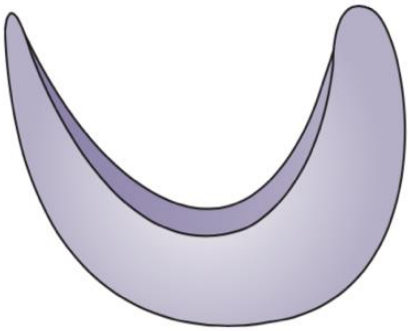

Ceci n'est pas un hippocampe

Ana Margarida Pinto da Fonseca Almeida Ribeiro

The role of hippocampal A_{2A} receptors in the generalization of conditioned fear

Dissertação de Mestrado em Biologia Celular e Molecular, orientada pela Doutora Paula Margarida Gomes Canas e pelo Professor Doutor Ângelo José Ribeiro Tomé e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

June 2018

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The role of hippocampal A_{2A} receptors in the generalization of conditioned fear

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O papel dos recetores A_{2A} para a adenosina do hipocampo na generalização do medo condicionado

Dissertação apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada pela orientação científica da Doutora Paula Margarida Gomes Canas (Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e do Professor Doutor Ângelo José Ribeiro Tomé (Departamento de Ciências da Vida, Universidade de Coimbra).

Coimbra, Junho de 2018

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Table of contents

List of figures

List of tables

Abbreviations

ACC Anterior cingulate cortex

ACSF Artificial cerebrospinal fluid

ADP Adenosine 5'-diphosphate

AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

ANOVA Analysis of variance

AR Adenosine receptor

A_1R Adenosine A_1 receptor

A_{2A}R Adenosine A_{2A} receptor

A_{2B}R Adenosine A_{2B} receptor

A_3R Adenosine A_3 receptor

ATP Adenosine 5'-triphosphate

CA *Cornu ammonis*

CaMKII Calcium/calmodulin-dependent kinase II

CS Conditioned stimulus

CNS Central nervous system

D_1R Dopamine receptor D1

D_2R Dopamine receptor D2

DAPI 4',6-diamidino-2-phenylindole

DG Dentate gyrus

dHPC Dorsal hippocampus

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DSM-5 *The Diagnostic and Statistical Manual of Mental Disorders*, fifth edition

EC Entorhinal cortex

fEPSP Field excitatory post-synaptic potential

GABA gamma-aminobutyric acid

GABAR gamma-aminobutyric acid receptor

GAD65 glutamic acid decarboxylase, 65 kDa isoform

HDAC histone deacetylase

HPC Hippocampus

IHC Immunohistochemistry

ITI Inter-trial intervals

LFS Low frequency stimulation

LG Licking/Grooming

LTD Long-term depression

LTP Long-term potentiation

mPFC Medial prefrontal cortex

mGluR Metabotropic glutamate receptor

NMDAR N-methyl-D-aspartate receptor

PBS Phosphate-buffered saline

PFA Paraformaldehyde

PFC Prefrontal cortex

PKA Protein kinase A

PKC Protein kinase C

PP2B Protein phosphatase 2B

PTSD Post traumatic stress disorder

RT Room temperature

SC Schaffer collateral cells

SCH 58261 5-amino-7-(2-phenylethyl)- 2- (2- furyl)- pyrazolo[4,3-e]- 1,2,4- triazolo-[1,5-c] pyrimidine

SEM Standard error of the mean

TSPT Transtorno de stresse pós-traumático

US Unconditioned stimulus

vHPC Ventral hippocampus

Abstract

Post-traumatic stress disorder (PTSD) is a condition that can develop in individuals exposed to an environmental, psychologically traumatic event. This disorder occurs in 5 to 10% of the population, ranking as the fourth most common psychiatric disorder in the world. A central feature of PTSD is over-generalized fear, which consists in the transference of fear from a particular stimulus to another one sharing similarities with the original stimulus. Research in this field is therefore gaining traction with many efforts being directed towards figuring out what are the behavioural and neural mechanisms of fear generalization.

Deficits in the hippocampus (HPC)-mediated pattern separation (that is to say the disability of the HPC to discriminate between two similar contexts) have been proposed to be the main process underlying the increase in fear generalization that occur as the memory ages.

Besides its already known role in emotional responses, the ventral HPC (vHPC) has been recently implicated in spatial memory, an ability manifested by its capacity to assemble the contextual information collected by the dorsal HPC (dHPC). However, although impairments of the vHPC have been suggested to be implicated has a putative mechanism underlying fear generalization, uncovering the exact hippocampal synaptic plasticity processes underlying fear generalization is still a challenge.

Despite the fact that long-term depression (LTD) in the CA1 hippocampal sub-region has been associated with contextual novelty exploration, no association between this type of synaptic plasticity and fear generalization has been dissected yet.

Regarding the adenosine system, A_{2A}Rs have been implicated in memory performance. Such an evidence has been correlated with the capacity of A_{2A}Rs to modulate long-term potentiation (LTP), under physiological and pathological conditions. Recently, a capacity of A_{2A}Rs to modulate LTD has been revealed under pathological conditions, in the dorsal-medial hippocampus. However, the role of ventral and dorsal A_{2A}Rs on LTD is still to be explored. Additionally, a substantial body of evidence suggests a therapeutic interest in A_{2A}Rs to manage stress and fear-related pathologies.

Thus, the present study aims to evaluate the hippocampal synaptic plasticity mechanisms that might explain fear generalization to later on, explore the involvement of A_{2A}Rs.

Taken together our data reveal an implication of the vHPC in generalized contextual fear memory – manifested by a disability of this region to produce LTD – while it did not reveal the dHPC as a possible structure implicated in contextual fear generalization.

Furthermore, we have shown that ventral and dorsal A_{2A}Rs have no effect on the LTD amplitude under physiological situations, however a gain-of-function of A_{2A}Rs to modulate LTD in such regions is revealed under pathological conditions, namely, in fear generalization. In detail, the acute blockade of A_{2A}Rs was able to rescue the LTD synaptic impairments observed in ventral slices from the animals that generalized their fear, indicating that A_{2A}Rs could indeed have a prominent role in fear generalization.

Despite the importance of biomarkers to help in the prevention, diagnosis and treatment of many diseases no biomarker for PTSD has been uncovered yet. Here, our results appear to suggest that alterations in the GABAergic system in vHPC could be related with fear generalization since the animals that generalized their fear seem to have a decrease in gephyrin immunoreactivity. Thus, this current work strongly proposes a therapeutic interest of using antagonists of A_{2A}Rs against fear generalization and suggests that gephyrin could be a promising synaptic biomarker for fear generalization in the vHPC.

Keywords: Fear generalization, ventral hippocampus (vHPC), long-term depression (LTD), A_{2A} receptors (A_{2A}Rs), gephyrin.

Resumo

O transtorno de stresse pós-traumático (TSPT) é uma doença psiquiátrica que pode desenvolver-se em indivíduos expostos a eventos psicologicamente traumáticos. Este transtorno mental ocorre em 5 a 10% da população, estando classificado como a quarta doença psiquiátrica mais comum em todo o mundo. Uma característica chave do TSPT é a sobre- generalização do medo, que consiste na transferência de um medo associado a um estímulo em particular para outro que partilhe semelhanças com o estímulo original.

A investigação nesta área tem direcionado os seus esforços na tentativa de descortinar quais os mecanismos comportamentais e neurológicos por detrás da sobre-generalização do medo.

Défices no processo de separação de padrões mediado pelo hipocampo (HPC) – incapacidade do HPC para descriminar entre dois contextos semelhantes – têm sido propostos como sendo o principal processo subjacente ao aumento da generalização do medo ao longo do tempo.

Além do seu conhecido papel nas respostas emocionais, o HPC ventral (vHPC) tem sido implicado na memória espacial, uma habilidade manifestada pela sua capacidade de agregar a informação contextual recolhida pelo HPC dorsal (dHPC).

Contudo, apesar de défices no desempenho funcional do vHPC serem apontados como uma possível explicação para a generalização do medo, a identificação dos mecanismos de plasticidade sináptica ao nível do hipocampo por detrás da generalização é ainda um desafio.

Apesar do processo de depressão de longa duração (LTD, do inglês *long-term depression*), na sub-região CA1 do HPC, ter sido associado à codificação de detalhes espaciais num novo contexto, nenhuma relação entre este tipo de plasticidade sináptica e a generalização do medo foi ainda postulada.

Em relação ao sistema adenosinérgico, os A_{2A}Rs têm sido implicados no desempenho da memória. Tal evidência tem sido correlacionada com a capacidade dos A_{2A}Rs em modular a potenciação de longa duração (LTP, do inglês *long- term potentiation*), em condições fisiológicas e patológicas. Recentemente, uma capacidade dos A_{2A}Rs para modular a LTD foi revelada sob condições patológicas, no hipocampo dorsal-medial. No entanto, o papel dos A_{2A}Rs ventrais e dorsais na LTD permanece inexplorado. Além disso, um corpo substancial de evidências sugere um interesse terapêutico nos A_{2A}Rs para gerir patologias relacionadas com stresse e medo.

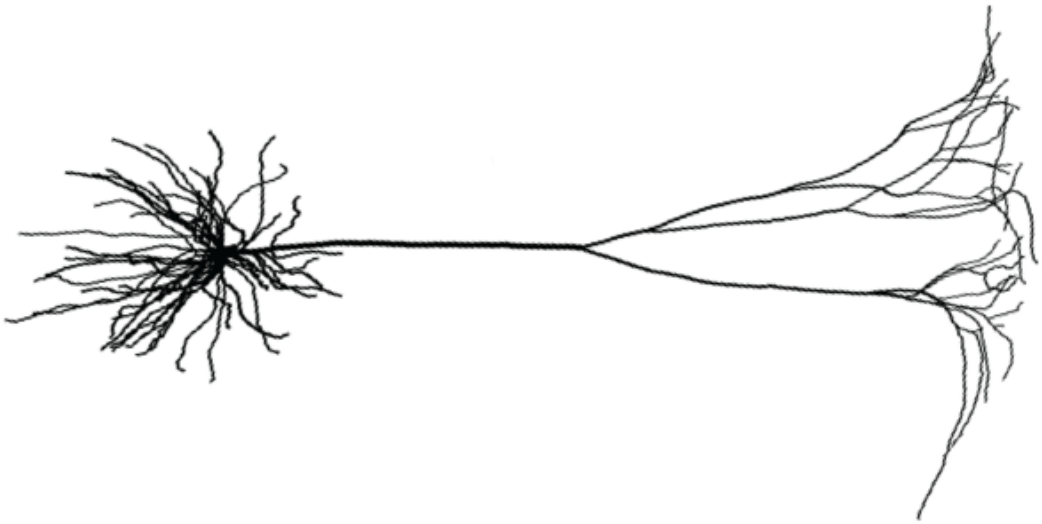
Assim, o presente estudo tem como objetivo principal avaliar os mecanismos de plasticidade sináptica do hipocampo que poderão explicar a generalização do medo para, mais tarde, explorar o envolvimento dos A_{2A}Rs.

Os nossos dados revelaram uma influência do vHPC na generalização de memórias contextuais relacionadas com o medo – manifestada pela incapacidade desta região em produzir LTD – enquanto não apontam o dHPC como um possível interveniente na generalização do medo contextual. Mais ainda, demonstrámos que os A_{2A}Rs ventrais e dorsais não estão envolvidos na LTD em situações fisiológicas, contudo um ganho-de-função destes recetores em modular a LTD nestas regiões foi revelado em condições patológicas, nomeadamente, na generalização do medo. Em detalhe, o bloqueio agudo dos A_{2A}Rs permitiu resgatar os défices sinápticos da LTD observados em fatias ventrais de animais que generalizaram o medo, indicando assim que os A_{2A}Rs podem de fato ter um papel proeminente na generalização do medo.

Apesar da importância do uso de biomarcadores para auxiliar na prevenção, diagnóstico e tratamento de várias doenças, não se conhece ainda nenhum biomarcador para TSPT. Nesse sentido, os nossos resultados parecem sugerir que alterações do sistema GABAérgico no vHPC podem estar associadas à generalização de medo, uma vez que os animais que generalizaram o medo parecem apresentar uma diminuição na imunoreactividade de gefirina no vHPC. Deste modo, tendo em vista uma aplicação terapêutica, este trabalho propõe fortemente o uso de antagonistas dos A_{2A}Rs para travar a generalização de medo, sugerindo ainda a gefirina como um biomarcador sináptico promissor da generalização do medo no vHPC.

Palavras-chave: Generalização do medo, hipocampo ventral (vHPC), depressão de longa duração (LTD), recetores A_{2A} para a adenosina (A_{2A}Rs), gefirina.

Chapter 1



Introduction

1.1- Some basic concepts – the ABC of neuropsychiatric disorders

In order to understand the clinical differences between psychiatric mood disorders and, consequently, why they are grouped in one way and not in another, it is first necessary to understand some basic concepts.

Are stress, anxiety and fear clinically different?

Although these three terms are typically used interchangeably in everyday conversations, they are, actually, clinically different since the states that they describe are very distinctive.

1.1.1- Stress *vs.* anxiety

Contrary to popular belief, there are differences between stress and anxiety existing, in fact, distinct animal models that are used to study anxiety disorders and stress (Campos *et al.*, 2013).

Stress is a response to one or more specific stressors, mainly, external pressures on us that are hard to cope, causing an unbalance of homoeostasis and, consequently, requiring a physical and physiological response to restore equilibrium (Johnson *et al.*, 1992; Sapolsky, 1996; Bremner, 1999).

There are two forms of stress: the eustress (the "good" type of stress) and the distress (the "bad" type). The eustress is "good" since it is a biological advantage, that occurs as a result of the exposure to short-term and controllable stressors, allowing us to adapt to new situations and making us achieve our goals, without imposing a burden on health. Thus, eustress is beneficial since it promotes adaptation to challenges and major life stressors, by stimulating the release of stress hormones and other mediators of allostasis, like cortisol and adrenaline, and its symptoms typically disappear after the stressful situation is over, thus being a temporary experience (figure 1) (McEwen, 2004, 2007 & 2012).

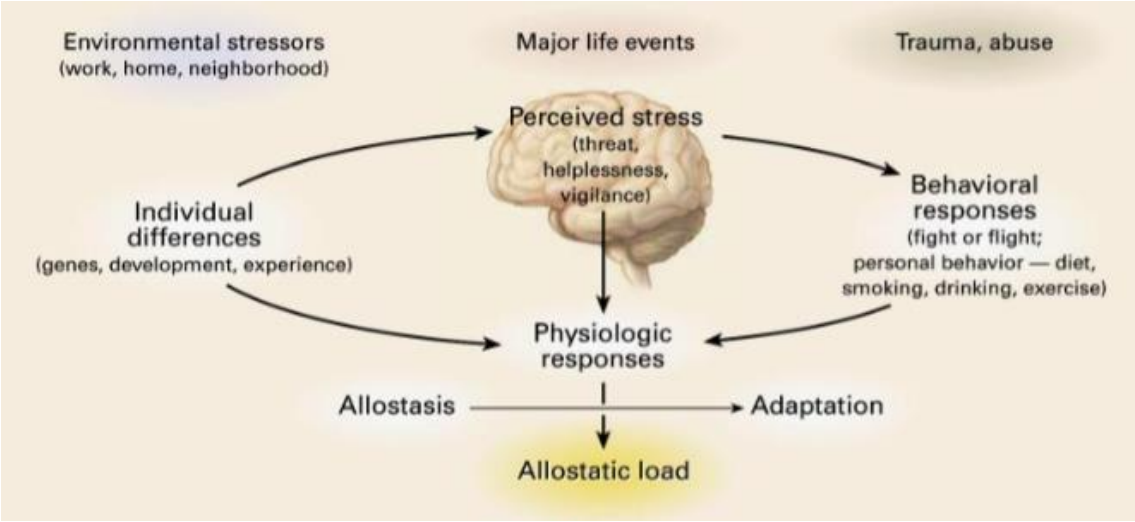


Figure 1- Central role of the brain in allostasis and the behavioural and physiological response to stressors (from McEwen, 2007).

However, when the stressor persists for a long time throughout life (chronic stressor), acting as a homoeostatic disruptor, affecting our health and leading to anxiety and mood disorders, we are facing distress (Shaw, 2003; Schneiderman *et al.*, 2005; Sotiropoulos *et al.*, 2008; Campos *et al.*, 2013).

Consequently, whereas stress is a particularly major risk factor for neuropsychiatric disorders (such as major depression and anxiety disorders) and is aetiologically causal in post-traumatic stress disorder (PTSD), anxiety is established as one of the negative and adverse effects of stress (McEwen, 2004; Schneiderman *et al.*, 2005; McEwen, 2012; Sharma *et al.*, 2015).

1.1.2- Fear *vs*. Anxiety

Although these two states may overlap, anxiety is more often associated with vigilance in preparation for future danger, cautious or avoidant behaviours and muscle tension, and fear is frequently associated with surges of autonomic arousal necessary for fight or flight thoughts of immediate danger, and escape behaviours.

From a clinical and more precise point of view, anxiety is the anticipation of a future threat, whereas fear is the emotional response to real or perceived imminent threats.

It is important to retain that although fear learning is an evolutionarily advantageous response mechanism aimed at survival in the face of life threatening circumstances, when fear becomes excessive it becomes a serious problem impairing the quality of life of the individual and may lead to devastating psychiatric consequences (DSM- 5; Am. Psychiatr. Assoc., 2013).

1.2- Neuropsychiatric disorders

In 2013, the most recent edition (fifth edition) of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) created a new category titled trauma and stressor-related disorders in addition to the already existing anxiety disorders (ADs) and obsessive-compulsive and related disorders categories. (DSM-5; Am. Psychiatr. Assoc., 2013; Calhoon & Tye, 2015).

1.2.1- Trauma-and stressor-related disorders

In the past, diseases like PTSD belonged to the group of ADs but as of the 2013 edition of DSM, they now belong to the trauma and stressor-related disorders.

Nevertheless, although PTSD and other disorders related with traumatic incidents are usually characterized by a phenotype of excessive fear and anxiety, the psychological distress following exposure to a traumatic or stressful event is in fact quite variable, with many individuals exhibiting anhedonic and dysphoric symptoms and externalizing angry and aggressive or dissociative signs rather than anxiety or fear-based symptoms, or both.

Thereby, since these disorders present variable and heterogeneous phenotypes, the fifth edition of the DMS has drawn a distinction between stress and anxiety, separating trauma and stressor-related disorders, such as PTSD, from anxiety disorders.

Nowadays trauma and stressor-related disorders cover PTSD, reactive attachment disorder, disinhibited social engagement disorder, acute stress disorder and adjustment disorders, with exposure to a traumatic or stressful event being an imperative diagnostic criterion to diagnose a person with one disease from this subgroup of disorders (DSM-5; Am. Psychiatr. Assoc., 2013).

1.2.2- Post-traumatic stress disorder (PTSD)

PTSD is a debilitating condition that can be developed in individuals exposed to a psychologically traumatic environmental event, such as interpersonal violence, combat, life-threatening accidents and natural disasters (Pitman *et al.*, 2012; Yehuda *et al.*, 2015; Howlett & Stein, 2015; Rutten *et al.*, 2017).

PTSD is the most predominant psychopathological consequence of the exposure to traumatic events and occurs in 5-10% of the general population (Sundin *et al.*, 2014;

Yehuda *et al.*, 2015; Shalev *et al.*, 2017). Thanks to Vietnam veterans and their advocates, PTSD finally made its way into the American psychiatric nomenclature as a formal diagnostic entity in 1980.

Nowadays, according to the DSM-5, in addition to exposure to a stressor, PTSD requires four clusters of symptoms that have to persist for at least 1 month: intrusive re-experiencing of the trauma, avoidance of stimuli associated with the trauma, negative cognition and mood associated with the trauma, and excessive arousal or reactivity (table 1) (DSM-5; Am. Psychiatr. Assoc., 2013; Howlett & Stein, 2015; Smoller, 2016; Shalev *et al.*, 2017). In more than 50% of the cases, PTSD co-occurs with mood or anxiety disorders, substance abuse, impulsive or dangerous behaviour or self-harm disorders, which contribute to the severity of the disease (Kessler & Wang, 2008; Yehuda *et al.*, 2015).

Table 1- DSM-5 criteria for PTSD (Yehuda *et al.*, 2015).

Criterion*	Description	Specific examples	Requirements	Compared with DSM-IV
Criterion A	Exposure to stressor	• Direct exposure • Witnessing trauma • Learning of a trauma • Repeat or extreme indirect exposure to aversive details	DSM-5 recognizes that exposure to trauma can occur either by direct or indirect confrontation with extreme trauma	Specific definition of details of the stressor needed, including repeated experience or extreme exposure to details of events
Criterion B	Intrusion symptoms	• Recurrent memories • Traumatic nightmares • Dissociative reactions (flashbacks) • Psychological distress at traumatic reminders • Marked physiological reactivity to reminders	At least one of these five examples is required	No change, but further clarification of the dissociative quality of flashbacks needed
Criterion C	Persistent avoidance	• Trauma-related thoughts or feelings • Trauma-related external reminders such as people, places or activities	At least one of these two examples is required	DSM-IV did not separate the avoidance criterion
Criterion D	Negative alterations in cognitions and mood	• Dissociative amnesia • Persistent negative beliefs and expectations • Persistent distorted blame of self or others for causing trauma • Negative trauma-related emotions: fear, horror, guilt, shame and anger • Diminished interest in activities • Detachment or estrangement from others • Inability to experience positive emotions	At least two of these seven examples are required	DSM-IV noted social estrangement and restricted the range of affect; numbing redefined to positive rather than all affects
Criterion E	Alterations in arousal and reactivity	• Irritable and aggressive behaviour • Self-destructive and reckless behaviour • Hypervigilance • Exaggerated startle • Problems concentrating • Sleep disturbance	At least two of these six examples are required	Self-destructive and risk-taking behaviours were not defined in DSM-IV
Criterion F	Duration	Must experience criteria B, C, D and F for >1 month	Acute stress disorder is diagnosed for symptoms occurring for <1 month post trauma	No change
Criterion G	Functional significance	Impairment in social, occupational or other domains	Disability in at least one of these domains is required	No change
Criterion H	Exclusion	Not attributable to medication, substance use or other illness	Symptoms must not be secondary to other causes	Not stated in DSM -IV
Subtypes	• Dissociative subtype: used when depersonalization and derealization occur in tandem with other symptoms described above. • Delayed subtype: used to describe the emergence of symptoms following a period post trauma in which symptoms were not present or were present at a subthreshold level.			

Altered fear acquisition (over-consolidation), abnormalities in fear extinction, abnormalities in threat detection systems and/or fear over-generalization constitute the four most robust conditioning correlates of PTSD (Liberzon & Abelson, 2016).

The conditional probability to develop this disorder varies according to the intensity

and number of traumatic events. However, this evidence does not explain two of the most critical current questions: why do some trauma victims develop PTSD whereas others experiencing the same trauma appear to be resilient, and why those who develop PTSD vary widely in their symptom severity and in the type of symptoms they experience. Currently, it is well established that although trauma exposure is the precipitating event for PTSD to develop, genetic and environmental risk factors (such as social support network and early life experiences) also have a huge impact in the development of this disorder, indicating that PTSD is a polygenic disorder (Mahan & Ressler, 2012) (figure 2).

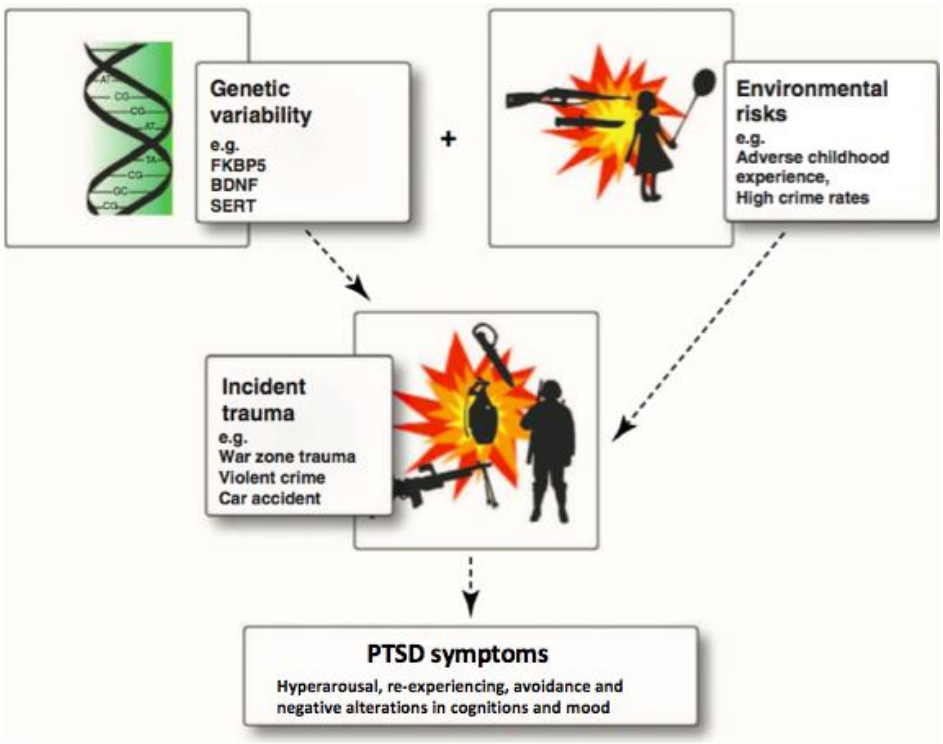


Figure 2- Risk factors to develop PTSD as well as the four clusters of symptoms of PTSD (adapted from Mahan and Ressler, 2012).

Current therapies for PTSD include psychological, pharmacologic, and innovative interventions. However, despite many existing treatments for PTSD alleviating symptoms they rarely induce remission, allowing for a substantial risk of relapse on discontinuation (Shalev *et al.*, 2017).

Therefore, a better understanding of the genetics and underlying neural molecular mechanisms of PTSD will hopefully lead to more successful treatments and to better predictions of which individuals might be more susceptible to develop PTSD (Mahan & Ressler, 2012).

1.2.2.1- Neurocircuits behind PTSD

To improve and develop more specific strategies for PTSD treatment, a thorough understanding of the neural circuits behind this disorder is crucial. In this sense, many efforts have been done to find out what are the neural system abnormalities thought to be responsible for the development and/or maintenance of PTSD.

It is very unlikely that any psychiatric disorder can be fully explained by dysfunction of a single neurobiological circuit (Liberzon & Abelson, 2016). Actually, dysfunctionalities in four specific neural circuits have already been identified to play a part in the PTSD psychopathology: the neurocircuits related with fear learning, threat detection, function and emotion regulation, and the neurocircuit related with contextual processing (figure 3) (Shalev *et al.*, 2017). Each circuit has distinguished focus in different aspects of the disorder, explaining a different subset of biological abnormalities or PTSD phenotypes (Liberzon & Abelson, 2016; Shalev *et al.*, 2017).

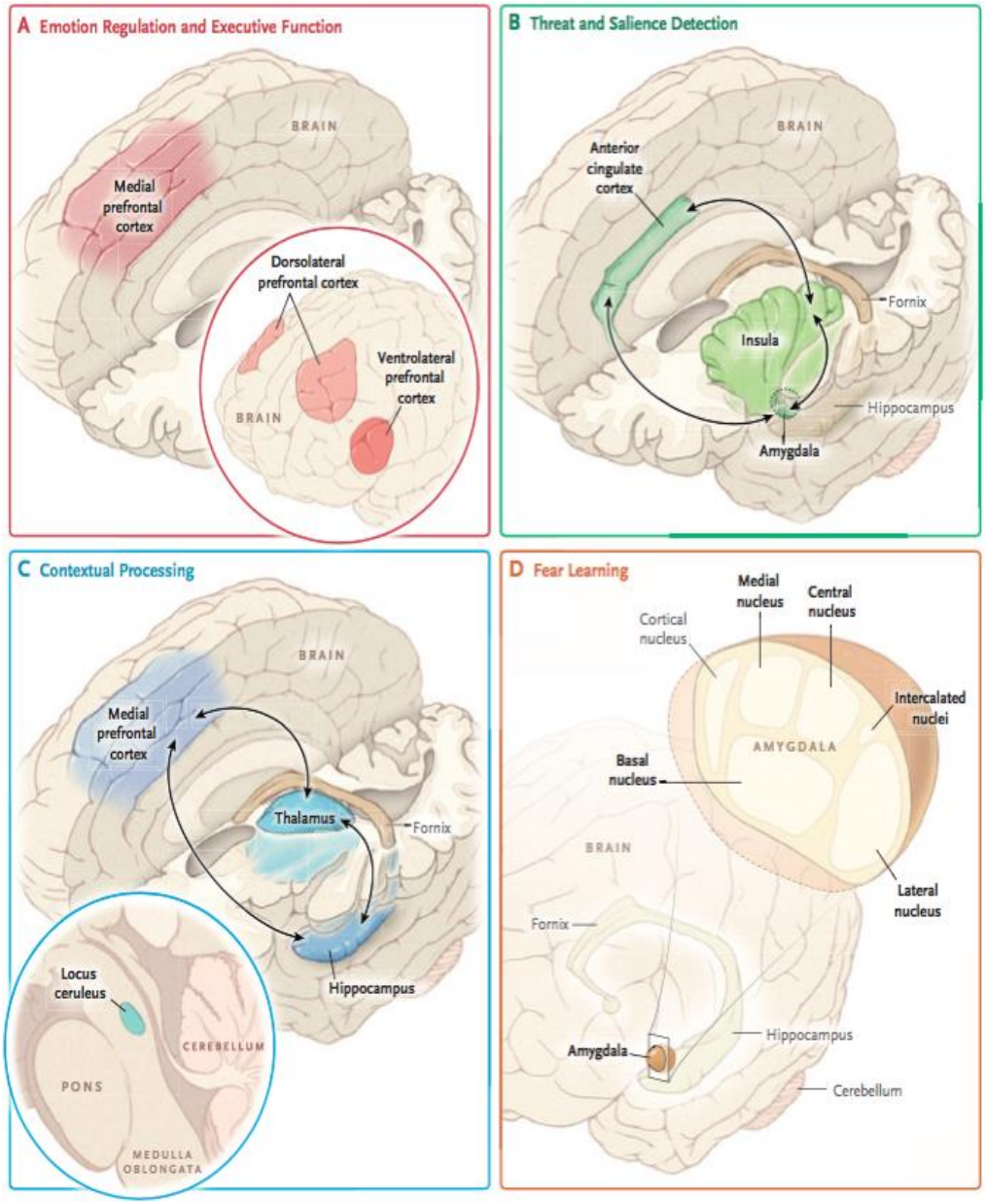


Figure 3- The known connectivity paths within four dysfunctional circuits that play a part in the psychopathology of PTSD: emotion regulation and executive function, threat detection, contextual processing, and fear learning (Shalev *et al*., 2017).

Despite their differences, all these circuits share similarities between them, since they overlap in identifying the hippocampus (HPC), amygdala and prefrontal cortex (PFC) as the most clearly altered structures involved in PTSD (Liberzon & Abelson, 2016).

Briefly, the earliest neuroimaging and neurochemistry studies in PTSD have focused on hippocampal dysregulation since deficits in memory performance and information processing were observed in patients with PTSD (Pitman *et al*., 2012; Yehuda *et al*., 2015). Since then, great interest was generated around the HPC due to pioneering structural magnetic resonance imaging (sMRI) studies finding that there are significantly smaller hippocampi in subjects with PTSD compared to trauma-exposed and non-

trauma-exposed (Gurvits *et al*. 1996; Bremner *et al*., 1997). Such a smaller HPC volume has been shown to reflect a risk factor for PTSD (Gilbertson *et al*., 2002; Pitman *et al*., 2012) and, consequently, a vulnerability factor in the persistence of this disorder (Van Rooij *et al.*, 2015).

Subsequent research projects expanded the focus from the HPC to other regions and have identified alterations in the amygdala and medial prefrontal cortex (mPFC) structures in patients with PTSD (Shin & Liberzon, 2010; Pitman *et al*., 2012; Yehuda *et al*., 2015; Liberzon & Abelson, 2016). For instance, functional neuroimaging studies have reported diminished prefrontal inhibition of fear circuitry (Lanius *et al.*, 2003; Etkin & Wager, 2007; Gold *et al*., 2011; Pitman *et al*., 2012) and exaggerated amygdala activation in PTSD patients (Liberzon *et al*., 1999; Etkin & Wager, 2007; Pitman *et al*., 2012). In agreement, a large number of studies have shown a volume reduction in prefrontal brain regions in individuals with PTSD (Kasai *et al*., 2007; Carrion *et al*., 2010).

In conclusion, an abnormal interaction of these three systems has been recognised to underlie PTSD, postulating that the reduced activation of the PFC and HPC could be intimately correlated with the increased amygdala activity. In detail, since the amygdala receives projections from the HPC and PFC it is possible that, as the result of the reduction of its activity, these two structures fail to control amygdala activity, resulting in a reduced top-down control of such structure, that ultimately leads to a hyper-responsive amygdala signal to fearful stimuli (Elzinga & Bremner, 2002; Rauch *et al*., 2006; Pitman *et al*., 2012). Consequently, since the amygdala has a crucial role in modulating fear processing and fear expression (Davis, 1992; Davis *et al*., 1994; Campeau & Davis, 1995), exaggerated amygdala activation eventually results in disrupted fear regulation in PTSD.

1.3- Hippocampus

The HPC, a medial temporal lobe structure, is a structure essential for contextual learning and episodic memory – a type of memory that is characterized by representations of relations between events and the context in which they were experienced (Scoville & Milner, 1958; Holland & Bouton, 1999; Strange *et al*., 2014; Eichenbaum, 2017).

1.3.1- Hippocampus (HPC) – hippocampal anatomy and circuit

The HPC is a small but complex anatomical structure that together with dentate gyrus (DG), the subicular complex and the entorhinal cortex (EC) forms the hippocampal formation (Schultz & Engelhardt, 2014).

Meaning *seahorse* due to its shape, the HPC exhibits a long and curved form, with its longitudinal axis being defined as ventrodorsal in rodents and anteroposterior in primates, and the same basic intrinsic circuitry is conserved across species (figure 4) (Strange *et al*., 2014).

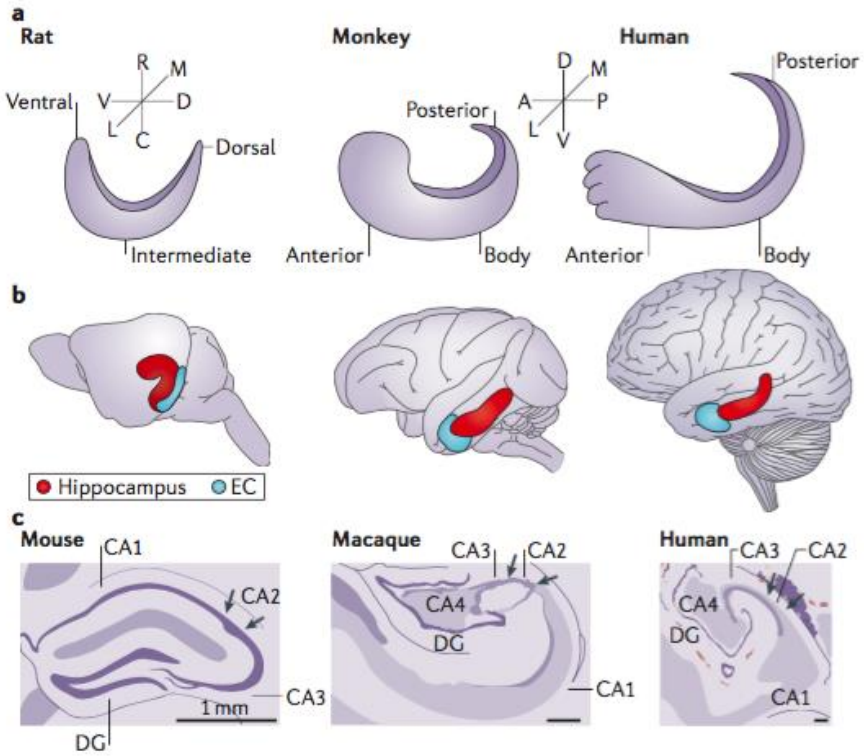


Figure 4- Cross-species comparison of hippocampal anatomy: **a)** Representation of the orientation of the hippocampal long axis in rats, macaque monkeys and humans; **b)** Representation of the hippocampus (red) and entorhinal cortex (EC) (blue) localization in rat, macaque monkey and human brains; **c)** Representation of the basic hippocampal circuit in mouse, rhesus and human brain (Strange *et al.*, 2014).

In brief, the HPC can be divided along its curve into four different *Cornu Ammonis* (CA) fields or regions, namely, CA4, CA3, CA2, and CA1 that are all filled with densely packed pyramidal cells (the main excitatory neurons of the HPC) (Agster *et al*., 2013; Schultz & Engelhardt, 2014).

Regarding the hippocampal connectivity, the intrinsic flow of hippocampal information occurs in a sequential, largely unidirectional and glutamatergic (excitatory) manner that ultimately forms part of a closed circuit called trisynaptic loop (Schultz & Engelhardt, 2014) (figure 5).

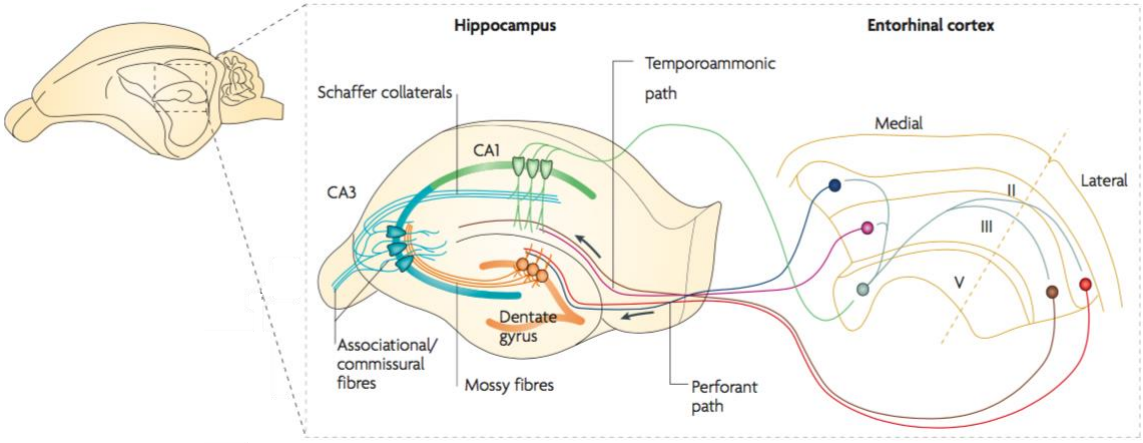


Figure 5- Illustration of the hippocampal trisynaptic loop (Adapted from Neves *et al*., 2008).

Succinctly and in a simple way, the EC gives rise to projections to all constituents of the HPC, with its strongest projections occurring via the perforant path to the DG region (Synapse 1 – entry point of the trisynaptic pathway). Subsequently, the DG projects to the CA3 region via the mossy fibre pathway (Synapse 2) and then CA3 projects to the CA1 region via the Schaffer Collaterals cells (SC) (Synapse 3). Finally, CA1 projects back to the entorhinal cortex, completing the loop (Ramón & Cajal, 1911; Amaral & Witter, 1989; Neves *et al.*, 2008; Agster *et al*., 2013; Knierim, 2015).

1.3.2- Synaptic plasticity at CA1 hippocampal sub-region

One of the most fascinating and important properties of the mammalian brain is its remarkable ability to modify, in an activity-dependent way, the strength/effectiveness of synaptic transmission in pre-existing synapses – a process known as **synaptic plasticity** (Hebb, 1949; Hughes, 1958; Malenka, 1994; Bliss & Cooke, 2011; Malinow & Malenka, 2002; Lüscher & Malenka, 2012).

This capacity to change the strength of synaptic communication has been an object

of intense investigation over the last three decades since this phenomenon is widely believed to underlie learning and memory formation (Hebb, 1949; Lisman, 1989; Alkon & Nelson, 1990).

Between the many forms of activity-dependent synaptic plasticity already identified, **long-term potentiation** (LTP) – in which the efficacy of synaptic transmission is enhanced – and **long-term depression** (LTD) – which results in a persistent downgrading of synaptic transmission – have received the focus of attention as cellular models of long- term information storage in the central nervous system (CNS), being the best described forms of synaptic plasticity in the brain (Malenka, 1994; Bliss & Cooke, 2011; Lüscher & Malenka, 2012) .

The most common types of LTP and LTD, in the HPC and throughout the CNS, are the NMDA receptor (NMDAR)-dependent forms (the canonical ones) (Malinow & Malenka, 2002; Bliss & Cooke, 2011) although it is clear that there are a wide range of other types of LTP/LTD that do not rely upon the NMDARs, such as a LTD form that is induced through activation of the metabotropic glutamate receptor (mGluR) (Kemp & Bashir, 1999; Huber *et al*., 2000).

Since LTP and LTD have opposite impacts in synaptic strength, it is not surprising that the molecular mechanisms underlying these processes are distinctive as well, with NMDAR-dependent forms of LTD resulting, in a large part, from a reversal of the processes that mediate LTP (figure 6). Current evidence suggests that the specific properties of intracellular calcium signalling (concentration and temporal profile), achieved by the activation of NMDARs, dictate whether LTP or LTD is generated, with LTD requiring a modest but prolonged rise in calcium, and LTP requiring a brief yet large rise beyond some critical threshold values (Winder & Sweatt, 2001; Malinow & Malenka, 2002; Bliss & Cooke, 2011; Lüscher & Malenka, 2012).

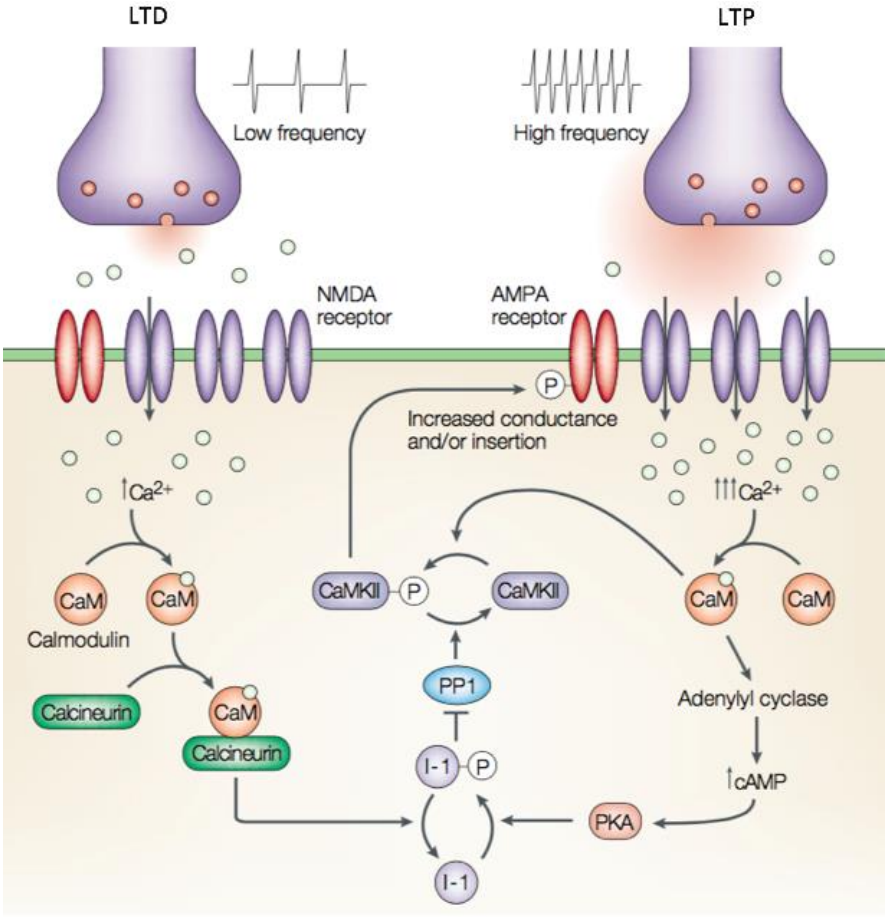


Figure 6- Schematic illustration of postsynaptic expression mechanisms of LTP and LTD (Winder & Sweatt, 2001).

1.3.2.1- Long-term potentiation (LTP)

LTP is the most studied form of activity-dependent plasticity and results in a persistent enhancement of synaptic transmission (Hebb, 1949; Bliss & Lømo, 1973; Malinow & Malenka, 2002).

As mentioned already, the induction of an NMDA-dependent form of LTP requires the influx of large amounts of calcium through NMDARs. This is accomplished by strong activity of presynaptic neurons which leads to a pronounced depolarization that, consequently, relieves the Mg^{2+} block of NMDARs ultimately allowing for the influx of calcium. As a consequence, the robust rise in calcium activates intracellular signalling cascades involving activation of Ca^{2+}/calmodulin-dependent protein kinases, such as calcium calmodulin-dependent kinase 2 (CaMKII), protein kinase A and C (PKA and PKC, respectively), that when activated phosphorylate other proteins that are involved in the expression of LTP such as AMPA receptors (AMPARs) (increasing channel conductance) and promotes the insertion of AMPARs in the plasma membrane, thus enhancing the synaptic transmission (reviewed in Malinow & Malenka, 2002; Lüscher & Malenka, 2012) .

1.3.2.2- Long-term depression (LTD)

LTD is another form of synaptic plasticity tightly related to memory performance and consists in an activity-dependent reduction of the efficacy of neuronal synapses (Dudek and Bear, 1992; Malenka, 1994).

NMDAR-dependent LTD at SC-CA1 synapses is normally generated by prolonged (3 to 15 minutes) low-frequency (1 – 5 Hz) afferent stimulation or by a pairing protocol whereby, during low-frequency activation of axons (0.1 – 1 Hz), individual cells are held at depolarized membrane potentials. Because at resting membrane potentials the driving force for Ca^{2+} entry is very large and the voltage-dependent block of NMDARs by Mg^{2+} is not 100% effective, the application of low-frequency stimulation protocols is capable of allowing the entrance of a moderate amount of Ca^{2+} (the exact trigger for LTD), by affording a modest and repetitive activation of NMDARs (Malenka, 1994; Bliss & Cooke, 2011; Malinow & Malenka, 2002).

If LTP involves the activation of protein kinases, and LTD represents the inverse of LTP, a reasonable hypothesis is that LTD is caused by preferential activation of protein phosphatases as a consequence of modest increases in Ca^{2+} influx. In brief, the small rise in Ca^{2+} favours the preferential activation of calcium/calmodulin-dependent phosphatase calcineurin (also known as protein phosphatase 2B or PP2B) because calcineurin has a much higher affinity for calcium/calmodulin than does CaMKII. Then, PP2B indirectly increases the activity of protein phosphatase 1 (PP1), that once activated leads to, for example, the internalization of synaptic AMPARs, thereby decreasing the synaptic transmission (reviewed in Bliss & Cooke, 2011; Lüscher & Malenka, 2012).

1.4- How to study PTSD? Animal models to study PTSD

Stress, anxiety and fear are normal emotions with great adaptive value that are conserved across most vertebrate species (Calhoon & Tye, 2015). Therefore, since PTSD is usually characterized by a phenotype of excessive fear and anxiety, this raises the possibility to study the mechanisms of PTSD in other mammals (mainly rodents), by using fear and anxiety models/paradigms (figure 7).

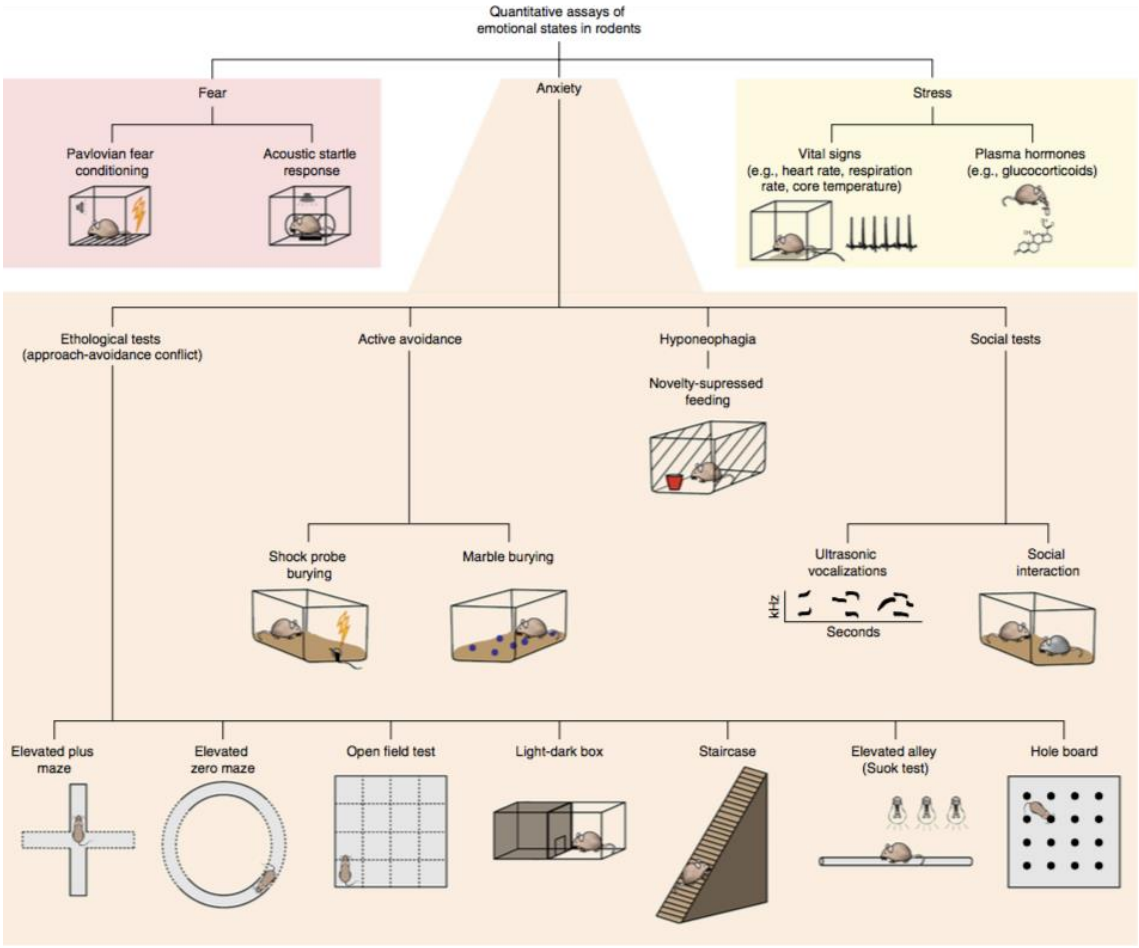


Figure 7- Validated tests to assay anxiety (beige), fear (pink) and stress (yellow) (Calhoon & Tye, 2015).

Actually, the development of animal models of stress, anxiety or fear have produced a significant contribution to the discovery of new drugs and the understanding of the neurobiological mechanisms behind psychiatric diseases.

Nevertheless, it is important to realise that a model cannot reproduce all features of a psychiatric disorder, but rather generate an emotional state that could be related to the disorder under investigation (Campos *et al*., 2013).

1.4.1- Fear paradigms

1.4.1.1- Pavlovian fear conditioning or classical conditioning

Classical conditioning or Pavlovian fear conditioning is a commonly employed method to study fear responses underlying traumatic memories, such as PTSD (Graff *et al.*, 2014).

Pavlovian fear conditioning experiments (figure 8) consist in an associative learning process that teaches an animal or human to associate a neutral conditional stimulus (CS) – such as a specific tone, light or context– with an aversive unconditioned stimulus (US) – such as an electrical footshock. To achieve this goal a CS is repeatedly paired with an US (Campos *et al.*, 2013).

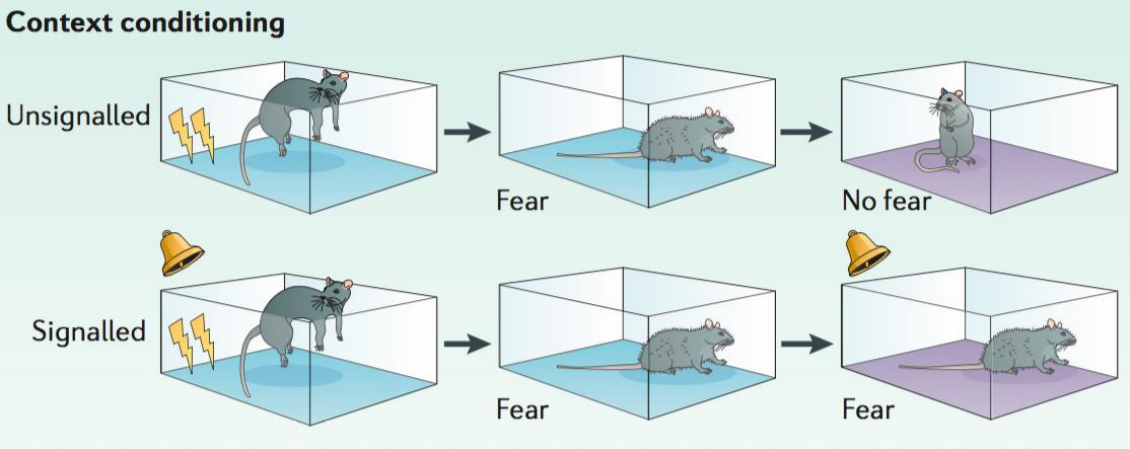


Figure 8- Representation of contextual fear conditioning (top) and cue fear conditioning (bottom) (Calhoon & Tye, 2015).

Then, after the repeated pairings, when the animals are later tested for their fear memory, the CS alone should be capable to induce a conditioned fear response similar to the one obtained in the presence of danger, such as: freezing (complete immobility except as required for breathing), reflex expression (characterized by fear-potentiated startle) and autonomic (increase in heart rate and in the mean arterial pressure) and endocrine (stress-related hormone release) responses (LeDoux, 2000; Rudy *et al.*, 2004).

However, context encoding is necessary for context conditioning which means that during a typical context fear-conditioning experiment, first it is necessary to encode a representation of the context (explore the context before a footshock) and only then associate that representation with the US (Maren *et al.*, 2013).

With these experiments, it is thus possible to simulate a real situation of conditioned

fear in which a subject suffers a traumatic event that leads to maladaptive fear responses that underlie neuropsychiatric disorders such as PTSD.

1.5- Fear generalization

As we known, a poisonous snake has a different meaning when it is encountered in the wild (being life threatening) compared to when it is seen behind glass in a zoo (where it can appear "exciting"). Contextual information is thus essential to allow us to decide if we should react in one way or another (freeze or enjoy, in this particular case) and impairments in the ability to distinguish between two or more similar contexts could result in serious health problems.

Over-generalized fear is one of the biggest symptoms of PTSD and consists in the transference of learned fear from a traumatic event to situations "resembling" the distressing event, but that would normally be considered safe, resulting in autonomic hyperarousal to inappropriate situations (Mahan & Ressler, 2012). For example, in PTSD subjects with war zone traumas, the sound of fireworks in a safe context could be experienced as the sound of a gunshot (since the two stimuli share similarities) resulting in an exacerbated reaction to the context, like unnecessary duck and cover.

Fear generalization is usually seen as an adaptive process that facilitates protective responses to situations that are similar to the situations previously learned to be dangerous, but when a disruption in generalization occurs (over-generalization) this process becomes unproductive and harmful (Lissek & Van Meurs, 2015).

The intensity of the adverse stimuli (US) seems to impact the breadth of generalization, with a very strong US by itself being capable to produce broad generalization, undermining discrimination between dangerous and safe cues and leading to autonomic hyperarousal (Baldi *et al.*, 2004; Ghosh & Chattarji, 2014).

Furthermore, it is also well established that fear generalization increases over time since as time goes by most memories become less precise and more generalized– conveying the "gist" of a context, rather than its precise identity. Such fact is supported by several animal studies using a contextual fear conditioning paradigm, which have proven that animals tested at a recent time point are able to discriminate between the novel context and the context where they have received the shocks (training context) (Feinberg & Riccio, 1990; Zhou & Riccio, 1996; Wiltgen & Silva, 2007; Wiltgen *et al.*, 2010; Ruediger *et al*., 2011; Jasnow *et al*., 2012), but when tested at a remote time point they are not, generalizing their fear (Perkins & Weyant, 1958; Mcallister & Mcallister, 1963; Richardson *et al*., 1984; Gisquet-Verrier & Alexinsky, 1986; Zhou & Riccio, 1996; Metzger & Riccio, 2008).

Over-generalization of fear is a serious burden to daily life since PTSD subjects are always vulnerable to a broad variety of stimuli that present similarities to the initial trauma making exposure therapies ineffective. Thus, it is urgent to find effective

therapies for such a symptom.

In this sense, research in this field is therefore gaining traction with many efforts being directed towards figuring out what are the behavioural and neural mechanisms of fear generalization. Recent studies including neuroimaging works have begun to examine the human neurocircuitry of fear generalization, implicating interactions between microcircuits within the amygdala (Duvarci & Pare, 2014), the PFC (Chavez *et al.*, 2009; Dunsmoor *et al.*, 2011; Courtin *et al.*, 2014; Likhtik *et al.*, 2014) and hippocampus (Bergado-Acosta *et al.*, 2008; Kaouane *et al.*, 2012; Xu & Südhof, 2013) in the generalization of fear.

However, neurobiological studies of generalization in fear conditioning paradigms have been sparse (Likhtik & Paz, 2015) so more research is required to understand the fear generalization process.

1.5.1- Role of hippocampus (HPC) in contextual fear generalization

Most of the work done so far about fear generalization has focused in HPC thanks to is crucial function in contextual learning and formation of episodic memories (Scoville & Milner, 1958; Holland & Bouton, 1999).

Hereupon, many studies have shown that the HPC appears to be critical for the retrieval or reconstruction of vivid and highly detailed memories for events and certain forms of spatial representations (Rosenbaum *et al*., 2000; St-Laurent *et al.*, 2014 & 2016). In depth, the increase in contextual fear generalization that seems to occur as the memory ages has been linked with impairments in the recruitment of the HPC when a memory is recalled by contextual cues at remote time points. Consequently, as a result of the absence of a hippocampal trace at remote time points, the remaining cortical memory will lack spatial detail that only the hippocampal system allows to emerge, thus resulting in a generalized fear memory (reviewed in Doron & Goshen, 2017; Hardt & Nadel, 2017).

Moreover, it has been proposed that deficits in HPC-mediated pattern separation may underlie contextual fear over-generalization. Pattern separation is a process by which similar experiences or events are transformed into non-overlapping representations thus allowing the discrimination between two highly similar contexts and, consequently, the identification of safe contexts. In accordance, McHugh and colleagues (2007) have shown that the absence of NMDARs in the DG did not affect the performance of the mutant mice in the extinction of fear in standard contextual fear

conditioning, but impaired the ability to discriminate between two similar contexts, providing evidence that NMDARs within the DG are essential for discrimination learning.

Such functional alterations are compatible with the most replicated structural abnormality found in PTSD which is a lower volume of the HPC (reviewed in Pitman *et al*., 2012; Yehuda *et al*., 2015).

1.5.2- Ventral hippocampus (vHPC) and contextual fear generalization

Until now, based on the assumption that dHPC projects to associational cortical regions whereas its ventral portion projects to regions implicated in motivational, neuroendocrine and autonomic responses – such as hypothalamus and amygdala (Swanson & Cowan, 1977; Moser & Moser, 1998a; Fanselow & Dong, 2010; Kheirbek *et al.*, 2013) – it was presumed that dorsal parts of the HPC mediate cognitive functions (spatial memory) whereas ventral portions are involved in emotional responses (Bannerman *et al*., 2004).

However, this standard interpretation based on a dorsal-ventral dichotomy model recently started to be questioned. Growing evidences have reported that the vHPC also plays its part in the spatial processing functions, suggesting a more graded action of the HPC during spatial learning (Moser & Moser, 1998b). Such assumption is supported by the existence of place cells – cells that participate in multiple and independent spatial representations – also in the vHPC (Jung *et al*., 1994; Kjelstrup *et al*., 2008).

Moreover, it has been shown that ventral and dorsal cells display distinct firing characteristics, further suggesting that different sub-regions of the HPC may have different spatial functions. Cells in the dHPC fire in specific circumscribed locations, whereas ventral cells have large and overlapping receptive fields (O'Keefe & Dostrovsky, 1971; Kjelstrup *et al*.,2008; Keinath *et al*.,2014). Based on these distinct firing characteristics, it has been proposed that the dHPC is more associated with spatial recognition of certain aspects of the context such as objects and cues, while the ventral region appears to be crucial for the formation of the context as a whole and, consequently, for the discrimination of similar places, through its capacity for assembling the contextual information collected by the dHPC (Maurer *et al*., 2005; Kjelstrup *et al*., 2008; Royer *et al*., 2010; Komorowski *et al*., 2013; Keinath *et al*.,2014).

In line with all these facts, recent articles have insinuated that the dorsal region may be important for minimizing memory interference and generalization by coding specific aspects of the context, while the vHPC may be more vulnerable to contextual

generalization since there is a higher probability of occurring errors during the assembly of the contextual cues (Komorowski *et al*., 2013; Keinath *et al*.,2014; Yuan *et al*., 2015).

Indeed, a large variety of publications have implicated the vHPC in fear generalization (McHugh *et al*., 2013; Weeden *et al.*, 2014; Yuan *et al*., 2015; Cullen *et al*.; 2015; Nguyen *et al*., 2018). For instance, it was demonstrated that the bilateral infection of a histone deacetylase (HDAC) inhibitor in the vHPC of C57BL/6 mice, after context pre-exposure, elicited predator odour fear generalization to a neutral context (Yuan *et al*., 2015). In agreement, Cullen and colleagues (2015) showed that in C57BL/6 mice, contrarily to dHPC, the CA1 region of vHPC is involved in the expression of a contextual generalized fear memory (Cullen *et al*., 2015). Furthermore, a very recent article has reported that, compared to offspring of low licking/grooming mothers (low LG), the adult male offspring of high LG displayed reduced ventral hippocampal LTP expression and froze significantly less in response to a neutral tone than to the conditioned tone following cued fear conditioning. Additionally, Nguyen and colleagues (2018) have also demonstrated that the selective blockade of ventral hippocampal LTP increases generalized freezing in offspring of low but not high LG mothers.

Altogether, these findings suggest that an intact ventral hippocampal function is required for the discrimination of conditioned *vs*. neutral stimuli. However, although impairments of vHPC have been implicated has a putative mechanism underlying fear generalization, the discovery of the exact hippocampal synaptic plasticity processes that underlie fear generalization is still a challenge.

1.5.2.1- Ventral hippocampus- medial pre-frontal cortex (vHPC-mPFC) communication and contextual fear generalization

The PFC is also an essential structure for episodic memory (Jones & Wilson, 2005; Siapas *et al.*, 2005; Barker *et al.*, 2007; Benchenane *et al*., 2010; Chao *et al*., 2016; Barker *et al*., 2017), with human and animal studies indicating that HPC and mPFC have complementary roles in memory processing (reviewed in Eichenbaum, 2017). Thus, it has been proposed that the expression of a contextually precise memory involves the interaction between the HPC and the PFC (Cullen *et al*., 2015; Eichenbaum, 2017).

Regarding the ventral hippocampal pole, a recent study found that inhibition of the vHPC reduces the synchronization between the PFC and the dHPC, important for spatial working memory tasks (O'Neill *et al*., 2013). Such a result together with the fact that vHPC is the only hippocampal area that directly projects to the mPFC (Jay & Witter, 1991; Hoover & Vertes, 2007) suggests that the vHPC could work as the key mediator of the communication between dHPC and mPFC, thus conveying spatial information between

these two regions.

Recently, Cullen and colleagues (2015) have proposed that a possible interaction between the anterior cingulate cortex (ACC) and vHPC could control the expression of fear generalization, since when ACC or the ventral CA1 were inactivated at remote time points animals reduced their freezing in the novel context.

1.6- GABAergic and dopaminergic systems in fear generalization

A substantial body of evidence has suggested that an imbalance of the gamma-aminobutyric acid (GABA) system in the amygdala may underlie fear generalization. Bergado- Acosta and colleagues (2008) have shown that the genetic ablation of the enzyme glutamic acid decarboxylase 65 (GAD65) – an enzyme that catalyses the conversion of glutamic acid to GABA – results in a pronounced context-independent, generalization of learned conditioned fear responses during long-term memory retrieval. In line with these previous findings, Lange and colleagues (2014) have shown that a deficiency in GAD65 affects synaptic transmission and plasticity in the lateral amygdala thus resulting in an impairment of the cue specificity of conditioned fear responses. About GABA receptors (GABARs), there are evidences that the presynaptic inhibition through $GABA_{B(1a,2)}$ receptors may be important to prevent the generalization of conditioned fear (Shaban *et al*., 2006). Furthermore, it was recently demonstrated that the modulation of $GABA_A$ receptors in the basolateral amygdala complex is critical for the facilitatory effect of stress on fear memory generalization (Bender *et al*., 2018).

Regarding dopaminergic system, evidences have suggested that midbrain dopaminergic neurons are important for aversive Pavlovian conditioning (Fadok *et al*., 2010; Bromberg-Martin *et al*., 2010; Zweifel *et al*., 2011). Moreover, hippocampal encoding of novel and contextual information has been linked to dopamine release via excitation of dopamine neurons of the midbrain (Schultz *et al*., 1997; Lisman & Grace, 2005; Luo *et al.,* 2011). In line with this evidence, it was recently demonstrated that dopamine receptor D1 (D_1R) knock out mice (that lack D1R in DG granule cells) exhibit contextual fear generalization while DG D_1R activation decreases generalization of the conditioned fear response to novel contexts (Sariñana *et al*., 2014). Finally, a recent study has shown that the blockade of D_2R in the amygdala induces generalized threat responses (De Bundel *et al*., 2016).

1.7- Adenosine

Adenosine is a ubiquitous purine nucleoside composed by an adenine linked to a ribose sugar molecule and it is involved in many biological processes (reviewed in Fredholm *et al*., 2005).

Adenosine plays a critical role in cellular viability and adaptability processes since it is involved in energy transfer, redox control, building block for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), signal transduction and epigenetic control (Cunha, 2016).

Definitively, all cells have an intracellular metabolism based on adenosine. However, there has been a particular interest in the role of this purine nucleoside in the brain, where it acts mainly as a synaptic modulator. Thus, adenosine does not directly activate or inhibit synaptic transmission but instead it actually acts as a modulation system to fine-tune the flow of information in neuronal circuits (Cunha, 2016).

Conventionally, adenosine is considered an inhibitory neuromodulator since it usually decreases the activity of excitatory synapses by a negative feedback mechanism (Dunwiddie & Masino, 2001). Yet adenosine, a full agonist of all its receptors (Fredholm *et al*., 2011), acts as an inhibitory or excitatory neuromodulator depending on the extracellular adenosine source and, consequently, of the preferential activation of distinct ARs (Gomes *et al.*, 2011).

1.7.1- Adenosine receptors (ARs)

Adenosine's actions are mediated by four distinct plasma membrane adenosine receptors – denoted A_1Rs, A_{2A}Rs, A_{2B}Rs and A_3Rs – that are metabotropic. (Fredholm *et al*., 2011). These receptors are widely expressed, but A_1Rs and A_{2A}Rs are the main receptors responsible for the effects of adenosine in the brain (Fredholm *et al*., 2005), with both mostly located in synapses (Tetzlaff *et al*., 1987; Rebola *et al*., 2003 & 2005a) namely, excitatory synapses (glutamatergic) (Tetzlaff *et al*., 1987; Rebola *et al*., 2005b) although they are also present in GABAergic (Cunha & Ribeiro, 2000; Shindou *et al*., 2002; Rombo *et al*., 2015) cholinergic (Cunha *et al*., 1995a; Rodrigues *et al*., 2008) among others types of synapses.

In particular, A_1Rs are more expressed in the cerebral cortex, HPC, cerebellum, thalamus, brain stem, and spinal cord (Reppert *et al*., 1991; Dixon *et al*., 1996) and A_{2A}Rs are highly enriched in the striatum (Fink *et al*., 1992; Chen, 2014) being also weakly expressed in other brain regions such as the HPC and cortex (Chen, 2014). In the dHPC

even though A_{2A}Rs are weakly expressed there (Jarvis & Williams, 1989; Sebastião & Ribeiro, 1992; Dixon *et al*., 1996), they display a functional excitatory action (Sebastião & Ribeiro, 1992; Cunha *et al*., 1994; Rombo *et al.*, 2015). In addition, despite the lack of neurochemical data on the levels of A_{2A}Rs in the vHPC, there are indications of the existence of functional A_{2A}Rs. (Moschovos *et al*., 2012). Finally, A_{2A}Rs expression is not exclusive to neurons, for there is evidence of these receptors also being expressed in glial cells (Chen, 2014).

A_1Rs are more abundant presynaptically (Tetzlaff *et al*., 1987; Rebola *et al*., 2003) and work as inhibitory receptors since they inhibit synaptic transmission, by usually decreasing the activity of excitatory synapses (Barrie & Nicholls, 1993; Ambrósio *et al*., 1997; Wu *et al*., 1994), in order to control basal synaptic transmission and maintain homoeostasis. However, A_1R-mediated inhibition seems to become less efficient the more intense the recruitment of neuronal circuits, as demonstrated by the lower ability of A_1R to control high-frequency-induced synaptic plasticity (Costenla *et al*., 2011; Rex *et al*., 2005).

In contrast, A_{2A}Rs show their efficiency at higher frequencies of stimulation since they require a disproportional pool of adenosine formed by the extracellular catabolism of ATP (Cunha *et al*., 1996; Rebola *et al*., 2008), which is mainly released upon higher frequencies of stimulation (reviewed in Cunha, 2016). Thus, if at lower frequencies of stimulation, the predominant role of adenosine is an A_1Rs-mediated inhibition of synaptic transmission (Dunwiddie & Masino, 2001; Fredholm *et al*., 2005), at higher frequencies of stimulation the A_{2A}Rs (that are not engaged at lower frequencies) are the type of receptors that stands out, being selectively activated and contributing to high-frequency-induced synaptic plasticity (Rebola *et al*., 2008; Costenla *et al*., 2011). So, A_{2A}Rs work as facilitator receptors by triggering the enhancement of synaptic efficiency (figure 9), namely by enhancing the evoked release of glutamate (Marchi *et al*., 2002; Ciruela *et al*., 2006; Shen *et al*., 2013; Matsumoto *et al.*, 2014; Machado *et al*., 2017) and the function of ionotropic glutamate receptors (Wirkner *et al*., 2004; Guntz *et al*., 2008; Rebola *et al*., 2008; Azdad *et al*., 2009; Dias *et al*., 2012; Di Angelantonio *et al*., 2015; Sarantis *et al*., 2015) and, in this way, contributing to synaptic plasticity, e.g. LTP (D'Alcantara *et al*., 2001; Fontinha *et al*., 2008; Rebola *et al*., 2008; Costenla *et al.*, 2011; Li *et al*., 2015; Viana da Silva *et al*., 2016). A_{2A}Rs activation also decreases the efficiency of presynaptic inhibitory systems such as the one assured by A_1Rs (through its desensitization) (Lopes *et al*., 1999; Ciruela *et al*., 2006) having the ability to switch presynaptic modulation from inhibitory to facilitatory (Cunha, 2016).

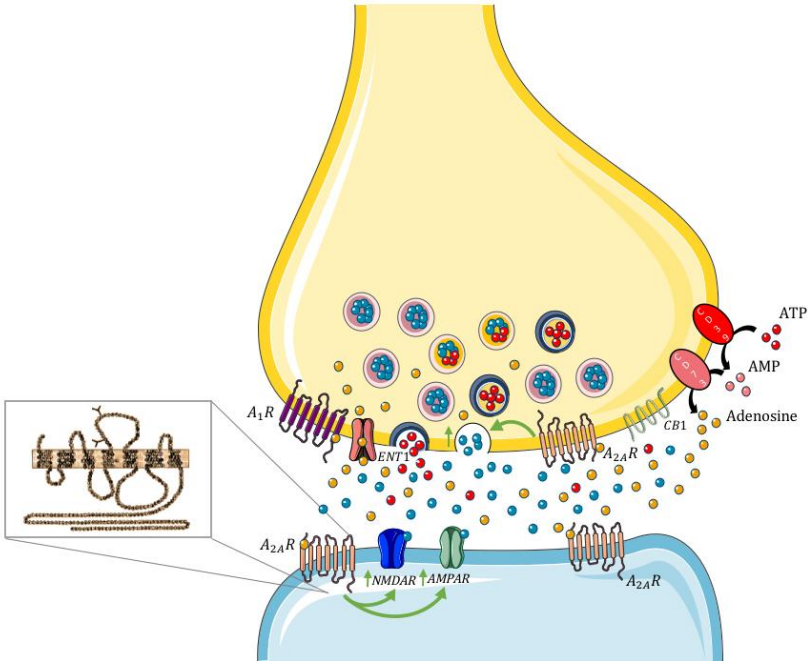


Figure 9- Schematic illustration of the role of neuronal A_{2A}Rs under high-frequency stimulation.

ARs are implicated in many biological functions, showing promise as important therapeutic targets. For example, taking into account the glutamate hypothesis of excitotoxicity-mediated neurodegeneration (Choi, 1992; Lipton & Rosenberg, 1994; Mattson, 2003), which postulates that an excessive glutamate signalling leads to neuronal damage, it is expected that by using A_1Rs agonists it could be possible to control neurodegeneration, since A_1Rs inhibit synaptic transmission. However, although many studies have documented that the acute administration of A_1Rs agonists decrease neurodegeneration by using different models such as slices or *in vivo* animals (Parkinson *et al*., 1994; Von Lubitz *et al*., 1995; Fredholm, 1997; Dunwiddie & Masino, 2001; Ribeiro *et al*., 2002; Boison, 2006 & 2013), this promising supposition presents many restrictions and contradictory information that limits their clinical use. For example, A_1Rs agonists have marked cardiovascular effects (Peart & Headrick, 2007; Stella *et al*., 1993).

In contrast, the use of A_{2A}Rs appears to be a more realistic and promising therapeutic strategy. In fact, with ageing (Cunha *et al*., 1995b; Canas *et al*., 2009; Costenla *et al*., 2011) and in many disorders there is an upregulation of A_{2A}Rs (Albasanz *et al*., 2006; Batalha *et al*., 2013; Crema *et al*., 2013; Villar-Menéndez *et al*., 2014; Kaster *et al*., 2015; Cunha, 2016; Simões *et al*., 2016) which inadvertently leads to a glutamate excitotoxicity and, consequently, to neuronal damage. This explains why A_{2A}Rs facilitate "healthy" synaptic plasticity under physiological conditions, whereas their upregulation in disease conditions triggers an aberrant synaptic plasticity that leads to neuronal damage, and also suggests that by using A_{2A}Rs antagonists it is possible to afford a robust neuroprotection against brain damage (Chen *et al*., 2007; Stone *et al*., 2009; Gomes *et*

al., 2011; Chen *et al*., 2013; Cunha *et al*., 2016). Hence, the A_{2A}Rs blockade is a potential treatment against many brain disorders, such as Parkinson's and Alzheimer's diseases as well as neuropsychiatric disorders (reviewed in Cunha *et al*., 2008; Cunha, 2016).

1.7.2- A_{2A} receptors (A_{2A}Rs) and mood-related disorders

In the last years ARs, and especially A_{2A}Rs, started to emerge as promising target candidates in the management of neuropsychiatric disorders based on three premises: adenosine may act as go-between glutamate and dopamine, two of the key players in mood processing; the consumption of coffee, in particular, caffeine (AR antagonist) modifies the mood profile; and the positive effects on mood disorders obtained by different therapeutic strategies seem to be related to the adenosine modulation system (Cunha *et al.,* 2008).

Since then, many are the human and animal studies that have implicated A_{2A}R in mood-related disorders, such as anxiety and depression. For example, human studies had suggested a genetic linkage between A_{2A}R polymorphisms and anxiety related-conditions after caffeine administration (Alsene *et al*., 2003) and also with the susceptibility to develop panic disorder, which can be envisioned as a situation of anticipatory anxiety (Deckert *et al.*, 1998; Hamilton *et al*., 2004).

Likewise, other studies indicate that increased levels of adenosine (Minor *et al*., 1994; Woodson, 1998; Hunter *et al*., 2003) and the overexpression of A_{2A}Rs in the HPC, cortex and striatum triggers depressive-like behaviour (Coelho *et al*., 2014). In agreement, it has been demonstrated that caffeine (A_{2A}R antagonist) consumption correlates inversely with the incidence of depression (Smith, 2009; Lucas *et al*., 2011) and the risk of suicide (Kawachi *et al*., 1996; Lucas *et al*., 2014) and it also has been determined that A_{2A}Rs antagonists are potential antidepressants (El Yacoubi *et al*., 2001).

Still, another recent study indicates that A_{2A}Rs are a candidate target to treat chronic unpredictable stress (CUS), a risk factor for neuropsychiatric disorders, namely depression (Kim & Diamond, 2002; McEwen, 2007), since it was demonstrated that the consumption of caffeine and the pharmacological or genetic blockade of adenosine A_{2A}Rs were capable of alleviating the behavioural, neurochemical, and electrophysiological alterations on brain function caused by chronic stress (Batalha *et al*., 2013; Kaster *et al*., 2015).

Nevertheless, although there are some studies implicating A_{2A}Rs in mood-related disorders it is not yet well established if A_{2A}Rs can control fear behaviour.

1.7.3- A_{2A} receptors (A_{2A}Rs) and regulation of fear responses

Besides being involved in emotional processes such as anxiety and depression, and taking part in LTP as mentioned above, (D'Alcantara et al., 2001; Fontinha et al., 2008; Rebola et al., 2008; Fontinha et al., 2009; Li et al., 2015; Pagnussat et al., 2015; Viana da Silva et al., 2016) A_{2A}Rs have been implicated in a large variety of studies linking this type of receptors with memory performance. In detail, it was demonstrated that the pharmacological or genetic blockade of A_{2A}R impedes memory deterioration (Dall'Igna *et al*., 2007; Canas *et al*., 2009; Cognato *et al.*, 2010) while the abnormal activation of A_{2A}Rs signalling in HPC is sufficient to impair memory function (Li *et al*., 2015; Pagnussat *et al*., 2015). Thus, keeping all this information in mind it is expected that A_{2A}Rs also have an important role in fear memory.

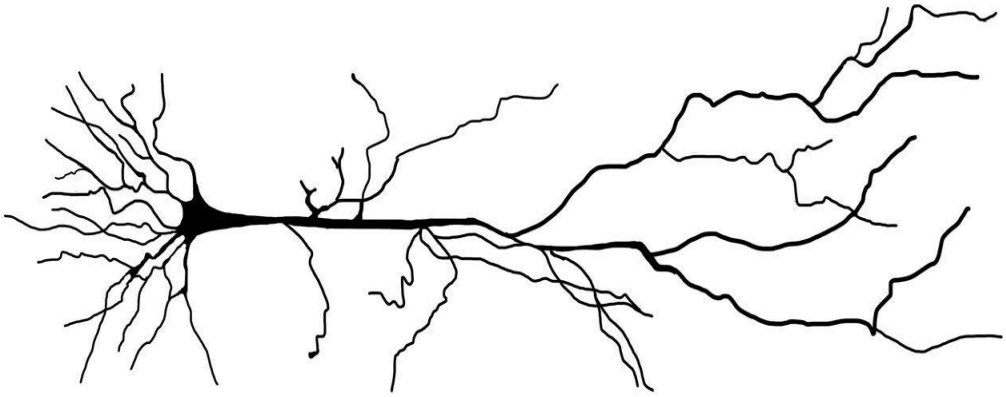
In fact, there are evidences, for example, that the acute administration of caffeine disrupts fear memory (Corodimas et al., 2000). Furthermore, a recent study has provided combined pharmacological and genetic evidence that amygdalar A_{2A}Rs control fear memory, by regulating synaptic plasticity in that region (Simões *et al*., 2016). More precisely, Simões and colleagues (2016) have demonstrated that the selective downregulation of A_{2A}Rs in the basolateral complex of the amygdala impairs fear acquisition, as well as Pavlovian fear retrieval in agreement with the A_{2A}Rs' blockade ability to selectively dampen the amplitude of amygdalar LTP. Additionally, the putative role of A_{2A}Rs in the acquisition and preservation of contextual fear memories was bolstered by the observation of an upregulation of A_{2A}Rs in the amygdala and a gain-of-function of A_{2A}Rs to control amygdala LTP after fear acquisition. Such upregulation was also detected in other brain regions such as the HPC and the striatum, and is in agreement with the previous observation that stressful events upregulate A_{2A}Rs (Fredholm et al., 2005; Cunha & Agostinho, 2010).

Converging evidences suggest that A_{2A}R action in fear conditioning depends on its brain location. For example, a recent study has demonstrated that striatal and extrastriatal A_{2A}Rs in the entire forebrain exert opposite control over fear conditioning, since the deletion of striatal A_{2A}Rs seems to facilitate context and tone fear conditioning (without affecting anxiety-like behaviour), while deleting A_{2A}Rs in the entire forebrain (striatum, HPC, and cortex) normalized or reversed both, produced an anxiolytic phenotype and increased the startle response. Nevertheless, focal deletion of hippocampal A_{2A}Rs selectively attenuated context but not tone-fear conditioning (Wei *et al*., 2014). Thus, since selective deletion of A_{2A}Rs in the forebrain yields alterations in three of the defining features of PTSD in rodent models (fear conditioning, startle response, and anxiety) this indicates that forebrain A_{2A}Rs could be potential and novel therapeutic targets for PTSD.

Together this data indicates that although amygdala A_{2A}Rs have an important role in fear conditioning its impact is unlikely to be restricted to the amygdala, with A_{2A}Rs located in other brain regions, such as HPC, also performing relevant functions in fear expression. In fact, since the amygdala is not the only brain structure involved in fear memory it makes sense the idea that A_{2A}Rs beyond the amygdala could participate in acquisition and recall of conditioned fear.

All the evidences gathered so far point in the direction that A_{2A}Rs could be an attractive target to manage diseases associated with an abnormal fear such as PTSD, however their involvement and impact in different brain regions still remains to be detailed and their role should be further explored.

Chapter 2



Objectives

2.1- Aim of the work

Accumulating evidences have implicated the HPC in fear generalization. Nevertheless, there is a lack of information about the elusive hippocampal synaptic plasticity processes underlying fear generalization.

A_{2A}Rs have been implicated in many pathologies, including conditions associated with stress or fear, thus suggesting those receptors as attractive candidates for the management of diseases like PTSD. However, despite the potential involvement of hippocampal A2ARs and LTD (thanks to its participation in novelty exploration) in PTSD this relationship has not been explored yet.

Thus, the core purpose of this study is to:

Assess the impact of dorsal and ventral hippocampal A_{2A}Rs in contextual fear generalization.

In this sense, in order to explore this subject we intend to:

1- Optimize an LTD protocol in our laboratory;

2- Dissect the role of dorsal and ventral A_{2A}Rs on LTD under a physiological situation;

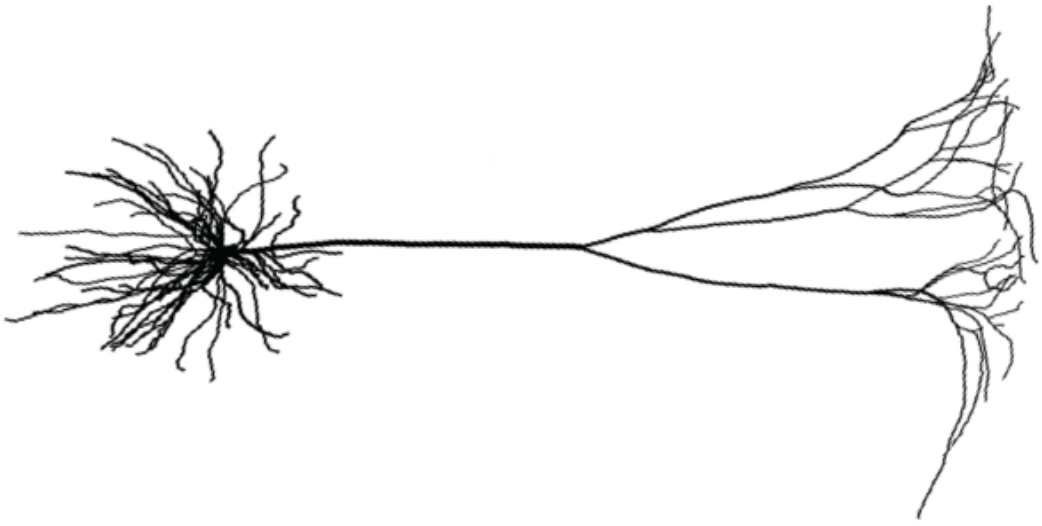
3- Optimize a time-dependent contextual fear generalization protocol;

4- Understand if alteration in LTD in vHPC and dHPC could underlie fear generalization;

5- Investigate the impact of hippocampal A_{2A}Rs in fear generalization mechanisms.

With the present study, we expect to discover that alterations in LTD in the CA3-CA1 pathway of ventral and/or dorsal HPC underlie fear generalization and that by blocking A_{2A}Rs in such regions we could revert said alterations.

Chapter 3



Materials & Methods

3.1- Mice and ethical considerations

All experiments were conducted on 8-12 weeks old male C57BL/6 mice originated from Charles River Laboratories (Barcelona, Spain). The animals were single-housed in standard cages with free access to food and water in a room maintained under controlled standard conditions: fixed 12:12h light/dark cycle, controlled temperature (23 ± 2°C) and humidity (40-60%).

All efforts were made in order to try to minimize the number of animals used as well as their suffering/discomfort. In that way, the experimental procedures were carried out in conformity with standard animal welfare guidelines and European legislation (ORBEA 138-2016/15072016) and the certification of Direção Geral de Alimentação e Veterinária (DGAV 0421/000/000/2016; 25/07/2016).

3.2- Materials

3.2.1- Chemical reagents, antibodies and their manufacturers/ suppliers

For electrophysiological recordings, the $A_{2A}R$ antagonist 5-amino-7-(2-phenylethyl)-2- (2- furyl)-pyrazolo[4,3-e]- 1,2,4- triazolo- [1,5-c] pyrimidine (SCH 58261) was purchased from Tocris (Bristol, UK). Carbogen mixture (95% O_2/5% CO_2) was obtained from Linde (Lisbon, Portugal).

For immunohistochemistry studies, two primary antibodies were used: 1) rabbit polyclonal anti-D_2R from Frontier Institute (Hokkaido, Japan); 2) mouse monoclonal anti-Gephyrin from Synaptic Systems (Goettingen, Germany). The anti-rabbit and anti-mouse polyclonal secondary antibodies were obtained from Invitrogen-ThermoFisher Scientific (Oeiras, Portugal). Additionally, the ultrapure low melting point agarose was acquired from Invitrogen-ThermoFisher Scientific (Oeiras, Portugal). The nuclear dye 4',6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen-ThermoFisher Scientific (Oeiras, Portugal) and the fluorescence mounting medium was obtained from Agilent Technologies (Santa Clara, CA, USA).

Furthermore, the reagents needed to prepare the solutions for electrophysiological and/or immunohistochemistry studies, such as artificial cerebrospinal fluid (ACSF), phosphate buffered saline (PBS), paraformaldehyde (PFA), sucrose, anti-freezing, permeabilization and blocking solutions were all acquired from Sigma-Aldrich (Sintra, Portugal).

Table 2- Solutions prepared for electrophysiological and immunohistochemistry studies.

	ACSF (pH= 7.4) (mM)	**PBS** (pH=7.4) (mM)	**PFA** (pH=7.4) (mM)	**Anti-Freezing** (pH= 7.4) (mM)
$CaCl_2$	2			
Ethylene Glycol				4.8
Glucose	10			
Glycerol				3.3
KCl	3	2.7	2.7	
KH_2PO_4		1.9	1.9	
$MgSO_4$	1			
NaCl	124	137	137	
$NaHCO_3$	26			
$Na_2HPO_4.7H_2O$		10	10	
$NaH_2PO_4.H_2O$	1.25			12.3
$NaHPO_4.2H_2O$				20.3
PFA			1.3	

Table 3- Drugs used for Electrophysiological Recordings.

Drug	**Dissolved in**	**Concentration** (nM)
SCH 58261	ACSF + 0.01% DMSO	50

Table 4- Antibodies used in immunohistochemistry.

Antibodies		**Dilution**	**Origin**
Primary Antibodies	Anti-D_2R	1:200	Rabbit
	Anti-Gephyrin	1:500	Mouse
Secondary Antibodies	Anti-Rabbit AlexaFluor 594	1:2000	Donkey
	Anti-Mouse AlexaFluor 594	1:2000	Donkey

Table 5- Blocking solutions used in immunohistochemistry.

Blocking Solutions	**Constitution**
Blocking Solution for D_2R Immunostaining	10% Horse Serum + 0.1% Triton x100 (in PBS)
Blocking Solution for Gephyrin Immunostaining	10% Horse Serum + 0.2% Triton x100 (in PBS)

3.3- Methods

3.3.1- Behavioural experiments

Based on the fact that animals can develop emotions similar to humans, rodent paradigms have historically been used in basic research and drug development to model fear learning, stress and anxiety.

Therefore, to investigate the impact of hippocampal A_{2A}Rs on fear generalization, we performed a time-dependent contextual fear generalization paradigm, inspired by the Pavlovian fear conditioning model.

3.3.1.1- Time-dependent Contextual Fear Generalization Protocol

To assess generalization, animals were trained in a contextual fear conditioning paradigm in order to teach the mice to associate the CS – training context – with the aversive US – electrical footshocks.

3.3.1.2- Context fear conditioning apparatus

Behavioural procedures were performed in 2 identical conditioning chambers (17 Width x 17 Depth x 25 Height cm) containing 2 plexiglas walls (front and back), 2 aluminum sidewalls and a stainless steel shock-grid floor (46003 Mouse Cage Model).

In those chambers two different contexts were created for the protocol for time-dependent contextual fear generalization: the training and the novel context. The training context was constituted by a polka-dot insert attached to the rear plexiglas wall, white noise (65 decibels, dB), dim illumination and the steel grid floors were cleaned with 70% ethanol. The novel context contained no polka dot wall background, no white noise and was illuminated by infrared lights. Furthermore, a flat-grey plexiglas floor replaced the grid floor and was washed with 10% acetic acid (adapted from Cullen *et al.*, 2015).

3.3.1.3- Procedure

All animals received 5 min of pre-exposure to the training context following 5 min of

handling, on the two days prior to fear conditioning, in order to allow mice to encode the contextual cues (figure 10).

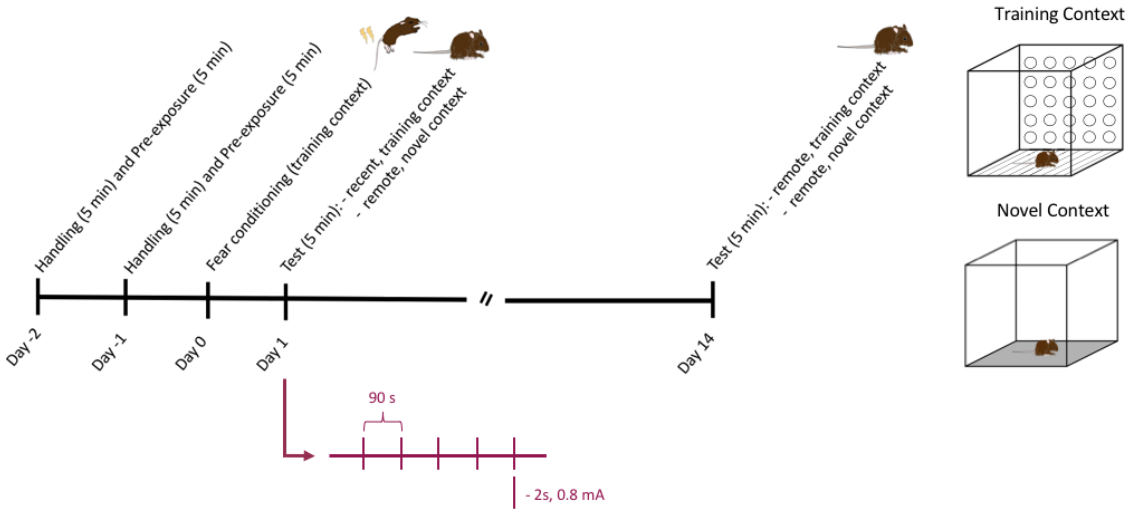


Figure 10- Schematic illustration of the time-dependent contextual fear generalization protocol as well as the contexts used (training and novel contexts).

The fear conditioning paradigm was employed in the training context, consisting in 5 footshocks (2s, 0.8 mA) separated by 90s inter-trial intervals (ITIs).

Afterwards, fear conditioning mice were divided into four groups and fear memory was tested during 5 min through three different parameters: percentage of freezing (motionless state most often characterized by a crouching posture), number of tail rattlings (high-frequency shaking of the tail) and travelled distance. Briefly, tail rattling is a behavioural response associated with elevated emotional state during aggressive encounters (Beilharz & Beilharz, 1975) or fear (Fitch *et al.,* 2002) while freezing is a natural response to fear or pain (Blanchard & Blanchard, 1969; Bolles & Collier, 1976). The percentage of freezing and the number of tail rattlings were quantified manually by visual observation while the distance was measured by ANY-maze (software).

Two of the four groups were tested one day after fear conditioning (recent time point): one group in the training context (recent, training context group) in order to ensure that fear conditioning was successful and the second group (recent, novel context group) was tested in the novel context in order to be sure that fear generalization did not occur at this recent time point.

Since it has been reported that a 14 days period after fear conditioning is sufficient for fear generalization to occur, the last two groups were tested at a remote time point of 14 days after fear conditioning. One group was tested in the training context (remote, training context group) in order to be sure that the animals maintained the fear until this

time point and the other group was tested in the novel context (remote, novel context group) to evaluate if mice generalized their fear (adapted from Cullen *et al.*, 2015).

3.3.2- Electrophysiological experiments

Synaptic plasticity became one of the most intensively researched topics in all of neuroscience because it has been recognised as a key property of brain function likely to underlie learning and memory (Hebb, 1949; Alkon & Nelson, 1990; Lisman, 1989; Lüscher & Malenka, 2012).

The CA1 sub-region of the HPC is of particular interest because of its association with impaired LTP and memory consolidation (Zola-Morgan *et al.*, 1996; Granger *et al*., 1996; Bliss & Cooke, 2011). Thus, although LTP and LTD have been studied throughout the CNS, the vast majority of experimental work aimed at understanding such mechanisms have focused on excitatory synapses in the HPC, specifically, at SC-pyramidal cell synapses in the CA1 region of the rodent transverse hippocampal slices (Remondes & Schuman, 2004).

Regarding LTD, it a has been suggested that the induction of this type of synaptic plasticity in the CA1 hippocampal sub-region is important to encode fine spatial details in a new environment (Kemp & Manahan-Vaughan, 2004 & 2007).

For this work, electrophysiological recordings were performed to assess LTD in SC-pyramidal cell synapses in the CA1 region (figure 11) of ventral and dorsal hippocampal brain slices.

To achieve that goal, animals were sacrificed by cervical dislocation, decapitated and the brain was rapidly removed and placed into a petri dish with ice-cold, oxygenated ACSF, gassed with 95% O_2/5% CO_2 mixture. Next, the two hippocampi were isolated and then transversely sectioned into 400 µm-thick slices (figure 11), using a McIlwain tissue chopper (Brinkmann Instruments, New York, NY, USA).

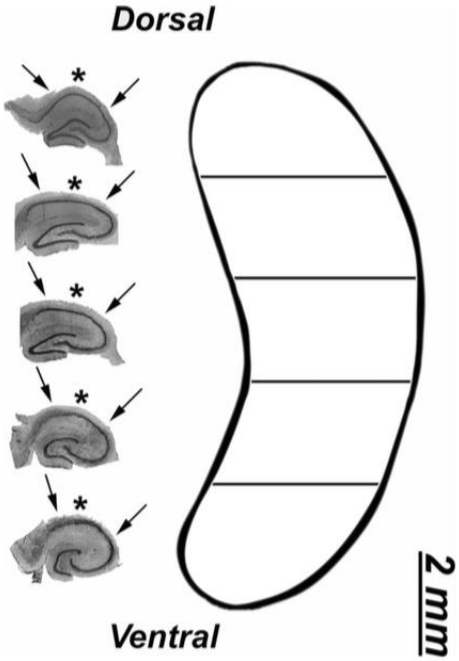


Figure 11- Hippocampal sections spaced across the septotemporal axis- from the most dorsal part (top) to the most ventral one (below)- obtained after the transversal slicing procedure. (Maggio & Segal, 2007).

Subsequently, transverse slices were kept for at least 1h30 prior to any recordings into a resting chamber (BSC-PC prechamber, Harvard Apparatus, Massachusetts, USA) with gassed ACSF at 32°C (Van der Jeugd *et al*., 2011), in order to recover both functionally and energetically. After a 90 min incubation, one ventral or dorsal slice was arbitrarily selected for recording, being transferred to a 1 mL capacity submersion-type recording chamber (BSC-ZT Zbicz Top, Harvard Apparatus, Massachusetts, USA) and continuously superfused with ACSF (control) or SCH 58261, 50 nM (condition tested) at a constant flow rate of 3 mL/min at 32ºC (TC-202A Bipolar Temperature Controller, Harvard Apparatus, Massachusetts, USA). For the tested condition, ventral and dorsal hippocampal slices were superfused with SCH 58261, at a supra-maximal concentration of 50 nM, with the objective to virtually block all A_{2A}Rs without compromising the activity of the other adenosine receptor subtypes (Lopes *et al*., 2004).

For electrophysiological recordings, the stimulation electrode was placed in the SC and the evoked field excitatory post-synaptic potentials (fEPSPs) were recorded in the dendrites of the CA1 pyramidal neurons (figure 12A) through a glass recording electrode (filled with ACSF, 1-2 MΩ) formed by micro pipettes obtained by a Flaming/Brown micropipette puller system, model P-97 (Sutter Instruments, USA).

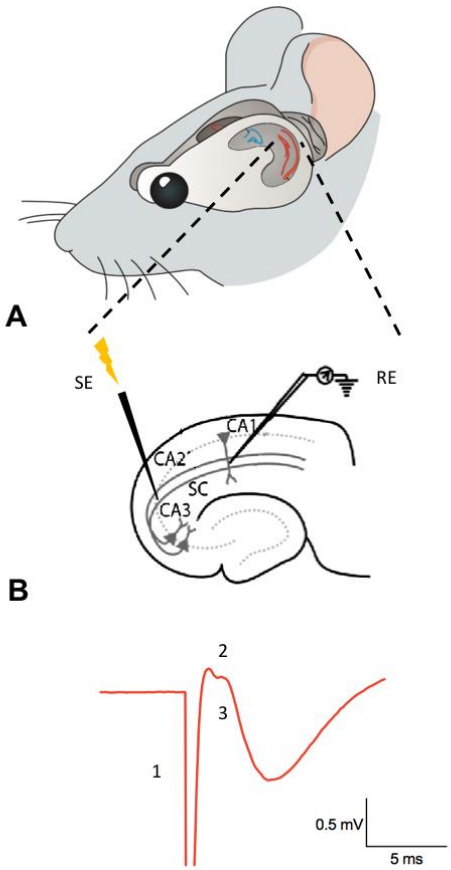


Figure 12- Diagram showing how electrophysiological recordings were obtained. **A)** Schematic representation of an electrophysiological recording performed in Schaffer collateral-pyramidal cell synapses in the CA1 region of a ventral hippocampal slice. **B)** Example of a representative trace obtained after stimulation: 1) stimulus artefact; 2) pre-synaptic volley and 3) field excitatory post-synaptic potential (fEPSP) (Mouse illustration obtained from Bannerman *et al.*, 2004).

The stimulation was performed using either a Grass S44 or Grass S48 square pulse stimulator (Grass Technologies, Warwick, RI, USA), the signal was amplified by an amplifier (ISO-80, World Precision Instruments, Hertfordshire, UK) and finally after amplification the recordings were digitized using an analogue-to-digital converter (BNC-2110, National Instruments, Newbury, UK) (Lopes *et al.*, 2015).

The data acquisition and analysis software was performed using the WinLTP version 2.20.1 (WinLTP Ltd., Bristol, UK) (Anderson & Collingridge, 2001). To quantify changes in the fEPSPs, the criteria used was the signal slope measured right after the presynaptic volley (figure 12B).

Input/output curves (I/O curves) were generated for each slice in the control condition and in the treated condition (before and following SCH 58261 superfusion) by measuring the slope of fEPSPs elicited by stimuli of graded intensities (approximately 20 µA increments) from that which produced no detectable post-synaptic response to a stimulus that produced a maximal post-synaptic response. The objective of doing I/O

curves was to determine the adequate level of electrical stimulation for the remainder of the experimental protocol – 60% of the maximum fEPSP slope (Habib & Dringenberg, 2009; Maggio & Segal, 2009) with no apparent contamination – as well as to evaluate changes in basal synaptic transmission due to pharmacological manipulations (by comparing the curves after and before SCH 58261 superfusion).

In detail, in the control conditions after the I/O curve (figure 13A), a steady baseline of at least 10 min was obtained (by adjusting the stimulation intensity to an adequate level of electrical stimulation previously stipulated) and LTD was induced – three trains of low frequency stimulation (LFS), each one consisting of 1500 pulses at 2 Hz, separated by a 10-min interval – and recorded for 60 min. On the other hand, in the treated slices after the first I/O curve and after achieving a steady baseline of at least 10 min, SCH 58261 was superfused (figure 13B). Then, after 20 min of pharmacological exposure, a second I/O curve was obtained, followed by a second steady baseline of at least 10 min and finally LTD was induced.

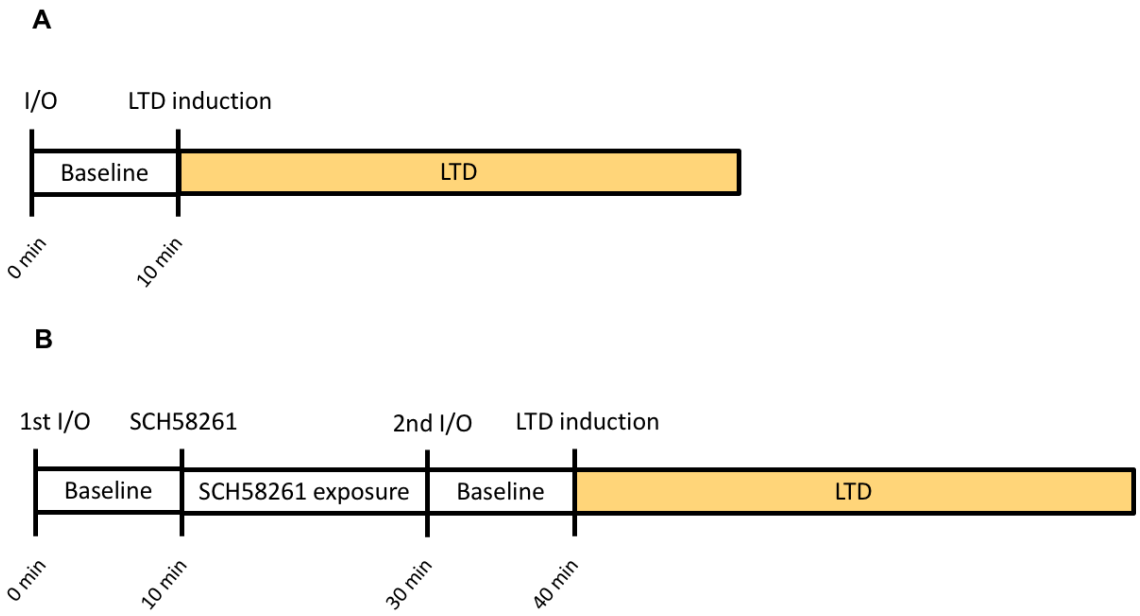


Figure 13- Schematic representation of the protocols used for extracellular electrophysiological recordings. **A)** Protocol for controlled condition **B)** Protocol for the tested condition.

Regarding the effect of SCH 58261 on basal synaptic transmission it was quantified as the percentage of change of fEPSP slope of the last 5 min (after the superfusion) in relation to the average of fEPSP slope during the 10 min immediately before the addition of the drug.

LTD was quantified as the percentage of change between two values: the average slope of the ten potentials taken between 50 and 60 min after LTD induction in relation

to the average of the fEPSP slopes measured during the 10 min that preceded LTD induction (earlier recorded baseline).

The effect of SCH 58261 on LTD was assessed by comparing the amplitude of LTD in untreated *vs*. treated slices.

3.3.3- Neurochemical studies

After performing the behavioural and electrophysiological tasks, immunohistochemistry studies were performed by using ventral and dorsal hippocampal slices with a thickness of 50 µm.

3.3.3.1- Sectioning hippocampal slices for immunohistochemistry

For immunohistochemistry studies, after performing the electrophysiological recordings, the remaining ventral and dorsal hippocampal slices (that were not selected for electrophysiological recordings) were fixated by immersion in a 4% PFA solution for 2 days and then were transferred into a 30% sucrose solution for dehydration. Three days later (at least), hippocampal slices were washed in PBS and were stored at -20°C embedded in an anti-freezing solution until being used in immunohistochemistry analysis.

After a while, when it was necessary to perform the immunohistochemistry studies, the transverse slices were again washed in PBS to completely remove the anti-freezing solution. Then, they were transferred into a plastic mould and immersed in a 3% ultrapure low melting point agarose solution (figure 14). After the agarose solidified completely, the excess of agarose was removed in order to obtain little individual cubes (with the slice inside) that were then placed in the vibratome (Leica VT1200S, Leica Biosystems, Germany) in order to obtain 50 µm-thick slices. The parameters used to cut the slices in the vibratome were: 0.22 mm/s of speed and 0.55 mm of amplitude. The 50-µm thick slices were temporarily stored until the start of the immunohistochemistry studies.

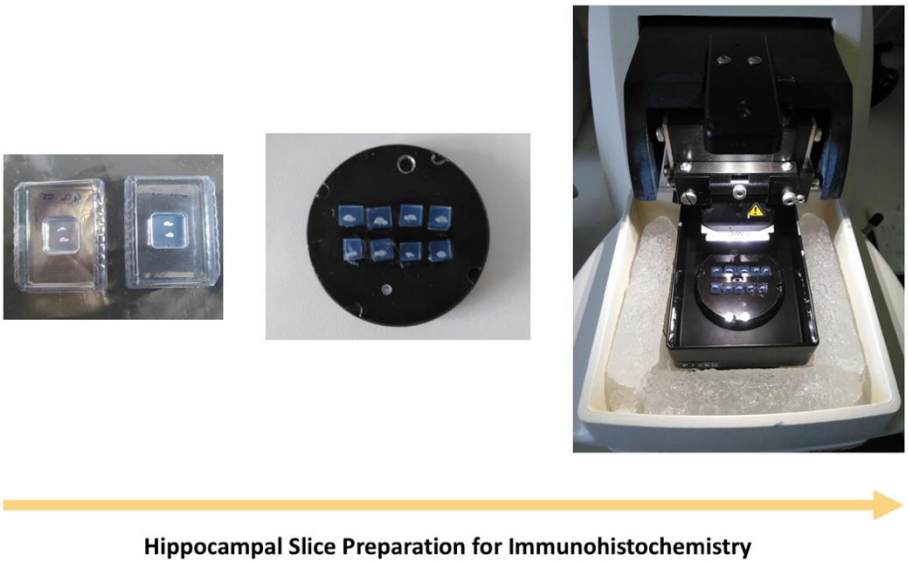


Figure 14- Illustration of the process to obtain 50 µm-thick hippocampal slices for immunohistochemistry.

3.3.3.2- Fluorescent immunohistochemistry

Immunohistochemistry (IHC) is a powerful microscopy-based technique used for visualizing the presence and location of an antigen of interest, such as proteins, in tissue samples.

The immunohistochemical staining is based on an antibody-antigen interaction, being subsequently necessary the use of antibodies to recognize the target antigen.

In our study, we tried to visualize two different proteins: 1) D_2R and 2) Gephyrin (a scaffold protein known for anchoring $GABA_A$ and glycine receptors) on 50 µm-thick ventral and dorsal hippocampal slices. For that goal, we performed two individual immunohistochemical stainings, one for D_2R and the other for gephyrin stainings.

First, 50 µm-thick ventral and dorsal hippocampal slices were washed 3 times with PBS. Then, the slices were incubated for 1h in the case of gephyrin and for 20 min in the case of D_2R with the respective blocking solution (described above). This step is very important to allow cell permeabilization and to prevent the nonspecific binding of the antibodies. After the blocking step, hippocampal slices were incubated with primary antibodies under gentle agitation at 4ºC for 2 days. Afterwards, the slices were washed 3 times for 10 min with PBS and then incubated with the secondary antibodies (in blocking solution) for 2h at room temperature (RT), under agitation. For each IHC, some slices were only incubated with the secondary antibody (not being incubated with the primary antibody) serving as negative controls of the experiments. Later, the slices were

again washed 3 times for 10 min with PBS, stained with nuclear dye DAPI (1:5000) for 10 min at RT, culminating in another set of washes.

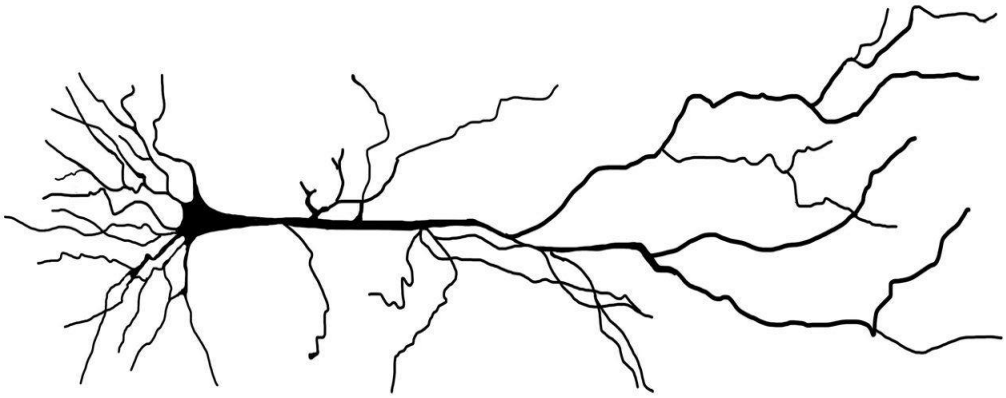
Finally, hippocampal slices were mounted in gelatin-coated slides using flurorescence mounting medium. After completely dried, hippocampal slices were visualized in the epifluorescence microscope (Zeiss Imager Z2, Oberkochen, Germany) and the images of the different hippocampal sub-divisions (CA1, CA3 and DG) were obtained at 20x magnification.

3.4- Statistical analysis

Data are expressed as mean ± the standard error of the mean (SEM) of n different animals (n= number of animals).

The comparison between the two experimental conditions was performed using either a paired or unpaired Student's *t*-test, as indicated in each case. One sample *t*-test compared with hypothetical value of 0 was performed to evaluate the effect of SCH 58261 superfusion on basal transmission. When doing comparisons among more than two experimental groups, one-way analysis of variance (ANOVA) followed by Dunnett post hoc test (comparing the mean values with habituation) or two-way ANOVA followed by Newman-Keuls post hoc test were performed. Exceptionally, since statistical analysis cannot be performed when the n value is less than three, in such cases unpaired Student's *t*-test was performed between the remaining groups instead of performing ANOVA. Statistical significance was set as $P<0.05$. All statistical analyses were performed with GraphPad Prism software (v.7).

Chapter 4



Results

4.1- Impact of hippocampal A_2ARs on long-term depression under physiological conditions

Long-term changes in hippocampal synaptic transmission (LTP and LTD) are widely considered to be required for spatial learning and memory (Malenka, 1994; Tsien *et al*., 1996).

With respect to LTD, increasing evidence has indicated that stress situations affect this type of synaptic plasticity in the dorsal and ventral HPC (Wong *et al*., 2007; Maggio & Segal, 2009). Moreover, LTD induction in the CA1 hippocampal sub-region has been recently associated with the encoding of fine spatial details in a new environment (Kemp & Manahan-Vaughan, 2004 & 2007).

On the other hand, information about the impact of A_{2A}Rs on LTD is sparse, with the existing information about such topic exploring only the dorsal-medial HPC.

In this sense, the hypothesis was born: study the impact of dorsal and ventral hippocampal A_{2A}Rs in fear generalization, a well-defined stress situation.

However, since the existing data about the potential role of A_{2A}Rs on LTD have focused only on dorsal-medial HPC we went to explore the impact of A_2ARs on LTD in the ventral and dorsal HPC, under a naïve situation, before conducting any other experiment (that is before exploring a stress situation).

4.1.1- The blockade of ventral and dorsal hippocampal A_{2A}Rs decreased LTD amplitude under physiological conditions

After optimizing an LTD protocol, and in order to investigate if A_{2A}Rs are active participants in LTD of dorsal and ventral HPC, we took advantage of a selective antagonist of these receptors (SCH 58261).

Our results reveal that the I/O curves are not significantly different between the control and the treated groups (figure 15A) and that SCH 58261 superfusion produced no effect in synaptic transmission (figure 15B).

The present data also show that there are no significant differences in LTD amplitude between the SCH 58261-treated slices and the non-treated slices (Ctrl) (figure 15D). Specifically, in Ctrl slices the amplitude of LTD was of -23.08 ± 8.26 % (n=5) whereas in SCH 58261-treated slices it was of -17.35 ± 4.90 % (n=5).

These observations clearly indicate that A_{2A}Rs do not participate in LTD in the vHPC.

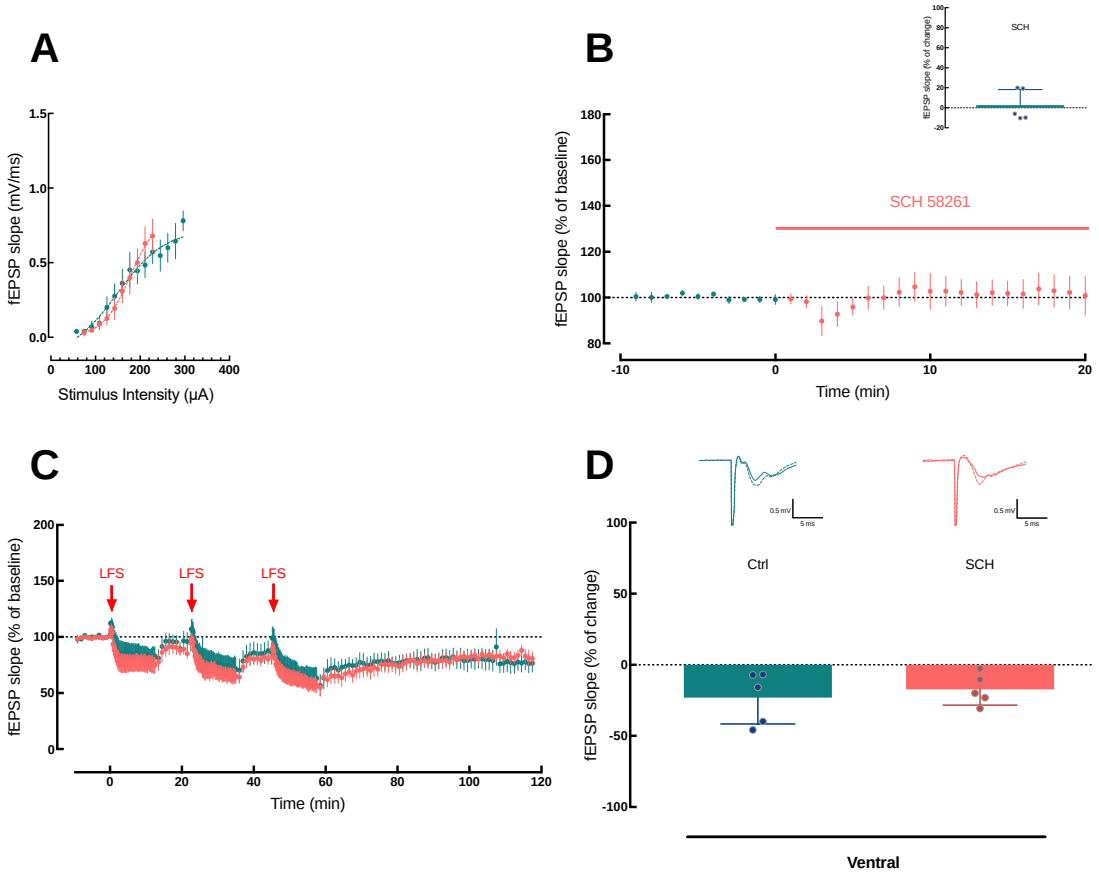


Figure 15- Effect of the selective A_{2A}R antagonist SCH 58261 in LTD of ventral hippocampus. A) Input/output (I/O) curves obtained by plotting the slope of fEPSPs in the CA1 area of the ventral hippocampus as a function of the stimulation intensity. I/O curves are similar, before (control, Ctrl) and after the application of SCH 58261 (50 nM, superfused for 20 min). **B)** Effect of SCH 58261 (50 nM) on basal synaptic transmission. Hippocampal slices were exposed to SCH 58261 20 min prior the LTD induction until the end of the recordings. As shown in the bar, no statistical alterations in basal synaptic transmission are observed following SCH 58261 superfusion in the system. **C)** Averaged time course changes of fEPSP slope produced by LTD induction (1500 pulses at 2 Hz repeated three times, with 10-min interval) in hippocampal slices from young adult mice. **D)** LTD amplitude corresponding to the average fEPSP slope 50–60 min after LTP induction is not significantly different in slices acutely treated with SCH 58261 comparing with the control situation. A) Nonlinear fit – Boltzmann sigmoidal curve; B) One sample *t*- test compared with hypothetical value of 0; D) Unpaired Student's *t*-test. All values are mean ± SEM of 3-7 mice per group.

With respect to the dHPC, the I/O curves do not display significant differences between the two groups (figure 16A) and SCH 58261 has no meaningful effect on basal transmission (figure 16B).

Once again, the acute blockade of dorsal hippocampal A_{2A}Rs (8.77 ± 7.34 %, n=6) did not yield any significant alteration in LTD amplitude when compared to Ctrl (-9.30 ± 7.54 % n=6) (figure 16D).

Therefore, A_{2A}Rs also appear do not modulate LTD in the dHPC.

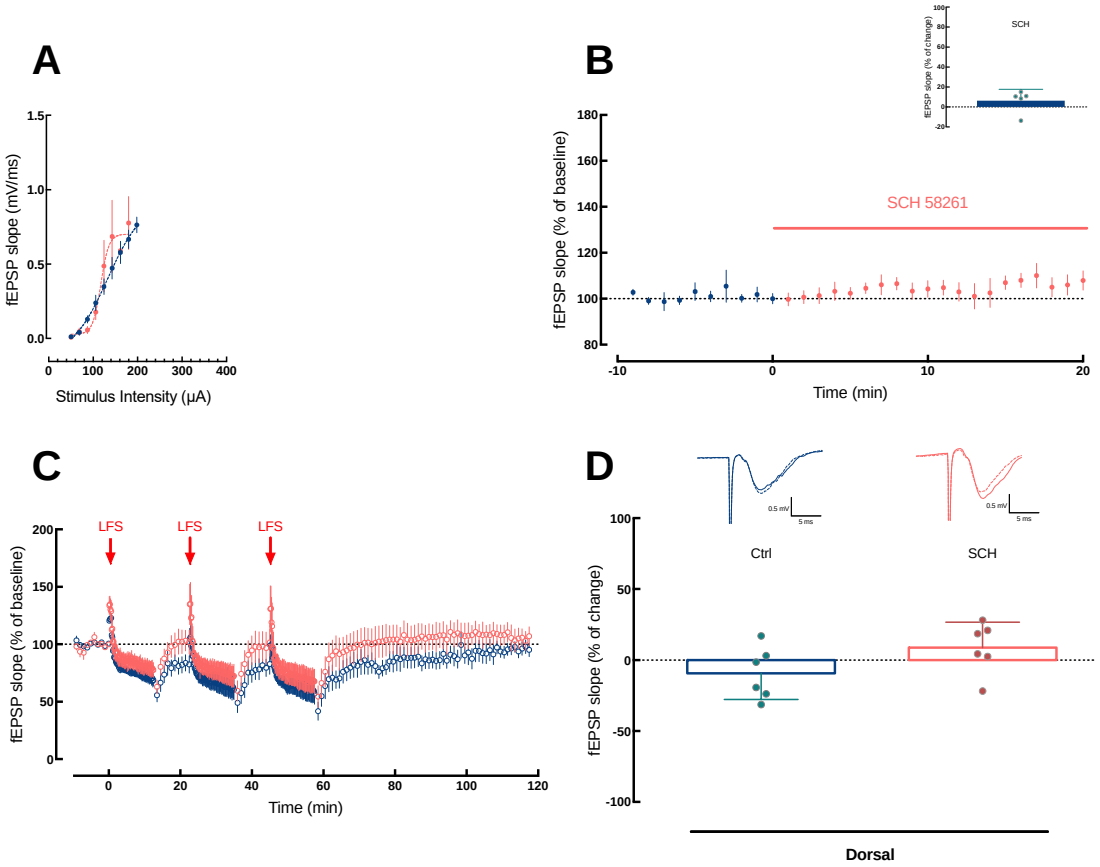


Figure 16- Effect of SCH 58261 on field-potential LTD of dorsal hippocampal slices. A) Averaged input/output (I/O) curves of the CA3–CA1 fEPSP slope from hippocampal slices treated with ACSF or SCH 58261 (50 nM, superfused for 20 min). I/O curves, where the fEPSP slope was plotted versus the stimulus intensity, do not present significant differences. **B)** Effect of SCH 58261 (50 nM) on basal transmission. No alteration in basal transmission is shown in the bar graph (average of the last 5 min) following SCH 58261 superfusion in the system. **C)** Time course of changes in fEPSP slope after LTD induction. **D)** LTD amplitude from the averaged fEPSP slope 50–60 min after LTD induction, revealed no significant alteration in slices acutely treated with SCH 58261 when compared to the control situation. A) Nonlinear fit – Boltzmann sigmoidal curve; B) One sample *t*- test compared with hypothetical value of 0; D) Unpaired Student's *t*-test. All values are mean ± SEM of 3-10 animals.

4.2- Optimization of time-dependent contextual fear generalization protocol

Recent studies have implicated the generalization of conditioned fear as one of the biggest symptoms of PTSD (Mahan & Ressler, 2012). Posing as a serious burden to daily life, it is urgent to find effective therapies for such a symptom. Therefore, it is of interest to investigate what are the behavioural and neural mechanisms underlying fear generalization.

However, in order to explore the role of A_{2A}Rs in that symptom, we first had to implement a fear generalization protocol in the laboratory.

Thus, since it is well established that fear generalization increases over time and our focus of study is the HPC (a structure essential for spatial and memory recognition), we optimized a protocol for time-dependent contextual fear generalization (HPC-dependent paradigm) (Granger *et al*., 1996; Zola-Morgan *et al*., 1996; Wiltgen & Silva, 2007; Cullen *et al*., 2015).

4.2.1- Memory for contextual cues becomes less specific over time

After some attempts with several approaches, we were finally successful (as evidenced in our results) by implementing an adaptation of the protocol by Cullen *et al*., 2015.

Here we show, through the acquisition curve (figure 17A), that mice increased their percentage of freezing as the number of shocks increased, beginning with a percentage of freezing of 5.07 ± 0.85 during habituation and finishing with a percentage of 53.18 ± 3.23 after the 5^{th} shock. Such result poses as a good indicator of a successful fear acquisition, a supposition that was further confirmed when animals were tested in the training context at a recent time point of 1 day after fear conditioning (Figure 17B, first bar).

As expected, figure 17B shows that 1 day after fear conditioning, mice tested in the novel context (13.24 ± 2.67 %) froze significantly for less time than animals tested in the context where they have received the footshocks (49.28 ± 3.40 %) (training context), suggesting that animals express a contextually precise memory at a recent time point following fear training. However, at a remote time point of 14 days after fear conditioning, animals in both the novel context (41.39 ± 5.42 %) and the training context

(54.41 ± 8.74 %) froze at equivalent levels, indicating that animals exhibited a contextually imprecise memory and had generalized their fear from the training context to the novel context.

Since many laboratories have reported that fear memories sometimes become stronger over time (Houston *et al.*, 1999; Balogh *et al.*, 2002; Frankland *et al*., 2004) – in a phenomenon known as fear incubation – it was necessary to clarify whether the observed generalization was actually due to a loss of contextual details or rather due to an increase in fear with the passage of time. Importantly, we demonstrated that contrarily to what happens in the novel context, the fear of the training context was stable over time which confirms that the increase in generalization was due to the loss of contextual details and not due to fear incubation (figure 17B).

Although freezing is often the most quantified behaviour in fear conditioning studies, we also evaluated fear memory through other two parameters (travelled distance and number of tail rattlings) in order to reinforce our results. Thus, although 2 of the 3 parameters were quantified manually by visual observation, by using three parameters we managed to reduce the probability of bias. Not surprisingly, at a recent time point, the travelled distance was significantly higher in the novel context (1.80 ± 0.17 m) comparing to the training context (0.77 ± 0.13 m) (figure 17C). There were no statistical differences between the training (4.17 ± 1.70) and the novel (0.86 ± 0.55) contexts at a recent time point with regard to the number of tail rattlings (figure 17D).

Altogether our results confirm previous suggestions (Perkins & Weyant, 1958; Mcallister & Mcallister, 1963; Richardson *et al*., 1984; Gisquet-Verrier & Alexinsky, 1986; Zhou & Riccio, 1996; Metzger & Riccio, 2008) that contextual memories become less specific with time, which reveals that the optimization of the protocol for time-dependent contextual fear generalization was accomplished.

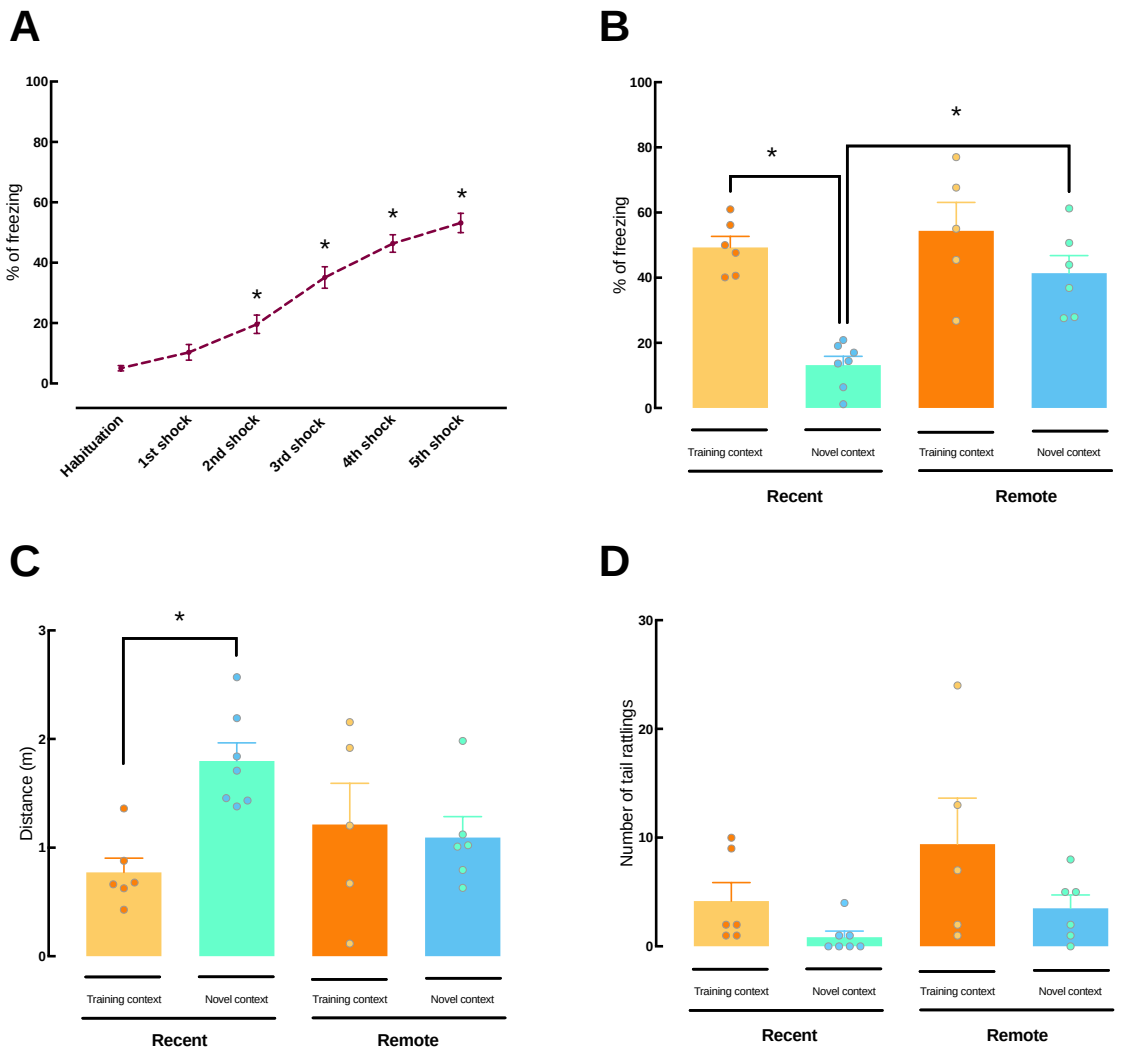


Figure 17- Fear memory levels for C57BL/6 mice during recent (1 day post training) and remote (14 days post training) tests. At the recent time point, mice tested in both training (yellow) and novel context (turquoise) exhibit a contextually precise fear memory. However, mice tested in the novel context at a remote time point (blue) generalized fear from the training context (orange) to the novel context. **A)** Acquisition Curve obtained from mice during the training session in which the percentage of freezing was plotted versus the number of shocks. During the habituation time (1.30 min) mice displayed no considerable percentage of freezing, however their percentage for this parameter increased with higher number of shocks given, suggesting a successful fear acquisition. **B)** Percentage of freezing levels between the four groups. The animals tested in the novel context at a remote time point display a freezing percentage significantly higher than the animals tested in the same context 1 day after fear conditioning, indicating that fear generalization increased over time. **C)** Locomotor activity among the four groups of mice. The results are expressed as travelled distance (m). **D**) Number of tail rattling. No significant difference is observed among the four groups. *P< 0.05: A) One-way ANOVA followed by Dunnett post hoc test; B, C and D) Two-way ANOVA followed by Newman-Keuls post hoc test. All values are mean ± SEM of 5-7 animals per group.

4.3- Hippocampal mechanisms underlying fear generalization

It has been proposed that impairments in hippocampal-mediated pattern separation (disability of the HPC to discriminate between two similar contexts) may underlie the over-generalization of fear (Kheirbek *et al*., 2012; Lopresto *et al*., 2015). Moreover, LTD induction in the CA1 hippocampal sub-region is associated with the encoding of fine spatial details in a new environment (Kemp & Manahan-Vaughan, 2004 & 2007).

Nevertheless, there is a lack of information about the possible hippocampal synaptic plasticity processes that could be involved in fear generalization.

Thus, after performing the behavioural experiments, animals were sacrificed and electrophysiological recordings in the CA3-CA1 pathway of ventral and dorsal hippocampal slices were performed in order to try to understand if LTD impairments could be involved in this model of fear generalization in such regions.

4.3.1- Alterations in ventral hippocampal synaptic plasticity underlie fear generalization

The I/O curve of the remote, novel context group is significantly different from the I/O curves of the other groups (figure 18A). For the same stimulus intensity, slices from the animals that generalized their fear produced lower values of fEPSP slope when compared to the remaining groups.

Our data revealed that in the vHPC animals that generalized their fear (remote, novel context group) exhibited alterations in synaptic plasticity when compared to animals that did not generalize (recent, novel context group) figure 18C). In detail, in the ventral hippocampal slices of the animals tested in the novel context at day 1, the amplitude of LTD was of -38.40 ± 8.34 % (n=5) while in the remote, novel context group there was an impairment of LTD (10.93 ± 8.46 %, n=3) in response to an LTD protocol.

Curiously, the remaining groups also displayed robust LTDs: recent, training context group (-24.05 ± 11.03 %, n=6) and remote, training context group (-26.31 ± 5.11 %, n=2).

Thus, these results suggest that an impairment of LTD in vHPC could be responsible for the expression of an imprecise fear memory (remote, novel context group) instead of a precise one (recent, training context group; recent, novel context group and remote, training context group).

Together, these observations suggest that alterations in synaptic plasticity in vHPC appear to underlie the expression of an imprecise memory pointing towards an implication of this structure in fear generalization.

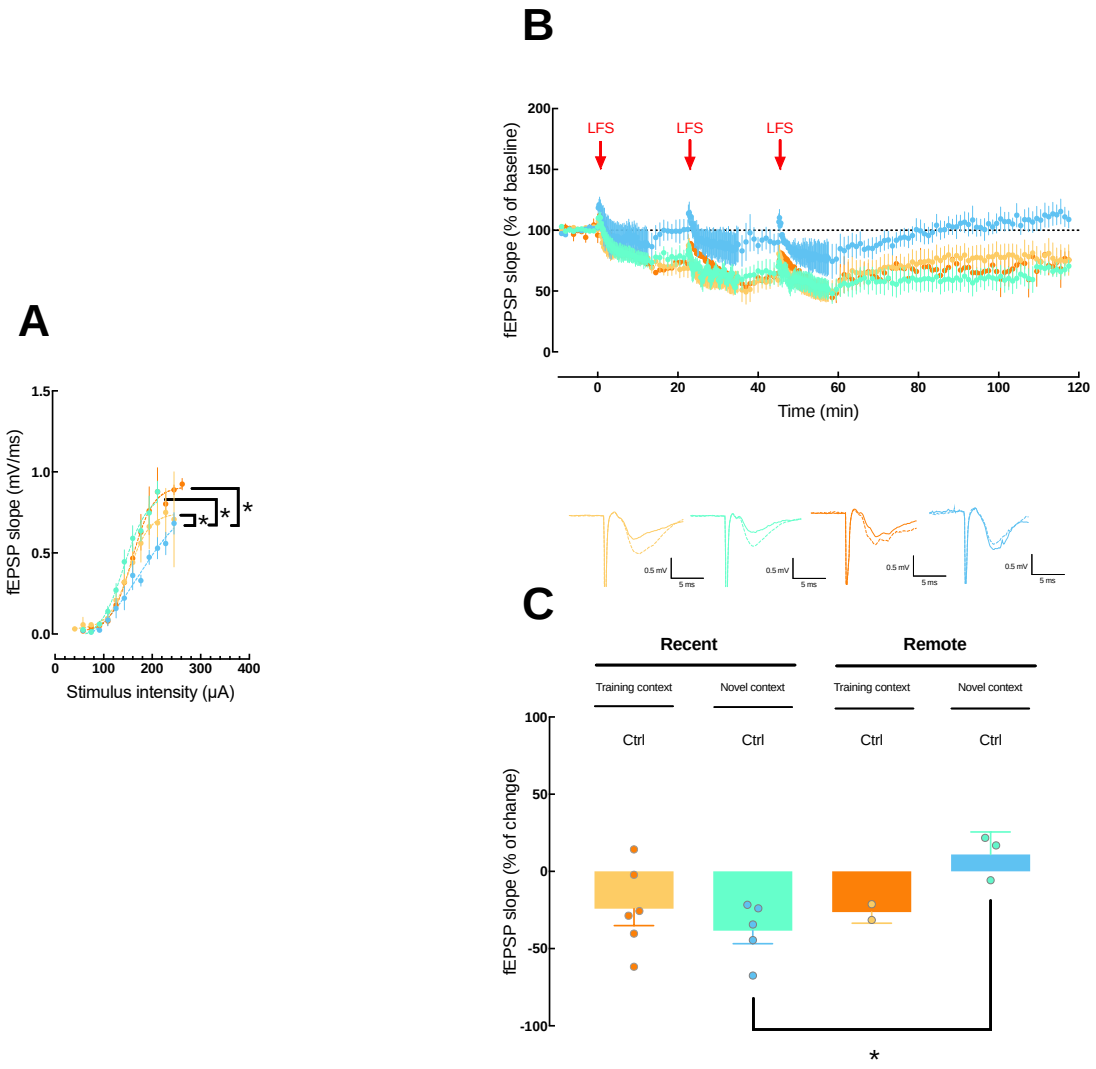


Figure 18- Impairments in long-term depression (LTD) could underlie fear generalization. A) Input/output curves, presenting fEPSP slope in response to increasing stimulus input, of adult C57BL/6 mice tested at recent (1 day post training) or remote (14 days post training) time points. Statistical differences are observed in the I/O curve of the remote, novel context group when compared to the remaining groups. For the same stimulus intensity, slices from the animals that generalized their fear produced lower values of fEPSP slope. **B)** Averaged time course fEPSP slope compared to baseline from the four distinct groups. fEPSP amplitude was recorded during 60 min after the LTD induction. **C)** LTD amplitude corresponding to the average fEPSP slope 50–60 min after LTP induction. Contrarily to the groups that displayed a contextual precise memory recall, the slices from the animals that generalized their fear were not capable of producing LTD in response to the application of an LTD protocol. *P < 0.05: A) Nonlinear fit – Boltzmann sigmoidal curve; C) Unpaired Student's *t*-test. All values are mean ± SEM of 2-6 mice per group.

4.3.2- Dorsal hippocampus (dHPC) does not appear to be implicated in fear generalization

Conversely, similar results are not observed in dHPC. Our data shows that there is no significant difference in synaptic plasticity (namely LTD) between the animals that express a contextual precise memory and the animals that do not (figure 19C). In accordance, the I/O curves do not display significant differences (figure 19A).

Our data appear to exclude the possibility that alterations in LTD in the dHPC could underlie fear generalization.

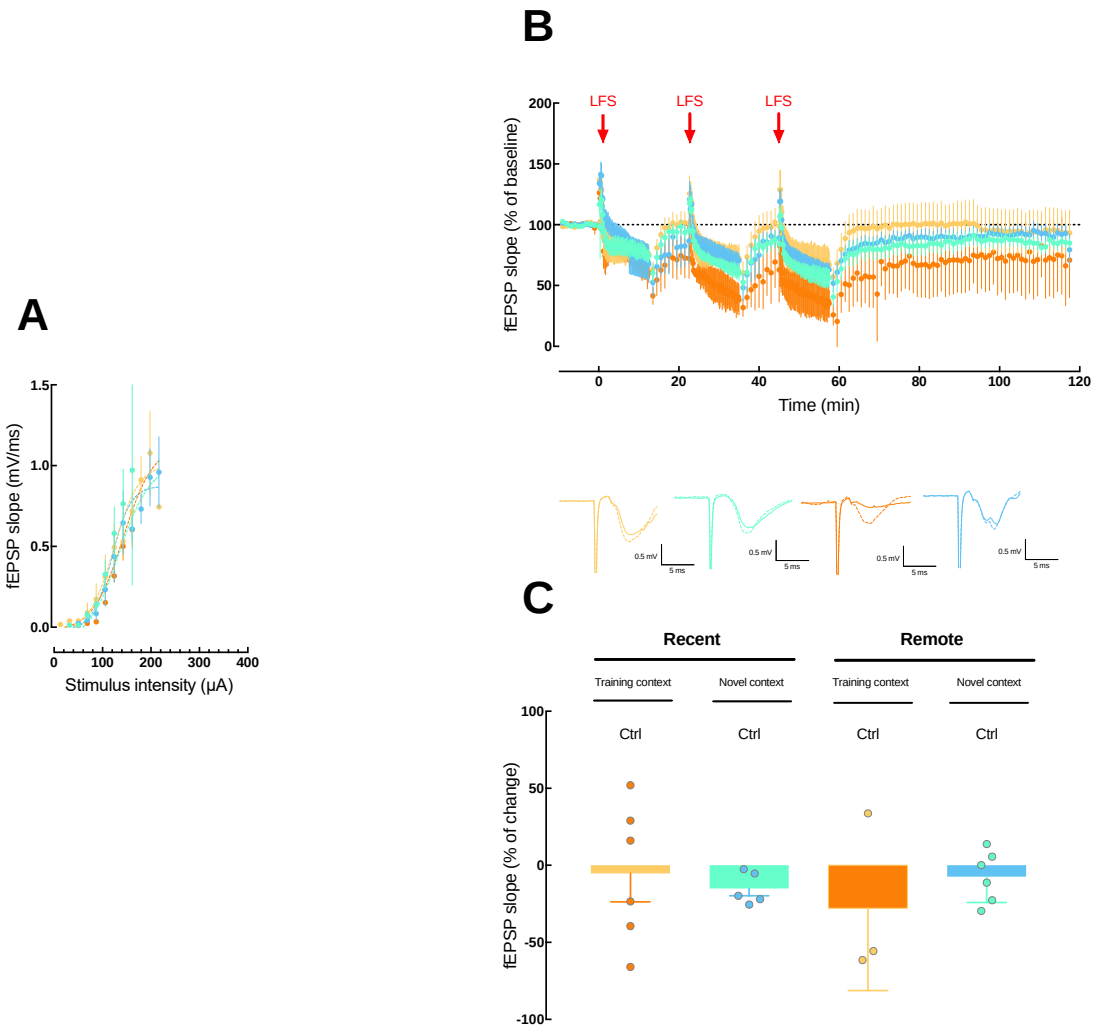


Figure 19- In the dorsal hippocampus, alterations in LTD do not appear to underlie fear generalization. A) Input/output (I/O) curves obtained by plotting the slope of fEPSPs in the CA1 area of the dorsal hippocampus as a function of the stimulation intensity. No significant differences are observed in the I/O curves from the four groups. **B)** Time course of changes in fEPSP slope after LTD induction. **C)** LTD amplitude corresponding to the average fEPSP slope 50–60 min after LTP induction are not significantly different among the four groups. A) Nonlinear fit – Boltzmann sigmoidal curve; C) Two-way ANOVA. All values are mean ± SEM of 3-6 mice per group.

4.4- Role of ventral hippocampal A_{2A}Rs on long-term depression (LTD) in fear generalization and in the animals that expressed a precise memory

Up to this point, we have shown that: 1) A_{2A}Rs do not seem to modulate LTD in dorsal and ventral HPC, under physiological conditions; 2) alterations in vHPC synaptic plasticity could underlie fear generalization.

Furthermore, there is compelling evidence that the acute blockade of A_{2A}Rs is sufficient to rescue the LTD-to-LTP shift observed in pathological conditions such as Alzheimer's disease, stressing out A_{2A}R as a pathological mediator involved in memory disruption (Ferreira *et al*., 2018).

Thus, having all this information in mind, we postulated the next question: Could the acute selective blockade of A_{2A}Rs revert the ventral hippocampal synaptic alterations observed in the animals that generalized their fear to values similar to those observed in animals that do not generalize?

Of note, the impact of ventral and dorsal hippocampal A_{2A}Rs in the synaptic plasticity of the remaining groups (recent, training context group; recent, novel context group; remote, training context group) was also evaluated.

4.4.1- The acute blockade of ventral hippocampal A_{2A}Rs rescued the long-term depression (LTD) impairments in animals that generalized their fear

To investigate if A_{2A}Rs are implicated in the LTD impairment observed in mice that generalized their fear, we took again advantage of the selective antagonist of these receptors (SCH 58261). Thus, ventral hippocampal slices from the animals that generalized their fear were superfused with the SCH 58261 (50 nM) and the LTD amplitude was compared to the respective control (remote, novel context group).

Regarding the I/O curves, statistically differences were observed between the SCH 58261 condition and the remote, novel context group (figure 20A). Indeed, the input-output function of evoked fEPSPs is significantly elevated in the tested condition when compared to the control situation. In agreement, the basal synaptic transmission was also statistically altered after SCH 58261 exposure (35.20 ± 9.05 %) (figure 20B).

When using an unpaired Student's *t*-test no statistical differences were observed between the tested condition and the respective control condition (10.93 ± 8.46 %, n=3

vs. -21.82 ± 14.28 %, n=4, respectively) (figure 20E). However, when we checked the results in more detail and paired them we observed that SCH 58261 reverted the impairments of LTD (figure 20D).

Hence, we observed that the acute blockade of A_{2A}Rs rescued the synaptic plasticity deficits observed in the animals that generalized their fear, allowing the occurrence of LTD and, consequently, normalizing the values back to the ones seen in the animals that did not generalize.

These results further reinforce the therapeutic interest of this molecular target in multiple pathologies associated with memory deficits.

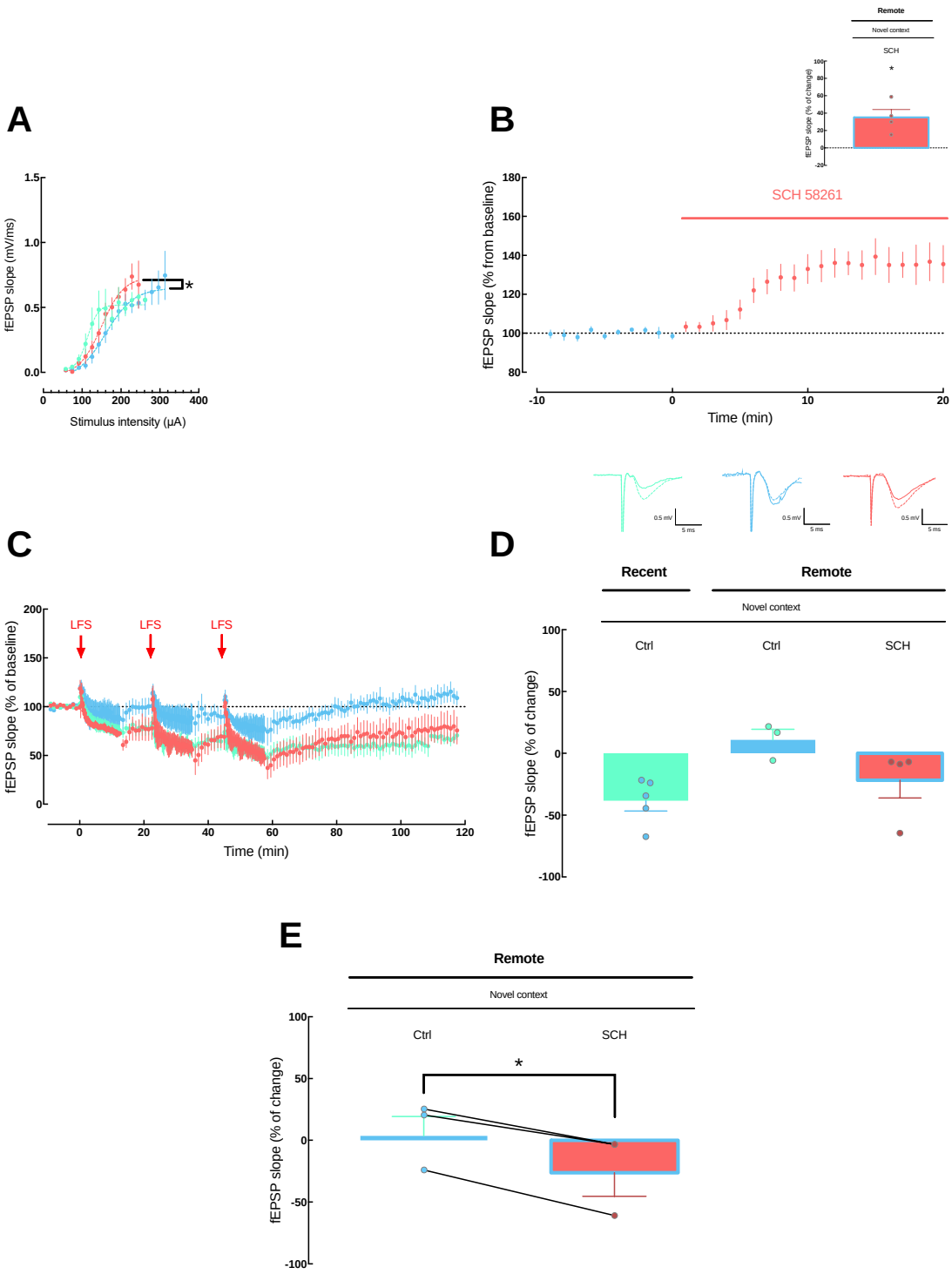


Figure 20- The acute blockade of A_{2A}Rs is sufficient to prevent the ventral hippocampal plasticity impairments observed in the group of mice that generalized their fear. A) Stimulus-response curves presenting fEPSP slope in response to increasing stimulus. The acute treatment with SCH 58261 rescued the stimulus-response function back to the standards observed in the animals that did not generalize their fear. **B)** Effect of SCH 58261 (50 nM) on basal synaptic transmission of the remote, novel context group. As shown in the bar, statistical alterations in basal synaptic transmission are observed following SCH 58261 superfusion in the system (average of the last 5 min). **C)** Averaged time course changes of fEPSP slope produced by LTD induction (1500 pulses at 2 Hz repeated three times with 10-min interval) in ventral hippocampal slices, recorded for 60 min. **D and E)** LTD amplitude corresponding to the average fEPSP slope 50–60 min after LTP induction. **E)** In a paired comparison of the results, the acute blockade of A_{2A}Rs is sufficient to rescue the LTD deficits observed in the animals that generalized their fear. *P < 0.05. A) Nonlinear fit – Boltzmann sigmoidal curve; B) One sample *t*- test compared with hypothetical value of 0; D) Unpaired Student's *t*-test; E) Paired Student's *t*-test. All values are mean ± SEM of 3-5 animals per group.

4.4.2- The acute blockade of ventral hippocampal A_{2A}Rs does not appear to have any impact in the synaptic plasticity of the animals that expressed a precise memory

The impact of the acute blockade of ventral A_{2A}Rs was also evaluated in the remaining groups in order to evaluate if the role of A_{2A}Rs on LTD is only manifested when there is a synaptic dysfunction.

No statistical differences are observed between the I/O curves of the recent, training context group and the recent, novel context group when compared to the respective drug tested condition (SCH 58261 superfusion) (figure 21, A and E). However, the basal transmission is statistically altered in those two groups following SCH 58261 exposure - recent, training context group: 30.22 ± 7.54 %, n=4; recent, novel context group: 18.73 ± 6.28 %, n=5 (figure 21, B and F).

Regarding LTDs, contrarily to what was observed in the group that generalized fear, no significant alteration in synaptic plasticity was visualized in the groups tested at a recent time point of 1 day after fear conditioning, following the acute blockade of ventral hippocampal A_{2A}Rs (figure 21, D and H). In detail, in the recent, training context group (-24.05 ± 11.03 %, n=6) SCH 58261 superfusion produced an LTD amplitude of -29.91 ± 6.77 % (n=5), while in the recent, novel context group (-38.40 ± 8.34 %, n=5) the same treatment induced an LTD value of -22.85 ± 30.54 % (n=3).

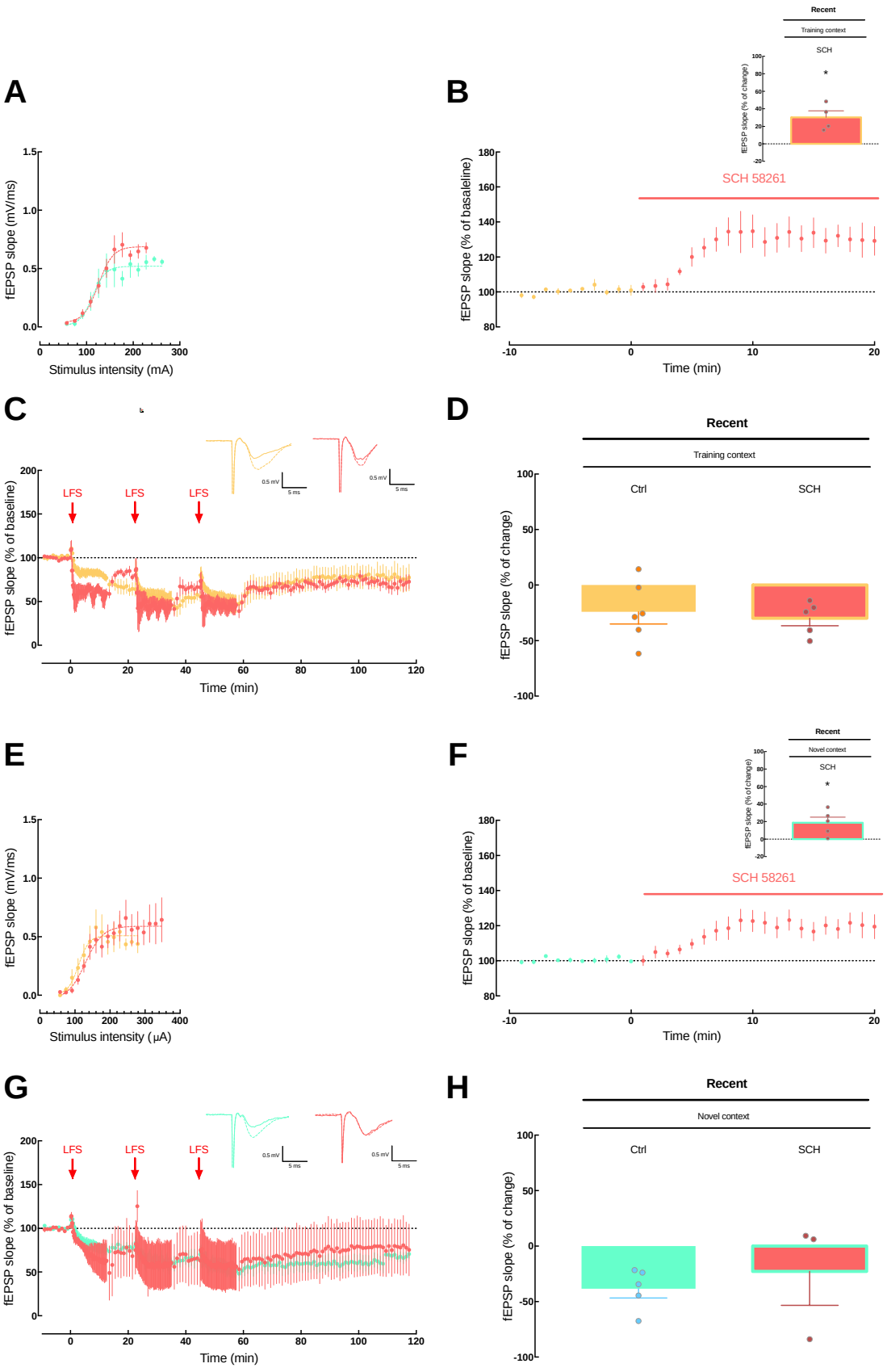


Figure 21- Effect of the selective $A_{2A}R$ antagonist SCH 58261 in ventral hippocampal slices from the animals tested at a recent time point of 1 day following fear conditioning. A-D) Recent, training context group; **E-H)** Recent, novel context group. **A and E)** Input–output (I/O) function measured at CA3-CA1 pathway in ventral hippocampal slices from the animals tested on day after fear conditioning. No statistical difference is observed among the I/O curves

obtained after and before SCH 58261 superfusion (50 nM), in the recent, training context group. However, significant alterations are visualized between the I/O curves of the control (Ctrl) condition and the tested condition, in the recent, novel context group. **B and F)** Effect of SCH 58261 (50 nM) on basal transmission. Alterations in basal transmission are shown in the bar graph (average of the last 5 min) in the two groups, following SCH 58261 superfusion in the system. **C and G)** Time course showing the effects of SCH 58261 on fEPSP slope in acute slices of mice tested at a recent time point. fEPSP slopes were normalized in each experiment using the averaged slope value during the baseline (-10 to 0 min). **D and H)** LTD amplitude corresponding to the average fEPSP slope 50–60 min after LTP induction, is not significant different among the slices treated with SCH 58261 and the slices treated with ACSF, in both groups. *P < 0.05. A and E) Nonlinear fit – Boltzmann sigmoidal curve; B and F) One sample *t*- test compared with hypothetical value of 0; D and H) Unpaired Student's *t*-test. All values are mean ± SEM of 3-6 animals per group.

In the remote time point, regarding the I/O curves, statistical differences are observed between SCH 58261 condition and the remote, novel context group (figure 22A). Likewise, the acute blockade of A_{2A}Rs yielded significant alterations in basal transmission (40.50 ± 18.94%, n=3) (figure 22B).

In the remote, training context group no statistical analysis was performed to compare the LTD amplitude of the tested condition (A_{2A}Rs blockade) with the control since the number of experiments is low (figure 22D).

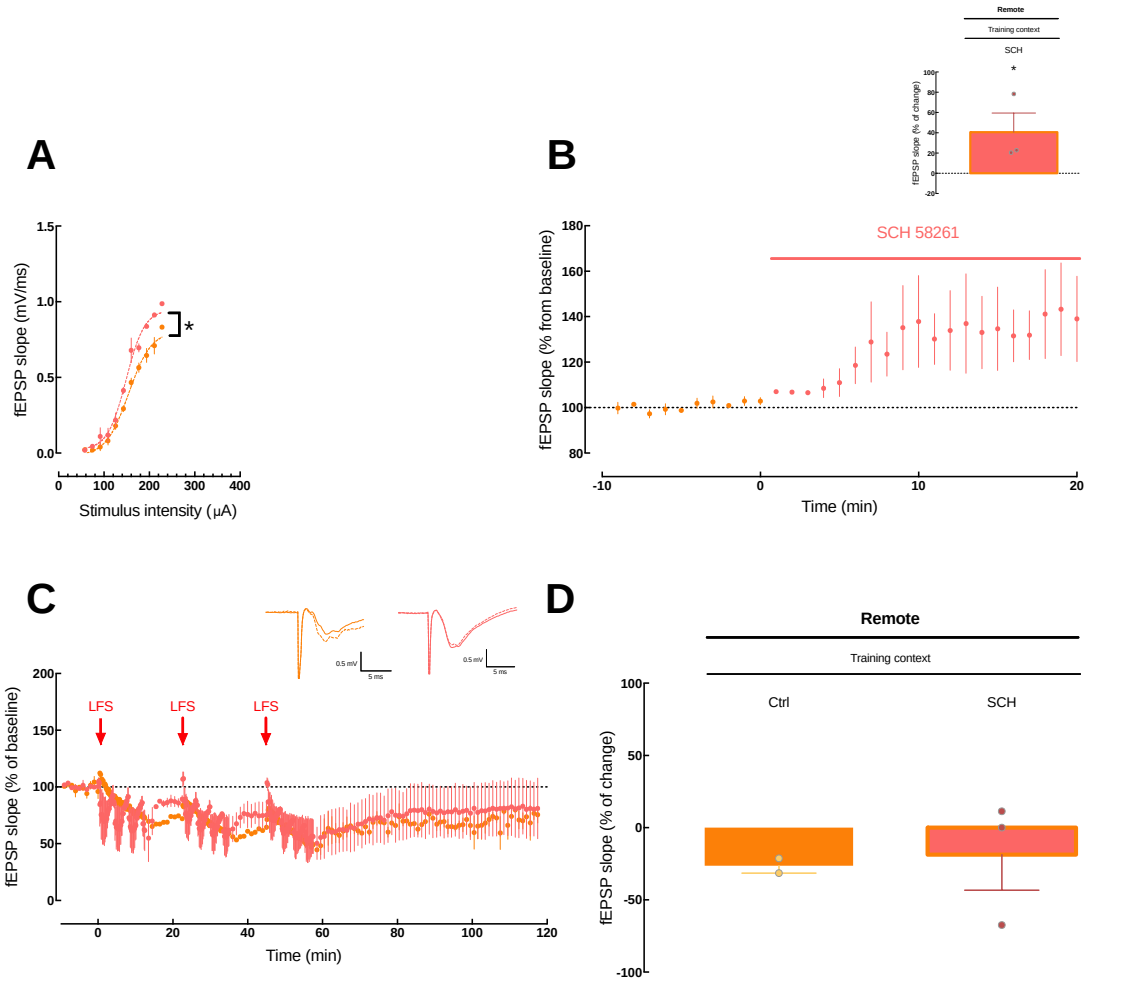


Figure 22- Effect of SCH 58261 on LTD of remote, training context group. A) Input/output (I/O) curves obtained by plotting the slope of fEPSPs in the CA1 area of the ventral hippocampus as a function of the stimulation intensity. Significant differences are observed in the I/O curves obtained after and before SCH 58261 superfusion of ventral

slices. **B)** Effect of SCH 58261 (50 µM) on basal synaptic transmission. Hippocampal slices were exposed to SCH 58261 20 min prior the LTD induction until the end of the recordings. Alterations in basal synaptic transmission following SCH 58261 exposure are shown in the bar graph (average of the last 5 min). **C)** Time course of changes in fEPSP slope after LTD induction. fEPSP slopes were normalized in each experiment using the averaged slope value during the baseline (-10 to 0 min). **D)** The SCH 58261 superfusion did not produce any change in the LTD amplitude of the remote, training context group. LTD amplitude corresponds to the average fEPSP slope 50–60 min after LTP induction. *P < 0.05. A) Nonlinear fit – Boltzmann sigmoidal curve; B) One sample *t*- test compared with hypothetical value of 0; D) Unpaired Student's *t*-test. All values are mean ± SEM of 2-3 animals per group.

4.5- Role of dorsal hippocampal A_{2A}Rs on long-term depression (LTD) in the animals that expressed an imprecise or precise Memory

For the same reason enunciated in the 4.4.2 section, we also explored the impact of dorsal A_{2A}Rs on LTD of the different groups, although no statistical differences were found between them.

4.5.1- The acute blockade of dorsal hippocampal A_{2A}Rs does not appear to have any impact in the synaptic plasticity of the animals tested at a recent or remote time point

The I/O curves are not significant different between the tested condition (SCH 58261) and the respective control (recent, training context group and recent, novel context group) (figure 23, A and E). The SCH 58261 exposure statistically increased the basal synaptic transmission of both groups - recent, training context group: 21.02 ± 7.39 % (n=6) and recent, novel context group: 30.09 ± 6.24 % (n=6) (figure 23, B and F).

Similarly to what happened in the vHPC, the acute blockade of dorsal hippocampal A_{2A}Rs did not yield any statistical alteration in synaptic plasticity of the groups tested at a recent time point of 1 day following fear conditioning (figure 23, D and H). In detail, in the recent, training context group (-5.27 ± 18.32 %, n=6) the superfusion of SCH 58261 produced an LTD amplitude of -20.26 ± 5.42 % (n=6) while in the recent, novel context group (-14.99 ± 4.65 %, n=5) the same treatment induced an LTD amplitude of -14.85 ± 17.15 % (n=5).

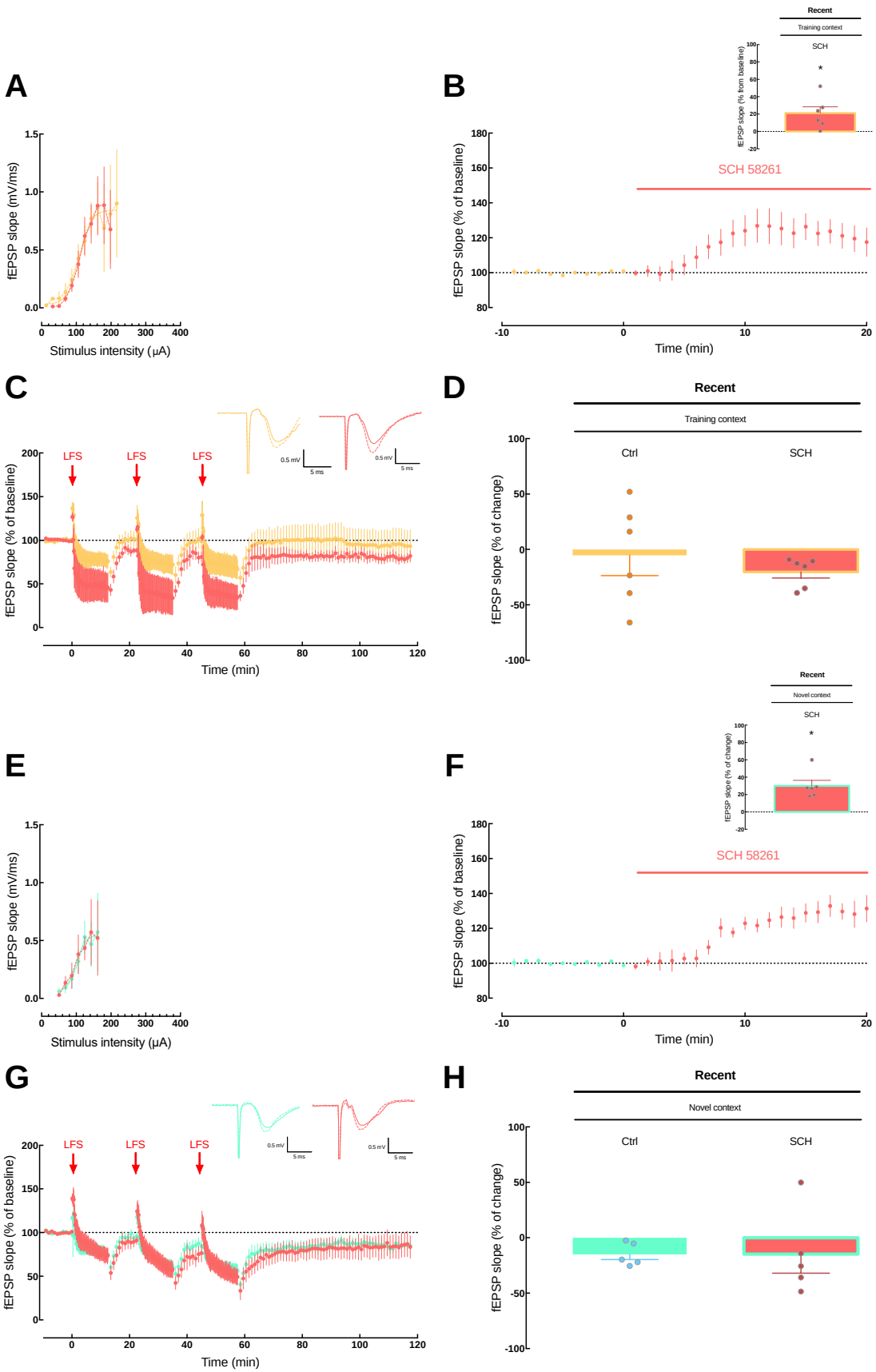


Figure 23- Effect of the acute blockade of dorsal hippocampal A_{2A}Rs on synaptic plasticity of mice tested at a recent time point of 1 day after fear conditioning. A-D) Recent, training context group; **E-H)** Recent, novel context group. **A and E)** Averaged input/output (I/O) curves of the CA3–CA1 fEPSP slope from hippocampal slices treated with ACSF or

SCH 58261 (50 nM, superfused for 20 min). No statistical difference is observed among the I/O curves obtained after and before SCH 58261 superfusion (50 nM), in the animals tested at a recent time point. **B and F)** Effect of SCH 58261 (50 nM) on basal transmission. Hippocampal slices were exposed to SCH 58261 during the time indicated by the yellow or turquoise line until the end of the recordings. Significant alteration in basal transmission are shown in the bar graph (average of the last 5 min) in the two groups, following SCH 58261 superfusion in the system. **C and G)** Time course showing the effects of SCH 58261 on fEPSP slope in acute slices of mice tested at a recent time point. **D and H)** LTD amplitude, corresponding to the average fEPSP slope 50–60 min after LTP induction, is not significantly different among the slices treated with SCH 58261 and the slices treated with ACSF, in both groups. A and E) Nonlinear fit – Boltzmann sigmoidal curve; B and F) One sample *t*- test compared with hypothetical value of 0; D and H) Unpaired Student's *t*-test. All values are mean ± SEM of 3-6 animals per group. All values are mean ± SEM of 5-6 animals per group.

In accordance, no significant alterations in synaptic plasticity were observed in the groups tested in the training or novel contexts at a remote time point of 14 days following fear conditioning, after the acute blockade of A_{2A}Rs (figure 24, D and H). In detail, in the remote, training context group (-27.79 ± 30.81 %, n=3) the SCH 58261 superfusion produced an LTD amplitude of -2.09 ± 9.28 % (n=4) while in the recent, novel context group (-7.30 ± 6.86 %, n=6) the same treatment induced an LTD value of -24.68 ± 15.18 % (n=6).

Statistical differences were not observed between the I/O curves of the recent, training context group and the respective tested condition (figure 24A). In contrast, the input-output function of the remote, novel context group is significantly different from the respective tested condition (figure 24E).

Once again, the acute blockade of A_{2A}Rs yielded statistical alterations in basal synaptic transmission – remote, training context group: 13.12 ± 4.73 % (n=4); remote, novel context group: 28.11 ± 12.28 % (n=6) (figure 24, B and F).

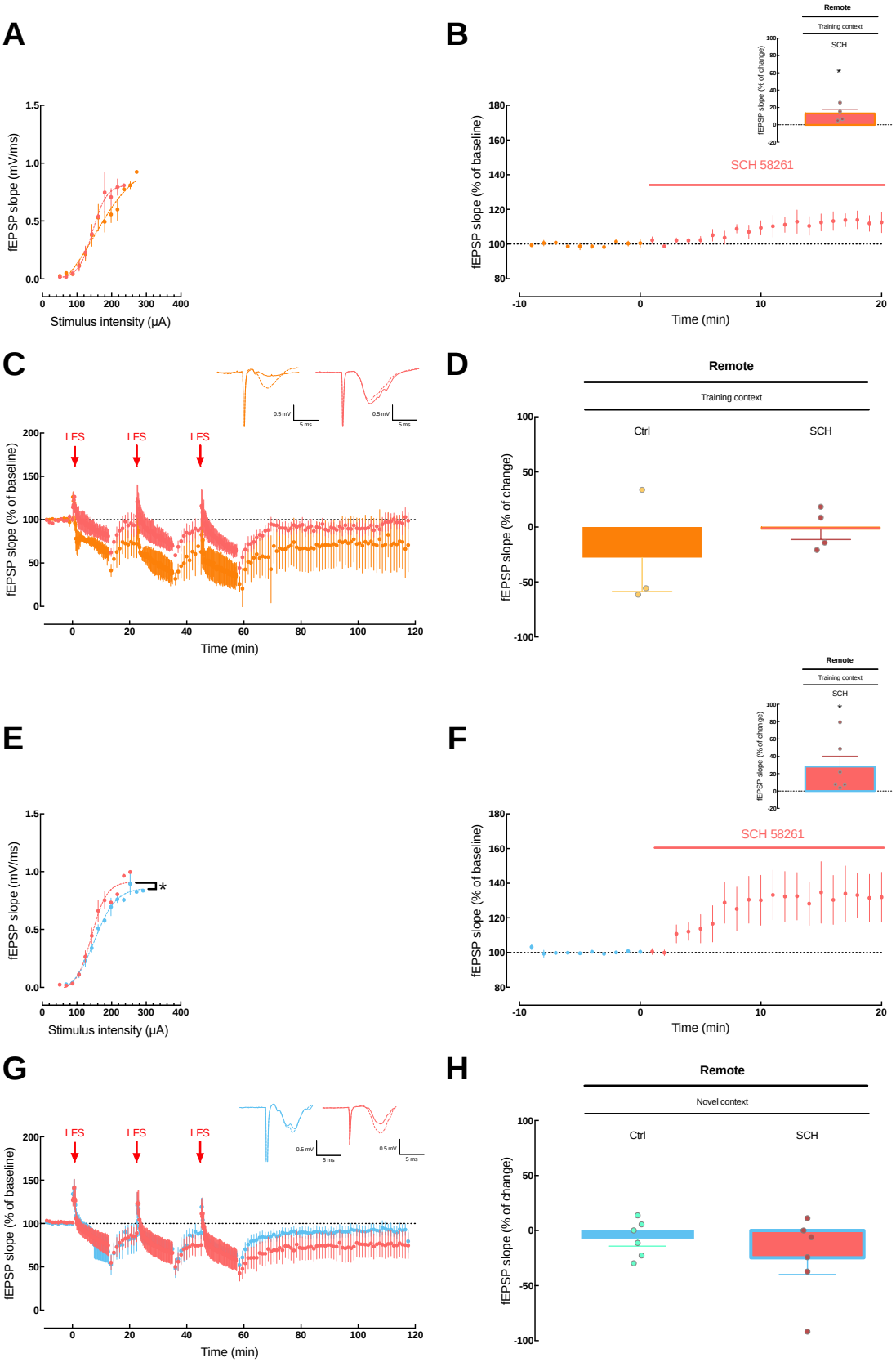


Figure 24- Effect of the selective $A_{2A}R$ antagonist SCH 58261 in dorsal hippocampal slices from the animals tested at a remote time point of 14 day following fear conditioning. A-D) Remote, training context group; **E-H)** Remote, novel context group. **A and E)** Input–output (I/O) function measured at CA3-CA1 pathway in dorsal hippocampal slices

from the animals tested on 14 after fear conditioning. No statistical difference was observed among the I/O curves obtained after and before SCH 58261 superfusion (50 nM), in the remote, training context group. Nevertheless, significant alterations are visible between the I/O curves of the control (Ctrl) condition and the tested condition, in the remote, novel context group. **B and F)** Effect of SCH 58261 (50 nM) on basal transmission. Hippocampal slices were exposed to SCH 58261 20 min prior the LTD induction until the end of the recordings. Alterations in basal synaptic transmission following SCH 58261 superfusion are shown in the bar graph (average of the last 5 min). **C and G)** Averaged time course changes of fEPSP slope produced by LTD induction in dorsal hippocampal slices from young adult mice. **D and H)** LTD amplitude corresponding to the average fEPSP slope 50–60 min after LTP induction. The acute blockade of $A_{2A}R$ did not yield any significant alteration in synaptic plasticity of mice tested at a remote time point. *P < 0.05. A and E) Nonlinear fit – Boltzmann sigmoidal curve; B and F) One sample *t*- test compared with hypothetical value of 0; D and H) Unpaired Student's *t*-test. All values are mean ± SEM of 3-6 animals per group.

4.6- Potential biomarkers of fear generalization

The research of potential biomarkers is essential to aid in the prevention, diagnosis and adequate treatment selection of many disorders. However, despite many efforts, no putative biomarker for PTSD has been uncovered yet.

Thus, we also performed immunohistochemical assays using the transverse hippocampal slices that were not used for electrophysiological recordings in order to look for potential biomarkers of fear generalization, namely, gephyrin and D_2Rs.

However, it is important to mention that this study is exploratory, no quantification of the results was performed and only the most obvious visible differences were mentioned.

4.6.1- Alterations in D_2Rs immunoreactivity do not seem underlie fear generalization in ventral and dorsal hippocampus (HPC)

Regarding vHPC, in the remote time point it was observed an increased in the D_2R immunoreactivity in the training context when compared with the novel context (figure 25). However, no visual differences are found between the animals that generalized their fear (remote, novel context group) and the animals that did not in any of hippocampal sub-region (recent, novel context group).

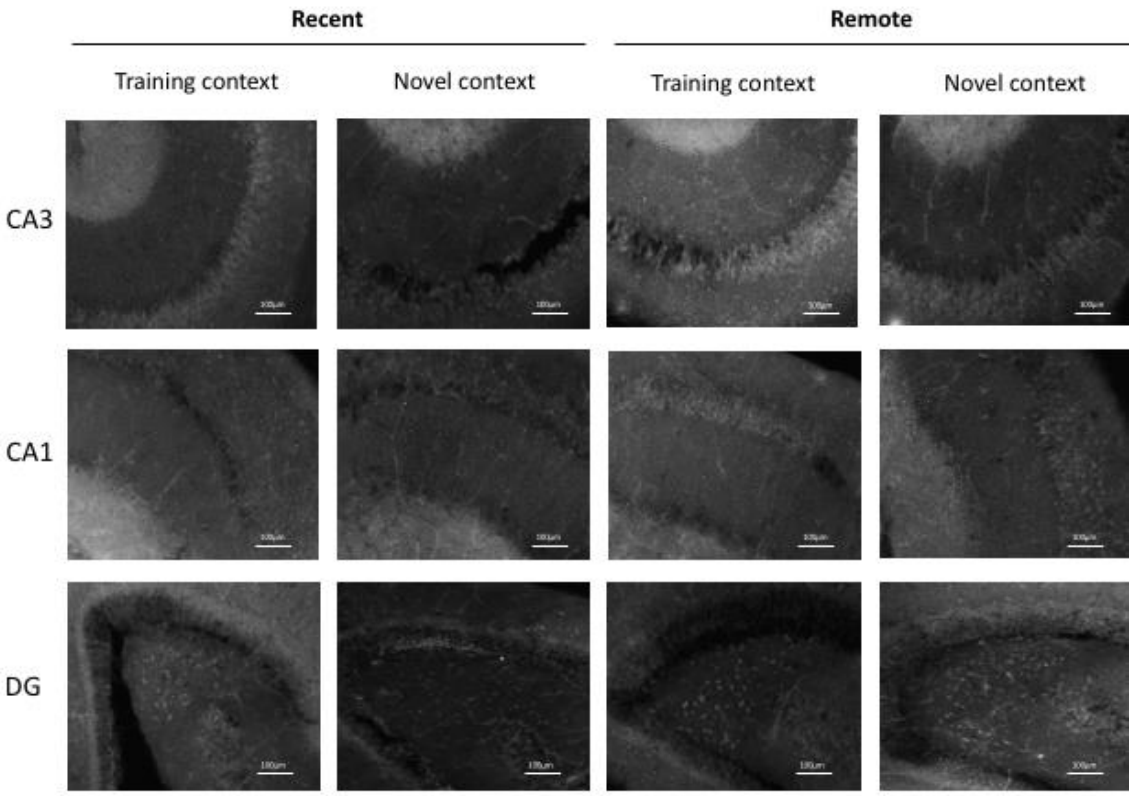


Figure 25- Representative photomicrographs of D_2R immunohistochemistry in the different hippocampal sub-divisions (CA1, CA3 and DG) from ventral hippocampal slices (50 µm-thick) from the different groups: recent, training context group (n=4), recent, novel context group (n= 3), remote, training context group (n=5) and remote, novel context group (n= 6). Scale bar of 100 µm for all panels. All images were obtained using a fluorescent microscope, with an objective of 20x.

In the dorsal hippocampal, no alterations are visualized between the four groups in the CA3, CA1 and DG hippocampal subregions (figure 26).

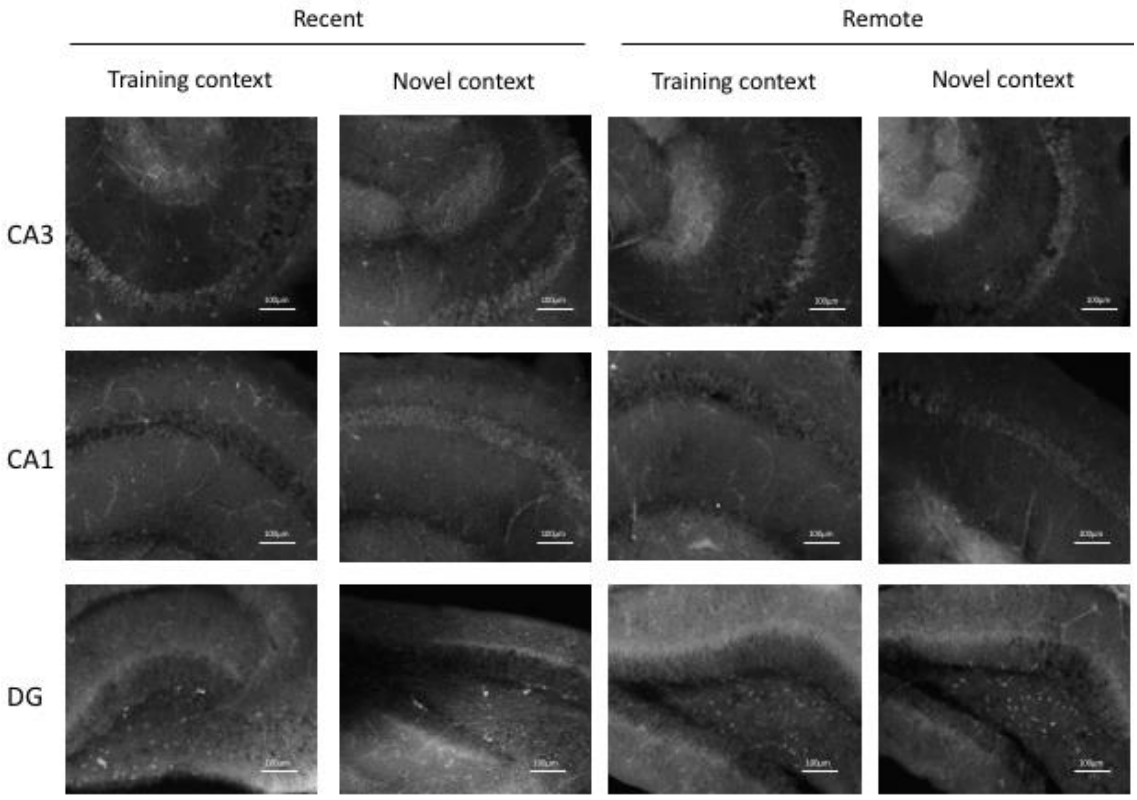


Figure 26- Representative photomicrographs of D_2R immunohistochemistry in the different hippocampal sub-divisions (CA1, CA3 and DG) from dorsal hippocampal slices (50 µm-thick) from the different groups: recent, training context group (n=4), recent, novel context group (n= 3), remote, training context group (n=5) and remote, novel context group (n= 6). Scale bar of 100 µm for all panels. All images were obtained using a fluorescent microscope, with an objective of 20x.

4.6.2- Alterations in gephyrin immunoreactivity appear underlie fear generalization in ventral but not in dorsal hippocampus (dHPC)

In the CA3 and CA1 sub-regions of the vHPC, it appears to be a lack of gephyrin staining in the animals that generalized their fear when compared to the animals that expressed a precise memory (figure 27).

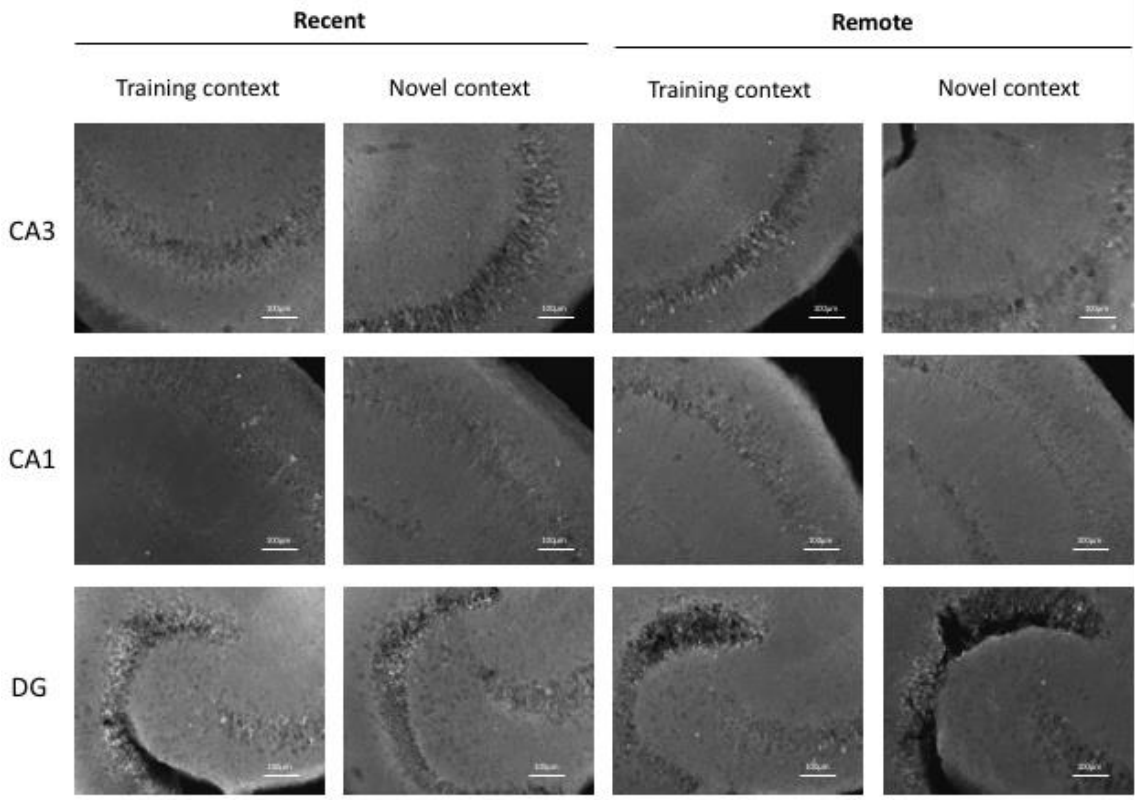


Figure 27- Representative photomicrographs of gephyrin immunohistochemistry in the different hippocampal sub-divisions (CA1, CA3 and DG) from ventral hippocampal slices (50 µm-thick) from the different groups: recent, training context group (n=4), recent, novel context group (n= 3), remote, training context group (n=5) and remote, novel context group (n= 6). Scale bar of 100 µm for all panels. All images were obtained using a fluorescent microscope, with an objective of 20x.

In addition, in the dHPC, in the remote, training context group there is an increase in the gephyrin immunoreactivity when compared to the remaining groups in the CA3 sub-region (figure 28). In the CA1 sub-region it seems to be a lack of staining in all groups.

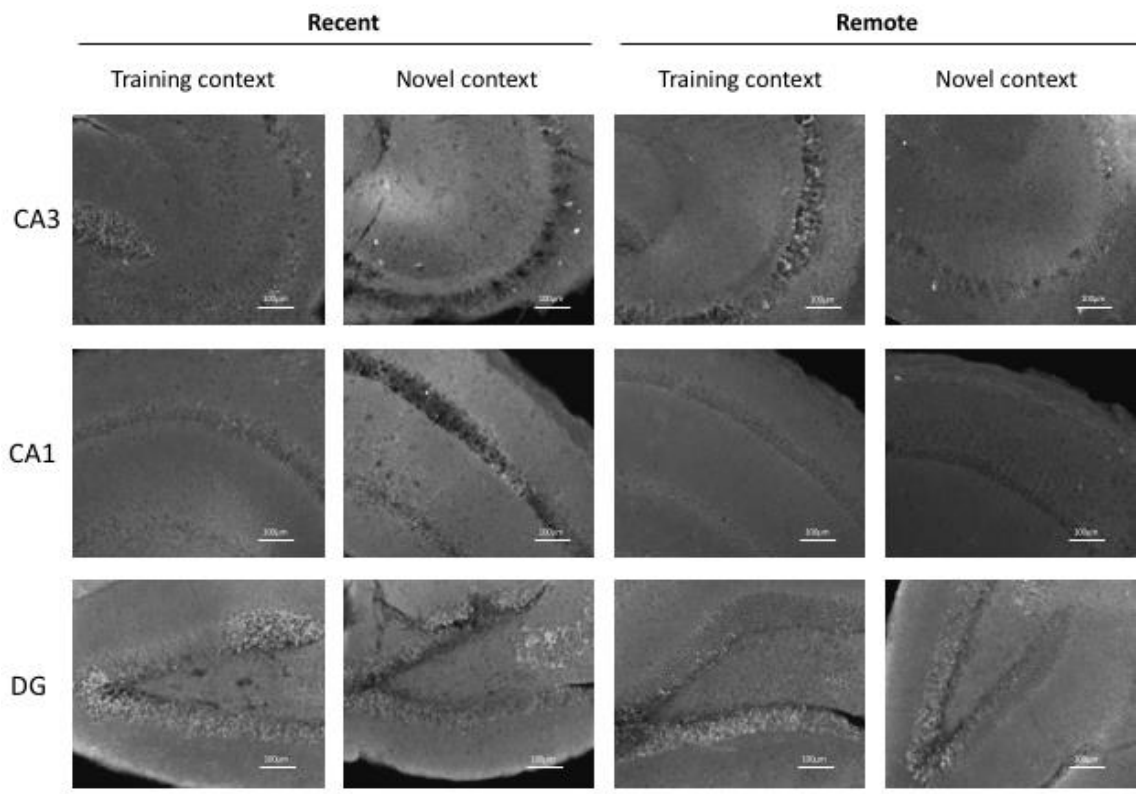
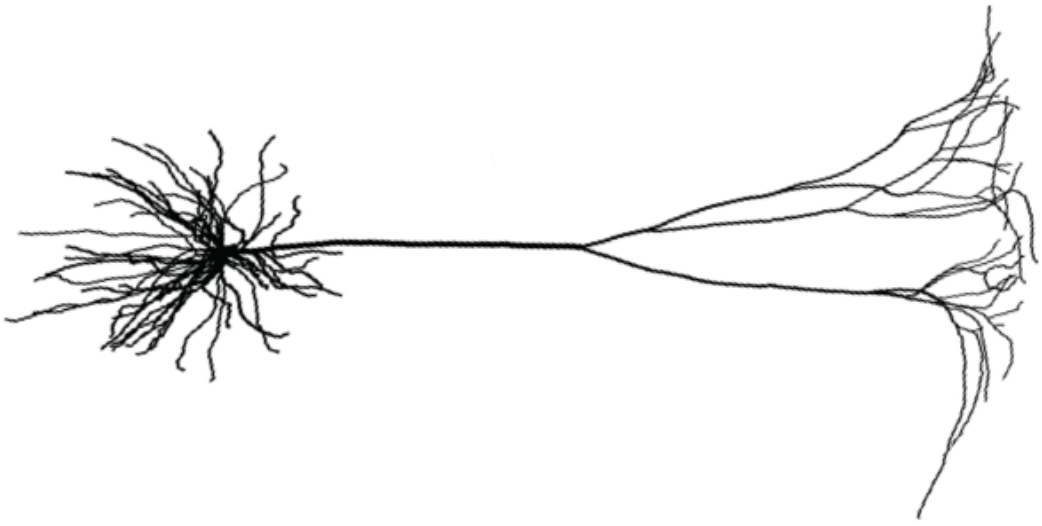


Figure 28- Representative photomicrographs of gephyrin immunohistochemistry in the different hippocampal sub-divisions (CA1, CA3 and DG) from dorsal hippocampal slices (50 µm-thick) from the different groups: recent, training context group (n=4); recent, novel context group (n= 3); remote, training context group (n=5) and remote, novel context group (n= 6). Scale bar of 100 µm for all panels. All images were obtained using a fluorescent microscope, with an objective of 20x.

Chapter 5



Discussion

Post-traumatic and stress disorder (PTSD) is a disease that occurs in 5 to 10% of the population, ranking as the fourth most common psychiatric disorder in the world. Recent studies have implicated generalization of conditioned fear as one of the most robust conditioning correlates of PTSD, being urgent to find effective therapies for such a symptom (Mahan & Ressler, 2012; Lopresto *et al*., 2015).

Additionally, it is already known from previous findings that in hippocampal circuits A_{2A}Rs are selectively engaged to control synaptic plasticity, in particular, LTP (D'Alcantara *et al*., 2001; Rebola *et al*., 2008; Fontinha *et al*., 2009; Costenla *et al*., 2011). For instance, in animal models of several pathologies, there is a clear correlation of A_{2A}R upregulation with abnormal synaptic plasticity and, consequently, cognitive deficits, as seen in acute or chronic stress (Batalha *et al*., 2013; Kaster *et al.*, 2015) and it has been observed that A_{2A}R genetic deletion rescues such stress-related synaptic dysfunction (Kaster *et al*., 2015). Moreover, recent findings have suggested a therapeutic interest in A_{2A}Rs to manage fear related pathologies (Wei *et al*., 2014; Simões *et al*., 2016), with the focal deletion of hippocampal A_{2A}Rs by AAV5-Cre injection selectively attenuating context (but not tone) fear conditioning (Wei *et al.,* 2014).

Furthermore, there are also robust evidences that an altered hippocampal LTD is tightly related to memory performance (Ge *et al*., 2010; Van der Jeugd *et al*., 2011; Dong *et al*., 2013), as shown in animal models of Alzheimer's disease (Van der Jeugd *et al*., 2011; Laurent *et al*., 2016) and stress (Wong *et al*., 2007; Maggio & Segal, 2009).

The present study provides the first evidence of an altered synaptic plasticity (namely LTD) in the vHPC of animals that generalized their fear and suggests a possible involvement of A_{2A}Rs in the deficits observed in LTD.

5.1. Role of ventral and dorsal hippocampal A_2ARs on long- term depression (LTD) in naïve C57BL/6 mice

Perhaps due to the inherent difficulty of performing electrophysiological recordings in the two hippocampal poles, existing data exploring the role of A_{2A}Rs on LTD have only focused in the dorsal-medial HPC, remaining unknown any potential involvement of A_{2A}Rs on LTD in ventral and dorsal HPC, under physiological conditions. Thus, the lack of information about such topic led to the need to first investigate the impact of A_{2A}Rs on LTD in such regions under a naïve situation, before exploring a stress condition. In this sense, taking advantage of the selective A_{2A}Rs antagonist (SCH 58261) we explored, for the first time to our knowledge, such a gap in information.

Based on our first results, the acute blockade of dorsal and ventral hippocampal A_{2A}Rs was not able to yield any alteration in LTD amplitude, indicating that ventral and dorsal A_{2A}Rs do not have any influence upon LTD phenomena, under physiological conditions. Such data are in agreement with the results collected in the dorsal-medial HPC in which recent observations have demonstrated that, under physiological conditions, neither the blockade of A_{2A}Rs, nor the A_{2A}Rs knockout *per se* impact LTD amplitude (Laurent *et al.*, 2016; Rodrigues *et al*., 2014; Ferreira *et al*., 2018).

Also, as expected the acute blockade of A_{2A}Rs by SCH 58261 did not alter basal synaptic transmission as well as the input-output function, results that are in line with the previous findings that A_{2A}Rs are rather discrete under basal transmission (Lupica *et al.*, 1990; Cunha *et al*., 1997; Rebola *et al*., 2008; Costenla *et al*., 2011).

5.2. Fear generalization: hippocampal synaptic plasticity mechanisms and role of A_{2A}Rs

Later, after exploring the role of A_{2A}Rs on ventral and dorsal LTD under physiological conditions, we moved on to a time-dependent contextual fear generalization model.

Thus, either 1 or 14 days after fear conditioning, animals were tested in the training or novel contexts in order to evaluate fear memory. Although freezing is often the most common behaviour to be quantified in fear conditioning studies, we recorded other two parameters that are thought to be related with fear behaviours: travelled distance and number of tail rattlings. Together, the percentage of freezing and travelled distance reveal that, by using C57BL/6 mice and context fear conditioning, we were able to replicate the gradual increase in fear generalization that occurs over time. The fact that non-significant alterations were observed in the number of tail rattlings may indicate that this is not a good parameter to test fear memory. As an alternative, the evaluation of the relative duration of tail rattlings could be a more promising approach (Fitch *et al*., 2002).

In brief, the current results are in agreement with previous findings (Richardson *et al.,* 1984; Zhou & Riccio, 1996; Cullen *et al*., 2015) demonstrating that a period of 14 days after fear conditioning is sufficient for fear generalization to occur, albeit Wiltgen and Silva's observation (2007) that at this time point mice are still able to discriminate the training and the novel contexts, being necessary to use a later time point (28-36 days). It is then important to point out that the fear acquisition protocol used by Wiltgen and Silva (2007) was weaker (mice only received 1 footshock), an observation that could justify their need to use a longer time window, since it is known that the intensity of the adverse stimuli seems to impact the breadth of generalization (Baldi *et al*., 2004; Ghosh & Chattarji, 2014).

Thus far, most of the research in this field has implicated deficits in HPC-mediated pattern separation as a putative mechanism underlying contextual fear generalization (reviewed in Kheirbek *et al*., 2012), however nothing is known about the synaptic hippocampal plasticity processes that could be involved in contextual fear generalization. Moreover, it has been suggested that the induction of LTD in the CA1 hippocampal sub-region may play an essential role in detection of novelty, since several studies have reported that hippocampal LTD is facilitated by novelty exposure (Manahan-Vaughan & Beaunewell, 1999; Kemp & Manahan-Vaughan, 2004). Thus, we performed extracellular recordings on the CA3-CA1 hippocampal pathway in order to try to correlate the behaviour results with plasticity events, namely, LTD, in the ventral and dorsal HPC.

Remarkably, our data suggests an implication of the vHPC in generalized contextual fear memory, manifested by a disability of this region to produce LTD. With the aforementioned LTD role in mind and the knowledge that this type of synaptic plasticity specifically serves to encode fine spatial details in a new environment (Kemp & Manahan-Vaughan, 2004, 2007 & 2008) it is not surprising that animals that generalized their fear were not capable to produce LTD. In other words, it is possible that the animals tested in the novel context 14 days after fear conditioning were not capable to discriminate the novel context of the training context since they were not able to detect the spatial details of the new environment due to impairments in LTD.

On the other hand, our results did not associate dHPC as a possible structure implicated in fear generalization, since no significant alterations in LTD amplitude were noticeable between the animals that presented a precise memory and the ones that did not. Such collected data is in agreement with a recent publication showing that in C57BL/6 mice the activity of the CA1 region of vHPC is required for the expression of a contextual generalized fear memory (Cullen *et al*., 2015). Furthermore, recently Nguyen and colleagues (2018) have presented evidence for an effect of early life maternal care on ventral hippocampal synaptic function and plasticity (namely LTP) of Long-Evans rat dams, which in turns influenced tone-generalization of conditioned fear, thus suggesting a role of ventral hippocampal synaptic plasticity in the specification/generalization of fear memories. Finally, another group has shown that the bilateral injection of a histone deacetylase (HDAC) inhibitor in the vHPC of C57BL/6 mice, after context pre-exposure, elicited predator odour fear generalization to a neutral context (Yuan *et al*., 2015).

It has been proposed that the dHPC is more associated with spatial recognition of certain aspects of the context such as objects and cues, while the ventral region appears to be crucial for the formation of the context as a whole and, consequently, for the discrimination of similar places, through its capacity for assembling the contextual information collected by the dHPC (Maurer *et al*., 2005; Kjelstrup *et al*., 2008; Royer *et al*., 2010; Komorowski *et al*., 2013; Keinath *et al*., 2014). These different functions of the dorsal and ventral HPC in the spatial information processing suggest that the dorsal region may be important for minimizing memory interference and generalization, by coding specific aspects of the context. Conversely, the vHPC may be more vulnerable to contextual generalization since there is a high probability for errors during the assembly of the contextual cues (Komorowski *et al*., 2013; Keinath *et al*., 2014), thus supporting our results which indicate that contrarily to the dorsal hippocampal pole, the vHPC does play a role in contextual generalization.

Moreover, there are indications that the vHPC region must directly or indirectly convey spatial information by controlling the transfer of information between the dHPC and the PFC (a structure that together with the HPC is essential for episodic memory

and memory retrieval) (O'Neill *et al*., 2013; Keinath *et al*., 2014). This is based on the fact that the vHPC is the only hippocampal area that directly projects to the mPFC (Jay & Witter, 1991; Hoover & Vertes, 2007). Thus, since PFC-hippocampal interactions have been suggested to be important to distinguish a context during memory retrieval (Place *et al*., 2016), it is likely that alterations in vHPC synaptic plasticity could compromise such communication and, consequently, contribute to fear generalization, thus placing the vHPC in a key position to modulate contextual learning. Finally, as it is known, the modulation of fear expression is achieved by the amygdala, a structure also involved in fear generalization according to Shaban, *et al*., 2006; Ghosh and Chattarji, 2014. With this in mind, and again due to the vHPC being the only area of the HPC that projects to the amygdala (Pitkänen *et al*., 2000), this interaction between spatial information and fear expression significantly reinforces the vHPC role on fear generalization.

Animals that generalized their fear also displayed a reduced input-output curve in the vHPC when compared to animals that expressed a precise memory, thus suggesting that alteration in the basal synaptic transmission could indirectly influence the alteration in synaptic plasticity observed. Curiously, in the dHPC similar to what happens with synaptic plasticity, no significant difference is visualized between the animals that displayed a precise memory and the animals that did not.

Remarkably, a gain-of-function of A_{2A}Rs to modulate LTD under pathological conditions has been suggested. For instance, it was shown that the deletion or the selective blockade of A_{2A}Rs rescued the deficits in LTD observed in a mouse model of Alzheimer's disease (Laurent *et al*., 2016) and, more recently, Ferreira and colleagues (2018) have demonstrated that the selective blockade of A_{2A}Rs completely rescued the LTD-to-LTP shift observed in Tg(CaMKII-hA_{2A}R) animals (a model selectively overexpressing those receptors in neurons) and in a APP/PS1 mouse model of Alzheimer's disease. Therefore, we took advantage of a selective A_{2A}R antagonist (SCH 58261) to examine if such blockade could restore the LTD deficits observed in ventral slices from the animals that generalized their fear. Indeed, our findings showed that the acute blockade of A_{2A}Rs repaired the LTD amplitude back to the LTD characteristic of the mice that expressed a precise memory, thus emphasizing A_{2A}Rs as the key mediators involved the LTD impairments observed in the animals that generalized their fear.

A_{2A}R are pleiotropic receptors activating multiple transducing pathways depending on their density and the biological system (reviewed in Cunha, 2016), with several articles suggesting that the gain-of-function of A_{2A}Rs observed in many pathological conditions is a consequence of an A_{2A}Rs upsurge or a consequence of an increase in the adenosine levels. Thus, we could speculate that this gain-of-function of A_{2A}Rs to modulate LTD observed in our results could be the result of an over-expression of A_{2A}Rs or an increase in the particular source of adenosine (that ultimately leads to an over-

activation of A_{2A}Rs) in the animals that generalized their fear. In fact, it was shown that the upregulation of A_{2A}Rs driven by the CaMKII promoter in rat forebrain neurons is sufficient to mimic ageing-like memory impairments and to reveal an LTD-to-LTP shift, in the HPC (Ferreira *et al*., 2018), thus indicating that something similar may be happening in the animals that present a generalized fear memory.

It is well established that the A_{2A}R activation directly increases Ca^{2+} intracellular levels in a NMDAR-dependent manner due to the enhancement of glutamate release (if they are presynaptic located) or through the improvement of NMDAR conductance (if they are postsynaptic). Thus, it is possible that the gain-of-function of A_{2A}Rs in the modulation of LTD could be the result of a greater NMDAR recruitment/NMDAR over-activation that ultimately leads to a larger calcium influx and, consequently, to the abrogation of LTD. Our idea is supported by previous findings collected by Ferreira and colleagues (2018) that hinted an A_{2A}R-NMDAR interaction as the key mediator responsible for the LTD-to-LTP shift observed in the Tg(CaMKII- hA2AR) animals. Therefore, since the protocol used by us to induce LTD is an adaptation of the protocol used by them there is a high probability of our LTD be also NMDA-dependent.

Furthermore, it has been provided compelling evidence of an A_{2A}R-mGluR5 synergistic interaction in the modulation of NMDAR-mediated effects (Tebano *et al*., 2005; Sarantis *et al*., 2015; Kouvaros & Papatheodoropoulos, 2016), so maybe the potential effect of A_{2A}R on NMDAR-mediated responses could require the mGluR5 involvement.

It is important to mention that we are aware that such hypothesis are only assumptions that require further development, so as a future perspective we plan to dissect the exact mechanism of action by which A_{2A}Rs compromise the LTD phenomena in animals that generalized their fear, by making use of a NMDAR or mGluR5 antagonist.

The acute treatment with SCH 58261 rescued the stimulus-response function in animals that manifested a generalized fear memory, restoring it back to the profile observed in animals that expressed a precise memory (figure 20), suggesting that A_{2A}Rs are implicated in I/O curve modifications. The increase in basal synaptic transmission after the superfusion of SCH 58261 confirmed such an assumption, indicating that the alterations in the I/O curve are a reflection of a decrease of basal synaptic transmission under the control of A_{2A}Rs.

Since A_{2A}Rs are rather discrete under basal transmission (Lupica *et al*., 1990; Cunha *et al*., 1997; Rebola *et al*., 2008; Costenla *et al*., 2011) it was not expected that the acute blockade of A_{2A}Rs yielded any alteration in this type of communication. However, even though it is often found in the literature that A_{2A}Rs do not participate in basal synaptic transmission, both our experiment and the one conducted by Ferreira and colleagues (2018) suggest some alterations at the basal transmission level after the

acute blockade of SCH 58261. Although our experiment indicates an increase in basal transmission, surprisingly, Ferreira and colleagues (2018) report a reverse observation – a decrease in fEPSPs of basal transmission. A result like this decrease is more expected, since it can be easily explained by over-recruitment of NMDARs in response to an over-expression of A_{2A}Rs, resulting in NMDARs becoming direct contributors of excitatory synaptic transmission, due to a close management from A_{2A}Rs. Moreover, such a result is also supported by the fact that in the HPC, associated to the A_{2A}R overexpression, there is a loss of the A_{2A}R-A_1R cross-talk upon ageing resulting in a facilitation of basal transmission (Lopes *et al*., 1999). Taking all these evidences into account, our results may appear a little bit puzzling and unexpected. Nevertheless, it should be considered that the adenosine system plays a crucial role in modulating the synaptic transmission and that signal transduction pathway(s) of a specific receptor are largely dependent on the environment (in insult, for instance, A_{2A}Rs can recruit alternative signalling pathways instead of the canonical pathway (Canas *et al.,* 2009)). Therefore, there is a possibility that, in animals that generalize their fear, A_{2A}Rs act on one of those pathways, diminishing the basal synaptic transmission in order to try to compensate possible deficits of the GABAergic system (figure 27).

In conclusion, we postulate that, contrarily to Ferreira and colleagues (2018), the gain-of-function of A_{2A}Rs to control basal synaptic transmission is different from the one observed upon LTD induction. Although less likely, it is important to mention that the use of different animal models could also explain these opposite results, since Ferreira and Colleagues (2018) have used rats for this particular trial, while our experiments were all performed in C57BL/6 mice.

In the remaining groups, either in the dorsal or ventral HPC, the acute blockade of A_{2A}Rs did not entice any alteration in LTD amplitude. This indicates that the role of A_{2A}Rs on LTD is only manifested when there is a synaptic dysfunction, thus stressing out A_{2A}Rs as a pathological mediator involved in such LTD impairments. In other words, A_{2A}R appear to shift the adenosine neuromodulation towards a synaptic pathology manifested through an LTD impairment that, ultimately, could result in fear generalization. Regarding the effect of SCH 58261 on the basal synaptic transmission and of the remaining groups, the result observed was similar to the one observed in ventral slices from the animals that generalized their fear, thus indicating that the gain-of-function of A_{2A}Rs to control basal transmission is transversal to all groups.

5.3. Possible biomarkers for fear generalization

In this work, all experiments were performed to try to gather as much information as possible, so we also performed immunohistochemical assays using the transverse hippocampal slices that were not used for electrophysiological recordings in order to look for potential biomarkers of fear generalization, namely, gephyrin and D_2Rs.

Previous pharmacological and genetic studies have suggested a role of DA in the establishment of aversive memory traces and in the modulation of threat response generalization (Fadok *et al*., 2010; Zweifel *et al*., 2011). Furthermore, a recent study has shown that the blockade of D_2R in the amygdala induces generalized threat responses (De Bundel *et al*., 2016). However, nothing was done in terms of D_2R immunoreactivity and fear generalization. In our study differences in D_2R immunoreactivity were not detected in dorsal and ventral HPC, between the animals that generalized their fear and the ones that expressed a precise memory. Many hypotheses could explain our results. For instance, it is possible that fear generalization could involve alterations in DA concentration rather than alterations in the density of D_2Rs. Moreover, Bundel and colleagues (2016) performed their experiments in the amygdala so it would be interesting to investigate other brain regions thought to be involved in fear generalization. On the other hand, in our immunohistochemistry study, a step for cell permeabilization was performed, so it is likely that possible alterations in the D_2R immunoreactivity in the plasma membrane could be camouflaged by opposite differences in D_2R cellular pool. Thus, it would be interesting to perform another immunohistochemical study that does not comprise a cellular permeabilization step in order to clarify such doubts. Finally, a recent article demonstrated that the D1/5 receptor agonism during novel environmental exploration promotes LTD in the CA1 region (Lemon & Manahan- Vaughan, 2012), so it would be interesting to verify if any alteration in density of D1/5 receptors in vHPC could be found in animals that generalized their fear.

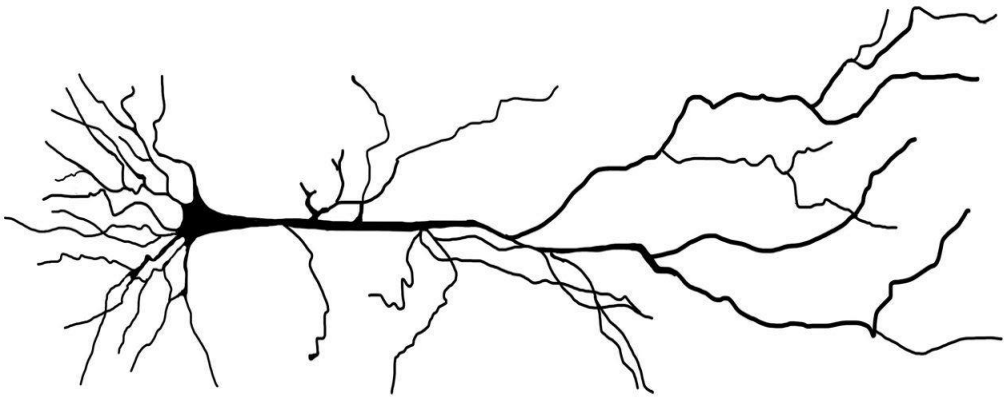
Regarding gephyrin immunoreactivity, alterations between the animals that generalized their fear and the animals that expressed a precise memory were visualized in CA1 and CA3 ventral hippocampal sub-regions, suggesting that gephyrin could be a promising synaptic biomarker for fear generalization in the vHPC. Furthermore, since gephyrin is a scaffold protein that anchors $GABA_A$Rs, such results hint that alterations in the GABAergic system could underlie fear generalization. Indeed, such results are in line with previous findings establishing a correlation between alteration in the GABAergic signalling and fear generalization, however these studies are focused in the amygdala (Shaban *et al*., 2006; Lange *et al*., 2014; Bender *et al*., 2018).

Pavlov and colleagues (2004) have shown that the activation of $GABA_A$Rs promotes induction of NMDAR-dependent LTD of glutamatergic synapses in the newborn rat hippocampal area CA1, while its inhibition affected LTD induction. Thus, our gephyrin results obtained in the vHPC from animals that generalized their fear appear to closely match to the respective electrophysiological results obtained in the vHPC in the same animals, since in the animals where we observed an impairment of LTD we then observed a decrease in gephyrin immunoreactivity. It is important to keep in mind that our imnunohistochemical results are from a preliminary experiment in which we mentioned only the most obvious visible differences since the existence of artefacts in the images have compromised its quantification. So as future perspective we plan on continuing this study in order to validate our results.

In summary, our data suggests an implication of the vHPC in generalized contextual fear memory (manifested by a disability of this structure to produce LTD) while it did not reveal the dHPC as a possible structure implicated in fear generalization. Furthermore, we have shown that ventral and dorsal A_{2A}Rs have no effect on the LTD amplitude under control situations, however a gain-of-function of A_{2A}Rs to modulate LTD in such regions is revealed under pathological conditions, namely, in fear generalization. Thus, our results show that the acute blockade of A_{2A}Rs was able to rescue the LTD synaptic impairments observed in ventral slices from the animals that generalized their fear, indicating that A_{2A}Rs are responsible for shifting the adenosine neuromodulation towards a pathology-related status, manifested by LTD deficits, that ultimately could be the cause of fear generalization. Moreover, our results appear to suggest that alterations in the GABAergic system in vHPC could be related with fear generalization since the animals that generalized their fear seem to have a decrease in gephyrin immunoreactivity.

In conclusion, together our data strongly propose a therapeutic interest of using antagonists of A_{2A}Rs against fear generalization and suggest that gephyrin could be a promising synaptic biomarker for fear generalization in vHPC.

Chapter 6



Conclusions

6.1- Highlights

⇒ Ventral and dorsal A_{2A}Rs have no impact on LTD under physiological conditions;

⇒ Alterations in LTD in ventral but not in dorsal HPC appear to underlie fear generalization;

⇒ A gain-of-function of ventral A_{2A}Rs to modulate LTD is revealed in fear generalization, thus stressing out A_{2A}Rs as a pathological mediator involved in the LTD impairments observed in the vHPC;

⇒ Gephyrin appears to be a potential synaptic biomarker of fear generalization in the vHPC, since a decrease in gephyrin immunoreactivity in this region is observed in animals that generalized their fear.

Table 6- Summary of the electrophysiological results from the vHPC.

	vHPC	
	Ctrl	**SCH 58261**
Recent, training context	-24.05 ± 11.03 %	-29.91 ± 6.77
Recent, novel context	-38.40 ± 8.34 %	-22.85 ± 30.54
Remote, training context	-26.31 ± 5.11 %	-18.65 ± 24.56
Remote, novel context	**3.61 ± 15.73#**	**-26.17 ± 19.23#**

#values from paired results.

Table 7- Summary of the electrophysiological results from the dHPC.

	dHPC	
	Ctrl	**SCH 58261**
Recent, training context	-5.27 ± 18.32 %	-20.26 ± 5.42 %
Recent, novel context	-14.99 ± 4.65 %	-14.85 ± 17.15 %
Remote, training context	-27.79 ± 30.81 %	-2.09 ± 9.28 %
Remote, novel context	-7.30 ± 6.86 %	-24.68 ± 15.18 %

6.2- Future Perspectives

Should our results be confirmed by increasing the number of experiments, as future perspectives it would be interesting to:

⇒ Evaluate if ventral hippocampal A_{2A}Rs are in fact implicated in fear generalization by injecting SCH 58261 directly into the vHPC;

⇒ Investigate if the blockade of A_{2A}Rs could prevent generalization by chronic intraperitoneal injection of SCH 58261 in mice (topic of clinical interest);

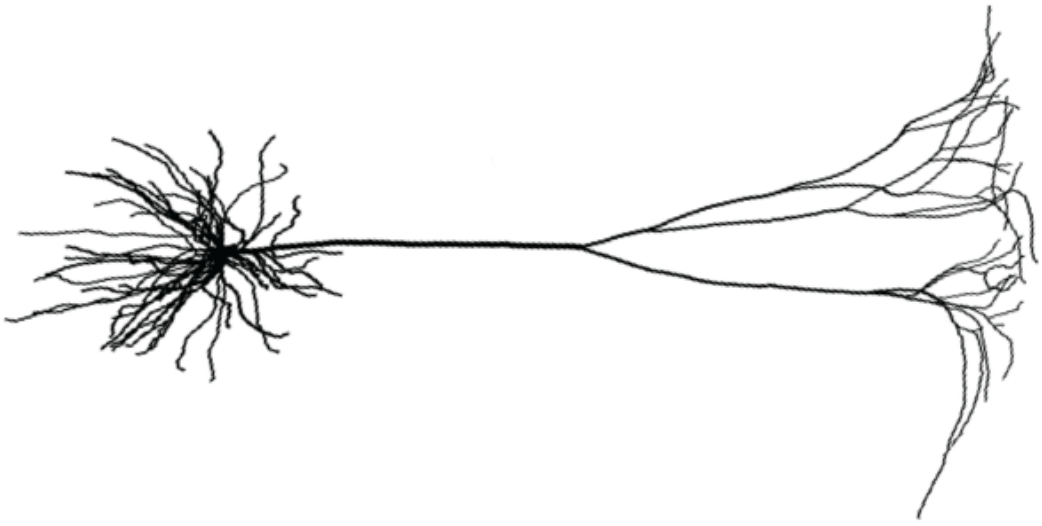
⇒ Assess the possible mechanism by which A_{2A}Rs impact on basal transmission;

⇒ Study the possible mechanisms by which A_{2A}Rs impair LTD in the animals that generalized their fear;

⇒ Study the role of the vHPC-PFC interaction in fear generalization;

⇒ Validate the results related with gephyrin immunoreactivity in perfused animals.

Chapter 7



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