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BEHAVIORAL PHENOTYPES OF MICE LACKING CANNABINOID CB1 RECEPTORS IN DIFFERENT NEURONAL SUBPOPULATIONS – FOCUS ON SEX-RELATED SOCIAL INTEREST

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To my family, that despite being an ocean away was always near.

I don’t wanna go.

— The Tenth Doctor

ABSTRACT

Abnormalities in social behavior are found in almost all psychiatric disorders, such as anxiety, depression, autism and schizophrenia. Thus, comprehension of the neurobiological basis of social interaction is important to better understand numerous pathologies and improve treatments. Several evidences suggest that an alteration of cannabinoid CB1 receptor function could be involved in the pathophysiology of such disorders. However, the role of CB1 receptor is still unclear and its localization on different neuronal subpopulations may produce distinct outcomes. To dissect the role of CB1 receptor on different neuronal population, male mice were used – knockout mice and their respective control littermates [total deletion ($CB1^{-/-}$); specific deletion on cortical glutamatergic neurons ($GluCB1^{-/-}$); on GABAergic neurons of the forebrain ($GABACB1^{-/-}$); or on dopaminergic D1 receptor expressing neurons ($D1CB1^{-/-}$)], and wild-type (WT) mice treated with CB1 antagonist/inverse agonist SR141716A (3mg/kg). To elucidate the behavioral effects of specific CB1 receptor deficiency, $D1CB1^{-/-}$ mice were submitted to a battery of behavioral tests which included exploration-based tests, depressive-like behavioral tests, and fear-related memory paradigms. It was demonstrated that $D1CB1^{-/-}$ mice exhibited significantly increased contextual and auditory-cued fear, with attenuated within-session extinction. Also, when all mice lines were submitted to different social tasks, involving male or female as the stimulus subject, $GluCB1^{-/-}$ mice showed reduced interest for the social stimulus, as $CB1^{-/-}$ or WT treated with SR141716A mice. $D1CB1^{-/-}$ showed moderate changes in social interest, and $GABACB1^{-/-}$ mice showed the opposite phenotype by spending more time investigating the social stimulus. In conclusion, specific reduction of endocannabinoid signaling in D1-expressing neurons is able to affect acute fear adaptation. Moreover, CB1 receptors specifically modulate social investigation of female mice in a cell-specific manner. These findings support the involvement of cannabinoid signaling in social alterations in psychiatry disorders.

Keywords: endocannabinoid system, CB1 receptor, GABA, glutamate, dopamine, affective disorders, social behavior

Abbreviations List

2-AG – 2-arachidonoyl glycerol

ANOVA – Analysis of variance

AM404 – *N*-arachidonoylaminophenol

Ca^{+2} – íon Calcium

CA1 – *Cornu Ammonis* area 1

CA3 – *Cornu Ammonis* area 3

cAMP– Cyclic adenosine monophosphate

CB1 – Cannabinoid receptor type 1

CB2 – Cannabinoid receptor type 2

CCK – Cholecystokinin

CNS – Cetral neurvous system

COX – Cyclooxygenase

D1 – Dopaminergic receptor type 1

D2 – Dopaminergic receptor type 2

EPM – Elevated plus maze

EPSCs – Excitatory postsynaptic currents

FAAH – Fatty acid amide hydrolase

FST – Forced swim test

GABA – γ-Aminobutyric acid

Glu – Glutamate

IPSCs– Inhibitory postsynaptic currents

KO – Knockout

LD – Light-dark

LOX – lipoxygenase

MGL – Monoacylglycerol lipase

NAc – Nucleus accumbens

NGT – Novelty-induced grooming test

NOI – Novel object investigation

OF – Open Field

OVX – Ovariectomized

OVX + E – Ovariectomized females with hormone replacement

OX1R – Orexin 1 receptor

PTX – Pertussis toxin

SC – Sucrose consumption test

SI – Social interaction

SInv – Social investigation

THC – Delta-9-tetrahydrocannabinol

TRPV1 – Transient receptor potential vanilloid type 1

WT – Wild type

SUMMARY

CB1 receptors gate sex-dependent social interest in mice *European Journal of Neurose* , 2014

Modulation of defensive behavior by Transient Receptor Potential Vanilloid Type-1 (TRPV1) Channels. Neurosc. Biobeh. Rev. 2014

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1 INTRODUCTION

Marijuana and other derivatives of the plant *Cannabis sativa* have been used for thousands of years for their therapeutic and mood-altering properties. Nowadays, *Cannabis sativa* belongs to the most popular illicit drugs used for recreational purposes in contemporary societies all around the world (Ehrenreich et al. 1999; Watson et al. 2000; Kano et al, 2009). Cannabis smoking ("marijuana") is common particularly among adolescents and young adults. The pervasiveness of this drug worldwide, along with its relatively low lethality, has led many to believe that it is of little harm. Indeed, the use of cannabis currently exceeds that of tobacco smoking among adolescents in the some countries (Chadwick et al, 2013). However, evidence accumulates that chronic cannabis abuse - in particular during sensitive phases of development - may have long-lasting psychopathological consequences. Such effects are mostly associated with *Cannabis sativa* component delta-9-tetrahydrocannabinol (THC), identified in 1964 (Pertwee 1997). Research focused on the relationship between mental health and cannabinoid components is thus important, particularly considering that psychiatric illnesses are complex disorders with multiple factors contributing to vulnerability and eventual expression of symptoms (Ashton and Moore, 2011; Schier et al, 2012), and cannabinoids are associated with the onset of certain diseases (Andreasson et al, 1987; Hambrecht and Hafner, 2000; Arseneault et al, 2002; Segev and Lev-Ran, 2012; Zamberletti et al, 2012; Robson et al, 2013). For example, several studies imply correlation between cannabis exposure during adolescence and vulnerability to drug addiction (Murray et al, 2007; Malone et al, 2010; Chadwick et al, 2013), schizophrenia (Andreasson et al, 1987; Hall et al. 2004; Malone et al, 2010) and may also produce long-lasting effects on anxiety and mood disorders (Croxford, 2003; Mechoulam and Parker, 2013).

1.1 *The endocannabinoid system*

The psychoactive effects of cannabis, principally mediated by THC, occur via its interaction with the endocannabinoid system, which regulates numerous biological processes involved in development and neuroplasticity. The endocannabinoid system consists of lipid-derived ligands, receptors, and enzymes that orchestrate intercellular communication and intracellular metabolism (Di Marzo, 2009).

In the early-1990s a marked advance has been made in the cannabinoid research by the discovery of the receptors that bind THC in animal tissues. The presence of a cannabinoid receptor in the brain was demonstrated, followed by its cloning (Matsuda et al, 1990). The first cannabinoid receptor (CB1) was cloned and characterized in 1991 (Matsuda et al, 1990), and the second receptor (CB2) was identified in 1993 (Munro et al, 1993). They are both G protein-coupled seven transmembrane domain receptors and differ in their tissue distributions. The CB1 receptor is abundantly expressed in the central nervous system (CNS), whereas the CB2 receptor is also present in the CNS, but mainly in the immune system. The discovery of these high-affinity, stereoselective and pharmacologically distinct cannabinoid receptors in a rat brain tissue (Matsuda et al, 1990; Munro et al, 1993) led to a search for natural endogenous ligands, which bind to these cannabinoid receptors. It was assumed, as previously for other systems (as for opioid / morphine; Laux-Biehlmann et al, 2013), that the cannabinoid receptors in the brain are not present just to bind a plant constituent, but to be activated by specific endogenous ligands.

Isolated from the porcine brain, the first endogenous compound presenting the similar potency to THC was identified as *N*-arachidonoylethanolamide (Devane et al, 1992). This endocannabinoid is better known by the name of anandamide, derived from the Sanskrit word for bliss, *ananda*. Anandamide behaves as a partial agonist of the two different cannabinoid

receptors, CB1 and CB2, and also binds to the transient receptor vanilloid receptor type 1 (TRPV1). Therefore, it can activate both the endocannabinoid and endovanilloid systems. Another major endocannabinoid, 2-arachidonoyl glycerol (2-AG) was originally isolated from canine gut (Mechoulam et al, 1995) and rat brain (Sugiura et al, 1995). 2-AG is a rather common molecule and is present in the brain at higher concentrations than anandamide (Sigiura et al, 2006). 2-AG acts as a full agonist in various assay systems and is strictly recognized by CB1 and CB2 receptors, suggesting that 2-AG is a true natural ligand for the cannabinoid receptors (Sugiura et al, 2006). There is enough evidence to show that these endocannabinoids are synthesized from lipid membrane precursor molecules and released from neurons in an activity- and calcium-dependent manner, however are not stored in vesicles as classical neurotransmitters. They also play physiological roles as intercellular signaling molecules. Other putative endocannabinoids include dihomolinolenoyl ethanolamide (Hanus et al, 1993), docosatetraenoyl ethanolamide (Hanus et al, 1993), 2-arachidonyl glycerol ether (noladin ether; Hanus et al, 2001), *O*-arachidonoylethanolamine (virodhamine; Porter et al, 2002), and *N*-arachidonoyldopamine (Huang et al, 2002).

The year 2001 was the turning point of the cannabinoid research. In this year, endocannabinoids were discovered to mediate retrograde signaling at central synapses (Maejima et al, 2001; Wilson and Nicoll, 2001). Accordingly, endocannabinoid production and diffusion from the postsynaptic cell would stimulate CB1 receptors on presynaptic terminals, where they are frequently located, leading to decreased release of neurotransmitters (Wilson and Nicoll 2001). These enabled a new phase for the cannabinoid research, and also established a new concept of how diffusible messengers such as endocannabinoids (postsynaptic release of the endogenous agonist and receptor activation on presynaptic terminal) modulate synaptic efficacy and neural activity.

The degradation of endocannabinoids happens through two different pathways, hydrolysis and oxidation (Vandevoorde and Lambert, 2007). The enzymes that catalyze the first pathway include fatty acid amide hydrolase (FAAH) for anandamide and monoacylglycerol lipase (MGL) for 2-AG. The second pathway involves the well-known cyclooxygenase (COX) and lipoxygenase (LOX), which induce oxidation of the arachidonic moiety of the endocannabinoids. FAAH proteins are 579 amino acids in length among different species, such as rat, mouse, and human. FAAH is detected in many organs including brain. The enzyme is able to recognize a variety of fatty acid amides, but its preferred substrate is anandamide. It also catalyzes the hydrolysis of the ester bond of 2-AG in vitro. MGL was identified in 1976 (Tornqvist and Belfrage, 1976) and first cloned from a mouse adipocyte cDNA library (Karlsson et al, 1997), being now recognized as the main enzyme catalyzing the hydrolysis of 2-AG in vivo (Dinh et al, 2002; Dinh et al, 2004; Vandevoorde and Lambert, 2007). The removal of endocannabinoids from the extracellular space occurs by a two-step process: the transport into cells and the subsequent enzymatic degradation (Hillard and Jarrahian, 2000; Fowler and Jacobsson, 2002; McFarland and Barker, 2004). Anandamide uptake has been observed in a number of preparations including primary neuronal cell cultures (Beltramo et al, 1997; Di Marzo et al, 2004; Hillard et al, 1997). Anandamide uptake is saturable and temperature dependent. Several structural analogs of anandamide, such as *N*-arachidonoylaminophenol (AM404), have been reported to inhibit the anandamide uptake (Beltramo et al, 1997; Piomelli et al, 1999), and they are called anandamide transport inhibitors. However, their molecular identities have not been fully clarified yet. Recently, Fu et al (2012) identify a partly cytosolic variant of FAAH-1, termed FLAT that lacks amidase activity but binds anandamide with low micromolar affinity and confers anandamide transport on cells that are engineered to express it. Such effect is suppressed by AM404 and other anandamide transport inhibitors. In contrast, a new study (Leung et al, 2013) fail to detect

FLAT expression in DRGs and in other tissues examined, possibly suggesting that FLAT may exhibit a low tissue expression profile that is below the detection limit of these approaches. The reason for this discrepancy could be related to antibodies sensitivity, however further studies would be necessary to clarify this matter (Leung et al, 2013). In contrast to the intensive studies on the mechanisms of anandamide uptake, there is relatively little information concerning 2-AG uptake. Yet, there are several studies suggesting that 2-AG and anandamide are transported by the same system (Bisogno et al, 2001, Kano et al, 2009).

The endocannabinoid system in involved in various functions of the CNS under physiological and pathological conditions, which suggests that the molecules involved in endocannabinoid signaling may be promising targets for clinical management of disturbed neural functions or pathological conditions.

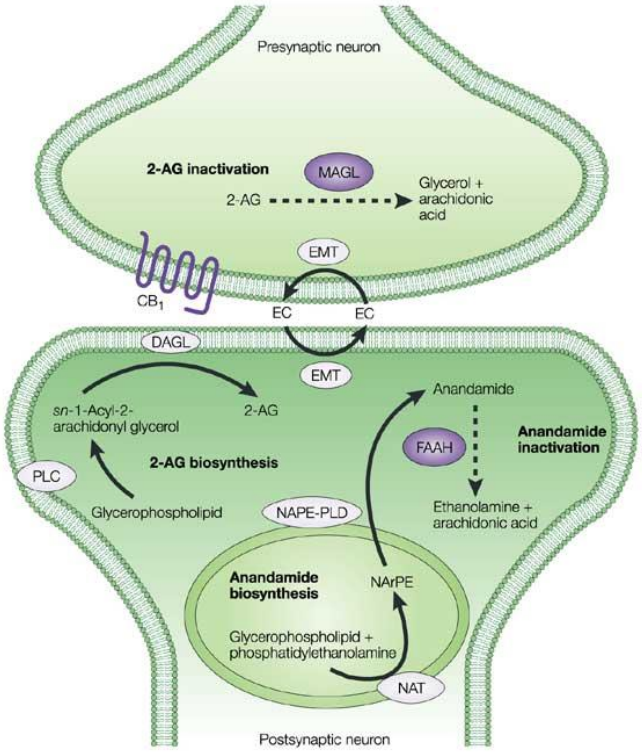

Figure 1. Major signalling pathways associated with cannabinoid receptor activation by agonists. Hydrolytic enzymes are involved in both the biosynthesis of endocannabinoids and in their inactivation. The enzymes for 2-arachidonoylglycerol (2-AG) biosynthesis, the

phospholipases C (PLC) and the sn-1-selective diacylglycerol lipases (DAGLs) seem to be mostly localized on the plasma membrane. The DAGLs, in particular, are located on postsynaptic neurons in the adult nervous system, whereas the monoacylglycerol lipase (MAGL) for 2-AG inactivation is localized in presynaptic neurons, which supports a possible role as retrograde messenger at presynaptic CB1 receptors for this compound. The anandamide biosynthetic enzymes and the inactivating enzyme fatty acid amide hydrolase (FAAH) are all located on intracellular membranes. FAAH seems to be most abundant on neurons postsynaptic to CB1 receptors, indicating that anandamide acts principally on these neurons. An as yet uncharacterised endocannabinoid membrane transporter seems to facilitate both endocannabinoid release and re-uptake, and might be localized on both pre- and postsynaptic neurons (Adapted from Di Marzo et al, 2004).

1.2 *CB1 receptors*

As mentioned above, during the 1990s CB1 receptor (Matsuda et al, 1990) was first cloned from rat brain cDNA. Its structure consisted of a 473-amino acid G protein-coupled receptor. Later, a human homolog of 472 amino acids (Gerard et al, 1990) and a mouse homolog of 473 amino acids (Chakrabarti et al, 1995) have also been reported. These three CB1 receptors share 97–99% amino acid sequence similarity. Also, a number of genetic polymorphisms have been described in the CB1 receptor, and their correlation with various conditions has been examined (Norrod and Puffenbarger, 2007). Some of the polymorphisms have been reported to be linked to several disorders, as obesity-related phenotypes (Gazzerro et al, 2007), schizophrenia (Chavarria-Siles et al, 2008; Ujike and Morita, 2004), childhood attention deficit/hyperactivity disorder (Ponce et al, 2003), and depression in Parkinson's disease (Barrero et al, 2005), but results still need confirmation in independent samples.

With the use of site-directed mutagenesis, binding properties of cannabinoids to the CB1 receptor have been elucidated, leading to the knowledge of transmembrane helices of the receptor (Song and Bonner, 1996). It is proposed that the CB1 receptor likely exists as a homodimer in vivo (Wager-Miller et al, 2002). The magnitude of CB1 dimerization could be regulated by agonists' actions (Martin and Alger, 1999). The CB1 receptor can also exist as a

heteromer (Mackie, 2005). One example is the heteromer between CB1 and D2 (Kearn et al, 2005). It was demonstrated that receptor stimulation promotes the formation of CB1/D2 complex and alters the CB1 signaling. Another example is the heteromer between CB1 and orexin 1 receptor (OX1R). The CB1 activation potentiated the OX1R signaling (Hilairet et al, 2003), suggesting the interaction of these two receptors.

In the CNS, cannabinoid receptors distribution was first demonstrated by ligand binding using the radiolabeled synthetic cannabinoid [^{3}H]CP55,940 (Herkenham et al, 1990; 1991; Mailleux and Vanderhaeghen, 1992). Ligand binding sites are widely distributed in the brain at various levels depending on the regions and also the neuron types within a given region. High levels of [^{3}H]CP55,940 binding are observed in layers of the olfactory bulb, hippocampus (particularly high in the dentate gyros and CA3 region), striatum and cerebellar molecular layer (Herkenham et al, 1990). Moderate levels are noted in cerebral cortex, septum, amygdala, hypothalamus and spinal dorsal horn. The thalamus, other nuclei in the brainstem and spinal ventral horn present lower ligand binding. These overall binding properties are preserved across mammals (Herkenham et al, 1990). These high levels of ligand binding sites in the telencephalic and cerebellar regions are compatible with the effects of cannabinoids on motor and cognitive functions. In contrast, generally low levels of ligand binding in the lower brain stem areas that control cardiovascular and respiratory functions may explain the lack of lethal effect of high doses of cannabinoids (Herkenham et al, 1991; Mailleux and Vanderhaeghen, 1992). Likewise, the ventromedial hypothalamic nucleus is the satiety center for controlling appetite and feeding behavior, moderate levels in these nuclei seem to explain anti-anorexic and antiemetic actions of cannabinoids. For this reason, drugs that activate the cannabinoid system are used in treatments for nausea and vomiting associated with cancer chemotherapy and for appetite suppression and cachexia in acquired immunodeficiency syndrome (AIDS) patients (Kano et al, 2009).

Considering the widely expression of CB1 in the CNS, two distinct patterns of CB1 mRNA expression appears. CB1 mRNA can be present in uniform and nonuniform labelings, depending on brain regions (Mailleux and Vanderhaeghen, 1992, Matsuda et al, 1993). Uniform labeling is found in the striatum, thalamus, hypothalamus, cerebellum, and lower brain stem in major neuronal populations. For example, CB1 mRNA is expressed in medium spiny neurons and parvalbumin-positive interneurons within the striatum, and in cerebellar granule cells, basket cells, and stellate cells within the cerebellar cortex. In contrast, non-uniform expression is found in the cerebral cortex, hippocampus, and amygdala, reflecting the presence of a few cell types expressing high CB1 mRNA. In these regions, strong expression is seen in cholecystokinin (CCK)-positive GABAergic interneurons, whereas no expression in parvalbumin-positive interneurons and generally low expression in principal (or excitatory glutamatergic) neurons are noted (Marsicano and Lutz, 1999; Tsou et al, 1999; Katona et al, 2000; Katona et al, 2001; Kawamura et al., 2006; Monory et al., 2006; Azad et al., 2008; Morozov et al., 2009). However, sometimes the endocannabinoid system appears to be functionally very important in regions or cell types where the density of CB1 receptor is relatively low (Walker et al., 1999; Hohmann et al., 2005 Marsicano et al., 2003; Monory et al., 2006).

Still on CB1 receptor location, inhibitory synapses generally have higher levels of CB1 than excitatory synapses among CB1-expressing synapses within given neural regions. Moreover, the enrichment of CB1 receptors at inhibitory synapses varies greatly depending on brain regions. For example, the density of CB1 on inhibitory synaptic elements is higher than excitatory synapses for hippocampal CA1 pyramidal cells, cerebellar Purkinje cells, and striatal medium spiny neurons (Kawamura et al, 2006; Uchigashima et al, 2007). The difference in distribution, density, and regulation of CB1 expression between excitatory and inhibitory synapses provides molecular and anatomical bases for biphasic psychomotor and

perceptual actions of marijuana that appear in time- and dose-dependent manners. It is also worth mentioning that while most expression CB1 receptor occurs in the limbic areas arises from GABAergic and glutamatergic neurons, the receptor has also been identified on serotonergic and noradrenergic terminals, suggesting other manners on how endocannabinoids influence limbic circuits (Häring et al., 2007; Oropeza et al., 2007).

Concerning the mechanism of action of the CB1 receptor, multiple signal transduction pathways are activated by pharmacological stimulation of CB1 receptors primarily via the Gi/o family of G proteins, supported by the studies examining [35S]GTPγS binding and pertussis toxin (PTX) sensitivity of cannabinoid effects (Pertwee, 1997). This results in inhibition of adenylyl cyclase or cAMP production. Moreover, CB1 activation evokes a transient Ca^{2+} elevation in a phospholipase C (PLC)-dependent manner through either Gi/o (Sugiura et al, 1997) or Gq proteins (Lauckner et al, 2005). Activation of CB1 receptors modulates various types of ion channels and enzymes in a cAMP-dependent or-independent manner.

The activation of CB1 receptor usually works as feedback response for several neurotransmitters activations, acting as a *break* on their release, thus protecting the brain from being overwhelmed by excessive excitatory or inhibitory activity. Glutamate release can be suppressed by cannabinoid agonists, as reported in several brain regions, including hippocampal, cerebellar, striatal, and cortical neurons (Shen et al, 1996; Schlicker and Kathmann, 2001). The cannabinoid agonist WIN55,212-2 suppress excitatory postsynaptic currents (EPSCs), which was shown to be sensitive to the CB1-specific antagonist SR141716A, confirming the involvement of CB1 receptors. The inhibitory effects of cannabinoids on GABA release were reported, e.g. in striatum, hippocampus, cerebellum, and nucleus accumbens (NAc; Chan et al, 1998; Szabo et al, 1998; Schlicker and Kathmann, 2001). In these neurons, WIN55,212-2 suppressed GABAergic inhibitory postsynaptic

currents (IPSCs), but not the postsynaptic response to exogenously activation after GABA or the $GABA_A$-receptor agonist administration, indicating a presynaptic site of action. Such response was antagonized by SR141716A, thus confirming the involvement of CB1 receptors (Chan and Yung, 1998; Szabo et al, 1998). WIN55,212-2 also reduced EPSCs in wildtype animal. However, in slices from conditional mouse mutants lacking CB1 in all principal forebrain neurons but not in GABAergic interneurons, WIN no longer affected glutamatergic synaptic transmission (Domenici et al, 2006). Modulation of voltage gated Ca^{2+} channels is the proposed mechanism involved in the suppression of both GABA (Hoffman and Lupica, 2000) and glutamate release (Huang et al, 2001) in the synapses.

Due to its broadly abundance in the CNS, association with a variety of synaptic location and influence on several neurotransmitters release, CB1 is implied as the primarily responsible for most, if not all, of the psychotropic of exogenous cannabinoids and physiological actions of endocannabinoids in the CNS (Elphick and Egertova, 2001). Such responses could be modulated based on the action of CB1 on principal projecting neurons and interneurons. The release and the rapid inactivation of endocannabinoids, together with the above mentioned differential expression of CB1, could suggest that the cannabinoid system has different, and occasionally opposite, modulatory functions depending on particular physiological or pathophysiological conditions.

In addition, development of selective CB1 receptor antagonists, such as SR141716A (Rinaldi-Carmona et al, 1994) and the generation of genetically engineered mice lacking CB1 ubiquitously or in specific neuronal populations allowed researchers to better investigate the relative contribution of the CB1 receptor to pharmacological effects of cannabinoids and confirm its importance and different actions in an cell-specific fashion (Ledent et al, 1999; Zimmer et al, 1999; Marsicano et al, 2002; 2003; Monory et al, 2006; 2007). These studies have revealed that the endocannabinoid system is involved in numerous aspects of neural

functions. For example, blocking the endocannabinoid system suppresses the extinction of aversive memory (Marsicano et al, 2002), relearning of the water maze test (Varvel and Lichtman, 2002), cerebellum-dependent eyeblink conditioning (Kishimoto and Kano, 2006), drug addiction (Maldonado et al, 2006), feeding behavior (Pagotto and Pasquali, 2006), a certain form of stress-induced analgesia (Hohmann et al, 2005), and the recovery of neurobehavioral function after brain injury (Panikashvili et al, 2005).

1.3 *Endocannabinoids in affective disorders*

The exposure to cannabis is currently being related to the outcome of several affective disorders, such as major depression, anxiety and schizophrenia. Many of the adverse effects of cannabis result from high doses or rapid administration of THC or chronic cannabis use. For example, intravenous infusion of THC in normal subjects can induce transient psychotic symptoms, anxiety, perceptual distortion and cognitive impairment (D'souza et al, 2004; Morrison et al, 2009). Smoked cannabis, especially varieties with high concentrations of THC, can cause acute psychosis, sometimes accompanied by hypomanic features in normal individuals and may precipitate schizophrenia in genetically predisposed individuals (Johns, 2001). Some longitudinal studies could even demonstrated an association between early-life exposure to cannabis and affective disorders, showing that frequent adolescent cannabis use increased depression and anxiety in early adulthood (Murray et al, 2007).

As stated in previous section, CB1 receptors are densely located in brain areas involved in emotional states, including amygdala, hippocampus and other limbic sites (Herkenham et al, 1990). As the endocannabinoid system acts as a modulator of brain excitatory (i.e. glutamatergic) or inhibitory (i.e. GABAergic) activity, extreme dysfunctional excitation or inhibition may occur leading to neuropsychological states, such as cortical malfunction causing mania or hyperarousal at one extreme and depression, anhedonia or apathy on the

other. The intrinsic tone of the endocannabinoid system may also be a major contributor to the spectrum of personality characteristics in normal individuals and their vulnerability to mood disorders. Many of these effects are biphasic and bidirectional depending on dose, mode of administration, environment, expectation, personality, degree of tolerance, time after dose and other individual factors. For instance, small to moderate doses produce euphoria, anxiolytic, sedative/hypnotic, myorelaxant and analgesic effects, whereas higher doses induce dysphoria and anxiogenic effects (Ashton and Moore, 2011). Also, patients using cannabis or synthetic THC compounds, like dronabinol or nabilone, for chronic pain conditions or multiple sclerosis reported improvement of mood and increased general well-being and mental health as well as improved sleep, relief of pain and spasticity and anxiolytic effects (Fabre and Mclendon, 1981; Ilaria et al, 1981; Martyn et al, 1995; Wade et al, 2003; Svendson et al, 2004). However, chronic or repeated use of cannabis is also associated with tolerance, dependence, a withdrawal syndrome and possibly long-term cognitive impairment (Hall and Solowij, 1998; Ashton, 1999).

Some of the aspects related to emotional alterations caused by cannabinoids or stressful situations can be modeled in laboratory (Cryan and Holmes, 2005), which facilitates the investigation of the neurobiology of both stress-related responses and the involvement of the cannabinoid system in such situations. When confronted with aversive situation, individuals present a behavior repertoire that serves to alert the organism of potentially dangerous or harmful stimuli, hence increasing the chances of survival. However, when anxiety and fear responses are disproportional in intensity, duration, and/or not associated with any actual risk, they can impair physical and psychological functions (LeDoux, 2000; Blanchard et al, 2001). Such overreactions may be symptomatic of anxiety-related neuropsychiatric disorders such as generalized anxiety, phobia and post-traumatic stress disorder (Graham et al., 2011).

Considering animal models of anxiety and fear, most of them take into consideration two main aspect of anxiety/fear behavior – one, more ethological, related to exploratory approach-avoidance behavior that is based on the animal's innate conflict between the natural drive to explore a new environment and the necessity to avoid possible dangerous situations, such as open, high and bright areas or contact with a predator. The other aspect is based on learned situation (Cryan and Holmes 2005), as learned association between a given neutral space or stimulus that is paired with a harmful stimulus like electrical shock. An important matter in animal models is the use of multifactorial analyses that reveals that a behavior measured in one test of anxiety may not have a similar ethological significance than the one measured in another test although both tests are thought to capture the anxiety dimension. Thus, anxiety-related behaviors measured in on animal test such as the elevated plus-maze do not reflect a similar emotionality dimension than those measured in the social interaction test or in neophobia tests (Cryan and Holmes, 2005; File and Seth, 2003).

Bearing that in mind, several animal studies point to the involvement of the endocannabinoid system in anxiety and fear, especially the role of the CB1 receptor in these behaviors. Typically, increased transmission at CB1 receptors decreases anxiety, whereas inhibition at CB1 receptors induces an anxiogenic-like effect (Ruehle et al, 2012; Moreira and Wotjak, 2010). Thus, considering the action of CB1 receptors in the suppression of neurotransmitter release, the endocannabinoid system is seen as one of the key regulatory elements of anxiety and fear behavior, which, in turn, could provide new alternatives for treatment and better understand the mechanism behind affective disorders.

As in humans, animal studies reported that CB1 receptor agonists induce biphasic effects, with a bell-shaped dose-response curve, with lower doses being anxiolytic and higher doses being anxiogenic (Viveros et al, 2005, Moreira et al, 2012). In addition, similar bimodal responses were found using CB1 receptor antagonists and other drugs interfering with the

molecular machinery of the endocannabinoid system (Lafenetre et al., 2007). These results extent to various anxiety paradigms, such as the Vogel conflict test, light/dark box and elevated plus-maze, where different components of the anxiety state can be measured (Cryan and Holmes, 2005; Rey et al, 2012; Ruehle et al, 2012).

In different anxiety-related test, animals treated with low doses of CB1 receptor antagonist showed no alteration in behavior (Balerio et al, 2006), however when treated with high doses some studies described ineffective (Rodgers et al, 2003; Griebel et al, 2005), anxiogenic (Patel and Hillard, 2006; Navarro et al, 1997; Arevalo et al, 2001) or anxiolytic (Haller et al, 2004) responses, which could be a result of different experimental conditions or animal species/ strains. For ubiquitous genetic ablation of CB1 receptor, some heterogeneity was also found, which is likely to be due to differences in the mouse lines background used, and also to experimental conditions. CB1 knockout (CB1-KO) mice seems to behave as control animals when tested in a non-aversive environment, but display higher anxiety levels than controls under aversive situations (Haller et al, 2004). This demonstrates the on-demand characteristic of the endocannabinoid system, and how only particular conditions are able to trigger the system.

The endocannabinoid system is also involved in fear responses. CB1-KO mice show impairment extinction of the fear conditioning response when a cue was associated with footshock (Marsicano et al, 2002). CB1 antagonist injected in wild type mice confirmed these results, which are also observed in the fear potentiated startle paradigm (Chhatwal et al, 2005). It was also described that the genetic disruption of the CB1 receptor abolished the contextual conditioned fear response and that the administration of CB1 receptor antagonist AM251 before the test session reduced it in a less efficient manner (Mikics et al, 2006), suggesting a differential role of the endocannabinoid system according to the blockage method. The use of different contextual fear conditioning protocols allowed discriminating

whether the endocannabinoid system modulated the acquisition/initial consolidation of fear memory, its reconsolidation or its extinction (Suzuki et al, 2004). Some studies suggest that the endocannabinoid system is involved in the acquisition of fear conditioning in particular conditions, with no effect in the consolidation of fear memory in either conditioning versions (Arenos et al, 2006; for review see Riebe et al, 2012).

Several evidence also points to the participation of the cannabinoids system in depression-like behavior (Serra and Fratta, 2007). It has been shown that cannabinoid receptor agonists could have antidepressant activity. Also, pharmacological stimulation of CB1 receptors elicits antidepressant-like effects in the rat forced swimming test (Gorzalka and Hill, 2011). Such responses are blocked by CB1 receptor antagonists, which *per se* can induce a state analogous to depression in the rodents, including reduced food intake, heightened anxiety, increased wakefulness, deficits in the extinction of aversive memories and hypersensitivity to stress (Gorzalka and Hill, 2011).

The bidirectional action of CB1 receptors on emotional-related responses may be related to the modulatory role of these targets on GABA and glutamate release across amygdala and other forebrain areas (Katona et al, 2000; 2001; Azad et al, 2008). As these two major neurotransmitters affect emotional-related in an opposite fashion, different doses of cannabinoids and synthetic CB1 receptor agonists may indeed produce highly divergent effects in relation to their ability to affect the homeostasis and the balance of GABA and glutamate (Bortolato and Piomelli, 2008). Furthermore, CB1 receptors have been shown to play critical roles in the regulation of most neurochemical substrates of stress-related behavior, including the neurotransmitters dopamine, serotonin, norepinephrine and acetylcholine, as well as stress hormones, cholecystokinin and opioid peptides (Szabo and Schlicker, 2005; Bortolato and Piomelli, 2008). Further, several studies using CB1 receptor knockout mice have reported anxiogenic / reduced fear expression responses in classical

paradigms (Haller et al., 2004; Martin et al., 2002). Nevertheless, contradictory data also do exist. Together with the susceptibility of the endocannabinoid system to environmental variables, the presence of CB1 receptors on glutamatergic and GABAergic neuronal subpopulations could provide an explanation, at least in part, for these contradictory findings. Depending on whether the experimental conditions predominantly modulate excitatory or inhibitory transmission (i.e. glutamatergic or GABAergic), the effect of the absence of CB1 receptor signaling will lead to different behavioral outcomes.

Nevertheless, not only GABA and glutamate are involved in the expression of emotions modulated by the cannabinoid system. The role of the dopaminergic neurotransmitter system in the processing of emotional behavior is also well established and supported by several preclinical and clinical data showing that dopamine, acting on dopaminergic postsynaptic D1- or presynaptic D2-like receptors, is of highly importance as a neuromodulator for fear and anxiety (LeDoux, 2000). The interaction between dopaminergic and cannabinoid system appears at different anatomical levels (i.e., amygdala, nucleus accumbens and striatum) and seems to be involved in several neurophysiological responses. More specifically, it has been suggested that CB1 receptor signaling modulates dopaminergic pathways by influencing directly or indirectly the activity of dopaminergic neurons through either post- or pre-synaptic mechanisms (Laviolette and Grace, 2006). However, both the mechanisms through which dopaminergic and endocannabinoids signaling cross-talk and the role played by the dopamine receptor positive neurons still remain unclear. The dopamine D1 receptors which belong to the “D1-like” group are expressed in brain regions involved in aversive learning and memory such as nucleus accumbens, hippocampus, and amygdala (Kamei et al., 1995; El-Ghundi et al., 2001; Nagai et al., 2007). Interestingly, the colocalization of CB1 receptors with D1 receptors indicates that these receptors may interact by potentially modifying their respective

functions with important behavioral and pharmacological consequences (Hermann et al., 2002).

Another significant, but yet scarcely explored participation of CB1 receptor is in social paradigms. Social disruption is observed in many psychiatric disorders, especially those presenting emotion-related dysfunctions as core symptom conditions, such as social withdrawal, increased aggressiveness, decreased affiliative behavior and social cognition or loss of sexual interest, which are described in schizophrenia, autism, anxiety and major depression (American Psychiatric Association, 2000). Considering social aspects, rodents, like humans, present a large social repertoire with conspecifics, characterized by high levels of complexity in type and number of interactions (Crawley et al, 2007; File & Seth, 2003; Choleris et al, 2009). Such similarities between humans and animals underline the potential translational value of studies on social behavior in mice in respect to the clinical situation.

In summary, many studies investigated the role of the cannabinoid system, in particular of the CB1 receptor in stress-related situation, however cellular substrates of these effects with regard to specific neuronal subpopulation involved (i.e., GABAergic-, glutamatergic- and D1-expressing neurons) is still largely unexplored. Also, little is known about CB1 receptor participation in social behaviors in rodents. For this reason, conditional CB1 knock-out animals, lacking CB1 receptor specifically in GABAergic, glutamatergic and D1- positive neurons provide an important tool to answer these questions.

2 OBJECTIVE

Considering all the above mentioned, the present work had as main objectives:

- To investigate the role of CB1 receptor located in the dopamine receptor D1-expressing neurons in emotional behavior. For this reason, conditional CB1 mutant mice, lacking CB1 receptors expression in neurons containing dopamine D1 receptors ($D1CB1^{-/-}$), were submitted to a battery of behavioral tests, which included exploration-based tests, depressive-like behavioral tests, and fear-related memory paradigms.

- Secondly, as social engagement is an important feature for species that present large and complex social repertoire with conspecifics, and can be disrupted in several psychiatry disorders where the endocannabinoid system might be involved, it was also evaluated the role of CB1 receptor located in different neuronal subpopulations in social approach tests.

3 MATERIALS & METHODS

3.1 Animals

A total of 247 adult male mice (10-20 weeks) were used. Animals were bred in our breeding facility (Max-Planck Institute of Biochemistry, Martinsried, Germany) and transferred to our experimental building. Mice were single housed and maintained in standard conditions with food and water *ad libitum* under a 12-h inverse light–dark cycle (lights off at 9 a.m.) for at least 14 days before starting the experiments. Total and conditional CB1 mutant mice were obtained and genotyped as described previously (Marsicano et al, 2002; Monory et al, 2006; Jacob et al, 2009). We used total CB1 receptor knockout ($CB1^{-/-}$; from heterozygous breeding pairs); CB1 receptor knockout on cortical glutamatergic neurons ($GluCB1^{-/-}$; from breeding pairs where only the fathers expressed the Cre-recombinase); CB1 receptor knockout on forebrain GABAergic neurons ($GABACB1^{-/-}$; from breeding pairs where only the fathers expressed the Cre-recombinase); CB1 receptor knockout on dopamine D1-expressing neurons ($D1CB1^{-/-}$; from breeding pairs where only the fathers expressed the Cre-recombinase) and their wildtype littermates ($CB1^{loxP/loxP}$ = WT); alongside with wildtype mice for pharmacological treatment. All animals were from C57Bl/6N background, including females (total of 25 females were used as stimulus subject). For the social behavior-related experiments ovariectomized females were uses. Females underwent ovariectomization surgery at least 15 days before the beginning of experiments. Some females also received ß-estradiol (micro-osmotic pump delivery) for 1 week before the beginning of experiments. Mice of a given experiment and line derived from at least four different litters. All behavioral experiments were performed during the active (dark) phase of mice between 9:30 a.m. and 5 p.m.

Laboratory animal care and experimental procedures were in compliance with the European Union recommendations for the care and use of laboratory animals (86/609/EEC). All experimental procedures were approved by the Committee on Animal Health and Care of the State of Upper Bavaria (AZ55.2-1-54-2532-44-09), and efforts have been made to minimize animal suffering and reduce the number of animals used.

3.1.1 Mutant mice

CB1$^{-/-}$ mice

Mice with ubiquitously deletion of CB1 receptor were obtained as described previously (Marsicano et al, 2002). Briefly, mice bearing the floxed-neo allele were crossed with transgenic mice expressing Cre recombinase ubiquitously. Those carrying a germ-line transmissible deletion of CB1 were backcrossed for five generations into C57BL/6N (Charles River). Homozygous CB1-deficient mice (CB1$^{-/-}$) and littermate controls (CB1$^{+/+}$) from heterozygous breedings were used for the experiments. Experimental animals were genotyped by PCR, using primers G50 (5'-GCT GTC TCT GGT CCT CTT AAA-3'); G51 (5'-GGT GTC ACC TCT GAA AAC AGA-3') and G54-neo-null 3' (5'-CCT ACC CGG TAG AAT TAG CTT-3').

Glu-CB1$^{-/-}$ mice

Mice with conditional deletion of CB1 receptor on glutamatergic neurons were obtained as described previously (Monory et al, 2006). Briefly, transgenic mice (NEX-Cre; where, in the adult brain, NEX is expressed in mature glutamatergic cortical neurons, but not in cortical GABAergic interneurons; Goebbels et al, 2006) were crossed with CB1$^{f/f}$ mice to obtain CB1$^{f/f;Nex\text{-}Cre}$ (GluCB1$^{-/-}$) and littermate CB1$^{f/f}$ controls (GluCB1$^{+/+}$). Experimental animals were genotyped by PCR, using primers G50 (5'-GCT GTC TCT GGT CCT CTT AAA-3'); G51 (5'-GGT GTC ACC TCT GAA AAC AGA-3'); G53 (5'-CTC CTG TAT GCC ATA GCT

CTT-3'); G100 (5'-CGG CAT GGT GCA AGT TGA ATA-3') and G101 (5'-GCG ATC GCT ATT TTC CAT GAG-3').

GABA-CB1$^{-/-}$ mice

Mice with conditional deletion of CB1 receptor on GABAergic neurons were obtained as described previously (Monory et al, 2006). Briefly, transgenic mice (Dlx5/6-Cre; where Dlx5/6 is expressed in GABAergic neurons) were produced and then crossed with *CB1*$^{f/f}$ mice to obtain *CB1*$^{f/f;Dlx5/6\text{-}Cre}$ (GABACB1$^{-/-}$) and littermate CB1$^{f/f}$ controls (GABACB1$^{+/+}$). Experimental animals were genotyped by PCR, using primers G50 (5'-GCT GTC TCT GGT CCT CTT AAA-3'); G51 (5'-GGT GTC ACC TCT GAA AAC AGA-3'); G53 (5'-CTC CTG TAT GCC ATA GCT CTT-3'); G100 (5'-CGG CAT GGT GCA AGT TGA ATA-3') and G101 (5'-GCG ATC GCT ATT TTC CAT GAG-3').

D1-CB1$^{-/-}$ mice

Mice with conditional deletion of CB1 receptor on neurons expressing dopaminergic D1 receptors were obtained as described previously (Monory et al, 2007). The D1CB1$^{-/-}$ line was generated by crossing CB1$^{f/f}$ mice with dopamine receptor D1-Cre line, in which the Cre recombinase was placed under the control of the dopamine receptor D1A gene *(Drd1a)* regulatory sequences (Lemberger et al, 2007). Experimental animals were genotyped by PCR, using primers G50 (5'-GCT GTC TCT GGT CCT CTT AAA-3'); G51 (5'-GGT GTC ACC TCT GAA AAC AGA-3'); G53 (5'-CTC CTG TAT GCC ATA GCT CTT-3'); G100 (5'-CGG CAT GGT GCA AGT TGA ATA-3') and G101 (5'-GCG ATC GCT ATT TTC CAT GAG-3').

CB1$^{f/f}$ mice

CB1$^{f/f}$ mice were obtained by crossing mice carrying the CB1-floxed-neo allele with flipase deleter mice, carrying the germ-line expression of the recombinase flipase in order to delete the FRT-PGK-Neo selection cassette. Experimental animals were genotyped by PCR, using primers G50 (5'-GCT GTC TCT GGT CCT CTT AAA-3'); G51 (5'-GGT GTC ACC TCT GAA AAC AGA-3') and G53 (5'-CTC CTG TAT GCC ATA GCT CTT-3').

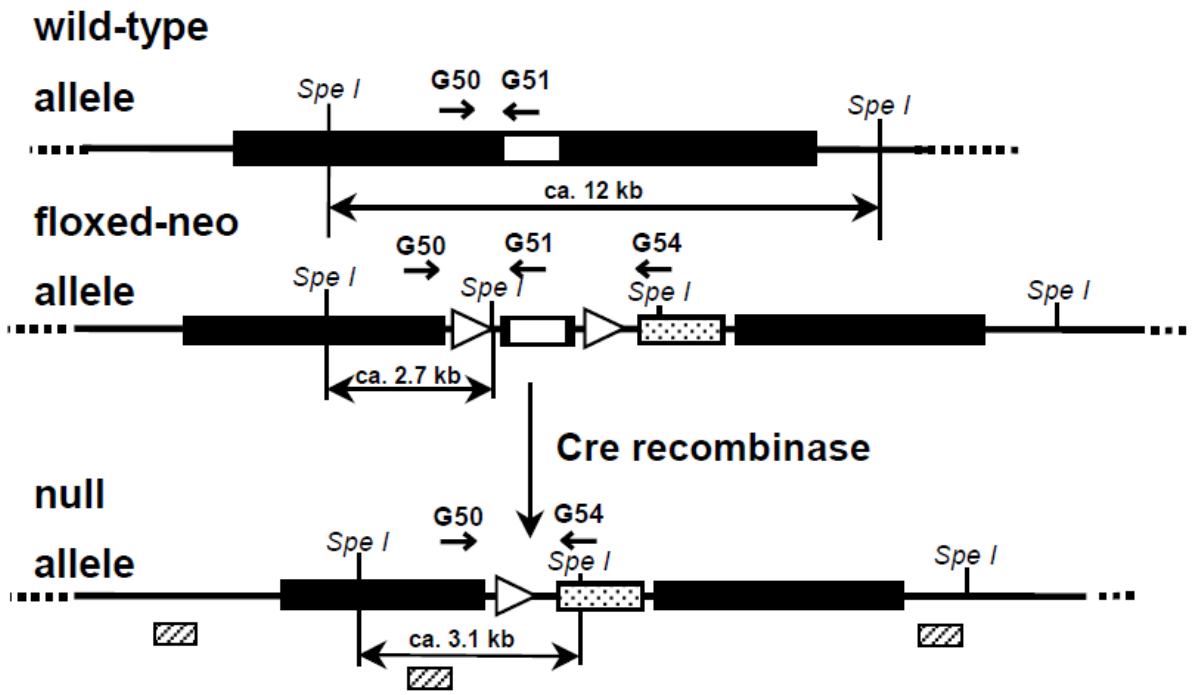

Figure 2. **Schematic representation of the CB1 receptor allele**. Generation of CB1-deficient mice (homozygous CB1null/null, called CB1-/-).Wild-type, floxed-neo and null allele of CB1, respectively. Black boxes, homology arms; white box, CB1 open reading frame; stippled box, PGK-neo cassette; white triangles, loxP sites; dashed bars, probes used for Southern blot experiments; small arrows, primers for PCR analyses (Adapted from Marsicano et al, 2002)

3.2 Drugs

Rimonabant [SR141716A; N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; Cayman Chemical, USA] was dissolved in vehicle solution (one drop Tween-80; 2.5% dimethylsulfoxide in saline) to a final concentration of 3 mg/kg and injected i.p. at 10 ml/kg body weight, 30 minutes before testing. ß-estradiol (Sigma-Aldrich, Germany) was dissolved in 0.5% ethanol : 99.5% propyleneglycol for administration via micro-osmotic pumps [model 1002, pumping rate: 0.25µL/hr, total volume: 90±10µL; Alzet, USA] to final concentration of 36mg/mL (Ström et al, 2012)

3.3 Behavioral Tests

Specifically for the D1CB1 mice, to minimize the use of animal, they were submitted to a battery of behavioral tests, which was divided in three main categories, in the following order: (1) low- or mild-stress situation (a) exploratory-based approach avoidance conflict tests: open field, elevated plus maze, light/dark, novel object investigation, and novelty- induced grooming (b) depressive-like behavior paradigms: sucrose consumption and forced swim test (2) social approach: social interaction and social investigation test (3) high-stress situation: fear conditioning tests. The order of tests within the battery was designed in such manner that mice would be evaluated on what were thought to be least invasive tests before being tested on more invasive assays. This design was developed with the assumptions that testing from least to most invasive would allow for recovery time (4-5 days) between tests and would reduce the likelihood that behavioral responses would be influenced by previous testing experience.

3.3.1 Anxiety- and fear-like behavioral tests

Animal tests of anxiety and fear are based on innate animal responses or in leaned association with aversive conditions. Many anxiety-like tests are based on exploratory 'approach–avoidance' tasks. They take into account that mice are naturally exploratory species, and exploration-based tasks exploit the conflicting tendencies to approach novelty versus avoid a potentially dangerous area. The aversive area takes different forms in different tests: open, elevated arms (elevated plus-maze), a light compartment (light/dark test) the central area of a brightly lit open field (open field test). The second sort of test is based on learned associations between a neutral stimulus or area and a harmful stimulus, such as a footshock, whereas subsequent presentation of the before-neutral stimulus induces aversive responses even when the harmful stimulus is not presents, as observed in the fear conditioning test. As different tests may not address ethological significance, it is important to submit animals to a battery of test that includes several characteristic of anxiety-like behavior.

Open field test

Exploratory and locomotor activity of D1CB1$^{-/-}$ and control littermate mice was evaluated in the open field (OF) test. The experiment was performed in a squared box (26 cm × 26 cm), in which the animal was placed in the central zone of the apparatus equipped with infrared beams (TruScan; Coulbourn Instruments, Allentown, PA, USA) and allowed to explore for 30 min at 300 lux. All sensor rings were connected via interface to a computer equipped with TruScan Software Version 99 (Coulbourn Instruments). Boxes and sensor rings (infrared beam spacing is 0.76cm) were surrounded by an additional box made of opaque Plexiglas side walls (47 cm × 47 cm × 38 cm) without roof and floor. Horizontal locomotion (total, margin, or central distance moved) vertical movements (exploratory rearing) and time spent at rest

were analyzed during the 30-min monitoring period with a sampling rate of 4 Hz. After each session, the apparatus was cleaned with a solution containing neutral soap.

Elevated plus maze test

The apparatus consisted of two opposite open arms, (30 cm × 5 cm) and two arms with walls (30 cm × 5 cm × 14 cm) that were attached to a central platform (5 cm × 5 cm) to form a cross. The maze was elevated 50 cm from the floor. Illumination measured at the center of the maze was 300 lux. The animal was placed in the center of the maze facing one of the closed arms, and observed for 5 min, according to the following parameters: number of entries in the open or closed arms and time of permanence in each arm (i.e., the time spent by the animal in the open or closed arms). An entry was defined as all four paws having crossed the line between an arm and the central area. It is accepted that the anxiolytic effect of a given treatment/condition is illustrated by increased parameters in open arms (time and/or number of entries). The augmented percentage of entries in open arms over the total entries in both arms is a good indicator of reduced anxious-like phenotype as well. Entries in closed arms and total entries reflect the motor component of the exploratory activity. In the end of each session, the maze floor was carefully wiped with a wet towel. All trials were recorded and scored off-line by an experienced observer blind to the genotype, using ANY-MAZE (Stoelting).

Novelty-induced grooming test (NGT)

The mice were placed individually into a clean unfamiliar Plexiglass box (27 cm × 16 cm × 12 cm) without bedding for 10 min. Three ethological measures of grooming activity were scored: latency to start grooming, grooming episodes (washing, general grooming, scratching,

licking of paws, or genital grooming), and total time spent grooming. All trials were recorded for subsequent video analysis.

Light/dark test

The light-dark (LD) box was divided in two compartments: (1) one dark compartment (15 cm × 20 cm × 38 cm) with black walls and (2) one lit compartment (30 cm × 20 cm × 38 cm) with white plastic walls. Both compartments were connected by a 4-cm long tunnel. Light intensity was 600 lux in the light compartment and 15 lux in the dark compartment measured at floor level. Mice were placed into the corner of the dark compartment at the start of the experiment which lasted for 5 min. After each test, the LD box was thoroughly cleaned with soap and water. Entries and time spent in the light compartment were assessed by video analysis by a trained observer blind to the genotype. These two variables were expressed as percentage of the total observation period and the total number of LD transitions, respectively. Videos were analyzed using ANY-MAZE (Stoelting).

Novel object investigation test

The novel object investigation (NOI) test was performed at 30 lux (which provides low aversiveness to the environment, and still allowed the assessment of exploration of the objects) for 10 min. Experimental subjects were habituated to the test arena (36 cm × 22 cm × 14 cm, with sawdust bedding material and transparent walls) for 2 days for 10 min (one cage per mouse without cleaning or changing of bedding). On the third day, mice were transferred into the same test cages and two identical objects (cone made of aluminum: ∅ 6 cm + H 13 cm) were placed in a symmetrical position at the short walls of the cages. Between animals, objects were thoroughly cleaned with water containing detergent to eliminate olfactory cues. Objects were heavy enough that a mouse could not displace them. Every trial was video

recorded and analyzed using ANY-MAZE (Stoelting). Investigation was defined as follows: directing the nose toward the object at a distance of not more than 2 cm and/or touching the object with the nose and paws (Jacob et al., 2009).

Fear conditioning task

For the fear conditioning, two different protocols were used. The first experiment was performed in two contexts: (1) the shock context – a cubic-shaped box with a metal grid for shock application – and (2) the neutral test context – a cylinder made of transparent Plexiglas, lined with wood bedding (Kamprath and Wotjak, 2004). For conditioning (d0), mice were placed in the conditioning context. Three minutes later, a tone (80 dB, 9 kHz sine-wave, 10 ms rising, and falling time) was presented to the animals for 20 s that co-terminated with a 2-s scrambled electric footshock of 0.7 mA. Mice were returned to their home cages 60 s later. On day 1 (d1), mice were exposed to the neutral context and on day 2 (d2) to the shock context for 7 and 3 min, respectively. To test the freezing response to the tone, mice were placed in the neutral context, which differed from the conditioning context in material, shape, surface texture, and odor of the cleaning solution (Kamprath and Wotjak, 2004). After an initial 3 min of habituation, a 180-s permanent tone [9 kHz, 80 dB, sine-wave] was delivered. To test the contextual freezing, animals were re-exposed to the shock chamber for 3 min without tone presentation and without further shock presentation, and immediately returned to their home cages afterward. In the second experiment, mice were conditioned as described for the first experiment. On day 1 (d1) and on day 7 (d7), mice were exposed to the 180-s tone in the neutral test context. Animals' behavior was video recorded and analyzed off-line by a trained observer blind to the groups (EVENTLOG software, designed by Robert Henderson, 1986). Freezing behavior was defined as immobility except for respiration movements.

3.3.2 Depression-like behavior tests

Animal models and tests for assessing depression-related behavior in rodents usually involve exposure to stressful situations. Of these experimental procedures, the forced swim test (FST) (also known as Porsolt's test; a behavioral despair test) is probably the most widely and most frequently. It is based on the observation that rodents placed in an enclosed filled with water will initially engage in vigorous escape-orientated movements, but when they realized that it is not possible to escape from this situation, they exhibit increasing bouts of immobility, as if they are "giving up the fight". Another phenotype measured is anhedonia (loss of interest in normally pleasurable and rewarding activities), that can be assessed by the simple preference for highly palatable solution, such as sucrose, over water.

Forced swim test

In the forced swim test (FST; Porsolt et al., 1978), mice were individually placed into transparent cylinders (height 23.5 cm; diameter 16.5 cm) containing 15 cm water at 25 ± 1°C for 6 min. The water was changed after each trial. After vigorous activity, swimming attempts cease and the animal adopts a characteristic immobile posture. A mouse is considered immobile when it floats in upright position and makes only small movements to keep its head above water. The duration of mobility was recorded during the last 4-min of the 6-min testing period. All trials were recorded and scored off-line by an experienced observer blind to the genotype.

Sucrose consumption test

During this test, mice are given a free choice between two bottles for 10 h – one filled with 2.5% sucrose solution and the other with tap water – for two consecutive days (Strekalova and Steinbusch, 2010). To prevent possible effects of side preference in drinking behavior, the

bottles position was switched in the mid-point of testing. Animals were not food or water-deprived before the test. For habituation, 1 day prior to the first testing day, animals were allowed to drink a 2.5% sucrose solution for 2 h. The consumption in water, sucrose solution, and total intake of liquids were estimated simultaneously in the both groups by weighing the bottles before and after each trial. The preference for sucrose was calculated as a percentage of the consumed sucrose solution from the total amount of liquid drunk, by the formula: Sucrose Preference = V(Sucrose solution)/[V(Sucrose solution)+V(Water)]×100%.

3.3.3 Social-related tests

For these experiments, two different approaches were selected. In the first one, bidirectional free-contact was allowed (social interaction test), meaning that regardless of which animal initiated the contact there was some interaction between them. On the second task, only the test subject was able of initiating contact (social investigation test), which allowed a more specific measured of the social intention.

Social interaction test

Experiments were performed in a new cage (27 cm × 16 cm × 12 cm) with fresh bedding at 5 lux (i.e., red light). The wall of the cage was extended by 12.5 cm with semi transparent plastic. In this test, pairs of unfamiliar mice were placed into the cage for 5 min. Depending on the test session, each test subject was paired either with an ovariectomized female (OVX); an ovariectomized female with hormonal replacement (OVX + E); or a male mouse of the same genotype. The time spent in social interactions (SI; active contact such as sniffing, licking, close following, and grooming) and aggressive behavior (fighting and biting, causing injury) was recorded for each pair of mice. Each session was video recorded and analyzed off-line using ANY-MAZE (Stoelting).

Social investigation test

Social investigation (SInv) was performed inside a rectangular box made of white PVC walls and with a dark gray PVC floor. The box was divided into three equal compartments (30 cm × 30 cm × 30 cm) that were interconnected by small opening (6 cm × 5 cm) with guillotine doors. Each animal was allowed to free exploration of the apparatus for 10 min (habituation). An empty perforated 50 ml falcon tube (length: 11.4; diameter: 2.8 cm^2; with 22 holes of 0.5 cm diameter to provide ventilation and olfactory cues to the test animals) was placed in each side of the box. This 10 min exposure was designed to familiarize the test subject with the box environment. After habituation session, the animal was kept in the center compartment and one of the tubes was replaced by a tube containing either an ovariectomized female (OVX); an ovariectomized female with hormonal replacement (OVX + E); or male mice. For the next 10 min test session, the mouse was allowed to explore all three compartments and the time spent in contact with the tubes (active contact such as sniffing) was evaluated. All trials were recorded and scored off-line by an experienced observer blind to the genotype.

3.4 Statistical Analysis

Data were analyzed using unpaired *t*-test or two-way ANOVA. Newman–Keuls test was used as *post hoc* test, if appropriate. Data are presented as mean ± SEM. Statistical significance was accepted if $p < 0.05$.

4 RESULTS

In this first part, it is shown the role of CB1 receptor signaling in the dopamine receptor D1-expressing neurons on emotional behavior. D1CB1 mice underwent a test battery to access different behaviors, which are considered to be related to anxiety, depression and fear-responses. All results will be discussed separately in the following sections.

ANXIETY-LIKE BEHAVIOR

Open field test

In the open field test, there was no difference in the exploratory activity between D1CB1$^{-/-}$ and control group (Figures 3A–F). Both groups showed the same horizontal activity (n= 6-9; total distance: $t = 1.246$; $p = 0.2348$; central distance: $t = 1.501$; $p = 0.1574$, margin distance: $t = 0.2401$; $p = 0.8140$), total duration of movement ($t = 1.217$; $p = 0.2452$), rearing ($t = 1.715$; $p = 0.1101$), and jumping episodes ($t = 1.344$; $p = 0.2021$). This response indicates that in our test conditions, genetic deletion of CB1 in neurons expressing D1 receptors did not alter basal locomotor activity of mice.

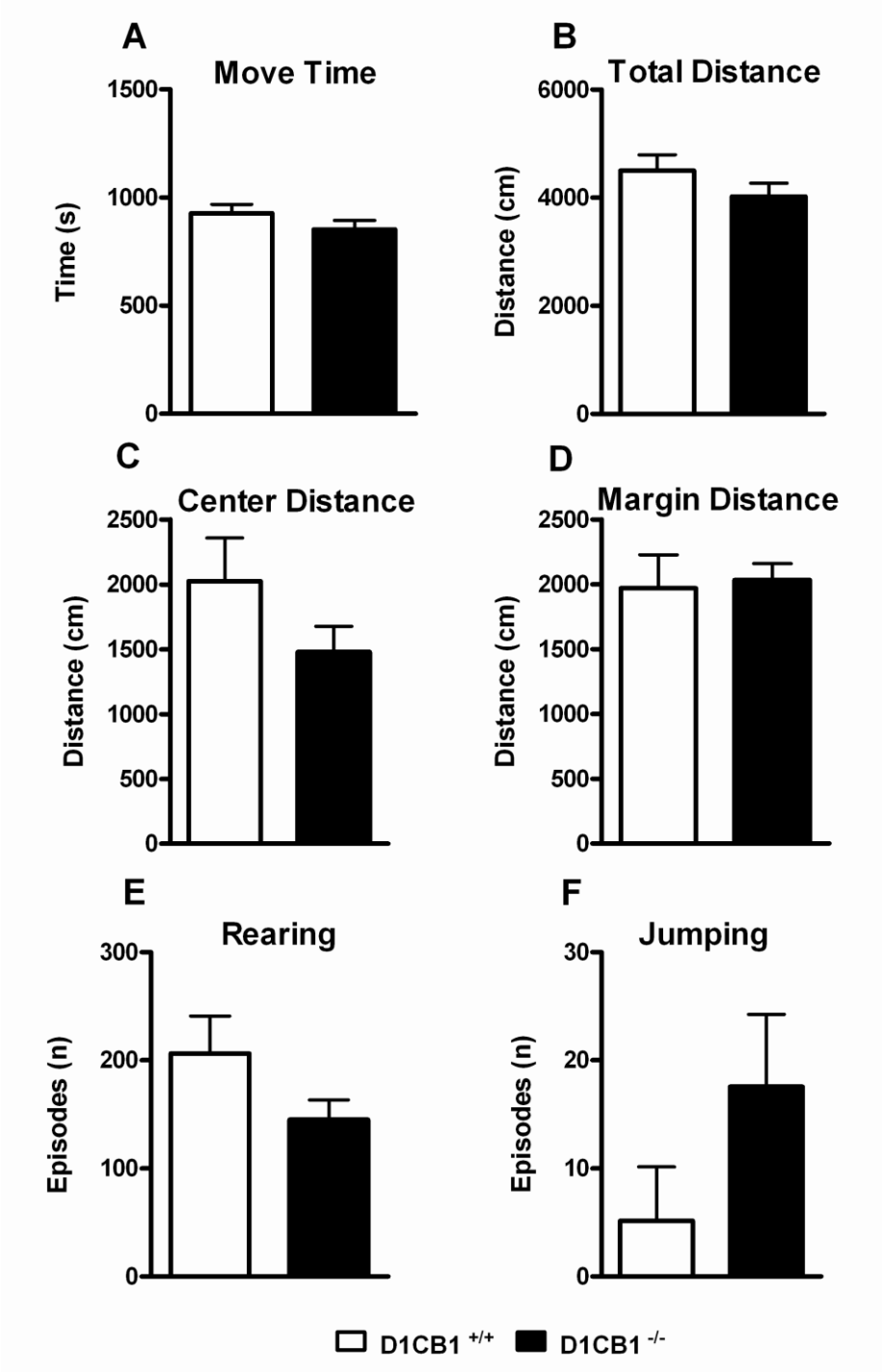

Figure 3. Assessment of general locomotor activity of D1CB1$^{-/-}$ mice in the open field (OF) test. Conditional D1CB1$^{-/-}$ mutant mice and their respective control littermates were tested in an open field for 30 min (n= 6-9). Values are mean ± SEM in terms of horizontal activity **(A–D)** rearing **(E)** and jumping episodes **(F)**.

Elevated plus maze and light/dark test

As described in Figures 4A–D, statistical analysis did not reveal any significant difference between D1CB1$^{-/-}$ and control group both in the time spent (n=7-8; t = 0.5568; p = 0.5871) or in the number of entries (t = 0.6133; df = 14; p = 0.5502) into open arms of the EPM test. Also, there was no difference in the time spent (n= 8-9; t = 0.2827; p = 0.7813) or in number of entries (t = 0.9739; df = 15; p = 0.3430) into light compartment of the LD test. No locomotion difference was found, considering the total arm entries (t = 0.7276; p = 0.4798) and the total LD transitions (t = 0.8154; p = 0.4255) as index.

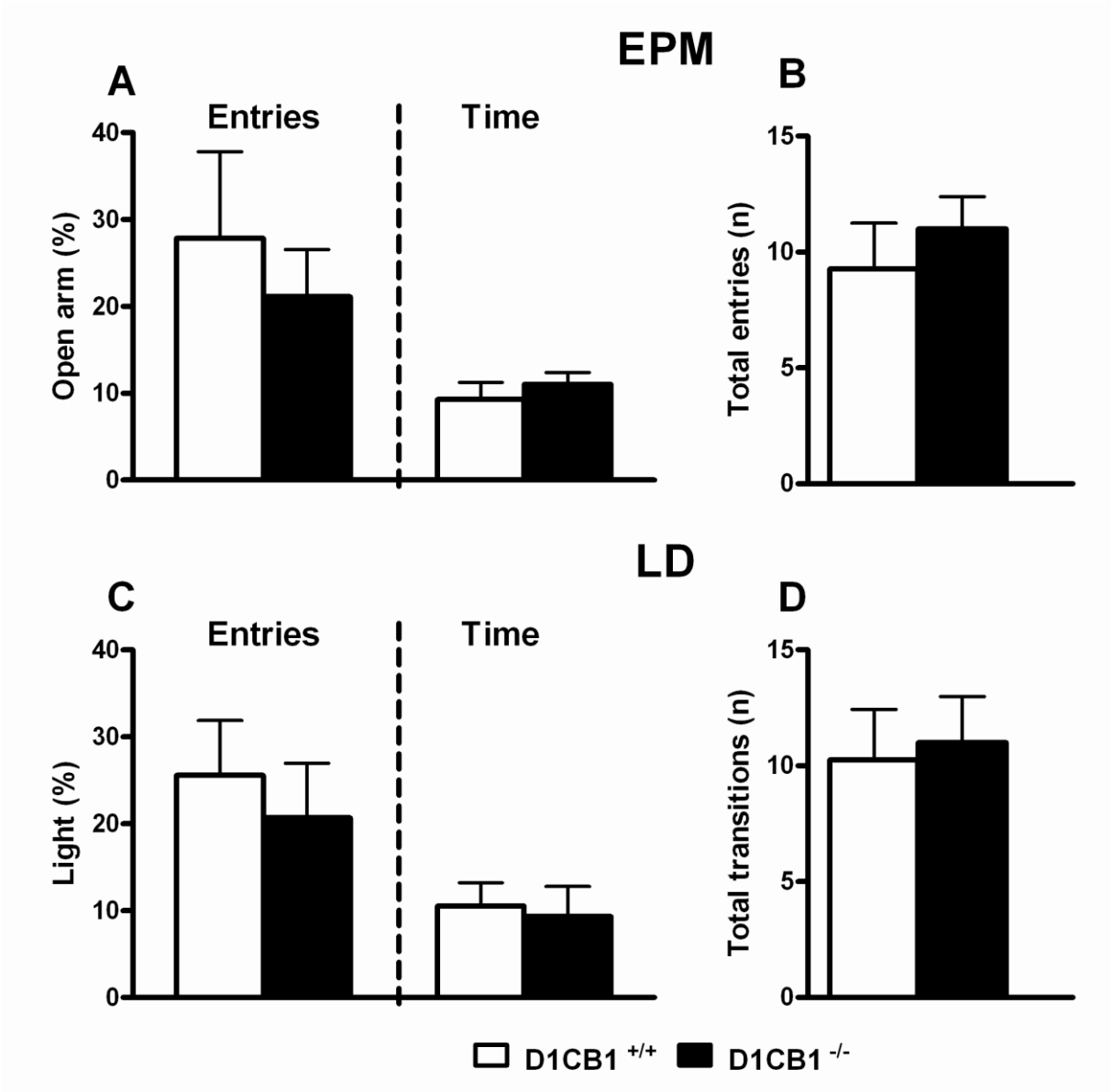

Figure 4. Anxiety-like behaviors of D1CB1$^{-/-}$mice. Conditional D1CB1$^{-/-}$ mutant mice and their respective control littermates were tested for 5 min in the elevated plus maze (upper panel; n=7-8) or in the light/dark box (lower panel; n= 8-9). Data are presented as mean ± SEM regarding open arm entries and open arm time **(A)** the total arm entries**(B)**, light compartment entries and light compartment time **(C)**, and total compartment transitions **(D)**.

Novel object investigation

Unpaired *t*-test showed that D1CB1$^{-/-}$ and control group, during the 10-min test, spent the same amount of time investigating the pair of novel objects (n=8-10; $t = 0.5887$; $p = 0.5643$), as well as they approached them with the same frequency ($t = 0.5705$; $p = 0.5762$; Figure 5).

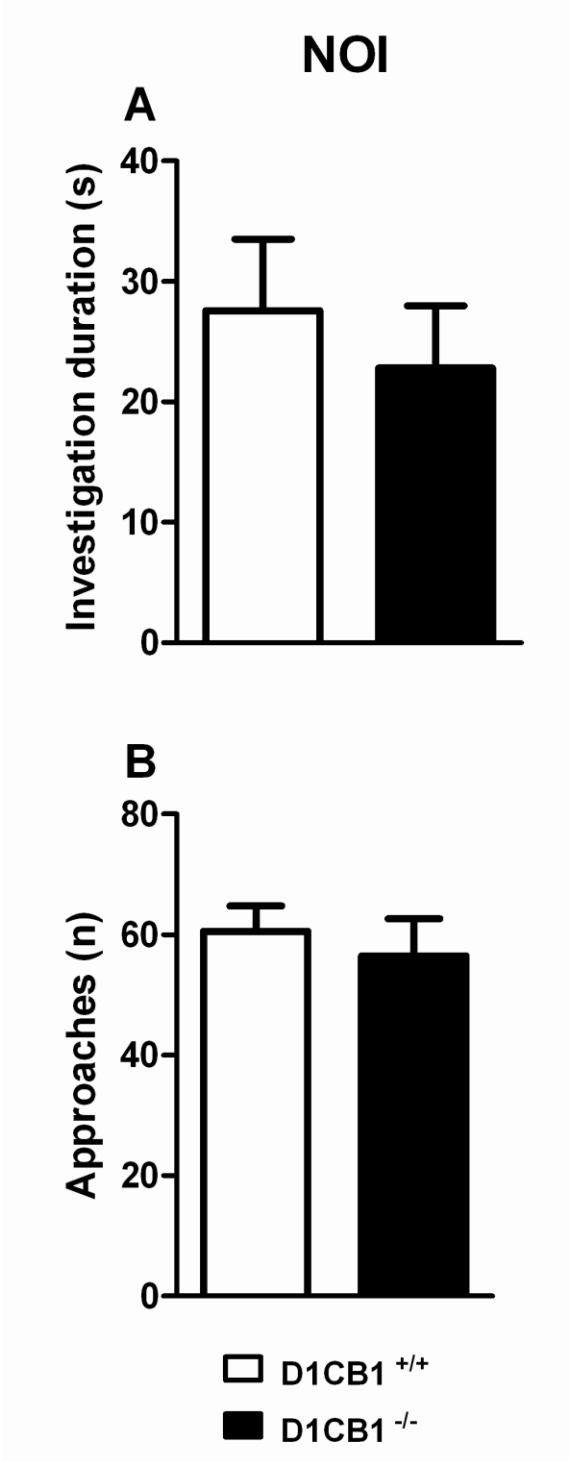

Figure 5. Novel object investigation test (NOI). Conditional D1CB1$^{-/-}$ mutant mice and their respective control littermates were exposed to two novel objects for 10 min (n=8-10). Data are presented as mean ± SEM regarding total investigation duration **(A)** and number of approaches **(B)**.

Novelty-induced grooming activity test

As described in Figures 6A–C, D1CB1$^{-/-}$ mice performed more grooming episodes (n=8-9; $t = 2.240$; $p < 0.05$) as well as they spent more time grooming as compared to control animals ($t = 2.568$; $p < 0.05$). However, the latency to start grooming was not significantly different between the two groups ($t = 1.170$; $p = 0.2603$).

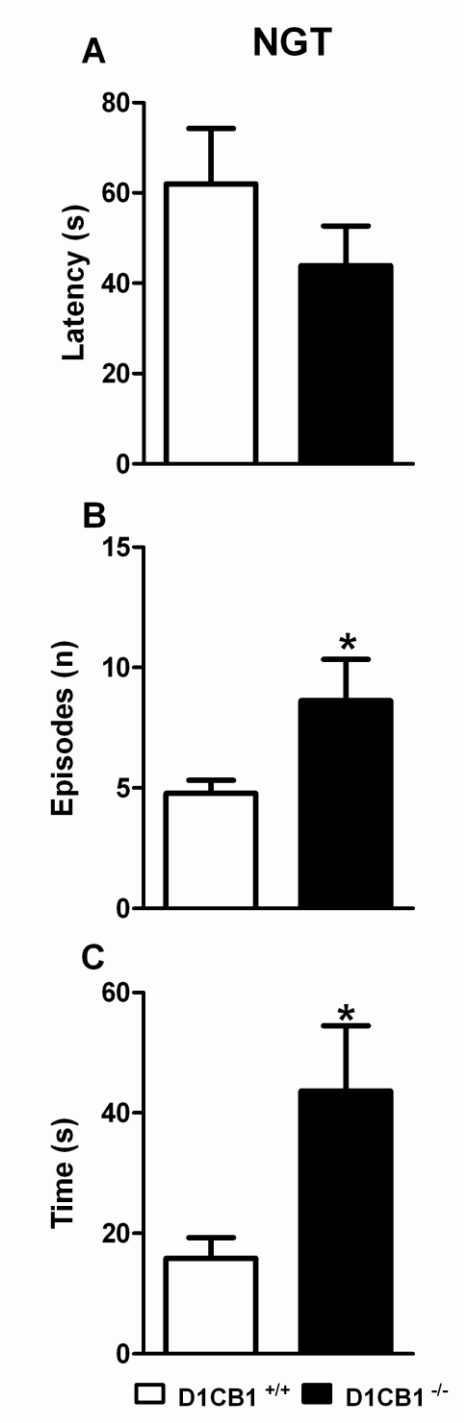

Figure 6. Novelty-induced grooming behavior in D1CB1$^{-/-}$mice (NGT). Conditional D1CB1$^{-/-}$ mutant mice and their respective control littermates were tested for the grooming activity measure (n=8-9). Data are presented as mean ± SEM regarding latency to start grooming **(A)**, number of grooming episodes **(B)** and total grooming duration **(C)**. *$p < 0.05$ as compared to WT mice (unpaired *t*-test).

DEPRESSIVE-LIKE BEHAVIOR

Forced swim test and sucrose consumption

As described in Figure 7, D1CB1$^{-/-}$ mice showed a significant lower sucrose consumption as compared to control group on the first (n=10; t = 2.868; p < 0.05), but not on the second testing day (t = 0.3575; p = 0.7249). Both, D1CB1$^{+/+}$ and D1CB1$^{-/-}$ mice, showed a high percentage of sucrose consumption as compared to the total amount of liquid consumed. In the FST, although D1CB1$^{-/-}$ mice showed a decrease in the mobility as compared to control animals, the difference between the two genotypes did not reach statistical significance (n=7-9; t = 1.904; p = 0.0777).

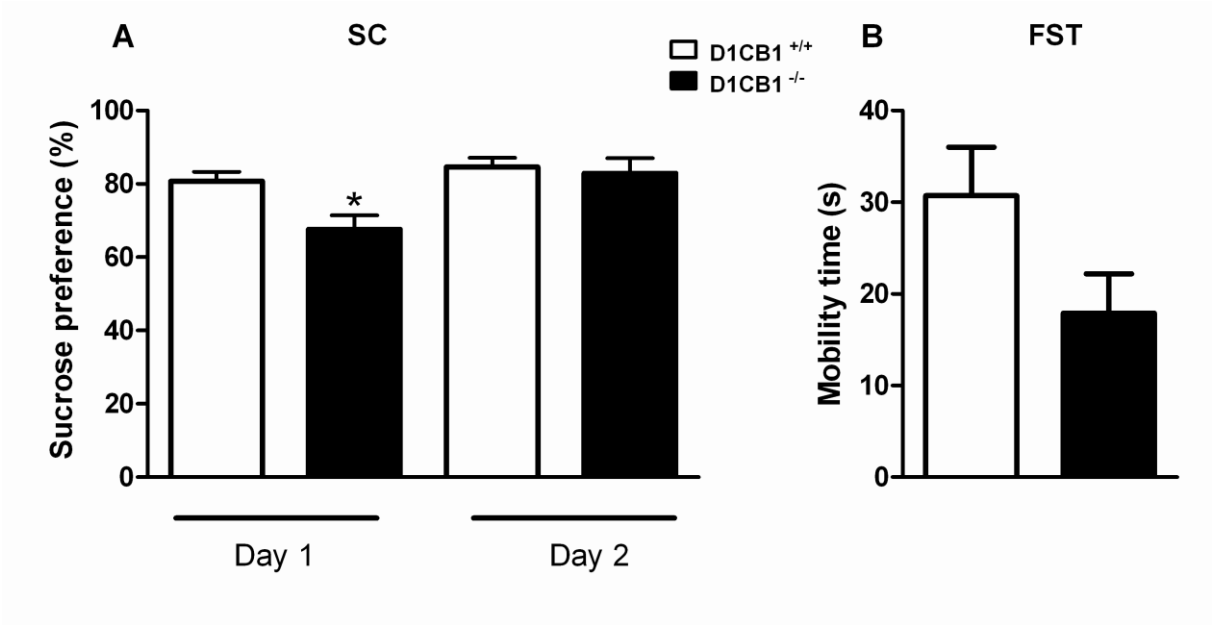

Figure 7. Depressive-like behaviors of D1CB1$^{-/-}$ mice. Conditional D1CB1$^{-/-}$ mutant mice and their respective control littermates were tested in the sucrose consumption (SC; n=10) test **(A)** or in the forced swim test (FST; n=7-9) paradigm **(B)**. Data are presented as mean ± SEM regarding percentage of sucrose consumption or mobility time expressed in seconds. *$p < 0.05$ as compared to WT mice (unpaired *t*-test).

FEAR-LIKE BEHAVIOR

Fear conditioning

As shown in Figure 8A, unpaired *t*-test revealed that D1CB1$^{-/-}$ showed a significant increase on freezing response to the tone at day 1 (n= 8-9; $t = 2.497$; $p < 0.05$) and to the context at day 2 ($t = 3.210$; $p < 0.01$) as index of increased auditory-cued and contextual fear responses, respectively. When analyzed in 20-s intervals, all mice showed the same initial freezing response on day 1. However, whereas control animals showed a rapidly waning freezing response during the tone presentation, D1CB1$^{-/-}$ mice showed a deficit in acute fear adaptation (B). The second experiment, largely confirmed their phenotype (C, D): D1CB1$^{-/-}$ mice showed a significant increase on freezing response to the tone on day 1 ($t = 4.234$; $p < 0.001$) and on day 7 ($t = 2.923$; $p < 0.01$), which again results from impaired acute fear adaptation over the course of tone presentation (D). Freezing before tone presentation on day 1 was low and indistinguishable between the two groups.

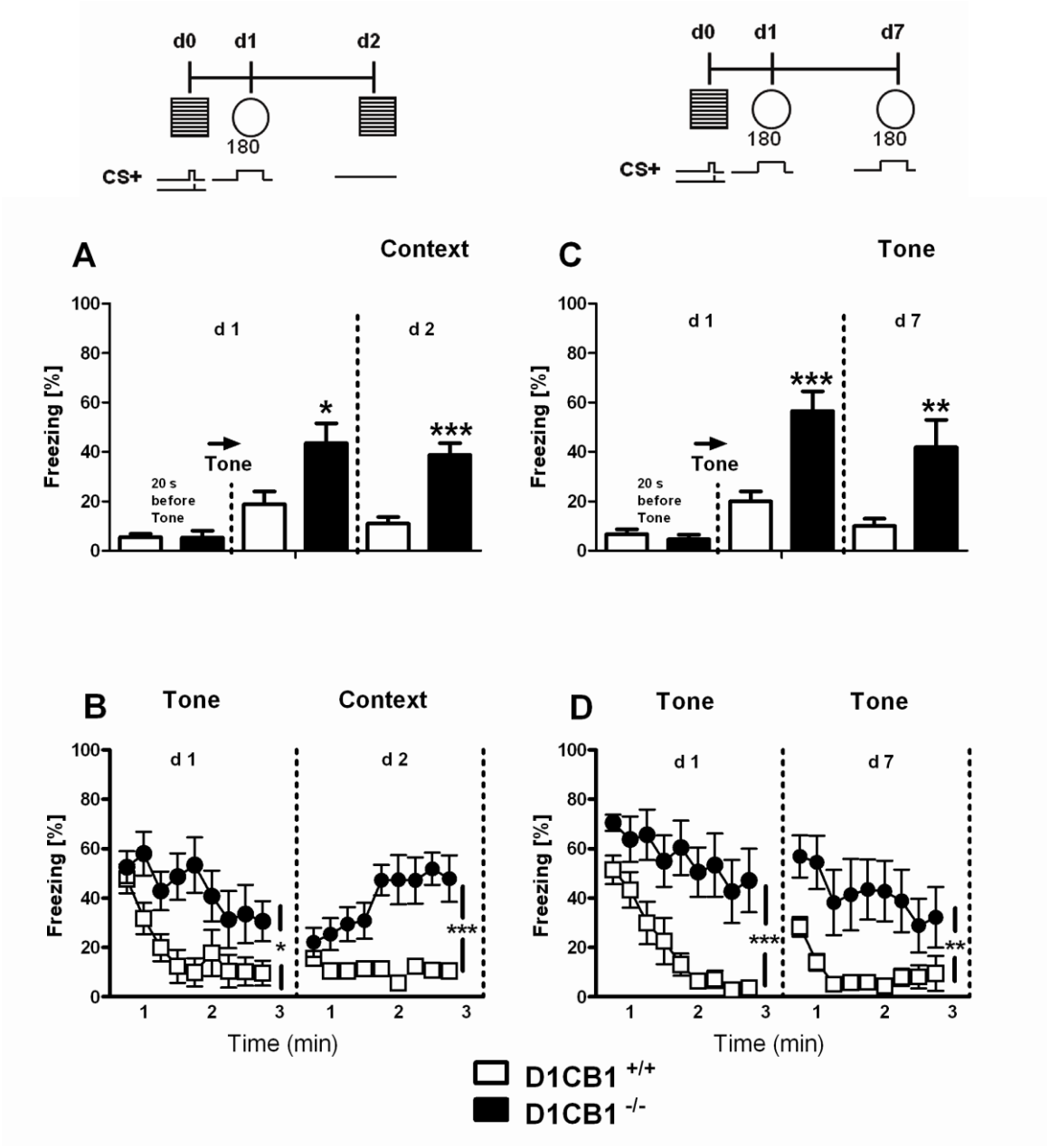

Figure 8. Fear memory in D1CB1$^{-/-}$ mice. Auditory-cued (Tone) and contextual (Context) fear memory assessed by freezing responses (mean ± SEM) of conditional D1CB1$^{-/-}$ mutant mice and their respective control littermates in two independent sets of experiments (A/B, C/D; n= 8-9)**.** If not stated otherwise, freezing was averaged over the entire 180 s observation periods (**A, C**) or analyzed in 20 s intervals (**B, D**)**.** **p* < 0.05, ***p* < 0.01, ****p*< 0.001 as compared to WT mice (ANOVA for repeated measures followed by Newman–Keuls post hoc test).

SOCIAL BEHAVIOR

In the second part of this thesis, it is presented results related to the role of CB1 receptor localized in different neuronal sub-populations in the modulation of social activity. The following results were separeted by social task, where animals were directly or indirectly allowed to interact with a male or ovariectomized female stimulus subjects.

Social Interaction

For the social activity, all lines were tested. In the social interaction test, when two free-moving male mice were confronted in the same cage, none of the tested lines presented differences in the contact time compared to their control group (Figure 9), even though GABACB1$^{-/-}$ showed slightly increased social interest (CB1: t=0.29; WT: t=1.20; GluCB1: t=1.15; GABACB1: t=2.00; D1CB1 : t=0.25; All p>0.05). Aggressive behavior was only observed in one pair of GluCB1$^{-/-}$ and one pair of GluCB1$^{+/+}$. Therefore, this parameter was not further considered. Still in the social interaction test, to verify if the two main opposite genotype (GluCB1 and GABACB1) and the D1CB1 would present differences in interaction when confronted with female subjects, males were introduced to ovariectomized females with or without ß-estradiol treatment.

In case of GluCB1, a 2-way ANOVA (genotype, stimulus subject) revealed significant main effects of genotype ($F_{1,28}$ = 30.72, p < 0.0001) and subject ($F_{2,25}$ = 26.91, p < 0.0001) as well as a significant genotype x subject interaction ($F_{2,25}$ = 8.563, p < 0.005). Post-hoc tests confirmed that GluCB1$^{-/-}$ showed less exploration of OVX females without or with estradiol replacement than control group, whereas exploration of male subjects was similar. Interestingly, GluCB1$^{+/+}$, but not KO, spent significantly more time investigating the females

than the males. For the GABACB1, it was observed significant main effects of genotype ($F_{1,44}$ = 8.160, $p < 0.01$) and subject ($F_{2,41}$ = 6.907, $p < 0.005$), but no significant genotype x subject interaction ($F_{2,41}$ = 0.8315, p = 0.442). Thus, GABACB1$^{-/-}$ spent more time in investigating social stimuli than GABACB1$^{+/+}$, irrespective of the sex of the stimulus subjects. D1CB1$^{-/-}$ it was observed significant main effects of stimulus subject ($F_{2,40}$ = 16.25 $p < 0.01$), but no significant effect of genotype ($F_{1,43}$ =0.56, p=0.46), nor genotype x subject interaction ($F_{2,40}$ = 1.42 p = 0.25).

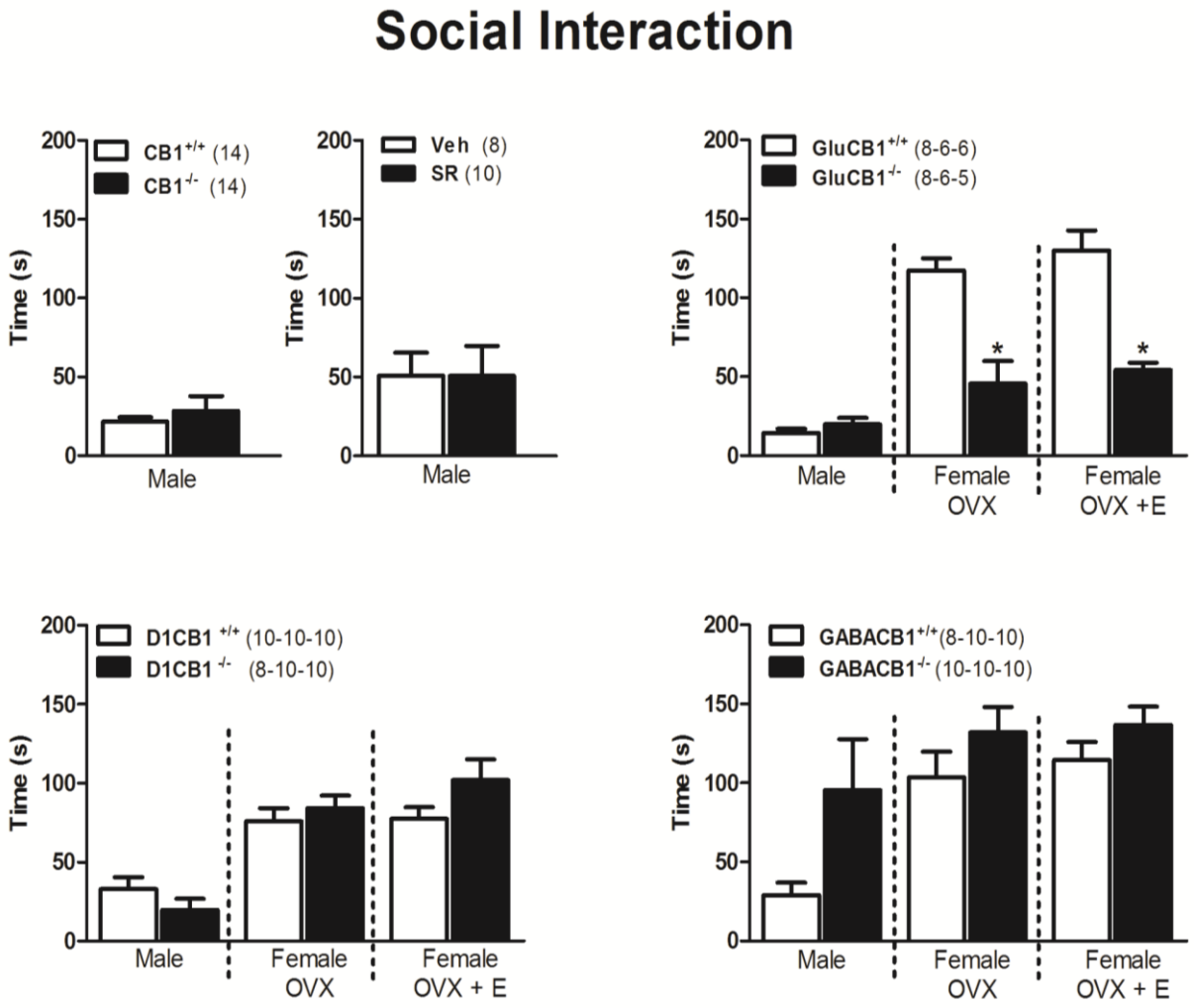

Figure 9. Social Interaction with a male, an OVX female or an OVX female treated with ß-estradiol. Effects of pharmacological blockage (SR141716) or genetic deletion of CB1 receptor on social interaction behavior between a pair of unfamiliar mice - male mice of the same genotype/pharmacological treatment, ovariectomized females (female OVX) or ovariectomized females with hormonal replacement (female OVX + E). Values are represented as means ± S.E.M. *$p< 0.05$.

Social Investigation

In the 3-chamber social investigation test, when the stimulus subject was a male mouse (Figure 10), $CB1^{-/-}$ mice presented a lower interest for the social stimulus relative to the control group, however in a non-significant manner (n=6-7; genotype: $F_{11,1}$=1.84; p=0.20; genotype x time in contact: $F_{11,1}$=3.24, p=0.09). Both groups showed preference for the tube containing the stimulus mouse compared to the empty one (time in contact: $F_{11,1}$= 36.16, p<0.001). Similarly, animals that received vehicle or SR141716 preferred to explore the tube containing the unknown mouse instead of the empty tube (time in contact: $F_{17,1}$=26.18, p<0.001). Yet, differently from the $CB1^{-/-}$, animals treated with the CB1 receptor blocker/inverse agonist exhibited a significant reduction in time spent investigating the social stimulus (n=9-10; genotype x time in contact: $F_{1,17}$=4.52; p<0.05). As observed for the $CB1^{-/-}$ animals, in the presence of a male mouse stimulus, $GluCB1^{-/-}$ showed no significant difference in investigation as compared to the control group (n=10; genotype x time in contact: $F_{1,18}$=0.07; p=0.93). The same applies to $GABACB1^{-/-}$ mice, (n=8-10; genotype x time in contact: $F_{1,16}$=0.60; p=0.45), and to $D1CB1^{-/-}$ (n=9-10; genotype x time in contact: $F_{1,17}$=1.30; p=0.27). All three mutant lines showed preference for the social stimulus when compared to the inanimate object.

In presence of a female stimulus subject (Figure 10), $CB1^{-/-}$ spent significantly less time in contact with the social stimulus compared to control littermates (n= 13-16; genotype x time in contact: $F_{1,27}$ = 7.17, p<0.05). WT mice treated with SR141716 also spent less time investigating the female stimulus compared to the vehicle group (n=6-7; genotype x time in contact: $F_{1,11}$ =6.53, p<0.05). The same applies to $GluCB1^{-/-}$ (n=8-10; genotype x time in contact: $F_{1,16}$=4.65; p<0.05). $GABACB1^{-/-}$, in contrast, showed the opposite behavior by spending significantly more time in contact with the female stimulus (n=11; genotype x time

in contact: $F_{1,20}$=16.05, p<0.01). Yet, D1CB1$^{-/-}$ investigated the OVX female with similar intensity as their control group (n=9-10; genotype x time in contact: $F_{1,17}$=1.85, p>0.05).

Again, to better understand the role of CB1 receptor on specific neuronal population, D1CB1, GABACB1 and GluCB1 mice were presented to a tube containing a female OVX + E. In this situation, GluCB1$^{-/-}$ showed reduced interest for the female tube, yet non-significant compared to the control group (n=6; genotype x time in contact: $F_{1,10}$=0.93, *p*=0.35), oppositely to the GluCB1$^{-/-}$, but in the same direction as previously with the OVX females, GABACB1$^{-/-}$ demonstrated increased investigation for the female treated with ß-estradiol (n=10; genotype x time in contact: $F_{1,19}$=5.23, *p*<0.05). While, D1CB1$^{-/-}$ showed for the first time a significant increase in time of investigation when compared to control group (n=9-10; genotype x time in contact: $F_{1,18}$=4.79, *p*<0.05).

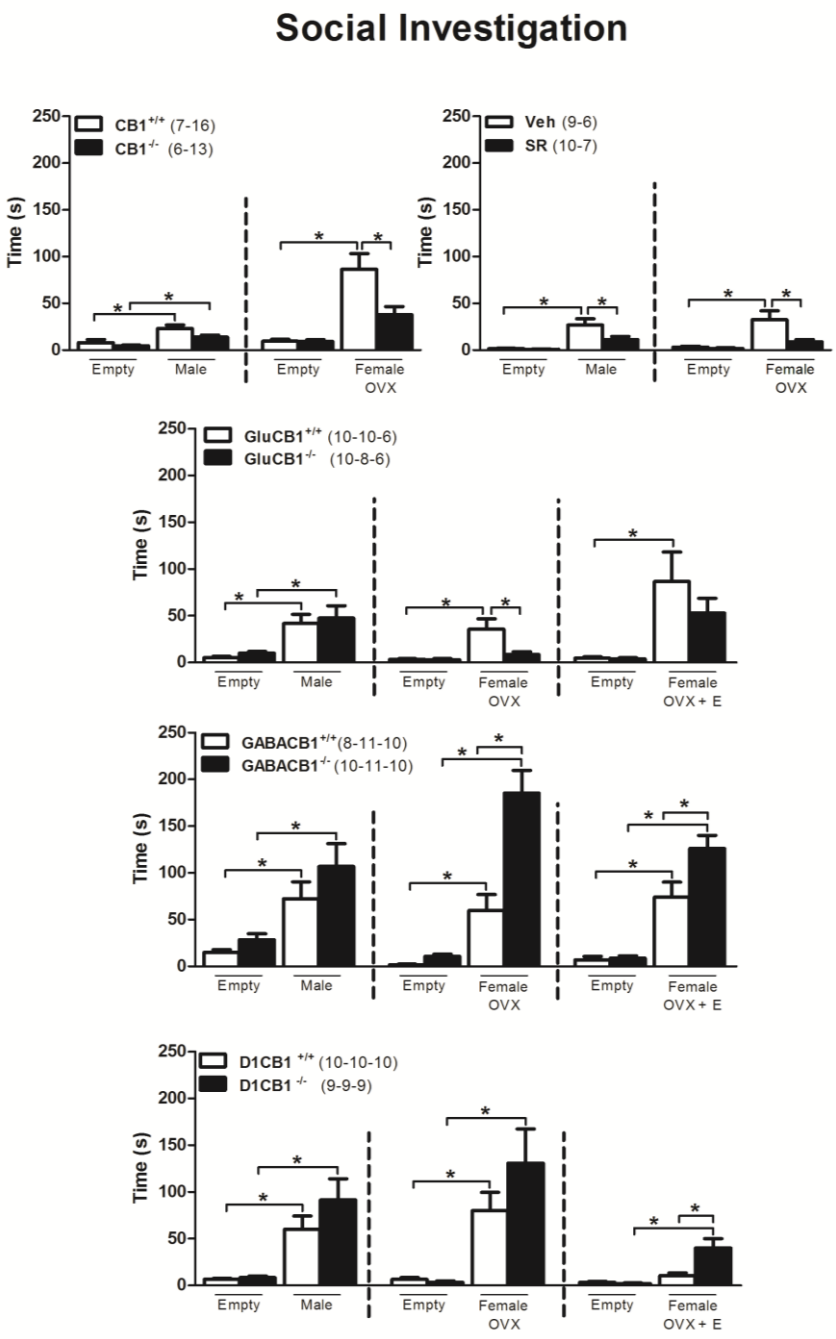

Figure 10. Social Investigation of social stimulus subject – Effects of pharmacological blockage (SR141716) or genetic deletion of CB1 receptor on social investigation of a male stimulus mouse; ovariectomized females (female OVX) or ovariectomized females with hormonal replacement (female OVX + E) in the 3-chamber box. Values are represented by means ± S.E.M. *$p< 0.05$; Newman–Keuls *post hoc* test.

5 DISCUSSION

The first half of the present study provide novel evidence that the genetic deletion of cannabinoids CB1 receptors in dopamine D1 receptor-expressing neurons is able to affect the emotional behavior in mice in highly selective manner. The development of conditional CB1 mutant mice lacking CB1 receptors specifically from neurons expressing D1 receptors (Monory et al., 2007) has been a useful tool to better understand the role of the endocannabinoid system in emotional behavior.

Firstly, it should be reminded that growing evidence indicates that measures of anxiety from different tests reflect different states of anxiety. This encouraged the usage of different behavioral paradigms such as exploration-based tests and learned fear paradigms, to assess different aspects related to several psychiatric disorders in humans, as agoraphobia, social phobia or post-traumatic stress disorder (Lister, 1990; File, 1993; Cryan and Holmes, 2005). Some of the tests used for this work primarily focus on interest to investigate novel environment, others emphasizes social interaction, while a third kind targeted situations that involve a strong mnemonic component, such as fear based tests. Even though animal studies allow the possibility to directly test the causal relationship between cannabinoid exposure and subsequent risk for several psychiatric disorders or to better understand the neurobiological background of certain conditions independent of subject-specific factors or previous individual history that confound human investigations, it presents some caveats. Animal studies do not mimic the complex nature of psychiatric disorder, thus only specific phenotypes relevant to a given disorders can be examined. Still, animal models should present characteristic that make them valid for use. Any model should be valid if shares structure with a behavior or pathology, which means that whenever a relation holds between an animal model, a correspondent relation should be seen in the modeled species, usually human

(Kaplan and Saccuzzo, 1997). This improves the reliability of the model and obtained results. It has been proposed that the validity of a model should be based on *face validity*, *predictive validity* and *construct validity* (Willner, 1986). Face validity has many advantages and can be easily verified, however, high emphasis on face validity could delay or even prevent the development of new animal models because it considers the similarity between the behavior observed in an non-human animal and the one observed in human, therefore the usage of such an anthropomorphic focus could be a barrier for the use of animals more distant from human in the phylogenetic scale (Willner 1986; van der Staay, 2006). Predictive validity considers the ability of a model to estimate a behavior outcome, which can be exclusively based on empirical evidences without understanding the behavior itself or even its biological background. Also, predictive validity is vastly used to evaluate pharmacological properties of certain compounds, especially to investigate antidepressant and anxiolytic properties of new drugs (Bourin et al, 2002, 2001; Epstein et al, 2006), however in some situations, predictive validity shows limitations due to the distinct pharmacodynamics and pharmacokinetic properties of each species. Construct validity refers to the theoretical aspects of what the model measures, and is consider the most important criterion for animal models. It is based on the similarity between the mechanisms underlying animal and human behavior, and requires an etiological relationship between the species. Thus, it is theory-driven, where behavioral, pathophysiological and neural components are taking into consideration; reflecting the degree in which the theory and the model explain the true nature of the symptoms or syndrome studied. Construct validity also defines a relationship between dependent and independent variables with the theoretical hypothesis tested (Lubow, 2005; van der Staay et al, 2009). It is easier to apply this concept in models that investigate "normal behavior" aspects, as in anxiety, that in different species it can be induced by a threatening stimulus, considering those that can produce disturbance in the subject's homeostasis. However, it becomes more difficult

when considering pathological processes, due to the unknown and complex nature of many disorders (Sarter and Bruno, 2002).

The first novel result of the present study was that D1CB1$^{-/-}$ mice did not show any anxiety-like phenotype when tested in exploratory behavioral paradigms such as elevated plus-maze, light-dark test or novel object investigation. These procedures mostly reflect the conflict between exploration and avoidance of a novel environment; thus, the inhibition of exploratory behavior observed by the reduced open arms or light compartment entries and novel object exploration is commonly associated with high emotionality or anxiety. D1CB1$^{-/-}$ mice also did not show any alteration in spontaneous exploration and locomotor behavior. These findings are in line with previous data showing no anxiety-like phenotype in mice with total CB1 receptors deletion and with specific CB1 receptors deletion on glutamatergic neurons (Marsicano et al., 2002; Lafenêtre et al, 2007 Jacob et al., 2009). However, the D1CB1$^{-/-}$ mice showed increased grooming activity. Grooming is considered a "maintenance" behavior, a common species-characteristic movement pattern with readily definable components (Bolles, 1960) that serves a range of adaptive functions, including stress reduction and social interplay (Kalueff and Tuohimaa, 2005). In rodents, spontaneous grooming behavior may occupy as much as 25–40% of the wakeful time, but it is specifically elicited in situations (i.e., novelty-induced grooming) where an animal is under a stress-induced conflict or frustration. It is also affected by pharmacological treatment, being reduced following anxiolytic treatment (Dunn et al., 1981; Gispen and Isaacson, 1981; van Erp et al., 1994; Micale et al., 2008). Thus, the present results suggest that if the exposure to novelty cannot be controlled by the animals, the novel environment is able to influence the emotionality of D1CB1$^{-/-}$ mice.

The D1CB1$^{-/-}$ mice exhibited a decreased preference for sweet solutions on the first but not on the second day of the sucrose preference test under basal conditions, indicating a mild

anhedonia-like state. Although anhedonia is commonly associated with depression-like behavior phenotype, mutant mice performed as controls in FST, a procedure widely used for evaluating behavioral despair in rodents (Cryan and Holmes, 2005). Thus, the modulation of depressive-like behaviors may be mediated by distinct neuronal circuits in D1CB1$^{-/-}$ mice evaluated in a task-specific or dependent manner. On the other hand, the lower sucrose preference test was only evident upon the first confrontation with the novel taste (day 1) and disappeared on the next day, suggesting a significant contribution of neophobia. Interestingly, D1CB1$^{-/-}$ mice showed sustained auditory-cued and contextual fear responses, thus resembling the phenotype of impaired fear adaptation observed in mice with complete deletion of CB1 receptors (Marsicano et al., 2002; Kamprath et al., 2006) or selective deletion from principal neurons of the forebrain (Kamprath et al., 2009). Since Monory et al. (2007) showed that the deletion of CB1 in D1-expressing neurons did not alter the analgesic effects of THC, it can be excluded the possibility that the phenotype of D1CB1$^{-/-}$ mice in the fear conditioning paradigms could be due to different nociceptive thresholds.

Currently, it is not precisely known the mechanism behind the CB1 receptors modulation of D1 receptors-mediated emotional behavior. However, due to their coexpression, it is tempting to assume that a direct or indirect receptor–receptor interaction, via intracellular signaling pathways, might be involved (Glass et al., 1997; Gangarossa et al., 2011). Previous studies showed D1 receptor encoding gene (*Drd1a*) associated with Cre-recombinase is robust in the major projections areas of the dopaminergic system, including striatum, nucleus accumbens, olfactory tubercles and prefrontal cortex. Lower expression was observed in layer VI of the cortex, hippocampus, thalamic and hypothalamic nuclei (Lemberger et al, 2007). To better study the role of D1 receptor in different pathologies, a mutant mouse line using used *Cre*-mediated recombination to ablate neurons expressing the *Drd1a* gene during development was developed (Drago et al, 1998; Wong et al, 2000; Padungchaichot et al, 2000); however

most animals died before completing 1 week. Those that survive until 3 weeks old displayed hyperkinetic syndrome with gait abnormality, as observed in basal ganglia diseases. Recently, some studies use a *Cre*-mediated strategy to ablate *Drd1a*-expressing neurons after birth.These animals present a better survival rate and showed a relatively mild distonia phenotype (Gantois et al, 2007).

Still in the hypothesis of interaction between D1 and CB1 receptors, Martín et al. (2008) showed in rats that pharmacological CB1 receptors blockade or activation could facilitate or inhibit animal behavior, respectively; and this latter effect was absent in D1 receptors knock-out mice, demonstrating a D1 receptors dependence on CB1-mediated actions. Thus, in the highly aversive situations of fear conditioning paradigms, where a strong stimulus as a footshock was delivered, the endocannabinoid signaling failed. This happens due to the CB1 deletion that negatively modulates the D1 receptors emotional-related behavior, leading to an impaired fear adaptation.

In the second part of the present study, it was evaluated the involvement of CB1 receptors in social behavior in mice. For that purpose, direct contact between two animals was investigated, with the attempt to create a more ethological situation. Given previous reports about social interaction after pharmacological blockage or genetic deletion of CB1 receptor (Jacob et al, 2009; Häring et al, 2011, Litvin et al 2013) it was surprising not to observe any treatment / genotype differences. One explanation for the discrepant findings might be the previously observed interaction between aversiveness of the test situation and involvement of CB1 receptors signaling in social behavior (Jacob et al, 2009). In the present study the emotional load was maintained at a minimum by using less aversive conditions like red light. Higher light intensity, however, would have confounded our interpretation of “social intention” by differences in anxiety levels (Jacob et al, 2009). Also, these experiments, for all mutant lines and treatment, failed to observed differences in aggression, differently than

reported for the resident-intruder test where GluCB1$^{-/-}$ showed increased aggression (Häring et al, 2011) – mice did not show any territorial / aggressive behavior in the novel environment, independent of the genotype. While testing the speficic mutante mice lines (D1CB1, GABACB1 and GluCB1) on direct contact with females (OVX and OVX + E), D1CB1$^{-/-}$ showed same levels of interaction as control littermates. Also, GABACB1$^{-/-}$ presented a similar phenotype as observed in the interaction between two males, they showed increased, yet not significant, in time of interaction. On the other hand, GluCB1$^{-/-}$ demonstrated reduced levels of interaction in both female conditions (OVX and OVX + E). This could be related to the level of salience provocated by female subjects that are not observed during a less interested pairing.

Continuing on the involvement of CB1 receptors in social behavior, animals were submitted to the 3-chamber social investigation test, where the test subject had the option of investigating or not the stimulus animal in comparison with exploration of an inanimate object located in another compartment. When mice were presented with a male mouse as stimulus subject, all groups showed a higher interest for the social stimulus compared to the empty tube. However, only animals treated with SR141716 showed significantly reduced exploration of the social stimulus when compared with vehicle-treated control. Yet, in the presence of a more interesting stimulus subject, such as an OVX female mouse, all groups differed from their control, except for the D1CB1$^{-/-}$. Again, SR141716 treated animals showed a reduction in time investigating the social stimulus; the same was observed for total CB1$^{-/-}$ and GluCB1$^{-/-}$. This suggests that activation of CB1 receptors in glutamatergic neurons is involved in the promotion of sexual interest. The opposite was observed for GABACB1$^{-/-}$, which presented an increase in social interest for the female stimulus. When tested with an OVX + E female, GABACB1 and GluCB1 presented the same pattern of behavior, with GABACB1$^{-/-}$ showing higher time investigating the OVX + E female, whereas GluCB1$^{-/-}$

showed a tendency to reduced investigation time towards the stimulus female. Interestingly, in the presence of the OVX + E female, D1CB1$^{-/-}$ showed an increased investigation towads the stimulus mouse, a phenotype more related to GABACB1 animals. Therefore, CB1 in glutamatergic versus GABAergic neurons control sexual interest in an opposite manner, similar to exploration of novel objects (Lafenêtre et al, 2009; Häring et al, 2011), active versus passive avoidance (Metna-Laurent et al, 2012) and cannabinoid-induced anxiolytic versus anxiogenic effects (Rey et al, 2012), while CB1 in D1 expressing neurons involment depends on the value or intensity of the situation. The sex dependency of differences in social interest is remarkable since it was used ovariectomized females, which may not be the most arousing stimulus for a male subject. However, Pankevich et al (2004) showed that even though males generally prefer to investigate female in estrous cycle, odor of ovariectomized females still promotes interest for investigation when compared to a neutral odor. Still, the opposite situation (i.e. female investigating male stimulus subject) is rarely investigated, which provides an interesting area for future studies. These results corroborate with previous studies (Häring et al, 2011; Jacob et al, 2009) that showed the different involvement of CB1 receptor depending on its localization. An important point to highlight in the current study is that differences observed were dependent on the gender of the stimulus subject, i.e. male *vs* female mice.

Several studies reported increased anxiety-related behaviors after impaired CB1 receptors signaling only when aversive stimulus cannot be avoided (Haller et al., 2004, 2009; Thiemann et al., 2007; Kamprath et al., 2009; Jacob et al, 2009). However, little is known about how the endocannabinoid system modulation of the dopaminergic system could be involved in this effect. It is accepted that the endocannabinoid system modulates several neurotransmitter systems (glutamatergic, GABAergic, and dopaminergic) at multiple levels (Piomelli, 2003; Katona and Freund, 2008). In the brain, where exogenously administered and endogenously

released cannabinoids exert most of their behavioral effects, the CB1 receptors are expressed at different levels at different neuronal subpopulations. More specifically, they are present at very high levels in GABAergic interneurons, where they mediate cannabinoid-dependent inhibition of GABA release, and to a minor extent, in glutamatergic terminals (Marsicano and Lutz, 1999). In the glutamatergic neuronal subpopulation, they play a pivotal role in both neuroprotection and fear extinction in highly aversive situations, through the modulation of glutamate release, further confirming that the fear-alleviating effects of CB1 became evident primarily under highly aversive conditions (Monory et al., 2006; Kamprath et al., 2009; Moreira and Wotjak, 2010). Several lines of evidence suggest that dopamine is released in several brain regions such as the amygdala and prefrontal cortex under stress conditions. By acting on D1- or D2-like receptors, dopamine is involved in physiological processes facilitating affective behaviors and emotional learning (LeDoux, 2000). Although, coexpression of the cannabinoid CB1 and D1 receptors supports the idea of the cannabinoid–dopaminergic system cross-talk, as in forebrain basal ganglia and piriform cortex, the exact role of D1 receptors is not fully understood. Nevertheless, the present data add a new facet to the cross-talk between different neurotransmitter systems (i.e. GABAergic, glutamatergic and dopaminergic) and the endocannabinoid systems, within the attention being draw to different levels of social interaction. Several animal studies investigated the participation of the cannabinoid system in sexual interest and reproductive processes. They observed that direct or indirect CB1 receptor activation significantly impairs sexual-related behavior (Shrenker and Bartke 1985; Ferrari et al. 2000; Hill and Gorzalka, 2006), indicating reduction in sexual motivation. However, human studies suggested a facilitatory effect of cannabis on subjective indices of sexual function, which may be related to altered sensation and/or perception or indirectly enhanced through cannabis-induced disinhibition and reduction in anxiety levels (Hill and Gorzalka, 2006; Klein et al, 2012). Currently, it is proposed that the biphasic effect

produced by CB1 receptor activation is dependent on the balance between the receptors populations located either on GABAergic or glutamatergic neurons (Moreira et al, 2012; Rey et al, 2012). Meaning, high dose of cannabinoid produces anxiogenic-like behavior by CB1 receptor activation on GABAergic neurons; whereas, activation of CB1 receptors located in glutamatergic neurons by low dose of cannabinoid produce an anxiolytic-like effect (Rey et al, 2012).

Considering that the neural circuits regulating emotions and social behavior are highly interconnected, it is not surprising that changes in anxiety levels would influence social interactions. Disturbances in emotional regulation can lead to deficits in social behavior and excessive aggression (Davidson et al., 2000). Abnormalities in sexual drive are also affected by emotional alterations, leading to situations of anxious arousal and aggression or lack of sexual interest and anhedonia (Ace, 2007; Laurent and Simons, 2009), corresponding to anxiety and depression, respectively (Kalmbach et al, 2012). In animal studies, social investigation of a female subject can reflect positive hedonic behavior. Accordingly, diminished interest in pleasurable activities (i.e., anhedonia) predicts depressive-like disorders (Cryan and Holmes, 2005; Micale et al, 2013 Willner, 1986). In contrast, contact with a male subject typically evoked behaviors characterized as competitive (territorial or partner dominance), that are highly related to anxiety-like states (Cryan and Holmes, 2005; Paré, 2000).

However, it cannot be ruled out the involvement of different pathways as well as the potential compensatory mechanisms occurring during embryonic or early postnatal development, which represents a limitation of experiments with mutant mice in general. Since the 1980s, much of today's basic biomedical research, including neuropsychiatric research, make intense use of genetic mouse models as a powerful tool for investigating gene function *in vivo*. However, a lot of attention must be given to its potential limitations. To start, the function of a

gene essential for embryonic or early postnatal development cannot be studied in adulthood. Next, the disruption of ubiquitously expressed genes might result in rather complex phenotypes with too much unclear outcomes for a good understanding of the specific function of a gene. Particularly in the case of psychiatric disorders, it is compulsory to address the function of a gene of interest in the central nervous system or even more precisely in specific neuronal or glial cell populations. Also, early ablation of a gene might activate compensatory mechanisms that possibly mask or blur the direct effects of the gene of interest. In addition, difficulty that occurs in conjunction with the original gene targeting strategies is the methodologically unavoidable introduction of positive selection markers, which are used to enrich for homologous recombination events and often for gene disruption. These selection markers, comprising their own regulatory elements necessary for efficient transcription, have been demonstrated to interfere with the expression of the target gene and with genes adjacent to the target site (Olson et al. 1996; Meyers et al. 1998; Matthaei, 2007; Deussing, 2013).

The most common method, and the one used in this work, to obtain tissue-specific control of gene deletion is the Cre*LoxP* system modified from bacteriophage. Briefly, *LoxP* sites are short DNA sequences that are recognized by a specific DNA recombinase enzyme called Cre (*cyclization recombination* or c*auses* re*combination*) that deletes any DNA between the two sequences. An exon in the gene of interest is flanked by these *LoxP* (floxed) sites and the modified gene is introduced into its correct location in embryonic stem cells by homologous recombination. The stem cells are injected into mouse blastocysts and mice generated in which the gene is ‘floxed’ but fully functional until it is inactivated by the Cre recombinase due to the removal of the exon. This is achieved by breeding the ‘floxed’ mice with a mouse transgenic for the Cre recombinase usually under the control of a tissue-specific promoter (Gu et al. 1994, Rajewsky et al. 1996; Kwan, 2002; Matthaei, 2004). The result is deletion of the gene in a specific tissue. A major problem with this method is the ‘position’ effect, whereby

Cre is expressed non-specifically in other tissues, or the promoter driving Cre is active early during development so that the gene is ablated at the wrong time or in the wrong tissue. A further difficulty, that has not been enough addressed, is that the mammalian genome contains many 'pseudo' LoxP sites (Thyagarajan et al. 2000) that potentially could be recombined by Cre recombinase with totally unknown effects. Also, many transgenic cre strains exhibit "off-target" activity. For well-characterized promoters, however, traditional transgenesis might be an ideal option as it allows the flexibility to incorporate a fragment of promoter sequence with known functionality. Provide temporal control of Cre expression through the use of an inducible promoter that has been modified to be 'off' until the mice are treated with the inducer (i.e. tamoxifen) is a great improvement to the technique (Kellendonk et al. 1996, Zheng et al. 2002), although side-effects in other tissues, such as in neurons, may continue to be a problem (Hendry et al. 1987). It would also be an improvement to have a regulatory system that would allow the reliable switching 'on' and 'off' of endogenous genes in a repeatedly reversible manner, especially considering that the major problem with the Cre recombinase system is that it is not reversible (Matthaei, 2007). Ideally, a system to test the effects of a gene deletion should incorporate complete reversibility, totally tissue specific and not modify the host genome or be modified by the host genome. Currently none of the commonly used methods satisfy these requirements since they rely on the use of transgenic mice with their inherent limitations. Nevertheless, none of the common psychiatric disorders is the consequence of a single null mutation. As in many other common disease areas, psychiatric research encounters the challenge that susceptibility to this type of diseases arises from the complex interaction of many genes or of genetic variants of a relatively small effect size with largely unknown environmental factors rather than from a single gene. All these points should be taken into account when modeling psychiatric disorders or when evaluating the potential underlying disease mechanisms (Deussing, 2013). Even considering all caveats

from these techniques, simple deletions of gene function will continue to have a place in various situations as will transgenic mice using minimal promoters. However, it is imperative to understand the limitations of these systems so that appropriate controls can be included and correct conclusions can be made (Hagg 1999; Matthaei, 2007). Also, so far, research involving genetic mouse models has significantly contributed to the field of neurobiology in general and to our understanding of psychiatric disorders in particular.

Another approach recently used to better understand modulatory function of the CB1 receptor in neuronal circuits, is the conditional rescue mouse line generated by a the loxP-flanked stop cassette (Stop-CB1 allele) controlled by its endogenous promoter, which allows selective reactivation of the CB1 receptor in specific cell populations (Ruehle et al, 2013). Before the rescue process, mice lack CB1 receptor protein similar to CB1 receptor-null mutants. They also reproduced behavior and electrophysiological phenotypes described for CB1 receptor-null mutant animals (Marsicano et al., 2002, 2003; Haller et al., 2004). While animals with rescue of the CB1 receptor throughout the whole organism did not differ from WT animals in any of the tested procedures, indicating the validity of this experimental approach. Thus, this newly generated mouse line can be a valuable tool to investigate sufficient CB1 receptor function in any given cell type to which Cre recombinase can be delivered specifically, to improve the knowledge about different subpopulations role and the involvement of CB1 receptors in different situations.

In conclusion, the present work provided direct evidence for a cross-talk between dopaminergic D1 receptors and endocannabinoid system in terms of controlling negative affect, suggesting that a specific reduction of endocannabinoid signaling in neurons expressing dopamine D1 receptors is able to affect acute fear adaptation. It also demonstrated an opposite role of CB1 receptors on glutamatergic versus GABAergic neurons in regulation of social interaction with female, but not male conspecifics. The present data imply that

selective enhancement of endocannabinoid signaling at glutamatergic synapses may promote sexual interest, rendering it an interesting option for the treatment of psychiatric diseases related with its loss.

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7 APPENDIX

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Cannabinoid receptor type 1 receptors on GABAergic vs. glutamatergic neurons differentially gate sex-dependent social interest in mice

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Keywords: CB1, depression, GABA, glutamate, sexual interest, social behavior

Abstract

Abnormalities in social behavior are found in almost all psychiatric disorders, such as anxiety, depression, autism, and schizophrenia. Thus, comprehension of the neurobiological basis of social interaction is important for a better understanding of numerous pathologies and improved treatments. Several findings have suggested that an alteration of cannabinoid receptor type 1 (CB1) receptor function could be involved in the pathophysiology of such disorders. However, the role of CB1 receptors is still unclear, and their localisation on different neuronal subpopulations may produce distinct outcomes. To dissect the role of CB1 receptors in different neuronal populations, we used male knockout mice and their respective control littermates [total deletion ($CB1^{-/-}$); specific deletion on cortical glutamatergic neurons (Glu-$CB1^{-/-}$) or on GABAergic interneurons (GABA-$CB1^{-/-}$), and wild-type (WT) mice treated with the CB1 antagonist/inverse agonist SR141716A (3 mg/kg). Mice were required to perform different social tasks – direct social interaction and social investigation. Direct interaction of two male mice was not modified in any group; however, when they were paired with females, Glu-$CB1^{-/-}$ mice showed reduced interaction. Also, exploration of the male stimulus subject in the three-chamber social investigation test was almost unaffected. The situation was completely different when a female was used as the stimulus subject. In this case, Glu-$CB1^{-/-}$ mice showed reduced interest in the social stimulus, mimicking the phenotype of $CB1^{-/-}$ or WT mice treated with SR141716A. GABA-$CB1^{-/-}$ mice showed the opposite phenotype, by spending more time investigating the social stimulus. In conclusion, we provide evidence that CB1 receptors specifically modulate the social investigation of female mice in a neuronal subtype-specific manner.

Introduction

Cannabis is known to affect psychological states in humans (and animals). For this reason, it is the most frequently used recreational drug, mainly influencing emotional processes (Murray *et al.*, 2007). However, the effects of cannabinoids appear in a biphasic manner (Viveros *et al.*, 2005), as observed in several behaviors, including motivational processing (Maldonado & Rodríguez de Fonseca, 2002; Ruehle *et al.*, 2011; Moreira *et al.*, 2012), locomotion and exploration (Haller *et al.*, 2004), novelty-seeking (Lafenêtre *et al.*, 2009), and feeding behavior (Bellocchio *et al.*, 2010). The wide distribution of cannabinoid receptor type 1 (CB1) in the central nervous system (Marsicano & Lutz, 1999) and its location in different neural subpopulations (Monory *et al.*, 2007) increase the difficulties in understanding the mechanisms underlying its biphasic effect. This is particularly challenging because the endocannabinoid system controls the activation of both inhibitory (GABA) and excitatory (glutamate) neurotransmission (Monory *et al.*, 2006).

Alterations in the endocannabinoid system are observed in several psychiatric disorders, especially those with emotion-related dysfunctions as a core symptom. In those, social disruption is observed – conditions such as social withdrawal, increased aggressiveness, decreased affiliative behavior and social cognition and loss of sexual interest are described in schizophrenia, autism, anxiety, and major depression (American Psychiatric Association, 2000). With regard to social aspects, rodents, like humans, show a large repertoire of social interactions with conspecifics, characterised by high levels of complexity in the type and number of interactions (File & Seth, 2003; Crawley *et al.*, 2007; Choleris *et al.*, 2009). Such similarities between humans and animals underscore the potential translational value of studies on social behavior in mice with respect to the clinical situation.

Although several CB1 receptor mutants and pharmacological approaches have been used to study the contribution of the endocannabinoid system to the control of emotions (Moreira & Wotjak, 2010; Micale *et al.*, 2013a), the cellular substrates of such responses with regard to the specific neuronal subpopulations involved are still largely unexplored. Likewise, little is known about the role played by

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CB1 receptors in the repertoire of different social behaviors. With the development of total ($CB1^{-/-}$) (Zimmer *et al.*, 1999; Marsicano *et al.*, 2002; Martin *et al.*, 2002) and conditional mutant mice lacking the CB1 receptor on either GABAergic (GABA-$CB1^{-/-}$) or glutamatergic (Glu-$CB1^{-/-}$) neurons (Monory *et al.*, 2006), we are starting to understand the role of CB1 receptors on different neuronal populations in emotional behavior (Jacob *et al.*, 2009; Häring *et al.*, 2011). Hence, we subjected male mice with pharmacological blockade or ubiquitous respective cell-specific genetic deletion of CB1 receptors to different social behavior tests. For the social interaction test, we placed pairs of unfamiliar mice in a novel environment for bidirectional investigation. The direct contact allowed assessment of social investigation and aggressive behavior at the same time. To assess the interest in social stimuli, we confronted the experimental subjects with a social (a tube containing a male or an ovariectomised female with or without estradiol replacement) and a non-social (empty) stimulus in the three-chamber social investigation task. This allowed us to distinguish between general social interest (male stimulus) and sexual interest (female stimulus).

Materials and methods

Animals

Adult male mice (aged 8–12 weeks) were used. Mice (n = 5–11 per group) were bred in our breeding facility (Max-Planck Institute of Biochemistry, Martinsried, Germany), and transferred to our experimental building. Mice were housed singly and maintained in standard conditions with food and water available *ad libitum* under a 12-h inverse light–dark cycle (lights off at 09:00 h) for at least 14 days before the experiments were started. Total and conditional CB1 receptor mutant mice were obtained and genotyped by polymerase chain reaction (PCR) with specific primers, as previously described (Marsicano *et al.*, 2002; Monory *et al.*, 2006; Jacob *et al.*, 2009). We used mice with total CB1 receptor knockout ($CB1^{-/-}$; from heterozygous breeding pairs) [PCR primers: G50 (5′-GCT GTC TCT GGT CCT CTT AAA-3′); G51 (5′-GGT GTC ACC TCT GAA AAC AGA-3′); and G54-neo-null 3′ (5′-CCT ACC CGG TAG AAT TAG CTT-3′)], and mice with CB1 receptor knockout on cortical glutamatergic neurons [Glu-$CB1^{-/-}$; mice with conditional deletion of CB1 receptors on glutamatergic neurons were obtained as described previously (Monory *et al.*, 2006)]. Briefly, NEX-Cre mice were crossed with $CB1^{f/f}$ mice to obtain $CB1^{f/f;Nex\text{-}Cre}$ mice, from breeding pairs where only the fathers expressed Cre-recombinase [PCR primers: G50 (5′-GCT GTC TCT GGT CCT CTT AAA-3′); G51 (5′-GGT GTC ACC TCT GAA AAC AGA-3′); G53 (5′-CTC CTG TAT GCC ATA GCT CTT-3′); G100 (5′-CGG CAT GGT GCA AGT TGA ATA-3′), and G101 (5′-GCG ATC GCT ATT TTC CAT GAG-3′)]. We also used mice with CB1 receptor knockout on forebrain GABAergic neurons [GABA-$CB1^{-/-}$; mice with conditional deletion of CB1 receptors on GABAergic neurons were obtained as described previously (Monory *et al.*, 2006)]. Briefly, Dlx5/6-Cre mice were crossed with $CB1^{f/f}$ mice to obtain $CB1^{f/f;Dlx5/6\text{-}Cre}$ mice, from breeding pairs where only the fathers expressed Cre-recombinase [PCR primers: G50 (5′-GCT GTC TCT GGT CCT CTT AAA-3′); G51 (5′-GGT GTC ACC TCT GAA AAC AGA-3′); G53 (5′-CTC CTG TAT GCC ATA GCT CTT-3′); G100 (5′-CGG CAT GGT GCA AGT TGA ATA-3′); and G101 (5′-GCG ATC GCT ATT TTC CAT GAG-3′)]. Control littermates of knockout mice were also used, along with wild-type (WT) mice for pharmacological treatment ($CB1^{f/f}$ = WT) [PCR primers: G50 (5′-GCT GTC TCT GGT CCT CTT AAA-3′); G51 (5′-GGT GTC ACC TCT GAA AAC AGA-3′); and G53 (5′-CTC CTG TAT GCC ATA GCT CTT-3′)]. Mice of a given experiment and line were derived from at least four different litters. All stimulus subjects were from a C57BL/6N background. For the social behavior experiments, ovariectomised females were used. Females were ovariectomised at least 15 days before the beginning of experiments. Some females also received β-estradiol for 1 week before the experiments started (see below). All behavioral experiments were performed during the active (dark) phase of mice, between 09:30 h and 17:00 h.

Drugs

Rimonabant [SR141716A; *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; Cayman Chemical, Ann Arbor, MI, USA] was dissolved in vehicle solution (one drop of Tween-80; 2.5% dimethylsulfoxide in saline) to a final concentration of 3 mg/kg, and injected intraperitoneally at 10 mL/kg body weight, 30 min before testing. β-Estradiol (Sigma-Aldrich, Steinheim, Germany) was dissolved in 0.5% ethanol/99.5% propyleneglycol for administration via micro-osmotic pumps (Model 1002; pumping rate, 0.25 μL/h; total volume, 90 ± 10 μL; Alzet, Cupertino, CA, USA) to a final concentration of 36 μg/mL (Ström *et al.*, 2012).

Behavioral tests

During experiments and video analysis, experimenters were blind to the genotype. All behavioral tests took place in an experimental room with the same light–dark cycle and environmental conditions (i.e. humidity and temperature) as in the housing facility. Laboratory animal care and experimental procedures were in compliance with the European Union recommendations for the care and use of laboratory animals (86/609/EEC). All experimental procedures were approved by the Committee on Animal Health and Care of the State of Upper Bavaria (AZ55.2-1-54-2532-44-09 and AZ55.2-1-54-2532-142-12).

Social interaction test

The social interaction experiment was performed as previously described (Terzian *et al.*, 2011). Briefly, all experiments were performed in a new cage (27 × 16 × 12 cm) with fresh bedding at 5 lux (i.e. red light). The wall of the cage was extended by 12.5 cm with semi-transparent plastic. In this test, pairs of unfamiliar mice were placed into the cage for 5 min. Depending on the test session, each test subject was paired either with an ovariectomised female, an ovariectomised female treated for 1 week with β-estradiol, or a male mouse of the same genotype. The time spent in social interactions (active contact such as sniffing, licking, close following, and grooming) and aggressive behavior (fighting and biting, causing injury) was recorded for each pair of mice. Each session was video-recorded, and analysed off-line with ANY-MAZE (Stoelting, Wood Dale, IL, USA).

Three-chamber social investigation test

The three-chamber social investigation task was conducted as previously described (Terzian *et al.*, 2011). The three-chamber apparatus consists of a PVC rectangular box divided into three equal compartments (30 × 30 × 30 cm) interconnected by small openings (6 × 5 cm) that could be closed with guillotine doors. Mice were tested under 5 lux (i.e. red light). During the habituation phase, each mouse freely explored the apparatus for 10 min, and empty perforated 50-mL Falcon tubes (length, 11.4 cm; diameter, 2.8 cm^2;

22 holes of diameter 0.5 cm were used to provide ventilation and olfactory cues to the test mice) were placed in each side of the box. After the habituation session, the test mouse was kept in the center compartment, and one of the tubes was replaced by a tube containing a stimulus C57BL/6N mouse – an adult male, an ovariectomised female, or an ovariectomised female treated for 1 week with β-estradiol. In this second stage, the mouse had 10 min to explore all three compartments, and the time spent in direct active contact (with the tubes) was recorded. Independent groups were exposed to the male or female stimulus subjects. Each session was video-recorded, and analysed off-line with ANY-MAZE (Stoelting).

Data analysis

Data were analysed with an unpaired *t*-test or two-way ANOVA (genotype/treatment; time in contact) for repeated measures (time in contact with the tube containing the social stimulus vs. time spent in the empty tube). The Newman–Keuls test was used as a *post hoc* test, if appropriate. Data are presented as means ± standard errors of the mean (SEMs). Statistical significance was accepted if $P < 0.05$.

Results

In the social interaction test, where two freely moving male mice were in the same cage, none of the tested lines showed differences from the control group in contact time (CB1$^{-/-}$, $t = 0.29$; WT, $t = 1.20$; Glu-CB1$^{-/-}$, $t = 1.15$; Fig. 1), even though GABA-CB1$^{-/-}$ mice showed slightly increased social interest; however, this difference failed to reach statistical significance (GABA-CB1$^{-/-}$, $t = 2.00$, $P > 0.05$; Fig. 1). Aggressive behavior was observed in only one pair of Glu-CB1$^{-/-}$ mice and one pair of Glu-CB1$^{+/+}$ mice. Therefore, this parameter was not considered further. However, in the social interaction test, to verify whether the two main opposite genotypes (Glu-CB1 and GABA-CB1, the terms 'Glu-CB1' and 'GABA-CB1' refer to the different groups related to their location of CB1 deletion, involving both knockout (-/-) and control (+/+) groups) would show differences in interaction when confronted with female subjects, males were introduced to ovariectomised females with or without β-estradiol treatment. In the case of Glu-CB1, a two-way ANOVA (genotype; stimulus subject) revealed significant main effects of genotype ($F_{1,28} = 30.72$, $P < 0.0001$) and subject ($F_{2,25} = 26.91$, $P < 0.0001$), as well as a significant genotype × subject interaction ($F_{2,25} = 8.563$, $P < 0.005$). *Post hoc* tests confirmed that Glu-CB1$^{-/-}$ mice showed less exploration of ovariectomised females without or with β-estradiol replacement than the control group, whereas exploration of male subjects was similar. Interestingly, Glu-CB1$^{+/+}$ mice, but not Glu-CB1$^{-/-}$ mice, spent significantly more time investigating the females than the males. For GABA-CB1, significant main effects of genotype ($F_{1,44} = 8.160$, $P < 0.01$) and subject ($F_{2,41} = 6.907$, $P < 0.005$) were observed, but no significant genotype × subject interaction ($F_{2,41} = 0.8315$, $P = 0.442$). Thus, GABA-CB1$^{-/-}$ mice spent more time in investigating social stimuli than GABA-CB1$^{+/+}$ mice, irrespective of the sex of the stimulus subjects.

In the three-chamber social investigation test, when the stimulus subject was a male mouse (Fig. 2), CB1$^{-/-}$ mice showed less interest in the social stimulus than did the control group, but the difference was not significant manner ($n = 6$–7; genotype, $F_{11,1} = 1.84$, $P = 0.20$; genotype × time in contact, $F_{11,1} = 3.24$, $P = 0.09$). Both groups showed a preference for the tube containing the stimulus mouse as compared with the empty tube (time in contact, $F_{11,1} = 36.16$, $P < 0.001$). Similarly, mice that received vehicle or SR141716A preferred to explore the tube containing the unknown

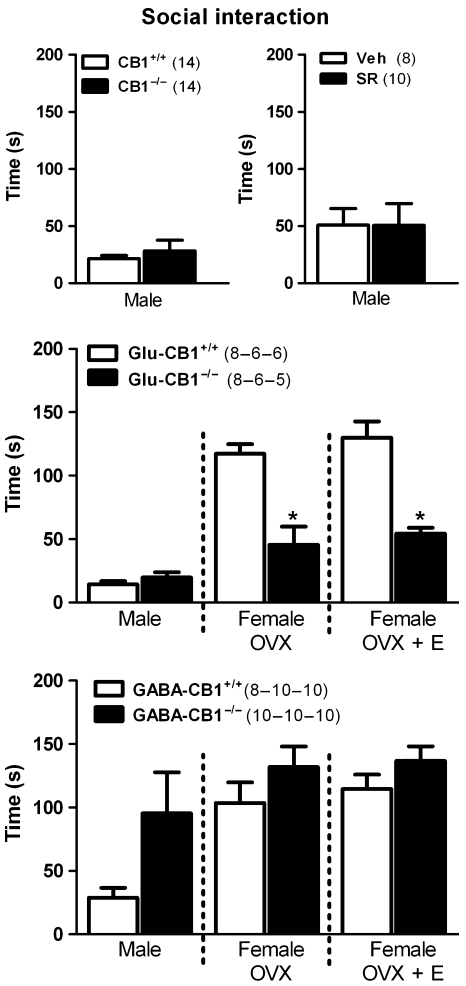

FIG. 1. Direct social interaction: effects of pharmacological blockage [SR141716A (SR)] or genetic deletion of CB1 receptors on social interaction between a pair of unfamiliar mice – male mice of the same genotype/receiving the same pharmacological treatment, ovariectomised females (OVX), or ovariectomised females with hormone replacement (OVX + E). Values are means ± SEMs. *$P < 0.05$. Numbers next to each group represent the number of animals per group (n) for each experiment. Newman–Keuls *post hoc* test. Veh, vehicle.

mouse rather than the empty tube (time in contact, $F_{17,1} = 26.18$, $P < 0.001$). However, mice treated with the CB1 receptor blocker/inverse agonist showed a significant reduction in time spent investigating the social stimulus ($n = 9$–10; genotype × time in contact, $F_{1,17} = 4.52$, $P < 0.05$).

As observed for the CB1$^{-/-}$ mice, in the presence of a male mouse stimulus, Glu-CB1$^{-/-}$ mice showed no significant difference in investigation from the control group ($n = 10$; genotype × time in contact, $F_{1,18} = 0.007$, $P = 0.93$). The same was found for GABA-CB1$^{-/-}$ mice ($n = 8$–10; genotype × time in contact, $F_{1,16} = 0.60$, $P = 0.45$). Both mutant lines showed a preference for the social stimulus as compared with the inanimate object (statistics not shown).

In presence of an ovariectomised female stimulus subject (Fig. 2), CB1$^{-/-}$ mice spent significantly less time in contact with the social stimulus than did control littermates ($n = 13$–16; genotype × time in contact, $F_{1,27} = 7.17$, $P < 0.05$). WT mice treated with SR141716A also spent less time investigating the female stimulus than did the vehicle group ($n = 6$–7; genotype × time in contact, $F_{1,11} = 6.53$, $P < 0.05$). The same was found for Glu-CB1$^{-/-}$ mice ($n = 8$–10; genotype × time in contact, $F_{1,16} = 4.65$, $P < 0.05$).

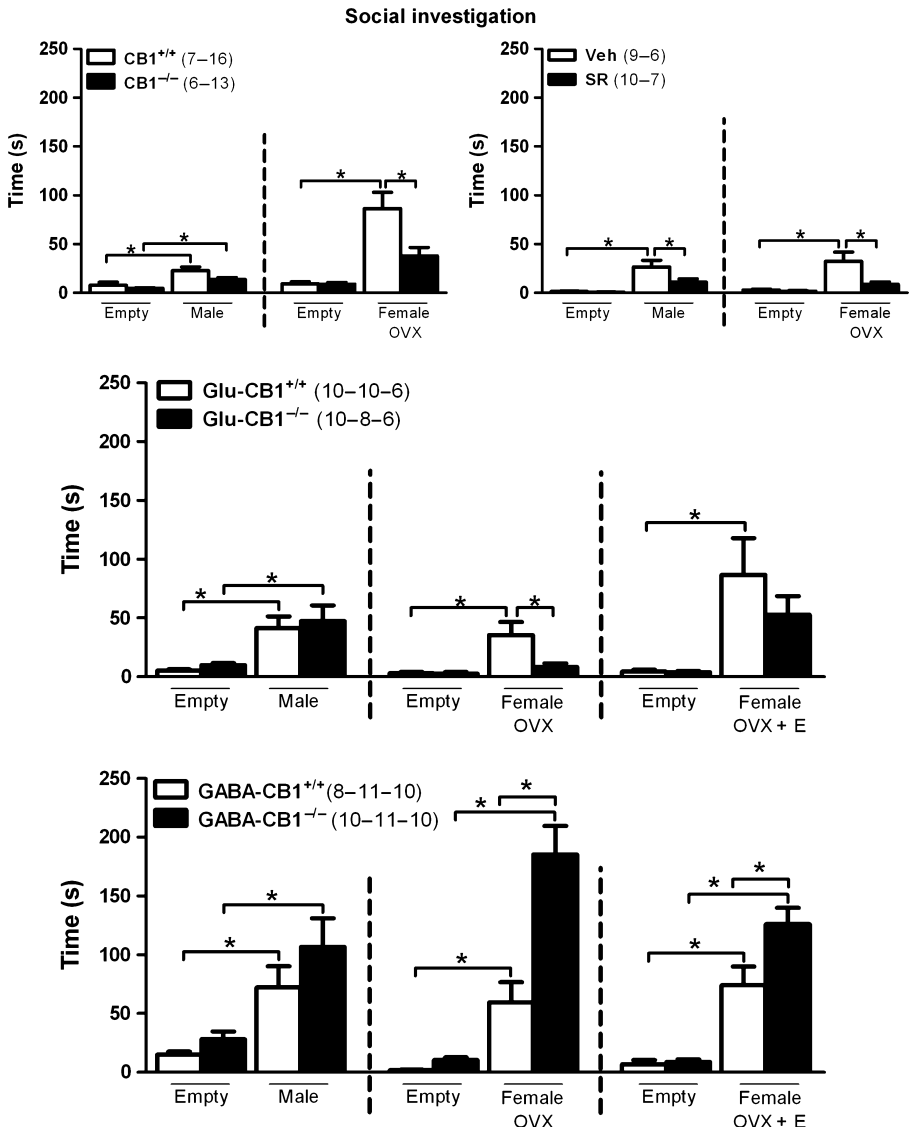

FIG. 2. Social investigation of social stimulus subject: effects of pharmacological blockage [SR141716A (SR)] or genetic deletion of CB1 receptor on social investigation of a male stimulus mouse by ovariectomised females (OVX) or ovariectomised females with hormone replacement (OVX + E) in the three-chamber box. Graphs represent different mutant mice and treatment with SR141716, with respective control groups. Values are means ± SEMs. *$P < 0.05$. Numbers next to each group represent the number of animals per group (n) for each experiment. Newman–Keuls *post hoc* test. Veh, vehicle.

GABA-CB1$^{-/-}$ mice, in contrast, showed the opposite behavior, by spending significantly more time in contact with the female stimulus ($n = 11$; genotype × time in contact, $F_{1,20} = 16.05$, $P < 0.01$).

To further substantiate the role of CB1 receptors in the two opposite neuronal populations, new batches of GABA-CB1 and Glu-CB1 mice were presented with a tube containing an ovariectomised female treated for 1 week with β-estradiol. In this situation, GluCB1$^{-/-}$ mice showed reduced interest in the female tube, but the difference from the control group was not significant ($n = 6$; genotype × time in contact, $F_{1,10} = 0.93$, $P = 0.35$). GABA-CB1$^{-/-}$ showed increased investigation of the ovariectomised female treated for 1 week with β-estradiol ($n = 10$; genotype × time in contact, $F_{1,18} = 5.23$, $P < 0.05$).

Discussion

The present study evaluated the involvement of CB1 receptors in social behavior in mice. For this purpose, we first investigated direct contact between two mice, in an attempt to create a more ethological situation. Given previous reports about social interaction after pharmacological blockage or genetic deletion of CB1 receptors (Jacob *et al.*, 2009; Häring *et al.*, 2011; Litvin *et al.*, 2013), we were surprised to not observe any treatment/genotype differences between two males of the same genotype. One explanation for the discrepant findings might be the previously observed relationship between aversiveness of the test situation and the involvement of CB1 receptor signaling in social behavior (Jacob *et al.*, 2009). In the present study, we kept the emotional load to a minimum by using less aversive conditions, such as red light. Higher light intensity, however, would have confounded our interpretation of 'social intention' by differences in anxiety levels (Jacob *et al.*, 2009). We also failed to observed differences in aggression. Other than that reported for the resident–intruder test, where Glu-CB1$^{-/-}$ mice showed increased aggression (Häring *et al.*, 2011), mice did not show any territorial/aggressive behavior in the novel environment, independently of the genotype. When both mutated lines (GABA-CB1 and Glu-CB1)

were tested upon direct contact with females (ovariectomised females or ovariectomised females treated for 1 week with β-estradiol), GABA-CB1$^{-/-}$ mice showed a similar phenotype as observed in the interaction between two males; that is, they showed a non-significant increase in interaction. Glu-CB1$^{-/-}$ mice, in contrast, showed reduced levels of interaction with both groups of females. We relate the sex-dependent differences in free social interaction shown by GluCB1$^{-/-}$ mice to different levels of salience provoked by female and male stimulus subjects.

In free social interaction, interaction times are determined not only by the test, but also by the stimulus subjects. This potentially confounding factor could be circumvented in the second experiment, the three-chamber social investigation test, where the stimulus subject was exposed in a Falcon tube. This time, the test mice had the option of investigating or not investigating the stimulus mouse in comparison with exploration of an inanimate object located in another compartment. When mice were presented with a male mouse as the stimulus subject, all groups showed higher interest in the social stimulus than in the empty tube. However, only mice treated with SR141716A showed significantly reduced exploration of the social stimulus as compared with vehicle-treated controls. In the presence of a more interesting stimulus subject, such as an ovariectomised female mouse, all groups differed from their controls. Again, SR141716A-treated mice showed a reduction in time spent investigating the social stimulus; the same was observed for CB1$^{-/-}$ and Glu-CB1$^{-/-}$ mice. This suggests that activation of CB1 receptors in glutamatergic neurons is involved in the promotion of sexual interest in female stimulus subjects. The opposite was observed for GABA-CB1$^{-/-}$ mice, which showed an increase in social interest in the female stimulus subject. When tested with the ovariectomised female treated for 1 week with β-estradiol, GABA-CB1 and Glu-CB1 mice showed the same pattern of behavior, with GABA-CB1$^{-/-}$ mice spending more time and GluCB1$^{-/-}$ mice less time in investigating the female. Therefore, CB1 receptors in glutamatergic and in GABAergic neurons control sexual interest in an opposite manner, similarly to exploration of novel objects (Lafenêtre *et al.*, 2009; Häring *et al.*, 2011), active vs. passive avoidance (Metna-Laurent *et al.*, 2012), and cannabinoid-induced anxiolytic vs. anxiogenic effects (Rey *et al.*, 2012). The sex dependency of differences in social interest is remarkable, as we used ovariectomised females, which may not constitute the most arousing stimulus for a male subject. However, Pankevich *et al.* (2004) showed that, even though males generally prefer to investigate females in the estrous cycle, the odor of ovariectomised females still promotes interest in investigation as compared with a neutral odor. However, the opposite situation (i.e. a female investigating a male stimulus subject) is rarely investigated, which provides an interesting area for future studies.

Our results are in agreement with previous studies (Jacob *et al.*, 2009; Häring *et al.*, 2011) showing the differential involvement of CB1 receptors, depending on their localisation. An important point to highlight in the current study is that we observed such differences according to the sex of the stimulus subject, i.e. male vs. female mice. Endocannabinoids are known to modulate several neurotransmitter systems at different levels of complexity (Piomelli, 2003). In the central nervous system, CB1 receptors are expressed at different levels in different neuronal subpopulations – higher expression levels in GABAergic interneurons, where they mediate cannabinoid-dependent inhibition of GABA release; and lower expression levels in glutamatergic terminals (Marsicano & Lutz, 1999). In the latter neuronal subpopulation, they modulate glutamate release, influencing fear and anxiety levels in threatening situations (Monory *et al.*, 2006; Kamprath *et al.*, 2009; Hill *et al.*, 2010; Moreira & Wotjak, 2010). It is also important to note that conditional CB1 receptor mutants could show different phenotypes from those observed in CB1$^{-/-}$ animals (Jacob *et al.*, 2009; Bellocchio *et al.*, 2010; Häring *et al.*, 2011; Terzian *et al.*, 2011). Even though the majority of CB1 receptors in the brain are located on GABAergic neurons, many behaviors observed after CB1 receptor ablation are related to action upon those located on glutamatergic neurons (Hill *et al.*, 2010; Rey *et al.*, 2012). Further studies are necessary to identify the main structures implicated in the alterations in social interest observed in the present study.

Several animal studies have investigated the participation of the cannabinoid system in sexual interest and reproductive processes. They found that direct or indirect CB1 receptor activation significantly impairs sex-related behavior (Shrenker & Bartke, 1985; Ferrari *et al.*, 2000; Gorzalka & Hill, 2006), indicating a reduction in sexual motivation. However, human studies have suggested a facilitatory effect of cannabis on subjective indices of sexual function, which may be related to altered sensation and/or perception, or indirect enhancement through cannabis-induced disinhibition and reduction in anxiety levels (Gorzalka & Hill, 2006; Klein *et al.*, 2012). Currently, it is proposed that the biphasic effect produced by CB1 receptor activation is dependent on the balance between the receptor populations located either on GABAergic or glutamatergic neurons (Rey *et al.*, 2012); a high dose of cannabinoid produces anxiogenic-like behavior by CB1 receptor activation on GABAergic neurons, whereas activation of CB1 receptors located on glutamatergic neurons by a low dose of cannabinoid produces an anxiolytic-like effect (Rey *et al.*, 2012).

Considering that the neural circuits regulating emotions and social behavior are highly interconnected, it is not surprising that changes in anxiety levels influence social interactions. Disturbances in emotional regulation can lead to deficits in social behavior and excessive aggression (Davidson *et al.*, 2000). Abnormalities in sexual drive are also affected by emotional alterations, leading to situations of anxious arousal and aggression, or lack of sexual interest and anhedonia (Ace, 2007; Laurent & Simons, 2009), corresponding to anxiety and depression, respectively (Kalmbach *et al.*, 2012). In animal studies, social investigation of a female subject can reflect positive hedonic behavior. Accordingly, diminished interest in pleasurable activities (i.e. anhedonia) predicts depressive-like disorders (Willner, 1991; Cryan & Holmes, 2005 Micale *et al.*, 2013b). In contrast, contact with a male subject typically evoked behaviors characterised as competitive (territorial or partner dominance), which are highly related to anxiety-like states (Paré, 2000; Cryan & Holmes, 2005).

Together, the findings of this study demonstrate opposite roles of CB1 receptors on glutamatergic and on GABAergic neurons in the regulation of social interaction with female, but not male, conspecifics. Our data imply that selective enhancement of endocannabinoid signaling at glutamatergic synapses may promote sexual interest, making it an interesting option for the treatment of psychiatric diseases related to its loss, such as depression.

Disclosure/conflicts of interest

The authors declare no conflicts of interests.

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Abbreviations

CB1, cannabinoid receptor type 1; PCR, polymerase chain reaction; SEM, standard error of the mean; WT, wild-type.

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Review

Modulation of defensive behavior by Transient Receptor Potential Vanilloid Type-1 (TRPV1) Channels

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ABSTRACT

The Transient Receptor Potential Vanilloid Type-1 (TRPV1) was first characterized in primary afferent fibers as a receptor for capsaicin (the pungent ingredient of chili peppers). Later on, this cation-permeable ion channel was also described in the central nervous system, where its main putative endogenous ligand is N-arachidonoyl ethanolamide (an endocannabinoid, also known as anandamide). Recent results employing genetic, pharmacological and histochemical techniques indicate that TRPV1 tonically modulate anxiety, fear and panic responses in brain regions related to defensive responses, such as the dorsal periaqueductal gray, the hippocampus and the medial prefrontal cortex. Genetic deletion or antagonism of this ion channel induces anxiolytic-like effects in several animal models. The main mechanism responsible for TRPV1-mediated effects on anxiety seems to involve facilitation of glutamatergic neurotransmission. In addition, there is evidence for interactions with other neurotransmitter systems, such as nitric oxide and endocannabinoids.

Contents

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1. The Transient Receptor Potential Type-1 (TRPV1) Channel

1.1. Structure and function

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the main pungent constituent of the native American plant *Capsicum sp*, popularly known as the hot chili pepper, was first isolated and denominated by Thresh in 1846 (Szallasi and Blumberg, 1990b). Initial studies showed that it could induce a burning pain perception (Bernstein et al., 1981; Stjarne et al., 1989) and skin hyperalgesia (Bartho et al., 1990; Baumann et al., 1991). These effects result from stimulation and depolarization of primary afferent sensory neurons (Holzer, 1991) followed by the release of pro-inflammatory mediators (Southall et al., 2003). After repeated administration, however, these capsaicin-induced responses show a rapid desensitization (Dray, 1992; Green, 1989; Hayes et al., 1984).

Biochemical and electrophysiological studies indicated that capsaicin facilitates excitability and electrical conduction in sensory unmyelinated C-fibers and neural cell bodies by increasing sodium and calcium permeability (Erdelyi et al., 1987; Marsh et al., 1987; Wood et al., 1988). Capsaicin-induced calcium influx results in exocytosis of excitatory amino acids and other neuromodulators such as substance P (Go and Yaksh, 1987; Li et al., 2004; Marinelli et al., 2002, 2003; Sasamura et al., 1998). In addition, it was initially observed that capsaicin can also bind to specific sites in the peripheral terminals of thin-myelinated Aδ fibers (Chung et al., 1985; Hiura and Sakamoto, 1987; Matsumiya et al., 1983; Szolcsanyi et al., 1988). This subset of bipolar neurons is involved in the development of chronic pain. Their cellular bodies are located in the dorsal root (DRG), nodose and trigeminal ganglion, from where they send terminals that make synapses with second-order neurons in the central nervous system (CNS; Chad et al., 1983; Holzer, 1991; Szallasi, 1995; Williams and Zieglgansberger, 1982).

It was later found that capsaicin effects were antagonized in a competitive way by a drug named capsazepine (Walpole et al., 1994). This fact, together with its biological effects, led to the proposal of an orphan "capsaicin receptor" in mammalian organisms. This receptor was indeed identified by autoradiographic studies using [3H] resiniferatoxin (RTX), a potent capsaicin analog, in tissues of several species, including humans (Acs et al., 1994; Szallasi, 1994; Szallasi and Blumberg, 1990a,b). Since capsaicin and RTX share a vanillyl group as the key structural recognition site the receptor was initially denominated "vanilloid" (Szallasi and Blumberg, 1990b). In 1997 the vanilloid receptor was cloned in rat cells from the DRG and demonstrated to be a subtype of non-selective cation channels related to the Transient Receptor Potential (TRP) family of ion channels (Caterina et al., 1997). This receptor was named by the authors as 'vanilloid receptor subtype 1' (VR1). It was later renamed by the IUPHAR Nomenclature Committee the 'Transient Receptor Potential Vanilloid Type 1' (TRPV1; Clapham et al., 2005). Structurally, it is composed by four subunits, each having six-transmembrane-spanning domains and a short hydrophobic pore between the fifth and sixth segments (Ramsey et al., 2006; Wu et al., 2010).

TRPV1 channels can be activated by several stimuli in addition to capsaicin, including noxious heat, low pH (Caterina et al., 1997; Tominaga et al., 1998) and inflammatory mediators (Moriyama et al., 2005; Tang et al., 2004; Vyklicky et al., 1998). Lipid endogenous agonists of these receptors have also been proposed and named endovanilloids. The most studied endovanilloid is, by far, anandamide (N-arachidonoyl ethanolamine), an arachidonic acid derived neuromodulator. Anandamide is primarily known as the endogenous agonist at the CB1 receptors, being the first described endocannabinoid/endovanilloid (Di Marzo et al., 2001; Smart and Jerman, 2000; Zygmunt et al., 2000). Other proposed endovanilloids are N-arachidonoyl dopamine (Huang et al., 2002; Toth et al., 2003), N-oleoyl dopamine (Chu et al., 2003) and some products of lipoxygenases, such as 12-HPETE (12-hydroperoxyeicosatetraenoic acid) and leukotriene B4 (Hwang et al., 2000). Due to their lipophilic nature, these TRPV1 agonists are able to freely cross the cell membrane and interact with the intracellular binding site of the receptor (De Petrocellis et al., 2001; Jung et al., 1999).

1.2. Brain localization

In addition to their presence in sensory neurons and some tissues such as the respiratory and urinary bladder epithelium (Szallasi et al., 1993; Ahmed et al., 2009; Watanabe et al., 2005), skin, mast cells (Stander et al., 2004) and the enteric system (Horie et al., 2005; Matsumoto et al., 2011), a large number of studies employing [3H]RTX autoradiography, immunohistochemistry, electron microscopy, electrophysiology and molecular biological (western blot, RT-PCR and in situ hybridization) techniques, as well as genetically-modified mice, indicated that TRPV1 receptors are located in several CNS regions, including the dorsal horn of the spinal cord (Acs et al., 1994; Szallasi and Blumberg, 1991), hindbrain, midbrain and forebrain areas, being present not only in neurons but also in astrocytes (Toth et al., 2005). These studies are summarized in Table 1. Some studies, however, have failed to detect the presence of TRPV1 channels in the CNS (Benninger et al., 2008; Caterina et al., 1997; Szallasi, 1995; Tominaga et al., 1998). Corroborating these latter findings, a recent study using a sophisticated genetic strategy to visualize TRPV1, complemented with the use of TRPV1 knockout mice, was also unable to detect the expression of these receptors in most of the brain areas analyzed except for a discrete expression in few regions that include areas within and adjacent to the caudal hypothalamus and rostral midbrain (Cavanaugh et al., 2011). How much these contradictory results depend on methodological issues needs to be further clarified. Only the generation of brain-specific TRPV1 deficient mouse mutants may ultimately resolve this puzzle. In addition, its cellular localization (e.g., pre- versus post-synaptic) in specific neuronal populations needs to be further investigated. Finally, further quantitative experiments need to be performed to compare TRPV1 expression across different regions of the CNS. Anyhow, as reviewed below, even if the expression of TRPV1 channels in the brain is much lower than in other areas such as the DRG (Cavanaugh et al., 2011; Sanchez et al., 2001), pharmacological, electrophysiological and behavioral studies strongly indicate that brain-located TRPV1 plays an important role on the control of emotional responses.

2. TRPV1 channels modulate defensive behavior: systemic and genetic studies

The development of pharmacological tools has been instrumental for studying the involvement of TRPV1 channels in diverse physiological processes. Among them, capsazepine (a thiourea derivate), one of the first antagonists to be discovered, was initially employed to investigate the involvement of these channels in defensive responses. This compound, however, lacks selectivity, since it can also bind to voltage-gated calcium channels and nicotinic acetylcholine receptors. The capsaicin halogenated derivate, 6-iodo-nordihydrocapsaicin, is a more selective and potent (4-fold) antagonist than capsazepine (Appendino et al., 2003). Finally, cinnamide analogs, such as SB-366791, also exhibit a very high selectivity to TRPV1 channels (for a review, see Szallasi et al., 2003).

Regarding their effects in defensive responses, an initial study employed systemic injection of capsazepine and observed

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Table 1
Studies supporting the presence of TRPV1 receptors in mammalian brain regions.

Brain region	Subregion	Localization	Co-expression	Species	Reference
Amygdala	NS	NS	NS	M	Roberts et al. (2004)
	Central	NS	NS	R	Mezey et al. (2000)
	Basolateral/lateral	NS	NS	M	Zschenderlein et al. (2011)
Basal Ganglia	Striatum	NS	NS	R, M	de Lago et al. (2004), Maccarrone et al. (2008), Mezey et al. (2000), Sasamura et al. (1998) and Tzavara et al. (2006)
	Substantia nigra	NS	NS	R	Musella et al. (2009, 2010)
		NS	CB1	M	Cristino et al. (2006)
		Pre-synaptic	CB1	R	Marinelli et al. (2007)
	Caudate putamen	NS	NS	M	Roberts et al. (2004)
		NS	CB1	M	Cristino et al. (2006)
	Globus palidus	NS	CB1	M	Cristino et al. (2006)
	Nucleus accumbens	Post-synaptic	NS	M	Grueter et al. (2010)
	Ventral Tegmental Area	NS	NS	R	Marinelli et al. (2005)
BNST	NS	Post-synaptic	CB1	R	Puente et al. (2011)
Cerebellum	NS	NS	NS	H, MK, R	Cortright et al. (2001), Hayes et al. (2000), Mezey et al. (2000), Sanchez et al. (2001), Sasamura et al. (1998), Schumacher et al. (2000), Szabo et al. (2002) and Toth et al. (2005)
	Cerebellar cortex	NS	NS	M	Roberts et al. (2004)
		NS	CB1	M	Cristino et al. (2006)
Cortex	NS	NS	NS	R	Mezey et al. (2000), Sanchez et al. (2001), Sasamura et al. (1998), Schumacher et al. (2000)
		Post-synaptic	NS	R	Toth et al. (2005)
	Parietal	NS	NS	H, R, M	Mezey et al. (2000) and Sun et al. (2013)
	Somatosensory	NS	NS	MK	Szabo et al. (2002)
		NS	TRPV2	R	Liapi and Wood (2005)
	Somatomotor	NS	TRPV2	R	Liapi and Wood (2005)
		NS	NS	MK	Szabo et al. (2002)
	Frontal	NS	NS	H, R, M	Hayes et al. (2000) and Roberts et al. (2004)
	Prelimbic	Probably post-synaptic	Not found	R	Fogaca et al. (2012)
	Entorhinal	NS	NS	M	Cavanaugh et al. (2011)
	Parietal	NS	NS	R, M	Mezey et al. (2000) and Roberts et al. (2004)
	Piriform	NS	NS	R, M	Roberts et al. (2004) and Szabo et al. (2002)
	Retrosplienal	NS	NS	M	Roberts et al. (2004)
	Insular	NS	TRPV2	R	Liapi and Wood (2005)
	Cingulate	NS	TRPV2	R	Liapi and Wood (2005)
		NS	NS	R	Steenland et al. (2006)
Habenula	Medial, lateral	NS	NS	R	Mezey et al. (2000)
Hippocampus	NS	NS	NS	H, R, M	Cavanaugh et al. (2011), Hayes et al. (2000), Sanchez et al. (2001), Sasamura et al. (1998), Schumacher et al. (2000)
	Dentate gyrus	NS	NS	H, R, M	Roberts et al. (2004) and Sun et al. (2013)
		Post-synaptic	NS	R, M	Toth et al. (2005)
		NS	CB1	M	Cristino et al. (2006)
	CA1	Post-synaptic	NS	R, H	Roberts et al. (2004), Sun et al. (2013) and Toth et al. (2005)
		NS	NS	R, M	Al-Hayani et al. (2001), Marsch et al. (2007), Roberts et al. (2004) and Li et al. (2008)
		NS	CB1	M	Cristino et al. (2006)
		Pre-synaptic	NS	M	Gibson et al. (2008)
	CA2	NS	NS	M, H	Roberts et al. (2004) and Sun et al. (2013)
		NS	CB1	M	Cristino et al. (2006)
	CA3	NS	CB1	M	Cristino et al. (2006)
		NS	NS	M	Cristino et al. (2008) and Roberts et al. (2004)
Hypothalamus	–	NS	NS	R	Sasamura et al. (1998) and Schumacher et al. (2000)
	Suprachiasmatic and anterior	NS	NS	R	Mezey et al. (2000)
	Supraoptic	Pre-synaptic	NS	R, M	Yokoyama et al. (2010)
	Medial-basal	NS	NS	MK	Szabo et al. (2002)
	Medial	NS	NS	R, H, M	Acs et al. (1994) and Roberts et al. (2004)
	Periventricular, ventral medial	NS	NS	M	Roberts et al. (2004)
	Dorsomedial	NS	NS	M, R	Cavanaugh et al. (2011), Mezey et al. (2000) and Roberts et al. (2004)
	Posterior	NS	NS	M, R	Cavanaugh et al. (2011)
	Arcuate	NS	NS	M, R, MK	Mezey et al. (2000), Roberts et al. (2004) and Szabo et al. (2002)
	Preoptic area	NS	NS	R, H, MK	Acs et al. (1994), Hori et al. (1988) and Szabo et al. (2002)
		Pre-synaptic	NS	R	Karlsson et al. (2005)
	Paraventricular	NS	NS	R, M	Mezey et al. (2000) and Roberts et al. (2004)
Locus coeruleus	NS	NS	NS	MK, H, M, R	Acs et al. (1994), Hajos et al. (1987), McGaraughty et al. (2003), Mezey et al. (2000), Roberts et al. (2004) and Szabo et al. (2002)

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Table 1 (*Continued*)

Brain region	Subregion	Localization	Co-expression	Species	Reference
		Probably pre-synaptic	NS	R	Marinelli et al. (2002)
		Pre-synaptic	NS	R	Li et al. (2004)
		NS	CB1	M	Cristino et al. (2006)
Olfactory bulb	NS	NS	NS	R, M	Sanchez et al. (2001) and Sasamura et al. (1998)
Olive	Inferior	NS	NS	R	Mezey et al. (2000)
Periaqueductal gray matter	NS	NS	NS	M	Cavanaugh et al. (2011) and Roberts et al. (2004)
		NS	CB1	M	Cristino et al. (2006)
	Dorsolateral	Pre-synaptic	NS	R, M	Xing and Li (2007)
	Ventral	NS	NS	R	McGaraughty et al. (2003)
	Dorsal	NS	NS	R	McGaraughty et al. (2003)
		NS	CB1	R	Casarotto et al. (2012)
	Ventrolateral	Probably pre-synaptic	CB1	R, M	Maione et al. (2006)
Raphe	NS	NS	NS	M, R	McGaraughty et al. (2003) and Roberts et al. (2004)
	Dorsal	NS	NS	R	Cavanaugh et al. (2011) and Mezey et al. (2000)
Reticular formation	NS	NS	NS	R, H, MK	Acs et al. (1994), Mezey et al. (2000) and Szabo et al. (2002)
Several Nuclei	Cochlear, mammillary, suprammamillary, interpeduncular, olfactory, intrafascicular	NS	NS	R, M	Cavanaugh et al. (2011), Mezey et al. (2000) and Roberts et al. (2004)
Septum	Dorsal, fimbrial septal nucleus	NS	NS	R	Mezey et al. (2000)
	Lateral, medial septal nucleus	NS	NS	M, R	Mezey et al. (2000) and Roberts et al. (2004)
Solitary tract nucleus	NS	NS	NS	R	Guo et al. (1999) and Mezey et al. (2000)
		NS	$P2X_3$	R	Guo et al. (1999)
		Pre-synaptic	NS	R	Doyle et al. (2002)
Superior colliculus	NS	NS	NS	M, R	Roberts et al. (2004)
		Probably pre-synaptic	NS	M	Maione et al. (2009a)
Thalamus	–	NS	NS	R	Sasamura et al. (1998)
	Paracentral, central lateral, ventromedial, medial habenular, zona incerta	NS	NS	M	Roberts et al. (2004)
	Paraventricular	NS	NS	R, M	Mezey et al. (2000) and Roberts et al. (2004)
	Centromedian and midline	NS	NS	R	Mezey et al. (2000)
	Ventral	NS	NS	MK, H, R	Acs et al. (1994) and Roberts et al. (2004)
	Reticular	NS	NS	M	Roberts et al. (2004)
		NS	CB1	M	Cristino et al. (2006)
	Ventral posterolateral, laterodorsal thalamic nucleus ventrolateral	NS	CB1	M	Cristino et al. (2006)
Trigeminal tract	NS	NS	NS	R	Guo et al. (1999) and Mezey et al. (2000)

NS: Not Shown; R: Rat; M: Mouse; MK: Monkey; H: Human.

anxiolytic-like effects in rats submitted to the elevated plus maze (EPM), suggesting that tonic activation of these receptors facilitates anxious states (Kasckow et al., 2004). This result has been recently confirmed by a study showing that, besides being anxiolytic by itself, i.c.v. injected capsazepine potentiates the effects of an anxiolytic benzodiazepine in the social interaction test in mice (Manna and Umathe, 2011).

Experiments investigating the phenotype of knockout animals also point to TRPV1 channel as an important modulator of fear and anxiety-related responses. TRPV1-KO mice explore more the open arms of the EPM and the lit compartment of the light-dark box, in comparison to wild-type littermate, indicating reduced anxiety-like behavior. Moreover, deletion of this channel results in impaired stress sensitization as well as decreased cue and contextual fear conditioning (Marsch et al., 2007). Thus, the genetic approach converges with pharmacological data suggesting that TRPV1 tonically controls both innate and learned defensive responses.

3. Brain sites involved in TRPV1 modulation of defensive responses

Although the aforementioned results indicate that pharmacological or genetic interference with TRPV1 channels modifies defensive-like responses, it was only recently that the brain sites involved in these effects began to be investigated. As discussed below, studies using intra-cerebral injections of TRPV1 agonists/antagonists show that these channels modulate normal defensive responses both to potential (anxiety) or clearly threatening stimuli (fear; Blanchard and Blanchard, 2008) in brain areas that include the medial prefrontal cortex (mPFC), the ventral hippocampus (vHPC) and the midbrain periaqueductal gray (PAG) (Canteras et al., 2010; McNaughton and Corr, 2004; Millan, 2003).

3.1. TRPV1 and the medial prefrontal cortex

The mPFC, particularly its ventral portion (vmPFC), coordinates defensive behaviors (Bishop, 2007; Vertes, 2006) via its connections with limbic structures that include the bed nucleus of stria terminalis (BNST), amygdaloid complex, ventral hippocampus, medial thalamus and brainstem (Peters et al., 2009). Intra-vmPFC injection of the TRPV1 antagonist capsazepine decreased anxiety in the EPM and Vogel conflict (VCT) tests and attenuated the expression of contextual fear conditioning in rats (Rubino et al., 2008; Aguiar et al., 2009; Terzian et al., 2014). This anxiolytic effect was confirmed with 6-iodo-nordihydrocapsaicin (Fogaca et al., 2012; Terzian et al., 2014), a more potent and selective TRPV1 antagonist (Appendino

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Table 2
Pharmacological targeting of TRPV1 receptors in animal tests of anxiety.

Administration route	Drug (dose range tested) [effective doses]	Possible mechanism	Animal model	Effect on defensive behaviors	Species	References
i.p.	Olvanil (0.2–5.0 mg/kg) [0.2, 1 and 5 mg/kg]	TRPV1 agonist	EPM	↑	Rat	Kasckow et al. (2004)
i.c.v.	Capsaicin (1–100 μg) [10 and 100 μg]	TRPV1 agonist	Social interaction test	↑	Mice	Manna and Umathe (2011)
i.c. (mPFC)	Methanandamide (0.1–10 μg) [10 μg[a]]	CB1 and TRPV1 agonist	EPM	↑	Rat	Rubino et al. (2008)
i.c. (mPFC)	Capsaicin (1–10 μg and 1 nmol) [5 and 10 μg, 1 nmol]	TRPV1 agonist	EPM, contextual fear conditioning	↑	Rat	Rubino et al. (2008) and Terzian et al. (2014)
i.c. (dHPC)	Capsaicin (0.003, 0.03 and 0.3 μg) [0.003 and 0.03 μg]	TRPV1 agonist	EPM	↑	Rat	Hakimizadeh et al. (2012)
i.c. (dPAG)	Capsaicin (0.01, 0.1 and 1 nmol) [0.1 and 1 nmol]	TRPV1 agonist	EPM	↑	Mice	Mascarenhas et al. (2013)
i.c. (dlPAG)	Capsaicin (0.01, 0.1 and 1 nmol) [1 nmol]	TRPV1 agonist	EPM and VCT	↓[b]	Rat	Terzian et al. (2009)
i.p.	Capsazepine (1–10 μg/kg) [1, 5 and 10 μg/kg]	TRPV1 antagonist	EPM	↓	Rat	Kasckow et al. (2004)
i.p.	SB366791 (0.1–2.5 mg/kg) [1 mg/kg]	TRPV1 antagonist	EPM	↓	Mice	Micale et al. (2009)
i.p.	AA-5-HT (0.1–5 mg/kg) [0.1, 0.5, 1 and 2.5 mg/kg]	TRPV1 antagonist and FAAH inhibitor	EPM	↓	Mice	Micale et al. (2009)
i.c. (BLA)	AA-5-HT (0.25–0.5 nmol) [0.25 and 0.5 nmol]	TRPV1 antagonist and FAAH inhibitor	EPM	↓	Rat	John and Currie (2012)
i.c.v.	Capsazepine (100 μg)	TRPV1 antagonist	Social interaction test	↓	Mice	Manna and Umathe (2011)
i.c. (mPFC)	Capsazepine (10 μg)	TRPV1 antagonist	EPM	↓	Rat	Rubino et al. (2008)
i.c. (mPFC)	Capsazepine (1, 10 and 60 nmol) [1, 10 and 60 nmol]	TRPV1 antagonist	EPM, VCT and contextual fear conditioning	↓	Rat	Aguiar et al. (2009) and Terzian et al. (2014)
i.c. (mPFC)	6-I-CPS (3 nmol)	TRPV1 antagonist	EPM, VCT and contextual fear conditioning	↓	Rat	Fogaca et al. (2012) and Terzian et al. (2014)
i.c. (vHPC)	Capsazepine (0.2–2 nmol) [2 nmol]	TRPV1 antagonist	EPM	↓	Rat	Santos et al. (2008)
i.c. (dHPC)	AMG 9810 (0.003, 0.03 and 0.3 μg) [0.03 and 0.3 μg]	TRPV1 antagonist	EPM	↓	Rat	Hakimizadeh et al. (2012)
i.c. (dlPAG)	Capsazepine (1–60 nmol) [60 nmol in the ETM, EPM or VCT and 1, 10, 30 or 60 nmol in dlPAG chemical or electrical stimulation]	TRPV1 antagonist	ETM, EPM, VCT and escape induced by dPAG electrical or chemical (NMDA, NO) stimulation	↓	Rat	Almeida-Santos et al. (2013), Casarotto et al. (2012), Lisboa and Guimaraes (2012) and Terzian et al. (2009)
i.c. (dPAG)	SB366791 (1 nmol)	TRPV1 antagonist	Escape induced by dPAG electrical stimulation	↓	Rat	Casarotto et al. (2012)

i.p.: intraperitoneal, i.c.v.: intracerebroventricular, i.c.: intracerebral, dlPAG: dorsolateral periaqueductal gray, dPAG: dorsal periaqueductal gray, ETM: elevated T-maze, EPM: elevated plus-maze, mPFC: medial prefrontal cortex, vHPC: ventral hippocampus, dHPC: dorsal hippocampus, BLA: basolateral amygdala, AA-5HT: N-arachidonoyl-serotonin, 6-I-CPS: 6-iodonordihydrocapsaicin, SB366791: N-(3-Methoxyphenyl)-4-chlorocinnamide, VCT: Vogel conflict test; NO: nitric oxide.
[a] Anxiolytic at 0.1 μg.
[b] Desensitization of TRPV1 receptors?

et al., 2003; Table 2). Capsazepine was also able to reverse the anxiogenic-like effect induced by higher doses of anandamide in rats (Rubino et al., 2008).

3.2. TRPV1, the ventral hippocampus, BNST and amygdaloid complex

Specific lesions of the ventral hippocampus (vHPC), BNST and amygdaloid complex attenuate anxiety-like behaviors (Bannerman et al., 2004; Bertoglio et al., 2006; Engin and Treit, 2007; Pentkowski et al., 2006). These regions are proposed to cooperate with the vmPFC to regulate emotional responses (Bannerman et al., 2004; Kjelstrup et al., 2002; Verwer et al., 1997). Similar to the vmPFC, blockade of TRPV1 channels in the vHPC was anxiolytic in rats tested in the EPM (Santos et al., 2008). Capsaicin was recently shown to modify long-term potentiation (LTP), the physiological correlate of fear memories (Sigurdsson et al., 2007), in slices obtained from the lateral amygdala of mice (Zschenderlein et al., 2011). TRPV1channels are also involved in long-term depression (LTD) induced by anandamide in the BNST of rats (Puente et al., 2011). Since these regions are related to defensive behavior and aversive conditioning learning (Canteras et al., 2010), TRPV1 could modulate anxiety and fear learning mechanisms. However, the effects of TRPV1-related drugs injected into the amygdala or BNST on anxiety responses remain to be investigated.

3.3. TRPV1, the periaqueductal gray and panic-like behavior

The PAG is proposed to control emotional coping strategies (Blanchard et al., 2005; McNaughton and Corr, 2004). Among its regional subdivisions, the dorsal portion (dPAG), particularly the dorsolateral column (dlPAG), is part of a neural defensive system that coordinates freezing, flight and fight behaviors in threatening situations, such as the presence of a predator (Blanchard and Blanchard, 2008; Canteras and Blanchard, 2008). Several pieces of evidence indicate that this region is closely associated with

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panic attacks (Del-Ben and Graeff, 2009; Gray and McNaughton, 2000; Schenberg et al., 2001). For example, electrical stimulation of the dPAG in humans induces symptoms similar to a panic attack (Schenberg et al., 2001), such as feelings of intense fear and imminent death, together with autonomic arousal (Nashold et al., 1969). Corroborating this finding, neuroimaging studies show increased dPAG activity in panic patients (Del-Ben and Graeff, 2009) or healthy volunteers exposed to a proximal threatening stimulus such as predator exposure (Mobbs et al., 2007). In rodents, electrical or chemical stimulation of the dPAG causes intense flight responses accompanied by autonomic changes (Beckett and Marsden, 1995; Krieger and Graeff, 1985; Schenberg et al., 2001), which are attenuated by systemic or local administration of panicolytic drugs (Hogg et al., 2006; Jenck et al., 1995; Moreira et al., 2013; Schenberg et al., 2001; Schutz et al., 1985). Taken together, these findings suggest that local electrical or chemical stimulation of the dPAG is a putative model of panic attacks (Moreira et al., 2013; Schenberg et al., 2001). Moreover, these panic-like responses can be modulated by several neurotransmitters including serotonin, GABA, glutamate and nitric oxide (Guimaraes et al., 1991; Moreira and Guimaraes, 2004; Schenberg et al., 2001).

Considering that several studies indicate the presence of TRPV1 in the PAG (Cavanaugh et al., 2011; Cristino et al., 2006; Maione et al., 2006; McGaraughty et al., 2003; Roberts et al., 2004; Xing and Li, 2007), we have recently started to investigate the possible role of dlPAG-located TRPV1 channels in defensive responses. In line with our initial hypothesis, intra-dlPAG administration of capsazepine in rats reduced anxiety-like behavior in the EPM and the VCT (Terzian et al., 2009). In this study, however, a similar anxiolytic effect was observed after intra-dlPAG administration of capsaicin, which could have involved TRPV1 desensitization (De Petrocellis et al., 2011). Contrasting with this latter result, anxiogenic effects of capsaicin have been reported in mice after dlPAG administration (Mascarenhas et al., 2013).

We have also investigated TRPV1 antagonists in animal tests associated with panic attacks. Local injection of capsazepine and the selective TRPV1 antagonist, SB366791, attenuated panic-like behavior induced by electrical stimulation of the dorsal PAG in rats (Casarotto et al., 2012). This result was reproduced in three other tests: escape responses induce by (i) local injection of the excitatory amino acid N-methyl-D-aspartate (NMDA) or (ii) the nitric oxide donor SIN-1, and (iii) exposure to the open arms of the elevated T-maze (Almeida-Santos et al., 2013; Lisboa and Guimaraes, 2012). Taken together, these results indicate that, under threatening situations, TRPV1 channels facilitate defensive responses in the dlPAG.

4. Mechanisms of TRPV1 interference in defensive behavior

The anti-aversive effects of TRPV1 antagonists in the dlPAG strongly resemble those described for antagonists of glutamate ionotropic receptors (NMDA and non-NMDA) (Aguiar and Guimaraes, 2011; Guimaraes et al., 1991; Molchanov and Guimaraes, 2002; Resstel et al., 2008; Tonetto et al., 2009). Modification of glutamate-mediated synaptic transmission (Feinmark et al., 2003; Gibson et al., 2008; Kauer and Gibson, 2009) has been proposed as the main mechanism by which TRPV1 channels influence several physiological conditions, including neural plasticity (Bennion et al., 2011; Chavez et al., 2010; Gibson et al., 2008; Grueter et al., 2010; Li et al., 2008; Marsch et al., 2007), descending modulation of nociception (McGaraughty et al., 2003) and facilitation of aversive states (Terzian et al., 2009).

Early electrophysiological data obtained in rat brain slices of the hypothalamus, substantia nigra or locus coeruleus showed that TRPV1 stimulation evokes glutamate release (Marinelli et al., 2002, 2003; Sasamura et al., 1998). Corroborating these findings, an increase in glutamatergic synaptic transmission was also observed after capsaicin infusion into the dPAG, an effect that was prevented by TRPV1 antagonists (Xing and Li, 2007). These results agree with findings suggesting that TRPV1 channels are present in glutamatergic terminals in the PAG and adjacent colliculi (Cristino et al., 2006; Maione et al., 2006, 2009a, 2009b; McGaraughty et al., 2003; Starowicz et al., 2007). Anandamide can also tonically facilitate glutamate release through TRPV1 activation in the substantia nigra (Marinelli et al., 2003). Indeed, it has recently been reported that, when anandamide degradation was inhibited along cannabinoid type 1 (CB1) blockade, glutamatergic activity increases in the PAG through a presynaptic TRPV1 mechanism (Kawahara et al., 2011).

In addition to presynaptic, post-synaptic effects of TRPV1 channels on glutamate-mediated neurotransmission can also be important. Actually, it was recently shown that the long-term depression (LTD) induced in the nucleus accumbens by mGluR-5 activation depends on postsynaptic TRPV1 activation by anandamide (Grueter et al., 2010). Similar results have been reported in the dentate gyrus (Chavez et al., 2010). Also, in the BNST an increased anandamide release induced by glutamate-mediated activation of mGluR-5 could act on post-synaptic TRPV1 to induce LTD (Puente et al., 2011). In this region TRPV1 channels were present for the most part in dendrites next to the synaptic cleft (Puente et al., 2011). Anandamide has a lower affinity for TRPV1 than CB1 receptors (Piomelli, 2003), and facilitation of glutamate release by TRPV1 activation might be related to the bell-shaped dose–response curves observed with high doses of anandamide and other cannabinoids (Moreira et al., 2007, 2012; Campos and Guimaraes, 2009; Rubino et al., 2008). In line with this, we hypothesized that the lack of anxiolytic-like effects observed with higher doses of anandamide injected into the dlPAG of rats was due to TRPV1-mediated facilitation of glutamatergic neurotransmission. Accordingly, local blockade of NMDA receptors unveiled the anxiolytic-like effect of higher doses of this substance, which was prevented by a CB1 receptor antagonist (Fogaca et al., 2013).

Considering that anandamide can bind to both TRPV1 and CB1, we also hypothesized the existence of a reciprocal interaction between these two receptors in the modulation of panic-like responses. At electrophysiological and neurochemical levels, TRPV1 and CB1 mediate opposite responses regarding neural firing and glutamate release (Kawahara et al., 2011). Accordingly, we found that local TRPV1 antagonism or CB1 activation increases the current threshold to induce panic-like behaviors after electrical stimulation of the dlPAG (Casarotto et al., 2012). Furthermore, CB1 antagonism prevented the anti-panic effects of TRPV1 antagonists. We also observed that these receptors are co-expressed in synaptic terminals in the dlPAG (Casarotto et al., 2012). Similar reciprocal interactions have been observed in the modulation of anxiety-like behavior in rats tested in the EPM and VCT after anandamide administration into the vmPFC (Fogaca et al., 2012).

Based on these observations, we put forward a hypothesis that anandamide, CB1 and TRPV1 could behave as a set-point system in the modulation of panic-like responses (Moreira et al., 2012; Moreira and Wotjak, 2010). We proposed that anandamide, which can tonically activate both receptors, has its actions entirely shift to CB1 after TRPV1 blockade. This would change the balance, resulting in CB1-mediated anti-aversive effects of TRPV1 antagonists. One premise of this hypothesis is that anandamide could simultaneously activate both receptors, which is supported by their co-localization in the PAG and other brain regions. Therefore, the mechanisms though which TRPV1 modulate defensive responses seem to be tightly linked to the functioning of the endocannabinoid system (Di Marzo, 2008; Moreira et al., 2012; Moreira and Wotjak, 2010).

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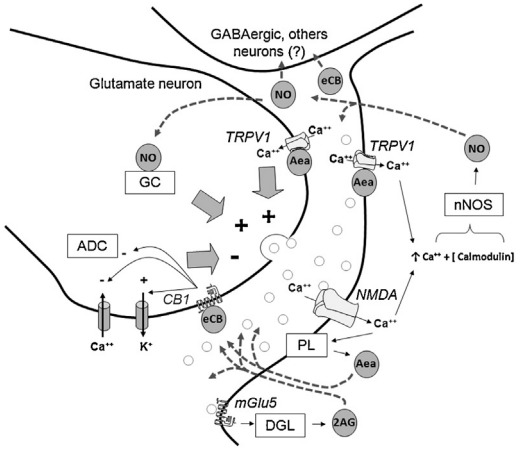

Fig. 1. Possible mechanisms involved in TRPV1-mediated effects in defensive responses. Activation of TRPV1 receptors by anandamide facilitates glutamate (open circles) release, which drives defensive responses in anxiety-related regions such as the vmPFC and dlPAG. However, recent evidence suggests that other neurotransmitters, including NO, and GABA, are also involved in the modulatory influence of these receptors in anxiety (see main text for details). 2AG: 2-arachidonoyl glycerol, ADC: adenylyl cyclase, CB1: cannabinoid type-1 receptor, DGL: diacylglycerol lipase, GC: guanylyl cyclase, mGlu5: glutamate metabotropic type-5 receptor, NMDA: N-methyl-D-aspartate receptor, nNOS: neuronal nitric oxide synthase, NO: nitric oxide, PL: N-acylphosphatidylethanolamine-hydrolyzing phospholipase.

Although most of the evidence reviewed so far suggests that TRPV1 channels interfere with defensive responses by facilitation of glutamate release, the role of these receptors is probably more complex and could involve other neurotransmitters such as nitric oxide (NO). In the CNS, NO is synthesized by the neuronal nitric oxide synthase (nNOS) (Bredt and Snyder, 1994). This enzyme is activated by calcium influx trough NMDA receptors, indicating a close relationship between NO and glutamate-mediated neurotransmission (Bredt and Snyder, 1994). Recent electrophysiological data by Zschenderlein et al. (2011) suggest that anandamide stimulates NO synthesis via TRPV1. NO could then act presynaptically as a retrograde messenger and enhance the release of glutamate (Zschenderlein et al., 2011). Corroborating a possible TRPV1-NO-glutamate interaction, systemic administration of capsaicin in rats increased NO synthesis in the paraventricular nucleus of hypothalamus, medial amygdala and dlPAG, brain regions related to defensive responses (Okere et al., 2000a, 2000b; Okere and Waterhouse, 2006). Therefore, TRPV1 could modulate aversive responses by facilitating the glutamate/NMDA receptor/NO pathway. To test this hypothesis we verified if defensive responses induced by increasing NO action in the dlPAG of rats would be blocked by pretreatment with a TRPV1 antagonist. In accordance to our expectations, local injection of capsazepine prevented the defensive reaction induced by the NO donor 3-morpholinosylnomine hydrochloride (SIN-1) in this brain region (Lisboa et al., 2013). This result is in line with that obtained in a previous study showing that the flight reactions induced by SIN-1 in the dlPAG depend on glutamate release (Moreira et al., 2004). Moreover, similar to the effects observed with TRPV1 antagonists, local blockade of NMDA receptors in the dlPAG turned a high, ineffective dose of anandamide, into an anxiolytic one (Fogaca et al., 2013). Together, these findings suggest that NO and endovanilloids interact to control glutamate-mediated defensive responses (Fig. 1).

Some authors have also proposed that TRPV1 can interfere with GABAergic neurotransmission (Bennion et al., 2011; Maccarrone et al., 2008). However, this interference has been found in processes involving synaptic plasticity and there is still controversy if they depend on a direct effect on GABA neurons or if TRPV1 receptors facilitate GABAergic neurotransmission indirectly through a glutamate-mediated mechanism (Chavez et al., 2010; Gibson et al., 2008; Maione et al., 2009a). Linking this latter proposal with the endocannabinoid system, a recent electrophysiological study using ventrolateral PAG slices suggested that local TRPV1 activation, by facilitating glutamate release, activates post-synaptic metabotropic type-5 glutamate receptor (mGlu-5) and increases 2-arachidonoyl glycerol (2-AG) formation (Liao et al., 2011). This endocannabinoid would then act presynaptically on CB1 receptors to decrease GABA release (Fig. 1). This would disinhibit the vlPAG and activate the descending pain inhibitory pathway. This result suggests that TRPV1 and CB1 interact to control not only glutamate but also GABA-mediated neurotransmission and may help to explain why, although sharing endogenous ligands such as anandamide, these receptors seem to play opposite roles in several physiological responses, including those related to fear and anxiety (Di Marzo, 2008; Moreira et al., 2012; Moreira and Wotjak, 2010).

At the present most studies have investigated the effects of single, acute interference with TRPV1 channels on defensive responses. However, TRPV1 channels can undergo plastic changes after prolonged exposure to aversive stimuli such as chronic pain. For example, de Novellis and co-workers (2011) showed that neuropathic pain induced by spinal nerve ligation causes an increase in extra-cellular glutamate in the vmPFC accompanied by up-regulation of fatty acid amide hydrolase (FAAH, the enzyme responsible for anandamide hydrolysis) and TRPV1 channels (de Novellis et al., 2011). Increased expression of TRPV1 channels during chronic pain has also been described in the periphery (Akbar et al., 2010). Although the mechanisms involved in these changes are unknown, a recent study in rats showed that repeated corticosterone administration or exposure to a psychological stressor (water avoidance, 1 h daily) for 10 days resulted in visceral hyperalgesia and increased and decreased expression of TRPV1 and CB1 receptors, respectively, in DRG neurons. The stress effects were prevented by co-treatment with the glucocorticoid receptor (GR) antagonist RU-486. In vitro incubation of DRG neurons with corticosterone produced similar results (Hong et al., 2011). Together, these results suggest that stress exposure, by enhancing corticosterone levels and activating GRs, changes the balance between TRPV1 and CB1 receptors, increasing the former and decreasing the latter. Other stressful interventions such as exposure to volatile anesthetics may also result in TRPV1 sensitization (Cornett et al., 2008; Harrison and Nau, 2008; Zschenderlein et al., 2011). These results highlight the importance of further investigating the plastic changes of the endovanilloid system under chronic conditions such as repeated drug administration or prolonged exposure to aversive situations.

5. Conclusions

Several pieces of evidence suggest that TRPV1 channels promote defensive responses. The underlying mechanisms of this effect seem to involve facilitation of glutamate actions in specific brain areas that include the dlPAG, vmPFC and vHPC. Key questions, however, remain to be answered, including TRPV1 role in other brain regions related to defensive responses, such as the amygdala and BNST, its differential involvement in physiological versus pathological anxiety and its multiple interactions with other transmitter systems. Its endogenous ligands and their release mechanisms under acute and repeated aversive situations also remain to be determined. An additional aspect to be considered is the potential clinical use of TRPV1 desensitization by capsaicin analogs (De Petrocellis et al., 2011). Also, the possible side effects of acute and chronic administration of TRPV1-related drugs remain to be further investigated. TRPV1 channels play an important role in body temperature regulation (Moran et al., 2011) and its antagonists cause

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hyperthermia in laboratory animals (Gavva et al., 2008). Accordingly, human studies indicated that hyperthermia could be a side effect of these drugs (Gavva et al., 2007; Krarup et al., 2011). Thus, an important step toward therapeutic applications would be developing compounds that are able to block specific sites of TRPV1 channels, without interfering with its modulatory role upon body temperature (Moran et al., 2011).

Finally, TRPV1 should also be considered for the development of drugs that interfere with endocannabinoids. Compounds that inhibit the anandamide-hydrolyzing enzyme, FAAH, have anxiolytic properties by enhancing the actions of this endocannabinoid upon CB1 receptors (Kathuria et al., 2003; Moreira et al., 2008). Their effects, however, are partially occluded by the parallel action of anandamide upon TRPV1. In this way, simultaneous blockade of FAAH and TRPV1 induced by drugs such as N-arachidonoyl-serotonin (AA-5-HT) represents an interesting strategy to increase anandamide levels and redirect its action entirely to CB1 receptors, resulting in a more robust anxiolytic effect (Micale et al., 2009).

Thus, drug development in this field could be based either on selective blockade of TRPV1 or rely upon interactions between this ion channel and the endocannabinoid system. If their putative side effects are circumvented, drugs targeting TRPV1 could represent a new and promising strategy for the treatment of anxiety disorders.

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ORIGINAL INVESTIGATION

Medial prefrontal cortex Transient Receptor Potential Vanilloid Type 1 (TRPV1) in the expression of contextual fear conditioning in Wistar rats

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Abstract
Rationale Contextual fear is evoked by re-exposing an animal to an environment that has been previously paired with an aversive or unpleasant stimulus. It can be assessed by freezing and cardiovascular changes such as increase in mean arterial pressure and heart rate. A marked increase in neuronal activity is associated with contextual fear conditioning, especially in limbic structures involved with defense reactions, such as the ventral portion of medial prefrontal cortex.
Objective Given the fact that transient receptor potential vanilloid type 1 (TRPV1) receptors could be involved in the expression of defensive behavior, the present work tested the hypothesis that TRPV1 manipulation in the ventromedial prefrontal cortex (vMPFC) modulates the expression of contextual conditioned fear.
Methods Male Wistar rats received bilateral microinjections into the vMPFC of the TRPV1 receptor antagonists capsazepine (1, 10, and 60 nmol/200 nL) or 6-iodonordihydrocapsaicin (3 nmol/200 nL), and the TRPV1 agonist capsaicin (1 nmol/200 nL) preceded by vehicle or 6-iodonordihydrocapsaicin before re-exposure to the experimental chamber for 10 min, 48 h after conditioning in two different protocols distinct by their aversiveness.
Results Both antagonists reduced the freezing and cardiovascular responses in the high aversive protocol. Capsaicin caused an increase in fear-associated responses that could be blocked by 6-iodonordihydrocapsaicin.
Conclusions Our results indicate that TRPV1 receptors located in the vMPFC have a tonic involvement in the modulation of the expression of contextual fear conditioning.

Keywords Contextual fear · TRPV1 receptors · Prefrontal cortex · Cardiovascular system · Rats

Introduction

Contextual fear responses are evoked when an animal is re-exposed to an environment previously paired with an electric footshock (Blanchard and Blanchard 1969; Fanselow 1980; LeDoux et al. 1988; Resstel et al. 2008a, c). It is characterized by freezing and cardiovascular responses such as mean arterial pressure (MAP) and heart rate (HR) increases. Different shock intensities can be applied to better understand the relation between fear responses and environment aversiveness, i.e., higher shock intensities are usually used to overload the subject, thus the search for manner to reduce or alleviate the aversive response; less aversive shock intensities can be used to avoid the ceiling effect and investigate how a certain system could be involved in the fear expression and the potential enhancer effect of some drugs (Quigley et al. 1994; Baldi et al. 2004; Lisboa et al. 2010).

Fear-related responses are associated with increased neuronal activity in the ventral portion of medial prefrontal cortex

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(vMPFC) (Beck and Fibiger 1995). This region comprises infralimbic (IL) and prelimbic (PL) cortices (Verberne and Owens 1998; Resstel and Correa 2006) that are involved with cardiovascular, neuroendocrine, and behavioral responses, including those elicited by contextual fear conditioning (Frysztak and Neafsey 1994; Schulkin et al. 2005; Sierra-Mercado et al. 2006; Radley et al. 2009; Tavares et al. 2009).

A study using immunohistochemistry and in situ hybridization was the first to describe the existence of transient receptor potential type 1 (TRPV1)-expressing neurons in the brain (Mezey et al. 2000). Next, it was unveiled that their pro-depolarization characteristic led to neural firing—mainly on glutamatergic transmission (Starowicz et al. 2007; Xing and Li 2007; Kawahara et al. 2011). These cation-permeable channel receptors were first described in peripheral afferent fibers related to nociceptive processes (Caterina et al. 1997). Also, its activation is usually followed by receptor desensitization (Szallasi and Blumberg 1999; Szallasi and Di Marzo 2000). Since their first description, the presence of TRPV1 receptors has been recognized in several regions, including those involved in defensive behavior expression (Mezey et al. 2000; Sanchez et al. 2001; Roberts et al. 2004; Toth et al. 2005). More specifically, recent studies have described the specific expression of TRPV1 receptors in both portions of vMPFC, PL, and IL (de Novellis et al. 2011; Giordano et al. 2012).

Kasckow et al. (2004) suggested for the first time the possible involvement of TRPV1 receptors on emotional responses, by submitting animals to an anxiety model after systemic injection of the TRPV1 antagonist capsazepine. After that, Rubino et al. (2008) demonstrated that administration of an analogue of the TRPV1 agonist anandamide into the prefrontal cortex produces biphasic effects—in high dose-promoted anxiogenic-like behavior in animals submitted to the elevated plus maze which was blocked by capsazepine. Also, TRPV1 knockout mice present reduced anxiety-like behavior and impaired fear conditioning (Marsch et al. 2007). Our group also showed reduction in anxiety level after local blockade of TRPV1 receptors in different brain regions (Aguiar et al. 2009; Terzian et al. 2009; Fogaça et al. 2012), thus supporting the hypothesis that TRPV1 receptors play a role in defensive behavior. Although capsazepine has been employed in most part of these studies as TRPV1 receptor antagonist, its selectivity is limited and shows several off-target effects. For this reason, a more selective antagonist was employed in the present work (6-iodonordihydrocapsaicin; Appendino et al. 2003).

Considering the abovementioned evidence, the present work aimed to evaluate the pharmacological role of TRPV1 receptors located in the vMPFC during contextual fear expression. For this reason, we administrated two different TRPV1 antagonists and one TRPV1 agonist to rats submitted to fear conditioning protocols with different levels of aversiveness that would enable a bidirectional manipulation of the vanilloid system—where a high aversive protocol allows a substantial activation of TRPV1 receptors and a more clear result after its blockade, and a less aversive protocol still permits an increase in the system activation after pharmacological treatment with the agonist capsaicin. Our results demonstrated that depending on the aversiveness level, TRPV1 receptors located in the vMPFC have a partial or complete influence on the expression of contextual fear responses.

Materials and methods

Animal preparation

Seventy-two male Wistar rats weighing 230–270 g were used. Animals were maintained in the Animal Care Unit of the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo. Rats were housed individually in plastic cages with free access to food and water and under a 12-h light/dark cycle (lights on at 0630 hours). The Institution's Animal Ethics Committee approved housing conditions and experimental procedures.

Seven days before the first context exposure (habituation), rats were anesthetized with tribromoethanol (250 mg/kg, IP). After local anesthesia with 2 % lidocaine, the skull was surgically exposed, and stainless steel guide cannulae (26G) were implanted bilaterally into the vMPFC using a stereotaxic apparatus (Stoelting, Wood Dale, Illinois, USA) as described by Lisboa et al. (2010). Coordinates for cannulae implantation were selected from the rat brain atlas of Paxinos and Watson (1997). Cannulae were fixed to the skull with dental cement and one metal screw. After surgery, the animals received a poly-antibiotic (Pentabiotico®, Fort Dodge, Brazil), with streptomycins and penicillins, to prevent infection and the nonsteroidal anti-inflammatory flunixine meglumine (Banamine®, Schering Plough, Brazil) for postoperative analgesia.

One day before the re-exposure to the context, rats were anesthetized with tribromoethanol and a catheter (a 4 cm PE-10 segment heat-bound to a 13-cm PE-50 segment, Clay Adams, Parsippany, New Jersey, USA) was inserted into the abdominal aorta through the femoral artery for cardiovascular recording. The catheter was tunneled under the skin and exteriorized on the animal's dorsum.

Drugs

Capsazepine and capsaicin (TOCRIS, Ellisville, Missouri, USA) were dissolved in dimethyl sulfoxide (DMSO), as previously described (Terzian et al. 2009). The doses (capsazepine, 1, 10, and 60 nmol/200 nL; capsaicin, 1 nmol/200 nL) were chosen based on previous results from our group. 6-Iodonordihydrocapsaicin (6-iodo, TOCRIS, Ellisville, Missouri, USA) dose of 3 nmol/200 nL was prepared in DMSO (100 %). The dose of 6-iodo was selected

based on the study of Appendino et al. (2003) and Fogaça et al. (2012). All solutions were prepared immediately before use.

Fear conditioning

Habituation, conditioning, and re-exposure were carried out in 25×22×22 cm footshock chambers. The chambers had a grid floor composed of 18 stainless steel rods (2 mm in diameter), spaced 1.5 cm apart and wired to a shock generator (Insight, Brazil). The chamber was cleaned with 70 % ethanol, before and after each animal. The procedure started 1 week after guide cannulae implantation. It consisted of one 10-min habituation session to the footshock chamber (no shock presentation). The conditioning session (shock presentation) was performed 24 h after the habituation session, which consisted of six electric 1.5 mA/3 s footshocks (Resstel et al. 2008a, b) delivered at 20 s to 1 min intervals, starting 3 min after placing the animal into the chamber.

An additional group of animals was submitted to a different conditioning protocol to obtain reduced conditioned fear responses as described before (Baldi et al. 2004; Lisboa et al. 2010). In this condition, three electric footshocks of 0.85 mA/3 s were delivered at 20 s to 1 min intervals.

Twenty-four hours after the conditioning session, for both protocols, a catheter was implanted into the femoral artery for cardiovascular recording. Forty-eight hours after the conditioning, cardiovascular and behavioral (freezing) responses to context were evaluated in the re-exposure session.

In this re-exposure session, the animal was placed for 10 min to the same chamber but without any shock presentation. Animals were transferred from the animal room to the procedure room (a different room was used for conditioning) in their home box. MAP and HR were recorded using an HP-7754A amplifier (Hewlett Packard, Palo Alto, CA, USA) connected to a signal acquisition board (Biopac M-100, Goleta, CA, USA) and computer-processed. Rats were tested one at a time. After connecting the animal to the setup, drugs were administrated into the vMPFC, and 5 minutes before re-exposure to the context, MAP and HR baseline were recorded in the home box. Two 33G needles (Small Parts, Miami Lakes, FL, USA) 1-mm longer than the guide cannula and connected to a 10-μL syringe (7002-H, Hamilton Co., Reno, NV, USA) through PE-10 tubing were used. The needles were carefully inserted into the guide cannulae, and the solutions were infused over a 30-s period with a rate of 400 nL/min. They remained in place for an additional 20-s period to prevent reflux. The re-exposure session started 10 min after injection.

Freezing was evaluated during the re-exposure session by an experimenter blind to the treatment located 30 cm from the experimental chamber. It was defined as the complete absence of movement except for breathing, while the animal assumed a characteristic tense posture (Fanselow 1980; Resstel et al. 2006, 2008c).

Experimental design

In the first experiment, all animals were randomly assigned to treatment groups. Each animal received 200 nL bilateral injections into the vMPFC of capsazepine (1, 10, or 60 nmol), 6-iodo (3 nmol), or vehicle. The animals were tested 10 min after the microinjection.

In the second experiment, to evaluate the effects of TRPV1 receptor activation in the vMPFC in a less aversive conditioning protocol, each animal received two bilateral injections of 200 nL into the vMPFC of the drugs or vehicle. The experimental groups were as follows: vehicle+vehicle (n=6), vehicle+capsaicin (1 nmol, n=5), 6-iodo (3 nmol)+vehicle (n=5), or 6-iodo (3 nmol)+capsaicin (1 nmol, n=5). The interval between the first and second microinjection was 5 min, and the animals were tested 10 min after the last microinjection.

Histology

After the behavioral tests, the rats were anesthetized with urethane, and 200 nL of 1 % Evans blue dye was bilaterally injected into the vMPFC as a marker of injection sites. After this procedure, the animals were perfused through the left ventricle of the heart with isotonic saline followed by 10 % formalin solution. The brains were removed and, after a minimum period of 5 days immersed in a 10 % formalin solution, 50-μm sections were obtained in a Cryostat (Cryocut 1800, Leica). The injection sites were identified in diagrams from the Paxinos and Watson's atlas (Paxinos and Watson 1997) and are illustrated in Fig. 1.

Data analysis

MAP and HR values were continuously recorded in the home cage during the 5-min period prior and the 10-min period during re-exposure to the context. Data were expressed as means ± SEM of MAP or HR changes (ΔMAP and ΔHR, respectively) sampled at 60-s intervals. Average points sampled during the 5-min before re-exposure were used as control baseline value. MAP and HR changes were analyzed using two-way analysis of variance (ANOVA) with treatment as main independent factors and time as repeated measurement. When interaction between the factors was observed, the main independent factors were compared at specific times using the Bonferroni's post hoc test.

Freezing was expressed as percentage of the whole test period. Freezing was analyzed using one-way or two-way ANOVA with treatment as the main independent factor followed by the Bonferroni's post hoc test or Student's t test. $p<0.05$ was assumed as statistically significant.

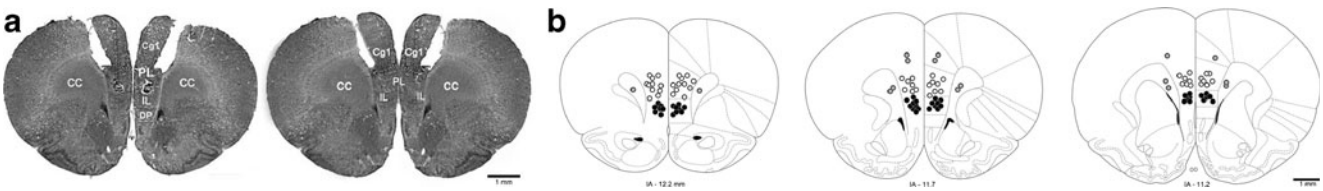

Fig. 1 **a** Photomicrographs of a coronal brain section showing bilateral microinjections sites in the IL and PL regions of vMPFC. **b** Diagrammatic representation based on the rat brain atlas of Paxinos and Watson (1997) indicating the injection sites in the IL (*black circles*) and IP (*white circles*). Animals with drug injection sites outside the vMPFC were represented by *gray circles*. *Cg1* cingulate cortex area 1, *PL* prelimbic cortex, *IL* infralimbic cortex, *DP* dorsal peduncular córtex, *cc* corpus callosum, *IA* interaural

Results

Schematic diagram indicating the injection sites of vehicle and drugs and photomicrographs showing a representative bilateral injection site in the IL and PL regions of vMPFC are presented in Fig. 1.

Effects of the capsazepine microinjected into the vMPFC on behavioral and cardiovascular responses to contextual fear conditioning

The administration of capsazepine (10 and 60 nmol) into the vMPFC significantly reduced the freezing ($F_{3,30}$=30.75, p<0.001) of conditioned animals (n=10/group) but not the dose of 1 nmol (n=6, p>0.05), when compared with vehicle-treated conditioned animals (n=8, Fig. 2a, left panel). Moreover, a dose dependency was demonstrated by nonlinear regression analysis, showing a significant correlation between capsazepine doses and attenuation of the freezing (r^2=0.71, df=23, p<0.05, Fig. 2a, right panel), confirming a dose-dependent effect.

The bilateral injection of capsazepine into the vMPFC had no effect on basal levels of both MAP ($F_{3,30}$=0.87, p>0.05) and HR ($F_{3,30}$=0.92, p>0.05) when compared to vehicle control group. The re-exposure to a context previously paired with footshocks induced a marked and sustained increase in both MAP ($F_{14,450}$=104.4, p<0.001) and HR ($F_{14,450}$=84.2, p<0.001) during the 10 min of session. Capsazepine (10 and 60 nmol) attenuated the cardiovascular response increases of conditioned animals (MAP: $F_{3,450}$=130.5, p<0.001, and HR: $F_{3,450}$=108.4, p<0.01), but not the dose of 1 nmol (p>0.05), when compared with vehicle-treated animals (Fig. 2b, left panels). Again, a nonlinear regression analysis showed a significant correlation between capsazepine doses and attenuation of both MAP (r^2=0.7, df=23, p<0.05) and HR (r^2=0.65, df=23, p<0.05) (Fig. 2b, right panels).

The capsazepine effects (10 and 60 nmol doses) were similar when it was administered in PL (n=5) or IL (n=5) of conditioned rats (freezing: $F_{1,16}$=0.03, p>0.05; MAP: $F_{3,240}$=0.76, p>0.05; and HR: $F_{3,240}$=0.058, p>0.05). In these two areas, capsazepine reduced these responses (IL—freezing: $F_{2,15}$=37.4, p<0.001; MAP: $F_{2,225}$=75.2, p<0.001; and HR: $F_{2,225}$=88.3, p<0.001; PL—freezing: $F_{2,15}$=17.5, p<0.001; MAP: $F_{2,225}$=101.5, p<0.001; and HR: $F_{2,225}$=91.3, p<0.01).

Finally, no changes on behavioral or cardiovascular responses were observed when capsazepine (10 or 60 nmol) was microinjected into structures surrounding the vMPFC of animals (n=8; freezing, p>0.05; MAP, p>0.05; and HR, p>0.05, respectively).

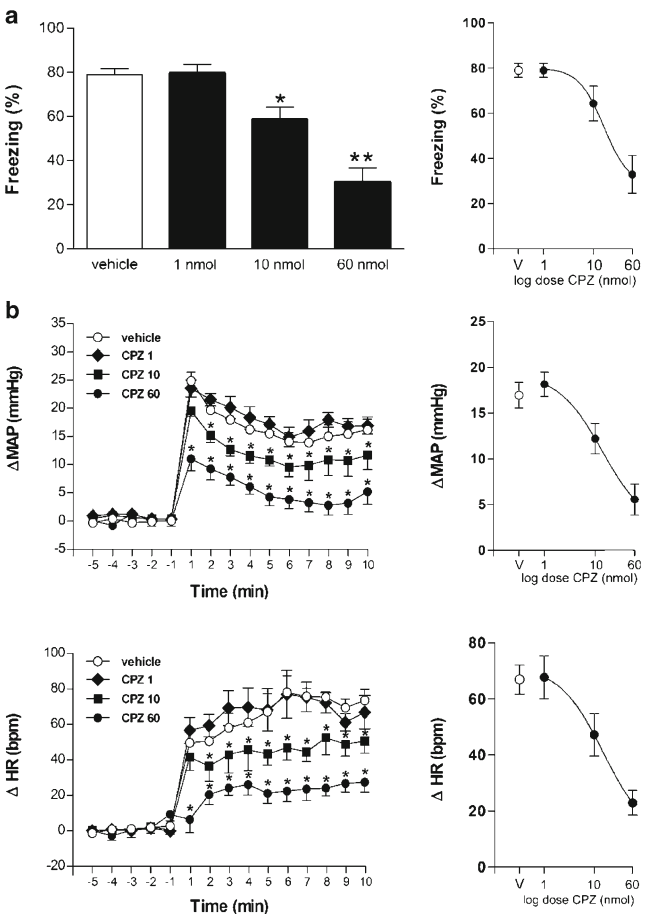

Fig. 2 Effects of bilateral injection of 200 nL of vehicle or capsazepine (CPZ, 1, 10, or 60 nmol) in conditioned animals (n=6–10) during chamber exposition **a** on the percentage of time spent freezing behavior (*left panel*) and nonlinear regression analysis of percentage of time spent freezing (*right panel*). **b** Time course of mean arterial pressure (*ΔMAP*) and heart rate (*ΔHR*) increases (*right panels*), and nonlinear regression analysis of mean values of mean arterial pressure (*ΔMAP*) and heart rate (*ΔHR*) increases. Values are represented by means ± SEM. *p<0.05 and **p<0.01 compared to vehicle group, Bonferroni's post hoc test

Effects of 6-iodonordihydrocapsaicin microinjected into the vMPFC on behavioral and cardiovascular responses to contextual fear conditioning

To confirm the involvement of vMPFC-TRPV1 receptors on expression of contextual fear conditioning, we administrated the selective TRPV1 receptor antagonist, 6-iodo (3 nmol) into the vMPFC of a new group of animals. The administration of 6-iodo into the vMPFC significantly reduced the freezing of conditioned animals (n=5, t=8.6, p<0.001) when compared with vehicle-treated animals (n=5, Fig. 3a). Also, 6-iodo attenuated the increase in cardiovascular response of conditioned animals (MAP: $F_{1,120}$= 83.6, p<0.001 and HR: $F_{1,120}$=66.4, p<0.001) when compared with the control group (Fig. 3b), without affecting the basal levels of MAP (t=0.16, p>0.05) and HR (t=0.07, p>0.05).

Effects of the capsaicin microinjected into the vMPFC on behavioral and cardiovascular responses to contextual fear conditioning in a less aversive protocol

The group conditioned with a less aversive conditioning protocol (n=5) spent less time freezing (48±3.4 % vs 80.3±2.5 %, t=7.6, p<0.001) and presented reduced cardiovascular responses (MAP=$F_{1,120}$=89.2, p<0.001 and HR=$F_{1,120}$=81.3, p<0.001) during the re-exposure to the context in comparison to the group submitted to the more aversive conditioning protocol (n=5).

Compared with vehicle-treated animals (n=5), the administration of capsaicin (1 nmol) into the vMPFC of conditioned animals submitted to the less aversive conditioning protocol (n=5) increased freezing ($F_{1,16}$=10.3, p<0.001) and cardiovascular responses (MAP: $F_{3,240}$=107.9, p<0.001 and HR: $F_{3,240}$=127.1, p<0.001; Fig. 4). The bilateral injection of capsaicin into the vMPFC had no effect on basal levels of both MAP ($F_{3,19}$=0.07, p>0.05) and HR ($F_{3,19}$=0.1, p>0.05) when compared to the vehicle control group.

In this less aversive conditioning protocol, the bilateral administration of 6-iodo (3 nmol, n=5) had no effects on both freezing ($F_{1,16}$=2.2, p>0.05) and cardiovascular responses (MAP: $F_{3,240}$=0.03, p>0.05 and HR: $F_{3,240}$=0.1, p>0.05) when compared to the vehicle-treated group. However, the pretreatment with 6-iodo blocked the effects of capsaicin in increasing both freezing ($F_{1,16}$=11, p<0.001) and cardiovascular responses (MAP: $F_{3,240}$=82.8, p<0.001 and HR: $F_{3,240}$=98.7, p<0.001).

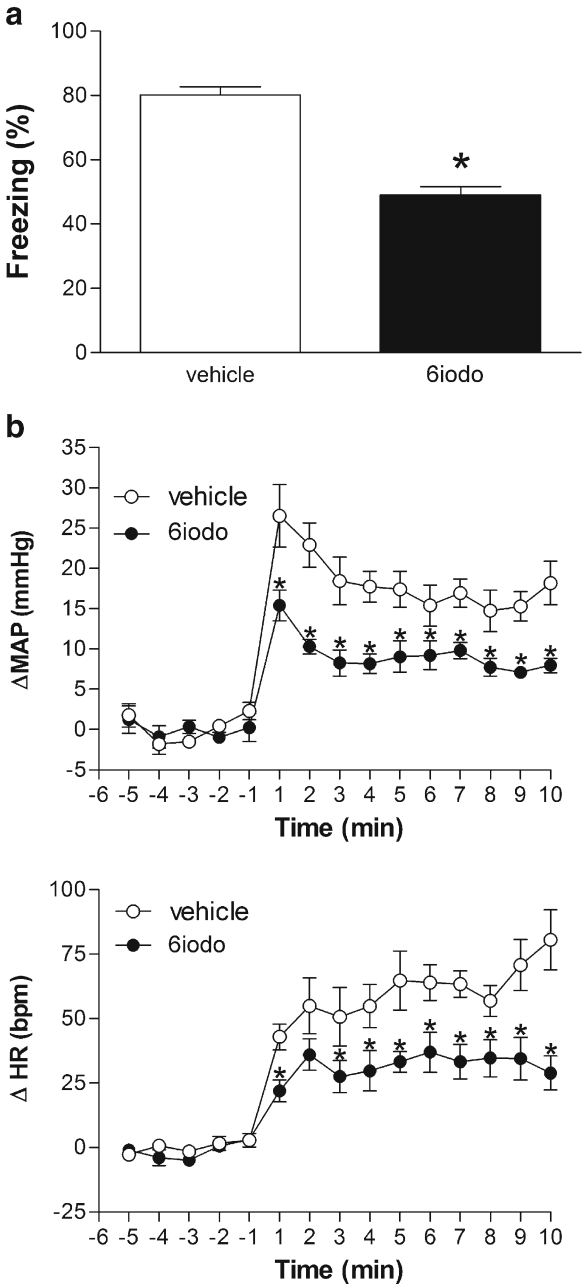

Fig. 3 **a** Effects of bilateral injection of 200 nL of vehicle or 6-iodonordihydrocapsaicin (6-iodo, 3 nmol) in conditioned animals (n=5 per group) on the percentage of time spent freezing behavior. **b** Time course of the effects of bilateral microinjection of 200 nL of vehicle 6-iodonordihydrocapsaicin (6-iodo, 3 nmol) recorded in conditioned animals (n=5 per group) on mean arterial pressure (*ΔMAP*, *upper panel*) and heart rate (*ΔHR*, *lower panel*) increases. Values are represented by means ± SEM. *p<0.05 compared to vehicle group, Student's t test, Bonferroni's post hoc test

Discussion

The present study demonstrated that TRPV1 receptors located into the vMPFC are involved in the expression of contextual fear conditioning. This involvement seems to be tonically mediated, since TRPV1 blockade is sufficient to decrease fear-related freezing and cardiovascular responses. Corroborating these results, another important finding is that direct activation of TRPV1 receptors by capsaicin can increase conditioned fear response, an effect that was blocked by pretreatment with TRPV1 antagonist.

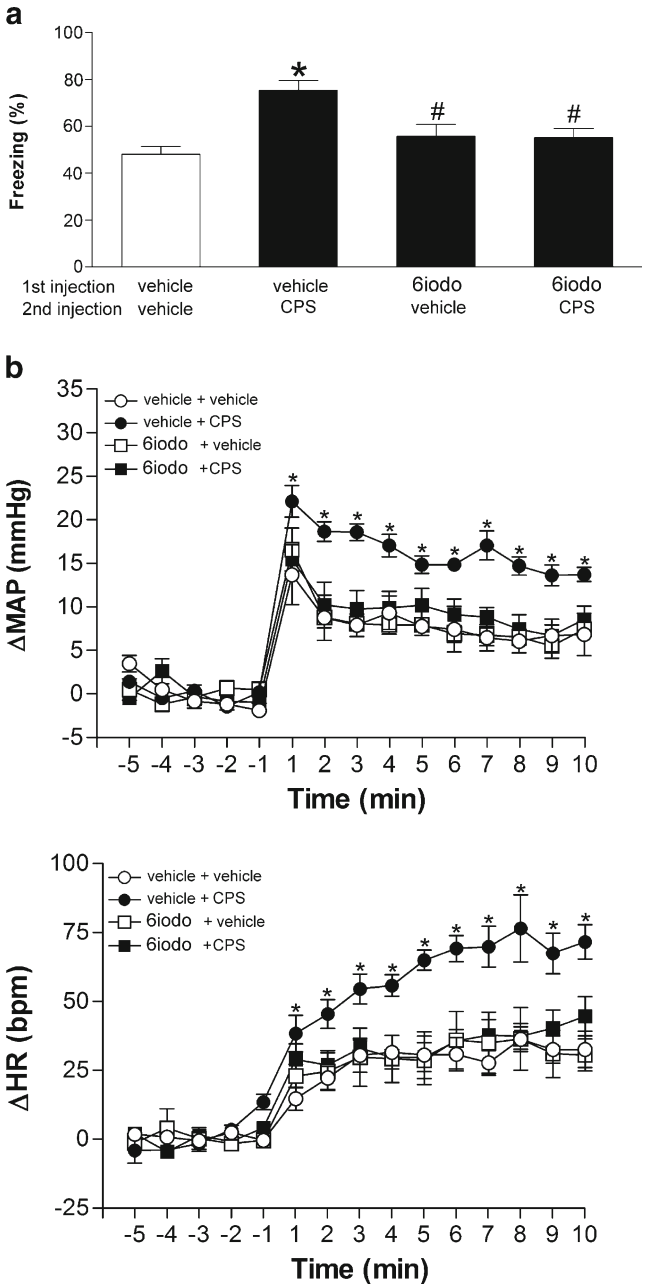

Fig. 4 **a** Effects of bilateral injection of 200 nL of vehicle or 6-iodonordihydrocapsaicin (6-iodo, 3 nmol) followed by a second injection of vehicle or capsaicin (CPS, 1 nmol) in conditioned animals (n=5 each group) on the percentage of time spent in freezing behavior. **b** Time course of the effects of bilateral microinjection of vehicle or 6-iodonordihydrocapsaicin (6-iodo, 3 nmol) followed by a second injection of vehicle or capsaicin (CPS,d 1 nmol) recorded in conditioned animals (n=5 each group) on mean arterial pressure (*ΔMAP*, *upper panel*) and heart rate (*ΔHR*, *lower panel*) increases. Values are represented by means ± SEM. *p<0.05 compared to vehicle group; *p<0.05 compared to capsaicin group. Student's t test, Bonferroni's post hoc test

Intra-vMPFC administration of capsazepine attenuated the expression of contextual fear conditioning in a dose-dependent manner, suggesting the involvement of local TRPV1 receptors in this response. However, capsazepine may have a nonspecific action in addition to its effects on TRPV1 receptors, which is characterized by inhibition of calcium current via blockade of voltage-dependent calcium channels usually associated with TRPV1 receptors activation (Docherty et al. 1997). Therefore, to confirm TRPV1 receptors involvement in the expression of contextual fear conditioning, we microinjected the selective TRPV1 receptor antagonist 6-iodo (Appendino et al. 2003; Fogaça et al. 2012). The bilateral administration of 6-iodo was able to reduce both behavior and autonomic responses evoked by exposure to an aversive context, as observed after capsazepine administration. Binding studies described that 6-iodo has two- to tenfold higher affinity than capsazepine for TRPV1 receptors, without affinity for voltage-dependent calcium channels (Appendino et al. 2003). Thus, the present findings about 6-iodo, together with those obtained with capsazepine, indicate that TRPV1 receptors in vMPFC are involved on the modulation of contextual fear conditioning expression.

Previous studies described the differences between IL and PL, areas that comprise the vMPFC, in the modulation of cardiovascular and behavioral responses in several models and different treatments (Jaskiw and Weinberger 1990; Frysztak and Neafsey 1991, 1994; Jinks and McGregor 1997; Vidal-Gonzalez et al. 2006; Tavares et al. 2009; Sierra-Mercado et al. 2011). Therefore, to inquire if the TRPV1 receptors in both the PL and IL cortex could modulate the expression of contextual fear differently, animals that received a dose of 10 or 60 nmol of the TRPV1 receptor antagonist capsazepine were separated according to the site of microinjection into two groups, PL and IL. It must be acknowledged that in some occasions, it is difficult to separate IL and PL, especially when dealing with injection volume spreading through the tissue. The same effects of TRPV1 receptor antagonism on contextual fear conditioning were observed when capsazepine was administrated in PL or IL. Moreover, the bilateral administration of TRPV1 receptor antagonist into structures surrounding the vMPFC, which are near to both areas, did not affect the expression of contextual fear conditioning. Therefore, it is less probable that the effects of drugs would be due to their spread from the site of injection to one unspecific point, in this case, located between the IL and PL. These results are in agreement with other observations that the IL or PL has the same influence on both cardiovascular and behavior responses (Owens and Verberne 2001; Resstel and Correa 2005, 2006; Resstel et al. 2008a,b,c; Lisboa et al. 2010; Ferreira-Junior et al. 2012). Moreover, studies showed that IL and PL had the same role in defensive responses to contextual fear conditioning, including cardiovascular responses (Corcoran and Quirk 2007; Lisboa et al. 2010; Resstel et al. 2006). Thus, our findings show a homogeneous influence of the vMPFC subarea TRPV1 receptors on contextual fear conditioning expression.

The participation of TRPV1 receptors is proposed to depend on the environment aversiveness (Marsch et al. 2007). Corroborating this proposal, in higher shock conditions, both capsazepine (10 and 60 nmol) and 6-iodo (3 nmol) were able to reduce freezing and cardiovascular responses associated with fear expression, which indicates the active participation of TRPV1 receptors in high-threatening situations. Meanwhile, when a lower shock intensity was given, the same dose of 6-iodo (3 nmol) was no longer capable to reduce fear-related responses. Also, in the low-shock protocol, TRPV1 receptors activation by capsaicin increased the fear responses, an effect that was prevented by pretreatment with a TRPV1 antagonist. This suggests that, under this condition, the endovanilloid system in this area is not fully engaged. Altogether, these results indicate that the role of the endovanilloid system depends on environmental conditions.

They agree with the findings observed in TRPV1 knockout animals, where differences between the mutant and the wild-type animals were only observed under aversive conditions in several behavioral tests but not under standard conditions (Marsch et al. 2007). In this case, TRPV1 KO animals presented less anxiety and fear levels compared to the wild-type, although their extinction capability was maintained.

Considering the prefrontal cortex, previous works have extensively shown its participation in aversive responses (Frysztak and Neafsey 1994; Resstel and Correa 2006). More recently, studies evaluating vMPFC interplay between cannabinoid and vanilloid system demonstrated that both systems play a role in suppressing and promoting fear/anxiety behavior, respectively. Rubino et al. (2008) demonstrated that inhibition of local TRVP1 was sufficient to reduce anxiety-like behavior in the elevated plus maze, which was also reported by Aguiar and collaborator (2009). Furthermore, in the first study, TRPV1 antagonist was capable of abolishing the anxiogenic-like affect observed after methanandamide treatment.

On another hand, increased endogenous tone by rising levels of anandamide or NADA (N-arachidonyl dopamine) could also activate both TRPV1 and the cannabinoid type 1 (CB1) receptors in different extents (Di Marzo et al. 2001; Di Marzo 2010). Whereas higher levels of these compounds would result in a predominant drive towards TRPV1 receptors, the pharmacological blockade or genetic ablation of these receptor causes a shift of activation onto CB1 receptor direction (Casarotto et al. 2011; Moreira et al. 2011), which has been shown to reduce aversive responses in different animal models (Lisboa et al. 2010; Rubino et al. 2008; Pamplona et al. 2006).

Recent pieces of evidence have also shown that TRPV1 receptors are able to modulate the glutamate releases in the central nervous system (Karlsson et al. 2005; Sikand and Premkumar 2007). Moreover, the antagonism of local vMPFC glutamatergic NMDA receptors reduces the contextual fear conditioning responses (Resstel et al. 2008b). Therefore, blockade of TRPV1 could culminate in a sort of indirect antagonist of glutamatergic neurotransmissions in the vMPFC. These results and our present findings suggest that endovanilloids could play a major role on the expression of contextual fear conditioning by controlling glutamatergic synaptic transmission in the vMPFC by activation of TRPV1 receptors.

In conclusion, our work demonstrated the involvement of TRPV1 receptor located in the vMPFC in fear expression. The results also suggest that the vanilloid system is tonically activated during threatening situations accordantly with the situation aversiveness. Together, these results suggest that vanilloid system could be a possible target for pharmacological treatment of anxiety disorders.

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Conflict of interest The authors declare no conflicts of interests.

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frontiers in
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The dopamine and cannabinoid interaction in the modulation of emotions and cognition: assessing the role of cannabinoid CB1 receptor in neurons expressing dopamine D1 receptors

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Although cannabinoid CB1 receptors (CB1Rs) are densely expressed in neurons expressing dopamine D1 receptors (D1Rs), it is not fully understood to what extent they modulate emotional behaviors. We used conditional CB1R knock-out animals lacking CB1Rs in neurons expressing D1R (D1–CB1$^{-/-}$) in order to answer this question. To elucidate the behavioral effects of CB1R deficiency in this specific neuronal subpopulation, we subjected D1–CB1$^{-/-}$ mice to a battery of behavioral tests which included exploration-based tests, depressive-like behavioral tests, social behavior, and fear-related memory paradigms. D1–CB1$^{-/-}$ did not show any difference in the exploration-based paradigms such as open field, elevated plus maze, or novel object investigation test, except for an increase in novelty-induced grooming. By contrast, they showed a mild anhedonia-like state as described by the slightly decreased preference for sweet solution, as compared to wild-type control group. This decrease, however, could be observed only during the first day of exposure, thus suggesting increased neophobia as an alternative explanation. Accordingly, mutant mice performed normally in the forced swim test, a procedure widely used for evaluating behavioral despair in rodents. However, weak- to moderate anxiety-like phenotypes were evident when D1–CB1$^{-/-}$ mice were tested for social behavior. Most strikingly, D1–CB1$^{-/-}$ mice exhibited significantly increased contextual and auditory-cued fear, with attenuated within session extinction, suggesting that a specific reduction of endocannabinoid signaling in neurons expressing dopamine D1Rs is able to affect acute fear adaptation. These results provided first direct evidence for a cross-talk between dopaminergic D1Rs and endocannabinoid system in terms of controlling negative affect.

Keywords: cannabinoids, CB1R, dopamine, D1R, social behavior, aversive memories, anxiety, fear extinction

INTRODUCTION

In the central nervous system (CNS), endogenous cannabinoids compounds activate cannabinoid CB1 receptors (CB1Rs), which are located pre-synaptically in several brain regions such as prefrontal cortex, hippocampus, amygdala, and basal ganglia. They act as inhibitory retrograde signaling messengers at glutamatergic and GABAergic synapses, modulating the release of several neurotransmitters such as acetylcholine or dopamine (DA) (Marsicano and Lutz, 1999; Piomelli, 2003). Thus, the endocannabinoid system (ECS), through its neuromodulating activity, could be involved in several physiological functions as memory processing, pain perception, locomotion, and inflammation; additionally, its dysregulation could underlie several pathological conditions known to accompanying psychiatric disorders (Di Marzo, 2008).

The role of the dopaminergic (DAergic) neurotransmitter system in the processing of emotional behavior is well established and supported by several preclinical and clinical data showing that DA, acting on D1- or D2-like receptors, is one of the most important neuromodulators of fear and anxiety (LeDoux, 2000). A DAergic and EC interaction at different anatomical levels (i.e., amygdala, nucleus accumbens and striatum) seems to be involved in several neurophysiological responses. More specifically, it has been suggested that CB1R signaling modulates DAergic pathways by influencing directly or indirectly the activity of DAergic neurons through either post- or pre-synaptic mechanisms (Laviolette and Grace, 2006). However, both the mechanisms through which DAergic and EC signaling cross-talk and the role played by the dopamine D1 receptor positive neurons still remain unclear. The dopamine D1 receptors (D1Rs), which belong to the "D1-like" group, are expressed in brain regions involved in aversive learning and memory such as nucleus accumbens, hippocampus, and amygdala. (Kamei et al., 1995; Bernabeu et al., 1997; El-Ghundi et al., 2001; Nagai et al., 2007). Interestingly, the colocalization of CB1Rs with D1Rs indicates that these receptors may interact by potentially modifying their respective functions with important behavioral and pharmacological consequences (Hermann et al., 2002).

Although the use of complete CB1 knock-out mice together with pharmacological approaches suggest that ECS controls fear and anxiety primarily under highly aversive situations (Moreira and Wotjak, 2010), the cellular substrates of these effects with regard to specific neuronal subpopulation involved (i.e., dopamine receptor D1-expressing neurons) is still largely unexplored, except

for a specific contribution of principal neurons of the forebrain (Kamprath et al., 2009). Thus, conditional CB1 knock-out animals, lacking CB1Rs specifically in D1R positive neurons provide an important tool to answer these questions.

Based on the above premises, this study was undertaken to investigate the role of CB1R signaling in the dopamine receptor D1-expressing neurons in affecting emotional behavior. For this purpose, conditional CB1 mutant mice, lacking CB1Rs expression in neurons containing dopamine D1Rs (D1–CB1$^{-/-}$; Monory et al., 2007), were submitted to a battery of behavioral tests, which included exploration-based tests, depressive-like behavioral tests, and fear-related memory paradigms. Since it has been hypothesized that ECS is a relevant modulator of dopamine D1Rs-mediated behaviors including social activity (Martín et al., 2008; Zenko et al., 2011), we also evaluated the phenotype of these mice in social approach tests.

MATERIALS AND METHODS

ANIMALS

Male mice at the age of 8–16 weeks were used throughout the experiments. Conditional D1–CB1 knock-out mice (D1–CB1$^{-/-}$ or KO) and their respective wild-type (WT) littermate controls (D1–CB1$^{+/+}$ or WT) were generated and genotyped as previously described (Monory et al., 2007). Animals (n = 6–10 per group) were single housed and maintained in standard conditions with food and water *ad libitum* under a 12-h inverse light–dark (LD) cycle (lights off at 9 a.m.) for at least 14 days before starting the experiments. All behavioral experiments were performed during the active (dark) phase of mice between 9:30 a.m. and 5 p.m. Experimenters were always blind to the genotype. All behavioral tests took place in an experimental room with the same LD cycle and environmental conditions (i.e., humidity, temperature) as in the housing facility. All experiments were carried out according to the European Community Council Directive 86/609/EEC and efforts have been made to minimize animal suffering and reduce the number of animals used.

BEHAVIORAL TESTING

Novelty-induced grooming test

Grooming behavior was observed under the same environmental conditions as previously described (Kalueff and Tuohimaa, 2005). The mice were placed individually into a clean unfamiliar Plexiglass box (27 cm × 16 cm × 12 cm) without bedding for 10 min. Three ethological measures of grooming activity were scored: latency to start grooming, grooming episodes (washing, general grooming, scratching, licking of paws, or genital grooming), and total time spent grooming. All trials were recorded for subsequent video analysis.

Open field test

Exploratory activity of D1–CB1$^{-/-}$ and WT mice was evaluated in the open field (OF) test, as previously described (Jacob et al., 2009). The experiment was performed in a squared box (26 cm × 26 cm), in which the animal was placed in the central zone of the apparatus equipped with infrared beams (TruScan; Coulbourn Instruments, Allentown, PA, USA) and allowed to explore for 30 min at 300 lux. All sensor rings were connected via interface to a computer equipped with TruScan Software Version 99 (Coulbourn Instruments). Boxes and sensor rings were surrounded by an additional box made of opaque Plexiglas side walls (47 cm × 47 cm × 38 cm) without roof and floor. Horizontal locomotion (total, margin, or central distance moved) vertical movements (exploratory rearing) and time spent at rest were analyzed during the 30-min monitoring period with a sampling rate of 4 Hz. After each session, the apparatus was cleaned with a solution containing neutral soap.

Elevated plus maze test

The apparatus consisted of two opposite open arms, (30 cm × 5 cm) and two arms with walls (30 cm × 5 cm × 14 cm) that were attached to a central platform (5 cm × 5 cm) to form a cross. The maze was elevated 50 cm from the floor (Pellow et al., 1985). Illumination measured at the center of the maze was 300 lux. The animal was placed in the center of the maze facing one of the closed arms, and observed for 5 min, according to the following parameters: number of entries in the open or closed arms and time of permanence in each arm (i.e., the time spent by the animal in the open or closed arms). An entry was defined as all four paws having crossed the line between an arm and the central area. It is accepted that the anxiolytic effect of a drug treatment is illustrated by increased parameters in open arms (time and/or number of entries; Pamplona et al., 2011: for pharmacological validation of our current set-up). The augmented percentage of entries in open arms over the total entries in both arms is a good indicator of reduced anxious-like phenotype as well. Entries in closed arms and total entries reflect the motor component of the exploratory activity. On removal of each mouse, the maze floor was carefully wiped with a wet towel. All trials were recorded on a HDD using a video-camera and then scored off-line by an experienced observer by means of a video/computer system ANY-MAZE (Stoelting).

Light/dark test

Set-up and test procedure were essentially the same as previously described (Jacob et al., 2009). The LD box was divided in two compartments: (1) one dark compartment (15 cm × 20 cm × 38 cm) with black walls and (2) one lit compartment (30 cm × 20 cm × 38 cm) with white plastic walls. Both compartments were connected by a 4-cm long tunnel. Light intensity was 600 lux in the light compartment and 15 lux in the dark compartment measured at floor level. Mice were placed into the corner of the dark compartment at the start of the experiment which lasted for 5 min. After each test, the LD box was thoroughly cleaned with soap and water. Entries and time spent in the light compartment were assessed by video analysis by a trained observer. These two variables were expressed as percentage of the total observation period and the total number of LD transitions, respectively.

Novel object investigation test

The novel object investigation (NOI) test was performed at 30 lux (which still allowed the assessment of exploration of the objects) for 10 min. Experimental subjects were habituated to the test arena (36 cm × 22 cm × 14 cm, with sawdust bedding material and transparent walls) for 2 days for 10 min (one cage per mouse without cleaning or changing of bedding). On the third day, mice were transferred into the same test cages and two identical objects (cone

made of aluminum: ∅ 6 cm + H 13 cm) were placed in a symmetrical position at the short walls of the cages. Between animals, objects were thoroughly cleaned with water containing detergent to eliminate olfactory cues. Objects were heavy enough that a mouse could not displace them. Every trial was video recorded and analyzed using ANY-MAZE (Stoelting). Investigation was defined as follows: directing the nose toward the object at a distance of not more than 2 cm and/or touching the object with the nose and paws (Jacob et al., 2009).

Sucrose consumption test

During this test, mice are given a free choice between two bottles for 10 h – one filled with 2.5% sucrose solution and the other with tap water – for two consecutive days (Strekalova and Steinbusch, 2010). To prevent possible effects of side preference in drinking behavior, the bottles position was switched in the mid-point of testing. Animals were not food or water-deprived before the test. For habituation, 1 day prior to the first testing day, animals were allowed to drink a 2.5% sucrose solution for 2 h. The consumption in water, sucrose solution, and total intake of liquids were estimated simultaneously in the both groups by weighing the bottles before and after each trial. The preference for sucrose was calculated as a percentage of the consumed sucrose solution from the total amount of liquid drunk, by the formula: Sucrose Preference = V(Sucrose solution)/[V(Sucrose solution)+V(Water)]×100%.

Forced swim test

The forced swim test (FST) employed here was essentially similar to that described elsewhere (Porsolt et al., 1978). Mice were individually placed into transparent cylinders (height 23.5 cm; diameter 16.5 cm) containing 15 cm water at 25 ± 1°C for 6 min. The water was changed after each trial. After vigorous activity, swimming attempts cease and the animal adopts a characteristic immobile posture. A mouse is judged to be immobile when it floats in upright position and makes only small movements to keep its head above water. The duration of mobility was recorded during the last 4-min of the 6-min testing period. All trials were recorded for subsequent off-line analysis.

Social interaction test

The procedure was adopted from (Smit-Rigter et al., 2010). Experiments were performed in a new cage (27 cm × 16 cm × 12 cm) with fresh bedding at 5 lux (i.e., red light) or 700 lux (light intensity measured at the level of test cages). The lid of the new cage was removed and the walls elongated by 12.5 cm of semi transparent plastic. Briefly, pairs of unfamiliar mice of the same genotype ($n = 7$ pairs of D1–CB1$^{-/-}$ and WT) were placed into the cage for 5 min. The time spent in social interactions (SI; active contact such as sniffing, licking, close following, and grooming) was recorded for each pair of mice. Each session was video recorded and analyzed off-line using ANY-MAZE (Stoelting).

Social investigation test

Social investigation (SInv) task was conducted as previously described with slight modifications (Crawley et al., 2007). It took place in a rectangular box made of white PVC walls and with a dark gray PVC floor. The box was divided into three equal compartments (30 cm × 30 cm × 30 cm) that were interconnected by small opening (6 cm × 5 cm) with guillotine doors. Each animal was allowed to free exploration of the apparatus for 10 min (habituation). An empty perforated 50 ml falcon tube was placed in each side of the box. This 10 min exposure was designed to familiarize the subject mouse with the testing environment. After habituation session, the animal was kept in the center compartment and one of the tubes was replaced by a tube containing an ovariectomized female. For the next 10 min session, the mouse was allowed to explore all three compartments and the time spent in the SInv (active contact such as sniffing) was recorded.

Fear conditioning task

The set-up has been described and displayed in detail before (Kamprath and Wotjak, 2004; Plendl and Wotjak, 2010). Two different protocols were programmed and carried out. The first experiment was performed in two contexts: (1) the neutral test context – a cylinder made of transparent Plexiglas, lined with wood shavings – and (2) the shock context – a cubic-shaped box with a metal grid for shock application. For conditioning (d0), mice were placed in the conditioning context. Three minutes later, a tone (80 dB, 9 kHz sine-wave, 10 ms rising, and falling time) was presented to the animals for 20 s that coterminated with a 2-s scrambled electric footshock of 0.7 mA. Mice were returned to their home cages 60 s later. On day 1 (d1), mice were exposed to the neutral context and on day 2 (d2) to the grid context for 7 and 3 min, respectively. Briefly, mice were placed in the test context, which differed from the conditioning context in material, shape, surface texture, and odor of the cleaning solution. After an initial 3 min of habituation, a 180-s permanent tone [9 kHz, 80 dB, sine-wave] was delivered. To test the contextual freezing, animals were re-exposed to the shock chamber for 3 min without tone presentation and without further shock application, and immediately returned to their home cages afterward.

In the second experiment, mice were conditioned as described for the first experiment. On day 1 (d1) and on day 7 (d7), mice were exposed to the 180-s tone in the neutral test context. Animals' behavior was video recorded by small CCD cameras (Conrad Electronics, Hirschau, Germany) and rated off-line by a trained observer (EVENTLOG, designed by Robert Henderson, 1986). Freezing behavior was defined as immobility except for respiration movements.

EXPERIMENTAL DESIGN

Behavioral experiments were conducted in two screens to reduce the number of animals used for the study with separate cohorts of animals for every screen (**Table 1**). If not stated otherwise, the different screens were accomplished with 4–5 days in between two consecutive tests. Animals were submitted to a battery of behavioral tests, which was divided in three main categories, in the following order: (1) low- or mild-stress situation (a) exploratory-based approach avoidance conflict tests: open field, elevated plus maze, light/dark, novel object investigation, and novelty-induced grooming (b) depressive-like behavior paradigms: sucrose consumption and forced swim test (2) social approach: social interaction and social investigation test (3) high-stress situation: fear conditioning (FC) tests. The order of tests within the battery was designed in such manner that mice would be evaluated on what were thought to be least invasive tests before

Table 1 | Comprehensive behavioral test battery of D1–CB1 knock-out mice.

Test	Age (weeks)	Days	*n*	Results
FIRST GROUP				
OF	8–10	1	6–9	**Figures 1A–F**
EPM	8–10	5	7–8	**Figures 2A, B**
LD	9–11	9	8–9	**Figures 2C,D**
SI	9–11	13	6–8	**Figures 6A,B**
FST	10–12	20	7–9	**Figure 5B**
FC	11–13	27	8–9	**Figures 7A,B**
SECOND GROUP				
NGT	8–10	2	8–9	**Figures 4A–C**
NOI	8–10	1	8–10	**Figure 3**
SC	8–10	5	10	**Figure 5A**
SInv	9–11	9	9–10	**Figures 6C,D**
FC	11–13	27	9–10	**Figures 7C,D**

n, Animal number; OF, open field; EPM, elevated plus maze; LD, light/dark; SI, social interaction; FST, forced swim test; FC, fear conditioning; NOI, novel object investigation; SC, sucrose consumption; SInv, social investigation; NGT, novelty-induced grooming test.

being tested on more invasive assays. This design was developed with the assumptions that testing from least to most invasive would allow for recovery time between tests and would reduce the likelihood that behavioral responses would be influenced by previous testing experience.

STATISTICAL ANALYSIS

Data were analyzed using unpaired *t*-test or two-factor ANOVA by means of Sigma Stat 3.5 (Systat Software Inc., San Jose, CA, USA). Newman–Keuls test was used as *post hoc* test, if appropriate. Data are presented as mean ± SEM. Statistical significance was accepted if $p < 0.05$.

RESULTS

EXPLORATORY AVOIDANCE CONFLICT TESTS

Open field test

In the OF test, there was no difference in the exploratory activity between D1–CB1$^{-/-}$ and WT mice (**Figures 1A–F**). Both groups showed the same horizontal activity (total distance: $t = 1.246$; $p = 0.2348$; central distance: $t = 1.501$; $p = 0.1574$, margin distance: $t = 0.2401$; $p = 0.8140$), total duration of movement ($t = 1.217$; $p = 0.2452$), rearing ($t = 1.715$; $p = 0.1101$), and jumping episodes ($t = 1.344$; $p = 0.2021$). This response indicates that in our test conditions, genetic deletion of CB1 in neurons expressing D1Rs did not alter basal locomotor activity of mice.

Elevated plus maze and light/dark test

As described in **Figures 2A–D**, statistical analysis did not reveal any significant difference between D1–CB1$^{-/-}$ and WT mice both in the time spent ($t = 0.5568$; df = 14; $p = 0.5871$) or in the number of entries ($t = 0.6133$; df = 14; $p = 0.5502$) into open arms of the EPM test. Also, there was no difference in the time spent ($t = 0.2827$; df = 15; $p = 0.7813$) or in number of entries ($t = 0.9739$; df = 15; $p = 0.3430$) into light compartment of the LD test. No locomotion

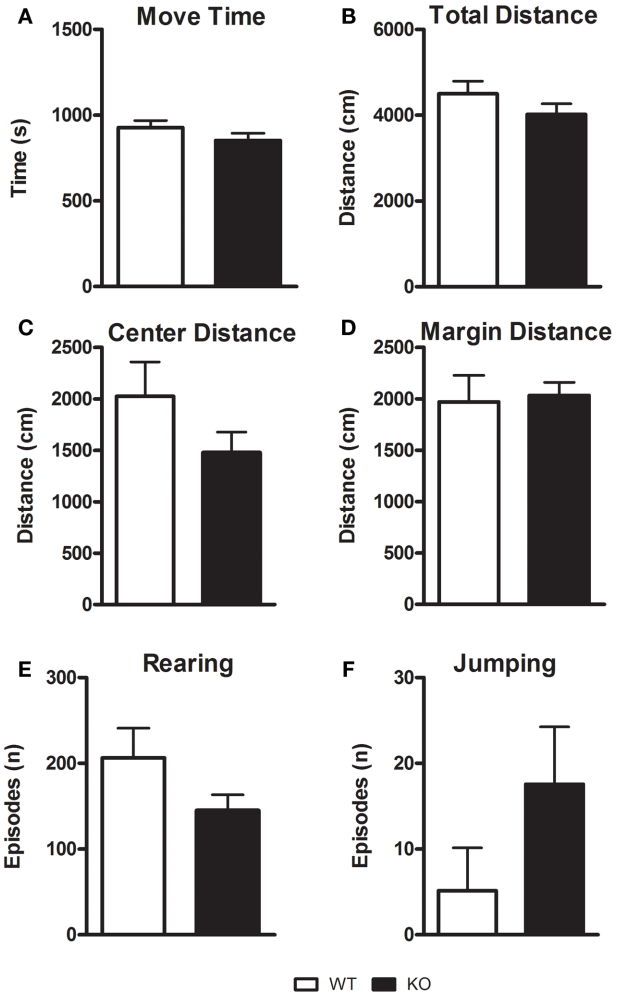

FIGURE 1 | Assessment of general locomotor activity of D1–CB1$^{-/-}$ mice in the open field (OF) test. Conditional D1–CB1$^{-/-}$ mutant mice (KO) and their respective wild-type (WT) littermates were tested in an open field for 30 min. Values are mean ± SEM in terms of locomotor activity **(A–D)** rearing **(E)** and jumping episodes **(F)**.

difference was found, considering the total arm entries ($t = 0.7276$; df = 14; $p = 0.4798$) and the total LD transitions ($t = 0.8154$; df = 15; $p = 0.4255$) as index.

Novel object investigation

Unpaired *t*-test showed that D1–CB1$^{-/-}$ and WT mice, during the 10-min test, spent the same amount of time investigating the pair of novel objects ($t = 0.5887$; $p = 0.5643$), as well as they approached them with the same frequency ($t = 0.5705$; $p = 0.5762$; **Figure 3**).

Novelty-induced grooming activity test

As described in **Figures 4A–C**, D1–CB1$^{-/-}$ mice performed more grooming episodes ($t = 2.240$; $p < 0.05$; df = 15) as well as they spent more time grooming as compared to WT animals ($t = 2.568$; $p < 0.05$; df = 15). However, the latency to start grooming was not significantly different between the two groups ($t = 1.170$; $p = 0.2603$; df = 15).

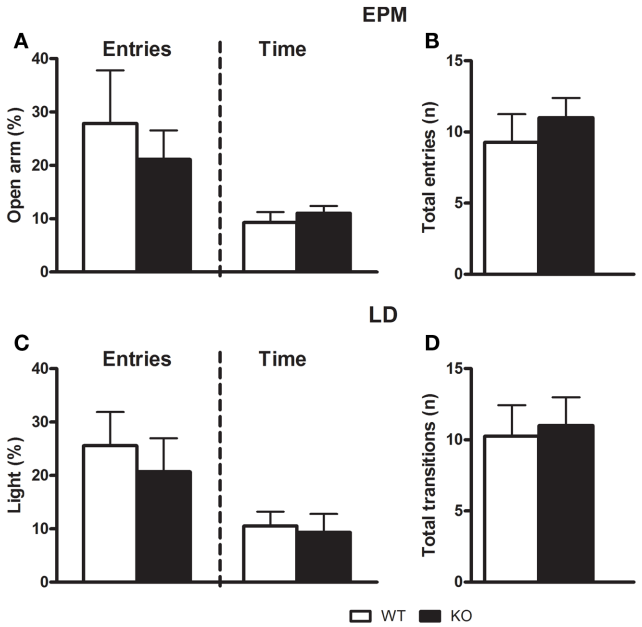

FIGURE 2 | Anxiety-like behaviors of D1–CB1$^{-/-}$ mice. Conditional D1–CB1$^{-/-}$ mutant mice (KO) and their respective wild-type (WT) littermates were tested for 5 min in the elevated plus maze (upper panel) or in the light/dark box (lower panel). Data are presented as mean ± SEM regarding open arm entries and open arm time **(A)** the total arm entries **(B)**, light compartment entries and light compartment time **(C)**, and total compartment transitions **(D)**.

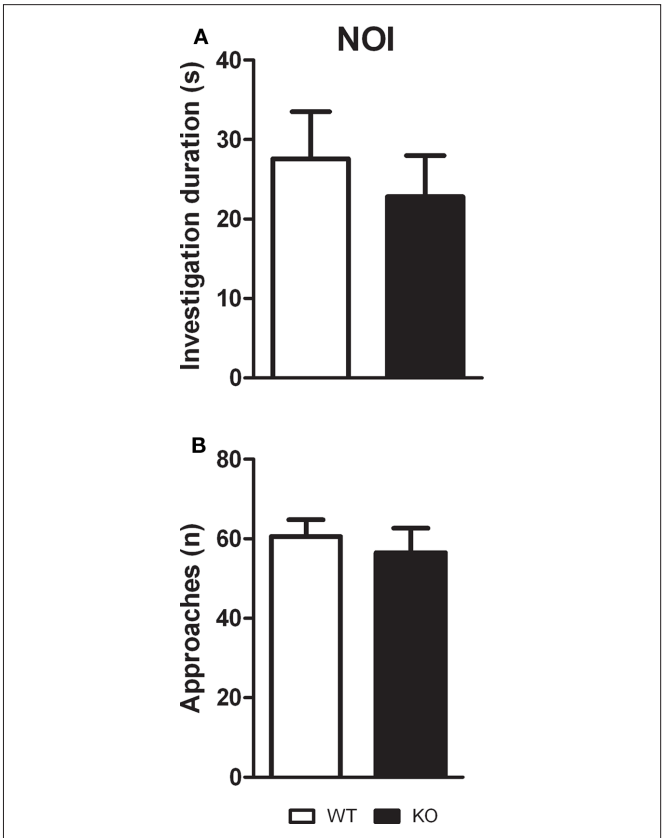

FIGURE 3 | Novel object investigation test. Conditional D1–CB1$^{-/-}$ mutant mice (KO) and their respective wild-type (WT) littermates were exposed to two novel objects for 10 min. Data are presented as mean ± SEM regarding total investigation duration **(A)** and number of approaches **(B)**.

DEPRESSIVE-LIKE BEHAVIOR

Forced swim test and sucrose consumption

As described in **Figure 5**, D1–CB1$^{-/-}$ mice showed a significant lower SC as compared to WT mice on the first ($t = 2.868$; $p < 0.05$), but not on the second testing day ($t = 0.3575$; $p = 0.7249$). However, WT and D1–CB1$^{-/-}$ mice showed a high percentage of SC as compared to the total amount of liquid consumed. In the FST, although D1–CB1$^{-/-}$ mice showed a decrease in the mobility as compared to WT animals, the difference between the two genotypes did not reach statistical significance ($t = 1.904$; $p = 0.0777$).

SOCIAL ACTIVITY TESTS

Social interaction

As described in **Figures 6A,B**, two-way ANOVA (factor 1: light intensity, factor 2: genotype) revealed a main effect of light intensity ($F_{1,13} = 14.656$; $p < 0.01$) genotype ($F_{1,13} = 6.366$; $p < 0.05$) and a light intensity × genotype interaction ($F_{1,13} = 10.904$; $p < 0.01$) for time of interaction. There were also a main effect of light intensity ($F_{1,13} = 18.472$; $p < 0.01$) genotype ($F_{1,13} = 5.285$; $p < 0.05$) and a light intensity × genotype interaction ($F_{1,13} = 12.947$; $p < 0.01$) for the frequency of interaction. *Post hoc* analysis showed that in the less aversive environment (0 lux), D1–CB1$^{-/-}$ expressed a lower SI during the 5-min test than WT mice as described by the decreased number and time of interactions ($p < 0.05$). WT approached the low level of performance seen in D1–CB1$^{-/-}$ under aversive conditions (700 lux).

Social investigation

Figures 6C,D describe the time and the number of active approaches toward the empty falcon tube vs. the tube containing the ovariectomized female. Two-way ANOVA analyses (factor 1: object, factor 2: genotype) revealed a main effect of object (number of interaction: $F_{1,34} = 35.588$; $p < 0.001$; time of interaction: $F_{1,34} = 25.023$; $p < 0.001$), but no main effect of genotype (number of interaction: $F_{1,34} = 0.0182$; $p = 0.893$; time of interaction: $F_{1,34} = 1.402$; $p = 0.245$) or a object × genotype interaction (number of interaction: $F_{1,34} = 0.839$; $p = 0.366$; time of interaction: $F_{1,34} = 1.780$; $p = 0.191$), indicating that mice of both genotype display a preference for the ovariectomized female. Additional *t*-test was performed separately for each genotype. D1–CB1$^{-/-}$ and WT mice showed higher interest for the tube containing the female, as described by the significant increase of time of investigation (WT: $t = 3.782$; $p < 0.01$; D1–CB1$^{-/-}$: $t = 3.489$; $p < 0.01$) and by number of interactions (WT: $t = 3.904$; $p < 0.01$; D1–CB1$^{-/-}$: $t = 4.459$; $p < 0.001$).

Fear conditioning

As shown in **Figure 7A**, unpaired *t*-test revealed that D1–CB1$^{-/-}$ showed a significant increase on freezing response to the tone at day 1 ($t = 2.497$; $p < 0.05$) and to the context at day 2 ($t = 3.210$; $p < 0.01$) as index of increased auditory-cued and contextual fear responses, respectively. When analyzed in 20-s intervals, all mice

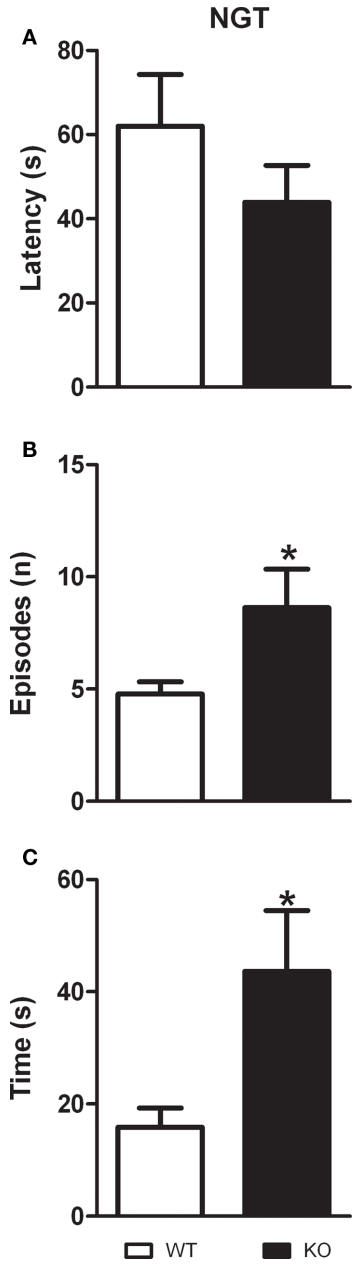

FIGURE 4 | Grooming behavior in D1–CB1$^{-/-}$ mice. Conditional D1–CB1$^{-/-}$ mutant mice (KO) and their respective wild-type (WT) littermates were tested for the grooming activity measure. Data are presented as mean ± SEM regarding latency to start grooming **(A)**, number of grooming episodes **(B)** and total grooming duration **(C)**. *$p < 0.05$ as compared to WT mice (unpaired *t*-test).

showed the same initial freezing response on day 1. However, whereas WT mice showed a rapidly waning freezing response during the tone presentation, D1–CB1$^{-/-}$ mice showed a deficit in acute fear adaptation (**Figure 6B**). The second experiment, largely confirmed their phenotype (**Figures 6C,D**): D1–CB1$^{-/-}$ mice showed a significant increase on freezing response to the tone on day 1 ($t = 4.234$; $p < 0.001$) and on day 7 ($t = 2.923$; $p < 0.01$), which again results from impaired acute fear adaptation over the course of tone presentation (**Figure 6D**). Freezing before tone presentation on day 1 was low and indistinguishable between the two groups.

DISCUSSION

In the present study we provide first evidence that the genetic deletion of cannabinoids CB1Rs in dopamine D1Rs-expressing neurons is able to affect the emotional behavior in mice in highly selective manner. Several studies reported increased anxiety-related behaviors after impaired CB1R signaling only when aversive stimulus cannot be avoided (Haller et al., 2004, 2009; Thiemann et al., 2007; Kamprath et al., 2009). However, little is known about how ECS modulation of the DAergic system could be involved in this effect.

It is accepted that ECs modulate several neurotransmitter systems (glutamatergic, GABAergic, and DAergic) at multiple levels (Piomelli, 2003). In the brain, where exogenously administered (Δ^9-tetrahydrocannabinol [THC]) and endogenously released cannabinoids exert most of their behavioral effects, the CB1Rs are expressed at different levels at different neuronal subpopulations. More specifically, they are present at very high levels in GABAergic interneurons, where they mediate cannabinoid-dependent inhibition of GABA release, and to a minor extent, in glutamatergic terminals (Marsicano and Lutz, 1999). In the glutamatergic neuronal subpopulation, they play a pivotal role in both neuroprotection and fear extinction in highly aversive situations, through the modulation of glutamate release, further confirming that the fear-alleviating effects of CB1 became evident primarily under highly aversive conditions (Monory et al., 2006; Kamprath et al., 2009; Moreira and Wotjak, 2010).

Several lines of evidence suggest that DA is released in several brain regions such as the amygdala and prefrontal cortex under stress conditions. By acting on D1- or D2-like receptors, DA is involved in physiological processes subserving affective behaviors and emotional learning (LeDoux, 2000). Although, coexpression of the cannabinoid CB1Rs and D1Rs supports an ECS–DAergic system cross-talk, as in forebrain basal ganglia and piriform cortex, the exact role of D1Rs is not fully understood. Thus, the development of conditional CB1 mutant mice, in which the CB1Rs are specifically deleted in neurons expressing D1Rs (Monory et al., 2007) has been an useful tool to understand their role in the emotional behavior.

It should be recalled that growing evidence indicates that measures of anxiety from different tests could reflect different states of anxiety. This prompted us to use different behavioral paradigms such as exploration-based tests and social paradigms, that primarily focus on reciprocal SI and on the preference for social novelty, respectively, as well as tasks involving a strong mnemonic component, such as fear based tests, to assess different aspects that could mimic symptoms of human anxiety disorders as agoraphobia, social phobia or post traumatic stress disorder (Lister, 1990; File, 1992; Cryan and Holmes, 2005).

The first novel result of the present study was that D1–CB1$^{-/-}$ mice did not show any anxiety-like phenotype when tested in exploratory behavioral paradigms such as EPM, LD, or NOI. These procedures mostly reflect the conflict between exploration and avoidance of a novel environment; thus, the inhibition of exploratory behavior given by the reduced open arms or light compartment entries and novel object exploration is commonly associated with high emotionality or anxiety. D1–CB1$^{-/-}$ mice also failed to show alteration in spontaneous

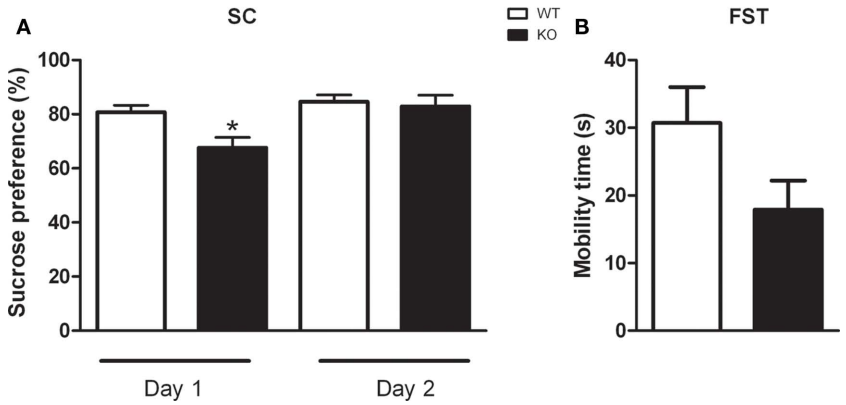

FIGURE 5 | Depressive-like behaviors of D1–CB1$^{-/-}$ mice. Conditional D1–CB1$^{-/-}$ mutant mice (KO) and their respective wild-type (WT) littermates were tested in the sucrose consumption test **(A)** or in the forced swim test (FST) paradigm **(B)**. Data are presented as mean ± SEM regarding percentage of sucrose consumption or mobility time expressed in seconds. *$p < 0.05$ as compared to WT mice (unpaired *t*-test).

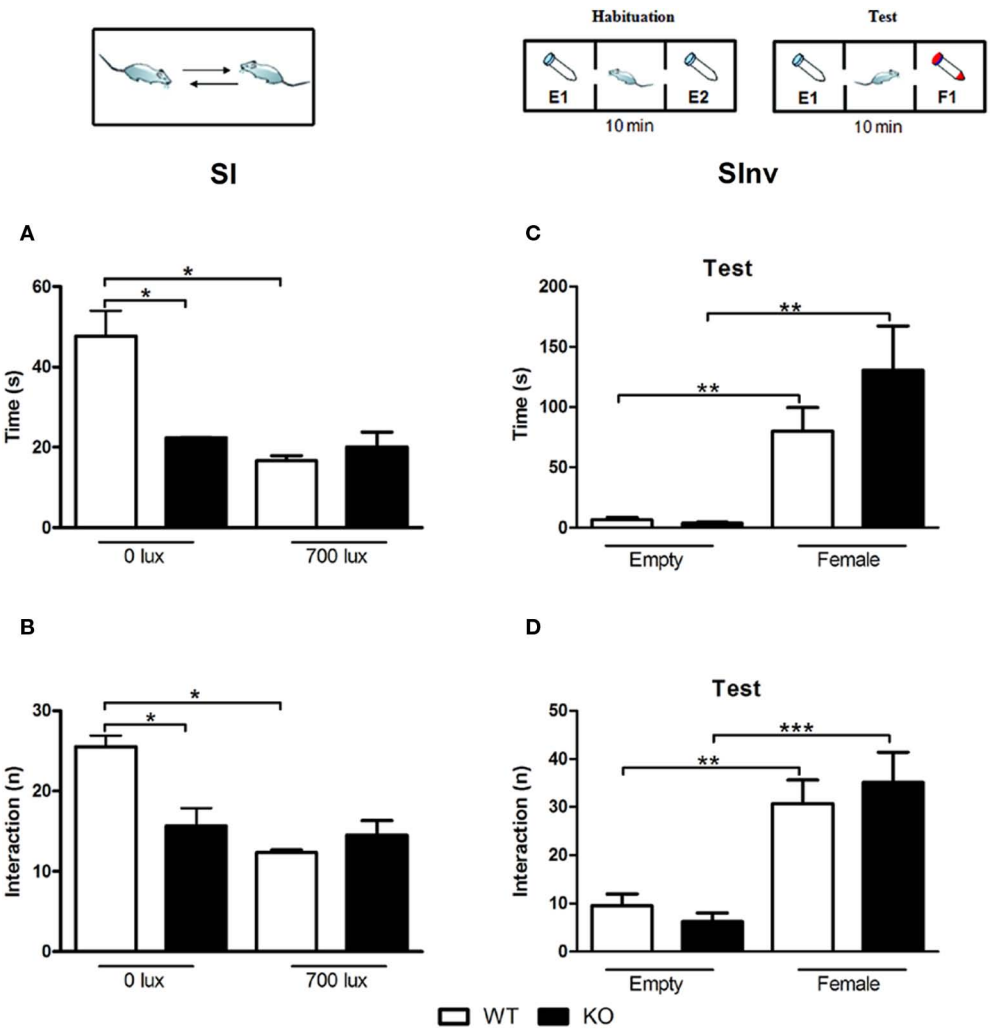

FIGURE 6 | Social behaviors in D1–CB1$^{-/-}$ mice. Conditional D1–CB1$^{-/-}$ mutant mice (KO) and their respective wild-type (WT) littermates were tested in the social interaction **(A,B)** or in the social investigation **(C,D)** test. Data are presented as mean ± SEM regarding time in interaction and number of interactions. E1: empty tube 1; E2: empty tube 2; F1: tube with female. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ (Newman–Keuls *post hoc* test or unpaired *t*-test).

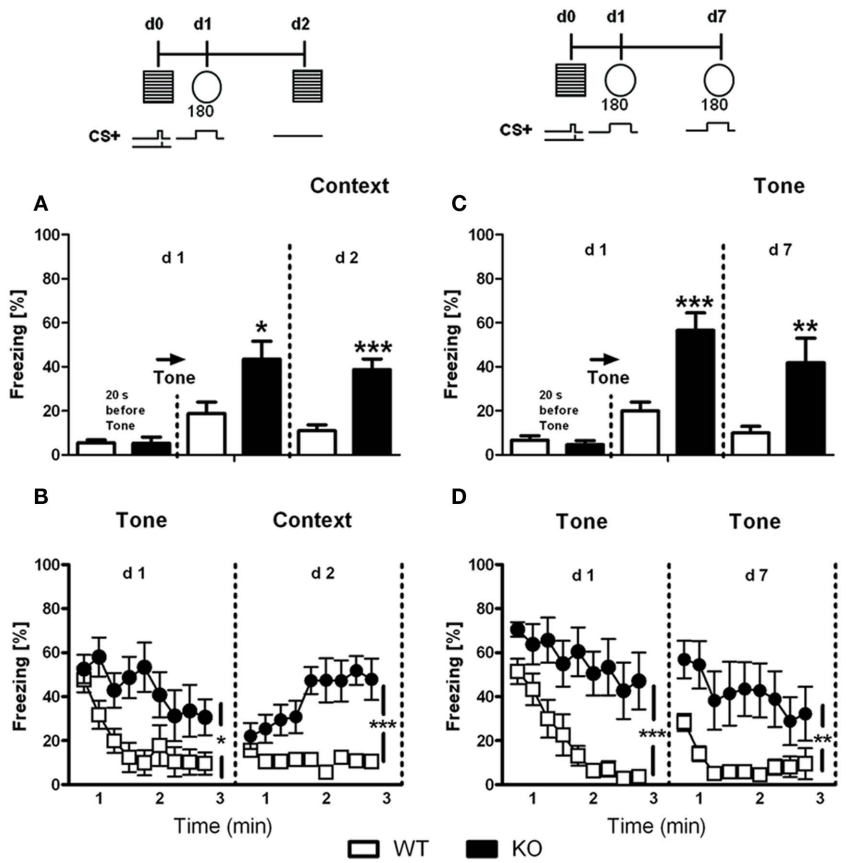

FIGURE 7 | Fear memory in D1–CB1$^{-/-}$ mice. Auditory-cued (Tone) and contextual (Context) fear memory assessed by freezing responses (mean ± SEM) of conditional D1–CB1$^{-/-}$ mutant mice (KO) and their respective wild-type (WT) littermates in two independent sets of experiments **(A/B, C/D)**. If not stated otherwise, freezing was averaged over the entire 180 s observation periods **(A,C)** or analyzed in 20 s intervals **(B,D)**. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared to WT mice (unpaired *t*-test).

exploration and locomotor behavior. These findings are in line with previous data showing no anxiety-like phenotype in mice with total CB1Rs deletion and with specific CB1Rs deletion on glutamatergic neurons (Marsicano et al., 2002; Jacob et al., 2009). However, the D1–CB1$^{-/-}$ mice showed increased grooming activity. Grooming is considered a "maintenance" behavior, a common species-characteristic movement pattern with readily definable components (Bolles, 1960) that serves a range of adaptive functions, including stress reduction and social interplay (Kalueff and Tuohimaa, 2005). In rodents, spontaneous grooming behavior may occupy as much as 25–40% of the wakeful time, but it is specifically elicited in situations (i.e., NGT) in which an animal is in stress-induced conflict or frustration, as well as being reduced following anxiolytic treatment (Dunn et al., 1981; Gispen and Isaacson, 1981; van Erp et al., 1994; Micale et al., 2008). Thus, our results suggest that if the exposure to novelty cannot be controlled by the animals, the novel environment is able to influence the emotionality of D1–CB1$^{-/-}$ mice.

The D1–CB1$^{-/-}$ mice exhibited a decreased preference for sweet solutions on the first but not on the second day of the SC test under basal conditions, indicating a mild anhedonia-like state. Although anhedonia is commonly associated with depression-like behavior phenotype, mutant mice performed normally in the FST, a procedure widely used for evaluating behavioral despair in rodents (Cryan and Holmes, 2005). Thus, the modulation of depressive-like behaviors in D1–CB1$^{-/-}$ mice evaluated in different tasks may be mediated by distinct neuronal circuits. On the other hand, the lower SC was only evident upon the first confrontation with the novel taste (day 1) and disappeared on the next day, suggesting a significant contribution of neophobia. In fact, a weak- to moderate anxiety-like phenotype of D1–CB1$^{-/-}$ mice became evident when the animals were tested under low (0 lux) aversive conditions in an unavoidable situation (i.e. SI test), where the WT control mice demonstrated social approach (intense interaction). These findings suggest that the deletion of CB1Rs specifically in D1Rs-expressing neurons elicited SI impairments, similarly to those observed in mice lacking CB1 in cortical glutamatergic neurons (Jacob et al., 2009). By contrast, it did not affect the preference for social novelty with female stimulus.

Interestingly, D1–CB1$^{-/-}$ mice showed sustained auditory-cued and contextual fear responses, thus resembling the phenotype of impaired fear adaptation observed in mice with complete deletion of CB1Rs (Marsicano et al., 2002; Kamprath et al., 2006) or selective deletion from principal neurons of the forebrain (Kamprath et al., 2009). Since Monory et al. (2007) showed that the deletion of

CB1 in dopamine D1-expressing neurons did not alter the analgesic effects of THC, we can exclude the possibility that the phenotype of D1–CB1$^{-/-}$ mice in the FC paradigms could be due to different nociceptive thresholds.

Currently, we do not know exactly how the CB1Rs modulate D1Rs-mediated emotional behavior. However, due to their coexpression, it is tempting to assume that a direct or indirect receptor–receptor interaction, via intracellular signaling pathways, might be involved (Glass et al., 1997; Gangarossa et al., 2011). In support of this hypothesis, Martín et al. (2008) showed in rats that pharmacological CB1R blockade or activation could facilitate or inhibit animal behavior, respectively; and this latter effect was absent in D1Rs knock-out mice, demonstrating a DR1s dependence on CB1-mediated actions. Thus, in the highly aversive situations of FC paradigms, where a strong stimulus as a footshock was delivered, the EC signaling failed. This happens due to the CB1 deletion, to negatively modulate the D1Rs-emotional behavior, leading to an impaired fear adaptation. However, we cannot rule out the involvement of different pathways as well as the potential compensatory mechanisms occurring during development, which represents a limitation of experiments with mutant mice in general. Nevertheless, the present data add a new facet to the cross-talk between DAergic and the EC systems, within the framework of fear adaptation.

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Opposing Roles for Cannabinoid Receptor Type-1 (CB_1) and Transient Receptor Potential Vanilloid Type-1 Channel (TRPV1) on the Modulation of Panic-Like Responses in Rats

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The midbrain dorsal periaqueductal gray (dPAG) has an important role in orchestrating anxiety- and panic-related responses. Given the cellular and behavioral evidence suggesting opposite functions for cannabinoid type 1 receptor (CB_1) and transient receptor potential vanilloid type-1 channel (TRPV1), we hypothesized that they could differentially influence panic-like reactions induced by electrical stimulation of the dPAG. Drugs were injected locally and the expression of CB_1 and TRPV1 in this structure was assessed by immunofluorescence and confocal microscopy. The CB_1-selective agonist, ACEA (0.01, 0.05 and 0.5 pmol) increased the threshold for the induction of panic-like responses solely at the intermediary dose, an effect prevented by the CB_1-selective antagonist, AM251 (75 pmol). Panicolytic-like effects of ACEA at the higher dose were unmasked by pre-treatment with the TRPV1 antagonist capsazepine (0.1 nmol). Similarly to ACEA, capsazepine (1 and 10 nmol) raised the threshold for triggering panic-like reactions, an effect mimicked by another TRPV1 antagonist, SB366791 (1 nmol). Remarkably, the effects of both capsazepine and SB366791 were prevented by AM251 (75 pmol). These pharmacological data suggest that a common endogenous agonist may have opposite functions at a given synapse. Supporting this view, we observed that several neurons in the dPAG co-expressed CB_1 and TRPV1. Thus, the present work provides evidence that an endogenous substance, possibly anandamide, may exert both panicolytic and panicogenic effects via its actions at CB_1 receptors and TRPV1 channels, respectively. This tripartite set-point system might be exploited for the pharmacotherapy of panic attacks and anxiety-related disorders.

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INTRODUCTION

Panic disorder is a subtype of anxiety spectrum disorders characterized by recurrent episodes of panic attacks, which comprise intense feelings of fear and distress, accompanied by tachycardia, hyperventilation and increased blood pressure (Katon, 2006; Roy-Byrne *et al*, 2006). Although its precise neural basis has remained uncertain, evidence indicates that it might result from a malfunction in brain systems related to defense reactions (McNaughton and Gray, 2000). Accordingly, panic attacks exhibit isomorphism with flight responses regarding their autonomic, behavioral and psychological aspects (Bandler *et al*, 2000; McNaughton and Gray, 2000).

Among the brain regions that may participate in the elaboration of both panic and innate defense reactions is the midbrain dorsal periaqueductal gray (dPAG), comprising dorsomedial and dorsolateral columns (Del-Ben and Graeff, 2009; Schenberg *et al*, 2001). In humans, electrical stimulation of this structure induces an intensive feeling of aversion, fear and imminent death along with autonomic changes (Nashold *et al*, 1969). Moreover, neuroimaging studies detected increased activity in this region in patients suffering from panic (Del-Ben and Graeff, 2009) and in healthy volunteers exposed to a proximal threatening stimulus (Mobbs *et al*, 2007). In laboratory rats, local electrical or chemical dPAG stimulation induces autonomic changes along with escape responses (Beckett and Marsden, 1995; Krieger and Graeff, 1985; Schenberg *et al*, 2001). In addition, systemic or intradPAG injections of panicolytic drugs increase the threshold required to induce escape responses (Hogg *et al*, 2006; Jenck *et al*, 1995; Schenberg

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et al, 2001; Schütz *et al*, 1985). Due to its robust face, construct and predictive validity, the escape reaction resulting from electrical stimulation of the rat dPAG has been proposed as a model of panic attacks (Beckett and Marsden, 1995; Del-Ben and Graeff, 2009; Jenck *et al*, 1995; Schenberg *et al*, 2001).

Diverse types of receptors are expressed in the PAG, including the G-protein coupled cannabinoid type 1 receptor (CB_1) and the transient receptor potential type-1 (TRPV1) channel (Cavanaugh *et al*, 2011; Cristino *et al*, 2006; Herkenham *et al*, 1990, 1991; Mezey *et al*, 2000; Tóth *et al*, 2005; Tsou *et al*, 1998). These receptors may bind common endogenous ligands, which are thus termed endocannabinoids/endovanilloids, such as arachidonoyl ethanolamide (anandamide) (for reviews, see Di Marzo, 2008; Di Marzo *et al*, 2001 and Starowicz *et al*, 2007a). Despite this similarity, electrophysiological and neurochemical pieces of evidence indicate that they may have opposite functions. In the PAG, local CB_1 activation inhibits calcium channels and excitatory neuronal activity (Vaughan *et al*, 2000), whereas TRPV1 promotes calcium influx and glutamate release (Starowicz *et al*, 2007b; Xing and Li, 2007). At the behavioral level, CB_1 knockout mice show deficits in extinction and habituation of conditioned fear (Kamprath *et al*, 2006; Marsicano *et al*, 2002) and exacerbated anxiety-like behavior (Haller *et al*, 2002, 2004; Jacob *et al*, 2009; Martin *et al*, 2002). On the contrary, TRPV1 knockout mice show reduced fear- and anxiety-like responses (Marsch *et al*, 2007). Finally, either local activation of CB_1 or blockade of TRPV1 at level of the dorsolateral columns of the PAG-induced anxiolytic-like effects (Lisboa *et al*, 2008; Moreira *et al*, 2007, 2009; Terzian *et al*, 2009). The same is observed with either systemic or intraprefrontal cortex injections (Aguiar *et al*, 2009; Micale *et al*, 2009; Rubino *et al*, 2008).

So far, most studies have investigated the roles of CB_1 and TRPV1 separately. However, there is evidence that they may act in concert to modulate behavioral responses (Maione *et al* 2006; Micale *et al*, 2009; Rubino *et al*, 2008). They are co-expressed in several regions related to aversion, such as prefrontal cortex, amygdaloid complex and hippocampus, where they may modulate anxiety-related responses (Cristino *et al*, 2006; Micale *et al*, 2009), as well as in the ventrolateral PAG (vlPAG), where they control nociceptive reactions (Cristino *et al*, 2006; Maione *et al*, 2006).

Considering this background, the present study tested the hypothesis that CB_1 and TRPV1 exert opposite effects on panic-like responses at level of the dPAG and looked for interdependences of the two signaling systems.

MATERIALS AND METHODS

Animals

Male Wistar rats were obtained from the animal facilities at the University of São Paulo, Ribeirão Preto. The animals, weighing 300–330 g, were housed in groups of 4–6 per cage under a 12-h dark/12-h light cycle (lights on at 0700 h) at $22 \pm 1\,^{\circ}C$, and given free access to food and water. All experiments were carried in accordance with the Brazilian Society of Neuroscience and Behavior for the care and use of laboratory animals and were approved by the Experimental Animal Ethical Committee of University of São Paulo (protocol number: 114/2007). All efforts were made to minimize animal suffering.

Surgery

Rats were anesthetized with 2,2,2 tribromoethanol (250 mg/kg i.p.) combined with local anesthesia (2% lidocaine with a vasoconstrictor; Harvey, Brazil), and fixed in a stereotaxic frame (David Kopf) for implantation of a chemitrode in the dPAG (incisor bar 3.3 mm below the interaural line, 1.7 mm lateral to lambda at an angle of 22° with the sagittal plane, 5.0 mm below the surface of the skull; according to Paxinos and Watson, 1997). The chemitrode consisted of a stainless steel guide cannula (outer diameter: 0.6 mm, length: 13 mm) glued to a brain electrode made of stainless steel wire (diameter: 250 μm) that was insulated by enamel except for the cross-section of the tip. The electrode reached 1 mm below the end of the cannula. It was connected to a male pin parallel to the outer end of the cannula that could be plugged into an amphenol socket at the end of a flexible electrical cable. The chemitrode was fixed to the skull by means of acrylic resin and two stainless steel screws. A stylet was inserted into the guide cannula in order to prevent obstruction. At the end of the surgery, animals were treated with an antibiotic (Pentabiótico, Fort Dodge, Brazil; 1.0 ml/kg, i.m.) and fluxinin meglumine (Schering-Plough, Brazil; 2.5 mg/kg, s.c.), which has analgesic, antipyretic and anti-inflammatory properties. The animals were left undisturbed for 5–7 days after the surgery.

Drugs and Treatment

The CB_1 agonist, arachidonyl-2-chloro-ethylamide (ACEA; Tocris) was dissolved in Tocrisolve (vehicle). The CB_1 antagonist, *N*-(piperidin-1yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251; Tocris) and the TRPV1 antagonists, capsazepine (Tocris) and 4′-Chloro-3-methoxycinnamanilide (SB366791; Tocris), were dissolved in DMSO.

For local drug treatment, an injection needle (outer diameter: 0.3 mm) was inserted through the guide into the dPAG to a final depth of 6.0 mm below the surface of the skull. A volume of 0.2 μl was injected for 60 s using a 10-μl microsyringe (Hamilton) attached to a microinfusion pump (KD Scientific). The needle was left in place for another 60 s before removal.

Escape Threshold Determination

Escape behavior induced by dPAG electrical stimulation was performed in accordance with previous work (Casarotto *et al*, 2010). The animals were evaluated in a circular arena (40 cm in diameter) with 40 cm high walls made of transparent Plexiglas. Brain stimuli were generated by a sine-wave stimulator. The stimulation current (peak-to-peak) was monitored on the screen of an oscilloscope (Minipa, Brazil). The brain electrode was connected to the stimulator by means of an electromechanical swivel and a flexible cable, allowing ample movement of the animal inside the experimental cage. The escape threshold was determined in response to an electrical stimulus (AC, 60 Hz, 10 s)

presented through the chemitrode. The interstimulus interval was 10 s. The current intensity started at 20 μA and was increased by steps of 4 μA until the rat started running around the circular arena (escape behavior). In most cases, this vigorous reaction was accompanied by vertical jumps. When these behaviors were observed, electrical stimulation to the dPAG was interrupted by the experimenter that was blind to the treatment groups. The basal escape threshold was defined as the lowest current intensity that evoked escape in three successive trials of electrical stimulation. Animals with basal thresholds >150 μA were excluded from the study. Following basal escape threshold determination, independent groups of animals were injected with the drugs or vehicle into the dPAG, and the escape threshold was re-analyzed 10 min later. The variation in escape threshold (Δ) was then calculated for each animal and refers to the difference between escape threshold values obtained post- and pre-treatment.

Experiments

Independent batches of animals were used in each experiment. In experiment 1, the rats were treated with ACEA (0.01, 0.05 and 0.5 pmol) or vehicle ($n=6$–7). In experiment 2, new groups of animals received AM251 (75 pmol) or its vehicle followed by ACEA (0.05 pmol) or its respective vehicle 5 min later ($n=5$–6). In experiment 3, they were injected with capsazepine (0.1, 1 and 10 nmol) or vehicle ($n=6$–9). In experiment 4, capsazepine (0.1 nmol) or vehicle injections were followed by ACEA (0.5 pmol) 5 min later ($n=5$ per group). In experiment 5, the rats were treated with AM251 (75 pmol) or vehicle followed by capsazepine (10 nmol) or vehicle 5 min later ($n=5$–7). Finally, in experiment 6, they were treated with AM251 (75 pmol) or vehicle followed by SB366791 (10 nmol) or vehicle 5 min later ($n=5$–7). The doses of ACEA, AM 251 and capsazepine were selected based on previous works performing injections into the dPAG (Moreira *et al*, 2007; Terzian *et al*, 2009). The dose of SB366791 was selected based on the affinity of this substance for TRPV1 as compared with other antagonists (Gawa *et al*, 2005; Gunthorpe *et al*, 2004).

Histology

After the experiments, animals were deeply anesthetized with chloral hydrate. The brain was perfused through the heart with saline solution followed by 10% formalin solution, before being removed and fixed in 10% formalin. Frozen sections of 55 μm were cut using a microtome to localize the positions of the electrode tips according to the atlas by Paxinos and Watson (1997). Only data from rats having chemitrode tips inside the dPAG were included in the statistical analysis.

Immunofluorescence

Male Wistar rats were anaesthetized with urethane and transcardially perfused with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PBS, pH 7.4). The brain was removed and post-fixed over 2 h in PFA 4% and stored for at least 30 h in 30% sucrose for cryoprotection. Coronal sections (25 μm) were obtained in a cryostat and were washed in PBS 0.01 M. After this, the sections were incubated in glycine 0.1 M, washed again and incubated for 1 h in 5% bovine serum (BSA, sigma) in PBS 0.01 M, pH 7.4 containing 0.3% Triton X-100. Thereafter, the sections were incubated for 2 days at 4 °C in a mixture of anti-CB1 receptor N-terminus (1 : 250; Abcam raised in rabbit) to anti-TRPV1 receptor N-terminus (1 : 100; Santa Cruz raised in goat) diluted in PBS 0.01 M containing BSA 5% and 0.3% Triton X-100. After incubation in the primary antiserum, the tissue sections were washed in PBS and sequentially incubated in a mixture of Alexa 594 donkey anti-rabbit IgGs (1 : 1000; Molecular Probes, Eugene, OR) and Alexa 488 donkey anti-goat IgGs (1 : 1000; Molecular Probes) for 1 h. Slides were rinsed in PBS and coverslipped with Fluormount-G (Electron Microscopy Sciences, Hatfield, PA). Images were first obtained using an Olympus BX50 microscope through computerized image system (Image Pro-Plus 4.0, Media Cybernetics). For a more appropriate analysis of double-stained cells, the slides were re-examined using a modified Olympus BX61WI confocal microscope in an upright configuration, using an Argonium laser at 488 nm. Immunostained slides (Alexa Fluor 488-coupled anti-CB_1 receptors; Alexa Fluor 546-coupled anti-TRPV1 receptors) were exposed for 2.71 s and green and red channels were merged digitally by Fluoview software. Images were further exported in TIFF format. The specificity of the primary antibodies has been demonstrated before by means of CB_1 and TRPV1 knockout mice (Maione *et al*, 2006). Nevertheless, we additionally performed negative controls for non-specific labeling. The protocol was the same as described above, except for the fact that the primary antibodies for CB_1 and TRPV1 were switched, so that each protocol would include a primary antibody for one receptor and a secondary for the other.

Statistical Analysis

The effects of intradPAG drug injections were analyzed by Student's *t*-test or by one-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test, when appropriate. Data were expressed as mean ± SEM. Statistical significance was accepted if $p<0.05$.

RESULTS

The sites of electrical stimulation and drug injection can be seen in Figure 1. Electrical stimulation of the dPAG (black dots), but not of surrounding regions (gray dots), caused an explosive panic response, which was characterized by running and jumping. Activation of CB_1 receptors by local administration of ACEA modified the effects of electrical stimulation in a bell-shaped manner. It increased the threshold for inducing escape behavior at 0.05 pmol, though not at 0.01 or 0.5 pmol ($F(3,23)=4.06$; $p<0.05$; Figure 2). The panicolytic-like effect of 0.05 nmol ACEA was mediated by CB_1 receptors, since it could be blocked by pre-treatment with the selective antagonist AM251 (75 pmol) ($F(3,17)=13.86$; $p<0.01$; Figure 3). Local blockade of TRPV1 channels by capsazepine also raised the escape threshold at 1 and 10 nmol ($F(3,25)=16.58$; $p<0.05$; Figure 4).

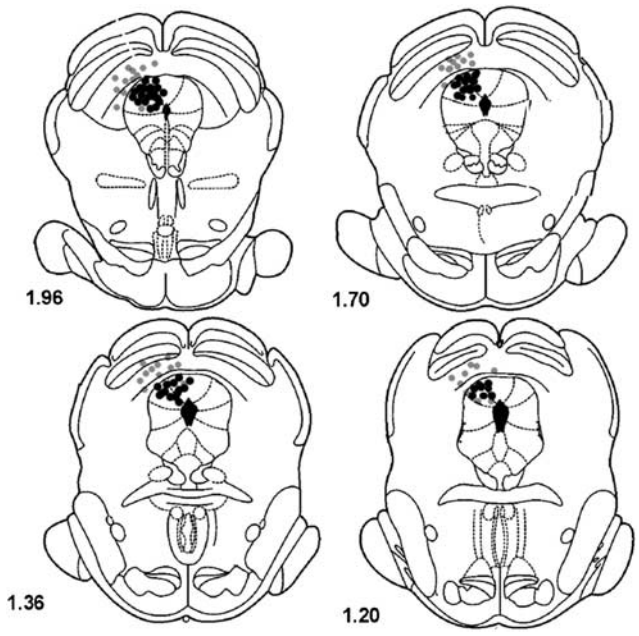

Figure 1 Schematic representation of coronal sections from interaural line of the rat brain (Paxions and Watson, 1997) showing the injection/electrical stimulation sites inside (dark circles) of the dPAG. Sites where electrical stimulation failed to induce escape responses are also presented (gray circles). Due to overlaps, the number of points represented is fewer than the number of rats actually employed in the experiments.

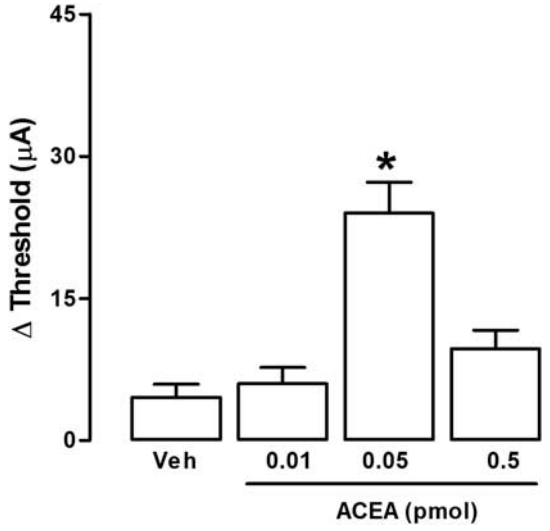

Figure 2 Local injection of the selective CB_1-receptor agonist, ACEA, into the dPAG increases the threshold of electric current necessary for inducing panic-like response ($n = 7, 6, 7, 7$). Following basal escape threshold determination, independent groups of animals were injected with vehicle or ACEA (0.01–0.5 pmol), and the escape threshold was re-analyzed 10 min later. The variation in escape threshold (Δ) was then calculated for each animal and refers to the difference between escape threshold values obtained post- and pre-treatment. $^*p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test).

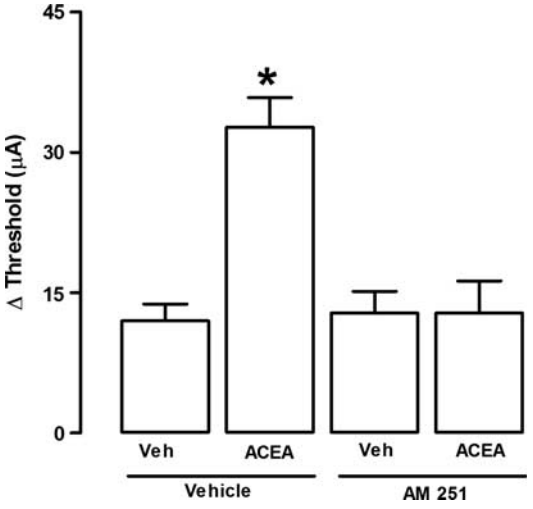

Figure 3 Local pre-treatment (5 min) with the CB_1 antagonist, AM251 (75 pmol), prevents ACEA (0.05 pmol)-induced panicolytic-like effect ($n = 5, 6, 5, 5$). $^*p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

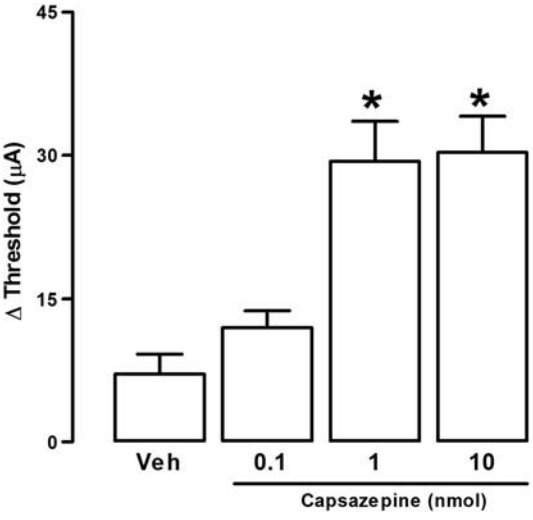

Figure 4 Local injection of the TRPV1 antagonist, capsazepine (CPZ; 0.1–10 nmol), into the dPAG increases the threshold of electric current required for inducing panic-like response ($n = 9, 7, 6, 7, 7$). $^*p < 0.05$ Compared with vehicle-treated rats (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

These results suggest that TRPV1 has diametrically opposite functions as compared with CB_1. Considering that certain cannabinoids may also bind TRPV1 at higher concentrations (Huang *et al*, 2002; Price *et al*, 2004; Silveira *et al*, 2010; Smart *et al*, 2000; Zygmunt *et al*, 1999), simultaneous CB_1 and TRPV1 activation could explain the bell-shaped effects observed with ACEA in the first experiment (Figure 2). High-dose ACEA could activate both CB_1 and TRPV1, occluding the CB_1-mediated effects. Indeed, an ineffective dose of capsazepine (0.1 nmol) unmasked the panicolytic-like effect of ACEA (0.5 pmol) ($t(8) = 3.48$; $p < 0.01$; Figure 5).

Finally, we tested the hypothesis that the panicolytic-like effect of TRPV1 blockade would occur because it would shift the action of a common endogenous agonist toward CB_1 activation. Supporting this hypothesis, the panicolytic-like effect of capsazepine at higher doses (10 nmol) could be

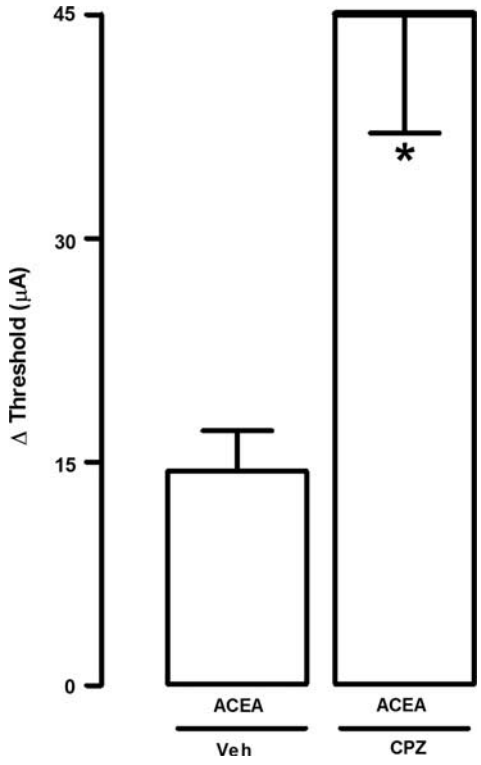

Figure 5 Local pre-treatment (5 min) with the TRPV1 antagonist, capsazepine (CPZ; 0.1 nmol), unmasks the panicolytic-like effect of high-dose ACEA (0.5 pmol) ($n = 5$ per group). *$p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

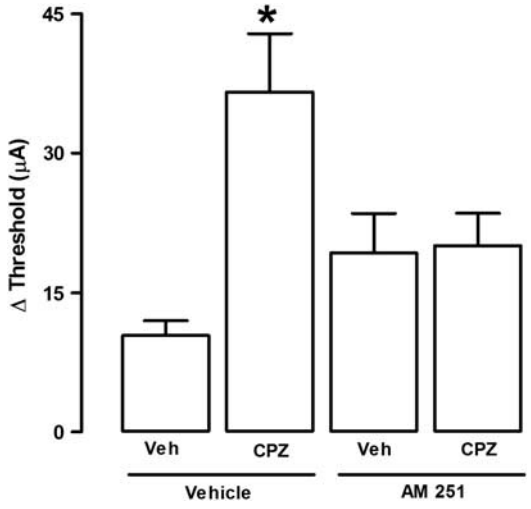

Figure 6 Local pre-treatment (5 min) with the CB_1 antagonist, AM251 (75 pmol), prevents the panicolytic-like effect of capsazepine (CPZ; 10 nmol)-induced ($n = 5, 7, 5, 7$). *$p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

prevented by pre-treatment with AM251 (75 pmol), demonstrating its dependency on CB_1 signaling ($F(3,20) = 5.74$; $p < 0.01$; Figure 6). Remarkably, this data could be reproduced with a more selective TRPV1 antagonist, SB366791 (10 nmol), which increased the threshold for

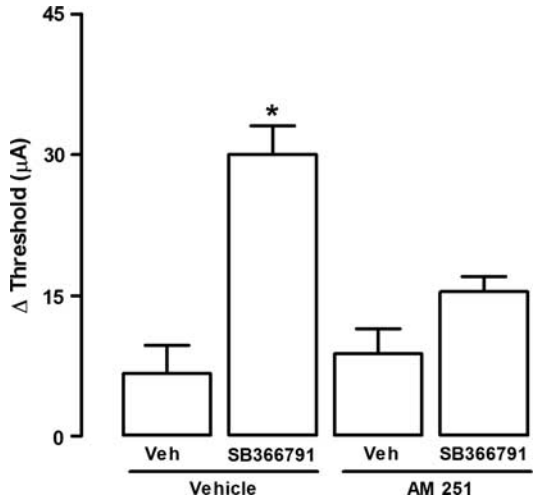

Figure 7 Local pre-treatment (5 min) with the CB_1 antagonist, AM251 (75 pmol), prevents the panicolytic-like effect of SB366791 (10 nmol) ($n = 6, 6, 5, 7$). *$p < 0.05$ Compared with all other groups (one-way ANOVA followed by Newman-Keuls *post hoc* test). For further details, see legend to Figure 2.

panic-like responses to the same extent as capsazepine, again in a CB_1-dependent manner (75 nmol), as presented in Figure 7 ($F(3,20) = 16.3$; $p < 0.01$; Figure 7).

At the cellular level, both CB_1 and TRPV1 are expressed in neurons within the dPAG (Figure 8). Immunofluorescent labeling revealed neurons that were exclusively CB_1 or TRPV1 positive. However, many neurons co-expressed both receptors in their cell bodies. This could be certified through confocal microscopy examination, which allowed us to distinguish double-stained neurons from pairs of mono-stained neurons located in adjacent plans. There was a medium degree of colocalization throughout the dorsolateral and dorsomedial aspects of the PAG. This pattern of co-expression supports the notion that they could be simultaneously activated at a given synapse. Importantly, no staining was observed in protocols in which the antibodies were switched, so that the primary for one receptor was combined with the secondary for the other (Figure 9). This re-assures the specificity of the antibodies used in the present protocols.

DISCUSSION

The present work provides evidence for opposite functions of CB_1 and TRPV1 in an animal model of panic attacks, the electrical stimulation of the dPAG. In this model, local application of antipanic drugs increases the current threshold necessary for the induction of explosive escape behaviors. We demonstrated that the selective CB_1 agonist, ACEA, increased the threshold for inducing panic-like reaction in a bell-shaped manner. This panicolytic-like effect was completely prevented by the CB_1 receptor antagonist, AM251. Although this effect was lost in a higher dose, it could be unmasked by previous treatment with an inactive dose of capsazepine, a TRPV1 antagonist. In addition, pharmacological blockade of TRPV1 *per se* also exerted panicolytic-like effects, suggesting the existence of

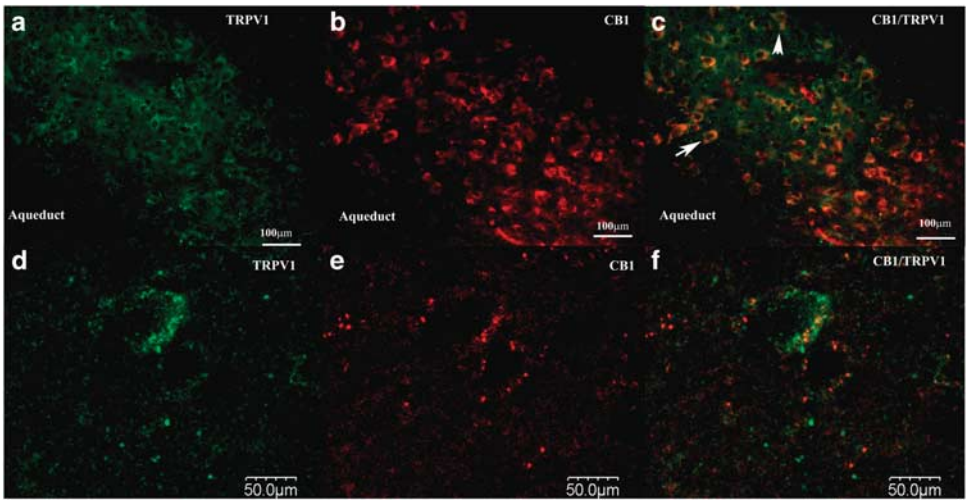

Figure 8 CB_1 receptors and TRPV1channels are co-expressed in neurons in the dPAG. The photomicrographs (25 μm thick section) show image of double-label immunofluorescence of CB_1 (red, Alexa Fluor 594; a, d) and TRPV1 receptors (green, Alexa Fluor 488; b, e), as revealed by fluorescence (a–c) and confocal (d–f) microscopy. Panel (c) (the composite images of (a, b)) shows neurons expressing both receptors. One representative double-stained neuron is shown in higher magnification in f (arrow). Scale bars: 100 μm (a–c) and 50 μm (d–f).

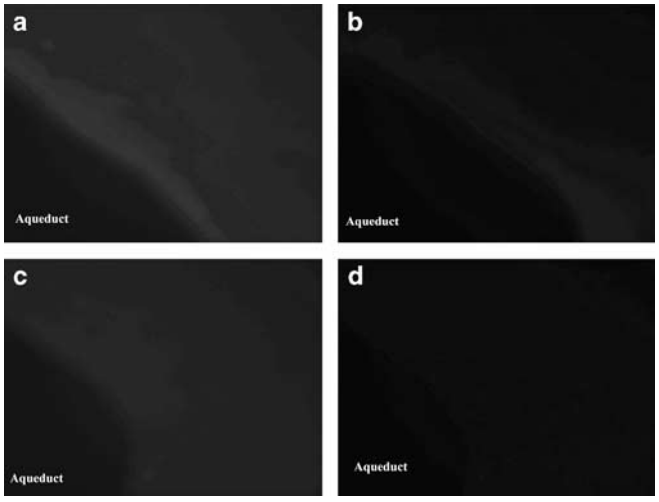

Figure 9 Negative controls for the double-labeled immunofluorescence. The photomicrographs (25 μm thick section) show images of the dPAG from protocols combining TRPV1 secondary (Alexa Fluor 488) with CB_1 primary antibodies (a) or CB_1 secondary (Alexa Fluor 594) with TRPV1 primary antibodies (b). Images resulting from protocol in which the primary antibodies were omitted as seen in the microscopy though filters for Alexa Fluor 488 (c) and Alexa Fluor 594 (d) are also shown.

tonic modulation by TRPV1, whose function opposed that of CB_1. Remarkably, capsazepine effect could be prevented by AM251, revealing that the effect of TRPV1 blockade was completely dependent on CB_1 signaling. Noteworthy, capsazepine effect could be mimicked by another TRPV1 antagonist, SB366791, whose action was also prevented by AM251. Together with the co-expression of CB_1 and TRPV1 observed at level of the dPAG, these data suggest the existence of a set-point system, whereby activation of TRPV1 *vs* CB_1 balances the induction of panic-like responses.

The dPAG model of panic has some limitations in terms of a broader interpretation in relationship to human psychopathology. It mimics only the panic attacks, which actually occur with several other symptoms of panic disorder, such as behavioral and physiological signs of intense anxiety, fear and distress (Katon, 2006; Roy-Byrne *et al*, 2006). Moreover, it is based on the measure of an all-or-none response to electrical stimulation of a specific brain structure, which is only part of a more complex neural circuitry generating fear, anxiety and panic responses (Del-Ben and Graeff, 2009). Despite of these drawbacks, this model presents face, construct and predictive validities (Jenck *et al*, 1995; Beckett and Marsden, 1995; Del-Ben and Graeff, 2009; Schenberg *et al*, 2001). The face validity is supported by the fact that the behavioral and autonomic responses observed in this model resemble those seen in panic attacks (Schenberg *et al*, 2001). As for the theoretical construct, the PAG is a midbrain structure activated during escape responses induced by natural aversive stimulus either in laboratory animals or in healthy volunteers (Dielenberg *et al*, 2001; Mobbs *et al*, 2007). Neurosurgery patients, in whom this structure has been electrically stimulated, reported feelings of fear and despair similar to those described in panic patients (Nashold *et al*, 1969). Finally, and more relevant for the present study, the pharmacological predictability of the model is supported by the observations that drugs used to treat panic disorder increase the threshold of escape responses induced by electrical stimulation of the dPAG (Jenck *et al*, 1995; Schenberg *et al*, 2001; Schütz *et al*, 1985).

In this model, ACEA induced a panicolytic-like effect in an intermediary dose, which could be prevented by AM251, in line with previous experiments in anxiety-related reactions (Moreira *et al*, 2007). These results support the concept of general anti-aversive effects of CB_1 signaling at level of the PAG. Accordingly, local injections of other cannabinoids inhibit the escape responses induced by local injection of an excitatory amino acid (Finn *et al*, 2003) and

promote anxiolytic-like effects in several animal models (for a review, see Moreira *et al*, 2009).

Contrary to CB_1, TRPV1 seems to mediate pro-aversive effects, since we detected a clear panicolytic-like effect after its blockade with capsazepine or SB366791. Moreover, an ineffective dose of capsazepine was able to unmask the panicolytic-like effects of higher dose of ACEA. Therefore, the bell-shaped dose–response curve of ACEA seems to result from an activation of CB_1 at lower concentrations (panicolytic), whereas higher concentration of ACEA also activates the less affine TRPV1 channels (panicogenic), counteracting CB_1-mediated actions, resulting in a net null effect. This is in line with ACEA affinities profile, binding CB_1 in lower dose (Gawa *et al*, 2005) and both CB_1 and TRPV1 in higher dose (Huang *et al*, 2002; Price *et al*, 2004; Silveira *et al*, 2010; Smart *et al*, 2000; Zygmunt *et al*, 1999).

The panicolytic consequences of TRPV1 antagonists speak for a tonic activation of TRPV1 under basal conditions. This is in accordance with previous studies, which reported decreased anxiety upon pharmacological or genetic interruption of TRPV1 signaling. For instance, systemic injection of capsazepine reduced anxiety-like behavior in rats (Kasckow *et al*, 2004). Moreover, a detailed investigation on the behavior of TRPV1 knockout mice unveiled a phenotype of reduced anxiety-like behavior in the elevated plus maze and in the light-dark box, as well as reduced fear conditioning (Marsch *et al*, 2007). In addition, the effect of systemically administered capsazepine is mimicked by local injection into specific structures, namely the ventral hippocampus (Santos *et al*, 2008), the dorsolateral PAG (Terzian *et al*, 2009) and the prefrontal cortex (Aguiar *et al*, 2009; Rubino *et al*, 2008). This is consonant with neurochemical and electrophysiological data showing that this ion channel stimulates calcium influx and glutamate release in the PAG, increasing local neuronal activity (Starowicz *et al*, 2007b; Xing and Li, 2007). In addition, it has been demonstrated in other brain regions related to anxiety and emotional processing, such as the hippocampus and the nucleus accumbens, that anandamide acts upon TRPV1 modulates long-termed depression of synaptic activity (Chavez *et al*, 2010; Grueter *et al*, 2010). Although it remains unclear how these electrophysiological events would reflect behavioral responses, these studies suggest that anandamide could be the endogenous substance activating this ion channel (Di Marzo, 2010; Chavez *et al*, 2010; Grueter *et al*, 2010). Other lipids, including *N*-arachidonoyl dopamine, N-oleoyldopamine and some lypooxigenase products have also been among the main candidates proposed as endovanilloids (Di Marzo *et al*, 2001; Starowicz *et al*, 2007a).

Remarkably, the effects of TRPV1 blockade could be fully prevented by pre-treatment with the selective CB_1 antagonist, AM251. Thus, we have shown that the effect of TRPV1 antagonists is ultimately mediated by CB_1 receptor, possibly because the actions of an endogenous agonist would be entirely shifted to CB_1, after TRPV1 blockade. This finding adds to previous observations about opposite roles for CB_1 and TRPV1 in various behavioral responses (Maione *et al*, 2006; Micale *et al*, 2009; Rubino *et al*, 2008). We suggest that CB_1 and TRPV1 might be simultaneously activated at a given synapse, since we observed co-expression of CB_1 and TRPV1 at level of the dPAG. Similar observations were obtained before in the prefrontal cortex, amygdala complex, hippocampus and vlPAG (Cristino *et al*, 2006; Maione *et al*, 2006; Micale *et al*, 2009). In accordance with previous studies, we also detected dPAG neurons that were either exclusively CB_1 positive (Herkenham *et al*, 1990, 1991; Tsou *et al*, 1998) or TRPV1 positive (McGaraughty *et al*, 2003; Mezey *et al*, 2000; Tóth *et al*, 2005).

Our pharmacological and histological experiments support a scenario in which TRPV1 and CB_1 would counteract each other to balance the threshold for panic-like responses in multiple ways. For instance, the opposite effects of CB_1 and TRPV1 could be mediated by different neurons that converge on the same output system. Alternatively, they could result from divergent influences on intracellular signaling, neural firing rates and neurotransmitter release within the same neurons (Kawahara *et al*, 2010; Starowicz *et al*, 2007b; Vaughan *et al*, 2000; Xing and Li, 2007). Regarding the physiological modulation of these mechanisms, there might be an endogenous agonist, with affinity for both, which would be part of a tripartite system to establish set points for behavioral responses. One candidate would be anandamide, which has been proposed as an endocannabinoid/endovanilloid (Zygmunt *et al*, 2000), with higher affinity for CB_1 as compare with TRPV1 (Di Marzo *et al*, 2001; Starowicz *et al*, 2007a).

Despite of the facts that anandamide has lower affinity for TRPV1 and has its actions limited by the enzyme fatty acid amide hydrolase (FAAH; Piomelli, 2003), the local levels of this substance are significantly increased by electrical stimulation of the dPAG (Walker *et al*, 1999). Therefore, it is conceivable that, in the present model, anandamide levels would increase and act upon both receptors, whereby TRPV1-mediated actions may prevail over CB_1 (Tognetto *et al*, 2001), thus facilitating panic-like behavior. Blockade of this ion channel would increase the threshold by shifting anandamide action entirely to CB_1 receptors, whose selective activation is panicolytic. This hypothesis is reinforced by recent electrophysiological and behavioral experiments. In PAG slices, anandamide could either facilitate or inhibit excitatory transmission through TRPV1 and CB_1, respectively, in the presence of the hydrolysis inhibitor URB597 (Kawahara *et al*, 2010). Accordingly, FAAH knockout mice have a phenotype of lower anxiety-like behavior due to higher CB_1 activation by anandamide. However, CB_1-antagonist treatment induced anxiogenic-like effects in these animals, but not in wild-type controls, possibly by shifting anandamide activity toward TRPV1 (Cassano *et al*, 2010).

In conclusion, we have shown opposite functions for TRPV1 and CB_1 receptors in the modulation of panic-like responses at the level of the dPAG. Anandamide actions via CB_1 *vs* TRPV1 may constitute a set-point system that represents a promising target for the pharmacotherapy of panic and other anxiety spectrum disorders.

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DISCLOSURE

The authors declare no conflict of interest.

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REVIEW

CANNABINOID TYPE 1 RECEPTORS AND TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 CHANNELS IN FEAR AND ANXIETY—TWO SIDES OF ONE COIN?

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Abstract—The transient receptor potential vanilloid type 1 channel (TRPV1; originally vanilloid receptor VR1) is activated in peripheral terminals of nociceptive fibers by noxious heat, low pH, and natural products such as capsaicin, the pungent ingredient of red-hot chilli peppers. Evidence has been accumulating that TRPV1 is expressed also in the brain, where it seems to be involved in antinociception, locomotor control, and regulation of affective behaviors. This ion channel might be activated by arachidonoyl ethanolamide (anandamide), the endogenous agonist of the cannabinoid type 1 (CB_1) receptor. However, while CB_1 activation leads to a decrease in intracellular calcium and attenuation of synaptic transmission, anandamide binding to TRPV1 results in elevated calcium levels and potentiated synaptic transmission. This suggests a tripartite regulatory system with antagonistic effects of CB_1 and TRPV1, which are tied together by the same endogenous ligand. Such a system may have important implication for the modulation of behavioral responses. The present commentary elaborates on this interplay between CB_1 receptors and TRPV1 channels in the context of fear- and anxiety-related behaviors.

Key words: anandamide, cannabinoid, vanilloid, anxiety, fear, stress.

Contents

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Abbreviations: CB_1, cannabinoid type 1; EPM, elevated plus maze; PAG, periaqueductal gray; PFC, prefrontal cortex; TRPV1, transient receptor potential vanilloid type 1 channel; 2-AG, 2-arachidonoyl glycerol.

TRANSIENT RECEPTOR POTENTIAL VANILLOID TYPE 1 (TRPV1) CHANNELS AND CANNABINOID TYPE 1 (CB_1) RECEPTORS: BIOCHEMISTRY, BINDING PARTNERS, AND INTRACEREBRAL EXPRESSION

Both TRPV1 channels and CB_1 receptors were initially identified as molecular targets of natural products (capsaicin and tetrahydrocannabinol (THC), respectively) (Caterina et al., 1997; Devane et al., 1988). They were cloned as "orphan receptors," meaning that no endogenous ligands were known by that time (Caterina et al., 1997; Matsuda et al., 1990). Today, a number of endogenous binding partners could be identified. Most prominent ligands of CB_1 are fatty acid derivates, such as anandamide and 2-arachidonoyl glycerol (2-AG), termed endocannabinoids (Devane et al., 1992; Mechoulam et al., 1995). Remarkably, anandamide also binds to TRPV1 (Zygmunt et al., 1999; Di Marzo et al., 2001; van der Stelt et al., 2005; Vriens et al., 2009). Although it is difficult to compare affinities of a substance for such different molecular targets, it has been suggested that anandamide would bind TRPV1 with lower affinity and intrinsic efficacy as compared to what is observed for this compound at CB_1 (Pertwee et al., 2010; Ross, 2003). Other endogenous binding partners of TRPV1 include *N*-arachidonoyldopamine (NADA), *N*-oleoylethanolamine (OLEA) and hydroperoxyeicosatetraenoic acids (HPETE; for review see van der Stelt and Di Marzo, 2004).

The CB_1 receptor is a seven-transmembrane receptor (Matsuda et al., 1990) coupled to $G\alpha_{i/o}$ proteins. Extracellular binding of endocannabinoids inhibits adenylate cyclase, leads to a decrease in intracellular calcium, and activates inwardly rectifying K+ channels (Howlett et al., 2002). In concert, these actions result in a decrease in transmitter release at both GABAergic (e.g. Katona et al., 1999) and glutamatergic synapses (e.g. Domenici et al., 2006; Melis et al., 2004; Monory et al., 2006; Shen et al., 1996; Takahashi and Castillo, 2006; Vaughan et al., 2000). Unlike CB_1 receptors, TRPV1 channels belong to the transient receptor potential superfamily, with six-transmembrane domains and a nonselective cation-permeable pore (Clapham et al., 2003, 2005; Owsianik et al., 2006; Wu et al., 2010). These channels are activated at intra-

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cellular binding sites, leading to an increase in intracellular calcium and a potentiation of glutamatergic transmission (e.g. McGaraughty et al., 2003; Starowicz et al., 2007a,b; Xing and Li, 2007; Kawahara et al., 2011). Both, CB_1 receptor and TRPV1 channel properties might be modified by a variety of factors, including desensitization/internalization, heterodimerization, and phosphorylation (Hudson et al., 2010; Premkumar and Ahern, 2000). In fact, the observation that TRPV1 channel became activated only at temperatures >43 °C seemed to preclude a significant involvement in signaling processes within the brain. However, phosphorylation of TRPV1 by protein kinase C (PKC) shifts the activation threshold to the physiological range (Premkumar and Ahern, 2000) and could, thus, enable TRPV1 signaling or facilitate its activation at body temperature.

Receptor autoradiography and expression analyses revealed that CB_1 belongs to the most abundantly expressed G-protein coupled receptors in the brain (Herkenham et al., 1990; 1991; Tsou et al., 1998). It is present in a variety of neuronal subpopulations with most prominent expression in cholecystokinin-positive interneurons, but considerable expression also in serotonergic and glutamatergic neurons (Häring et al., 2007; Marsicano and Lutz, 1999). CB_1 receptors can be detected throughout the brain, including cortex, hippocampus, amygdala, dorsal raphe nucleus, periaqueductal gray (PAG), and cerebellum. At ultrastructural level, CB_1 is primarily localized at presynaptic terminals (Egertová et al., 1998). The fact that CB_1 receptors were found presynaptically, whereas its endogenous ligands were primarily synthesized at postsynaptic sites led to the assumption that endocannabinoids may act as retrograde messengers from the postsynaptic to presynaptic terminals. This possibility has been further supported by electrophysiological means: depolarization of postsynaptic neurons leads to mobilization of endocannabinoids and, after binding to presynaptic CB_1 receptors, to a decrease in GABA (depolarization-induced suppression of inhibition, DSI) and glutamate (depolarization-induced suppression of excitation, DSE) release (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001).

Unlike CB_1, it is still a matter of debate to what extent TRPV1 channels are expressed in the brain. In particular, a recent study with mutant mice expressing TRPV1 gene reporters speaks against a broad expression of TRPV1 within the brain. This study reported a highly restricted expression in the posterior caudal hypothalamus, the rostral midbrain, the PAG and, in a very limited manner, the hippocampus (Cavanaugh et al., 2011). These observations stand in contrast to the results of other studies, which reported much stronger expression patterns by immunohistochemistry, *in situ* hybridization, RT-PCR, and receptor autoradiography (Cristino et al., 2006, 2008; McGaraughty et al., 2003; Mezey et al., 2000; Tóth et al., 2005), with prominent expression in the cortex, hippocampus, and the PAG. Those studies revealed that TRPV1 and CB_1 were occasionally co-expressed either within the same neurons or within the same synapses (Cristino et al., 2006; Maione et al., 2006; Micale et al., 2009). In most cases, TRPV1 knockout mice were used for control experiments to confirm the specificity of the methodological approaches. We currently do not know the reasons for the discrepancies of the anatomical studies but assume that dynamic changes in TRPV1 expression due to differences in rearing and housing conditions might play a role.

Strong support for an intracerebral expression of TRPV1 comes from electrophysiological studies, which revealed an involvement of TRPV1, among others, in facilitation of glutamatergic transmission (McGaraughty et al., 2003; Starowicz et al., 2007a; Xing and Li, 2007; Kawahara et al., 2011), long-term potentiation in the hippocampus (Marsch et al., 2007; Li et al., 2004), and long-term depression in this structure (Chávez et al., 2010; Gibson et al., 2008) as well as in the nucleus accumbens (Grueter et al., 2010) and in the developing superior colliculus (Maione et al., 2009). This diversity in TRPV1 actions may depend on its expression in postsynaptic vs. presynaptic nerve terminals. Remarkably, pharmacological blockade and genetic deletion of TRPV1 or CB_1 often revealed opposite changes in synaptic plasticity (Heifets and Castillo, 2009).

The results of the anatomical, biochemical, and electrophysiological studies can be integrated in the following hypothetical scenario (see also Di Marzo, 2008): CB_1 receptors, TRPV1 channels, and their endogenous agonist anandamide form a tripartite regulatory system. The action of anandamide can occur upon presynaptic neurons co-expressing TRPV1 and CB_1. Alternatively, anandamide may bind TRPV1 at postsynaptic neuron and interfere with 2-AG synthesis (Maccarrone et al., 2008), which would restrain neurotransmitter release upon binding to presynaptic CB_1 receptors. The following section will elucidate to what extent these antagonistic effects of CB_1 vs. TRPV1 apply to the behavioral regulation of fear and anxiety.

OPPOSING ROLES FOR CB_1 AND TRPV1 IN MODULATION OF FEAR AND ANXIETY-RELATED RESPONSES

Several brain regions expressing CB_1 receptor are part of the fear and anxiety matrix of the brain. This comprises, among others, the prefrontal cortex (PFC), the hippocampus, the amygdala, the hypothalamus, and the PAG (McNaughton and Gray, 2000; Corr and McNaughton, 2004; Canteras et al., 2010). The existence of CB_1 receptor in all these structures may explain the extensively described behavioral and emotional changes induced by Cannabis and cannabinoids in humans and experimental animals (D'Souza et al., 2009; Moreira and Wotjak, 2010; Moreira et al., 2009; Viveros et al., 2005). Most studies have dissected these mechanisms in laboratory rodents by gene deletion or pharmacological treatment, followed by behavioral analyses of innate anxiety or learned fear responses. Innate anxiety was typically assessed in approach/avoidance conflict tasks, such as the elevated plus maze (EPM) and the light–dark box (LDB). Conditioned fear was measured in classical conditioning tasks, where the animals associated an

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explicit stimulus (e.g. tone, light) or a complex set of different stimuli (i.e. context) with an electric foot shock.

In low doses, cannabinoids tend to induce anxiolytic-like effects (for a review, see Moreira and Wotjak, 2010). In turn, pharmacological blockade of CB_1 (e.g. by rimonabant or AM251) or gene deletion result in exacerbated anxiety-like behavior (Haller et al., 2002, 2004; Jacob et al., 2009; Martin et al., 2002). Another prominent effect of attenuated/abolished CB_1 signaling refers to deficits in acute fear adaptation and fear extinction (Marsicano et al., 2002; Kamprath et al., 2006; Chhatwal et al., 2005; Pamplona et al., 2006; Niyuhire et al., 2007; Lisboa et al, 2010; Plendl and Wotjak, 2010; for review see; Resstel et al., 2009; Pamplona et al. in this Special issue). Noteworthy, the involvement of CB_1 in memory extinction seems to be restricted to aversive memories, since it cannot be observed under appetitive conditions (Hölter et al., 2005; Niyuhire et al., 2007). Considering the neuromodulatory aspects of the endocannabinoid system, a possible mechanism through which CB_1 activation would attenuate aversive responses could be the modulation of glutamatergic activity (Jacob et al., 2009; Kamprath et al., 2009). Because glutamate is widely distributed in the brain and has a well-established role in facilitating aversive responses (Millan, 2003), cannabinoids could ultimately act by inhibiting glutamatergic synapses in the anxiety and fear circuitry (for review see Moreira and Wotjak, 2010).

TRPV1 is also present in glutamatergic synapses, but its role in fear and anxiety is by far less well explored than that of CB_1. Based on the accumulating histological, electrophysiological, biochemical, and pharmacological evidence, it was tempting to assume that this ion channel facilitates anxiety and fear, contrary to the effects of CB_1 receptors. One of the first reports about such an involvement of TRPV1 goes back to Kasckow and co-workers (2004), who demonstrated that systemic injection of the TRPV1 antagonist capsazepine reduced anxiety-like behavior in rats in the EPM. Behavioral analyses of TRPV1 knockout mice strengthened the notion that TRPV1 affects anxiety diametrically opposite to CB_1, since TRPV1 knockout mice showed decreased anxiety in the EPM and LDB and displayed impaired fear expression in response to a tone or a context previously paired with electric foot shocks (Marsch et al., 2007).

A number of pharmacological studies confirmed and extended the notion of anxiolytic effects of CB_1 receptor vs. anxiogenic effects of TRPV1 channel activation. Indeed, compounds with affinity for both receptors, such as anandamide analogs, (i) exert anxiolytic effects in low doses, but (ii) promote anxiety at higher doses, (iii) likely via activating TRPV1 channels. (iv) Pharmacological blockade of TRPV1 not only abolished the effects of high doses of CB_1 agonists but also resulted in an anxiolytic phenotype without concomitant CB_1 agonist treatment. For instance, local administration of anandamide or the CB_1 receptor agonist WIN 55,212–2 into the dorsolateral PAG induced anxiolytic-like effects in rats in the EPM (Moreira et al., 2007; Campos and Guimarães, 2009). These effects were mimicked by local injection of arachidonoyl chloroethylamide, an anandamide analogue, and prevented by AM251, pointing to a role for CB_1 receptor. Interestingly, arachidonoyl chloroethylamide and WIN 55,212-2 showed bell-shaped dose–response curves (Moreira et al., 2007; Campos and Guimarães, 2009), which led us to conclude that the anxiolytic-like effects mediated via CB_1 receptors are lost at higher doses upon activation of TRPV1 channels (Moreira et al., 2009). Indeed, blockade of TRPV1 within the dorsolateral PAG with capsazepine consistently induced anxiolytic-like effects (Terzian et al., 2009) and prevented the anxiogenic-like effects of higher doses of WIN 55,212-2 (Campos and Guimarães, 2009). These effects could be confirmed in the Vogel conflict test (Lisboa et al., 2008; Terzian et al., 2009), a model that involves a different type of conflict-based anxiety (Sousa et al., 2006). However, because of the complex nature of CB_1 and TRPV1 pharmacology, we cannot rule out that some of the effects of CB_1 receptor agonists were mediated via direct desensitization of TRPV1 (at least in case of WIN 55,212-2; Patwardhan et al., 2006).

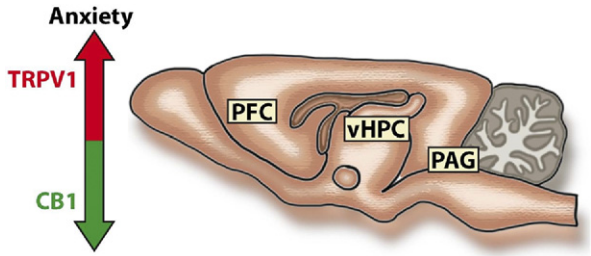

Fig. 1. Bidirectional modulation of anxiety-like behavior via CB_1 and TRPV1. Studies combining intracerebral drug treatment with behavioral analyses revealed that activation of CB_1 receptors reduces, whereas activation of TRPV1 channels promotes anxiety-like behavior at level of the prefrontal cortex (PFC), ventral hippocampus (vHPC), and periaqueductal gray (PAG). For details see text.

Studies with local drug treatment in other parts of the fear/anxiety matrix revealed essentially the same findings as obtained in the dorsolateral PAG (Fig. 1): injection of both THC and the anandamide analogue methanandamide, into the medial PFC of rats, reduced anxiety-like behavior in the EPM (Rubino et al., 2008a,b), with biphasic effects of methanandamide (Rubino et al., 2008b). The anxiolytic effects of lower doses and the anxiogenic effects of higher doses were mediated by CB_1 and TRPV1, respectively (Rubino et al., 2008b). Local blockade of TRPV1 within the medial PFC had an anxiolytic profile in rats both in the EPM and the Vogel conflict test (Aguiar et al., 2009). Finally, within the ventral hippocampus, both activation of CB_1 by THC (Rubino et al., 2008a) and blockade of TRPV1 by capsazepine (Santos et al., 2008) decreased anxiety-like responses in the EPM.

CONCLUSIONS

This commentary compiles a number of biochemical, anatomical, electrophysiological, and behavioral studies, which suggest opposite roles of CB_1 receptors and TRPV1 channels in synaptic transmission, fear, and

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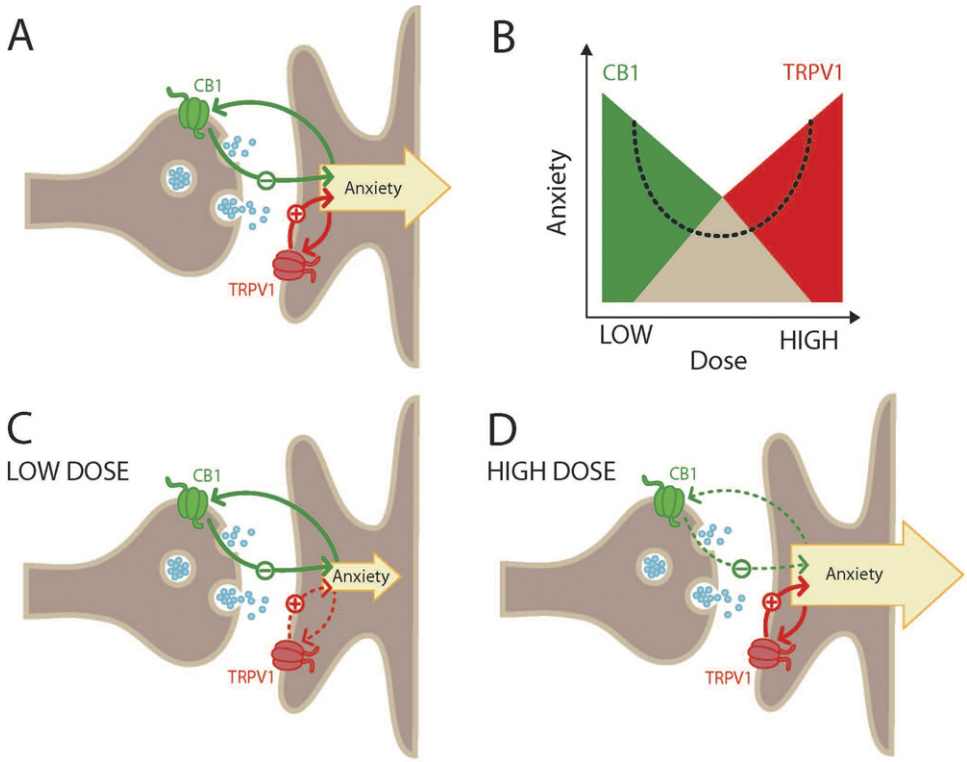

Fig. 2. Hypothetical scenario for the bidirectional modulation of anxiety-like behavior via CB_1 and TRPV1. (A) Activation of presynaptic CB_1 receptors leads to a decrease in transmitter release (e.g. glutamate) and, in consequence, to a reduction in anxiety. In contrast, activation of postsynaptic TRPV1 channels promotes depolarization and anxiety. Both CB_1 and TRPV1 might be activated by the same endogenous ligands (e.g. anandamide). Noteworthy, this hypothetical scenario is further complicated by presynaptic expression of TRPV1 channels and the involvement of different transmitter systems. (B) Agonists of CB_1 often exert bimodal effects on anxiety-like behavior, with lower doses causing anxiolytic and higher doses anxiogenic effects. Co-administration of selective antagonists revealed that this bimodality results from an activation of CB_1 receptors at lower and TRPV1 channels at higher concentrations. (C) At lower concentrations, the CB_1 agonists primarily bind to CB_1, thus resulting in a decrease in anxiety. (D) At higher concentrations, the agonist binds to TRPV1 in addition to CB_1. In consequence, activation of the TRPV1 loop prevails (direct activation of the postsynaptic site bypassing trans-synaptic regulatory loops) and results in an increase in anxiety.

anxiety: whereas activation of CB_1 receptors promotes acute fear adaptation and decreases anxiety, TRPV1 channels exert anxiogenic actions. The co-existence of anxiolytic and anxiogenic transmitter systems is reinforced by the several pieces of evidence indicating a close interaction between CB_1 receptors and TRPV1 channels (i.e. endocannabinoid and endovanilloid actions), such as shared binding partners (i.e. anandamide) and co-expression within the same neurons and/or synapses. Even though the mechanisms of how anandamide acts at CB_1 receptors and TRPV1 channels are far from being clear, there might be, at least, indirect effects via changes in the synthesis/mobilization of other endocannabinoids (e.g. 2-AG). Anyhow, the promiscuous binding capabilities of some cannabinoids could explain the well-known bell-shaped dose–response curves and biphasic effects of some of these compounds by activation of CB_1 receptors at lower and of TRPV1 channels at higher doses (Fig. 2; see also Di Marzo et al., 2008; Di Marzo, 2008). These effects ask for a careful investigation of the receptor types involved in a particular behavioral study. The same applies to compounds that supposedly interfere with endocannabinoid uptake and degradation, but may directly affect TRPV1 channels, such as AM404 (Mallet et al., 2010; Zygmunt et al., 2000a).

There is an urgent need for detailed studies on the role of anandamide as a potential endocannabinoid/endovanilloid, given the steadily increased application of FAAH inhibitors in animal models of psychopathology (Moreira and Wotjak, 2010). The same applies to our poor knowledge about dynamic regulation of TRPV1 channels within the brain, including phosphorylation and desensitization, in particular, in light of the contradictory studies about TRPV1 expression.

On the basis of our current knowledge, antagonists of TRPV1 channels represent promising compounds for the treatment of anxiety disorders. Moreover, the opposing actions of CB_1 and TRPV1 may guide the development of novel therapeutic drugs. For instance, increasing brain levels of anandamide (through FAAH-inhibition) induces CB_1-mediated anxiolytic-like effects (Moreira et al., 2008; Kathuria et al., 2003; Patel and Hillard, 2006), although this effect can be subtle in certain models (Naidu et al., 2007) and depends on the aversiveness of the test situation (Haller et al., 2007). Nonetheless, the effects of FAAH-inhibition can be potentiated by simultaneous antagonism

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of TRPV1 channels, a mechanism achieved, for instance, with arachidonoyl serotonin (Micale et al., 2009). Thus, dual FAAH/TRPV1 blockers represent a particularly promising novel class of anxiolytic drugs.

Taken together, CB_1 and TRPV1 seem to embody the two sides of the same coin (Zygmunt et al., 2000b) also in the context of fear and anxiety. Despite the significant progress in the field, further experiments are required for a better understanding of the relevance of the proposed tripartite CB_1–anandamide–TRPV1 system in physiology and pathology. These studies might lead to the development of novel drugs for the treatment of psychiatric disorders that will allow us to influence what side of the coin is laying face-up: CB_1 or TRPV1.

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I hereby confirm that the dissertation entitle "*Behavioral phenotypes of mice lacking cannabinoid CB1 receptors in different neuronal subpopulations – focus on sex-related social interest*" is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

Munich, 04.02.2014,

Ana Luisa B. Terzian
PhD candidate

Ana Luisa B. Terzian participated on the development of the project and experimental design, performed all experiments, data analysis, collaborated with papers production and wrote the thesis.

Vincenzo Micale participated on the development of the project and experimental design, performed some experiments, data analysis, and collaborated with papers production.

Carsten T. Wotjak is the group leader, coordinated the projects and collaborated with papers and thesis production.

Munich, 04.02.2014

Carsten T. Wotjak
Supervisor

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List of publication – Terzian, AL

1. **Terzian AL**, Micale V, Wotjak CT. CB1 receptors gate sex-dependent social interest in mice *European Journal of Neuroscience* , 2014
2. Aguiar DC, Moreira FA, **Terzian AL**, Fogaça MV, Lisboa SF, Wotjak CT, Guimarães FS Modulation of defensive behavior by Transient Receptor Potential Vanilloid Type-1 (TRPV1) Channels.. *Neurosci Biobehav Rev*., 2014
3. **Terzian AL**, Dos Reis DG, Guimarães FS, Corrêa FM, Resstel LB Medial prefrontal cortex Transient Receptor Potential Vanilloid Type 1 (TRPV1) in the expression of contextual fear conditioning in Wistar rats. *Psychopharmacology* (Berl). 2013
4. Moreira FA, Aguiar DC, **Terzian AL**, Guimarães FS, Wotjak CT. Cannabinoid type 1 receptors and transient receptor potential vanilloid type 1 channels in fear and anxiety-two sides of one coin? *Neuroscience*. 2011
5. Casarotto PC, **Terzian AL**, Aguiar DC, Zangrossi H, Guimarães FS, Wotjak CT, Moreira FA. Opposing Roles for Cannabinoid Receptor Type-1 (CB(1)) and Transient Receptor Potential Vanilloid Type-1 Channel (TRPV1) on the Modulation of Panic-Like Responses in Rats. *Neuropsychopharmacology*. 2011
6. **Terzian AL**, Drago F, Wotjak CT, Micale V. The Dopamine and Cannabinoid Interaction in the Modulation of Emotions and Cognition: Assessing the Role of Cannabinoid CB1 Receptor in Neurons Expressing Dopamine D1 Receptors. *Front Behav Neurosci*. 2011
7. Tonetto LL, **Terzian AL**, Del Bel EA, Guimarães FS, Resstel LB. Inhibition of the NMDA receptor/Nitric Oxide pathway in the dorsolateral periaqueductal gray causes anxiolytic-like effects in rats submitted to the Vogel conflict test. *Behav Brain Funct*. 2009
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12. Leite-Panissi CR, Ferrarese AA, **Terzian AL**, Menescal-de-Oliveira L. Serotoninergic activation of the basolateral amygdala and modulation of tonic immobility in guinea pig. *Brain Res Bull*. 2006

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CARRIER

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Graduate School of Systemic Neuroscience – Ludwig Maximilian Universität
Degree: PhD *Fellowship:* DAAD / CNPq
Research Group: Neural Plasticity *Supervisor:* Dr. Carsten T. Wotjak
"Behavioral phenotypes of mice lacking cannabinoid CB1 receptors in different neuronal subpopulations – focus on sex-related social interest "

2008 - 2009 **School of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP)**
Degree: MSc *Fellowship:* CNPq
Research Group: Psychopharmacology *Supervisor:* Dr. Francisco S. Guimarães
"Involvement of Transient Receptor Potential Vanilloid Type 1 (TRPV1) in periaqueductal gray and medial prefrontal cortex on the modulation of defensive behavior in rats".

2006 - 2007 **School of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP)**
Degree: Scientific Initiation *Fellowship:* FAPESP
Research Group: Psychopharmacology *Supervisor:* Dr. Francisco S. Guimarães
"Participation of Transient Receptor Potential Vanilloid Type 1 (TRPV1) in defensive behavior mediated by periaqueductal grey in rats."

2003 - 2005 **Dental School of Ribeirão Preto, University of Sao Paulo (FORP-USP)**
Degree: Scientific Initiation *Fellowship:* FAPESP
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"*Evaluation of serotoninergic activation in the basolateral nucleus of amygdala in the guinea pig tonic immobility behavior."*

2003 - 2007 **School of Pharmaceutical Science of Ribeirão Preto, University of Sao Paulo (FCFRP-USP)**
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PUBLICATIONS

1. **Terzian AL**, Micale V, Wotjak CT. Cannabinoid receptor type 1 receptors on GABAergic vs. glutamatergic neurons differentially gate sex-dependent social interest in mice. *Eur J Neurosci*. 2014

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7. Tonetto LL, **Terzian AL**, Del Bel EA, Guimarães FS, Resstel LB. Inhibition of the NMDA receptor/Nitric Oxide pathway in the dorsolateral periaqueductal gray causes anxiolytic-like effects in rats submitted to the Vogel conflict test. *Behav Brain Funct*. 2009

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10. **Terzian AL,** Aguiar DC, Guimarães FS, Moreira FA. Modulation of anxiety-like behaviour by Transient Receptor Potential Vanilloid Type 1 (TRPV1) channels located in the dorsolateral periaqueductal gray. *Eur Neuropsychopharmacol*. 2009

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12. Leite-Panissi CR, Ferrarese AA, **Terzian AL**, Menescal-de-Oliveira L. Serotoninergic activation of the basolateral amygdala and modulation of tonic immobility in guinea pig. *Brain Res Bull*. 2006

AWARDS

2014 Travel Grant – 9th FENS Forum of Neuroscience. Milan, Italy

2012 Travel Grant – 8th FENS Forum of Neuroscience. Barcelona, Spain

2011 Travel Grant **–** Cannabinoid Conference. Bonn, Germany

2011 Travel Grant – 24th ECNP. Paris, France

2008 Travel Grant – NIDA-ICRS, SfN, Washington D.C., USA

2008 Travel Grant – 1a. Escola de Altos Estudos da Capes, IINN-ELS / UFRN. Natal, Brazil

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INTERNATIONAL MEETINGS

2014 **9th FENS** Forum of Neuroscience. Milan, Italy

Oral Presentation: *CB1 receptors modulate social interest in a sex-dependent manner in mice*

2013 **6th European Workshop on Cannabinoid Research**. Dublin, Ireland

2012 **8th FENS** Forum of Neuroscience. Barcelona, Spain

2011 **24th ECNP Congress**. Paris, France.

Targeted Expert Meeting of Anxiety disorders and Anxiolytics. Paris, France.

Cannabinoid Conference. Bonn, Germany.

Oral Presentation: *The cross-talk between dopamine and cannabinoid in emotions and cognition: the role of cannabinoid CB1 receptor in neurons expressing dopamine D1 receptors*.

ECNP Workshop in Psychopharmacology. Nice, France

Poster Presentation: *CB1 and TRPV1 receptors located in periaqueductal gray matter mediate opposite effects in panic-like responses in rats*.

Interact 2011. Munich, Germany

Oral Presentation: *CB1 and TRPV1 receptors located in periaqueductal gray matter mediate opposite effects in panic-like responses in rats*.

2010 **International Behavioral Neuroscience Society** (IBNS). Vilasimius, Italy.

Poster Presentation: *Antagonism of transient receptor potential vanilloid type-1 (TRPV1) receptors in the medial prefrontal cortex reduces the expression of contextual fear conditioning in rats*.

2008 **I Congresso IBRO/LARC de Neurociências da América Latina, Caribe e Península Ibérica** (Neurolatam). Buzios, Brazil.

Poster Presentation: *Antagonism of TRPV1 receptors in the medial prefrontal cortex induces anxiolytic-like effects*.

Society for Neuroscience. Washington D.C., USA.

Poster Presentation: *Anxiolytic-like effect of TRPV1 receptor antagonism in the medial prefrontal cortex and central gray*.

NIDA's Frontiers in Addiction Research. Washington D.C., USA.

Poster Presentation: *Anxiolytic-like effect of TRPV1 receptor antagonism in the medial prefrontal cortex and central gray*.

2007 **39° Congresso da Sociedade Brasileira de Farmacologia e Terapêutica Experimental.** Ribeirao Preto, Brazil. 2007.

Poster Presentation: *Anxiolitic-like effect of capsazepine in the rat dorsolateral periaqueductal gray.*

VII São Paulo Research Conferences - Cérebro e Pensamento. São Paulo, Brazil.

2006 **IX Congresso Médico-Acadêmico** - Medicina Forense. Ribeirão Preto, Brazil.

2005 **XX Reunião da Federação das Sociedades de Biologia Experimental**. Águas de Lindóia, Brazil.

5th International Congress of Pharmaceutical Science. Ribeirão Preto, Brazil.

Simpósio de Neurodesenvolvimento Infantil. Ribeirão Preto, Brazil.

IV Semana de Física Médica. Ribeirão Preto, Brazil.

2004 **XIX Reunião da Federação das Sociedades de Biologia Experimental**. Águas de Lindóia, Brazil

ADDITIONAL TRAINING

2014 **FENS-SfN School –** Neurodevolopmental Psychiatry Disorders. Bertinoro, Italy

Young Investigator Training Programme (YITP) – FENS-Internship program – Lab of Prof Laura Ballerini. SISSA. Trieste, Italy

2012 **FENS-CSHL School –** Cellular Biology of Addiction. Barcelona, Spain

The Young Investigator Training Programme (YITP) – FENS-Internship program – Lab of Prof. Andrés Ozaita. PRBB. Barcelona, Spain

FENS School – Drugs and the Brain: an Update in Psycho-pharmacology from Experimental to Clinic. Braga, Portugal

Workshop in Electrophysiology. Max-Planck Institute of Psychiatry. Munich, Germany

2011 **ECNP Workshop in Psychopharmacology.** Nice, France

Workshop on Coping with the challenges of a PhD study, (16h), Ludwig-Maximilian Universität. Munich, Germany.

Workshop on Learning How to Teach (16h), Ludwig-Maximilian Universität. Munich, Germany.

2010 **Workshop on Brain Oscillation, GSN-LMU (16h),** Ludwig-Maximilian Universität. Munich, Germany.

2008 **1a Escola de Altos Estudos CAPES, IINN-ELS/UFRN. (171h).** Instituto Internacional de Neurociências de Natal (IINN-ELS). Natal, RN, Brasil.

2007 **III IBRO School in Brazil (ISBRA),** University of São Paulo. Ribeirão Preto, SP, Brazil.

2005 **Symposia of Child Neurodevelopment (4h),** School of Medicine of Ribeirão Preto, FMRP-USP. Ribeirão Preto, SP, Brazil.

Nervous System Comunication (4h), Reunião da Federação das Sociedades de Biologia Experimental (FESBE). Águas de Lindóia, SP, Brazil.

Chronic Liphoproliferative Illness (3h), International Congress of Pharmaceutical Science (CIFARP). Ribeirão Preto, SP, Brazil.

Neural Network (3h), IV Semana da Física Médica. Ribeirão Preto, SP, Brasil.

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KEY SKILLS

Strong communicative skills: lab meetings and conferences
Initiative, adaptability, creativity and problem solving
Strong interactive skills: successfully training and coaching people
Independent and cooperative
International mobilit

ADDITIONAL COMPETENCES

Operating systems & Softwares	Windows; Microsoft Office: Word, Excel, PowerPoint
Statistical programs	SPSS, StatWin, Statistica, Prism
Graphical and image processing programs	SigmaPlot, PhotoShop
Tracking Software	ANY-Maze, Ethovision

REFERENCES

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