UNIVERSIDADE FEDERAL DE MINAS GERAIS

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

LIA PARADA IGLESIAS

ROLE OF HIPPOCAMPAL TRPV1 CHANNELS IN CONTEXTUAL FEAR MEMORY

BELO HORIZONTE

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LIA PARADA IGLESIAS

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Tese apresentada ao Programa de Pós-Graduação em Neurociências do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do Grau de Doutor em Neurociências.

Orientador: Prof. Dr. Fabrício de Araújo Moreira

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LIA PARADA IGLESIAS

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A Comissão considerou a tese: Aprovada

Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.

Belo Horizonte, 01 de julho de 2022.

Carlos Magno Machado Dias - Secretário

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

FOLHA DE APROVAÇÃO

Envolvimento do canal TRPV1 no hipocampo na memoria de medo contextual

LIA PARADA IGLESIAS

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em NEUROCIÊNCIAS, como requisito para obtenção do grau de Doutor em NEUROCIÊNCIAS, área d concentração NEUROCIÊNCIAS BÁSICAS.

Aprovada em 01 de julho de 2022, pela banca constituída pelos membros:

Profa. Cristina Aparecida Jark Stern

UFPR

Profa. Elaine Cristina Gavioli

UFRN

Profa. Fabiola Mara Ribeiro

UFMG

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“Every material system can exist as an entity only so long as its internal forces balance the external forces acting upon it. […] Being a definite circumscribed material system, it can only continue to exist so long as it is in continuous equilibrium with the forces external to it: so soon as this equilibrium is seriously disturbed the organism will cease to exist as the entity it was”. (Ivan P. Pavlov, Conditioned reflexes: An invesitgation of the physiological activity of the cerebral cortex, 1927).

RESUMO

Alguns transtornos psiquiátricos podem ser conceptualizados como maladaptações nos mecanismos envolvidos no aprendizado associativo. O hipocampo dorsal (dHPC) é uma das estruturas chave no processamento de estímulos contextuais na memória associativa. O envolvimento de TRPV1, um canal catiônico ativado por anandamida (AEA), neste processo permanece pouco estudado. O objetivo deste estudo foi testar a hipótese de que o envolvimento dos canais TRPV1 em memórias de medo contextual é dependente de intensidade e do sistema endocanabinoide (eCB) no dHPC. Camundongos C57BL/6J foram testados no medo condicionado ao contexto usando diferentes intensidades. O bloqueador TRPV1, SB366791 (SB), foi administrado diretamente no dHPC; um subgrupo de animais recebeu AM251, um antagonista CB_1, como pré-tratamento. Os níveis de AEA no HPC foram quantificados usando cromatografia liquida de alta performance seguido de espectrometria de massas; a expressão de TRPV1 e CB_1 foi avaliada por imunofluorescência. Fatores envolvidos com plasticidade como Zif-268 (Zif), Arc, TrkB e BDNF foram analisados por PCR ou ELISA. Bloqueadores TRPV1 inibiram a expressão da memória aversiva de forma dependente da intensidade, efeito prevenido pelo AM251. Os níveis de AEA foram correlacionados com os níveis de congelamento e a co-expressão de TRPV1 e CB_1 foi observada no dHPC. Finalmente, houve um aumento nos níveis de fatores envolvidos com a plasticidade após a expressão do medo condicionado nos animais tratados com o bloqueador TRPV1, que também apresentou um padrão dependente de intensidade. Os canais TRPV1 hipocampais parecem especificamente envolvidos na expressão de memórias contextuais aversivas. Os nossos resultados sugerem que o recrutamento dependente de intensidade do canal TRPV1 está relacionado com o aumento da disponibilidade de AEA em procedimentos mais intensos. Ademais, o bloqueio de TRPV1 induziu o recrutamento de vias relacionadas com plasticidade, o que pode subjazer os efeitos protetivos a longo prazo conferidos pelo bloqueador.

Palavras-chave: hipocampo, memória, condicionamento, TRPV1, receptor CB_1, endocanabinoides, medo.

ABSTRACT

Certain psychiatric disorders can be conceptualized as maladaptation in associative learning mechanism. The dorsal hippocampus (dHPC) is one of the key structures in processing contextual stimuli in associative memory. The role of TRPV1, a cationic channel activated by anandamide (AEA), in this process remains poorly understood. The aim of this work was to test the hypothesis that the role of TRPV1 channels in contextual fear memory depends on intensity and the endocannabinoid system in the dHPC. C57BL/6J mice were submitted to contextual fear conditioning (CFC) using different intensities. A TRPV1 blocker, SB36679 (SB) was administered into the dHPC, a subset of animals received AM251, a CB_1 antagonist, as pre-treatment. Hippocampal levels of AEA were quantified using high performance liquid chromatography followed by Mass Spectrometry (HPLC-MS) and TRPV1- CB_1 expression was assessed by double-immunofluorescence. Plasticity factors such as Zif-268 (Zif), Arc, TrkB and BDNF were analysed by polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA). TRPV1 blockers impaired specifically the retrieval of aversive memory in an intensity-dependent manner, effect prevented by AM251 pre-treatment. The levels of AEA were correlated with freezing levels, co-expression of TRPV1 and CB_1 was confirmed in the dHPC. Finally, plasticity factors were up-regulated after retrieval by the TRPV1 blocker also following an intensity-dependent pattern. Hippocampal TRPV1 channels seem specifically involved in retrieval of aversive contextual memory in an intensity-dependent manner. Our results suggest that the intensity-dependent recruitment of TRPV1 is due to the increased availability of AEA in more aversive procedures. In addition, TRPV1 blockers recruit plasticity pathways that may underlie their long-term protective effect.

Keywords: hippocampus, memory, conditioning, TRPV1, CB_1 receptor, endocannabinoids, fear.

FIGURES AND TABLES INDEX

ABREVIATION LIST

2-AG 2-arachidonoylglycerol

6-I-NC 6-iodo-nordihidrocapsaicin

AA-5-HT N-araquidonil-serotonin

ACC anterior cingulate cortex

AEA anandamide

AFC auditory fear conditioning

AM251 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide

AMG amygdala

Anti-ODN antisense-oligodeoxynucleotides

Arc activity-regulated cytoskeleton-associated protein

BDNF brain-derived neurotrophic factor

BLA basolateral amygdala

BSA bovine serum albumin

CamK calmodulin-dependent kinase

CB_1 cannabinoid receptor type 1

CB_2 cannabinoid receptor type 2

CBD cannabidiol

CFC contextual fear conditioning

CPP conditioned place preference

CPS capsaicin

CREB cAMP response element-binding protein

CS conditioned stimulus

DG dentate gyrus

dHPC dorsal hippocampus

eCB endocannabinoids

Egr-1 early growth response protein 1

ELISA enzyme-linked immunosorbent assay

ERK1-2 extracellular signal-regulated kinase 1-2

FAAH fatty acid amide hydrolase

GC glucocorticoids

GPCR G-protein-coupled receptors

HI high intensity

HPA hypothalamic pituiray adrenal

HPC hippocampus

HPLC-MS high performance liquid chromatography followed by Mass Spectrometry

IEG immediate-early genes

LI low intensity

LTD long-term depression

LTP long-term potentiation

MAGL monoacylglycerol lipase

MAPK mitogen-activated protein kinase

MI moderate intensity

mTOR mammalian target of rapamycin

NC not conditioned

NOR novel object recognition

PAG periaqueductal gray

PBS phosphate buffered saline

PCR polymerase chain reaction

PFA paraformaldehyde

PFC pre-frontal cortex

PKA protein kinase A

PKC protein kinase C

PTSD post-traumatic stress disorder

RI reinstatement intensity

SB SB366791

THC delta-9-tetrahydrocannabinol

Trkb tropomyosin receptor kinase

TRPV1 transient receptor potential, family V type-1

US unconditioned stimulus

vHPC ventral hippocampus

Zif Zif-268

SUMARY

Introduction

1. Associative Memory

1.1. Theoretical aspects, health and disease

During the course of life, all individuals make certain types of associations; when a stimulus follows a specific behaviour, the process is known as operant conditioning (KONORSKI; MILLER, 1937; SKINNER, 1937; THORNDIKE, 1898). These associations can also occur when events or stimuli are experienced together or close in time and the behavioural response does not exert control over the stimulus, forming the so-called pavlovian conditioning or classic conditioning (I . P . PAVLOV ; G . V . ANREP, 1927). This form of learning receives its name after the classic studies by Pavlov in 1927 (I . P . PAVLOV ; G . V . ANREP, 1927). In its seminal experiment, Pavlov trained a dog presenting a neutral stimulus, a tone, followed by an appetitive stimulus, the food. After the paired presentation of both stimuli, every time the dog was exposed to the tone, it elicited a reflex previously evoked by the food, salivation. Pavlov`s concluded that the set of innate reflexes is insufficient to provide optimal responses to certain environments (I . P . PAVLOV ; G . V . ANREP, 1927). Accordingly, he stablished that experience along life can modulate and adapt some reflexes, when a “significant biological stimulus” (aversive or appetitive), changes the natural response to another neutral stimulus (IZQUIERDO, 2018; PAVLOV, 1928).

Therefore, four elements constitute the pavlovian conditioning: I) the conditioned stimulus (CS), an otherwise neutral stimulus that acquires valence because of the experience; II) the unconditioned stimulus (US) which has *per se* the ability to induce an innate response; III) the unconditioned response, an innate reflex induced by the US and IV) the conditioned response, displayed after the experience, when the CS is presented even in the absence of the US. Thus, the conditioned response can be defined as an adaptive behavioural response derived from the cognitive expectation of the US induced by its predictor, the CS (BOLLES, 1972; BOLLES; FANSELOW, 1980; FANSELOW; WASSUM, 2016).

Schematically, the whole learning and memory process can comprise the following phases: The first phase is called **acquisition**, it involves the

conditioning itself, when the two stimuli are presented together, in this first step takes place the processing and initial encoding of memory. It is immediately followed by the **consolidation of memory**, a complex molecular process lasting for some hours and crucial for the longevity of this memory (ASOK et al., 2019; DE OLIVEIRA ALVARES; DO-MONTE, 2021; FRANKLAND; BONTEMPI, 2005). Synaptic consolidation implies the stabilization of the memory trace (ASOK et al., 2019; DE OLIVEIRA ALVARES; DO-MONTE, 2021; FRANKLAND; BONTEMPI, 2005) which is encoded by an assembly of neurons called engram (JOSSELYN; KÖHLER; FRANKLAND, 2015). Later, when the animals are re-exposed to the CS, they will display the conditioned response, the underlying phenomenon is the **retrieval of memory**, which involves the activation of the engram encoding the memory previously acquired (JOSSELYN; KÖHLER; FRANKLAND, 2015; JOSSELYN; TONEGAWA, 2020; SIGWALD; DE OLMOS; LORENZO, 2020). Likewise, the exposition to the CS can trigger different processes, depending, at least, on the duration of this exposition (AUBER et al., 2013; LEE; NADER; SCHILLER, 2017). For instance, brief expositions reactivate the memory inducing destabilization of the memory trace which is again labile, and susceptible to updating and re-stabilization, this phase is called **reconsolidation** (ASOK et al., 2019; DE OLIVEIRA ALVARES; DO-MONTE, 2021). The reconsolidation often leads to strengthen the memory trace and to exacerbate the performance. However, longer expositions to the CS can induce **extinction**, in this phase, a new memory trace able to inhibit the original one is formed, in consequence a decrease in the conditioned response can be observed (ASOK et al., 2019; DE OLIVEIRA ALVARES; DO-MONTE, 2021; LUCHKINA; BOLSHAKOV, 2019). In theory, the duration of the re-exposition determines which process is engaged (CAHILL; MILTON, 2019; SANTIAGO; TORT, 2020). A third phenomenon, often called *limbo*, a transition phase between reconsolidation and extinction, can be inferred when the experimental design was not able to induce any change at least in the observable performance (VAVERKOVÁ et al., 2020). After extinguished, conditioned responses can be re-stored, by **reinstatement, renewal** or **spontaneous recovery.**

Functionally, associative memory enables to assign motivational valence to neutral cues, allowing environmental stimuli to become predictors of dangerous or beneficial situations; then, modifying future behavioural responses increasing fitness and survival (KRAUSE; DOMJAN, 2017). As any other mechanism or process governing vital functions and promoting survival, memory misfunctioning can thereby lead from health to disease. For instance, post-traumatic stress disorder (PTSD) and phobias were the firsts disorders to be conceptualized as a result of associative memory maladaptation (FOA; STEKETEE; ROTHBAUM, 1989; SELIGMAN, 1971). More recently, anxiety and substance use disorder were also included as potentially associated to memory maladaptation (EVERITT; DICKINSON; ROBBINS, 2001; ITZHAK; PEREZ-LANZA; LIDDIE, 2014; MILTON; EVERITT, 2012; MINEKA; OEHLBERG, 2008; VAVERKOVÁ et al., 2020). Since associative memory and pavlovian conditioning play a crucial role in health and disease, the study of this process can bring both, a better understanding of a biological function, and new therapeutical targets. Among common research models for studying conditioned aversive responses in experimental animals are the auditory fear conditioning (AFC) and the CFC, whereas conditioned appetitive responses can be investigated using the conditioned place preference (CPP).

1.2. Contextual Fear Conditioning

Despite fear was presented for some authors as a human-exclusive subjective emotion (LEDOUX; PINE, 2016), fear can be defined as a “coordinate reaction to danger involving autonomic, behavioural and cognitive responses” (FANSELOW; PENNINGTON, 2018) that depends on predatory imminence (FANSELOW, 1994; FANSELOW; LESTER; HELMSTETTER, 1988). In the CFC, a classic conditioning paradigm, animals are exposed to a context, CS, where they receive one or several footshocks, US. Later, when the animals are re-exposed to the context, they will elicit a fear-response, freezing.

Neuroanatomy of CFC: emphasis in the hippocampus

The hippocampus (HPC) can be divided in dorsal and ventral portions. Each one comprises a neuronal circuit including three different subregions, namely CA1, CA3 and the dentate gyrus (DG). In the principal pathway, the information is provided through the entorhinal cortex to the DG; from there, mossy fibres

reach CA3 which in turn send projections, Schaffer collaterals, to CA1. CA1 projections close the circuit returning again to the entorhinal cortex. In addition, there is also a monosynaptic connection between the entorhinal cortex and CA1 (AMARAL; SCHARFMAN; LAVENEX, 2007; MARKS et al., 2022). The HPC is connected with other structures important for fear memory as the amygdala (AMG), the anterior cingulate cortex (ACC) and the prefrontal cortex (PFC) (AMARAL; SCHARFMAN; LAVENEX, 2007; MARKS et al., 2022).

Lesion studies suggested that the HPC is involved in acquisition, consolidation, retrieval and extinction of CFC (ANAGNOSTARAS; MAREN; FANSELOW, 1999; CHEN et al., 1996; KIM; RISON; FANSELOW, 1993; KJELSTRUP et al., 2002; LEHMANN; LACANILAO; SUTHERLAND, 2007; MAREN; AHARONOV; FANSELOW, 1997; YOUNG; BOHENEK; FANSELOW, 1994). This was later confirmed by opto- / chemo-genetic technics (CHEN et al., 2019; KRUEGER et al., 2020; LACAGNINA et al., 2019; PARK et al., 2016). Specifically, lesions of the dorsal but not the ventral portion of this structure (ventral HPC, vHPC), impaired contextual conditioned fear but not unconditioned fear (KIM; RISON; FANSELOW, 1993; KJELSTRUP et al., 2002) while the ventral portion seems related to unconditioned fear (KJELSTRUP et al., 2002; MAREN; HOLT, 2004). These findings suggested that the dHPC would be involved in the mnemonic process and specially in encoding the context, while the vHPC would be related with emotional-related responses. Regarding the three subregions of the dHPC: CA1, CA3 and DG are involved in memory acquisition (DAUMAS; HALLEY; LASSALLE, 2004; LEE; KESNER, 2004), CA1 and DG in retrieval (LEE; KESNER, 2004) and CA1 and CA3 are necessary during consolidation (DAUMAS et al., 2005; DAUMAS; HALLEY; LASSALLE, 2004).

Bidirectional projections between the HPC and the AMG are involved in sustaining the emotional information related to the context (MARKS et al., 2022). The AMG is responsible for triggering conditioned fear responses (COUSENS; OTTO, 1998; HARALAMBOUS; WESTBROOK, 1999; HELMSTETTER; BELLGOWAN, 1994) and unconditioned fear (AMMASSARI-TEULE et al., 2000; KIM; RISON; FANSELOW, 1993; LI et al., 2004; PHILLIPS; LEDOUX, 1992), this is sustained by different projections providing fear- and

sensory-related information from the periaqueductal gray (PAG), the parabrachial nucleus and the thalamus (MARKS et al., 2022). Moreover, from the central nucleus of the AMG leave projections recruiting different downstream effectors controlling fear responses, for instance the PAG (KIM; RISON; FANSELOW, 1993; VIANNA; LANDEIRA-FERNANDEZ; BRANDÃO, 2001) responsible for freezing behaviour. In addition, cortical structures, specially, the PFC and the ACC are involved in some phases of the CFC. Indeed the ACC participates in consolidation, extinction and maintenance of remote memories (FRANKLAND et al., 2004; VETERE et al., 2011a, 2011b) while the PFC, particularly, the infralimbic portion is involved in extinction (THOMPSON et al., 2010).

Neurotransmission and molecular pathways

Several molecular mechanisms have been implicated in fear memory in the dHPC. The glutamatergic system (Fig.1), including NMDA, AMPA and metabotropic glutamatergic receptors are crucially involved in learning and memory (RIEDEL; PLATT; MICHEAU, 2003). Furthermore, other neurotransmission systems such as gabaergic (LUCAS; CLEM, 2018), noradrenergic (GIUSTINO; MAREN, 2018) and dopaminergic (STUBBENDORFF; STEVENSON, 2021) also participated in CFC. In addition to neurotransmitters, the aversive experience activates the hypothalamic-pituitary-adrenal (HPA) axis, leading to the release of glucocorticoid (GC) hormones. GC are secreted after acquisition and retrieval of fear memory (DE QUERVAIN; SCHWABE; ROOZENDAAL, 2017) and they are responsible for regulating or inducing a plethora of responses among them the enhancement of neurotrophic signalling (NOTARAS; VAN DEN BUUSE, 2020; SURI; VAIDYA, 2013). Neurotrophic factors (Fig.1), and specially the brain-derived-neurotrophic factor (BDNF) is released after neuronal depolarization (BRIGADSKI; LESSMANN, 2020) and induces dimerization of Tropomyosin receptor kinase B (TrkB) receptor modulating a number of molecular effectors (ANDERO; CHOI; RESSLER, 2014).

Sequentially, the combination of the mentioned events converges to recruit several pathways (Fig.1). Together, the Ca^{2+} influx and engagement of G-proteins lead to the recruitment of downstream effectors. Particularly, the

activation of several kinases; calmodulin-dependent kinase II (CamKII) (FRANKLAND et al., 2004; KIMURA; SILVA; OHNO, 2008; LEPICARD et al., 2006), protein kinase A (PKA) (ABEL et al., 1997; BOURTCHOULADZE et al., 1998; SZAPIRO et al., 2003), protein kinase B (PKC) (LI; INOUE; KOYAMA, 2002; WEEBER et al., 2000) and extracellular signal-regulated kinase 1-2 (ERK1-2) (SHALIN et al., 2004; SZAPIRO et al., 2003). This triggers a series of concomitant events: I) phosphorylation and modulation of receptors and channels, II) activation of mammalian target of rapamycin (mTOR) inducing protein synthesis (BLUNDELL; KOUSER; POWELL, 2008), III) activation of neuronal nitric oxide synthase (ARAKI et al., 2020), IV) enhancement of histone acetylation (LEVENSON et al., 2004; MILLER; CAMPBELL; SWEATT, 2008), V) activation of transcription factors such as cAMP response element-binding protein (CREB) (SINDREU; SCHEINER; STORM, 2007) and VI) transcription of immediate-early genes (IEG) as activity-regulated cytoskeleton-associated protein (Arc) (CZERNIAWSKI et al., 2011; HUFF et al., 2006), Early Growth Response Protein 1 (EGR-1, also called Zif-268) (MALKANI; ROSEN, 2000a, 2000b) and Fos (MILANOVIC et al., 1998; STREKALOVA et al., 2003) involved in the modulation of transcription and dendritic re-structuration (DUCLOT; KABBAJ, 2017).

2. The endocannabinoid system

2.1. Cannabis and the endocannabinoid system

Cannabis sativa is a plant known as a drug of abuse capable of causing euphoria, pleasure and relaxation (HALL; SOLOWIJ, 1998; PATON, 1975). The medical, recreational and ceremonial used of this plant was popular for at least the last 5000 years (PERTWEE, 2006). However, it was only in 1964 that its main psychoactive compound, delta-9-tetrahydrocannabinol (THC), was characterized (GAONI; MECHOULAM, 1964). Since then, dozens of compounds have been identified (ELSOHLY, 2002). The description of THC and other cannabinoids made it possible more accurate research of the mechanism of action underlying the effects of Cannabis consumption. Several theories arose (HOWLETT, 2003; PERTWEE, 2006), but the capacity of THC to inhibit adenylate cyclase acting through Gi/o proteins was an early discovery supporting the view that cannabinoids exert their effects through activation of

specific receptors in the brain (DEVANE et al., 1988). Indeed in 1990, Matsuda et al. described for the first time the cannabinoid receptor type 1, CB_1 receptor (MATSUDA et al., 1990). The remaining question was if there were endogenous compounds able to activate the cannabinoid receptor. AEA was the first endogenous ligand discovered (DEVANE et al., 1992) followed by 2-arachidonoylglycerol (2-AG) (MECHOULAM et al., 1995). These compounds were termed endocannabinoids (eCB). Three years after the characterization of CB_1, another cannabinoid receptor was revealed, CB_2 (MUNRO; THOMAS; ABU-SHAAR, 1993) and the mechanisms underlying eCB metabolism were also identified. AEA and 2-AG are metabolized by fatty acid amide hydrolase (FAAH) (DEUTSCH; CHIN, 1993) and monoacylglycerol lipase (MAGL) (TORNQVIST; BELFRAGE, 1976) respectively, and their membrane transport is mediated by the eCB transporter (HILLARD et al., 1997). Altogether, the cannabinoid receptors, the eCB and the related enzymes constitute a highly preserved system across species (ELPHICK; SATOU; SATOH, 2003) called the endocannabinoid system. This system may include other components, such as the transient receptor potential, family V, type-1, (TRPV1) channel (CATERINA et al., 1997; ZYGMUNT et al., 1999).

A remarkable characteristic of the eCB system that makes it different from other neurotransmitters is that their ligands are synthesized and released on demand (PERTWEE, 2004). While AEA can be synthesized by the pre- or the postsynaptic neuron, 2-AG is mostly exclusively produced in postsynaptic terminals (HOWLETT, 2003; PIOMELLI; MABOU TAGNE, 2022). Once released, eCBs can act as retrograde messengers (postsynaptic release – presynaptic modulation) or as autocrine feedback modulators (BUSQUETS-GARCIA; BAINS; MARSICANO, 2017; UCHIGASHIMA et al., 2007). The synthesis and release of eCB can be triggered by several factors such as Ca^{2+} influx, activation of NMDA, cholinergic and mGluR receptors (E. ALGER, 2002). Once in the synaptic cleft eCBs activate CB_1, among others, which in turn inhibits the release of other neurotransmitters and modulated short- and long-term synaptic plasticity (WINTERS; VAUGHAN, 2021). Probably, 2-AG modulates gabaergic neurotransmission while AEA acts in glutamatergic neurons (MIZUNO; MATSUDA, 2021).

2.2. CB_1 receptors

CB_1 characterization and pathways

The brain expression of CB_1 was initially confirmed in the HPC and cerebral cortex (MATSUDA et al., 1990). Later, it was also found in the AMG, the HPA axis, and the striatum (GALIEGUE et al., 1995; ONG; MACKIE, 1999; TSOU et al., 1998). In addition to its wide distribution at the neuroanatomical level, CB_1 is also broadly expressed among different cell types, this includes neurons, microglia and astrocytes. In neurons, CB_1 receptors are highly expressed in gabaergic interneurons and in a lesser extent in glutamatergic neurons (BUSQUETS-GARCIA; BAINS; MARSICANO, 2017; PIOMELLI; MABOU TAGNE, 2022; WINTERS; VAUGHAN, 2021), CB_1 can also be colocalized with dopaminergic (MARTÍN et al., 2008) and serotoninergic receptors (LAU; SCHLOSS, 2008). In addition, CB_1 can also be found in different positions at the synapse been more common in the presynaptic terminal, but a post-synaptic location was also described (BUSQUETS-GARCIA; BAINS; MARSICANO, 2017; BUSQUETS GARCIA et al., 2016). Finally, different cellular compartments such as mitochondria (AQUILA et al., 2010; TEDESCO et al., 2010) or the cell nucleus (BOIVIN et al., 2008) can express CB_1.

In terms of signal transduction, CB_1 receptors are G-protein-coupled receptors (GPCR) (GONZALEZ, S., SAGREDO, O., GÓMEZ, M., RAMOS, 2002) and are one of the GPCR most expressed in the brain (BUSQUETS-GARCIA; BAINS; MARSICANO, 2017; HERKENHAM et al., 1990; HOWLETT, 2003). CB_1 receptor was firstly characterized as coupled to Gi/o proteins due to its capacity to inhibit adenylate cyclase and in turn decrease cAMP (HOWLETT, 2003). However, CB_1 also inhibits Ca^{2+} channels (HOWLETT, 2003; LOZOVAYA et al., 2009; MACKIE et al., 1995; PIOMELLI, 2003; TWITCHELL; BROWN; MACKIE, 1997), activates K^+ channels (CHILDERS; DEADWYLER, 1996; HOWLETT, 2003; MACKIE et al., 1995; PIOMELLI, 2003) and Mitogen-Activated Protein Kinase (MAPK) (PIOMELLI, 2003). Likewise, under certain circumstances, CB_1 receptors can also act coupled to Gs (ABADJI et al., 1999; GLASS; FELDER, 1997) or Gq subunits (LAUCKNER; HILLE; MACKIE, 2005; NAVARRETE; ARAQUE, 2008; REDMOND et al., 2016). The pathway engaged

seems to be influenced by the ligand (DIEZ-ALARCIA et al., 2016; LAUCKNER; HILLE; MACKIE, 2005; REDMOND et al., 2016) and the tissue type (TURU; HUNYADY, 2010) Finally, CB_1 receptors can also act through β-arrestin leading to the activation of the ERK pathway, CB_1 desensitization and internalization (AHN et al., 2013; WOUTERS et al., 2019), this seems dependent on exposition time and strength of the stimulus (HOWLETT; ABOOD, 2017).

CB_1 and their endogenous ligands in contextual fear conditioning

Early observations suggested that knockout animals for CB_1 presented alterations selectively in the CFC (JACOB et al., 2012; MIKICS et al., 2006) since other types of conditioned fear, such as AFC, was spared (MARSICANO et al., 2002a). Different from genetic manipulations, the administration of AM251, a CB_1 inverse agonist/antagonist impaired memory acquisition in the AFC (ARENOS; MUSTY; BUCCI, 2006; SINK et al., 2010). In the CFC, pharmacological manipulations using AM251 showed contradictory results, including no effect (ARENOS; MUSTY; BUCCI, 2006) or enhancements in fear memory (LIN et al., 2011; SINK et al., 2010). In contrast, treatment with CB_1 agonists, but not the blockage of the hydrolysis of AEA (LARICCHIUTA; CENTONZE; PETROSINI, 2013) or 2-AG (KISHIMOTO et al., 2015), impaired fear memory (NASEHI et al., 2016a, 2016b; PAMPLONA; TAKAHASHI, 2006), this was prevented by CB_1 antagonism (PAMPLONA; TAKAHASHI, 2006). Regarding consolidation, the administration of CB_1 antagonists had no effect (ARENOS; MUSTY; BUCCI, 2006). However, Hu-210, a CB_1 agonist, impaired this memory phase (MAĆKOWIAK et al., 2009). Similarly, when administered after a brief reactivation, CB_1 antagonists had no effect (SUZUKI et al., 2004) but CBD and THC impaired memory reconsolidation (STERN et al., 2012, 2015) this was mimicked by a combination of subeffective doses of a CB_1 agonist and a kappaB inhibitor (LEE; FLAVELL, 2014). Likewise, neither CB_1 antagonism nor blocking the hydrolysis of AEA or 2-AG interfere with memory retrieval (ARENOS; MUSTY; BUCCI, 2006; KISHIMOTO et al., 2015; LARICCHIUTA; CENTONZE; PETROSINI, 2013; MIKICS et al., 2006). Curiously, Mikicks et al. (2006) showed that, AM251 administered before the test reduced freezing, the opposite was observed when the treatment was done with an agonist, Win-55,212-2 (MIKICS et al., 2006).

Differently from other memory phases, increasing AEA availability led to facilitation of extinction (BITENCOURT; PAMPLONA; TAKAHASHI, 2008; CHHATWAL et al., 2004; LARICCHIUTA; CENTONZE; PETROSINI, 2013) this was abolished by CB_1 antagonism (BITENCOURT; PAMPLONA; TAKAHASHI, 2008; CHHATWAL et al., 2004; LARICCHIUTA; CENTONZE; PETROSINI, 2013), ACTH enhancements (BITENCOURT; PAMPLONA; TAKAHASHI, 2014) or antagonism of GC receptor (BITENCOURT; PAMPLONA; TAKAHASHI, 2014) but not by TRPV1 blocking (BITENCOURT; PAMPLONA; TAKAHASHI, 2008; LARICCHIUTA; CENTONZE; PETROSINI, 2013). Equally important, the elevation of AEA during extinction seems to induce a sustained effect since these animals were resistant to fear reinstatement (CHHATWAL et al., 2004). Similar to the enhancement of AEA, the treatment with a CB_1 agonist also facilitated extinction in the CFC (PAMPLONA et al., 2006; SIMONE et al., 2015) but this was not observed in the fear potentiated startle test (CHHATWAL et al., 2004). On the other hand, the antagonism/inverse agonism of CB_1 receptors lead to impairments in the extinction of AFC (MARSICANO et al., 2002b; SIMONE et al., 2015), CFC (NIYUHIRE et al., 2007; PAMPLONA et al., 2006; SUZUKI et al., 2004), potentiated startle test (CHHATWAL et al., 2004) and passive avoidance (NIYUHIRE et al., 2007). Moreover antagonism of CB_1 receptors prevented the impairments of extinction induced by GC receptor antagonists, suggesting that endocannabinoid and GC signalling can be synergically involved in fear extinction (BITENCOURT; PAMPLONA; TAKAHASHI, 2014). Regarding generalization, CB_1 knock out conditioned using highly intense footshocks presented high rates of generalization which was not observed using moderate intensities (JACOB et al., 2012).

Studies using central administration of synthetic cannabinoids into fear-related structures show that intra-AMG infusion of a CB_1 agonist prevented consolidation (KUHNERT; MEYER; KOCH, 2013), retrieval (KUHNERT; MEYER; KOCH, 2013), reconsolidation (LIN; MAO; GEAN, 2006) and reinstatement (LIN; MAO; GEAN, 2006) of fear memory. Similarly, AEA levels are increased in the basolateral AMG (BLA) after retrieval of fear memory (GASPAR et al., 2022). Furthermore, intra-PAG infusion of AEA or AM404 before retrieval of CFC reduced behavioural and cardiovascular responses to

the context, this effect was prevented by local pre-treatment with AM251 (RESSTEL et al., 2008). 2-AG administration into the dorsolateral PAG impaired retrieval and this seems to be mediated by CB_1 receptors (BRIANIS et al., 2022). In consonance, AM251 treatment before retrieval increases freezing in the CFC (ULIANA et al., 2016). This was prevented by NMDA antagonism, by modulating the nitric oxide pathway and by TRPV1 antagonism (ULIANA et al., 2016).

Moreover, CB_1 involvement was also studied in the HPC. Intra-HPC administration of AM404 prevented memory acquisition and this was dependent on CB_1 since AM281 attenuated AM404 effects, probably modulating LTP in gabaergic neurons (LIN et al., 2011). The administration of AM251 into the CA1 region of the dHPC impaired fear extinction (DE OLIVEIRA ALVARES et al., 2008), facilitated reconsolidation (DE OLIVEIRA ALVARES et al., 2008) and enhanced retrieval (SPIACCI et al., 2016). In this last phase, the effect was prevented by NMDA antagonism and neuronal nitric oxide synthase inhibition (SPIACCI et al., 2016). Also, when subeffective doses of AM251 and a GABA A antagonism were combined they presented a synergic effect in the enhancement of memory retrieval (SPIACCI et al., 2016). In contrast with the antagonism, increasing AEA availability in CA1 during consolidation using a blocker of the AEA transporter, decreased freezing time and this effect was dependent on the HPA axis, CB_1 and muscarinic receptors but not TRPV1 (SCIENZA-MARTIN et al., 2022). The effects of AM404 were accompanied by a disruption in long-term potentiation (LTP) in the dHPC and replicate in pre-retrieval administrations (SCIENZA-MARTIN et al., 2022). Moreover, AEA blocks reconsolidation and facilitates extinction, effect prevented by pre-treatment with AM251 (DE OLIVEIRA ALVARES et al., 2008). Similarly, increasing AEA availability or the administration of a CB_1 agonist into the dHPC also facilitates extinction (ABUSH; AKIRAV, 2010). In the same vein, the treatment with an agonist impaired memory reconsolidation (SANTANA et al., 2016).

CB_1 seems also relevant for the modulation of CFC in other structures like the PFC (KUHNERT; MEYER; KOCH, 2013; LISBOA et al., 2010; ULIANA et al., 2020) or the nucleus accumbent (PEDROZA-LLINÁS et al., 2013).

2.3. Limits and perspectives of the eCB system as a therapeutical target

CB_1 involvement in the modulation of fear memory lead to proposed this receptor as a potential target for the treatment of certain disorders such as PTSD (LISBOA et al., 2019; MIZUNO; MATSUDA, 2021; RESSTEL; MOREIRA; GUIMARÃES, 2009). In fact, beyond fear memory, the endocannabinoid system was proposed as a target for the treatment of other psychiatric and neurologic disorders including addiction, anxiety or epilepsy (ASTH et al., 2019; LUTZ et al., 2015; MOREIRA; LUTZ, 2008). The idea that the manipulation of the endocannabinoid system would be interesting for psychopharmacology was first derived from the capacity of Cannabis to induced altered emotional states (HALL; SOLOWIJ, 1998; LUTZ et al., 2015; MOREIRA; LUTZ, 2008) and the high expression of CB_1 receptors in limbic and stress-related structures (LUTZ et al., 2015). Later, several preclinical studies endorse the role of the eCB system in stress-coping behaviours, anxiety responses and maladaptive memory (LISBOA et al., 2019; LUTZ et al., 2015; MOREIRA; LUTZ, 2008; MOREIRA; WOTJAK, 2010). However, the direction of this modulation was highly controversial. A topic extensively reviewed in the past (LUTZ et al., 2015; MOREIRA; LUTZ, 2008; MOREIRA; WOTJAK, 2010) is that in the same way that Cannabis is able to induced relaxation but also anxiety (Hall & Solowij, 1998; Moreira & Lutz, 2008), the modulation of the eCB system can induced both, anxiolytic and anxiogenic effects, panic or panicolitic, memory enhancements and deficits, then, bidirectional effects. Indeed, one of the main functions of the eCB system is to protect homeostasis, and, in this sense, homeostasis can be perturbed in both directions. The first attempt to reconcile the opposite effects induced by *Cannabis* pointed to the plethora of compounds included in the plant which may induce opposite consequences, then the effect of the main psychoactive compound could be counteract by another phytocannabinoid. Indeed, the discovery of cannabidiol (CBD), reinforced this idea. However, the purification of THC allowed studies with this substance isolated, and in the absence of other cannabinoids THC also induced bidirectional effects. This fact can be explained by four key elements of the endocannabinoid system biology: I) The high expression of CB1 in different neuroanatomical structures, II) the capacity of this receptor to modulate conflicting neurotransmitter systems such as gabaergic and glutamatergic, III)

the on-demand release of eCB and IV) the promiscuity of AEA (MOREIRA; LUTZ, 2008; MOREIRA; WOTJAK, 2010); AEA is able to activate other receptors, such as the calcium channel TRPV1, with opposite effects than CB_1.

The difficulties in predict the response of CB_1 modulators together with the relevant psychiatric side-effects of Rimonabant, an inverse agonist of CB_1 that reach the market for the treatment of obesity, brought certain mistrust in the therapeutic potential of the eCB system. In contraposition, other pharmacological strategies emerged to overcome this situation. Among them, the enhancement of eCB availability by using inhibitors of FAAH and MAGL (BATISTA et al., 2014; MOREIRA; WOTJAK, 2010) and, the modulation of other receptors of the eCB system such as CB_2 or TRPV1.

3. TRPV1

3.1. General characteristics

The TRPV1 channel was first described in 1997 and named vanilloid receptor 1 for being the target of the vanilloid capsaicin (CPS), a compound from chilli peppers of the genus Capsicum, and responsible for the burning pain associated to this substance (CATERINA et al., 1997). It was described as a non-selective cation channel with high permeability to Ca^{2+} and expressed in peripheral afferent neurons (CATERINA et al., 1997). TRPV1 is formed by six transmembrane domains with a permeable central pore of 6Å between S5 and S6 (Caterina et al., 1997). The N-terminal presents a ankyrin repeated domain with an ATP-binding site associated to protein stabilization and desensitization by ATP and Ca^{2+}-calmodulin respectively (LIAO et al., 2013).

CPS activates the channel in sensitive neurons inducing excitation followed by fast desensitization (SZALLASI; BLUMBERG, 1999). In addition to CPS, TRPV1 is modulated by a long list of stimuli of different natures (KANEKO; SZALLASI, 2014). TRPV1 can be considered **a detector of painful or dangerous stimuli** (SHUBA, 2021): noxious heat (CATERINA et al., 1997), voltage (VLACHOVA et al., 2003), low levels of pH (TOMINAGA et al., 1998), pro-inflamatory mediators (SUGIURA et al., 2002), tarantula vanilloid toxin (CROMER; MCINTYRE, 2008). In addition, TRPV1 is modulated by endo- and phytocannabinoids: AEA (ZYGMUNT et al., 1999), N-arachidonoyldopamine

(HUANG et al., 2016) or CBD (BISOGNO et al., 2007). Furthermore, there are several intracellular ligands that modulate the channel: Ca^{2+}-calmodulin complex (NUMAZAKI et al., 2016), ATP (LISHKO et al., 2007), PKA and PKC (PETROCELLIS et al., 2001; PREMKUMAR; AHERN, 2000) and phosphatidylinositol 4,5 biphosphate (YAO; QIN, 2009).

The TRPV1 channel is expressed in neurons, glia cells (MARRONE et al., 2017; NAM et al., 2015; TOTH; BOCZA; BLUMBERG, 2005) and neuronal precursor cells (STOCK et al., 2014). At the neuroanatomical level its expression was demonstrated in several areas: cortical structures, limbic system, striatum, thalamic nuclei, substancia nigra, locus coeruleus, cerebellum, AMG, ventral tegmental area, nucleus accumbent, striatum and thalamic nucleus (HENG et al., 2014; KAUER; GIBSON, 2009; MEZEY et al., 2000; VIERECKEL et al., 2016). TRPV1 is also expressed in neurogenic regions, where it seems involved in neurogenesis, cell migration and survival (RAMIREZ-BARRANTES et al., 2016). Finally, there are evidence from preclinical research indicating a strong association between TRPV1 expression and developmental stage, for example, in C57Bl6/J TRPV1 has its peak of expression in the eighth-nine postnatal week (HUANG et al., 2014).

Since AEA is one of the main endogenous ligands of TRPV1, this channel can be considered a part of the extended eCB system (Fig.1). Moreover, TRPV1 is coexpressed with CB_1 in the HPC and AMG (CRISTINO; PETROCELLIS; PRYCE, 2006; KAUER; GIBSON, 2009). In contrast with the canonical presynaptic position of CB_1, in the HPC, TRPV1 is predominantly found in postsynaptic neurons (TOTH; BOCZA; BLUMBERG, 2005). Moreover, CB_1 is a Gi/o-protein coupled receptor (MATSUDA et al., 1990), while TRPV1 is a cationic channel (CATERINA et al., 1997). They also differ regarding AEA binding, which has twenty times more affinity for CB_1 when compared to TRPV1 (DEVANE et al., 1992; ROSS, 2003; STELT et al., 2005). However, when AEA reaches concentrations high enough to activate both targets, it acts as a partial agonist at CB_1 and a full agonist at TRPV1 (ROSS, 2003; ZYGMUNT et al., 1999).

Furthermore, several studies show that TRPV1 is highly regulated. Thereby, in a large influx of Ca^{2+}, Calmodulin can bind to TRPV1, forming a

Ca^{2+}/Calmodulin complex and inducing the channel inactivation and desensitization (NUMAZAKI et al., 2016). ATP is also capable to bind TRPV1 preventing this effect (LISHKO et al., 2007). On the other hand, calcineurin seems to dephosphorylate the channel leading to its desensitization (DOCHERTI; YEATS; BEVAN, 1996). On the contrary, activation of PKA and PKC increases the activity of the channel and modify its response to, for example, AEA (PETROCELLIS et al., 2001; PREMKUMAR; AHERN, 2000). Moreover, hydrolysis of phosphatidyl-inositol-bis-phosphate may potentiate TRPV1 channels activation (CHUANG et al., 2001).

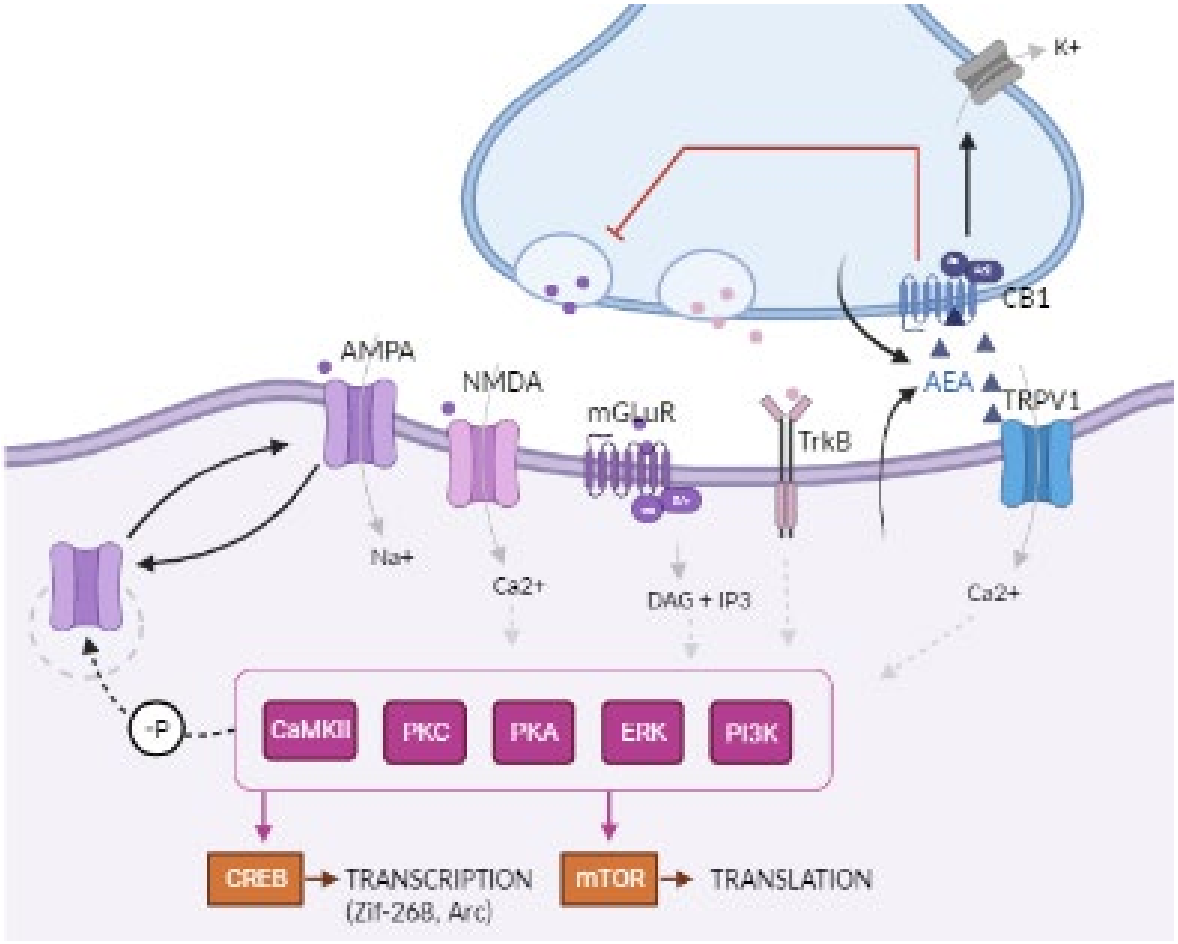

Figure 1: Activity-dependent release of AEA activates CB1 and TRPV1 in the excitatory circuit. CB1 receptors are located in the presynaptic neuron, after activated they inhibit neurotransmitters release. TRPV1 channels are located in postsynaptic neurons, when activated they allowed Ca^{2+} influx.

3.2. TRPV1 in contextual fear conditioning

One of the first observations implicating TRPV1 in conditioned fear was obtained from studies with knock out mice. TRPV1 deletion results in reduced conditioning to context and tone, together with weaker LTP in CA1 (MARSCH et al., 2007). In addition, local TRPV1 blockade in the HPC impaired consolidation of fear memory when the conditioning was performed with stimulus of high intensity (GENRO; ALVARES; QUILLFELDT, 2012). Similar results were obtained by blocking TRPV1 in the vmPFC (TERZIAN et al., 2014). The role of TRPV1 in structures associated with fear related behaviours has also been investigated. Pre-treatment with 6-iodo-nordihidrocapsaicin (6-I-NC) a TRPV1 blocker, in the PAG, prevented the increase in freezing induced by CB_1 antagonism (ULIANA et al., 2016). Moreover, the administration of CPS into the vmPFC enhanced fear responses while the administration of a blocker decreased them (ULIANA et al., 2020). Gobira et al. also observed a decrease in freezing when N-araquidonil-serotonin (AA-5-HT), a dual blocker of FAAH and TRPV1, was administered in the dHPC before retrieval, this effect was prevented by CB_1 antagonist pre-treatment and mimic by co-administration of SB, a TRPV1 blocker and a FAAH inhibitor (GOBIRA et al., 2017a). AA-5-HT also attenuates fear generalization decreasing dopamine release in BLA and the nucleus accumbent (FREELS; LESTER; COOK, 2019).

Aims

This project was designed to test the hypothesis that hippocampal TRPV1 channels are involved in the retrieval of CFC in an intensity-dependent manner.

Objective 1: Characterizing the intensity-dependent recruitment of hippocampal TRPV1 channels in the retrieval of fear memory and its relation with the endocannabinoid system.

Objective 2: Characterizing the effects of TRPV1 blockers in different phases and types of memory.

Objective 3: Characterizing plasticity pathways triggered by TRPV1 blockers in the HPC and their potential long-term effects.

Material and Methods

1. Animals

The experimental animals were 9 weeks-old male mice from C57B/L6J strain, with the exception of one experiment where the animals were 20-weeks-old. They were provided by the vivarium of UFMG (Biotério Central). They were located in plastic cages and kept in a temperature-controlled room, 24±2 C, with standard dark-light cycle of 12h and free access to water and food. At the end of the experiments the animals were euthanized through CO_2 inhalation, except those designed to molecular tests. The protocols were approved by the local ethics committee (CEUA) under the protocol number 176/2020.

2. Apparatus

The CFC procedure was performed in a chamber with dimensions 20 x 20 x 22 cm. The chamber floor consists of 23 rods of stainless steel with 2mm of diameter. They are separated by 0.7cm and linked to a shock generator. All the walls are metallic except the frontal and the superior ones, which are made of an acrylic material. In front of the chamber was located a camera CANON® powershot SX520.

The CPP was performed in a chamber with dimensions 45.6 x 13 x 17 cm, divided in three compartments. A small aperture (3x4cm) communicates them. All the walls are opaque and the roof is made of transparent material. The middle compartment is black. One of the lateral compartments has black and white horizontal stripes in the walls and the floor is white and covered by holes. The other lateral compartment has black and white vertical stripes and its floor is formed by white rods intercalated with empty spaces. Between the compartments, there are apertures that allows the animal to move from one compartment to the other. These apertures can be closed. Over the chamber was positioned a camera LG720p coupled to a computer containing the behavioural analysis software Any-Maze.

3. Drugs

The TRPV1 blocker, N-(3-Methoxyphenyl)-4-chlorocinnamide (SB366791, SB; Tocris, Bioscience®) was administered at the doses of 1, 3 and 10 nmol (CASAROTTO et al., 2012) for the dose-response curve; the intermediary dose

of 3nmol was later selected for most of the experiments. 6-iodo-nordihidrocapsaicin (6-I-NC; Tocris, Bioscience®) another selective TRPV1 blocker, was administered at the dose of 3 nmol, the dose was selected based on the similar IC50 between SB (IC50=651.9nM) and 6-I-NC (IC50=638.6nM) against CPS (APPENDINO et al., 2003; VARGA et al., 2005). TRPV1 blockers were administered 5min before acquisition or retrieval or immediately after retrieval or reactivation. The CB_1 antagonist/inverse agonist, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide (AM251; Tocris, Bioscience®), was administered at the sub-effective dose of 75pmol (GUIMARA et al., 2012; HARTMANN et al., 2019) 5 min before SB. The drugs were administered bilaterally into the dHPC and diluted in ethanol:cremophor:saline (1:1:18). Cocaine (Merck) was used in the CPP experiment, administered via intraperitoneal route, diluted in saline, at the dose of 15mg/kg (THOMSEN; CAINE, 2011). A combination of ketamine (Vetnil®) and xylazine (Sedanew, Vetnil®) diluted in saline was used for general anaesthesia. The dose for stereotaxic surgery was 100mg/kg, 10mg/kg i.p, and for transcardial perfusion 150mg/kg, 15mg/kg i.p respectively. During surgical procedures the animals received 0.06ml s.c Banamine (50mg/ml, Intervet®) and 0.1ml i.m Pentabiotic (Agrosil 5 Mega, Vansil®) diluted in saline 1:10.

4. Procedures

4.1. Transcardial perfusion

The animals were anesthetized with ketamine and xylazine (i.p, 150mg/kg,15mg/kg, respectively) and the thoracic cavity was opened to leave the heart exposed. A needle coupled to a peristaltic bomb was inserted in the left ventricle and the aorta was sectioned. The animals were perfused with 100ml of phosphate buffered saline (PBS) and 50ml of paraformaldehyde (PFA) 4%. Once concluded the perfusion, the brain was removed and kept it in PFA 4% during 2h. Later, it was maintained in PFA 2% at 4ºC.

4.2. Stereotaxic surgery and intra-HPC administration

The surgical procedures were performed 7 days before behavioural tests. The animals were anesthetized with ketamine and xylazine (i.p., 100mg/kg, 10mg/kg respectively) and received Banamine (0.06ml, 1:10) and Pentabiotic (0.1ml, 1:10). They were positioned in the stereotaxic apparatus;

asepsis of the surgical field was performed with iodine and the skullcap was exposed retiring part of the skin; the skullcap was cleaned with oxygenated water 10%. The coordinates used to access the dHPC were: P: -1.9mm, ML: +1.5mm, DV: -1.3mm (PAXINOS; FRANKLIN, 2003). Later, two apertures were opening, two cannulas of 0.7 mm (made from needle 20x5.5-26x^3/415 0.5x19mm) were implanted. A mixture of resin (JET®) and acrylic (TDV®) was used to cover the skullcap. Both cannulas were sealed by steel wire.

In order to perform the bilateral administration into the dHPC, the animal was immobilized and an injector needle of 0.8mm (made from gingival needle 3mmx22mm, 30G) was located into each cannula. Injector needles were coupled to a polyethylene catheter (P10) linked to Hammilton microsyringes. It was administered a volume of 0.25µl using an infusion bomb in a flux of 0.25µl/min. The injector needles were removed 30s after the administration was concluded to avoid reflux.

After the experiment, the animals were euthanized by CO_2 inhalation and 0.25µl of Evans blue ink 10% diluted in saline was administered in a flux of 0.25µl/min. The brains were removed and storage in PFA 4% at 4ºC to posterior verification of the administration site.

4.3. Hippocampus dissection

In order to dissect the HPC the animals were euthanatized by cervical dislocation. The tissue was carefully removed and three longitudinal cuts were performed, one following the sagittal suture and the other two bellow the right and left hemisphere. The brain was removed, hydrated with saline and kept on ice. The cortex was carefully separated until the HPC was exposed. The entire HPC was removed and frozen with liquid nitrogen. The samples were kept at -80ºC.

5. Behavioural procedures.

All the behavioural experiments were performed in an isolated room; the temperature was kept at ±24ºC, the illumination of the room was without any brightness and with low intensity. The experiments were performed between 8:00am and 3:00pm, during the light phase of the cycle. The animals were

located in the experimental room at least 1h before the procedure. All the equipment was clean with alcohol 70% between animals.

5.1. Contextual fear conditioning

The protocol was based on previous report (Gobira et al., 2017). On the first day, conditioning, the animals were gently placed in the chamber and submitted to one of the protocols named Not-conditioned, low, moderate or high intensity based on the **aversiveness** of the experience (see Table 1). The first shock was carried 3min after the animal was in the chamber, the second 60s after the first one, and the third 40s after the second. The animal was kept in the chamber for one more minute after the last shock. Twenty-four hours later was

Name	Conditions
Not-Conditioned (NC)	0
Low Intensity (LI)	X3 shocks, 0.5 mA, 1s
Moderate Intensity (MI)	X3 shocks, 0.5 mA, 2s
High Intensity (HI)	X3 shocks, 0.8 mA, 1s
Reinstatement Intensity (RI)	X1 shock, 0.5 mA, 1s

Table 1: Contextual Fear Conditioning intensities

performed the test. The animal was placed into the chamber during 5min without any intervention and recorded.

Some experiments involved a second retrieval session 24h after the first one which also consisted in a 5min exposition to the chamber. The experiments involving the reinstatement were based on previous reports (HITORA-IMAMURA et al., 2015; VOUIMBA; MAROUN, 2011). Briefly, one week after the test, the animals were submitted to an extinction session where they were exposed to the chamber for 20 min. Twenty-four hours later, it was performed the reinstatement session; the animals were put into the chamber where they received one shock of 0.5 mA for 1s, after that the animals were kept in the chamber for one more minute. Twenty-four hours after the reinstatement session the animals were re-tested; they were exposed to the chamber for 5 min and recorded.

The evaluated parameter was the freezing time defined as a complete absence of movement except for those movements related to breathing. The percentage of freezing was interpreted as a measure of memory evocation in response to the conditioned element, in this case, the context (FANSELOW, 1980). The evaluation of freezing was performed manually and without previous knowledge of the experimental group.

5.2. Conditioned place preference:

In the first day, pre-test, the animal was located in the middle compartment and allowed to explore the entire apparatus for 15 minutes. The procedure was recorded and analysed with the software Anymaze. The exploration time of each compartment was assessed. It was calculated the exploration index (EI) for the compartment with vertical stripes (EI_v) and the compartment with horizontal stripes (EI_h):

$$EI_v : \frac{\text{tvertical}}{\text{tvertical} + \text{thorizontal}} \text{x } 100 \qquad \text{EI}_\text{h} : \frac{\text{thorizontal}}{\text{tvertical} + \text{thorizontal}} \text{x } 100$$

Where EI is expressed in percentage and t_i corresponds to time in seconds. The animals that spent more than 70% of the time in one of the compartments were excluded. The animals were conditioned in the compartment with lower EI.

The conditioning phase corresponded to days 2, 3, 4, 5, 6 and 7. On days 2, 4 and 6 the animals received 15mg/kg cocaine i.p and they were immediately confined in the respective compartment during 30min. On days 3, 5 and 7 the animals received saline i.p and were immediately confined in the opposite compartment during 30min. The control group received saline every day.

On day 8 the test was performed; the procedure was identical to the first day. The animals were recorded by a video-camera coupled to a computer and the behaviour was analysed with the software Anymaze. The exploration time of each compartment was assessed and it was calculated the EI for the compartment paired with cocaine. The CPP index was calculated using the EI value from day 1, EI_1, and the value from day 8, EI_2, of the chamber paired with cocaine: $CPPindex = EI_2 - EI_1$

5.3. Novel Object Recognition:

The protocol for novel object recognition (NOR) was based on previous reports (LUEPTOW, 2017). In the first day, habituation, the animal was placed in the box for 5 min without any object. Twenty-four hours later, in the familiarization phase, the animal was placed in the box for one minute, after that, two identical objects were put in opposite corners. The animal was free to explore the objects up to 30 seconds. Twenty-four hours later, in the test phase, the animal was placed again in the box for one minute, after this time two objects, one from the familiarization phase and one new, were placed in opposite corners. The animal was free to explore the objects up to 30 seconds. The evaluated parameter was the exploration time defined as the time the animal spent in direct contact with the object, excluding the time the animal was sitting on the object or climbing it. The time that the animal spent at a range of 2cm of the object facing it was also considered (LEGER et al., 2013).

The quantification of exploration was performed manually and without previous knowledge of the experimental group. The Preference Index was calculated as (LUEPTOW, 2017): $IP = \frac{t_n}{30} x100$

Where t_n is the time in seconds that the animal spent exploring the novel object.

6. Imaging and molecular procedures

6.1. HPLC-MS

As previously described by de Oliveira et al., (2020). The samples were homogenized in 500µl of MilliQ H_2O using a Bead ruptor (10min 30Hz). Later, 400µl were mixed with 1000µl of methanol and 10µl of the internal pattern (2AG-d5 and AEA-d4, 1000ng/ml). After a brief homogenization 500µl of chloroform was added. All the volume was transferred to a solution containing 500µl of chloroform and 500µl of MilliQ water. The samples were centrifugated during 10 min at 4ºC, 3000rpm. The aqueous phase was collected and 500µl of chloroform was added. The samples were concentrated for 1h and resuspended in 100µl of methanol and water (7:3) and injected into the HLPC followed by the MS. The mobile phases were water and acetonitrile containing 0.1% of formic acid. The MS was operated in positive mode.

6.2. Immunofluorescence

Coronal sections of 50 µm obtained in a vibratome were washed three times for 5 min at RT in washing solution, PBS 0.01M + triton X-1000 0.3%. After that, the sections were kept in a citrate buffer (pH=6) for 1h at 70ºC for antigen retrieval. After washing three times with washing solution at RT, the sections were submerged in a solution of PBS 0.01M, triton X-1000 and tween 20 (1000:10:1) for 20 min at RT in order to permeabilized the sections. Later, the sections were blocked with glycine 0.1M in PBS for 20min followed by an incubation in blocking solution, Bovine Serum Albumin (BSA) 5% in PBS + 0.3% of triton X-1000. The sections were incubated in primary antibodies diluted in blocking solution for 72h at 4ºC: VR1 in goat (Santa Cruz, 1:30) + CB_1 in rabbit (Invitrogen, 1:1000).

After 72h of incubation the sections were washed 6 times and incubated with secondary antibodies diluted in blocking solution for two hours: Alexa 594 anti-rabbit (Invitrogen, 1:1000), Alexa 488 High Cross Absorbed (Invitrogen, 1:1000). After washing three times the sections were incubated in DAPI (1:1000) for 20 min. Finally, the sections were washed 6 times, mounted in gelatinize slides and covered with Flouroumnt G.

The images were acquired with the confocal microscopy LSM 880 Zeiss and processed by airyscan and the software ZEN2, images were obtained using a magnification of x68.

6.3. PCR

All the steps were performed in ice in RNAse and DNAse free material unless otherwise noted.

The lyse and homogenization of the samples was performed in accordance with TRIzol® Reagent (Invitrogen) User Guide. First of all, 500 µl of Trizol® was added to the tissue which was homogenised with an ultrasonic homogenizer (pulse mode, 60 W), samples were kept at -20°C overnight. The day after, samples were completely defrosted and 100µl of chloroform was added (Merk Millipore). The samples were centrifugate for 15min at 12000g, the aqueous phase was transferred to a new Eppendorf containing 250 µl of isopropanol (Sigma Aldrich, molecular grade) and after a brief homogenization,

the samples were centrifuged 10min at 12000 g. The supernatant was discarded by inversion and the pellet was washed in 500 µl of ethanol (Sigma Aldrich, molecular grade) and again centrifuged for 5min at 7500g. The supernatant was discarded and the RNA pellet air dried for 5min. The pellet was resuspended in 35uL of DEPC water (LGC biotecnologia) and then incubated in a heat block for 15min at 57°C. Total RNA concentration was assessed using a nanodrop spectrophotometer (NanoDrop™ Lite, Thermo Fisher Scientific). The purity of RNA samples was determined by the ratio of absorbance 260/280 (1.8 – 2 ratios were accepted). The samples were stored at -80°C until use.

Target gene	Primers	Sequence 5´→ 3'	Thermal cycling protocol
Trpv1	Foward	CCGGCTTTTTGGGAAGGGT	95ºC/30s 95ºC/5s 60ºC/30s (x40) 65ºC/ 5s +0.5ºC/cycle +0.5ºC/1s (melting)
	Reverse	GAGACAGGTAGGTCCATCCAC	
Arc	Foward	GTTAGCCCCTATGCCATCACC	
	Reverse	CTGGCCCATTCATGTGGTTCT	
Erg1 (Zif268)	Foward	TCGGCTCCTTTCCTCACTCA	
	Reverse	CTCATAGGGTTGTTCGCTCGG	
Ntrk2	Foward	CCGCTAGGATTTGGTGTACTG	
	Reverse	CCGGGTCAACGCTGTTAGG	
Rpl32	Foward	GCTGCCATCTGTTTTACGG	
	Reverse	TGACTGGTGCCTGATGAACT	

Table 2: Sequence of the primers and termal cycling protocol for PCR

M-MLV reverse transcriptase kit (Invitrogen) was used to first strand cDNA synthesis. Following manufacturer's instructions, 1 µg of total RNA was used as template. All sample were diluted with DEPC water to obtained a final volume of 1000ng/µl. Later, it was added 2.5 µl of MIX 1 (oligo dT, dNTP, DEPC water, 1:1:3) and the samples were kept 5 min at 65ºC. After that, the mixture was quickly chilled on ice and 4.5 µl of the MIX 2 was added (MML-V, DTT, DEPC water and M-MLV buffer, 1:2:2:3) and put in a bath at 37ºC for 50min. The reaction was inactivated by heating at 70°C for 15min. The cDNA was stored at -20ºC until used.

Quantitative PCR was performed using the iTaq™ Universal SYBR® Green Supermix (BioRad) in the CFX96 Touch™ Real Time detection system (BioRad). The PCR reaction consist on 5µl of iTaq SYBR green supermix (2x), 2µl of DNAse free water, 0.5µl of forward primer 10µM, 0.5µl of reverse primer 10µM and 2µl of cDNA 10ng/µl (10µl final volume). A mix was prepared with all reagents, except the samples that were added later in the plate already containing 8µl of mix. The plate was sealed and centrifuged at 400g for 5 min. The PCR running conditions as well as primer sequences are described in the table 2. The running results were analysed by CFX manager and Ct values used to calculate the relative mRNA levels.

6.4. ELISA

The quantification of BDNF was assessed by ELISA, the procedure was performed as indicated by the manufacture R&D Systems kit. The samples were processed in 175 µl of lysis buffer (Tris-HCl 20mM, NaCl 137mM, igepal 1%, glycerol 10%, EDTA 10mM, E-64 10mM, PMSF 1mM, pesptatin A 1µM, sodium vanadate 500mM) and homogenised with an ultrasonic homogenizer (pulse mode, 60 W). Then, they were centrifugate at 16000 rpm for 20min at 4ºC. The supernatant was collected and stored at -80°C. In order to sensibilize the plate it was added 100µl/well of capture antibody diluted 1:190 in sterile PBS and kept sealed o.n at RT. In the next day, the plate was washed three times with 300µL/well of washing buffer (PBS + tween20 0.05%). After that, the plate was blocked with 200 µl/well of blocking solution (PBS + BSA 1%) for 1h at RT followed by three washes with washing buffer. The samples were diluted 1:10 in PBS + BSA 0.1% and 50 µl/well were added. The correspondent standard curve was added in this step. The sealed plate was kept at 4ºC o.n. The day after, the plate was washed 3 times and 100 µl of detection biotinylated antibody was added, the antibody was diluted 1:190 in PBS+BSA 0.1%. The plate was kept at RT for 2h. After washing three times, it was added 100 µl/well of streptavidin diluted 1:40 in PBS+BSA 0.1% and kept 30 min at RT. After washing three times 100 µl/well of 0.3 µg/ml of OPD diluted in citrate buffer and H_2O_2 (5:1) was added and the plate was incubated for 30min protected from light. The reaction was interrupted with 50 µl/well of stop solution, H_2SO_4 1M in distilled water. The plate was read at 490nm.

7. Statistical analysis

The statistical analysis was performed using the software GraphPad Prism 8.0.1. The results were analysed by the student's t-test or by analysis of variance (ANOVA) followed by Bonferroni post-hoc test, as appropriate. Correlation analysis was performed using Pearson's. Outliers were identified using Grubbs. The statistical significance was set at $p<0.05$. The data are presented as mean and s.e.m., the post-hoc results were represented graphically.

Results

Intensity-dependent recruitment of TRPV1

The experimental design proposed for investigating the effects of TRPV1 blockage after conditioning to aversive stimuli of increasing intensity is presented in Fig. 2A. In animals exposed to LI, the TRPV1 blocker SB was not able to reduce freezing levels ($F_{3,22} = 1.161$, $p=0.3468$, Fig.2B). Under higher intensity of conditioning, MI, we observed that all three doses of the TRPV1 blocker were able to impair memory retrieval ($F_{3,23} = 6.6468$, $p=0.0025$, Fig.2C). In order to further investigate the effect of training intensity on TRPV1 recruitment, we used a third training protocol, HI, and we observed that 3nmol was able to decrease freezing also at this intensity ($t=2.518$, $df=11$, $p=0.0286$, Fig. 2D). Later, to verify that the impairments observed in the retrieval were due to TRPV1 blocking, we repeated the experiment (MI) using another selective blocker, 6-I-NC. This compound reduced the retrieval of fear memory in animals conditioned with the MI ($t=2.687$, $df=20$, $p=0.0142$, Fig. 2E). Taken together these results support the hypothesis that blocking TRPV1 channels impair retrieval of fear memory in an intensity-dependent manner. Since it was previously reported that TRPV1 expression in the HPC is affected by age (HUANG et al., 2014), we performed an experiment using the LI protocol in aged animals, as it can be observed, Fig. 2F, in this case, SB was able to impair retrieval ($F_{3,32} = 2.85$, $p=0.052$, Fig. 2F).

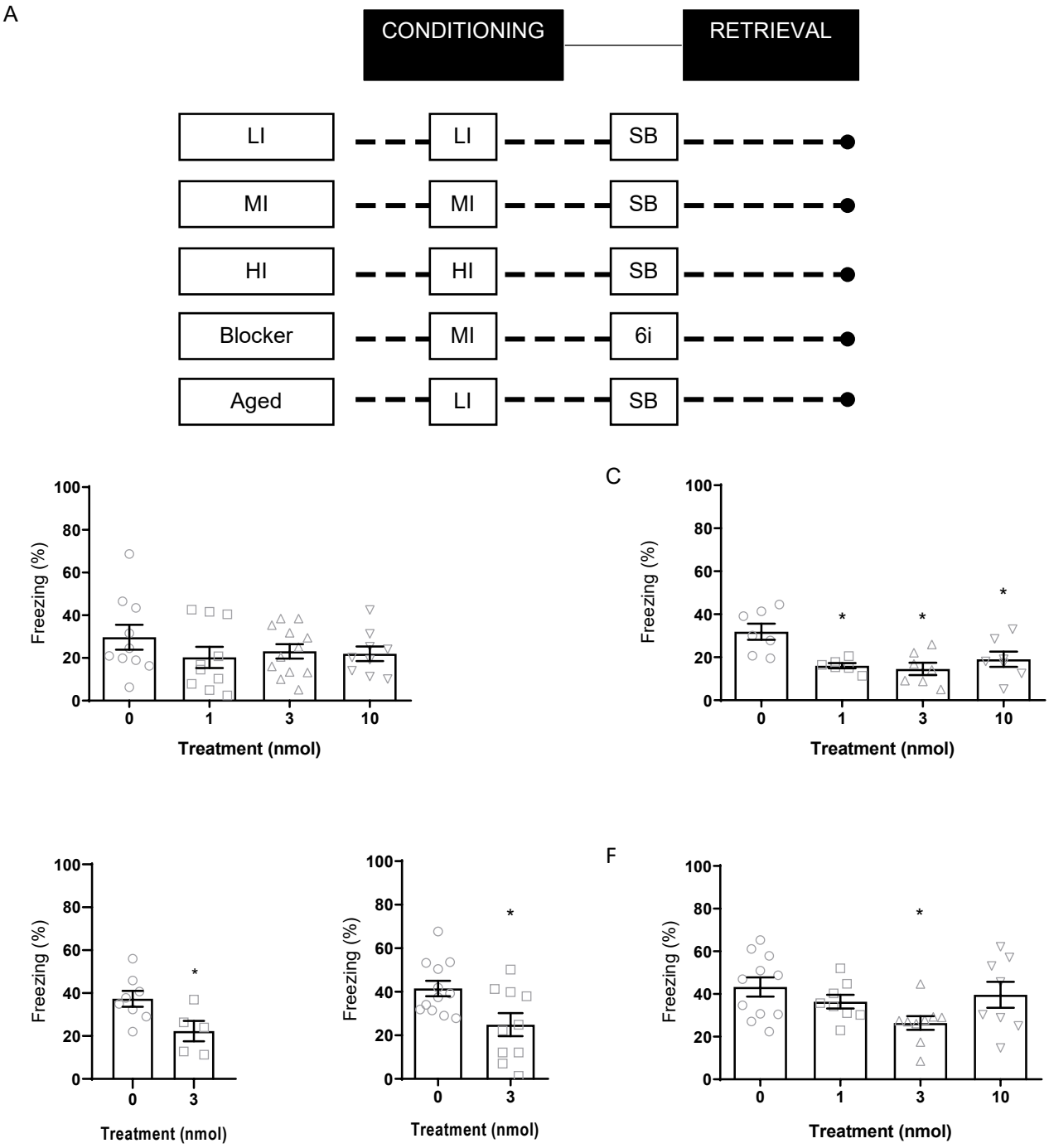

Figure 2: Intensity-dependent recruitment of hippocampal TRPV1 in the retrieval of the CFC. A) Experimental design. B) Animals conditioned using a LI protocol, SB was administered 5min before retrieval, one-way ANOVA followed by Bonferroni post-hoc n=10-10-12-9. C) Animals conditioned using a MI protocol, SB was administered 5min before retrieval, one-way ANOVA followed by Bonferroni post-hoc n=7-6-7-7. D) Animals conditioned using a HI protocol, SB was administered 5min before retrieval, t-Student n=8-5. E) Animals conditioned using the MI protocol, the animals were treated with 6-I-NC, another TRPV1 blocker, t-Student n=12-10. F) Twenty-weeks-old animals conditioned using a LI protocol, SB was administered 5min before retrieval, one-way ANOVA followed by Bonferroni post-hoc n=11-8-9-8. *p<0.05 compare to control

Involvement of endocannabinoid signalling in TRPV1 modulation of fear memory

In order to determine the relationship between TRPV1 and AEA/CB_1 signalling, we first evaluated the pattern of expression of CB_1 and TRPV1 in the dHPC. As it can be observed in Fig.3, TRPV1 and CB_1 are colocalized in all the three regions of the dHPC; CA1, CA3 and DG. Later, we quantified the levels of AEA released in the dHPC immediately after the retrieval (Fig. 4A). The animals were conditioned using LI, MI or HI, or NC (the animals were exposed to the context but not submitted to the footshock) ($F_{3,15} = 20.36$, $p<0.0001$, Fig. 4B). The HPC was dissected immediately after the test. The levels of AEA correlated with the levels of freezing (Pearson r=0.4707, R=0.2216, p=0.0486, Fig. 4C), in contrast, 2-AG levels remained unaltered (Pearson r=-0.1618, R=0.02617, p=0.5213, Fig. 4C). Finally, we studied the involvement of CB_1 receptors in SB effect. The animals were conditioned using the MI and pre-treated before the test with a subeffective dose of AM251, a CB_1 antagonist, followed by the treatment with 3nmol of SB (Fig. 4D). As expected, the group treated with SB displayed lower levels of freezing as compared to the control group. AM251 by itself did no interfere with freezing. Finally, the pre-treatment with AM251 prevented the retrieval impairments induced by SB (Pre-treatment: $F_{1,28} = 0.3097$, p=0.5823. Treatment: $F_{1,28} = 4.367$, p=0.0458. Interaction: $F_{1,28} = 1.564$, p=0.2214, Fig. 4E). This suggests that CB_1 is mediating the effects of TRPV1 blocking.

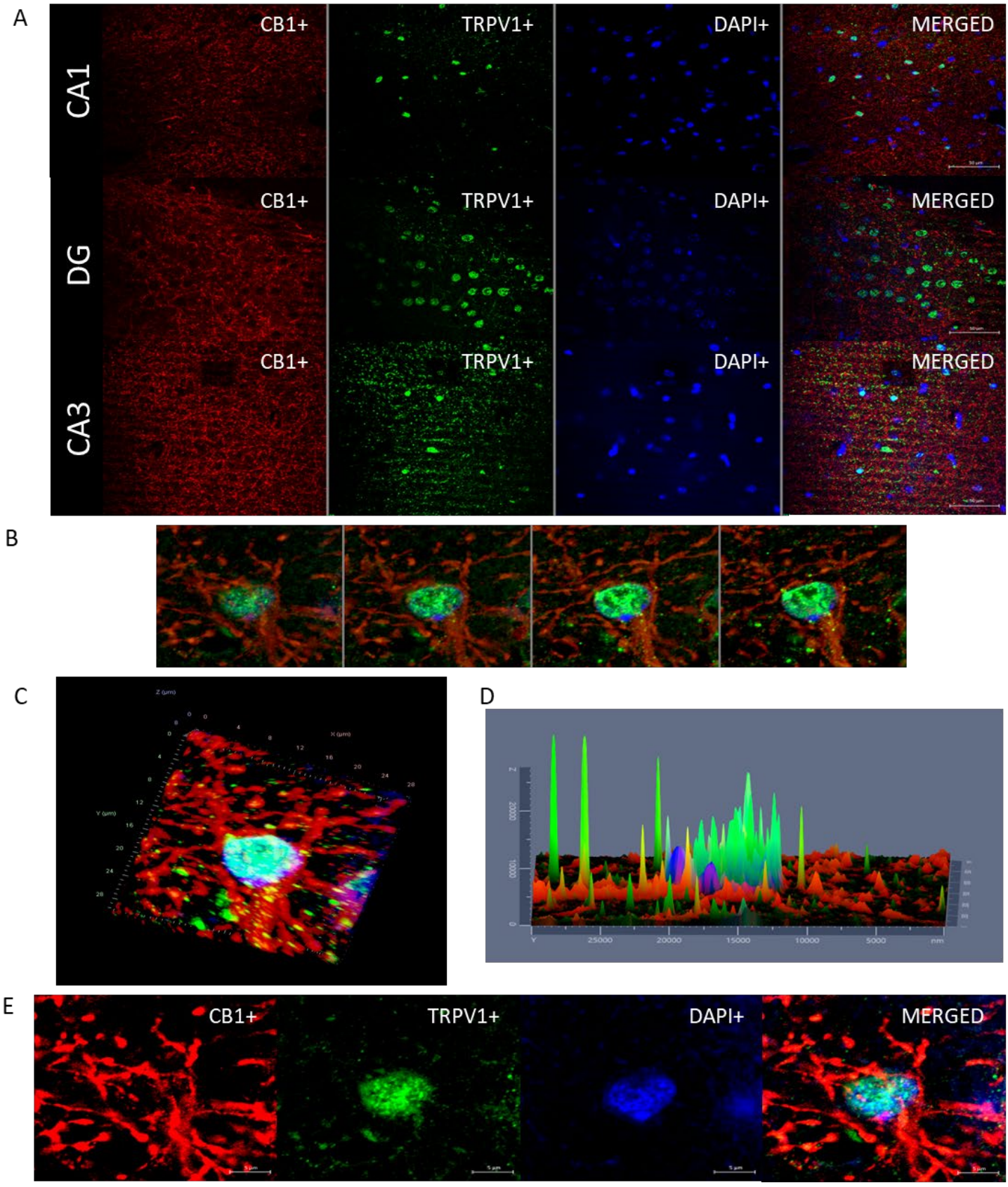

Figure 3. Co-expression of TRPV1 channels, CB1 receptors and DAPI in the dorsal hippocampus as revealed by triple immunofluorescence staining. A) The photomicrographs show representative images of CB1 (secondary antibody Alexa 594; first column), TRPV1 (secondary antibody Alexa 488; second column, DAPI (third column) and triple stained (composite images; fourth column) positive cells in the CA1, dentate gyrus (DG) and CA3 subregions, scale bar 50um. B) Z-stack planes of 1 um of a CA1 TRPV1+/CB1+/DAPI+ cell obtained using a x68 objective and airyscan. C) 3D image obtained from the merge of the z-stack planes (B). D) 2.5D image of one plane (1um) of the z-stack (E). E) Photomicrographs of the separate channels of one z-stack (B) plane (1 um), scale bar 5um.

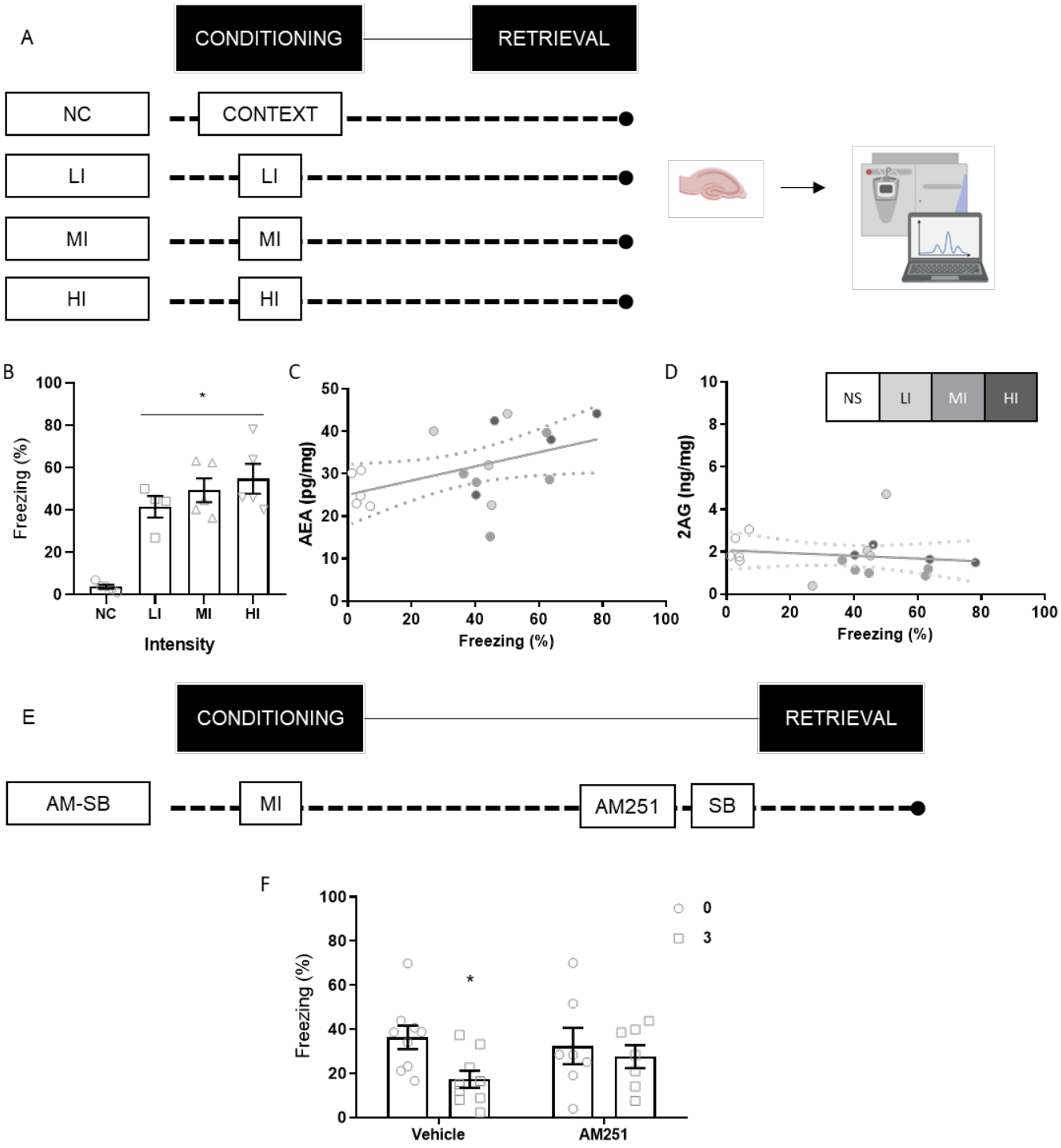

Figure 4: The involvement of AEA and CB_1 in TRPV1 modulation of contextual fear conditioning. A) Experimental Design B and C. B) Freezing levels from animals conditioned with different intensities, one-way analysis of variance followed by Bonferroni post-hoc n=5-4-5-5. C) AEA levels in the HPC in relation to freezing n=18. D) 2-AG levels in the HPC in relation to freezing n=18. E) Experimental Design of F. F) Freezing levels of animals submitted to the CFC test (MI) and pre-treated with 75pmol of AM251 before administration of 3nmol of SB 5min before retrieval, two-way ANOVA followed by Bonferroni's post-hoc n=9-9-7-7. *p<0.05 compare to control.

Characterization of TRPV1 channels in dHPC-dependent memories

Since the dHPC is involved in processing and stored contextual memories not only related to aversive stimulus, but also to appetitive ones and not conditioned contextual memory, we investigated the involvement of hippocampal TRPV1 channels in the cocaine-induced CPP (Fig. 5A) and NOR task (Fig. 5C).

As it can be observed in Fig. 5B, the TRPV1 blocker failed to induce any effect in the retrieval of appetitive memories, the animals conditioned with cocaine displayed a preference by the compartment paired with the drug but the treatment with SB before the test was no able to reduce this preference (Drug: $F_{1,39} = 3.96$, $p=0.053$. Treatment: $F_{1,39} = 0.5195$, $p=0.4754$. Interaction: $F_{1,39} = 0.09645$, $p=0.7578$, Fig. 5B). Similarly, SB did not impair the recognition of the novel object in the NOR test ($t=0.9311$, $df=12$, $p=0.3702$, Fig. 5D). Our results suggested that, at least in our experimental conditions, hippocampal TRPV1 channels are not involved in the retrieval of these types of memory.

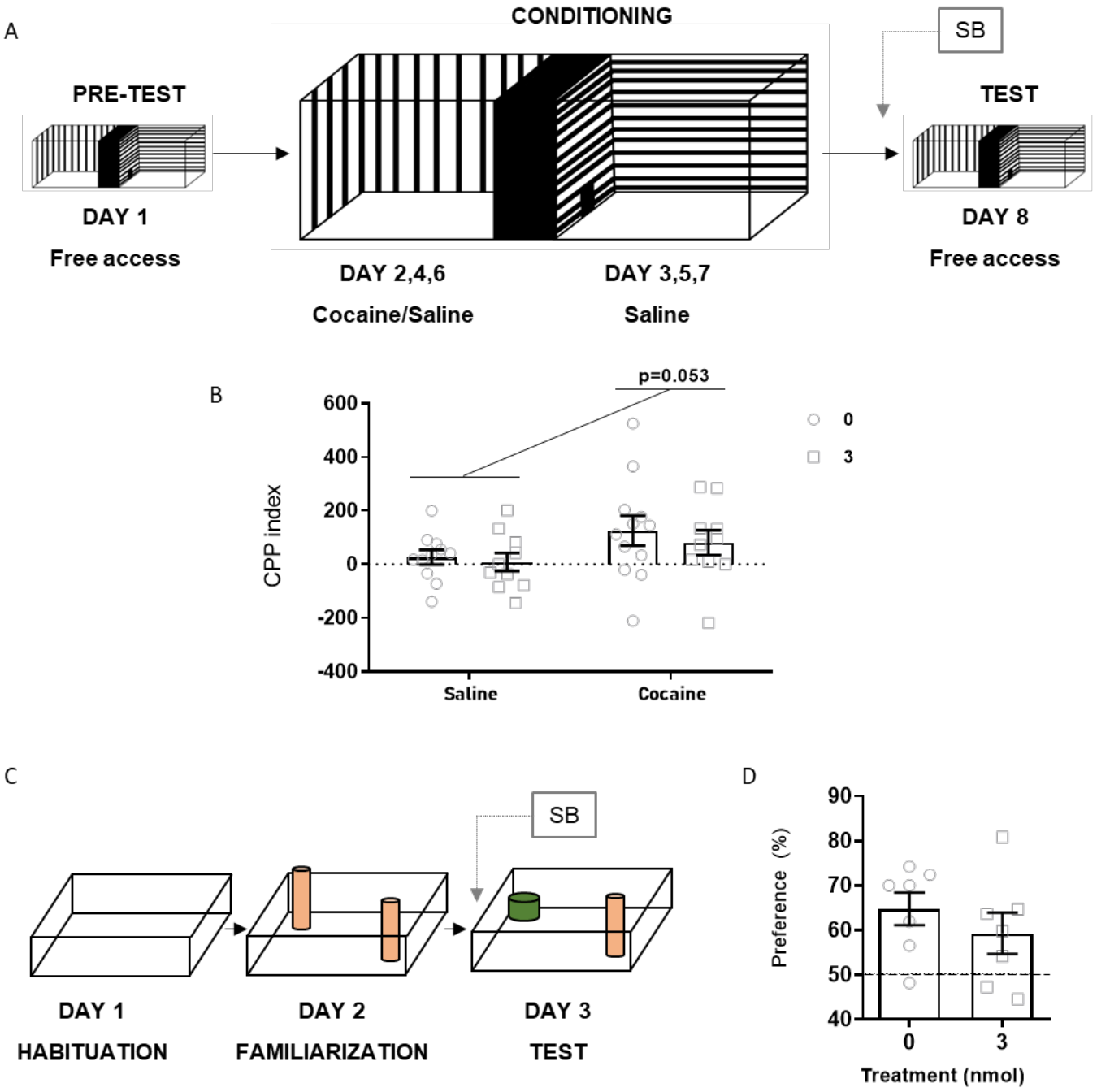

Figure 5: Characterization of TRPV1 channels in the retrieval of dHPC-dependent memories. A) Experimental Design of CPP. B) CPP index of animals submitted to the cocaine-induced CPP, two-way ANOVA followed by Bonferroni post-hoc n=11-10-12-10. C) Experimental Design of NOR. D) Preference index of animals submitted to the NOR task, t-student test n=7-7.

Characterization of TRPV1 channels in different phases of the contextual fear conditioning

First, we wonder if 24h after the acute treatment the animals will still display impairments in retrieval (MI). Thus, animals conditioned with the MI and treated before the test were submitted to a second drug-free test session 24h after the first one. As we can observed in Fig. 6B, the animals treated with the blocker did not present an increase in freezing levels when re-tested 24h after the acute effect of the drug (Treatment: $F_{1,28} = 15.42$, $p=0.0005$. Time $F_{1,28} = 1.479$, $p=0.2340$. Interaction: $F_{1,28} = 5.911$, $p=0.0217$, Fig. 6B).

Then, we investigated the involvement of hippocampal TRPV1 channels in the acquisition, consolidation and reactivation of contextual fear memory (MI). The drug was administered 5min before the acquisition of fear memory (acquisition), immediately after (early-consolidation) or immediately after a brief reactivation (reactivation). The TRPV1 blocker was not able to induced a reduction in freezing during the test when the drug was administered in a phase different than retrieval: acquisition ($t=0.3680$, $df=26$, $p= 0.103$, Fig. 6C), consolidation ($t=1.264$ $df=16$, $p= 0.2243$, Fig. 6D) and reactivation (Treatment: $F_{1,28} = 0.2796$, $p=0.6012$. Time $F_{1,28} = 3.347$, $p=0.0780$. Interaction: $F_{1,28} = 0.001658$, $p=0.9678$, Fig. 6E).

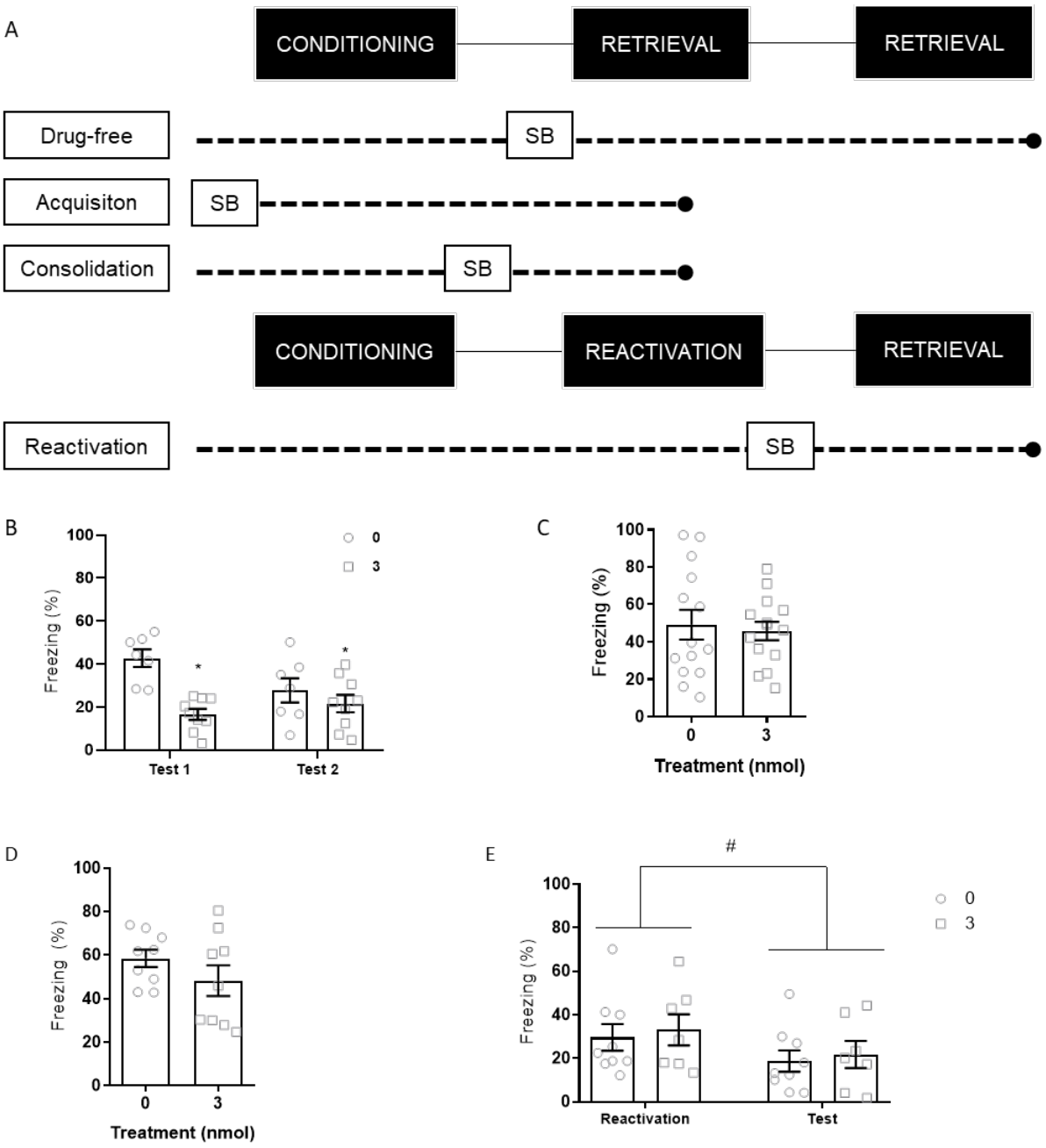

Figure 6: Involvement of HPC TRPV1 channels in different phases of the CFC. A) Experimental design. B) Freezing levels of animals submitted to the CFC test (MI) and treated with 3nmol of SB 5min before retrieval and re-tested 24h later, two-way ANOVA followed by Bonferroni post-hoc n=7-9-7-9. C) SB 3nmol or vehicle was administered into the dHPC 5min before acquisition, t-Student n=14-14. D) SB 3nmol or vehicle was administered into the dHPC immediately after acquisition, t-Student n=9-9. E) SB 3nmol or vehicle was administered into the dHPC immediately after 3min memory reactivation and the animals were tested 24h later, two-way ANOVA followed by Bonferroni post-hoc n=9-7-9-7. * differences compare to control group test 1 0nmol, # differences between the same treatment in different groups.

Molecular pathways engaged by TRPV1 blockers

We investigated plasticity pathways potentially triggered by hippocampal administration of SB before retrieval (MI), Fig. 7A. We focused on BDNF, TrkB, Arc and Zif. The HPC was collected 30min after different phases, processed and TrkB, Arc, Zif and TRPV1 mRNA levels were assessed by PCR, BDNF levels by ELISA.

First, we compared the levels of these targets after acquisition and retrieval in control animals. We did not observe differences between these memory phases regarding TRPV1 ($t=1.178$, $df=10$, $p=0.266$, Fig. 7B), Arc ($t=0.2572$, $df=10$, $p=0.8023$, Fig. 7C), Trkb levels ($t=2.055$, $df=9$, $p=0.0700$, Fig. 7E) or BDNF ($t=1.116$, $df=10$, $p=0.2904$, Fig. 7F). However, Zif levels were decreased after the retrieval ($t=3.630$, $df=9$, $p=0.0055$, Fig. 7D).

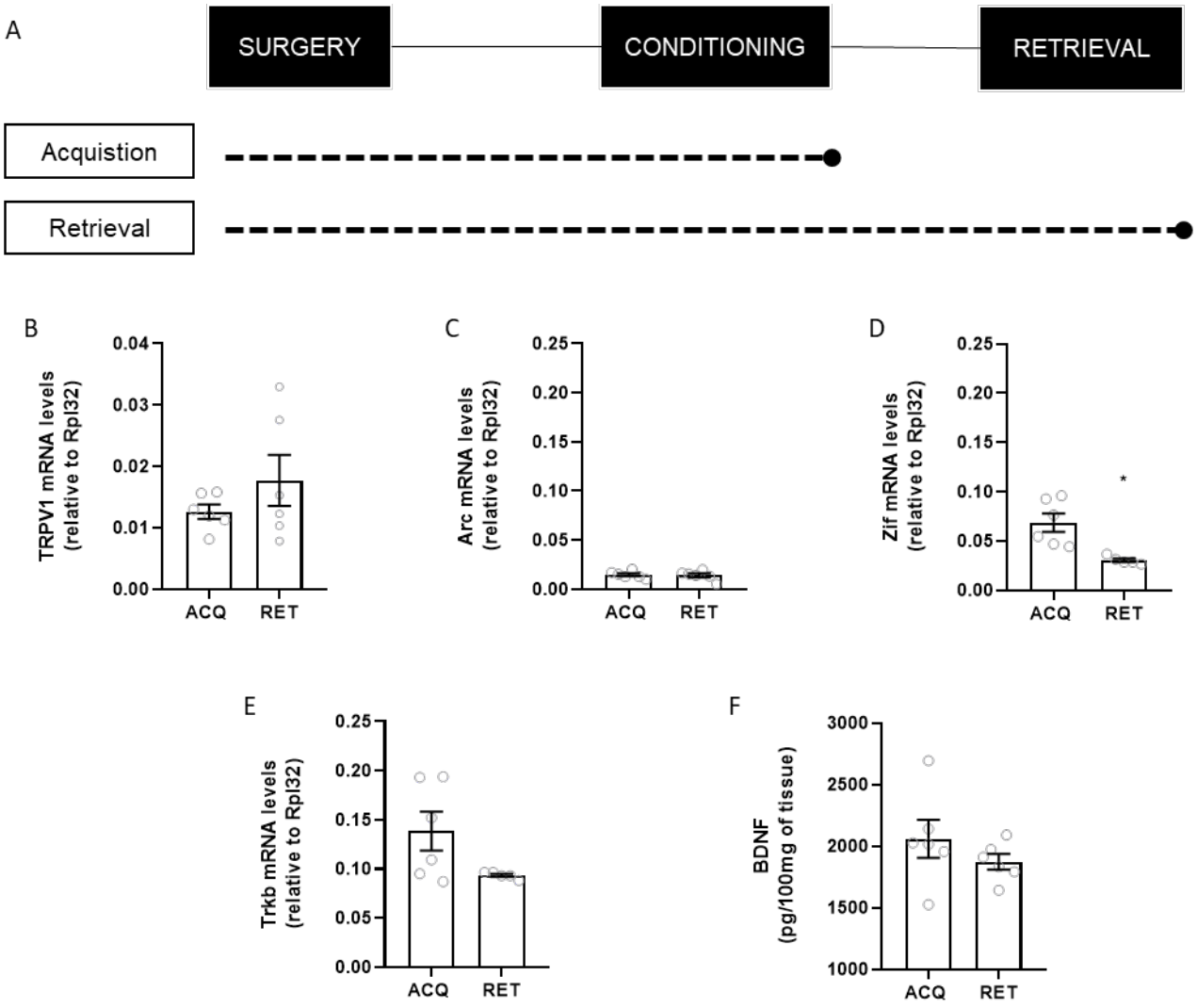

Figure 7: Early genes and neurotrophic signaling in the HPC 30min after acquisition or retrieval in animals conditioned with MI. A) Experimental Design. B) TRPV1 mRNA levels, t-Student n=6-6. C) Arc mRNA levels, t-Student n=6-6. D) Zif mRNA levels, t-Student n=6-5. E) Trkb mRNA levels, t-Student n=6-5. F) BDNF levels, t-Student n=6-6. *p<0.05 compare to control.

Later, we investigated if the stereotaxic surgery and the intrahippocampal drug administration would induce changes in the levels of these factors or in the behavioural responses, hence considered as confounding factors Fig. 8A. We did not find differences between groups in any of the parameters evaluated: freezing levels ($t=0.1246$, $df=9$, $p=0.9036$, Fig. 8B), TRPV1 ($t=0.06133$, $df=9$, $p=0.952$, Fig. 8C), Arc ($t=0.6375$, $df=9$, $p=0.5397$, Fig. 8D), Zif ($t=1.367$, $df=7$, $p=0.2139$, Fig. 8E), Trkb ($t=0.9955$, $df=8$, $p=0.3486$, Fig. 8F) and BDNF ($t=0.816$, $df=9$, $p=0.3958$, Fig. 8G).

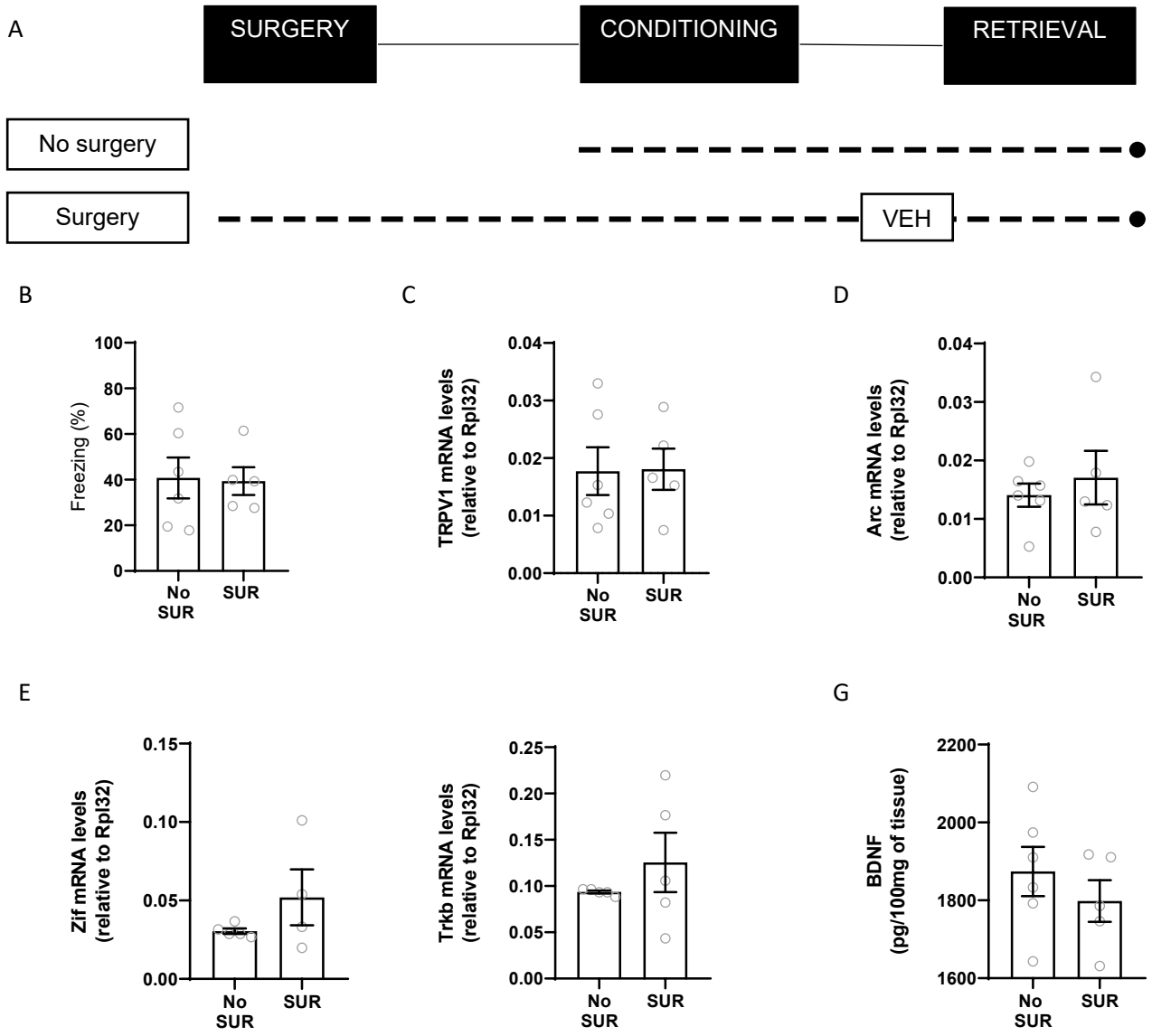

Figure 8: Early genes and neurotrophic signaling 30min after retrieval in the HPC in animals conditioned with MI and submitted (SUR) or not (No SUR) to surgery + intra-HPC administration. **A)** Freezing levels of animals with and without surgery t-Student n=6-5. **B)** TRPV1 mRNA levels, t-Student n=6-5. **C)** Arc mRNA levels, t-Student n=6-5. **D)** Zif mRNA levels, t-Student n=5-4. **E)** Trkb mRNA levels, t-Student n=5-5. **F)** BDNF levels, t-Student n=6-5. *p<0.05 compare to control.

Thereafter, we compared the levels of Trpv1, Arc, Zif, Trkb, and BDNF 30min and 24h after the test in animals conditioned with the MI and treated with vehicle or SB, Fig. 9A. The tissue was dissected 30min after the test1 or immediately after the test 2 (24h after treatment). We evaluated TRPV1 mRNA levels (Treatment: $F_{1,17} = 0.1842$, $p=0.6732$. Time: $F_{1,17} = 1.438$, $p=0.2469$. Interaction: $F_{1,17} = 0.005418$, $p=0.9422$, Fig. 9B). Arc mRNA levels (Treatment: $F_{1,19} = 25.9$, $p<0.0001$. Time: $F_{1,19} = 33.33$, $p<0.0001$. Interaction: $F_{1,19} = 24.77$, $p<0.0001$, Fig. 9C), Zif mRNA level (Treatment: $F_{1,16} = 87.48$, $p<0.0001$. Time: $F_{1,16} = 147.5$, $p<0.0001$. Interaction: $F_{1,16} = 89.43$, $p<0.0001$, Fig. 9D), Trkb mRNA levels (Treatment: $F_{1,18} = 5.836$, $p=0.0265$. Time: $F_{1,18} = 23.81$, $p=0.0001$. Interaction: $F_{1,18} =7.946$, $p=0.0114$, Fig. 9E) and BDNF levels (Treatment: $F_{1,19} = 8.946$ $p=0.0075$. Time: $F_{1,19} = 8.035$, $p=0.0106$. Interaction: $F_{1,19} = 1.059$, $p=0.3164$, Fig. 9F).

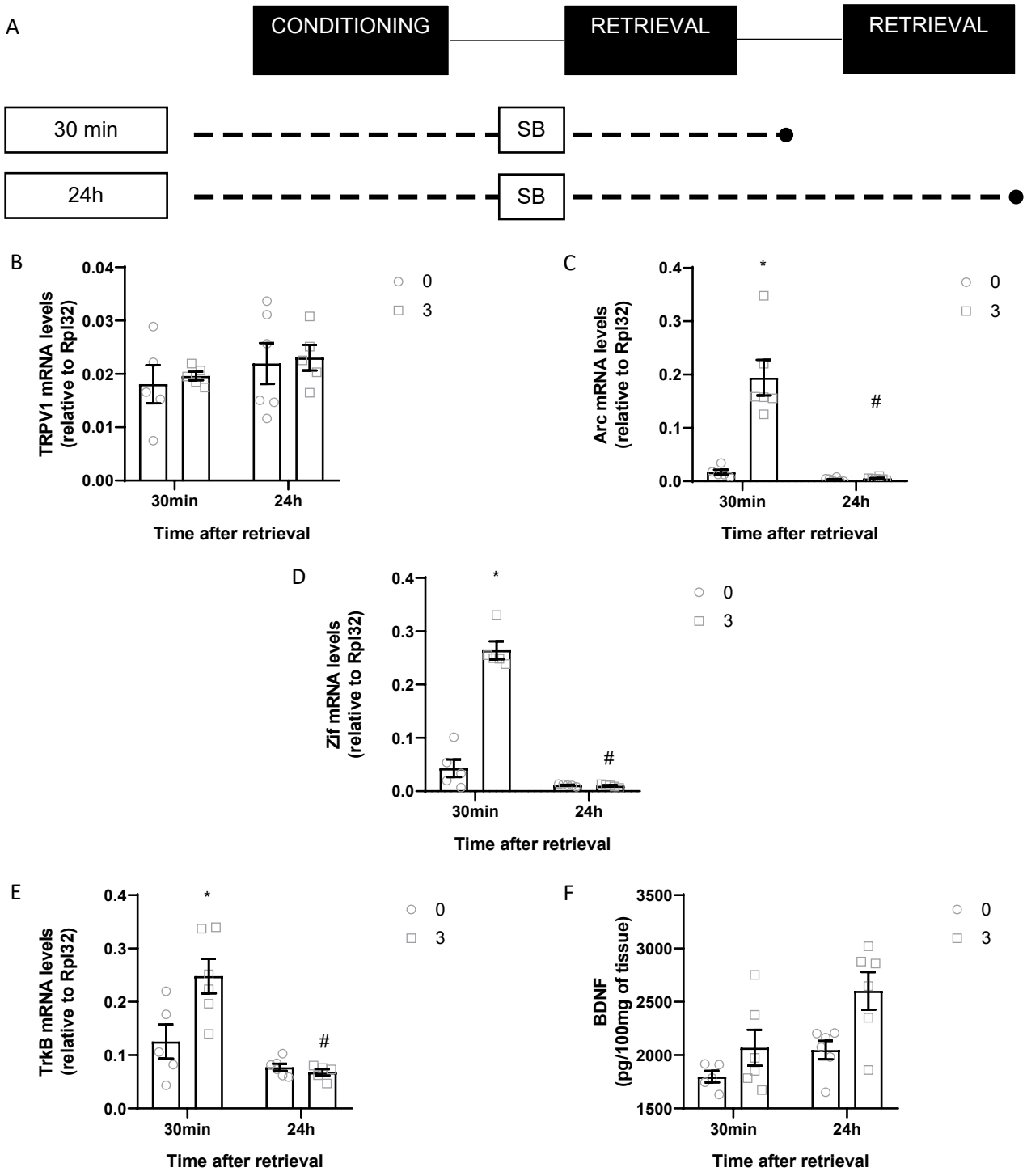

Figure 9: Early genes and neurotrophic signalling in the HPC 30min or 24h after the test in animals conditioned with the MI and treated with SB 3nmol or vehicle, two-way ANOVA followed by Bonferroni post-hoc. A) Experimental Design. B) TRPV1 mRNA levels n=5-5-6-5. C) Arc mRNA levels n=5-6-6-6. D) Zif mRNA levels n=5-5-5-5. E) Trkb mRNA levels n=5-6-6-5. F) BDNF levels n=5-6-6-6. * differences inside the group, # differences between the same treatment in different groups.

Our results showed that intensity seems a key factor determining the recruitment of TRPV1 in the retrieval of fear memory. In this sense, SB impaired memory retrieval in animals conditioned with MI and HI but has no effect on freezing in animals conditioned with LI. However, remains unknown if intensity may bias the molecular pathways recruited by SB. In order to address this question, we investigated the levels of early genes and neurotrophic signalling after the retrieval of fear memory in animals treated with SB or vehicle and conditioned with the MI or the HI. In addition, we also evaluated the levels of these factors in a group that was exposed to the context but not to the footshock, NC (Fig. 10A). The HPC was collected 30min after the test.

As expected, the freezing levels were lower in the group treated with SB in animals conditioned with MI and HI, no effect of the treatment was observed in the NC group (Treatment: $F_{1,32} = 18.18$, $p=0.0001$. Intensity: $F_{2,32} = 24.49$, $p<0.0001$. Interaction: $F_{2, 32} = 10.95$, $p=0.0002$, Fig. 10B). Trpv1 mRNA levels were not modulated by the treatment or the intensity (Treatment: $F_{1,26} = 2.978$, $p=0.0963$. Intensity: $F_{2,26} = 1.254$, $p=0.3021$. Interaction: $F_{2,26} = 0.6268$, $p=0.5422$, Fig. 10C). However, Arc mRNA levels were increased by the treatment in the MI but not in the NC group or in the HI group (Treatment: $F_{1,27} = 23.15$, $p<0.0001$. Intensity: $F_{2,27} = 8.182$, $p=0.0017$. Interaction: $F_{2,27} = 11.50$, $p=0.0002$, Fig. 10D), the same pattern was observed regarding Trkb mRNA levels (Treatment: $F_{1,27} = 5.550$, $p=0.0260$. Intensity: $F_{2,27} = 4.311$, $p=0.0237$. Interaction: $F_{2,27} = 3.480$, $p=0.0452$, Fig. 10F). However, Zif mRNA levels were increased by the treatment in the NC and the MI group but not in the HI group (Treatment: $F_{1,25} = 60.53$, $p<0.0001$. Intensity: $F_{2,25} = 11.30$, $p=0.0003$. Interaction: $F_{2,25} = 11.34$, $p=0.0003$, Fig. 10E). Finally, BDNF levels were moderated by intensity (Treatment: $F_{1,27} = 1.877$, $p=0.1820$. Intensity: $F_{2,27} = 6.823$, $p=0.0040$, Interaction: $F_{2,27} = 0.7236$, $p=0.4942$, Fig. 10G).

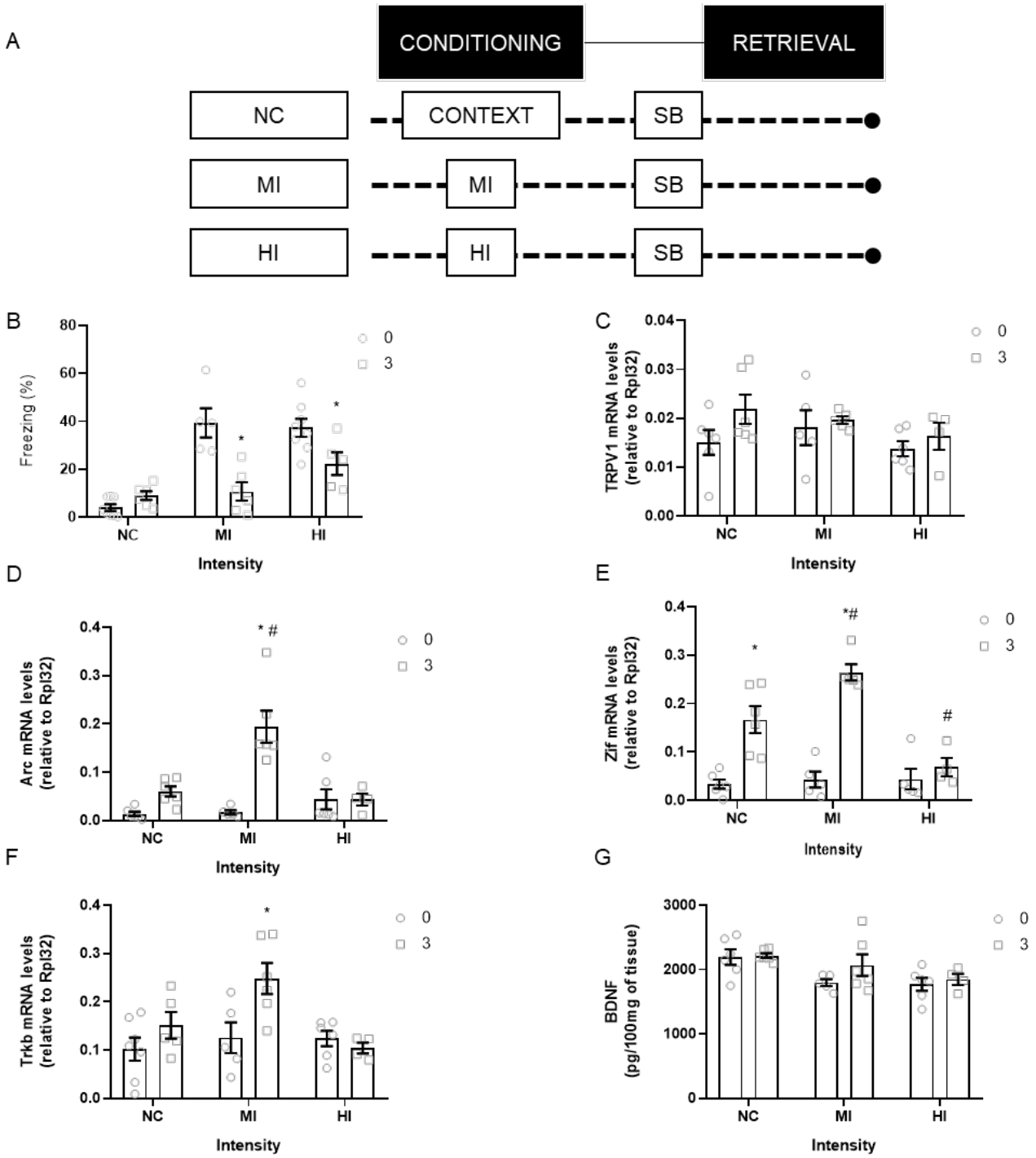

Figure 10: Early genes and neurotrophic signalling 30min after retrieval in animals treated with 3nmol of SB or vehicle and conditioned with the MI, HI or NC, two-way ANOVA followed by Bonferroni post-hoc. A) Experimental Design. B) Freezing levels of animal treated with SB or vehicle before retrieval n=8-6-5-6-8-5. C) Trpv1 mRNA levels n=6-6-5-5-6-4. D) Arc mRNA levels n=6-6-5-6-6-4. E) Zif mRNA levels n=6-6-5-5-5-4. F) Trkb mRNA levels n=7-5-5-6-6-4. G) BDNF n=6-6-5-6-6-4. * differences inside the group, # differences between the same treatment conditions in different groups.

Intensity modulates the long-term effects of SB366791

Our results suggested that intensity may determine whether blocking TRPV1 channels is going to recruit plasticity pathways after memory retrieval. Since, Arc, Zif, BDNF and Trkb are involved in synapse remodelling and transcription regulation which in turn may lead to modifications in memory, we investigated if intensity may influence the long-term effects of the treatment. In order to address this question, we investigated the extinction and reinstatement of animals conditioned with MI or HI and treated with the blocker before the retrieval of memory.

First of all, we assessed the freezing levels induced by the intensity proposed for the reinstatement session, since it should not be sufficient to induce high levels of freezing by itself. We also evaluated if one extinction session was sufficient to decrease freezing levels in animals conditioned with the MI or the HI. As it can be observed in Fig. 11, the levels of freezing displayed by animals conditioned with the intensity proposed for the reinstatement session are lower than those observed in animals conditioned with the other intensities ($F_{2,15} = 31.23$, $p<0.0001$, Fig. 11B). Moreover, after the extinction session, test 2, the animals from both groups presented lower levels of freezing when compared to those observed before extinction, test 1 (Test: $F_{1,20} = 82.06$, $p<0.0001$. Intensity: $F_{1,20} = 8.064$, $p=0.0101$. Interaction: $F_{1,20} = 2.890$, $p=0.1047$, Fig. 11C). During the test 2 the freezing levels were uniformly distributed along time in both groups (Time: $F_{4,40} = 0.3836$, $p=0.8191$. Intensity: $F_{1,10} = 1.147$, $p=0.3094$. Interaction: $F_{4,40} = 1.054$, $p=0.3920$, Fig. 11D).

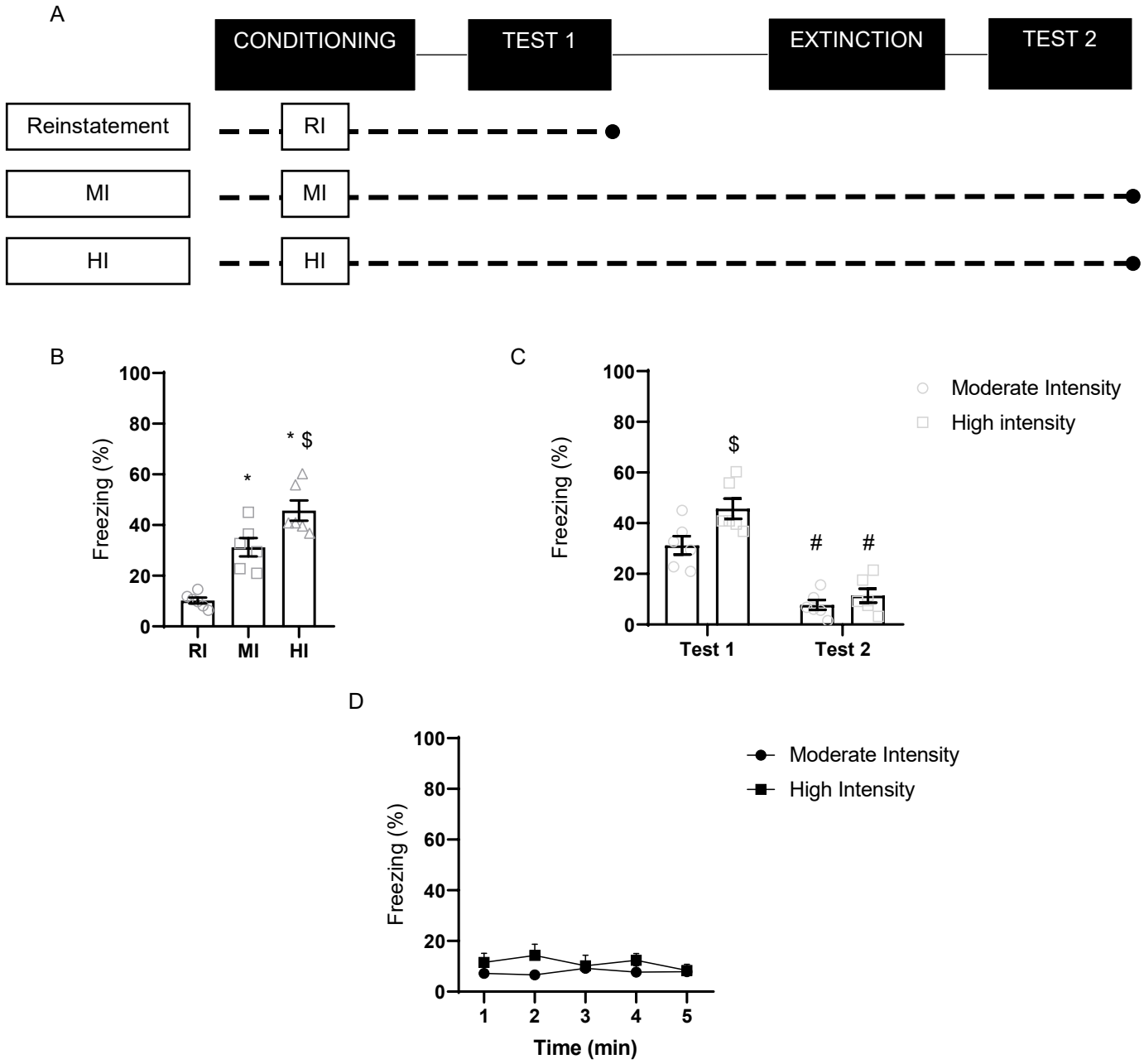

Figure 11: Evaluation of the protocol proposed to study the long-term effects of SB. A) Experimental Design. B) Freezing levels during the test 1 in animals conditioned with the intensity of the reinstatement protocol, the MI or the HI protocol, one-way ANOVA followed by Bonferroni post-hoc n=6-6-6. C) Freezing levels during the test 1 and 2 (after extinction) of animals conditioned with the MI or HI, two-way ANOVA followed by Bonferroni post-hoc n=6-6-6-6. D) Freezing levels by time during the test 2 in animals conditioned with the moderate intensity or the high intensity protocol two-way ANOVA followed by Bonferroni post-hoc n=6-6-6-6-6. *p<0.05 compare to NC, $p<0.05 compare to MI, #p<0.05 between the same intensity in different tests.

Finally, we evaluated the effect of reinstatement in animals conditioned with the MI or the HI and treated with SB before the retrieval. As it can be observed in Fig. 12, in the first experiment, in which the animals were conditioned with the MI, the treatment was able to decrease freezing levels in the retrieval phase. In addition, after the extinction, no differences were observed between vehicle and SB group, but only the animals treated with the TRPV1 blocker were resistant to memory reinstatement (Treatment: $F_{1,29} = 23.40$, $p>0.0001$. Time: $F_{2,29} = 11.41$, $p=0.0002$. Interaction: $F_{2,29} = 2.888$, $p=0.0718$, Fig. 12B). On the other hand, in the second experiment, where the animals were conditioned using the HI, the blocker was able to reduce freezing levels in the retrieval, test 1, and both groups, vehicle and SB-treated animals were able to extinct the memory, however, after the reinstatement no differences were observed between the vehicle and the treated group (Treatment: $F_{1,27} = 25.94$, $p<0.0001$. Time: $F_{2,27} = 18.96$, $p<0.0001$. Interaction: $F_{2,27} = 1.574$, $p=0.2256$, Fig. 12C). Moreover, when analysing for a potential interaction between intensity of conditioning and response to the reinstatement shock, it was a tendency in the MI group (Treatment: $F_{1,9} = 4.622$, $p=0.0600$. Time: $F_{3,27} = 4.371$, $p=0.0449$. Interaction: $F_{3,27} = 2.509$, $p=0.0801$, Fig. 12D). This was statistically significant in the group conditioned with the HI protocol (Treatment: $F_{1,9} = 8.249$, $p=0.0184$. Time: $F_{3, 27} = 18.57$, $p<0.0001$. Interaction: $F_{3,27} = 2.745$, $p=0.0625$, Fig. 12E).

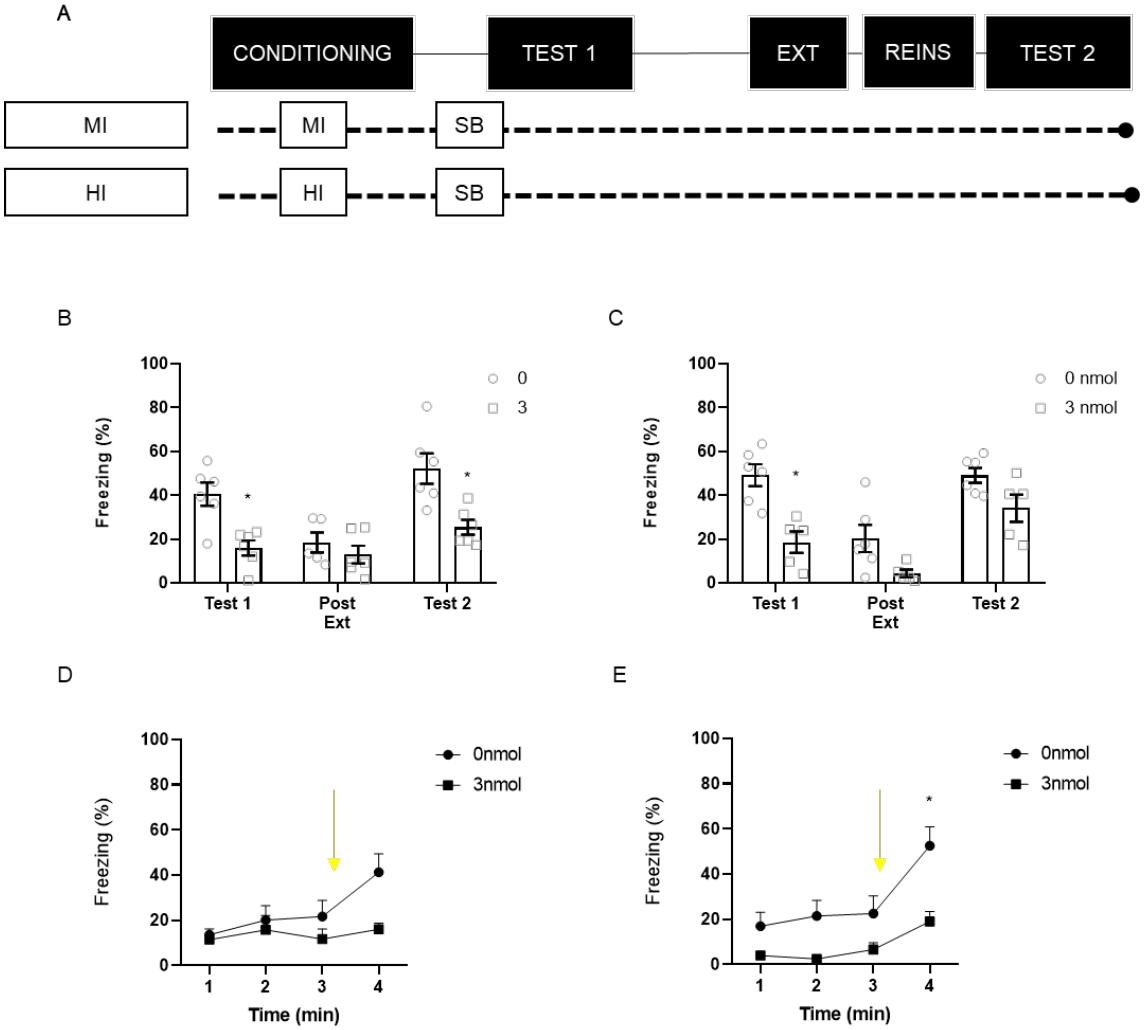

Figure 12: Long-term effects of SB treatment in the CFC. A) Experimental design. B) Freezing of animals conditioned with MI, two-way ANOVA followed by Bonferroni post-hoc n=6-6-5-6-6-6. C) Freezing of animals conditioned with HI, two-way ANOVA followed by Bonferroni post-hoc n=6-5-6-5-6-5. D) Freezing along time from animals conditioned with the MI, two-way ANOVA followed by Bonferroni post-hoc n=5-6-5-6-5-6-5-6. E) Freezing along time from animals conditioned with the HI, two-way ANOVA followed by Bonferroni post-hoc n=6-5-6-5-6-5-6-5. *p<0.05 compare to control. Yellow arrow represents the time of the shock.

Discussion

In this work we characterized the involvement of hippocampal TRPV1 channels in contextual fear memory. First, using the CFC protocol, we observed that local administration of TRPV1 blockers into the dHPC impaired fear memory retrieval in an intensity-dependent manner. Second, the present results show co-localization between TRPV1 and CB_1 in the dHPC, a positive correlation between behaviour and the local levels of AEA after exposure to the conditioned context, and evidence that the actions of TRPV1 blockers in the dHPC depend on CB_1. Overall, this part of the study implicates endocannabinoid signalling in the protective effects of TRPV1 blockers. Third, we determine that the involvement of this channel is modulated by the memory phase and type. Hence, we identify that TRPV1 blockers are not able to impair acquisition or consolidation of fear memory. Likewise, we assessed the effect of this drug in the retrieval of cocaine-induced CPP and the NOR task, showing no role of hippocampal TRPV1 in memory associated with drugs of abuse neither in HPC-dependent not-conditioned memory. Finally, by PCR and ELISA we demonstrated that the administration of the TRPV1 blocker before memory retrieval enhanced the expression of plasticity factors and confers long-term protection against the reinstatement of fear memory.

Previous research found that AA-5-HT, a dual TRPV1 and FAAH blocker, was able to impair retrieval in the CFC, an effect mediated by CB_1-signalling facilitation in the dHPC (GOBIRA et al., 2017b). Using the same intensity of footshock to promote CFC, we treated the animals with SB, a selective TRPV1 blocker, infused directly into the dHPC before retrieval. Our results suggest that, at least in this intensity of conditioning, blocking TRPV1 channels without increasing AEA levels was not enough to impaire memory retrieval. This is in contrast with early reports indicating that knock out animals for this channel presented decreased freezing levels in the CFC (MARSCH et al., 2007). However, TRPV1 blockers effect may depend on stimulus intensity (GENRO; DE OLIVEIRA ALVARES; QUILLFELDT, 2012; TERZIAN et al., 2014) and a similar pattern seems to underly CB_1 recruitment during retrieval (MIZUNO; MATSUDA, 2021). Based on these findings we repeated our experiment increasing the aversiveness of the conditioning; in this case we observed that all the three doses, 1, 3 and 10 nmol, impaired retrieval. Moreover, to further explore the intensity-dependency of TRPV1 blockers effect, we used a third

conditioning protocol with a higher intensity; the animals were infused into the dHPC with 3nmol of SB before the test and, again, we observed an impairment in retrieval. In order to confirm that these effects relayed on TRPV1 blocking, we repeated the experiment (MI) using 6-I-NC, another selective and potent inhibitor of the channel and we observed the same effect. Lastly, we repeated the LI experiment using aged animals which were related with lower levels of TRPV1 channels in the HPC (HUANG et al., 2014), surprisingly, in this case the TRPV1 blocker impaired fear memory retrieval.

Afterwards, we wondered which mechanism underly the intensity-dependent recruitment of TRPV1. The selective TRPV1 blocker was ineffective when the animals were exposed to the same shock intensity in which a dual TRPV1 and FAAH blocker impaired CFC, suggesting that, under this protocol, local levels of AEA may not be sufficient to significantly activate TRPV1. Indeed, previous studies established that AEA is increased in certain fear-related structures after the exposition to the CS (MARSICANO et al., 2002b; OLANGO et al., 2012). In particular, after retrieval, AEA is enhanced in CA1 (SEGEV et al., 2018) while, after training, AEA levels in the HPC depend on shock intensity (MORENA et al., 2014). In parallel, some authors already hypothesized that, given the on demand nature of eCB synthesis and the different affinity for TRPV1 and CB_1, lower concentrations of AEA would recruit CB_1 while higher concentrations would activate both CB_1 and TRPV1 (MOREIRA et al., 2012; MOREIRA; WOTJAK, 2010). Based on this theoretic framework and experimental findings, we hypothesized that the intensity-dependent recruitment of TRPV1 would be determined by the intensity-dependent release of AEA. To test our hypothesis, first we confirmed the co-localization of TRPV1 and CB_1 in dHPC synapses, using double immunofluorescence. These findings are in agreement with previous studies showing CB_1-TRPV1 co-localization in several brain regions (CASAROTTO et al., 2012; CRISTINO; PETROCELLIS; PRYCE, 2006; FOGAÇA et al., 2012). Then, using HPLC-MS, we observed that freezing corelates with the levels of hippocampal AEA, but not 2-AG. Finally, taking together the raise in local AEA and the co-localization of TRPV1 and CB_1, we proposed that the anti-aversive effects of TRPV1 blockers would occur by favouring the CB_1-mediated actions of anandamide, which in turn will decrease excitatory neurotransmission. Therefore, we pre-treated the animals with the CB_1 antagonist AM251 infused directly into the dHPC. Supporting

our hypothesis, AM251 prevented the impairment in retrieval induced by local blocking of TRPV1. These results are in consonance with previous reports suggesting that AEA enhancement is able to impair fear retrieval dependent on CB_1 in glutamatergic neurons (LLORENTE-BERZAL et al., 2015) probably related with the modulation of LTP but not to depolarization induced suppression of inhibition or excitation (ZIMMERMANN et al., 2019). Interestingly, CB_1 agonism by 2-AG enhancements was related to an increased in retrieval through the modulation of gabaergic neurotransmission (LLORENTE-BERZAL et al., 2015). The proposed mechanisms underlying our hypothesis is illustrated in Figure 13.

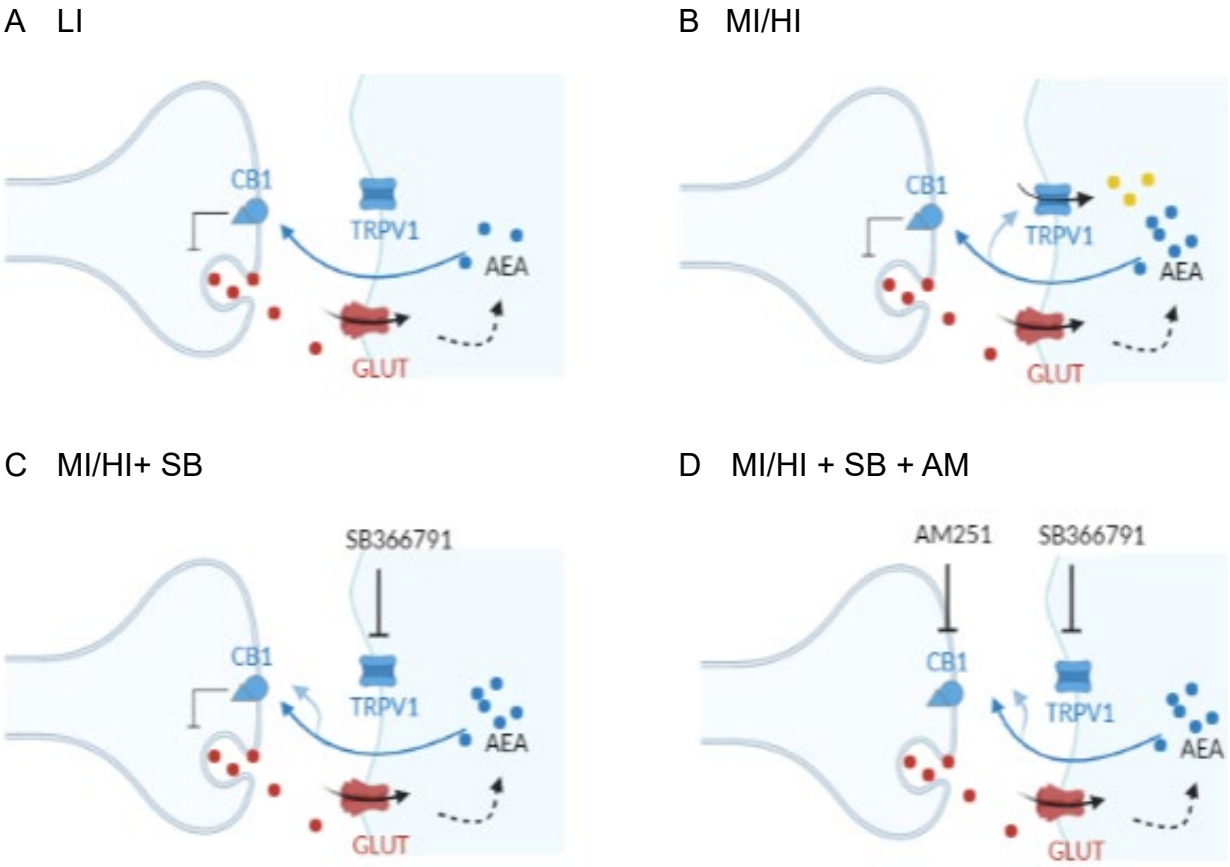

Figure 13: The AEA/CB_1/TRPV1 interplay in the modulation of contextual fear memory in the HPC. A) When the US has LI, the exposition to the CS during retrieval induces a moderate release of AEA, which activates CB_1 but not TRPV1. B) When there is an increase in the US intensity (MI/HI), more AEA is released in response to the CS activating presynaptic CB_1 receptors but also postsynaptic TRPV1 channels. C) Under MI/HI, SB reduces freezing by preventing AEA effects through TRPV1 and favouring CB_1-signalling. Thus, D) If CB_1 is antagonized, the protective effects of the TRPV1 blockage are abrogated.

Since we had observed that TRPV1 recruitment depends on the intensity of the aversive stimulus, we investigated if it also depends on the nature of the stimulus. We used two memory tests related to dHPC functioning, namely the cocaine-induced CPP (CASTILLA-ORTEGA et al., 2016; HITCHCOCK; LATTAL, 2018; MEYERS et al., 2006), to asses retrieval of associative memories related to appetitive stimulus, and the NOR task (BROADBENT et al., 2010; COHEN; STACKMAN, 2015), to evaluate hippocampal-dependent non-associative memory. The local infusion into the dHPC of the TRPV1 blocker in a dose able to impair the retrieval of fear memory, did not interfere with the animal performance in neither of these tasks. This suggests, that at least in our experimental conditions, the nature of the US and the type of memory influences the recruitment of TRPV1, probably influencing AEA levels. In this sense, Wise, Harloe and Lchtiman (2009) showed that FAAH -/- mice presented deficits in aversive but not appetitive-related spatial memory using a modified Barnes maze task (WISE; HARLOE; LICHTMAN, 2009). Although TRPV1 is involved in other actions of psychostimulants (TIAN et al., 2018), it seems to have no effect in the retrieval of contextual memory associated with them. In relation to the not-conditioned memory, our results extended prior findings. For instance, administration of agonist or blockers of TRPV1 had no effect on retrieval in the NOR task (YOU et al., 2012) and the Morris water maze (AMIRESMAILI; SHAMSIZADEH; ALLAHTAVAKOLI, 2014; LI et al., 2008). In this line, the absence of effect of SB in the NOR is in agreement with the hypothesis that eCB role in memory is subordinated to certain level of aversiveness or stress (MORENA; CAMPOLONGO, 2014).

TRPV1 blockers were previously studied in unconditioned anxiety tests. These tests explore the contradictory motivational states derived from a natural aversion of rodents for certain situations such as high illuminated environments or altitudes and their natural drive for the exploration of new contexts (CAMPOS et al., 2013). The elevated plus-maze or the dark-light box are embraced in this category. Using these tests, it was stablished the capacity of TRPV1 blockers to induce anxiolytic-like effects either when they are systemically administered (FARAGI et al., 2017; KASCKOW; MULCHAHEY; GERACIOTI, 2004; SOCALA; WLA; KATARZYNA, 2016; TERZIAN et al., 2009) or locally administered into the dorsal PAG (BATISTA; FOGAÇA; GUIMARÃES, 2015; CAMPOS; GUIMARÃES, 2009; CARDOZO;

SANTOS; NUNES-DE-SOUZA, 2013; CASAROTTO et al., 2012; TERZIAN et al., 2009), the vHPC (SANTOS; STERN; BERTOGLIO, 2008) or the PFC (AGUIAR et al., 2009). Since some authors presented evidences suggesting that pharmacological manipulations in the dHPC can modulate anxiety like-behaviours (CAMPOS-CARDOSO et al., 2021; REZAYAT et al., 2005; ROOHBAKHSH et al., 2007) and anxiolytic-like behaviours are usually related to the acute effect of the drug, we decided to evaluate the performance of SB-treated animals in a drug-free test. We conditioned the animals with the MI and before the retrieval they received either vehicle or SB infusions into the dHPC, twenty-four hours after the first test the animals were submitted to a second drug-free test. Our results suggested that TRPV1 blockers act interfering with the mnemonic process rather than inducing acute and transitory anxiolytic-like effects or state-dependent learning.

Later, we investigated whether TRPV1 blockers are involved in other memory phases. Using the MI and the 3nmol dose of SB, we found that TRPV1 blockade does not impair memory acquisition or consolidation. To the best of our knowledge, this is the first study addressing the involvement of TRPV1 in CFC acquisition. In line with our result, FAAH blocking has no effect on CFC acquisition (LARICCHIUTA; CENTONZE; PETROSINI, 2013). Regarding consolidation, it was found that a TRPV1 blocker, impaired consolidation in a dose-dependent manner (SCIENZA-MARTIN et al., 2022). Genro et al. (2014) observed that capsazepine injected into the HPC after training induced memory impairments. This effect was restricted only to highly intense protocols (GENRO; DE OLIVEIRA ALVARES; QUILLFELDT, 2012), in agreement with the intensity-dependent post-training levels of AEA (MORENA et al., 2014). Thus, our results may be explained by the intensity-dependent levels of AEA underlying this phase in our experimental conditions. Another possible and complementary explanation for the lack of effect of SB in acquisition and consolidation relays on the own regulation of this channel. As mentioned before, TRPV1 is highly regulated and PKA or PKC can modify the response of TRPV1 to AEA (PETROCELLIS et al., 2001; PREMKUMAR; AHERN, 2000). At the same time PKA and PKC activity increases after training in the CFC (ATKINS et al., 1998). Therefore, it is possible that the enhanced activity of these kinases after conditioning increased the susceptibility of TRPV1 channels to be activated by AEA in the subsequent test, acting such as an emotional priming. However further research is

needed to fully elucidate which mechanism is orchestrating the differences in TRPV1 recruitment observed in this work.

Afterwards, we assessed the effect of SB after a brief reactivation of memory in order to evaluate potential effects in reconsolidation. However, our experimental design was not able to induce reconsolidation as it can be inferred by the levels of freezing of vehicle-treated animals in the subsequent test. Then, our experiment did not overcome the boundary conditions necessary for the inducement of reconsolidation (VAVERKOVÁ et al., 2020), hindering any conclusion about the involvement of this channel in this phase.

Later, we evaluated potential plasticity pathways that may be altered by TRPV1 blockers in the dHPC, particularly IEG and neurotrophic factors. IEG are key factors in gene-environment interactions actin as a fast response modulators of plasticity (DUCLOT; KABBAJ, 2017; GALLO et al., 2018). The transcription of IEG is related with several pathways including BDNF-TRkB, NMDA, MAPK, PKA, phosphoinositide 3-kinase, RhoA-actin, mGluRs and transcription factors (BAHRAMI; DRABLØS, 2016; CARTER; MIFSUD; REUL, 2015; COLE et al., 1989; DUCLOT; KABBAJ, 2017; GALLO et al., 2018; HERDEGEN; LEAH, 1998; PANJA et al., 2014; SHEPHERD; BEAR, 2011; STEWARD; WORLEY, 2001; WALTEREIT et al., 2001). However, some IEG, such as Zif, had a basal expression in several areas including the HPC (CULLINAN et al., 1995; HUGHES; LAWLOR; DRAGUNOW, 1992). Functionally, Zif is related to LTP persistence (ABRAHAM et al., 1993; RICHARDSON et al., 1992) and it modulates the expression of genes related to plasticity and cellular growth (DUCLOT; KABBAJ, 2017; GALLO et al., 2018). Interestingly among all the genes regulated by Zif, it is included Arc, another IEG (LI et al., 2005). Arc shares with Zif several of their functions (DUCLOT; KABBAJ, 2017; SHEPHERD; BEAR, 2011) but it can also be involved in long-term depression (LTD) probably by modulating AMPA endocytosis (WAUNG et al., 2008). Differently from Zif, Arc is exclusively post-synaptic (KOBAYASHI et al., 2005) and expressed only in CaMKII+ glutamatergic neurons (VAZDARJANOVA et al., 2006). On the other hand, BDNF is released by neurons and glia (BRIGADSKI; LESSMANN, 2020) and modulated by GC (BENNETT; LAGOPOULOS, 2014). BDNF acting via TrkB enhances synaptic plasticity and dendritic remodelling through, for example, Arc and MAPK (BENNETT; LAGOPOULOS, 2014; LEAL; BRAMHAM; DUARTE, 2017) been

a key piece in hippocampal synaptic plasticity (LEAL; BRAMHAM; DUARTE, 2017). For instance, BDNF is related to the increase in AMPA trafficking underlying BDNF-dependent LTP (EDELMANN et al., 2015).

First, we observed that, in animals conditioned with the MI and treated with vehicle, there were no differences in the levels of TRPV1, Arc, TrkB mRNA and BDNF between acquisition and retrieval. However, Zif RNA levels were decreased after test when compare to acquisition. More importantly, the levels of these targets did not change when compare animals submitted to surgery with no-surgery. Treatment with the TRPV1 blocker before the test did not influence the mRNA levels of TRPV1 but enhanced the hippocampal levels of Zif, Arc, Trkb mRNA and BDNF quantified 30min after retrieval. Only BDNF levels were enhanced 24h after retrieval. Since TRPV1 blockers seem to modulate these factors in the HPC we wonder if this effect could be dependent on conditioning intensity. We compare the levels of TRPV1, TrkB, Arc, Zif mRNA and BDNF in the HPC 30 min after retrieval in animals treated with SB 3nmol or vehicle and conditioned with MI and HI or exposed to the context without receiving footshocks. We can observe that the increased in plasticity-related factors, TrkB, Arc and BDNF, induced by SB was not observed in animals conditioned with HI or exposed to the context. However, animals exposed to the context and treated with SB presented an increased in Zif.

In the CFC these targets were extensively studied after acquisition and usually, increases in Zif, Arc and BDNF in CFC are crucial for memory consolidation. For instance, BDNF reduction after training in this structure seems to mediated amnesic effects induced by certain interventions (GONZALEZ et al., 2013; HEIN et al., 2007; UWAYA et al., 2016). Similarly, TrkB overexpression enhanced CFC (TAKEI et al., 2011). Regarding Arc, antisense-oligodeoxynucleotides (anti-ODN) infused into the HPC impaired CFC (CZERNIAWSKI et al., 2011). Likewise, anti-ODN against Zif infused into the AMG impaired conditioning but not retrieval (MALKANI et al., 2004) and Zif -/- presented impairments in memory consolidation (BESNARD; CABOCHE; LAROCHE, 2013). However, in our work we did not observed differences in Arc, TrkB or BDNF levels when compare vehicle-treated animals conditioned and not conditioned. This can be related to the phase studied (retrieval) or the fact that the dHPC is involved in encoding contextual information, then even in the absence of the footshock, the animals created a memory of the

context which may underly the equal enhancement of these plasticity factors among groups treated with vehicle.

Surprisingly, blocking TRPV1, a cationic channel, lead to an increase in Zif, Arc, TrkB RNA and BDNF in the MI group but not in the HI group. As we showed before, the effect of TRPV1 blockers on behaviour seems enabled by favouring the CB1-mediated actions of AEA. In this sense, the enhancement of AEA in the HPC was related with an increase in CaMK-IV activity and CREB phosphorylation, a transcription factor involved in IEG expression (BASAVARAJAPPA et al., 2014). Likewise, acute treatment with CB_1 agonists, THC and CP-55,940, increased CREB but also Zif and cFOS in several brain regions (LAZENKA; SELLEY; SIM-SELLEY, 2013). CB_1 seems to induce the transcription of IEG through the activation of MAPK pathway, a mechanism described for other receptors coupled to Gi proteins (DERKINDEREN et al., 2003; HOWLETT; MUKHOPADHYAY, 2000). Then, AEA-CB_1 signalling can also be involved in the molecular events derived from TRPV1 blockers. Interestingly, Arc, a postsynaptic target is also enhanced, which can be in discrepancy with the presynaptic location of the receptor. However, CB_1 mediating post-synaptic plasticity has been previously described (BUSQUETS-GARCIA; BAINS; MARSICANO, 2017; MAROSO et al., 2016). A potential explanation for the postsynaptic effects is that AEA released is able to increase TRkB phosphorylation (DINIZ et al., 2019); we observed an increase in trkb mRNA and BDNF induced by SB in the MI. BDNF acting through TrkB can induce an increase in post-synaptic transcription (BENNETT; LAGOPOULOS, 2014; LEAL; BRAMHAM; DUARTE, 2017). Regarding the intensity-dependent effect of SB on plasticity factors, it remains to be determined why in the HI the treatment was unable to induce an enhancement of the evaluated targets, more research is needed to further elucidate this question.

The increased transcription of BDNF, Arc and Zif usually happens 15-30 min after a given event (ANTOINE; SERGE; JOCELYNE, 2014; BARRIENTOS et al., 2004; CHEN et al., 2007; MIZUNO et al., 2006). Thus, it seems unlikely that they underly the behaviour observed during retrieval. Since SB was able to mediated a post-retrieval increase in plasticity factors, we wonder if it would be able to induced long-term effects on memory. First, we validate our protocol for the study of memory reinstatement. We observed that our extinction protocol was sufficient to decreased the levels of freezing. Then, we demonstrate that the shock used in the reinstatement

was not able to induce fear conditioning by itself. In this experiment, the animals were conditioned with MI or HI and received intra-dHPC infusions of SB or vehicle before retrieval, then they were submitted to extinction, reinstatement and test. Curiously, during the reinstatement session, animals treated with vehicle presented high levels of freezing in response to the shock when compared with SB-treated animals. Moreover, the same group that presented an enhancement in plasticity factors, animals conditioned with MI and treated with SB, were resistant against reinstatement. However, in the HI group the treatment failed in inducing these long-term protective effects, which may be related with the incapacity of SB to increase plasticity after retrieval at this intensity of conditioning. With the extent of our data is not possible to stablished a direct relation between the molecular and the behavioural effects of SB, neither why the increased in this plasticity factors by SB would confer protection against reinstatement. A possibility is that the treatment before retrieval, in an intensity-dependent manner, is enhancing extinction.

Protection against reinstatement, renewal or spontaneous recovery is usually more consistent with reconsolidation interferences (KUIJER et al., 2020; LEE et al., 2015; MILTON, 2019; MONFILS; HOLMES, 2018; VAVERKOVÁ et al., 2020) than with extinction enhancements (BOUTON, 2004). However, AEA enhancements during extinction training facilitates extinction (BITENCOURT; PAMPLONA; TAKAHASHI, 2008; CHHATWAL et al., 2004; LARICCHIUTA; CENTONZE; PETROSINI, 2013) promoting protection against reinstatement (CHHATWAL et al., 2004). Although, different from CB_1 (BITENCOURT; PAMPLONA; TAKAHASHI, 2008; CHHATWAL et al., 2004; LARICCHIUTA; CENTONZE; PETROSINI, 2013), TRPV1 seems not involved in this effect (BITENCOURT; PAMPLONA; TAKAHASHI, 2008; LARICCHIUTA; CENTONZE; PETROSINI, 2013). Moreover, BDNF has a consistent role as a extinction facilitator (HELDT et al., 2007; PETERS et al., 2010; PSOTTA; LESSMANN; ENDRES, 2013). On the other hand, Zif was highly related with reconsolidation (BESNARD; CABOCHE; LAROCHE, 2013; KIRTLEY; THOMAS, 2010; LEE, 2010; LEE; HYNDS, 2013; MADDOX; MONSEY; SCHAFE, 2011), but it seems also involved in fear extinction (HAN et al., 2014; HERRY; MONS, 2004). Reductions in Arc were also related to extinction impairments (ONOUE et al., 2014).

As mentioned above, in a mechanistic point of view BDNF-TrkB, Zif and Arc are able to enhance plasticity through several pathways and then potentiated the

new memory trace underlying extinction (LEAL; BRAMHAM; DUARTE, 2017; MINATOHARA; AKIYOSHI; OKUNO, 2015; SHEPHERD; BEAR, 2011). In parallel, Arc is also able to mediated LTD, then it could induce a decrease in the synaptic efficacy of the original memory trace. Specifically, Arc was involved in inverse synaptic tagging, in this process after memory retrieval the absence of a large influx of Ca^{2+} drives the interaction of Arc towards inactive forms of CaMK-IIB, the accumulation of Arc in these synapses induces the internalization of AMPA receptors (MORIN; GUZMÁN-RAMOS; BERMUDEZ-RATTONI, 2015; OKUNO et al., 2012; OKUNO; MINATOHARA; BITO, 2018; SHEPHERD; BEAR, 2011; ZHANG; BRAMHAM, 2021). Thus, Arc could depotentiate the original memory trace. To summarize, increases in Arc, Zif and BDNF can sustain long-term plastic effects. These targets could theoretically, underly the protection conferred by SB treatment against reinstatement. However, it remains unknown which specific mechanism is mediating the long-term effects on plasticity induced by SB.

Conclusion

Overall, this work characterized the involvement of hippocampal TRPV1 channels in the modulation of associative memory, including how different factors can contribute for the recruitment of this channel: memory type, phase, age or intensity. Our results suggest that blocking TRPV1 favours AEA effects through CB_1 to impair the retrieval of fear memory, triggering plasticity pathways that may result in long-term protective effects. TRPV1 blockers were previously studied in humans regarding its analgesic properties and presented a favourable safety profile (IGLESIAS; AGUIAR; MOREIRA, 2020). These previous findings, together with our data, suggest that TRPV1 channels could be an interesting target for the selective modulation of fear memories, sparing other memory types.

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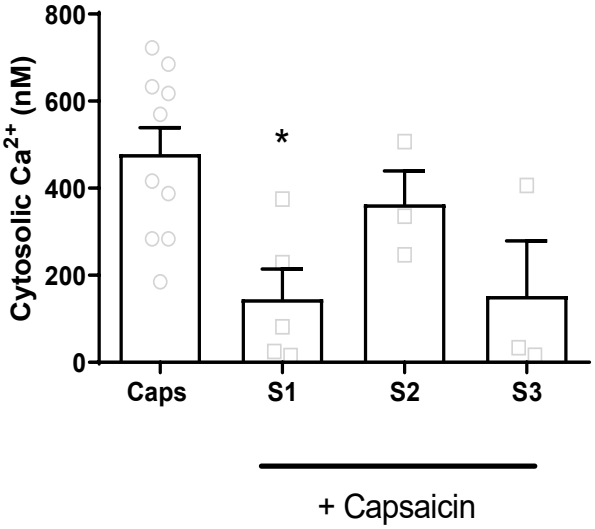
ANEX I - Complementary results

Calcium synaptosomes

The doses of SB366791 used to ex vivo calcium assay were 1, 10 and 100 µl as described previously (LI et al., 2008), and the CPS concentration was 50 µM (LU et al., 2017). Both drugs were diluted in DMSO 3% and Krebs-Ringer-HEPES solution (KRH).

The HPC was dissected and homogenised in 3 ml of gradient solution. It was centrifuged for 10 min, x3000 rpm at 4ºC and 3ml of supernatant was collected. It was placed in a discontinued gradient of Percoll (23%, 15%, 10%, 3%) and centrifuged x18000 rpm for 15 min at 4ºC. This procedure allows the identification of 4 bands, the band 2 and 3 were collected a centrifuged 15min, x18000rpm at 4ºC. The pellet was resuspended in KRH and FURA 2 (200:1) was added. The mixture was kept in a bath for 30 min at 35.5ºC, after that it was centrifuged and the pellet resuspended in in KRH and FURA 2 (200:1) and placed in the bath for 30 min, finally it was centrifugate and the pellet resuspended with KRH and FURA 2 (200:1). The Mix was prepared containing 170 µl of KRH, 30 µl of synaptosome and 0.1% of CaCl2. The assay included five continuous lectures basal (mix) 5 min, the drug SB366791 diluted in KRH (1, 10 or 100 µM) 10min, capsaicin 50mM 5min, SDS (10%) 5min and Tris/EGTA (3:1) 5min. Finally, the solution was analysed by a fluorimeter Cytation 5 (CAPI-UFMG), the synaptosomes were activated by CPS.



Effects of SB in Calcium influx in hipocampal synaptosomes incubated with Caps. one-way ANOVA followed by Bonferroni post-hoc [F(3,17)=4,985, p=0.0116] n=10-5-3-3. F

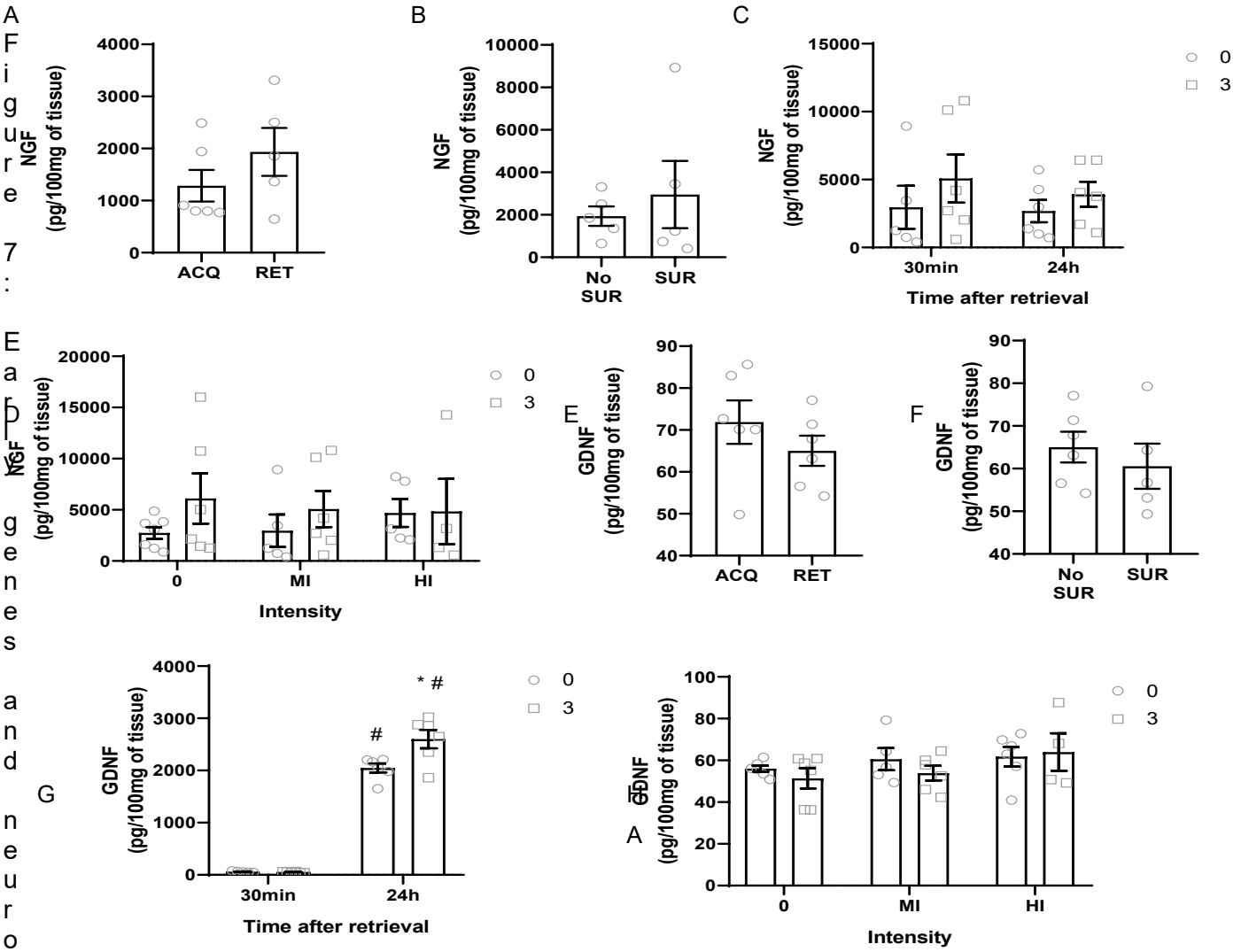
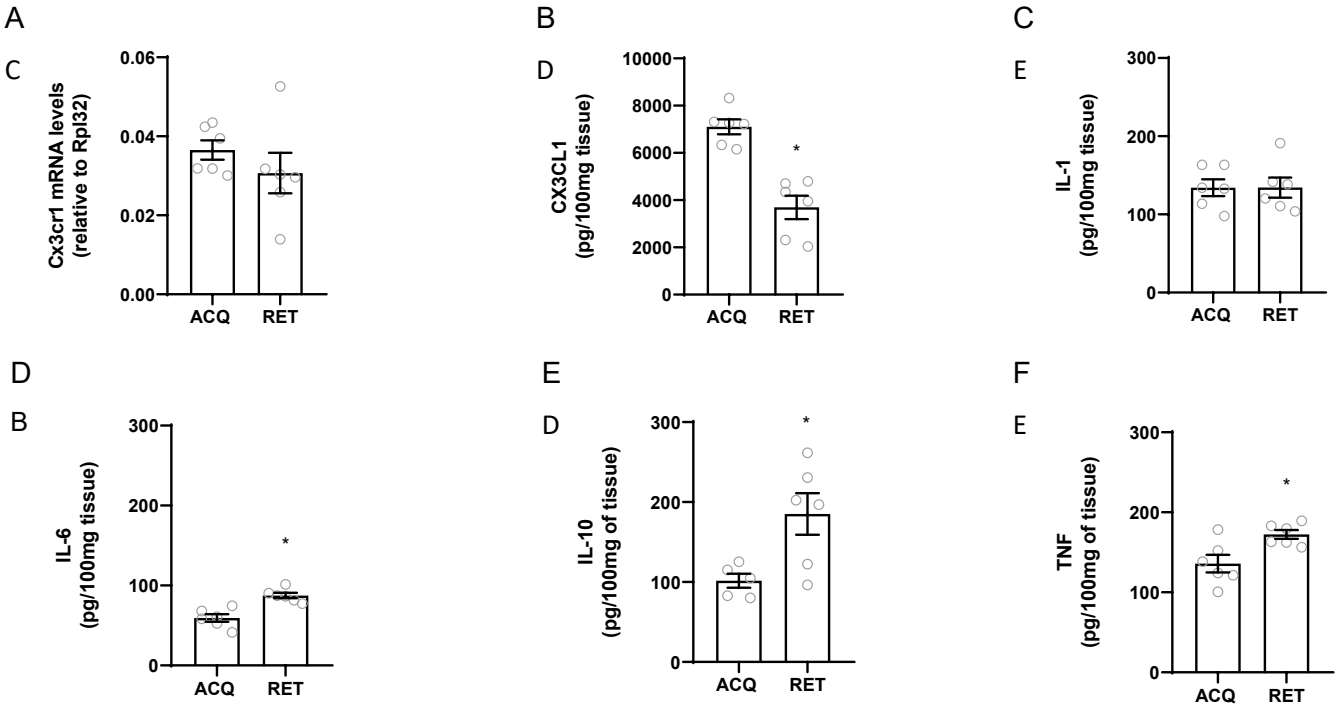
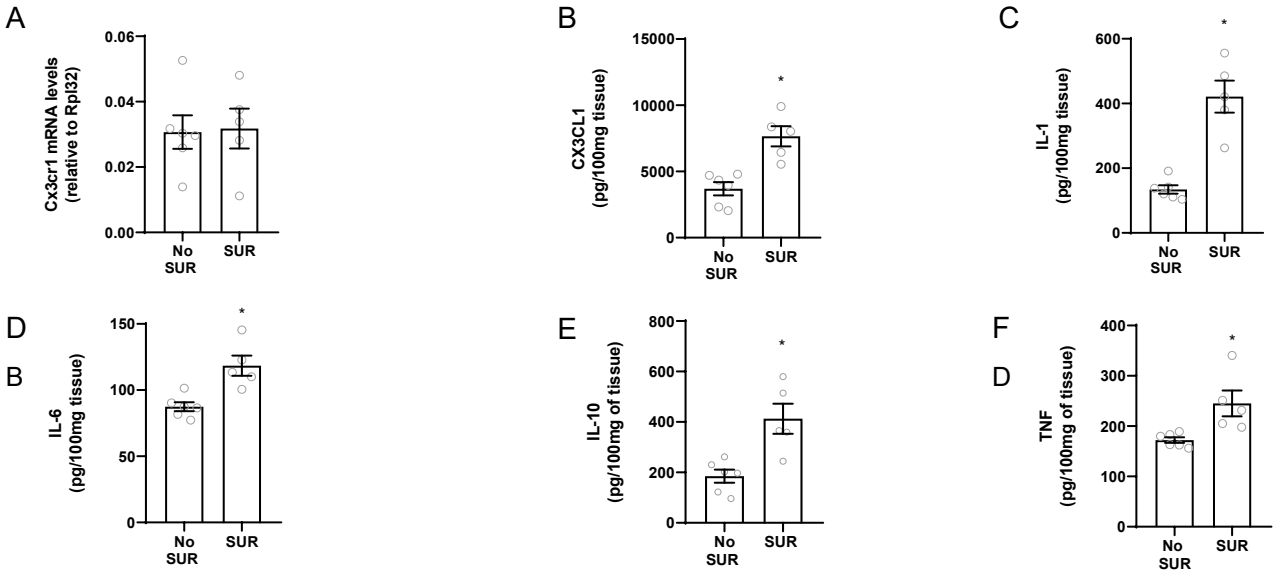


Figure 7: Early genes and neurotrophic signaling in the HPC 30min after acquisition or retrieval in animals conditioned with MI. A) Experimental Design. B) TRPV1 mRNA levels, t-Student n=6-6. C) Arc mRNA levels, t-Student n=6-6. D) Zif mRNA levels, t-Student n=6-5. E) Trkb mRNA levels, t-Student n=6-5. F) BDNF levels, t-Student n=6-6. *p<0.05 compare to control. = 1. 520, p=0.2283. Intensity: F (2,27) = 0.07576, p=0.9242, Interaction: F (2, 27) =0.3695, p=0.6945] n=4-7. E) GDNF, t-Student [t=1.087, df=10, p=0.3027] n=6. F) GDNF, t-Student [t=0.7230, df=9, p=0.4881] n=5-6. G) GDNF, two-way ANOVA followed by Bonferroni post-hoc [Treatment: F (1,19) = 7.061 p=0.0156. Time: F (1, 19) = 483,1, p<0.0001, Interaction: F (1,19) = 7.408, p= 0.0135] n=5-6. H) GDNF, Two-way ANOVA followed by Bonferroni post-hoc [Treatment: F (1,27) = 0.6003, p=0.4452. Intensity: F (2,27) = 1.830, p=0.1797, Interaction: F (2, 27) =0.4419 p=0.6474] n=4-6. *p<0.05 compare to control. # differences between the same treatment conditions in different groups.

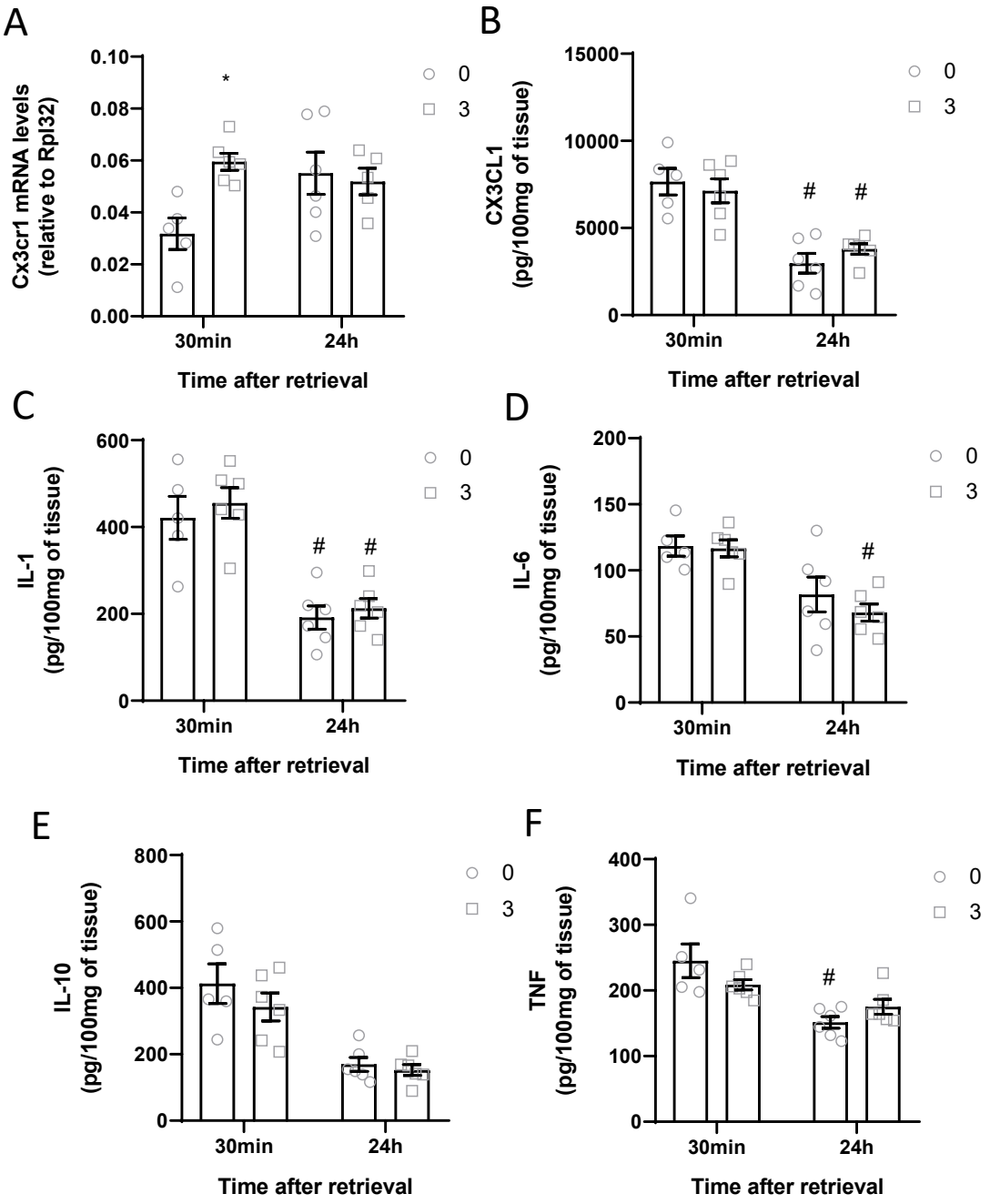
NGF and GDNF levels in different prFigure 7: Early genes and neurotrophic signaling in the HPC 30min after acquisition or retrieval in animals conditioned with MI. A) Experimental Design. B) TRPV1 mRNA levels, t-Student n=6-6. C) Arc mRNA levels, t-Student n=6-6. D) Zif mRNA levels, t-Student n=6-5. E) Trkb mRNA levels, t-Student n=6-5. F) BDNF levels, t-Student n=6-6. *p<0.05 compare to control. = 1. 520, p=0.2283. Intensity: F (2,27) = 0.07576, p=0.9242, Interaction: F (2, 27) =0.3695, p=0.6945] n=4-7. E) GDNF, t-Student [t=1.087, df=10, p=0.3027] n=6. F) GDNF, t-Student [t=0.7230, df=9, p=0.4881] n=5-6. G) GDNF, two-way ANOVA followed by Bonferroni post-hoc [Treatment: F (1,19) = 7.061 p=0.0156. Time: F (1, 19) = 483,1, p<0.0001, Interaction: F (1,19) = 7.408, p= 0.0135] n=5-6. H) GDNF, Two-way ANOVA followed by Bonferroni post-hoc [Treatment: F (1,27) = 0.6003, p=0.4452. Intensity: F (2,27) = 1.830, p=0.1797, Interaction: F (2, 27) =0.4419 p=0.6474] n=4-6. *p<0.05 compare to control. # differences between the same treatment conditions in different groups.



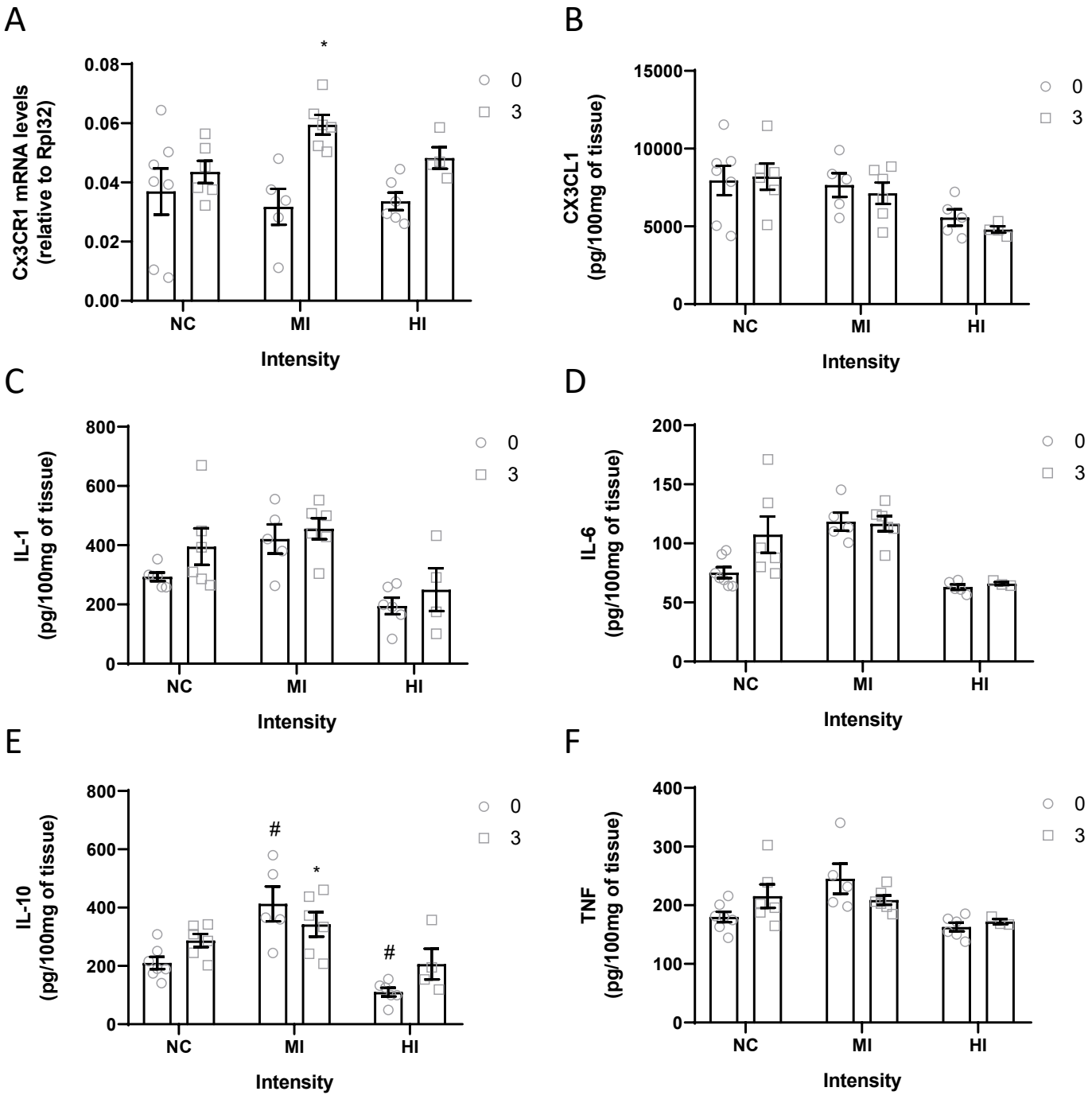
Immune factors 30min after acquisition or retrieval in animals conditioned with moderate intensity. **A)** Cx3cr1 mRNA levels, t-Student [t=1.028, df=10, p=0.3280] n=6. **B)** CX3CL1 levels, t-Student [t=5.791, df=10, p=0.0002] n=6. **C)** IL-1β levels, t-Student [t=0.006285, df=10, p=0.9951] n=6. **D)** IL-6 levels, t-Student [t=4.808, df=10, p=0.0007] n=6. **E)** IL-10 levels, t-Student [t=2.803, df=9, p=0.0206] n= 5-6. **F)** TNFα levels, t-Student [t=2.952, df=10, p=0.0145] n=6. *p<0.05 compare to control.



Immune factors 30min after retrieval in animals with (SUR) and without surgery (No SUR) and conditioned with the moderate intesity. A) Cx3cr1 mRNA levels, t-Student [t=0.1373, df=9, p=0.8938] n=5-6. B) CX3CL1 levels, t-Student [t=4.507, df=9, p=0.0015] n=5-6. C) IL-1β levels, t-Student [t=6.117, df=9, p=0.0002] n=5-6. D) IL-6 levels, t-Student [t=3.959 df=9, p=0.0033] n=5-6. E) IL-10 levels, t-Student [t=3.727, df=9, p=0.0047] n=5-6. F) TNFα levels, t-Student [t=3.052 df=9, p=0.0137] n=5-6.*p<0.05 compare to control.



Immune factors 30min or 24h after retrieval in animals conditioned with the moderate intensity and treated with 3nmol of SB or vehicle, two-way ANOVA followed by Bonferroni post-hoc. A) Cx3cr1 mRNA levels [Treatment: F (1,18) = 4.141, p=0.0569. Time: F (1, 18) = 1.696, p=0.2092. Interaction: F (1,18) = 6.566, p=0.0196] n=5-6. B) CX3CL1 levels [Treatment: F (1,19) = 0.06, p=0.8091. Time: F (1, 19) = 45.54, p<0.0001. Interaction: F (1,19) = 1.299, p=0.2685] n=5-6. C) IL-1β levels [Treatment: F (1,19) = 0.6733, p=0.422. Time: F (1, 19) = 49.06, p<0.0001. Interaction: F (1,19) = 0.03912, p=0.8453] n=5-6. D) IL-6 levels [Treatment: F (1,19) = 0.7345 p=0.4021. Time: F (1, 19) = 22.23, p=0.0002. Interaction: F (1,19) = 0.4346, p=0.5177] n=5-6. E) IL-10 levels [Treatment: F (1,19) = 1.426 p=0.2471. Time: F (1, 19) = 35.12, p<0.0001. Interaction: F (1,19) = 0.5392, p=0.4717] n=5-6. F) TNFα levels [Treatment: F (1,19) = 0.2093 p=0.6525. Time: F (1, 19) = 20.92, p=0,0002. Interaction: F (1,19) = 4.682, p= 0.0434] n=5-6. * differences inside the group, # differences between the same treatment conditions in different groups.



Immune factors 30min after retrieval in animals treated with 3nmol of SB or vehicle and conditioned with the moderate intensity, high intensity or not-conditioned, two-way ANOVA followed by Bonferroni post-hoc. A) Cx3cr1 mRNA levels [Treatment: F (1, 28) = 13.65, p=0.0009. Intensity: F (2,28) = 0.6041, p=0.5536. Interaction: F (2, 28) = 2.067, p=0.1455] n=5-6. B) CX3CL1 levels [Treatment: F (1, 27) = 0.2970, p=0.5902. Intensity: F (2,27) = 6.832, p=0.004. Interaction: F (2, 27) = 0.2412, p=0.7873] n=4-7. C) IL-1β levels [Treatment: F (1, 27) = 3.010, p=0.0941. Intensity: F (2,27) = 10.98, p=0.0003. Interaction: F (2, 27) = 0.3165, p=0.7313] n=4-6. D) IL-6 levels [Treatment: F (1,26) = 2.253 p= 0.1454. Intensity: F (2,26) = 15.64, p<0,0001. Interaction: F (2, 26) = 2.412, p= 0.1094] n=3-7. E) IL-10 levels [Treatment: F (1,28) = 1.363, p=0.2529. Intensity: F (2,28) = 17.80, p<0,0001. Interaction: F (2, 28) = 3.188, p=0.0566] n=4-6. F) TNFα levels [Treatment: F (1,27) = 0.04761, p=0.8289. Intensity: F (2,27) = 6.949, p=0.0037. Interaction: F (2, 27) = 3.3254, p=0.0542] n=3-5. * differences inside the group, # differences between the same treatment conditions in different groups.

ANEX II - Complementary production

Published

Röpke J., Ferreira-Vieira TH., **Iglesias LP**., Asth L., Ribeiro FM, Moreira FA. (2021). Protective role of endocannabinoid signalling in an animal model of haloperidol-induced tardive dyskinesia. Pharmacology Biochemistry and Behavior, 173193. Journal pre-proof. https://doi.org/10.1016/j.pbb.2021.173193.

Asth, L., **Iglesias, L. P**., Briânis, R. C., Marçal, A. P., Soares, N. P., Aguiar, D. C., & Moreira, F. A. (2021). Effects of the monoamine stabilizer, (-)-OSU6162, on cocaine-induced locomotion and conditioned place preference in mice. Naunyn-Schmiedeberg's archives of pharmacology. Advance online publication. https://doi.org/10.1007/s00210-021-02053-x

Iglesias LP, Aguiar DC, Moreira FA. TRPV1 blockers as potential new treatments for psychiatric disorders. Behav Pharmacol. 2020 Oct 28. Epub ahead of print. https://doi.org/10.1097/FBP.0000000000000603

Asth, L., **Iglesias, L. P**., De Oliveira, A. C., Moraes, M. F. D., & Moreira, F. A. (2019). Exploiting cannabinoid and vanilloid mechanisms for epilepsy treatment. Epilepsy and Behavior, 106832. https://doi.org/10.1016/j.yebeh.2019.106832

Accepted

Lia P. Iglesias, Lucas Bedeschi, Daniele C. Aguiar, Laila Asth, Fabrício A. Moreira. Effects of delta-9-THC and related cannabinoids in the elevated plus maze test of anxiety: A systematic review and meta-analysis. (Cannabis and Cannabinoids Research).

Submitted

Lia P. Iglesias, Heliana B. Fernandes, Aline S. de Miranda, Malena M. Perez, Lucia H. Faccioli, Carlos A. Sorgi, Leandro J. Bertoglio, Daniele C. Aguiar, Carsten T. Wotjak, Fabrício A. Moreira. TRPV1 modulation of contextual fear memory depends on stimulus intensity and endocannabinoid signaling in the hippocampus. (Neuropharmacology)

Maria Carolina Machado da Silva, **Lia Parada Iglesias**, Eduardo Candelario-Jali, Habibeh Khoshbouei, Fabrício A. Moreira, Antônio Carlos Pinheiro de

Oliveira. Role of microglia in psychostimulant addiction. (Current Neuropharmacology)

Manuscript in preparation

Lia P. Iglesias*, Nicia Soares*, Laila Asth, Fabricio A Moreira, Daniele C Aguiar. Minocycline as a potential target for anxiety - Systematic Review and Metanalysis from rodent anxiety models