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CB1-dependent Neuromodulation in the Nucleus Accumbens core

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List of Abbreviators

2-AG – 2-arachynodoil-glycerol

AC – Adenylate Cyclase

AEA - Anandamide

aCSF – artificial cerebral-spinal fluid

BLA – Basolateral Amygdala

CB_1 – cannabinoid receptor type-1 (or 2)

CNS – central nervous system

$CB_1^{-/-}$ - CB_1 knock-out

DAG - Diacylglicerol

eCBs – endocannabinoids

ECS – Endocannabinoid system

GABA-$CB_1^{-/-}$ - CB1 knock-out in GABAergic neurons

Glut-$CB_1^{-/-}$ - CB1 knock-out in glutamatergic neurons

MSN – Medium Spiny neuron

mtCB_1 – Mitochondrial CB_1

NAc – Nucleus Accumbens

PFC – Prefrontal Cortex

RT - Room temperature

Resumo

A Canábis tem sido utilizada pelas populações ao longo dos milénios, tanto para fins medicinais, como para fins recreativos. Apesar da nossa relação de longa data com a planta, os seus efeitos fisiológicos e mecanismos moleculares só foram estudados nas últimas décadas, havendo ainda muito por desvendar. A primeira grande descoberta neste campo foi na década de 1930 com o isolamento de Canabinol (o primeiro derivado da planta), que despoletou o aumento do interesse da comunidade científica pelo potencial medicinal da Canábis. Nos anos noventa, descobriram-se recetores canabinóides do tipo 1 em humanos e pouco depois, o tipo 2 foi também caracterizado. Durante a primeira década dos anos dois mil, a descoberta do primeiro antagonista seletivo do CB_1 (Rimonabant) e a produção de ratinhos CB_1 *knock-out* permitiram-nos começar a entender alguns dos efeitos e mecanismos destes recetores. Hoje, sabe-se que ambos os recetores canabinóides podem estar patologicamente expressos em variadíssimos tipos de doenças, como neurológicas (Alzheimer e Esclerose múltipla), psiquiátricas (depressão e esquizofrenia), cardiovascular (Arteriosclerose) e também gastrointestinal (cirrose). Tanto o facto do sistema endocanabinóide estar relacionado com inúmeras patologias, como a descriminalização da Canábis ajudaram a atenuar alguma desconfiança na planta e consequentemente, descobriu-se um novo mundo de possíveis terapias associadas a canabinóides.

O sistema endocanabinóide é composto por endocanabinóides, proteínas recetoras e enzimas para a síntese e degradação de endocanabinóides. No cérebro, a sinalização endocanabinóide implica a ativação de CB_1, que exerce um importante papel em funções neuronais relacionadas com memória e aprendizagem, controlo motor, sono, entre outros. Em neurónios, CB_1 estão acoplados a proteínas $G_{i/o}$ e encontram-se maioritariamente expressos em membranas pré-sinápticas, particularmente de sinapses Glutamatérgicas. Nestas, a ativação de CB_1 e $G_{i/o}$ medeiam a sinalização retrógrada de endocanabinóides, através da supressão da atividade da enzima Adenil Ciclase que por consequência leva não só à diminuição de cAMP mas como também à inativação de PKA. Desta forma, ocorre o influxo pré-sináptico de Ca^{2+} e a inibição da libertação de neurotransmissores. Para além da sua expressão em neurónios, os CB_1 também estão expressos em astrócitos, onde se encontram acoplados a G_q. A sua ativação leva à cascata MAPK/ERK e regula a excitabilidade neuronal, a transmissão sináptica e plasticidade, ao estimular a libertação de Glutamato.

Apesar do mecanismo pré-sináptico da sinalização de endocanabinóides já ter sido extensivamente estudado, alterações pós-sinápticas também podem ocorrer, já que os CB_1 conseguem modular a transmissão sináptica mediada através de recetores AMPA e NMDA. É de interesse perceber a relação entre recetores AMPA, NMDA e CB_1 pois são essenciais, não só para a

transmissão sináptica mas também na plasticidade sináptica. Os recetores AMPA são recetores ionótrópicos de glutamato e estão localizados em terminais pós sinápticos, mais especificamente na densidade pós-sináptica. Quando ativos, contribuem para a abertura de canais iónicos, induzindo assim a despolarização membranar e sendo por isso essenciais para a plasticidade sináptica, aprendizagem e memória. AMPARs são tetraméricos, estando organizados em dois dímeros, cujas subunidades podem variar entre GluA1 – GluA4. Para além de serem permeáveis a Na^{+}, AMPARs sem a subunidade GluA2 são permeáveis a Ca^{2+}, o que possibilita a ativação de cascatas dependentes de Ca^{2+}. Estas cascatas de eventos levam ao *trafficking* de AMPARs nas membranas pós-sinápticas, processo essencial para a plasticidade sináptica. O aumento de Ca^{2+} pode também contribuir para a ativação de NMDARs e cascatas MAPK/ERK. Os recetores de NMDA são ionotrópicos e ativados por Glutamato, sendo essenciais para a neurotransmissão excitatória rápida. Tal como AMPARs, as funções de NMDARs nas sinapses são extremamente complexas e diferem entre áreas do cérebro. Esta complexidade é o que permite mecanismos de plasticidade sináptica, não só a iniciação mas também a sua manutenção. NMDARs são tetrâmeros que contêm obrigatoriamente duas subunidades GluN1 e duas subunidades reguladoras, GluN2 ou GluN3. Para além de serem permeáveis a Na^{+}, NMDARs com a subunidade GluN2A têm alta sensibilidade a bloqueios por Mg^{2+}, o que contribui para o influxo de Ca^{2+}. Para além disto, esta subunidade é responsável por controlar os estados aberto/fechado dos canais de NMDAR, o que induz cascatas de CamKII. Estas têm a capacidade de regular o *tráfego* de NMDARs nas membranas, contribuindo também para a iniciação de cascatas MAPK/ERK.

O NAc é um dos principais componentes da via mesocorticolímbica, sendo constituído por duas partes que diferem em morfologia e função – um *core* e uma *shell*. Esta zona é responsável não só por emoções como desejo e motivação, mas também emoções associadas ao prazer, como a felicidade e a euforia. Mais especificamente, o *core* do NAc é responsável pelo processamento cognitivo de funções motoras relacionadas com reforço e recompensa, sendo também responsável pelo vício de Anfetaminas e Cocaína. Para além disto, o NAc está também envolvido em doenças psiquiátricas como a esquizofrenia e a depressão. O NAc é composto por 90% de *medium spiny neurons* GABAérgicos e o restante corresponde a *medium spiny neurons* Glutamatérgicos e interneurónios Colinérgicos. Recebe *inputs* glutamatérgicos do Córtex Pré-frontal, do Hipocampo e da Área Tegmental Ventral, enquanto que os neurónios do output enviam projeções axonais para a Área Tegmental Ventral, a Hipocampo, o Córtex Pré-frontal, entre outros. Os circuitos e neurotransmissores do NAc já foram extensivamente estudados, contudo ainda existem muitas questões quanto à influência do CB_1, recetor que já mostrou ser abundante nesta região, particularmente em terminais glutamatérgicos de corpos celulares GABAérgicos. Para além disto, já foi mostrado que a ativação farmacológica de CB_1 diminui a transmissão glutamatérgica evocada, sugerindo que estes têm um papel fisiológico fundamental na excitabilidade do NAc. Os mecanismos pré-sinápticos já foram

estudados, contudo, alterações pós-sinápticas também são possíveis já que os CB_1 podem modular AMPARs e NMDARs. É importante perceber a relação entre CB_1, AMPARs e NMDARs já que estes recetores são essenciais para a transmissão e plasticidade sináptica, afetando os *outcomes* comportamentais.

De forma a investigar se a ausência crónica de CB_1 afeta a transmissão glutamatérgica no núcleo do NAc, foi utilizada eletrofisiologia *whole-cell patch clamp ex vivo* combinada com farmacologia e linhas de ratinho transgénicas. Primeiro, verificou-se que a deleção total de CB_1 afeta a transmissão sináptica espontânea ao aumentar a frequência de eventos sinápticos. Estes resultados sugerem que CB_1 são essencias para controlar a libertação e *clearence* de Glutamato. De seguida, verificou-se que os endocanabinóides não são tónicamente libertados no *core* do NAc, sugerindo que os resultados anteriormente são causados pela falta crónica de CB_1. Em terceiro, verificamos que não existem alterações no ratio de AMPA/NMDA, sugerindo que podem existir alterações nas subunidades de recetores AMPA e NMDA. Depois, verificou-se que a deleção total de CB_1 prejudica a atividade de AMPARs permeáveis a Ca^{2+} em *medium spiny neurons* no core do NAc, sugerindo que os mecanismos de tráfego podem estar afetados. Por último verificou-se que a deleção total de CB_1 aumenta a presença da subunidade GluN2A em NMDARs no core do NAc, sugerindo que pode estar a compensar a falta de CB_1. Para além disto, pode também haver um aumento crónico de PKA, PKC ou CaMkII. Com este projeto podemos então concluir que a ausência de CB_1 induz alterações na atividade sináptica que permitem o aumento da libertação de glutamato. A ausência de CB_1 também causa alterações pós-sinápticas ao modificar o tipo de subunidades presente nos recetores AMPA e NMDA. Seria interessante perceber se estas alterações são diretamente causadas pela ausência de CB_1 ou por outro mecanismo. Para além disto, devemos também tentar perceber como é que estas alterações afetam a maturação sináptica e os mecanismos de plasticidade.

Palavras-chave: Recetores Canabinóides do tipo 1, recetores AMPA, recetores NMDA, *core* do Nucleus Accumbens, eletrofisiologia.

Abstract

The Endocannabinoid System (ECS) is mainly composed by endocannabinoids (eCBs) and cannabinoid receptor proteins. The endocannabinoid signaling plays a major role in neural functions, regulating emotional and motivational states mostly through the activation of Cannabinoid type-1 receptors (CB_1), the main effectors of the ECS in the brain. The Nucleus Accumbens (NAc) is a major component of the Mesocorticolimbic pathway, being a key structure in mediating emotional and motivation processing, modulating reward and also pleasure. The NAc core receives glutamatergic inputs and as CB_1 have been shown to be essential to maintain evoked glutamatergic transmission, it suggests that they play a relevant physiological role for the NAc core excitability. Moreover, manipulation of CB_1 signaling within this brain region triggers robust emotional/ motivational alterations related to drug addiction and other psychiatric disorders (CB1 expressing neurons in the Nac, 2012). Although the associated presynaptic mechanism of endocannabinoid signaling has already been studied, postsynaptic changes may occur, as CB_1 are able to modulate AMPAR and NMDAR-mediated synaptic transmission. It is of interest to understand the relationship between AMPA, NMDA and CB_1 receptors in the NAc because they are essential not only to synaptic transmission but also plasticity, which can affect certain behaviors. Using *ex vivo* whole-cell patch clamp electrophysiology combined with pharmacology and transgenic mouse lines, we aimed at investigating whether the chronic lack of CB_1 affects spontaneous and evoked glutamatergic transmission in the NAc core. Our results show that full CB_1 knock-out mice ($CB_1^{-/-}$) have an increased frequency of miniature synaptic events without changes in their amplitude, while blocking CB_1 with the antagonist Rimonabant shows no effect. Moreover, $CB_1^{-/-}$ lack Calcium-permeable AMPARs and have an increase in GluN2A-containing NMDARs. Our results confirm the CB_1 presynaptic mechanism of action but also suggest a complementary postsynaptic mechanism. Altogether these results show that the chronic lack of CB_1 is able to induce postsynaptic changes in medium spiny neurons (MSNs) from the NAc core, specifically in AMPAR and NMDAR subunit composition.

Keywords: Cannabinoid type-1 receptors, AMPA receptors, NMDA receptors, Nucleus Accumbens core, electrophysiology.

1. Introduction

1.1. CB_1 and the Endocannabinoid system

1.1.1. First glimpse of the Endocannabinoid system

Cannabis has been used for medical and recreational purposes for at least eight thousand years, even though, its underlying physiological and molecular mechanisms have been unknown until these last few decades. The first observed medical benefits included encompassed anesthetic, airway opening and antihypertensive as well as antiemetic action. A century ago, cannabinol, the first plant-derived cannabinoid was isolated and, in the 1930s its chemical structure was elucidated[1]. In the following years tetrahydrocannabinols and cannabidiols were discovered and isolated but only in 1963 was the naturally occurring structure and stereochemistry of (-) – trans - Δ^9 – tetrahydrocannabinol (Δ^9 - THC), the main psychoactive constituent of Cannabis[2], unraveled.

At the time, the underlying mechanisms of cannabinoids were mostly believed to result from "non-specific" interactions between the lipophilic Δ^9 - THC and the cell membranes, changing the fluidity and structure of the latter, hence affecting most cell types[3],[4]. However, the unexpected recognition of cannabinoid-specific binding sites in the brain[5] led to the discovery of CB_1 in humans[6] as well as its high density in the brain[7]. CB_2 was then soon characterized[8], followed by the first endocannabinoid, Anandamide (AEA)[9], as well as the first selective CB_1 antagonist, Rimonabant[10]. Another famous eCB was also identified a year later, 2 - arachynodoilglycerol (2-AG)[11,12].

Recent technologies allowed us to engineer CB_1 knockout mice strains[13,14] and the vast majority of behavioral and physiological responses to cannabinoid ligands in the brain were no longer observed, compared to CB_2 knockout mice[15]. These findings, did not only underline CB_1 as the major cannabinoid receptor in the CNS, but also opened a whole new plethora of possibilities of cannabinoid involvement in synaptic transmission. As the conditional knockout technology became novel and widespread, neuron-specific conditional CB_1 knockout mice were also generated[16]. With all these discoveries in the "Cannabinoid World", the major phytocannabinoid structure was then identified as a tricyclic ring constituted from a phenol ring, having a 5-carbon alkyl chain meta to the hydroxyl, a central pyran ring, and a mono-unsaturated cyclohexyl ring[17]. Recently, the Endocannabinoid System surprised us once again and proved its ubiquity with the discovery of mitochondrial CB_1 ($mtCB_1$) and its direct involvement in both cellular respiration and synaptic plasticity[18].

CB_1 and CB_2 receptors have shown to be pathologically expressed in many diseases, not only nervous system related, such as neurodegenerative (Alzheimer disease[19], Multiple Sclerosis and Amyotrophic Lateral Sclerosis[20]) and psychiatric (Depression[21] and Schizophrenia[22]), but also in cardiovascular (Arteriosclerosis[23]), gastrointestinal (Cirrhosis[24]), among many others. The fact that the Endocannabinoid System is involved in many pathologies, along with the Cannabis decriminalization managed to break the wheel of mistrust on cannabinoids, hence opening a whole new field of

opportunities to be explored. Great efforts have been put into investigating Cannabis, mainly in the last century (**Fig. 1**). Such interest, managed to increase exponentially not only our knowledge, but also the methods available to study both the plant and the Endocannabinoid System.

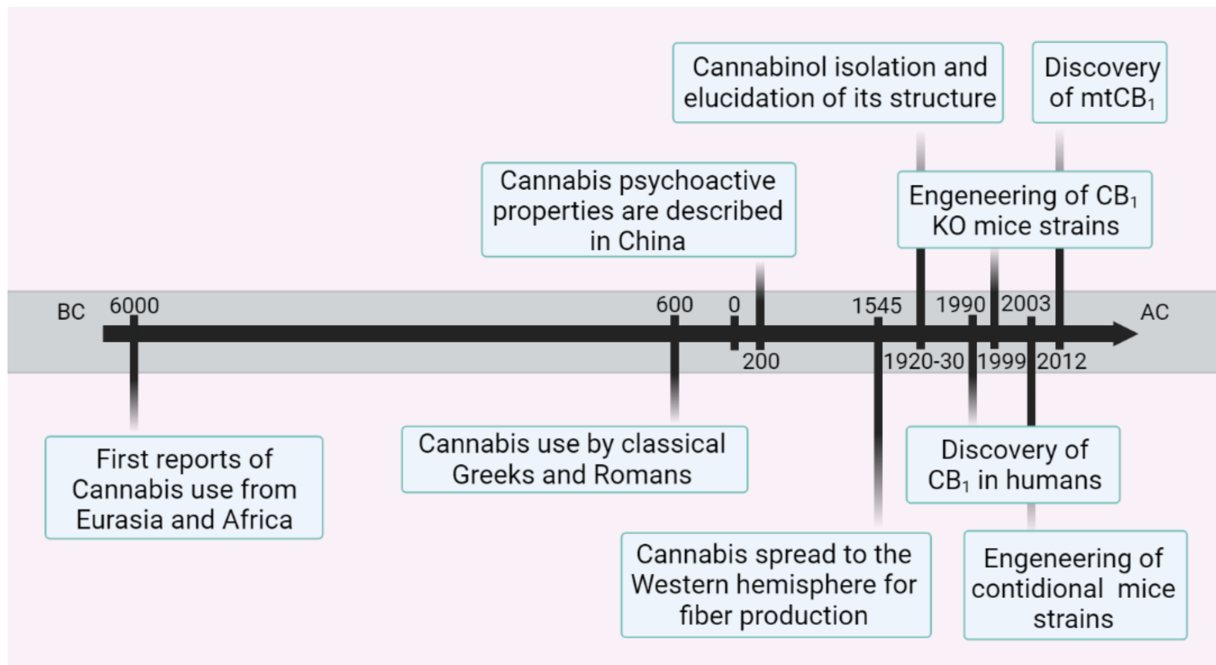


Fig. 1: Timeline of the events that marked the Cannabis history. Image was created with BioRender.com.

1.1.2. Cannabinoid Type-1 Receptors

CB_1 are G-protein coupled receptors (GPCRs)[1,25] that mediate many cannabinoid-induced effects, being involved in learning and memory[26, 27], cognition[28, 29], motor control[30], sleep[31], neuroprotection[32] and many more functions. They are encoded by the *cnr1* gene, located in the 6th human chromosome[33] and acquires a monomeric form composed of 472 aminoacids[34] (**Fig. 2A**), while only sharing 44% protein sequence homology with CB_2[35]. In recent years, two more isoforms of CB_1 have been identified, both resulting from alternative splicing[36]. These isoforms have shorter N-terminus hence changing ligands biding constants[37].

It is known that the full-length CB_1 dominates in the brain and skeletal muscle, whereas shorter isoforms are highly expressed in the liver and pancreatic islet cells, being involved in metabolism[38–40] (**Fig. 2B**). In the brain, CB_1 is the most-widely expressed receptor from the GPCR family[25,41], being highly present in neurons from the Olfactory bulb, Hippocampus, Basal Ganglia and Cerebellum. Moderate CB_1 expression is found in Cerebral Cortex, Amygdala and Hypothalamus, whereas regions like Thalamus and ventral horn of spinal cord have lower expression[41] (**Fig. 2C**). Several studies have suggested that CB_1 are highly expressed in presynaptic terminals, where it mediates retrograde signaling of eCBs[42,43]. Despite this, there is also evidence of postsynaptic CB_1 that act by mediating self-inhibition in neocortical neurons by endocannabinoids[44–46]. In addition to neural expression, CB_1R

are also expressed in astrocytes, where its activation regulates neuronal excitability, synaptic transmission and plasticity[47–50].

Cell membrane CB_1 are the most abundant and their activation decreases cAMP formation and intracellular Ca^{2+} influx[51]. Furthermore, recent studies have reported the presence of CB_1 associated to mitochondria[52,53], where it decreases mitochondrial respiration and cAMP production, hence regulating cellular energy metabolism[18], synaptic transmission, plasticity[54] and animal behavior[39]. Other than this, there is evidence of CB_1R in the membrane of acid-filled endo/ lysosomes[55,56].

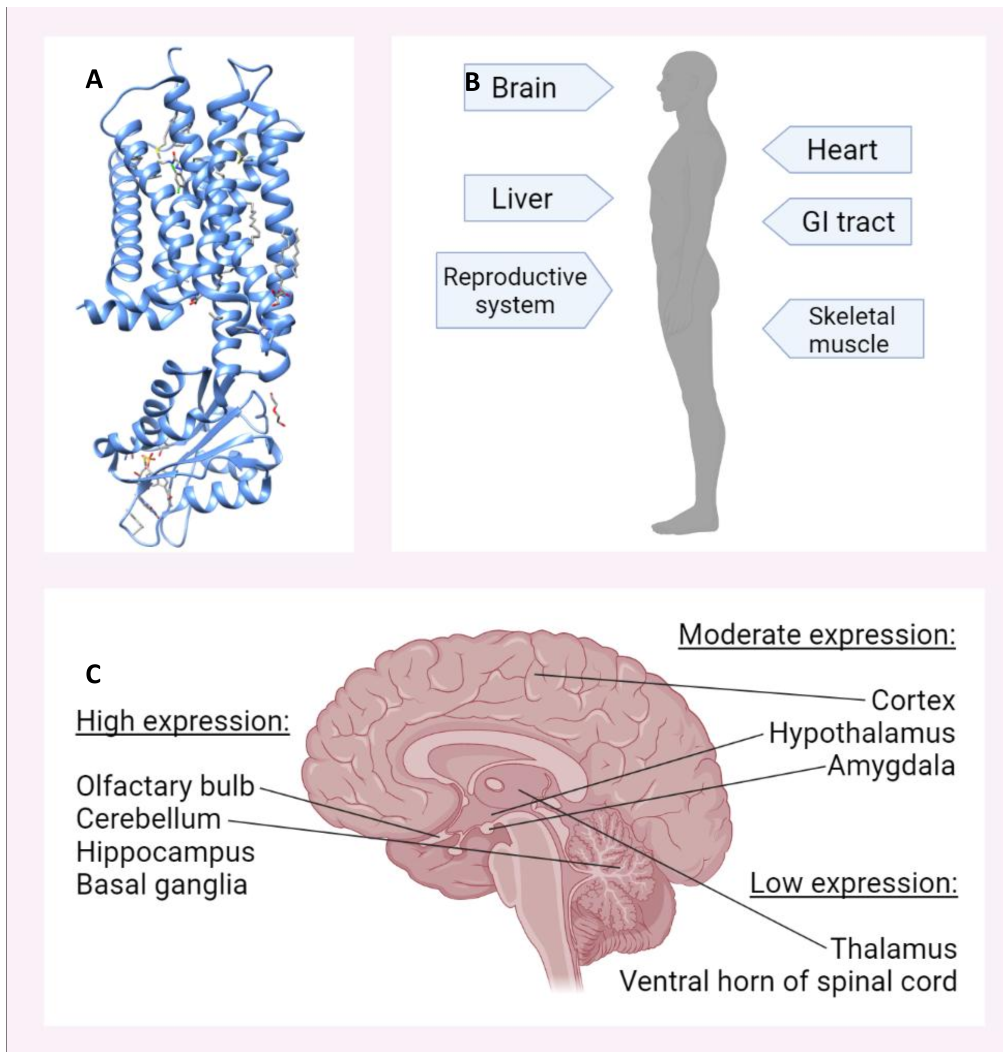


Fig. 2: CB_1 structure, their location in the body and expression levels in the brain. (A) Predicted CB_1 structure, acquired with X-ray diffraction and a resolution of 2.8 Å. Image was edited using Chimera software (PDB - 5TGZ) **(B)** CB_1 plays a role both in the brain and peripheral organs, where it is essential to regulate local tissue functions. Image was created with BioRender.com. **(C)** Depending on the brain region, CB_1 has different levels of expression. Image was created with BioRender.com.

1.1.3. CB_1 signaling in the Central Nervous System

CB_1-mediated signaling starts with the production of eCBs which is triggered by postsynaptic membrane depolarization[57], through the activation of postsynaptic receptors and consequent increase of intracellular Ca^{2+}. Once postsynaptic depolarization occurs, 2-AG is produced from Diacylglycerol and AEA is produced from N-acyl-phosphatidylethanolamine, two lipids commonly found in cell membranes[58]. Produced eCBs are then mobilized from the postsynaptic membrane and travel retrogradly to bind CB_1 in both presynaptic membranes[25,44] and nearby astrocytes [50,59] **(Fig. 3)**.

In neurons, CB_1 is coupled to $G_{i/o}$ and once activated it is able to modulate major signaling pathways by suppressing Adenyl cyclase activity, which then leads to the decrease of cAMP and consequent inhibition of PKA[44]. Altogether, these events lead to the decrease of calcium influx in the presynaptic cell, hence suppressing the release of neurotransmitters. On the other hand, astrocytic CB_1 is coupled to G_q and its activation leads to the increase of astrocytic calcium levels hence stimulating the release of glutamate[50,60].

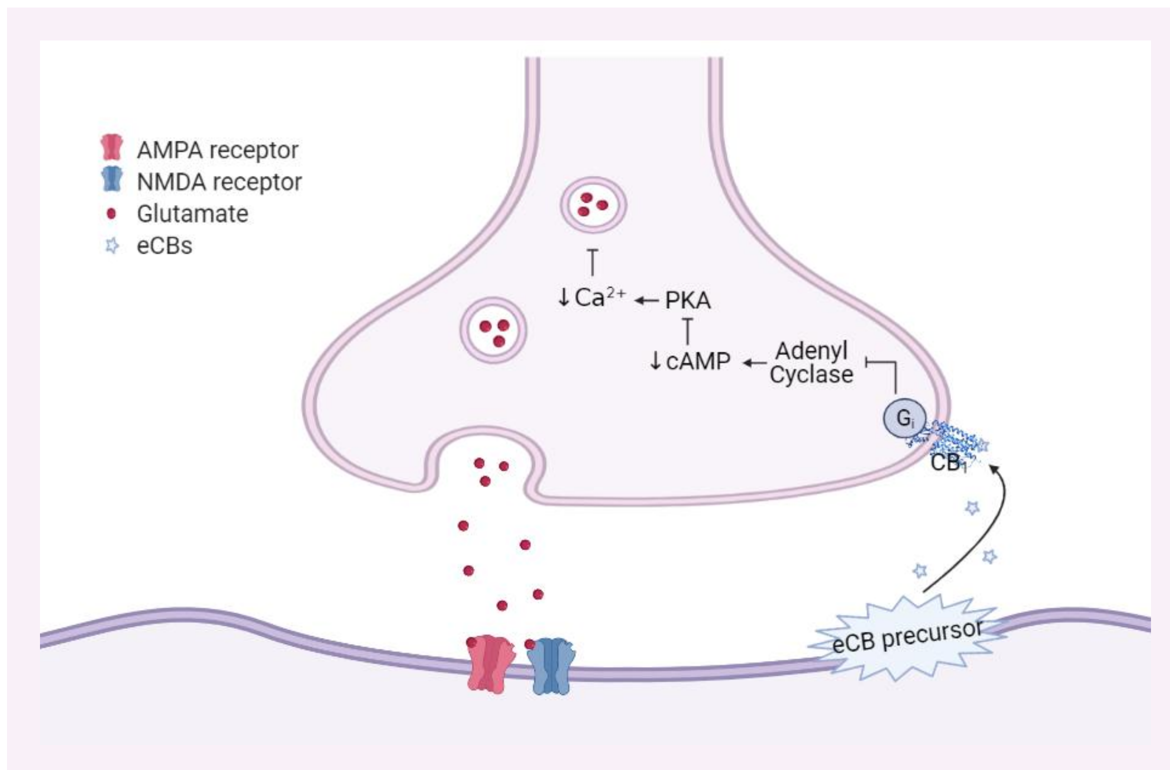


Fig. 3: CB_1 activation inhibits neurotransmitter release in neurons from the central nervous system. The depolarization of postsynaptic membranes is mediated by calcium increase and triggers endocannabinoid production. eCBs then travel retrogradly to bind and activate CB_1 which is coupled to $G_{i/o}$, being able to suppress Adenyl cyclase activity. The consequent decrease of cAMP and inactivation of PKA leads to a decrease of calcium influx in the presynaptic cell, which results in the inhibition of neurotransmitter release. Image was created with BioRender.com.

1.2. AMPA receptors

In the mammalian brain, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are responsible for the vast majority of fast excitatory neurotransmission. These receptors are encoded by the gene family *gria* in the 4th human chromosome and are usually located in postsynaptic terminals, more specifically in the postsynaptic density (PSD). AMPARs are ionotropic glutamate receptors while also being activated by AMPA. Once active, AMPARs contribute to the opening of cation channels, hence inducing membrane depolarization. Overall, such receptors are essential to allow for synaptic plasticity, learning and memory.

1.2.1. Structure and Subunit composition

The AMPAR signaling complex is typically composed by a tetrameric AMPAR[61–63] (**Fig. 4A**) and also a broad range of auxiliary proteins that modulate the trafficking, gating, pharmacology and permeation of receptors leading to a spatial and fine tuning of AMPARs function[64]. The tetrameric AMPAR structure is composed by a dimer of dimers, which includes a total of four neurotransmitter binding sites[65,66]. The tetramer is composed of various combinations of 4 different subunits (GluA1 – GluA4)[67] which differ mostly in their c-terminal sequence, responsible for interactions with scaffolding proteins[64]. This receptor is Na^{+}- permeable due to its porous structure. Moreover, AMPARs that lack the subunit GluA2 are also permeable to Ca^{2+}, being known as Calcium permeable AMPARs (Cp-AMPARs)[68–70]. Ca^{2+} impermeability is caused by a mRNA subunit switch from a conserved glutamine codon (neutral charge) to an arginine (positive charge)[69,70], allowing porous resistance to Ca^{2+}. The subunit GluA1 is also of interest since it is the most abundant, and along with GluA2, forms the most abundant AMPAR dimer[71]. Even though AMPARs were considered passive conduits for current flux across the membrane, recent evidence suggests that these receptors can also have metabotropic roles as cell surface signal transducers[72–74].

1.2.2. Location and Trafficking

AMPARs are diffusely located throughout dendrites during development and later become concentrated in the synapses[75], mostly in the PSD[76]. This type of receptor is particularly mobile and its trafficking is carefully determined by a balance of endocytosis[77], exocytosis[78] and lateral diffusion[76] (**Fig. 4B**). Due to its tight regulation, AMPARs can be rapidly translocated in and out of synapses in a NMDAR-dependent manner[79], leading to an increase or decrease in AMPAR expression depending on the stimulus.

Recent evidence shows that some synaptic plasticity mechanisms depend not only in NMDAR, but also in AMPAR subunit composition[80,81]. Interestingly, in some brain regions the switch mechanism of Cp-AMPARs by Calcium impermeable AMPARs (Ci-AMPARs) (or vice-versa) is essential to initiate synaptic plasticity[82,83]. Such switch seems to be controlled by Cp-AMPARs themselves, though the exact mechanism is yet to be understood. Such trafficking is believed to be

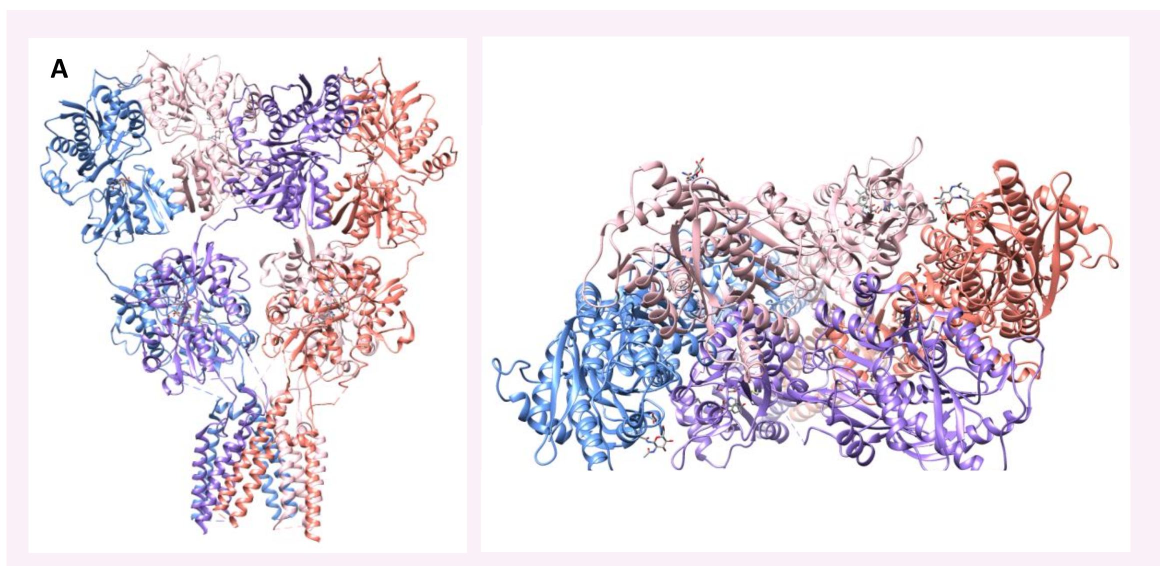
carried mainly through NMDAR activation, followed by increase in CaMKII and PKA[84] or PKC[85] activity. The activation of such protein kinases is known for phosphorylation insertion in AMPAR's Serines, which causes receptor activation and anchoring in the membrane of the PSD[86].

1.2.3. Function

AMPARs serve mainly as ion channels, contributing to synaptic transmission and plasticity, processes that are possible due to the carefully controlled receptor trafficking, introduced previously.

AMPARs pathway begins with their activation, followed by porous opening, ion influx and consequential postsynaptic depolarization[63,87]. In certain conditions, these events lead to NMDARs activation which in turn initiate Ca^{2+}-dependent signaling pathways that modulate the surface presence of AMPAR. Ultimately, AMPAR activation and consequent cascade of events, leads to the activation of MAPK/ERK pathway[74,88]. Curiously, CB_1 and NMDAR activation can also culminate in such pathway and thought the difference among such mechanism is believed to exist, it is yet to be clarified.

By allowing ion influx, these receptors are known to play a crucial role during long-term potentiation (LTP) and depression (LTD), two common forms of synaptic plasticity mechanisms. The occurrence of such processes depends not only on the brain region, but also on the type of neuron and its circuitry. Other than this, the availability of Ci and Cp - AMPARs, as well as the correct functioning of trafficking mechanisms, are also of extreme importance to successfully ensure synaptic plasticity mechanisms.



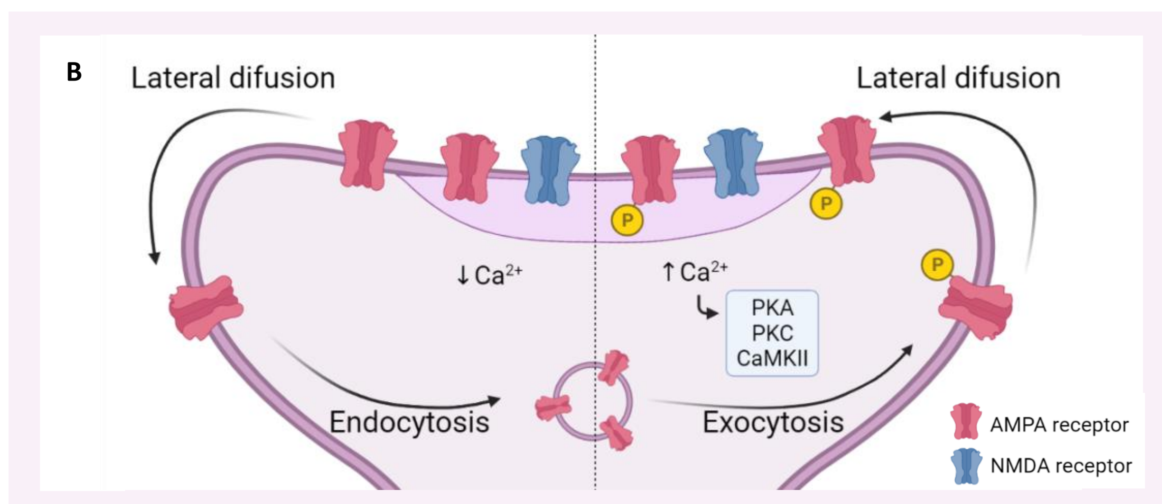


Fig. 4: AMPA receptor tetramer structure and its trafficking mechanisms. **(A)** Two different viewpoints of the predicted AMPA receptor structure, acquired with X-ray diffraction and a resolution of 3.5 Å. On the right, we can see the pore opening right in the middle of the receptor. Image was edited using Chimera software (PDB – 4U2Q). **(B)** AMPA receptor endocytosis is believed to be caused by a reduction of postsynaptic calcium influx through NMDARs. On the other hand, exocytosis is potentiated by calcium influx increase through NMDARs, which leads to the activation of PKA, PKC and CaMKII. Such proteins promote the insertion and anchoring of AMPARs in the PSD by phosphorylation in certain AMPAR residues, such as Serines. Image was created with BioRender.com.

1.3. NMDA receptors

N-methyl-D-aspartate receptors (NMDARs) are Ionotropic Glutamate receptors, encoded by the gene family *grin* in the 9th human chromosome. They bind Glutamate, NMDA and Glycine, being essential for fast excitatory neurotransmission[89]. Like AMPARs, NMDAR's functions in the synapses are extremely complex, finely tuned and differ between brain regions. Such complexity along with subunit composition is essential to ensure synaptic plasticity mechanisms[90], not only initiation, but also to ensure its maintenance.

1.3.1. Structure and subunit composition

NMDARs are heterotetramers (**Fig. 5A**) composed of two obligatory GluN1 and two regulatory subunits[91] - GluN2 and/or GluN3. NMDARs have a total of three binding sites and similarly to AMPARs, each subunit has 3 main parts: intracellular C–terminus binding associate scaffolding proteins, a transmembrane region comprising three segments and the extracellular N–terminus, which comprises the binding site[92]. GluN2 is mostly expressed in the PSD and is known for conferring high sensibility to Mg^{2+} blocking[93], while GluN3 is mostly expressed extra-synaptically and has very low Ca^{2+} influx, being resistant to Mg^{2+} blocking[94,95].

The subunit GluN2 is of special interest here, not only because it is the most abundant in mature neurons, but also because of its ability to greatly increase Ca^{2+} influx. Furthermore, GluN2A is

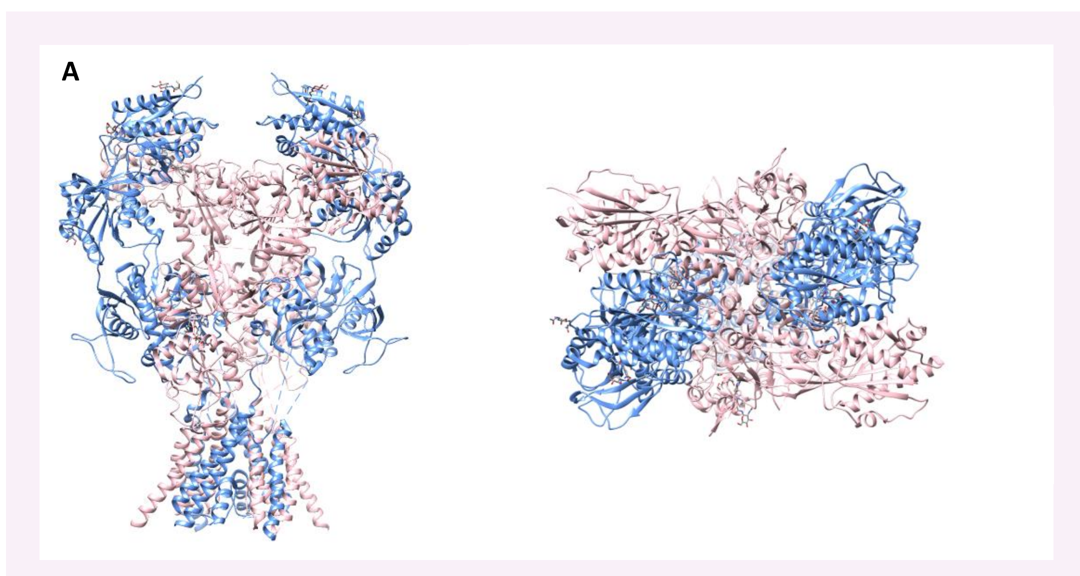
responsible for controlling open/close state of the NMDAR's channel, by allowing a strong Ca^{2+} influx[96] and on the other side GluN2B is responsible for initiating CamKII cascades[97] (**Fig. 5B**). The switch between these two subunit types have shown to be essential for synaptic plasticity mechanisms[98], such as LTP.

1.3.2. Location and trafficking

NMDARs are usually located in the PSD but can also be found pre-[99] and extra-synaptically[100], depending on their subunit composition. Like AMPARs, NMDARs are trafficked through endocytosis, exocytosis and lateral diffusion. The main control mechanism is believed to be through the activation of PKA or PKC which then leads to NMDAR phosphorylation[101,102]. While Tyrosine phosphorylation affects the surface expression of the receptors, Serine phosphorylations, as well as CAMKII-dependent phosphorylation of GluN2A subunits have also shown to increase ion flux through NMDAR pores.

1.3.3. Function

NMDARs have ion flux as their main function: opening of the NMDAR channel allows K^{+} efflux and also Na^{+} and Ca^{2+} influx, which consequently triggers intracellular signaling pathways (**Fig. 5B**). Similarly to AMPARs, NMDAR activation by Glutamate binding leads to open channel state, hence favoring Ca^{2+} influx, especially in GluN2-containing NMDARs. Such increase in the intracellular Ca^{2+} will induce the activation of CaMKII followed by the MAPK/ERK pathway[103]. Ultimately, such pathway can lead to nuclear gene expression by controlling the transcription factor ARC[104,105]. The proteins produced are essential to allow for the growth and stabilization of newly formed spines, therefore contributing to synaptic strengthening.



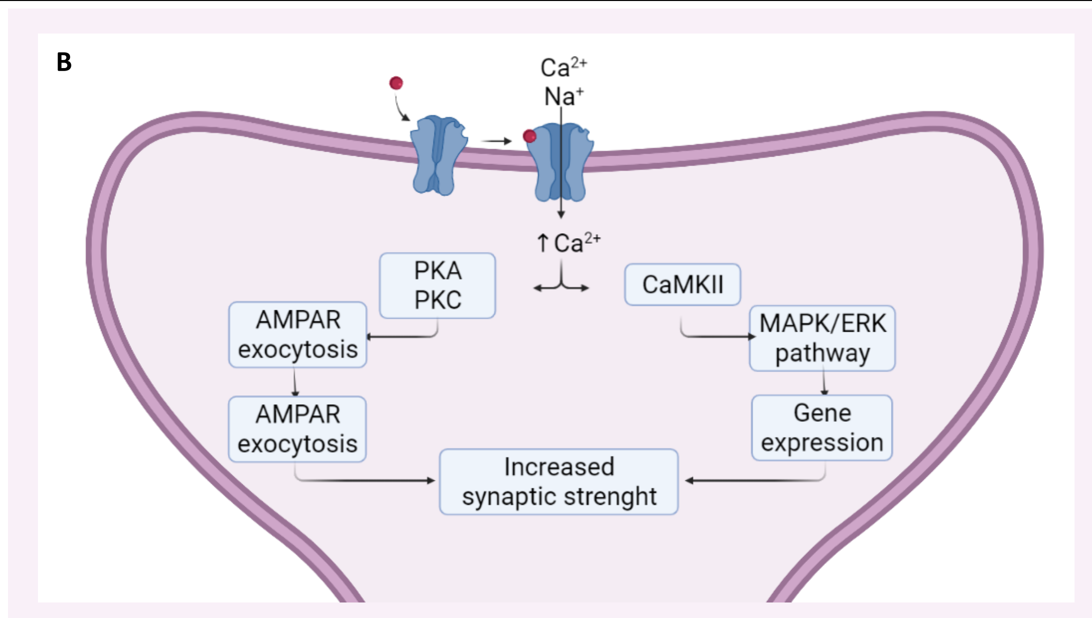


Fig. 5: NMDA receptor tetramer structure and related functions. (A) Two different viewpoints of the predicted NMDA receptor structure, acquired with X-ray diffraction and a resolution of 3.8 Å. On the right, we can see the pore opening right in the middle of the receptor. Image was edited using Chimera software (PDB – 4TLM). **(B)** Once Glutamate binds NMDARs, these get activated and in open-channel state. Consequently, there is an increase in postsynaptic calcium influx which can activate CaMKII and both PKA and PKC. While CaMKII leads to MAPK/ERK pathway followed by gene expression, PKA and PKC contribute to AMPAR exocytosis. Altogether, these events lead to increased synaptic strength. Image was created with BioRender.com.

1.4. Nucleus Accumbens core

1.4.1. Location and Function

The Nucleus Accumbens (NAc) is located in the basal forebrain and is part of the ventral striatum. Each cerebral hemisphere has its NAc, which is formed by two structures differing in function and morphology - an outer shell and an inner core[106]. It is anatomically located in a way that serves emotional and behavioural components of feelings, while being considered as a neural interface between motivation and action[107].

Functionally, the NAc is a major player in the Mesocorticolimbic circuit (or Reward System), a group of neural structures responsible for incentive salience (motivation and desire), associative learning (positive reinforcement and classical conditioning) and also positively valenced emotions, particularly pleasure-associated (joy and euphoria)[108–111]. More specifically, the NAc core is responsible for the cognitive processing of motor functions related to reward and reinforcement, while also being responsible specifically for cocaine and amphetamine addiction[112–114]. Other than playing a major role in cognitive, emotional and psychomotor functions, the NAc is involved in psychiatric disorders, not only addiction but also depression, schizophrenia, among many others.

1.4.2. Morphology and Circuitry

The NAc core is composed of 90% GABAergic medium spiny neurons (MSNs) (**Fig. 6A**), while the other 10% are GABAergic and Cholinergic interneurons. Furthermore, in this brain region, MSNs can be divided into those expressing dopamine receptor D1 (D1R) or dopamine receptor D2 (D2R)[115,116].

As a central part of the mesolimbic pathway, the NAc receives Dopamine inputs from the Ventral Tegmental Area and Glutamatergic inputs from the Basolateral Amygdala (BLA), Ventral Subiculum, Hippocampus and Prefrontal Cortex (**Fig. 6B**). The output neurons of the NAc send axonal projections to the Ventral Pallidum, Hypothalamus, Midbrain and also effector motor

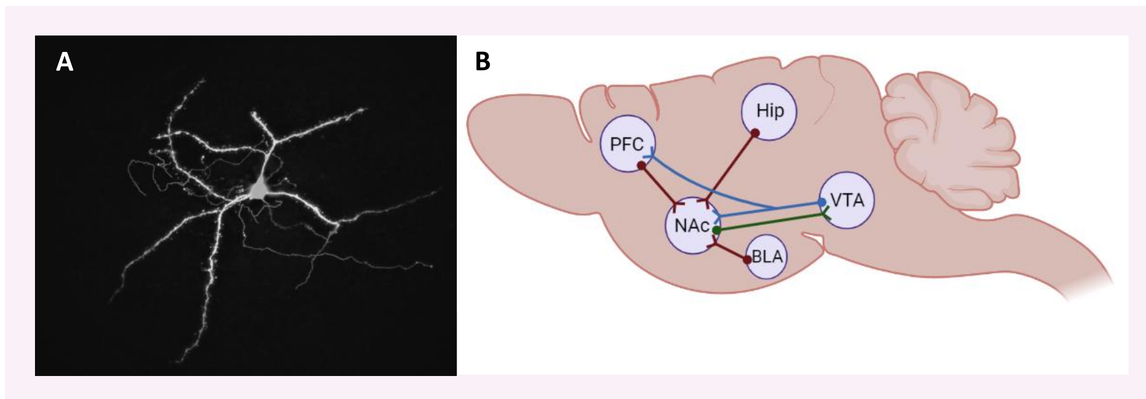


Fig. 6: (A) Example of a Biocytin filled MSN, after Streptavidin-AF646 counterstaining. Image was obtained with a confocal microscopy system and reconstructed using Las X and Photoshop softwares. **(B)** NAc circuitry involves Glutamatergic inputs from the hippocampus, Prefrontal cortex and Basolateral amygdala and Dopaminergic inputs from the Ventral Tegmental Area. The major GABAergic output is to the Ventral Tegmental Area. Image was created with BioRender.com.

1.4.3. Addiction and the NAc

The NAc plays a key role in the neural circuitry related to motivation and drug addiction[119,120]. Moreover, we now know that drug addiction involves activity-dependent plasticity at glutamate synapses within neural circuits important for motivated behaviors[121,122]. Other than the fact that drugs of abuse can influence fundamental cellular processes that regulate synaptic strength, considerable evidence suggests that activation of NAc MSNs by glutamate inputs originating from limbic and cortical regions is the final common pathway for drug seeking in many animal models of addiction[120]. The activation of NAc MSNs by glutamate inputs is primarily mediated by AMPARs[123], so it is not surprising that cocaine-elicited drug seeking depends on AMPARs transmission in the NAc[124–126]. Other than this, it is also known that enhanced AMPAR transmission in the NAc is associated with

enhanced drug seeking[127,128] and that the strength of AMPAR transmission in the NAC represents a critical determinant of intensity of drug seeking[113]. Altogether and along with the fact that Cp-AMPAR formation in the NAc mediates incubation of cocaine craving[126], it is extremely important to investigate GluA2-lacking AMPARs, in order to fully understand addiction mechanisms.

1.4.4. CB_1 influence in the NAc

Even though the circuitry and neurotransmitters in the NAc have been extensively described, there are still many questions concerning CB_1 influence in this brain region. Even though these receptors are sparsely expressed in the NAc, they play an important role in its core[129]. Furthermore, they are usually expressed in presynaptic membranes, specifically in glutamatergic terminals that contact GABAergic cell bodies[7,129].

It has been previously shown that pharmacological CB_1 activation decreases evoked glutamatergic transmission, in both *ex vivo* and *in vivo* experiments, suggesting that it plays a relevant physiological role for NAc excitability[130,131]. Such presynaptic mechanism has already been extensively studied, however postsynaptic changes are also believed to occur. It is important for us to understand the relationship between AMPA, NMDA and CB_1 receptors in the NAc because they are essential for synaptic transmission and plasticity, hence affecting many behavioral outcomes.

1.5. Goal

The first goal of this investigation project is to determine whether the chronic lack of CB_1 affects glutamatergic synaptic activity in the Nucleus Accumbens core of adult mice. Secondly, to investigate whether there are any synaptic changes in the postsynaptic membrane, specifically concerning AMPAR and NMDAR subunit composition. Lastly, to check which type of neurons is involved in such mechanisms.

2. Materials and Methods

2.1. Animals

Throughout the study, animals had *ad libitum* access to food and water. They were housed under controlled temperature (20–22°C), humidity (50–55%) and 12 hour light/dark cycle. All animals used in the experiments were adults (*ca.* 8-12 weeks old). Adequate measures were taken to minimize pain and discomfort of the animals, as well as the number of animals used in the experiments, on the basis of the 3Rs (replacement, reduction and refinement) principle. All experiments were conducted in strict compliance with the European Union recommendations and were approved by the French Ministry of Agriculture and Fisheries and the local ethical committee.

We used full CB_1 knockout ($CB_1^{-/-}$) mice [29], conditional knockout animals lacking CB_1 in GABAergic Dlx positive neurons (GABA-$CB_1^{-/-}$) [16, 132] and also knockout animals lacking CB_1 in Glutamatergic Nex positive neurons (Glut-$CB_1^{-/-}$) [16, 132]. As controls we used their wild-type littermates. All the animals used were breed and born in the facility where the experiments took place.

2.2. Electrophysiology Recordings

2.2.1. Brain slice preparation

Whole-cell electrophysiology recordings were obtained in acute brain slice preparations (**Fig. 7A**) from mice previously described. Animals were euthanized under isoflurane anesthesia after which the head was separated, followed by a sagittal cut along the skull and a coronal cut along the eye sockets. After brain extraction, it was placed in the specimen holder of the Vibratome which was then filled with oxygenated (95% O_2; 5% CO_2) and halfway-frozen cutting solution (in mM: 180 Sucrose, 2.5 KCl, 1.25 NAH_2PO_4, 26 $NaHCO_3$, 11 Glucose, 0.2 $CaCl_2$, 12 $MgCl_2$). After cutting 350 µm-thick coronal slices, these were transferred into a chamber filled with continuously oxygenated (95% O_2; 5% CO_2) artificial Cerebral-spinal fluid (aCSF, in mM: 123 NaCl, 26 $NaHCO_3$, 11 Glucose, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 NaH_2PO_4) and left incubating at 34ºC, for 30 minutes. After a minimum of 20 minutes recovery at room temperature (RT, 22-25ºC), slices were transferred to a recording chamber with continuously perfused oxygenated aCSF, at RT.

2.2.2. Whole-cell patch-clamp electrophysiology recordings

Recordings were performed using Multiclamp 700B amplifier (Molecular devices) in Medium Spiny neurons (MSNs) of the NAc core, clamped with 4-6 MU borosilicate glass pipettes. For recordings at - 70 and - 80 mV, experiments were performed with Potassium Gluconate - based intracellular solution (in mM: 130 KCl, 10 HEPES, 1 EGTA, 2 $MgCl_2$, 0.3 $CaCl_2$, 7 Phosphocreatin, 3 Mg_2-ATP, 0.3 Na_2-GTP; pH = 7.2; 290mOsm). For recordings of NMDA receptors at + 40 mV, a Cs^+-based intracellular solution was used (in mM: 20 HEPES, 0.4 EGTA, 5 TEA-Cl, 2.5 Mg_2-ATP, 0.25

Na_2-GTP, 2.8 NaCl, 117 CH_3CsO_3S, 290 mOSM, pH 7.2 – 7.4). The brain region was identified based on its relative position to the Anterior Commissure (**Fig. 7B**). MSNs were distinguished from interneuron cell types based on morphological (size, shape) and biophysical properties [132,133] (**Fig. 7C**). Electrophysiological data were acquired and filtered at 1Hz using an amplifier and fed to a computer using a Digidata. Neurons were recorded in voltage clamp mode of the patch clamp technique and the resting membrane potential and an access resistance was continuously monitored. Cells in which the access resistance changed more than 20% were discarded. Miniature EPSC recordings were recorded at -80 mV and isolated in the presence of TTX (1µM), Picrotoxin (50µM) and CGP54626 (1µM) to block Na^+ channels[134], $GABA_A$[135] and $GABA_B$[136] receptors, respectively.

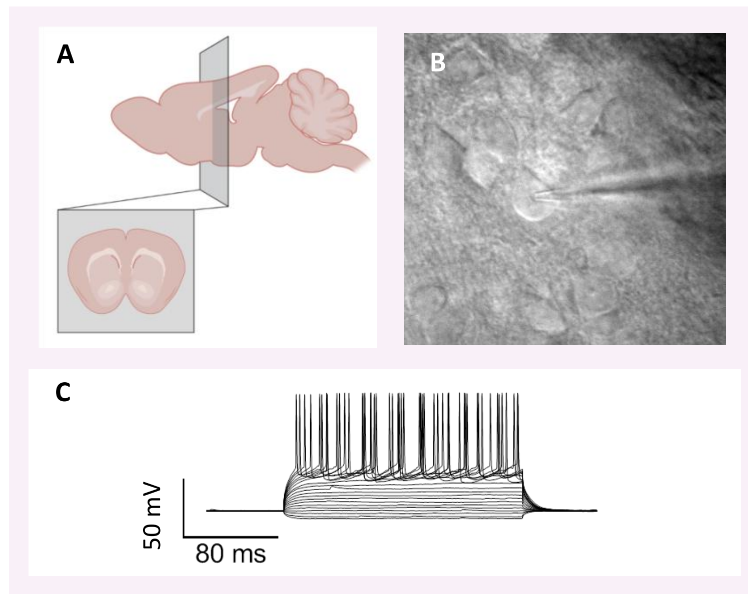


Fig. 7: (A) The NAc is located in the basal forebrain, surrounding the Anterior Commissure. Image was created with BioRender.com. **(B)** Pipette applying positive pressure in a MSN in the NAc. The dark region on the top left corresponds to the Anterior Commissure. (**C**) Representative traces of a current clamp recording of a NAc core MSN showing its response to hyperpolarizing and depolarizing steps.

2.2.3. Synaptic stimulation

Stimulator was connected to an aCSF-filled borosilicate glass pipette that was used for bipolar stimulation in MSNs of the NAc core. EPSCs were isolated using Picrotoxin (50µM) and CGP54626 (1µM). NMDA currents were measured in the presence of NBQX (10µ) which blocks AMPAR[137]. The AMPA/NMDA ratio was measured by stimulating at -80mV and +40mV which allow us to obtain AMPA currents and AMPAR+NMDAR currents, respectively. D-AP5 (50 µM, NMDAR antagonist) was then applied to block NMDARs[138] and isolate the AMPA current at +40mV[139].

$$\frac{AMPA}{NMDA}\,ratio = \frac{EPSC_{-80mv}}{EPSC_{+40mv} - EPSC_{+40\,mV/DAP5}}$$

2.3. Streptavidin staining

Slices from mice brains were obtained as described in **2.2.1.** and MSNs in the NAc were patched with a pipette containing internal solution with 0.05% biocytin. To perform immunostaining of biocytin filled neurons, the slices were fixed at 4% PFA overnight at 4ºC. After fixation, slices were transferred and washed in PBS, 3 times for 10 minutes. Biocytin filled MSNs were blocked (1x PBS, 0.3% Tritox-X) for an hour at RT before a 4-hour incubation with Streptavidin-AF647 (1:500) at RT. Brain slices were then washed in PBS, 3 times for 10 minutes before being mounted on gelatin-coated slides. Finally the cover slips were mounted with anti-fade prolong gold mounting medium and led to dry for 24h. Slices were imaged using a multiphoton SP5 from Leica.

2.4. Analysis and Statistics

2.4.1. Electrophysiology data analysis

Electrophysiological data were filtered at 1kHz by a Digidata and collected during the full length of each condition. Electrical properties of mEPSCs were analyzed with Axograph in which a temple function was created (5ms baseline, 30 ms length, -25 pA amplitude, 0.5 ms rise, 3 ms decay). Parameters were detected with a variable amplitude template (0 minimum event separation, 5 ms capture baseline, 30 ms length). mEPSC amplitudes were tested and synaptic events smaller than 5 pA and bigger than 100 pA were discarded. The measures analyzed for mEPSCs were mean amplitude of response, frequency of events, rise-time and decay-time (**Fig. 8B**). To compare mEPSCs, data were analyzed 5 minutes before and 15 after Rimonabant treatment. The measures analyzed for EPSCs were mean amplitude of EPCS response. Baseline mean EPSC amplitude was obtained by averaging mean values obtain within 5 minutes of baseline recordings and mean EPSC amplitudes were normalized to baseline. NASPM effect was statistically tested by comparing the mean of normalized EPSCs amplitude recorded during the 5 minute of baseline and 20 minutes after the drug, for 5 minutes. PEAQX effect was statistically tested by comparing the mean of normalized EPSCs amplitude recorded during the 3 minute baseline and 15 minutes after the drug, for 5 minutes.

2.4.2. Statistics

Electrophysiological data expressed as mean ± SEM.was analyzed and represented with Prism Software. Paired or unpaired statistical analyses were obtained with Student's t test. Statistical differences were established with $p < 0.05$ (*) and $p < 0.01$ (**).

Table 1: Chemicals, animal models, softwares/ algorythms and other materials used in the investigation project.

Name	Source	Identifier
Chemicals		
aCSF and Cutting solution		
Sucrose	Sigma-Aldrich	S0389
KCl	Sigma-Aldrich	P9541
NAH_2PO_4	Merck	1063461000
$NaHCO_3$	Sigma-Aldrich	S6014
Glucose	Sigma-Aldrich	G5767
$CaCl_2$	Sigma-Aldrich	C3881
$MgCl_2$	Sigma-Aldrich	M0250
NaCl	Sigma-Aldrich	746398
Internal solutions		
KCl	Sigma-Aldrich	P9541
HEPES	Sigma-Aldrich	H3375
EGTA	Sigma-Aldrich	E4378
$MgCl_2$	Sigma-Aldrich	M0250
$CaCl_2$	Sigma-Aldrich	C3881
Phosphocreatin	Sigma-Aldrich	P7936-5G
Mg-ATP	Sigma-Aldrich	A9187-500MG
Na-GTP	Sigma-Aldrich	G8877-25G
TEA-Cl	Sigma-Aldrich	T-2265
CH_3CsO_3S	Sigma-Aldrich	C1426
Drugs		
Picrotoxin	Sigma	P1675
CGP 54626 Hydrochloride	TOCRIS Bioscience	1088
Tetodrotoxin Cytrate	TOCRIS Bioscience	1069
Biocytin	Sigma-Aldrich	B4261
NASPM	TOCRIS Bioscience	2766
PEAQX	TOCRIS Bioscience	5018
Rimonabant	Cayman Chemical Company	9000484
NBQX	Abcam Biochemicals	ab120046
D-AP5	Abcam Biochemicals	ab120003

Experimental Model: Organisms/ strains		
Mice: $CB_1^{-/-}$	N/A	N/A
Mice: GABA-$CB_1^{-/-}$	N/A	N/A
Mice: Glut-$CB_1^{-/-}$	N/A	N/A
Software/ Algorythms		
Axograph	Axograph software	N/A
Chimera	RBVI	1.11.2
Clampfit	Molecular devices	Pclamp10
Clampex	Molecular devices	Pclamp10
Graphpad Prism 6.0	Graphpad software	Prism 6.0
Other		
Carbogene	Messer	105112501
Digidata	Molecular devices	1440 A
Humbug	Quest scientific	N/A
MultiClamp amplifier	Molecular devices	700 B
Patch-clamp microscope	Zeiss	Examiner A
Stimulator	Digitimer LTD	DS2A-mk.II
Vibratome	Leica	VT 1200 S
Micropipettes	Sutter Instrument Company	BT-150-10
Micropipette puller		P1000

3. Results

3.1. Total CB_1 deletion affects synaptic activity in the NAc core

To study the effect of total CB_1 deletion in synaptic activity, miniature excitatory synaptic potentials (mEPSCs) were recorded in both WT mice and $CB_1^{-/-}$ mice. The experiments were carried in a bath with the presence of TTX (1µM) to inhibit cell firing and Picrotoxin and CGP.

The results show that the frequency of mEPSCs is increased in the $CB_1^{-/-}$ compared to the WT (**Fig. 8C**). Both populations show similar mEPSCs amplitude (**Fig. 8D**) and rise-time (**Fig. 8E**). Moreover, the mEPSCs decay-time is significantly reduced in the $CB_1^{-/-}$ compared to their WT littermates (**Fig. 8F**).

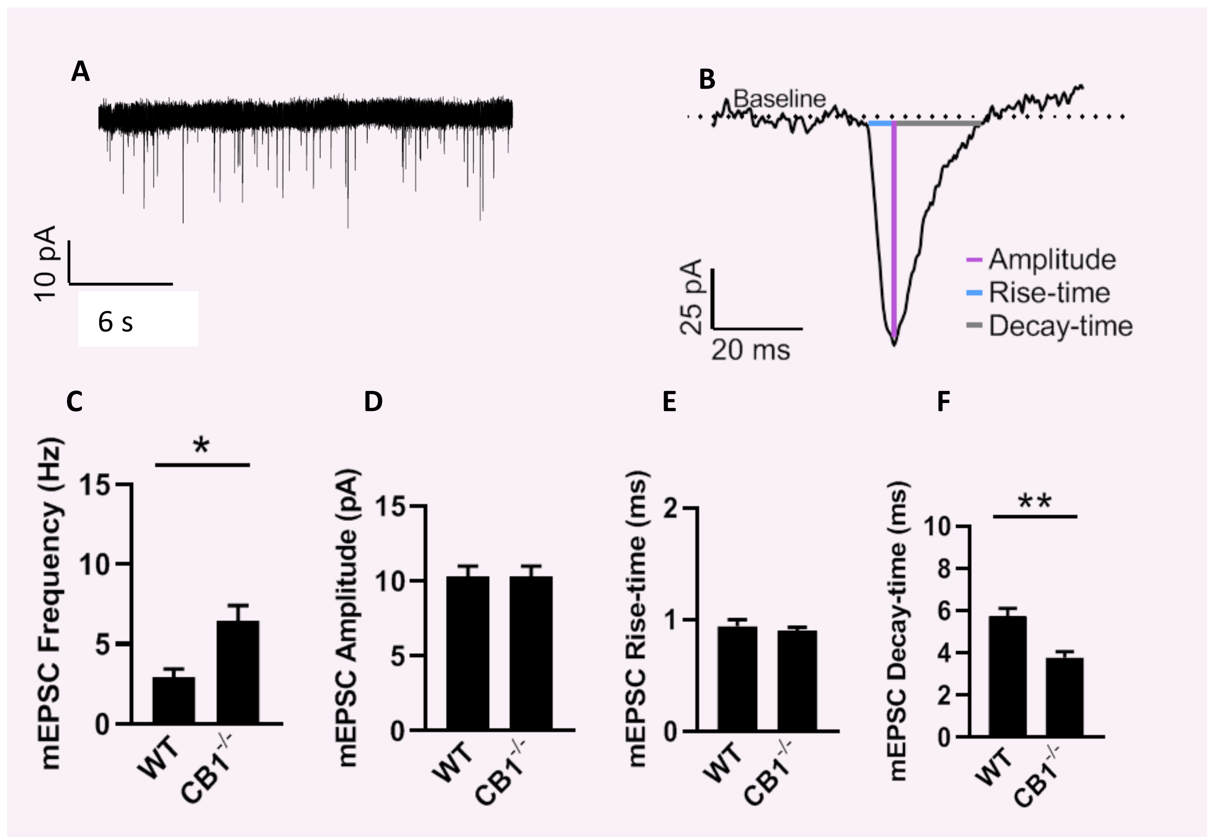


Fig. 8: Full CB_1 deletion increases the frequency and decay-time of mEPSCs, at glutamatergic synapses of the NAc core. (**A**) Representative trace of mEPSCs in the NAc core. (**B**) Representative trace of a single mEPSC in the NAc core and the parameters analyzed. (**C**) Average mEPSC frequency recorded at glutamatergic synapses. (**D**) Average mEPSC amplitude recorded at glutamatergic synapses. (**E**) Average mEPSC rise-time recorded at glutamatergic synapses, showing no difference between mice populations. (**F**) Average mEPSC decay-time recorded at glutamatergic synapses.

3.2. CB_1 blocking with Rimonabant does not affect basal synaptic activity in the NAc core

In order to understand if eCBs are being tonically released, CB_1 was blocked in WT mice, with the antagonist Rimonabant[10] (1µM). mEPSCs were recorded and the parameters were measured both before and after the treatment. The experiments were carried in the presence of TTX, CGP and Picrotoxin in the bath.

Blocking CB_1 with Rimonabant showed no changes in any of the parameters analyzed, when compared to the baseline (**Fig. 9**). Such results suggest that eCBs are not being tonically released in this brain region and make us wonder if basal activity is only affected by the chronic lack of CB_1.

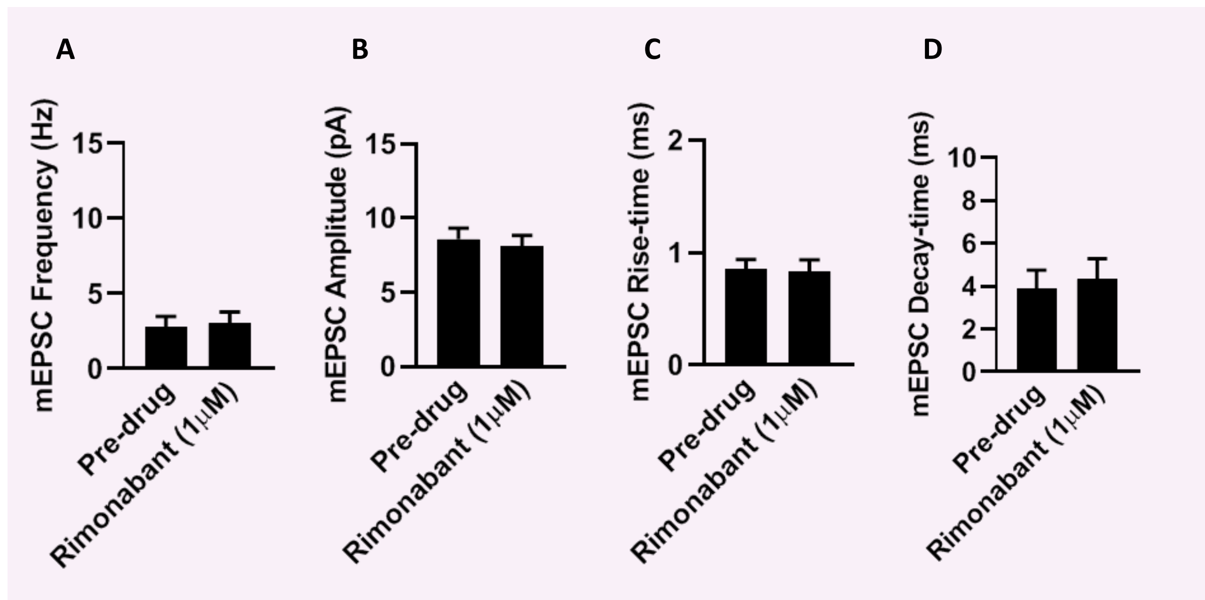


Fig. 9: Rimonabant treatment in NAc slices does not affect basal synaptic activity of the NAc core. (**A**) Average mEPSC frequency in the presence of Rimonabant, showing no difference between pre and after treatment. (**B**) Average mEPSC amplitude in the presence of Rimonabant, showing no difference between pre and after treatment. (**C**) Average mEPSC rise-time in the presence of Rimonabant, showing no difference between pre and after treatment. (**D**) Average mEPSC decay-time in the presence of Rimonabant, showing no difference between pre and after treatment.

3.3. Total CB_1 deletion does not affect AMPA/NMDA ratio in the NAc core

To check if the lack of CB_1 is affecting the post-synapse we first measured the AMPA/NMDA ration as an indicator of synaptic plasticity. Particularly, this ratio would change if the amount of AMPARs and/or NMDARs changes due to receptor motility or synapse remodeling[139,140]. NMDARs are blocked by Mg^{2+} at normal resting membrane potentials but this blockade is removed at depolarizing potentials[141]. Thus, stimulations at -80 mV and +40 mV allowed us to obtain AMPA currents ($EPSC_{-80mV}$) and AMPAR+NMDAR ($EPSC_{+40mV}$), respectively. To isolate the AMPA current during our recordings at +40 mV, D-AP5 (concentration, a NMDA antagonist) was then used to block

the NMDAR component of the EPSC ($EPSC_{+40mV/AP5}$). The NMDA component of the EPSC was then calculated by subtracting the $EPSC_{+40mV/AP5}$ from the $EPSC_{+40mV}$. The AMPA/NMDA ratio was calculated as seen in the equation below. The experiments were carried using a bath with CGP and Picrotoxin to isolate excitatory synaptic transmission.

$$\frac{AMPA}{NMDA}\,ratio = \frac{EPSC_{-80mv}}{EPSC_{+40mv} - EPSC_{+40\,mV/DAP5}}$$

The results show that there are no changes in the AMPA/NMDA ratio, between $CB_1^{-/-}$ mice and their WT littermates, indicating that either there are no differences in receptor numbers in the synapses of these two mouse lines, or that both receptors increase or decrease in similar way.

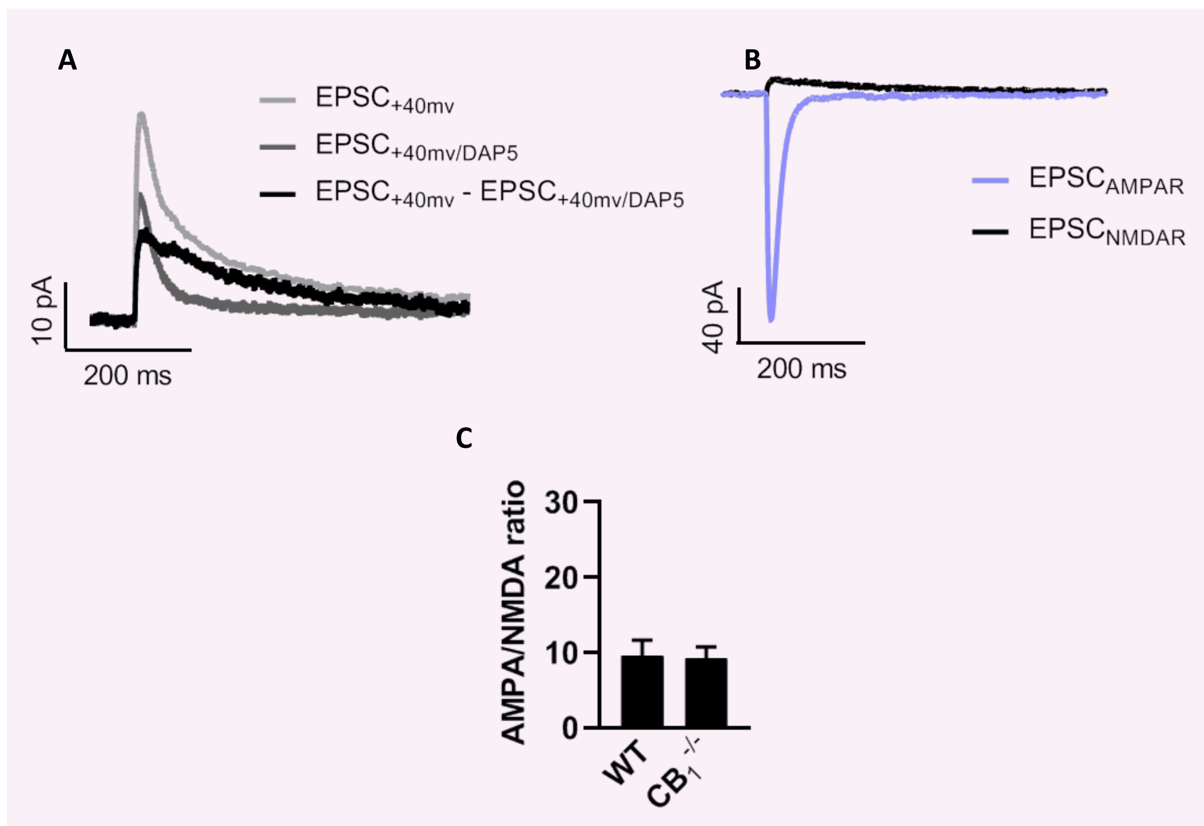


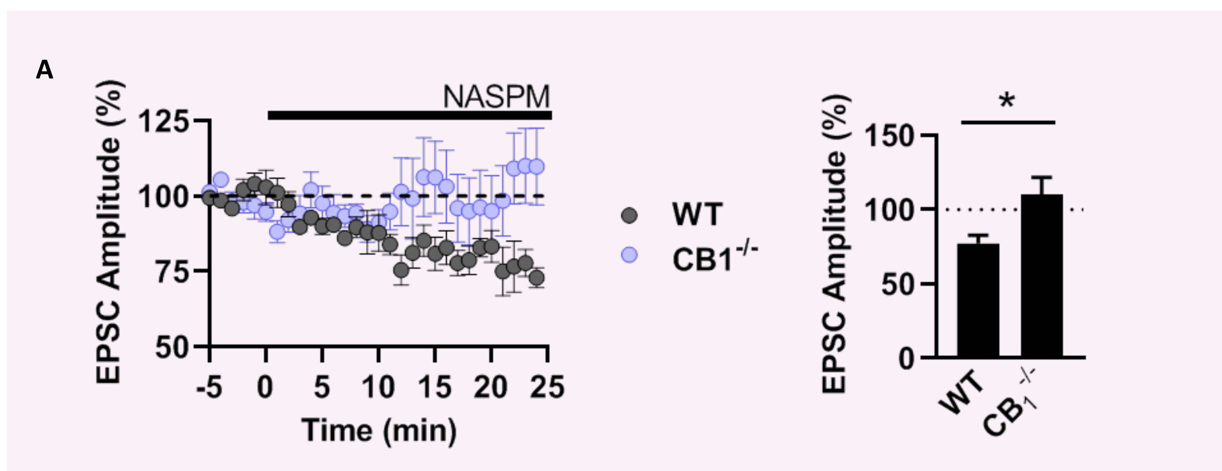
Fig. 10: The lack of CB_1 in the NAc core does not affect the AMPA/NMDA ratio nor changes the AMPAR I-V relationship. **(A)** Representative traces of the EPSCs used to determine the trace associated to NMDAR currents. **(B)** Representative traces of the EPSCs used to calculate the AMPA/NMDA ratio. **(C)** Average AMPA/NMDA ratios in MSNs from the NAc core, showing no difference between mice populations.

3.4. Total CB_1 deletion impairs Cp-AMPARs activity in NAc core MSNs

Next, we used another approach to assess postsynaptic modifications in the MSNs. During synaptic plasticity Ci-AMPAR are replaced in the synapse by Cp-AMPAR due to changes in their subunit composition. We therefore studied the subunit composition of AMPARs by recording EPSCs at -70 mV before and after the application of NASPM 25µM (GluA2-containing AMPAR antagonist) which blocks Cp-AMPARs[142].

Blockage of Cp-AMPARs by NASPM showed a decrease in EPSC amplitude in WT mice, indicating that WT mice contain both CP and Ci-AMPARs. However, in $CB_1^{-/-}$ mice NASPM did not have an effect, indicating that $CB_1^{-/-}$ mice lack Cp-AMPARs. These results suggest that while WT mice undergo normal synaptic changes during development, $CB_1^{-/-}$ mice lack certain mechanisms that allow for these synaptic modifications, probably due to the lack of endocannabinoid signaling.

CB_1 receptors are expressed in different cell types including the excitatory inputs that arrive to the NAc, but also in local GABAergic interneurons. In order to understand which cell types are involved in the AMPA modification, NASPM was then applied to brain slices from different CB_1conditional mutants. Because CB_1 is enriched in presynaptic terminals, we first used a mouse line that lacks CB_1 in glutamatergic neurons (Glut-$CB_1^{-/-}$), however, we did not find a significant difference between these mice and their WT littermates in the effect of NASPM. These results show that presynaptic CB_1 in the glutamatergic terminals is not involved in the AMPA receptor remodeling deficits observed in the $CB_1^{-/-}$. Next we tested a mouse line that lacks CB in the GABAergic neurons and, surprisingly, we did not find a significant difference between these mice and their WT littermates in the effect of NASPM, indicating that CB_1 expressed in the MSN or in GABAergic interneurons is not involved in the AMPA receptor remodeling deficits observed in the $CB_1^{-/-}$. Altogether these results show that the impairment of Cp-AMPARs replacement in the synapse that we observed in $CB_1^{-/-}$ mice is caused either by the global CB_1 deletion or by other cell type such as Acetilcholinergic interneurons, Dopaminergic terminals or astrocytes.



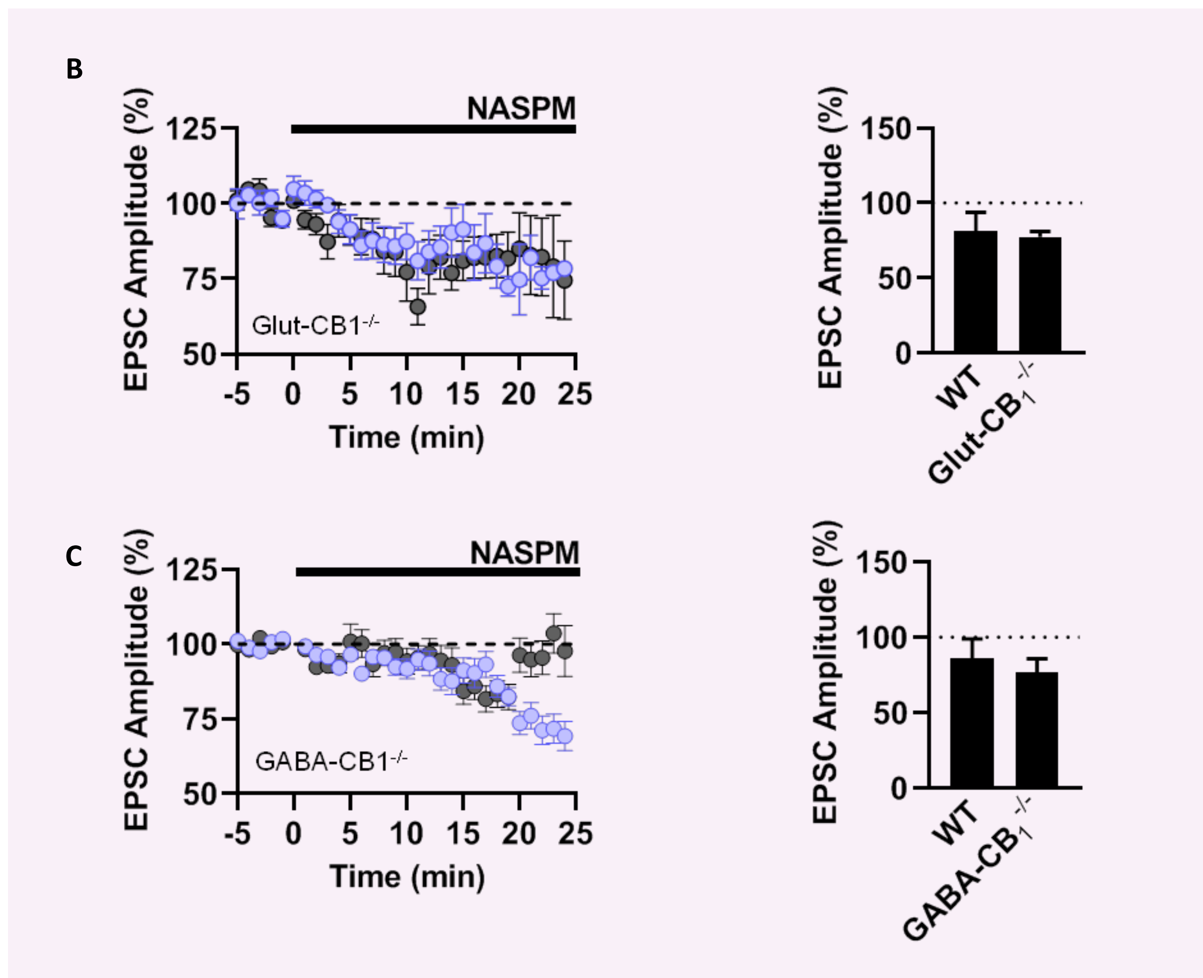


Fig. 11: The lack of CB_1 impairs Cp-AMPARs activity in full knock-outs but not in Glut-$CB_1^{-/-}$ nor in GABA-$CB_1^{-/-}$. (A) Time course of normalized EPSC amplitude and quantification of average EPSC amplitude during NASPM bath application, in both $CB_1^{-/-}$ and their WT littermates. **(B)** Time course of normalized EPSC amplitude and quantification of average EPSC amplitude during NASPM application, in Glut-$CB_1^{-/-}$ mice. **(C)** Time course of normalized EPSC amplitude and quantification of average EPSC amplitude during NASPM application, in GABA-$CB_1^{-/-}$ mice.

3.5. Total CB_1 deletion alters the subunit composition of NMDARs in NAc core MSNs

Next, we use a similar approach to assess more postsynaptic modifications in the MSNs. During synaptic plasticity, there are changes in the Ca^{2+} influx through NMDARs, due to changes in their subunit composition. We therefore studied the subunit composition of NMDARs by recording EPSCs at +40mV before and after the application of PEAQX 0.5 µM (GluN2A-containing NMDARs antagonist) which blocks highly Ca^{2+}-permeable NMDARs[143].

Blockage of GluN2A-containing NMDARs by PEAQX showed decreased EPSC amplitude in WT and $CB_1^{-/-}$ mice, however the PEAQX effect was higher in the $CB_1^{-/-}$ mice, indicating that $CB_1^{-/-}$ mice are enriched in GluN2A-containing NMDARs compared to WT. These results suggest that while

WT mice undergo normal synaptic changes during development, $CB_1^{-/-}$ mice have certain mechanisms altered, probably due to the lack of endocannabinoid signaling.

CB_1 is pre-synaptically expressed, including in the excitatory inputs that arrive to the NAc. In order to understand if glutamatergic neurons are involved in the NMDAR modification, PEAQX was then applied to brain slices from Glut-$CB_1^{-/-}$. The results show that Glut-$CB_1^{-/-}$ mice have no significant difference from their WT littermates, indicating that CB_1 expression in glutamatergic neurons is not involved in the enrichment of GluN2A-containing NMDARs found in $CB_1^{-/-}$ mice. Altogether these results show that the impairment of highly Ca^{2+}-permeable NMDAR replacement in the synapse that we observed in $CB_1^{-/-}$ mice is caused either by the global CB_1 deletion or by other cell type such as GABAergic cells, Acetilcholinergic interneurons, Dopaminergic terminals or astrocytes.

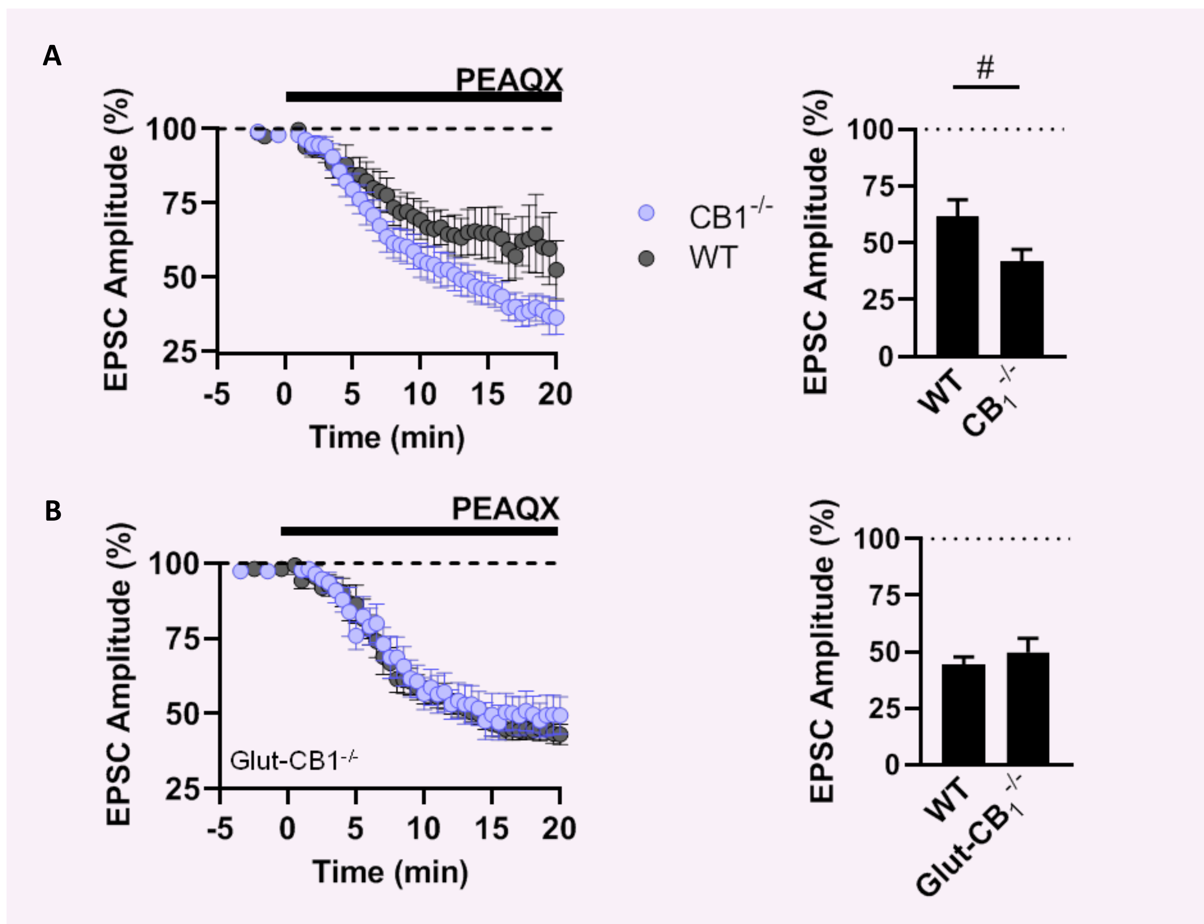


Fig. 12: The lack of CB_1 increases the activity of GluN2A-containing NMDARs in NAc core MSNs from full knock-outs but not in Glut-$CB_1^{-/-}$. (A) Time course of normalized EPSC amplitude and quantification of average EPSC amplitude during PEAQX bath application, in both $CB_1^{-/-}$ and their WT littermates. **(B)** Time course of normalized EPSC amplitude and quantification of average EPSC amplitude during PEAQX bath application, in both Glut-$CB_1^{-/-}$ and their WT littermates.

4. Discussion

Our results show that full CB_1 knock-out mice have a frequency increase of miniature synaptic events without changes in their amplitude. However, blocking CB_1 with the antagonist Rimonabant shows no effect. Moreover, $CB_1^{-/-}$ lack Calcium-permeable AMPARs and have an increase in GluN2A-containing NMDARs. Our results confirm the CB_1 presynaptic mechanism of action but also suggest a complementary postsynaptic mechanism.

4.1. Total CB_1 deletion affects synaptic activity in the NAc core

Changes observed in event frequency suggest that $CB_1^{-/-}$ mice have a chronic increase in the number of released glutamate-containing vesicles, which goes accordingly to the CB_1 presynaptic mechanism. The lack of change in amplitude suggests that the amount of receptors in the post-synapse is unchanged between $CB_1^{-/-}$ mice and their WT littermates. Moreover, the lack of change in the rise-time suggests that the amount of Glutamate in each vesicle is unchanged between the two mice populations. Furthermore, the decrease in the decay-time in $CB_1^{-/-}$, suggests that the clearance of Glutamate from the synaptic cleft is decreased in this population. This effect may be due to a smaller coverage of the synapse by astrocytes or to a down-regulation of glutamate transporters in the astrocytes. More experiments are necessary to elucidate what is the cause of such changes.

Altogether, these results suggest that CB_1 is essential to control the release and clearance of Glutamate in the NAc core. Glutamatergic release seems to be controlled by presynaptic CB_1 while clearance is suspected to be controlled possibly by astrocytic CB_1, since they are the ones usually responsible for Glutamatergic clearance.

4.2. Endocannabinoids are not tonically mobilized in the NAc core

The lack of changes seen with the Rimonabant treatment suggests that endocannabinoids are not being tonically produced and mobilized. Our results are supported by previous literature[129,131] and one may hypothesize that the changes seen in the basal synaptic activity in the $CB_1^{-/-}$ mice are due to a chronic lack of CB_1, instead of a simple blockade.

To further understand if the changes are due to CB_1 in the NAc, one can administer a chronic Rimonabant treatment, by microinjecting it directly into the NAc core (via neurosurgery). Moreover, mice can be intraperitoneally injected with Rimonabant, in order to achieve CB_1 blocking in the entire body.

4.3. Total CB_1 deletion does not affect AMPA/NMDA ratio in the NAc core

There was no change in the AMPA/NMDA ratio, suggesting the relative quantity of these receptors is unchanged between WT and $CB_1^{-/-}$ mice. The lack of change in these results, together with the results in **3.1.** and **3.2.** led us to think that there may be changes in the subunit composition of

AMPA and/or NMDA receptors. Such changes may be caused by a defective AMPAR switch mechanism, which might be useful to compensate the lack of CB_1.

4.4. Total CB_1 deletion impairs Cp-AMPARs activity in NAc core MSNs

The lack of Cp-AMPARs in $CB_1^{-/-}$ mice led us to think that there may be an impairment in AMPAR switch mechanisms. Such situation can be caused by excessive AMPAR phosphorylation, which has shown to affect receptor movement in and out of the PSD. With CB_1 deletion and consequential impairment of its cascade, a chronic increase in CamKII and PKA might occur, due to lack of AC inhibition. PKA activation is known to cause GluA1-containing AMPARs phosphorylation and thus their insertion in the PSD and activation. As such, one might hypothesize that CB_1 deletion chronically increases PKA activity, hence inhibiting GluA1-containing AMPARs dephosphorylation and internalization, which can affect the switch mechanisms. This being said, CB_1 may be indirectly affecting synaptic regulation and influencing AMPARs trafficking by potentiating phosphorylation/ dephosphorylation.

As future experiment and to determine if AMPAR phosphorylation levels are altered, one can use antibodies, specific for phosphorylated AMPARs. These antibodies can be specific to PKA or PKC induced phosphorylation, which would be even more elucidating.

4.5. Total CB_1 deletion alters the subunit composition of NMDARs in NAc core MSNs

The increase of GluN2A-containing NMDARs in $CB_1^{-/-}$ compared to the WT, suggests that NMDARs are increasing the postsynaptic Calcium influx, due to its increased probability of open channel state. Such effect might be happening to compensate the increased neurotransmission caused by the chronic lack of CB_1. On the other hand, it can be a consequence of the increased activation of PKA, PKC and CaMKII which can further increase the probability of open channel state by phosphorylation. To test this hypothesis, we can determine the phosphorylation levels of NMDARs with antibodies.

It would be interesting to understand if the lack of CB_1 is directly affecting AMPARs and NMDARs subunit composition or if they are caused by another indirect mechanism. The changes seen are not due to CB_1 expression in Glut- or GABAergic cells so it can be due to other cell types like astrocytes, acetilcholinergic interneurons or Dopaminergic inputs coming from the VTA. While CB_1 expression in acetilcholinergic interneurons is very high, the Dopaminergic inputs are improbable as CB_1 expression is very low. In astrocytes it is very difficult to detect, however their functional role has already been described in other brain regions and it would not be surprising that it plays a role here too. Furthermore, we should also try to understand how such changes affect mice behaviors. Because the changes in AMPA are the basis of synaptic plasticity and they can also be induces after exposure to drugs of abuse, it would not be surprising that $CB_1^{-/-}$ mice have altered behavioral response to drugs of abuse but also to hedonic rewards. Other than this, the NAc receives inputs from different brain

regions and whether these changes are happening in all those synapses needs to be elucidated. We also don't know if these changes occur either in D1 or D2 MSNs and even perhaps in both. If the changes observed are synapse or cell-type specific, it is possible that the effects observed here are diminished as the effect may be concealed by the synapses and/or cells that were not undergoing the same synaptic changes. If the changes are also synapse or cell-type specific it is possible that it will impact certain behaviors related to those brain areas or cell-types while leaving other behaviors unchanged.

5. Conclusions

With this investigation project we can conclude that the lack of CB_1 induces changes in the presynaptic activity, allowing the increase of glutamate release. Furthermore, the chronic lack of CB_1 induces postsynaptic changes by modifying the composition of AMPA and NMDA receptor subunits. The type of AMPAR subunits does not depend on the presence of CB_1 in Glutamatergic or in GABAergic neurons, while the type of NMDA subunits does not depend on the presence of CB_1 in Glutamatergic neurons. In summary, our findings suggest that the chronic lack of CB_1 affects synaptic maturation possibly due to deficits in CB_1-dependent synaptic plasticity mechanisms.

6. References

1. Pertwee, R. G. Cannabinoid pharmacology: the first 66 years: Cannabinoid pharmacology. *Br. J. Pharmacol.* **147**, S163–S171 (2006).
2. Gaoni, Y. & Mechoulam, R. Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *J. Am. Chem. Soc.* **86**, 1646–1647 (1964).
3. Lawrence, D. K. & Gill, E. W. The effects of delta1-tetrahydrocannabinol and other cannabinoids on spin-labeled liposomes and their relationship to mechanisms of general anesthesia. *Mol. Pharmacol.* **11**, 595–602 (1975).
4. Hillard, C. J., Harris, R. A. & Bloom, A. S. Effects of the cannabinoids on physical properties of brain membranes and phospholipid vesicles: fluorescence studies. *J. Pharmacol. Exp. Ther.* **232**, 579–588 (1985).
5. Devane, W. A., Dysarz, F. A., Johnson, M. R., Melvin, L. S. & Howlett, A. C. Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.* **34**, 605–613 (1988).
6. Gérard, C., Mollereau, C., Vassart, G. & Parmentier, M. Nucleotide sequence of a human cannabinoid receptor cDNA. *Nucleic Acids Res.* **18**, 7142 (1990).
7. Herkenham, M. *et al.* Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J. Neurosci. Off. J. Soc. Neurosci.* **11**, 563–583 (1991).
8. Munro, S., Thomas, K. L. & Abu-Shaar, M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365**, 61–65 (1993).
9. Devane, W. A. *et al.* Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**, 1946–1949 (1992).
10. Rinaldi-Carmona, M. *et al.* SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.* **350**, 240–244 (1994).
11. Mechoulam, R. *et al.* Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50**, 83–90 (1995).

12. Sugiura, T. *et al.* 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* **215**, 89–97 (1995).

13. Zimmer, A., Zimmer, A. M., Hohmann, A. G., Herkenham, M. & Bonner, T. I. Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 5780–5785 (1999).

14. Ledent, C. Unresponsiveness to Cannabinoids and Reduced Addictive Effects of Opiates in CB1 Receptor Knockout Mice. *Science* **283**, 401–404 (1999).

15. Buckley, N. E. *et al.* Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur. J. Pharmacol.* **396**, 141–149 (2000).

16. Marsicano, G. CB1 Cannabinoid Receptors and On-Demand Defense Against Excitotoxicity. *Science* **302**, 84–88 (2003).

17. Howlett, A. C. *et al.* Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology* **47**, 345–358 (2004).

18. Bénard, G. *et al.* Mitochondrial CB1 receptors regulate neuronal energy metabolism. *Nat. Neurosci.* **15**, 558–564 (2012).

19. Mulder, J. *et al.* Molecular reorganization of endocannabinoid signalling in Alzheimer's disease. *Brain J. Neurol.* **134**, 1041–1060 (2011).

20. Yiangou, Y. *et al.* COX-2, CB2 and P2X7-immunoreactivities are increased in activated microglial cells/macrophages of multiple sclerosis and amyotrophic lateral sclerosis spinal cord. *BMC Neurol.* **6**, 12 (2006).

21. Hungund, B. L. *et al.* Upregulation of CB1 receptors and agonist-stimulated [35S]GTPγS binding in the prefrontal cortex of depressed suicide victims. *Mol. Psychiatry* **9**, 184–190 (2004).

22. Dean, B., Sundram, S., Bradbury, R., Scarr, E. & Copolov, D. Studies on [3H]CP-55940 binding in the human central nervous system: regional specific changes in density of cannabinoid-1 receptors associated with schizophrenia and cannabis use. *Neuroscience* **103**, 9–15 (2001).

23. Steffens, S. *et al.* Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice. *Nature* **434**, 782–786 (2005).

24. Teixeira-Clerc, F. *et al.* CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat. Med.* **12**, 671–676 (2006).

25. Kano, M., Ohno-Shosaku, T., Hashimotodani, Y., Uchigashima, M. & Watanabe, M. Endocannabinoid-mediated control of synaptic transmission. *Physiol. Rev.* **89**, 309–380 (2009).

26. Miller, L. L. & Branconnier, R. J. Cannabis: effects on memory and the cholinergic limbic system. *Psychol. Bull.* **93**, 441–456 (1983).

27. Terral, G. *et al.* CB1 Receptors in the Anterior Piriform Cortex Control Odor Preference Memory. *Curr. Biol.* **29**, 2455-2464.e5 (2019).

28. Terranova, J. P. *et al.* Improvement of memory in rodents by the selective CB1 cannabinoid receptor antagonist, SR 141716. *Psychopharmacology (Berl.)* **126**, 165–172 (1996).

29. Marsicano, G. *et al.* The endogenous cannabinoid system controls extinction of aversive memories. *Nature* **418**, 530–534 (2002).

30. El Manira, A. & Kyriakatos, A. The Role of Endocannabinoid Signaling in Motor Control. *Physiology* **25**, 230–238 (2010).

31. Murillo-Rodríguez, E. The role of the CB1 receptor in the regulation of sleep. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **32**, 1420–1427 (2008).

32. van der Stelt, M. & Di Marzo, V. Cannabinoid receptors and their role in neuroprotection. *Neuromolecular Med.* **7**, 37–50 (2005).

33. Mungall, A. J. *et al.* The DNA sequence and analysis of human chromosome 6. *Nature* **425**, 805–811 (2003).

34. Hua, T. *et al.* Crystal Structure of the Human Cannabinoid Receptor CB1. *Cell* **167**, 750-762.e14 (2016).

35. Montero, C., Campillo, N. E., Goya, P. & Páez, J. A. Homology models of the cannabinoid CB1 and CB2 receptors. A docking analysis study. *Eur. J. Med. Chem.* **40**, 75–83 (2005).

36. Shire, D. *et al.* An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. *J. Biol. Chem.* **270**, 3726–3731 (1995).

37. Ryberg, E. *et al.* Identification and characterisation of a novel splice variant of the human CB1 receptor. *FEBS Lett.* **579**, 259–264 (2005).

38. González-Mariscal, I. *et al.* Human CB1 Receptor Isoforms, present in Hepatocytes and β-cells, are Involved in Regulating Metabolism. *Sci. Rep.* **6**, 33302 (2016).

39. Bellocchio, L. *et al.* Bimodal control of stimulated food intake by the endocannabinoid system. *Nat. Neurosci.* **13**, 281–283 (2010).

40. Soria-Gómez, E. *et al.* The endocannabinoid system controls food intake via olfactory processes. *Nat. Neurosci.* **17**, 407–415 (2014).

41. Mackie, K. Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb. Exp. Pharmacol.* 299–325 (2005) doi:10.1007/3-540-26573-2_10.

42. Katona, I. *et al.* Presynaptically Located CB1 Cannabinoid Receptors Regulate GABA Release from Axon Terminals of Specific Hippocampal Interneurons. *J. Neurosci.* **19**, 4544–4558 (1999).

43. Tsou, K., Brown, S., Sañudo-Peña, M. C., Mackie, K. & Walker, J. M. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* **83**, 393–411 (1998).

44. Castillo, P. E., Younts, T. J., Chávez, A. E. & Hashimotodani, Y. Endocannabinoid signaling and synaptic function. *Neuron* **76**, 70–81 (2012).

45. Marinelli, S. *et al.* The endocannabinoid 2-arachidonoylglycerol is responsible for the slow self-inhibition in neocortical interneurons. *J. Neurosci. Off. J. Soc. Neurosci.* **28**, 13532–13541 (2008).

46. Marinelli, S., Pacioni, S., Cannich, A., Marsicano, G. & Bacci, A. Self-modulation of neocortical pyramidal neurons by endocannabinoids. *Nat. Neurosci.* **12**, 1488–1490 (2009).

47. Covelo, A. & Araque, A. Lateral regulation of synaptic transmission by astrocytes. *Neuroscience* **323**, 62–66 (2016).

48. Gómez-Gonzalo, M. *et al.* Endocannabinoids Induce Lateral Long-Term Potentiation of Transmitter Release by Stimulation of Gliotransmission. *Cereb. Cortex N. Y. N 1991* **25**, 3699–3712 (2015).

49. Navarrete, M. & Araque, A. Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. *Neuron* **68**, 113–126 (2010).

50. Navarrete, M. & Araque, A. Endocannabinoids mediate neuron-astrocyte communication. *Neuron* **57**, 883–893 (2008).

51. Howlett, A. C. *et al.* International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.* **54**, 161–202 (2002).

52. Hebert-Chatelain, E. *et al.* Studying mitochondrial CB1 receptors: Yes we can. *Mol. Metab.* **3**, 339 (2014).

53. Hebert-Chatelain, E. *et al.* Cannabinoid control of brain bioenergetics: Exploring the subcellular localization of the CB1 receptor. *Mol. Metab.* **3**, 495–504 (2014).

54. Hebert-Chatelain, E. *et al.* A cannabinoid link between mitochondria and memory. *Nature* **539**, 555–559 (2016).

55. Brailoiu, G. C., Oprea, T. I., Zhao, P., Abood, M. E. & Brailoiu, E. Intracellular cannabinoid type 1 (CB1) receptors are activated by anandamide. *J. Biol. Chem.* **286**, 29166–29174 (2011).

56. Grimsey, N. L., Graham, E. S., Dragunow, M. & Glass, M. Cannabinoid Receptor 1 trafficking and the role of the intracellular pool: implications for therapeutics. *Biochem. Pharmacol.* **80**, 1050–1062 (2010).

57. Hillard, C. J. The Endocannabinoid Signaling System in the CNS. in *International Review of Neurobiology* vol. 125 1–47 (Elsevier, 2015).

58. Di Marzo, V., Melck, D., Bisogno, T. & De Petrocellis, L. Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci.* **21**, 521–528 (1998).

59. Navarrete, M., Díez, A. & Araque, A. Astrocytes in endocannabinoid signalling. *Philos. Trans. R. Soc. B Biol. Sci.* **369**, 20130599 (2014).

60. Min, R. & Nevian, T. Astrocyte signaling controls spike timing–dependent depression at neocortical synapses. *Nat. Neurosci.* **15**, 746–753 (2012).

61. Song, I. & Huganir, R. L. Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci.* **25**, 578–588 (2002).

62. Shi, S. Rapid Spine Delivery and Redistribution of AMPA Receptors After Synaptic NMDA Receptor Activation. *Science* **284**, 1811–1816 (1999).

63. Rosenmund, C. The Tetrameric Structure of a Glutamate Receptor Channel. *Science* **280**, 1596–1599 (1998).

64. Chen, S. & Gouaux, E. Structure and mechanism of AMPA receptor — auxiliary protein complexes. *Curr. Opin. Struct. Biol.* **54**, 104–111 (2019).

65. Ward, S. E. *et al.* Discovery of *N* -[(2 *S*)-5-(6-Fluoro-3-pyridinyl)-2,3-dihydro-1 *H* -inden-2-yl]-2-propanesulfonamide, a Novel Clinical AMPA Receptor Positive Modulator. *J. Med. Chem.* **53**, 5801–5812 (2010).

66. Koller, M. *et al.* Quinazolinedione sulfonamides: A novel class of competitive AMPA receptor antagonists with oral activity. *Bioorg. Med. Chem. Lett.* **21**, 3358–3361 (2011).

67. Hollmann, M. & Heinemann, S. Cloned Glutamate Receptors. *Annu. Rev. Neurosci.* **17**, 31–108 (1994).

68. Cull-Candy, S., Kelly, L. & Farrant, M. Regulation of Ca2+-permeable AMPA receptors: synaptic plasticity and beyond. *Curr. Opin. Neurobiol.* **16**, 288–297 (2006).

69. Hollmann, M., Hartley, M. & Heinemann, S. Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science* **252**, 851–853 (1991).

70. Burnashev, N., Monyer, H., Seeburg, P. H. & Sakmann, B. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* **8**, 189–198 (1992).

71. Huganir, R. L. & Nicoll, R. A. AMPARs and Synaptic Plasticity: The Last 25 Years. *Neuron* **80**, 704–717 (2013).

72. Huettner, J. E. Spine-Tingling Excitement from Glutamate Receptors. *Sci. Signal.* **2003**, pe53–pe53 (2003).

73. Hayashi, T., Umemori, H., Mishina, M. & Yamamoto, T. The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. *Nature* **397**, 72–76 (1999).

74. Rao, V. R. & Finkbeiner, S. NMDA and AMPA receptors: old channels, new tricks. *Trends Neurosci.* **30**, 284–291 (2007).

75. Rao, A., Kim, E., Sheng, M. & Craig, A. M. Heterogeneity in the Molecular Composition of Excitatory Postsynaptic Sites during Development of Hippocampal Neurons in Culture. *J. Neurosci.* **18**, 1217–1229 (1998).

76. Borgdorff, A. J. & Choquet, D. Regulation of AMPA receptor lateral movements. *Nature* **417**, 649–653 (2002).

77. Carroll, R. C. *et al.* Dynamin-dependent endocytosis of ionotropic glutamate receptors. *Proc. Natl. Acad. Sci.* **96**, 14112–14117 (1999).

78. Park, M. Recycling Endosomes Supply AMPA Receptors for LTP. *Science* **305**, 1972–1975 (2004).

79. Hayashi, Y. Driving AMPA Receptors into Synapses by LTP and CaMKII: Requirement for GluR1 and PDZ Domain Interaction. *Science* **287**, 2262–2267 (2000).

80. Man, H.-Y. GluA2-lacking, calcium-permeable AMPA receptors — inducers of plasticity? *Curr. Opin. Neurobiol.* **21**, 291–298 (2011).

81. Anggono, V. & Huganir, R. L. Regulation of AMPA receptor trafficking and synaptic plasticity. *Curr. Opin. Neurobiol.* **22**, 461–469 (2012).

82. McCormack, S. G., Stornetta, R. L. & Zhu, J. J. Synaptic AMPA Receptor Exchange Maintains Bidirectional Plasticity. *Neuron* **50**, 75–88 (2006).

83. Lüscher, C. *et al.* Role of AMPA Receptor Cycling in Synaptic Transmission and Plasticity. *Neuron* **24**, 649–658 (1999).

84. Esteban, J. A. *et al.* PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. *Nat. Neurosci.* **6**, 136–143 (2003).

85. Boehm, J. *et al.* Synaptic Incorporation of AMPA Receptors during LTP Is Controlled by a PKC Phosphorylation Site on GluR1. *Neuron* **51**, 213–225 (2006).

86. Diering, G. H., Heo, S., Hussain, N. K., Liu, B. & Huganir, R. L. Extensive phosphorylation of AMPA receptors in neurons. *Proc. Natl. Acad. Sci.* **113**, E4920–E4927 (2016).

87. Armstrong, N., Jasti, J., Beich-Frandsen, M. & Gouaux, E. Measurement of Conformational Changes accompanying Desensitization in an Ionotropic Glutamate Receptor. *Cell* **127**, 85–97 (2006).

88. Gu, Y. & Stornetta, R. L. Synaptic plasticity, AMPA-R trafficking, and Ras-MAPK signaling. *Acta Pharmacol. Sin.* **28**, 928–936 (2007).

89. Furukawa, H., Singh, S. K., Mancusso, R. & Gouaux, E. Subunit arrangement and function in NMDA receptors. *Nature* **438**, 185–192 (2005).

90. Li, F. & Tsien, J. Z. Memory and the NMDA Receptors. *N. Engl. J. Med.* **361**, 302–303 (2009).

91. Salussolia, C. L., Prodromou, M. L., Borker, P. & Wollmuth, L. P. Arrangement of Subunits in Functional NMDA Receptors. *J. Neurosci.* **31**, 11295–11304 (2011).

92. Traynelis, S. F. *et al.* Glutamate Receptor Ion Channels: Structure, Regulation, and Function. *Pharmacol. Rev.* **62**, 405–496 (2010).

93. Ruppersberg, J. P., Kitzing, E. v. & Schoepfer, R. The mechanism of magnesium block of NMDA receptors. *Semin. Neurosci.* **6**, 87–96 (1994).

94. Pachernegg, S., Strutz-Seebohm, N. & Hollmann, M. GluN3 subunit-containing NMDA receptors: not just one-trick ponies. *Trends Neurosci.* **35**, 240–249 (2012).

95. McClymont, D. W., Harris, J. & Mellor, I. R. Open-channel blockade is less effective on GluN3B than GluN3A subunit-containing NMDA receptors. *Eur. J. Pharmacol.* **686**, 22–31 (2012).

96. Franchini, L., Carrano, N., Di Luca, M. & Gardoni, F. Synaptic GluN2A-Containing NMDA Receptors: From Physiology to Pathological Synaptic Plasticity. *Int. J. Mol. Sci.* **21**, 1538 (2020).

97. Barcomb, K., Hell, J. W., Benke, T. A. & Bayer, K. U. The CaMKII/GluN2B Protein Interaction Maintains Synaptic Strength. *J. Biol. Chem.* **291**, 16082–16089 (2016).

98. McKay, S. *et al.* The Developmental Shift of NMDA Receptor Composition Proceeds Independently of GluN2 Subunit-Specific GluN2 C-Terminal Sequences. *Cell Rep.* **25**, 841-851.e4 (2018).

99. Banerjee, A., Larsen, R. S., Philpot, B. D. & Paulsen, O. Roles of Presynaptic NMDA Receptors in Neurotransmission and Plasticity. *Trends Neurosci.* **39**, 26–39 (2016).

100. Petralia, R. S. *et al.* Organization of NMDA receptors at extrasynaptic locations. *Neuroscience* **167**, 68–87 (2010).

101. Lau, C. G. & Zukin, R. S. NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. *Nat. Rev. Neurosci.* **8**, 413–426 (2007).

102. Chen, B.-S. & Roche, K. W. Regulation of NMDA receptors by phosphorylation. *Neuropharmacology* **53**, 362–368 (2007).

103. Haddad, J. J. N-methyl-d-aspartate (NMDA) and the regulation of mitogen-activated protein kinase (MAPK) signaling pathways: A revolving neurochemical axis for therapeutic intervention? *Prog. Neurobiol.* **77**, 252–282 (2005).

104. Krapivinsky, G. *et al.* The NMDA Receptor Is Coupled to the ERK Pathway by a Direct Interaction between NR2B and RasGRF1. *Neuron* **40**, 775–784 (2003).

105. Valjent, E. *et al.* From The Cover: Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. *Proc. Natl. Acad. Sci.* **102**, 491–496 (2005).

106. Zahm, D. S. & Brog, J. S. On the significance of subterritories in the “accumbens” part of the rat ventral striatum. *Neuroscience* **50**, 751–767 (1992).

107. Mogenson, G., Jones, D. & Yim, C. From motivation to action: Functional interface between the limbic system and the motor system. *Prog. Neurobiol.* **14**, 69–97 (1980).

108. Salamone, J. D. *et al.* Mesolimbic Dopamine and the Regulation of Motivated Behavior. in *Behavioral Neuroscience of Motivation* (eds. Simpson, E. H. & Balsam, P. D.) vol. 27 231–257 (Springer International Publishing, 2015).

109. Saddoris, M. P., Cacciapaglia, F., Wightman, R. M. & Carelli, R. M. Differential Dopamine Release Dynamics in the Nucleus Accumbens Core and Shell Reveal Complementary Signals for Error Prediction and Incentive Motivation. *J. Neurosci.* **35**, 11572–11582 (2015).

110. Berridge, K. C. & Kringelbach, M. L. Pleasure Systems in the Brain. *Neuron* **86**, 646–664 (2015).

111. Berridge, K. C., Robinson, T. E. & Aldridge, J. W. Dissecting components of reward: 'liking', 'wanting', and learning. *Curr. Opin. Pharmacol.* **9**, 65–73 (2009).

112. Soares-Cunha, C., Coimbra, B., Sousa, N. & Rodrigues, A. J. Reappraising striatal D1- and D2-neurons in reward and aversion. *Neurosci. Biobehav. Rev.* **68**, 370–386 (2016).

113. Wolf, M. E. Regulation of AMPA Receptor Trafficking in the Nucleus Accumbens by Dopamine and Cocaine. *Neurotox. Res.* **18**, 393–409 (2010).

114. Blum, K. *et al.* Sex, Drugs, and Rock 'N' Roll: Hypothesizing Common Mesolimbic Activation as a Function of Reward Gene Polymorphisms. *J. Psychoactive Drugs* **44**, 38–55 (2012).

115. Kawaguchi, Y. Neostriatal cell subtypes and their functional roles. *Neurosci. Res.* **27**, 1–8 (1997).

116. Gerfen, C. *et al.* D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**, 1429–1432 (1990).

117. Floresco, S. B. The Nucleus Accumbens: An Interface Between Cognition, Emotion, and Action. *Annu. Rev. Psychol.* **66**, 25–52 (2015).

118. Yager, L. M., Garcia, A. F., Wunsch, A. M. & Ferguson, S. M. The ins and outs of the striatum: Role in drug addiction. *Neuroscience* **301**, 529–541 (2015).

119. Kelley, A. E. Memory and Addiction. *Neuron* **44**, 161–179 (2004).

120. Kalivas, P. W. & Volkow, N. D. The Neural Basis of Addiction: A Pathology of Motivation and Choice. *Am. J. Psychiatry* **162**, 1403–1413 (2005).

121. Kauer, J. A. & Malenka, R. C. Synaptic plasticity and addiction. *Nat. Rev. Neurosci.* **8**, 844–858 (2007).

122. Wolf, M. E., Sun, X., Mangiavacchi, S. & Chao, S. Z. Psychomotor stimulants and neuronal plasticity. *Neuropharmacology* **47**, 61–79 (2004).

123. Hu, X. T. & White, F. J. Glutamate receptor regulation of rat nucleus accumbens neurons in vivo. *Synap. N. Y. N* **23**, 208–218 (1996).

124. Cornish, J. L., Duffy, P. & Kalivas, P. W. A role for nucleus accumbens glutamate transmission in the relapse to cocaine-seeking behavior. *Neuroscience* **93**, 1359–1367 (1999).

125. Di Ciano, P. Dissociable Effects of Antagonism of NMDA and AMPA/KA Receptors in the Nucleus Accumbens Core and Shell on Cocaine-seeking Behavior. *Neuropsychopharmacology* **25**, 341–360 (2001).

126. Conrad, K. L. *et al.* Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. *Nature* **454**, 118–121 (2008).

127. Suto, N. *et al.* Previous Exposure to Psychostimulants Enhances the Reinstatement of Cocaine Seeking by Nucleus Accumbens AMPA. *Neuropsychopharmacology* **29**, 2149–2159 (2004).

128. Anderson, S. M. *et al.* CaMKII: a biochemical bridge linking accumbens dopamine and glutamate systems in cocaine seeking. *Nat. Neurosci.* **11**, 344–353 (2008).

129. Winters, B. D. *et al.* Cannabinoid receptor 1-expressing neurons in the nucleus accumbens. *Proc. Natl. Acad. Sci.* **109**, E2717–E2725 (2012).

130. Pedroza-Llinás, R., Méndez-Díaz, M., Ruiz-Contreras, A. E. & Prospéro-García, Ó. CB1 receptor activation in the nucleus accumbens core impairs contextual fear learning. *Behav. Brain Res.* **237**, 141–147 (2013).

131. Melis, T. *et al.* The cannabinoid antagonist SR 141716A (Rimonabant) reduces the increase of extra-cellular dopamine release in the rat nucleus accumbens induced by a novel high palatable food. *Neurosci. Lett.* **419**, 231–235 (2007).

132. Manz, K. M. *et al.* Calcium-Permeable AMPA Receptors Promote Endocannabinoid Signaling at Parvalbumin Interneuron Synapses in the Nucleus Accumbens Core. *Cell Rep.* **32**, 107971 (2020).

133. de Rover, M. *et al.* Long-lasting nicotinic modulation of GABAergic synaptic transmission in the rat nucleus accumbens associated with behavioural sensitization to amphetamine. *Eur. J. Neurosci.* **19**, 2859–2870 (2004).

134. Bane, V., Lehane, M., Dikshit, M., O'Riordan, A. & Furey, A. Tetrodotoxin: Chemistry, Toxicity, Source, Distribution and Detection. *Toxins* **6**, 693–755 (2014).

135. Rho, J. M., Donevan, S. D. & Rogawski, M. A. Direct activation of GABAA receptors by barbiturates in cultured rat hippocampal neurons. *J. Physiol.* **497 (Pt 2)**, 509–522 (1996).

136. Bittiger, H., Froestl, W., Mickel, S. J. & Olpe, H.-R. GABAB receptor antagonists: from synthesis to therapeutic applications. *Trends Pharmacol. Sci.* **14**, 391–394 (1993).

137. Randle, J. C., Guet, T., Cordi, A. & Lepagnol, J. M. Competitive inhibition by NBQX of kainate/AMPA receptor currents and excitatory synaptic potentials: importance of 6-nitro substitution. *Eur. J. Pharmacol.* **215**, 237–244 (1992).

138. Morris, R. G. Synaptic plasticity and learning: selective impairment of learning rats and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist AP5. *J. Neurosci. Off. J. Soc. Neurosci.* **9**, 3040–3057 (1989).

139. Moussawi, K. *et al.* Reversing cocaine-induced synaptic potentiation provides enduring protection from relapse. *Proc. Natl. Acad. Sci.* **108**, 385–390 (2011).

140. Boudreau, A. C. & Wolf, M. E. Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *J. Neurosci. Off. J. Soc. Neurosci.* **25**, 9144–9151 (2005).

141. Kampa, B. M., Clements, J., Jonas, P. & Stuart, G. J. Kinetics of Mg^{2+} unblock of NMDA receptors: implications for spike-timing dependent synaptic plasticity: Mg^{2+} unblock of NMDA receptors and STDP. *J. Physiol.* **556**, 337–345 (2004).

142. Twomey, E. C., Yelshanskaya, M. V., Vassilevski, A. A. & Sobolevsky, A. I. Mechanisms of Channel Block in Calcium-Permeable AMPA Receptors. *Neuron* **99**, 956-968.e4 (2018).

143. Frizelle, P. A., Chen, P. E. & Wyllie, D. J. A. Equilibrium Constants for (*R*)-[(*S*)-1-(4-Bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic Acid (NVP-AAM077) Acting at Recombinant NR1/NR2A and NR1/NR2B *N* -Methyl-d-aspartate Receptors: Implications for Studies of Synaptic Transmission. *Mol. Pharmacol.* **70**, 1022–1032 (2006).