Neurobiological mechanisms involved in memory function and dysfunction: focus on the endocannabinoid system and associated signaling pathways

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A totes les persones que m'han acompanyat al llarg d'aquest camí

La única persona con la que deberías compararte es con la persona que eras ayer. Esa es la persona a la que debes superar y en la que debes fijarte para ser mejor

(Sigmund Freud)

If any one faculty of our nature may be called more wonderful than the rest, I do think it is memory

(Jane Austen, Mansfield Park)

Abstract

Memory is a complex brain function that constitutes a crucial feature of individuals. Understanding the precise neurobiological mechanisms involved is a fundamental challenge in Neuroscience, where multiple brain areas and signaling pathways interact to regulate different stages and kinds of memory. In this thesis we used specific murine mouse models to reveal the cellular and molecular mechanisms involved in different physiopathological conditions that course with memory impairment focusing our attention in the endocannabinoid system (ECS) and its associated intracellular signaling pathways. Specifically, we described that the ECS is a suitable therapeutic target for the treatment of the intellectual disability present in the fragile X syndrome and that it plays a pivotal role in the memory deficits produced by a stressful situation. Moreover, we identified that the protein kinase C (PKC) signaling pathway is involved in the deleterious effects on short-term memory produced by delta9-tetrahydrocannabinol (THC). We pinpointed the PKC gamma isoform to play a crucial role in the modulation of memory in both normal conditions and after THC administration. Overall, we combined behavioral, biochemical and pharmacological approaches to advance in the understanding of the neurobiological substrates of memory and the specific role of the ECS in this function.

Resum

La memòria és una funció cerebral complexa que constitueix una característica crucial dels individus. Entendre els mecanismes neurobiològics precisos involucrats és un repte per la Neurociència, on múltiples regions cerebrals i vies de senyalització interactuen per regular les diferents etapes i tipus de memòria. En aquesta tesi hem utilitzat models murins específics per revelar els mecanismes cel·lulars i moleculars involucrats en diferents condicions patològiques que cursen amb problemes de memòria centrant la nostra atenció en el sistema endocannabinoid (SEC) i les vies de senyalització associades. Concretament, hem descrit que el SEC és una diana terapèutica adequada pel tractament del dèficit intel·lectual present en la síndrome del cromosoma X fràgil i que

juga un paper essencial en els dèficits de memòria produïts per una situació estressant. També hem identificat que la via de senyalització de la proteïna kinasa C (PKC) està involucrada en els efectes deleteris del delta9-tetrahydrocannabinol (THC) sobre la memòria a curt termini. Hem determinat amb precisió que la isoforma PKC gamma juga un paper clau en la modulació de la memòria tant en condicions normals com després de l'administració de THC. En general, hem combinat tècniques comportamentals, bioquímiques i farmacològiques per avançar en l'enteniment dels substrats neurobiològics de la memòria i del rol específic que juga el SEC en aquesta funció.

Abbreviations

2-AG: 2-arachidonoylglycerol

ACTH: Adrenocorticotropic hormone

AEA: Anandamide: N-arachidonoylethanolamide

A-loop: Activation loop

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ASD: Autism spectrum disorder

BLA: Basolateral amygdala

BSA: Body surface area

Ca^{2+}: Calcium

CamKII: Calcium/calmodulin-dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

CB1R: CB1 cannabinoid receptor

CB2R: CB2 cannabinoid receptor

CBD: Cannabidiol

CNS: Central nervous system

CR: Conditioned response

CRH: Corticotropin releasing hormone

CREB: cAMP responsive element binding protein

CS: Conditioned stimuli

DAG: Diacylglycerol

DAGL: Diacylglycerol lipase

DBH: dopamine β-hydroxilase

DI: Discrimination Index

ECS: Endocannabinoid system

ERK: Extracellular signal-regulated kinase

FAAH: Fatty-acid amide hydrolase

Fmr1: fragile X mental retardation gene

Fmr1 KO: fragile X mental retardation I knockout

FMRP: fragile X mental retardation protein

FXS: fragile X syndrome

FXTAS: Fragile X-associated tremor/ataxia syndrome

GABA: γ-aminobutyric acid

GPCR: G protein-coupled receptor

GR: Glucocorticoid receptor

HM: Hydrophobic motif

HPA: hypothalamic-pituitary-adrenal

KO: knockout

LTD: Long-term depression

LTM: Long-term memory

LTP: Long-term potentiation

MAGL: Monoacylglycerol lipase

MAPK: Mitogen-activated protein kinase

MARCKS: Myristoylated alanine-rich C kinase substrate

mGluR: Group I metabotropic glutamate receptors

MR: Mineralocorticoid receptor

mTOR: mammalian target of rapamycin

NMDA: n-methyl-D-aspartate

NOR: Novel object-recognition

NPR: Novel place-recognition

PI3K: Phosphatidylinositol 3-kinase

PDK1: Phosphoinositide-dependent kinase 1

PFC: Prefrontal cortex

PKA: Protein kinase A

PKC: Protein kinase C

PKM: Protein kinase M

PLC: Phospholipase C

PTSD: Post-traumatic stress disorder

PVN: Paraventricular nucleus

STM: Short-term memory

THC: Delta9-tetrahydrocannabinol

TM: Turn-motif

US: Unconditioned stimuli

WM: Working memory

WT: Wild-type

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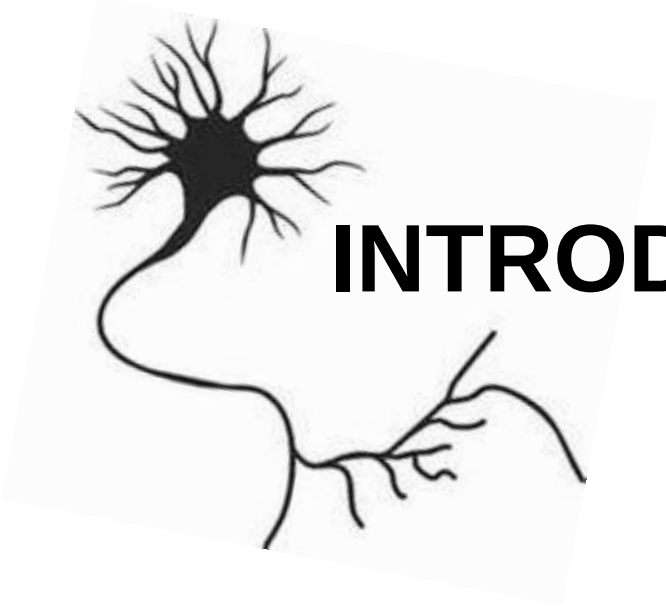


INTRODUCTION

1. Memory

Memory is a physiological brain function that classifies, encodes, stores, and recovers relevant information for the subject. It is responsible for the changes produced in animal and human behavior, some time after learning (Abel and Lattal 2001; Kandel 2001; Squire 1986).

1.1 Memory stages

Memory processes can be classified depending on the amount of time the information is available for the subject (Tetzlaff et al. 2012) (Fig 1).

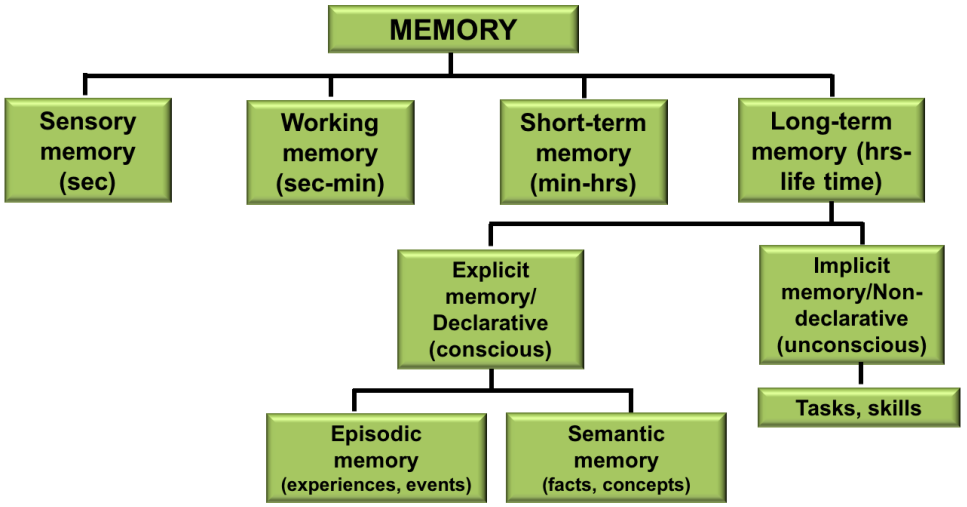


Figure 1. **Schematic representation of the different memory types depending on the time the information is available for the subject.**

Sensory memory, which lasts only for a few seconds, can be considered in between perceptive and memory processes and it is constantly being used. This type of memory involves visual and auditory processes and allows the sensory information to be processed by the central nervous system (CNS) once the stimulus has disappeared.

Introduction

Working memory (WM) was first described by Miller, Galanter and Pribram in 1960 (Miller et al. 1960). It is defined as a brain function that temporarily stores small and limited amounts of information that are readily accessible for use providing temporary storage and manipulation of the information (Baddeley 1992; Cowan 2010; Jeneson and Squire 2012). According to Baddeley and Hitch, WM can be divided into three subcomponents: the central executive (involved in processing and attention), the visuospatial sketchpad (involved in the manipulation of visual information) and the phonological loop (based on verbal rehearsal in order to handle the verbal information) (Baddeley and Hitch 1974). This kind of memory is necessary for the successful completion of different cognitive tasks such as understanding a language for communication or solving an arithmetical problem. WM time scale goes from miliseconds to minutes (Tetzlaff et al. 2012).

WM has been frequently confused with the next type of memory described according to the time scale, short-term memory (STM) (Cowan 2016). In fact, Atkinson and Shiffrin first described STM as a component identified with WM affirming that “the short-term store is the subject’s working memory; it receives selected inputs from the sensory register and also from long-term store” (Atkinson & Shiffrin 1968). Nowadays WM and STM are considered as different memory types. Some authors defend that they are confused because of the absence of clarity in definitions of WM (Cowan 2016; Postle 2015; Cowan 2009). Compared to WM, STM lasts from minutes to days in humans and from minutes to few hours in rodents. Miller, in 1956 proposed that the capacity for short-term memory was limited to 7 ± 2 items (Miller 1956) although more recent studies reduced this number to 4 ± 1 items (Mathy and Feldman 2012). Considering that,

the main features of STM are the limitations in duration and capacity: only small amounts of information are available for a short period of time. Moreover, this type of memory is still susceptible to perturbations (Cowan 2009; Kumaran 2008).

Lastly, long-term memory (LTM) can last from days to years (or a lifetime) in humans and from hours to days in mice. It constitutes what is considered a vast amount of knowledge (Cowan 2009). This kind of memory involves many brain regions and requires dynamic and plastic changes (Xu et al. 2010; Costa-Mattioli et al. 2009). LTM has been classified into two major groups: declarative memory and non-declarative memory (Figure 2). Declarative memory, also known as explicit memory, is defined as a conscious memory and it is acquired after a few exposures to the material or information to be learned. In humans, it can be divided into episodic memory, related to personal experiences and events (episodes of an individual lifetime), and semantic memory which makes reference to the knowledge of facts and concepts (commonly defined as the knowledge acquired at school) (Squire and Zola 1996; Squire 1992; Zola-Morgan and Squire 1993). In animals, declarative memory is described as the processing of spatial, configural, contextual and relational information (Richter-Levin 2004). On the other hand, non-declarative memory, or implicit memory, is an unconscious memory, more complex, that requires longer acquisition phases. It is related to the skills and habits of the individual and it is used to perform some task without full awareness (for example, going by bike) (Schacter and Cooper 1993; Zola-Morgan and Squire 1993).

Both declarative and non-declarative memories involve the activation of cortical and subcortical structures but the specific brain regions involved are different (Figure 2) (Yang and Li 2012).

It is also important to take into account that memories can be classified as emotional memory, when some components are involved in fear or stress (it will be explained in chapter 4), or non-emotional memory.

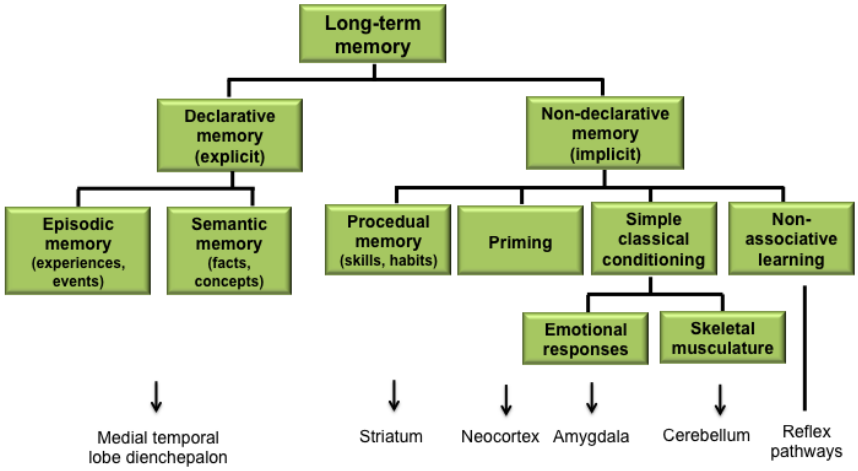


Figure 2. **Schematic representation of declarative and non-declarative memory processes and their anatomical representation.** A taxonomy of long-term memory systems and the brain structures involved in each of them (Adapted from Squire and Zola 1996).

The most important stages involved in the establishment and remembrance of a memory are acquisition (also known as encoding), consolidation (also known as storage) and retrieval. Acquisition occurs when animals or humans learn new information and new memories are established (Abel and Lattal 2001). Consolidation is the process that makes this memory move from a labile to a permanent state, becoming a long-term memory. Before being consolidated, memories are sensitive to disruptions (Abel and Lattal 2001; Walker et al. 2003). Retrieval is the process by which memories stored in our brain are recovered. It is the use of learned information, and makes stable memories return to a labile state

(Ben-yakov et al. 2015; Miller and Matzel 2000). Another interesting stage in memory is extinction, which consists of the suppression of learned information (Abel and Lattal 2001) . Finally, reconsolidation can be defined as the process that takes place when a memory previously consolidated goes back to a labile state requiring a new consolidation (Walker et al. 2003)

1.2 Neuroanatomical substrates of memory

There is an important relationship between declarative or explicit memories and structures in the medial temporal lobe, including the hippocampal formation and the parahippocampal region (Moscovitch et al. 2006; Remondes and Schuman 2002; van Strien et al. 2009). The hippocampal formation is the most important brain region involved in learning and memory processes. The rodent hippocampus is a C-shaped structure situated in the caudal part of the brain that includes the dentate gryrus, the hippocampus proper (CA fields: CA3, CA2 and CA1) and the subiculum cortex (Figure 3). The parahippocampal region involves the presubiculum, the parasubiculum, the entorhinal cortex, the perirhinal cortex and the postrhinal cortex. The first explorations about the parahippocampal-hippocampal network were performed by Ramón y Cajal more than a century ago. Since then, many studies have been done in order to clarify how the information flows into the hippocampal formation from the enthorinal cortex, which is the main input to the hippocampus (van Strien et al. 2009; Amaral and Witter 1989).

The hippocampal formation receives information from all regions of the cingulate cortex and the association cortex via the perirhinal cortex that projects to the entorhinal cortex (Richter-Levin 2004). The entorhinal cortex projects through the perforant pathway (layer II) to

the dentate gryrus and the CA3 field while simultaneously projecting to the CA1 and subiculum through layer III. The granule cells of the dentate gyrus, also project to CA3 via the mossy fibers, which provides the major input to CA1 field through the Schaffer-collaterals (Amaral and Witter 1989; van Strien et al. 2009; Steward and Scoville 1976). Thus, CA1 pyramidal neurons receive two excitatory inputs that seem to be crucial for memory formation, consolidation and retrieval in the hippocampus (Remondes and Schuman 2004; Eichenbaum 2001). A diagram representing the hippocampal neuronal circuitry is presented in Figure 3 (Deng et al. 2010).

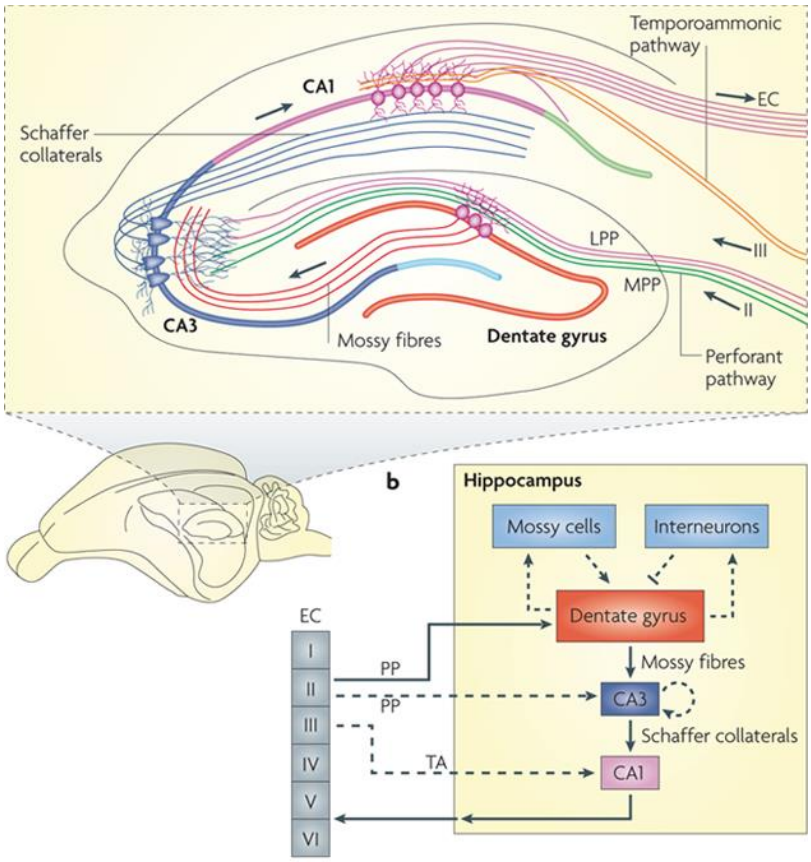


Figure 3. **The hippocampal network.** Illustration of the hippocampal circuitry and diagram of hippocampal neural network**.** The traditional excitatory trisynaptic pathway (entorhinal cortex (EC) – dentate gyrus – CA3 – CA1 – EC) is depicted by solid arrows. The axons of layer II neurons in the enthorinal cortex project to the dentate gyrus through the perforant pathway (PP), including the lateral and the medial perforant pathway. The dentate gyrus sends projections to the pyramidal cells in CA3 through mossy fibers. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons through the PP. CA1 receives direct input from EC layer III neurons through the temporoammonic pathway (TA). The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the dentate gyrus (Deng et al. 2010) .

Although the role of the hippocampus in declarative memory formation can not be argued, it is important to consider the relevance of other brain regions in memory formation such as the amygdala, more specifically, the basolateral amygdala (BLA). The amygdala is considered as the site for some aspects of the emotional memory (Richter-Levin 2004). The BLA is suggested to be crucial in the development of conditioned fear and other forms of affective memory by experiments done in monkeys and rats (McGaugh 2002; McIntyre et al. 2003; Zola-Morgan and Squire 1993). There are two different hypotheses on the role of amygdala in fear memory formation, but some studies have demonstrated that both of them can be assumed. One hypothesis suggests that the amygdala modulates memory-related processes in other brain regions such as the hippocampus, so the amygdala and the hippocampus may support different aspects of the same experience. The interconnections between these two brain regions are very complex, dynamic and the projections between them are mainly reciprocal (Figure 4) (Richter-Levin 2004; LeDoux 2000; Mcdonald and Mott 2016; Huff et al. 2016). The second hypothesis says that apart from the hippocampus, the amygdala also presents direct and indirect projections to other brain regions involved in different memory processes, including the prefrontal cortex or the cerebellum, among others (Figure 4). The amygdala also promotes the release of stress hormones through the hypothalamic-pituitary-adrenal (HPA) axis that will modulate memory storage (Figure 4) (LaBar and Cabeza 2006; Roozendaal and McGaugh 2011).

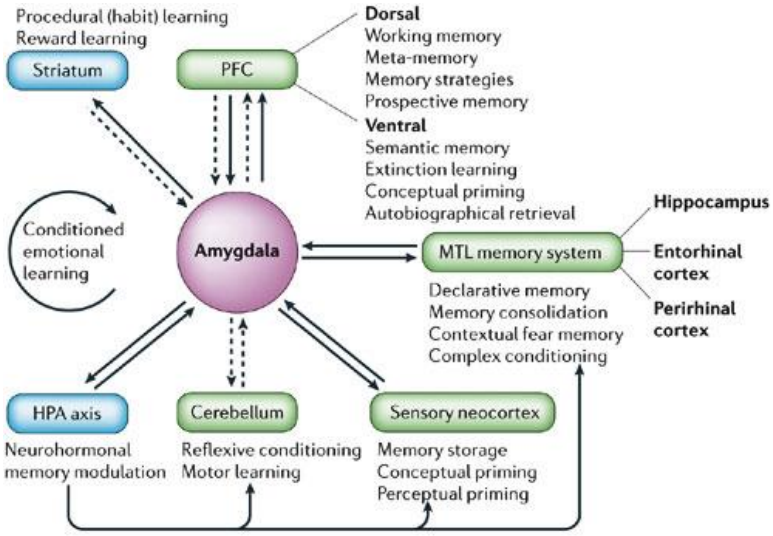


Figure 4. **Mechanisms by which the amygdala mediates the influence of emotional arousal on memory**. In addition to the emotional learning that takes place intrinsically in the amygdala, direct and indirect neural projections target several memory systems in the brain, including those that subserve working memory, declarative memory and various non-declarative forms of memory. Complex conditioning refers to various higher-order conditioning procedures that are hippocampal-dependent, including trace fear-conditioning and conditional discrimination learning. The amygdala also triggers the release of stress hormones by way of the hypothalamic-pituitary-adrenal (HPA) axis, which feed back onto memory consolidation and storage sites as well as the amygdala itself to enhance memory over longer time intervals. Solid arrows indicate direct connections, dashed arrows indicate indirect connections. Blue labels indicate connections with subcortical structures (LaBar and Cabeza 2006).

1.3 Molecular substrates of memory

Many neurobiological processes need to be taken into account when talking about memory. LTM storage requires controlled activation and changes in gene expression including the regulation of hippocampal mRNA translation, a process also necessary for the formation of long-term memory (Bekinschtein et al. 2007; Donnelly

et al. 2010). Secondly, protein synthesis is also important for memory consolidation. It has been observed that the use of an inhibitor of protein synthesis does not prevent the learning of a task but impairs long-term memory, suggesting that protein synthesis is required for long-term memory consolidation but not for short-term memory. Moreover, protein synthesis is also required for the formation of long-term synaptic plasticity (Alberini 2008; McGaugh 2000). Synaptic plasticity, including both functional (alterations in the efficacy of the synaptic transmission) and structural (changes in the number or the structure of synaptic connections) plasticity is also crucial in learning and memory processes as the formation of new connections is required. In fact, the necessity of new connections for memory storage was first described in 1894 by Santiago Ramón y Cajal (Kandel 2001; Korte and Schmitz 2016). The changes at the synaptic level produce the modulation of some, but not all the synapses. This can be explained by a putative mechanism known as "synaptic tagging and capture hypothesis" described by Fred and Morris in 1997. It consists of local and persistent protein modifications that mark the synapses that will be modified. In this sense, the synapses that are "tagged" will be these able to incorporate the products of gene expression that are delivered throughout the neuron (Korte and Schmitz 2016; Redondo and Morris 2011; Lesburguères et al. 2011).

There are multiple intracellular signaling pathways involved in memory function. Within them, we are going to pay special attention to two important signaling pathways involved in memory formation and storage: the mammalian target of rapamycin (mTOR) signaling cascade and the protein kinase C (PKC) signaling pathway.

Several pharmacological studies have demonstrated that mTOR is crucial in memory processes as the inhibition of this signaling pathway by rapamycin produces long-term memory impairment affecting the consolidation process without affecting acquisition (Bekinschtein et al. 2007; Mac Callum et al. 2014; Parsons et al. 2006). A possible explanation is that mTOR plays a key role in translational efficacy and the formation of LTM requires hippocampal mRNA translation (Bekinschtein et al. 2007).

The second signaling pathway involved in learning and memory that is going to be studied in this thesis is the PKC signaling pathway. PKCs activation has been demonstrated to facilitate long-term potentiation (LTP) and plays a crucial role in the acquisition and maintenance of several types of learning and memory (Sun and Alkon 2014). Moreover CamKII, an enzyme that phosphorylates multiple proteins important in synaptic plasticity, is involved in both consolidation and LTP as its inhibition in the amygdala or the CA1 region of the hippocampus produces impairments in this process (Izquierdo 1997; McGaugh 2000). Furthermore, CaMKII mutant mice present an impairment of spatial learning and a selective loss of CA1 LTP (Fukunaga and Miyamoto 2000).

Besides the previously presented signaling pathways, there are other pathways also important in memory formation and storage.

One of the most well characterized members of the mitogen-activated protein kinase (MAPK) family, the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, is involved in the regulation of many physiological processes in the nervous system. These include the regulation of synaptic plasticity, memory formation and brain development and repair (Sun and Nan 2017). Its role on memory formation was described by Atkins *et al.* (Atkins et al. 1998)

demonstrating the involvement of ERK1/2 signaling in the consolidation of both short-term and long-term memories (Feld et al. 2005; Igaz et al. 2006).

Another interesting factor in memory formation is the transcription factor cAMP responsive element binding protein (CREB). Studies performed in different animal models have demonstrated that CREB activation is involved in the long-term, but not in the short-tem memory formation (Silva et al. 1998; Wang and Peng 2016).

Finally, it is important to consider that the activation of PKA is also required for LTM storage (Kandel 2001).

1.4 Behavioral mouse models to study learning and memory

Multiple tests, protocols and schedules have been developed in order to study learning and memory in different murine models, including rats and mice and based in different responses.

First of all, a set of tests have been designed which are believed to mimic the natural behavior of the mice for example, the T- or Y-maze alternation tasks (Gerlai 1998a), the novel object-recognition task (Dere et al. 2007) or the social-recognition task (Thor et al. 1982), among others. More complex learning tasks involve positive reinforcers such as food, sweetened water or the possibility to stay in a safe compartment, that can be used in the radial arm maze, (Sharma et al. 2010) or in the classical models of operant behavior acquisition (Baron and Meltzer 2001). Finally, other memory tests involve the presence of an aversive component or an aversive reinforcement (for example a footshock or a loud noise), such as the active avoidance test, fear-conditioning or conditioned taste aversion tasks (Gerlai 1998b).

A brief summary of some differential behavioral paradigms designed to study learning and memory are presented in box 1 (Lee and Silva 2009).

Novel object-recognition task

It is a non-aversive and non-spatial test that requires hippocampal function. Animals are allowed to freely explore two identic objects placed one at each corner of a v-shaped maze or in an open field. In the test session, one of the objects is replaced for a new one. The time spent exploring each of the objects is used to calculate a discrimination index (DI); a high discrimination index indicates good memory. Short-term and long-term memory can be measured by using this task.

Novel place-recognition task

It is a non-aversive, spatial test that works similarly to the novel object-recognition task. In this case, two objects are presented to the mice during the training session in an open field. During the test session, the same objects are presented to the mice but one of them is changed of place. As in the novel object-recognition test, a discrimination index is obtained from the time the mice spends exploring the objects. This test also allows studying short-term and long-term memory processes.

Fear-conditioning

Pavlovian aversive learning task in which animals associate a non-aversive conditioned stimuli (CS; for example a tone, a light or a context) with an aversive unconditioned stimulus (US, for example a footshock). Conditioned responses, usually freezing behavior, are measured as an indicative of memory. There are different versions of the test depending on the nature of the stimulus and depending on the time point when the animal is exposed to the conditioned stimuli. Fear memories can last a lifetime or can be extinguished by repeated exposure to the CS without the US.

T-maze or Y-maze task

Mice are placed in a maze with three arms (T-shaped or Y-shaped) where working memory can be studied with this spatial test. It permits the study of spontaneous alternation.

Morris water maze

It is one of the most used spatial learning and memory tasks that depend on the hippocampus. Animals are placed in a pool full of water to find the location of a hidden platform. To escape the water, mice use a variety of cues and strategies placed around the pool, in the room. Animals are trained for several days and the time/path length they take to find the platform is usually measured as a learning index.

Active avoidance

In this test mice are placed in a box with two compartments separated by an open door. Mice need to learn that five seconds after the light is on, they will receive a footshock unless they change to the other compartment. If mice learn the association between the light and the footshock they will change of compartment and avoid the footshock.

Passive avoidance

The animals learn to inhibit their natural tendency to step into an apparently safer compartment (a dark compartment) that has previously been associated with a footshock.

Radial arm maze

It is another spatial learning task with various versions. The apparatus has several arms (most commonly eight) that can contain food pellets at the end. Food-deprived animals are allowed to enter the arm and search for the hidden food. Arms can be blocked (commonly during the first phase). After a retention interval mice go back to the maze with access to all the arms. Hippocampus and prefrontal cortex seem to be the most important brain regions for this task

Conditioned taste aversion

Aversive learning task where animals associate a food source (CS) with malaise usually induced by lithium chloride injection (US). Avoidance of the food previously associated with malaise is used as a memory index.

Box 1. **Behavioral tests for the study of learning and memory** (Modified from (Lee and Silva 2009).

Most of the tests mentioned before have been used during this thesis. However, two of them are of particular interest for this work so they are going to be more deeply described: the novel object-recognition task and the fear-conditioning task.

1.4.1 Novel object-recognition (NOR) test

Ennaceur and Delacour first described this memory task in 1988, performing their experiments in rats by using an open box made of wood. The conclusions obtained by these authors pointed to three main features of this test: it allows interspecies comparison, it is based in the spontaneous behavior of animals and it does not involve reinforcements such as food or electric shock, making it more comparable to the tests used in humans (Ennaceur and Delacour 1988).

In general terms, the object-recognition memory task is a one-trial common test to study non-emotional declarative memory, considering recognition as a judgement of the prior occurrence (Winters et al. 2008). Recognition memory deficits are present in many human disorders, which points to the interest in the NOR test, as it is an experimental tool used in a large variety of animal models of human diseases where cognition is affected. Moreover, it can also be used to study the effect of different drugs or evaluate the efficacy of novel therapeutic targets (Bengoetxea et al. 2015; Grayson et al. 2014). In conclusion, the NOR test is a good tool that allows to study the neural mechanisms underlying learning and memory (Antunes and Biala 2012; Bengoetxea et al. 2015).

The NOR test has been demonstrated to be useful in different animal species used in research, such as monkeys (Peissig et al. 2007),

rabbits (Weiss and Disterhoft 2015) or zebrafish (May et al. 2016) which present the particularity to prefer the familiar object to the novel one. However, our interest is focused in rodents and, specifically, in mouse models. In 1950, Berlyne described that rats spend more time exploring a novel object than one that had been presented before (Berlyne 1950). Since then, many studies have described and demonstrated that rodents present an innate preference for novelty making it a spontaneous behavior (Bengoetxea et al. 2015; Vogel-Ciernia and Wood 2014), and suggesting that this task is suitable for this memory model. Moreover, it has been observed that there are no differences on the relative discrimination indexes between mouse strain although some differences in the absolute times of exploration activity are observed (Şık et al. 2003).

The task consists in three different sessions, all of them lasting for 10 minutes in our experimental conditions: habituation, training and test (Figure 5). During the habituation phase, mice are placed in the V-shaped maze and are allowed to explore it in order to get used to this novel environment. The V-shaped maze presents the advantage to reduce the exploratory field so it has been observed that mice spend higher total times exploring the objects in the following phases. During the training session 2 identical objects are placed at the end of the arms and the mice can freely explore them. Finally, 3 hours (when STM is studied) or 24 hours (when LTM is studied) after the training session, the test is performed. In this session, one of the familiar objects has been replaced for a novel one. The total exploration time of both objects is recorded and different indexes can be calculated to study memory performance, such as discrimination

index, index of global habituation or novel object preference (Gaskin et al. 2010).

In our laboratory, the measure used to study memory in the NOR TEST is the discrimination index (DI) obtained as follows:

$$DI = \frac{\text{Time spent exploring novel object} - \text{Time spent exploring familiar object}}{\text{Total exploration time (Novel+Familiar)}}$$

Discrimination indexes higher than 0.3 are considered indicators of good memory performance.

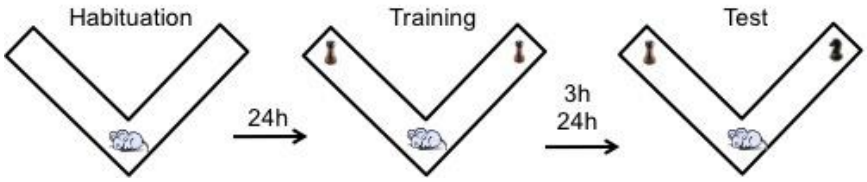


Figure 5. **Schematic representation of the protocol and maze used to perform the novel object-recognition task.**

Some aspects that need to be considered when using the NOR test are the following: experimental room, which has to be different from the one where the animals are housed, adjust for correct lighting in the experimental room, avoid odors that can affect the test as well as external noises, use of an appropriate maze taking into account the material, size, shape and color of the apparatus, use of appropriate objects that had been previously validated by experimented observers, being careful of the mouse position inside the maze, age and sex of the animals and handle appropriately the animals to avoid the appearance of anxiety or stress responses (Antunes and Biala 2012; Vogel-Ciernia and Wood 2014). Table 1 presents a summary of the main advantages and troubleshooting of

this memory test (Bevins and Besheer 2006; Ennaceur 2010; Grayson et al. 2014; Vogel-Ciernia and Wood 2014).

Advantages	Disadvantages
Easy to operate	Difficult to automatize
Rapid data	Expertize from the researcher
Economic	Possible low exploration times
Relies on innate exploratory behavior	Objects needs validation before being used
Avoid stress induced in other memory test	Low discrimination indexes (due to anxiety or stress)
Avoid the use of positive or negative reinforces	

Table 1. **Summary of the advantages and troubleshooting in the novel object-recognition task.**

There are two main brain regions involved in object recognition memory which play different roles and interact between themselves: the hippocampus and the perirhinal cortex (Antunes and Biala 2012; Cohen and Stackman 2015). There has been some controversy about the involvement of the hippocampus in this memory process. However, many studies have demonstrated that normal recognition performance depends on the integrity of the hippocampus (Broadbent et al. 2010; Cohen et al. 2013). In fact, it seems that this brain region, located in the medial temporal lobe, is involved in the maintenance of novel object preference after long but not short (few minutes between training and test) term delays (Antunes and Biala 2012; Clark et al. 2000; Kumaran 2008). The perirhinal cortex, which is part of the parahippocampal region, presents direct and indirect connections with the hippocampus via the entorhinal cortex. Lesions

in the perirhinal cortex have demonstrated its crucial role in object recognition memory in rodents and primates. Memory deficits have also been observed when there are long intervals between the training and the test sessions (LTM) (Albasser et al. 2009; Antunes and Biala 2012; Cowell et al. 2010; Murray et al. 2013).

1.4.2 Fear-conditioning

Fear-conditioning memory is an emotional memory task initially described in rats. In this experiment, a single 2-seconds footshock evoked a particular natural response in animals, that can be observed and quantified as immobility or freezing (Blanchard and Blanchard 1969; Gerlai 1998b). Freezing is defined as the absence of movement in the subject except for respiration.

The basis of this paradigm consists in the association between a neutral stimulus, such as a context, a sound or a light (conditioned stimulus; CS) with an aversive stimulus, for example a footshock (unconditioned stimulus; US). If mice learn and remember this association, a new exposure to the CS will produce a conditioned response, which will be represented by the freezing behavior.

Three different protocols based on the fear-conditioning paradigm have been used in this thesis:

1. Trace fear-conditioning: During the first day (training phase) mice are placed in a shuttle box with electrifiable floor. After a period of 2 minutes of free exploration, the animal hears a sound (CS) that lasts for 1 minute. Fifteen seconds after the end of the sound, the animal receives a footshock (US: 2 seconds, 0.35 mA intensity) and remain in the chamber for 30 additional seconds. Three hours or 24 hours later (STM and LTM respectively) mice are placed in a new environment (for example, in a glass cylinder that allows

observation). After 2 minutes, animals are exposed to the CS (the sound) and freezing behavior is counted (Figure 6a)

2. Delayed fear-conditioning: The protocol is similar to the one presented for the trace fear-conditioning. The difference remains in the fact that the footshock (US) is received just at the moment that the sound (CS) stops during the training session (Figure 6b).

3. Context-recognition: During the first day (training phase) mice are placed in a shuttle box with electrifiable floor. After a period of 2 minutes of free exploration, mice receive a footshock (US: 2 seconds, 0.35 mA intensity) and then remain in the chamber for 30 seconds. Three or 24 hours later (STM and LTM respectively), mice are placed again in the shuttle box for 5 minutes in absence of the shock. The association between the CS (the context) and the US will be learnt and the freezing behavior is counted (Figure 6c).

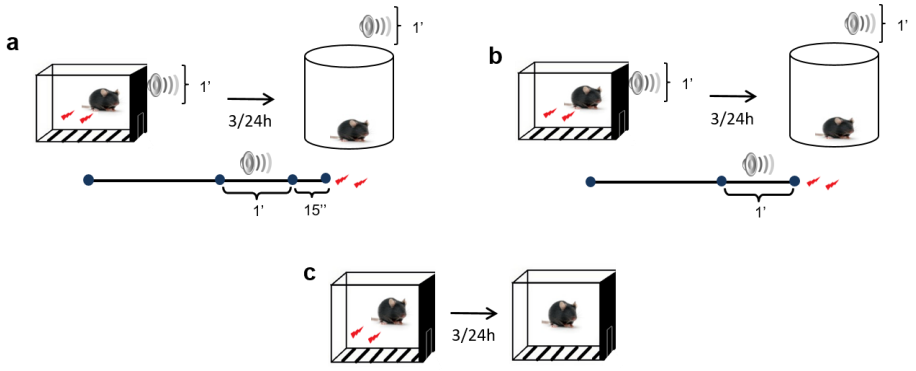


Figure 6. **Schematic representation of different protocols based on the fear-conditioning paradigm.** a) Trace fear-conditioning (association between a sound (CS) and an electric shock (US); 15 seconds between the CS and the US). b) Delay fear-conditioning (association between a sound (CS) and an electric shock (US); the US appears just when the CS stops). c) Context fear-conditioning (association between the context (CS) and an electric shock (US)).

In general, memory tasks involving emotional situations, like the presence of fear responses, are regulated by the amygdala (LaBar and Cabeza 2006). However, it has been seen that although all the tasks described are sensitive to lesions in the amygdala, it is generally accepted that contextual fear-conditioning depends also on the hippocampus (Holland and Bouton 1999; Kim et al. 1993; Phillips and LeDoux 1992). Moreover, differences have also been observed within the cued fear-conditioning, as delay fear-conditioning requires the basolateral amygdala while, the appearance of a gap between the CS and the US stimulus (trace fear-conditioning), seems to be amygdala-independent and depends only on the hippocampus (Raybuck and Lattal 2011).

1.5 Cognitive alterations

Memory or cognitive alterations may be described as deficits in the processes by which people perceive, encode, consolidate, retrieve or use memory. The degree of affection, the brain region affected or the extension of the lesion can produce different kinds of alterations. There is a wide range of processes that can lead to memory alterations (Buffum et al. 2007):

- Genetic disorders coursing with intellectual disability including Down syndrome, fragile X syndrome or Williams-Beuren syndrome, among others (Conners et al. 2011).
- Neurodegenerative disorders being some of the most representative Alzheimer's disease, Parkinson's disease, Huntington's disease and cerebellar degeneration (Perry and Hodges 1996).
- Vascular disorders such as stroke or cerebral embolic disease (Jellinger 2014).

- Traumatic events that can produce acute or chronic effects in memory, such as stressful events leading to traumatic stress disorder (Golier and Yehuda 2002).
- Toxics: the consumption of drugs of abuse, such as cannabis, has been widely demonstrated to produce amnesic-like effects (Lundqvist 2005) as well as the prolonged exposure to other types of chemicals or gases.
- Infectious processes, such as encephalitis and sepsis (Habbas et al. 2015).
- Normal aging processes that have been demonstrated to produce memory deficits in different species including humans, primates or rodents (Erickson and Barnes 2003).

In this thesis we will focus our attention in studying memory performance ans deficits associated with specific situations and involving different signaling pathways:

1. Cognitive impairment associated to a genetic disorder, the fragile X syndrome.
2. Effects of a stressful situation on a non-emotional declarative memory.
3. Effects of THC in short-term memory processes and the possible pathways involved.
4. Study of the possible role of the protein kinase C gamma in the regulation of learning and memory processes.

2. Endocannabinoid system

2.1 Natural and synthetic cannabinoids

Cannabis sativa is possibly one of the oldest plants cultivated by man and their derivates, such as marijuana, has been used for millenia for both recreational and medical purposes. The first documented use of *Cannabis sativa* was in the ancient China (2,727 BC) and it was described in the Pên-ts'ao Ching, the oldest known pharmacopoeia. It was prescribed for several diseases but its excessive use produced some side effects considered as "seeing the devil". Since then, it has been used in many cultures and regions (India, Persia and Arabia, among others), and it has been considered a sacred plant. For example, it was thought to be a "source of happiness and freedom" in the Indus Valley civilization (Mechoulam et al. 2014; Murray et al. 2007; Russo 2007). Over the past 4,000 years, *Cannabis sativa* preparations have been used for its analgesic, anti-spasmodic, anti-epileptic, anti-emetic and orexigenic properties (Ben Amar 2006).

During the last century, at least 85 different compounds have been isolated, and some of them extracted, from the *Cannabis sativa* plant, which are known as phytocannabinoids (ElSohly and Slade 2005). Among them, delta9-tetrahydrocannabinol (THC) is the main psychoactive component of the plant. Its structure was first determined and synthetized in the 1960s by Raphael Mechoulam and colleagues (Gaoni and Mechoulam 1964). Another important phytocannabinoid is cannabidiol (CBD) that does not present the psychoactive effects of THC. For that reason, it is an interesting agent for potential therapeutic use presenting an excellent tolerability profile in humans, anti-inflammatory effects and

promising effectiveness in the treatment of epileptic seizures (Iuvone et al. 2009; O'Connell et al. 2017). Other phytocannabinoids extracted from the plant include cannabinol, delta8-tetrahydrocannabinol, cannavidivarin or cannabigerol, among others (reviewed by Maldonado et al. 2011). Since the isolation of THC, a number of synthetic active analogs of this compound, presenting cannabimimetic properties, have been synthetized displaying different selectivity and affinity for the cannabinoid receptors. Synthetic analogs can be differentiated according to its activity between cannabinoid agonist such as HU-210, CP55,940 or WIN55,212-2 and antagonist being some of the most relevant the SR141716A (rimonabant), AM251 (both of them acting on the type-1 cannabinoid receptor (CB1R)) and AM630 (acting on type-2 cannabinoid receptor (CB2R)) (Figure 7) (Pop 1999; Gatley et al. 1996; Pertwee et al. 1995).

The isolation of THC, followed by the development of synthetic cannabinoids, has permitted the advances in the study of the endocannabinoid system, the identification of its components and the physiological functions that it exerts. The most relevant topics will be described in the following sections.

2.2 Components of the endocannabinoid system

The endocannabinoid system (ECS) is an endogenous neuromodulatory system involved in many physiological functions. It is composed by the cannabinoid receptors, their endogenous ligands (also known as endocannabinoids) and the enzymes responsible for their synthesis and degradation.

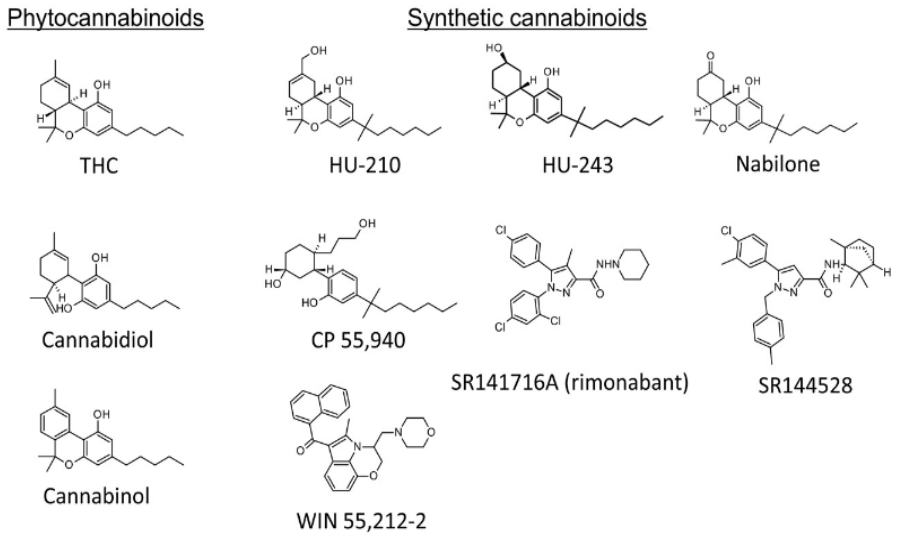


Figure 7. **Chemical structure of representative phytocannabinoids and synthetic cannabinoids** (Adapted from Maldonado et al. 2011).

2.2.1 Cannabinoid receptors

Endogenous and exogenous cannabinoids exert their physiological or pharmacological functions, respectively, through the activation of the cannabinoid receptors, being the most importants the cannabinoid receptor type-1 (CB1R) and the cannabinoid receptor type-2 (CB2R). Both are G-protein coupled receptors and present seven hydrophobic trans-membrane domains connected by alternating extracellular and intracellular loops (Childers and Deadwyler 1996; Svíženská et al. 2008). A schematic representation of the CB1R is shown in Figure 8. The existence of cannabis receptors was discovered around 30 years ago, in 1988, by Howlett and Devane (Devane et al. 1988). Two years later, in 1990, the first cannabinoid receptor, CB1, was cloned (Matsuda et al. 1990) while CB2 receptor was cloned three years later, in 1993 (Munro et al. 1993).

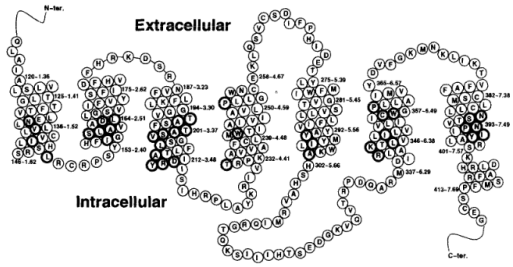


Figure 8. **Representation of the CB1 receptor.** Highly conserved residues are in bold. (Bramblett et al. 1995).

Cannabinoids present different affinities for their receptors. In Table 2, a summary of the specific binding affinities (represented as K_i values) of the most studied cannabinoid ligands for the CB1R and CB2R are presented (Pertwee et al. 2010).

The CB1R and CB2R have been both identified in different species including mice, rats, dogs, pigs, monkeys and humans. However, they present many differences regarding their localization at both the CNS and the peripheral tissues (Svíženská et al. 2008; Mackie 2005). In general terms, CB1R are highly expressed in certain brain areas of the CNS, while the CB2R are mainly expressed in the immune system.

Cannabinoid Receptor Ligand	K_i	
	CB_1	CB_2
	nM	
Section II.C.1		
(−)-Δ^9-THC	5.05–80.3	3.13–75.3
HU-210	0.06–0.73	0.17–0.52
CP55940	0.5–5.0	0.69–2.8
R-(+)-WIN55212	1.89–123	0.28–16.2
Anandamide	61–543	279–1940
2-AG	58.3, 472	145, 1400
Section II.C.2		
Agonists with higher CB_1 than CB_2 affinity		
ACEA	1.4, 5.29	195, >2000
Arachidonylcyclopropylamide	2.2	715
R-(+)-methanandamide	17.9–28.3	815–868
Noladin ether	21.2	>3000
Agonists with higher CB_2 than CB_1 affinity		
JWH-133	677	3.4
HU-308	>10000	22.7
JWH-015	383	13.8
AM1241	280	3.4
Section II.C.3		
Rimonabant (SR141716A)	1.8–12.3	514–13,200
AM251	7.49	2290
AM281	12	4200
LY320135	141	14,900
Taranabant	0.13, 0.27	170, 310
NESS 0327	0.00035	21
O-2050	2.5, 1.7	1.5
Section II.C.4		
SR144528	50.3–>10,000	0.28–5.6
AM630	5152	31.2
JTE-907	2370	35.9
Section II.C.5		
11-OH-Δ^8-THC	25.8	7.4
Ajulemic acid	5.7, 32.3	56.1, 170.5
Cannabinol	120–1130	96–301
Cannabigerol	81	2600
Cannabidiol	4350–>10,000	2399–>10,000
N-Arachidonoyl dopamine	250	12,000
Virodhamine	912	N.D.

Table 2. **Some K_i values of cannabinoid CB1/CB2 receptor ligands for the *in vitro* displacement of a tritiated compound from specific binding sites on rat, mouse or human CB1R and CB2R** (Modified from Pertwee et al. 2010).

CB1R distribution in the CNS has been well characterized in both rodents (Tsou et al. 1998) and humans (Westlake et al. 1994) (Figure 9) and it is considered the most abundant seven trans-membrane metabotropic receptor in the brain (Herkenham et al. 1991). The high expression of the CB1R in the CNS makes it

responsible from the psychoactive effects produced by THC and other exogenous and endogenous cannabinoid ligands. Different techniques have been used to map the distribution of the cannabinoid receptors in the CNS including quantitative autoradiography, *in situ* hybridization, immunohistochemistry, as well as GTPgamma-S binding to study the CB1R function (Mackie 2005).

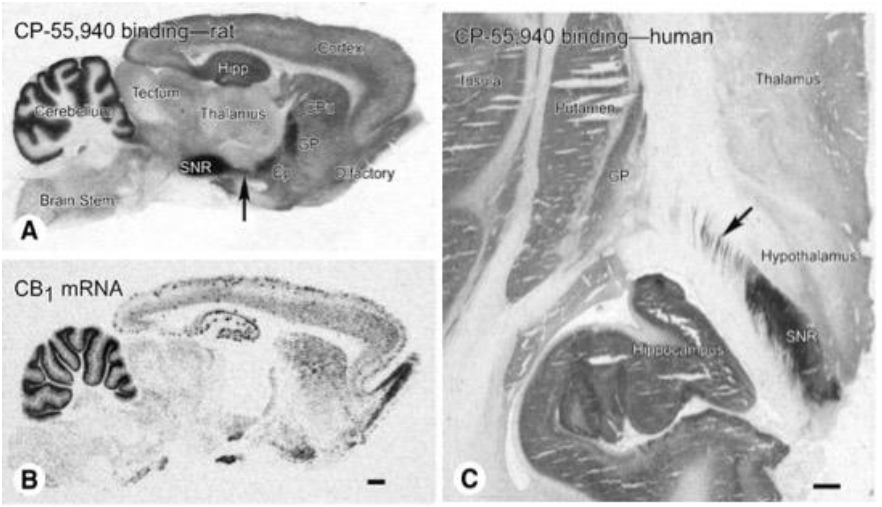


Figure 9. **Distribution of CB1R in the brain.** Autoradiography images showing cannabinoid receptor in rat (A) and human (C) brain marked with the tritiated ligand CP-55,940. Sagittal section of rat brain hybridized with a CB_1-specific oligonucleotide probe (B) shows locations of neurons that express mRNA at this levels. High levels of receptor protein are observed in different basal ganglia structures and the cerebellum. Moderate binding levels can be found in the hippocampus, cortex and caudate putamen. Low binding is seen in the brain stem and thalamus (Modified from Freund et al. 2003).

The CNS areas showing the highest CB1R expression are the cortex, some olfactory regions, the hippocampus, the amygdala, some subcortical structures, such as the basal ganglia, the cerebellum, the substantia nigra of the midbrain and the

periaqueductal grey. Moderate levels can be found in the basal forebrain, the nucleus accumbens, the hypothalamus and the spinal cord. Finally, low levels have been identified in the thalamus or in the brainstem (Mackie 2005; Svíženská et al. 2008; Tsou et al. 1998; Freund et al. 2003). Moreover, CB1R are expressed in peripheral tissues including gastrointestinal tract, adipose tissue, liver (Matias et al. 2006), urinary bladder (Walczak et al. 2009), skeletal muscle, pancreas (Tam et al. 2010), reproductive organs (Gérard et al. 1991) and retina (Porcella et al. 2000), among others.

Ultra structural studies have shown that at the cellular level, CB1R are mainly expressed at neuronal presynaptic terminals where they play a major role regulating the release of different excitatory and inhibitory neurotransmitters, mainly γ-aminobutyric acid (GABA) and glutamate but also noradrenaline, acetylcholine, dopamine and cholecystokinin, among others (Pertwee and Ross 2002; Szabo and Schlicker 2005).

The distribution of these receptors is heterogeneous within cellular populations. Thus, CB1R are more highly expressed in GABAergic than glutamatergic terminals (Katona et al. 2006; Kawamura et al. 2006; Katona 2009; Gutierrez-Rodríguez et al. 2017). Although CB1R are mainly expressed in neurons, basically on axons and presynaptic terminals (Mackie 2005), recent studies have demonstrated that CB1R are also expressed in astrocytes, (Navarrete and Araque 2008; Stella 2010) and in a particular subcellular compartment, the mitochondria, where they control neuronal energy metabolism (Bénard et al. 2012) and may be involved in the modulation of memory formation (Hebert-Chatelain et al. 2016). The differences between the classic view of CB1R

localization, limited to their presynaptic location, and the current view is shown in Figure 10.

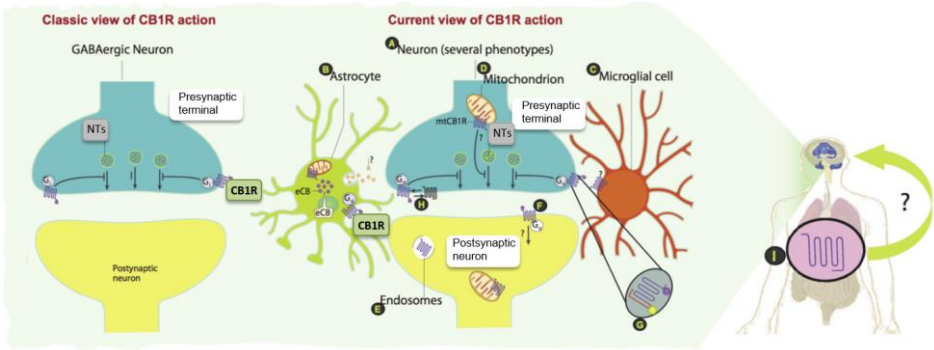


Figure 10. **Schematic comparison between the classic and the current view of CB1R functional expression**. In the classic view, CB1R was thought to be exclusively localized in GABAergic neurons where its function was to inhibit neurotransmitter release. In the current view, several advances have completely changed the figure demonstrating that CB1R is present in different neuronal types (A) and in glial cells including both astrocytes (B) and potentially the microglia (C). Moreover it has been found in intracellular compartments such as the mitochondria (D). Beyond the brain, the CB1R is widely express in the periphery (I) where it can modulate the periphery-brain connection (Modified from Busquets-Garcia et al. 2016).

CB2R are mainly expressed in peripheral tissues, specifically in the immune system and, in particular, they are mainly present in leucocytes and immune cells of the spleen and tonsils (Sívženská et al. 2008). The presence of these receptors in the CNS has been controversial. Many studies, until few years ago, demonstrated that CB2R were not present in the CNS (Munro et al. 1993; Galiegue et al. 1995). However, recent studies show evidence that these receptors are also expressed in microglia and, more interestingly, in neurons (Morgan et al. 2009). The functional presence of CB2R has been suggested in the mammalian brain in regions such as the cerebellar Purkinje cells or the hippocampal pyramidal cells (Van Sickle et al. 2005). Moreover, these studies suggest the possible

postsynaptic localization of the CB2R as the cells that present positive immunoreactivity for CB2 were not immunostained for CB1 (Gong et al. 2006; Onaivi et al. 2006). However, the levels detected of CB2R in the brain are much lower than those of CB1R (Svíženská et al. 2008). The neuronal expression of CB2R has been suggested to contribute in pain attenuation (Shang and Tang 2016). Other studies have suggested that these receptors can be involved in neuropsychiatric disorders such as depression and substance abuse (Onaivi et al. 2012).

Besides the well-known cannabinoid receptors previously presented, other receptors could also explain the effects of cannabinoid compounds that are not mediated by CB1R nor CB2R. One of them is the orphan G-protein coupled receptor GPR55 that is considered to be targeted by a number of cannabinoids. This receptor binds a number of synthetic and plant-derived cannabinoid ligands and it is activated by anandamide (Pertwee 2007; Ryberg et al. 2007). GPR55 is expressed in the CNS and in the periphery pointing to its possible involvement in multiple actions (Tudurí et al. 2017). Other potential cannabinoid receptors are G protein-coupled receptor 3 (GPR3), G protein-coupled receptor 6 (GPR6) and G protein-coupled receptor 12 (GPR12) all of them sphingosine-1-phosphate lipid receptors (Yin et al. 2009), as well as the transient receptor potential vanilloid type-1 (TRPV1) that bind anandamide (Di Marzo and De Petrocellis 2010).

2.2.2 Endocannabinoids

Once the first cannabinoid receptors were identified and cloned in the nineties, the next step was to find possible endogenous cannabinoid ligands (known as endocannabinoids). The first endocannabinoids to be described were N-arachidonoylethanolamine (commonly called anandamide (AEA)) and 2-arachidonoylglycerol (commonly called 2-AG) (Figure 11) (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995). AEA was the first endocannabinoid discovered, in 1992, and its name comes from the sacred word “ananda” that means “bliss” (Murray et al. 2007). It behaves as a partial agonist to the CB1R and CB2R (Table 2) and can also bind to the vanilloid receptor TRPV1 (Cristino et al. 2008). 2-AG was discovered three years later, in 1995 (Mechoulam et al. 1995; Sugiura et al. 1995). This endocannabinoid acts as a full agonist to the CB1R and CB2R (Table 2), suggesting that it is the true natural ligand for the cannabinoid receptors and is present in the brain in a higher concentration than AEA (Murray et al. 2007; Takayuki Sugiura et al. 2006). Other putative endocannabinoids have been identified although their physiological function is still unknown. Some examples are 2-arachydonoylglycerol ether (noladin ether), N-arachidonoyldopamine, methanadamide and N-arachidonoylethanolamine (virodhamine). Their structure is presented in Figure 11 (Matias and Di Marzo 2007).

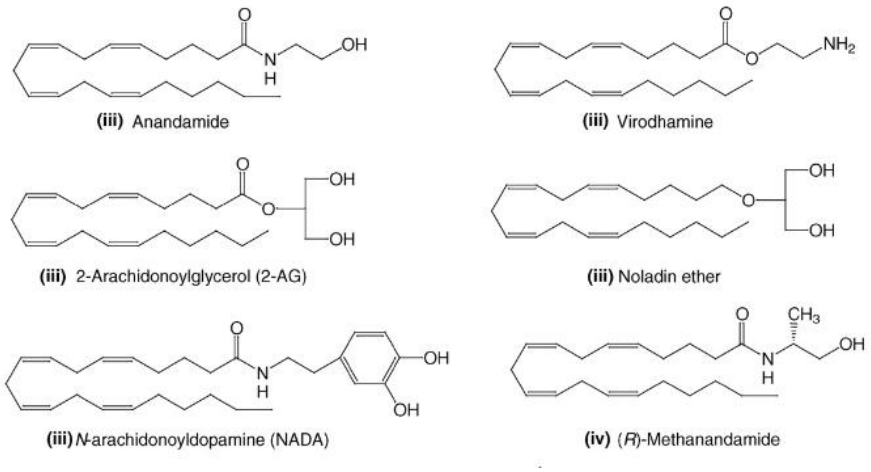


Figure 11. **Endocannabinoid structures** (Modified from Matias and Di Marzo 2007).

Endocannabinoids are lipid molecules and their expression levels are regulated by a balance between their synthesis and inactivation, performed by specific enzymes for each endogenous ligand. In contrast to other neurotransmitters, endocannabinoids are not prestored in secretory vesicles. In fact, they are biosynthetized de novo, “on demand”, responding to the increases in calcium intracellular concentration (Matias and Di Marzo 2007). Once synthetized, endocannabinoids are released from the postsynaptic terminal. They act as retrograde messengers in the CNS, travelling across the synapse to bind to the CB1R in order to prevent an excessive neuronal activity and maintain the homeostasis in physiological and pathological conditions (Figure 12). Thus, the activation of the CB1R will decrease the neurotransmitters release at both excitatory and inhibitory synapses in either a transiently or persistently manner (Wilson and Nicoll 2002; Mechoulam and Parker 2013; Kano 2014).

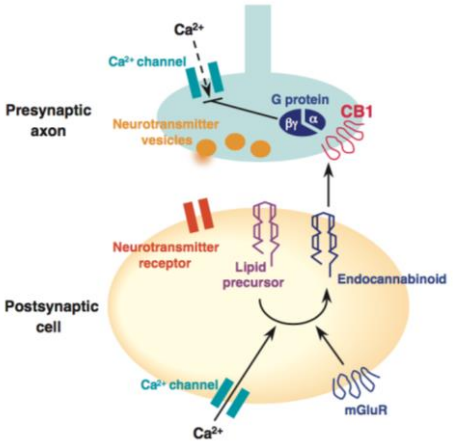


Figure 12. **Retrograde signaling by endocannabinoids**. The increase of intracellular calcium concentrations activates enzymes that synthetize endocannabinoids from lipid precursors. Endocannabinoids are released from the postsynaptic cell and activate presynaptic CB1R, that regulate the release of neurotransmitters (Wilson and Nicoll 2002).

The persistent suppression of neurotransmitters release produces the endocannabinoid-mediated long-term depression (eCB-LTD), which will facilitate the induction of long-term potentiation (LTP) at excitatory neurons when it occurs at inhibitory terminals (eCB-LTDi). This phenomenon is widely expressed in different brain regions (Figure 13) (Kano 2014; Castillo et al. 2012; Heifets and Castillo 2009).

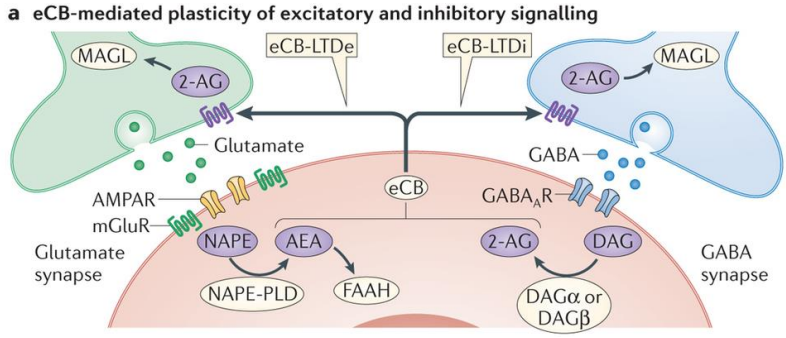


Figure 13. **Schematic illustration of the endocannabinoid-mediated long-term depression.** eCB-LTD is induced by afferent stimulation with or without postsynaptic depolarization, resulting in the synthesis of endocannabinoids. These endocannabinoids activate CB1R, which together with other events such as increased calcium concentrations or n-methyl-D-aspartate (NMDA) receptor stimulation results in persistently decreased neurotransmitters release. Depending on brain region, eCB-LTD of both excitatory (eCB-LTDe) and inhibitory (eCB-LTDi) afferents has been described (Modified from Parsons and Hurd 2015).

2.2.3 Enzymes involved in the biosynthesis and degradation of endocannabinoids

AEA and 2-AG are produced from the hydrolysis of precursors via different pathways and involving different enzymes (Figure 14).

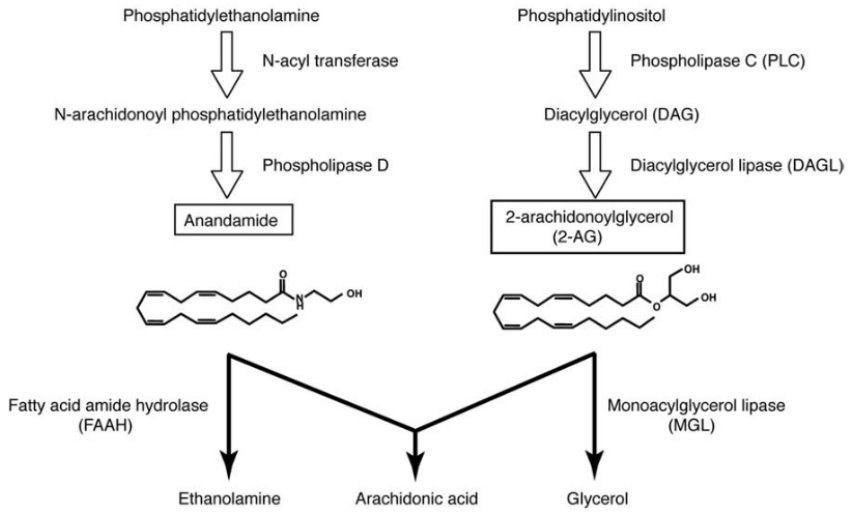


Figure 14. **Main pathways and enzymes involved in the biosynthesis and degradation of the two main endocannabinoids** (anandamide and 2-arachidonoylglycerol) (Hashimotodani et al. 2007).

AEA is synthetized by the action of two enzymatic reactions. In a first step, phosphatidylethanolamine is converted into N-arachidonoyl phosphatidylethanolamide (NAPE) by calcium-dependent N-acyltransferase. Secondly, the phosphodiester bond of NAPE is hydrolysed to AEA by the action of a phospholipase D (NAPE-PLD), an enzyme identified in the 1890s (Hashimotodani et al. 2007; Di Marzo et al. 2004; Di Marzo et al. 1994).

2-AG is also synthetized as a result of two enzymatic reactions. Phospholipase C (PLC) is responsible for the production of the 2-AG precursor, diacylglycerol (DAG) from phosphatidylinositol. DAG, at its time, is hydrolysed by two diacylglycerol lipase (DAGL-α and

DAGL-β) producing 2-AG (Hashimotodani et al. 2007; Di Marzo et al. 2004).

Once AEA and 2-AG have activated their target receptors, endocannabinoid signaling is reduced by specific enzymes. In order to be degraded, they first need to be cleared from the cannabinoid receptors and be taken up by the cell, which takes place via rapid re-uptake through the cell membrane although the specific proteins mediating this uptake have not been identified yet (Guindon and Hohmann 2009). Different enzymes are involved in the degradation of each endocannabinoid. AEA degradation is produced by the fatty-acid amide hydrolase (FAAH) resulting in arachidonic acid and ethanolamine (Cravatt et al. 1996; Hashimotodani et al. 2007; Di Marzo et al. 1994). On the other hand, 2-AG degradation involved the action of the monoacylglycerol lipase (MAGL) and its hydrolysis results in the production of arachidonic acid and glycerol (Hashimotodani et al. 2007; Dinh et al. 2002). Other enzymes seem to be responsible for about 15% of the 2-AG degradation, such as the α/β hydrolase domain 6 and 12 (ABHD6 and ABHD12) (Kano 2014).

FAAH and MAGL, apart from being involved in the degradation of a different endocannabinoid, present also some differences regarding their subcellular localization. FAAH is expressed in the soma and dendrites of postsynaptic neurons, whereas MAGL is localized in the presynaptic terminals (Gulyas et al. 2004; Di Marzo et al. 2004; Parsons and Hurd 2015) (Figure 15).

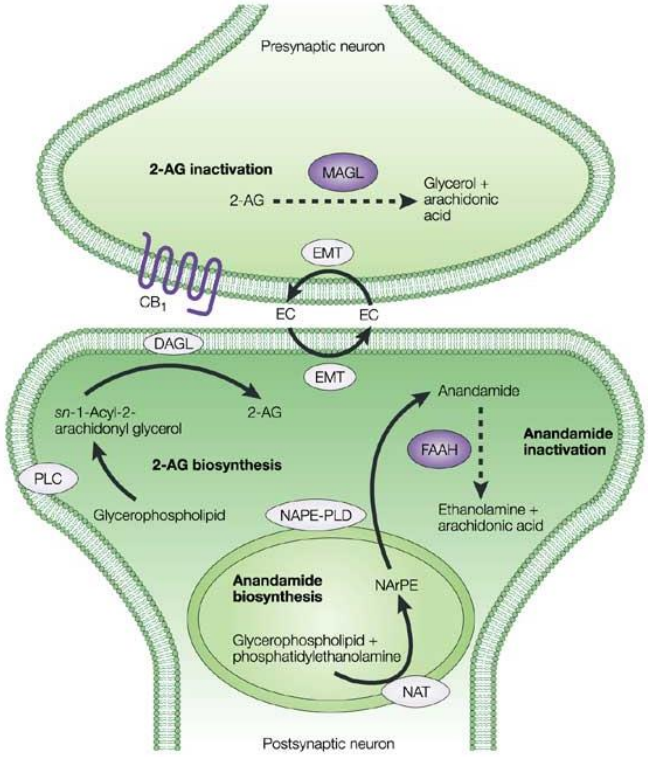


Figure 15. **Main pathways involved in the synthesis and degradation of endocannabinoids and their most likely subcellular localization**. The monoacylglycerol lipase (MAGL) for 2-AG inactivation is mainly localized in presynaptic neurons while the inactivating enzyme fatty acid amide hydrolase (FAAH) seems to be most abundant on neurons postsynaptic to CB1R (Di Marzo et al. 2004).

2.3 Cannabinoid receptors signaling

The stimulation of the cannabinoid receptors produces the activation of multiple signaling pathways that will cause a great variety of effects (Figure 16). As mentioned before, CB1R and CB2R are members of the G protein-coupled receptor superfamily. It has been suggested that cannabinoid receptors, as well as other GPCRs, are able to signal in three different spatiotemporal waves: the first one is transient (less than 10 seconds) and is mediated by heterotrimeric G proteins, the second wave is mediated by beta-arrestins and

finally, the last one, can be mediated by both G proteins and beta-arrestins (Lohse and Calebiro 2013; Nogueras-Ortiz and Yudowski 2016). Cannabinoid receptors mediate their biological effects by activating heterotrimeric Gi/o type G proteins (α, β and γ) although they can also couple with other proteins (Bosier et al. 2010). As a consequence, there is an inhibition of the adenylyl cyclase activity together with a decrease of the cyclic adenosine monophosphate (cAMP) and the protein kinase A (PKA) activity (Howlett 2005). CB1R can also couple to G$\beta\gamma$i/o producing the phosphorylation and activation of multiple members of the mitogen-activated protein kinase (MAPK) family such as extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) (Howlett 2005). Moreover, *in vitro* assays have demonstrated that cannabinoids can also activate PKC signaling (Hillard and Auchampach 1994). Finally, other proteins that seem to be modulated by CB1R stimulation include phosphatidylinositol 3-kinase (PI3K) (Bouaboula et al. 1995), the mammalian target of rapamycin (mTOR) (Puighermanal et al. 2009), focal adhesion kinases (Derkinderen et al. 1996) and some enzymes involved in energy metabolism (Guzmán and Sánchez 1999).

On the other hand, CB1R can also modulate different types of ion channels, as the inhibition of N-type and P/Q-type calcium current or activation of A-type potassium channels that regulates neurotransmitter release in a negative manner (Bosier et al. 2010; McAllister and Glass 2002).

Finally, for the regulation of signaling transduction pathways triggered by GPCRs like CB1R, the possible formation of heteromers (Pertwee et al. 2010) and the lipid composition of the cellular

membrane surrounding the receptor (Maccarrone 2010) seem to be important.

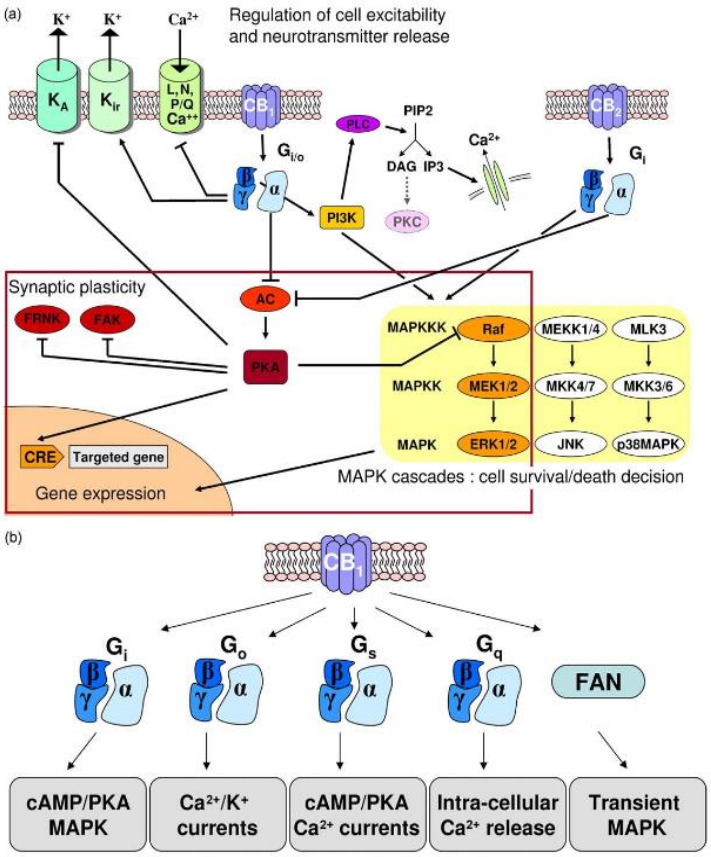


Figure 16. **Complexity of the cannabinoid receptor signaling pathway**. (a) CB1R and CB2R are both associated to protein $G\alpha_{i/o}$-dependent inhibition of adenylyl cyclase activity and $G\beta\gamma$-dependent activation of the different MAPK cascades. CB1R negatively regulate voltage-gated Ca^{2+} channels and positively regulates inwardly rectifying K^+ channels, thereby inhibiting neurotransmitter release. Cross-talk between signaling pathways are illustrated by the variety of responses requiring cannabinoid-mediated inhibition of PKA. (b) Preferential activation of different intracellular effectors by each G protein contributes to diversity and selectivity of responses regulated by cannabinoid receptors (Modified from Bosier et al. 2010).

2.4 Physiological role of the endocannabinoid system

The ECS is involved in a large number of physiological functions that have been well described by using genetic (such as different knock out animal models) and pharmacological tools (use of agonists and antagonists) (Table 3) (Grotenhermen 2003). In consequence, the dysregulation of the ECS will be present in different pathological states. Moreover, as mentioned previously, the ECS presents a widespread expression in the CNS and peripheral tissues, which will be of crucial relevance for the different functions mediated by this system.

Body system	Effects
Psyche and perception	Fatigue, euphoria, enhanced well-being, dysphoria, anxiety, reduction of anxiety, depersonalisation, increased sensory perception, heightened sexual experience, hallucinations, alteration of time perception, aggravation of psychotic states, sleep
Cognition and psychomotor performance	Fragmented thinking, enhanced creativity, disturbed memory, unsteady gait, ataxia, slurred speech, weakness, deterioration or amelioration of motor coordination
Nervous system	Analgesia, muscle relaxation, appetite stimulation, vomiting, antiemetic effects, neuroprotection in ischaemia and hypoxia
Body temperature	Decrease of body temperature
Cardiovascular system	Tachycardia, enhanced heart activity, increased output, increase in oxygen demand, vasodilation, orthostatic hypotension, hypertension (in horizontal position), inhibition of platelet aggregation
Eye	Reddened conjunctivae, reduced tear flow, decrease of intraocular pressure
Respiratory system	Bronchodilation
Gastrointestinal tract	Hyposalivation and dry mouth, reduced bowel movements and delayed gastric emptying
Hormonal system	Influence on luteinising hormone, follicle-stimulating hormone, testosterone, prolactin, somatotropin, thyroid-stimulating hormone, glucose metabolism, reduced sperm count and sperm motility, disturbed menstrual cycle and suppressed ovulation
Immune system	Impairment of cell-mediated and humoral immunity, immune stimulation, anti-inflammatory and antiallergic effects
Fetal development	Malformations, growth retardation, impairment of fetal and postnatal cerebral development, impairment of cognitive functions
Genetic material and cancer	Antineoplastic activity, inhibition of synthesis of DNA, RNA and proteins

Table 3. **Effects of cannabinoid agonists** observed in clinical studies, *in vitro* and *in vivo* (Modified from Grotenhermen 2003).

The ECS has been implicated in other functions in the CNS, beyond the control of synaptic plasticity, for example in neurogenesis by the regulation of neural progenitor cells proliferation, pyramidal specification and axonal navigation. Moreover, CB1 mRNA is expressed in many regions of the developing brain. The ECS is also

involved in neuronal synaptic communication, in the modulation of final brain maturation and connectivity and in the control of neuron survival and neuroprotection against neuronal damage (Fowler et al. 2010; Galve-Roperh et al. 2009; Mechoulam and Parker 2013; Skaper and Di Marzo 2012). All together, the ECS is crucial for the control of synaptic homeostasis and for the maintenance of the correct brain circuitry function.

At the central level, the CB1R are located in multiple brain regions with a high or a moderate expression, where they regulate different functions. Some of them are represented in Figure 17.

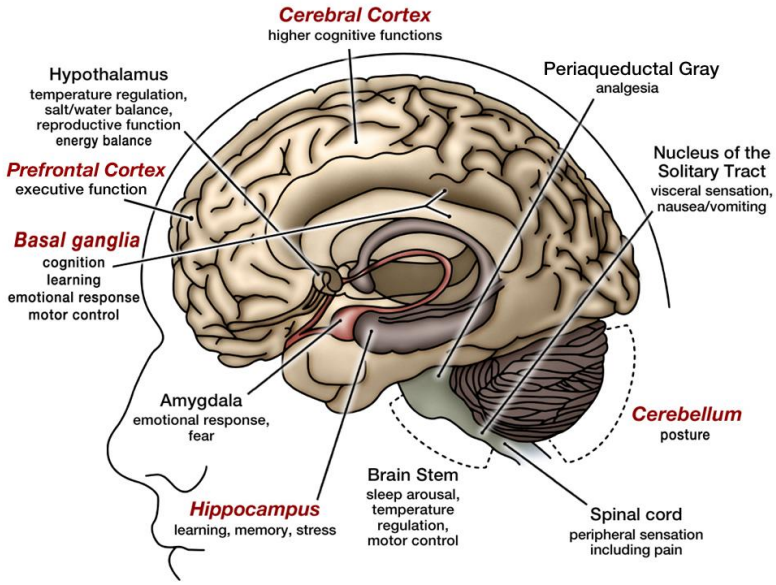


Figure 17. **Main brain regions where ECS is expressed in association with its main physiological function.** Red = High expression of CB1R. Black = Moderate expression of CB1R

CB1R expressed in the olfactory system are involved in the modulation of olfaction in humans and rodents (Egertova and Elphick 2000). CB1R also play a key role in anxiety and it has been shown that cannabinoid agonists influence anxiety in a biphasic manner. Thus, high doses of cannabinoid agonist produce anxiogenic-like effects that are mediated by CB1R on forebrain GABAergic neurons while low doses of cannabinoid agonists produce anxiolytic-like effects due to the modulation of CB1R on cortical glutamatergic neurons (Lutz et al. 2015). Related to that, there is also a clear relation between the ECS and stress responses. This topic will be analyzed in more detail in chapter 4. Moreover, the expression of CB1R in the cerebellum and the basal ganglia are involved in the fine control of movement and motor coordination (Rodríguez de Fonseca et al. 1998). The ECS is also important in the modulation of pain and analgesic processes (Guindon and Hohmann 2009; La Porta et al. 2014), in the control of nausea and vomiting (Sharkey et al. 2014), in the regulation of energy balance (including the search, metabolism and storage of calories) (Gatta-Cherifi and Cota 2016) and it is involved in motivation and reward functions such as food intake and drug addiction (Maldonado et al. 2011; D'Addario et al. 2014), among other central physiological functions. Finally, high expression of CB1R can also be found in the hippocampus, a brain region that has been highly related with learning and memory processes. In fact, the deleterious effects of cannabinoids in these brain functions have been widely studied (Mishima et al. 2001; Puighermanal et al. 2012). The effects of cannabinoids in memory and cognition will be developed in more detail in the following section. In addition, some central effects could be also regulated by the CB2R, together with the CB1R, such as emesis and cocaine

rewarding effects (Van Sickle et al. 2005; Xi et al. 2011). On the other hand, the presence of the ECS in peripheral tissues has also been described in the modulation of the immune system, vascular beds, reproductive organs in relation to male and female fertility, gastrointestinal motility, energy balance and metabolism, among others (Grotenhermen 2003; Bellocchio et al. 2008; Aizpurua-Olaizola et al. 2016).

Finally, different pathological states including neurological and metabolic disorders, among others, present alterations in some components of the ECS (Table 4).

Pathology	Endocannabinoid system alteration
Alzheimer's disease	Decreased CB1R expression in hippocampus and basal ganglia and overexpression of CB2R and FAAH in glial cells observed in post-mortem brains of human patients
Parkinson's disease	CB1R down-regulation at early stages Overactivation of the endocannabinoid system in advanced stages of the disease
Huntington's disease	Low levels of CB1R in post-mortem brains from patients
Brain ischemia	Increased levels of endocannabinoids after traumatic injury and increased CB1R expression in rodent brain cortex after an hypoxic-ischemic insult
Obesity	Upregulation of the endocannabinoid system: Increased levels of 2-AG and increased expression of CB1R in genetic animal models
Gastrointestinal disorders	Concentration of AEA and/or expression of CB1R increased in different mouse models
Reproductive disorders	Increased levels of AEA related with premature abortion or failure of implanted oocytes fertilized *in vitro*

Table 4. Dysregulation of the endocannabinoid system in pathological states. Based on Martínez-Orgado et al. 2009; Di Marzo et al. 2004.

2.5 Endocannabinoid system and memory

CB1R are widely expressed in the hippocampus, a brain region that plays a key role in the regulation of learning and memory processes, as it is supported by many experiments and clinical studies. Moreover, the hippocampus is one of the brain regions with highest amount of AEA in rodents (Mechoulam and Parker 2013).

The memory-related effects produced by cannabinoids may vary depending on the kind of cannabinoid compound tested (agonists/antagonists, direct/indirect agonists), the dosage, the route of administration and the memory task performed (Kruk-Slomka et al. 2017). It is believed that cannabinoid agonists produce memory and learning impairments while cannabinoid antagonists may produce the opposite effect. However, recent works have demonstrated that the regulation of learning and memory processes by cannabinoids is not that simple (Abush and Akirav 2010).

2.5.1 Effects of cannabinoid ligands (endogenous and exogenous) on memory

It has been known, for many years, that cannabis consumption provoke deficits in several aspects of learning and memory in humans and in laboratory animals. To understand the role of the endocannabinoid system in these processes, many studies have been performed using natural and synthetic exogenous ligands of the cannabinoid receptors. Moreover, other approximations used for these studies involved the modulation of the endocannabinoid tone by using inhibitors of the endogenous ligands degradation, among others.

Regarding the use of CB1R agonists, it is mostly believed that they disrupt different memory processes, such as working memory, while

do not affect memory retrieval (Lichtman et al. 2002). The exogenous natural cannabinoid THC or the synthetic cannabinoid agonist WIN55,212, among others, have been described to disrupt working memory (Mechoulam and Parker 2013). In that sense THC, as well as different synthetic CB1R agonists, increases the number of errors in a spatial working memory task, the eight-arm maze. Moreover these deficits could be blocked by the pre-treatment with the compound SR141716A (rimonabant), a CB1R antagonist/inverse agonist (Lichtman et al. 2002). Other studies, recapitulated in a recent review from Kruk-Slomka et al. (2017) described that synthetic CB1R agonists produce deficits in the acquisition and the consolidation of several memory tasks including the contextual fear-conditioning, the Morris water maze test and the novel object-recognition task (Kruk-Slomka et al. 2017).

On the other hand, as mentioned previously, CB1R antagonists are believed to produce an amelioration of the memory processes, an effect confirmed in numerous studies. The use of CB1R antagonists has demonstrated an increase in the mice olfactory memory in a social recognition test (Terranova et al. 1996). Moreover, in the elevated T-maze, rimonabant produced an enhancement of memory when administered before or immediately after the training, but not when administered before the test. Thus, rimonabant was involved in improving the consolidation, but not the memory retrieval (Takahashi et al. 2005). In addition, these effects seem to be dose-dependent, a variable that had been described also by other authors (Takahashi et al. 2005; Wolff and Leander 2003). It has been hypothesized that the improving and enhancing memory effects of rimonabant may be due to its action as an inverse agonist of the CB1R (Lichtman et al. 2002).

AM251 is another CB1R antagonist/inverse agonist that has demonstrated controversial effects in different memory paradigms. It demonstrated a dose-dependent effect in the novel object-recognition memory task in rats where the lowest dose tested improved significantly acquisition and consolidation of the object memory (Bialuk and Winnicka 2011). The effect of this compound was also studied in the active avoidance test. In this case, AM251 was injected bilaterally in the hippocampus after the training session and amnesic-like effects where observed. These deficits where associated to the consolidation process, while memory acquisition and retrieval were not affected by the hippocampal-infusion of this antagonist (De Oliveira Alvares et al. 2008).

Finally, the involvement of the endocannabinoid system in learning and memory has also been studied by using CB1 knock out (KO) mice. This genetic model seem to present better social abilities and an increased cognitive performance in the NOR test, the contextual fear-conditioning and the active avoidance task (Kruk-Slomka et al. 2017; Reibaud et al. 1999; Litvin et al. 2013). Morever, in the NOR test, CB1 KO mice retain memory for longer periods (48 hours) than the wild-type (WT) controls (Mechoulam and Parker 2013). However, they present short-term and long-term extinction impairments in auditory fear-conditioning tests, indicating that CB1R are necessary for memory extinction (Marsicano et al. 2002).

Altering the levels of the endogenous cannabinoid ligands can also modulate the endocannabinoid system activity. Previous works of our research group studied the effect on memory of increasing the levels of the two main endocannabinoids, separately. The main observations were that the increase of AEA levels by the use of URB597, an inhibitor of the FAAH enzyme, interfered on the

consolidation of contextual fear-conditioning as well as short-term and long-term object recognition memory (Busquets-Garcia et al. 2011). Other studies have been performed evaluating the modulation of AEA and have demonstrated that AEA effects on cognitive processes might be strain-dependent (Fride 2002; Castellano et al. 1999). The different effects previously studied, can be consequence of the activation of different CB1R populations located in different brain regions, which could explain the opposite effects observed in some cases (Riedel and Davies 2005). In this thesis we are particularly interested in the amnesic-like effects produced by the natural cannabinoid agonist THC in STM.

2.5.2 Possible mechanisms underlying memory impairment by cannabinoids

The involvement of the hippocampal CB1R in the memory alterations produced by THC has been reported by pharmacological, genetic and electrophysiological studies (Zanettini et al. 2011; Kendall et al. 2017). However, the involvement of other brain regions in these deleterious effects cannot be discarded. In addition, the ECS modulates a large number of neurotransmitter systems, some of them involved in the cognitive impairment (Puighermanal et al. 2012). In this sense, cannabinoid-induced memory deficits have been related to an inhibition of the cholinergic activity in the CNS (Braida and Sala 2000) or an inhibition of cholecystokinin release (Harro and Oreland 1993). Moreover, it is known that CB1R are highly expressed in GABAergic terminals and that THC acts as a full agonist in these sites (Kawamura et al. 2006; Laaris et al. 2010). Consequently, the administration of THC will decrease GABA release and, in consequence, increase excitatory firing (Katona and Freund 2012) resulting in a deregulation of the excitatory/inhibitory

neurotransmission in the hippocampus (Puighermanal et al. 2009) (Figure 18).

After the stimulation of the cannabinoid receptors, several signaling pathways are activated including the phosphatidylinositol-3-kinase/protein kinase B (Akt)/glycogen synthase kinase-3 signaling pathway, as described by our group in 2007 (Ozaita et al. 2007). One of the downstream pathways of Akt is the mTOR pathway, which is required for proper memory storage. Its over-activation due to THC administration has been associated to the amnesic-like effects produced by this compound in the NOR test and in the context-recognition test when LTM processes were studied (Puighermanal et al. 2009). However, the specific signaling pathways involved in the STM deficits produced by an acute administration of THC are still unknown.

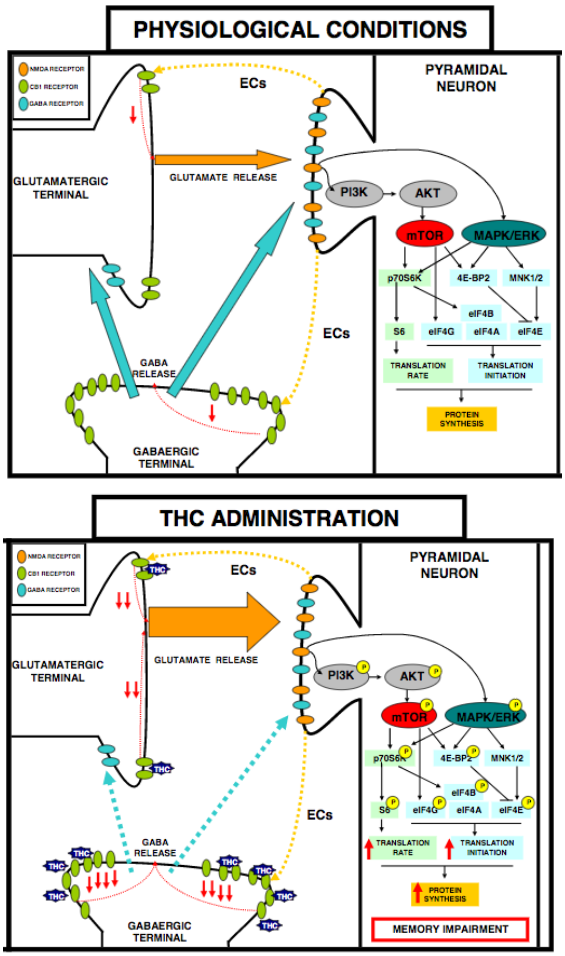


Figure 18. **Schematic diagram showing a possible mechanism involved in THC amnesic-like effects.** In physiological conditions CB1R are mainly localized in GABAergic neurons and to minor extent in glutamatergic neurons. Endocannabinoid system, through CB1R, modulates the neurotransmitter release in GABAergic and glutamatergic terminals. Post-sinaptically, mTOR and MAPK/ERK pathways are activated by glutamate receptors and modulate protein synthesis regulating the translation initiation. When THC is administrated, it acts mainly on CB1R located in GABAergic neurons producing an unbalance between GABAergic and glutamatergic neurotransmission. This unbalance leads to a glutamatergic activation of the mTOR pathway resulting in the phosphorylation of different downstream targets, an increase of the protein synthesis and the consequent amnesic-like effects promoted by THC (Puighermanal et al. 2009).

2.6 Therapeutic applications of the endocannabinoid system

Although marijuana has been used over the history for recreational, magical or spiritually purposes, its therapeutic potential has also been known and exploited during millennia. During the last decades, cannabinoids are gaining weight as promising therapeutic tools despite the public concern related to the negative consequences of their recreational use.

Due to the large distribution of the endocannabinoid system in the body, cannabis preparations have demonstrated beneficial properties for many conditions including stimulation of appetite, antiemesis, muscle relaxation, analgesia and sedation, improve mood, decrease intraocular pression, bronchodilatation, neuroprotection, anti-inflammatory and antineoplastic effects (Pertwee 2005; Pertwee 2009; Pertwee 2012). Cannabinoid synthetic derivatives targeting the endocannabinoid system (both agonists and antagonists of the CB1R and CB2R) have also demonstrated good results in some neurological and neurodegenerative disorders (Wright 2007; Fernández-Ruiz et al. 2015), such as multiple sclerosis and amyotrophic lateral sclerosis (basically alleviating spasticity) (Pryce and Baker 2015), Parkinson's disease (Sieradzan et al. 2001), Tourette's syndrome (Müller-Vahl 2003), Alzheimer's disease (Aso and Ferrer 2014), Huntington's disease (Sagredo et al. 2012) or schizophrenia (Manseau and Goff 2015). Moreover, the efficacy of these compounds as therapeutic agents has also been suggested in other emotional disorders and in autism related disorders such as in the fragile X syndrome (Busquets-Garcia et al. 2013). In reference to the fragile X syndrome, we have recently reported that the antagonism of the endocannabinoid system by the use of rimonabant is able to rescue

some of the phenotypes of the syndrome in a mouse model of the disease, including the deficits in learning and memory. In this thesis, the therapeutical targeting of the endocannabinoid system in the treatment of the cognitive deficits associated to this syndrome has been further characterized. The results obtained in our previous work opens the door to study the modulation of the ECS for the amelioration of the cognitive deficits present in other diseases coursing with mental retardation, such as Down syndrome.

Rimonabant was commercialized in the year 2006 for the treatment of obesity, overweight and metabolic disorders. It acts by blocking the CB1R at both central and peripheral levels promoting body weight loss, a decrease in the waist circumference and an amelioration of lipid and glucose balance (Patel and Pathak 2007). However, in 2008, this drug was suspended due to the appearance of unwanted side effects affecting the CNS including depression and anxiety, among others (Cheung et al. 2013). In the present, the use of rimonabant is being explored for the treatment of other disease at lower doses than the ones used for the treatment of obesity in order to avoid the unwanted side effects. As mentioned earlier, one of the objectives of this thesis is to further explore the potential beneficial effects of CB1R blockade in the treatment of the cognitive deficits present in the fragile X syndrome using doses as low as possible of rimonabant. Recently, the use of neutral antagonists of the CB1R for the treatment of obesity is emerging as safer alternatives to rimonabant (Meye et al. 2013), being NESS0327 one of them (Ruiu et al. 2003). In consequence, we are also going to explore the possibility to ameliorate the cognitive deficits of the fragile X syndrome by the use of neutral antagonists of the CB1R.

Nowadays, there are multiple pharmaceutical companies with high interest in the identification of new cannabinoid compounds to treat several diseases and some drugs have already been approved.

Nabilone (Cesamet®) is a synthetic derivative of THC used as an antiemetic in cancer chemotherapy patients reducing vomiting frequency, nausea severity and increasing food intake.

Dronabinol (Marinol®) is a THC oral preparation used to prevent nausea and vomiting provoke by cancer chemotherapy as well as to treat anorexia associated with weight loss in patients with AIDS.

In 2005 Sativex®, an oromucosal spray containing equal amounts of THC and CBD (proportion 1:1), was licensed in Canada by the company GW pharmaceuticals* for the treatment of spasticity in multiple sclerosis patients. Since then, Sativex® has been approved for this therapeutic effect in more than 15 countries, including Spain, where it was approved in 2011. Moreover, this cannabis-derivative compound is being studied in the present for the treatment of other diseases and some clinical trials are under development. In that sense, a clinical trial performed in 2015 for the treatment of cancer pain failed at phase III.

Another drug developed by GW pharmaceuticals is Epidiolex®, which has been granted Orphan Drug Designation by the Food and Drug Administration (FDA). It is an oral solution containing pure plant-derived CBD, which is under clinical trials for the treatment of resistant epilepsy syndromes such as Dravet syndrome, Lennox-Gastaut syndrome, tuberous sclerosis complex and infantile spams. In this sense, the pharmaceutical company has recently announced (December 2016) positive results in phase III clinical trials in Dravet syndrome and Lennox-Gastaut syndrome, demonstrating a

statistically significant difference in seizure frequency comparing Epidiolex® to placebo.

Finally, when talking about therapeutic compounds obtained from derivatives of the *Cannabis sativa plant*, it is very important to take into account the differences between these preparations and the ones used for recreational purposes, including the route of administration or the dose adminestered, among other. A summary on the main differences is presented in table 5.

	Recreational use	**Therapeutic use**
Num. of compounds	> 60 compounds	1-2 known compounds
Route of administration	Usually smoked (mixed with tobacco)	Oral /Sublingual spray
Doses	Unknown doses	Known regulated doses
Target population	Healthy people	People with diagnosed health problems

Table 5. **Main differences between the recreational and the therapeutic use of cannabis.**

*www.gwpharm.com

3. Fragile X syndrome

3.1 General features and preclinical models

The fragile X syndrome (FXS), which was originally called Martin-Bell syndrome (Wijetunge et al. 2013), is the most common monogenic cause of inherited intellectual disability and autism (de Vries et al. 1998; Penagarikano et al. 2007). It is caused by a CGG trinucleotide expansion located in the 5'-untranslated region of the *Fragile X Mental Retardation 1 (FMR1)* gene on the X chromosome (Verkerk et al. 1991; Penagarikano et al. 2007). This abnormal trinucleotide expansion, consisting in more than 200 CGG repeats (full mutation of the allele) (Hagerman and Hagerman 2007; de Vries et al. 1998) is responsible for the hypermethylation and the consequent transcriptional silencing and loss of its encoded protein, the fragile X mental retardation protein (FMRP) (Jin and Warren 2003; O'Donnell and Warren 2002). The FMRP is a sinaptically expressed RNA-binding protein regulating translation (Darnell et al. 2011) (Figure 19). The FXS appears as a consequence of the FMRP silencing. This protein loss impairs normal synaptic plasticity that seems to be the cause of intellectual disability in FXS patients (Penagarikano et al. 2007). The prevalence of the syndrome is about 1/4000 in males and 1/6000-8000 in females (Turner et al. 1996; Jin and Warren 2003).

As mentioned earlier, the FXS is associated with a trinucleotide expansion of more than 200 CGG repeats while, in normal conditions, the number of this CGG repeats is between 7 and 54, being 30 repeats the most commonly found (Jin and Warren 2003). However, there are also individuals with a pre-mutation of the allele presenting expanded repeated lengths varying from 50 to 200 CGG

repeats. Some individuals present a disorder known as Fragile X-associated tremor/ataxia syndrome (FXTAS) (Figure 19), associated to deficits in executive functions, ataxia and slowly progressive neurodegenerative disorders (Berman et al. 2014; Van Esch 2006; Hagerman and Hagerman 2007).

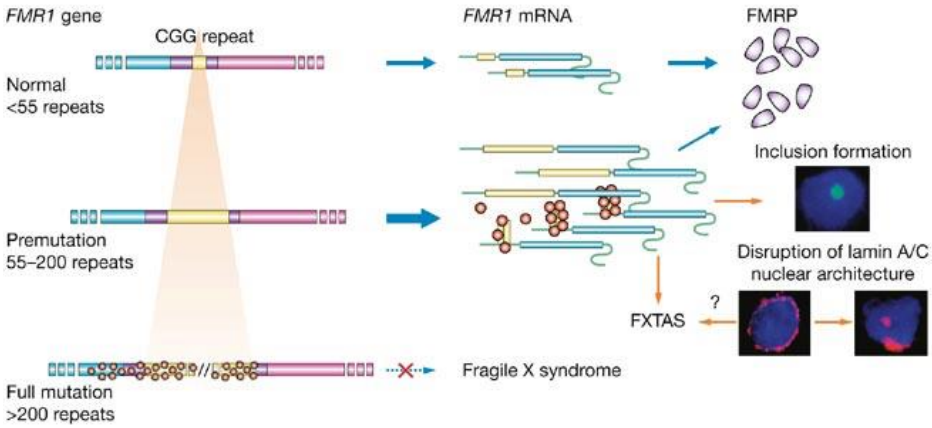


Figure 19. **Clinical and pathogenic consequences of expanded CGG repeat in the FMR1 gene**. The CGG repeat, in yellow, is located within the 5' untranslated portion of the gene. For full-mutation alleles (>200 CGG repeats), the promoter and CGG repeat region are usually methylated (red spots), which leads in turn to gene silencing. Absence of mRNA and fragile X mental retardation 1 protein (FMRP) gives rise to fragile X syndrome. By contrast, pre-mutation alleles (50-200 CGG repeats) are associated with substantial increases in fragile X mental retardation 1 gene (*FMR1*) mRNA. The excess mRNA itself results in inclusion formation through excess binding of a number of nuclear proteins and the clinical manifestations of fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman and Hagerman 2007).

Nowadays, numerous mouse models (table 6) are available reproducing some of the most important features of the syndrome. These genetic tools are of huge utility for studying the different behavior, cellular and molecular alterations present in the disease. Among them, the most used animal model for the FXS is the *Fmr1*

KO mice, obtained by interrupting the murine Fmr1.

Although the *Fmr1* KO mice is not representative of the CGG expansion, it keeps the loss of FMRP production (Bakker et al. 1994; Kooy 2003), recreating the same situation found in human patients. Some mouse models have also been created to study the fragile X-associated tremor/ataxia syndrome (Berman et al. 2014; Hagerman and Hagerman 2007).

Genetic approach	Mouse model	Modification	References
Knockout model	Fragile X knockout mice	*Fmr1* knockout	Bakker, 1994
		Fmr1 knockout 2	Mientjes et al, 2006
Paralogous genes	FXR1	FXR1 knockout	H.Siomi (pers. commun.)
	FXR2	FXR2 knockout	Bontekoe et al, 2002
Repeat expansion	Transgenic	$(CGG)_{60}$	Bontekoe et al, 1997
		$(CGG)_{43}$	Lavedan et al, 1997
		$(CGG)_{97}$	Lavedan et al, 1998
	Knock-in	$(CGG)_{98}$	Bontekoe et al, 2001
	Transgenic rescue	*FMR1* cDNA	C.E. Bakker (pers. commun.)
		FMR1 YAC	Peier et al, 2000

Table 6. Mouse models of the fragile X syndrome (Adapted from Kooy 2003 and Wijetunge et al. 2013). FXR1 and FXR2 = FMR1 autosomal homolog 1 and 2.

3.1.1 Physical and behavioral alterations in FXS

Fragile X syndrome patients present an important number of physical and behavioral alterations, although the clinical presentation of these alterations can vary considerably within patients.

In relation to the physical alterations, the patients suffering this syndrome present elongated faces with large prominent ears, prominent jaw, hyperextensible joints, macroorchidism and flat feet, among others (Kooy 2003; Mineur et al. 2002; Belmonte and Bourgeron 2006).

Regarding the behavioral abnormalities, some of the most commonly found alterations are anxiety-like behaviors with hypersensitivity to stimuli and concentration difficulties, delayed verbal development, repetitive behavior, socialization difficulties, low stress tolerance and automutilation due to a decreased nociceptive sensitivity (Kooy 2003; Fryns et al. 1984; Mineur et al. 2002). Moreover, around 10 to 20% of FXS patients have been reported to suffer epileptic seizures as FMRP loss seems to produce an increased neuronal excitability and, therefore, a higher susceptibility to epilepsy (Berry-Kravis 2002; Kluger et al. 1996; Musumeci et al. 1999). Autistic-like behaviors are also commonly observed in FXS patients. In fact, this syndrome is considered the most common monogenetic cause of autism (Schaefer and Mendelsohn 2008). However, the most prominent phenotype found in FXS patients is the intellectual disability, that present IQ values usually between 20 and 70, with problems mainly affecting working and STM, executive function and visuo-spatial abilities (de Esch et al. 2014; Penagarikano et al. 2007). These memory deficits can be a

consequence of the altered synaptic plasticity produced by the deficiency of the FMRP (O'Donnell and Warren 2002).

The *Fmr1* KO mouse reproduces some of the previously mentioned features mainly including the behavioral alterations but also presenting some physical abnormalities such as macroorchidism (Bakker et al. 1994; Slegtenhorst-Eegdeman et al. 1998). The behavioral phenotype of FXS has been largely studied using the different mouse models shown before (table 6). The *Fmr1* KO mice model is the most commonly used. This mouse model, as well as human patients, presents a higher susceptibility to suffer epileptic seizures than sane controls, elicited by auditory stimuli, in both males and females with higher intensity and frequency at around 21 days of age. However, it does not seem to present spontaneous seizures (Musumeci et al. 2000). Another phenotype reproduced in this mouse model is the decreased nociceptive sensitivity, demonstrating that FMRP may also play a crucial role in pain processing (Price et al. 2007). It is important to notice that this behavior is not related to a higher aggression tendency as when *Fmr1* KO mice were tested for this feature, no differences were observed compared to their WT littermates (Mineur et al. 2002). In the case of anxiety-like behaviors there is still some controversy. Some studies demonstrated that *Fmr1* KO models also present increased anxiety-like responses (Spencer et al. 2005) as observed in human patients, while others have demonstrated that *Fmr1* KO mice present decreased anxiety by the use of different behavioral tests (Peier et al. 2000).

The intellectual disability is one of the most important features present in FXS patients. In the *Fmr1* KO mice some mild cognitive deficits have been found in different spatial tasks, such as in the

Morris water maze task (D'Hooge et al. 1997) and in the radial arm maze task (Mineur et al. 2002), usually attributed to hippocampal defects (Kooy 2003). Moreover, memory deficits in the *Fmr1* KO mice have also been observed in a leverpress avoidance task (Brennan et al. 2006) and in the trace fear-conditioning test (Zhao et al. 2005). Finally, our research group demonstrated that this mouse model also presents an impaired novel object-recognition memory (Busquets-Garcia et al. 2013). In this thesis, we have used this cognitive task to explore the possible therapeutic effects of distinct CB1R antagonists at different doses. Our objective is to promote the amelioration of the intellectual deficit observed in the *Fmr1* KO mice, one of the most limiting features that characterize this syndrome.

3.1.2 Cellular and molecular alterations

Despite the anxiety, learning and memory problems present in FXS patients, it is interesting to note that post mortem studies demonstrated no pathological brain abnormalities in these patients, at least by routine neuroimaging and gross inspection during autopsy. In a similar way, no major brain anatomical differences have been recorded in the *Fmr1* KO mice (Kooy 2003; He and Portera-Cailliau 2013; Reyniers et al. 1999). However, microscopic neuropathological abnormalities in dendritic spine density and maturation have been later demonstrated in both human patients and *Fmr1* KO mice (Bakker et al. 1994; He and Portera-Cailliau 2013). These alterations in dendritic spines are closely related to the deficits in synaptic plasticity, which will finally be the cause for the intellectual disability described in the FXS. In fact, alterations in spine morphology and density have been reported in post-mortem neurons of patients with different intellectual disabilities (Kaufmann and Moser 2000). In general terms, the most commonly found alteration

associated to the loss of FMRP in both humans and mice is an increase of immature dendritic spines (Figure 20) also known as dendritic protusions or filopodia, usually accompanied by a lower proportion of mature mushroom spines (Figure 20) (Wijetunge et al. 2013). These alterations in dendritic spine structure and/or density have been described in several brain regions, such as the neocortex (Nimchinsky et al. 2001), the cerebellar Purkinje cells (Koekkoek et al. 2005) and most importantly the hippocampus, in a region specific manner, showing alterations in the CA1, but not in the CA3 region of this brain area (Levenga et al. 2011).

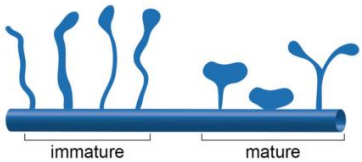


Figure 20. **Morphology of dendritic spines.** Graphical representation of dendritic spine morphologies defined as mature (mushroom, bifurcated) or immature (thin, stubby) (Modified from de Esch et al. 2014).

Studies using animal models have shown that possible differences regarding the alteration in the morphology and density of dendritic spines may appear depending on the genetic background and age of the animals, the brain region studied, the differences in experimental design and/or techniques or the cell type being examined (He and Portera-Cailliau 2013; Portera-Cailliau 2012).

In relation to the abnormalities in spine morphology and density, it has been observed in the *Fmr1* KO mice that the loss of FMRP produces alterations in synaptic plasticity consisting in enhanced Gq-coupled receptor-dependent LTD and impaired cortical LTP (Pfeiffer and Huber 2009).

Disturbances in synaptic transmission linked to altered spine morphology have also been related to altered signaling in the

excitatory metabotropic glutamate receptor 5 (mGluR5) pathway (Levenga et al. 2011). In fact, there is an uncontrolled activity of group I metabotropic glutamate receptors (mGluR1 and mGluR5), mainly mGluR5 in absence of FMRP (Bear et al. 2004; Michalon et al. 2012). This is confirmed by the fact that genetic reduction of mGluR5 expression (50%) is sufficient to normalize some features of the FXS in the *Fmr1* KO mouse model (Dölen et al. 2007). In normal conditions, glutamate stimulates mGluR to induce local mRNA translation, producing new protein synthesis and consequently the internalization of AMPA receptors, a phenomenon important for long-term synaptic plasticity (Levenga et al. 2010). However, the uncontrolled activity of mGluR5 that characterizes the FXS, in absence of FMRP, leads to an exaggerated AMPA internalization in *Fmr1* KO, weakening the synapse as well as to an excessive protein synthesis in brain areas such as the hippocampus and to an exaggerated LTD (Bear et al. 2004; Levenga et al. 2010; Osterweil et al. 2010; Sidorov et al. 2013). This theory was described in 2004, by Bear and colleagues, under the name of mGluR theory of the Fragile X syndrome (Figure 21) (Bear et al. 2004).

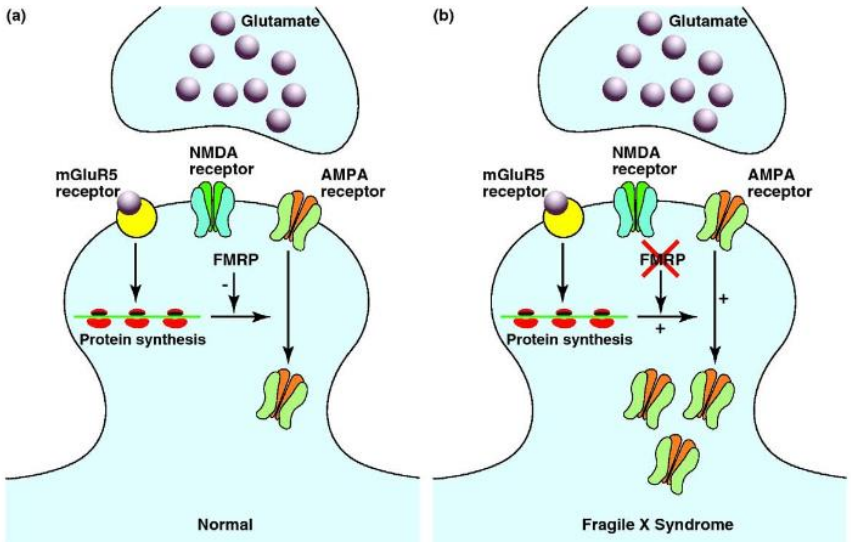


Figure 21. **The mGluR theory of fragile X syndrome**. a) Stimulation of mGluR5 by glutamate induces local mRNA translation in the synapse. Local protein synthesis stimulates the internalization of AMPA receptors, which is essential for long-term synaptic plasticity. FMRP negatively regulates transcription and reduces the internalization of AMPA receptors. b) Thanks to the extrapolation from studies in the *Fmr1* KO mice, it has been demonstrated that neurons from patients with FXS present increased internalization of AMPA receptors in the absence of FMRP, which weakens the excitatory synapse (Levenga et al. 2010).

It has also been proposed a possible alteration of the gamma-aminobutyric acid (GABA) receptor signaling in FXS. Briefly, different studies have demonstrated decreased mRNA and protein levels of several $GABA_AR$ subunits in the *Fmr1* KO mice as well as decreased mRNA expression of GAD67, the GABA synthesizing enzyme glutamate decarboxylase, all together leading to a reduced GABAergic transmission (Levenga et al. 2010; Paluszkiewicz et al. 2011; D'Hulst and Kooy 2009). These alterations in the GABAergic system have been described in several brain regions such as the

amygdala, the cerebral cortex, the hippocampus and the striatum, all of them relevant for the FXS phenotype (Paluszkiewicz et al. 2011).

Taken together, both the mGluR and the GABA theory suggest the presence of an excitatory/inhibitory unbalance due to an exaggerated excitatory mGluR signaling and a decreased GABA signaling that may be responsible for most of the features that characterize the FXS.

Many other mechanisms have been described that can be involved in the molecular and cellular alterations explaining the pathological features of the FXS. Among others:

- In the *Fmr1* KO mice, a clear overactivation of the mTOR signaling pathway has been described. This overexpression in the hippocampus will produce an aberrant synaptic protein synthesis and exaggerated protein synthesis-dependent mGluR LTD being a possible mechanism explaining the impaired cognition in FXS (Levenga et al. 2010; A. Sharma et al. 2010; Busquets-Garcia et al. 2013)
- The absence of FMRP could be responsible of some alterations in miRNA expressions that can contribute to the molecular pathology of the syndrome (Liu et al. 2015)
- The neuronal nitric oxide synthase 1 (NOS1) is severely diminished in the neocortex of human FXS patients producing a dysregulated nitric oxide signaling. As nitric oxide is involved in several neural processes, alterations at this level can contribute to the etiology of the disorder (Colvin and Kwan 2014).

3.2 Therapeutic targets in preclinical models

As it has been seen in the previous section, many alterations are present in the FXS that can be responsible, at least in part, for the phenotypes that define the syndrome. For this reason, many therapeutic targets have been described and studied for treating the alterations of FXS. A summary of the main therapeutic targets suggested for the treatment of FXS is presented in Figure 22.

Taking into account the previously exposed mGluR theory for FXS, the first therapeutic strategies trying to find a treatment were focused in the regulation of the uncontrolled activity of mGluR5. With this purpose, preclinical studies have been done using mGluR5 inhibitors such as MPEP, fenobam or CTEP, that demonstrated the amelioration of some fragile X phenotypes in several animal models (Michalon et al. 2012; Krueger and Bear 2011). Moreover, the genetic reduction of mGluR5 also rescues some phenotypes in the *Fmr1* KO mice (Dölen et al. 2007). Due to these promising results, the first clinical trial using a mGluR5 inhibitor, fenobam, was performed in 2009 (Berry-Kravis et al. 2009). Since then, many clinical trials have been or are being performed using different mGluR5 inhibitors such as AFQ056, which has been tested in phase II clinical trial in adolescents and adults (clinicaltrials.gov; Id: NCT01433354 and NCT01348087). Moreover, some studies have been performed targeting signaling pathways that are upstream or downstream of mGluRs (Krueger and Bear 2011). In that sense, PI3K is overactive in absence of FMRP and PI3K antagonists were able to normalize three FXS-associated phenotypes in *Fmr1* KO mice: dysregulated synaptic protein synthesis, excessive AMPA receptor internalization and increased spine density (Gross et al. 2010).

In the same direction, and as expected, the levels of ERK and MEK1/2 phosphorylation were significantly increased in both patient brain tissue and *Fmr1* KO brain tissue. As ERK is highly involved in synaptic plasticity, it has also been suggested as a possible therapeutic target for the treatment of some FXS phenotypes. The use of selective inhibitors of MEK1/2 in *Fmr1* KO (Figure 22) mice was able to abolish the audiogenic seizure activity (Wang et al. 2012).

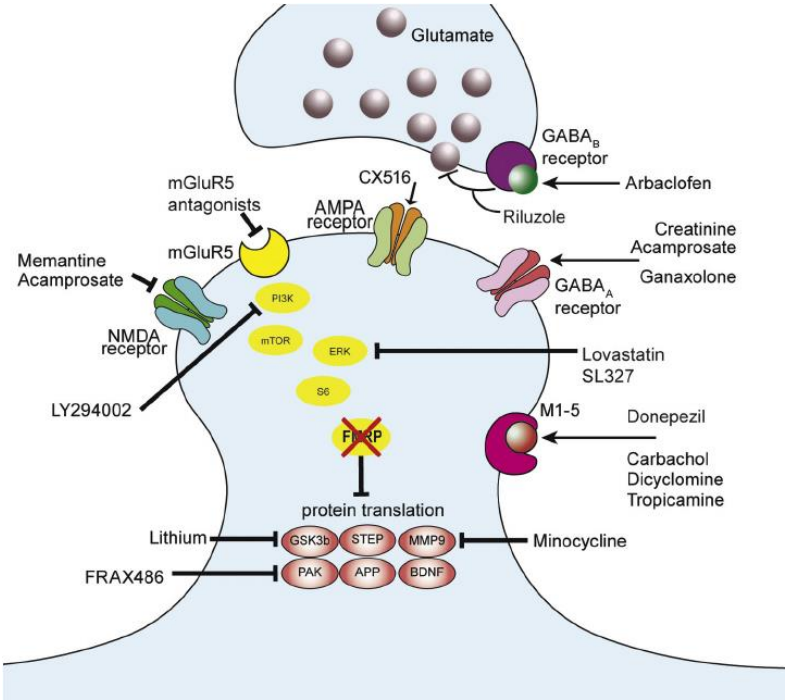


Figure 22. **Synaptic targets of the therapeutic interventions in fragile X syndrome**. Compounds acting on the glutamate receptors (NMDA, AMPA, mGluR5), downstream mGluRs, GABA receptors and muscarinic receptors (M1-5) are represented (de Esch, Zeidler, and Willemsen 2014).

Other target proteins of FMRP, such as the matrix metalloproteinase 9 (MMP-9) or GSK3b, have also been considered as possible therapeutic targets. Their inhibition by minocycline (Bilousova et al.

2009) or lithium (Liu and Smith 2014), respectively, have also demonstrated efficacy in normalizing some features associated with synaptic plasticity present in the *Fmr1* KO mice. A trial is under way to study the effects of minocycline, lovastatin (ERK inhibitor) or the combination of both. Patients are beingrecruited to start phase II (clinicaltrials.gov; Id: NCT02680379).

Interestingly, some studies have focused their attention on mTOR or its downstream effector p70 ribosomal s6 kinase 1 (S6K1). Briefly, the use of temsirolimus, a specific mTOR inhibitor, reversed the cognitive deficits observed in the *Fmr1* KO mice when tested in the NOR test (Busquets-Garcia et al. 2013), while two different S6K1 inhibitors reversed the excessive protein synthesis, the altered dendritic spine morphology and macroorchidism, among others (Bhattacharya et al. 2015).

Besides the alterations observed in mGluR5 and its downstream signaling pathways, it has also been discussed that FXS is characterized by a decreased GABAergic signaling and some therapeutic strategies have been also proposed in this direction (Wijetunge et al. 2013). The use of a $GABA_BR$ agonist corrected the elevated protein synthesis and reduced the deficits observed in social behavior in the *Fmr1* KO mice (Qin et al. 2015). $GABA_BR$ agonists have also been tested in some phase II clinical trials (Berry-Kravis et al. 2012; Veenstra-Vanderweele et al. 2016) showing promising results. However, results from phase III trials did not meet the primary outcome in relation to social avoidance (Berry-kravis et al. 2017). Some $GABA_AR$ have also been tested demonstrating beneficial effects by attenuating hyperactivity and rescuing audiogenic seizures in the *Fmr1* KO mice (Heulens et al. 2012; Olmos-Serrano et al. 2011). Phase II clinical trials are also under

way using a $GABA_AR$ agonist (ganaxolone) (clinicaltrials.gov; Id: NCT01725152).

The ECS has also been studied as a possible therapeutic target for the FXS as it will be discussed in the following section.

All these data indicate that FXS is a complex disorder in which several signaling pathways seem to present alterations. For that reason, it seems that the best form of treatment will consist on combining different drugs targeting the different pathways involved to ameliorate all the symptoms observed in this syndrome (de Esch et al. 2014).

3.2.1 Fragile X syndrome and the endocannabinoid system

A crosstalk between mGluR5 and CB1R has been described regulating several physiological functions indicating that both GPCRs are potential therapeutic targets (Olmo et al. 2016).

Opposite results have been found targeting the endocannabinoid system in the *Fmr1* KO mice (Busquets-Garcia et al. 2014). The pharmacological enhancement of 2-AG signaling, with the use of a specific irreversible MGL inhibitor (JZL184) produces the normalization of anomalous synaptic plasticity as well as the correction of some behavioral abnormalities including hyperlocomotion and reduced anxiety-like behaviors (Jung et al. 2012). In contrast, our research group has studied the effect of blocking the CB1R or the CB2R in the *Fmr1* KO mice. We concluded that CB1R blockade through pharmacological (rimonabant, 1mg/kg) or genetic (*Fmr1* KO mice presenting genetic attenuation of CB1R) approaches were able to normalize several phenotypes of the FXS including cognitive impairment, decreased nociceptive sensibility, susceptibility to audiogenic seizures, altered spine morphology and

overactivated mTOR signaling (Busquets-Garcia et al. 2013) (Figure 23).

Considering the previously exposed results, and taking into account that this thesis is mainly focusesd on the study of memory processes, one main objective of the thesis was to further clarify the interest of targeting the endocannabinoid system as a therapeutic strategy for the treatment of the cognitive deficits associated to the FXS.

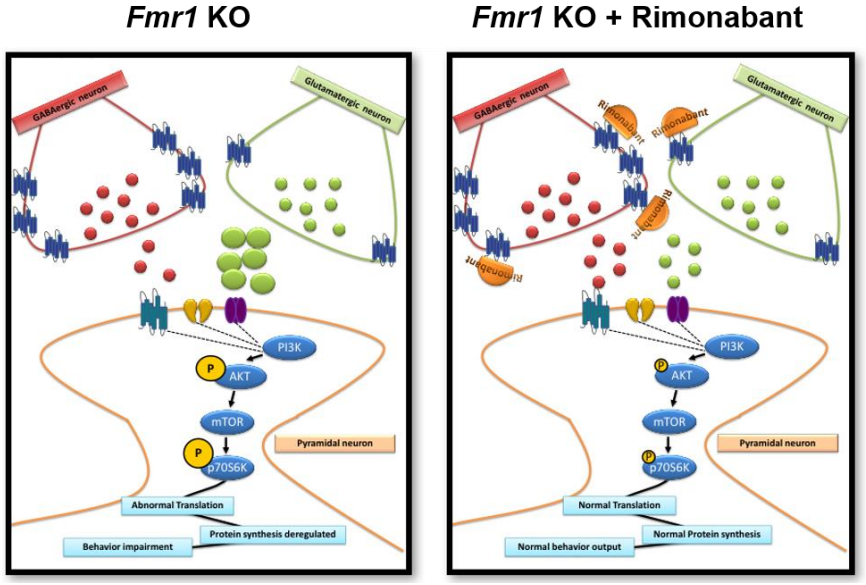


Figure 23. **Schematic diagram showing the possible therapeutic site of action of rimonabant in FXS.** In WT animals, CB1R are mainly localized in GABAergic terminals in the hippocampus and to a minor extent in the glutamatergic terminals, regulating neurotransmitters release. FMRP regulates the translation and synthesis of several proteins at the synaptic level that will contribute to the nomal behavioral output. In FXS conditions, the uncontrolled overactivity of mGluR5 and the reduced GABAergic transmission lead to the activation of different signaling pathways and the enhanced protein synthesis and synaptic plasticity. Rimonabant treatment or genetic reduction of CB1R, may contribute to the normalization of the excitatory/inhibitory balance in the hippocampus and, consequently, normalize the behavioral performance in the cognitive test (Modified from Busquets-Garcia et al. 2013).

4. Stress response

4.1 Definition of stress

Stress can be defined as the subjective state of sensing any stimulus that presents a challenge for the organism's homeostasis or suppose a threat to its well-being. The possible physical or psychological stimuli, called stressors, can be either an actual or a future potential disturbance/modification of the environment. The stress response produced by the organism to this new situation, including behavioral and physiological responses, will promote the adaptation to this new situation through the release of multiple molecules known as stress mediators (Akirav 2013; Joëls and Baram 2009; de Kloet et al. 2005; Ulrich-Lai and Herman 2009).

Many factors can influence the pattern and the magnitude of the stress response, in both humans and animals, including the duration of the stress exposure, the type of stress or the context, among others (Joëls and Baram 2009) (Figure 24).

Some authors have differentiated between "good stress" and "bad stress", considering the first as experiences of small duration that are easily overcomed by the subject while the second kind is referred to the prolonged experiences where the subject loses control and the situation becomes emotionally and physically exhausting (McEwen 2007). It is also important to notice that repeated or chronic life stress may induce or precipitate different mental illnesses such as depression, anxiety disorders or toxic substances abuse (Hillard 2008).

4.1.1 Stressors used in animal research

Stress, stress mediators and stress responses have been widely studied using animal models. In animal research we can divide stressors depending on their nature between physical stressors, psychosocial stressors, psychological stressors, physiological stressors and pharmacological stressors. Table 7 summarizes the main stressors used in animal models included in the previously presented classification of stressors.

Type of stressor	Examples	References
Physical stressors	Restraint stress	Keim and Sigg, 1975 Xu et al, 2017
	Immobilization	Elias and Redgate, 1975 Uwaya et al, 2016
	Footshock	Bali and Jaggi, 2015
Psychosocial stressors	Maternal separation	Banqueri, Méndez and Arias, 2017
	Social isolation	Haj-Mirzaian, 2017
	Social defeat	Huang et al, 2015
Psychological stressors	Tail-suspension	Heinrichs, 2010
	Forced-swimming	Morello et al, 2012
Physiological stressors	Continuous light 24h	Voiculescu et al, 2016
	Food restriction	Sedki et al, 2013
	Sleep-deprivation	Krishnan et al, 2016
Pharmacological stressors	Corticosterone injection	Stanic, 2017

Table 7. **Some examples of the most common types of stressors used in animal research.**

In this thesis, we have used one kind of physical stressor, also classified as an emotional stressor, the footshock. It presents the

advantage to be easy to apply and it allows to study at the same time emotional memory (the remembrance of the shock) and non-emotional memory when it is combined with the NOR test.

4.2 Physiology, function and pathways involved

As mentioned previously, stress responses require the release of different molecules that will transmit to the CNS the stress signal including noradrenaline, corticosteroids and dopamine, among others (Joëls and Baram 2009) (Figure 24).

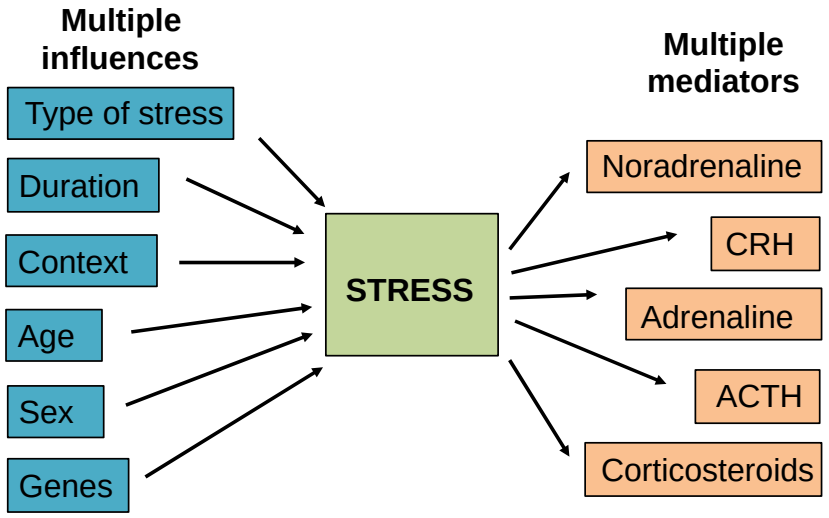


Figure 24. **Different stressors require different responses.** Many factors influence the pattern and magnitude of the response to stress, including the duration, the type or the context of the stress, the developmental stage of the animal and the animal's sex and background. Multiple stress mediators are involved in the stress response, so each combination of mediators addresses the specific aspects of a stressor. The molecules that transmit the stress signal to the CNS include monoamines, neuropeptides and steroid hormones (Modified from Joëls and Baram 2009).

In general terms, the acute exposure to a stressful situation will produce the activation of two biological systems highly conserved within vertebrates: the autonomic nervous system and the

hypothalamic-pituitary-adrenal (HPA) axis (Hill and Tasker 2012; Joëls et al. 2006) (Figure 25).

The autonomic nervous system provides the most immediate response to the stressful stimuli or in anticipation to stress (Ulrich-Lai and Herman 2009; Myers et al. 2012) by a rapid activation of the sympathetic nervous system, which represents the classical “fight or flight” response to stress (Cannon 1929). The activation of the sympathetic nervous system is responsible for the release of noradrenaline, from widely distributed synapses and adrenaline, primarily from the adrenal medulla (de Kloet et al. 2005; Schwabe et al. 2012). Moreover, the sympathetic activation will produce alterations in some physiological states producing the commonly known symptoms of stress, such as increased heart rate and blood pressure (Ulrich-Lai and Herman 2009). There is also a parasympathetic response to stress to control the duration of the autonomic response (Ulrich-Lai and Herman 2009).

The neuroendocrine response, consisting of the activation of the HPA axis, takes place some minutes after stress exposure and, in general terms, results in an increase of glucocorticoids circulation (mainly corticosterone in rodents and cortisol in humans) (Hill and Tasker 2012; Schwabe et al. 2012; Ulrich-Lai and Herman 2009). Activation of HPA axis involves a neuroendocrine cascade that starts with the activation of a small set of neurons located in the hypothalamic paraventricular nucleus (PVN) which release corticotropin releasing hormone (CRH) and, in some conditions, also vasopressin into the portal circulation (Herman et al. 2012; Herman et al. 2016). These hormones stimulate cells on the anterior pituitary to promote the secretion of adrenocorticotropic hormone (ACTH), which is released to the systemic circulation within minutes of

stimulation. ACTH will finally stimulate the synthesis and release of glucocorticoids in the cortex of the adrenal glands which are secreted to the blood and bind to high-affinitiy mineralocorticoid receptors (MR) or lower-affinity glucocorticoid receptors (GR) (Herman et al. 2016; Hill and Tasker 2012; Ulrich-Lai and Herman 2009). This increase in circulating glucocorticoids will directly act as a negative feedback on the HPA axis (Hill and Tasker 2012; Tasker and Herman 2011). In that sense, at the adrenal and pituitary levels, there is a negative feedback initiated by glucocorticoids that acts to suppress the release of CRH from the PVN, ACTH from the pituitary and glucocorticoids from the adrenal gland cortex to limit the release of HPA hormones (Herman et al. 2016; Hill and Tasker 2012; Myers et al. 2012). Moreover, circulating glucocorticoids will also contribute, indirectly, to the negative feedback of the HPA axis acting through upstream limbic structures such as the hippocampus, where MR and mainly GR are highly expressed and are required for inhibition of the HPA axis (Hill and Tasker 2012; Reul and De Kloet 1985; Tasker and Herman 2011). In fact, it has been demonstrated that hippocampal lesions produce a diminished feedback efficacy (Herman and Mueller 2006). Other brain regions indirectly involved in the negative feedback of the HPA axis include the medial prefrontal cortex and the amygdala (Myers et al. 2012).

There is also a delayed feedback mediated by the GR and the MR. Although MR has higher affinity for endogenous glucocorticoids, stress levels of glucocorticoids also bind to GR, densely expressed in the PVN, which is necessary for the inhibition of the HPA stress responses (Herman et al. 2012; de Kloet, Joëls, and Holsboer 2005; Myers, Mcklveen, and Herman 2012). Different factors modulating glucocorticoid response to stress have been studied including sex,

development and aging and corticosteroid-binding proteins (Herman and Mueller 2006).

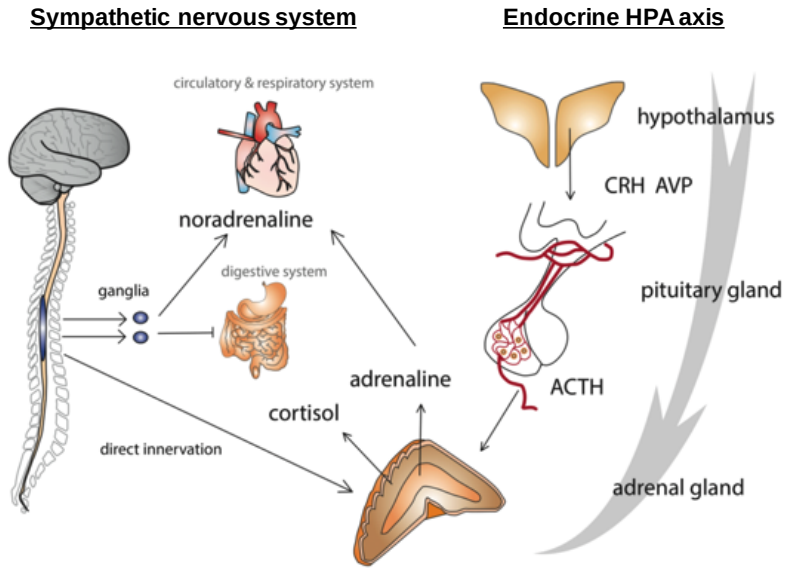


Figure 25. **HPA axis and autonomic nervous system responses to stress.** The sympatho-adrenomedullary and hypothalamic-pituitary-adrenocortical (HPA) axes are the primary systems for maintaining or reinstating homeostasis during stress. Stressor exposure results in activation of the sympathetic neurons representing the classical “fight or flight” response. It produces an increase of the circulating levels of adrenaline (primarily from the adrenal medulla) and noradrenaline (primarily from the sympathetic nerves). This results in an increase of the heart rate, force of contraction, peripheral vasoconstriction and energy mobilization. For the endocrine HPA axis, stressor exposure will produce the release of CRH and vasopressin. They act on the anterior pituitary to promote the secretion of ACTH that will act in the adrenal cortex to produce the synthesis and release of glucocorticoid hormones, which will provide a feedback signal to regulate HPA axis activity (Ulrich-Lai and Herman 2009; Myers et al. 2012; Even et al. 2012).

The balance between these two systems is very important as an excessive or inadequate autonomic or adrenocortical function can be dangerous for the subject’s health (McEwen 2007). Thus, abnormal elevation or decrease of glucocorticoids due to an altered

negative feedback efficacy can lead to multiple pathological conditions, such as neuropsychiatric disorders and metabolic dysregulation (Herman et al. 2012; Myers, Mcklveen, and Herman 2012). Similarly, dysregulation of the HPA axis is related to cardiovascular disorders (Myers et al. 2012).

Briefly, chronic exposure to stress can produce functional alterations of different brain regions that play a key role in the control of the autonomic and the HPA axis responses, including the hippocampus and the PVN (Ulrich-Lai and Herman 2009). When stress responses are inadequate, they can produce some pathological conditions, increasing the risk to suffer mental disorders such as post-traumatic stress disorder (de Kloet et al. 2005). Some aspects that must be taken into account when talking about the impact of chronic stress are the severity of the stressor, the modality and the extent to which the organism can predict the challenge (Herman et al. 2016).

4.3 Stress and the endocannabinoid system

The presence of a stressor will produce the activation of some pathways and the release of different hormones leading to the stress responses. In this sense, acute exposure to stress will evoke different endocrinal and behavioral responses in mice, including anhedonia or reduced exploration, among others.

Moreover, stress will also produce a physiological response of the ECS. In response to stress, the ECS acts as a buffer of the endocrinal and behavioral responses previously mentioned. It contributes to the decrease of the HPA axis activation and to the decrease of the behavioral responses. The relationships between

stress and the ECS are bidirectional (Hillard 2008; Patel et al. 2004) (Figure 26).

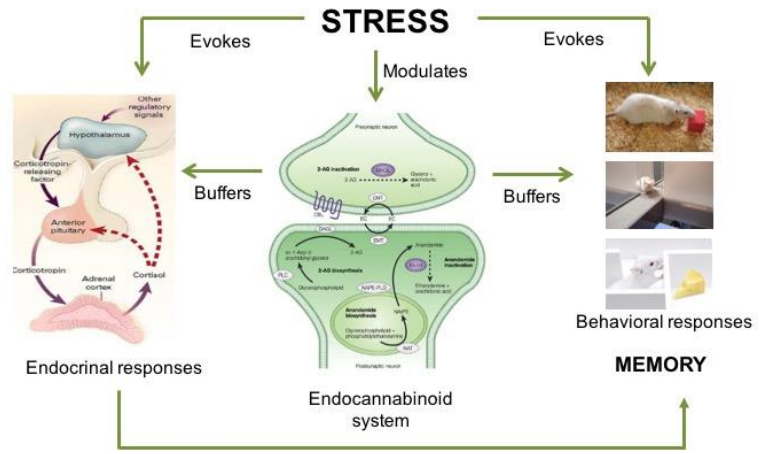


Figure 26. **Model of the interactions between the endocannabinoid signaling, stress and cognitive function.** Interactions between ECS and stress are bidirectional: the presence of a stressor produces the alteration of the ECS besides evoking endocrinal and behavioral responses. In parallel the ECS will buffer and contribute to these endocrinal and behavioral responses providing a negative feedback to the stress circuit (Modified from Hillard 2008).

Acute stress, produces distinct time-dependent changes in the two main endocannabinoids, AEA and 2-AG, leading to an alteration of the CB1R signaling (Lutz et al. 2015). In fact, there is a relation between the changes in the ECS, the HPA axis regulation and the glucocorticoid-mediated feedback (Figure 26).

In basal conditions without stress, there is an AEA tone in the basolateral amygdala that suppresses its own activity. After exposure to stress, a rapid decrease of the AEA content occurs (Figure 27a) in the BLA that will lead to the HPA axis activation and the secretion of the glucocorticoid hormone into circulation (Hill and

Tasker 2012; Hill et al. 2009; Lutz et al. 2015). Once glucocorticoids penetrate the brain, they will bind to membrane-associated receptors in the PVN and the amygdala where endocannabinoid synthesis is induced (Figure 27b) (Myers et al. 2012). This release of endocannabinoids will contribute to the fast-feedback inhibition of the HPA axis (Evanson et al. 2010; Hill and Tasker 2012). Moreover, glucocorticoids will also act in the prefrontal cortex (PFC) and the hippocampus producing a delayed increase in 2-AG content (Figure 27a and 27b), which will contribute to glucocorticoid-mediated negative feedback of the HPA axis (Hill and Tasker 2012; Lutz et al. 2015). The ECS plays a key role in the regulation of the HPA axis at different levels and during different phases of the stress response (Hill and Tasker 2012) and an inhibition of this system would produce psychopathological consequences (Roberts et al. 2014)

The relationships between the ECS and stress have also been studied by using CB1 KO mice and CB1 receptor antagonists. In this genetic or pharmacologic situations, there is a basal increase of the HPA axis, an elevated CRH expression in the PVN and increased plasma levels of ACTH and glucocorticoids, demonstrating that the ECS plays a role in the regulation of the stress response (Ginsberg et al. 2010; Myers et al. 2012; Patel et al. 2004).

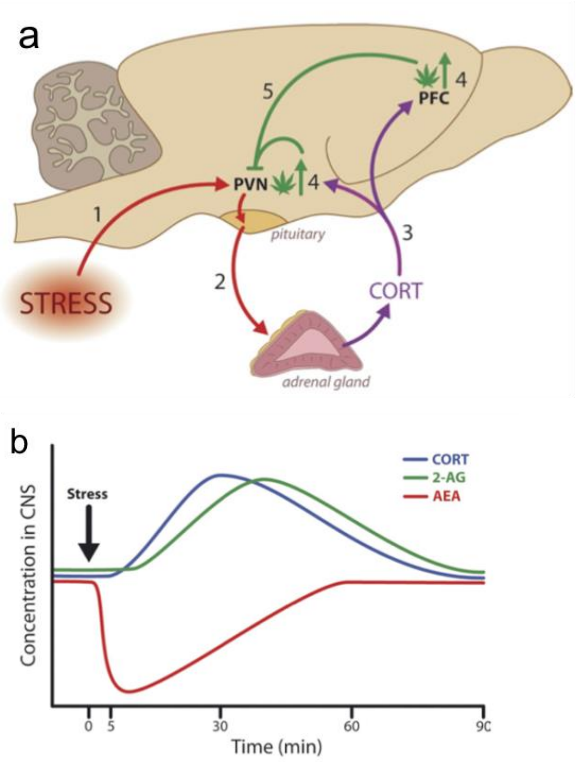


Figure 27. **AEA and 2-AG relationship with HPA axis activity.** Under basal conditions, AEA tonically suppresses HPA axis activity. In response to stress, AEA levels rapidly decline within the amygdala, disinhibiting HPA axis activity and resulting in an increase in glucocorticoid hormone secretion. Glucocorticoid hormones act to increase 2-AG production, which then acts to suppress HPA axis activity through the hypothalamus and prefrontal cortex. In addition, glucocorticoids also normalize AEA levels within the amygdala and thus, remove the disinhibition on the HPA axis and help return HPA function to basal levels (Hill and Tasker 2012) .

This effect is suggested to be centrally mediated as the intracerebroventricular administration of a CB1R antagonist activates the HPA axis (Manzanares et al. 1999).

Chronic stress produced by a single stressor can lead to an habituation process of HPA axis activation consisting in a decrease of the hormonal and the behavioral consequences of stress, a

process where the ECS is also involved (Hillard 2008; Lutz et al. 2015). Repeated exposure to stress will increase the activity of the ECS leading to an attenuation of the stress response (Hillard 2008; Lutz et al. 2015). However, there are some cases where chronic exposure to the stressor does not lead to habituation. In these circumstances, a down-regulation of the CB1R signaling has been described in several brain regions involved in emotional processing, such as the hippocampus (Hill et al. 2005) or the amygdala (Hill et al. 2013), which can help understand the human psychopathologies associated to stress (Lutz et al. 2015).

Taken into account the importance of the ECS in the regulation of the stress response, several reports support this system as a possible target in the treatment of anxiety like-behaviors in patients with post-traumatic stress disorders (Korem et al. 2016).

4.4 Stress, learning and memory

Stress modulates learning and memory processes. It is known that memory consolidation is sensitive to manipulation due to emotion-related events after acquisition (Roozendaal and McGaugh 2011). Different studies have demonstrated that emotions, such as those related to stressful events, can produce both enhancing and impairing effects on memory (Joëls et al. 2006; Kim and Diamond 2002; Sandi and Pinelo-Nava 2007; Schwabe et al. 2012). In contrast, other studies have shown that cognitive functions are not affected by stress (Warren et al. 1991). These differences in learning and memory modulation observed in both animal models and humans may depend on the different factors such as the source of stress (intrinsic vs. extrinsic), the stressor intensity, the stressor duration (acute vs. chronic stress), the different time courses of

stress hormones and the kind of memory studied (Roozendaal and McGaugh 2011; Finsterwald and Alberini 2014; Schwabe et al. 2012; Sandi and Pinelo-Nava 2007). Moreover, stress can differentially affect all memory phases including encoding, consolidation, retrieval, reconsolidation and extinction (Schwabe et al. 2012).

For the interest of this thesis, we are going to focus our attention in the study of how extrinsic stress affects memory consolidation of a non-emotional memory task. Extrinsic stress is defined as a stressful experience not related to the cognitive task at hand, that takes place before or after the learning period (Sandi and Pinelo-Nava 2007).

It has been typically believed that the emotional arousal associated to a stressful event produces an enhancement of memory consolidation while memory retrieval processes are impaired (Roozendaal 2002; LaLumiere et al. 2017). Nowadays, the available information regarding the effects of stress exposure on memory consolidation is not clear and opposite results can be found (Sandi and Pinelo-Nava 2007). Thus, exposure to a high intensity stressor after training using the eyeblink conditioning task in rats, does not influence the retention level (Beylin and Shors 1998). However, the exposure of rats to social isolation after being trained in the contextual fear-conditioning task impairs the retention levels. Strikingly, this same stressor had no effect when applied after the training in the auditory fear-conditioning (Rudy 1996). A most recent study showed that the post-training exposure to stress in a passive avoidance task produces a clear impairment of memory consolidation in rats (Sardari et al. 2015).

Effects of stress have also been widely studied in humans. Memory consolidation of a list of words (considered a non-emotional memory) was studied in participants who were exposed to cold pressor

stimulation (stressor). In contrast to what authors expected, exposure to stress immediately after learning produce an impairment of the long-term memory consolidation (Tramell and Clore 2014).

Most of the research done in this topic has studied the effects of stress over emotional memories (using task as the fear-conditioning, passive avoidance or eyeblink condition) while the effects of stressful events over non-emotional memories are poorly understood. In this thesis, we have studied how a stressful stimulus can affect a non-emotional and hippocampal-dependent memory, and the possible involvement of the endocannabinoid system in its effect.

5. Protein Kinase C signaling

Protein kinase C (PKC) is a family of serine/threonine kinases that are involved in many physiological functions including cell growth and proliferation, differentiation, immune responses, apoptosis, angiogenesis and processes of learning and memory, among others (Mackay and Twelves 2007; Newton 1995)

5.1 Classification and isoforms

Yasutomi Nishizuka is considered the father of the PKC family initially described in the late 1970s (Nakamura and Yamamura 2010). The PKC family consists of at least 11 different isoforms presenting differences in their subcellular localization, tissue distribution, structure, the way in which they are activated and their substrate specificity (Mackay and Twelves 2007). They can be divided into three subgroups depending on two of the previously mentioned features, their structure and the way they are activated. The conventional group (also known as classical group) or calcium-dependent, are activated by both calcium and diacylglycerol (DAG), is the most well characterized and includes the isoforms α, βI, βII and γ. The second group are the novel or calcium-independent PKCs, that are activated only by DAG and include the isoforms δ, ε, η, θ and μ. Finally, the atypical group, which is the less understood and does not require neither calcium nor DAG to be activated, but it is sensitive to phospholipids. The isoforms ζ and λ (ι in humans) are included in this group (Newton 1995; Newton 2010).

All PKCs share a similar structure composed by a C-terminal catalytic domain (approximately 45 KDa) conserved within all

isoforms coupled to a N-terminal regulatory domain (approximately 20-40 KDa), which differs between the different subgroups (Figure 28) (Freeley et al. 2011; Newton 1995). Four domains composed the PKC structure (Coussens et al. 1986) (Figure 28):

- C1: located in the N-terminus, contains a diacylglicerol/phorbol ester binding site thanks to the presence of a Cys-rich motif.
- C2: also located in the N-terminus, contains a recognition site for acid lipids and, in some enzymes, the calcium-binding site.
- C3 and C4: conform the catalytic domain and contain the ATP- and the substrates-binding sites, respectively.

There is also a pseudosubstrate peptide sequence in the regulatory domain that is released when PKCs are activated, allowing the subsequent binding and phosphorylation of downstream substrates (Gould and Newton 2008).

Conventional PKCs contain a putative calcium-binding site in the C2 domain that although being structurally similar in the novel PKCs, in this second subgroup does have the functional group involved in the calcium binding. In the atypical PKCs, the key residues in charge of the C2 folding are not present. Another difference within subgroups is that conventional and novel PKCs contain two Cys-rich motifs in the C1 domain, whereas atypical PKCs only contains one, making them unable to respond to DAG (Newton 1995; Gould and Newton 2008).

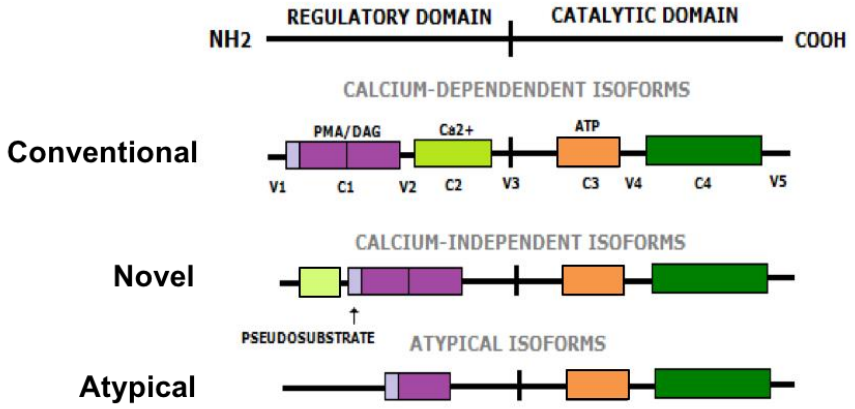


Figure 28. **Schematic representation of the primary structure of conventional, novel and atypical protein kinase C.** Indicated are the pseudosubstrate domain (light purple), C1 domain comprising one or two Cys-rich motifs (dark purple), C2 domain (light green) in the regulatory half, and the ATP-binding loop (C3; orange) and substrate-binding loop (C4; dark green) of the catalytic region (Modified from Freeley et al. 2011).

In the C-terminus, there are three conserved phosphorylation sites important for the PKC function: the activation-loop (A-loop), the turn-motif (TM) and the hydrophobic-motif (HM), all of them involved in controlling the PKC catalytic activity, stability and intracellular localization. These phosphorylation sites are also conserved in other kinases (Freeley et al. 2011).

5.2 PKC life cycle

In general terms, the PKC function and activation is regulated by two mechanisms, both of them necessary and highly important (Newton 2003). However, the sustained activation of PKC will finally lead to its downregulation and to the termination of its life cycle, a process less understood than its activation (Gould and Newton 2008).

The activation of PKCs requires the action of second messengers as well as the translocation from the cytosol to the cell membrane, which requires specific anchoring proteins. This response of PKC to second messengers requires its previous maturation that involves the phosphorylation of the three sites previously mentioned, the A-loop, the TM and the HM (Freeley et al. 2011). Briefly, the steps that take place in the maturation of the PKCs are the following (Figure 29):

- Phosphorylation of the A-loop (Thr500 in PKC beta II) by the upstream kinase phosphoinositide-dependent kinase 1 (PDK1), also responsible for the phosphorylation of other kinases. This step is critical for the correct maturation of the PKC as unphosphorylated or dephosphorylated forms of PKC are rapidly degraded (Balendran et al. 2000; Gould and Newton 2008; Newton 2003).
- Once the A-loop is phosphorylated, the enzyme suffers a rapid phosphorylation at the TM (Thr641 in PKC beta II), which is required for the maintenance of the catalytic competence of the enzyme (Gould and Newton 2008). The mammalian target of rapamycin complex 2 (mTORC2) plays here an important role as it is required for the phosphorylation of the PKC TM, a function that is conserved from yeast to mammals (Facchinetti et al. 2008; Ikenoue et al. 2008).
- The last step in PKC maturation requires the autophosphorylation at the HM (Ser660 in PKC beta II). Although this autophosphorylation is not functionally necessary, it affects the subcellular localization and stability of PKC. Again, mTORC2 is involved in the phosphorylation of the HM (Ikenoue et al. 2008; Gould and Newton 2008).

In the conventional PKCs, which will be the subgroup of main interest in this thesis, the phosphorylation at the TM and the HM is

constitutive, but it can also take place in response to agonist-evoked signaling (Gould and Newton 2008; Antal and Newton 2014). Moreover, PKC members from all subgroups can also be activated through a tyrosine phosphorylation, as an additional mechanism to regulate PKC activity (Konishi et al. 1997).

At this point, PKCs are processed but still inactive at the cytosol. As mentioned in the previous section, conventional PKCs are activated by two second messengers, calcium and DAG, both of them produced from the phosphatidylinositol 4,5-biphosphate (PIP_2) hydrolysis and that will be responsible for the initiation of the membrane translocation and activation of PKC. Briefly, calcium binds the C2 domain (pretargeting PKC to the plasma membrane) and then DAG (and also phorbol esters) bind the C1 domain promoting the pseudosubstrate to be released and allowing the open conformation of PKC. In the novel PKCs, a C1 domain with higher affinity to DAG than the one of conventional PKCs, compensates the lack of a calcium-binding C2 domain. It will also take place the translocation to the cell membrane (or other cellular membranes), which occurs through a specific kind of proteins, the receptors for activated C-kinase (RACKS). The interaction takes place in the C2 domain and determines the proper location of the PKCs for their cellular function (Gould and Newton 2008; Callender and Newton 2017; Antal and Newton 2014).

Once activated, PKCs will be able to phosphorylate its large variety of substrates and initiate their downstream signaling cascades. The downstream events following PKC activation involve multiple pathways, such as mitogen-activated protein kinases (MAPKs) including ERK, p38 and JNK, the PI3K-Akt pathway and the calcium-calmodulin-dependent protein kinase II (CamKII) signaling pathway

(Gould and Newton 2008; Mackay and Twelves 2007; Sun and Alkon 2014).

As mentioned previously, the sustained activation of PKC will finally lead to its downregulation and termination of its life cycle. The active and therefore open conformation of PKC makes it sensitive to phosphatases and, in consequence, sensitive to dephosphorylation. In this regard, the PH domain leucine-rich repeat protein phosphatase (PHLPP) is responsible for the HM dephosphorylation and plays an important role in the regulation of PKC levels and stability. PKC dephosphorylation makes them unstable, targeted for ubiquitination and prone to degradation. However, once dephosphorylated, the molecular chaperone HSP70 can bind to the TM promoting a rephosphorylation and stabilization of PKC, which will be again active and competent (Newton 2010; Gould and Newton 2008; Callender and Newton 2017).

Perturbations or modifications in any of the previously described processes (phosphorylation states, localization, and others) can disrupt the signalling events derivate from PKC activation leading to altered physiological states found in different diseases such as metabolic and cardiovascular disorders and CNS disorders, among others (Gould and Newton 2008).

A summary of the PKC life cycle previously described is represented in Figure 29.

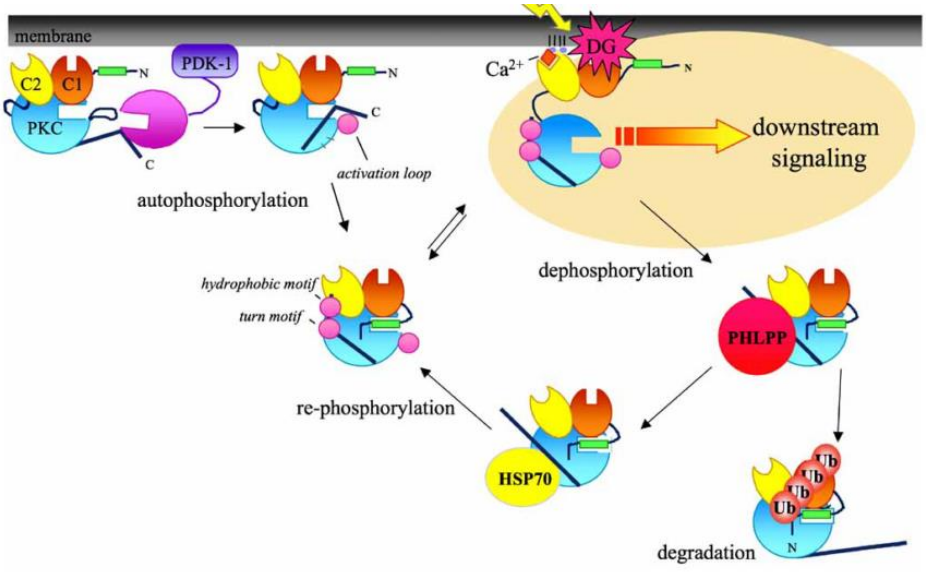


Figure 29. **Model showing the life cycle of PKC, from biosynthesis to degradation.** PDK1 phosphorylates the activation-loop and is released from the C-terminus. When it is free, PKC can autophosphorylate the TM and HM with the participation of mTORC2. Once PKC has been processed and maturated, it is released into the cytosol and maintained in an inactive conformation with the pseudosubstrate lodged into the substrate-binding cavity. The generation and action of the second messengers, calcium and DAG (expressed as DG in the diagram), cause the translocation of PKC to the membrane and provides the energy to release the pseudosubstrate from the active site, allowing the downstream signaling. In this open, active conformation PKC is susceptible to dephosphorylation by phosphatases like PHLPP and then targeted for ubiquitination and degradation. However, molecular chaperones like HSP70 can rephosphorylate the TM and stabilize PKC, allowing it to re-enter the pool of signaling-competent enzymes (Modified from Gould and Newton 2008).

5.3 PKC and memory

PKCs seem to play a crucial role in the regulation of synaptic plasticity, specifically, PKC activation facilitates LTP. Due to the known relation between synaptic plasticity and memory it seems that PKC also plays an important role in many types of learning and memory processes, such as acquisition and maintenance (Sun and Alkon 2014).

As reported in the literature, many types of memory and learning processes can be related with the different PKC isoforms (Sun and Alkon 2014) in both a positive or a negative way. Thus, working memory in rodents is improved by the administration of a calcium-sensitive PKC isoforms inhibitor (Dash et al. 2007). However, contextual memory consolidation (long-term contextual fear-conditioning task) is negatively affected in rats when a PKC inhibitor is infused in the dorsal hippocampus demonstrating the existence of a critical time window (Wallenstein et al. 2002). Similar results were reported when studying acquisition and consolidation in a spatial memory task (Bonini et al. 2007). Many other learning tasks have also been related with PKC. Moreover, deficits in the PKC signalling cascade have been described as one of the earliest abnormalities in the brains of patients suffering from Alzheimer's disease (Alkon, Sun, and Nelson 2007; Sun and Alkon 2014).

This PKC-related role in the maintenance, consolidation and storage of memory seems to be produced by an isoform known as PKM zeta (PKMζ) (Giese and Mizuno 2015). It is an autonomous and active fragment of the PKC zeta isoform that lacks the regulatory domain and remains active until it is degraded (Glanzman 2013; Furini et al. 2013). The pharmacological inhibition or the suppression on the

production of PKMζ prevents the formation of long-term memories and can erase well-established memories (Glanzman 2013; Morris 2016). On the other hand, overexpression of PKMζ has demonstrated to enhance memory (Shema et al. 2011).

Due to the importance of the PKC signaling pathway in the formation and regulation of memory, we have studied in this thesis the role of this signaling pathway in the deleterious effects produced by THC on short-term memory consolidation.

5.4 PKC gamma isoform

5.4.1 PKC gamma expression and function

As mentioned previously, the PKC gamma isoform belongs to the conventional or classical group. In consequence, it has C1 and C2 domains, which bind DAG and calcium respectively to promote its activation (Saito and Yasuhito 2002). The structure of the C2 domain within the different conventional isoforms presents low homology and apparently, PKC gamma presents higher affinity to calcium than other isoforms (Kohout et al. 2002).

PKC gamma isoenzyme can be found, within the CNS, primarily in the dendrites and cell body of neurons (Abeliovich et al. 1993a; Saito and Yasuhito 2002) presenting an intracellular localization that differs from those of other conventional PKCs (Saito and Yasuhito 2002). In the brain, it is highly expressed in some regions such as the cortex, the amygdala, the hippocampus (in the pyramidal cells) and the cerebellum (in the Purkinje cells) (Figure 30) (Saito et al. 1988; Tanaka and Saito 1992).

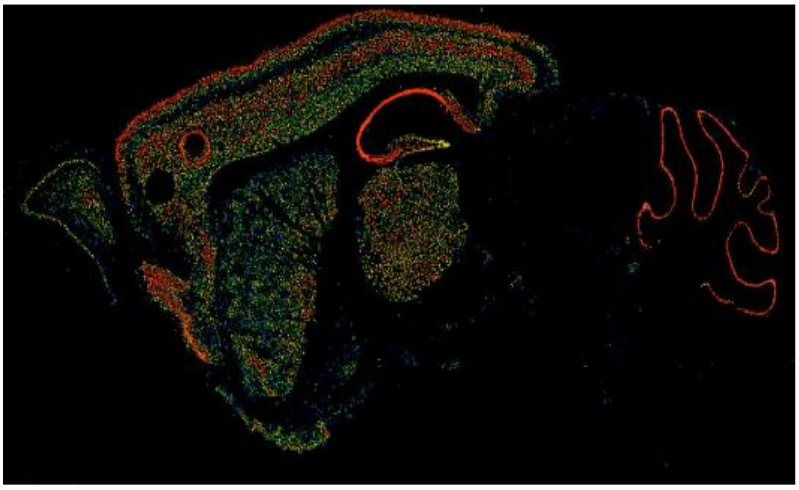


Figure 30. **PKC gamma isoform is highly expressed in the brain, mainly in the cortex, the hippocampus and the cerebellum** (Allen Brain Atlas).

This isoform has been reported to phosphorylate the n-methyl-D-aspartate receptor (NMDAR1) subunit preferentially at residue Ser890, but also at residue Ser896, which plays a role in synaptic plasticity, synaptogenesis, excitotoxicity, memory acquisition and learning. This is in contrast to other conventional PKCs such as PKC alpha that phosphorylates preferentially NMDAR1 subunit at residue Ser896 (Sánchez-Pérez and Felipo 2005). Moreover, activation of mGluRs activates PKC gamma, but not other classical isoforms in cultured cerebellar neurons (Ramakers et al. 1999).

Mice deficient in PKC gamma (PKC gamma KO mice) were generated in 1993 by embrionic stem cell gene targeting technique (Abeliovich et al. 1993a) and it is an excellent tool for the study of its function and its involvement in different physiological processes (Abeliovich et al. 1993a; Saito and Yasuhito 2002). PKC gamma KO mice are viable, present an apparently normal behavior and normal development and their brain anatomy and synaptic transmission are

apparently not altered (Abeliovich et al. 1993a; Saito and Yasuhito 2002).

Among the features that characterize mice lacking the PKC gamma isoform, it has been described that they exhibit decreased anxiety levels (Bowers et al. 2000) and reduced neuropathic pain after partial sciatic nerve section (Malmberg et al. 1997). Moreover, PKC gamma mutant mice present impaired motor coordination in the rotarod test and mild ataxic gait (Chen et al. 1995).

Finally, the possible involvement of PKC gamma in the modulation of learning and memory processes is poorly understood. Some studies have shown that PKC gamma KO mice correctly learn to carry out different hippocampus-dependent tasks although some mild to moderate deficits in the performance of a context-dependent fear-conditioning task are reported (Abeliovich et al. 1993b). Moreover, PKC gamma KO mice also present diminished hippocampal LTP, although other forms of synaptic plasticity are normal, suggesting that PKC gamma may be a regulatory component of LTP (Abeliovich et al. 1993a).

One objective of this thesis was to further investigate the possible involvement of the PKC gamma isoform in the formation of short-term and long-term memories.

5.4.2 PKC gamma preferential substrates

The activation of the PKC gamma isoform, produces the phosphorylation of three preferential substrates: neurogranin (also known as release candidate 3, RC3), myristoylated alanine-rich C kinase substrate (MARCKS) and neuromodulin (also known as growth associated protein-43, GAP-43) (Ramakers et al. 1999).

Neurogranin (RC3) is a neuron specific and postsynaptic small protein. It is abundantly expressed in brain regions involved in cognitive functions, such as the cerebral cortex, the amygdala and the hippocampus where it is mainly concentrated in dendritic spines (Díez-Guerra 2010; Domínguez-González et al. 2007). It is implicated in synaptic plasticity through the regulation of calcium/calmodulin signaling, since neurogranin is the most abundant calmodulin-binding protein in the brain and therefore modulates calmodulin (CaM) availability (Díez-Guerra 2010). Thus, neurogranin KO mice present severe deficits in LTP and in the performance of hippocampal-dependent tasks (Huang et al. 2004). In the postsynaptic environment of resting synapses neurogranin would be bound to calmodulin (Huang et al. 2004; Domínguez-González et al. 2007; Zhong and Gerges 2012). However, modifications such as an increase of the calcium concentration or the phosphorylation by PKC gamma at Ser36 IQ motif (Kumar et al. 2013) would reduce the binding affinity between neurogranin and calmodulin (Díez-Guerra 2010). Then, calmodulin binds to calcium and active calcium-calmodulin-dependent protein kinase II (CamKII) signaling cascades, relevantly involved in learning and memory (Gaertner et al. 2004; Fukunaga and Miyamoto 2000) (Figure 31).

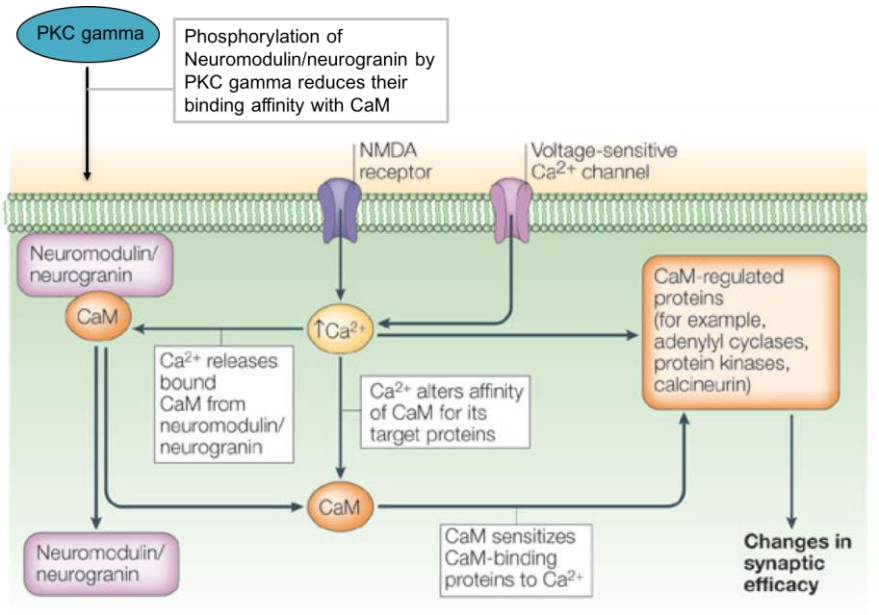


Figure 31. **Neurogranin and neuromodulin, two preferential substrates of PKC gamma, regulate the calcium/calmodulin signaling, an important process for synaptic plasticity**. Increases in intracellular calcium, generated through the activity of NMDA receptors or voltage-sensitive calcium channels or the phosphorylation of neurogranin/neuromodulin by PKC gamma reduce the binding affinity between these proteins and CaM. Then, CaM binds to calcium to stimulate several enzymes that are required for changes in synaptic plasticity (Modified from Xia and Storm 2005).

MARCKS is a postsynaptic protein highly enriched in the brain that acts as an important regulator of the cell shape by binding actin to the cell membrane, as well as a key factor in the maintenance of the dendritic spines and of calcium-dependent changes in the cortical actin cytoskeleton (Calabrese and Halpain 2005; Callender and Newton 2017). When MARCKS is phosphorylated, mainly by the PKC gamma activity, but also by other conventional and novel PKCs, the interaction between this protein and the membrane is prevented, resulting in the shrinkage and collapse of the dendritic spines

(Calabrese and Halpain 2005; Hartwig et al. 1992) (Figure 32). This protein is essential for the brain development and postnatal survival, as it has been demonstrated in mice with MARCKS depletion (Stumpo et al. 1995).

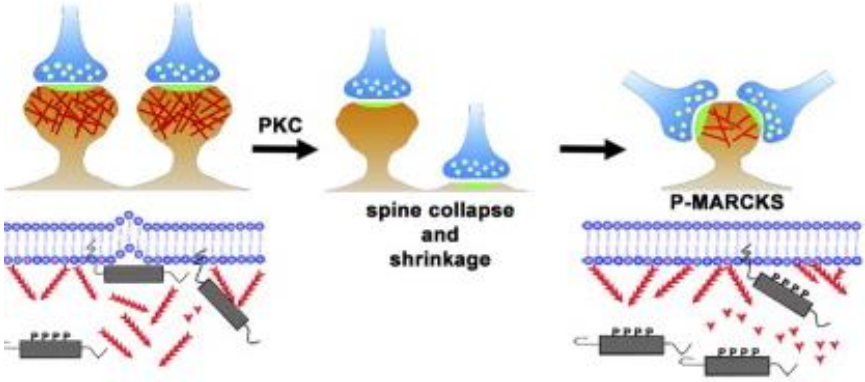
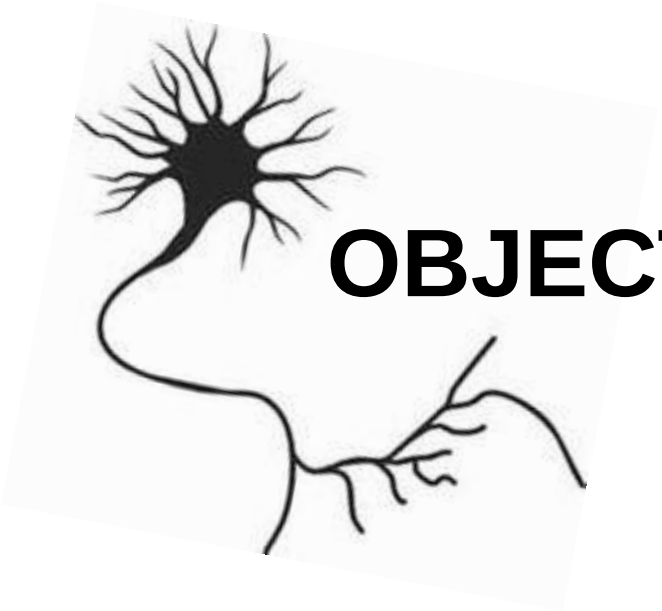


Figure 32. **A model for the function of MARCKS in dendritic spine morphology when it is phosphorylated by PKC.** Under resting conditions, MARCKS (shown in grey) exists in equilibrium between dephosphorylated and phosphorylated states. When PKC becomes activated through neuronal activity, the balanced is shifted towards the phosphorylated state of MARCKS. Release of membrane bound MARCKS stimulates a net decrease in F-actin content (shown in red), but an increase in the tethering of the cytoskeleton to the plasma membrane. As a result, spines shrink in size or collapse altogether; presynaptic terminals reorganize onto the remaining spines, which also show less morphing (Modified from Calabrese and Halpain 2005).

Neuromodulin (GAP-43) is a presynaptic protein, which plays a key role in guiding the growth state of axon terminals and in modulating the formation of new synaptic connections. Consequently, its suppression has adverse effects on axon outgrowth. As neurogranin, neuromodulin sequesters calmodulin in the absence of calcium modulating calcium/calmodulin signaling at the presynaptic terminal (Biewenga et al. 1996; Benowitz and Routtenberg 1997; Holahan and Routtenberg 2008; Larsson 2006) (Figure 31).



OBJECTIVES

Objective 1

To investigate the endocannabinoid system as a potential therapeutic target in the treatment of the cognitive impairment that characterize the fragile X syndrome.

Article #1

Possible therapeutic doses of cannabinoid type 1 receptor antagonist reverses key alterations in fragile X syndrome mouse model

Maria Gomis-González, Arnau Busquets-Garcia, Carlos Matute, Rafael Maldonado, Susana Mato, Andrés Ozaita

Genes. 7(9): E56 (2016)

Objective 2

To study the involvement of the central and the peripheral endocannabinoid system in the modulation of the non-emotional memory impairment produced by stressful stimuli.

Article #2

Peripheral and central CB1 cannabinoid receptors control stress-induced impairment of memory consolidation

Arnau Busquets-Garcia*, Maria Gomis-González*, Raj Kamal Srivastava*, Laura Cutando, Antonio Ortega-Álvaro, Sabine Ruehle, Floortje Remmers, Laura Bindila, Luigi Bellocchio, Giovanni Marsicano, Beat Lutz, Rafael Maldonado, Andrés Ozaita

Proc Natl Acad Sci. 113(35): 9904-9 (2016)

** Equal contribution*

Objective 3

To describe the signaling pathways involved in the short-term memory deficits produced by THC and determine the similarities and differences between the previously described effects on long-term memory

Article #3

Hippocampal protein kinase C signaling mediates the short-term memory impairment induced by delta9-tetrahydrocannabinol

Arnau Busquets-Garcia*, Maria Gomis-González*, Victòria Salgado-Mendialdúa, Lorena Galera-López, Emma Puighermanal, Rafael Maldonado, Andrés Ozaita.

Neuropsychopharmacology (2017)

*Equal contribution

Objective 4

To elucidate the significance of PKC gamma signaling in memory processes by using a mouse model lacking this specific PKC isoform and studying the effect of THC.

4a) Protein kinase C gamma is involved in short-term but not long-term memory performance

Maria Gomis-González, Arnau Busquets-Garcia, Emma Puighermanal, Rafael Maldonado, Andrés Ozaita

4b) Effect of low doses of THC in the PKC gamma mouse

Maria Gomis-González, Arnau Busquets-Garcia, Rafael Maldonado, Andrés Ozaita

RESULTS

OBJECTIVE 1

To investigate the endocannabinoid system as a potential therapeutic target in the treatment of the cognitive impairment that characterize the fragile X syndrome

Article #1

Possible therapeutic doses of cannabinoid type 1 receptor antagonist reverses key alterations in fragile X syndrome mouse model

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Gomis-González M, Busquets-Garcia A, Matute C, Maldonado R, Mato S, Ozaita A. Possible therapeutic doses of cannabinoid type 1 receptor antagonist reverses key alterations in fragile X syndrome mouse model. *Genes (Basel)*. 2016 Aug 31; 7(9). DOI: 10.3390/genes7090056

OBJECTIVE 2

To study the involvement of the central and the peripheral endocannabinoid system in the modulation of the non-emotional memory impairment produced by stressful stimuli

Article #2

Peripheral and central CB1 cannabinoid receptors control stress-induced impairment of memory consolidation

Arnau Busquets-Garcia*, Maria Gomis-González*, Raj Kamal Srivastava*, Laura Cutando, Antonio Ortega-Álvaro, Sabine Ruehle, Floortje Remmers, Laura Bindila, Luigi Bellocchio, Giovanni Marsicano, Beat Lutz, Rafael Maldonado, Andrés Ozaita

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Busquets-Garcia A*, Gomis-González M*, Srivastava RK*, Cutando L, Ortega-Alvaro A, Ruehle S et al. Peripheral and central CB1 cannabinoid receptors control stress-induced impairment of memory consolidation. *Proc Natl Acad Sci U S A.* 2016 Aug 30; 113(35): 9904-9. DOI: 10.1073/pnas.1525066113

OBJECTIVE 3

To describe the signaling pathways involved in the short-term memory deficits produced by THC and determine the similarities and differences between the previously described effects on long-term memory

Article #3

Hippocampal protein kinase C signaling mediates the short-term memory impairment induced by delta9-tetrahydrocannabinol

Arnau Busquets-Garcia*, Maria Gomis-González*, Victòria Salgado-Mendialdúa, Lorena Galera-López, Emma Puighermanal, Elena Martín-García Rafael Maldonado, Andrés Ozaita

Neuropsychopharmacology (2017)

* *Equal contribution*

Busquets-Garcia A*, Gomis-González M*, Salgado-Mendialdúa V, Galera-López L, Puighermanal E, Martín-García E, Maldonado R, Ozaita A. Hippocampal protein kinase C signaling mediates the short-term memory impairment induced by delta9-tetrahydrocannabinol. *Neuropsychopharmacology* accepted article preview 17 August 2017. DOI: 10.1038/npp.2017.175

OBJECTIVE 4

To elucidate the significance of PKC gamma signaling in memory processes by using a mouse model lacking this specific PKC isoform, and studying the effects of THC

Supplementary results

a) Protein kinase C gamma is involved in short-term but not long-term memory performance

Maria Gomis-González, Arnau Busquets-Garcia, Emma Puighermanal, Rafael Maldonado, Andrés Ozaita

b) Effect of low doses of THC in the memory performance of PKC gamma KO mice

Maria Gomis-González, Arnau Busquets-Garcia, Rafael Maldonado, Andrés Ozaita

a) Protein kinase C gamma is involved in short-term but not long-term memory performance

Short-term non-emotional memory performance is impaired in the PKC gamma KO mice

Non-emotional memory performance was evaluated in the PKC gamma KO mice compared to wild-type (WT) controls in two memory tasks: the novel object-recognition (NOR) test (Figure 33a) and the novel place-recognition (NPR) test (Figure 33b). In both cases short-term memory (STM) performance was impaired in the PKC gamma KO mice while no deficits were observed when long-term memory (LTM) was assayed in a different set of mice.

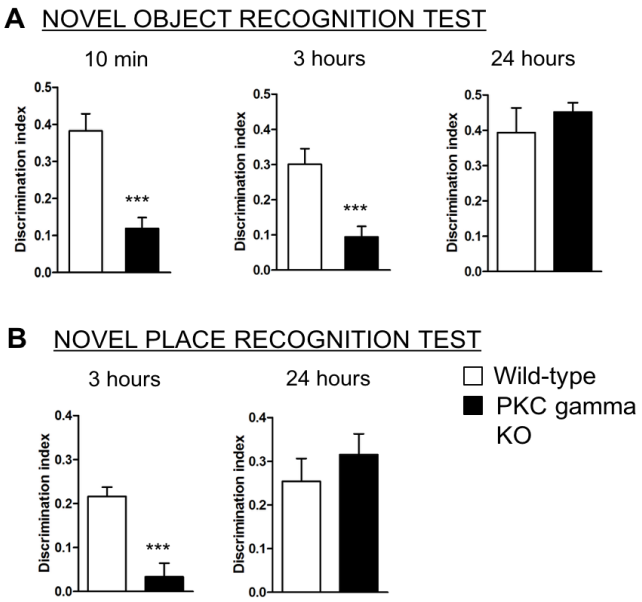


Figure 33. **Short-term, but not long-term non-emotional memories are affected in PKC gamma KO mice.** Short-term (10 min and/or 3h) and long-term memory (24h) were studied in the novel object-recognition test (A) and in the novel place-recognition test (B) comparing the PKC gamma KO mice with wild-type. Data are expressed as mean ± s.e.m. *** $p < 0.001$ (PKC gamma KO compared to WT).

Moreover, both STM and LTM were tested in the same animal combining the two non-emotional memory tasks previously described. One set of animals performed the NOR test to study STM and the NPR test to study LTM, while another set of animals did it in an inverted order (Figure 34a). The results demonstrate again that PKC gamma KO mice present STM deficits, independently on the memory task performed in the first place (Figure 34b-c)

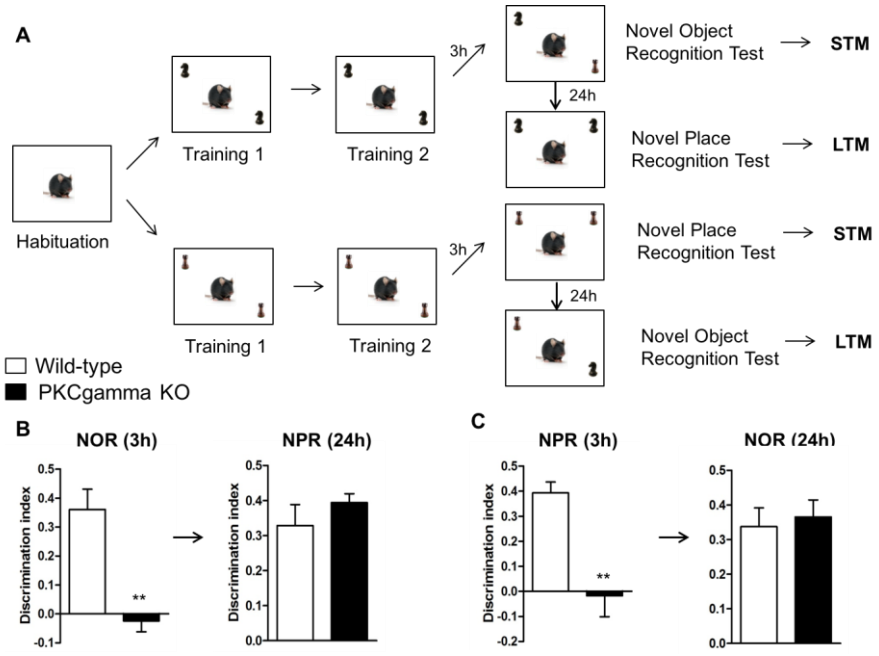


Figure 34. **Short-term and long-term memories were studied in the same animal by using a different memory test.** (A) Protocol used that combined the novel object-recognition (NOR) test and the novel place-recognition (NPR) test in order to study short-term and long-term memory in the same animal. (B) Half of the animals performed the NOR test to study STM and the NPR test to study LTM while the other half (C) performed the NPR test to study STM and the NOR test to study LTM. In both cases, PKC gamma KO animals only present memory impairments when STM was studied. Data are expressed as mean ± s.e.m. ** $p < 0.01$ (PKC gamma KO mice compared to WT).

PKC gamma KO mice present deficits in short-term emotional memory only in hippocampal-dependent tasks.

PKC gamma KO mice, compared to WT, were also tested in the following emotional tasks: the context recognition test (Figure 35a), the trace fear-conditioning (Figure 35b) and the delay fear-conditioning (Figure 35c). Again, memory performance was correct when LTM was studied. However, differences were observed in STM depending on the main brain area involved in the memory task. While PKC gamma KO mice present STM impairment in the context recognition and in the trace fear-conditioning, both of them hippocampal-dependent tasks, no deficits were observed in the delay fear-conditioning, an amygdala-dependent task.

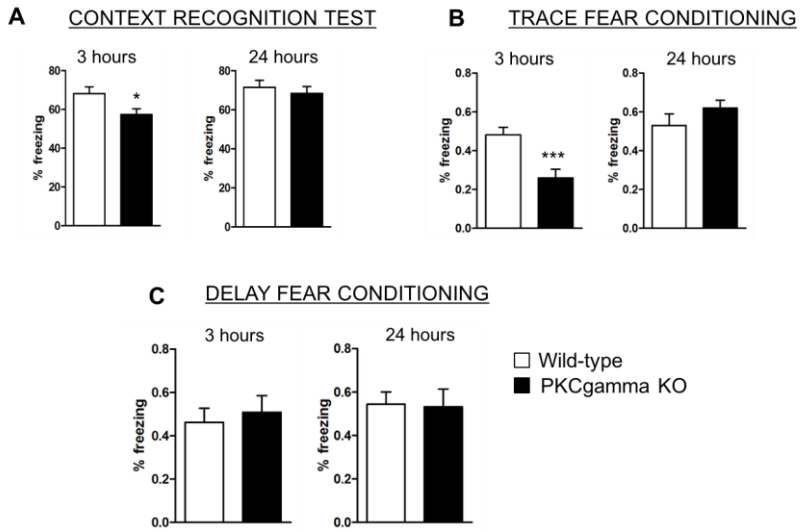


Figure 35. **Short-term and long-term emotional memories were studied in the PKC gamma KO mice by using three different memory tests.** Short-term and long-term memories were studied in the context recognition test (A), in the trace fear-conditioning tests (B) and in the delayed fear-conditioning test (C) comparing the PKC gamma KO mice with the wild-type controls. Data are expressed as mean ± s.e.m. * $p < 0.05$, *** $p < 0.001$ (PKC gamma KO compared to WT).

Working memory is also impaired in the PKC gamma KO mouse

Working memory was tested by spontaneous alternation, using the same protocol but two different shaped mazes: the Y-maze (Figure 36a) and the T-maze (Figure 36b). In both cases, memory deficits were observed in the PKC gamma KO mice.

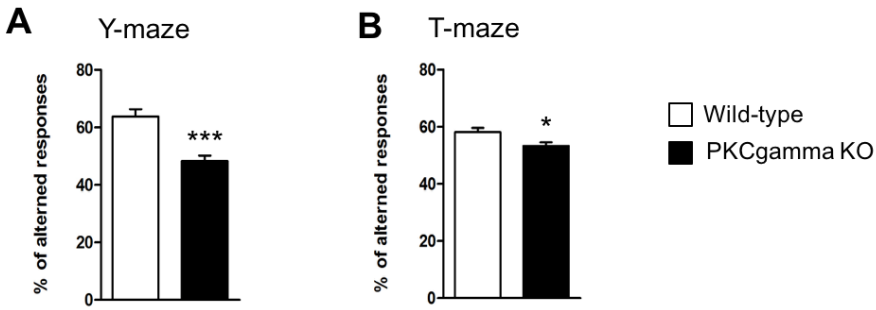


Figure 36. **Working memory was studied in the PKC gamma KO mice**. Working memory was studied in the PKC gamma KO mice compared to wild-types in two different shaped mazes, the Y-maze (A) and the T-maze (B). Data are expressed as mean ± s.e.m. * $p < 0.05$, *** $p < 0.001$ (PKC gamma KO compared to WT).

General behavior in the PKC gamma KO animal was similar than in the wild-type animals

Sensitivity to painful stimuli was studied by testing thermal hyperalgesia in the plantar test, in order to discard a possible bias in those tests that involved a footshock. No differences in the plantar test were observed between genotypes (Figure 37a). Moreover, locomotor activity was also checked as some deficits in motor coordination have been described in the PKC gamma KO mice (Chen et al. 1995). The objective is to anticipate a possible bias in those tests that involved an explorative behavior. Again, no differences were observed between genotypes (Figure 37b).

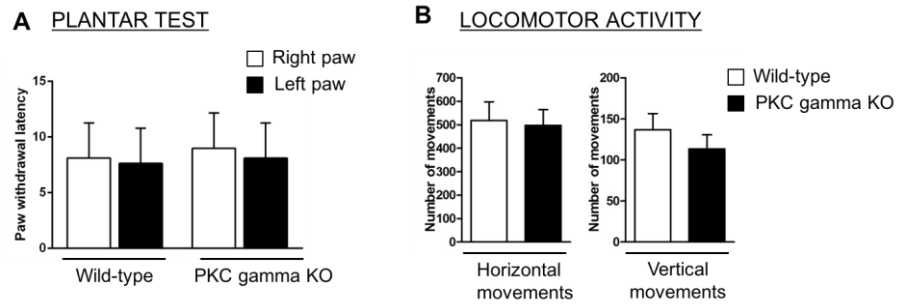


Figure 37. **Nociception and locomotion were studied as control measures in the PKC gamma KO mice.** No differences were observed between the PKC gamma KO and wild-type mice when nociception (A) and locomotion (B) were studied. Data are expressed as mean ± s.e.m.

c-Fos expression was analyzed in relation to memory acquisition in the trace fear-conditioning task

Trace fear-conditioning was performed following the same protocol (Figure 38a). Immediately after performing the STM test, animals were perfused in order to study c-Fos expression related to memory acquisition (Figure 38b). Three different conditions were compared in both genotypes: Animals directly sacrificed from the homecage (controls), animals that go through all the steps but did not receive the footshock (control + sound), and animals that performed the whole test (sound + shock). Freezing behavior was analyzed in the second and the third group (Figure 38c) obtaining the expected results. c-Fos quantification in the dentate gyrus of the hippocampus demonstrated that the amount of c-Fos activation was higher in the WT animals that listened to the sound compared to home cage controls and even higher in those wild-type animals that also received the footshock (Figure 38d-e). However, similar levels of c-Fos were observed in the PKC gamma KO mice that received the footshock compared to the control PKC gamma KOs (Figure 38d-e).

These results indicate that PKC gamma KO animals may present deficits in the acquisition of short-term hippocampal-dependent memories.

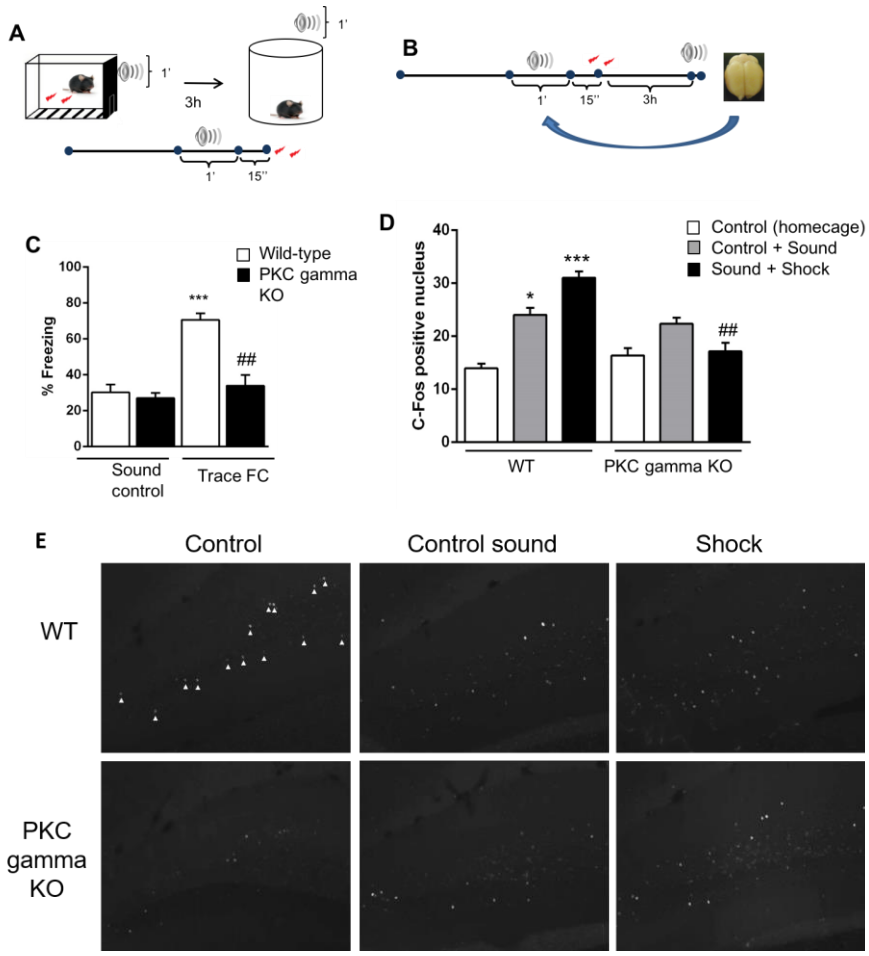


Figure 38. **c-Fos expression in the dentate gyrus of the hippocampus was studied for memory acquisition using the trace fear-conditioning paradigm in PKC gamma KO mice.** (A) Protocol followed to perform the memory test. (B) Time-course followed to obtain the brain samples. (C) Freezing behavior observed 3 hours after the training phase. (D) c-Fos quantification related to memory acquisition. (E) Representative immunofluorescences obtained for c-Fos expression. Data are expressed as mean ± s.e.m. * $p < 0.05$, *** $p < 0.001$ (control + sound or sound + shock compared to controls) $^{\#\#}$ $p < 0.01$ (PKC gamma KO compared to WT).

Molecular signatures of retrieval are not affected in PKC gamma KO mice

We evaluated in WT and PKC gamma KO mice the phosphorylation of ERK, which has been described to respond during the retrieval session of a fear-conditioning paradigm (Huang et al. 2010). In this regard, phosphorylation of ERK in hippocampal samples of mice was studied to check the retrieval for the long-term memory trace fear-conditioning. Similar results were obtained for both genotypes when they were compared to control mice (Figure 39).

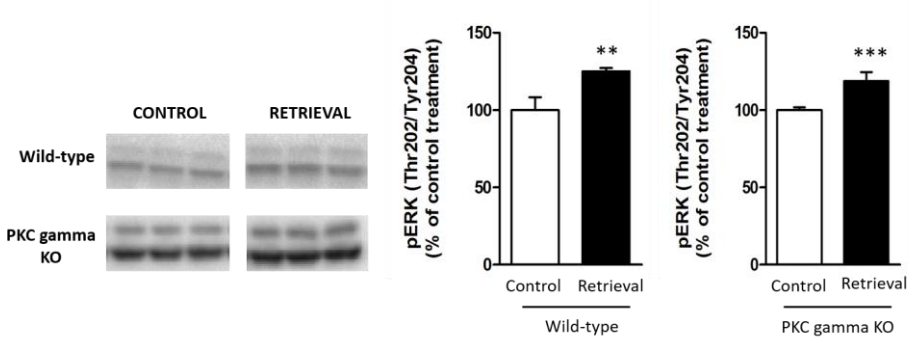


Figure 39. **No differences were found between WT and PKC gamma KO mice when retrieval for long-term trace fear-conditioning was studied**. Data are expressed as mean ± s.e.m ** $p < 0.01$, *** $p < 0.001$ (Retrieval compared to control).

b) Effect of low doses of THC in the memory performance of PKC gamma KO mice

PKC gamma KO mice do not display the amnesic-like effects of THC in hippocampal-dependent memory tasks

We studied if THC elicits the same amnesic-like effects in mice lacking the PKC gamma isoform. Memory performance was assessed in five different memory tasks. In the novel object-recognition test, an amnesic dose of THC (3 mg/kg) reduced the

discrimination index in the WT mice but did not affect PKC gamma KO mice (Figure 40a). However, when animals were treated chronically with THC and the test was performed also chronically, using each test as the training for the following one, we observed that repeated doses can finally also produced a memory deficit in the PKC gamma KO mice (Figure 40b). In the active avoidance test, composed by 4 sessions on four consecutive days, mice received the pharmacological treatment at the end of the daily session and approximately 24 hours before the performance of the following session. In this case, WT animals treated with THC displayed a lower number of conditioned responses than mice treated with vehicle, only in the last session. Instead, no differences were observed in the number of conditioned responses when comparing KO animals treated with vehicle and THC (Figure 40c). In the context recognition test, animals received THC or its vehicle after conditioning and 24 hours before the test. As expected, significant differences appear in the percentage of freezing behavior comparing WT animals treated with vehicle or THC, while PKC gamma KO mice did not show any effect of THC on memory (Figure 40d). Finally, two different protocols regarding cue fear-conditioning were performed, the trace and the delay fear-conditioning. Surprisingly, while PKC gamma KO mice treated with THC do not present the memory deficits observed in WT animals in the trace fear-conditioning paradigm (Figure 40e), no differences in behavior between genotypes were observed following the delay protocol (Figure 40f). In this case, both WT and KO animals treated acutely with THC presented a decreased percentage of freezing behavior. These results indicate that PKC gamma KO animals do not display the expected THC amnesic-like effects only when hippocampal-dependent tasks are performed.

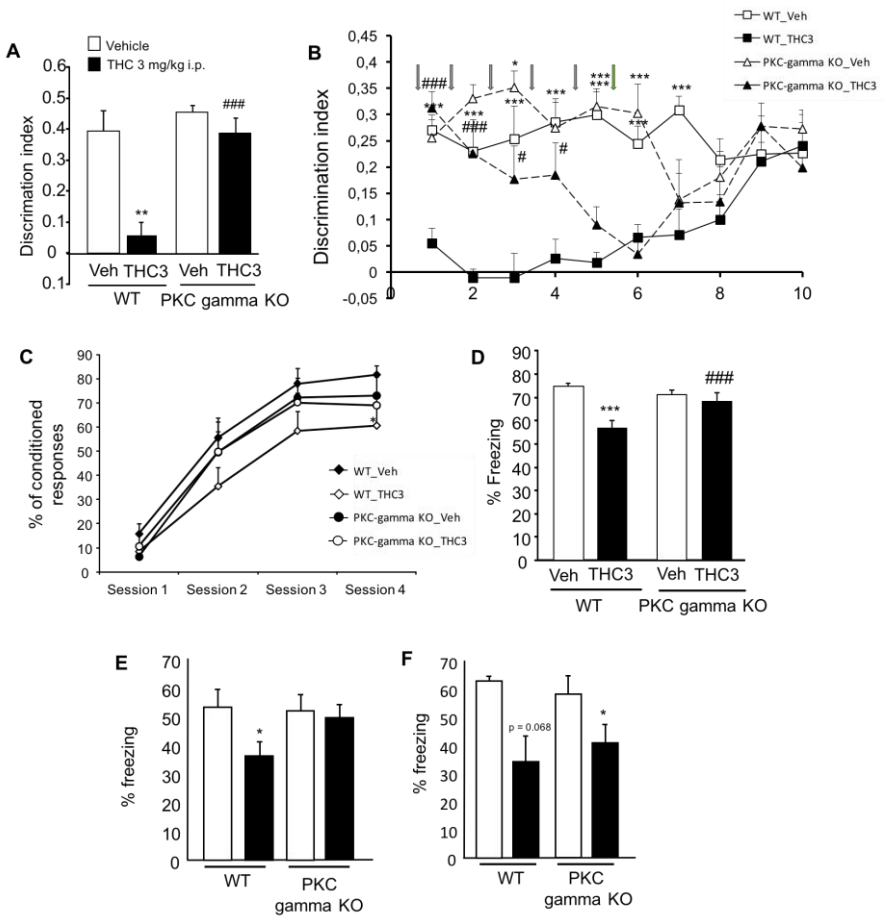


Figure 40. **PKC gamma KO animals do not present memory deficits when treated with an amnesic dose of THC in hippocampal-dependent tests**. (A) In the NOR test, WT animals treated with THC present a significant decrease in the discrimination index value whereas this effect is not observed in the PKC gamma KO mice. (B) After 4-5 chronic administrations of THC, PKC gamma KO also present memory deficits in the NOR test. (C) The number of conditioned responses during the last session of the active avoidance test is decreased in WT animals treated with THC while this effect is not observed in the PKC gamma KO mice. Similar results are also observed in the percentage of freezing performed in (D) the context recognition test and (E) the trace fear-conditioning task. However, no differences were observed between genotypes in mice treated with THC in the delay fear-cognition task (F), a task that is not dependent on the hippocampus. Data are expressed as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (THC compared to vehicle); ## $p < 0.01$, ### $p < 0.001$ (PKC gamma KO compared to WT).

PKC gamma is not involved in other pharmacological effects produced by THC

No differences were found between WT and PKC gamma KO mice on the hypothermic effect of THC. Indeed, there were no changes in body temprature in any of the genotypes one hour after THC administration (Figure 41a). On the other hand, using the acetic acid test, a model of visceral pain, we observed a similar number of writhing responses for both genotypes, which were similarly reduced by the THC treatment (Figure 41b). These results drive us to the conclusion that PKC gamma KO mice are as sensitive as WT mice to other effects of THC, independently from those mechanisms involved in memory modulation.

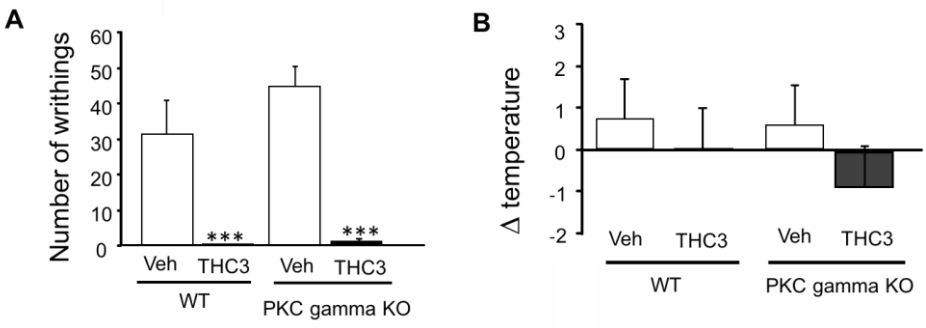


Figure 41. **PKC gamma KO mice and WT controls treated with THC are equally affected in other behavioral tests**. (A) The decrease in body temperature is observed in both genotypes one hour after THC administration. (B) The number of writhings observed in a model of visceral pain is significantly decreased in both genotypes after acute THC injection. Data are expressed as mean ± s.e.m. *** $p < 0.001$ (THC compared to vehicle).

Specific PKC gamma substrates are modulated after an acute administration of THC

PKC gamma is one of the PKC isoforms heavily expressed in the hippocampus. We evaluated the phosphorylation of its three preferential substrates after acute THC administration: neurogranin, MARCKS and neuromodulin. Compared to control group, mice treated with THC present an enhanced phosphorylation for p-neurogranin and p-MARCKS, both of them postsynaptic proteins, but not for p-neuromodulin (Figure 42).

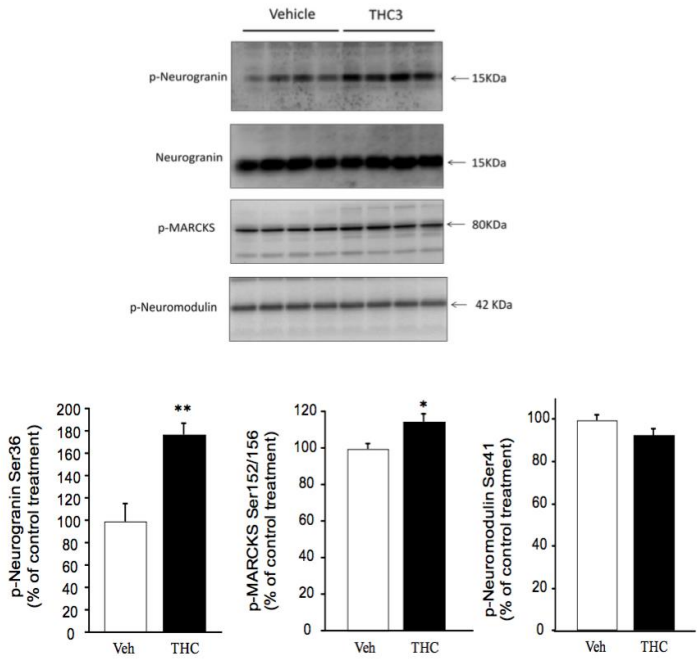
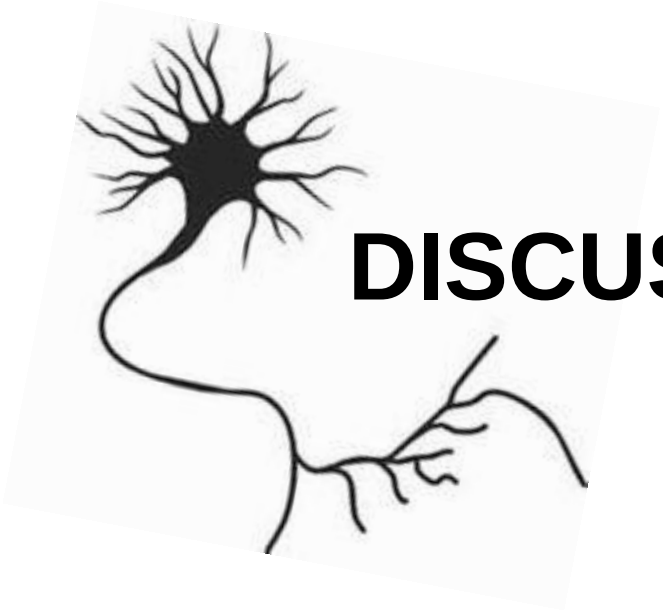


Figure 42. **Modulation of preferential PKC gamma substrates in the hippocampus after acute THC exposure**. Representative immunoblot and quantification of the modulation of the three preferential PKC gamma substrates. The phosphorylation of neurogranin and MARCKS is significantly higher when mice are treated with THC. Instead, neuromodulin is not modulated after THC administration. Data are expressed as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$ (THC compared to vehicle).



DISCUSSION

The general aim of this thesis was to investigate the neural substrates of several physiological and phyisiopathological aspects of memory by addressing the role of the endocannabinoid system and of related intracellular signaling pathways. Using relevant mouse models, we have focused our attention in four specific objectives to explore different inter-related venues contributing to memory function where the endocannabinoid system could play a relevant role. In these objectives, we have investigated physiopathological conditions with a different etiology, but all of them leading to memory impairment and with an involvement of the endocannabinoid system.

In Objective 1, we have studied the memory deficits present in an inherited pathology, the fragile X syndrome (FXS), which is the most common cause of inherited intellectual disability (Penagarikano et al. 2007). Based on previous findings of our research group (Busquets-Garcia et al. 2013), we have now clarified the benefits of low doses of the CB1R antagonism (using the antagonist/inverse agonist rimonabant and the neutral antagonist NESS0327) to prevent the memory deficits in a mouse model of FXS. In Objective 2, we have investigated how stress exposure affects memory performance. Stress is a situation that can be considered adaptive for our organism to face negative contexts, environments or situations but leading to pathological conditions when chronified. We have described the role and the specific cell population of the central and the peripheral endocannabinoid system involved in this stress-induced effect. Finally, in objectives 3 and 4, we have studied the effects of THC administration in two different contexts considering drug consumption as another condition producing a potential pathological situation. In objective 3 we have studied the

consequences of THC administration on short-term memory and we have described the involvement of the protein kinase C signaling pathway in the short-term memory deficits produced by the exogenous activation of the CB1R. In objective 4, we have addressed the physiological role of a PKC isoform, the PKC gamma, in short-term and long-term memory hippocampal-dependent memories, and in the deleterious effects produced by THC on memory by using a mouse model lacking this specific PKC isoform.

In this section, we will further discuss the main results obtained in each of the objectives of this thesis.

1. The endocannabinoid as a possible therapeutic target for the memory impairment that characterize the fragile X syndrome

Many therapeutic approaches have been suggested for the treatment of the main symptoms of the fragile X syndrome (FXS). Nowadays, a number of drugs are being tested in preclinical models of the syndrome as well as in clinical trials at different phases, including modulators of the mGluR system, GABAergic agents or GSK3 inhibitors, among others (Munshi et al. 2017). In our research group, we previously demonstrated using a preclinical mouse model of the FXS, the *Fmr1* KO mice, that the ECS is a potential therapeutic target for this disease. In this regard, we showed that the pharmacological blockade of the CB1R by rimonabant, a CB1R antagonist/inverse agonist, in the *Fmr1* KO mice or the downregulation of CB1R expression, normalized the cognitive impairment, the altered dendritic spine morphology and the high susceptibility to suffer epileptic seizures, among other features

(Busquets-Garcia et al. 2013). In this thesis, we have focused our attention, specifically, in the memory deficits present in the *Fmr1* KO mice with the objective to use doses as low as possible of the CB1R antagonist rimonabant.

We have demonstrated that lower doses of rimonabant than the previously described (Busquets-Garcia et al. 2013), even given in a discontinuous schedule (once every two day) or the use of low doses of NESS0327, a CB1R neutral antagonist, managed to normalize this memory deficit.

The use of low doses of rimonabant to reveal its pro-cognitive effect is relevant due to its previously described unwanted effects. Rimonabant, is an antagonist/inverse agonist of the CB1R and it was first approved and commercialize in 2006, for the treatment of obesity, overweight and metabolic disorders demonstrating weight loss and a reduction in the waist circumference (Dibble et al. 2007; Pi-sunyer et al. 2007). However, after two years, it was removed from the market due to the appearance of unwanted psychiatric side effects such as anxiety, depression and suicidal ideation as well as other side effects including headache and sleep disorders, among others (Cheung et al. 2013; Butler and Korbonits 2009). In the obese human patients, the dose that produces the previously mentioned side effects was 20 mg/day of rimonabant, which corresponds to a dose of 3.5 mg/kg in mice, according to the dose conversion between species proposed by Reagan-Shaw et al., a conversion that takes into account the body surface area (BSA) normalization method. BSA considers several parameters, such as the basal metabolism or the caloric expenditure (Reagan-Shaw et al. 2007). In our study we demonstrate that the subchronic treatment (7 days) with a dose of 0.1 mg/kg, a dose approximately 35 times lower than

the one used in human patients, or the same number of administration (7 administrations), given every two days (in a total of 14 days), are able to completely reverse the cognitive deficit observed in the novel object-recognition task in the *Fmr1* KO mice. All together, our data demonstrate that a dose of rimonabant far low from those producing adverse effects in humans is able to normalize the cognitive deficits present in the *Fmr1* KO mice. This low dose of rimonabant presents the advantage that has been described to produce anxiolytic properties in mice (Zádor et al. 2015) compared to the high doses needed for its anti-obesity effects.

Another well-described feature of the *Fmr1* KO mice is the enhanced hippocampal mGluR5-LTD due to the loss of FMRP (Huber et al. 2002), a form of synaptic plasticity important for learning and memory processes (Simonyi et al. 2005). This aberrant synaptic plasticity has been linked to the intellectual disability in FXS (Bear et al. 2004). As mentioned above, inhibition of group I mGluRs in order to reduce excitatory neurotransmission, specially mGluR5, has been widely proposed as another possible therapeutic target for the treatment of FXS (Davenport et al. 2016). Although preclinical studies have demonstrated promising results (Michalon et al. 2012; Michalon et al. 2014), the clinical trials performed until today following this approach, as well as using GABA agonists, did not reach the primary outcomes (Scharf et al. 2015). This may be due to an inadequate utility or quality of the studied endpoints as well as due to the validation criteria of some instruments (Budimirovic et al. 2017; Duy and Budimirovic 2017; Davenport et al. 2016). In that sense, the tools used to study cognitive and behavioral problems present limited reliability and validity (Budimirovic et al. 2017). Moreover, it has been suggested that future trials may give more

importance to the objective measures that reflect the improvements in the quality of FXS patient's life (Budimirovic et al. 2017).

In our pre-clinical study, rimonabant treatment was administered subchronically since the day before recording, so its effect will be related to the long lasting alterations in brain circuits. We found that mGluR5-LTD was normalized in *Fmr1* KO mice after rimonabant treatment using the same dose of rimonabant that was effective in the behavioral studies, 0.1 mg/kg.

Finally, it has been described that rimonabant, apart from being a CB1R antagonist, can also act as an inverse agonist (Pertwee 2005), a property that has been associated to the side effects of high doses of rimonabant (Bergman et al. 2008) when treating obese patients. The use of a neutral antagonist may attenuate the incidence of the unwanted side effects due to its different pharmacodynamic profile compared to inverse agonists. CB1R inverse agonists, such as rimonabant, increase GABAergic transmission in hippocampal slices (Lee et al. 2015) due to the suppression of CB1R constitutive activity (Meye et al. 2013). In contrast, neutral antagonists, such as NESS0327, do not affect this GABAergic transmission (Lee et al. 2015). For that reason, we tested whether NESS0327, a neutral and highly selective CB1R antagonist (Ruiu et al. 2003), would also reverse the cognitive deficits observed in the *Fmr1* KO mice. As expected, the subchronic treatment (7 days) with this drug demonstrated similar effects to those observed with rimonabant when similar low doses were tested, producing a clear increase in the discrimination index values obtained in *Fmr1* KO mice.

All together, our results strongly support that the endocannabinoid system may be a good therapeutic target for the treatment of the cognitive deficits in the FXS, normalizing the altered synaptic

plasticity. Here we have used lower doses or alternative drugs to the one that produced adverse effects in human obese patients. Future studies should also investigate the potential of these doses and these drugs to prevent other features that characterize the FXS in the *Fmr1* KO mice such as the lower nociceptive sensitivity or the higher susceptibility to suffer epileptic seizures. Targeting the endocannabinoid system at different levels than the blockade of CB1R can also be considered. Some beneficial effects in the amelioration of the different features that characterize the FXS have been observed using different approaches such as the inhibition of FAAH, that have demonstrated to improve the *Fmr1* KO mice performance in the passive avoidance test (Qin, Zeidler, et al. 2015) or the enhancement of 2-AG signaling, that normalizes two behavioral phenotypes of the *Fmr1* KO mice consisting on a lower aversion for open places and an elevated motor activity (Jung et al. 2012). Finally, the use of other animal models such as the rat model of FXS (Till et al. 2015), an animal model with high translational value, would be of great interest as a complement for the existing mouse models.

2. Involvement of the endocannabinoid system in the modulation of non-emotional memory impairment produced by stressful stimuli

The main objective of this work was to study the influence of stress exposure, that is known to mobilize the ECS (Hillard 2008), on non-emotional memory consolidation. It has been widely described that stress affects memory performance in opposite ways depending on different factors, such as the source of stress, the intensity of the stress or the kind of memory studied (Joëls et al. 2006; Schwabe et

al. 2012; Finsterwald and Alberini 2014; Roozendaal and McGaugh 2011). For this study, we used the novel object-recognition test to analyze the consolidation phase in a non-emotional memory paradigm. Regarding the ECS, we have studied, in the previous objective, its role in a pathological and inherited condition, the FXS, which is characterized by coursing with intellectual disability. In the present work, we studied the possible involvement of the ECS in the effect of stress over memory consolidation as the ECS has been widely related with both cognitive functions (Zanettini et al. 2011; Abush and Akirav 2010) and with the modulation of stress responses (Hillard 2008; Riebe and Wotjak 2011)

We have observed that just an acute exposure to stress can generate non-emotional memory deficits. Following our experimental design, mice were exposed to a footshock, considered as a physical stressor, just after the training phase on the novel object-recognition task. The results obtained demonstrated that this stressful stimulus produced long-term non-emotional memory impairment in the NOR test while it did not affect emotional memory when freezing behavior associated to the shock was studied. Similar results were obtained when other kinds of stressors were tested including psychological stressors such as tail suspension. These stressful stimuli only affected memory performance when applied within a critical time window after the training session indicating an effect on memory consolidation. In contrast, no short-term memory deficits were observed in mice exposed to the footshock, a result in accordance to what observed in human declarative memory, which is not affected by emotional arousal (Quevedo et al. 2003).

Our results also showed that the systemic or local (directly infused in the hippocampus) blockade of the CB1R with rimonabant reversed

the cognitive deficit induced by stress. Similarly, the complete inactivation of this receptor (CB1 KO mice) could also reverse the memory impairment, indicating that the CB1R play a crucial role in this effect of stress.

However, other approaches have also shown effectiveness in preventing the memory deficits produced by stress. Adrenal glands, as part of the HPA axis and being in charge of the synthesis and release of glucocorticoids, play an important role in stress response (Herman et al. 2016; Hill and Tasker 2012). Interestingly adrenalectomy also prevented the stress-induced amnesia, pointing to the adrenal gland as a key peripheral tissue involved in the acute effects of stress over cognition. In fact, it has been previously described the relationships between the HPA axis and the cognitive performance (Tasker and Herman, 2011). In our study, the relevance of the peripheral ECS was revealed with the peripherally restricted CB1R antagonist AM6545, which also blocked the deleterious effects of stress. The results obtained using this drug reproduced those obtained with rimonabant confirming a major role of the peripheral CB1R.

Different conditional mutant mice for the CB1R have been developed lacking these receptors only in specific brain regions or cell population. Moreover, there are also some mice models with cell-type-specific conditioned rescue of CB1R (Zimmer 2015). Both of them represent excellent tools for the study of the ECS and the function of specific CB1R cell populations. Using these genetic approaches, we identified that CB1R located at the dopamine β-hydroxylase expressing adrenergic/noradrenergic cells (DBH+ cells) are necessary, but also sufficient, for the memory deficits observed in the novel object-recognition test after stress exposure. Mice

lacking CB1R on DBH+ cells (DBH-CB1-KO mice) did not present the memory impairment observed in WT mice after the footshock exposure, whereas mice expressing CB1R only in DBH+ cells (DBH-CB1-RS mice) present similar results on the NOR test than WT. CB1R in other cell populations highly abundant in the brain were not involved in the stress response as memory deficits were still present in mice lacking CB1R in forebrain GABAergic neurons, in dorsal telencephalic glutamatergic neurons, in both GABAergic and glutamatergic neurons or in central serotonergic neurons. This is in contrast to the memory deficits produced by THC which are mediated by the CB1R expressed on GABAergic interneurons (Puighermanal et al. 2009). Notably, no behavioral, sensibility to footshock or pain differences was detected between the different genotypes studied in this work.

Taken into account all the results previously presented, we considered that CB1R present in DBH+ cells of the hippocampus, as it is one main brain region involved in the performance of the NOR test, and CB1R present in the DBH+ cells of the adrenal glands are relevant for the memory impairment observed after a footshock.

At a central level, the intrahippocampal administration of rimonabant, which demonstrated to be effective in preventing the memory deficits produced by stress in the WT mice, also prevents this memory deficit in the DBH-CB1-RS mice. Those results demonstrate that although different brain regions have been described to participate in the performance of the novel object-recognition test, such as the prefrontal cortex (Akirav and Maroun 2006) and the perirhinal cortex (Antunes and Biala 2012), the hippocampus plays a major role in regulating the impact of endocannabinoid signaling in the deleterious effects of stress over the consolidation of a non-emotional memory.

In addition, CB1R were detected by immunofluorescence in DBH+ fibers of the *stratum radiatum* and the pyramidal layer of the hippocampus in DBH-CB1-RS mice, confirming that hippocampal CB1R in DBH+ cells are in part responsible for the memory deficit produce by stress stimuli.

In relation to the peripheral tissues, we have observed that the peripherally restricted inhibitor AM6545 also prevents the memory impairment in the DBH-CB1-RS mice. Moreover, immunofluorescence analysis reveals a decreased DBH expression in the adrenal medulla of DBH-CB1-KO mice, which was recovered in the DBH-CB1-RS mice. Finally, a relation between the ECS and adrenergic receptors has been demonstrated in other brain regions, such as the nucleus accumbens (Carvalho et al. 2010). In our work, we showed an important beta-adrenergic receptor signaling downstream CB1R blockade as the peripherically restricted beta-adrenergic antagonist sotalol, prevented the effects of rimonabant on memory recovery. These results point to the CB1R expressed in the DBH^+ cells located in the adrenal glands as important players in mediating the amnesic effects produced by stress.

In relation to the endocannabinoid tone, an enhancement on the endocannabinoid levels in the hippocampus, specifically 2-AG, after an acute stress has been reported (Akirav 2013; Hill and Tasker 2012). Accordingly, we observed in our work an increase on 2-AG levels after stress exposure. These endocannabinoids may act on the CB1R expressed in noradrenergic fibers where they would control noradrenaline transmission through projections from the *locus coeruleus* or the *nucleus tractus solitarius* (Scavone et al. 2010). Based on these data, we hypothesize that the decrease in noradrenaline release at hippocampal noradrenergic terminals,

which is controlled by the endocannabinoid tone, would be an important step in the memory consolidation of the novel object-recognition task.

All together, we have demonstrated that central and peripheral adrenergic/noradrenergic transmission determines the consolidation of non-emotional memories, a function that is under the direct control of CB1R expressed in DBH+ cells. A summary on the general observations of this study that have been previously explained can be found in Figure 43.

The interplay between peripheral and central processes tightly controlled by CB1R, open new perspectives in the development of possible therapeutic strategies for the treatment of both memory- and stress-related disorders such as post-traumatic stress disorders.

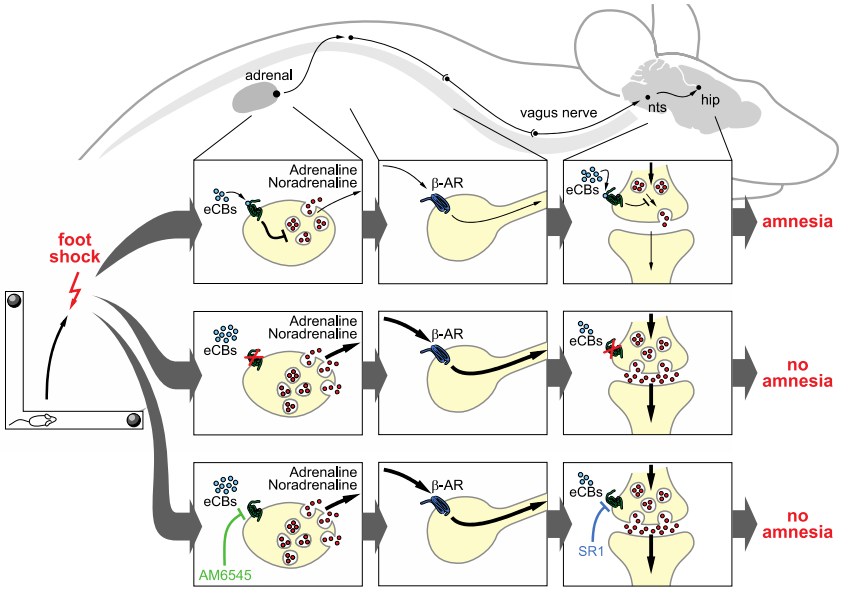


Figure 43. **Schematic representation showing how the endocannabinoid system is involved in the amnesic-like effects produced by acute stress exposure.** Exposure to a footshock after the training session in the novel object-recogition test produces amnesia in WT mice. The blockade of the peripheral and the central CB1R using both pharmacological (peripherally restricted drugs and local intra-hippocampal administration) together with genetic tools has allowed to elucidate the involvement of the ECS in this amnesic effect. These results revealed that CB1R located in DBH+ cells are necessary and sufficient for the memory deficits observed in the novel object-recognition test after stress exposure (designed by Lutz et al. 2016). SR1 = Rimonabant

3. Signaling pathways involved in the short-term memory deficits produced by THC

THC produces deficits in different aspects of memory including long-term memory, short-term memory and working memory. Our previous studies, have precisely described the signaling pathways

involved in the long-term memory deficits produced by THC (Puighermanal et al. 2009).

This study showed that THC acts mainly on CB1R located at GABAergic terminals producing a suppression of GABA release from inhibitory terminals. Consequently, THC produces an unbalance between GABAergic and glutamatergic neurotransmission that leads to an enhanced glutamatergic tone in the hippocampus (Laaris et al. 2010; Katona & Freund 2012; Puighermanal et al. 2009). In turn, this enhancement in the glutamatergic tone will be responsible of the over-activation of the mTOR-signaling pathway (Puighermanal et al. 2009), which plays an important role for proper memory storage (Bekinschtein et al. 2007). Over-activation of the mTOR pathway will result in an increased phosphorylation of different downstream targets and an increased protein synthesis responsible for the consequent amnesic-like effects produced by THC. In this regard, the mTOR blockade by rapamycin or the protein synthesis inhibition by anisomycin prevented the deleterious effects produced by THC. Moreover, the pre-treatment with non-amnesic doses of the NMDA receptor antagonist MK801 also suppressed the THC-induced memory impairment (Puighermanal et al. 2009).

In the present work, we focused our attention in studying the signaling pathways involved in the cognitive deficits produced by THC in short-term memory to study the possible similarities and differences between short-term and long-term memory.

We have first demonstrated that THC dose-dependently impairs short-term memory in the novel object-recognition test when administered after the training session. Morover, the pre-treatment with the CB1R antagonist rimonabant prevented this memory

impairment, similarly to what observed when long-term memory was studied (Puighermanal et al. 2009). MK801, in a dose that did not produce memory deficits on its own, reversed this memory deficit. More specifically, we showed that both NR2A- and NR2B- preferring NMDA receptor antagonists can prevent the memory deficits produced by THC. This is in contrast to previous observations showing that spatial working memory impairments produced by HU210, another CB1R agonist, were only prevented with the NR2B-NMDA antagonist (Han et al. 2012).

In order to determine whether the mTOR pathway is also involved in the short-term memory deficits produced by THC, we tested the effects of the pre-treatment with rapamycin, temsirolimus (both inhibitors of the mTORC1 pathway) and anisomycin (a protein synthesis inhibitor). Strikingly, while all of them prevented THC-mediated long-term memory deficit, no effects on improving short-term memory performance were observed.

THC has been demonstrated to enhance several signaling pathways including the mTOR signaling pathway. We also demonstrated an increase in PKC phosphorylated substrates after THC administration indicating an enhancement on the PKC signaling system. Moreover, this effect was blocked by the pre-treatment with MK801, similarly to what was observed in mTOR signaling (Puighermanal et al. 2009). Taking into account these results, we tested the effect of PKC inhibitors at doses that do not affect memory performance on their own. Two different PKC inhibitors were evaluated: chelerythrine that acts on the catalytic domain of PKC (Herbert et al. 1990) and NPC-15437 that acts on the regulatory domain of PKC (Sullivan et al. 1991). Pre-treatment with both PKC inhibitors completely prevented THC short-term memory deficits when administered both

systemically or directly into the hippocampus. However, no amelioration of the THC deficit was observed on long-term memory when PKC inhibitors were tested. In accordance, the pre-treatment with a PKC inhibitor also normalized the enhancement in PKC phosphorylated substrates after THC administration. These results, together with those previously presented in spatial working memory, suggest the involvement of different molecular mechanisms in relation to the effects produced by cannabinoid agonists on short-term and long-term memory (Figure 44).

One important substrate of PKC is neurogranin, an extremely abundant postsynaptic protein highly involved in the regulation of synaptic function and widely expressed on brain areas involved in cognitive functions (Díez-Guerra 2010). Neurogranin is the most abundant calmodulin-binding protein in the brain. It would be bound to calmodulin in the postsynaptic environment of resting synapses regulating its availability for other signaling pathways (Zhong and Gerges 2012; Domínguez-González et al. 2007). However, modifications in this environment, such as the phosphorylation by PKC of neurogranin at Ser36 IQ motif (Kumar et al. 2013), would reduce the binding affinity between neurogranin and calmodulin. This mechanism allows calmodulin to bind to calcium which, in turn, will activate calcium-calmodulin-dependent protein kinase II (CamKII) signaling cascades, involved in learning and memory (Gaertner et al. 2004; Fukunaga and Miyamoto 2000). Our data showed that neurogranin phosphorylation is enhanced in hippocampal samples of mice treated with THC producing an unbalance in the ratio of phosphorylated/unphosphorylated neurogranin and, in consequence, preventing the buffering effect of unphosphorylated neurogranin, which leads to an anomalously long

calmodulin signaling (Pak et al. 2000; Díez-Guerra 2010) (Figure 44). Interestingly, the phosphorylation of neurogranin was reversed under those conditions of NPC-15437 pre-treatment that prevented the short-term memory deficit of THC.

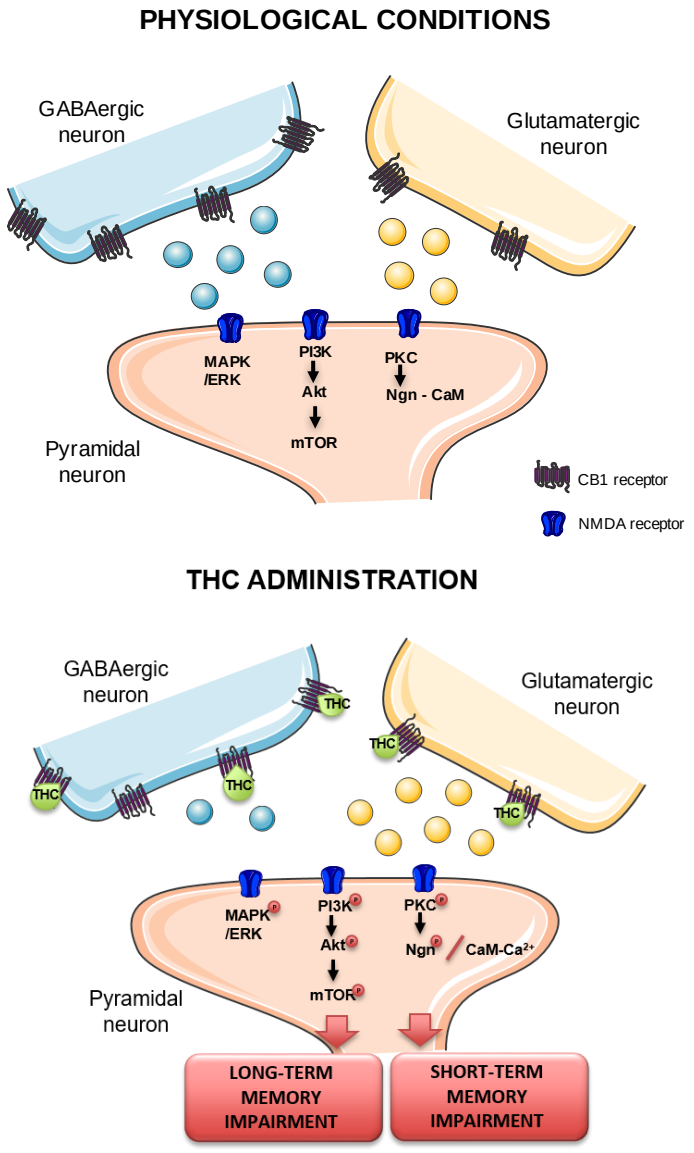


Figure 44. **Different signaling pathways are involved in the short-term and the long-term memory deficits produced by THC**.

PKC is a family of proteins involved in many physiological functions including the regulation of synaptic plasticity (Hsu et al. 2011) and the modulation of learning and memory processes (Glanzman 2013; Bonini et al. 2007). In fact, deficits in PKC signaling cascades have been related with diseases where cognition is affected, such as Alzheimer's disease (Sun and Alkon 2014). PKC family is composed by at least 11 different isoforms, all of them expressed in the hippocampus (Lein et al. 2007), and they are divided in three subgroups depending on their structure and the way they are activated (Mackay and Twelves 2007; Newton 2010). In order to determine the specific isoform/isoforms involved in the THC-induced short-term memory deficits, we studied the phosphorylation of a Ser residue located at the hydrophobic motif, a motif highly conserved within isoforms and involved in PKC maturation and activation (Freeley et al. 2011). We used an antibody phospho-PKC (pan) (βII Ser660), which detects PKC alfa, betaI, betaII, delta, epsilon, etha and theta (α, βI, βII, δ, ε, η and θ) isoforms only when phosphorylated at the carboxy-terminal residue homologous to Ser660 of PKC betaII (Figure 45).

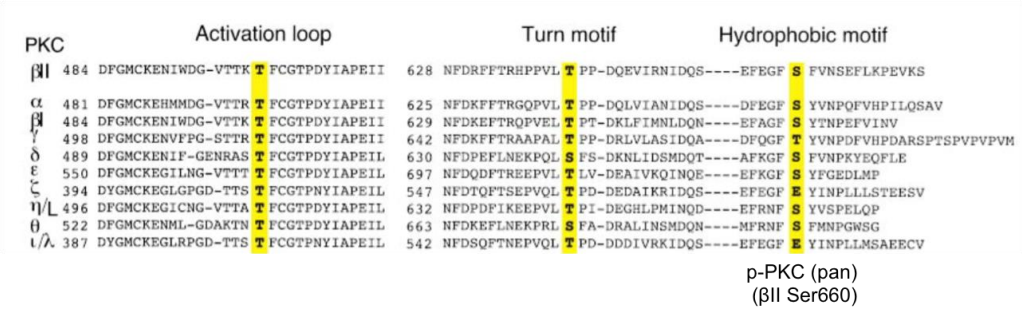


Figure 45. **Alignments of the activation loop, turn motif and hydrophobic motif phosphorylation sequences for the PKC isoforms.** In the present work, we are interested in studying the phosphorylation at the hydrophobic motif (Ser residue). (Modified from Newton 2003).

The results obtained using this antibody demonstrated a modulation at 60 kDa in hippocampal samples obtained 1h after THC administration, a modulation that can be partially normalized by the pre-treatment with the PKC inhibitor, NPC-15437. We then analyzed all isoforms containing a Ser660 residue in the hydrophobic motif, allowing us to select PKC theta with a molecular weight close to 60 kDa and discard the other isoforms due to its high molecular weight (75-110 kDa). Although additional studies would be of interest to confirm this result, we hypothesize that PKC tetha may play a major role in the short-term memory deficit produced by THC. In support to our results, it has been described that a mutation in the hydrophobic-motif of PKC theta, turning Ser to Ala, would reduce its activity (Liu et al. 2002).

In summary, these results identify the crucial contribution of PKC signaling in the cognitive impairment produced by THC, one of the most deleterious effects of cannabis abuse. Moreover, we reveal that independent molecular mechanisms, the mTORC1 and the PKC signaling pathways, are responsible for the long-term and short-term memory deficits, respectively, induced by THC administration.

4. Specific role of the protein kinase C gamma signaling in hippocampal associated memory processes

We have previously demonstrated that PKC signaling is crucial for the cognitive deficits produced by THC in short-term memory, but not in long-term memory. Other works have extensively reviewed the relevance of the PKC signaling pathway in cognition (Sun and Alkon 2014). We have now studied more extensively the role of one specific PKC isoform, the PKC gamma, to elucidate its possible

relevance in memory formation at both basal conditions and after THC administration.

a) Protein kinase C gamma is involved in short-term but not long-term hippocampal-dependent memory performance

The results obtained in this study point the PKC gamma isoform as an important player for the formation, performance or retrieval of short-term memory. PKC gamma isoform belongs to the conventional subgroup of PKCs and is highly expressed in some brain regions related to learning and memory such as the hippocampus (Tanaka and Saito 1992). In order to elucidate the possible involvement of the PKC gamma isoform in memory performance, we used a mouse model lacking this specific isoform. Mice deficient in the PKC gamma isoform (PKC gamma KO mice) were developed in 1993 by Abeliovich et al. This mouse model presents an apparently normal behavior. In our experimental conditions, they showed the same sensitivity to pain and similar motor activity than WT mice, although some deficits in motor coordination and mild ataxic gait have been previously described (Chen et al. 1995). These alterations do not prevent the animal to explore or respond to noxious stimulus, such as a footshock. Abeliovich et al. also described a normal brain anatomy in these mice, although PKC gamma KO mice showed larger lateral ventricles than their WT littermates in our histological observations. This feature has been observed in different neurological diseases in human patients including Parkinson's disease (Apostolova et al. 2012), amyotrophic lateral sclerosis (Westeneng et al. 2015) and pediatric epilepsies (Jackson et al. 2012)

Previous studies have shown that PKC gamma KO mice present mild deficits in the performance of a LTM context-dependent fear-conditioning paradigm (Abeliovich et al. 1993b) and a diminished hippocampal LTP (Abeliovich et al. 1993a), suggesting that this kind of synaptic plasticity might be dependent on PKC gamma. In contrast, no differences were found between WT and PKC gamma KO mice in the Morris water maze task, a measure of spatial learning, neither in the tone-dependent fear-conditioning (Abeliovich et al. 1993b). To better understand the role of PKC gamma in memory processes, we studied the behavior of the PKC gamma KO mice in different memory tests, including non-emotional tasks, such as the novel object-recognition test and the novel place-recognition test, emotional tasks, such as the context recognition test and two different forms of cued fear-conditioning, and working memory in the spontaneous alternation tasks. All tests were performed to study STM (3 hours) and LTM (24 hours).

Our experimental design did not reveal differences in LTM between genotypes in any of the memory tests performed, suggesting that other PKC isoforms might undertake the role of PKC gamma in basal conditions. The differences with the results of Abeliovich et al. in context fear-conditioning may be explained by differences in the experimental design and the number of footshocks received. Strikingly, PKC gamma KO mice present cognitive impairment in STM in all the memory tasks, except the delayed fear-conditioning, where PKC gamma KO behaves similarly to WT mice. We hypothesize that these results are due to the main brain regions involved in each memory task. While all of them are mainly hippocampal-dependent tasks, including the trace fear-conditioning, the amygdala is the most important area involved in the delayed fear-

cognition (Raybuck and Matthew Lattal 2011). In agreement, the inactivation of the amygdala produces deficits in delayed, but not in trace fear-conditioning, in contrast to the hippocampal inactivation that produces opposite effects (Raybuck and Matthew Lattal 2011).

Other studies comparing the role of PKC isoforms on STM and LTM conclude that only alpha and/or beta I, but not other PKC isoforms, are necessary for STM memory formation in the active avoidance test, another hippocampal-dependent memory task (Vianna et al. 2000). The authors considered that PKC is essential for LTM retrieval but is not necessary for STM retrieval (Vianna et al. 2000). This result is in contrast to our data as we showed that PKC gamma isoform is crucial for hippocampal-dependent STM, independently of the emotionality of the memory trace. However, the specific memory stage where PKC gamma is required is still unknown. It has been proposed that this isoform plays an important role in memory retrieval in the Morris water maze test consisting of an hippocampal-dependent task (Li et al. 2014). Taking into account these data, it is possible that PKC gamma KO mice present specific problems in the retrieval of STM, understanding this phenomenon as the use of learned memories stored in our brain (Ben-yakov et al. 2015). Considering that, we have produced some preliminary results studying the molecular signatures of retrieval by checking ERK phosphorylation. It has been reported that MAPK/ERK are involved in different kinds of memory retrieval (Barros et al. 2000; Huang et al. 2010). In that sense, biochemical analyses of ERK expression in hippocampal samples of control animals compared to those exposed to LTM retrieval did not reveal differences between genotypes. Future experiments will help to elucidate possible differences in ERK phosphorylation during STM retrieval. In addition, a phosphorylation

of the transcription factor CREB has been reported in the basal and lateral nuclei of the amygdala during retrieval for cued fear memory (Hall et al. 2001). Therefore, it will also be interesting to study CREB phosphorylation after STM retrieval in the PKC gamma KO mice in both hippocampal and amygdala samples.

To further investigate the memory stage affected, we studied the c-Fos activation in the hippocampus as c-Fos expression has been demonstrated to be effective on predicting memory retrieval in mice in other memory tests (Lüscher Dias et al. 2016). We compared c-Fos expression following the exposure to different behavioral protocols. Preliminary results for c-Fos quantification in the dentate gyrus of the hippocampus revealed that WT mice exposed to the trace fear-conditioning present higher amounts of c-Fos activation than controls during memory acquisition. However, the amount of c-Fos activation in the PKC gamma KO mice was similar in both experimental conditions. These results open the possibility that PKC gamma KO animals present deficits in the acquisition of short-term hippocampal-dependent memories. Further c-Fos quantifications must be performed to study (i) other memory stages, particularly memory retrieval, (ii) other experimental conditions, such as mice exposed to the delayed fear-conditioning, to compare the results in the two forms of cue fear-conditioning and (iii) the amount of c-Fos activation in other brain regions, such as the amygdala.

Therefore, we hypothesized that STM deficits may be explained by (i) different signaling involving STM and LTM and/or (ii) a problem in STM acquisition or retrieval.

These results suggest that PKC gamma signaling is important for hippocampal-dependent STM and underline the different molecular mechanisms involved in short-term and long-term memory

processes. However, the specific memory stage affected for the lack of PKC gamma is still unknown.

b) Effect of low doses of THC in the memory performance of PKC gamma KO mice

In the last part of this thesis, we have studied the involvement of PKC gamma activity in THC amnesic-like effects on LTM by using the PKC gamma KO mice. An acute amnesic dose of THC (3 mg/kg, i.p.) did not affect LTM performance in PKC gamma KO mice in four different hippocampal-dependent tasks: the novel object-recognition test, the active avoidance test, the context recognition test and the delayed fear-conditioning. However, similarly to what was observed when short-term and long-term memory were studied in this mouse model, similar amnesic-like effects produced by THC were observed in WT and PKC gamma KO mice in a hippocampal-independent task, the delayed fear-conditioning. Object-recognition memory was also evaluated after a sub-chronic THC treatment. In this condition, PKC gamma KO mice were only sensitive to the amnesic-like effects of THC after 4 days of treatment while the WT mice were sensitive to THC amnesic-like effects from the first day of THC exposure. We suggest that the study of other PKC isoforms could be of interest in order to discard possible compensatory mechanisms.

THC administration to WT mice enhances in the hippocampus the phosphorylation of NMDAR1 subunits in Ser890 and Ser896, two residues modulated by PKC activity (Tingley et al. 1997) Since PKC gamma and NMDAR are mainly postsynapticaly expressed (Suen et al. 1998; Kose et al. 1990), while CB1R are largely presynaptic (Kawamura et al. 2006), a direct relation or interaction of all these players is unlikely. Instead, a possible explanation involve the fact

that CB1R are mainly localized on a subpopulation of GABAergic presynaptic terminals (Kawamura et al. 2006). CB1R activation by THC would reduce the GABAergic tone promoting an enhanced glutamatergic excitatory tone in the hippocampus (Puighermanal et al. 2009). This concomitant increase in the glutamatergic tone may lead to the activation of postsynaptic NMDAR, which would increase intracellular calcium. The activation of NMDAR is associated to the increase of postsynaptic PKC gamma activity (MacDonald et al. 2001). In turn, PKC phosphorylation of NMDAR would regulate their conductance or the expression of these receptors at the cellular membrane.

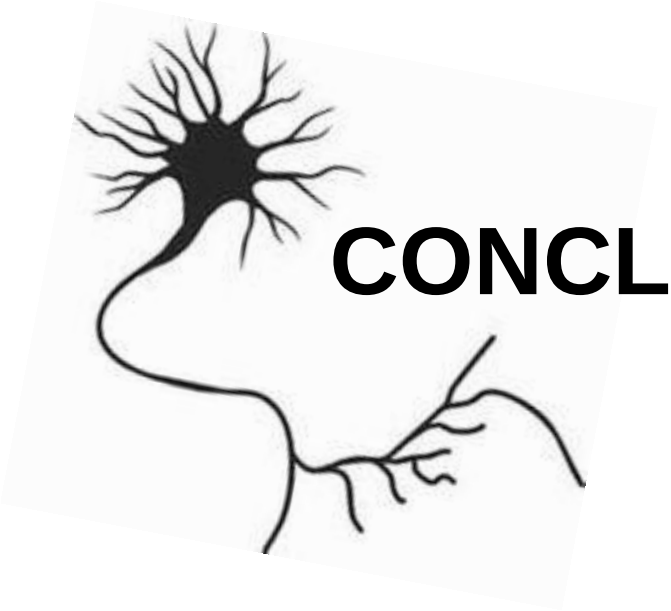
The over-activation of PKC gamma may increase the phosphorylation of other downstream substrates, which are also involved in the regulation of processes related to memory and synaptic plasticity. This hypothesis would explain the lack of memory impairing effects of THC in the PKC gamma KO mice.

Neurogranin, neuromodulin and MARCKS have been described as preferential substrates for the PKC gamma isoform (Ramakers et al. 1999), all of them related to synaptic plasticity. Consequently, an enhancement in PKC gamma activation will produce the phosphorylation of those preferential substrates. In the case of neurogranin and neuromodulin, their phosphorylation by PKC gamma would reduce their binding to calmodulin, which would drive to the activation of calmodulin-dependent processes. In the case of MARCKS phosphorylation, it would stimulate spine shrinkage and reduce the content of the filamentous form of actin (F-actin) (Calabrese and Halpain 2005). Our biochemical results demonstrated a significant increase in the phosphorylation levels of two of the preferential substrates of PKC gamma, neurogranin and

MARCKS, after acute THC treatment. However, no differences were observed in the phosphorylation of neuromodulin. This would indicate that the poll of PKC gamma activated after THC administration would be confined to the postsynaptic compartment considering the presynaptic localization of neuromodulin (Holahan and Routtenberg 2008) and the postsynaptic localization of neurogranin and MARCKS (Díez-Guerra 2010; Calabrese and Halpain 2005).

Interestingly, we have demonstrated that PKC gamma is not involved in two other pharmacological effects produced by THC. Indeed, no differences between genotypes were observed in the hypothermic and analgesic (studied in a model of visceral pain) effects of THC. Other actions of THC, such as the anxiolytic-like effects, were not studied since PKC gamma KO mice are less anxious than WT mice which would difficult the interpretation of the results (Bowers et al. 2000). Overall, these results demonstrate that PKC gamma isoform seems to be only a relevant isoform in the cognitive deficits produced by THC.

All together, our results show the crucial role of PKC gamma signaling in the hippocampal cognitive deficits produced by THC.



CONCLUSIONS

Conclusions

The main conclusions of the work presented in this thesis can be summarized as follows:

1. Low doses of the CB1R antagonist/inverse agonist rimonabant (0.1 mg/kg) are sufficient to resolve the cognitive deficit in the object-recognition memory test presented by a FXS mouse model, the *Fmr1 KO* mice, even when administered following an alternate-day protocol.

2. The same low dose of rimonabant normalizes, in the *Fmr1* KO mice, the altered hippocampal group I mGluR-LTD, a form of synaptic plasticity related with intellectual disability.

3. The subchronic treatment with the neutral CB1R antagonist NESS0327 also ameliorates the memory deficit in this FXS mouse model.

4. Exposure to an acute stressful event impairs the consolidation phase of a non-emotional memory through a peripheral response involving CB1R.

5. The memory impairment observed after acute stress exposure could also be prevented by the intrahippocampal administration of rimonabant demonstrating an interplay between peripheral and central processes.

6. CB1R in dopamine β-hydroxylase-expressing cells have been identified as necessary and sufficient for the memory impairment produced by stress.

7. Chelerytrine and NPC-15437, two PKC inhibitors, prevent the short-term memory deficit produced by THC revealing the involvement of PKC signaling in this amnesic-like effect of THC.

8. These PKC inhibitors do not prevent the long-term memory deficit produced by THC, an effect that is prevented by a mTORC1 signaling inhibitor.

9. Hippocampal PKC isoforms and the postsynaptic PKC substrate neurogranin are phosphorylated in the hippocampus after an acute dose of THC. These modulations are partially (PKC isoforms) or completely (neurogranin) reversed by the NPC-15437 pre-treatment.

10. PKC gamma KO mice present deficits in the performance of short-term memory hippocampal-dependent tasks. However, long-term memory is not modified in PKC gamma KO mice.

11. The deficits in short-term memory may be due to problems in the acquisition phase, as revealed by c-Fos studies using different memory paradigms. Further studies must be done to discard other possible alterations in short-term memory retrieval.

12. Two preferential substrates of PKC gamma, neurogranin and MARCKS, are modulated in the hippocampus after acute THC administration.

13. PKC gamma KO mice are less sensitive to the deleterious memory effects of a low dose of THC pointing to a critical role of this kinase in THC-induced cognitive deficits. No differences were observed between PKC gamma KO mice and WT in other pharmacological effects of THC.

This thesis has described novel key molecular events related with the impairment of different stages of memory processes, and the role that the ECS plays in the modulation of this brain function.2 bh

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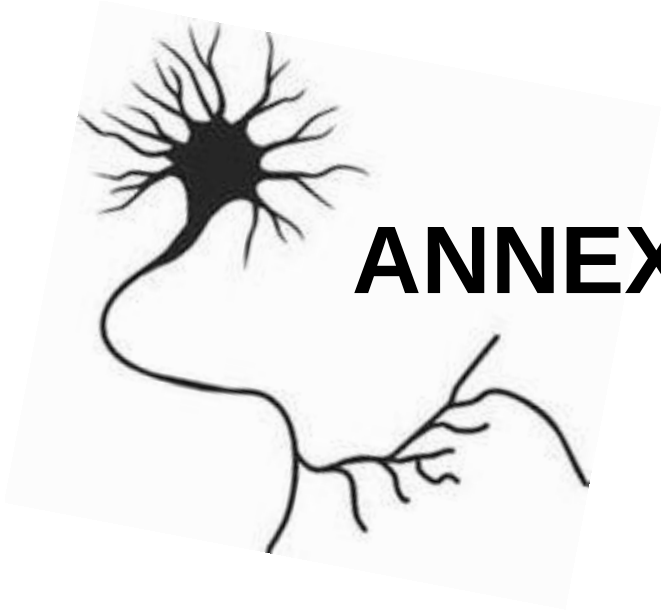

ANNEX

ARTICLE 1

Targeting the endocannabinoid system in the treatment of fragile X syndrome

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ARTICLE 2

Dissociation of the pharmacological effects of THC by mTOR blockade

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ARTICLE 3

Microglial activation underlies cerebellar deficits produced by repeated cannabis exposure

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ARTICLE 4

An improved technique to study sociability and preference for social novelty in mice

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(In preparation)

This article will be presented in the thesis of Sara Martínez

An improved technique to study sociability and preference for social novelty in mice

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Abstract

Background: Studying social behavior in mouse models empowers the understanding of the neurobiological mechanisms involved in this task, which is affected in neuropsychiatric disorders, allowing the evaluation of therapeutic strategies.

New Method: We validated a new reliable and sensitive method to study social behavior (sociability and preference for social novelty) in different mouse models using a modified version of the V-shaped maze.

Results: Using this novel procedure, we characterized the social performance of two mouse strains commonly used in biomedical research, the CD1 outbreed strain and the C57BL/6J inbreed strain. In addition, this approach revealed significant differences in the social behavior of two mouse models of genetic disorder: fragile X syndrome and Williams-Beuren syndrome.

Comparison with Existing Method: The V-maze for sociability and preference for social novelty improves time performance and reduces variability compared to the classical approach, the three-chamber apparatus.

Conclusions: Altogether, the V-maze allows evaluating the specific alterations of social behavior in mice in a time-efficient and reproducible manner.

Keywords: social behavior, mouse model, autism, sociability, preference for social novelty.

1. Introduction

Social behaviors are important in numerous species to establish the networks and relationships that define social communities (Berry et al., 1992). Among those behaviors, sociability is defined as the tendency to seek out social interaction (Caldwell, 2012). Some neuropsychiatric disorders, such as autism spectrum disorders (ASD) display a marked alteration in sociability combined with other features (American Psychiatric Association, 2013). This characteristic highlights the need for experimental behavioral settings in animal models to address the research on such complex multi-facetted disorders (Caldwell, 2012). The wide repertoire of mouse behaviors makes this specie suitable for modeling human disorders characterized by disruptions in social recognition and social behavior (Crawley, 2004; Yang et al., 2007). Such rodent models warrant the evaluation of potential therapeutic approaches for treatment (Moy et al., 2004).

Fragile X syndrome (FXS) is the most common monogenic cause of ASD (Hannan, 2010), and is caused by the lack of expression of the *FMR1* gene (Verkek et al., 1991). FXS patients display hyperactivity, attention deficits, intellectual disability and social behavior

disturbances (Symons et al., 2010). In this regard, the mouse model for FXS, the *Fmr1* knockout (*Fmr1* KO) mouse (Bakker et al., 1994) shows social deficits (Liu et al., 2009), which have been studied in the search for pharmacological normalization.

Social behavior might also be genetically predisposed to an outgoing personality. This is the case of Williams-Beuren syndrome (WBS). WBS is a rare disorder caused by a recurring spontaneous deletion of a sensitive region about 1.55-1.83 Mb in length containing 25-28 genes in chromosome band 7q11.23 (Pérez-Jurado, 2003; Schubert, 2009). Notably, several mouse models of WBS, among those one bearing a hemizigous removal of the 26 genes commonly deleted in WBS (complete deletion WBS, CD), reproduce the hypersocial behavior (Segura-Puimedon et al., 2014). Altogether, mouse models become important approaches to provide robust preclinical tools for understanding the biological basis of the behavioral traits in complex disorders and facilitate the development of potential treatment strategies.

Several paradigms have been described to measure social behavior in mouse models (Silverman et al., 2010). Among those, the most common is the Crawley's sociability test also called three-chamber

apparatus (Chadman et al., 2008; McFarlane et al., 2008; Moy et al., 2008). We have adapted a V-shaped maze (V-maze), previously used successfully to evaluate object-recognition memory (Puighermanal et al., 2009; Busquets-Garcia et al., 2011; Busquets-Garcia et al., 2013), to assess social behavior. We found that this new procedure provides an advantageous approach to reveal particular social phenotypes in different mouse lines such a reduction on the time performance or the variability of the results obtained.

2. Material and methods

2.1. Animals

C57BL/6J and CD1 mice (Charles River Laboratory) were used as experimental mice at 3-4 months of age. *Fmr*1 KO mice in C57BL/6J and FVB.129 backgrounds with their respective WT mice were used as mouse models of FXS at 3-4 months of age. *Fmr1* KO mice in C57BL/6J congenic background (B6.129P2-Fmr1tm1Cgr/J) were obtained from the Baylor College of Medicine Mouse Facility. *Fmr1* KO mice in FVB background (FVB.129P2-Pde6b+ Tyrc−chFmr1tm1Cgr/J) and WT mice (FVB.129P2-Pde6b+ Tyrc−ch/AntJ) were purchased from The Jackson Laboratory and crossed to obtain *Fmr*1 KO and WT littermates. Heterozygous CD (complete deletion; Gtf2i-Fkbp6) (Segura-Puimedon et al., 2014) mice with their respective WT littermates in 97% C57BL/6 background were used as a mouse model of WBS at 3-4 months of age. Juvenile (4 weeks old) male C57BL/6J mice (Charles River Laboratory) were used as stranger mice.

Mice were housed four per cage and maintained in standard environment conditions of temperature (21ºC ± 1ºC) and humidity

(55% ± 10%) with food and water *ad libitum*. All the experiments were performed during the light phase of the dark/light cycle (lights on at 8 a.m. and off at 8 p.m.). Before starting the experiment, mice were habituated in the experimental room and handled for 1 week. All animal procedures followed standard ethical guidelines (European Communities Directive 86/60-EEC) and were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB). The PRBB also has Animal Welfare Assurance (#A5388-01, Institutional Animal Care and Use Committee approval date 06/08/2009) granted by the Office of Laboratory Animal Welfare (OLAW) of the US National Institutes of Health. Behavioral tests were performed by researchers unaware of the different experimental groups.

2.2. Equipment Setup

2.2.1. V-maze. We used a modified version of the V-shaped maze (V-maze, Busquets-Garcia et al., 2011). It consists of two structures: the maze wall (150 mm high), made of black Plexiglas (**Fig. 1**), and the maze lead, made of transparent Plexiglas (**Fig. 1**). Corridors in the V-maze are 300 mm long and 45 mm wide (internal

measures). Two small chambers (65 mm long) were created at the end of the corridors when the lead was inserted into the V-maze. These chambers were used to allocate the juvenile stranger mice (**Fig. 1**).

The design was deliberately simple to enhance the exploratory activity of the experimental mouse under analysis, and to facilitate cleaning between sessions.

2.2.2. Three-chamber maze. It consisted in a rectangular box made of Plexiglas (600 mm wide x 405 mm long x 150 mm high) divided in three-identical-chambers (200 mm wide x 405 mm long x 150 mm high) by two Plexiglas walls containing small openings, which measure 100 mm wide x 50 mm high, to allow mouse access between chambers. The measures of each chamber were 200 mm (length) x 405 mm (width) x 220 mm (height). The juvenile stranger mice were enclosed in a round wire cage in the side chambers. The wire cage was 110 mm high x 105 mm diameter and vertical bars spaced 10 mm, which allow sniffing and exploration. There was a weighted cup on top of the wire cage to prevent the experimental animal from climbing.

2.3. Procedure

The mazes (V-maze and three-chamber maze) were used in a sound-attenuated room with dim illumination 4-7 lux. A digital camera on top of the maze was used to record the sessions. On-line image in a contiguous room was available to the observer through a close-circuit camera situated on top of the maze. All three phases of the social test (habituation, sociability and preference for social novelty) were performed consecutively (**Fig. 2**). V-maze phases lasted 5 min, compared to the three-chamber maze where sessions lasted 10 min, and exploratory behavior was computed at 5 min and 10 min. In a set of experiments objects substituted juvenile mice.

2.3.1. Habituation (Phase I)

Experimental mice were introduced into the central part of the V-maze for 5 min, where they freely explored the empty chambers at the end of the corridors. The experimenter recorded the exploration time for each chamber analyzing the image obtained by the closed-circuit camera. During the habituation phase, mice explored similarly both compartments. This measurement also informs about the activity of the mouse in the maze.

2.3.2. Sociability (Phase II)

The sociability session was performed just after the habituation session. In this phase, a juvenile mouse assigned as stranger-1 is placed in one of the chambers at the end of the corridors (both corridors were alternated during the experiments). The experimental mouse is allowed to explore both compartments for 5 min. The experimenter records the time that the experimental mouse spends exploring each chamber. In normal conditions, mice will prefer exploring the chamber containing the stranger mouse in comparison with the empty one. At the end of the sociability session the subject and stranger-1 are maintained in the V-maze to start the last phase of the test**.**

2.3.3. Preference for social novelty (Phase III)

The preference for social novelty phase is performed just after the sociability session, when a second novel mouse, assigned as stranger-2, is placed inside the previously empty chamber, while the stranger-1 remains inside the same chamber as in phase II. During 5 min, the experimental animal is allowed to explore the two strangers.

After this phase, animals are removed from the apparatus and returned to their home cage. Then, the maze is cleaned with 30% ethanol (vol/vol) to avoid odor cues between different subjects.

The experimenter records the time that the mouse spends exploring each chamber. In control conditions, mice will prefer to explore the chamber with the stranger-2 (social novelty), compared to the exploration of already familiar stranger-1.

2.4. Data analysis

Exploration was considered when the experimental mouse directed his nose in close proximity (1 cm) to the vertical bars at the end of the corridors (Yang et al., 2011). In each phase the time exploring both chambers (whether they are empty, or they hold a juvenile stranger mouse, or an object) was noted.

The mean time spent exploring both chambers were used to perform ANOVA repeated measures followed by Newman's Keuls *post hoc* test or Student's t test analysis. Comparisons were considered statistically significant when the level of significance was < 0.05.

3. Results

3.1. The V-maze as a new setting to characterize social behavior in mice

The V-maze has been successfully used to measure cognitive responses with the novel object-recognition test (Puighermanal et al., 2009; Busquets-Garcia et al., 2011; Busquets-Garcia et al., 2013). We hypothesized that the V-maze setting would improve those results obtained in the three-chamber maze for a social task. Therefore, we compared the exploration times in both settings, the V-maze and the three-chamber maze, using an inbreed and an outbreed mouse strain (C57BL/6J and CD1 mice, respectively). In order to compare time efficiency between mazes, both strains were analyzed for 5 min in the V-maze (**Fig. 3, A and C**), and for 5 min (**Fig. 3, B and D**) and 10 min (**Suppl. Fig. 1**) in the three-chamber maze. The V-maze setting provided quite similar results in the habituation session (Phase I) for both strains (**Fig. 3, A and C**). These results were variable for CD1 mice in the three-chamber maze (**Fig. 3D**). In the sociability phase (Phase II), both mouse strains demonstrated a significant preference ($P < 0.001$) for the chamber

containing the juvenile stranger-1 compared to the empty chamber (**Fig. 3 and Suppl. Fig. 1**). Such preference was evident in the 5 min period in both mazes. Finally, on the 5 min session to assess preference for social novelty (Phase III), only mice analyzed in the V-maze, independent of their strain, showed a significant predilection ($P < 0.001$) for exploring the new-unfamiliar juvenile mouse (stranger-2) compared to the now-familiar mouse (stranger-1) (**Fig. 3 and Suppl. Fig. 1**). C57BL/6J mice took longer to show a clear preference for social novelty in the three-chamber maze since such preference was only apparent after 10 min (**Fig. 3B** and **Suppl. Fig. 1A**).

3.2. Reduced preference for social novelty in the FXS mouse model

The aim of this experiment was to ascertain the social phenotype in the murine model of FXS using the V-maze setting. We used the *Fmr1* KO mutation in two different backgrounds, inbreed C57BL/6J and mixed FVB.129 background. We compared the social behavior of *Fmr1* KO mice to that of their WT littermates (**Fig. 4**).

As expected, there were no significant differences on the time mice spent exploring the empty chambers during Phase I (**Fig. 4. A and D**). In Phase II, WT and *Fmr1* KO mice in both genetic backgrounds

spent more time exploring the stranger-1 than the empty chamber ($P < 0.001$) **(Fig. 4. B and E).**

Data obtained on Phase III showed that *Fmr1* KO mice in C57BL/6J background (**Fig. 4C**) had a preference for exploring the unfamiliar mouse (stranger-2) ($P < 0.05$), but spent more time exploring stranger-1 than WT mice ($P < 0.05$). In agreement, *Fmr1* KO mice in FVB.129 background (**Fig. 4F**) did not show preference for the stranger-2 and spent less time exploring the novel mouse than WT mice did.

Overall, these results reveal a reduced preference for social novelty in both mouse models of FXS.

3.3. WBS mouse model shows increased social behavior independent of the novelty preference

The WBS model (CD) was analyzed in comparison to their littermate controls (WT) in the V-maze to characterize the social phenotype. Both CD and WT similarly explored the empty compartments on Phase I **(Fig.** 5A). Interestingly, CD mice spent more time sniffing the side with stranger-1 than WT did in Phase II ($P < 0.001$) (**Fig. 5B**). In addition, during the Phase III, CD mice did not show significant preference for social novelty since the time spent

exploring both strangers (stranger-1 and stranger-2 was similar ($P = 0.16$). Instead, the WT littermates showed the expected social novelty preference ($P < 0.05$) (**Fig. 5C**).

To further support the specificity of the social behavior analyzed in the V-maze for WBS mice, we analyzed the responses of CD and WT mice using the same 3-phase procedure but with unanimated objects instead of stranger mice inside the chambers (**Fig. 5, D-F**). In this case, both CD and WT mice spent comparable times exploring the novel object-1 in Phase II (**Fig. 5E**), and discriminated between novel object-2 and now-familiar object-1 in Phase III (**Fig. 5F**). These data discarded a potential bias related with novelty discrimination in Phase III and revealed that the behavior is motivated by social stimuli.

4. Discussion

In this study we present a new and improved approach, the V-maze sociability test, to measure social behavior in mouse models. Using this approach that we validate in comparison to the three-chamber maze, we demonstrate the characteristics of social behavior in five mouse models, three of them models of two genetic disorders with altered social traits.

Commonly, mice conserve a characteristic pattern of social behavior, initiating social contact and approach when exposed to an unfamiliar conspecific (Moy et al., 2004). We have demonstrated that this behavioral pattern is detected in the V-maze sociability test. The V-maze (without the lead) had been previously used to evaluate novel object-recognition memory in mice (Puighermanal et al., 2009; Busquets-Garcia et al., 2011; Puighermanal et al., 2013). This approach also allowed revealing the cognitive deficit in *Fmr1* KO mice (Busquets-Garcia et al., 2013), or in murine models of Alzheimer's disease (Aso et al., 2015). We hypothesized that the V-maze arrangement could improve also social testing.

In order to ascertain the advantages of our novel approach, we performed the social assessment, under the same experimental

conditions (lighting, room environment, experimental mouse strain and stranger strain, as well as overall procedure) using the V-maze and the three-chamber maze, the standard approach in this type of social paradigm in rodents (Moy et al., 2004). Interestingly, the social behavior in C57BL/6J and CD1 mice, the inbred and outbreed strains most frequently used in biomedical research, was surprisingly similar in 5 min sessions when using the V-maze, compared with the results obtained in the three-chamber maze at 5 or 10 min. Since the overall results obtained with 5 min sessions were robust, we concluded that the V-maze approach was more time efficient than the three-chamber approach.

We standardized the stranger mice to be juvenile (4 weeks old) C57BL/6J mice. In this regard, other authors have also observed that there is no apparent effect of the strain of stranger mouse on the sociability and on the preference for social novelty of experimental mouse under study (Nadler et al., 2004). Although it is reasonable to suppose that odors, appearance and behavioral responses of the stranger would influence the social behavior of the experimental subject, our results show that the exploration times obtained with different strains such as C57BL/6J, CD1, and those WT littermates

for the different mouse models of disorder in this study, were alike demonstrating in all cases a preference for mate encounter and for social novelty.

Disruption in social behavior is a tremendous setback in specific neuropsychiatric disorders. Deficits in social interaction and communication have become fundamental symptoms to diagnose ASD (American Psychiatric Association, 2013). Therefore, a reproducible model of social interaction in mice allows approaching, in experimental settings, the assessment for potential treatments, as well as the study of the biological mechanisms underlying social behavior. The *Fmr1* mutation was studied in two backgrounds, C57BL/6J and FVB.129. This mutation in both strains showed less preference for social novelty, while they did not show deficits in sociability. This phenotype is in agreement with that described previously (Liu et al., 2009), but does not fit with other reports (Heitzer el al., 2013; McNaughton et al., 2008; Mines et al., 2010). These discrepancies might be due to the different experimental conditions in each study (age of the experimental mice, age of the stranger mice or whether experimental mice were reared in isolation

or group housed). Such discrepancies warrant further studies in more controlled conditions.

In the other side of the sociability spectrum, WBS individuals also show a characteristic hypersocial behavior (Järvinen-Pasley et al., 2008). We analyzed the social behavior of a mouse model of WBS, the CD model. This model had been found to be hypersociable in a different experimental setting to assess sociability (Segura-Puimedon et al., 2014). Under our experimental conditions, CD mice spent more time than WT controls exploring the stranger mice, and performed similar to WT mice when strangers were substituted by objects in the same V-maze. This result allows discarding visual-spatial biases or enhanced novelty preference in CD mice. Interestingly, and using the three-chamber maze, the hyper-sociable phenotype in WBS has been related with the heterozygous expression of *Gtf2i* (Dai et al., 2009). Notably, rescuing the expression of *Gtf2i* through gene therapy has been shown to normalize the hypersocial phenotype in the CD mouse model (Borralleras et al., 2015), pointing to the interest of the behavioral paradigms assessing social phenotypes.

In conclusion, we propose the V-maze sociability test as a new paradigm to evaluate social behavior in mice, added to the benefits

of its suitability for cognition. We used this test to reveal the altered sociability in two strains of a mouse model of autism, the *Fmr1* KO model, and the hypersociability of a recently described model of WBS, the CD mouse. The new test described in this report affords the advantage in time efficiency and reproducibility, reducing the variability and strengthening the accuracy of the experimental results. Altogether, the V-maze is a versatile and reliable setting to study social behavior.

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Sponsors were not involved in study design, collection, analysis and interpretation of data, writing of the report or in the decision to submit the article for publication.

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Figures

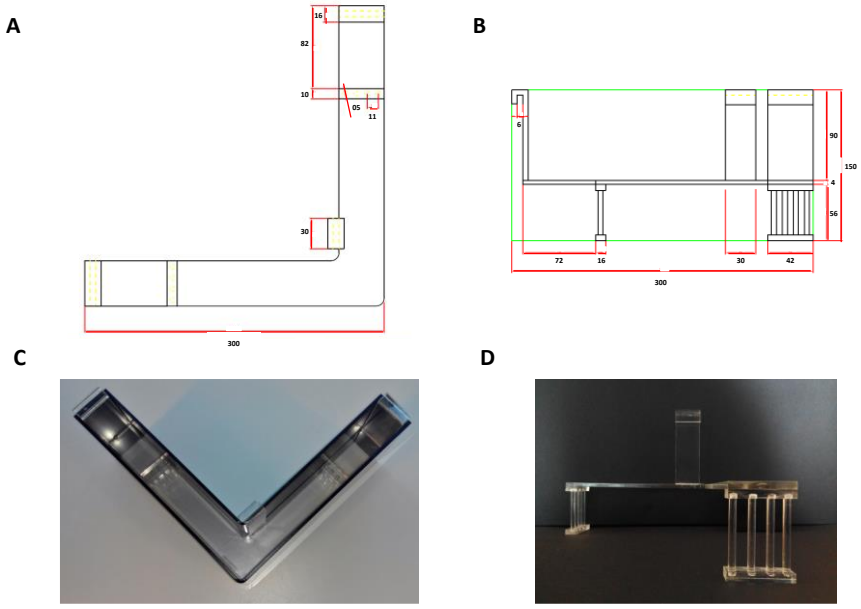


Figure 1. Design of the V-maze. Top view (**A** and **C**) and side view (**B** and **D**) of the V-maze and the Plexiglas transparent lead showing its dimensions (in mm). Photographs show the V-maze with the transparent lead (**D**), and a detail of the lead which includes the Plexiglas bars for mice to interact (**D**).

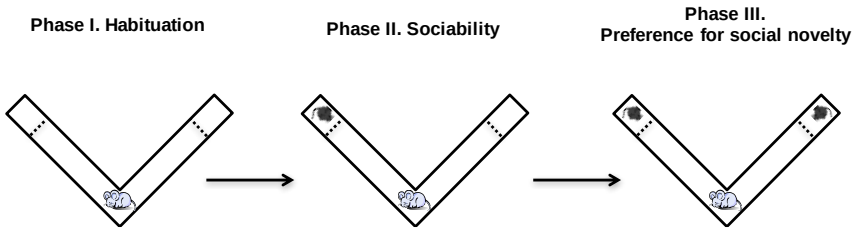


Figure 2. Scheme of the sociability test. There are three different phases: Phase I (habituation), Phase II (sociability) and Phase III (preference for social novelty). Each phase lasts 5 min. During this time the exploration of the experimental mouse towards the chambers at the end of the corridors was recorded under the three conditions: both chambers empty (Phase I), stranger-1 vs. empty chamber (Phase II) and stranger-1 vs. stranger-2 (Phase III).

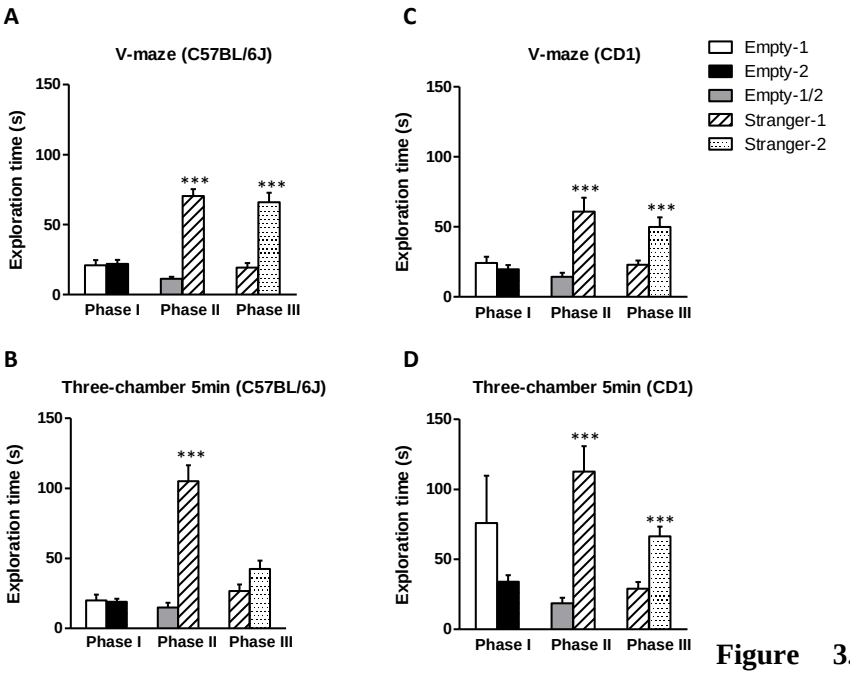


Figure 3. Comparison of strain behavior in the sociability assay using the V-maze and the three-chamber maze. Exploratory behavior of C57BL/6J mice (Phase I: n=8, t(14)=0.865; Phase II: n=8, t(14)=0.000; Phase III: n=8, t(14)=0.000) (**A**) and CD1 mice (Phase I: n=7, t(12)=0.080; Phase II: n=7, t(12)=0.000; Phase III: n=7, t(12)=0.003) (**B**) in the V-maze sociability test was recorded during 5 min per phase. Total exploration time of C57BL/6J mice (Phase I: n=6, t(10)=0.836; Phase II: n=6, t(10)=0.000; Phase III: n=6, t(10)=0.07) (**C**) and CD1 mice (Phase I: n=8, t(14)=0.240; Phase II: n=8, t(14)=0.000; Phase III: n=8, t(14)=0.000) (**D**) in the three-chamber maze during the first 5 min of each phase (data obtained in the same sessions after 10 min are depicted in **Suppl. Fig. 1**). Statistical significance was calculated by Student's t test. Data are expressed as mean ± s.e.m. ***P < 0.001 (compartment comparison).

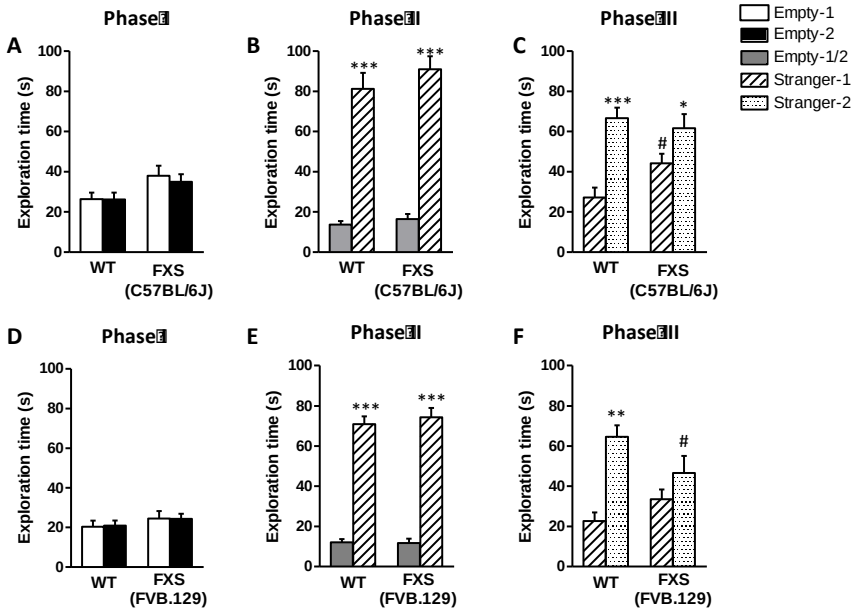


Figure 4. Two different strains of the FXS model produced the same social phenotype. Exploratory behavior of *Fmr1* KO and WT mice in C57BL/6J background (n =11-14; genotype: F(1,23)=3.196, P=0.088; chamber side: F(1,23)=0.843, P=0.371; interaction: F(1,23)=0.703, P=0.411) (**A**) (n=11-14; genotype: F(1,23)=1.087, P=0.308; chamber side: F(1,23)=248,654, P=0.000; interaction: F(1,23)=0.590, P=0.450) (**B**) (n=11-14; genotype: F (1,23)=0.826, P=0.373; chamber side: F(1,23)=37.082, P= 0.000; interaction: F(1,23)=5.526, P=0.028) (**C**) and FVB.129 background (n=8-9; genotype: F(1,15)=0.429, P=0.522; chamber side: F(1,15)=0.0001, P=0.993; interaction: F(1,15)=0.008, P=0.928) (**D**) (n=8-9; genotype: F(1,15)=0.236, P=0.634; chamber side: F(1,15)=242.612, P=0.000; interaction: F(1,15)=0.231, P=0.638) (**E**) (n=8-9; genotype: F(1,15)=0.380, P=0.547; chamber side: F(1,15)=18.678, P=0.0006; interaction: F(1,15)=5.121, P=0.039) (**F**) were analyzed for 5 min on each phase. Statistical significance was calculated by repeated measures ANOVA comparison followed by Newman's Keuls *post hoc* test. Data are expressed as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (chamber side comparison). $^{\#}$P < 0.05 (genotype comparison).

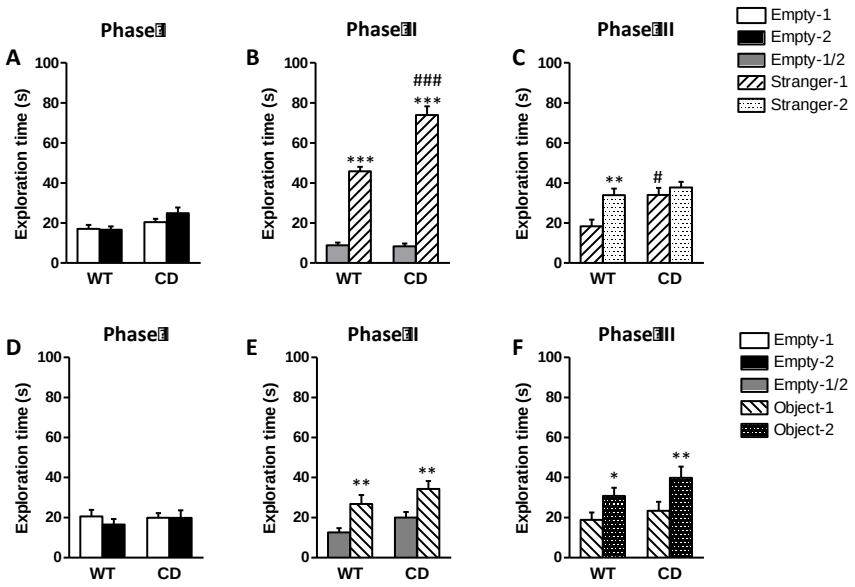
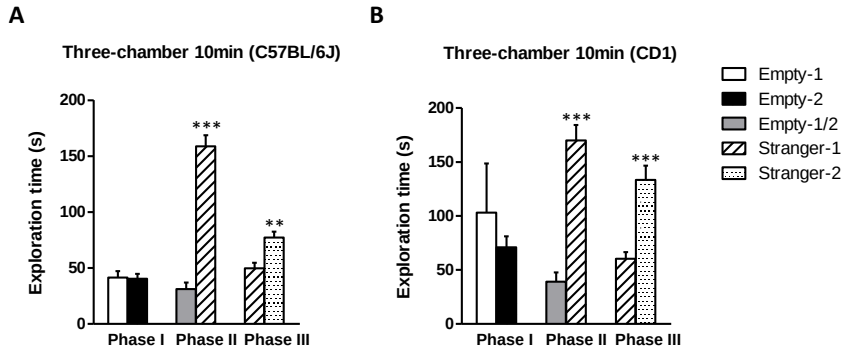


Figure 5. CD mice show a hypersocial phenotype that is not related to novelty. Exploratory behavior of CD and WT mice were analyzed for 5 min on each phase (n=10-11; genotype: F(1,19)=8.193, P=0.01; chamber side: F(1,19)=0.847, P=0.369; interaction: F(1,19)=1.344, P=0.261) (**A**) (n=10-12; genotype: F(1,20)=32.532, P=0.000; chamber side: F(1,20)= 282.045, P=0.000; interaction: F(1,20)=23.708, P=0.000) (**B**) (n=11-12; genotype: F(1,21)=6.806, P= 0.016; chamber side: F(1,21)=13.499, P= 0.001; interaction: F(1,21)=5.046, P= 0.035) (**C**). Exploratory behavior of CD and WT mice when novel objects were used instead of stranger mice (n=8-12; genotype: F(1,18)=0.001, P=0.975; chamber side: F(1,18)=0.993, P=0.332; interaction: F(1,18)=0.896, P=0.356) (**D**) (n=8-12; genotype: F(1,18)=2.877, P= 0.107; chamber side: F(1,18)=37.943, P= 0.000; interaction: F(1,18)=0.000, P= 0.999) (**E**) (n=8-12; genotype: F(1,18)=1.553, P=0.229; chamber side: F(1,18)= 24.674, P=0.0001; interaction: F(1,18)=0.605, P=0.447) (**F**). Exploratory behavior was analyzed during 5 min on each phase. Statistical significance was calculated by repeated measures ANOVA comparison followed by Newman's Keuls *post hoc* test. Data are expressed as mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001 (compartment comparison). $^{\#}$P < 0.05 (genotype comparison).

Supplementary Figures



Supplementary Figure 1. Total exploration time during the three phases of the three-chamber social test in 10 min. Exploratory behavior of C57BL/6J mice (phase I: n=6, t(10)=0.142; phase II: n=6, t(10)=0.000; phase III: n=6, t(10)=0.003) (**A**) and CD1 mice (phase I: n=8, t(14)=0.690; phase II: n=8, t(14)=0.000; phase III: n=8, t(14)=0.000) (**B**) in the three-chamber social test during the 10 min of each phase. Statistical significance was calculated by Student's t test. Data are expressed as mean ± s.e.m. **P < 0.01; ***P < 0.001 (compartment comparison).