Universidad Andrés Bello
Facultad de Ciencias Biológicas
Escuela de Ingeniería en Biotecnología

EFECTO DE LA ACTIVACIÓN ADRENÉRGICA INSULAR EN LA NEOFOBIA GUSTATORIA

Proyecto de Tesis presentado como parte de los requisitos para optar
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Director de tesis: Jimmy Stehberg Liberman
Centro de Investigaciones Biomédicas
Universidad Nacional Andrés Bello

Sebastián Andrés Rojas Silva
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1. Resumen

La respuesta frente al estrés corresponde a la capacidad de un organismo de responder frente a un estímulo estresante y volver a su homeostasis. Esta respuesta es iniciada por la respuesta autonómica, mediada principalmente por actividad adrenérgica, la cual es modulada por catecolaminas, entre las cuales se destacan la norepinefrina (NE) a nivel de sistema nervioso central, y epinefrina (EPI) a nivel periférico.

Se conoce una serie de áreas del cerebro asociadas a la respuesta al estrés, incluyendo la amígdala extendida, locus coeruleus, hipocampo, corteza prefrontal, entre varias. Una de las áreas del cerebro que ha sido propuesta muy recientemente como mediadora de la respuesta fisiológica frente al estrés es la corteza insular. Esta área recibe información emocional, visceral y gustatoria, y aún se desconoce por qué su actividad se ve alterada en trastornos de ansiedad. Es posible estudiar el rol de la ínsula en ansiedad usando conductas asociadas a gustos que sean sensibles al estrés y ansiedad, como la neofobia gustatoria. La neofobia gustatoria se describe como el miedo a un gusto nuevo, y es exacerbada en ambientes de estrés (hiponeofagia), siendo una medición de comportamiento tipo ansioso en animales.

Este trabajo tuvo como objetivo evaluar si la actividad adrenérgica en la corteza insular media el aumento en neofobia gustatoria inducido por la presentación de un gusto nuevo en un ambiente de alta exaltación. La hipótesis que sustenta este trabajo es que **la actividad adrenérgica en la corteza insular aumenta la neofobia gustatoria y modula el incremento de neofobia inducida por un ambiente de alta exaltación.** Para probar la hipótesis se usó una combinación de microinyecciones de NE y propranolol intra insular y de inyecciones subcutáneas de EPI y propranolol, antes de la presentación del gusto en contextos con alta y baja exaltación. Los resultados obtenidos apoyan la hipótesis. Nuestros resultados proponen que la actividad adrenérgica periférica media la respuesta de neofobia gustatoria en ambientes de alta exaltación, efecto que es modulado por actividad adrenérgica en la corteza insular.

2. Abstract

The stress response is the ability of an organism to respond to a stressor and return to homeostasis. The first body's response to stress is the autonomic response, primarily mediated by adrenergic activity, which is modulated by catecholamines, among which norepinephrine is the predominant in the central nervous system, and epinephrine peripherally. Studies show that a dysregulation in the levels of these hormones may lead to psychiatric disorders including anxiety disorders.

Among several brain areas involved in stress response, including the extended amygdala, locus coeruleus, hippocampus and prefrontal cortex, the insular cortex has been recently associated with physiological responses to stress. This area receives emotional, visceral and gustatory information, but it is still unknown why its activity is altered in anxiety disorders. It is possible to study the role of the insula in anxiety using behaviors associated with tastes which are sensitive to stress and anxiety, such as gustatory neophobia. The gustatory neophobia is the reluctance or fear to try a new taste, and is exacerbated in arousing contexts (also known as hyponeophagia), being a widely used to measure anxiety in animals.

This study aimed to evaluate to which extent the adrenergic activity in the medial insular cortex increases gustatory neofobia induced by arousing novel contexts. The hypothesis behind this study is that **adrenergic activity in the insular cortex increases taste neophobia and modulates the increment in neophobia induced by an arousing context.** To test this hypothesis we used a combination of intrainsular micro-injections of norepinephrine and propranolol and peripheral injections of epinephrine and propranolol before presentation of a novel taste in contexts of high and low arousal. Our results support the hypothesis and suggest that peripheral adrenergic activity mediates arousal induced increases in neophobia, effect that is mediated by adrenergifc activity in the insular cortex.

3. Abreviaturas

NE- Norepinefrina

EPI – Epinefrina

TEPT – Trastorno por estrés postraumático

IA – Índice de aversión

PFA – Paraformaldehido

HPA – Eje hipotálamo pituitaria adrenal

4. Introducción

4.1 La respuesta al estrés

El estrés es una condición en que la expectativa establecida por un aprendizaje previo o deducida de las circunstancias no coincide con las percepciones actuales o provistas del medio interno o externo, y esta divergencia entre lo que se observa o se sintió, provoca respuestas compensatorias (Goldstein y cols, 2006). Esta divergencia es producida por un estímulo estresante, el cual puede ser visto como un reto inminente para el equilibrio del cuerpo (Anisman y Matheson, 2005). El estrés afecta a todos los estratos sociales, y dependiendo de su intensidad o nivel de percepción puede llegar a desencadenar diversos tipos de molestias. El estrés incrementa el riesgo de padecer diabetes mellitus, ya que se altera la necesidad de insulina (Stenstrom y cols, 1993). Asimismo, el estrés aumenta los niveles de catecolaminas, glucocorticoides y supresores de células T, lo cual hace a nuestro cuerpo más susceptible a infecciones virales (Hafen y cols., 1991; Huebner, 1992).

Cada año, al sumar los días mundiales de trabajo de cada empleo, 13.4 millones de días perdidos de trabajo son atribuibles al estrés, ansiedad o depresión (Salleh, 2008). Además se estima que entre el 80% y 90% de los accidentes industriales están relacionados con problemas personales y a la incapacidad de manejar el estrés (Giunchi y cols., 2016, Jansen, 1986, Lu y Kuo, 2016). Esto también se refleja en datos entregados por la agencia europea de seguridad y salud en el trabajo, la cual reportó que cerca del 50% de ausencias laborales son causadas por el estrés (Simmons y Simmons, 1997). Además, en empleos cuyo estrés es elevado (policías, bomberos, paramédicos, etc) se ha encontrado un aumento de individuos con trastornos de ansiedad y que padecen un elevado riesgo de suicidio (Stanley y cols., 2016).

4.2 Catecolaminas

Frente a una situación estresante nuestro cuerpo genera una respuesta adaptativa, la cual en primera instancia está dirigida por catecolaminas (EPI, NE y dopamina). Estas catecolaminas pueden activar procesos fisiológicos y conductuales que facilitan la superación del estrés y de esta manera, mantener la homeostasis en el individuo (Cannon y de la Paz, 1991).

La cronología de esta respuesta adaptativa inicia cuando un estímulo estresante activa vías aferentes que desencadenan la activación de una respuesta autonómica (primordialmente simpática) y la concomitante liberación de catecolaminas desde la médula adrenal. Las catecolaminas liberadas tanto a nivel cerebral como periférico vía activación de aferencias vagales, activan a su vez una serie de centros integrativos del cerebro, los cuales abarcan el sistema límbico, corteza cerebral e hipotálamo. Si el estímulo estresante se mantiene en el tiempo, la activación simpática sostenida activará al eje hipotálamo-pituitaria-adrenal (HPA) gatillando la secreción de cortisol (en humanos) o corticosterona en roedores. También se activarán neuronas catecolaminérgicas eferentes en el tronco encefálico y espina dorsal que inervan neuronas preganglionares simpáticas para volver a activar al sistema nervioso simpático y la médula adrenal, incrementando la EPI circulante (Olson, 2011).

Ambas respuestas hormonales generan cambios fisiológicos rápidos, como el incremento en la presión sanguínea, ritmo cardiaco y aumento de la glicemia.

Las catecolaminas liberadas no solo cumplen un rol a nivel periférico sino que también a nivel de sistema nervioso central, como neurotransmisores. El Locus coeruleus es la zona cerebral que secreta NE en el sistema nervioso central. Korf y colaboradores, en 1973 demostraron que al eliminar la región antes mencionada, es posible prevenir los efectos del estrés en el cerebro y que al estimular el Locus coeruleus es posible generar un aumento de NE en la corteza cerebral (Korf y cols. 1973).

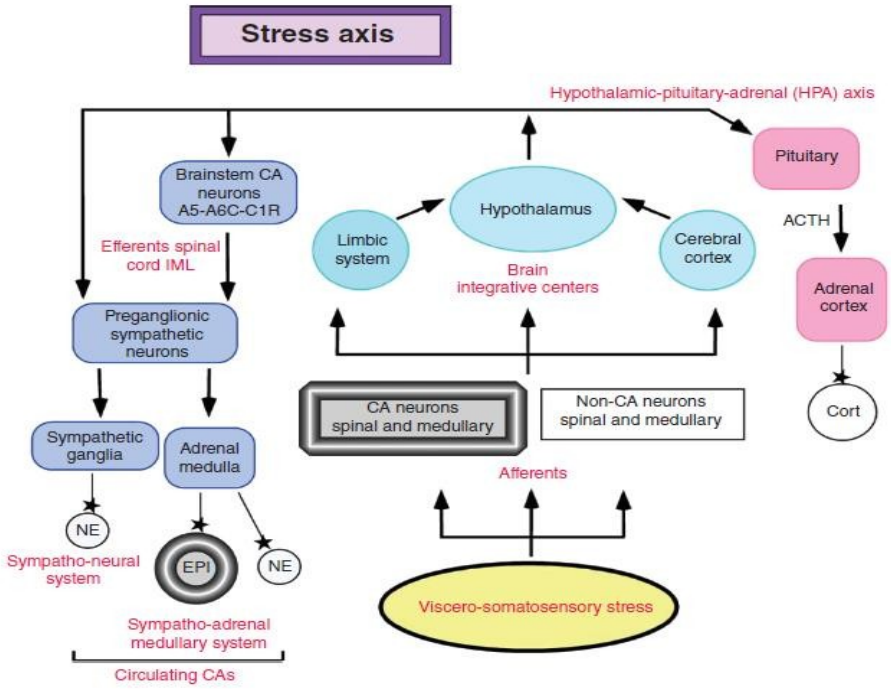

Figura 1 . Respuesta frente a un estrés. (Tank y Wong, 2015) Esquematización de la liberación de moléculas frente a una respuesta estresante.

4.3 Norepinefrina y Epinefrina

La NE participa activamente en la regulación de la respuesta cerebral al estrés, mientras que EPI comanda el efecto periférico de la actividad adrenérgica frente al estrés (Cameron y Nesse, 1988). La EPI circulante es secretada por las células cromafines de la médula adrenal. En humanos, el 80% de la médula adrenal está compuesta por células cromafines que expresan la enzima feniletanolamina N-metiltransferasa, y sintetizan y secretan EPI. La NE también es secretada por la médula adrenal pero en cantidades muy limitadas. Sus principales secretores son los nervios postglanglionares simpáticos (Goldstein y cols., 2003) y las proyecciones del núcleo Locus Coeruleus en el cerebro (Korf y cols. 1973).

Durante un estrés físico, psicológico o ambiental el sistema nervioso simpático es estimulado llevando a la secreción de NE desde los nervios simpáticos y un incremento en la secreción de EPI desde las células cromafines. La EPI es censada por aferencias

vagales que activan la secreción de NE desde el locus coeruleus. En humanos, EPI plasmática y NE incrementan entre 2 a 10 veces sus niveles basales en respuesta a un estímulo estresante (Brenner y cols., 1997; Chamberlain y cols., 1990; Tosti-Croce y cols., 1991).

La respuesta a EPI y NE está mediada por receptores adrenérgicos acoplados a proteína G (Bylund, 1992; Civantos y Aleixandre, 2001; Cotecchia, 2010). Existen diferentes tipos de receptores adrenérgicos; los α que se subdividen en α1 y α2; y los β, que se subdividen en β1 y β2. Estudios demuestran que antagonistas de estos receptores pueden ser usados para tratar variadas patologías, incluyendo estrés postraumático (TEPT) (Kukolja y cols., 2008; van Stegeren y cols., 2007). Pacientes que padecen de TEPT tienen problemas para dormir y al tratar estos pacientes con un antagonista β- adrenérgico se logra disminuir significativamente los problemas para conciliar el sueño (Krystaly Davidson, 2007; Raskindy cols., 2003, 2007; Taylor y cols., 2006).

La relevancia de estos receptores frente a enfermedades ansiosas se puede explicar por la afinidad de éstos a su ligando. Existe evidencia que indica que los receptores β- adrenérgicos poseen baja afinidad por NE pero una alta afinidad por EPI, siendo ésta incluso más elevada que su afinidad por los receptores α- adrenérgicos. Entre las funciones de los receptores β- adrenérgicos se encuentra la regulación del ritmo cardíaco y relajación de la musculatura, parámetros estrechamente relacionados con un aumento en la exaltación y ansiedad (Philipp y Hein, 2004; Wacher y Gilbert, 2012; Yang y cols., 2003). La inhibición de receptores β- adrenérgicos tanto de manera periférica como central interfiere en procesos de memoria, ansiedad y aprendizaje (Clayton y Williams, 2000; Gyires y cols., 2009; Parfitt y cols., 2012; Quirarte y cols., 1997; Roozendaal y cols., 2006; Schutsky y cols., 2011; Shepard, 1982).

La exaltación y ansiedad ha sido asociada a diversas zonas del cerebro. (Hollon y cols., 2015). Una de las zonas que ha sido asociada recientemente con ansiedad es

la corteza insular (Burguess, y cols., 2002 ;Cominski, y cols., 2014; James L. McGaugh, y cols., 1996; Paulus y Stein, 2006).

4.4 Corteza insular y neofobia

La corteza insular o ínsula es una zona del cerebro vinculada con diversas funciones tanto sensoriales como cognitivas (Devinskyy cols., 2003), siendo considerada de suma importancia para el nexo entre las emociones y los procesos cognitivos (Nitschke y cols., 2006). La ínsula posee conexiones bidireccionales con varias áreas límbicas incluyendo la amígdala cerebral, con la que comparten diversas funciones (Moraga-Amaro y Stehberg, 2012; Reynolds y Zahm, 2005), lo que la relaciona directamente con las emociones. Se ha propuesto que la ínsula posee la función de recibir la información emocional, evaluar su relevancia y valor, e integrarla para determinar el estado del cuerpo (Paulus y Stein, 2006).

Como se mencionó anteriormente se cree que la ínsula podría modular la respuesta al estrés y ansiedad. Esto se ve reflejado en modelos animales donde la modulación colinérgica de la ínsula es capaz de modular la respuesta ansiosa a un ambiente nuevo (Shepard y cols., 1982), como también en pacientes con TEPT, en los cuales se observa un aumento en la actividad de la corteza insular tras recordar el evento traumático (Rauch y cols., 1997).

Una de las funciones principales de la ínsula es recibir la información gustatoria (Lin y cols, 2009; Stehberg y Simon, 2011). Estudios han demostrado que una lesión en la corteza insular es capaz de atenuar el comportamiento condicionado al gusto, asociándola no solo con la función gustatoria sino también con el comportamiento aversivo (Bermudez-Rattoni y Gaugh, 1991). Entre los comportamientos gustatorios, uno de los más sensibles al estrés es la neofobia gustatoria, la que corresponde a una reducción del consumo de un gusto nuevo hasta determinar si es seguro (Dulawa, 2009). Cuando el gusto o comida se presenta en un ambiente nuevo o de alta

exaltación ocurre hiponeofagia o un aumento en la neofobia inducida por un ambiente nuevo, lo que se utiliza como medición de ansiedad en modelos animales (Hall, 1934; Poschel, 1971; Tye y cols., 1975).

Estudios previos sugieren que la hiponeofagia depende de la actividad adrenérgica tanto periférica como central (Shepard, y cols., 1982). En ese estudio se demostró una reducción en la alimentación del animal al ser sometido a un contexto nuevo o de alta exaltación, después de un bloqueo a nivel periférico con distintos bloqueadores adrenérgicos. Se desconoce aún la o las áreas del cerebro que modulan este comportamiento ansioso. Dado que estudios de nuestro laboratorio han demostrado que la ínsula es crítica para la neofobia gustatoria (Stehberg y Simon, 2011), es el área candidata perfecta para modular los efectos del estrés o exaltación de un ambiente nuevo en la neofobia (hiponeofagia), y con ello, modular los efectos en este comportamiento ansioso, tanto de la actividad adrenérgica periférica como cerebral.

4.5 Planteamiento del problema

Tanto la NE como la EPI se secretan durante situaciones de estrés y están involucradas en ansiedad. Una de las zonas recientemente propuestas en el control de la ansiedad es la corteza insular, pero se desconoce su rol en ansiedad. Uno de los modelos animales más usados para medir la ansiedad es la hiponeofagia, o el miedo a los gustos nuevos (neofobia) exacerbado por ambientes nuevos o estresantes, el que depende de actividad adrenérgica periférica y de la corteza insular. Por ello, aquí se propone que la actividad adrenérgica en la corteza insular modula el aumento en neofobia gustatoria inducida por ambientes estresantes o de exaltación.

5. Hipótesis

La hipótesis que sustenta la tesis es:

La actividad adrenérgica en la corteza insular aumenta la neofobia gustatoria y modula el incremento en neofobia inducida por un ambiente de alta exaltación.

6. Objetivos

El objetivo general de esta tesis es determinar el rol de la actividad adrenérgica en la corteza insular en el aumento de la neofobia gustatoria inducida por ambientes de alta exaltación.

Para llevar a cabo el objetivo general fue necesario cumplir con los siguientes objetivos específicos:

1. Determinar si la actividad adrenérgica periférica aumenta la neofobia gustatoria.
2. Determinar si la actividad adrenérgica en la corteza insular aumenta la neofobia gustatoria.
3. Determinar si el efecto periférico de la actividad adrenérgica está mediado por norepinefrina intrainsular.

7. Materiales y métodos

7.1 Metodología general

7.1.1 Manejo y cuidado de los animales

Todos los procedimientos con animales fueron aprobados por el comité de bioética de la Universidad Nacional Andrés Bello bajo el alero del Proyecto Fondecyt nº 1130724, acta de aprobación 001/2013 y acta de auditoría 004/2014. Los animales utilizados fueron ratas machos Sprague-Dawley de 250–300 grs.

Se separaron los animales en jaulas individuales y se acariciaron durante 4 días, 2-3 min diarios por animal, con la finalidad de habituarlos para que no se estresen al momento de su manipulación.

7.1.2 Implantación estereotáxica crónica de cánulas.

Este paso sólo se realizó a los animales designados para los experimentos que necesitaban microinyecciones intracraneales. Las ratas fueron anestesiadas como se describe en Taylor y cols., 2009, con una combinación de Ketamina/ Xilacina/acepromacina (60.6mg/kg; 0,6mg/kg; 6,67mg/kg, respectivamente), por inyección subcutánea. Se comprobó que el animal estuviese en un estado profundo de anestesia midiendo reflejos oculares, movimiento de bigotes, tipo de respiración, cianosis y respuesta al dolor por presión en la cola. Una vez anestesiado se fijó al animal en el aparato estereotáxico. Luego se determinaron los puntos de referencia Bregma y Lamda. Posteriormente se perforó el cráneo y se implantaron las cánulas en las coordenadas definidas para la corteza insular bilateral (1.2mm anterior a bregma, 5.4mm lateral a la línea media, 6.7mm ventral a la superficie del cráneo). Se fijaron 4 tornillos y se cubrió el cráneo con cemento dental. Finalmente se aplicó ungüento

dérmico, que contiene Bacitracina y Neomicina, entre la piel y el cemento dental. Ya finalizada la cirugía los animales recibieron una inyección de un anti-inflamatorio subcutáneo (ketoprofeno, 12 mg/kg) y se mantuvieron en observación por 48 horas, para luego ser llevados a una sala para recuperación post-operatoria durante 7 días. Las cánulas se mantuvieron bloqueadas por Dummies, los cuales son dispositivos de metal que van insertos en las cánulas y evitan que se contamine su lumen interno.

7.1.3 Modelo animal

Se utilizó el modelo de neofobia gustatoria descrito por Stehberg y Simon, 2011, el cual consiste en dos etapas; entrenamiento y el test. Tiene una duración de 4 días. En la etapa del entrenamiento se le enseña al animal a beber de pipetas plásticas. Para esto se les retira su botella durante 3 días y cada día a la misma hora se le dejan dos pipetas plásticas de 10 ml cada una, dándoles 10 min a los animales para beber, con el fin de que se acostumbren a beber su consumo diario de agua en 10 min. Al cuarto día se comienza el test, donde por 10 min se les da a elegir entre 6 pipetas plásticas alternadas cada una con 5 ml, 3 con agua y otras 3 con sacarina 0.1%. Se escogió esta concentración por ser un gusto preferido para los animales y no causar aversión que pudiese enmascarar nuestros resultados (Stehberg y Simon, 2011). Una vez terminado el test se midió la aversión al gusto, la cual fue medida como el índice de aversión. Este parámetro nos permite calcular el rechazo del animal hacia el gusto nuevo y está dado por la siguiente formula:

IA%= consumo de agua/ consumo total del liquido

Se presentó al gusto nuevo en dos contextos diferentes, uno de alta exaltación y otro de baja.

El contexto de alta exaltación consistió en una jaula sin viruta, con una luz fuerte (flujo luminoso de 380 lúmenes) sobre la jaula y en una habitación diferente. Tres

minutos después de exponer al animal al nuevo contexto comenzó el test de neofobia gustatoria. En el caso del uso de un contexto de baja exaltación, el test de neofobia gustatoria se realizó en la jaula (*homecage* en inglés) y habitación del animal, a la que ya está habituado.

7.1.4 Histología

Al terminar los experimentos se realizó una perfusión intracardiaca con salino (0.9%NaCl) y luego con 4% paraformaldehído (PFA) en buffer PBS. Este procedimiento se llevó a cabo sólo en animales implantados. Se extrajo el cerebro y se almacenó en sacarosa 30% disuelta en PFA al 4% y se dejó flotar hasta que su densidad igualó a la de la sacarosa. Estos cerebros se cortaron en el criostato, a un grosor de 30µm, recolectando sólo aquellos cortes contenían el implante de la cánula. Una vez cortados se montaron en un portaobjeto que contuvo 6 cortes por cerebro y se efectuó la tinción de Nissl mediante una solución de Cresyl Violeta Acetato 0.5% (Sigma,#C1791) y luego fueron deshidratados en una curva de etanol y tratados con cloroformo y xilol antes de ser cubiertos con cubreobjetos y entellan. La tinción de Nissl marca el núcleo celular y permite visualizar las áreas cerebrales y determinar posibles lesiones, infecciones o un mal posicionamiento de la cánula al momento de la cirugía, por microscopía óptica. Animales que presentaron lesiones más grandes que la cánula guía fueron excluidos. Se excluyó también del estudio animales que presentasen agresividad o estuviesen muy ansiosos antes del test. Todos los experimentos se realizaron bajo ciego, el que se abrió después de realizar la exclusión por histología.

La figura 2A muestra la zona de interés, el área disgranular de la corteza insular, en la figura 2B una tinción Nissl de un implante de cánula dentro de ella.

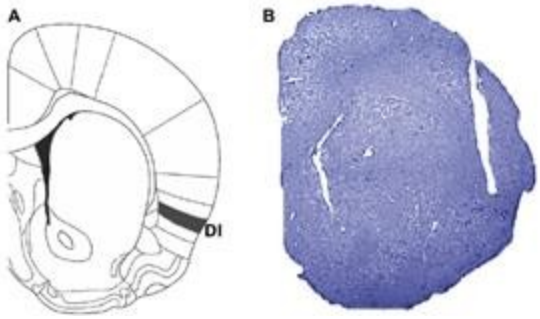

Figura 2. Localización de los implantes de cánulas dentro de la corteza insular. A. Esquema de una sección cerebral coronal mostrando el área disgranular de la corteza insular. B. Tinción Nissl representativa de un implante de cánula dentro del área disgranular de la corteza insular.

7.1.5 Curso Temporal

En esta figura podemos observar la metodología de los objetivos realizados. En rojo podemos ver la línea temporal para animales sin implante quirúrgico, y en azul podemos observar la línea temporal de animales con implantes quirúrgicos.

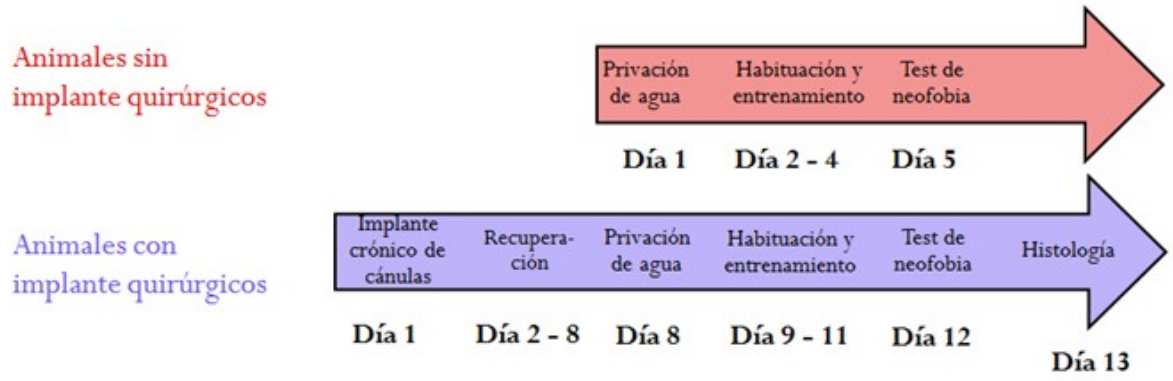

Figura 3. Curso temporal de experimento. Se observa la línea temporal experimental tanto para animales sin implante quirúrgico (rojo) y para animales con implantes quirúrgicos (azul)

7.2 Determinar si la actividad adrenérgica periférica aumenta la neofobia gustatoria.

Se probó el efecto periférico de la actividad adrenérgica en neofobia gustatoria. Para ello, se realizó una curva dosis respuesta de EPI subcutánea en diferentes animales, expuestos a un contexto de baja exaltación.

Para esto se habituó a los animales tal como fue descrito en 7.1.3 y el día del test se inyectaron subcutáneamente distintas dosis de EPI (0.001, 0.01, 0.1, 1 mg/Kg). Tras la inyección se esperó 30 min y se realizó el test de neofobia en un contexto de baja exaltación. Como grupo control se inyectó el vehículo de la EPI que correspondió a una solución salina 0.9%. Cada experimento consistió en dos repeticiones de 5 animales cada uno.

Las dosis utilizada se escogieron por una extensa búsqueda bibliográfica, al igual que los 30 min entre la inyección y el test de neofobia, los que permiten alcanzar el máximo plasmático de EPI (Flint y cols., 2007; Sadowski y cols., 2009).

7.3 Determinar si la actividad adrenérgica en la corteza insular aumenta la neofobia gustatoria.

Para determinar si la actividad adrenérgica en la corteza insular aumenta la neofobia gustatoria se llevaron a cabo dos experimentos. En el primero se microinyectó una curva dosis respuesta de 1, 10 y 100 µg intrainsular de NE y se compararon con un grupo control (solución salina 0.9%) en un ambiente de baja exaltación. El objetivo es determinar si la actividad adrenérgica en la ínsula puede aumentar la respuesta ansiosa en un ambiente de baja exaltación, simulando el efecto que tiene el contexto de alta exaltación. En un segundo experimento, se microinyectó una curva dosis respuesta de propanolol intrainsular (1, 5 y 10 µg) comparada con vehículo (solución salina 0.9%), con la finalidad de determinar si la ínsula modula el efecto de hiponeofagia inducido por el ambiente de alta exaltación. En ambos casos, se utilizaron animales con implantes crónicos de cánulas en la ínsula. Durante el entrenamiento se les limpió la cánula con un dummy diariamente. El día del test fueron inyectadas intracranealmente las dosis de NE o propranolol en la corteza insular, con un microinyector de Hamilton (kd Scientific). Se esperó 5 min y se procedió a realizar el procedimiento de neofobia. Cada experimento consistió en dos repeticiones de 5 animales cada uno.

Una vez realizado el experimento, se procedió a realizar la histología a través de los pasos descritos anteriormente en el punto 7.1.4. Las dosis a utilizadas se escogieron por una extensa búsqueda bibliográfica y corresponden a las más usadas en la literatura (Canal y cols., 2007; Tuinstra y cols., 2002; Roozendaal y Cools, 1994).

7.4 Determinar si el efecto periférico de la actividad adrenérgica está mediado por norepinefrina intrainsular.

Para determinar si el efecto sistémico de la actividad adrenérgica está mediado por actividad adrenérgica intrainsular se llevaron a cabo dos experimentos. En el primero, se inyectó EPI sistémica, seguida de propranolol intrainsular y se presentó el gusto en un ambiente de baja exaltación. Se usó la dosis más efectiva de EPI encontrada en 7.2 y una curva dosis respuesta de propranolol intrainsular, la misma usada en el 7.3. Basado en la literatura, las dosis que fueron utilizadas son 1, 5 y 10 µg de propranolol y 0.1 mg/Kg de EPI.

En un segundo experimento se administró oralmente propranolol y se microinyectó NE intrainsular en un ambiente de alta exaltación. Se utilizaron animales con implantes crónicos de cánulas, y se les limpio la cánula con un dummy diariamente. El día del test fue inyectado de forma subcutánea EPI o propranolol oral, 30 y 60 minutos antes del test, respectivamente. Cinco minutos antes del test fueron microinyectadas en la corteza insular las dosis de NE o propranolol, dependiendo de cuál fue el experimento, y se procedió a realizar el test de neofobia. Cada experimento consistió en dos repeticiones de 5 animales cada uno.

Al término de cada experimento se procedió a realizar la histología siguiendo los pasos descritos anteriormente en el punto 7.1.4.

7.5 Análisis estadístico.

Los datos se expresaron como media ± SEM. Las diferencias estadísticas fueron evaluadas por la prueba de Mann-Whitney y por análisis de la varianza (ANOVA) para comparaciones múltiples, seguido de un *post hoc* de Bonferroni. Las diferencias se consideraron significativas cuando $p < 0.05$. Los valores de p en el texto se escriben *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

8. Resultados

8.1 Hiponeofagia

Para determinar si el estrés genera un aumento en la aversión en el test de neofobia gustatoria, se tomó la decisión de utilizar un grupo de animales sin implantes crónicos de cánulas. Estos animales fueron habituados al investigador, para así disminuir el posible estrés y evitando de esta manera un enmascaramiento de los resultados.

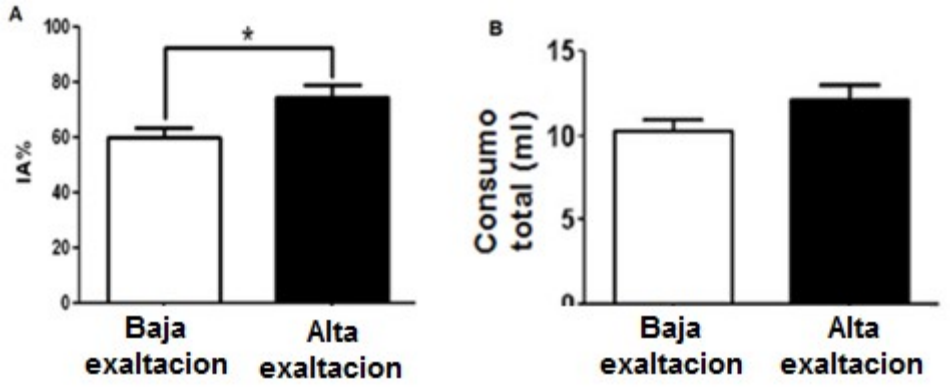

Figura 4. Efecto del estrés sobre el test de neofobia gustatoria. A. Efecto del estrés sobre el modelo de neofobia gustatoria. Se observa un aumento en el índice de aversión respecto al contexto de baja exaltación. (N= 10) B. Consumo total de líquido en un contexto de baja y alta exaltación.

Se observa un aumento significativo en la aversión provocado por un contexto estresante (hiponeofagía) al compararlo en un contexto de baja exaltación (Fig. 4). Esto se ve reflejado en el índice de aversión el cual fue cercano a un 60% en dicho contexto y aumento a un porcentaje cercano al 80% (Baja exaltación = 60% ± 3.6; Alta exaltación =75% ± 4.5; n = 10).

Una de las formas de asegurar que estas variaciones en la aversión fuesen realmente debidas a los compuestos, fue evaluando el consumo total de líquido. Se

utilizó este parámetro debido que existe la probabilidad que las drogas afecten la capacidad de los animales a beber o incluso afecten su necesidad de líquido.

No se observaron cambios significativos en el consumo de líquido (Baja exaltación = 12 ± 0.8 y alta exaltación = 10 ± 0.7; $p > 0.05$)

8.2 Objetivo específico 1: Determinar si la actividad adrenérgica periférica aumenta la neofobia gustatoria.

Para evaluar si la actividad adrenérgica periférica genera un aumento en la aversión se tomó la decisión de utilizar un grupo de animales sin implantes crónicos de cánulas, los cuales fueron sometidos al test de neofobia gustatoria. Estos animales también fueron habituados al investigador.

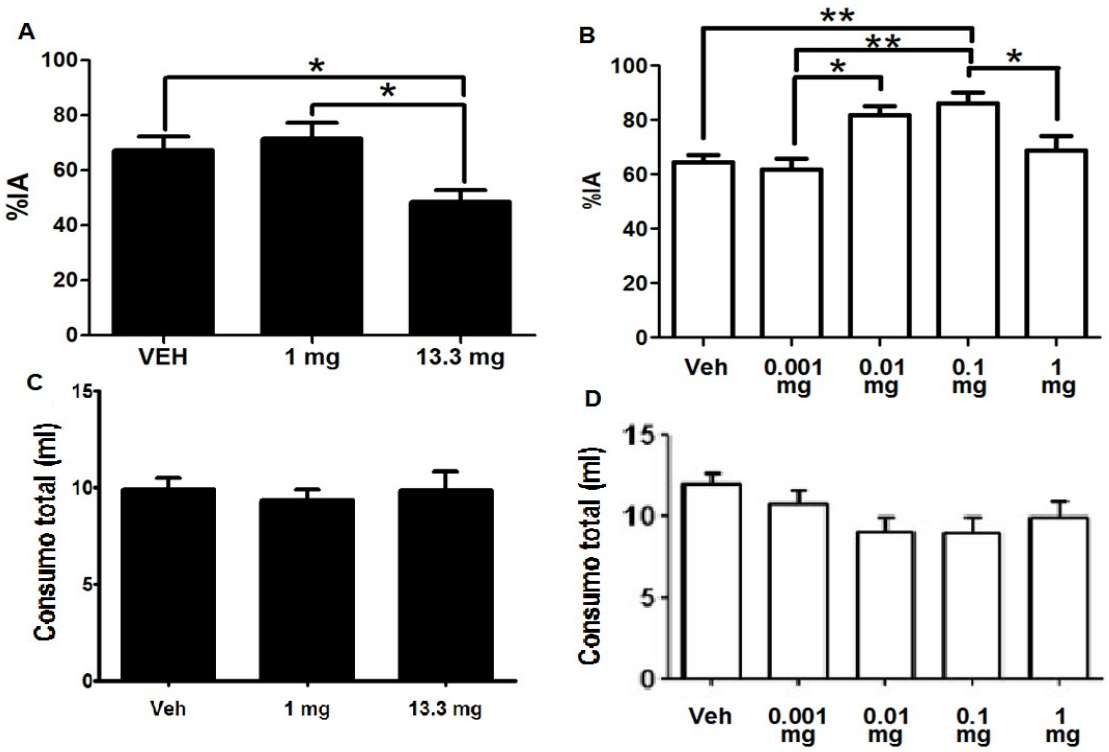

Figura 5. Efecto de epinefrina periférica en neofobia gustatoria. A. Diferentes dosis de propranolol oral en un contexto de alta exaltación. Dosis de 13.3 mg/Kg de propranolol inducen una disminución significativa de la neofobia gustatoria. (*N* = 12, 9, 8; ****p* < 0.001) B. Curva dosis respuesta de epinefrina periférica en un contexto de baja exaltación. Dosis de 0.1 mg de epinefrina inducen un aumento significativo de la neofobia gustatoria. (*N* = 9, 10, 9, 10, 10; ****p* < 0.001) C y D Consumo total de líquido en experimentos de propranolol oral y epinefrina periférica respectivamente.

En la figura 5A se muestran los resultados de la administración vía oral del antagonista β- adrenérgico, propranolol y se realizó el test de neofobia en un contexto de alta exaltación. Como se esperaba, la dosis de 13.3 mg/Kg logró disminuir la aversión en los animales, alcanzando valores de 50%, los cuales fueron significativos respecto al control (de 70%) y de la misma manera respecto a dosis de 1 mg/Kg. (Veh = 67 ± 4.8%; 1 mg/Kg = 72 ± 5.5%; 13.3 mg/Kg = 48.63 ± 4.2%; n = 12, 9, 8 respectivamente)

En otro grupo de animales se realizaron inyecciones subcutáneas de distintas dosis de EPI (Fig. 5B). Se observó una tendencia a aumentar la neofobia en los grupos que fueron inyectados de manera subcutánea con EPI. Sin embargo, solo la dosis de 0.1 mg logró ser significativamente distinta respecto al control, alcanzando una aversión alta (86%) y el grupo control alcanzó un índice de aversión cercano al 60%, lo que indicaría que la EPI de forma periférica es capaz de aumentar la aversión de los animales (Veh = 64 ± 2.8%; 0.001mg/Kg = 62 ± 4.3%; 0.01mg/Kg = 82 ± 3.3%; 0.1mg/Kg = 86 ± 3.8%; 1mg/Kg = 69 ± 5.6%; n = 9, 10, 9, 10, 10 respectivamente).

No hubo diferencia significativa en el consumo total al evaluar las diferentes dosis de propranolol oral (Veh Prop = 10 ± 0.8; Prop 1 mg = 9.3 ± 0.7; Prop 13.3 mg =10.4 ± 1.2, $p > 0.05$; Fig. 5C). De igual forma, las diferentes dosis de EPI periferica (Veh = 12 ± 0.6; 0.001 mg = 11 ± 0.8; 0.01 mg = 9 ± 0.9; 0.1 mg = 9 ± 1.0; 1 mg = 10 ± 1.0; $p > 0.05$; Fig. 5D) no variaron el consumo total de líquido.

8.3 Objetivo específico 2: Determinar si la actividad adrenérgica en la corteza insular aumenta la neofobia gustatoria.

Como se observa en la figura 6A, al analizar el efecto intrainsular del antagonista β-adrenérgico propranolol en un contexto de alta exaltación, podemos ver que la dosis de 5 µg produce una disminución significativa de la neofobia respecto al control de

vehículo en un contexto de alta exaltación (Veh = 58 ± 1.9%, 1 µg = 69 ± 2.6%; 10 µg = 60 ± 1.9%; 100 µg = 62 ± 2.7%; N = 12, 10, 10, 10 respectivamente).

Por otra parte, al examinar el efecto intrainsular de NE en un contexto de baja exaltación no se observan cambios ni tendencias en los grupos cuyas dosis fueron 10 µg y 100 µg (Fig. 6B), pero sí se observó un cambio significativo de la dosis de 1 µg con respecto al control, reflejado en un aumento en la neofobia gustatoria en el grupo con microinyección intrainsular de 1 µg (Veh = 67 ± 5.3%; 1 µg = 56 ± 4.2%; 5 µg = 48 ± 4.1%; 10 µg = 59 ± 4.5%; N = 12, 11, 11, 13 respectivamente)

También fue medido el consumo total de líquido de cada experimento. No hubo diferencias significativas tanto para la curva dosis respuesta de propranolol intrainsular (Veh=10 ± 0.7; 1 µg = 12 ± 0.9; 5 µg =10 ± 1.0; 10 µg =12 ± 1.2; $p > 0.05$; Fig. 6C), como para la microinyección intrainsular de NE (Veh = 12 ± 0.4; 1 µg = 14 ± 0.7; 10 µg = 13 ± 0.6; 100 µg = 11 ± 1.1; $p > 0.05$; Fig. 6D)

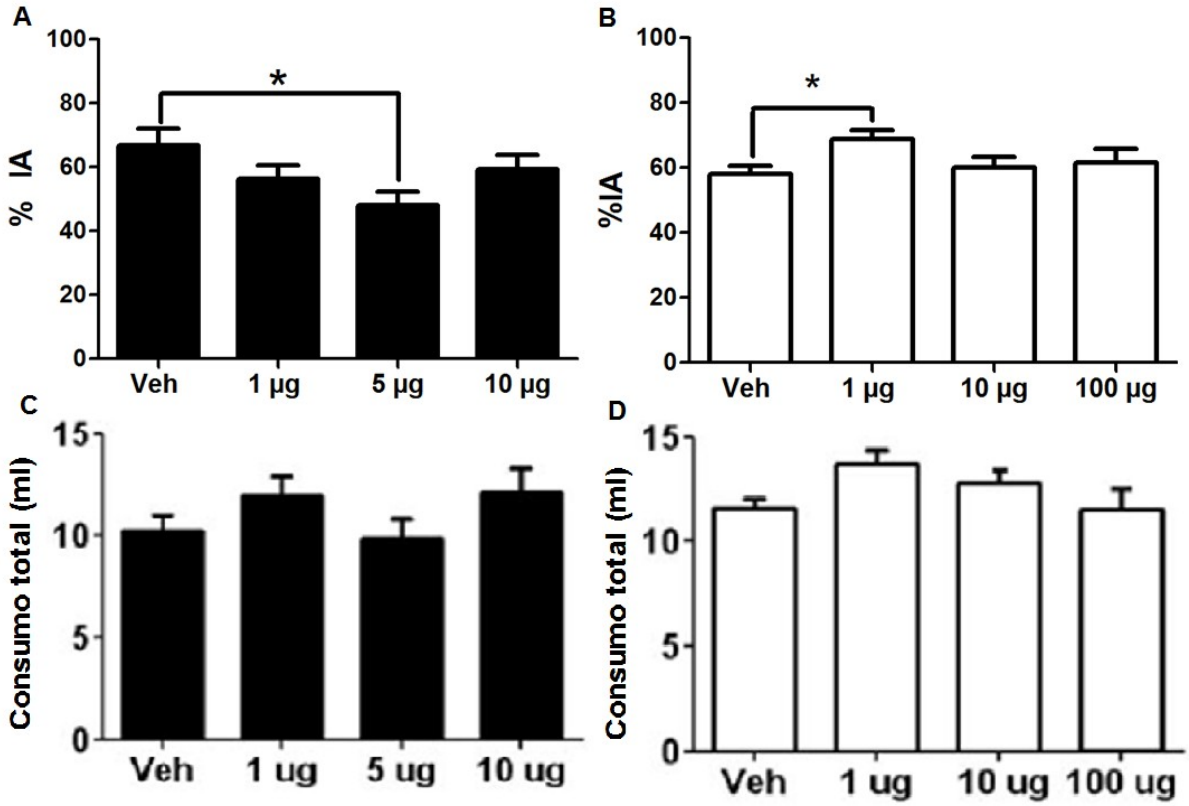

Figura 6. Efecto de la actividad adrenérgica insular en la neofobia gustatoria. A. Curva dosis respuesta de propranolol intrainsular en neofobia gustatoria presentada en un contexto de alta exaltación. Dosis de 5 µg induce una disminución significativa en la neofobia gustatoria (*N* = 12,10,10,10;*$p < 0.05$). B. Curva dosis respuesta de norepinefrina intrainsular en neofobia gustatoria presentada en un contexto de baja exaltación. Dosis de 1 µg induce un aumento significativo en neofobia (*N* = 12, 11, 11,13;**$p < 0.01$) C y D Consumo total de líquido en experimentos de propranolol intrainsular y norepinefrina intrainsular respectivamente.

8.4 Objetivo específico 3: Determinar si el efecto periférico de la actividad adrenérgica está mediado por norepinefrina intrainsular.

Una vez comprobada la participación de la actividad adrenérgica en la corteza insular y además sabiendo que esta actividad modula el comportamiento de neofobia gustatoria en un contexto de alta exaltación, se decidió explorar si la actividad adrenérgica periférica estaría mediando el aumento o disminución de la aversión vía modulación adrenérgica en la corteza insular.

Para realizar lo antes mencionado se procedió a la implantación crónica de cánulas en animales. En esta ocasión los animales no solo fueron microinyectados sino que, también se les suministró de manera periférica las dosis efectivas tanto de EPI como de propranolol encontradas en el punto 7.2, dosis de EPI 0.1 mg/Kg y dosis de propranolol 13.3 mg/Kg.

Como se puede observar en la figura 7A, al administrar propranolol de manera sistémica en un contexto de alta exaltación (13.3 mg/Kg) y microinyectar una dosis efectiva de NE intrainsular (1 µg/0.5 µl). La dosis efectiva de NE es capaz de bloquear el efecto de disminución de la aversión generado por el propranolol oral, el cual alcanzó una aversión cercana al 50%, al punto de igualar sus niveles al del control, el cual demostró un aversión cercana al 60 – 70 % (Veh prop + Veh NE = 64 ± 2.1; Prop + Veh NE = 52 ± 3.7; Prop + NE = 58 ± 5.2; *N* = 11, 10 y 7 respectivamente)

Para verificar que efectivamente la actividad adrenérgica periférica modula vía corteza insular el comportamiento de neofobia gustatoria, se decidió dar EPI de manera sistémica (0.1 mg/Kg) en un contexto de baja exaltación (Fig. 7B), se genera un aumento en la aversión cercana a 70%, el cual puede ser bloqueado por una

microinyeccion intrainsular de propranolol en sus distintas dosis, llegando a niveles de aversión cercanos al 50% (Veh Epi + Veh Prop = 61 ± 1.6%, Epi + Veh Prop = 70 ± 2.9%; Epi + Prop 1 mg = 48 ± 2.7%; Epi + Prop 5 mg = 54 ± 2.7%; Epi + Prop 10 mg = 55 ± 5.5%; *n* = 8, 7, 8, 7, 7 respectivamente).

Finalmente se midió el consumo total de líquido. Este no presento diferencias significativas ni para el experimento de propranolol sistémico y NE intrainsular (Veh Prop + Veh NE = 10 ± 0.6; Prop + Veh NE = 10 ± 0.7; Prop + NE = 8 ± 0.9, $p > 0.05$; Fig. 7C), ni para el experimento de inyección de EPI y propranolol intrainsular (Veh Epi + Veh Prop = 12 ± 0.6; Epi + Veh Prop = 13 ± 1.0; Epi + Prop 1 µg = 13 ± 1.2; Epi + Prop 5 µg = 13 ± 0.8; Epi + Prop 10 µg = 14 ± 1.1, $p > 0.05$; Fig. 7D).

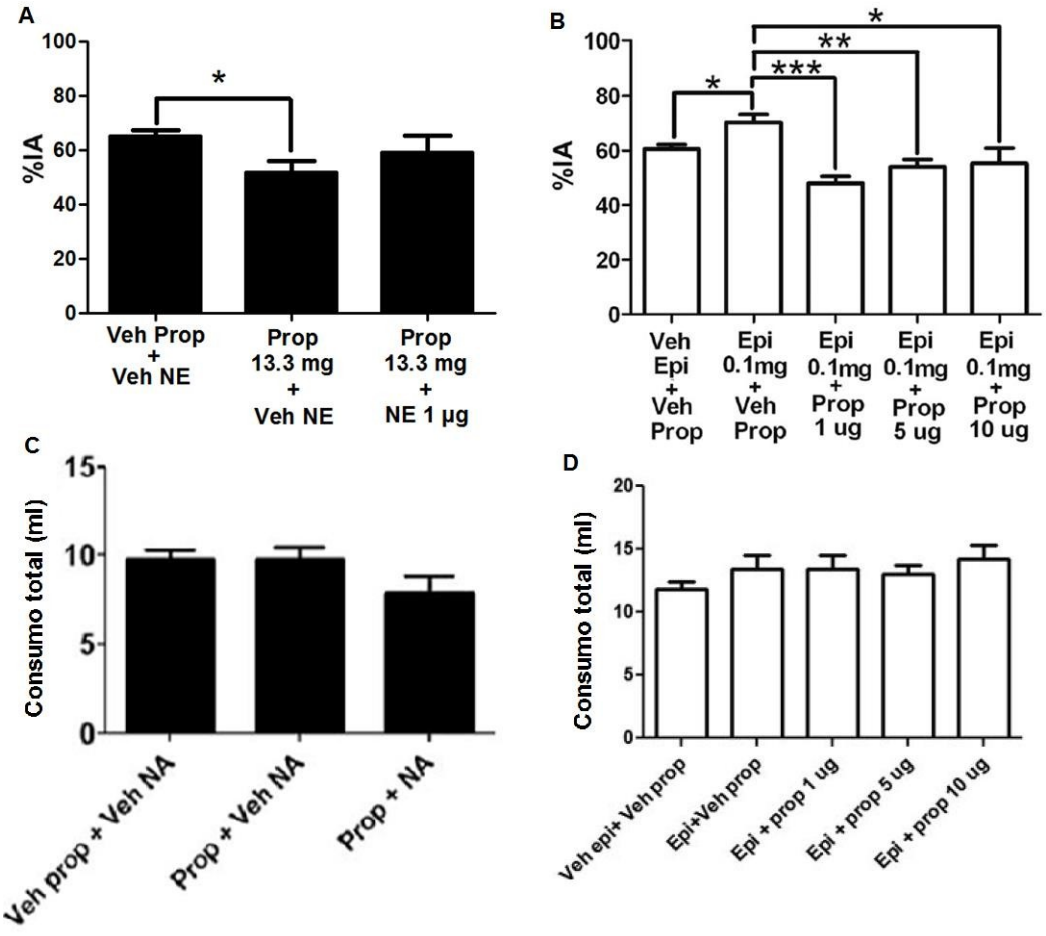

Figura 7. Efecto de epinefrina periférica en neofobia gustatoria y su modulación por la corteza insular. A. I Inhibición del efecto de 13.3 mg de propranolol oral por norepinefrina intrainsular. Propranolol oral induce una disminución significativa en la neofobia gustatoria, efecto que fue bloqueado por norepinefrina intrainsular. (Prop + NA; *N* = 11,10,7;**p* < 0.05; ***p* < 0.01; ****p* <

0.001). B. Inhibición del efecto de 0.1 mg de epinefrina periférica por propranolol intrainsular. Propranolol intrainsular induce un bloqueo significativo sobre la neofobia gustatoria, en animales con inyección subcutánea de epinefrina. (Epi+Veh Prop;(N = 8, 7, 8, 7, 7;**p < 0.01). C y D Consumo total de líquido en experimentos de propranolol oral con norepinefrina intrainsular y epinefrina periférica con propranolol intrainsular respectivamente.

9. Discusión

Nuestros resultados sugieren que neofobia gustatoria e hiponeofagia dependen de la actividad adrenérgica periférica. De hecho, neofobia gustatoria en un ambiente de baja exaltación fue incrementado por administración exógena de EPI a niveles similares a los obtenidos en ambientes de alta exaltación (Fig. 5B), mientras la administración oral de propranolol fue capaz de disminuir el aumento en neofobia producido por la presentación de gusto nuevo en el ambiente de alta exaltación (Fig. 5A).

Estudios previos han demostrado que la EPI es capaz de modular diferentes comportamientos en el individuo, como humor, vigía, memoria y respuesta al estrés (Charney y cols., 2004; Kudielka y cols., 2004; Mc Burnett y cols., 2005; McGaugh, 2000; Mezzacappa y cols., 1999). No obstante, esta hormona no es capaz de atravesar la barrera hematoencefalica (Kostrzewa, 2007) y por consiguiente requiere de mecanismos de señalización que le permitan lograr sus efectos centrales. El incremento de EPI circulante activa receptores β- adrenérgicos en la columna intermediolateral de la espina dorsal para estimular el nervio vago y evocar la liberación de NE desde proyecciones vagales aferentes al núcleo del tracto solitario o el locus coeruleus (Liang, y cols. 1990; Miyashita y Williams, 2006; Mravec, 2011; Williams y cols. 1998). La activación de estos núcleos genera un aumento de NE desde fibras eferentes, las cuales pueden estimular neuronas en la amígdala basolateral, incrementando su excitabilidad e iniciando cascadas de señalización activando muchas otras zonas cerebrales (Roozendaal y cols. 2009), incluyendo la corteza insular (revisado en Stehberg y Moraga-Amaro, 2012) y el hipotálamo, pudiendo aumentar la actividad simpática y gatillar la secreción de CRH con la consiguiente activación del eje HPA y secreción de glucocorticoides. Este modelo es el que mejor puede explicar nuestros resultados. Nuestros resultados sugieren que la manipulación adrenérgica de la insula modula no solamente neofobia (Fig. 6), sino también los efectos de la actividad adrenérgica periférica en neofobia (Fig. 7). Esto quiere decir que la activación adrenérgica periférica induce activación adrenérgica insular. También es posible que la regulación de este comportamiento esté mediado por interacción entre sistemas

monoaminérgicos. El propranolol, por ejemplo, a través de mecanismos serotoninérgicos podría atenuar el aumento de la neofobia inducido por un estrés inmediato (Shepard, y cols., 1982). Este punto se sustenta en trabajos en que ratones knock out, para el receptor de serotonina 5-HT$_{1a}$, incrementan su hiponeofagia (Gross y cols., 2000). Por otra parte agonistas de receptores 5-HT$_{1a}$ estimulan la liberación de NE en el cerebro (Hajós-Korcsok y cols., 1999), y de la misma manera la actividad adrenérgica mantiene la excitabilidad de las neuronas serotoninérgicas (Alojado y cols., 1994; Trulson y Fedrerickson, 1987) sugiriendo un trabajo en conjunto de ambos sistemas.

El que un aumento de EPI circulante produzca un aumento del temor o miedo, visto aquí como un aumento de neophobia tras la inyección de EPI, ya había sido reportado en humanos anteriormente, donde se propuso que podría deberse a un aumento de la activación simpática en respuesta a la EPI (Mezzacappa y cols., 1999).

Los resultados de la presente tesis no son suficientes para demarcar los mecanismos que subyacen el fenómeno descrito. Por un lado, el propranolol oral podría estar inhibiendo la respuesta emocional del ambiente de exaltación por medio de disminuir la respuesta cardíaca sin necesariamente estar afectando la actividad cerebral. Por ejemplo, atenolol, bloqueador beta-adrenérgico que no atraviesa la barrera hematoencefálica produce una disminución de la alerta subjetiva en humanos (Currie y cols 1988), posiblemente por una disminución en amplitud de la respuesta simpática. No obstante, los efectos observados podrían también deberse a un efecto ansiolítico del propranolol, el que ya ha sido descrito en humanos (Currie y cols., 1988).

La disminución en hiponeofagia por administración de propranolol también podría deberse a un bloqueo de los receptores β adrenérgicos en la columna celular intermediolateral, impidiendo la propagación de la respuesta adrenérgica (Liang, y cols., 1990; Miyashita y Williams, 2006; Mrave, 2011; Williams y cols., 1998). También el propranolol podría estar actuando en áreas del sistema límbico como la amígdala o la Insula bloqueando los efectos ansiogénicos del ambiente novedoso.

Nuestros resultados concuerdan con los de Shepard y cols. 1982, en donde se demostró una disminución en la neofobia en animales sometidos a un estrés contextual pero que anteriormente fueron inyectados de manera sistémica con antagonistas adrenérgicos.

Como se mencionó, las catecolaminas activan diversas zonas cerebrales, entre ellas la corteza insular. Se ha visto que microinyecciones de NE dentro de la corteza insular modulan la actividad barorrefleja (Alves y cols., 2009) y ritmo cardiaco (Alves y cols., 2011). Cambios en frecuencia cardíaca y presión arterial son procesos fisiológicos asociados a la actividad simpática, la cual se activa en situaciones estresantes (Tank y Wonk, 2015). Por lo tanto la actividad adrenérgica en la insula podría afectar ansiedad por medio de modular la respuesta visceral asociada al estrés.

La importancia de esta tesis es que es el primer estudio que muestra que la corteza insular es crítica en la respuesta ansiosa, conectando la insula con la la respuesta adrenérgica. Esto pone a la insula en una posición central en la regulación de la ansiedad, al menos en hiponeofagia. Son necesarios más estudios para poder determinar con exactitud los mecanismos y circuitos asociados al rol de la insula en ansiedad y su relación con el sistema adrenérgico.

10. Conclusiones

Este trabajo demuestra que la actividad adrenérgica periférica modula la hiponeofagia a través de la corteza insular. Esto indica que la corteza insular es un sitio cortical crítico para la modulación del efecto de exaltación de un ambiente nuevo. Además propone a la corteza insular como una nueva región cerebral crítica reguladora de la ansiedad o al menos a hiponeofagia y mediadora del efecto en ansiedad de la respuesta adrenérgica periférica a un ambiente novedoso.

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The insula modulates arousal-induced reluctance to try novel tastes through adrenergic transmission in the rat

*Sebastián Rojas, Raúl Diaz-Galarce, Juan Manuel Jerez-Baraona, Daisy Quintana-Donoso, Rodrigo Moraga-Amaro and Jimmy Stehberg**

Laboratorio de Neurobiologia, Centro de Investigaciones Biomedicas, Universidad Andres Bello, Santiago, Chile

Reluctance to try novel tastes (neophobia) can be exacerbated in arousing situations, such as when children are under social stress or in rodents, when the new taste is presented in a high arousal context (HA) compared to a low arousal context (LA). The present study aimed at determining whether adrenergic transmission at the Insula regulates the reluctance to try novel tastes induced by arousing contexts. To this end, a combination of systemic and intra-insular manipulations of adrenergic activity was performed before the novel taste (saccharin 0.1%) was presented either in LA or HA contexts in rats. Our results show that systemic adrenergic activity modulates reluctance to try novel tastes. Moreover, intra-insular microinjections of propranolol or norepinephrine (NE) were found to modulate the effects of arousing contexts on reluctance to try novel tastes. Finally, intra-insular propranolol blocked epinephrine-induced increased reluctance, while intra-insular NE blocked oral propranolol-induced decreases in reluctance and increased the reluctance to try novel tastes presented in low arousing contexts. In conclusion, our results suggest that the insula is a critical site for regulating the effects of arousal in the reluctance to try novel tastes via the adrenergic system.

Keywords: taste neophobia, reluctance, insular cortex, insula, adrenergic activity, arousal

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Federico Bermudez-Rattoni, Universidad Nacional Autónoma de México, Mexico

****Correspondence:***
Jimmy Stehberg, Laboratorio de Neurobiologia, Centro de Investigaciones Biomedicas, Universidad Andres Bello, Republica 217, Santiago, 8370146, Chile
jstehberg@unab.cl

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Introduction

Reluctance to novelty or Neophobia is a common adaptive behavior that ensures a cautious response to a novel stimulus until its safety has been ascertained. In animals including humans, consuming novel foods is usually accompanied by reluctance. For example, the experience of social pressure to consume novel foods in children can induce dislike for those foods (Batsell et al., 2002). The reluctance to try novel tastes was studied in the 60 s and 70 s when it was shown that domestic rats suffering from vitamin deficiency show strong decreases in their reluctance to try novel tastes, believed to ease the transition from vitamin deficient diets to novel ones (Rozin and Rodgers, 1967). Neophobia can be measured in a laboratory setting when animals (e.g., rodents) are exposed to a novel taste by itself or as a choice to water (Dunn and Everitt, 1988; Stehberg and Simon, 2011) and can be significantly increased if the novel taste is presented after stress (Dess, 1992) or in a novel context (high arousal context, HA) compared to a homecage (low arousal context, LA; De la Casa and Díaz, 2013).

This type of behavioral response has been used to measure anxiety in rodents. In fact, exposure to novel environments induces avoidance to consume novel foods (food chow rather than a particular taste dissolved in water) presented in those environments, behavior known as hyponeophagia and used to measure anxiety (Deacon, 2011).

Little is currently known about the brain areas and mechanisms that determine the reluctance to try novel tastes and how they are affected by stress and arousal. Lesion studies suggest that taste neophobia is modulated by the taste area within the Insular Cortex (or Insula, IC; Roman and Reilly, 2007; Lin et al., 2009; Stehberg et al., 2011; Moraga-Amaro et al., 2014). In the rat, the taste responsive area of the insula occupies the dysgranular and granular subregions dorsal to the rhinal fissure, from 1.5 mm posterior until 1.5 mm anterior to the Middle Cerebral artery (Paxinos and Watson, 2007), identified as taste responsive using intrinsic signal imaging (Accolla et al., 2007) as well as electrophysiologically (Yamamoto et al., 1980; Kosar et al., 1986; Ogawa et al., 1990; Bahar et al., 2004).

Although the neurotransmitters involved in the formation of novel taste memory, familiar taste memory and conditioned taste aversion have been studied in some detail (Berman et al., 2000; Bermúdez-Rattoni et al., 2004; Guzmán-Ramos et al., 2012), very few studies have attempted to study the neurotransmitters involved in taste neophobia and in arousal-induced increases in taste neophobia. This is probably due to the fact that the reluctance to try novel tastes only lasts seconds to minutes, but memories linger for days or even for a life time. Understanding the cortical mechanisms by which stress and arousal affects unconditioned spontaneous behaviors that depend directly on cortex, such as the reluctance to try novel tastes, could pave the way not only to the understanding of how stress directly affects behavior, including how it affects our perception of novelty, but may also lead to novel targets for the treatment of stress and anxiety disorders.

Early studies suggest that hyponeophagia depends on an intact brain norepinephrine system (Sahakian et al., 1983; Cole et al., 1988). During acute stress or arousal, epinephrine (EPI) is released by the adrenal medulla in response to an early sympathetic response, activating indirectly the release of brain norepinephrine (NE) from the locus coeruleus (McGaugh et al., 1996). Brain NE in turn activates the hypothalamic-adrenal axis (O'Connor et al., 2000), causes a shift from focused processing of sensory information to general scanning of the environment (Aston-Jones and Cohen, 2005; Roozendaal et al., 2009a; Sara, 2009) and enhances memory consolidation of stressful experiences (Liang et al., 1990; McGaugh and Roozendaal, 2002) in order to ensure a predictive or prompt response to a similar stressful situation in the future. NE enhancement of memory consolidation of emotionally arousing experiences is modulated by glucocorticoids (Roozendaal et al., 2009b), which have been shown to enhance memory in HA contexts or concomitant to NE administration in LA contexts (Roozendaal et al., 2006), suggesting that NE is capable of inducing high arousal experiences during low arousal situations.

Here we aim at studying the role of the adrenergic system at the insular cortex in modulating taste neophobia and arousal-induced increases in the reluctance to try novel tastes, by using a combination of systemic and intra-cortical manipulations of adrenergic activity before the presentation of saccharin as a novel taste, either at a LA (homecage) or HA context (lit novel clean cage).

Experimental Procedures

All procedures involving animals were in accordance with the U.S. National Institutes of Health guidelines and with approval of the Bioethical Committee of the Universidad Andres Bello. Male Sprague-Dawley rats (60 days old, 200–250 g) were caged individually with free access to food and water at 22°C, under a 12-h light-dark cycle. The rats remained in their homecage throughout the study and were removed only for surgery, and briefly for drug administrations and behavioral procedures.

Surgical Procedures

Cannulas were chronically implanted as described previously (Stehberg et al., 2012). In brief, animals were deeply anesthetized with a combination of ketamine/xylazine (0.02 μl/kg and 0.33 μl/kg, respectively), placed in a stereotaxic apparatus and their skull was surgically exposed after applying lidocaine subcutaneously into the scalp (2% HCl). Animals were then stereotaxically implanted bilaterally with a 21-gauge stainless steel guide cannulae positioned at 1.0 mm above the IC [1.2 mm anterior to Bregma, 5.4 mm lateral to the midline, and 6.7 mm ventral to the skull surface (Paxinos and Watson, 1986)]. The cannula was fixed to the skull using acrylic dental cement and secured by four screws. A stylus was placed inside the guide cannula to prevent clogging. After surgery, animals received a subcutaneous injection of ketoprofen 1% (Naxpet, laboratorio Drag pharma Chile Invetec S.A., Chile, 3 mg/Kg) and a dermal ointment consisting of a bacitracin/neomycin mixture (Laboratorio Chile, subsidiary of TEVA in Chile) was applied over the surgical area. Rats were given at least 7 days to recover from surgery before beginning experimental procedures and were handled for 10 min daily to habituate them to soft pressure on the implant throughout the recovery period to decrease microinfusion-related stress.

Intra-insular microinfusions were performed 10 min before taste presentation to avoid any behavioral effects from microinfusion discomfort. For microinfusion, the stylus was removed and a 25-gauge injection cannula was inserted through the guide cannula with its tip extending 1.0 mm beyond the guide cannula tip, into the dysgranular area of the insula, believed to be the taste responsive area according to previous literature using electrophysiology (Yamamoto et al., 1980; Kosar et al., 1986; Ogawa et al., 1990; Bahar et al., 2004) and intrinsic signal imaging (Accolla et al., 2007). Drugs were microinfused via the injection cannula, connected by PE20 tubing to Hamilton micro-syringes driven by an electronic microinfusion pump. Infusions consisted of 0.5 μl delivered at a rate of 0.25 ul/min to each hemisphere. Following drug microinfusion, the injection cannula was left in place for 3 min to allow the drug to diffuse away from the tip. Cannula placement was later determined by histology and maximal diffusion was verified by infusing 0.5 μl

of India ink in a group of five rats. The maximal diffusion spread observed included the caudal insular cortex (granular, dysgranular and agranular areas) and in some cases the claustrum, capsula externa, caudoputamen and somatosensory secondary cortex.

To evaluate to which extent adrenergic activity in the insular cortex modulates arousal-induced increases in the reluctance to try novel tastes, a combination of systemic and intra-insular manipulations of adrenergic activity were performed.

Drugs

All experimental groups were compared to a vehicle micro-infused control group. For the first experiment, intra insular propranolol (S(-)-Propranolol hydrochloride, Santa Cruz Biotechnology Inc., Dallas, TX, USA) was administered into the insular cortex at 1, 5 and 10 μg/0.5 μl dissolved in sterile saline. In the second experiment, an intra-insular microinfusion of 1 μg/0, 5 μl of NE (L-Norepinephrine hydrochloride, Sigma-aldrich, St. Louis, MO, USA) was performed. For the third experiment, systemic (oral) propranolol (Laboratorio Chile, subsidiary of TEVA, Chile) was administered dissolved in tap water for 1 h before taste presentation at a dose of 13.3 mg/kg, combined with an intra-insular microinfusion of NE at a dose of 1 μg/0.5 μl, 10 min prior to the neophobia test. For the fourth experiment, systemic epinephrine ((±) Epinephrine hydrochloride, Santa Cruz Biotechnology Inc., Dallas, TX, USA) was dissolved in sterile saline and administered i.p. 30 min before taste presentation using doses of 0.001, 0.01, 0.1 and 1 mg/kg. In the last experiment, systemic epinephrine (0.1 mg/kg) was followed by intra-insular microinjection of propranolol (1, 5 and 10 μg/0.5 μl).

Behavioral Testing

Rats underwent water restriction during behavioral procedures. On the training phase (day 1 to day 3), animals were trained to drink from two pipettes of 10 ml each and allowed to drink their daily fluid intake within a 10 min interval per day, for three consecutive days before the test. On the experimental day animals were randomly assigned to different groups and were exposed to saccharin 0.1% as the novel taste according to the method used in Stehberg and Simon (2011). In brief, 10 min long presentations of the taste (saccharin 0.1% dissolved in water) as a choice to water (taste presentation; six pipettes containing 5 ml each alternating taste or water). Free choice tests were used and preferred over one or two bottle tests to force rats to choose and drink from at least three pipettes to meet their daily needs of fluid intake (for a discussion on the benefits of using free choice tests see (Moraga-Amaro et al., 2014). During the test, animals were offered six pipettes of 5 ml each, with a total of 30 ml so that animals are exposed to a choice of the novel taste (saccharin) and water, but are not forced to drink from either. Thus, by giving them 30 ml to choose from, the animal will not be forced to drink from all the pipettes to quench its thirst, but may instead choose to drink up to 15 ml of any of the two tastes without needing to drink more than 5 ml of the other. This way, the animal is forced to choose and to try both tastes, but not forced to drink from any particular taste. To elicit the least neophobic response, the novel taste was presented in the LA context which was the animal's homecage. To elicit the greatest neophobic response a HA context was used, which consisted of a brightly illuminated (100 lux) clean cage void of wood shavings. Animals were put into the HA context 3 min before taste presentation and were monitored for any unusual behavior. Novel taste consumption was measured as aversion index, which represents the percentage of avoidance of the taste and is measured as the amount of water consumed divided by total liquid consumption.

Histology

At the end of all experiments, animals were anesthetized as above and perfused intracardially with saline and 4% buffered paraformaldehyde. Brains were extracted and left afloat in 30% sucrose until its density equaled that of sucrose. The brains were sectioned in a cryostat, Nissl-stained (cresyl violet) and examined using light microscopy for cannula placement and assessment of histological lesions, defined by tissue damage and/or gliosis. Animals with injection cannula tip outside the insular cortex or showing histological lesions beyond the size of the cannula tip and guide cannula diameter were excluded from the analysis.

Statistics

Data are expressed as mean ± standard error (SE). All experimental groups were tested for normality using the Kolmogorov-Smirnov test. Given that all data sets were found to be normally distributed, an unpaired Student's t test was used when comparing two different groups and considered significant at values of $p < 0.05$. For multiple comparisons, a One-way ANOVA with Bonferroni *post hoc* tests was used, considering α significance level of $p < 0.05$.

Results

Animals that showed a lesion larger than the size of the injection cannula or whose injection site was not located within the dysgranular or granular zones of the Insular cortex as described by Cechetto and Saper (1987) were excluded from analysis. For a scheme of the cannula locations included in the analysis see **Figure 1A** and for a representative microphotograph of a successful implant see **Figure 1B**.

As can be seen in **Figure 2A** (open bar), presentation of the novel taste in a LA context induced reluctance to consume the novel taste (neophobia), which is reflected by a slight aversion to the taste (drinking lower amounts of the taste compared to water) reaching 60% ± 3.6 aversion, which is greater than chance drinking (50%) or preferred drinking (any value <50%). However, when the novel taste is presented in a HA context, taste aversion increases significantly to 75% ± 4.5, which reflects what is here considered as arousal-induced increase in taste neophobia (see **Figure 2A**, closed bar; $N = 10$ each; $p < 0.05$). To ease viewing, in all graphs except for those showing total fluid intake, experiments performed in HA contexts are shown with closed bars, while those performed in LA contexts, with open bars.

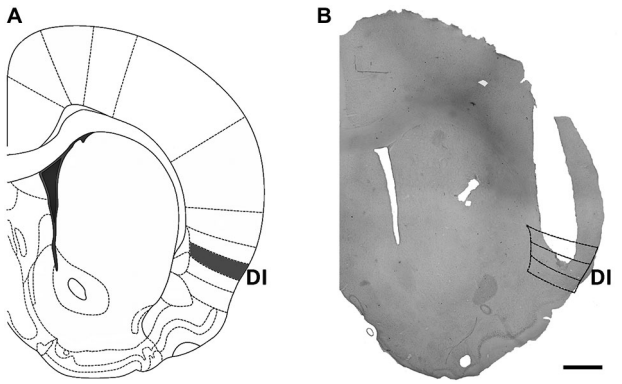

FIGURE 1 | Location of cannula implants within the insular cortex. **(A)** Scheme of a brain coronal section showing the dysgranular (gustatory) area of the insular cortex, which was targeted by cannula implants. **(B)** Microphotograph of a representative cannula implant into the dysgranular (taste) area of the insular cortex. Scale bar: 1 mm.

In the first experiment we aimed at testing whether adrenergic activity in the insula modulates arousal-induced increases in neophobia. For this aim propranolol was microinjected into the insular cortex prior to taste presentation in a HA context. As can be seen in **Figure 2B**, 5 μg of intra-insular propranolol produced a significant decrease in the reluctance to try the novel taste, suggesting that adrenergic activity at the insular cortex modulates arousal-induced increases in taste neophobia (for a curve-response graph see **Figure 2B**; Veh: 67 ± 5.3%, 1 μg: 56 ± 4.2% ($p < 0.05$), 5 μg: 48 ± 4.1%; 10 μg: 59 ± 4.5%; $n = 12$, 10, 10, 10 respectively).

To test if brain NE at the insular cortex can increase the reluctance to try novel tastes when the taste is presented in a LA context, in a manner similar to the arousal-induced increases in taste neophobia obtained when the taste is presented in a HA context, dose response curve of NE was microinjected into the Insular cortex, in a LA context (Exp. 2). As can be seen in **Figure 2C**, a significant increase in reluctance was obtained when microinjecting 1 μg of NE into the insula (for a dose-response curve see **Figure 2C**; veh: 58 ± 1.9%, 1 μg: 69 ± 2.6% ($p < 0.01$), 10 μg: 60 ± 1.9%, 100 μg: 62 ± 2.7%, $n = 15$, 11, 11, 13 respectively).

To test if the systemic effects of propranolol can be blocked by NE at the insular cortex, propranolol was administered orally (13.3 mg/kg) and 1 μg of NE was microinjected into

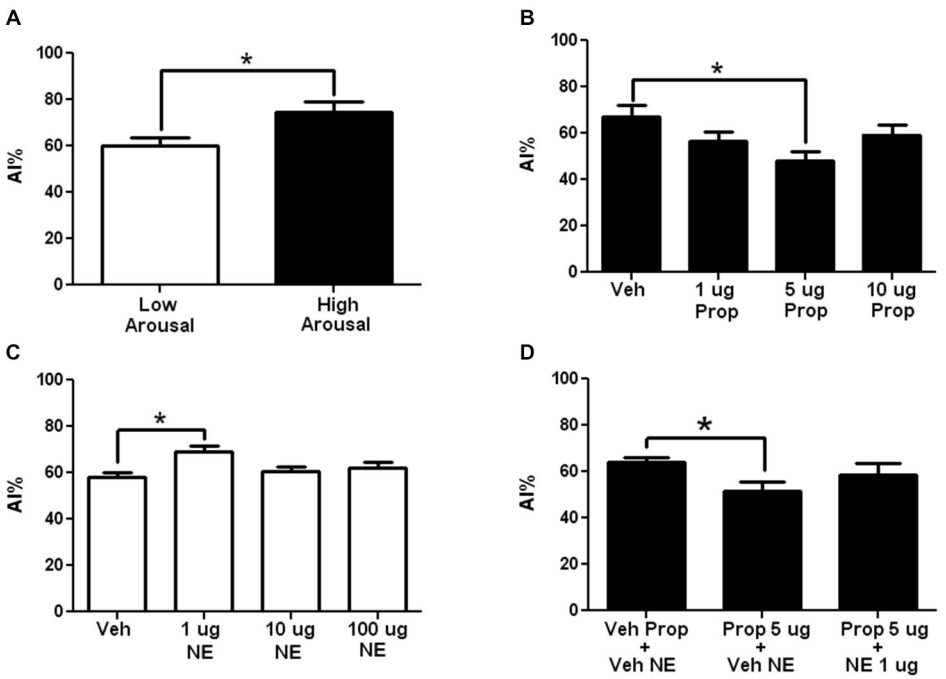

FIGURE 2 | The adrenergic antagonism by propranolol in arousal-induced taste neophobia and its modulation by the insular cortex. (A) Comparison of the neophobic response to a new taste (saccharin 0.1%) when it is presented in a low arousal context (LA) (open bar) compared to a high arousal context (HA) (closed bar). Note that arousal induces increases in taste neophobia ($N = 10$ each, $^*p < 0.05$). **(B)** Dose-response curve for Intra-insular propranolol in arousal-induced neophobia (taste presented in a HA context). Note that the 5 μg induced a significant decrease in arousal-induced neophobia ($N = 12$, 10, 10, 10; $^*p < 0.05$). **(C)** Dose response curve of Intra-insular norepinephrine in arousal-induced neophobia when taste is presented in a LA context. Note that 1μg induced a significant increase in neophobia. ($N = 15$, 11, 11, 13; $^{**}p < 0.001$) **(D)** Inhibition of the effects of 5 μg oral propranolol by intra-insular norepinephrine. Oral propranolol (Prop + Veh) induced a significant decrease in arousal-induced taste neophobia compared to Vehicle injected controls (Veh Prop-Veh NA), effect that was blocked when oral propranolol was followed by 5 μg of intra-insular norepinephrine (Prop + NA; $N = 11$, 10, 7; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

the insular cortex, in a HA context. A significant decrease in neophobia was found after propranolol was administered orally (see **Figure 2D**, Prop + Veh NE), effect that was blocked when oral propranolol was administered together with intra-insular NE (see **Figure 2D**; Veh prop + Veh NE: 64 ± 2.1, Prop + Veh NE: 52 ± 3.7 ($p < 0.05$), Prop + NE: 58 ± 5.2; $N = 11$, 10 and 7 respectively).

To test whether systemic adrenergic activity may affect taste neophobia, epinephrine was administered systemically in a LA context. Systemic administration of 0.1 mg/Kg epinephrine induced a statistically significant increase in neophobia to the taste, compared to vehicle injected controls (**Figure 3A**; Veh: 64 ± 2.8%, 0.001 mg/Kg: 62 ± 4.3%, 0.01 mg/Kg: 82 ± 3.3%; 0.1 mg/Kg: 86 ± 3.8% ($p < 0.01$); 1 mg/Kg: 69 ± 5.6%; $n = 9$, 10, 9, 10, 10 respectively). The 0.1 mg/kg dose had also significant differences from the 0.001 mg/Kg ($p < 0.01$) and 1 mg/Kg ($p < 0.05$) doses, while the dose of 0.01 mg/Kg showed a statistically significant difference with the 0.001 mg/kg dose ($p < 0.05$).

To determine whether the increase in reluctance to try novel tastes induced by systemic epinephrine is mediated by brain NE at the insular cortex, propranolol was microinjected into the insular cortex before taste presentation in a LA context with prior systemic injection of epinephrine. The vehicle injected group showed low neophobia as expected from a LA context (veh epi+veh prop), which was significantly increased in response to systemic epinephrine (epinephrine + Veh Prop; $p < 0.05$). All doses of intra-insular propranolol induced a significant decrease in neophobia, reaching chance drinking levels and blocking completely the effects of systemic epinephrine (for a dose-response curve see **Figure 3B**; Veh Epi + Veh Prop: 61 ± 1.6%, Epi + Veh Prop: 70 ± 2.9% (compared to veh; $p < 0.05$); Epi + Prop 1 μg: 48 ± 2.7% (compared to epi+veh prop; $p < 0.001$), Epi + Prop 5 μg: 54 ± 2.7% (compared to epi+veh prop; $p < 0.01$); Epi + Prop 10 μg: 55 ± 5.5% (compared to epi+veh prop; $p < 0.05$); $n = 14$, 8, 7, 8, 7 respectively). For a summary of results see **Table 1**.

To ensure that the drugs used did not affect the capacity of animals to drink or their thirst, total liquid intake (water + saccharin) was measured and compared between groups, for every experiment. No significant differences were found in total liquid intake between groups in the HA vs. LA neophobia experiment (HA = 10 ± 0.7; LA = 12 ± 0.8; $p > 0.05$; **Figure 3C** upper left), Propranolol dose response (Veh = 10 ± 0.7; 1 μg = 12 ± 0.9; 5 μg = 10 ± 1.0; 10 μg = 12 ± 1.2; $p > 0.05$; **Figure 3C** upper center), the systemic Propranolol and intra-insular NE experiment (Veh Prop + Veh NE = 10 ± 0.6; Prop + Veh NE = 10 ± 0.7; Prop + NE = 8 ± 0.9, $p > 0.05$; **Figure 3C** lower left), or the epinephrine and intra-insular propranolol experiment (Veh Epi + Veh Prop = 12 ± 0.6; Epi + Veh Prop = 13 ± 1.0; Epi + Prop 1 μg = 13 ± 1.2; Epi + Prop 5 μg = 13 ± 0.8; Epi + Prop 10 μg = 14 ± 1.1, $p > 0.05$; **Figure 3C** lower right). There were non-significant differences in total fluid intake after intra-insular NE injections (Veh = 12 ± 0.4; 1 μg = 14 ± 0.7; 10 μg = 13 ± 0.6; 100 μg = 11 ± 1.1; $p > 0.05$; **Figure 3C** upper right) and also non-significant differences in total fluid intake after systemic epinephrine (veh = 12 ± 0.6; 0.001 mg = 11 ± 0.8; 0.01 mg = 9 ± 0.9; 0.1 mg = 9 ± 1.0; 1 mg = 10 ± 1.0; $p > 0.05$; **Figure 3C** lower center).

TABLE 1 | Summary of results.

Figure	Experiment	*N*	Groups	AI (%)	*p* value
2A	HA vs. LA	10	HA	60 ± 3.6	
		10	LA	75 ± 4.5	*0.0193
2B	intra-insular Propanolol dose response curve (HA)	12	Veh	67 ± 5.3	
		10	1 μg	56 ± 4.2	
		10	5 μg	48 ± 4.1	*0.0470
		10	10 μg	59 ± 4.5	
2C	Intra-insular Noradrenaline dose response curve (LA)	15	Veh	58 ± 2.6	
		11	1 μg	69 ± 2.6	**0.0082
		11	10 μg	60 ± 1.9	
		13	100 μg	62 ± 2.7	
2D	Inhibition of oral Propanolol by intra-insular Noradrenline (HA)	11	Veh prop + Veh NA	64 ± 2.1	
		10	Prop + Veh NA	52 ± 3.7	*0.0470
		7	Prop + NA	58 ± 5.2	
3A	Systemic Epinephrine dose response curve (LA)	9	Veh	64 ± 2.8	
		10	0.001 mg	62 ± 4.3	
		9	0.01 mg	82 ± 3.3	
		10	0.1 mg	86 ± 3.8	***0.0002
		10	1 mg	69 ± 5.6	
3B	Epinephrine (0.1 mg) + intra-insular Propanolol dose response curve (LA)	8	Epi + Veh prop	70 ± 3.8	
		7	Epi + Prop 1 μg	48 ± 2.7	**0.0022
		8	Epi + Prop 5 μg	54 ± 2.7	
		7	Epi + Prop 10 μg	55 ± 5.5	

*Depicted from left to right are: the Figure panel where experiment is shown (Figure), a description of the experiment (Experiment), the number of animals per group (N), the groups compared (Groups), the aversion index obtained (AI%) and the P value if significant (*if $p < 0.05$; **if $p < 0.01$; ***if $p < 0.001$).*

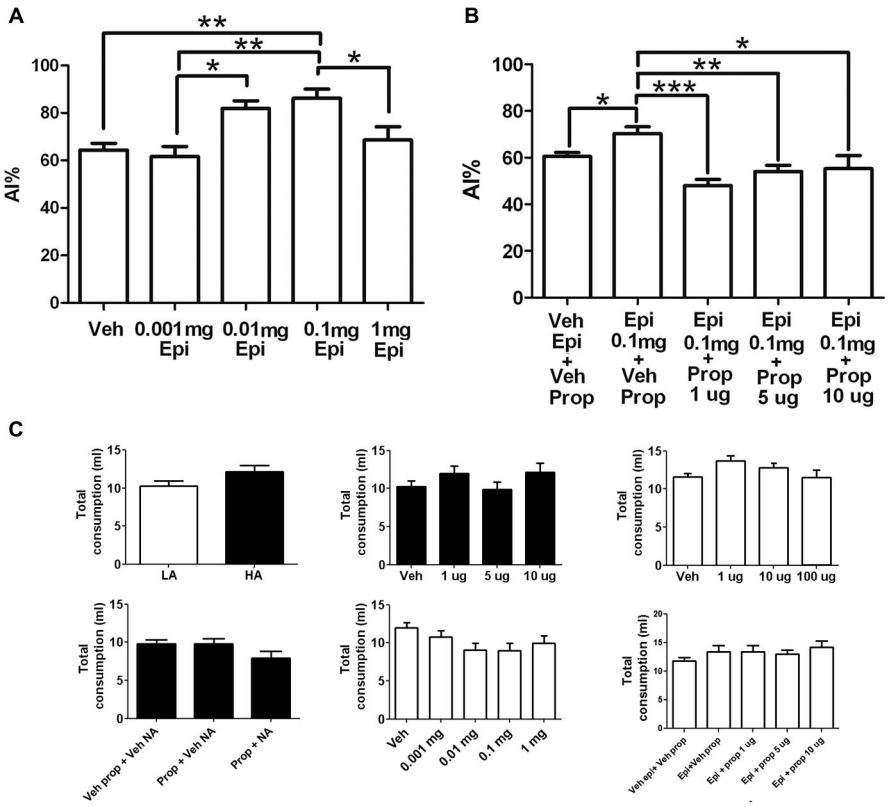

FIGURE 3 | Effects of systemic epinephrine in neophobia and its modulation by the insular cortex. (A) Dose response curve of systemic epinephrine in a LA context. Note that 0.1 mg of epinephrine induced a significant increase in taste neophobia (N = 9, 10, 9, 10, 10; $^{***}p < 0.001$) when the novel taste was presented in a LA context. **(B)** Dose response of intra-insular propranolol after a systemic epinephrine when novel taste is presented in a LA context. Note that 1μg of intra-insular propranolol (Epi + Prop 1μg) blocked the increase in neophobia induced by 0.1 mg of systemic epinephrine (Epi + Veh Prop; (N = 8, 7, 8, 7; $^{**}p < 0.01$). **(C)** Injections did not induce significant changes in total liquid consumption. Experiments in LA are shown in open bars, experiments performed in HA are shown in closed bars. Upper-middle: propranolol; left-upper right; noradrenaline; center-bottom: epinephrine. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Discussion

A summary of the experiments and results of this study can be found in **Table 1**. The present results suggest first, that adrenergic activity modulates the effects of arousal in taste neophobia, as systemic propranolol can reduce arousal-induced increases in neophobia (in a HA context), while epinephrine can increase neophobia in a LA context. Secondly, our results show that adrenergic activity at the insular cortex modulates arousal-induced increases in neophobia. This conclusion is supported by the fact that intra insular propranolol decreased reluctance to try novel tastes in HA contexts, while intra insular NE increased neophobia in a LA context. Both arousal-induced and epinephrine-induced increases in neophobia can be blocked by intra-insular propranolol, which may suggest that systemic adrenergic activity may be involved in arousal-induced increases in neophobia and that adrenergic activity at the insula may modulate this effect. This idea was further supported by the fact that intra-insular microinjections of NE produced an increase in taste neophobia when the taste is presented in LA contexts.

Evidence for a role of brain NE in food neophobia (or hyponeophagia) comes from studies showing that chemical NE depletion induces increased consumption of novel vs. familiar rat chow in novel contexts using 6-hydroxydopamine lesions of the noradrenergic bundle (Sahakian et al., 1983; Cole et al., 1988) or the olfactory bulb (Royet et al., 1983). Moreover, centrally acting propranolol and pindolol but not atenolol were able to decrease hyponeophagia (Shephard et al., 1982), while microinjections of NE into the basolateral amygdala induced an increase in food neophobia (Borsini and Rolls, 1984), suggesting that the Basolateral amygdala may also be involved in food neophobia. The amygdala shares several functions with the insular cortex and both interact extensively (Moraga-Amaro and Stehberg, 2012). In a study of Lin and Reilly (Lin and Reilly, 2012) using a combination of unilateral lesions of the insular cortex and amygdala, showed that Amygdala-gustatory insular cortex connections are necessary for taste neophobia.

From the present results it is possible to propose that during arousal, sympathetic activity will lead to the release of adrenal epinephrine, which in turn will trigger the release of

brain NE (McGaugh et al., 1996). Brain NE will be released at the insular cortex, triggering an increase in reluctance to try novel tastes. There may be other neurotransmitters, neuromodulatory systems and brain areas involved in this process, which have not been studied in the present report.

Distinguishing a role for adrenergic activity in neophobia *per se*, from arousal-induced increases in neophobia is challenging. When a taste is presented in the LA context (namely, the animal's homecage) the experience itself is—as the name implies—of "low arousal", not of "no arousal". This means that the decrease in neophobia induced by propranolol when the taste is presented in a LA context may reflect a role for the adrenergic system in neophobia *per se*, but may also reflect an effect of propranolol on the arousal—despite being low—that the presentation of the novel taste may induce.

In a study published by Roozendaal and Cools (1994), Wistar rats were placed in an openfield and divided according to their exploratory behavior (less or more than 8 min to habituate and 48 meters locomotion per 30 min) into high and low responders. Then they were microinjected into the basolateral amygdala with either beta-adrenergic antagonist propranolol or beta-adrenergic agonist isoproterenol and 5 min later presented with a choice of novel and familiar chow in a HA context. Interestingly, propranolol reduced food neophobia only in the high responders, while isoproterenol decreased food neophobia only in low responders (Roozendaal and Cools, 1994). This suggests that the effects of adrenergic manipulations may depend on the animals' responsiveness to stress or basal levels of anxiety. It remains to be determined whether this holds true for the insula adrenergic manipulations, or it may be unique to amygdala. If so, it may help distinguishing a role for the amygdala associating the taste response with the basal level of anxiety of each animal. In the present study no screening tests were performed to distinguish responders. More studies are required to assess this issue.

As can be seen in **Figure 3C**, the different injections did not interfere with the animals' capacity to drink or their thirst. This is important, as is widely known that exposure to novel contexts induces anxiety, while adrenergic activity also mediates anxiety (Nesse et al., 1984). Thus, here we show that the change in anxiety by contexts or drugs used affected the rats' preference for the novel taste, but not their capacity to drink or their overall thirst.

In conclusion, here we show that the adrenergic system modulates the effects of arousing contexts in the reluctance to try novel tastes via the insular cortex, suggesting that the insula may be a cortical site critical for modulating the effects of arousal in gustatory behavior. Given that hyponeophagia is used as a measure of anxiety, further studies are required to determine whether the role of the insular cortex may go beyond taste reluctance and modulate stress and anxiety.

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Restraint stress increases hemichannel activity in hippocampal glial cells and neurons

Juan A. Orellana[1], Rodrigo Moraga-Amaro[2], Raúl Díaz-Galarce[2], Sebastián Rojas[2], Carola J. Maturana[3], Jimmy Stehberg[2] and Juan C. Sáez[3,4]*

[1] Departamento de Neurología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile, [2] Laboratorio de Neurobiología, Centro de Investigaciones Biomédicas, Facultad de Ciencias Biológicas and Facultad de Medicina, Universidad Andres Bello, Santiago, Chile, [3] Departamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile, [4] Instituto Milenio, Centro Interdisciplinario de Neurociencias de Valparaíso, Santiago, Chile

Stress affects brain areas involved in learning and emotional responses, which may contribute in the development of cognitive deficits associated with major depression. These effects have been linked to glial cell activation, glutamate release and changes in neuronal plasticity and survival including atrophy of hippocampal apical dendrites, loss of synapses and neuronal death. Under neuro-inflammatory conditions, we recently unveiled a sequential activation of glial cells that release ATP and glutamate via hemichannels inducing neuronal death due to activation of neuronal NMDA/P2X$_7$ receptors and pannexin1 hemichannels. In the present work, we studied if stress-induced glia activation is associated to changes in hemichannel activity. To this end, we compared hemichannel activity of brain cells after acute or chronic restraint stress in mice. Dye uptake experiments in hippocampal slices revealed that acute stress induces opening of both Cx43 and Panx1 hemichannels in astrocytes, which were further increased by chronic stress; whereas enhanced Panx1 hemichannel activity was detected in microglia and neurons after acute/chronic and chronic stress, respectively. Moreover, inhibition of NMDA/P2X$_7$ receptors reduced the chronic stress-induced hemichannel opening, whereas blockade of Cx43 and Panx1 hemichannels fully reduced ATP and glutamate release in hippocampal slices from stressed mice. Thus, we propose that gliotransmitter release through hemichannels may participate in the pathogenesis of stress-associated psychiatric disorders and possibly depression.

Keywords: hemichannels, connexins, pannexins, stress, hippocampus, glia, neuron

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Andrei Belousov, University of Kansas Medical Center, USA

****Correspondence:***
Juan A. Orellana, Departamento de Neurología, Escuela de Medicina, Pontificia Universidad Católica de Chile, Marcoleta 391, Santiago 8330024, Chile
jaorella@uc.cl

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Introduction

Major depression disorder (MDD) is a disabling illness that adversely affects subject's family, behavior, mood, activity and physical health. In developed countries, around 3% of MDD patients commit suicide, whereas several studies show that around 60% of all suicide victims had previously suffered from MDD (Arsenault-Lapierre et al., 2004). Interestingly, ample evidence indicates that stressful life events increase the risk for MDD, including acute and chronic stress (Kessler, 1997; Kendler, 1998; Hammen, 2005; Hammen et al., 2009). The term stress defines all physiological and/or psychological responses to events that

require behavioral adjustment to overcome them (Sorrells et al., 2009; Popoli et al., 2011). Acute stress includes adaptive mechanisms necessary for survival, while chronic stress induces over-activation and dysfunction of stress-activated systems, resulting in further brain damage and depressive-like behavior (Sorrells et al., 2009; Popoli et al., 2011).

Restraint stress impairs both spatial hippocampal-dependent memory (Luine et al., 1994; Kleen et al., 2006) and hippocampal long-term potentiation (LTP; Pavlides et al., 2002; Alfarez et al., 2003). Such effects have been associated to retraction of apical dendrites as well as loss of synapses in the CA3 subregion of the hippocampus (Magariños and McEwen, 1995; Magariños et al., 1997). A proposed explanation is that these changes may be associated with dysregulated release of glutamate and NMDA receptor dysfunction (McEwen, 1999). Congruent with this idea, enhanced glutamate release in response to stress has been described (Gilad et al., 1990; Lowy et al., 1993), while NMDA but not AMPA receptors are reportedly involved in stress-related morphological changes in the hippocampus (Magariños and McEwen, 1995). Recently, we showed that amyloid-β peptide induces glutamate and ATP release via glial cell hemichannels, enhancing cell neuronal death by activation of NMDA/$P2X_7$ receptors (Orellana et al., 2011a,b). In the central nervous system (CNS), gliotransmitter release is in part mediated by the opening of hemichannels formed by connexins or pannexins (Wang et al., 2013b). These unopposed membrane channels serve as aqueous pores permeable to ions and small molecules, providing a diffusional pathway of exchange between intra- and extracellular compartments. In glial cells, hemichannels allow the release of gliotransmitters that are necessary for different brain functions including glucosensing (Orellana et al., 2012), ischemic tolerance (Lin et al., 2008), fear memory consolidation (Stehberg et al., 2012), neuron-glia crosstalk (Torres et al., 2012) and chemoreception (Huckstepp et al., 2010). However, several independent studies have pointed out that onset and progression of homeostatic imbalances observed during neurodegeneration could be associated to enhanced hemichannel activity in the CNS (Takeuchi et al., 2006; Thompson et al., 2008; Karpuk et al., 2011; Orellana et al., 2011a,b; Gulbransen et al., 2012; Burkovetskaya et al., 2014).

Stress activates microglia (Tynan et al., 2010), which release glutamate and/or ATP via hemichannels (Shijie et al., 2009; Sáez et al., 2013), whereas proinflammatory cytokines released by activated microglia enhance hemichannel activity of astrocytes (Orellana et al., 2011b). Astroglial hemichannels in turn mediate the release of gliotransmitters (Orellana and Stehberg, 2014), which are critical for synaptic transmission and plasticity (Perea et al., 2009). Thus, stress may alter glial cell hemichannel activity, leading to important alterations in neuronal networking and possibly contributing to stress-induced functional and morphological changes in neurons. Therefore, we decided to investigate whether stress modulates the functional activity of hemichannels in glial cells and neurons in the hippocampus. Here, restraint stress is shown to increase differentially the opening of hemichannels in glial cells and neurons depending on the restraint protocol. Interestingly, these responses were associated with increased release of glutamate and ATP through these channels.

Materials and Methods

Reagents and Antibodies

Gap26, TAT-L2 and ^{10}panx1 peptides were obtained from Genscript (New Jersey, USA). HEPES, DMEM, DNAse I, poly-L-lysine, CPP, A74003, MRS2179, brilliant blue G (BBG), oATP, ethidium (Etd) bromide, and probenecid (Prob) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT, USA). Penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Normal goat serum (NGS) was purchased from Zymed (San Francisco, CA, USA). $Cx43^{E2}$, a Cx43 hemichannel antibody to the second extracellular loop was kindly provided by Dr. Jean Jiang, Department of Biochemistry, University of San Antonio, USA (Siller-Jackson et al., 2008).

Animals and Restraint Stress Protocols

All animal experimentation were conducted in accordance with the guidelines for care and use of experimental animals of the National Institute of Health (NIH) and local guidance documents generated by the *ad hoc* committee of the Chilean Research Organization (CONICYT). The studies were performed according to protocols approved by the bioethical committee of Universidad Andrés Bello, Chile.

Wild-type C57BL/6 or $Panx1^{-/-}$ male mice weighting between 25 and 35 g were used. The generation of $Panx1^{-/-}$ (KO) mice has been described previously (Anselmi et al., 2008). Mice were housed individually in plastic homecages in a temperature controlled room at 24°C, under a 12 h:12 h illumination cycle (lights on at 8:00 AM). All animals were kept in individual cages throughout the study and had ad libitum access to standard rodent food pellets and tap water. Animals were maintained under standard laboratory conditions for at least 2 weeks before starting the stress protocol. To stress animals, we used a modified version of the restraint protocol described by Mozhui et al. (Mozhui et al., 2010). Animals were segregated in three groups: acute stress, chronic stress and control. For acute stress, animals were placed in ventilated 50 ml Falcon tubes only once for 2 h, prior to behavioral tests. For chronic stress, each mouse was placed into a tube for 2 h per day (14:00 P.M. to 16:00 P.M.) for 10 consecutive days before behavioral evaluations. Non-restrained mice (control group) remained in the home cage until behavioral evaluations.

Behavioral Evaluations

Open Field Test

Thigmotaxis was evaluated in the open field test, as reported previously (Takemoto et al., 2008; Ito and Ito, 2011). Animals were placed in the central zone of a plexiglas rectangular box ($40 \times 60 \times 60$ cm) and allowed to explore for 5 min, while being recorded digitally for subsequent off-line analysis. For analysis, the recorded trial was analyzed by a blinded investigator and the floor of the open field was virtually divided in the screen into

10 × 10 cm squares. Time spent in the periphery (thigmotaxis) and time spent in the center of the open field were measured.

Dark and Light Exploration Test

This test was performed as reported elsewhere (Crawley, 1981; Mathis et al., 1995). The dark and light box consisted of a plexiglas apparatus (50 × 30 × 20 cm) separated by two compartments: one dark (lacking illumination) with black walls (20 × 15 × 20 cm) and one lit compartment with transparent walls. Both compartments were connected by a small opening (6 × 6 cm) at the floor level. The lit compartment was brightly illuminated (∼1000 Lux) by a lamp from above. Mice were placed on the lit compartment looking opposite to the dark compartment and allowed to freely explore the apparatus for 5 min. Difference between total time in the lit compartment and the latency to enter the dark compartment for the first time was measured and plotted as "time in the lit" compartment. All trials were recorded digitally for subsequent off-line analysis by a blinded investigator.

Acute Hippocampal Slices

Mice were decapitated and brains were dissected and placed in ice-cold artificial cerebral spinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$, bubbled with 95% O_2/5% CO_2, pH 7.4. Hippocampal coronal slices (400 μm) were cut using a vibratome (Leica, VT 1000GS; Leica, Wetzlar, Germany) filled with ice-cold ACSF. The slices were transferred at room temperature (20–22°C) to a holding chamber and immersed in oxygenated ACSF, pH 7.4, for a stabilization period of 30 min before using them.

Dye Uptake and Confocal Microscopy

For "snapshot" experiments, acute slices were incubated with 100 μM Etd for 15 min in a chamber oxygenated by bubbling gas mixture (95% O_2 and 5% CO_2) into ACSF, pH 7.4. Slices were then washed five times with ACSF, fixed at room temperature with 4% paraformaldehyde for 30 min, rinsed extensively in PBS and stored overnight at 4°C in a cryoprotectant solution (30% sucrose). Next day, slices were frozen and dissected in 12–16 μm-thick sections with a cryostat. The sections were then mounted in Fluoromount and incubated in 0.1% PBS-Triton X-100 containing 10% NGS for 30 min. Afterwards, sections were incubated overnight at 4°C with either anti-Iba-1 polyclonal antibody (1:300, Wako), anti-GFAP monoclonal antibody (1:300, Sigma) or anti-NeuN monoclonal antibody (1:400, Chemicon) to detect microglia, astrocytes or neurons, respectively. All antibodies were diluted in 0.1% PBS-Triton X-100 with 2% NGS. After five rinses in 0.1% PBS-Triton X-100, sections were then incubated for 1 h at room temperature with goat anti-rabbit Alexa Fluor 488 (1:1,500), goat anti-mouse Alexa Fluor 488 (1:1,500) or goat anti-mouse Alexa Fluor 647 (1:1,500) antibody. After several washes, slices were mounted in Fluoromount, coverslipped and examined in a confocal laser-scanning microscope (Olympus Fluoview FV1000, Tokio, Japan). Stacks of consecutive confocal images taken with a 63 X objective at 500 nm intervals were acquired sequentially with two lasers (argon 488 nm and helium/neon 543 nm), and Z projections were reconstructed using Fluoview software. Dye uptake ratio was calculated as the subtraction (F−F0) between the fluorescence (F) from respective cell and the background fluorescence (F0) measured where no labeled cells were detected. At least six cells per field were selected from at least three fields in each hippocampal slice.

Measurement of Extracellular ATP and Glutamate Concentration

Acute hippocampal slices were immersed in oxygenated ACSF (as above), pH 7.4, at room temperature (20–22°C) for 30 min under control conditions or exposed to different agents. Then, extracellular ATP was measured using a luciferin/luciferase bioluminescence assay kit (Sigma-Aldrich), while extracellular levels of glutamate were determined using an enzyme-linked fluorimetric assay (Sigma-Aldrich). The amount of glutamate and ATP in each sample was inferred from standard curves as described previously (Orellana et al., 2011a,b). Briefly, after the experiments, the slices were washed twice with ACSF solution and sonicated in ice-cold PBS containing 5 μM EDTA, Halt (78440) and T-PER protein extraction cocktail (78510) according to manufacturer instructions (Pierce, Rockford, IL). Total proteins from tissue homogenates were measured using the Bio-Rad protein assay.

Data Analysis and Statistics

For each data group, results were expressed as mean ± standard error (SEM); *n* refers to the number of independent experiments. For statistical analysis, each treatment was compared with its corresponding control, and significance was determined using a one-way ANOVA followed, in case of significance, by a Tukey *post hoc* test.

Results

Restraint Stress Enhances Cx43 and Panx1 Hemichannel Activity in Brain Cells

Restrain stress impairs hippocampus-dependent spatial memory and hippocampal synaptic plasticity, inducing LTP deficits (Luine et al., 1994; Pavlides et al., 2002; Alfarez et al., 2003; Kleen et al., 2006), whereas hemichannel opening has been linked to glial and neuronal dysfunction (Takeuchi et al., 2006; Orellana et al., 2011a,b, 2013; Shestopalov and Slepak, 2014). Therefore, we investigated whether restrain stress affects the functional activity of hemichannels in hippocampal microglia, astrocytes and neurons. Anxiety-like symptoms increased as result of restraint stress using different models. A non-significant tendency for increased thigmotaxis was found in animals that underwent acute restraint stress, increment that became significant in mice subjected to chronic restraint stress, when compared to control mice (from 268.3 ± 4.3 s to 280.6 ± 2.7 s and 289.7 ± 2.7 s, respectively, $n = 7$, $p < 0.05$) (**Figure 1A**). Moreover, in the open field test, the time spent in the center was significantly reduced in mice subjected to acute restraint stress compared to control mice,

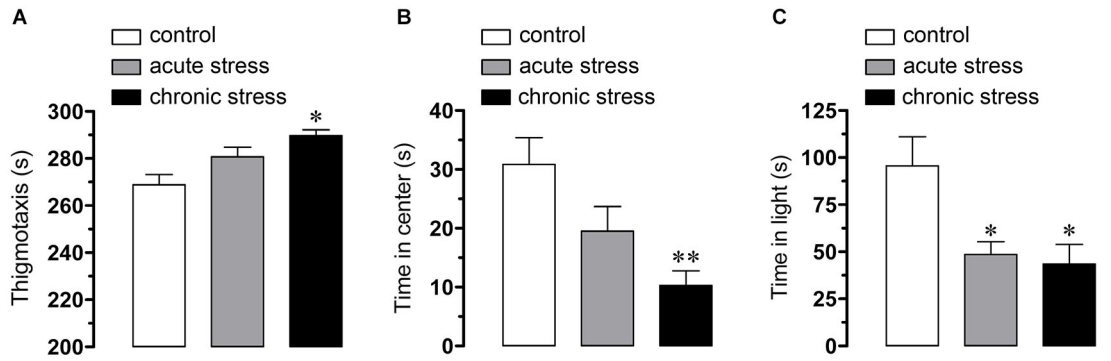

FIGURE 1 | Behavioral indicators of stress. Averaged data of anxiety-like symptoms as assessed by thigmotaxis **(A)** and time in the center **(B)** of the open field as well as time in the lit compartment of the dark and light box **(C)** in mice under control conditions (white bars), after acute (gray bars) or chronic (black bars) restraint stress. *$p < 0.05$; **$p < 0.01$. Averaged data were obtained from seven animals for each group.

a decrease that was even larger after chronic restraint stress (from 30.8 ± 4.6 s to 19.5 ± 3.8 s and 10.3 ± 2.7 s, respectively, $n = 7$, $p < 0.05$) (**Figure 1B**). In addition, in the dark and light exploration test, both acute and chronic restraint stress induced a significant reduction in the time spent in the lit compartment compared to control mice (from 95.5 ± 15.5 s to 48.5 ± 7 s and 43.4 ± 11.2 s, respectively, $n = 7$, $p < 0.05$) (**Figure 1C**). These results are indicative of anxiety-like symptoms in mice subjected to acute and chronic restrain confirming that they were stressed and suggesting that chronic stress induces more anxiety-like symptoms than acute stress.

To address whether restrain stress affects hemichannel activity of brain cells, Etd uptake was measured in hippocampal slices of mice that underwent each experimental condition. Etd crosses the plasma membrane of healthy cells passing through poorly selective channels, including connexin and pannexin hemichannels (Schalper et al., 2008). Upon binding to intracellular nucleic acids, Etd becomes fluorescent, and inhibition of this signal with specific blockers is indicative of dye uptake through hemichannels (Schalper et al., 2008; Sáez and Leybaert, 2014). Etd uptake was evaluated in "snapshot" experiments in Iba-1-positive microglia, GFAP-positive astrocytes and NeuN-positive neurons in hippocampal slices. All three cell types from control mice showed a low Etd uptake ratio (**Figures 2A–C**, **3A–C**, **4A–C**) as demonstrated previously (Karpuk et al., 2011; Orellana et al., 2011b). However, acute restraint stress increased drastically the amount of Etd uptake in microglia and astrocytes (401 ± 78.6% and 204.4 ± 8.9%; respectively, compared to control, $n = 3$, $p < 0.05$) (**Figures 2J**, **3J**), but not in pyramidal neurons (**Figure 4J**). Microglia have been shown to express functional unopposed pannexin1 (Panx1) and connexin43 (Cx43) hemichannels (Orellana et al., 2011b, 2013; Sáez et al., 2013). The possible role of Panx1 hemichannels in acute stress-evoked Etd uptake was studied using probenecid and the mimetic peptide ^{10}panx1 with an amino acid sequence homologous to the second loop of Panx1 (Pelegrin and Surprenant, 2006; Silverman et al., 2008). Probenecid (500 μM) and ^{10}panx1 (200 μM) nearly abolished the increase in microglial Etd uptake triggered by acute restraint stress (from 401 ± 78.6% to 110.3 ± 5.9% and 98.3 ± 15.9%, respectively, $n = 3$, $p < 0.005$) (**Figures 2G–J**). In contrast, mimetic peptides homologous to the cytoplasmic (TAT-L2), first (Gap26) or second (Gap27) extracellular loop of Cx43 (Wang et al., 2013a) and a Cx43 hemichannel antibody ($Cx43^{E2}$) (Siller-Jackson et al., 2008), did not affect acute stress-induced Etd uptake by microglia (**Figure 2J**). Astrocytes express functional unopposed hemichannels formed by Cx43 (Contreras et al., 2002) and Panx1 (Iglesias et al., 2009), thereby we used TAT-L2, $Cx43^{E2}$, Gap26, probenecid and ^{10}panx1 to determine the contribution of each channel type in acute stress-induced Etd uptake in astrocytes. TAT-L2, $Cx43^{E2}$, Gap26 and Gap27 fully reduced astroglial cell Etd uptake evoked by acute restraint stress (from 204.4 ± 8.9% to 103 ± 3.8%; 108.4 ± 4.5%, 101.4 ± 5.9% and 101.8 ± 0.3%, respectively, $n = 3$, $p < 0.005$) (**Figures 3G–J**). In contrast, ^{10}panx1 and probenecid did not affect the stress-induced Etd uptake (**Figure 3J**).

Responses to acute stress are generally adaptive, but long lasting stress can cause persistent changes and even irreversible damage (Millán et al., 1996; Dhabhar and McEwen, 1997). In agreement with this notion, we found that Etd uptake (% compared to control conditions) induced by chronic stress in microglia and astrocytes was stronger than that found after acute stress (401 ± 78.6% vs. 811.1 ± 124.1%; respectively; and 204.4 ± 8.7% vs. 525.3 ± 4.5%; respectively; $n = 3$, $p < 0.05$) (**Figures 2D–F,J**, **3D–F,J**). Probenecid and ^{10}panx1 nearly abolished the increase in microglial cell Etd uptake triggered by chronic restraint stress (from 811.1 ± 124.1% to 100.9 ± 13.9% and 97.5 ± 7.7%, respectively, $n = 3$, $p < 0.005$) (**Figure 2J**), whereas TAT-L2, $Cx43^{E2}$, Gap26 and Gap27 failed to affect this response (**Figure 2J**). The above findings suggest that in microglia, Panx1 but not Cx43 hemichannels, mediate the restraint stress-induced Etd uptake. This interpretation was supported by the absence of chronic stress-induced microglia hemichannel activation in hippocampal slices from $Panx1^{-/-}$ mice (**Figure 2J**). On the other hand, TAT-L2, $Cx43^{E2}$, Gap26 and Gap27 partially reduced astroglial Etd uptake evoked by chronic restraint stress (from 525.3 ± 4.6% to 287 ± 13.1%;

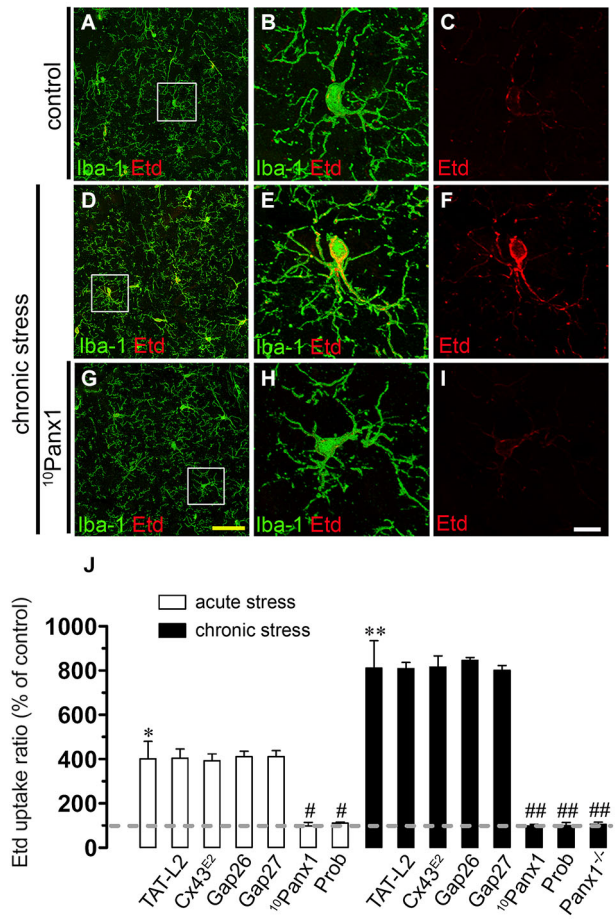

FIGURE 2 | Restraint stress increases Panx1 hemichannel activity in microglia. Representative images showing Iba-1 (green) and Etd (red) uptake of acute hippocampal brain slices from control mice **(A–C)** and mice subjected to chronic restraint stress **(D–F)**. In addition, the effect of ^{10}panx1 on Etd uptake is shown in mice subjected to chronic restraint stress **(G–I)**. Images of hippocampal microglia were taken from the area depicted within the white square in panels **(A,D)** and **(G)**. **(J)** Averaged data of Etd uptake ratio normalized to control conditions (dashed line) of microglia from mice subjected to acute (white bars) and chronic (black bars) restraint stress. Also shown are the effects of the following blockers applied during the Etd uptake essay: TAT-L2 (100 μM), Cx43^{E2} (1:500), Gap26 (100 μM), Gap27 (100 μM), ^{10}panx1 (100 μM) or probenecid (Prob, 500 μM). Also it is shown data of microglia of hippocampal slices from Panx1$^{-/-}$ mice. $^{*}p < 0.05$, $^{**}p < 0.01$, for the effect of restraint stress compared to control conditions; $\#p < 0.05$, $\#\#p < 0.01$, effect of each blocker compared to the respective effect induced by restraint stress. Averaged data was obtained from three independent experiments. Calibration bars: yellow bar = 70 μm; white bar: 10 μm.

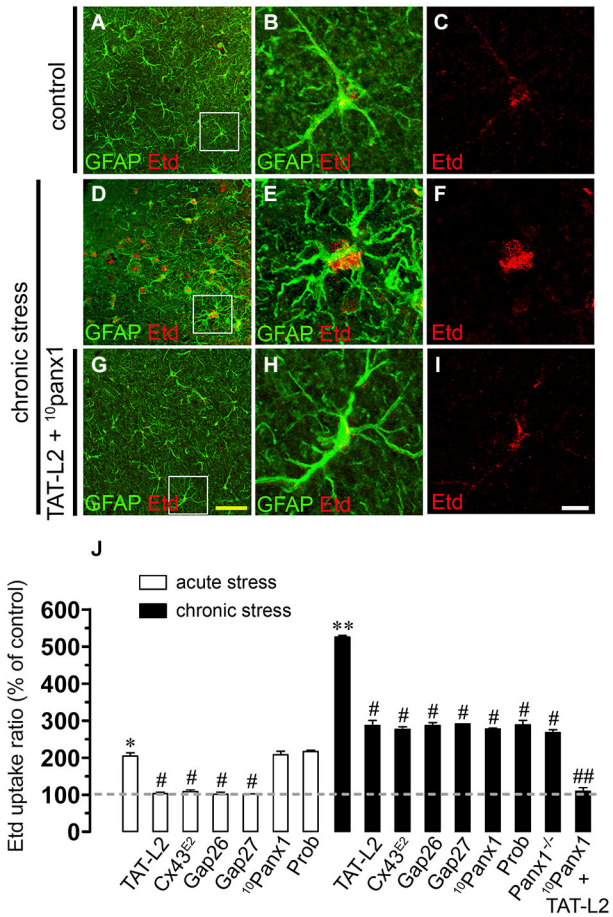

FIGURE 3 | Restraint stress increases Cx43 and Panx1 hemichannel activity in astrocytes. Representative images showing GFAP (green) and Etd (red) uptake of acute hippocampal slices made from control mice **(A–C)** and mice subjected to chronic restraint stress **(D–F)**. In addition, the effects of TAT-L2 and ^{10}panx1 on Etd uptake was tested in mice subjected to chronic restraint stress **(G–I)**. Images of hippocampal astrocytes were taken from the area depicted within the white square in panels **(A,D)** and **(G)**. **(J)** Averaged data for Etd uptake ratio normalized to control conditions (dashed line) of astrocytes from mice subjected to acute (white bars) and chronic (black bars) restraint stress. Also shown are the effects of the following blockers applied during the Etd uptake essay: TAT-L2 (100 μM), Cx43^{E2} (1:500), Gap26 (100 μM), Gap27 (100 μM), ^{10}panx1 (100 μM) or probenecid (Prob, 500 μM). Also it is shown data of astrocytes of hippocampal slices from Panx1$^{-/-}$ mice. $^{*}p < 0.05$, $^{**}p < 0.01$ for the effect of restraint stress compared to control conditions; $\#p < 0.05$, $\#\#p < 0.01$, effect of each blocker compared to the respective effect induced by restraint stress. Averaged data are obtained from three independent experiments. Calibration bars: yellow bar = 150 μm; white bar: 20 μm.

276.2 ± 13.1%, 286 ± 7.7% and 290.8 ± 0.6%, respectively, $n = 3$, $p < 0.05$) (**Figure 3J**). Moreover, contrary to the results observed in astrocytes from acute stress mice, ^{10}panx1 and probenecid inhibited prominently the chronic stress-induced Etd uptake (from 525.3 ± 4.6% to 277.3 ± 2.5% and 288.6 ± 12.1% respectively, $n = 3$, $p < 0.005$) (**Figure 3J**). These data were in agreement with the fact that chronic stress triggered a partial increase of astroglial hemichannel activity in hippocampal slices from Panx1$^{-/-}$ mice (**Figure 3J**). Moreover, consistent with the idea that both Cx43 and Panx1 hemichannels were the main contributors to chronic stress-induced Etd uptake in astrocytes, simultaneous blockade of these channels with TAT-L2 and ^{10}panx1 fully reduced the response (from 525.3 ± 4.6% to 109.2 ± 9.8%, respectively, $n = 3$, $p < 0.005$) (**Figure 3J**). In contrast to the lack of effect of acute stress on neuronal hemichannel activity, chronic stress evoked a prominent increase on Etd uptake in pyramidal neurons (641.9 ± 61.7%, $n = 4$) (**Figures 4A–F**). Since most available evidence support the

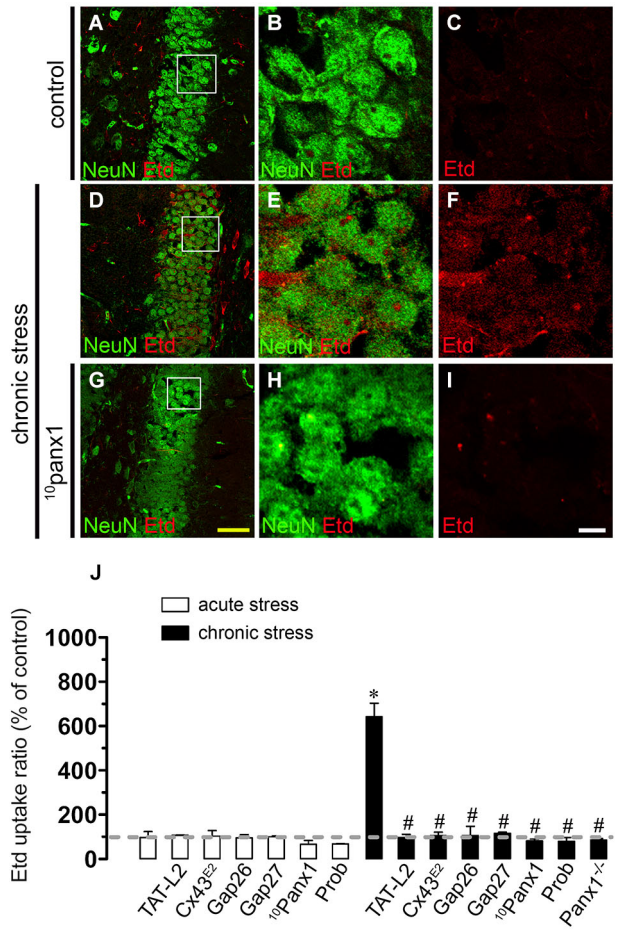

FIGURE 4 | Restraint stress increases Panx1 hemichannel activity in pyramidal neurons. Representative images showing NeuN (green) and Etd (red) uptake of acute hippocampal brain slices from control mice **(A–C)** and mice subjected to chronic restraint stress **(D–F)**. In addition, the effect of ^{10}panx1 on Etd uptake was tested in mice subjected to chronic restraint stress **(G–I)**. Images of hippocampal neurons were taken from the area shown within the white square in panels **(A,D)** and **(G)**. **(J)** Averaged data of Etd uptake ratio normalized to control conditions (dashed line) of neurons from mice subjected to acute (white bars) and chronic (black bars) restraint stress. Also shown are the effects of the following blockers applied during the Etd uptake essay: TAT-L2 (100 μM), Cx43^{E2} (1:500), Gap26 (100 μM), Gap27 (100 μM), ^{10}panx1 (100 μM) or probenecid (Prob, 500 μM). Also it is shown data of neurons of hippocampal slices from Panx1$^{-/-}$ mice. *$p < 0.05$; effects of restraint stress compared to control conditions; #$p < 0.05$, effects of each blocker compared to the respective effect induced by restraint stress. Averaged data are obtained at from three independent experiments. Calibration bars: yellow bar = 80 μm; white bar: 10 μm.

notion that neurons express hemichannels formed by Panx1 (Thompson et al., 2006), we used ^{10}panx1 and probenecid to determine the contribution of these channels on chronic restraint stress-induced neuronal Etd uptake. ^{10}panx1 and probenecid strongly reduced the stress-induced Etd uptake observed in pyramidal neurons (from 641.9 ± 61.7% to 81.8 ± 11% and 80.1 ± 17%, respectively, $n = 3$, $p < 0.005$) (**Figures 4G–J**), whereas TAT-L2, Gap19 and Gap26 caused a similar inhibition (from 641.9 ± 61.7% to 96.5 ± 14.9%; 105.1 ± 15.4% and 106 ± 41.5%, respectively, $n = 3$, $p < 0.005$) (**Figure 4J**). Accordingly, chronic restraint stress failed on evoke Etd uptake in hippocampal neurons from Panx1$^{-/-}$ mice (**Figure 4J**). Moreover, basal levels of Etd uptake in microglia, astrocytes or neurons from Panx1$^{-/-}$ mice were similar to that observed in wild type mice (data not shown). Overall, these data indicate that both acute and chronic restraint stress increase hemichannel opening of glial cells and neurons, being chronic restraint much more powerful than acute restraint stress in evoking this response.

Chronic Restraint Stress Increase Panx1 Levels in Astrocytes and Neurons

Given that pathological conditions affect the expression of connexins and pannexins in the CNS (Rouach et al., 2002; Orellana et al., 2009), we examined whether chronic or acute restraint stress could modulate Cx43 and Panx1 levels in brain cells by confocal analysis. Interestingly, chronic but not acute restraint stress evoked a significant increase on Panx1 levels in astrocytes and neurons when compared to control conditions (**Figures 5A–H,J,K**). However, neither Cx43 nor Panx1 levels were affected in microglia in mice subjected to chronic restraint stress (**Figure 5I**). Similarly, for all tested conditions, Cx43 remained unchanged in astrocytes (**Figure 5J**).

Hemichannel Opening Evoked by Chronic Restraint Stress Depends on Glutamatergic/Purinergic Signaling

Under activated state, glial cells release relevant amounts of gliotransmitters including glutamate and ATP, which underlie glia-to-glia and glia-to-neuron communication via glutamatergic and purinergic receptors, respectively (Perea et al., 2009; Perea and Araque, 2010). Because opening of hemichannels has been asociated with purinergic and glutamatergic signaling (Locovei et al., 2006; Thompson et al., 2008; Orellana et al., 2011a,b), we examined if NMDA and P2X$_7$ receptors are involved in chronic restraint stress-induced Etd uptake. We found that CPP, a NMDA receptor blocker, strongly abolished the Etd uptake evoked by chronic restraint stress in astrocytes (from 100% of stress-induced effect to 28.7 ± 5.4%, $n = 3$, $p < 0.05$) (**Figure 6**), whereas in microglia and pyramidal neurons caused a small inhibition (from 100% of stress-induced effect to 69.7 ± 3.8% and 49.5 ± 0.4%, respectively, $n = 3$) (**Figure 6**). Moreover, blockade of P2X$_7$ receptors with BBG, oATP and A740003 induced a prominent reduction on chronic stress-induced Etd uptake in microglia (from 100% of stress-induced effect to 29.6 ± 2.2%, 30.3 ± 0.7% and 30.5 ± 5.6%, respectively, $n = 3$, $p < 0.05$) and in neurons (from 100% of stress-induced effect to 44.2 ± 4.7%, 47 ± 3.4% and 53.6 ± 2.2%, respectively, $n = 3$, $p < 0.05$), with a lesser but significant decrease in astrocytes (from 100% of stress-induced effect to 67.3 ± 10.5%, 63 ± 10.2% and 60.5 ± 4.9%, respectively, $n = 3$) (**Figure 6**). To elucidate if in addition to P2X$_7$ receptors, metabotropic purinergic receptors might also be involved in chronic restraint stress-induced hemichannel opening, we used MRS2179, a blocker of P2Y$_1$ receptors, which has been previously linked to hemichannel opening in the

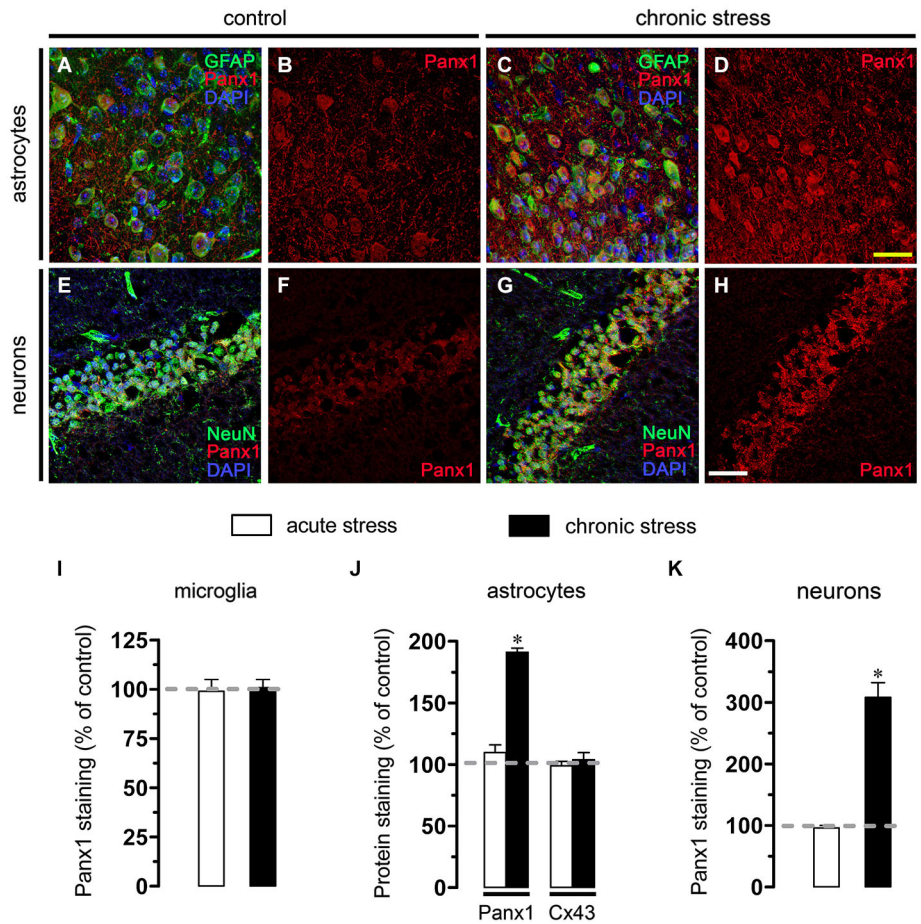

FIGURE 5 | Chronic restraint stress enhance Panx1 levels in astrocytes and neurons. (A–H) Representative images showing Panx1 (red) and DAPI (blue) staining in GFAP positive astrocytes (green, **A–D**) and NeuN positive neurons NeuN (green, **E–H**) of acute hippocampal slices made from control mice (left panels) or subjected to chronic restraint stress (right panels). Calibration bars: yellow = 70 μm and white = 110 μm. **(I–K)** Averaged data of Panx1 and Cx43 staining normalized to control conditions (dashed line) in microglia **(I)**, astrocytes **(J)** and neurons **(K)** of hippocampal slices from mice subjected to acute (white bars) or chronic restraint stress (black bars). *$p < 0.05$, effect of chronic restraint stress compared with control conditions. Averaged data were obtained from three independent experiments.

CNS (Orellana et al., 2012; Sáez et al., 2013). MRS2179 did not affect the Etd uptake induced by chronic restraint stress in all brain cells studied (**Figure 6**). In agreement with the idea that both NMDA and $P2X_7$ receptors are involved in hemichannel opening induced by chronic restraint stress, blockade of both receptors with CPP and A740003, respectively, fully reduced this response in microglia, astrocytes and neurons (from 100% of stress-induced response to 8.0 ± 1.0%, 12.1 ± 0.4% and 10.3 ± 0.1%, respectively, $n = 3$, $p < 0.05$) (**Figure 6**). Taken together these data indicate that Etd uptake induced by chronic restraint stress depends on NMDA/$P2X_7$ receptor signaling.

Chronic Restraint Stress Induces Cx43 and Panx1 Hemichannel-Dependent Release of Glutamate and ATP in Brain Cells

Recently, gliotransmitters were shown to elicit their own release in an autocrine manner, via Cx43 and Panx1 hemichannels (Orellana et al., 2012, 2013). Given that NMDA/$P2X_7$ receptors are involved in the Etd uptake observed in hippocampal cells of mice subjected to restraint stress, we evaluated whether this condition affects the glutamate and ATP release from hippocampal slices via Cx43 and/or Panx1 hemichannels. Acute and chronic stress strongly increased the release of glutamate and ATP (from 32.5 ± 4.4 pmol/mg to 56.4 ± 8.5 pmol/mg and 138.8 ± 25.9 pmol/mg, respectively and from 13.5 ± 4.2 pmol/mg to 30.7 ± 4.6 pmol/mg and 75.3 ± 9.6 pmol/mg, respectively, $n = 3$, $p < 0.05$) (**Figure 7**). Interestingly, TAT-L2, Gap26, ^{10}panx1 and probenecid prominently reduced the release of glutamate (from 138.8 ± 25.9 pmol/mg to 20.4 ± 3.3 pmol/mg, 31.7 ± 7.8 pmol/mg, 35.7 ± 7.5 pmol/mg and 25.5 ± 2.3 pmol/mg, respectively, $n = 3$) and ATP (from 75.3 ± 9.6 pmol/mg to 11.6 ± 2.3 pmol/mg, 13.8 ± 3.6 pmol/mg, 16.5 ± 5.2 pmol/mg and 12.5 ± 1.8 pmol/mg, respectively, $n = 3$, $p < 0.05$) induced by chronic restraint stress (**Figure 7**). These findings indicate that chronic stress increases the

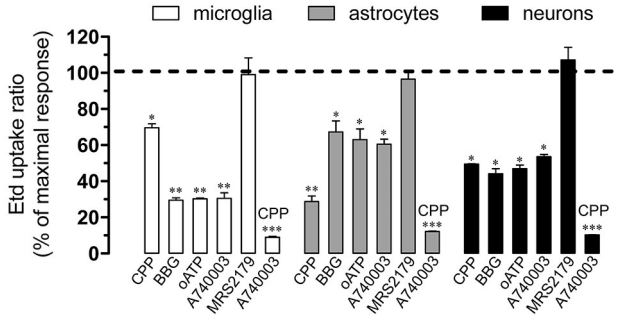

FIGURE 6 | Cx43 and Panx1 hemichannel activity evoked by chronic stress on NMDA/P2X$_7$ receptor signaling. Averaged data normalized to maximal Etd uptake ratio (dashed line) obtained after chronic restraint stress in microglia (white bars), astrocytes (gray bars) and neurons (black bars) treated with the following blockers during the Etd uptake essay: CPP (20 μM), BBG (10 μM), oATP (200 μM), A740003 (10 μM), MRS2179 (10 μM) or CPP (20 μM) plus MRS2179 (10 μM). *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$, effect of each blocker compared to the respective effect induced by chronic restraint stress. Averaged data were obtained from four independent experiments.

release of glutamate and ATP via opening of Cx43 and Panx1 hemichannels.

In support for the notion that gliotransmitters can elicit their own release, we found that CPP, BBG, oATP, A740003, CPP plus A74003, but not MRS2179 abolished almost completely the release of glutamate (from 138.8 ± 25.9 to 38.7 ± 5.9 pmol/mg, 40.4 ± 7.9 pmol/mg, 36.3 ± 7.9 pmol/mg, 45.6 ± 3.5 pmol/mg, 25.6 ± 5.6 pmol/mg and 145.6 ± 10.5 pmol/mg, respectively, $n = 3$, $p < 0.05$) and ATP (from 75.3 ± 9.6 pmol/mg to 34.9 ± 6.5 pmol/mg, 33.7 ± 8.5 pmol/mg, 30.7 ± 11.9 pmol/mg, 48 ± 17.1 pmol/mg, 17.4 ± 3.2 pmol/mg and 83.1 ± 17.9 pmol/mg, respectively, $n = 3$, $p < 0.005$) induced by chronic restraint stress (**Figure 7**). This evidence suggests that both glutamate and ATP evoke their own release by an autocrine pathway possibly mediated by unopposed Cx43 and Panx1 hemichannels.

Discussion

In this study, we showed that restraint stress increases the opening of Cx43 and Panx1 hemichannels in astrocytes; whereas Panx1 hemichannels are primarily activated in microglia and neurons. Moreover, the intensity of these responses depended on the duration of the restraint strees protocol and occurred by a mechanism linked to signaling via NMDA/P2X$_7$ receptors. Furthermore, hemichannel opening induced by restraint stress troggered the release of both glutamate and ATP, two major gliotransmitters in the CNS.

Previous studies have demonstrated that restraint stress impairs spatial hippocampus-dependent cognitive performance (Luine et al., 1994; Kleen et al., 2006) and LTP (Pavlides et al., 2002; Alfarez et al., 2003) and induces glial cell activation (Nair and Bonneau, 2006; Sugama et al., 2007; Kwon et al., 2008). The present results suggest that at least part of the above mentioned changes induced by restraint stress could be explained by enhanced gliotransmitter release and further increase in extracellular gliotransmitter concentration within the CNS. It has been shown that gliotransmitter release through hemichannels underlies crucial functions of brain physiology (Lin et al., 2008; Huckstepp et al., 2010; Orellana et al., 2012; Stehberg et al., 2012; Torres et al., 2012). Nevertheless, several studies indicate that uncontrolled opening of these channels results in exacerbated release of gliotransmitters, which in high concentrations can be toxic to neighboring cells (Takeuchi et al., 2006; Orellana et al., 2011a,b). Now, we found that 2 h of restraint stress is sufficient to enhance opening of hemichannels in glial cells, whereas an enhanced response in neurons was achieved with a more prolonged restraint stress protocol (2 h for over 10 days). These results are in agreement with the fact that the consequences of physiological stress are usually adaptive in short term, but can be damaging when stress is chronic and long lasting (Millán et al., 1996; Dhabhar and McEwen, 1997).

In agreement with their surveillance role in the CNS (Block et al., 2007), microglia showed the highest changes on Etd uptake evoked by chronic restraint stress compared to astrocytes and neurons. Since this response was absent in hippocampal slices from Panx1$^{-/-}$ mice and fully reduced by Panx1 blockers, hemichannels composed by the latter protein were mainly responsible of this phenomenon. In accordance with our results, recent studies have shown that pro-inflammatory conditions increase the opening of Panx1 channels in microglia (Orellana et al., 2013; Sáez et al., 2013). In our study, both astrocytes and neurons exhibited an evident increase in Etd uptake in mice subjected to chronic restraint stress as compared to control conditions. This response in astrocytes might be due to Cx43 and Panx1 hemichannels as mimetic peptides and blockers known to inhibit these channels (Pelegrin and Surprenant, 2006; Silverman et al., 2008; Wang et al., 2013a), completely inhibited the stress-induced Etd uptake. Another interpretation is that Panx1 hemichannels expressed by microglia or neurons could affect the opening of astroglial hemichannels by allowing the release of molecules that enhance their activity after restraint stress.

Neuronal Etd uptake induced by chronic restraint stress was strongly blocked by TAT-L2, Cx43^{E2}, Gap26 or Gap27, ^{10}panx1 and probenecid and absent in hippocampal slices from Panx1$^{-/-}$ mice, indicating the involvement of Panx1 and Cx43 hemichannels. Neurons have been reported to express hemichannels formed by Panx1 and Cx36, but not Cx43 (Thompson et al., 2006; Schock et al., 2008; Orellana et al., 2011a). The fact that Cx43 hemichannel blockade reduced neuronal Etd uptake, suggests that astroglial Cx43 hemichannel activity constitutes a pre-requisite condition for the effects of chronic stress on neuronal hemichannels. Consistent with this, a recent study showed that gliatransmitter release via astroglial Cx43 hemichannels is required to trigger the amyloid-β peptide-dependent activation of Panx1 hemichannels in hippocampal neurons (Orellana et al., 2011b).

Glutamate and ATP are considered crucial transmitters on neuron-glia crosstalk and thereby their release through membrane proteins and vesicles is tightly regulated (Fields and Burnstock, 2006; Perea and Araque, 2010). In fact, high

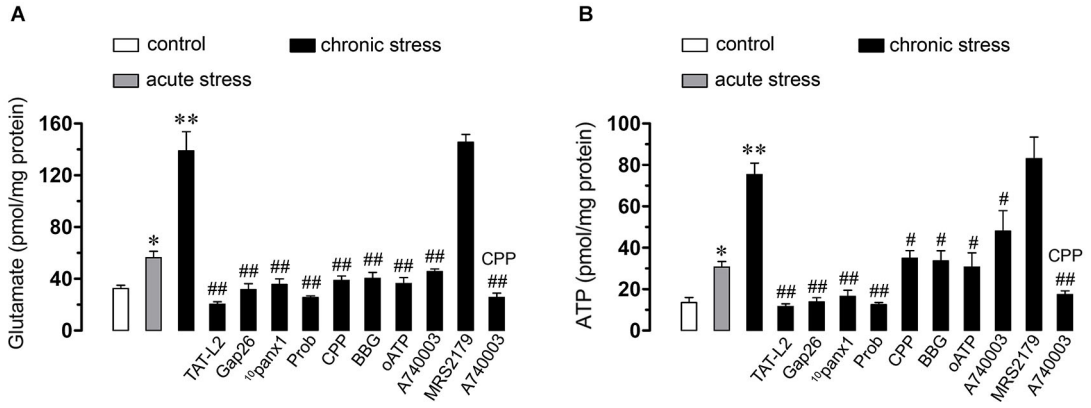

FIGURE 7 | Opening of Cx43 and Panx1 hemichannels allow the release of glutamate and ATP via NMDA/P2X$_7$ receptor signaling. Averaged data of glutamate **(A)** or ATP **(B)** release by hippocampal slices from mice under control conditions (white bars), subjected to acute (gray bars) or chronic (black bars) restraint stress. Also shown are the effects of the following blockers: TAT-L2 (100 μM), Gap26 (100 μM), ^{10}panx1 (100 μM), probenecid (Prob, 500 μM), CPP (20 μM), BBG (10 μM), oATP (200 μM), A740003 (10 μM), MRS2179 (10 μM) or CPP (20 μM) plus MRS2179 (10 μM). *$p < 0.05$, **$p < 0.01$; effect of restraint stress compared to control conditions; #$p < 0.05$, ##$p < 0.01$, effect of each blocker compared to chronic restraint stress. Averaged data are obtained from four independent experiments.

concentrations of glutamate and ATP at the synaptic cleft could be neurotoxic under pathological conditions (Lau and Tymianski, 2010; Arbeloa et al., 2012; Ashpole et al., 2013). As mentioned before, part of this neuronal damage could be the consequence of glutamate and ATP release via hemichannels (Takeuchi et al., 2006; Garré et al., 2010; Orellana et al., 2011a,b). Our findings indicate that chronic stress induced the release of hippocampal glutamate and ATP via Cx43 and Panx1 hemichannels, as their extracellular levels were reduced by TAT-L2, Cx43^{E2}, Gap26 or Gap27, ^{10}panx1 or probenecid.

What is the mechanism that underlies chronic stress-induced opening of Cx43 and Panx1 hemichannels? Previous studies have demonstrated that opening of these channels in glial cells relies on the rise of $[Ca^{2+}]_i$ linked to activation of NMDA, P2X$_7$ or P2Y$_1$ receptors (Orellana et al., 2011a,b, 2012; Sáez et al., 2013). Accordingly, in the present study Etd uptake and gliotransmitter release were both fully reduced by NMDA and P2X$_7$ but not P2Y$_1$ receptor blockers, suggesting that activation of Cx43 and Panx1 hemichannels likely occurs downstream in the NMDA/P2X$_7$ pathway. Since activation of NMDA/P2X$_7$ receptors raises $[Ca^{2+}]_i$ (Fields and Burnstock, 2006; Perea and Araque, 2010) and increased levels of $[Ca^{2+}]_i$ trigger gliotransmitter release via hemichannels (Locovei et al., 2006; Torres et al., 2012), it is plausible to suggest that stress induces NMDA/P2X$_7$ receptor activation and further glutamate and ATP release via hemichannels. The latter subsequently evokes re-activation of those receptors to promote hemichannel-dependent release of these gliotransmitters.

Here, we observed that chronic but not acute restraint stress increases Panx1 levels in astrocytes and neurons, whereas the amount of Cx43 protein remained unchanged in all conditions and brain cells studied. Surface hemichannels account for ∼11% of total Cx43 under resting conditions (Schalper et al., 2008), making them poorly detectable by immunofluorescence. Therefore, changes in Cx43 protein levels by immunodetection do not necessarily implicate change in surface hemichannels or in their activity, masked by a large amount of Cx43 forming gap junctions. Although it is still debated whether Panx1 hemichannels dock to form gap junctions (Sosinsky et al., 2011; Sahu et al., 2014), changes in Panx1 protein levels may reflect more surface hemichannels than in the case of Cx43. Thereof, it is possible that part of Etd uptake observed in astrocytes and pyramidal neurons could rely on the increase on surface levels of Panx1, whereas Cx43-dependent Etd uptake likely occurs via posttranslational modifications or changes in gating and sorting of Cx43 hemichannels (see previous paragraph). Further studies are required to elucidate whether changes in protein expression or degradation and sorting could contribute to the Cx43 and Panx1 hemichannel activity triggered by restraint stress.

Given the high expression of glucocorticoid (GC) receptors in the hippocampus, it may be one of the main target areas of GCs in the CNS (Popoli et al., 2011). During chronic restraint stress, blood and brain levels of GCs are persistently elevated, resulting in LTP and cognitive impairment and eventually promoting neuronal loss as well (Popoli et al., 2011). Moreover, both chronic stress and GCs increase glutamate levels (Moghaddam, 1993; Moghaddam et al., 1994) and $[Ca^{2+}]_i$ at hippocampal synapses (Elliott and Sapolsky, 1992, 1993). Taken altogether, we speculate that the chronic restraint stress protocol used in the present work increases GC brain levels, resulting in further activation of NMDA/P2X$_7$ receptors in microglia and astrocytes. In agreement with this interpretation, chronic stress evokes NMDA receptor-dependent proliferation of microglia associated to GC receptor activation (Nair and Bonneau, 2006), whereas GC exposure primes cytokine release from microglia *ex vivo* (Frank et al., 2007). Furthermore, stress also activates astroglial cells (Kwon et al., 2008), while GCs enhances astrocytic $[Ca^{2+}]_i$ and ATP release (Simard et al., 1999). Further research is needed

to unveil the exact mechanisms by which chronic stress affects hemichannels in glia and neurons and what the contribution of GCs on this process really is.

Although our working model does not recapitulate the mechanisms underlying the brain abnormalities induced by major depression and stress-associated psychiatric disorders, it allows us to dissect the specific contribution of hemichannels expressed by individual brain cell types. It must be noted that both chronic restraint stress and chronic GC administration are effective models for obtaining depressive-like symptoms in rodents (Levinstein and Samuels, 2014). In consequence, it is possible that hemichannel activation induced by chronic restraint stress may also contribute to the pathogenesis of depressive-like symptoms. Therefore, these findings may shed light into the early phases of neuronal dysfunction associated to stress, which may lead to major depression, post-traumatic stress disorder and other anxiety disorders. Our findings brings new vistas on the role of gliotransmitters on chronic stress and how hemichannels could arise as possible targets for developing novel pharmacological strategies to ameliorate different mental disorders associated to stress, anxiety and depression.

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