or sugar-rich food), in order to stimulate brain circuits of reward (Salamone et al. 2005). In fact, clinical evidence emphasizes the frequent use of palatable food to decrease drug craving during withdrawal (Cowan and Devine 2008).

Preclinical studies have also pointed to this relation. Orsini et al. (2014) reported that rats with a history of chronic amphetamine exposure increased their food consumption. In addition, Loebens and Barros (2003) observed that animals fed with a high-fat diet (HFD) are more prone to depression during cocaine withdrawal in the forced swimming test (Loebens and Barros 2003). Moreover, other studies suggest that fat intake may represent a competitive reward for drugs. The conditioned place preference (CPP) procedure evaluates the role of environmental cues associated with the rewarding effects of drugs of abuse, such as cocaine, which were decreased by previous exposure to HFD (Morales et al. 2012). In this line, the present work is a follow-up of our previous study reporting that continuous HFD administration during the extinction of cocaine-induced CPP reduced the sessions required to extinguish the preference and decreased the sensitivity to drug priming-induced reinstatement (Blanco-Gandía et al. 2017a).

Epidemiological studies have shown high levels of comorbidity between eating disorders (bulimia and binge eating disorder) and substance abuse (Becker and Grilo 2016; Conason et al. 2006; Holderness et al. 1994; Nøkleby 2012; Flores-Fresco et al. 2018). We speculate that this high comorbidity could in part be due to shared reinforcing properties between palatable foods and drugs of abuse. For example, certain foods, particularly those rich in sugar and fat, are potent rewards that promote eating even in the absence of energetic requirements (Lenoir et al. 2007), being able to trigger learned associations between environmental stimulus and reward (Volkow et al. 2011). In fact, several studies have pointed out that a continuous access to fat diminishes the rewarding effects of cocaine (Morales et al. 2012; Thanos et al. 2010; Blanco-Gandía et al. 2017a). Palatable food activates the reward system (DiLeone et al. 2012; Narayanaswami et al. 2013) through the activation of the mu-opioid receptor pathway in the VTA (Pitman and Borgland 2015) and the cannabinoid system (Parylak et al. 2012).

The present work relates to the idea of palatable food as an alternative reward. In the previous studies, a continuous high-fat diet produced harmful metabolic effects (Blanco-Gandía et al. 2017b). Here, we aim to study if an intermittent and limited access to a HFD could also diminish cocaine-associated memories without causing changes on bodyweight or metabolism, proving that a sporadic exposure could be sufficient to extinguish the preference and block reinstatement. Our study will be the first to evaluate the possible counteracting effects of intermittent and limited access to a HFD on the extinction and reinstatement of cocaine-induced CPP. Our general hypothesis is that the limited access to a HFD will accelerate the extinction of cocaine-associated memories and will reduce cocaine priming reinstatement. To assess metabolic disturbances, we will measure bodyweight and leptin and ghrelin changes after HFD exposure. Because the opioid and cannabinoid systems play a crucial role in food and drug reward (de Macedo et al. 2016), we also evaluated the effects of HFD administrations on the opioid mu receptor (Oprμ) and CB1 receptor gene expression (CB1r) in the striatum. Oprμ and CB1r are implicated in food reward processes and palatability (Kessler et al. 2016; Bello et al. 2014), as well as in various forms of learning and memory, including acquisition and reinstatement of cocaine-associated memory (Sticht et al. 2010; Hu et al. 2015). Given that sex differences in the vulnerability to drug seeking have been scarcely studied (Carroll et al. 2002), we will conduct our study in both male and female mice. For example, several data suggest a more intense response to cocaine in female rodents, with more psychomotor sensitization (Holly et al. 2012), faster acquisition of cocaine self-administration (Martini et al. 2014), and higher a breaking point of the progressive ratio and cocaine reinstatement (Lynch 2006). Sex-specific differences have also been described in response to HFD, with male rodents being more susceptible to physiological changes (Grove et al. 2010; Mela et al. 2012; Wang et al. 2018; Gelineau 2017).

Material and methods

Subjects

A total of 60 female and 47 male mice of the OF1 outbred strain were acquired commercially from Charles River (Barcelona, Spain). Animals were 42 days old when they arrived at the laboratory and were all housed under standard conditions in groups of 4–5 (cage size 28 × 28 × 14.5 cm). Mice were exposed to a reverse light cycle (white lights on from 19:30 to 7:30), and the vivarium was controlled for constant temperature (21 ± 2 °C). Food (standard diet) and water were available ab libitum except during the behavioral tests. All procedures involving mice and their care complied with national, regional, and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010, on the protection of animals used for scientific purposes. The Animal Use and Care Committee of the University of Valencia approved the present study.

Drug treatment

For CPP, animals were injected intraperitoneally (IP) with 10 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S.A., Madrid, Spain) diluted in 0.9% NaCl (saline) in a volume of 0.1 mL/10 g bodyweight.

Experimental design

Animals first underwent the 10 mg/kg cocaine-induced CPP procedure from postnatal day (PND) 43. From PND 53 onwards, all mice were, from this moment on, exposed twice a week to extinction sessions (Table 1). Female and male mice were randomly divided into four groups: standard diet (SD); daily high-fat diet (HFD-24h); Monday, Wednesday, and Friday high-fat diet (HFD-MWF); and high-fat diet 1h before extinction (HFD-Ext).

Apparatus and procedure

Conditioned place preference

For place conditioning, we employed sixteen identical Plexiglas boxes with two equally sized compartments (30.7 cm length × 31.5 cm width × 34.5 cm height) separated by a gray central area (13.8 cm length × 31.5 cm width × 34.5 cm height). The compartments have different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossing from one compartment to the other. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

Acquisition of CPP The procedure of place conditioning, unbiased in terms of initial spontaneous preference, was performed as described previously (Maldonado et al. 2006) and consisted in three phases. To summarize the main aspects, in the first phase, known as Pre-C, mice were allowed access to both compartments of the apparatus for 15 min (900 s) per day for 3 days. On day 3, the time spent in each compartment over a 900-s period was recorded, and animals showing a strong unconditioned aversion (less than 33% of the session time) or preference (more than 67%) for any compartment were excluded for the rest of the experiment (total excluded: 4). Half of the animals in each group received the drug or vehicle in one compartment and the other half in the other compartment. After assigning the compartments, no significant differences were detected between the time spent in the drug-paired and vehicle-paired compartments during the pre-conditioning phase. In the second phase (conditioning), which lasted 4 days, animals received an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30 min. After an interval of 4 h, they received an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. Confinement was carried out in both cases by closing the guillotine door that separated the two compartments, making the central area inaccessible. During the third phase, known as Post-C, the guillotine door separating the two compartments was removed (day 8), and the time spent by the untreated mice in each compartment during a 900-s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates that an aversion has been developed.

Extinction of CPP When the preference for the drug-paired compartment was established, mice underwent twice a week (Monday and Thursday) an extinction session that consisted of placing the animals in the apparatus (without the guillotine doors separating the compartments) for 15 min. The extinction condition was fulfilled when there was a significant difference between CPP scores and Post-C scores in two consecutive sessions and a lack of significant difference between CPP and Pre-C test values.

Reinstatement of CPP Twenty-four hours after extinction had been confirmed, the effects of a priming dose of cocaine were

Table 1 Experimental design. *PND* postnatal days, *HFD* high-fat diet

			PND 53 onwards				
	Male	Female	Monday	Tuesday	Wednesday	Thursday	Friday
SD	*n*=12	*n*=15	Standard diet	Standard diet	Standard diet	Standard diet	Standard diet
HFD-24h	*n*=11	*n*=15	HFD 1h	HFD 1h	HFD 1h	HFD 1h	HFD 1h
HFD-MWF	*n*=12	*n*=15	HFD 2h		HFD 2h		HFD 2h
HFD-Ext	*n*=12	*n*=15	HFD 1h before EXT			HFD 1h before EXT	

evaluated. Reinstatement tests were the same as those carried out in Post-C (free ambulation for 15 min), except that animals were tested 15 min after administration of the respective dose of cocaine. When reinstatement of the preference was achieved, after a subsequent weekly extinction process, a new reinstatement test was conducted with progressively lower doses of the drug until the CPP was completely extinguished. This procedure of extinction-reinstatement was repeated with decreasing doses (half the previous dose) until a priming dose was confirmed to be ineffective (5 mg/kg after extinction of CPP induced by 10 mg/kg and 2.5 mg/kg after extinction if animals showed reinstatement with 5 mg/kg). HFD conditions were maintained until the end of the experiment. Priming injections were administered in the vivarium, which constituted a non-contingent place to that of the previous conditioning procedure.

Feeding conditions

After the Post-C test in the CPP, each group began their intermittent access to the different HFD patterns. Our feeding procedure is based on the limited access model described by Corwin et al. (1998), in which non-food-deprived animals have sporadic and limited access to a HFD. Two different types of diet were used in this study: a standard diet (Teklad Global Diet 2014, 13 Kcal % fat, 67 Kcal % carbohydrates, and 20 Kcal % protein; 2.9 kcal/g) and the HFD (TD.06415, 45 Kcal % fat, 36 Kcal % carbohydrates and 19% Kcal protein; 4.6 kcal/g). Both diets were supplied by Harlan Laboratories Models, S. L. (Barcelona, Spain) and will be referred to from now on as the standard diet and the high-fat diet (HFD).

As we described on Table 1, on postnatal day (PND) 53, mice were randomly divided into 4 groups of males and 4 groups of females with similar average bodyweights and assigned to one of the following conditions: standard diet (SD); daily high-fat diet (HFD-24h); Monday, Wednesday, and Friday high-fat diet (HFD-MWF); and high-fat diet before extinction (HFD-Ext). All the groups had standard diet access during the whole procedure, and the HFD-24h group had a 1-h access to HFD from Monday to Friday; the HFD-MWF had a 2-h access to HFD on Monday, Wednesday, and Friday; and lastly, animals in the HFD-Ext had a 1-h HFD access prior to each extinction session (twice a week). The SD group remained undisturbed in their home cage.

All groups were fed ad libitum with the standard diet (Teklad Global Diet 2014) in their own cages, and water was freely available. After acquiring cocaine preference, during the extinction process, animals continued with SD in their home cages, but only the animals in the HFD groups were taken for a limited time into a separated plastic cage with access to the HFD. Exposure to the HFD took place in different individual plastic cages 2–3 h after the beginning of the dark phase. HFD intake was measured after each session. Animals were weighed every week throughout the study, at which point their intake of the standard diet in their home cage was also measured in grams.

Determination of plasma leptin and ghrelin levels

For leptin and ghrelin plasma quantification, an ELISA kit was employed for leptin and ghrelin (Merck-Sigma Aldrich, Saint Louis, USA) following the manufacturer's instructions. The sensitivity of the test is 0.2. All samples were run in duplicate.

Gene expression analyses: RNA isolation and quantitative RT-PCR

At the end of the experiments, animals were euthanized by cervical dislocation, and the brains were immediately removed from the skull and placed on a cold plaque. Cerebellum and olfactory bulbs were eliminated, and the striatum was dissected. Brain tissue samples were immediately stored at −80°C until the RT-PCR assay was performed. The whole striatum was employed for this analysis.

Total RNA from the striatum was isolated using the Tri Reagent Method (Sigma-Aldrich, St. Louis, MO, USA), as described in the manufacturer's protocol. Reverse transcription of 1 mg of total RNA was performed using the Transcriptor First Strand cDNA synthesis kit (Thermo Fisher Scientific, Madrid, Spain). Amplification of the target and housekeeping (b-glucuronidase) genes was performed using the Taqman Gene Expression Master Mix (Thermo Fisher Scientific, Madrid, Spain) in a LightCycler 480 System (Roche Diagnostics) following the manufacturer's instructions. The assay codes of the primers used are Mm01212171_s1, Mm01188089_m1, and Mm00446953_m1 corresponding to CB1r, Oprμ, and Gusb, respectively. Data were analyzed using the LightCycler 480 relative quantification software and were normalized to the amplification product of b-glucuronidase.

Statistics

To test for the acquisition of CPP, the time spent in the drug-paired compartment was analyzed with a (2)×2×4 mixed ANOVA. The two between factors were sex (male and female) and diet (SD, HFD-24h, HFD-MWF, and HFD-Ext). The within subjects factors tested the measurement differences between the pre- and post-CPP phases (Pre-C and Post-C). Since at this point of measurement all the animals had been maintained with the standard diet, the diet factor simply functions as a control check for the effectiveness of the random assignment to produce equivalent groups prior to exposure to the extinction-reinstatement manipulation (i.e.,

the expectation is that there will not be an effect for Diet). To compare whether extinction/reinstatement is achieved within the same group, data related to extinction, 2.5 mg/kg reinstatement, and 5 mg/kg reinstatement were analyzed by means of Student's *t*-test. The time required for the preference to be extinguished was analyzed by means of the Kaplan-Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate. The extinction analyses were performed within every group; therefore, no comparisons among groups were made. Ghrelin and leptin plasma levels and CB1r and Oprμ gene expression values were analyzed by a one-way ANOVA, considering the between variable –Sex–, with two levels (male and female), and the between variable –Diet–, with 4 levels (SD, HFD-24h, HFD-MWF, HFD-Ext). All data are presented as mean ± standard error of mean (SEM). A p-value <0.05 was considered statistically significant. Analyses were performed using SPSS v26.

Results[1]

Conditioned place preference

Results of the 10 mg/kg cocaine-induced CPP are presented in Fig. 1a (males) and b (females). Regarding the main effects, the ANOVA for the time spent in the drug-paired compartment revealed an effect of the variable days [$F(1,99)=138,410$; $p<0.001$], which indicates that all animals developed conditioned place preference (Post-C vs Pre-C). Bonferroni's post hoc comparisons showed that in all cases, males and females spent significantly more time in the drug-paired compartment in Post-C than in Pre-C ($p<0.001$ in all cases). No effect was found in the variables sex nor diet separately. Regarding the interactions, there was no significant effect in the three-way interaction days × sex × diet, but the ANOVA did reveal a significant effect of the interaction days × sex [$F(1,99)=3833$; $p<0.05$]. Bonferroni's post hoc comparisons showed that female mice spent more time in the drug-paired compartment in Post-C than male mice ($p<0.05$), showing a stronger CPP but only in two groups: those that will receive the HFD tree days a week (HFD-MWF) or previously to each extinction sessions (HFD-Ext).

To compare if there was a reinstatement into drug seeking after achieving extinction within the same group, Student's *t*-tests were performed. Reinstatement with a priming dose of 5 mg/kg cocaine (Fig. 1a) was achieved in the male SD group ($t=-2.772$; d.f. 11; $p=0.018$), but after subsequent extinction sessions, the Student's *t*-test showed that no reinstatement with a priming dose of 2.5 mg/kg cocaine was achieved ($t=-0.078$; d.f. 11; $p=0.939$). In females (Fig. 1b), the Student's *t*-test showed reinstatement with a priming dose of 5 mg/kg cocaine in SD ($t=-2.297$; d.f. 14; $p=0.038$), HFD-24h ($t=-2.529$; d.f. 14; $p=0.024$), and HFD-Ext ($t=-2.282$; d.f. 14; $p=0.039$). Extinction was confirmed with the Student's *t*-test, where no group of males and females showed any differences with respect to the Pre-C test (data not shown). After two subsequent extinction sessions, the Student's *t*-test showed that no reinstatement with a priming dose of 2.5 mg/kg cocaine was achieved in any group (SD: $t=-1.241$; d.f. 14; $p=0.235$; HFD-24h: $t=-0.684$; d.f. 14; $p=0.505$; and HFD-Ext: $t=-0.303$; d.f. 14; $p=0.767$).

With regard to the time required to extinguish the preference, we found that in males (Fig. 2), the SD, HFD-MWF, and HFD-24h groups required a total of 16, 21, and 17 sessions, respectively, to achieve extinction, while HFD-Ext required only 8 sessions to extinguish the preference. The Kaplan-Meier analysis showed that the HFD-Ext male group required significantly less time to achieve extinction than the SD group ($\chi^2=4.336$; $p<0.05$), with no significant differences with respect to the other groups.

In female groups, the SD, HFD-MWF, and HFD-Ext groups required 9 sessions to achieve extinction, while the HFD-24h group required a total of 15 sessions to achieve extinction (Fig. 2). The Kaplan-Meier analysis revealed that the HFD-24h group required significantly more time to achieve extinction than the SD group ($\chi^2=8.431$; $p<0.01$), HFD-MWF ($\chi^2=4.250$; $p<0.05$), and HFD-Ext ($\chi^2=4.289$; $p<0.05$).

Effects of different HFD eating patterns and sex on plasma leptin and ghrelin levels and CB1r and Oprμ gene expression

There were no significant effects of the different intermittent eating patterns or sex on circulating leptin [$F(3,56)=0.975$; $p=0.411$] and ghrelin levels [$F(3,56)=0.440$; $p=0.725$) (Table 2). Regarding the real-time PCR analyses, the ANOVA indicated in the CB1r gene expression (Fig. 3a) an effect of the interaction diet × sex [$F(3,56)=3.340$; $p<0.05$]. There was an increased CB1r gene expression only in males of the HFD-Ext group with significant differences with respect to the SD group ($p<0.01$), HFD-MWF group ($p<0.01$), and HFD-24h group ($p<0.001$). Moreover, the HFD-Ext male group showed a significant increase against their corresponding HFD-Ext female group ($p<0.05$).

Regarding Oprμ gene expression (Fig. 3b), the ANOVA revealed a significant effect in the variable sex [$F(1,55)=7,604$; $p<0.01$], as overall, HFD male mice overexpressed Oprμ with respect to females ($p<0.01$).

[1] Information relating to bodyweight and food intake can be found in the supplementary material S1. No bodyweight and food intake differences were found between the four diet groups within each sex group.

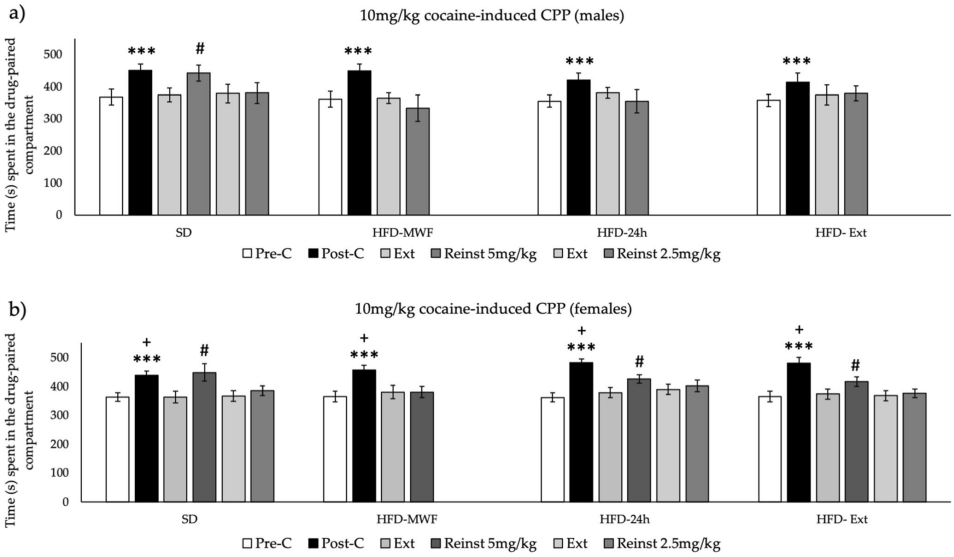


Fig. 1 Effects of HFD during the extinction-reinstatement process in conditioned place preference (CPP) in males (**a**) and females (**b**). CPP was induced by 10 mg/kg of cocaine in male and female mice fed with a standard diet and exposed after Post-C to different dietary conditions: SD group (standard diet throughout the procedure), HFD-24h group (1-h access to HFD from Monday to Friday), in HFD-MWF group (2-h access to high-fat diet on Monday, Wednesday, and Friday), and in the HFD-Ext group (1-h high-fat diet access twice a week, prior to extinction sessions). Bars represent the time (± SEM) in seconds spent in the drug-paired compartment in the pre-conditioning test (white bars), the post-conditioning test (black bars), in the last extinction session (light gray bars), and in the reinstatement test (dark gray bars). The first reinstatement test was evaluated 15 min after a priming dose of 5 mg/kg of cocaine, while the second reinstatement test was evaluated 15 min after a priming dose of 2.5 mg/kg of cocaine. ***p<0.001 significant difference with respect to the Pre-C (ANOVA); +p<0.05 significant difference in female Post-C vs male Post-C. #p<0.05 significant difference with respect to extinction (Student's t test)

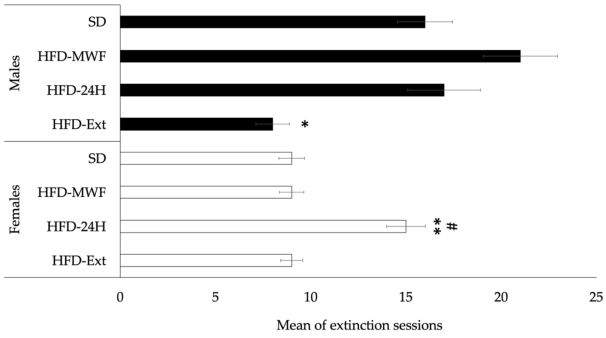


Fig. 2 Mean of extinction sessions. The bars represent the mean value (± SEM) of the number of sessions required for the preference to be extinguished after the Post-C test. Preference was considered to be extinguished when an animal spent 370s or less in the drug-paired compartment on two consecutive days. When the preference was not extinguished in a mouse, the number of days needed to achieve extinction in the whole group was assigned to that animal. *p<0.05, **p<0.01 with respect to the respective standard diet group (SD); #p<0.05 with respect to HFD-MWF and HFD-Ext

Table 2 Plasma leptin (ng/ml) and ghrelin (pg/ml) levels. Data are presented as mean values ± SEM (*n*=8/condition)

	Plasma leptin (ng/ml)		Plasma ghrelin (pg/ml)	
	Males	Females	Males	Females
SD	3.87 ± 0.49	3.64 ± 0.46	487 ± 51	356 ± 53
HFD-MWF	2.46 ± 0.77	4.03 ± 0.53	477 ± 78	471 ± 51
HFD-24h	3.07 ± 0.61	3.03 ± 0.70	475 ± 76	437 ± 60
HFD-Ext	3.01 ± 0.49	3.18 ± 0.79	482 ± 69	398 ± 50

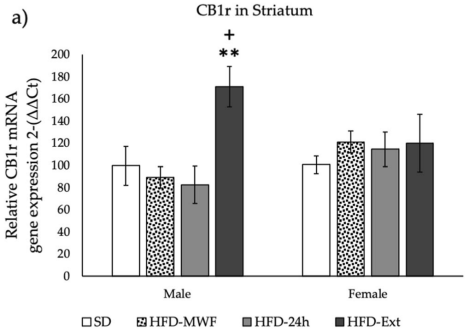
Discussion

The present study noticed that the intermittent and limited access to a HFD may prevent reinstatement of the conditioned rewarding effects of cocaine in males, and specific administration schedules can facilitate or disrupt the extinction of the cocaine-associated memories in males and females. Related to this, this study highlights the important role of sex in these effects, since there were great differences between the results obtained in male and female mice. Once 10 mg/kg cocaine-induced CPP was acquired, all the mice were exposed to a different intermittent administration of HFD. The main results of the present work indicate that in males, exposure to the HFD in any of the three patterns employed blocked the reinstatement of the preference with a priming dose of 5 mg/kg of cocaine. However, in female mice, only one HFD administration (HFD-MWF) was effective in blocking the reinstatement of cocaine-induced preference. Extinction of the memories associated with cocaine was faster when access to the HFD was prior to each extinction session, but this phenomenon occurred only in males.

As we mentioned in the "Introduction," this is a follow-up of a previous study where we confirmed that a continuous HFD administration during cocaine withdrawal undermined reinstatement of cocaine-induced CPP, thereby acting as an alternative reward. However, that administration pattern induced several metabolic consequences (Blanco-Gandía et al. 2017a). Therefore, the aim of the present study was to evaluate a reduced administration pattern of HFD that could have effects on cocaine reward without inducing metabolic disturbances, such as increased bodyweight or changes in circulating leptin or ghrelin levels. We have shown that the metabolic harm induced by HFD is not necessary to disrupt the extinction and reinstatement of the CPP induced by cocaine, given that a limited and intermittent exposure was sufficient to block cocaine-related effects in a sex-specific manner. Although the HFD did not modify these parameters or affect the Oprμ gene expression, we found changes in the CB1r gene expression in the striatum of male animals. Surprisingly, we observed that only males that showed a faster extinction of cocaine-related memories (HFD-Ext) exhibited an overexpression in CB1r.

Conditioned rewarding effects of cocaine

Most of the studies performed to date focus on the role of palatable food on the acquisition of the self-administration/CPP of drugs (Davis et al. 2008; Morales et al. 2012; Blanco-Gandía et al. 2017a, 2017b). Prevention of the reinstatement of cocaine self-administration in rats has been obtained by pairing cocaine-related stimuli with food (Kearns and Weiss 2007). In that study, one of the groups was submitted to extinction by pairing a tone with the presentation of food in a different context. When this group was exposed to the tone in the original context, their renewal was significantly lower than that observed in the group exposed to the tone but without alternative reward to cocaine. Kearns and Weiss (2007) suggested that pairing a drug-related stimulus, such as a tone, to another reward different from the drug enhances



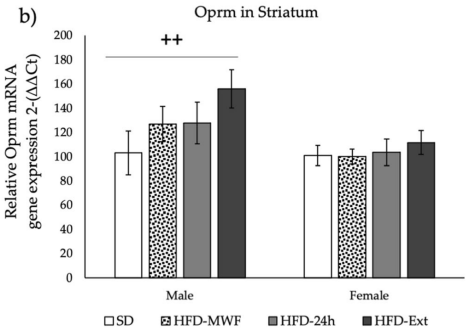


Fig. 3 Real-time PCR gene expression in the striatum. (**a**) CB1r relative gene expression evaluation in the striatum region (*n* = 8/condition). (**b**) Oprμ relative gene expression evaluation in the striatum region (*n*=8/condition). The columns represent means and the vertical lines ± SEM of relative (2-ΔΔCt method) gene expression in the striatum of OF1 mice. $^{**}p<0.01$ significant differences with respect to the rest of the groups. $^{+}p<0.05$; $^{++}p<0.01$ significant differences with respect to their corresponding female group

the reduction of the reinstatement into drug seeking and prevents relapse. To date, no data regarding food administration before being exposed to a drug-related context are available. In our study, we evaluated for the first time how intermittent access to HFD may modulate the extinction/reinstatement process.

We have previously shown that ad libitum access to a HFD accelerated the extinction process and blocked the reinstatement of cocaine seeking in adult mice (Blanco-Gandía et al. 2017a). However, an important weakness of that study was that continuous access to HFD induced bodyweight gain and dysregulation of hormone levels, with an increase in leptin and a decrease in ghrelin signaling, pointing to a metabolic syndrome and discarding the possibility of employing palatable food as an alternative reward to cocaine. However, in the present study, none of the intermittent and limited access to HFD schedules employed affected bodyweight, confirming that limited access to a HFD does not promote obesity (Corwin et al. 1998; Hudson et al. 2007; Blanco-Gandía et al. 2017b). In addition, there were no changes in leptin or ghrelin signaling in any of the groups, independently of HFD administration. These results confirmed previous studies (Blanco-Gandía et al. 2017b), showing that intermittent access to palatable food does not induce the negative consequences that were observed after ad libitum access (Davis et al. 2008; Morales et al. 2012; Blanco-Gandía et al. 2017a).

In agreement with previous reports indicating sex differences in the response to cocaine, we have observed a marked sex difference in the establishment of cocaine-induced CPP. Female mice spent more time in the drug-paired compartment during Post-C than males, suggesting a stronger sensitivity to the rewarding effects of cocaine. In line with our results, female rodents exhibit an increased sensitization to the locomotor effects of cocaine (Holly et al. 2012), faster acquisition of cocaine self-administration with persistence in the progressive ratio schedule (Martini et al. 2014; Lynch 2006), and develop cocaine-induced CPP with less pairing sessions than males (Russo et al. 2010).

However, after Post-C, female mice took less time to extinguish the preference than males, which suggests that cocaine-related memories are stronger in males. These results are in the line with previous studies showing that female mice require fewer extinction sessions than males to extinguish the CPP (Hilderbrand and Lasek 2014) and that estradiol administration facilitates extinction of cocaine CPP in female rats (Twining et al. 2013). However, no positive effects of the diet on the extinction process were observed in females, conversely to the results in males. Male mice exposed to HFD prior to each extinction session (HFD-Ext) exhibited a faster extinction of the drug-related memories. The HFD-Ext male group required significantly fewer sessions than the control group as well as fewer sessions than the HFD-MWF and HFD-24h groups. This effect could be related to the administration of HFD before every exposure to the context associated with cocaine reward. This is supported by previous data, where it has been suggested that pairing food with the old contextual cues related to the drug can prevent relapse (Kearns and Weiss 2007). An additional explanation is that the more rapid extinction in the HFD-Ext male group could be due to satiety and not be specific to HFD, as some studies have found that caloric restriction lowers the threshold dose for cocaine CPP and increases the persistence of extinction (Zheng et al. 2012; Jung et al. 2016). Thus, we cannot rule out that satiation in this group modulates the extinction process.

Hence, it can be argued that in order to diminish drug seeking in male mice, the moment in which HFD is administered plays a key role. Extinction recruits a new learning process (Lattal et al. 2006; Nic Dhonnchadha et al. 2013), especially in the hippocampus, which is particularly involved in the extinction of drug-associated memories (Szalay et al. 2013). Exposure to fat before each extinction session became a stimulus that predicted a non-reinforced context without cocaine and therefore accelerated the extinction of the preference. This effect is clear in males (HFD-Ext), but not in females, maybe due to their faster extinction of the preference. It is important to note that several studies have shown that HFDs lead to impairments in cognitive function such as memory or learning (Hwang et al. 2010; Valladolid-Acebes et al. 2013). In a previous study from our laboratory, we evaluated if a limited and intermittent access (exposed to the same conditions as the HFD-MWF group in this study) produced comparable impairments as the continuous access to a HFD (Blanco-Gandía et al. 2019). Our results showed that animals exposed to a limited and intermittent access to HFD showed no differences with respect to animals fed with a standard diet, which supports that the data obtained in the male HFD-Ext group are not due to a recall impairment.

As expected, 5 mg/kg of cocaine induced reinstatement in male and female mice fed with the standard diet. All three of the HFD administrations were effective in males, which did not reinstate their preference after a priming dose of cocaine. With a similar or lower number of extinction sessions than the control group, none of the male groups exposed to HFD reinstated the preference, suggesting that intermittent and limited HFD administration is a good alternative reinforcer. Therefore, the fact that the HFD blocked the reinstatement of preference in males, in all the schedules employed, independently of its administration schedule, suggests that fat could also act as an alternative reinforcer competing with cocaine.

Different results were observed in female mice, with only one group not reinstating the preference with 5 mg/kg cocaine, the female HFD-MWF group. The sex differences found in the response to HFD could be explained through the fact that female rats exhibited a higher cocaine priming-induced reinstatement response than males (Lynch 2006) with a greater

magnitude of reinstatement to cocaine-induced CPP (Bobzean et al. 2010). Several studies have suggested that the neural systems mediating cocaine reinforcement could also show sex differences, with dopamine response induced by cocaine in several brain areas being greater in female (Becker 1999; Becker and Ramirez 1981; Walker et al. 2001). Results even showed that this higher dopaminergic sensitivity in females could be independent of gonadal hormones (Bazzett and Becker 1994; Castner et al. 1993; McDermott et al. 1994). However, the remaining question is why only the HFD-MWF pattern was a protective pattern to reinstate cocaine preference in females. Firstly, it was only in this group where females ate significantly more fatty food. Secondly, we have previously shown that administration for several weeks is a risky pattern that induces neurobiological changes similar to those produced by chronic drug administration and could interfere in the reward system (Blanco-Gandía et al. 2017a; Corwin et al. 1998; Puhl et al. 2011). We can hypothesize that HFD-MWF females were protected from reinstatement into cocaine seeking because they may have developed another preference for HFD. Some studies have reported that, after drug withdrawal, there is increased overeating, and it is even recommended to counteract craving (Bane et al. 1993; Orsini et al. 2014). In this context, authors propose the concept of "addiction transfer," where one addiction is replaced by another, and could explain the behavioral outcomes exhibited by the HFD-MWF female group (Chechlacz et al. 2009).

Based on our results, we hypothesize that the administration of a natural reward, such as food, was not enough to block the potent effect of cocaine on female mice. However, in the case of males, who acquired the preference with less intensity than females, HFD became a good alternative reinforcer. Although preclinical studies are limited, several reports show that drug withdrawal induces an increase in food consumption. Orsini et al. (2014) showed that chronic exposure to amphetamine (9 injections) increased food consumption in male rats after cessation, discarding the possibility of a rebound from amphetamine-induced anorexia, as all the animals, control and amphetamine-treated, weighted the same when withdrawal began. In the same line, there is a reduction in the rewarding properties of drugs when a HFD is administered. For example, Wellman et al. (2007) demonstrated that a free access to HFD for 45 days diminished the acquisition of cocaine self-administration in male rats. Other findings on female rats showed that, after a 14-day exposure to cocaine, a specific increase in fat and carbohydrate consumption occurred, which was not seen in protein consumption (Bane et al. 1993). Most of these studies have been performed in male rodents, and our results highlighted the necessity of studying the response to palatable food in females as well as the limitation of the abovementioned results.

The different schedules of intermittent accesses to the HFD caused animals to receive a different number of rewarding experiences, which can be considered a limitation of this study. Those groups needing more sessions to extinguish the preference consequently were exposed to a higher number of fat administrations. However, a lack of reinstatement did not correlate to the number of HFD sessions in male or female mice. In addition, another important limitation to take into account in future studies is the variety of manipulations per week during the extinction process, given that, during this period, the SD groups were only moved to perform the extinction sessions, while the HFD groups were also moved for HFD access.

Neurobiology changes induced by intermittent and limited access to HFD

The endocannabinoid and the endogenous opioid systems are crucial in the addiction process and regulate feeding behaviors (Kessler et al. 2016; Bello et al. 2014). Opioid signaling is closely related to the rewarding properties of food, regulating palatability (Esch and Stefano 2004). For example, a continuous access to a HFD induces a significant reduction in Oprμ gene expression in the VTA (Blendy et al. 2005; Vucetic et al. 2011). Moreover, some studies point that CB1 activation in the NAcc and VTA modulates both dopaminergic and opioidergic pathways (Mellis et al. 2007). CB1 receptor antagonists are capable of reducing binge eating and mediate the extracellular dopamine release produced by a HFD (Parylak et al. 2012; Mellis et al. 2007). All these results support the idea that intermittent fat ingestion can modify reward pathways through different systems such as the opioid and cannabinoid systems. While the endogenous opioid system is related to the hedonic properties of food and modulates the release of DA-anticipating food, the endocannabinoid system is related to the homeostatic control of intake and positive feedback on the specific intake of fatty food (Koch 2001). In contrast with the numerous changes in gene expression induced by ad libitum administration of HFD (Blanco-Gandía et al. 2017a), the intermittent and the limited access to HFD during the extinction process practically did not induce variations. HFD administration did not induce any changes in the Oprμ gene expression, with the exception of a higher expression in males with respect to females. Results from previous studies are still controversial and depend on many factors, as some studies have reported that intermittent access to HFD for several weeks decreases mRNA expression of the Oprμ receptor in the NAcc in male mice (Blanco-Gandía et al. 2017b), while others reported the same reduction after a continuous access to a HFD or cafeteria diet in male mice (Ong et al. 2013; Vucetic et al. 2011) with no changes in females (Ong et al. 2013). The present results do not confirm that HFD administration changes Oprm gene expression, since we only found differences between sexes but not due to HFD intake. A previous study by Kawahara et al. (2013) showed that consumption of

palatable food increased DA release in the NAcc via activation of the Oprm pathway in the VTA, and it is well known that this projection has an influence on learning or performance of behaviors based on drug reward (Koob and Le Moal 2005). Regarding CPP studies, the Oprm antagonist naloxone was effective in blocking the reacquisition of a cocaine-induced CPP (Sticht et al. 2010). Although there are very few studies that focus on this relationship, this could account for the difference between males and females, indicating that male mice are modifying their learning processes during extinctions more efficiently than females.

Although HFD did not induce any changes in CB1r gene expression in females, an increase was detected in the HFD-Ext male group. Some studies have reported that HFD upregulates endocannabinoid levels (Massa et al. 2010; Higuchi et al. 2012) and that CB1 antagonists reduce binge eating (Parylak et al. 2012). We have only observed this upregulation in the HFD-Ext group, which could be related to the time required to extinguish the preference, and thus, males in this group could have quickly learned to notice the absence of the conditioned drug in the CPP. This would support that CB1 receptors are modulating the extinction and reinstatement processes, as previous studies show that CB1 antagonists such as rimonabant or AM251 are able to block cocaine and morphine extinction and CPP reinstatement (Yu et al. 2011; Khaleghzadeh-Ahangar and Haghparast 2015). A limitation of the present work is in the analysis of the complete striatum without differentiating the ventral from the dorsal part, which could have offered more specific changes.

Conclusion

The results of the present work support our initial hypothesis, showing that palatable food could act as an alternative reward to cocaine by decreasing its conditioned rewarding memories and blocking cocaine priming-induced reinstatement. These effects are conditioned by sex, as females were less sensitive to the protective action of HFD. Taken together, the present results point out that a sporadic access to HFD stimulates the same pathways activated by drugs, as well as decreasing craving and drug-related memories.

Currently, no controlled studies in humans have been performed to test the role of palatable food in attenuating drug withdrawal. It is well known that palatable food is generally used as self-medication to escape from a negative mood state or stressful situations (Groesz et al. 2012; Ifland et al. 2009; Kim et al. 2013). In 1989, Hatcher reported that substances such as sugar, aspartame, chocolate, and nutritional supplements were compulsively used by patients in rehabilitation centers (Hatcher 1989). Several reports highlighted that during drug withdrawal and abstinence, overeating is a well-known problem in rehabilitation centers (Edge and Gold 2011). For example, Cowan and Devine (2008) found that patients at different stages of recovery from substance addictions experienced an increase in food intake, weight changes, and used food in recovery. Overeating and post-addiction obesity are so common that most abstinence-oriented drug treatment programs schedule diet counseling and mandatory exercise programs (Cowan and Devine 2008). Our results support these clinical reports and suggest that, acting as an alternative reward, palatable food may attenuate drug craving and the vulnerability to relapse. In particular, a more effective reduction in the extinction of drug-related memories occurs when a HFD is consumed temporally close to a drug-related cue exposure.

Contrary to an ad libitum access, our feeding conditions do not have significant consequences on bodyweight or hormonal levels, with minimal changes in opioid and cannabinoid receptors gene expression. These results indicate that it would be beneficial to introduce the intermittent use of palatable food as a "rehabilitation tool" in drug treatment programs, allowing patients to eat it when drug craving is happening. The translational value of this study relies on the lack of metabolic adverse effects of any of the three HFD administrations. However, and no less important, this treatment was significantly less effective in females, which indicates the necessity of conducting more studies in females, since we cannot assume that environmental or nutritional interventions are equally effective in both sexes.

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Declarations

Conflict of interest The authors declare no competing interests.

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ANEXO 3: Estudio 4.

Cognitive profile of male mice exposed to a Ketogenic Diet.

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Cognitive profile of male mice exposed to a Ketogenic Diet

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ABSTRACT

In recent years, nutritional interventions for different psychiatric diseases have gained increasing attention, such as the ketogenic diet (KD). This has led to positive effects in neurological disorders such as Parkinson's disease, addiction, autism or epilepsy. The neurobiological mechanisms through which these effects are induced and the effects in cognition still warrant investigation, and considering that other high-fat diets (HFD) can lead to cognitive disturbances that may affect the results achieved, the main aim of the present work was to evaluate the effects of a KD to determine whether it can induce such cognitive effects. A total of 30 OF1 male mice were employed to establish the behavioral profile of mice fed a KD by testing anxiety behavior (Elevated Plus Maze), locomotor activity (Open Field), learning (Hebb Williams Maze), and memory (Passive Avoidance Test). The results revealed that the KD did not affect locomotor activity, memory or hippocampal-dependent learning, as similar results were obtained with mice on a standard diet, albeit with increased anxiety behavior. We conclude that a KD is a promising nutritional approach to apply in research studies, given that it does not cause cognitive alterations.

1. Introduction

In recent years, it has been widely discussed whether dietary modifications may be an important factor in several diseases [1,2]. Many of these new interventions modify one or more macronutrients, such as high-fat diets, low-carbohydrate or low-sugar diets [3,4]. An example that has been recently explored as a therapeutic target is the ketogenic diet (KD), which leads to changes in the body's own metabolism [5,6]. The KD has traditionally been used in epilepsy [7,8], but lately, it has also been used as a nutritional intervention to investigate its effects in other neurological disorders, such as Alzheimer's disease [9], Parkinson's disease [10], autism [11] and, most recently, addiction [12,13]. However, more research with randomized control trials is required to provide conclusive results.

The KD is a diet high in fat, low in carbohydrates and moderated in proteins. One of the main features of the KD is the reduction in carbohydrate intake, which reduces the production of glucose and induces the body to use fat stores, breaking down fatty acids and creating ketone bodies in the liver, like β-hydroxybutyrate (βOHB) [7,14]. This metabolic process is known as ketosis, which can be achieved by strict adherence to a KD [15], or by prolonged fasting [16,17]. βOHB is a non-volatile and stable compound released into the bloodstream [18] and constitutes up to 70% of the ketone bodies synthesized in liver mitochondria [19], being the main indicator of ketosis both in humans [13] and in animal models such as rats [20] and mice [12,21].

To date, numerous studies with high-fat diets have reported negative effects on behavior, such as locomotor activity [22,23], or cognition, such as learning deficits [24–27], impaired hippocampus-dependent memory [23,25,28,29] and even anxiety-like behavior [30]. These effects are crucial in animal model research, as a subtle deficit in these capabilities could be interfering with the results obtained on many levels. Sometimes, certain behavioral procedures in preclinical research require the animal to learn a task or remember an object. If dietary treatments like HFDs affect behavior and cognition, we could be attributing benefits, harms, or no significant results to other causes and not to the diet itself, leading us to contaminated conclusions.

Therefore, as the KD is increasing its popularity in research in several neurological diseases, such as anxiety, depression, bipolar disorder or attention deficit hyperactivity disorder [31], it is still necessary to establish a baseline of the behavioral and cognitive profile of the chronic

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administration of this type of diet in order to confirm if it is safe or, on the contrary, if it has similar effects to those of traditional high-fat diets [21].

As mentioned above, the main difference between the KD and HFDs is that traditional high-fat diets not only contain an important percentage of carbohydrates, with a significant proportion of sugar, but also contain fats obtained from lard and soybean oil. In previous behavioral studies, it has been reported that some of the main factors contributing to cognitive alterations are the metabolic effects of HFD exposure, such as increased fat intake and body weight gain and the general metabolic dysfunction, with alterations in insulin, ghrelin and leptin levels [24, 32–34].

Therefore, the aim of the present work was to evaluate the cognitive and behavioral differences observed in young and cognitively healthy mice exposed to a KD to explore the effects of this diet in standard animals. For this purpose, we provided the KD to OF1 mice and evaluated the differences in their anxiety behavior with the Elevated Plus Maze, their spontaneous locomotor activity using the Open Field test, their memory with the Passive Avoidance test and their hippocampal learning with the Hebb-Williams maze.

2. Material and methods

2.1. Subjects

A total of 30 male mice of the OF1 outbred strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4 (cage size 28 × 28 × 1 4.5 cm) for three days prior to initiating the experimental feeding condition (PND 25), at a constant temperature (21±2 °C), lights on from 8:00 to 20:00, and food and water available ad libitum (except during the behavioral tests). All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The Committee for the Use and Care of Animals of the University of Valencia approved the study (2019/VSC/PEA/0065). The size of the sample was determined with the G*Power program [35], estimating the need to include 13 mice per experimental group. An expected effect size of $d = 1.5$ ($\alpha = 0.05$ and statistical power = 0.95) was taken, based on the results of the previous study of Blanco-Gandía et al. [24], which employed a similar experimental design, strain of mice, age of the animals and behavioral tests.

2.2. Apparatus and procedure

2.2.1. Feeding conditions and experimental design

Two different types of diet were used in this study: the standard diet (SD) (Teklad Global Diet 2014, 13 kcal% fat, 67 kcal% carbohydrates and 20% kcal protein; 2,9 kcal/g) and the ketogenic diet (KD) (TD.96355, 90.5% kcal from fat [vegetable shortening and corn oil], 0.3% kcal from carbohydrates and 9.1% kcal from protein; 6.7 kcal/g). The different diets were supplied by Envigo Teklad Diets (Barcelona, Spain). In this experiment (Fig. 1a), OF1 male mice ($n = 30$) arrived in the laboratory on PND 21 and were randomly divided into 2 groups ($n = 15$/condition) with similar average body weights (25–26 g) and assigned either SD or KD feeding conditions. Tests were performed one week after the diet had been initiated in order to evaluate if it had induced alterations in anxiety behavior (Elevated Plus Maze on PND32), motor activity (Open Field on PND 33), memory (Passive Avoidance Test on PND 34) or learning (Hebb Williams Maze on PND 36). All animals were under their specific feeding conditions from one week before the behavioral tests began (PND 25) until the end of the experiment (PND 44). On PND 44, the KD group was switched back to the SD until the end of the experiment to reevaluate anxiety behavior with the standard diet (PND 51). Body weight and Beta-hydroxybutyrate (βOHB) plasma levels were measured every week throughout the study.

2.2.2. Ketosis status: β-hydroxybutyrate plasma levels

Plasma β-hydroxybutyrate was measured weekly from the tail vein with a On Call GK Dual monitor and ketone test strips (ACON Laboratories, Inc., San Diego, CA).

2.2.3. Elevated plus maze

The EPM consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm). The junction of the four arms formed a central platform (5 × 5 cm). The floor of the maze was made of black Plexiglas, and the walls of the enclosed arms of clear Plexiglas. The open arms had a small edge (0.25 cm) to provide additional grip for the animals. The entire apparatus was elevated 45 cm above floor level. In order to facilitate adaptation, mice were transported to the dimly

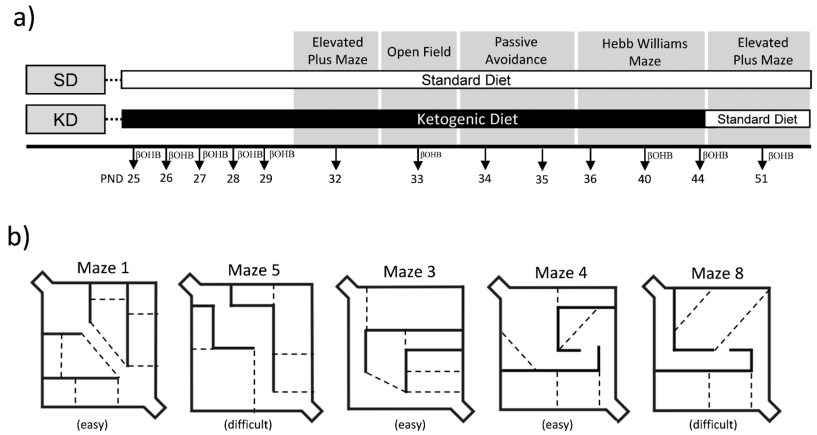

Fig. 1. (a) Experimental design. (b) Hebb-Williams Maze configuration and difficulty.

illuminated laboratory 1 h prior to testing. At the beginning of each trial, subjects were placed on the central platform facing an open arm, and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The behavior displayed by the mice was recorded automatically by an automated tracking control software (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The measurements recorded during the test period were frequency of entries, time and percentage of time spent in each section of the apparatus (open arms, closed arms, central platform). An arm was considered to have been visited when the animal placed all four paws on it. Number of open-arm entries, time spent in open arms and percentage of open-arm entries are generally used to characterize the anxiolytic effects of drugs [36,37].

2.2.4. Open field

The spontaneous locomotor behavior of the mice was quantified in an Open Field for a period of 1 hour. The Open Field test was performed in an opaque plastic box (30 × 30 × 15 cm) left open at the top. The animal was placed in the box and its activity was recorded automatically by tracking software (EthoVision 3.1; Noldus Information Technology, Leesburg, VA). The parameter studied was the total distance traveled (cm) and time near the wall and center (s).

2.2.5. Passive avoidance test

For the Passive Avoidance test, a step-through inhibitory avoidance apparatus for mice (Ugo Basile, Comerio-Varese, Italy) was employed. This cage is made of Perspex sheets and divided into two compartments (15 cm × 9.5 cm × 16.5 cm each one). The safe compartment is white and illuminated by a light fixture (10 W) fastened to the cage lid, whereas the "shock" compartment is dark and made of black Perspex panels. The two compartments are divided by an automatically operated sliding door at floor level. The floor is made of 48 stainless steel bars with a diameter of 0.7 mm and situated 8 mm apart.

Passive Avoidance tests were carried out following the procedure described in Aguilar et al. [38]. On the day of training, each mouse was placed in the illuminated compartment facing away from the dark compartment. After a 60 s period of habituation, the door leading to the dark compartment was opened. When the animal had placed all four paws in the dark compartment, a footshock (0.5 mA, 3 s) was delivered and the animal was immediately removed from the apparatus and returned to its home cage. The time taken to enter the dark compartment (step-through latency) was recorded. Retention was tested 24 h later, following the same procedure but without the shock. The maximum step-through latency was 300 s.

2.2.6. Hebb-Williams maze

The maze used in our experiment is made of black plastic and measures 60 cm wide x 60 cm long x 10 cm high. It contains a start box and a goal box (both 14 cm wide x 9 cm long), which are positioned at diagonally opposite corners. The maze contains cold water at a wading depth (15 °C, 3,5 cm high), while the goal box is stocked with fresh dry tissue. Several maze designs are produced by fixing different arrangements of barriers to a clear plastic ceiling. This apparatus allows the cognitive process of routed learning and the motivation of water escape to be measured.

The procedure followed was based on that employed by Galsworthy et al. [39], in which mice must navigate the maze and cross from the wet starting box to the dry goal box in order to escape the cold water. Animals underwent a 5-min habituation period (dry sand, no barriers) on day 1, and undertook problem A on day 2 and problem D on day 3 (4 trials/day) (practice mazes). Mice were subsequently placed in mazes 1, 5, 3, 4 and 8 on separate days (Fig. 1b), on which 8 trials took place (see Rabinovitch & Rosvold [40] for all maze designs). The time limit for reaching the goal box was 5 min, after which the mouse was guided to the box if necessary. The total latency score (sum of the latencies in all the problem trials in each maze) was registered.

2.3. Statistical analysis

Data relating to βOHB and body weight were analyzed by a mixed ANOVA with one between-subjects variable – "Diet", with 2 levels, (SD and KD) - and a within variable – "Days", with 9 (Baseline, Days 1–4, 7, 15, 19 and 25) or 4 levels (Baseline and weeks 1–3).

Data relating to the Elevated Plus Maze and Open Field test were analyzed by a one-way ANOVA with a between variable - "Diet", with 2 levels for Open Field (SD and KD) and three levels for the Elevated Plus Maze test (SD, KD and 7 days post-KD). The Passive Avoidance test was analyzed by a two-way ANOVA, with the same between variable and one within variable - "Days", with 2 levels (training day and test 24 h). The data of the Hebb-Williams maze were analyzed by a two-way ANOVA with one between subject variable – "Diet" - and one within subject variable – "Maze", with five levels. The Bonferroni adjustment was employed for post hoc comparisons. All results are expressed as mean ± S.E.M. Analyses were performed using SPSS v26.

3. Results

3.1. β-hydroxybutyrate (βOHB) and body weight

The ANOVA of the βOHB plasma levels (Fig. 2a) revealed a significant effect of the interaction "Days x Diet" [F(8224) = 25,594; $p < 0.001$], as the KD group showed increased levels of βOHB in comparison with the SD group at 24 h, 48 h, 72 h, 96 h, 7, 15 and 19 days ($p < 0.001$ in all cases), but showed no differences at 25 days ($p = 0.195$). There were also significant increases within the KD group on Days 1, 2, 3, 4, 7, 15 and 19 with respect to baseline ($p < 0.001$ in all cases), but no differences with respect to 25 days.

The ANOVA of body weight (Fig. 2b) revealed a significant effect of the interaction "Week x Diet" [F(3,84) = 14,364; $p < 0.001$] as mice on the KD displayed lower body weight at baseline and during week 1 compared to weeks 2 and 3 ($p < 0.001$, in all cases). Mice fed a SD showed higher body weight in Week 3 compared to Baseline, and Weeks 1 and 2 ($p < 0.001$, in all cases). In addition, the SD group exhibited higher body weight than the KD group at Baseline ($p < 0.05$) and in Week 1 ($p < 0.01$).

3.2. Elevated plus maze

The ANOVA (see Table 1) revealed an effect of the variable "Diet" for the time [F (2,42) =4.838; $p = 0.013$] and percentage of time [F (2,42) =4.944; $p = 0.012$] spent in the open arms of the maze. Animals belonging to the KD group spent less time and percentage of time in the open arms than the SD group ($p < 0.01$ in both cases).

There was also a significant effect of the variable "Diet" for the percentage of open entries [F (2,42) = 3.317 $p = 0.046$]. The KD group showed a lower percentage of open entries than the SD group ($p < 0.05$). There was no effect of the variable Diet on the total number of entries [F (2,42)= 0.428; $p = 0.655$]. There were no significant differences between the SD group and 7 days post-KD.

3.3. Open field

The ANOVA of the Open Field (Fig. 3 and Table 2) revealed no significant differences in the variable "Diet" for the distance traveled [F (1,18)= 0.175; $p = 0.681$], time near the wall [F(1,18)= 0.731; $p = 0.404$] and time in the center [F(1,18)= 0.038; $p = 0.848$].

3.4. Passive avoidance test

The results of the Passive Avoidance test are presented in Fig. 4. The ANOVA revealed an effect of the variable "Days" [F(1,28)=146.006; $p < 0.001$], with all the groups presenting longer step-through latencies in the 24 h test with respect to the training session ($p < 0.001$). The ANOVA

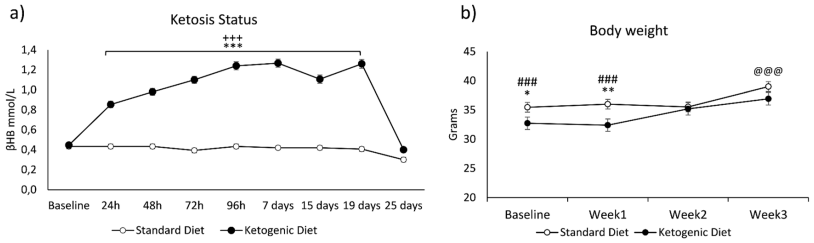

Fig. 2. β-hydroxybutyrate plasma levels and weekly body weight. (a) Ketosis status. Data are represented as the mean (±SEM) amount of βOHB. **(b) Weekly body weight**. Data are represented as the mean (±SEM) body weight measured weekly. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ significant difference with respect to the SD group. +++$p < 0.001$ significant difference with respect to baseline. ### $p < 0.001$ significant difference with respect to week 2 and 3 of KD. @@@$p < 0.001$ significant difference with respect to the rest of the weeks of SD.

Table 1
Effects of a KD on male mice in the Elevated Plus Maze ($n = 15$/group). Data are presented as mean values ± S.E.M. *$p < 0.05$; **$p < 0.01$ significant difference with respect to SD.

	Standard Diet	Ketogenic Diet	7 days post-Ketosis
Time OA	67 ± 15	20 ± 3 **	50 ± 11
% Time OA	33 ± 6	12 ± 2**	24 ± 5
% Open Entries	50 ± 4	31 ± 6*	43 ± 5
Total Entries	37 ± 4	37 ± 3	33 ± 4

did not show significant differences for the variable "Diet" [F(1,28)= 0.335; $p = 0.567$] or the interaction "Days x Diet" [F(1,28)=0.017; $p = 0.896$]. All animals remembered the footshock of the training session.

3.5. Hebb-Williams maze

The ANOVA for the total latency score (Fig. 5) revealed an effect of the variable "Maze" [F(4,96)=10.456 ; $p < 0.001$]. Maze 1 was significantly easier for all the groups than mazes 3, 4 and 5 ($p < 0.001$ in all cases) and 8 ($p < 0.01$), as the animals took less time to reach the goal. There were no significant differences in the variable "Diet" [F(1,24)= 0.040; $p = 0.843$] or the interaction "Maze x Diet" [F(4,96)=0.406; $p = 0.804$].

Table 2
Effects of a KD in the time (s) near the wall, center, rest of the field and total time in the Open Field by mice ($n = 10$/group). Data are presented as mean values ± S.E.M.

	Near the Wall	Center	Rest of the field	Total time
Standard Diet	1283 ± 42	519 ± 41	1798 ± 11	3600
Ketogenic Diet	1334 ± 47	510 ± 29	1756 ± 28	3600

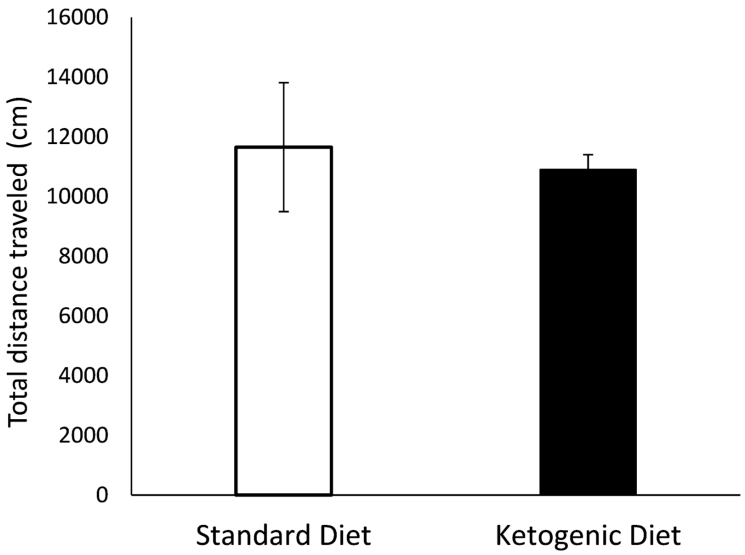

Fig. 3. Effects of a KD on the total distance covered in 1 h in the Open Field by mice ($n = 10$/group). Data are presented as mean values ± S.E.M.

Fig. 4. Effects of a KD on the time taken for male mice to enter the dark compartment in the Passive Avoidance test during training and 24 h after training ($n = 15$/group). Data are presented as mean values ± S.E.M. *** $p < 0.001$ significant differences with respect to training.

Fig. 5. Effects of a KD on the total latency score to reach the goal in the 8 trials of male mice in the Hebb-Williams maze. The mazes were classified as easy (1, 3 and 4) or difficult (5 and 8). ($n = 15$/group) ***$p < 0.001$ significant difference with respect to Maze 3, 4 and 5; ++ $p < 0.01$ significant difference with respect to Maze 8. Data are presented as mean values ± S.E.M.

4. Discussion

The ketogenic diet has been assessed to determine if it can be used as a nutritional treatment for numerous pathologies, such as epilepsy, Parkinson's disease, Alzheimer's disease, cancer, and more recently, drug addiction [7,9,13]. Although it has been observed that the KD could be an effective treatment option in certain pathologies, such as in children with drug-resistant epilepsy [41], further research is needed to achieve conclusive results in neurological disorders. Preclinical research in these fields studies the possible effects of nutritional interventions, and to draw conclusions, it is necessary to combine physiological results with behavioral outcomes. Experimental treatments, context or researchers can interfere with animal behavior, sometimes leading to inaccurate research conclusions.

As stated in the introduction, the main aim of the present work was to address whether the KD per se had any effects on anxiety, locomotor activity, memory, and learning. Our results confirmed that, even when the KD altered the animal's metabolism, with the increase in ketones,

this diet did not affect the cognitive profile of mice, as no significant changes were observed in locomotor activity, memory, or hippocampal-dependent learning with respect the group fed a standard diet. Interestingly, animals on a KD showed an increase in anxiety behavior 7 days after beginning the KD regimen.

Results related to metabolism confirmed that animals exposed to a KD rapidly displayed a ketotic state, as ketone body levels of βOHB significantly increased from the first 24 h onwards. When the individual is in a ketotic state, the reduction of carbohydrates in the diet leads to a drastic reduction in the levels of glucose, which ceases to be available as the main source of energy, inducing the body to generate ketone bodies in the liver [42,43]. This rise in blood ketone bodies induces a different metabolism [15] in which ketones, rather than sugar, become the main source of energy. In our study, these values remained stable while the animals were fed with the KD, but returned to normal 24 h after they were switched back to the SD. βOHB levels are widely used as a biomarker of ketosis in mice and humans [15], and our results are consistent with other studies that indicate that KD increases βOHB levels in both mice [21] and rats [20]. With respect to body weight and the KD, preclinical studies have revealed some disagreement, as different studies suggest that a KD can increase or decrease body weight in rats [12, 44–46]. This may be due to methodological issues, as it is necessary to match eucaloric diets. In our study, even when animals rapidly entered a ketotic state, their body weight did not differ from that of animals exposed to the SD, which also corresponds with previous results [21,47].

Our results showed that animals fed with the KD spent less time and percentage of time in the open arms of the EPM and made a lower percentage of open entries than those fed with the SD. This result is in contrast with the only other study that evaluated anxiety in animals fed on a KD, which reported no changes with respect to animals fed with a SD [21]. Another study reported that 8 weeks of ketone supplementation also did not induce any changes in anxiety [48]. A plausible explanation for the result obtained in our study is that, in every previous study, anxiety was evaluated 3 months after being on the KD and 8 weeks after supplementation, while we measured anxiety only 7 days after the beginning of KD administration. Adenosine receptors in GABAergic neurons play an essential role in anxiety regulation [49,50] and KD induces modifications in the adenosinergic systems [51], which could explain these initial alterations. The increase in anxiety observed in our study could be due to the short time of habituation to this type of diet. We confirmed that this increase in anxiety was due to the KD, as anxiety levels returned to normal when animals were switched back to the SD. To confirm the results obtained in the EPM, we assessed the time spent near the wall in the Open Field test and found no significant differences between both groups. This may be due to the fact that the EPM is much more sensitive [52], and that the EPM and the Open Field tests can measure different aspects of anxiety [53]. Indeed, several studies have reported symptoms of anxiety in mice using the EPM, but not with the Open field test [54]. Combining our results with those of previous studies, we could hypothesize that anxiety symptoms produced by the KD would be reduced over time. This result is an important issue to consider in future studies, as it indicates that it is prudent to lengthen the diet adaptation phase before the beginning of any behavioral test. For example, anxiety levels may be interfering if the animal is unable to complete the task of exiting a maze, and the researcher may misattribute this behavior to a lack of learning ability.

Focusing on the cognitive profile and the possible effects of a KD, no changes were observed in locomotion, learning or memory, with the exception of anxiety. In this line, the results of the present work showed that mice on the KD displayed similar locomotion abilities to animals fed the SD when evaluated in the Open Field test, which suggests that the KD does not alter locomotion behavior. The Open Field is a commonly used test, and several studies have confirmed that a KD does not affect general locomotor activity in rats [55,56] and mice [57]. Similar results have been reported by studies evaluating locomotor activity in mice receiving a ketone supplementation [48]. This result could be novel and promising, as studies employing common HFDs have reported alterations in locomotion, such as hyperlocomotion [24,58], or a decrease in activity [59,60]. This outcome would be crucial in studies on pathologies like epilepsy or Parkinson's disease, where a locomotor alteration may mask the real effects of nutritional interventions [10,61].

Regarding implicit memory, independently of the dietary treatment, all animals presented longer step-through latencies in the 24 h test with respect to the training session, confirming that they remembered the footshock received earlier. These results confirm those of a previous study in which a KD did not affect a contextual fear-conditioning task in rats [62].

In the same line are the results obtained in hippocampal-dependent learning, which showed that the KD group required the same time as the SD group to reach the goal in all the mazes, confirming that a KD does not alter acquisition of learning. Mice fed with the SD and the KD spent a similar amount of time in the easy or difficult (5) mazes. In general, difficult mazes can discriminate between groups when there is a cognitive deficit in any of them. In the present study, after 8 trials, regardless of diet, all mice always learned the task. A possible explanation for the reduction of time needed to complete Maze 8 despite being considered a difficult maze is the experience that had been previously acquired. The day before performing Maze 8, all mice completed Maze 4, which presents a comparable configuration (Fig 1b) and thus were familiarized with the spatial configuration and learned the task comparatively faster. The Hebb-Williams maze is a very sensitive test which is employed to detect spatial learning deficiencies, but there are no studies to date employing this maze to test KD effects. However, results from other studies have shown no deficits in the Y-maze or Water Maze in male mice kept on a KD for 3 months [21], or in the Novel Object Recognition test [63]. This result is in contrast with previous studies on common HFDs, where it has been shown that continuous exposure to a HFD induces marked memory and spatial learning deficits [24,29,64–67]. This affection might be triggered by leptin levels, which are significantly increased with HFDs [24,68]. It has been shown that when leptin levels increase, memory and learning can be affected [29, 69]. In fact, recent studies have suggested that not only does the KD not produce learning and memory impairments but it rescues hippocampal memory deficiencies in mice presenting impairments caused by age [70] or rats exposed to chronic stress environments [71]. Although the KD has different characteristics and metabolic properties from the common HFDs employed to date, it is still a high-fat diet. Thus, it is necessary to confirm the lack of detrimental effects induced by it. Conventional animal HFDs contain approximately 30–40% of carbohydrates, of which approximately 20% are sugars; while in the KD, the main component is fat (90%), followed by protein and less than 5% carbohydrates. These differences in sugar and carbohydrate composition are the main explanation for the physiological effects of KDs on several diseases. Although both diets are high in fat, the changes that they produce in metabolic status are significantly different.

In preclinical studies, anxiety and locomotor behavior are both commonly used as behavioral complements in several areas of knowledge. In addition, learning and memory are most employed in areas of neurodegenerative disorders and drug addiction, areas in which the KD is increasingly becoming a focus of interest [9,13]. A pharmacological or nutritional treatment can have a significant physiological effect (cells, metabolism), but this effect might not be reflected in a behavioral improvement. For example, a specific treatment could clear amyloid beta protein without an improvement in the learning or memory performance of the animals. Another example could be found in models of addiction, where animals have to learn an operant task to obtain the drug (models of self-administration) [72] or make a contextual association with the drug (models of conditioned place preference), which requires memory and learning abilities for the acquisition process [73]. If the diet per se is affecting learning and memory, the researcher might draw wrong conclusions about the drug, as food, rather than the drug itself, could be influencing the animals' cognitive ability. Therefore,

preclinical models of behavior help us to confirm whether the different pharmacological or nutritional treatments are effective. To achieve this end, we must ensure that the treatment per se does not affect behavior or cognition.

5. Conclusion

The present study shows that the KD is a nutritional intervention that does not affect behavior or cognitive performance in male mice. However, one important limitation of this study has been not exploring the effects of this diet in female mice. It should be noted that understanding sex differences in the clinical application of a KD is a very relevant factor to take into account, especially given that the cognitive and behavioral consequences of some psychiatric disorders differ between males and females, as in anxiety and depressive disorders [74]. Therefore, further research is needed to know the effects of a KD in females. In addition, in this study we have evaluated the short-term cognitive effects of a KD, but a longer exposure to this diet before the behavioral testing would verify the lack of cognitive alterations.

Future studies in the addiction and neurodegenerative disease fields that are exploring nutritional interventions with the KD can safely employ different tests of memory, learning and locomotor activity, as well as anxiety, always providing a prudent habituation time to the diet, as anxiety could otherwise alter the results of other tests.

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Author contributions

Francisco Ródenas-González: Methodology, Software, Validation, Formal Analysis, Investigation, Data Curation, Writing-Review and Editing, Visualization. **M. Carmen Blanco-Gandía**: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Data Curation, Writing-Review and Editing, Visualization. **José Miñarro:** Conceptualization, Resources, Supervision, Project Administration, Funding Acquisition. **Marta Rodríguez-Arias:** Conceptualization, Resources, Data Curation, Writing-Review and Editing, Visualization, Supervision, Project Administration, Funding Acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data Availability

Data will be made available on request.

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ANEXO 4: Estudio 5.

Effects of ketosis on cocaine-induced reinstatement in male mice.

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Research article

Effects of ketosis on cocaine-induced reinstatement in male mice

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ABSTRACT

In recent years, the benefits of the ketogenic diet (KD) on different psychiatric disorders have been gaining attention, but the substance abuse field is still unexplored. Some studies have reported that palatable food can modulate the rewarding effects of cocaine, but the negative metabolic consequences rule out the recommendation of using it as a complementary treatment. Thus, the main aim of this study was to evaluate the effects of the KD on cocaine conditioned place preference (CPP) during acquisition, extinction, and reinstatement. 41 OF1 male mice were employed to assess the effects of the KD on a 10 mg/kg cocaine-induced CPP. Animals were divided into three groups: SD, KD, and KD after the Post-Conditioning test. The results revealed that, while access to the KD did not block CPP acquisition, it did significantly reduce the number of sessions required to extinguish the drug-associated memories and it blocked the priming-induced reinstatement.

1. Introduction

The ketogenic diet (KD) is a high-fat, low-carbohydrate, and protein-balanced diet [1] that induces a specific metabolic status named ketosis. A ketosis status involves a significant change in the main source of energy used by the body and the brain, in which the reduction in carbohydrate intake reduces glucose production, leading the body to use up fat stores [2]. When carbohydrates are reduced to less than 5–10%, fatty acids break down and create ketone bodies in the liver, such as β-hydroxybutyrate (βOHB), which are indicators of nutritional ketosis [3]. Due to this special metabolic status that KD induces, in the last years this diet has been employed as complementary treatment in several neurological disorders, such as epilepsy or neurodegenerative diseases [4–6]. However, there are other diseases like drug addiction, in which the role of diet is just beginning to be studied, but the role of a KD is hardly explored.

Drugs of abuse and palatable diets affect common brain mechanisms, namely the reward system [7]. Both stimulate common brain regions like the lateral hypothalamus, ventral tegmental area, prefrontal cortex or amygdala [8], reduce dopamine active transporter density [9] and activate dopaminergic neurons of the nucleus accumbens [10,11]. This dopaminergic activation caused by palatable food affects neural pathways involved in motivation and reward, such as drugs of abuse [12–14]. For example, the downregulation of dopaminergic receptors in the nucleus accumbens, which is characteristic of the addictive process, is also found in obesity [7].

In recent years, some nutritional interventions have proved to be a modulating factor in the addiction process. For example, in a series of studies, it was observed that a high-fat diet (HFD) can be an important modulating factor of the rewarding properties of cocaine. This effect seems to be dependent on the access pattern of palatable diets, such as intermittently or continuously. While intermittent access in a vulnerable period, such as adolescence, increases sensitivity to cocaine in the conditioned place preference paradigm (CPP) [15,16], continuous HFD access seems to reduce it [17]. Likewise, HFD administration after acquisition of CPP reduces the number of sessions needed to achieve extinction, suggesting that the diet had a role as an alternative reinforcer and diminished the drug-related memories [17]. Recently, it was demonstrated that a HFD administered in an intermittent schedule, which does not affect metabolic indicators like ghrelin, leptin or body-weight, also reduced the time required to achieve extinction and blocked reinstatement of cocaine preference in adult male and female mice [18]. To date, studies regarding a possible beneficial interaction of the KD with substance use disorders are scarce. Thus, with all these results regarding HFDs, in the present study we asked ourselves whether other types of diet, such as the KD, could exert a modulation on the

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conditioned rewarding effects of cocaine.

For example, regarding alcohol, a recently published preclinical - clinical study [19] confirmed that people with an alcohol use disorder maintained on a KD manifested fewer withdrawal symptoms than those on a standard (American) diet. The preclinical data showed that access to a KD reduced ethanol consumption in rats [19] and, more recently, it has also been demonstrated in mice [20]. It seems that the KD could also be advantageous in decreasing ethanol withdrawal symptoms in rats and mice [20,21]. Regarding cocaine, to date only one study has reported decreased cocaine-induced stereotypies and sensitization in male and female rats maintained on a KD, suggesting that this nutritional intervention may act on the dopaminergic system [22].

The present work employed the CPP procedure, which evaluates the contextual cues related to the rewarding effects of a drug. Considering the previous results obtained with a HFD, we hypothesized that a KD, which changes the metabolic status in the individual, would block the cocaine-induced CPP acquisition and accelerate the extinction of cocaine-related memories in the mice that acquired CPP. Finally, KD may be able to block reinstatement of cocaine-seeking behaviour.

2. Material and methods

2.1. Subjects

A total of 45 male mice of the OF1 strain were acquired commercially from Charles River (France). Animals were 21 days old on arrival at the laboratory and were all housed under standard conditions in groups of 4–5 (cage size 28 × 28 x1 4.5 cm) at a constant temperature (21 ± 2 °C), lights on from 8:00 to 20:00, and food and water available *ad libitum*. All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of September 22, 2010 on the protection of animals used for scientific purposes. The Committee for the Use and Care of Animals of the University of Valencia approved the study (2019/VSC/PEA/0065).

2.2. Apparatus and procedure:

2.2.1. Experimental design

To avoid stressful social conditions in their home cages, animals arrived on PND 21 at the laboratory, but the experiment began during their young adulthood, on PND 42. Animals were randomly divided into 3 groups (Fig. 1) with similar average body weights (37–40 g): mice fed the standard diet throughout the whole procedure (SD, n = 12), mice fed the ketogenic diet throughout the procedure, from PND 42 (KD, n = 14), and mice fed the SD until the end of the CPP procedure and a KD after the Post-C test and until the end of the extinction sessions (PostCPP-KD, n = 15). Animals underwent a 10 mg/kg cocaine induced CPP procedure on PND 52, and then underwent an extinction session once a week in order to evaluate the effects of the KD on the extinction of the preference. Body weight and Beta-hydroxybutyrate (βOHB) plasma levels were measured before the Pre-C test, after the Post-C test, and 7 days after the Post-C.

2.2.2. Feeding conditions and ketosis

Two types of diet were administered in this study: the standard diet (SD) (Teklad Global Diet 2014, 13 kcal % fat, 67 kcal % carbohydrates and 20% kcal protein; 2,9kcal/g) and the ketogenic diet (KD) (TD.96355, 90.5 % kcal from fat, 0.3% kcal from carbohydrates and 9.1% kcal from protein; 6.7 kcal/g). Both diets were supplied by Envigo Teklad Diets (Barcelona, Spain).

To evaluate if animals were on a ketosis status, plasma β-hydroxybutyrate from the tail vein was measured weekly with an On Call GK Dual monitor and ketone test strips (ACON Laboratories, Inc., San Diego, CA).

2.2.3. Drug treatment

For CPP, animals were injected intraperitoneally (IP) with 10 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S.A., Madrid, Spain) diluted in 0.9% NaCl (saline) in a volume of 0.001 mL/kg body weight. The dose of 10 mg/kg cocaine has been demonstrated to be an effective dose that induces reinstatement with half the previous received dose in standard mice [18,23].

2.2.4. Conditioned place preference

For Place Conditioning, we employed sixteen identical Plexiglas boxes with two equally sized compartments (30.7 cm length × 31.5 cm width × 34.5 cm height) separated by a grey central area (13.8 cm length × 31.5 cm width × 34.5 cm height). The compartments have different coloured walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

2.2.5. Acquisition of CPP

The procedure of Place Conditioning, unbiased in terms of initial spontaneous preference, was performed as described previously [24] and consisted of three phases. To summarize the main aspects, in the first phase, known as Pre-C, mice were allowed access to both compartments of the apparatus for 15 min (900 s) per day for 3 days. On day 3, the time spent in each compartment over a 900-s period was recorded, and animals showing a strong unconditioned aversion (<33% of the session time) or preference (more than 67%) for any compartment were excluded from the rest of the experiment (number of mice excluded: 4). The procedure of assignment is unbiased, assigning half of the animals in each group to the drug or vehicle in one compartment (e.g. white), and the other half in the other compartment (e.g. black). Additionally, half of the animals are assigned to the initially preferred compartment and the other half to their non-preferred compartment.

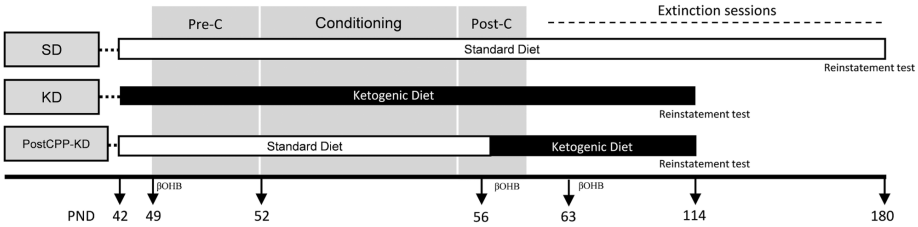


Fig. 1. Experimental design.

After assigning the compartments, no significant differences were detected between the time spent in the drug-paired and vehicle-paired compartments during the pre-conditioning phase. In the second phase (conditioning), which lasted 4 days, animals received an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30 min. After an interval of 4 h, they received an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. Confinement was made possible in both cases by closing the guillotine door that separated the two compartments, rendering the central area inaccessible. During the third phase, known as Post-C, the guillotine door separating the two compartments was removed (day 8) and the time spent by the untreated mice in each compartment during a 900-s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates that an aversion has developed.

2.2.6. Extinction of CPP

When preference for the drug-paired compartment had been established, all groups underwent a weekly extinction session in which they were placed in the apparatus (without the guillotine doors separating the compartments) for 15 min. Results were checked every week for each group to confirm if criteria had been satisfied. The extinction condition was fulfilled when there was a lack of a significant difference between CPP scores and Pre-C test values in two consecutive sessions.

2.2.7. Reinstatement of CPP

Twenty-four hours after extinction had been confirmed, the effects of a priming dose of cocaine were evaluated. The reinstatement test was the same as those carried out in Post-C (free ambulation for 15 min), except that animals were tested 15 min after administration of the respective dose of cocaine (5 mg/kg). Priming injections were administered in the vivarium, which constituted a non-contingent place to that of the previous conditioning procedure. If animals reinstated the preference, the extinction sessions continued in time and when the criteria were met again, the next half-dose (2.5 mg/kg) was administered. If they did not reinstate the preference, then the experiment finished. Therefore, each group can finish the procedure at different times.

2.3. Statistical analysis

Data relating to βOHB were analysed by a mixed ANOVA with one between-subjects variable – "Diet", with 3 levels (SD, KD and PostCPP-KD) - and a within variable – "Days", with 3 levels (Pre-C, Post-C and DAY7Post-C). Data relating to bodyweight were analysed by a mixed ANOVA with one between-subjects variable – "Diet", with 3 levels (SD, KD and PostCPP-KD) - and a within variable – "Weeks", with 8 levels (Baseline and Weeks 1–7). Body weight was compared until week 7 due to different extinction-reinstatement timings.

For the CPP procedure, the time spent in the drug-paired compartment was analysed by a repeated measures ANOVA, with the between-subjects variable - "Diet", with 3 levels (SD, KD and PostCPP-KD) - and a within variable – "Days", with two levels (Pre-C and Post-C). To compare whether extinction/reinstatement had been achieved within the same group, data relating to extinction and 5 mg/kg reinstatement were analysed by means of Student's *t*-test. The time required for the preference to be extinguished was analysed by means of the Kaplan-Meier test, with Breslow (generalized Wilcoxon) comparisons when appropriate. All results are expressed as mean ± S.E.M. Analyses were performed using SPSS v26.

3. Results

3.1. Increased β-hydroxybutyrate (βOHB) and body weight.

With respect to βOHB plasma levels (Fig. 2a), the ANOVA revealed a significant effect of the interaction "Days × Diet" [$F(4,76) = 34,714$; $p < 0.001$], as the KD group showed increased levels of βOHB with respect to SD and PostCPP-KD when measurements were taken in Pre-C ($p < 0.001$) and Post-C ($p < 0.001$). The KD and PostCPP-KD groups exhibited higher levels than the SD group 7 days after Post-C, ($p < 0,001$ in both cases). Moreover, the PostCPP-KD group's levels were higher 7 days after Post-C when compared to pre-C and post-C measures ($p < 0.001$ in both cases).

Regarding changes in body weight (Fig. 2b), the ANOVA revealed no significant differences in the variable "Diet" [$F(2,38) = 0.019$; $p = 0.981$], as all groups presented similar weight throughout the procedure. There was a significant effect of the variable "Week" [$F(7,266) = 254,571$; $p < 0.001$], since mice showed higher body weight in weeks 1 to 7 than at baseline ($p < 0.001$, in all cases).

3.2. Conditioned place preference

The ANOVA for the time spent in the drug-paired compartment (Fig. 3) revealed an effect of the variable "Days" [$F(1,38) = 111,919$; $p < 0.001$]. Bonferroni's post-hoc comparisons showed that the mice spent significantly more time in the drug-paired compartment in Post-C than in Pre-C ($p < 0.001$ in all cases). These results indicate that the three groups developed CPP.

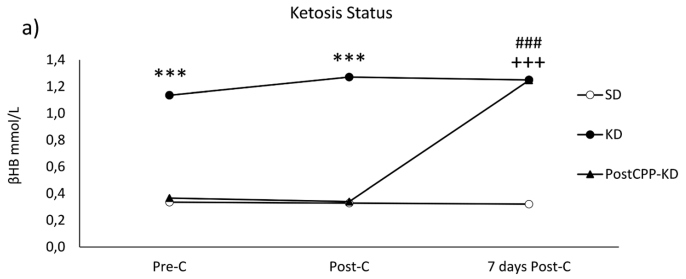
With regards to the time required to extinguish the preference (Fig. 4), the SD group required a total of 19 sessions, while the KD and the PostCPP-KD groups required only 8 sessions. The Kaplan-Meier analysis revealed that the SD group required significantly more sessions than the other two groups to extinguish the preference ($p < 0,05$ in both cases).

Reinstatement of drug-seeking after achievement of extinction was evaluated with Student's t-tests, which showed that reinstatement with a priming dose of 5 mg/kg cocaine was achieved only in the SD group ($t = -2.943$; d.f. 9; $p = 0.016$).

4. Discussion.

The aim of the present study was to evaluate whether a KD can modulate the conditioned rewarding effects of cocaine in two critical moments: during acquisition and/or during extinction/reinstatement of the preference. The results showed that all animals, regardless of being fed a KD or SD, developed a place preference for the cocaine-paired compartment after administration of 10 mg/kg of cocaine. However, during the extinction-reinstatement process both groups fed with the KD needed fewer sessions for the preference to be extinguished than the SD group. In the reinstatement test, induced by a priming dose of 5 mg/kg of cocaine (half the previously received dose), only the SD group exhibited preference for the drug-paired compartment, confirming that being on a KD blocked reinstatement with 5 mg/kg cocaine. To date, only one study has evaluated how access to a KD mediates the effects of cocaine. Martinez et al., [22] reported that access to a KD over three weeks reduced cocaine withdrawal symptoms in rats. In that study, rats received daily cocaine injections and after cessation, withdrawal symptoms such as stereotyped locomotor responses appeared. Their results showed that the animals fed a KD showed weaker cocaine-induced stereotyped response than those fed a SD.

Studies with other drugs of abuse, such as ethanol, have reported similar results, with rats or mice on KD displaying milder ethanol withdrawal symptoms [20,21]. As mentioned in the introduction, the KD reduces ethanol self-administration during acute withdrawal in rats and withdrawal symptoms during ethanol detoxification in humans [19]. In addition, previous studies by our research group have shown



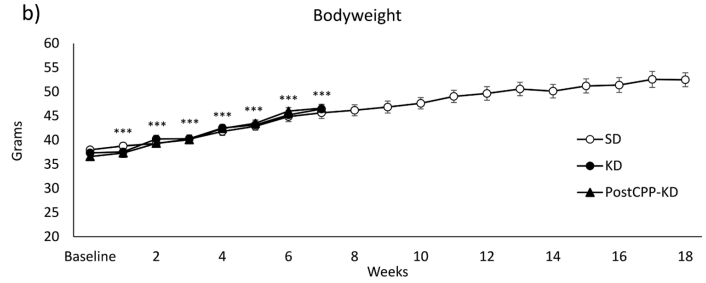


Fig. 2. β-hydroxybutyrate plasma levels and weekly body weight. (a) Ketosis status. Data are represented as the mean (±SEM) amount of βOHB. ***p < 0.001 significant difference with respect to the rest of the groups. +++p < 0.001 significant difference with respect to SD. ###p < 0.001 significant difference with respect to Pre-C and Post-C. **(b) Weekly body weight**. Data are represented as the mean (±SEM) body weight measured weekly. ***p < 0.001 significant difference with respect to Baseline.

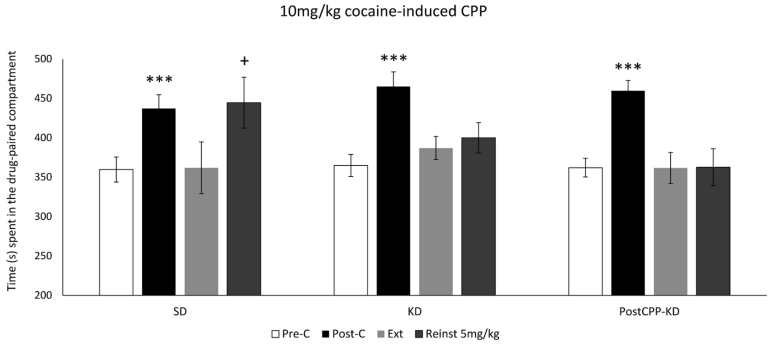


Fig. 3. Effects of KD during the extinction-reinstatement process in the Conditioned Place Preference (CPP) paradigm. Bars represent the time (±SEM) in seconds spent in the drug-paired compartment in the Pre-Conditioning test (white bars), the Post-conditioning test (black bars), the last extinction session (light gray bars) and the reinstatement test (dark gray bars). The reinstatement test was evaluated 15 min after a priming dose of 5 mg/kg of cocaine. ***p < 0.001 significant difference with respect to the Pre-C. + p < 0.05 significant difference with respect to Ext.

that access to a KD for 7 days prior to an ethanol self-administration test, and maintaining it for 4 weeks, reduces ethanol consumption compared to animals on a SD [25].

One of the main therapeutic effects of the KD is the increase that it produces in adenosine transmission, and one of the possible explanations for the effects of a KD on drug addiction is the relationship between adenosine and dopamine [22]. Several studies have demonstrated that there is an antagonistic interaction between the adenosine A1 - Dopamine D1 and adenosine A2A - dopamine D2 receptors [26], especially in GABAergic neurons. For example, D1 binding affinity is decreased by A1 agonists, suggesting that the A1 receptor modulates dopaminergic transmission [27]. It has been proposed that the response to drugs, such as psychostimulants, is also mediated by adenosine [28–30]. On the other hand, there are preclinical studies that have demonstrated that A2 agonists reduce cocaine and morphine locomotor sensitization [30,31] and decrease cocaine self-administration [32]. However, antagonist administration causes comparable effects to psychostimulants and enhances relapse into cocaine self-administration [33]. Thus, the main

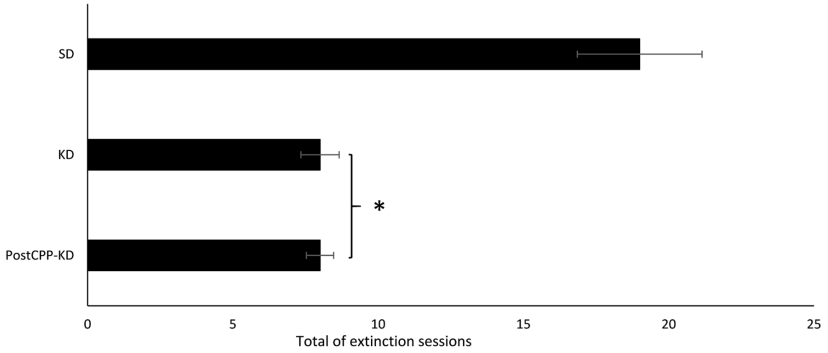


Fig. 4. Extinction. The bars represent the total value (±SEM) of the number of sessions required for the preference to be extinguished after the Post-C test. The Kaplan-Meyer analyses showed * $p < 0.05$ significant difference with respect to SD.

hypothesis of this work is that KD could attenuate dopaminergic transmission through activation of adenosine receptors [31,34]. In fact, in a previous study, we observed that a 4–5-week KD access led to alterations in the adenosine, dopamine and cannabinoid gene expression of mice [25]. Although the adenosine-dopamine modulation would not be strong enough to block the acquisition of cocaine-induced CPP, it could diminish the strength of the conditioning and therefore reduce the number of sessions needed to extinguish the preference, as well as the power of a cocaine-priming dose to reinstate drug-seeking behaviour. However, more extensive studies are needed to confirm the neurobiological mechanisms underlying these effects, especially ones considering female mice in them.

Our results, in line with those of Martínez et al., [22], also suggest another possible explanation for the KD modulation of addiction, which could be the role of differences in βOHB blood levels. Even when the KD contains more than double the calories as the SD, animals in the KD group did not gain more body weight than mice in the SD group. Previous results suggest differences in energy expenditure or lower food intake in KD-fed animals, with studies showing increases or decreases in body weight with respect to the control groups [21,25,35]. Nevertheless, some studies have reported that butyrate, which is a histone deacetylase inhibitor, keeps mice metabolically normal when maintained on a high-fat diet, with low glucose and insulin levels and normal body weight [36]. Butyrate is a product of bacterial anaerobic fermentation [37] and closely related to βOHB, the main source of energy for mammals during ketosis [38]. There are some studies that have reported that the overexpression of HDAC increases effects caused by cocaine [39]. Therefore, if βOHB could be acting as an endogenous HDAC inhibitor [40,41], it would contribute to the final effects of ketosis on cocaine extinction.

The KD may be considered as a promising nutritional approach in the treatment of cocaine addiction. Although the diet cannot be an exclusive treatment, it can contribute to the attenuation of the memories related to cocaine consumption, as well as the risk of relapse. This study supports what previous studies with other types of high-fat diets have suggested, and it is that nutritional interventions can modulate the conditioned effects of drugs like cocaine, which today does not yet have a definitive treatment.

CRediT authorship contribution statement

Francisco Ródenas-González: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **M. Carmen Blanco-Gandía:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **José Miñarro:** Funding acquisition, Investigation, Project administration, Resources, Writing – review & editing. **Marta Rodríguez-Arias:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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ANEXO 5: Estudio 6.

Ketogenic Diet Decreases Alcohol Intake in Adult Male Mice.

nutrients

MDPI

Article

Ketogenic Diet Decreases Alcohol Intake in Adult Male Mice

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Abstract: The classic ketogenic diet is a diet high in fat, low in carbohydrates, and well-adjusted proteins. The reduction in glucose levels induces changes in the body's metabolism, since the main energy source happens to be ketone bodies. Recent studies have suggested that nutritional interventions may modulate drug addiction. The present work aimed to study the potential effects of a classic ketogenic diet in modulating alcohol consumption and its rewarding effects. Two groups of adult male mice were employed in this study, one exposed to a standard diet (SD, $n = 15$) and the other to a ketogenic diet (KD, $n = 16$). When a ketotic state was stable for 7 days, animals were exposed to the oral self-administration paradigm to evaluate the reinforcing and motivating effects of ethanol. Rt-PCR analyses were performed evaluating dopamine, adenosine, CB1, and Oprm gene expression. Our results showed that animals in a ketotic state displayed an overall decrease in ethanol consumption without changes in their motivation to drink. Gene expression analyses point to several alterations in the dopamine, adenosine, and cannabinoid systems. Our results suggest that nutritional interventions may be a useful complementary tool in treating alcohol-use disorders.

Keywords: ketosis; alcohol; ketogenic; ketone; adenosine; dopamine

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1. Introduction

Motivation to seek drugs of abuse and highly palatable foods is regulated by the reward system [1]. Previous studies have shown psychological and biological commonalities between palatable food intake and drug addiction [2,3] and recent studies have indicated that nutritional habits are an important modulating factor in the development of cocaine [4,5] and alcohol addiction [6,7]. Palatable diets change metabolism and the reward system by increasing vulnerability to the rewarding effects of psychostimulants and depressants, such as cocaine and alcohol [4,6,8], but little is known about the protective effects that nutrition could have in the development of drug addiction. In a recent study, a high-fat diet was shown to reduce relapse into cocaine seeking in adult male mice [5]. However, the diet employed in those studies was rich in saturated fats and sugar, producing metabolic syndrome and obesity when the HFD administration was continuous. and therefore, it cannot be recommended as a drug-addiction combined therapeutic. This metabolic syndrome is avoided when the administration of HFD is intermittent, although the accelerated extinction and the blockade of reinstatement is maintained [9]. Nevertheless, this contribution opened a new gateway to focus on nutrition as a possible complementary treatment in the

field of drug addiction. For this reason, we considered the classic ketogenic diet (KD), a diet still high in fats, to prevent the development of escalation in alcohol consumption.

Today we can find several eucaloric protocols like the medium-chain triglyceride diet (MCT), the modified Atkins diet (MAD), the low glycemic index treatment (LGIT) or the classic ketogenic diet (KD). The KD is a high-fat, low-carbohydrate, and protein-balanced diet that induces a different metabolic state, in which ketone bodies are used as the main energy source [10,11]. The decrease in carbohydrates reduces the amount of glucose, which ceases to be available as the main source of energy [11]. This is where the body begins to use fat storages, breaking down fatty acids and creating ketone bodies in the liver (e.g., acetoacetate, β-hydroxybutyrate or βOHB to produce ATP, adenosine triphosphate) [11,12]. The rise in blood ketone bodies appears as a response to low glucose levels, known as metabolic state ketosis. Ketosis can be achieved in two ways: through diet (nutritional ketosis) or through fasting. In the present work we will focus on nutritional ketosis.

Ketosis is not new. Evolutionarily, humans have spent a good part of their existence in ketotic state (especially in winter, where carbohydrates such as fruits and vegetables were limited). Moreover, ketone bodies play a very important role in the normal fetal brain development [13,14], as breast milk is high in fat and medium-chain fatty acids, inducing a ketotic state in the new-born [15,16]. The KD is neuroprotective [17], since ketone bodies cross the blood-brain barrier without difficulty and increase metabolic efficiency by improving mitochondrial function [18,19]. It reduces oxidative stress, with antioxidant and anti-inflammatory effects, inhibiting inflammatory markers such as interleukins and tumour necrosis factor alpha [20]. However, the mechanism through which a KD is beneficial is still under study. The KD has been used successfully for different disorders such as epilepsy [21,22], Alzheimer's and Parkinson disease [23–27], brain cancer [28,29], autism [30,31], and amyotrophic lateral sclerosis [32]. Based on all of these studies, we can hypothesize that ketones induce a normalization process when metabolism functioning is dysregulated, which can account for its beneficial effects on all of these neurological disorders.

Diet and nutrients can exert great changes in neural plasticity, modifying circuits and normalizing their function [33–35]. One of the most important KD neural mechanisms is its modulation of ATP-sensitive potassium channels and increase of GABAergic and purine neurotransmission such as adenosine [36,37]. The KD activates adenosine receptors, which are the basis of its therapeutic effects on diseases such as epilepsy, as it inhibits the excitability of neurons [38–40]. Adenosine regulation is closely linked to the dopaminergic action, as its receptors are colocalized on GABAergic neurons together with dopamine D2 receptors, suggesting that modifying one may lead to the regulation of the other [41]. In fact, there is an antagonistic interaction between the heterodimers of the adenosine A1—Dopamine D1 vs. adenosine A2A—dopamine D2 receptors [42]. Although some of the results are controversial, there is evidence indicating that adenosine mediates the response of drugs such as opiates, cannabinoids, and psychostimulants [43–46]. For example, cocaine self-administration (SA) induces an upregulation of A2 receptors and a downregulation in D2 receptors as a compensation, a situation that is reversed with abstinence [47].

To date, only a few studies have shown the protective effect that a KD can have on drug addiction. Martínez and co-workers [48] showed that animals on a KD showed decreased cocaine-induced stereotypies and sensitization in male and female rats, suggesting that the KD modulates the dopaminergic system. Regarding alcohol, KD has been reported to decrease alcohol withdrawal symptoms in rats [49] and mice [50], as well as reduce alcohol consumption in rats [51]. The recently published work by Wiers and co-workers [51] combines a preclinical and a clinical study. In relation to the clinical study, Wiers and co-workers [51] also found that people with alcohol-use disorder who were on a KD had fewer withdrawal symptoms and required fewer medication the first week of detoxification than those following a standard American diet [51].

Therefore, the main aim of the present study is to evaluate whether a KD could modulate the rewarding effects of alcohol by acting through the adenosine-dopamine binomial. To achieve this objective, the behavioral effect of a KD on the oral ethanol SA was studied. Additionally, we evaluated the main changes in dopamine and adenosine receptor gene expression in the striatum, taking into account that diets and nutrition can disrupt the normal plasticity in the dorsal striatum [52]. Moreover, given the implication of the cannabinoid and opioid systems in the rewarding effects of not only alcohol but also high-fat foods [53,54], we included the analysis of the CB1r and Oprm expression.

2. Materials and Methods

2.1. Subjects

This study was performed with 47 OF1 male mice (Charles River, Écully France), which were housed under standard conditions (21 ± 2 °C) in groups of 4 (cage size 28 × 28 × 14.5 cm). Animals were 21-days old on arrival at the laboratory but initiated the experimental feeding condition on PND 42. Lights were turned on from 8:00–20:00, and food and water were available ad libitum. All procedures involving mice and their care complied with the national, regional, and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes. The Animal Use and Care Committee of the University of Valencia approved the study with the code 2019/VSC/PEA/0065 on 23 March 2019.

2.2. Drugs Treatment

Absolute ethanol (EtOH) (Merck, Madrid, Spain) was dissolved in water using a w/v percentage, i.e., a 6% (w/v) EtOH solution equivalent to a 7.6% (v/v) EtOH solution. Saccharin sodium salt (Sigma, Madrid, Spain) was diluted in water.

2.3. Apparatus and Procedure

2.3.1. Experimental Design

In the present study we employed two animal sets. In the first set, animals in the standard diet (SD, n = 15) group received the standard diet throughout the procedure, while animals in the ketogenic diet (KD, n = 16) group received the ketogenic diet from young adulthood, PND 42, until the end of the experiment. All the animals began the training phase on PND 49 and the 6% EtOH consumption phase on PND 66, when mice are already considered adults. Bodyweight and beta-hydroxybutyrate (βOHB) blood levels were measured every week throughout the study. During the SA procedure, animals had 1 h access to food per day. About 15% weight loss was produced by the food restriction schedule [55]. Brains were collected at the end of the SA for gene expression analysis.

In the second set, a total of 16 animals were used (n = 8 per diet). They had the same feeding conditions and initiated the diet conditions on the same PND as the first set, but without any behavioral manipulations. In order to test only the effects of KD, animals in this second set were not exposed to alcohol. They were euthanized for brain gene expression analysis on PND 77, as with the first set. A detailed description of the experimental procedure is shown in Figure 1.

2.3.2. Feeding Conditions

Two different types of diets were used in this study. The SD group was fed with the standard diet (Teklad Global Diet 2014, 13 kcal % fat, 67 kcal % carbohydrates and 20% kcal protein; 2,9 kcal/g) and the KD group with a ketogenic diet (TD.96355, 90.5% kcal from fat, 0.3% kcal from carbohydrates and 9.1% kcal from protein; 6.7 kcal/g). The different diets were supplied by Envoi Teklad Diets (Barcelona, Spain).

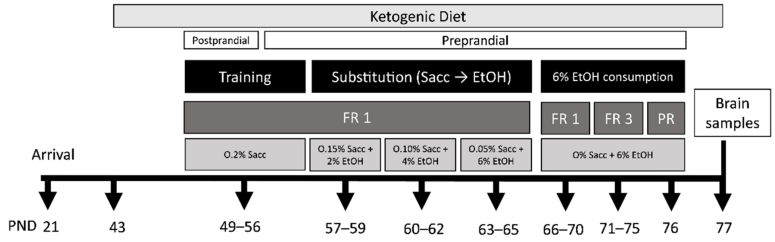

Figure 1. Experimental design for the 1st set of mice. FR, fixed ratio; PR, progressive ratio; PND, postnatal day; Sacc, saccharin; EtHO, ethanol.

2.3.3. Ketosis Status: β-Hydroxybutyrate Blood Levels

Blood β-hydroxybutyrate was measured weekly from the tail vein with an On Call GK Dual monitor and ketone test strips (ACON Laboratories, Inc., San Diego, CA, USA).

2.3.4. Oral Ethanol Self-Administration

Following the previously published protocol [55], eight modular operant chambers (Med Associates Inc., Georgia, VT, USA) and Med-PC IV were employed to carry out the oral EtOH SA. These cages contain two small holes with photocells that register nose-poke responses. Active nose-pokes activated a 0.5 s stimulus light and buzzer beep, which were followed by the delivery of 37 µL of EtOH, followed by a time-out of 6 s. Inactive nose pokes did not have any effect. This protocol consists of three phases: training phase, saccharin substitution phase, and 6% EtOH consumption phase.

- Training phase (8 days) In the training phase, animals had to nose-poke into the active wholes to obtain 37 µL of saccharin (0.2% (w/v)). To facilitate learning acquisition, two days before beginning the training, chow was restricted to 1 h/day and water was suspended for 24 h before the first session. Only during the subsequent 3 days, 1 h before initiating the operant session animals had access to food but not to water (postprandial). On the subsequent four days and during the course of the experiment, to avoid EtOH intake due to thirst, water was available anytime and food was available for 1 h after each training session (preprandial).
- Saccharin substitution (9 days) In this phase, saccharin percentage was progressively reduced as the EtOH concentration was gradually augmented [55,56]. Animals had access to each combination for three consecutive sessions (0.15% Sac −2% EtOH; 0.10% Sac −4% EtOH; 0.05% Sac −6% EtOH).
- 6% Ethanol consumption (11 days) This phase evaluates the number of active nose-poke responses, the 6% EtOH (w/v) intake and motivation to obtain it. First, animals were exposed to 5 days of fixed ratio 1 (FR1) sessions, in which the number of effective responses on the active nose-poke and EtOH consumption (all) was measured. After each session, the remaining fluid in the receptacle was collected and quantified with a micropipette. Following the FR1 sessions, animals were exposed to the fixed ratio 3 (FR3) schedule for 5 days, where they had to respond three times with an active nose poke to obtain one EtOH reinforcement. To set the breaking point for each animal, which is the maximum number of active nose-pokes the animal is capable of accomplishing to obtain one reinforcement, a progressive ratio (PR) session with a 2 h duration was carried out. The response requirement to achieve reinforcements increased corresponding to the series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The breaking point that the animal achieved was calculated based on this scale, which defines the animal's motivation toward EtOH consumption.

2.3.5. RNA Isolation, Reverse Transcription, and Quantitative RT-PCR

After the PR session, mice were euthanized by cervical dislocation, and their brains were extracted and striata dissected. Brain tissue samples were immediately stored at −80 °C until the qRT-PCR assay was performed.

Following the manufacturer's protocol, the Tri Reagent Method (Sigma-Aldrich, St. Louis, MO, USA) was employed to isolate the total RNA from the striatum. Reverse transcription of 1 mg of total RNA was performed via the Transcriptor First Strand cDNA synthesis kit (Thermo Fisher Scientific, Madrid, Spain). Amplification of the target and housekeeping (b-glucuronidase) genes was completed employing the Taqman Gene Expression Master Mix (Thermo Fisher Scientific, Madrid, Spain) in a LightCycler 480 System (Roche Diagnostics, Madrid, Spain). The assay codes of the primers used were Mm02620146 and Mm00438545 for dopamine receptors 1 and 2 (DrD1, DrD2), Mm01308023 and Mm00802075 for adenosine receptors A1 and A2 (ADORA1, ADORA2a), and Mm01188089 and Mm00446953 for cannabinoid receptor 1 (CB1r) and opioid receptor μ (Oprm), respectively. Data were analyzed using the LightCycler 480 relative quantification software and normalized to the amplification product of b-glucuronidase.

2.4. Statistics

Data relating to body weight and βOHB were analyzed by a mixed ANOVA with one between-subjects variable diet (with 2 levels, standard diet (SD) and ketogenic diet (KD)) and a within variable PND with 6 levels: Baseline and Weeks 1–5.

Regarding EtOH SA, a two-way ANOVA was performed with the variable diet (standard or ketogenic) as a between variable and days (5 levels for FR1 or FR3) as a within variable. To analyze breaking point values in the progressive ratio, a Student's test was performed. The gene expression data were analyzed by a two-way ANOVA with two between variables, diet (with 2 levels, standard diet (SD) and ketogenic diet (KD)) and EtOH (no-alcohol self-administration (NO-SA) and alcohol self-administration (SA). Bonferroni post-hoc tests were also analyzed. Data are presented as mean ± SEM. Analyses were performed using SPSS v26 (IBM SPSS Statistics for Windows, Version 26.0. IBM Corp, Armonk, NY, USA).

3. Results

3.1. Increased β-Hydroxybutyrate (βOHB) and Bodyweight in Mice Fed on KD

Results regarding βOHB blood levels (Figure 2) revealed a significant effect of the interaction week x diet [$F(5145) = 12{,}899$; $p < 0.001$]. The KD group showed increased levels of βOHB in comparison with the SD group in Weeks 1, 2, 3, 4, 5 ($p < 0.001$ in all cases). There were also significant increases within the KD group on weeks 1, 2, 3, 4, 5 with respect to the baseline ($p < 0.001$), as well as increases in the SD group in Weeks 3, 4, and 5 with respect to the baseline ($p < 0.05$), probably due to coincidence of food deprivation during the SA procedure.

Regarding changes in bodyweight (Figure 3), the ANOVA revealed a significant effect of the variable diet [$F(1,29) = 6.731$; $p < 0.05$], as the KD group presented a higher bodyweight than the SD group ($p < 0.05$, SD = 38 gr vs. KD = 40 gr). There was also a significant effect of the variable week [$F(5145) = 46.893$; $p < 0.001$], as all weeks showed lower bodyweight than baseline ($p < 0.001$). In addition, during Week 2 (when the food deprivation for oral SA started) bodyweight was significantly lower than the rest of the weeks ($p < 0.001$). Finally, there was an effect of the interaction week x diet [$F(5145) = 3.705$; $p < 0.01$]. The KD group exhibited higher body weight with respect to the SD group on weeks 2, 3, and 4 ($p < 0.001$; $p < 0.01$, and $p < 0.05$ respectively).

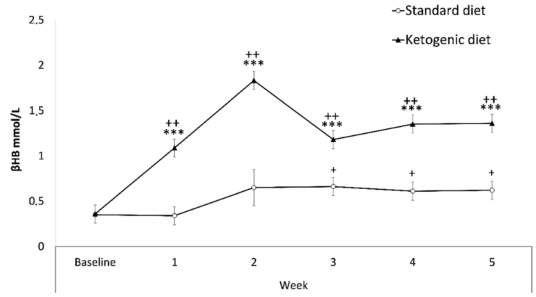

Figure 2. Weekly β-hydroxybutyrate blood levels. Data are represented as the mean (±SEM) amount of βOHB measured weekly. *** $p < 0.001$ significant difference with respect to SD group. ++ $p < 0.001$; + $p < 0.05$ significant difference with respect to the baseline.

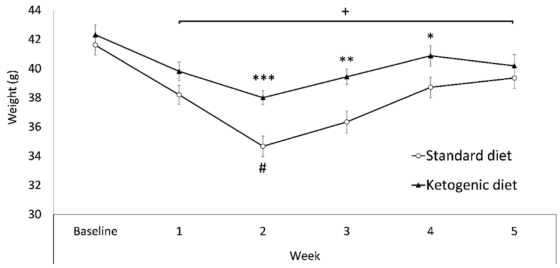

Figure 3. Weekly bodyweight. Data are represented as the mean (±SEM) bodyweight measured weekly. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ significant difference with respect to the SD group. + $p < 0.05$ significant difference with respect to the baseline. # $p < 0.001$ significant difference with respect to the rest of the weeks.

3.2. Ketogenic Diet Decreased Oral Ethanol Self-Administration

Regarding the number of active responses during the FR1 schedule of EtOH SA (Figure 4a), the ANOVA did not show any significant differences between SD and KD. With respect to EtOH consumption (g/kg) during the FR1 schedule (Figure 4b), the ANOVA reported a significant effect of the variable diet [F(1,29) = 10.554; $p < 0.01$], as the KD group exhibited a decreased oral SA of EtOH with respect to the SD group ($p < 0.01$; KD = 0.51 ± 0.04 g/kg vs. SD = 1.04 ± 0.08 g/kg).

During the FR3 schedule, the ANOVA for the number of active responses (Figure 4a) showed a significant effect of the variable day [F(4116) = 2942; $p < 0.05$], as all mice exhibited lower number of active responses on Day 6 with respect to Day 7 ($p = 0.076$). With regards to EtOH consumption (g/kg) during the FR3 schedule (Figure 4b), significant differences were reported in the variable diet [F(1,29) = 8142; $p < 0.01$], since the KD group showed a decreased oral SA of EtOH ($p < 0.01$; KD = 0.28 ± 0.03 g/Kg vs. SD = 0.51 ± 0.04 g/Kg). There was also an effect of the variable day [F(4116) = 2638; $p < 0.05$], as all mice showed higher EtOH intake in day 7 with respect to day 6 ($p < 0.05$).

Regarding the progressive ratio (Figure 4c–e), there were no significant differences in the breaking point (t = −0.005 d.f. 29; $p = 0.99$), EtOH consumption (t = 1674 d.f. 29; $p = 0.105$), and the number of rewards (t = −0.616 d.f. 29; $p = 0.54$).

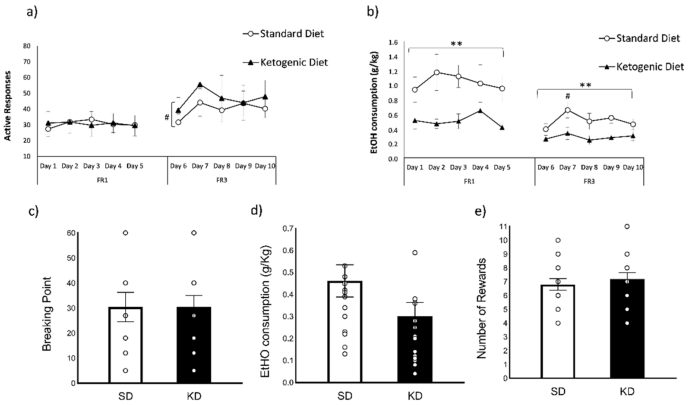

Figure 4. Oral EtOH self-administration (SD n = 15; KD n = 16). (**a**) The dots represent means and the vertical lines ± SEM of the amount of the number of active responses and (**b**) the volume of 6% EtOH consumption during FR1 and FR3 (in g/kg). (**c**) The columns represent means and the vertical lines ± SEM of breaking point values (**d**) the volume of 6% EtOH consumption (in g/kg) and (**e**) the number of rewards obtained during PR. ** $p < 0.01$, significant difference with respect to the SD group. # $p < 0.05$, significant differences with respect to Day 7.

3.3. Gene Expression Analyses

3.3.1. Ketogenic Diet Induced Increased Expression of DrD1 and DrD2 Gene Expression after Ethanol Self-Administration

For DrD1 gene expression (Figure 5a), the ANOVA revealed a significant effect of the variable diet [$F(1,28) = 14.652$; $p < 0.001$], EtOH [$F(1,28) = 8.378$; $p < 0.01$] and the interaction diet x EtOH [$F(1,28) = 8.142$; $p < 0.01$]. With regards to DrD2 expression (Figure 5b), the ANOVA revealed an effect of the variable EtOH [$F(1,28) = 8.625$; $p < 0.01$] and the interaction diet x EtOH [$F(1,28) = 8.639$; $p < 0.01$]. Exposure to a KD induced a significant increase in DrD1 and DrD2 expression in KD animals after the EtOH SA with respect to the rest of the groups ($p < 0.001$ in all cases).

3.3.2. Opposite Changes in ADORA1 and ADORA2 Gene Expression in Response to Ketogenic Diet and Ethanol Self-Administration

For the adenosine receptor A1 gene expression (ADORA1, Figure 5c), the ANOVA revealed a significant effect of the interaction diet x EtOH [$F(1,28) = 6.099$; $p < 0.05$]. Mice in the KD group that did not perform SA (KD-NO SA) showed an overexpression of ADORA1 with respect to the corresponding SD group ($p < 0.01$) as well as with respect to the KD-SA group ($p < 0.01$).

Regarding the expression of the ADORA2 gen (Figure 5d), the ANOVA revealed a significant effect of the variable EtOH [$F(1,28) = 10.587$; $p < 0.01$] and the interaction diet x EtOH [$F(1,28) = 10.615$; $p < 0.01$]. After the EtOH SA, mice exposed to the KD showed a significant overexpression in ADORA2 with respect to their corresponding SD-SA group ($p < 0.01$) and KD-NO SA group ($p < 0.001$).

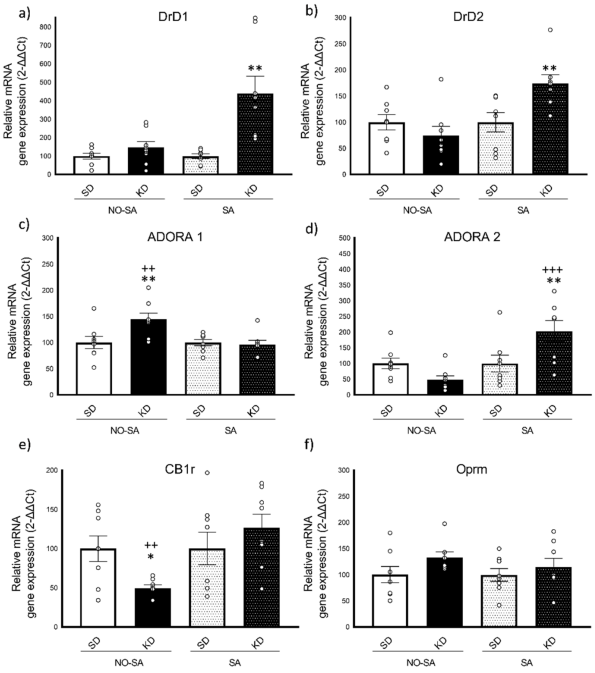

Figure 5. Real-time PCR Gene expression in the striatum (n = 8/condition). (**a**) Dopamine receptor D1 gene—DrD1, (**b**) Dopamine receptor D2 gene—DrD2, (**c**) Adenosin receptor A1 gene—ADORA1, (**d**) Adenosin receptor A2 gene—ADORA2, (**e**) Cannabinoid receptor 1—CB1r, (**f**) Opioid receptor μ—Oprm. The columns represent means and the vertical lines ± SEM of relative (2-ΔΔCt method) gene expression in the striatum of OF1 mice. * $p < 0.05$; ** $p < 0.01$ significant differences with respect to the corresponding SD group. ++ $p < 0.01$; +++ $p < 0.001$ significant differences with respect to their corresponding KD group.

3.3.3. Ketogenic Diet Decreases CB1r Gene Expression

With regards to CB1r gene expression (Figure 5e), the ANOVA revealed an effect of the variable EtOH [$F(1,28) = 5.851$; $p < 0.05$] and the interaction diet x EtOH [$F(1,28) = 5.862$; $p < 0.05$]. Bonferroni post-hoc analyses showed that KD-NO SA mice exhibited a significant decrease in CB1r gene expression in comparison with their corresponding SD group ($p < 0.05$) and the KD-SA group ($p < 0.01$). No significant differences were obtained in the gene expression of the opioid receptor mu (Figure 5f).

4. Discussion

The main aim of the present work was to evaluate whether a KD could modulate the rewarding and motivational effects of alcohol. In addition, the role in these diet-induced changes of receptors related to the reward process, such as adenosine, dopaminergic, cannabinoid, and opioid systems, were also considered through gene expression studies. The possible beneficial effects of nutrition as a complement to substance-use disorder treatments have been scarcely studied. Therefore, this work opens a new line of research in

drug addiction, given that to date only unhealthy diets such as cafeteria, high-sugar, and high-fat diets have been investigated in this field (for review see [57,58]).

The results found in the present work revealed that the KD induces an overall decrease in alcohol SA but does not affect the motivation to get the drug. Furthermore, the KD by itself induces opposite changes in the gene expression of ADORA1 and CB1r, which disappeared after alcohol consumption. Moreover, KD after EtOH SA induced striatal increases in the gene expression of ADORA2, DrD1, and DrD2 genes. No changes in Oprm gene expression were observed.

4.1. Ketogenic Diet Modulates Bodyweight and Increases β-Hydroxybutyrate Blood Levels

Animals in the KD group rapidly turned into a ketotic state and their βOHB levels stabilized after 7 days. The ketotic state was maintained throughout the experiment, even during EtOH SA, where a slight decrease in βOHB levels was observed. These could be due to a decrease of βOHB in the liver induced by alcohol, as it has been recently shown [59]. These authors showed that βOHB administration had an anti-inflammatory and hepatoprotective effect in mice with ethanol-induced liver disease, suggesting its possible therapeutic role for alcohol-use disorders. On the other hand, we have also observed a slight increase in βOHB levels in the control group as a result of food deprivation during EtOH SA, confirming that fasting also increases βOHB levels [60].

With respect to the bodyweight, our KD-exposed mice significantly increased their bodyweight in comparison to animals in the standard diet. Even though all groups experienced a decrease in bodyweight when SA food deprivation began (1 h/day access), the group exposed to KD maintained its weight above the SD group. To date, studies have shown conflicting results regarding the effects of KD on bodyweight. While some studies have found that KD for 2 weeks induces weight loss in rats [49], other studies show that bodyweight is increased by KD in rats when administered for 6 to 8 weeks, although the weight gain was slower than that observed in SD-fed animals [61,62]. Moreover, other studies reveal that male mice fed with a KD for a month showed an increase in brown adipose tissue (up to a 40% greater than controls) in agreement with our results [63].

4.2. Ketogenic Diet Diminishes Ethanol Self-Administration

Regarding EtOH SA, our results showed a general decrease in alcohol consumption in the KD group, both in FR1 and FR3 schedules. There were no differences in the number of effective responses between groups, meaning that animals on KD did not drink all the EtOH they had access to. This decrease in EtOH consumption was maintained across the ten days of self-administration, even during the FR3, where animals were demanded more work to obtain the drug.

Nevertheless, this decrease in alcohol intake did not modify the motivation to obtain the reward, as it was observed in the active responses and PR schedule, where no differences were observed between KD and SD. The PR indicates the breaking point or limit of active responses by the animals, that is, to what extent a mouse is willing to introduce its nose into the hole repeatedly to obtain one reinforcement (in this case, 38 µL 6% EtOH). Discarding an incorrect learning of the task (no differences in training or substitution), this result confirms that the mechanisms involved in FR1 and FR3 schedules (rates of operant responding) are not the same as those involved in PR (motivation) [64]. In fact, the PR schedule is complementary to the FR schedules and is indicative of the motivation for seeking the drug [65,66], while FR1 assesses the potential liability of a drug and the consumption based on its unconditioned psychopharmacological effects [67]. Moreover, these results support the fact that mice fed on KD do not show a motivational alteration towards reward.

To date, few studies have explored the relationship between alcohol consumption and KD, most of them focusing on ketoacidosis, a state in which the body's insulin is so low that the liver produces excess levels of ketone bodies that would have neurotoxic properties [68]. Nevertheless, only a few studies have evaluated the interaction between KD and EtOH, one of them focused on the reduction of alcohol intake in rodents [51]. In this

study, animals were first trained to lever press for alcohol in the vapor self-administration paradigm. Next, the rats were divided into two groups and exposed to a KD or SD for 8 weeks, after which both groups were returned to SD. Then, to induce alcohol dependence, half of the rats were exposed to chronic alcohol vapor for 7 weeks, and the other half, as a control group, were exposed to air. After this, animals were subjected again to a vapor self-administration paradigm. Their results showed that animals on KD-alcohol vapor did not exhibit an escalation in EtOH intake, as they self-administered EtOH to the same degree as non-dependent rats, suggesting that the KD exerted a protective effect. Regarding alcohol withdrawal symptoms, another study implemented a KD in rats before alcohol administration (intragastric administration) until the end of the procedure, although they did not verify whether the KD had increased ketone bodies [49]. Their results indicate that the KD was effective in reducing rigidity and irritability behaviors but the mechanism through which the KD had a positive effect on alcohol withdrawal symptoms remains unclear. Similar results have been obtained with administration of ketone monoester in mice [50], which reduces handling-induced convulsions and anxiety-like behaviors in early alcohol withdrawal, as with the KD.

Although the study focused on a psychostimulant drug, Martínez and co-workers [48] evaluated the potential of a KD as a therapy for cocaine addiction. The administration of a KD for three weeks to female and male rats was able to block cocaine sensitization, hyperlocomotion, and stereotypies, confirming a robust action of the KD on dopamine-mediated behaviors [48]. One of the explanations proposed by this study is that KD-mediated changes in adenosine may have therapeutic potential via actions at adenosine-dopamine receptor heterodimers.

Taking into account the different bodyweight of both groups, the results of EtOH consumption were calculated based on the individual weight of each subject (g/kg) in order for these differences to be avoided. A limitation of the present study could be the lack of control on the kcal intake between groups. Based on their bodyweight, we hypothesized that mice on KD ate a greater amount of kcal in an hour compared to the SD group during the SA procedure, indicating that the hypothesis of satiation as a contributor to the decrease in EtOH consumption should be taken into consideration. However, this possibility should be ruled out, as food intake occurred postprandially to SA, i.e., animals were introduced into the SA box 23 h after their last food intake. In addition, a recent study reported that food-deprived mice decreased their food intake after EtOH SA [69]. These results may explain why the SD group, which showed higher alcohol consumption, consumed fewer calories from food after consuming EtOH. In addition, food intake or satiation did not affect their EtOH motivation, as seen in the breaking point results. However, future studies should employ a eucaloric protocol to rule out this possible impact.

Nevertheless, even though studies report that a KD has beneficial effects, for example in weight loss in obese patients [70], the possible long-term effects of this diet remains unknown, probably due to the difficulty of adherence to a strict KD over time. Some studies have already reported that there can be adverse effects such as lipid abnormalities [71,72], hypoglycemia and dehydration [73,74], dysregulation of glucose levels [75,76] or nephrolithiasis [77]. Although some of these consequences have been seen in patients on the KD [77], the effects of a ketotic state in patients with an alcohol-use disorder may be pronounced, as they already present a poor nutritional status [78]. Thus, future studies should address this issue, considering the basic blood (cholesterol, low and high density lipoprotein, triglycerides, glucose) and liver parameters (transaminases) and investigate the possibility of using ketone esters as a supplement to a balanced diet, which also increase blood BHB [79,80].

4.3. Ketogenic Diet Diminishes Ethanol Intake through the Adenosine-Dopamine Binomial

Our initial hypothesis was that the administration of a KD would diminish the EtOH rewarding effects by acting through the adenosine-dopamine binomial. Our results showed that KD increased ADORA1 gene expression without affecting ADORA2 or dopaminergic

genes. Therefore, KD induced an overexpression of the ADORA1 gene with regards to the DrD1 gene. When animals on KD were exposed to EtOH, the changes in gene expression were completely different. KD and EtOH exposure increased the expression of the DrD1, DrD2, and ADORA2 genes. Under these circumstances, there was an overexpression of the DrD1 gene with respect to the ADORA1 gene and no change of balance was observed with regard to ADORA2 and DrD2, as both genes increased their expressions.

The binomial refers to the mutual antagonistic interactions between the heterodimer's A1-D1 and A2-D2 receptors [42]. Adenosine receptors are particularly expressed in GABAergic neurons in the striatum and are colocalized post-synaptically with dopamine receptors [41]. A1 agonists have been found to significantly decrease the binding affinity of D1, indicating that the function of the A1 receptor in the A1-D1 heterodimer is to inhibit dopamine signaling via the D1 receptor [81].

Although the activation of the A1 receptor has been demonstrated to be one of the main anti-seizure mechanisms of the KD [36], it seems that the A2 receptor collects more evidence in the addiction field, and this supports the results obtained in this study. For example, a mixed antagonism of both the A1 and A2 receptors, but with higher potency on the latter, produces similar effects for those generated by psychostimulants and enhances relapse into cocaine SA in baboons [82]. Likewise, A2 agonists decrease cocaine SA [83] and cocaine and morphine locomotor sensitization in rodents [46,84]. Finally, A2 knockout animals exhibit a significant decrease in cocaine SA, although conditioned place preference and sensitization were not affected [45,85].

There are also studies suggesting that adenosine mediates EtOH intake as well. Confirming our results, Feltmann and co-workers [86] reported an increase in the density of the A2-D2 heteroreceptor in the striatum of rats that voluntarily drank alcohol for 12 weeks, although a reduction of the striatal density of D2-D2 homoreceptor complexes was also observed. In addition, A2 receptor stimulation with A2 agonists attenuated alcohol intake, while the A2 antagonist increased EtOH intake in alcohol-preferring rats [87] and mice [88].

Moreover, studies about KDs and dopaminergic activity are scarce. However, it has been reported that 3 weeks of KD does not change DA in the Nucleus Accumbens [89]. A possible explanation for the decreased EtOH SA observed in mice on KD could be the relative increase of the ADORA1 gene, which in turn inhibits DrD1. With respect to the heterodimers A2-D2, KD did not induce any changes but both genes were overexpressed after oral EtOH SA, which indicates that there is no change in the balance of these genes.

As we mentioned, the GABAergic system is one of the most likely targets of EtOH [90] in addition to ketone bodies [91]. KD induces an increase in GABA-mediated inhibition and activates the GABA-B receptors [11,92]. Results showing the efficacy of GABA-B agonists decreasing EtOH SA [93–95] sustain the hypothesis that a KD modulates the GABAergic system through a change in the adenosine-dopamine heterodimer. Taking into account that both EtOH and KD interact with the GABAergic system and produce alterations in the adenosine-dopamine heterodimer, we can hypothesize that ketone bodies could induce a decrease in EtOH consumption through this mechanism.

The cannabinoid and opioid systems are both involved in fatty food intake and reward [4,5]. In this study we observed that the KD induced a significant underexpression of the CB1 gene, but after EtOH consumption, this underexpression was normalized. Calorically dense foods increase CB1r density in the Nucleus Accumbens, leading to their downregulation [96], which could be the case for KD. Moreover, it is important to note that most GABAergic inhibitory interneurons express presynaptic CB1r in abundance, which modulate the release of GABA at the synapses [97,98]. Regarding Oprm, this study reported no significant changes except for a trend to be overexpressed in the KD groups. Some studies suggest that Oprm presents an increase of expression in the brain areas processing reward associated with palatable foods [99,100], but there are no studies about the effects of KD on the opioid system.

5. Conclusions

KD may be a useful nutritional complement to the existing pharmacological therapies in alcohol addiction, especially considering the undernourishment that alcohol produces. In addition, we propose the adenosine-dopamine binomial as an interesting target that deserves to be explored in alcohol addiction. One important limitation of the KD is the difficulty to maintain adherence over time, and a permanent ketotic state is not considered realistic as a way of life. Stemming from this, there is a wide range of research that can be done, including testing other ketogenic-related therapies such as ketone esters, which promote an increase in blood βOHB levels. Furthermore, even when our results confirm that a KD could have a positive effect on reducing alcohol intake in mice, further research is needed to know the long-term effects of KD on metabolic health, such as basic blood and liver parameters. To date, although there is a scarce number of published studies highlighting the protective effects of KD on drug effects, the results obtained have been reported in preclinical studies, therefore more studies assessing the effect of different ketogenic diets on addiction in humans are needed. Many studies remain to be carried out with KD, and it is necessary to elucidate the exact neurobiological mechanisms through which KD modulates addiction. Even so, these results highlight once again the great relevance of nutritional interventions in mental and substance-use disorders.

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