Cannabinoid CB1 receptor in the regulation of sociability, stress coping, and its interaction with the serotonergic system

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Chapter 1

Summary & Zusammenfassung

1.1. Summary

Cannabinergic drugs have been found to inversely influence the behavioral outcome depending on the dose applied. This so-called biphasic effect might be caused by the distribution of the cannabinoid type 1 (CB_1) receptor on different neuronal populations. Two relevant populations in this respect are cortical glutamatergic and GABAergic neurons, as the two respective neurotransmitters opposingly influence excitability. Specific deletion of the CB_1 receptor in either of these two neuronal populations displayed opposite phenotypes, namely a decrease and increase in exploratory drive, respectively. No changes were observed for mice lacking CB_1 receptor specifically in dopamine receptor type 1 positive GABAergic medium spiny neurons, suggesting cortical glutamatergic and GABAergic CB_1 receptor to be responsible for a balanced exploratory behavior (**see Chapter 3**).

Such a dose dependent cannabinoid-induced biphasic effect on the behavioral performance was also observed in the forced swim test (FST). One possible mechanism underlying cannabinoid effects on stress-related responses might be by modulating of monoaminergic signaling. In order to evaluate the dependence of cannabinoid function on serotonin transmission, doses of CB_1 receptor agonist and antagonist mimicking anti-depressant effects were tested in the FST in combination with drugs which inhibit serotonin signalling. The results showed that only the cannabinoid agonist effect was reversed by blocking serotonin signaling. In addition, the dependence of the antagonist effect on functional CB_1 receptor on GABAergic neurons was shown. Interestingly, the phenotype induced by deletion of the receptor from cortical glutamatergic neurons is attenuated by blocking serotonin signalling (**see Chapter 4**).

These findings suggest that cannabinoids modulate GABAergic and glutamatergic neurons, which themselves regulate serotonergic neurons. However, it has remained unclear whether a direct connection for the cannabinergic effects on serotonin release exists. In 2007, our group could identify the mRNA expression and protein localization of CB_1 receptor in serotonergic neurons. The generation and analysis of a knock-out mouse line, lacking the CB_1 receptor specifically in these neurons revealed that male mice showed low but significant changes in anxiety in response to social and object stimuli. Thus, I could show for the first time that the direct influence of the eCB system on serotonin signalling is of physiological importance (**see Chapter 5**).

1.2. Zusammenfassung

Cannabinerge Substanzen können das Verhalten in einer dosisabhängigen, aber biphasischen Weise beeinflussen. Eine Erklärung für diese Art der Effekte könnte die Verteilung des CB_1 Rezeptors auf verschiedenen Neuronentypen sein. CB_1 Rezeptoren in glutamatergen und GABAergen Neuronen sind hier besonders wichtig, da die entsprechenden Neurotransmitter als Gegenspieler die neuronale Erregung kontrollieren. Spezifische Deletion des CB_1 Rezeptor-Gens von einer der beiden Populationen führte zu gegensätzlichen Phenotypen, genauer gesagt, einem erniedrigten, bzw. einem gesteigerten Interaktiondrang. Tiere, bei denen der CB_1 Rezeptor ausschließlich in striatalen, GABAergen „*Medium Spiny*" Neuronen deletiert wurde, zeigten keinen veränderten Phänotyp. Dies legt nahe, dass der CB_1 Rezeptor in kortikalen glutamatergen und GABAergen Neuronen für einen ausgeglichenen Interaktionsdrang entscheidend ist (**siehe Kapitel 3**).

Diese dosisabhängigen, biphasischen Effekte auf das Verhalten können auch im „*Forced Swim Test*" (FST) beobachtet werden. Ein möglicher Mechanismus, durch den Cannabinoide das Stressverhalten beeinflussen können, wäre die Regulierung der Monoaminausschüttung. Um die Abhängigkeit der Cannabinoideffekte von der Serotonintransmission zu untersuchen, wurden Dosen von CB_1 Rezeptoragonisten und –antagonisten mit antidepressiv-induzierenden Eigenschaften bei gleichzeitiger Inhibition der Serotonintransmission im FST getestet. Die Ergebnisse zeigten, dass lediglich der Agonisteffekt durch die Inhibition der Serotoninausschüttung beeinflusst wird. Zusätzlich konnte die Abhängigkeit des Antagonisteneffekts von funktionsfähigen GABAergen CB_1 Rezeptoren nachweisen werden. Interessanter Weise konnte der durch die Deletion von glutamatergen CB_1 Rezeptoren induzierte Phänotyp durch Inhibition der Serotoninausschüttung blockiert werden (**siehe Kapitel 4**).

Ein indirekter Einfluss auf Serotoninausschüttung scheint also wahrscheinlich zu sein. Bis jetzt blieb jedoch unklar, inwieweit cannabinerge Substanzen direkt auf serotonerge Neuronen wirken können. Im Jahr 2007 konnte unsere Gruppe die Expression des CB_1 Rezeptors in serotonergen Neuronen auf mRNA- und Proteinebene nachweisen. Die Züchtung und Analyse einer mutanten Mauslinie, in welcher der CB_1-Rezeptor spezifisch in serotonergen Neuronen ausgeschaltet wurde, zeigte bei männlichen Tieren eine schwache, aber signifikante Verhaltensänderungen, die durch soziale Stimuli und lebensbedrohlichen Situationen ausgelöst wurde. So ist es erstmals gelungen nachzuweisen, dass serotonerge CB_1-Rezeptoren eine physiologische Relevanz besitzen (**siehe Kapitel 5**).

Chapter 2

Introduction

2. Introduction

2.1. The endocannabinoid system

2.1.1. General overview

A tightly regulated neurotransmitter release is essential for proper functioning of the brain. It is therefore of greatest importance to prevent an imbalance in neuronal signaling, especially in stressful situations. One of the endogenous control mechanisms is the endocannabinoid (eCB) system, a molecular mechanism named for its sensitivity to Δ^9-tetrahydrocannabinol (THC), the main psychoactive substance of the hemp *Cannabis sativa*. Since its major constituent, the cannabinoid type 1 (CB_1) receptor, had been discovered (Matsuda et al., 1990), the interest in investigating the eCB system steadily increased, comprising nowadays different ligands, synthesizing and degrading enzymes as well as other cannabinoid receptors (Petrosino & Di Marzo, 2010). The CB_1 receptor belongs to the family of G-protein coupled seven-transmembrane receptors, primarily coupled to $G_{i/o}$, but coupling to alsoto G_q and G_s-proteins was reported (Bonhaus et al., 1998; Howlett et al., 2010). There are two major endogenous ligands (named endocannabinoids; eCBs), N-arachidonyl ethanolamine (anandamide, AEA) (Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG) (Sugiura et al., 1995; Mechoulam et al., 1995). Unlike classical neurotransmitters, the eCBs are not stored in vesicles of the presynapse, but are synthesized on demand from lipid membrane precursor molecules (Piomelli, 2003). The enzymes responsible for the synthesis are Ca^{2+} sensitive lipases in the postsynapse (see Fig. 2.1). The enzymes involved in the biosynthesis of 2-AG are phospholipase C and 1,2-diacylglycerol lipase. Anandamide, on the other hand, is synthesized by N-acyltransferase and phospholipase D. The ligands travel by a still unknown mechanism retrogradely to the presynaptically located CB_1 receptor (Kano et al., 2009). The activation of the CB_1 receptor induces the major neuronal effect of the eCB system, namely, the inhibition of neurotransmitter release by blockade of voltage activated, inwardly rectified Ca^{2+} channels and opening of outward rectified K^+ channels (Kano et al., 2009). Thus, upon CB_1 receptor activation, the cell becomes less excitable. Another consequence of the CB_1 receptor stimulation is an alteration of gene expression, which is achieved, among others, by the activation of the mitogen-activated protein kinase pathway (Di Marzo et al., 2004; Howlett et al., 2010).

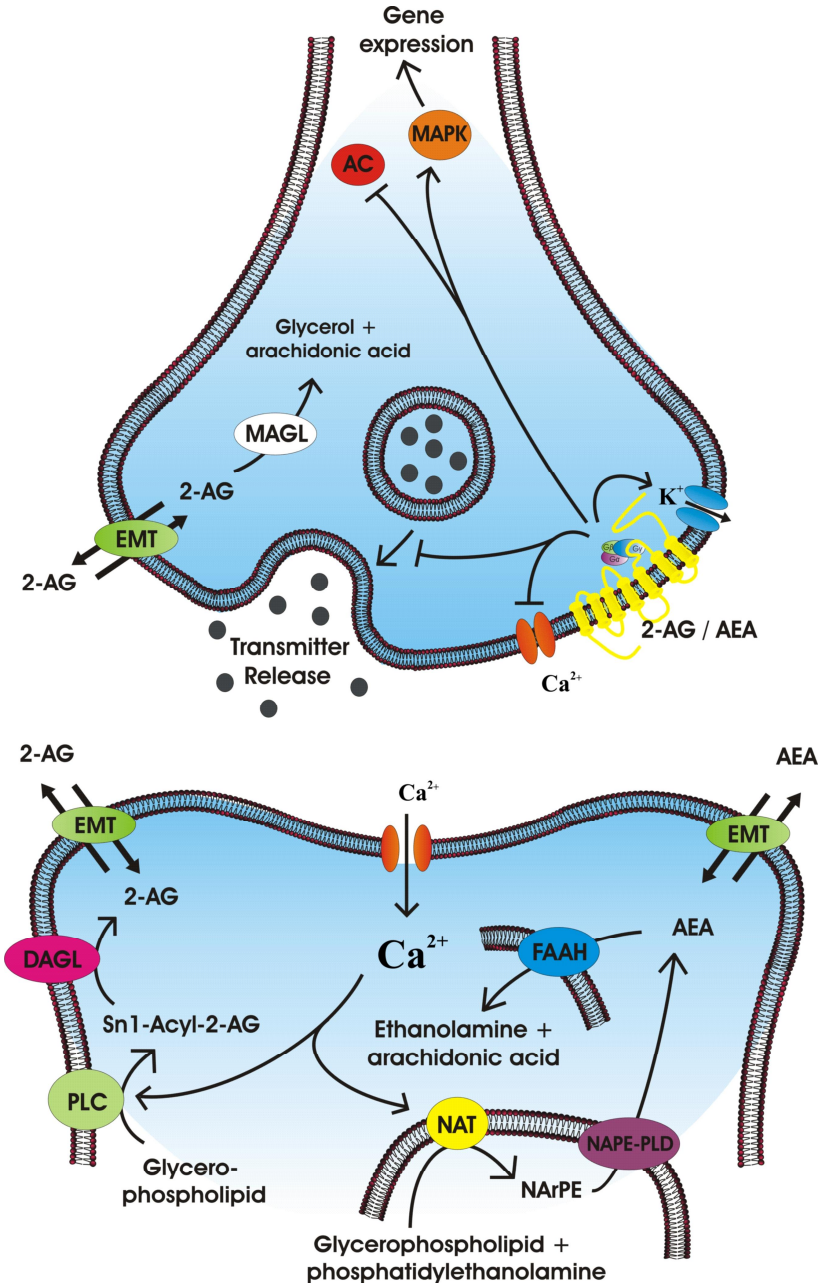

Fig. 2.1: Signaling, synthesizing and degrading pathways of eCB system and their localization in the cell. Synthesizing and degrading enzymes of endocannabinoids are in general membrane associated; however, the distribution on pre- and postsynapse can be different. The phospholipase C (PLC) and 1,2-diacylglycerol lipase (DAGL), which are the synthesizing enzymes for 2-arachidonoylglycerol (2-AG), are located at the plasma membrane of the postsynapse, while the degrading enzyme monoacylglycerol lipase (MAGL) is located in the presynapse. The enzymes responsible for the biosynthesis of anandamide (AEA), N-acyltransferase (NAT) and unspecific phospholipase D (PLD) are also located in the postsynapse. In constrast to the presynaptic localization of MAGL, the degrading enzyme of anandamide, the fatty acid amide hydrolase (FAAH), is located on intracellular postsynaptic membranes. The endocannabinoids are thought to be transported into and out of the synaptic cleft via an endocannabinoid membrane transporter (EMT), but might also be directly released into the synaptic cleft after their synthesis. How the endocannabinoids cross the synaptic cleft is not known, but they bind to the presynaptically located cannabinoid type 1 (CB_1) receptor 1 and induce the activation of the mitogen-activated protein kinase (MAPK) and K channels as well as the inhibition of Ca^{2+} channels and the adenylyl cyclase (AdCy).

Cannabinergic signaling is limited by a still badly defined uptake process and intracellular hydrolysis by fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol lipase (MAGL) as well as serine hydrolase α,β-hydrolase domain 6 for 2-AG (Hillard, 2000; Marrs et al., 2010). Interestingly, the degrading enzymes for the two endogenous ligands display distinct subcellular and synaptic localization, suggesting different signaling properties for AEA and 2-AG (Cristino et al. 2008). While FAAH is mostly found in the postsynape, MAGL is often colocalized with the CB_1 receptor in the presynaptic structure (Egertova et al., 2003; Gulyas et al., 2004). To date, there is clear evidence for other eCB receptors, such as the vanilloid receptor, TRPV1 (transient receptor potential cation channel subfamily V1), which makes understanding of distinct eCB system functions even more difficult (for review see De Petrocellis and Di Marzo, 2010).

Taken together, the eCB system with its regulatory properties is an interesting candidate to control neuronal activity and the resulting behavior (Kano et al., 2009). Consequently, various pharmacological and genetic studies targeting the eCB system show a variety of behavioral responses, in particular changes in mood and emotionality (Hill et al., 2010, Moreira & Wotjak, 2010), a fact which is underlined by the wide distribution of the eCB system in the CNS (Marsicano and Kuner, 2008; Häring et al., 2012).

2.1.2 Biphasic effects of cannabinoids

The eCB system functions as an endogenous control mechanism, regulating the release of different neurotransmitters. This widespread influence of CB_1 receptor signaling in combination with the multiple molecular targets of eCBs has continuously complicated the interpretation of contradictory behavioral data following the application of cannabinoids (Moreira and Lutz, 2008; Ruehle et al., 2012). Thus, agonist and antagonist treatment was found to induce similar effects in a dose dependent manner. Pharmacological approaches using selective drugs which target the major elements of the eCB system and the use of genetically modified mice have led to new insights into the mechanisms underlying the bimodal or contradictory actions of cannabinoids. Interestingly, this is in accordance with human reports on opposite (depressive/euphoric) experiences after cannabis use (Fusar-Poli et al., 2009). One explanation for the contradictory effects might be differences in the initial baseline stress level of an animal, which depends on a multitude of genetic, environmental and experimental factors. This baseline might modulate the activity of the eCB system and by this, even though induced by opposite pharmacological interventions, resulting in a similar outcome (Wotjak, 2005; Viveros et al., 2005). Recent findings support a different mechanism,

which could in addition explain the biphasic effects. The differential distribution of the CB_1 receptor could very likely be the reason for the described phenomenon. Interestingly, this dual role on physiological and behavioral outcome seem to be highly dependent on the concentration of the cannabinergic drug and could be evidenced by agonizing or antagonizing CB_1 receptor activation (Moreira and Lutz, 2008; Ruehle et al., 2012; Aparisi-Rey et al., 2012). Therefore, depending on its specific spatio-temporal modulation within neuronal circuits, the eCB system can act as a major bi-directional neuromodulatory system (Moreira and Lutz, 2008).

Dual role in behavioral response

On the behavioural level, several studies have shown this bimodal role of cannabinergic drugs on different behaviors (Moreira and Lutz, 2008, Ruehle et al., 2012). Thus, in stress and anxiety paradigms CB_1 receptor agonists are reported to induce biphasic effects with lower doses of CB_1 receptor agonist, resulting in antidepressant-like/anxiolytic effect and higher doses being depressive-like/anxiogenic (Viveros et al., 2005; Bambico et al., 2007; Ruehle et al., 2012). Additionally, similar biphasic responses were found using CB_1 receptor antagonists and other drugs interfering with the molecular machinery of the eCB system (Lafenetre et al., 2007; Ruehle et al., 2012). Thus, application of CB_1 receptor agonist resulted in dose dependent behavioral alterations in the forced swim test (FST), with lower doses possessing a antidepressive-like effect (Bambico et al., 2007). Accordingly, higher doses of CB_1 antagonists also resulted in an antidepressive-like phenotype in mice using the same paradigm (Steiner et al., 2008a). Similarly to the systemic blockade by rimonabant, the ubiquitous deletion of CB_1 receptor also resulted in an antidepressive-like effect. Interestingly, this phenotype occurred even when the CB_1 receptor was specifically deleted from cortical glutamatergic neurons. Contrary to this, the deletion of the CB_1 receptor from forebrain GABAergic neurons showed no phenotype (Steiner et al., 2008b) in FST. Studies using CB_1 receptor knockout mice have further reported to show anxiogenic responses in classical anxiety paradigms, such as elevated plus-maze (Haller et al., 2004b) and light/dark test (Martin et al., 2002), a phenotype again visible in mice lacking the receptor specifically in glutamatergic neurons (Jacob et al., 2009). Hence, the findings on anxiety alterations warrant a careful interpretation of the antidepressent-like phenotypes in these animals. To complete the picture, the opposite cannabinergic effects are not limited to anxiety and depressive behaviour, but also in food intake behavior (Bellocchio et al., 2008; Bellocchio et al., 2010).

Dual role on HPA axis activity

The hypothalamic-pituitary-adrenal axis (HPA) is the major circuit involved in the response to a stressful situation (Steiner and Wotjak, 2008; Hill et al., 2010). Upon exposure to stressful stimuli, neurons of the hypothalamic paraventricular nucleus (PVN) secrete corticotropin-releasing hormone (CRH) into the portal vessels of the median eminence. In the pituitary, CRH initiates the secretion of adrenocorticotropic hormone (ACTH), which in turn induces the synthesis and release of glucocorticoids (corticosterone in mice or rats and cortisol in humans) from the inner adrenal cortex.

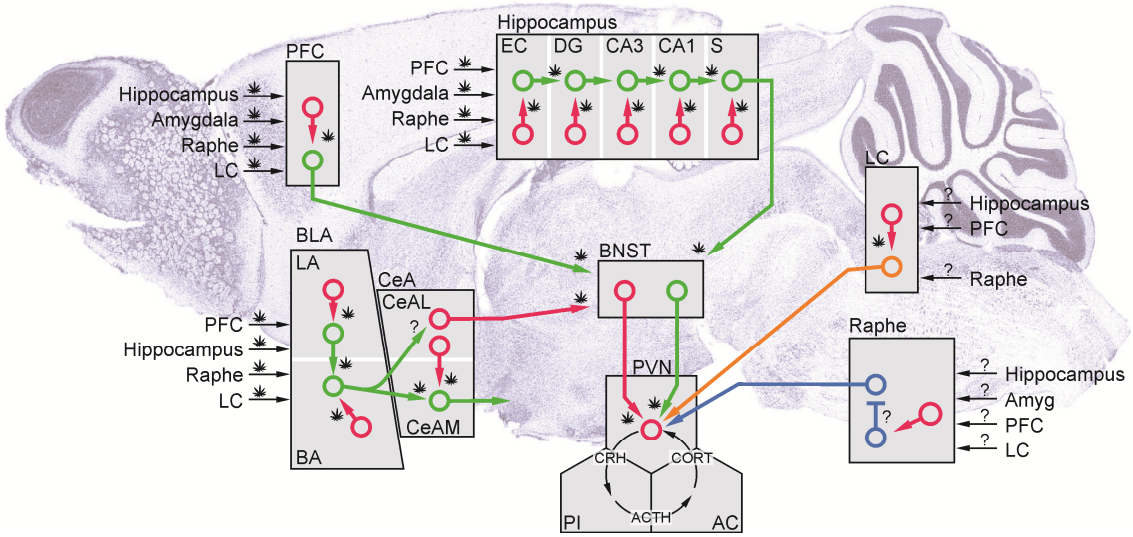

Fig. 2.2: Schematic illustration of the CB1 receptor distribution within the main stress circuits. The CB1 receptor, indicated as a *Cannabis sativa* leaf, is found at a majority of synaptic connections within and between each major brain region related to the activity of the hypothalamic-pituitary-adrenal axis, which is controlled by the subcortical structure paraventricular nucleus of the hypothalamus (PVN), the pituitary (PI), and the adrenal cortex (AC). A most dominant distribution of CB_1 receptor is found in GABAergic (red) and glutamatergic (green) neurons in limbic regions, i.e. prefrontal cortex (PFC), basolateral and central amygdala (BLA, CeA), bed nuclei of the stria terminalis (BNST), and hippocampus. Additionally, serotonergic (blue) and noradrenergic (orange) neurons from brainstem nuclei are also involved in the stress response. A projection to a particular brain region is depicted as an arrow with the specification where the projection originates from. Question mark (?) indicates that the presence of CB1 receptor at a given projection has not yet experimentally clearly been proven. Abbreviations: AC, adrenal cortex; ACTH, adrenocorticotropin; BNST, bed nuclei of the stria terminalis; BA, basolateral nucleus of BLA; BLA, basolateral amygdala complex; CA, cornu ammonis; CeA, central amygdala; CeAL, lateral part of CeA; CeAM, medial part of CeA; CORT, corticosterone; CRH, corticotropin-releasing hormone; DG, dentate gyrus; EC, entorhinal cortex; LA, lateral nucleus of BLA; LC, locus coeruleus; PFC, prefrontal cortex; PI, pituitary gland; PVN, paraventricular nucleus of the hypothalamus; S, subiculum. *Figure was taken from Häring et al., 2012*

Besides a fast mobilization of stored energy, released glucocorticoids inhibit HPA axis activity by a negative feedback mechanism via the activation of hypothalamic glucocorticoid receptors (Steiner et al., 2008a). Since the CB_1 receptor is one of the most abundant receptor in the mammalian CNS, it is not surprising that the receptor is also found in the major brain structures involved in the stress response, as illustrated in Figure 2.2 (for review see Häring et al., 2012). The dual role of cannabinoids was also found in respect to HPA activation. Thus, different studies showed that under stressful conditions cannabinoid agonist administration can elicit a dose-dependent biphasic effect on corticosterone secretion. While low agonist concentrations attenuated the stress-induced increase in plasma corticosterone levels, high agonist levels elevated the stress-induced increase in corticosterone secretion (Wenger et al., 2003; Patel et al., 2004). Activation of the CB_1 receptor by THC was also found to increase the plasma corticosterone levels, an effect blocked by a per se ineffective dose of the CB_1 antagonist rimonabant. Interestingly, higher doses of rimonabant induced HPA axis activation and were shown to enhance plasma corticosterone levels (Manzanares et al., 1999), a phenomenon described by several authors (Patel et al., 2004; Wade et al., 2006; Steiner et al., 2008a).

In order to dissect the involvement of different neurotransmitters on HPA axis activation, McLaughlin, et al. (2009) co-administered a cannabinoid agonist and specific antagonists of the serotonergic, noradrenergic and glutamatergic neurotransmitter system, respectively. This study revealed that activation of the HPA axis by cannabinoid treatment seemed to be mediated via serotonergic and noradrenergic, but not glutamatergic neurotransmission (McLaughlin et al., 2009), supporting previous results on the roles of monoamines on HPA axis activity (for review see, Forray and Gysling, 2004; Leonard, 2005; Pompili et al., 2010).

Two other investigations further observed an increased activity of monoaminergic neurons after enhancing CB_1 receptor signaling by application of a FAAH inhibitor or a CB_1 receptor agonist (Gobbi et al., 2005; Muntoni et al., 2006). Interestingly, these data suggest an indirect influence on serotonergic and noradrenergic neurons; hence, inhibitory interneurons within the brainstem nuclei seem to play an important role. In fact, Muntoni et al. (2006) could show that CB_1 receptor activation attenuated the inhibitory input from the nucleus prepositus hypoglossi, the main GABAergic input to the locus coeruleus (Muntoni et al., 2006). The missing effect of ionotropic glutamatergic receptor inhibition might be explained by the ubiquitous activation of CB_1 receptor, which also attenuates glutamatergic transmission (Kano, 2009).

However, strong evidence exists that also glutamatergic connections can be highly important for corticosterone release, at least in the connection of the prefrontal cortex (PFC) to the raphe nuclei. The dissection of the PFC-dorsal raphe nucleus pathway blocked the increase in serotonergic firing after local administration of a CB_1 receptor agonist (low doses) into the PFC (Bambico et al., 2007). It is possible that glutamatergic neurons innervate inhibitory brainstem neurons, which regulate serotonin release, a mechanism already proposed (Celada et al., 2001) and also plausible for the locus coeruleus. Genetic approaches using CB_1 receptor deficient mice gave additional insights. Thus, similarly to a systemic blockade by rimonabant, ubiquitous deletion of the CB_1 receptor resulted in an increase in plasma corticosterone levels (Steiner et al., 2008a). This increase was later pinpointed to different glutamatergic subpopulations, by using conditional transgenic mice, lacking the CB_1 receptor specifically in principal forebrain neurons (Steiner et al., 2008b).

GABAergic and glutamatergic CB_1 receptors: Essential for biphasic effect

CB_1 receptors on GABAergic and glutamatergic terminals might be of special interest for the biphasic effect of cannabinoids. Both neuronal populations possess significant amounts of the receptor and represent the two opposing neurotransmitter systems regarding neuronal excitability (Monory et al., 2006). In addition to the anatomical proof, different approaches also revealed the functionality of the CB_1 receptor in these neurons. Thus, different CB_1 receptor agonists, such as THC and WIN55,212-2, have recently been shown to inhibit GABA and glutamate release (Monory et al., 2006; Azad et al., 2008; Laaris et al., 2010; Hoffman et al., 2010). It should be mentioned that a different basal activation was demonstrated for CB_1 receptors expressed on glutamatergic neurons and GABAergic neurons. Electrophysiological studies found a lower basal activation of the CB_1 receptor on glutamatergic terminals, suggesting that their reactivity to an increase in the eCB tone would be higher than that of the CB_1 receptor on GABAergic terminals (Katona and Freund, 2008). Hence, depending on whether the experimental conditions predominantly modulate excitatory or inhibitory transmission (i.e. glutamatergic or GABAergic), opposite behavioral outcomes are achieved. In fact, a recent publications on mice lacking the CB_1 receptor in GABAergic and glutamatergic neurons, respectively, do show opposite phenotypes (Lafenetre et al. 2008; Bellocchio et al. 2010). Both studies included transgenic animals, lacking the CB_1 receptor either specifically in GABAergic or glutamatergic neurons (Monory et al., 2006). Particular interesting here, Lafenetre et al., (2009) concentrated on object recognition and exploration. They could show that, while deleting the receptor from GABAergic neurons had an anxiolytic

effect, the deletion from glutamatergic neurons had an anxiogenic effect. In another study, Jacob et al., (2009) underline these findings, as animals lacking the CB_1 receptor in gutamatergic neurons show a decreased exploratory drive towards an object or an juvenile interaction partner.

In this thesis (see Chapter 3 and 4), we are completely in line with the findings presented by Lafenetre and colleagues and with Jacob and co-workers. Thus, while a high dose of antagonist was found to require functional GABAergic CB_1 receptor to elicit its antidepressant effect in the forced swim test (FST), a low dose of the agonist THC was still functional in these mice. Furthermore, a detailed approach on object exploration and interaction partner interaction, using mice lacking the CB_1 receptor specifically either on glutamatergic or GABAergic neurons revealed an opposite role for the two CB_1 receptor subpopulations (Häring et al., 2011).

2.2. Cannabinoid function and the relation to the serotonergic system

Controlling serotonin release is of particular interest, as this monoaminergic transmitter is involved in multiple neuronal circuits and behavioral functions. As described above, the eCB system features all requirements to be a major regulatory mechanism for neurotransmitter release. During the last decade several studies suggested a functional connection between cannabinoid and serotonergic transmission. Thus, accumulating evidence supports the involvement of CB_1 receptor signaling in the regulation of serotonergic neurotransmission.

2.2.1. The serotonergic system

The monoamine serotonin, also named 5-HT (5-hydroxytryptamine), is already known for several decades and has become one of the most studied extracellular signaling molecules in various organisms. Its distribution ranges over many tissues, being most abundant in the gastrointestinal tract, in the

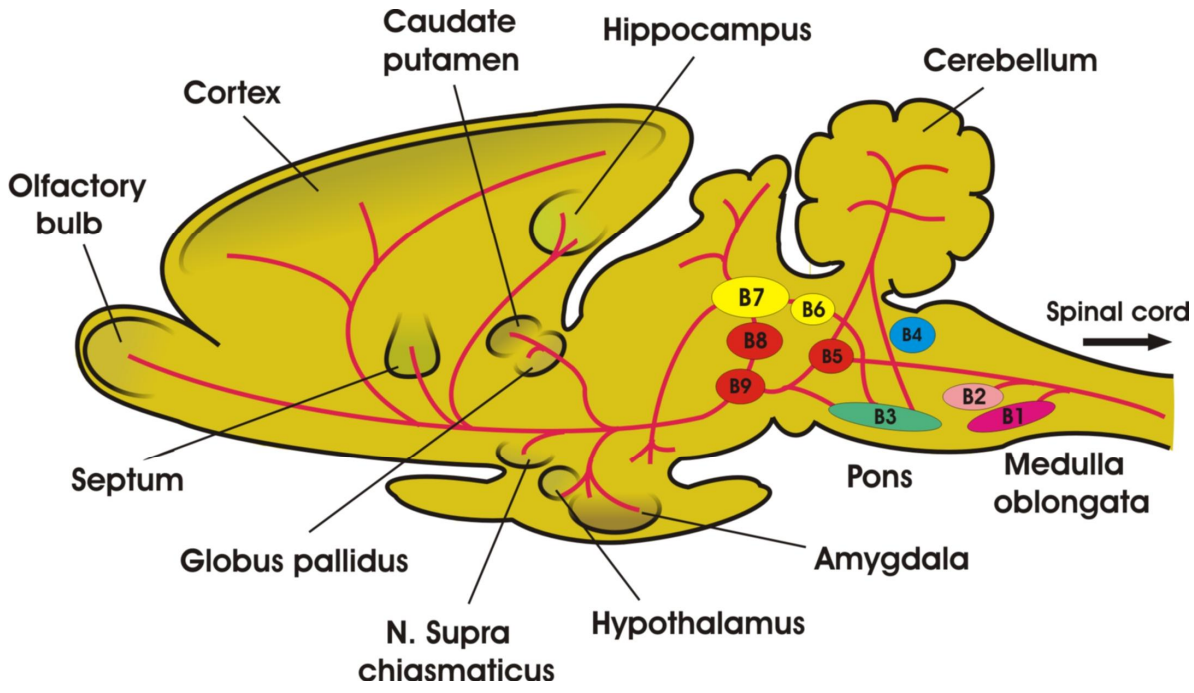

Fig. 2.3: Schematic overview on the serotonergic system; The center of the serotonergic system is formed by the raphe nuclei B1-B9 (Dahlström & Fuxe, 1964), which are located in the midbrain and the brainstem. From there, the serotonergic neurons innervate various brain regions (red lines) such as the amygdala, the hypothalamus, the hippocampus, the striatum and the olfactory bulbs, thus forming a widespread network over the whole CNS. Some of the nuclei are connected together (color code) rather than being separate nuclei, e.g. B5, B8 and B9 (red nuclei) are all parts of the median raphe, and B6 is more a caudal elongation of the dorsal raphe B7 (yellow nuclei).

central and peripheral nervous system as well as, in the cardiovascular system, where it functions as neurotransmitter or hormone. In this study, serotonin is of interest because of its influence on the central nervous system, where it was discovered by Twarog and Page in 1953 (Twarog & Page, 1953), but it was in fact first identified due to its cardiovascular effects (Rapport et al., 1948).

Serotonergic neurons can be detected throughout the brain, in highest concentrations in the midbrain and the brainstem areas called raphe nuclei, from where they innervate different parts of the CNS, such as the spinal cord, the hypothalamus, the cortex, the hippocampus, the amygdala and the striatum (Fig. 2.3). It is therefore not surprising that serotonin has been shown not only to influence various physiological mechanisms (e.g. primary haemostasis, respiration, vascular tone, thermoregulation, gut motility and cell mediated immune response), but also a wide range of behavioral responses (e.g. mood control, circadian rhythm, sleep-wake cycle, food intake and sexual behavior) (Lucki, 1998; Walther & Bader, 2003).

The synthesis of serotonin consists of two reactions, using the amino acid tryptophan as precursor molecule. In the serotonergic neurons tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the enzyme called tryptophan hydroxylase (TPH). TPH is considered to be the rate limiting enzyme in the biosynthesis of serotonin and can only be found in cells that synthesize serotonin (Sato et al., 1967; Malek et al., 2005). To date, two TPH isoforms are known, named TPH1 and TPH2 (Patel et al., 2003). Interestingly, the tissue distribution of the two isoforms is distinct. While TPH1 can mostly be found in the pineal gland, the thymus, the spleen and the gut, the second isoform, TPH2, is highly expressed in the raphe nuclei (Patel et al. 2003), suggesting a model of two distinct serotonergic systems. In the second step, the carboxyl group of the 5-HTP is removed by an aromatic L-amino acid decarboxylase, by which serotonin is finally generated. The uptake into vesicle is done by the vesicular monoamine transporter type-2, which is located at the vesicular membranes (Fig. 2.4).

The serotonergic signaling is transduced via several different serotonin receptors. To date, there are seven major groups of serotonin receptors, named 5-HT1-7 (Pandey et al., 1995). Except for the 5-HT3 receptor group, which are ion channels (Na^+ or Ca^{2+}), all other serotonergic receptors are G-protein coupled seven transmembrane receptors (Veenstra-VanderWeele et al., 2000). Interestingly, some serotonin receptors are located on the postsynapse and function as autoreceptors. Two isoforms of the 5-HT1 type, 5-HT1A and 5-HT1B, are representative for the presynaptic receptors. Both, 5-HT1A and 5-HT1B, are coupled to inhibitory G-proteins and act predominantly via the inhibition of adenylyl cyclase

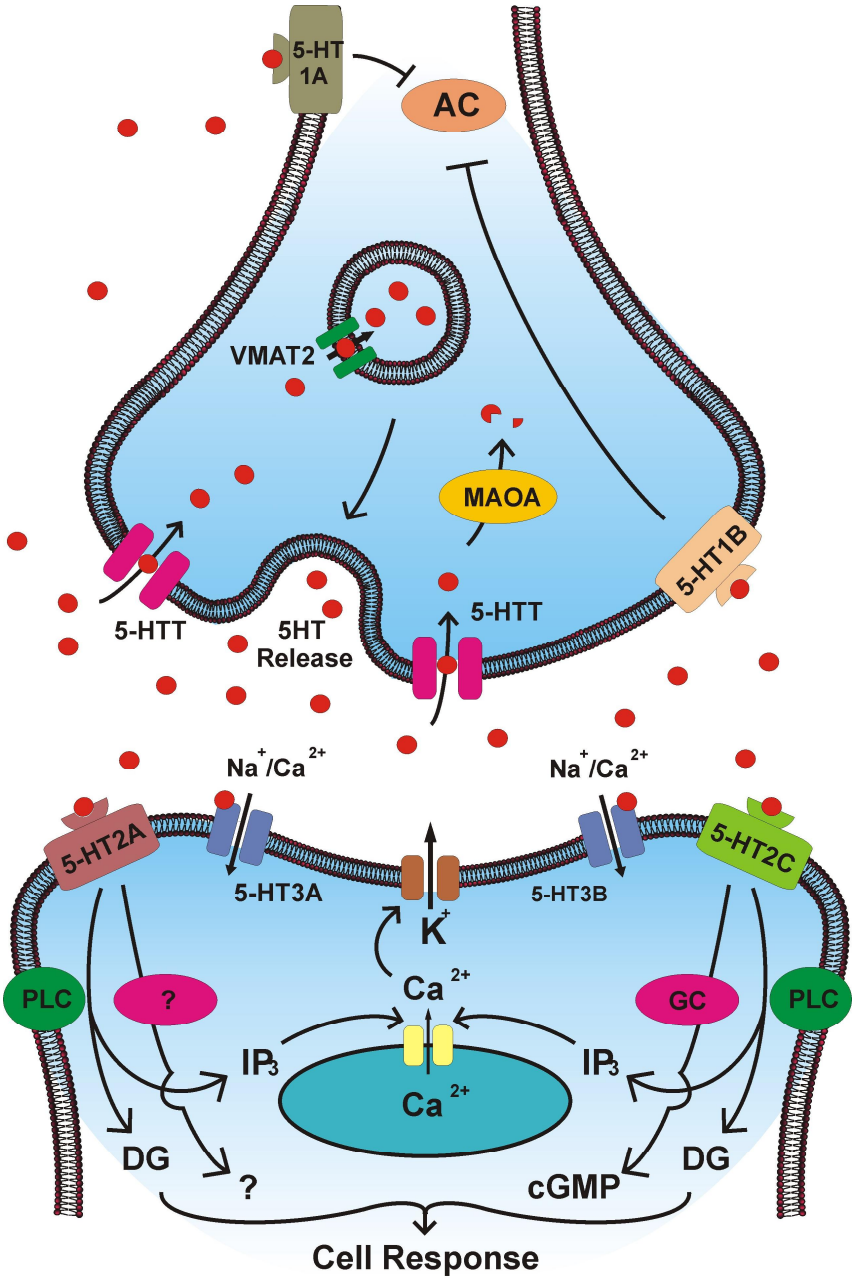

Fig. 2.4: Schematic overview on serotonergic signaling; After the synthesis of serotonin (red circles) it is packed into vesicles via VMAT2. An incoming action potential then leads to a release of serotonin into the synaptic cleft, where it can bind to several pre- and postsynaptic serotonin receptors. The presynaptic receptors of the receptor type 5-HT1 are G-protein coupled and negatively affect adenylyl cyclase. 5-HT2 receptors are also G-protein coupled; however increase phosphoinositide hydrolysis by activating phospholipase C (PLC). It is known that 5-HT2C also activates guanylyl cyclase thereby supports the synthesis of cGMP. cGMP and inositol-triphosphate (IP3) and diacylglycerol (DG), function as second messengers and are involved in important cellular pathways. IP3 is opening Ca^{2+} channels located on the membrane of intracellular Ca^{2+} stores, hereby releasing Ca^{2+}, which is thought to influence cell surface K^{+} channels. A more direct modulation of the membrane potential arises from the binding of serotonin to 5HT3 receptors, which are ion channels. For the degradation by monoamine oxidase A (MAOA), serotonin is transported via the 5HT-transporter (5-HTT) into the presynapse.

(Pandey et al., 1995). Thus, the activation of the two serotonin autoreceptors is able to modulate its own signaling, allowing a fine tuning of its release. Two well-studied postsynaptic serotonin receptors belong to the group of 5-HT2 receptors, 5-HT2A and 5-HT2C which mediate their effects by activating phospholipase C and therefore initiating phosphoinositide hydrolysis into the second messengers inositol-3-phosphat and diacylglycerol. Beside the induction of phosphoinositide hydrolysis, 5HT2 receptors might influence the activity of guanylyl cyclase, an enzyme responsible for the production of the second messenger cGMP (Fig. 2.4).

Another important protein in the serotonin signaling process is the monoamine transporter called serotonin reuptake transporter (5-HTT) (Hoffman et al., 1998). 5-HTT is responsible for the reuptake of serotonin from the synaptic cleft into the presynapse, where it can be degraded by the monoamine oxidase A. It is therefore essential for the termination of serotonin signaling, which made it a major target in pharmacological research (Fig. 2.4).

2.2.2. Endocannabinoids alter serotonin release

Several studies have addressed the question on whether cannabinoids can modulate serotonin release. Using different experimental approaches, these studies could clearly show an interaction of serotonin transmission and cannabinoid function. First investigations in this direction were done by Nakazi and colleagues. They could block the electrically stimulated release of [^{3}H]serotonin by CB_1 receptor activation, suggesting a direct inhibitory function of the CB_1 receptor on serotonergic neurons (Nakazi et al., 2000). This direct connection has been supported by the detection of the CB_1 receptor on serotonergic neurons (Häring et al., 2007). Various other studies indicated indirect mechanisms on how cannabinoids control serotonin release. Thus, the application of rimonabant increased the levels of serotonin release in brain tissue (Tzavara et al., 2003). In 2005, Haj-Dahmane and Shen provided evidence for the inhibitory function of cannabinoids on excitatory input onto serotonergic neurons within the dorsal raphe nucleus (Haj-Dahmane et. al, 2005). In a subsequent study, Haj-Dahmane and Shen underlined these findings and proposed a model where CB_1 receptor is located on glutamatergic terminals (Haj-Dahmane and Shen, 2009, 2011). By applying the CB_1 receptor agonists anandamide and WIN 55212-2, they were able to decrease evoked excitatory postsynaptic currents (eEPSCs), an effect blocked by rimonabant. They could also show that the CB_1 receptor gets activated by ligands produced by serotonergic neurons, as stimulation of these cells blocked eEPSCs, a phenomenon termed, depolarization-induced suppression of

excitation (DSE) (Haj-Dahmane and Shen, 2009). Thus, these studies suggest an innervation of excitatory, CB_1 receptor-positive synapses onto serotonergic neurons.

In contrast, i.p. application of FAAH inhibitors increased the firing rate of serotonergic neurons, suggesting an involvement of inhibitory circuits (Gobbi et al., 2005). This effect was mimicked by low doses of CB_1 receptor agonist (Bambico et al., 2007; Bambico and Gobbi, 2008). Microinjection of the CB_1 receptor agonist WIN 55,212-2 into the dorsal raphe region was shown to induce an antidepressive-like effect, suggesting the presence of the receptor in this region (Bambico et al., 2007). The same study also revealed the importance of CB_1 receptor function in the PFC and serotonin transmission. Interestingly, the increase in the firing rate of serotonergic neurons is accompanied by an increase in immobility behavior in the FST (Gobbi et al., 2005; Bambico et al., 2007). Consistent with these findings, i.p. application of higher doses of THC increased immobility behavior (Egashira et al. 2008).

Recently, Mendiguren and Pineda also suggested the involvement of the CB_1 receptor on GABAergic interneurons in the regulation of serotonin transmission in the dorsal raphe nuclei (Mendiguren and Pineda, 2009). Treatment of brain slices with the CB_1 receptor antagonists rimonabant and AM251, they could decrease the firing rate of serotonergic neurons. Surprisingly, WIN 55212-2 did not result in an opposite effect, and treatment of the slices with anandamide again decreased the firing. This effect could not be attenuated by the CB_1 receptor antagonists, suggesting the involvement of an additional cannabinoid target.

Despite the electrophysiological findings, anatomical evidence for CB_1 receptor distribution in the different raphe nuclei remains highly elusive (Mackie et al., 2005). Only one publication claims the CB_1 receptor to be present in the most pronounced serotonergic regions the dorsal and median raphe nuclei (Sanford et al., 2008). Downside of this otherwise well designed study in guinea pig is the rather untypical antibody staining, which appears to be most prominent in the cell body instead of being located at terminals and axon fibers (Mackie et al., 2005). Also clear anatomical proof for the existence of non-CB_1 receptor cannabinoid targets, as suggested by Mendiguren and Pineda (2009), in the raphe nuclei is still missing.

2.2.3. Serotonin alters eCB signaling

Despite the multiple ways on how cannabinoids seem to control serotonin release, this interaction between the cannabinoids and serotonin is not a unidirectional mechanism. As described above, serotonin acts via several G-protein coupled receptors, which can be linked to $G_{i/o,}$ G_s or $G_{q/11}$ proteins (Parrish and Nichols, 2006). Interestingly, the activation of $G_{q/11}$ by metabotropic glutamate mGluR1/5 and muscarinic acetylcholine receptors was

demonstrated to induce eCB synthesis from the dendrites (Chevaleyre et al., 2006; Oliet et al., 2007). Best and Wade aimed at investigating whether this is also true for the activation of serotonergic receptors (Best and Regehr, 2008). Using an electrophysiological approach they were able to block glutamate-induced excitatory postsynaptic currents in inferior olive neurons by the application of 5-HT1 and 5-HT2 receptor agonist or electrical stimulation of serotonergic neurons, a phenomenon attenuated by CB_1 receptor antagonist treatment. Thus, the authors could give clear evidence that eCB production can be promoted by serotonin.

Another possibility on how serotonin might influence eCB synthesis is the modulation of calcium currents, as eCB synthesizing enzymes are calcium dependent (Kano et al., 2009). In fact, the activation of the 5-HTR3 led to a calcium influx (Nayak et al., 1999). However, the influx was so far only detectable at presynaptic sites, where it enhances neurotransmitter release (Turner et al., 2004). Thus, whether this calcium channel properties of the receptor are also relevant for postsynaptic mechanism remains to be tested. Taken together, also serotonin via the interaction with its multiple receptor targets can modulate the production and therefore, signaling of eCBs.

2.3. Aim of the Thesis

During the last two decades, functional analysis on the eCB system revealed the tremendous importance for negatively regulating neuronal activity. This control mechanism functions via the activation of the CB_1 receptor which is located at the presynapse. The CB_1 receptor has been proven in several neuronal subtypes, what might account for the multiple effects of cannabinoids.
Interestingly, cannabinoids have been found to induce opposite effects depending on the dose applied (Ruehle et al., 2012). Recent findings suggest the involvement of two CB_1 receptor subpopulations, namely on GABAergic and glutamatergic neurons. As both neurotransmitters represent opposing players in excitability, the spatiotemporal activation of the CB_1 receptor on these neurons might induce opposite effects. Using two conditional mouse lines lacking the CB_1 receptor in either of these two neuronal populations, we aimed at investigating whether opposite phenotypes could be detected in these mutants as compared to control mice (**see Chapter 3**).

The dual role of cannabinoids has also been shown for the behavioral responses to stressful situations. In addition, an adequate stress response is strongly dependent on serotonin transmission. Interestingly, serotonin levels were found to be modulated by cannabinoids. Therefore, we aimed at analyzing the serotonin dependence of cannabinoid antidepressive effects in a model of depression, by applying a pharmacological approach (**see Chapter 4**).

A direct control function of cannabinoids via the CB_1 receptor has been demonstrated in GABA, noradrenaline, dopamine, glutamate and acetylcholine secreting neurons (Kano et al., 2009) and is also thought to be one way of action cannabinoids are regulating the release of serotonin. Being highly abundant in GABAergic and moderately in glutamatergic neurons, the CB_1 receptor could also be identified in very low levels in serotonergic neurons, suggesting a fine tuning of serotonin transmission by the eCB system (Häring et al., 2007). However, in contrast to the GABAergic and glutamatergic CB_1 receptor populations, the physiological importance of the receptor on serotonergic terminals has remained to be clarified. To target this problem, we aimed at generating and analyzing a tamoxifen-inducible conditional mutant mouse line, lacking CB_1 receptor specifically in serotonergic neurons (**see Chapter 5**).

Chapter 3

The dual role of the eCB system and the involvement of cortical glutamatergic and forebrain GABAergic neurons

3. The dual role of the eCB system and the involvement of cortical glutamatergic and forebrain GABAergic neurons

3.1. Introduction

Adequate novelty seeking and exploration are fundamental behaviors for survival. Dysfunctional exploratory profiles have been found in several distinct neuropsychiatric disorders, such as attention deficit disorder and schizophrenia-like diseases, expressed by altered social behavior and novelty seeking (Newcorn, 2001; Solanto, 2002; Perry et al., 2009; Kawa and Pisula, 2010; Patterson, 2011). Thus, identifying control mechanisms of exploratory behavior might allow new treatment strategies for such disorders. As described above, the eCB system with its major constituent, the CB_1 receptor, has emerged during the last two decades as a neuronal regulatory mechanism (Kano et al., 2009).

One important factor in respect to exploratory behavior is how a situation is evaluated. Brain regions involved in processing fear, such as amygdala, hippocampus, and prefrontal cortex, show high levels of CB_1 receptor mRNA and protein (Marsicano and Lutz, 1999; Tsou et al., 1998). These cortical areas possess two major neuronal subpopulations expressing the CB_1 receptor: GABAergic interneurons (with high CB_1 receptor levels) and glutamatergic neurons (with low CB_1 receptor levels) (Marsicano and Lutz, 1999; Monory et al., 2006, Katona et al., 2006). The two neuronal populations represent the two major opposing players regarding the excitation state of the brain, namely, GABAergic interneurons being inhibitory and glutamatergic neurons being excitatory. Therefore, a functional eCB system on these neuronal populations may have a protective role to prevent an imbalance of neuronal activity and inadequate behavioral responses. In accordance with this notion, it was shown that the inactivation of the CB_1 receptor gene from glutamatergic neurons leads to an increased vulnerability to kainic acid-induced seizures (Monory et al., 2006).

Furthermore, other behavioral studies indicated a bidirectional role of the eCB system in anxiety response, based on CB_1 receptor located on these two neuronal subpopulations (Lafenêtre et al., 2008; Jacob et al., 2009; for review see Ruehle et al., 2011).

The first two studies even indicated that the endocannabinoid (eCB) system in glutamatergic and GABAergic neurons might be important for a balanced response to novel situations (Lafenêtre et al, 2008; Jacob et al, 2009), but these studies elucidated only some aspects on the function of the eCB system in exploratory behavior. Lafentre et al., (2009) concentrated on object recognition with repeated exposure to a novel object and food pellet, thus, reducing the novelty factor strongly on every day. Jacob et al. (2009) performed multiple behavioral paradigms, including social interaction studies. However, the study was only performed with animals lacking the CB_1 receptor completely or specifically in cortical glutamatergic neurons. To this end, this study aimed at further detailing CB_1 receptor functions in investigatory drive and exploration behavior.

As anxiety plays a critical role in exploratory and investigatory behavior, we aimed at evaluating the importance of cell-type specific CB_1 receptor deletion in object exploration and social interaction. Several pharmacological studies showed the importance of the eCB system in social behavior (O´Shea et al., 2004, 2006; Schneider et al., 2008; Trezza and Vanderschuren, 2008a, 2008b, 2009). The results depended strongly on the treatment and experimental conditions, and they seemed to be contradictory at first sight. The acute and chronic administration of CB_1 receptor agonists (Δ^9-THC or WIN55,212-2), especially during adolescence, led to a decreased social interaction in rats. In opposition to this, treatment of adolescent rats with URB597, an inhibitor of anandamide degradation, and VDM11, a putative anandamide reuptake inhibitor, resulted in increased social play behavior (Trezza and Vanderschuren, 2008a, 2008b, 2009). These latter findings are supported by studies with transgenic mice lacking the CB_1 receptor ubiquitously or specifically in cortical glutamatergic neurons, where a decrease in object exploration and social interaction was shown, depending on the behavioral context (Haller et al., 2004; Jacob et al., 2009). Altogether, these data suggest that strong systemic activation of the eCB system has anti-social effects, whereas on-demand enhancement of anandamide signalling and subsequent activation of CB_1 receptor has a pro-social effect.

By using several conditional CB_1 receptor knock-out mice, we aimed at investigating whether CB_1 receptor on different neuronal cell types might explain the contradictory findings in social interaction and object exploration mentioned above. In order to address this question, we applied different behavioral paradigms to analyze inanimate (object) exploration and animate (interaction partner) exploration. Evaluating the results, we could detect a decreased exploratory drive in mice lacking CB_1 receptor in cortical glutamatergic neurons. Mice lacking CB_1 receptor in forebrain GABAergic neurons displayed opposite results, namely, an

increased exploratory drive. No changes in exploration were observed for mice lacking CB_1 receptor specifically in striatal D1 dopamine receptor-positive GABAergic medium spiny neurons. Thus, we hypothesize that forebrain GABAergic interneurons are important for the increased exploratory drive. Altogether, our results suggest that exploratory behavior (towards an object or an animal) is balanced by the eCB system via CB_1 receptor activation on the two opposing neuronal subpopulations.

3.2. Materials & Methods

3.2.1. Animals

This study was performed on adult (5-6 months old) male mutant mice and their respective wild-type littermates. Animals were housed in a temperature- and humidity-controlled room (22°C ±1; 50% ±1) with a 12 h light-dark cycle (lights on at 1 am) and had access to food and water *ad libitum*. The experimental protocols were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethical Committee on animal care and use of Rhineland-Palatinate, Germany. Generation, breeding and genotyping of the mutant lines were performed according to previous publications: $CB_1^{flox/flox;Nex\text{-}cre}$ mice (referred to as Glu-$CB_1^{-/-}$ mice; Monory et al., 2006), $CB_1^{flox/flox;Dlx5/6\text{-}cre}$ mice (referred to as GABA-$CB_1^{-/-}$ mice; Monory et al., 2006; Massa et al., 2010), and $CB_1^{flox/flox;D1\text{-}cre}$ mice (referred to as D1-$CB_1^{-/-}$ mice; Monory et al., 2007). While Glu-$CB_1^{-/-}$ mice lack the CB_1 receptor in cortical glutamatergic neurons, GABA-$CB_1^{-/-}$ mice lack the CB_1 receptor specifically in forebrain GABAergic neurons (Monory et al., 2006). In D1-$CB_1^{-/-}$ mice, the CB_1 receptor inactivation primarily occurred in GABAergic striatal medium spiny neurons, but also in a minor fraction of glutamatergic neurons in layer VI of the neocortex (Monory et al., 2007). Wild-type littermates do not possess the respective Cre recombinase transgenic allele, and contain the CB_1 floxed allele in a homozygous state. These mice were referred to as Glu-$CB_1^{+/+}$, D_1-$CB_1^{+/+}$ and GABA-$CB_1^{+/+}$. All mutant lines were bred for >10 generations on the background of C57BL/6N mice from Charles River, Germany. For detailed information on the anatomical differences in CB_1 receptor expression, see Monory, et al. (2007).

3.2.2. Experimental procedure

Animals were group-housed (3-5 animals per cage type 2 (26.5 x 20.5 x 14.0 cm), EBECO Germany) until one week before behavioral testing. Animals were then separated and single-housed to avoid behavioral differences between dominant and subordinate animals. The same animals were used in each paradigm. Between each experimental paradigm, animals were allowed to rest for one week (see Fig. 3.1). All experiments were performed one hour after turning off the lights (2 pm), in the active phase of the animals, with only a dim red light source in the room (0 lux).

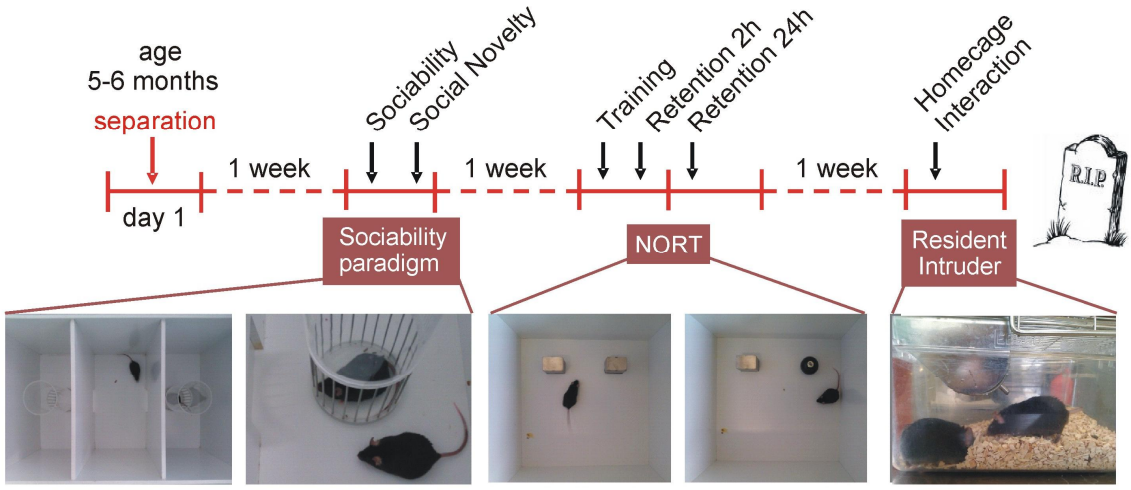

Fig. 3.1: Behavioral schedule. Schematic illustration showing the behavioural schedule. At the age of 5-6 months, mices were separated one week prior to behavioural testing in the sociability paradigm, novel object recognition test (NORT) and resident intruder paradigm.

3.2.3. Behavioral paradigms

Open Field and Novel Object Recognition Task: The novel object recognition task combined a general exploration test with a visual recognition memory paradigm. Therefore, it was used to evaluate object exploration and object recognition. The test was performed in a white plastic open field chamber (H40 cm x W40 cm x L40 cm). The protocol used was modified from Ennaceur and Delacour (1988), Tang et al. (1999), and Tordera et al. (2007) and performed by Nadine Kaiser, a Ph.D. student in the Lutz laboratory.

For habituation, the animals were placed into the empty open field and allowed to explore the box for 10 min once a day for two days. The first habituation session was analyzed according to a standard open field paradigm, hence, total distance moved and time spent in the center (defined as 20 cm x 20 cm) was evaluated using SMART software (PanLab, Spain). On day 3, two identical objects (O1 left, and O1 right; two metal cubes with 4 cm x 3 cm x 5 cm [H x W x L]) were placed symmetrically 6-7 cm from the walls and separated 16-18 cm from each other. The mouse was placed into the box at an equal distance from both objects and video-recorded for 10 min. After this first exposure to the object, the mouse was returned to its home cage.

2 h and 24 h later, the mouse was placed again into the open field and exposed to the familiar object (O1) and to a novel object (O2 for the 2 h time point, and O3 for the 24 h time point, respectively) each time for 10 min (retention tests). The novel object O2 was a plastic billiard ball (5.72 cm in diameter) fixed on a metal plate (0.2 cm) and O3 was a round glass flask (6 cm x 3 cm [H x W]), filled with sand and closed with a black rubber plug. The familiar object

was always positioned on the left side, while the new object was on the right side. Box and objects were cleaned with 70% ethanol after each trial to avoid olfactory cues. Experiment was video-recorded and the total time that the animal spent exploring each of the two objects in training and retention phase was evaluated by an experimenter blind to the genotype. Object exploration was defined as the orientation of the nose directly to the object at a distance <2 cm and/or touching the object with the nose and whiskers. Time spent climbing and sitting on the object were not regarded as exploration, and was therefore excluded from measurement (Ennaceur and Delacour, 1988), as these activities do not present a form of exploration. The discrimination index (DI) was calculated as the difference between the time spent exploring the new (N) and familiar (F) object, divided by the total time exploring the objects [(N - F)/(N + F)]. A positive DI is considered to reflect increased memory retention for the familiar object (Tordera et al., 2007).

Sociability Test: A modified sociability test was performed, based on a published protocol (Moy et al., 2004). In short, the test chamber (41 cm x 42 cm x 70 cm [H x W x L]) was divided into three compartments (40 cm x 40 cm x 22 cm [H x W x L]), all accessible by openings (7.5 cm x 10 cm [H x W]) in the dividing walls. Chambers and cages were cleaned with 70% ethanol between each trial to avoid olfactory cues. The total time that the test animals spent in each of the compartments during sociability and social novelty phase was measured. Male C57BL/6N animals (10-12 weeks old) were used as interaction partners for the sociability and social novelty phase.

Habituation Phase: The test animal was placed into the middle compartment for 5 min with entries to the side compartments blocked.

Sociability Phase: After the habituation phase, blockades of the entries were removed, allowing free access to the side compartments. By doing this, the animal tested was exposed to a novel C57BL/6N interaction partner and a novel object (round cage described below), positioned in the two side compartments. The position of the interaction partner (left vs. right compartment) was alternated between trials to avoid any bias. The interaction partner itself was enclosed in a round cage (10 cm in diameter; 30 cm high [upper 20 cm Plexiglass, lower 10 cm covered by metal bars 1 cm apart to allow interaction but prevent fighting]). To minimize stress levels of the animals used as interaction partners, they were habituated to the cages four times for 10 min. To counterbalance individual differences of these interaction partners they were equally used for wild-type and mutant test mice. The novel object control (empty cage, no animal) was always positioned in the opposite compartment to the cage with

the interaction partner. The DI was calculated as the difference between the time spent exploring the novel object (nO) and the novel animal (nA), divided by the total time exploring both [(nO - nA) / (nO + nA)]. A positive DI is considered to reflect increased preference for the social interaction partner.

Social Novelty Phase: 2 and 24 h after the sociability phase, an additional, unknown interaction partner (novel) was introduced. The interaction partner from the sociability phase (familiar) was again placed into the same cage and same compartment as before. The novel animal was placed into the former empty cage and positioned at the respective side compartment. Openings were unblocked. The test animal was placed into the middle compartment, and the test animal was allowed to freely explore for 10 min. The DI was calculated as the difference between the time spent exploring the new (N) and the familiar (F) animal, divided by the total time exploring both [(N - F) / (N + F)]. A positive DI is considered to reflect increased memory retention for the familiar animal.

Resident Intruder: The resident-intruder test was performed by placing a novel, group housed intruder into the home cage of the test animal for 10 min. This paradigm allows evaluating social exploration and aggressive behavior (Goyens and Noirot, 1975). To decrease interaction induced by the intruder, younger animals (males, 11-13 weeks) were used as intruders. Experiment was video-recorded, and the total interaction time of the animals spent exploring was measured by an experimenter blind to the genotype. Interaction was defined by any type of physical interaction clearly directed towards the partner. Duration and number of fights were evaluated separately. Fighting was defined by physical struggling between the interaction partners initiated by an attack of the resident towards the intruder.

3.2.4. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of individual data points. Results were considered to be significant at $p < 0.05$. All behavioral endpoints of the novel object recognition task were initially analyzed using two-way ANOVA, using genotype and object as variables and Bonferroni post-tests to correct for multiple comparisons. In some cases, to analyze the locomotion effects in the open field, the sociability in the sociability test and the aggression in the resident-intruder paradigm for each genotype, data were analysed using an unpaired Student's t-test or Kruskal-Wallis statistic. Additionally, in order to evaluate whether the DI of the genotypes deviated significantly from zero, we used the unpaired t-test with Welch´s correction. Graphs and statistics were generated by GraphPad Prism 4.03 (GraphPad Software; http://www.graphpad.com).

3.3. Results

3.3.1. Open Field

The evaluation of the locomotor activity in the open field revealed that only the GABA-$CB_1^{-/-}$ mice showed an increased locomotion ($T_{(18)} = 3.213$, $p = 0.0048$; Table 1). None of the other mutants showed any change in the distance moved as compared with their respective wild-type littermates in the open field (Glu-CB_1 line [$T_{(34)} = 1.609$, $p = 0.1169$]; D1-CB_1 line [$T_{(21)} = 0.5618$, $p = 0.5802$]). In regard to the time spent in the center region of the open field, we could not detect an alteration in any of the mutants (Glu-CB_1 line [$T_{(34)} = 0.8168$, $p= 0.4197$]; GABA-CB_1 line [$T_{(18)} = 1.418$, $p = 0.1733$]; D1-CB_1 line [$T_{(21)} = 0.9048$, $p = 0.3758$]; see Table 3.1).

3.3.2. Novel Object Recognition Task

The analysis of the novel object recognition task (referred to as NORT in Table 1) revealed a decrease in general object exploration in Glu-$CB_1^{-/-}$ mice as compared to wild-type littermate controls (Fig. 3.2A, D, G). We detected a significant decrease in time spent with the objects O1 in the training session ($F_{(1,62)} = 4.183$, $p = 0.0451$; Fig. 3.2A), but also in the 2 h ($F_{(1,66)} = 13.68$, $p = 0.0004$; Fig. 3.2D) and 24 h retention sessions ($F_{(1,66)} = 32.87$, $p < 0.0001$; Fig. 3.2G) for the novel object O2. In contrast, GABA-$CB_1^{-/-}$ mice displayed a general increase in exploration in all the sessions as compared to controls (training [$F_{(1,74)} = 17.88$, $p < 0.0001$], 2 h retention [$F_{(1,74)} = 8.411$, $p= 0.0049$)], 24 h retention [$F_{(1,74)} = 6.172$, $p = 0.0152$]; Fig. 3.2B, E, H). In the D1-CB_1 mutant line, no genotype differences were observed in the general object exploration (training [$F_{(1,44)} = 1.760$, $p = 0.1915$], 2 h retention [$F_{(1,44)} = 0.08051$, $p = 0.7721$], 24 h retention [$F_{(1,44)} = 3.317$, $p = 0.0754$]; Fig. 3.2 C ,F, I).

Evaluation of the DI revealed that all groups, independent of the line, showed no differences within the training session regarding the exploration of the left and the right object O1, respectively. (Glu-$CB_1^{+/+}$ [$T_{(20)} = 0.8230$, $p = 0.4202$]; Glu-$CB_1^{-/-}$ [$T_{(11)} = 0.9582$, $p = 0.3585$]; GABA-$CB_1^{+/+}$ [$T_{(15)} = 1.118$, $p= 0.2812$]; GABA-$CB_1^{-/-}$ [$T_{(22)} = 1.959$, $p = 0.0630$]; D1-$CB_1^{+/+}$ [$T_{(11)} = 1.447$, $p = 0.1758$]; D1-$CB_1^{-/-}$ [$T_{(11)} = 1.679$, $p = 0.1213$]; Table 1). Furthermore, no discrimination differences compared to their respective wild-type controls were found for all mutants within the training session (Glu-CB_1 line [$T_{(31)} = 1.407$, $p = 0.1693$]; GABA-CB_1 line [$T_{(37)} = 0.06488$, $p= 0.9486$]; D1-CB_1 line [$T_{(22)} = 1.951$, $p = 0.0639$]; Table 3.1).

	Glu-CB1		GABA-CB1		D1-CB1	
	+/+	-/-	+/+	-/-	+/+	-/-
Paradigm	Distance Moved (cm)					
Open Field	2824 ±209	2324 ±182	2621 ±306	3801 ±202**	4456 ±91	4368 ±131
Sociability Test						
Habituation Phase	2012 ±113	1569 ±162*	1597 ±59	1730 ±64	1669 ±81	1850 ±118
Sociability Phase	5171 ±205	4891 ±312	4945 ±127	5083 ±175	5055 ±162	5079 ±157
Social Novelty Phase	3973 ±211	3125 ±227*	4023 ±141	4623 ±175*	3863 ±292	3942 ±160
	Time in Center (sec)					
Open Field	63.3 ±12	80.9 ±20	156 ±48	82 ±21	65.4 ±11	82.4 ±15
	Discrimination Index					
NORT						
Training Session	0.01 ±0.01	-0.08 ±0.08	-0.03 ±0.03	-0.03 ±0.02	-0.08 ±0.05	0.03 ±0.02
2h Retention Session	0.16 ±0.03#	0.06 ±0.05	0.00 ±0.06	-0.03 ±0.03	0.15 ±0.06#	0.08 ±0.03#
24h Retention Session	0.25 ±0.06#	0.06 ±0.13	0.18 ±0.06#	0.15 ±0.05#	0.27 ±0.08#	0.18 ±0.05#
Sociability Test						
Sociability Phase	0.29 ±0.03#	0.12 ±0.07*	0.27 ±0.03#	0.35 ±0.04#	0.20 ±0.04#	0.30 ±0.04#
Social Novelty Phase	0.05 ±0.03	0.08 ±0.09	-0.01 ±0.05	0.09 ±0.03#	0.03 ±0.06	0.03 ±0.06

Table 3.1: Locomotion, anxiety and memory. Evaluation of locomotion (distance moved), anxiety (time in center) and memory (discrimination index) for all mutant lines; $^{+/+}$ (wild-type), $^{-/-}$ (mutant); t-test analysis: *p<0.05; **p<0.01 (significance between genotype); #p<0.05 (significant from 0; positive recognition of novel object).

In the 2 h retention phase, several groups lacked a significant discrimination between the familiar and the novel object. Only Glu-$CB_1^{+/+}$, D1-$CB_1^{+/+}$ and D1-$CB_1^{-/-}$ animals displayed a significant preference towards the novel stimulus (Glu-$CB_1^{+/+}$ [$T_{(21)}$ = 4.806, p < 0.0001]; Glu-$CB_1^{-/-}$ [$T_{(12)}$ = 1.220, p = 0.2458]; GABA-$CB_1^{+/+}$ [$T_{(15)}$ = 0.07097, p = 0.9444]; GABA-$CB_1^{-/-}$ [$T_{(22)}$ = 1.366, p = 0.1858]; D1-$CB_1^{+/+}$ [$T_{(10)}$ = 2.502, p = 0.0313]; D1-$CB_1^{-/-}$ [$T_{(10)}$ = 2.238, p = 0.0492]; Table 1). Comparison between the mutants and their respective wild-type littermates displayed no significant differences in all lines (Glu-CB_1 line [$T_{(33)}$ = 1.775, p = 0.0850]; GABA-CB_1 line [$T_{(37)}$ = 0.6235, p = 0.5368]; D1-CB_1 line [$T_{(20)}$ = 0.9965, p = 0.3309]; Table 1).

In the 24 h retention phase, independently of the genotype, all groups showed a significant preference towards the novel object, with the only exception of the Glu-$CB_1^{-/-}$ animals (Glu-$CB_1^{+/+}$ [$T_{(21)}$ = 4.472, p= 0.0002]; Glu-$CB_1^{-/-}$ [$T_{(12)}$ = 0.4328, p = 0.6729]; GABA-$CB_1^{+/+}$ [$T_{(15)}$ = 2.818, p = 0.0129]; GABA-$CB_1^{-/-}$ [$T_{(22)}$ = 3.072, p = 0.0056]; D1-$CB_1^{+/+}$ [$T_{(11)}$ = 3.601, p = 0.0042]; D1-$CB_1^{-/-}$ [$T_{(11)}$ = 3.540, p = 0.0046]; Table 1). Comparison between the mutants and

their respective wild-type littermates diplayed no genotype difference (Glu-CB_1 line [$T_{(33)}$ = 1.522, p = 0.1374]; GABA-CB_1 line [$T_{(37)}$ = 0.1255, p = 0.9008]; D1-CB_1 line [$T_{(22)}$ = 1.049, p = 0.3055]; Table 3.1).

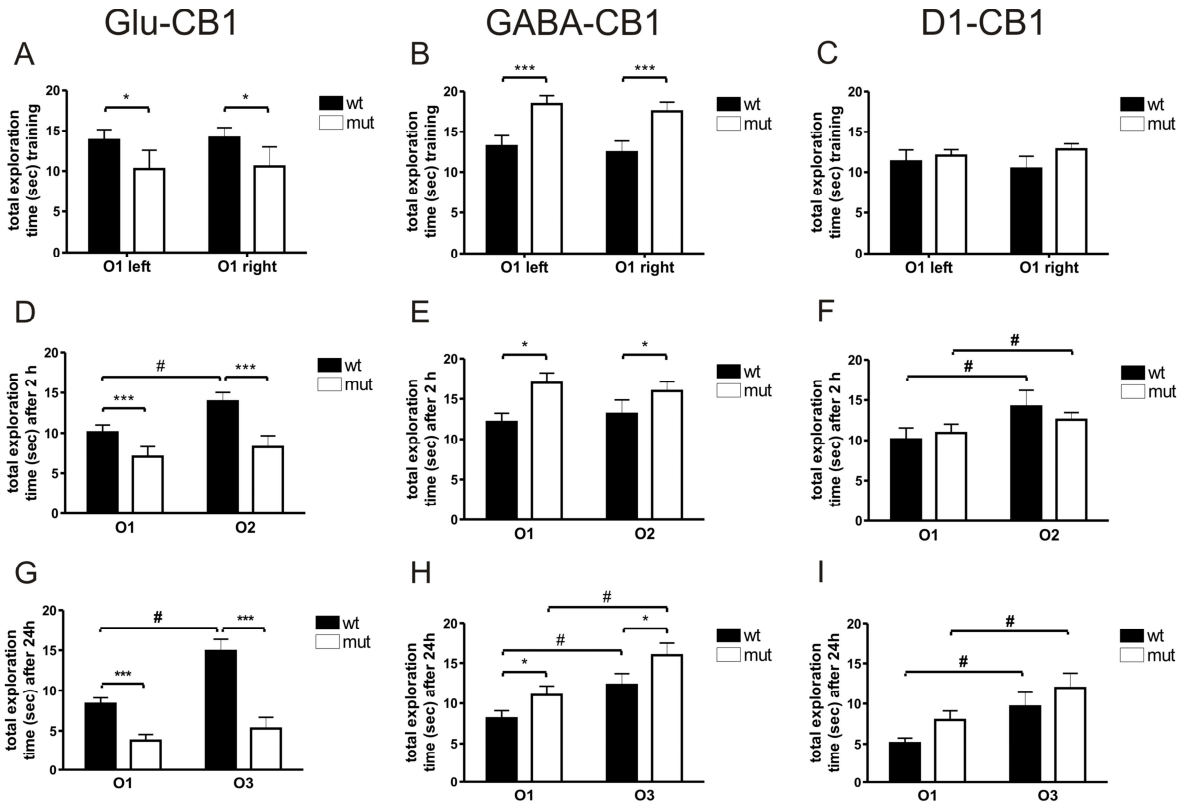

Fig. 3.2: Inanimate exploration in the novel object recognition task. (A-C) Total time of exploration of two identical objects (O1, both on left and right side) during the training session for three conditional CB_1 receptor mutant lines (Glu-CB_1 [n=23+13], GABA-CB_1 [n=18+23], D1-CB_1 [12+12]) and their wild-type control littermates. (D-F) Total time of exploration of familiar object (O1) and novel object (O2 or O3) during the retention session after 2 h or 24 h (G-I). Glu-$CB_1^{-/-}$ mice displayed a reduced exploration, while GABA-$CB_1^{-/-}$ mice showed an increased exploration both in the training and retention session as compared to their wild-type littermate controls. No significant genotype differences were observed in the D1-CB_1 mutant line. 2-way ANOVA (genotype differences) *p<0.05, ***p<0.001; Student´s t-test (discrimination index) $^{\#}$p<0.05.

The evaluation of object specific exploration (O1 left or O1-3 right) over the three sessions (training, 2 h retention and 24 h retention) revealed a significant difference for the Glu-$CB_1^{-/-}$ as compared to their littermate controls. Thus, the Glu-$CB_1^{-/-}$ mutants showed a steadily decreasing investigatory behavior for both, the left object (increasing familiarity) and the right object (always novel) (Glu-$CB_1^{-/-}$ interaction [object/time]: $F_{(2,48)}$ = 0.1537, p = 0.8580; Bonferroni post-test: training p > 0.05, 2 h p > 0.05, 24 h p > 0.05; Fig. 3.2A,D,G). This

phenomenon was only seen in the Glu-$CB_1^{+/+}$ mice for the left object (increasing familiarity), while the time spent investigating the right object (always novel) remained constant (Glu-$CB_1^{+/+}$ interaction [object/time]: $F_{(2,84)} = 4.851$, $p = 0.0101$; Bonferroni post-test: training $p > 0.05$, 2 h $p > 0.05$, 24 h $p < 0.01$; Fig. 3.2A,D,G). It was further possible to detect a significant difference between the genotypes in exploring the right object, but not the left object over the three sessions (left object interaction [genotype/time]: $F_{(2,48)} = 0.2283$, $p = 0.7965$; Bonferroni post-test: training $p > 0.05$, 2 h $p > 0.05$, 24 h $p > 0.05$; right object interaction [genotype/time]: $F_{(2,66)} = 3.522$, $p = 0.0352$; Bonferroni post-test: training $p > 0.05$, 2 h $p < 0.05$, 24 h $p < 0.001$; Fig. 3.2A,D,G).

3.3.3. Sociability Test

During the sociability phase, the Glu-$CB_1^{-/-}$ animals showed a significant increase in time spent in the middle compartment ($T_{(33)} = 2.247$, $p = 0.0314$; Fig. 3.3A). Accordingly, these mutants displayed a significant decrease in time spent with the interaction partner, but not with the object (mouse [$T_{(33)} = 3.734$, $p = 0.0007$]; object [$T_{(33)} = 1.412$, $p = 0.1672$]; Fig. 3.3A). A similar result was obtained, when the novel interaction partner was introduced during the social novelty test. While the Glu-$CB_1^{-/-}$ mice spent more time in the middle compartment, they spent less time with the familiar and novel partner as compared to the wild-type littermates (middle [$T_{(33)} = 3.772$, $p = 0.006$]; familiar [$T_{(33)} = 2.263$, $p = 0.0303$]; unknown [$T_{(33)} = 2.596$, $p= 0.0140$]; Fig. 3.3D). This phenotype was opposite to the findings with the GABA-CB_1 line. In the sociability phase as well as in the social novelty phase, the GABA-$CB_1^{-/-}$ mice showed a significant increase in time spent with the novel interaction partner as compared to controls (sociability [$T_{(57)} = 2.099$, $p = 0.0403$]; social novelty [$T_{(35)} = 3.063$, $p = 0.0042$]; Fig. 3.3B,E). The time spent in the middle compartment was consequently decreased (sociability [$T_{(57)} = 2.740$, $p = 0.0082$]; social novelty [$T_{(35)} = 2.168$, $p = 0.037$]). Interestingly, the time spent in the compartment with the empty cage (i.e. the object only) during the sociability phase as well as the time spent with the familiar animal (social novelty test) were not different between mutants and controls (object [$T_{(57)} = 1.114$, $p = 0.2699$]; familiar [$T_{(35)} = 1.017$, $p = 0.3162$]; Fig. 3.3B,E). The analysis of the D1-CB_1 line did not reveal any significant genotype differences in the 3 phases of the sociability test (Fig. 3.3C, F). Only a non-significant trend was observed in the sociability phase. Even though time spent in the middle area was not altered ($T_{(28)} = 0.9190$, $p = 0.3659$), it seemed that the mutants showed a slight preference to explore the interaction partner ($T_{(28)} = 1.909$, $p = 0.0666$) rather than the empty cage ($T_{(28)} = 1.859$, $p= 0.0736$), i.e. the object (Fig. 3.3C).

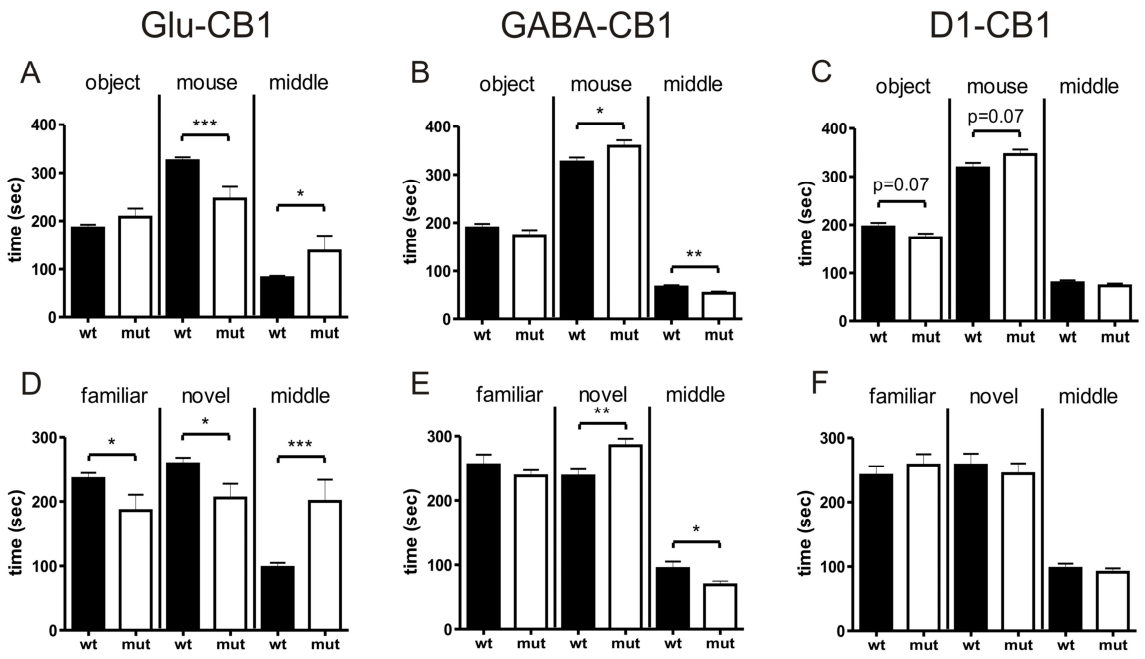

Fig. 3.3: Animate vs. inanimate exploration in the sociability test. (A-C) Comparison of animate (mouse) and inanimate (object, "empty") exploration for the three mutants lines (Glu-CB_1 [n=22+13], GABA-CB_1 [n=18+23], D1-CB_1 [16+16]) and their wild-type littermate controls during the sociability phase. (D-F) Exploration of the familiar and the novel interaction partner during the social novelty phase. Glu-$CB_1^{-/-}$ mice displayed no significant change in the exploration session, when there was a choice between the object and the interaction partner. In the social novelty phase, however, the interaction with a novel interaction partner was decreased as compared with their wild-type littermate controls. GABA-$CB_1^{-/-}$ mice showed an increased social interaction in both sessions. In the D1-CB_1 mutant line, no genotype differences were observed, neither in the sociability nor in the social novelty phase. n=11-20 animals; t-test *p<0.05, **p<0.01.

In the social novelty phase, not even a trend was detectable (familiar [$T_{(30)}$ = 0.7636 p = 0.4511]; novel [$T_{(30)}$ = 0.5840 p = 0.5636]; middle [$T_{(30)}$ = 0.6112 p = 0.5457]; Fig. 3.3F)

The evaluation of the discrimination index (DI) showed only minimal differences between the genotypes. In the sociability phase, the Glu-$CB_1^{-/-}$ animals showed an impaired preference towards the interaction partner as compared to their controls ($T_{(33)}$ = 2.537, p < 0.0161; Table 1). In contrast, the GABA-$CB_1^{-/-}$ mice and the D1-$CB_1^{-/-}$ mice showed no significant changes in the preference towards the interaction partner (GABA-$CB_1^{-/-}$ [$T_{(57)}$ = 1.507, p < 0.1373], D1-$CB_1^{-/-}$ [$T_{(28)}$ = 1.636, p < 0.1130]; Table 1). In the social novelty phase, no DI differences were observed in any of the lines (Glu-CB_1 line [$T_{(33)}$ = 0.3977 p= 0.6934]; GABA-CB_1 line [$T_{(34)}$ = 1.794 p= 0.0817]; D1-CB_1 line [$T_{(30)}$ = 0.6126 p= 0.547]).

For all lines and genotypes, except for the Glu-$CB_1^{-/-}$ mice, we observed a strong preference towards the social interaction partner over the object in the sociability phase (Glu-$CB_1^{+/+}$ [$T_{(21)}$

= 10.47, p < 0.0001]; Glu-$CB_1^{-/-}$ [$T_{(12)}$ = 1.559, p = 0.1450]; GABA-$CB_1^{+/+}$ [$T_{(27)}$ = 8.309, p < 0.0001]; GABA-$CB_1^{-/-}$ [$T_{(30)}$ = 8.187, p < 0.0001]; D1-$CB_1^{+/+}$ [$T_{(16)}$ = 5.017, p = 0.0002]; D1-$CB_1^{-/-}$ [$T_{(13)}$ = 7.458, p < 0.0001]; Table 1). In the social novelty phase, none of the groups, except for the GABA-$CB_1^{-/-}$ mice, showed any preference towards the novel over the familiar interaction partner (Glu-$CB_1^{+/+}$ [$T_{(21)}$ = 1.453, p < 0.1610]; Glu-$CB_1^{-/-}$ [$T_{(12)}$ = 0.8652, p = 0.4039]; GABA-$CB_1^{+/+}$ [$T_{(15)}$ = 0.2402, p = 0.8134]; GABA-$CB_1^{-/-}$ [$T_{(19)}$ = 2.674, p = 0.0150]; D1-$CB_1^{+/+}$ [$T_{(16)}$ = 0.4262, p = 0.6756]; D1-$CB_1^{-/-}$ [$T_{(14)}$ = 0.4437, p = 0.6841]; see Fig. 3.3).

The evaluation of the locomotor activity revealed no significant changes in the habituation phase of the sociability test, except for the Glu-$CB_1^{-/-}$ mice, which showed a decrease in locomotion (Glu-CB_1 line [$T_{(33)}$ = 2.312, p = 0.0271]; GABA-CB_1 line [$T_{(60)}$ = 1.506, p = 0.1374]; D1-CB_1 line [$T_{(29)}$ = 1.571, p = 0.1270]). In the sociability phase, no alteration in the distance moved was observed in any of the lines (Glu-CB_1 line [$T_{(29)}$ = 0.7833, p = 0.4398]; GABA-CB_1 line [$T_{(62)}$ = 0.6159, p = 0.5402]; D1-CB_1 line [$T_{(30)}$ = 0.1082, p = 0.9145]). However, a significant decrease and increase in the distance moved was detected in the social novelty phase for the Glu-$CB_1^{-/-}$ mice and the GABA-$CB_1^{-/-}$ mice, respectively (Glu-CB_1 line [$T_{(33)}$ = 2.575, p = 0.0146]; GABA-CB_1 line [$T_{(38)}$ = 2.591, p = 0.0135]). The D1-$CB_1^{-/-}$ mice again showed no change in the distance moved as compared to their respective wild-type littermates ($T_{(30)}$ = 0.2386, p = 0.8130).

3.3.4. Resident-Intruder Test

Glu-$CB_1^{-/-}$ mice displayed a significant decrease interacting with the intruder animals for the 10 min interaction phase as compared with wild-types ($T_{(35)}$ = 2.297, p = 0.0277). Splitting the 10 min period into two 5 min bins revealed that the difference in interaction was mainly visible for the first 5 min bin ($T_{(35)}$ = 3.106, p = 0.0038) (Fig. 3.4A). In addition, Glu-$CB_1^{-/-}$ mice displayed an altered aggressive behavior. Even though the number of fights was not different between the genotypes, the time during which Glu-$CB_1^{-/-}$ mice spent fighting the intruder was increased ($T_{(35)}$ = 2.249, p = 0.0309) (Fig. 3.4D). As observed in the previous experiments, we detected an opposite phenotype in the GABA-$CB_1^{-/-}$ animals, showing an increased interaction with the intruder animal ($T_{(29)}$ = 2.522, p = 0.0174) (Fig. 3.4B). The overall fighting with the younger intruder did not change as compared to the wild-type littermates ($T_{(26)}$ = 0.4227, p = 0.6760, $T_{(29)}$ = 0.6286, p = 0.5345) (Fig. 3.4E). D1-$CB_1^{-/-}$ mice again displayed no phenotype differences, neither in interaction time spent with the intruder ($T_{(20)}$ = 0.3481, p = 0.7314), nor in fighting behavior ($T_{(22)}$ = 0.0000, p = 1.0, $T_{(22)}$ = 0.8261, p = 0.4176) (Fig. 3.4C,F).

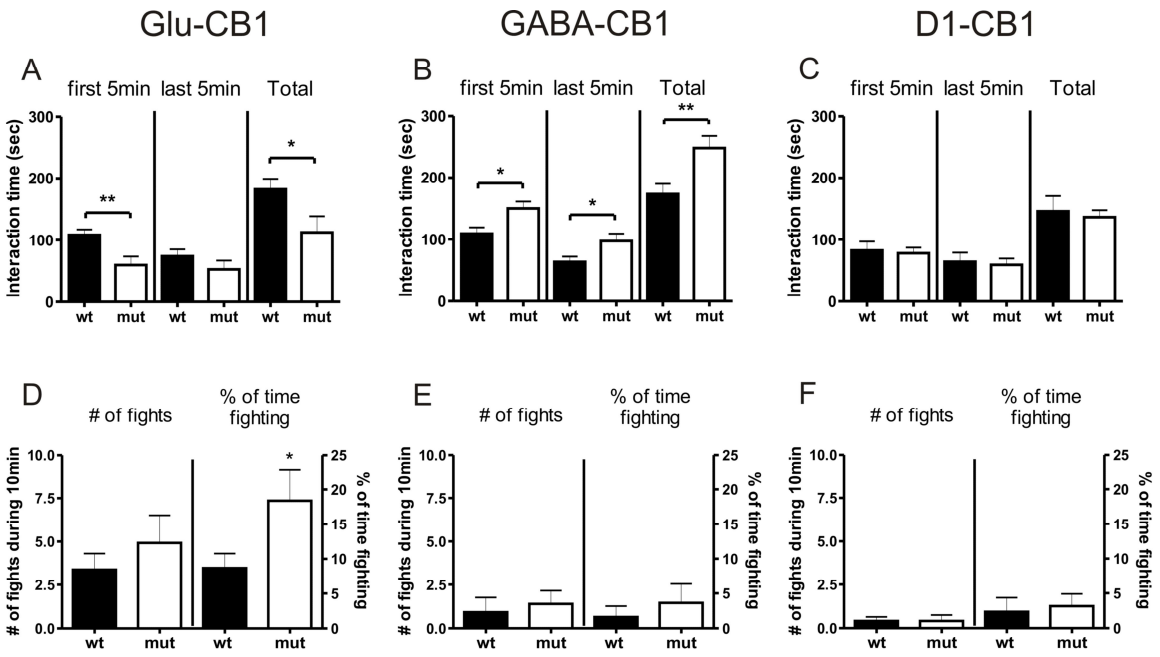

Fig. 3.4: Animate exploration in the resident-intruder test. (A-C) Social interaction with an unknown, younger intruder for all three mutant lines (Glu-CB_1 [n=23+13], GABA-CB_1 [n=18+23], D1-CB_1 [n=16+16]). (D-E) Number of fights induced by the resident is shown for all three mutant lines. Glu-$CB_1^{-/-}$ mice showed a significantly reduced exploration during the first 5 min observation period and an increased aggression towards the intruder as compared to wild-type littermate controls. GABA-$CB_1^{-/-}$ mice displayed an increased interaction with the intruder, but no difference in aggressive behaviour. D1-$CB_1^{-/-}$ mice showed no behavioural changes as compared to their wild-type littermate controls. t-test *p<0.05, **p<0.01.

Additional analysis revealed that Glu-$CB_1^{+/+}$ animals displayed a significant increase in aggression as compared to the other control groups, GABA-$CB_1^{+/+}$ and D1-$CB_1^{+/+}$. Thus, differences were detected in number of fights (Kruskal-Wallis statistic = 7.478, p = 0.0238; Dunn´s Multiple Comparison Post-Test: Glu-$CB_1^{+/+}$ vs GABA-$CB_1^{+/+}$ $p < 0.05$, Glu-$CB_1^{+/+}$ vs D1-$CB_1^{+/+}$ $p > 0.05$, GABA-$CB_1^{+/+}$ vs D1-$CB_1^{+/+}$ $p > 0.05$), as well as % of time fighting (Kruskal-Wallis statistic = 7.584, p = 0.0226; Dunn´s Multiple Comparison Post-Test: Glu-$CB_1^{+/+}$ vs GABA-$CB_1^{+/+}$ $p < 0.05$, Glu-$CB_1^{+/+}$ vs D1-$CB_1^{+/+}$ $p > 0.05$, GABA-$CB_1^{+/+}$ vs D1-$CB_1^{+/+}$ $p > 0.05$).

3.4. Discussion

Using different conditional CB_1 receptor mutant mice, we were able to show that the deletion of the CB_1 receptor from forebrain GABAergic and cortical glutamatergic neurons, respectively, resulted in an opposite behavioral outcome regarding animate and inanimate exploration. On the other hand, deletion of the CB_1 receptor from dopamine receptor D1-expressing GABAergic striatal medium spiny neurons did not result in any significant changes in these functions. These findings suggest a regulatory function of the eCB system in cortical GABAergic and glutamatergic circuits to prevent neuronal and behavioral imbalance.

Mice lacking the CB_1 receptor on glutamatergic neurons displayed a decreased exploratory behavior, both in animate interaction (the interaction with a partner) and inanimate interaction (the interaction with an object). A similar decrease in object and social exploration was found in earlier studies, which were related with increased fear (Lafenêtre et al., 2009; Jacob et al., 2009). In our study, the decrease in exploration was seen when the mouse was exposed to a social interaction partner and/or to an object, and seemed to be independent of novelty (Fig. 3.2, 3.3D). This phenotype was also visible in the resident-intruder test. However, the decreased social investigation was mainly based on a lower exploration during the first 5 min interval, a period important for information gathering (Fig. 3.4A).

The anxiogenic-like behavior associated with these mutants can also explain the significantly higher aggression level found in the resident-intruder paradigm (Fig. 3.4D), a behavior which was also observed in complete CB_1 receptor knock-out animals (Martin et al., 2002). The age and strength of the intruder as compared to the resident is highly important (Thor and Flannelly, 1976). In our case, the intruders were weaker and hence should not be regarded as a threat. We would therefore suggest that the deletion of the CB_1 receptor from cortical glutamatergic neurons might result in an inadequate aggressive response, suggesting an important role of CB_1 receptor on the neuronal population controlling aggression. CB_1 receptor in forebrain GABAergic interneurons appears to mediate an opposite behavior as compared to Glu-$CB_1^{-/-}$. While D1-$CB_1^{-/-}$ animals (CB_1 receptor loss primarily in the striatum) did not reveal any significant difference as compared to wild-type littermates, we observed that GABA-$CB_1^{-/-}$ mice (lacking CB_1 receptor from forebrain GABAergic interneurons) showed an increase in animate and inanimate exploration. Accordingly, increased investigatory behavior toward novel food and object was previously observed in the GABA-$CB_1^{-/-}$ mice (Lafenêtre et al., 2009). Interestingly, Glu-$CB_1^{+/+}$ control animals displayed an increased aggressive behavior in the resident intruder paradigm as compared to the other wild-

type controls, GABA-$CB_1^{+/+}$ and D1-$CB_1^{+/+}$. This elevated aggression might be explained by the fact that both the wild-type and mutant littermates are group-housed during growth. In case of the Glu-CB_1 line, the modulated social behavior of the Glu-$CB_1^{-/-}$ mutants might have an effect on their wild-type littermates.

Taken together, these results suggest an anxiolytic-like function of the CB_1 receptor on glutamatergic neurons and an anxiogenic-like function of the CB_1 receptor on GABAergic interneurons. However, a generalized conclusion on the involvement CB_1 receptor on cortical glutamatergic neurons in anxiety is not yet possible, as under our experimental conditions, the open field test was not congruent with this notion. Neither Glu-$CB_1^{-/-}$ nor GABA-$CB_1^{-/-}$ mutants spent a different period of time in the more aversive center zone as compared with their respective wild-type littermates (Table 1). In addition, studies with these animals on the elevated plus maze, an anxiety test, did not reveal any changes either (Jacob et al., 2009, Rühle et al., 2012). Also, levels of corticosterone under basal and stressful conditions were found to be similar between mutant and wild-type controls in both mutant lines (Steiner et al., 2008c). Thus, it seems that a respective exploratory stimulus, such as an object or interaction partner, is required to induce a phenotype in these mice.

An alternative explanation for the observed differences can be alterations in spontaneous locomotor activity. In fact, we observed for both the Glu-$CB_1^{-/-}$ and GABA-$CB_1^{-/-}$ changes in the distance moved, namely a decrease and increase, respectively. It seems unlikely that the difference in locomotion was the driving force underlying the exploration phenotypes, as the mutants, in contrast to the variation in animate and social investigation, did not always display the locomotor alterations (Table 1). We argue that a respective context (e.g. handling threshold, exploratory stimulus) is required for a detectable locomotion phenotype in our mutant lines. A similar situation seems to be true for the general investigatory drive. Thus, the clear differences in exploring object or interaction partner is not mirrored by the findings in the open field test, where we were not able to detect any alteration in the time spent in the more aversive center region (Table 1). This notion is supported by other studies with these mutant lines, where a behavioral change is only detectable in the presence of a respective stimulus or pharmacological modification of the eCB system (Lafenêtre et al., 2009; Jacob et al., 2009).

A further explanation for the behavioral differences might be memory alterations in the respective mutant. However, this might only account for the Glu-$CB_1^{-/-}$ mutants, as all other animals, independently of line and genotype, displayed a similar memory and recognition performance. Especially after 24 hours, mice recognized and distinguished strongly between

familiar and novel objects (Table 1). The low discrimination index to the familiar object after a 2 hour interval in several groups, however, is unexpected and cannot be explained at this point. Only Glu-$CB_1^{-/-}$ failed to show a clear preference towards the novel object in both retention sessions, indicating a memory deficit. Problematic for the interpretation is the overall low exploration for this mouse line, which is true for all three sessions of the novel object recognition test, as well as the other behavioral paradigms. The altered behavior of the mutants in response to the novel objects is of special interest. While wild-type littermates displayed a constant interest for the novel objects (O1-O3), the Glu-$CB_1^{-/-}$ animals showed a steadily decreasing exploration over the three sessions (Fig. 3.2A, D, G). For both genotypes, such a decrease was seen regarding the exploration of the familiar objects (O1), which is not surprising, as novelty of this object strongly decreased with each session. Thus, the Glu-$CB_1^{-/-}$ mice appeared to respond to the familiar and novel object in a similar way, suggesting rather a habituation to the context than a memory deficit. Nevertheless, a final conclusion cannot be made at this point.

As mentioned above, all groups, independently of the line and the genotype, showed a stronger preference for the social interaction partner as compared to the object in the sociability test (Table 1). This behavior was expected, as animals normally prefer social over non-social contacts (Moy et al., 2004). Surprisingly, we could not detect a significant preference towards the novel interaction partner in the social novelty phase (Table 1). While this preference was observed in several lines (Moy et al., 2004), in our hands it was only recognizable in the GABA-$CB_1^{-/-}$ mice. This finding could indicate that social discrimination was impaired in these mutants. However, comparable results from other studies suggest that a strong social preference does not necessarily predict a strong preference for social novelty. As a matter of fact, two different components of social behavior were postulated to underlie sociability and social novelty, respectively. In addition, life history and development are responsible for lower or higher novelty preference (Moy et al., 2009).

Taken together the strong differences observed in the GABA-$CB_1^{-/-}$ and Glu-$CB_1^{-/-}$ animals in respect to their wild-type littermates might be explained by anxiolytic and anxiogenic responses to novelty, respectively. Nevertheless, the eCB system has also been shown to be involved in learning and memory function, which should be kept in mind here (Moreira and Lutz, 2008; Marsicano and Lafenêtre, 2009). It may be even likely that both anxiety and memory components function together in our paradigms, but to solve this issue would require further investigations using other behavioral paradigms.

Our results, namely the increase of exploration following the deletion of GABAergic CB_1 receptor and the decrease of exploratory behavior following the deletion of glutamatergic CB_1 receptors, may explain the contradictory findings using Δ^9-THC, URB597 and VDM11, as described in the Introduction. We suggest that increased or decreased exploratory drive, respectively, as response to cannabinoid treatment depends on the predominant modulation of either GABAergic or glutamatergic CB_1 receptor, i.e. the activation of GABAergic CB_1 receptor decreases exploration, while the activation of glutamatergic CB_1 receptor leads to an increased investigatory drive. Thus, the decreased exploration induced by chronic and systemic activation of the eCB system with Δ^9-THC might be due to the exogenous activation of the CB_1 receptor in GABAergic interneurons (O´Shea et al., 2004; Schneider et al., 2008; Trezza und Vanderschuren 2008a). The increased exploratory profile after inhibition of anandamide degradation or reuptake could be explained by a specific on-demand activation of the CB_1 receptor on glutamatergic neurons (Trezza und Vanderschuren 2008a). On the other hand, the decreased animate and inanimate interaction as a result of the complete deletion of the CB_1 receptor might be caused by the increased GABAergic drive (Jacob et al., 2009; Haller et al., 2004). It seems that the GABAergic drive is the predominant factor for behavioral outcome, when the eCB system is activated or blocked in a chronic manner. This makes the increased social interaction after URB597 treatment even more interesting, as in this case, the glutamatergic drive seems to be the predominant component. To test this hypothesis, Glu-$CB_1^{-/-}$ or GABA-$CB_1^{-/-}$ would need to be injected with the respective drugs in comparable doses and tested in behavioral paradigms. Similar contradictory results were observed in pharmacological studies on anxiety and stress levels after cannabinoid administration, both being strongly involved in investigatory and exploratory drive [Hill and Gorzalka, 2005; Viveros et al., 2005). The opposite effects might also be based on cortical GABAergic or glutamatergic transmission. Therefore, depending on its specific spatiotemporal activation within neuronal circuits, this system can act as a major “bi-directional” neuromodulator (Rühle et al., 2011; Viveros et al., 2005).

Our results might also be interesting in respect to some disorders which are associated with inappropriate exploratory drive. Thus, a direct and indirect relation between these disorders and a dysregulation of GABAergic and/or glutamatergic transmission can be proposed. In animal models for autism, modulation of GABAergic transmission seems to be important (Chao et al., 2010; Sala et al., 2011). The induction of schizophrenia-like symptoms by administration of the NMDA receptor antagonist phencyclidine revealed an alteration of glutamatergic and GABAergic signalling in the prefrontal cortex (Amitai et al., 2011).

Interestingly, the effects of phencyclidine could be blocked by CB_1 receptor antagonist treatment (Del Arco et al., 2007). It was further shown that down-regulation of cortical glutamatergic drive resulted in an increase in dopamine levels and a hyperactive phenotype, which could be blocked by cortical GABA receptor activation (Guilali et al., 2011). These findings indicate a cortical control in these neuronal disorders, caused also by imbalanced GABAergic and glutamatergic transmission, a mechanism also suggested by our findings. Recent publications even suggest glutamatergic, instead of dopaminergic transmission to be the major factor of schizophrenia (Javitt, 2010).

In conclusion, our results indicate a major, but opposite role of the eCB system in cortical glutamatergic and forebrain GABAergic neurons in the regulation of exploration (Table 2). Hence, further investigations along this line should be able to detail the diverse effects of cannabinergic drugs on investigatory behavior. As investigatory drive is often associated with impulsive behavior, studies using respective paradigms would be of great interest. Lastly, in future studies, the regulatory properties of the eCB system on cortical excitatory and inhibitory drive should be exploited in psychiatric disorders, opening up a therapeutic avenue to restore a possible cortical imbalance pharmacologically.

Chapter 4

Antidepressant effects of cannabinoids depend on serotonin signaling

Chapter 4. Specific cannabinoid functions depend on serotonin release

4.1. Introduction

The herb *Cannabis sativa* induces a diversity of emotional responses, depending on the dose ranging from anxiolytic and relaxing effects to the induction of acute panic attacks (Hall and Solowij, 1998). Similarly, divergent emotional responses have been observed in both humans and rodents after the administration of THC, the main psychoactive compound from this plant (Berrendero and Maldonado, 2002; Patel and Hillard, 2006; Zuardi et al., 1982). Such a dose-dependent cannabinoid-induced biphasic effect on the behavioral performance can also be seen in the forced swim test (FST). Thus, depending on the dose, CB_1 receptor modulation using various agonist or antagonist concentrations can lead to a decrease or increase of immobility (Griebel et al., 2005; Bambico et al., 2007; Steiner 2008a), or an impairment of stress-coping behavior depending on the dose (Steiner et al., 2008c; Beyer et al., 2010). The FST is one of the most widely used behavioral paradigms to detect antidepressant-like activities of drugs (Lucki et al., 2001; Cryan and Mombereau, 2004). This test is based on the observation that rodents, when exposed to an inescapable situation (immersion in a beaker filled with water), will cease to engage in escape-oriented movements during several minutes and will adopt an immobile passive "floating" posture. Acquired immobility is often interpreted as "behavioral despair", mimicking psychomotor impairments experienced by depressed patients (Cryan and Mombereau, 2004). Specifically, a reduction of immobility time in the FST is observed after treatment with a broad range of antidepressants, which increase serotonergic and/or noradrenergic neurotransmission (Cryan and Mombereau, 2004). Nevertheless, it should be pointed out that the FST in mice should not be considered a marker of depression *per se*. In fact, there is no evidence for depression in mice, and the test is simply a measure of the ability of antidepressant compounds to modulate stereotypic behavior (Borsini and Meli, 1988).

The fact that cannabinergic drugs can have antidepressant effect supports the notion that this behavioral test may be a valid strategy for developing new drugs for the treatment of mood

disorders (Gobbi et al., 2005; Bambico et al., 2010; Gorzalka and Hill, 2010). Surprisingly, even though marihuana has been used for recreational purposes for centuries, studies on the antidepressant potentials of its major component, THC, are still sparse. Nonetheless, THC was also shown to elicit opposite responses, namely increase or decrease in immobility in the FST (Egashira et al., 2008; El-Alfy et al., 2010).

Interestingly, several studies connected the eCB system with serotonergic transmission. Indeed, the CB_1 receptor antagonist rimonabant was shown to increase the efflux of serotonin and noradrenaline in the rat prefrontal cortex (Tzavara et al., 2003). CB_1 receptor is expressed in mouse serotonergic raphe neurons (Häring et al., 2007) and in noradrenergic nerve terminals in the rat frontal cortex (Oropeza et al., 2007). In addition, CB_1 receptor activation influences the firing rate of serotonergic and noradrenergic neurons in the rat raphe nuclei and locus coeruleus, respectively (Muntoni et al., 2006; Bambico et al., 2010). Altogether, accumulating evidence supports the involvement of CB_1 receptor signalling in the regulation of monoaminergic neurotransmission, which could, in turn, mediate endocannabinoid effects in the FST.

Using a combination of pharmacological and genetic approaches, we aimed at investigating the contradictory findings on the modulation of emotions by the eCB system and how serotonergic transmission might be involved in. To this end, we studied the antidepressant-like effects of the CB_1 receptor agonist THC (0.1 mg/kg) and antagonist rimonabant (10 mg/kg) in combination with the drugs parachlorophenylalanine (pCPA) and WAY100635, which disrupt serotonergic transmission. In brief, pCPA blocks the serotonin synthesis, and WAY100635 functions as an antagonist for the $5HT_{1A}$ receptor. Furthermore, we included mice lacking the CB_1 receptor in specific neuronal subpopulations, namely in forebrain GABAergic neurons (GABA-CB_1 mouse line) and cortical glutamatergic neurons (Glu-CB_1 mouse line) in order to investigate the role of these cell types in the antidepressant effects induced by THC and rimonabant.

4.2. Materials & Methods

4.2.1. Animals

This study was performed on adult (3-5 months old) male C57BL/6N mice as well as in CB_1 receptor mutants and their littermate controls. Animals were housed in a temperature- and humidity-controlled room with a 12 h light-dark cycle (lights on at 1 am) and had access to food and water *ad libitum*. Generation, breeding and genotyping of the mutant lines were performed according to previous publications: $CB_1^{flox/flox;Nex\text{-}cre}$ mice (referred to as Glu-$CB_1^{-/-}$ mice; Monory et al., 2006), $CB_1^{flox/flox;dlx\text{-}cre}$ mice (referred to as GABA-$CB_1^{-/-}$ mice; Monory et al., 2006, Massa et al., 2010). All mutant lines were bred for > 10 generations on the background of C57BL/6N mice from Charles River, Germany.

4.2.2. Drugs

The % values below represent final concentrations. Stock solution of rimonabant (SR141716; NIMH Chemical Synthesis and Drug Supply Program) was prepared by dissolving the lyophilized drug in DMSO. Working solution contained the respective rimonabant concentration in 2% DMSO, 2.5% Tween-80, followed by dilution in 0.9% NaCl. THC (THC Pharm, Frankfurt, Germany) was dissolved in 100% ethanol. Working solution contained the respective THC concentration in 0.5% ethanol and 2.5% Tween-80, followed by dilution in 0.9% NaCl. WAY100635 (Sigma-Aldrich) was diluted in 0.9% NaCl solution. Parachlorophenylalanine (pCPA; Sigma-Aldrich) was suspended in 2.5% Tween-80, followed by dilution in 0.9% NaCl solution. All vehicle controls contained 0.9% NaCl and the respective concentration of polyoxyethylenesorbitan monooleate (Tween-80), dimethylsulfoxid (DMSO) and/or ethanol. The doses were selected based on previous publications (Braida et al., 2007; Egashira et al., 2008; Griebel et al., 2005; Kaster et al., 2005; Tzavara et al., 2003).

4.2.3. Experimental procedure

Animals were group housed (3-5 animals per cage) until one week before behavioral testing, when they were single housed to avoid behavioral differences between dominant and subordinate animals. All experiments were performed during the second half of the light phase. Injections were given intraperitoneally (i.p.) in a volume of 10 ml/kg body weight. Single drug injections were given 30 min prior to the behavioral analysis (Fig. 4.1B). If the mice were exposed to two different drugs, the first drug was applied 45 min and the second

drug 30 min before the behavioral analysis (Fig. 4.2C). pCPA was injected every 24 hours for 4 days with the last injection on the day of the FST (Fig. 4.1D). For each experiment, vehicle treatment was given as a control in the same injection schedule as the respective drug treated mice.

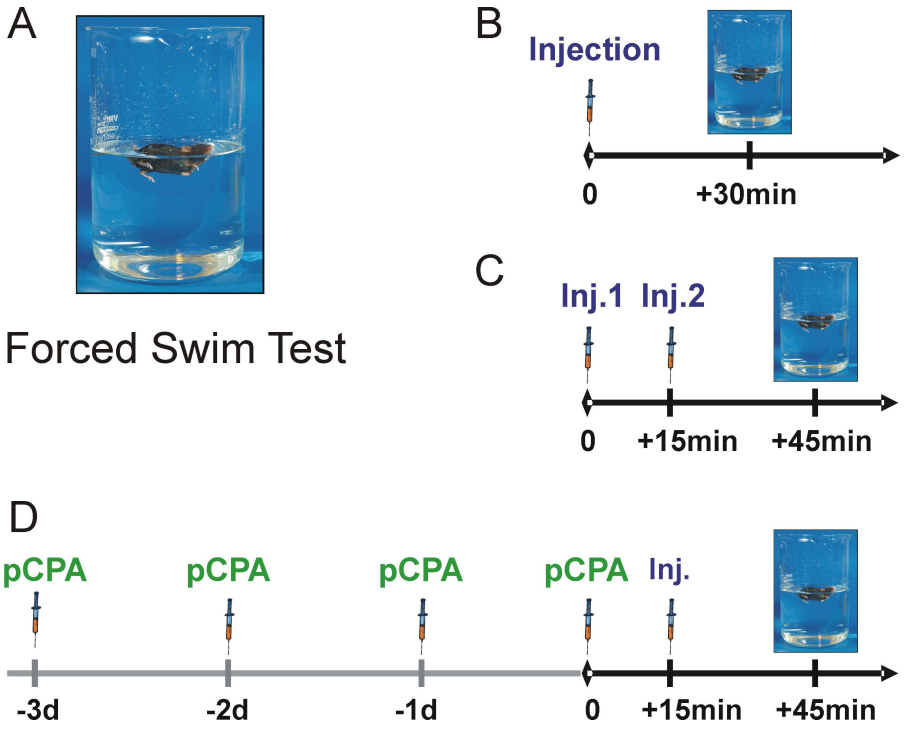

Fig. 4.1: Schematic illustration of injection procedures. **(A)** Forced swim test is performed in a water beaker filled with an appropriate water level, preventing the mouse from escaping and touching the beaker bottom. **(B-D)** Illustration of different injection procedures applied in this study. **(B)** Single drug injections were applied 30 min before starting the test. **(C)** If two drugs were applied the first drug was injected 15 min previous to the second drug, which was again injected 30 min before the test. **(D)** Treatment with the serotonin synthesis blocker pCPA required one injection daily for four days. The last injecting was 15 min before injection of the second drug 30 min before behavioral testing.

4.2.4. Behavioral Paradigms

Open field: To evaluate potential effects by the drugs on locomotor activity we performed an open field test. The open field was an 40cm x 40cm x 40cm (H x W x L) box, in which the animal was placed for 5 min to allow free exploration. The animal movement was recorded, and the distance moved was scored by the SMART program (PanLab, Spain).

Forced swim test: The paradigm was performed in a round glass beaker (18 cm in diameter) filled with tap water at 25 +/-0.5°C. The water level was approximately 20 cm to prevent the animals from touching the bottom of the glass. The mouse was also unable to climb off the beaker. The animal was carefully lowered into the water and recorded on DVD for 6 min. The first 2 min were not evaluated; however, floating behavior was scored for the following 4 min by an experimenter blind to genotype and treatment. Floating was defined by immobility of the animal and minimal movements to keep the body's balance. The functionality of the paradigm was successfully tested by an acute i.p. injection of the antidepressant drug imipramine (30 mg/kg), which resulted in a significant decrease in floating behavior as compared to saline treated animal (Fig. 4.2).

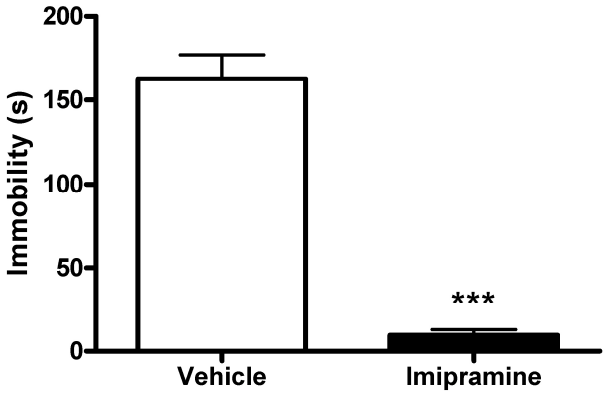

Fig. 4.2: Functional evaluation of the forced swim test. Treatment with the serotonin reuptake inhibitor imipramine (30 mg/kg) significantly decreased immobility compared to vehicle treatment (Veh) in the forced swim test. Data are expressed as means±SEM. n = 9; Students t-test, ***p < 0.001.

4.2.5. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). All behavioral endpoints of the open field and FST were analysed using Student t-test, one-way ANOVA followed by the Newman-Keuls test or two-way ANOVA, when genotype and treatment factors were combined in the same experiment. Graphs and statistics were generated by GraphPad Prism 4.03 Software. Results were considered to be significant at $p < 0.05$.

4.3. Results

4.3.1. Locomotor activity

To avoid potential confounding factors related to locomotor activity, all drugs were tested in the open field test. None of drugs and doses applied altered the distance moved, eliminating this otherwise confounding factor (Table 4.1).

Experimental Groups	Distance moved, Mean ± SEM	Statistics
Vehicle	1642 ± 182.6	$F_{4,38}$ = 0.59; ns
THC [0.1 mg/kg]	1574 ± 178.2	
THC [0.5 mg/kg]	1675 ± 189.1	
Rim [3 mg/kg]	1784 ± 190.9	
Rim [10 mg/kg]	1577 ± 135.9	
Vehicle + Vehicle	1752 ± 227.0	$F_{5,54}$ = 0.54; ns
Vehicle + THC [0.1 mg/kg]	1891 ± 171.0	
pCPA [100 mg/kg] + Vehicle	1697 ± 207.5	
pCPA [100 mg/kg] + THC [0.1 mg/kg]	1849 ± 161.4	
WAY [1 mg/kg] + Vehicle	1535 ± 116.3	
WAY [1 mg/kg] + THC [0.1 mg/kg]	1637 ± 176.2	
Vehicle + Vehicle	1668 ± 142.9	$F_{5,54}$ = 1.58; ns
Vehicle + Rim [10 mg/kg]	1740 ± 220.0	
pCPA [100 mg/kg] + Vehicle	1847 ± 115.0	
pCPA [100 mg/kg] + Rim [10 mg/kg]	1615 ± 128.5	
WAY [1 mg/kg] + Vehicle	1955 ± 102.1	
WAY [1 mg/kg] + Rim [10 mg/kg]	1435 ± 129.5	

Table 4.1: Evaluation of locomotor activity. Table illustrates the total distance moved during a 5 min period assessed in the open field. Data are expressed as standard error of means±SEM. n = 8-10; two way ANOVA, ns = not significant.

4.3.2. Antidepressant-like effect of THC requires serotonin transmission

Animals treated with a low dose of THC (0.1 and 0.5 mg/kg) showed a significant reduction in floating behavior ($F_{2,23}$ = 4.17; $p < 0.05$; Fig. 4.3A). The effect of THC (0.1 mg/kg) was prevented by a pre-treatment with a low dose (0.5 mg/kg) of rimonabant, (mean±SEM: Veh+Veh = 140±6.4 sec; Veh+THC = 113±8.9 sec; Veh+Rim = 141±7.8 sec; Rim+THC = 146±9.7 sec; $F_{3,62}$ = 3.16; $p < 0.05$; n = 17-18). In addition, THC effect was also prevented by a pre-treatment with the serotonin synthesis inhibitor pCPA 100 mg/kg ($F_{3,68}$ = 6.49; $p < 0.001$; Fig. 4.3C) or the 5-HT1A receptor antagonist WAY100635 1 mg/kg ($F_{3,35}$ = 3.48; $p < 0.05$; Fig. 4.3E). Applying rimonabant alone in high dose (3 and 10 mg/kg) also resulted in an antidepressant-like effect ($F_{2,19}$ = 10.74; $p < 0.001$; Fig. 4.2B). However, pCPA failed to block this effect ($F_{3,36}$ = 8.9; $p < 0.001$; Fig. 4.3D). Similarly, WAY100635 could not block rimonabant-induced reduction in immobility ($F_{3,34}$ = 12.68; $p < 0.001$; Fig. 4.3F).

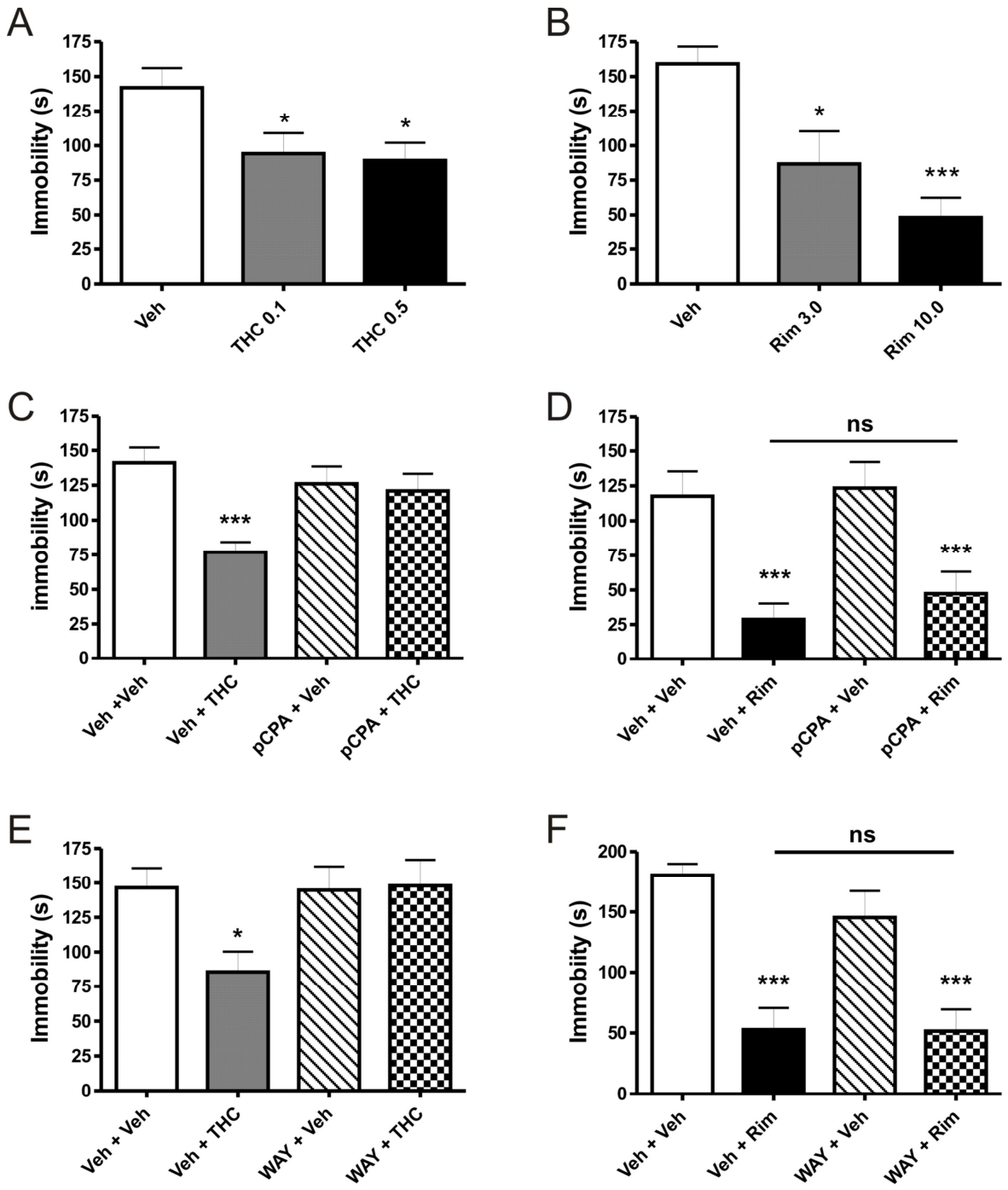

Fig. 4.3: Antidepressant-like effects of cannabinoids and the role of serotonin. Treatment with **(A)** THC (0.1 mg/kg and 0.5 mg/kg) and **(B)** rimonabant (Rim, 3 and 10 mg/kg) both decreased immobility in the forced swim test. The effect of THC (0.1 mg/kg) was attenuated when combined with **(C)** the serotonin synthesis inhibitor pCPA (100 mg/kg) or **(E)** the 5-HT_{1A} receptor antagonist WAY100635 (WAY, 1 mg/kg). The decrease in immobility induced by rimonabant (10 mg/kg), however, was not altered by **(D)** pCPA or **(F)** WAY100635. Data are expressed as mean±SEM. n = 9-15; *p < 0.05; ***p < 0.001; veh = vehicle; ns = non-significant. (These experiments were mainly performed by Max Grieb, a M.D. student in the Lutz' laboratory.)

4.3.3. Effect of THC is CB_1 receptor dependent

THC is known to bind other targets than the CB_1 receptor. In order to test whether the THC effects depend on CB_1 receptor activation, a *per se* ineffective dose of rimonabant was applied 15 min prior to the THC treatment. This ineffective dose attenuated the decrease in immobility induced by THC ($F_{3,62} = 3.16$; $p < 0.05$; Fig. 4.4)

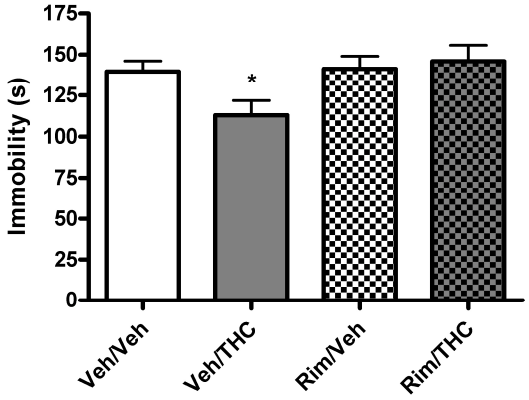

Fig. 4.4: THC effect depends on CB_1 receptor activity. Treatment with THC (0.1 mg/kg) significantly decreased immobility as compared to vehicle treatment (Veh) in the forced swim paradigm. This effect was blocked by a *per se* ineffective dose of rimonabant (Rim; 0.5 mg/kg). Data are expressed as means±SEM. n = 18; two way ANOVA, *$p < 0.05$.

4.3.4. Rimonabant effect depends on functional GABAergic CB_1 receptor expression

In order to test whether THC and rimonabant effects depend on CB_1 receptor activation on glutamatergic and GABAergic neuronal population, we tested these drugs in conditional mutant mice lacking CB_1 receptor specifically in either of these neuronal subpopulations. Analysis of the floating behavior in the conditional CB_1 receptor knock-out mice without treatment, revealed no significant change in floating time for GABA-$CB_1^{-/-}$ mutants (t_{11}=0.83, ns). These behavioral effects were still present in the subsequent tests, including vehicle treatments for the GABA-CB_1 (Fig. 4.5) and the Glu-CB_1 line (Fig. 4.6).

By testing drugs in these animals, we were able to show that the THC effect was still present in GABA-$CB_1^{-/-}$ mutants (Genotype factor: $F_{1,63} = 0.67$; ns; Treatment factor: $F_{1,63} = 17.9$; $p < 0.001$; Interaction: $F_{1,63} = 1.82$; ns; Fig. 4.5A). On the contrary, the antidepressant-like effect induced by a high dose of rimonabant was not detectable in GABA-$CB_1^{-/-}$ animals (Genotype factor: $F_{1,33} = 6.97$; $p < 0.05$; Treatment factor: $F_{1,33} = 14.64$; $p < 0.001$; Interaction: $F_{1,33} = 5.17$; $p < 0.05$; Fig. 4.5B).

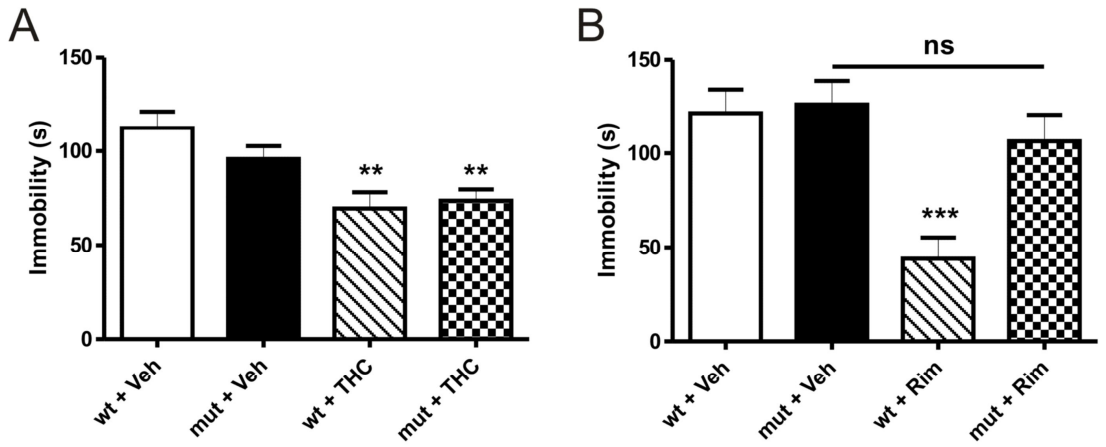

Fig. 4.5: CB_1 receptor deletion in GABAergic neurons blocks rimonabant but not THC effect. **(A)** While treatment with THC (0.1 mg/kg) still significantly decreased immobility in mice lacking the CB_1 receptor in forebrain GABAergic neurons (mut), **(B)** no decrease in immobility was detected in these mice after rimonabant (10 mg/kg) injection. Data are expressed as means±SEM. n = 12-15; two way ANOVA, **p < 0.01, ***p < 0.001. wt = GABA-$CB_1^{+/+}$, mut = GABA-$CB_1^{-/-}$. ns = non-significant

4.3.5. Effect of CB_1 receptor deletion from glutamatergic neurons requires serotonin

Analysis of the basic floating behavior without treatment, revealed a significant decrease in floating time for Glu-$CB_1^{-/-}$ mutants (t_{10} = 3.02; p < 0.01) without changes in open field activity (t_{16}=0.39, ns). This phenotype was unaltered in combination with vehicle injection (Fig. 4.6). Interestingly, the difference in phenotype in these animals was annulled by the pretreatment with the serotonin synthesis inhibitor pCPA (Genotype factor: $F_{1,46}$ = 1.47; ns; Treatment factor: $F_{1,46}$ = 1.72; ns; Interaction: $F_{1,46}$ = 4.33; p < 0.05; Fig. 4.6).

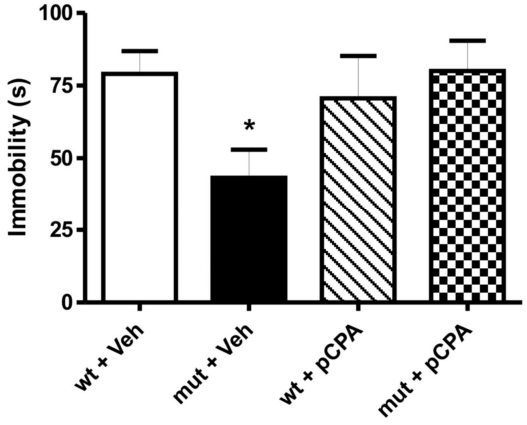

Fig. 4.6: Phenotype of genetic CB_1 receptor deletion in glutamatergic neurons depends on serotonin. Specific deletion of the CB_1 receptor in cortical glutamatergic neurons induced a decrease in imobility. Treatment with pCPA (100 mg/kg) blocked this effect. Data are expressed as means±SEM. n = 12-15; two way ANOVA, *p < 0.05. wt = Glu-$CB_1^{+/+}$, mut = Glu-$CB_1^{-/-}$.

4.4. Discussion

Our results confirm previous findings on the contradictory roles of the eCB system regarding stress coping. We could show that low dose of THC (0.1 and 0.5 mg/kg) or high dose of rimonabant (3 and 10 mg/kg) led to a decrease of immobility, indicating an increased stress coping behavior. THC effects were prevented by a *per se* ineffective dose of rimonabant, showing the CB_1 receptor dependence of the THC effect. Remarkably, inhibition of serotonin synthesis, and 5-HT1A receptor blockade, respectively, was also able to prevent the effects of THC, but not of rimonabant. On the other hand, using a genetic approach, we could show that the effects of rimonabant, but not of THC, for the doses used in this study, seemed to depend exclusively on CB_1 receptor in GABAergic neurons. Low doses of THC might act mainly via other CB_1 receptor populations, potentially on glutamatergic neurons, even though there is no final proof for this notion.

These findings suggest that, even though both drugs have antidepressant-like properties, they seem to interfere with different circuits, serotonergic transmission being important for the behavioral response to low doses of THC. The antidepressant-like effect of this drug is in line with previous data obtained with other cannabinoids (Jiang et al., 2005; Hill and Gorzalka, 2005; Bambico et al., 2007). In addition, the importance of serotonin for cannabinoid effects has also been proposed previously. For instance, Bambico et al. (2007) demonstrated that the antidepressant-like effects of WIN-55,212-2, a synthetic cannabinoid agonist, could be blocked by pCPA in rats. Likewise, WAY100635 blocked the effect of cannabidiol, a non-psychotomimetic phytocannabinoid, in mice in the FST (Zanelati et al., 2010). Finally, the same 5-HT1A receptor antagonist also blocked the anxiolytic-like effects of THC in rats (Braida et al., 2007). The latter result is highly congruent with our findings, as anxiolytic drugs can also have antidepressant-like effects and *vice versa* (Jiang et al., 2005; Höschl and Svestka, 2008).

A relevant neuronal circuit in respect to our findings might be the projection between prefrontal cortex (PFC) and serotonergic neurons in the raphe nuclei, which is modulated by cannabinoids, as proposed by Bambico et al. (2007). The PFC, a region centrally involved in the processing and evaluation of a stressful situation, has strong glutamatergic connections with the raphe nuclei (Jankowski and Sesack, 2004). Interestingly, the connection seems to be indirect, as decrease in excitatory drive leads to an increased serotonin transmission. Thus, the local CB_1 receptor activation on glutamatergic terminals in the PFC by WIN55,212 resulted in an increased firing of serotonergic neurons (Bambico et al., 2007). Earlier studies already

suggested that an impaired excitatory input from the PFC onto inhibitory neurons in the raphe nuclei, finally leading to an increased serotonin transmission and subsequently to decreased anxiety and stress levels (Celada et al., 2001).

Regarding the effects of high doses of rimonabant, this seems to be in contrast to the clinical effects of this drug, which can induce anxiety and depression in patients (for reviews, see Moreira and Crippa, 2009; Moreira et al., 2009). However, the antidepressant-like effect of this CB_1 receptor antagonist given acutely or subchronically was previously shown in rats which were exposed to the FST (Griebel et al., 2005; Steiner et al., 2008a). One explanation could be the chronic use in clinical applications, resulting in the negative side effects. Also, one should keep in mind the clinical intent to reduce obesity by the rimonabant treatment. Obesity might sensitize the body to an increased susceptibility towards depressive behavior. Nevertheless, our data strongly suggest that this antagonist acts via the inhibition of CB_1 receptor on GABAergic terminals, since the decrease in floating induced by rimonabant was abolished when injected into GABA-$CB_1^{-/-}$ mutant mice. Why inhibiting serotonin transmission had no effect on the action of rimonabant is not clear. This seems to be in contrast to neurochemical data, showing that similar doses of rimonabant increased serotonin and catecholamine release in the prefrontal cortex (Tzavara et al., 2003). One possibility could be the systemic increase in GABAergic transmission as the result of the loss of GABAergic CB_1 receptor, which could attenuate the effect of an increased serotonergic transmission downstream in the stress circuit. Also possible would be an assisting role of serotonin transmission after rimonabant treatment. Catecholamines, which are also released, could be the driving force, thus covering the behavioral effect of blocking serotonin transmission. Nevertheless, the absence of the behavioral effects of rimonabant in the GABA-$CB_1^{-/-}$ mice provides a most plausible mechanism for the action of this drug regarding the antidepressant-like effect.

Interestingly, contrary to the rimonabant treatment, which seemed to block CB_1 receptor on GABAergic neurons, specific deletion of this receptor in GABAergic neurons (GABA-$CB_1^{-/-}$ mice) did not induce an antidepressant-like behavior. One explanation for this phenomenon could be developmental compensation processes. Similar effects have already been seen, in fact, also for the serotonergic system, as result of acute or chronic serotonin reuptake-transporter (5-HTT) inhibition, which even resulted in opposite behavioral responses (Haenisch and Bönisch, 2011; see below). However, GABA-$CB_1^{-/-}$ mice are still responsive to THC, suggesting that GABAergic CB_1 receptor is unlikely to be the target of low doses of THC. Therefore, we suggest a possible connection to CB_1 receptor on other neuronal

populations. Recent findings in our group have highlighted the importance of glutamatergic CB_1 receptors, showing that comparable doses of CB_1 receptor agonist failed to induce an anxiolytic effect in Glu-$CB_1^{-/-}$ mutants tested in the elevated plus maze (Aparisi Rey et al., 2012. Due to the fact that Glu-$CB_1^{-/-}$ mice showed a decrease in floating behavior without treatment, it was not possible to test the corresponding hypothesis in the FST, namely whether the low dose of THC still has an antidepressive effect. A similar decrease in immobility has already been observed previously in Glu-$CB_1^{-/-}$ mice (Steiner et al., 2008b). This increased stress-coping behavior in Glu-$CB_1^{-/-}$ mice is in contrast with other studies in which these mutants actually showed increased anxiety-like responses (Lafenetre et al., 2009, Jacob et al., 2009). Thus, the decrease in floating behavior may rather be an inadequate fear response than a positive stress coping behavior. This situation would prevent a stringent interpretation of a possible antidepressant-like effect of THC in Glu-$CB_1^{-/-}$ mice.

The present data also suggest that the behavioral changes in the FST in Glu-$CB_1^{-/-}$ may depend on serotonergic transmission, as it was blocked by pCPA. Why the inhibition of serotonin transmission blocked this effect is not clear. One possibility might be a continuing over-excitation of the serotonergic neurons via a different pathway as suggested above, caused by a general elevated excitatory drive. A similar phenomenon was seen with pharmacological and genetic modulation of 5-HTT function. While an acute treatment with 5-HTT inhibitors showed antidepressant effects and led to a decreased anxiety level, the complete deletion of 5-HTT had opposite effects (Haenisch and Bönisch, 2011). Thus, life-long increased serotonin levels in mice lacking the 5-HTT were shown to result in elevated anxiety (Holmes et al. 2003; Ansorge et al. 2004; Kalueff et al., 2007) and stress level (Tjurmina et al., 2002; Jiang et al. 2009; Wellman et al. 2007). Interestingly, an early depletion of serotonin by pCPA treatment could block these effects of 5-HTT deletion (Persico et al., 2001; Alexandre et al., 2006).

In summary, by using pharmacological and genetic approaches, we could provide new insights into how to reconcile the contradictory findings on antidepressant-like effect of CB_1 receptor agonists and antagonist. The effects of low doses of cannabinoid agonists clearly depend on serotonin transmission. High doses of antagonists, on the other hand, dominantly act via CB_1 receptor on GABAergic neurons and depend, at least partly, not on serotonergic transmission. Our data further suggests a two-neuronal subpopulation model in which glutamatergic and GABAergic neurons, under the control of the CB_1 receptor, seem to be differently sensitive to cannabinergic drugs.

Chapter 5

Serotonin release is directly controlled by cannabinoids

Chapter 5. Serotonin release is directly controlled by cannabinoids

5.1. Introduction

Serotonin is present in many tissues, where it functions as a neurotransmitter or hormone. In the brain, the highest number of serotonergic neurons can be detected in midbrain and brainstem areas, called raphe nuclei (Baker et al., 1991; Carkaci-Salli et al., 2011). Projections from the raphe nuclei innervate nearly every regions of the CNS, including spinal cord, hypothalamus, cortex, hippocampus, amygdala and striatum. This dense anatomical distribution is in line with the various behavioral functions which can be modulated by serotonergic signalling. As pointed out in the Chapter 5 several investigations suggest the eCB system as a control system of serotonergic transmission, which might be the bases for new approaches for the treatment of anxiety disorders. Hence, both systems have been shown to influence numerous physiological functions and to control a wide range of behaviors and emotional states. Importantly, interaction had already been proven at the behavioral level: Specific cannabinoid-induced emotional changes were shown to depend on a functional serotonin transmission. Thus, a combined strategy of behavioral, electrophysiological and molecular approaches revealed major insights into the importance of serotonin in the anti-depressant effect of CB_1 receptor activation in the PFC (Gobbi et al., 2005; Bambico et al., 2007). The site of action of this effect has not yet clarified, as the CB_1 receptor is mainly expressed by GABAergic and glutamatergic neurons in cortical regions, such as the PFC, suggesting an indirect way on how cannabinoids modulate serotonin release. Interestingly, the administration of the fatty acid amide hydrolase inhibitor URB597 also increased the firing of serotonergic neurons in the dorsal raphe nucleus and increased hippocampal levels of serotonin after repeated URB597 administration (Gobbi et al., 2005).

CB_1 receptor presence was verified in GABAergic, adrenergic, glutamatergic and cholinergic neurons (e.g., Marsicano and Lutz, 1999; Kathmann et al,. 1999; Hajos & Freund 2002; Wallmichrath & Szabo, 2002; Monory et al., 2006). Our results presented in Chapter 4 suggest an indirect interaction between the eCB system and serotonin release.

Thus, CB_1 receptor activation regulates neuronal populations, which innervate serotonergic neurons in the raphe nuclei. Rather recently, however, anatomical evidence revealed a potential mechanism of cannabinoids to directly regulate serotonin transmission, as we could identify the expression of the CB_1 receptor in serotonergic neurons at mRNA and protein level (Häring et al., 2007). This suggests a possibility for cannabinoids to directly regulate the activity of serotonergic neurons. A dense distribution of serotonergic terminals can also be found in the PFC, which, if as our results suggest, express CB_1 receptor, offers an additional explanation for the results observed by Gobbi and colleagues (2007). Evidence for a direct interaction between the eCB system and the serotonergic system has already been suggested by earlier studies. Thus, the lack of CB_1 receptor reduces the responsiveness of mice to the anxiolytic drug buspirone, a serotonin (5-hydroxytryptamine, 5-HT) receptor agonist (Uriguen et al. 2004). In vitro studies showed that the release of serotonin can be altered by the CB_1/CB_2 receptor agonist WIN552122 and the CB_1 receptor antagonist rimonabant in mouse cortex slices (Nakazi et al., 2000).

Given that the CB_1 receptor activation can modify serotonin release (Nakazi et al., 2000; Gobbi et al., 2005), it is possible that this function is exerted either by direct CB_1 receptor-mediated control of serotonergic neurons and/or by indirect CB_1 receptor-dependent modulation of afferent fibers projecting to serotonergic neurons. A necessary prerequisite to support the first hypothesis is the presence of the CB_1 receptor on serotonergic neurons at single cell level. This could be achieved by detecting the coexpression of the CB_1 receptor and a marker gene for serotonergic cells, tryptophane hydroxylase 2, TPH2 (Patel et al., 2004), using double *in situ* hybridization experiments (Häring et al., 2007). Furthermore, double fluorescence immunohistochemistry and confocal analysis revealed also the existence of the CB_1 receptor protein on serotonergic terminals (Häring et al., 2007). Here, the serotonin reuptake transporter (5-HTT) was used as a marker for serotonergic neurons (Zhou et al., 1998). The results indicated that a low number of serotonergic neurons in the raphe nuclei and of serotonergic fibers in the hippocampus and amygdala contain the CB_1 receptor mRNA and protein, respectively.

The question has remained whether these very low CB_1 receptor levels are functionally important. In order to analyse the physiological role of the CB_1 receptor on serotonergic neurons we aimed at generating a conditional mutant mouse line specifically depleted of the CB_1 receptor in serotonergic cells of the CNS. To address this task we applied a strategy using the Cre-loxP system. It exploits the mechanism that the Cre recombinase initiates a recombination event at two specific loxP sites, allowing the deletion of desired sequences

between two loxP (locus of crossover (x) in P1) sites (Hoess et al., 1982; for review see Branda and Dymecki, 2004).

Therefore, we crossed two existing transgenic mouse lines in order to achieve a novel conditional mouse line, in which serotonergic neurons are depleted of the CB_1 receptor. One initial line possessed the CB_1 receptor coding region flanked by loxP sites (Marsicano et al., 2003), the other one possessed a tamoxifen-dependent Cre recombinase gene under the control of the TPH2 regulatory sequences (Weber et al., 2009). Tamoxifen dependence of the Cre recombinase is achieved by fusing a mutated ligand binding domain of the human estrogen receptor with the Cre recombinase enzyme, named Cre-ER^{T2} (Brocard et al., 1998; Indra et al., 1999, Branda and Dymecki, 2004). The mutation further prevents endogenous estrogen from being recognized as ligand. Taken together, this mouse model allowed a temporal control over the recombination event and by this allows avoiding potential disturbance from developmental effects.

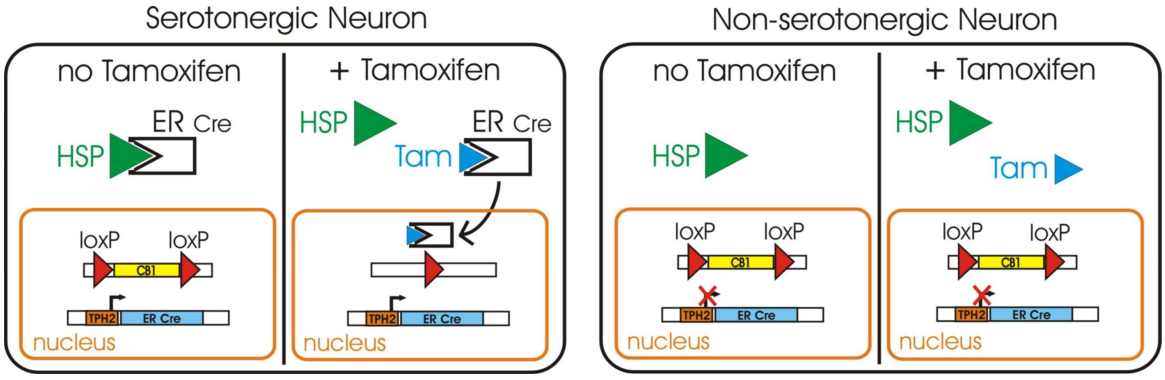

Fig. 5.1: Tamoxifen inducible and TPH2 specific deletion of CB_1 receptor. Cre-ER^{T2} recombinase under the control of TPH2 regulatory sequences is expressed specifically in serotonergic neurons. Mutated estrogen receptor ligand binding domain of Cre-ER^{T2} binds to heat shock protein (HSP), thus preventing the translocation to the nucleus. In presence of tamoxifen (Tam) HSP cannot bind, Cre-ER^{T2} is translocated into the nucleus and recombination (i.e. deletion of the cannbinoid receptor ORF) between the loxP sites can take place.

Molecular and behavioral analysis of mutants and wild-type littermates, both treated with tamoxifen, revealed the functionality of our system. Therefore, this study provides for the first time a clear evidence for the physiological relevance of the low CB_1 receptor levels in serotonergic neurons.

5.2. Materials & Methods

5.2.1. Breeding strategy

The novel conditional mutant mouse line, named $CB_1^{flox/flox;tph2\text{-}creERT2}$ and lacking the CB_1 receptor from central serotonergic neurons was generated through a three-step process. As a first step, homozygous CB_1-flox ($CB_1^{flox/flox}$) mice (Marsicano et al., 2003) were crossed with mice bearing a tamoxifen-inducible Cre-ER^{T2} recombinase expressed under the regulatory control of the mouse TPH2 gene locus (kindly provided by Tillmann Weber and Dusan Bartsch, Department of Molecular Biology, Central Institute of Mental Health, Mannheim; Weber et al., 2009). In a second step, heterozygous Cre-expressing/CB_1-flox mice ($CB_1^{flox/+;tph2\text{-}CreERT2}$) were again crossed with CB_1-flox mice to obtain homozygous Cre-expressing/CB_1-flox mice ($CB_1^{flox/flox;tph2\text{-}CreERT2}$). At last, male $CB_1^{flox/flox;tph2\text{-}CreERT2}$ mice were bred with $CB_1^{flox/flox}$ females to generate littermate experimental animals ($CB_1^{flox/flox;tph2\text{-}CreERT2}$, referred to as TPH2-$CB_1^{-/-}$ and $CB_1^{flox/flox}$, referred to as TPH2-$CB_1^{+/+}$). For this study adult (10-17 week old) male TPH2-$CB_1^{-/-}$ and TPH2-$CB_1^{+/+}$ mice were used. Animals were housed in a temperature- and humidity-controlled room with a 12 h light-dark cycle (lights on at 1 am) and had access to food and water *ad libitum*.

5.2.2. Genotyping

Genotyping of the mice were done by polymerase chain reaction (PCR) from genomic DNA, a technique allowing the amplification of specific DNA-sequences, provided that the flanking sequences are known (Mullis et al. 1986).

DNA preparation: Genomic DNA was prepared from tail tips (0.5-1cm). They were then treated o/n at 56°C with 550 µl tail buffer (50 mM Tris-HCl (pH 8), 0.1 M EDTA, 500 mM NaCl, 1% SDS) and 40 µl proteinase K (10 mg/ml stock-concentration) in a shaking (800 rpm) thermomixer (Eppendorf). On the next day, the probes were shaken vigorously for 5 min. Then, 300 µl 7 M NaCl were added and the probes were shaken again for 5 min. Afterwards, the probes were centrifuged at 13000 rpm (Centrifuge 5417C, Eppendorf) for 10 min at RT. 750 µl solution was then carefully transferred from top phase into a new tube. For precipitating the DNA, 500 µl isopropanol were added and the solution mixed by inversion. This was followed by a centrifugation at 13000 rpm for 5 min at RT (Centrifuge 5417C, Eppendorf). Then, the supernatant was removed as complete as possible, and the pellet was washed with 200 µl of 70% ethanol. After removing the ethanol completely (pipetting and

evaporation), the dried pellet was dissolved in 200 µl TE buffer (100 mM Tris-HCl, pH 7.2, 10 mM EDTA, pH 8) by vortexing it gently and subsequently stored at 4°C until use. The purified genomic DNA was then tested using the following PCR program and primers.

Primer for CreERT2-locus

Forward Primer G100	5´CGGCATGGTGCAAGTTGAATA-3´
Reverse Primer G101	5´-GCGATCGCTATTTTCCATGAG-3´

Primer for CB_1-flox-locus

Forward Primer G50	5´-GCTGTCTCTGGTCCTCTTAAA-3´
Reverse Primer G51	5´-GGTGTCACCTCTGAAAACAGA-3´

1x PCR-Reaction

5x Reaction Buffer	5.00 µl
$MgCl_2$ (25 mM)	2.50 µl
dNTPs (5 mM)	1.00 µl
Primer G100	1.25 µl
Primer G101	1.25 µl
Primer G50	1.75 µl
Primer G51	1.75 µl
Go Taq Polymerase	0.12 µl
H_2O	9.88 µl
Template DNA	1.00 µl

PCR program:

» 95° for 3 min (pre-heating)

» 37x [95°C for 1 min; 54°C for 1 min; 72°C for 1 min] (amplification)

» 72°C for 5 min

» 4°C storage until use

5.2.3. Induction of recombination

Due to the mutated estrogen receptor sequence, tamoxifen has to be present to allow the Cre recombinase access to the nucleus and induce the recombination. In order to prove this scheme, animals were injected with tamoxifen and evaluated for Cre recombinase localization and for recombination.

Tamoxifen dependence of the Cre recombinase localization: Before sacrificing the animals, each animal was injected 4 h, 2 h and 30 min, either with vehicle (90% sun flower seed oil and 10% ethanol; Sigma Aldrich) or 1 mg tamoxifen per injection (dissolved in vehicle solution).

For IHC, mice were deeply anesthetized with pentobarbital and trans-cardially perfused with 4% PFA solution (dissolved in phosphate buffered saline; PBS). After isolation, the brains were post-fixed for 24 h in 4% PFA solution, treated with 30% sucrose / PBS solution for 48 h and stored at -80°C until use. For section preparation, 30 µm thick brain slices were prepared on a Microm HM560 cryostat, and then stored at -20°C in cryoprotection solution (25 v/v % ethylene glycol; 25 v/v % glycerol; 50 v/v % PBS) until use. To determine the distribution of Cre recombinase protein in serotonergic cells, immunohistochemical experiments were performed, using antibodies against TPH (mouse monoclonal antibody, cat# T0678 Sigma Aldrich) and Cre recombinase (rabbit polyclonal antibody, kind gift from Matthias Klugmann). In short, all incubation steps were performed in wells of a 12-well-plate (100-400 µl solution per well) on a wave shaker (Heidolph) at RT. Sections were first rinsed from cryoprotection solution in (10 min) and then pre-incubated in blocking solution (4% normal goat serum, 0.3% Triton X-100 in PBS) for 1 h. After the blocking, the sections were treated o/n with the primary antibodies, which were dissolved in blocking solution. On the next day, the sections were washed in 1x PBS for 5x 10 min at RT and then incubated for 2 h with the matching secondary antibodies, also dissolved in diluted blocking solution; Alexa 488 labelled anti-mouse-IgG from goat 1:1000 (cat# A11001, Invitrogen) for anti-TPH-antibody, and Alexa 546 labelled anti-rabbit-IgG from goat 1:1000 (cat# A11081, Invitrogen) for anti-Cre-IgG. The incubation was followed by five washing steps in 1x PBS-T (1x PBS/0.1% Triton X-100). Sections were counterstained with Hoechst 33258 (2 µg/µl). After the counterstaining, the sections were washed for 2x 10 min in 1x PBS, then carefully transferred into a Petri dish filled with 1x PBS. Sections were then mounted on glass slides to dry for 2-4 h at 37°C. The remaining salt was washed away by dipping the slides for 2 sec into distilled water. Finally, the sections were dried overnight in a dust free environment at RT and covered with MOWIOL (cat# 475904, Calbiochem). Omission of primary anti-sera resulted in no detectable signal (data not shown).

Evaluation of recombination by PCR: The basic recombination event was analysed by PCR, using the following program and primers. Animals were treated with tamoxifen (2 mg/day; i.p.) or vehicle for 5 days. In the following week mice were sacrificed by decapitation after isofluran anesthesia. The brain and duodenum were removed. By using a brain matrix, the brain was cut into 1-mm sections. Using a 1-mm puncher, several brain regions were isolated. The duodenum was divided into two pieces and rinsed in 1x PBS to remove digested material. For both, brain and duodenum tissue, DNA isolation was performed as follows: samples were

treated o/n at 56°C with 500 µl tail buffer (100 mM Tris-HCl (pH 8), 5 mM EDTA, 200 mM NaCl, 0.2% SDS) and 10 µl proteinase K (10 mg/ml stock-concentration) in a shaking (800 rpm) thermomixer (Eppendorf). On the next day, the probes were centrifuged for 2 min at 13,000 rpm. The supernatant was collected and mixed by inversion with 500 µl phenol chloroform mixture (Sigma cat P2069). Following another centrifugation step for 5 min at 13.000 rpm, the upper (aqueous) phase was transferred and mixed by inversion with 500 µl chloroform. The probes were again centrifuged for 5 min at 13,000 rpm. Subsequently, the supernatant was transferred, mixed by inversion with 500 µl isopropanol and centrifuged for 5 min at 13,000 rpm. The supernatant was completely removed and the pellet washed with 70% ethanol at again centrifuged for 5 min at 13000 rpm. After removing the supernatant, the pellet was air dried at RT for 10 min and dissolved in 10 µl PCR grade H_2O. The DNA was tested for the recombination event by using the following PCR program and primer.

Primer for recombination in CB_1-flox locus

Forward Primer G50	5´-GCTGTCTCTGGTCCTCTTAAA-3´
Reverse Primer G53	5´-CTCCTGTATGCCATAGCTCTT-3´

1x PCR-Reaction

5x Reaction Buffer	5.00 µl
$MgCl_2$ (25 mM)	2.50 µl
dNTPs (5 mM)	1.00 µl
Primer G50	1.75 µl
Primer G53	1.75 µl
Go Taq Polymerase	0.12 µl
H_2O	9.88 µl
Template DNA	1.00 µl

PCR program:

» 95° for 3 min (pre-heating)

» 30x [95°C for 1 min; 54°C for 1 min; 72°C for 45 sec] (amplification)

» 72°C for 5 min

» 4°C storage until use

5.2.4. Design of behavioral experiments

Animals were group housed (3-5 animals per cage) in a temperature- and humidity-controlled room with a 12 h light-dark cycle. One week before behavioral testing, mice were single housed to avoid behavioral differences between dominant and subordinate animals. If not

stated otherwise, experiments were performed one hour after turning off the lights (2 pm), in the active phase of the animals, with only a dim red light source in the room (0 lux).

Adult male TPH2-$CB_1^{+/+}$ and TPH2-$CB_1^{-/-}$ animals were treated with tamoxifen (2mg/day for 5 days i.p.) between 10-17 weeks of age to induce recombination. A three-week waiting period followed to ensure the degradation of the remaining CB_1 receptor transcript and protein. In the 3rd week, mice were separated, followed by behavioral analysis, starting at the 4th week after the treatment (see Fig. 5.2). If not stated otherwise, behavioral experiments were performed in the active (dark) phase of the animals. All apparatuses and boxes used were cleaned with 70% ethanol after each test of a mouse and with water after each animal. Experiments were recorded on DVD, and the behavioral performance evaluated by hand or SMART software (Panlab, Spain).

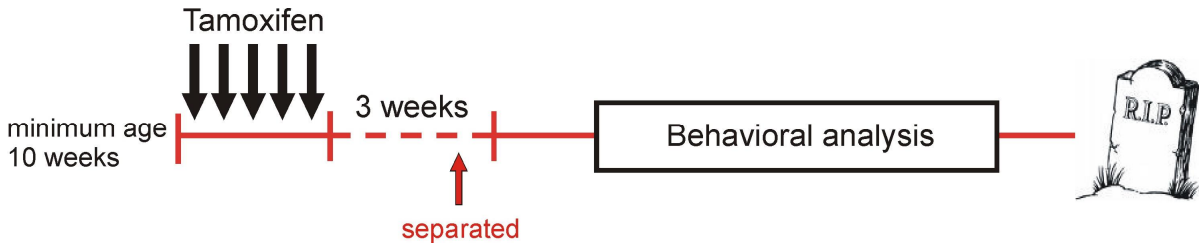

Fig. 5.2: Illustration of experimental schedule. Adult male wild-type (TPH2-$CB_1^{+/+}$) and mutant (TPH2-$CB_1^{-/-}$) animals were treated with tamoxifen (2mg/day for 5 days, i.p.) to induce recombination. A 3-week waiting period followed, in which remaining CB_1 receptor transcript and protein degraded. In the 3rd week, mice were separated, followed by behavioral analysis, starting the 4th week after the treatment.

5.2.5. Behavioral paradigms

Body weight and food consumption: Body weight and food consumption were evaluated during the whole behavioral analysis in weekly intervals.

Open Field: To evaluate locomotor activity an open field test was performed. The open field test is a general exploration test with a basic anxiety component. The test was performed in a plastic open field chamber (41 cm x 42 cm x 70 cm [H x W x L]).

Rota Rod: To evaluate motor skills animals were tested in a 5 day protocol on the RotaRod apparatus from "Ugo Basile" (38 cm high, 44 cm long and 32 cm deep). It consisted of five 3 cm diametric rotors with six flanges, dividing the rotor into five lanes, where five mice can walk simultaneously on the treadmill without seeing each other. Speed of the rod accelerates from 4 to 50 rpm (max. speed is reached after 180 sec). Time and speed until the animals fell down was registered. Cut off time of each trial: 210 sec. Each day the animals had to run 3 times with 5 min resting time between the trials. The RotaRod rotates at 4 rpm, while the mice was put on the bar.

Light Dark Test: The light dark test investigates basic anxiety behavior by using a box separated into one open white compartment (40 x 26 x 38.5 cm [H x L x W]) and one closed black compartment (40 x 13 x 38.5 cm [H x L x W]). Animals could move freely between the two compartments via an entrance into the closed compartment. Experiments were performed during the light phase (~ 200 lux above floor of light compartment). Animals were placed directly in front of the entrance. As soon as the animals entered the closed compartment, a 5 min test period was started. The entries into the open compartment as well as the time spent there were evaluated.

Novelty Suppressed Feeding: The novelty suppressed feeding paradigm measures basic anxiety behavior with acute food intake and body weight development. The test was performed in a brightly lit (450 lux) white plastic box (40 cm x 80cm x 80cm [H x W x L]) The floor was covered with a thin layer of saw dust. To not disturb the sleep-wake cycle, this test was performed during the light phase. In the center, a food pellet was placed bedded on a 18-cm filter paper. Animals were food deprived 24 h before the test, in order to increase the drive towards the presented food pellet. The animal was placed into the box for a maximum time of 10 min. Time was measured until the animal first touched the food pellet and until mice started eating. If the animal started eating, it was placed back into the home cage with food *ad libitum*. Food consumption was measured at different time points (5 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h).

Elevated Plus Maze: The elevated plus maze is 100 cm high; made of white plastic, with two closed and two open arms of 35 cm length and 6 cm width. The closed arms had additional black plastic walls of 20 cm height. Animal was placed into the center of the elevated plus

maze and was allowed to freely explore for a 5-min period. Time and entries on the different arms were measured.

Forced Swim Test: The paradigm was performed in a round glass beaker (18 cm in diameter) filled with tap water at 25 +/-0.5°C. The water level was approximately 20 cm to prevent the animal from touching the bottom of the glass. Mouse was unable to climb off of the beaker. The animal was carefully lowered into the water and recorded on DVD for 6 min. The first 2 min were not evaluated; however, floating behavior was scored for the following 4 min by an experimenter blind to genotype and treatment. Floating was defined by immobility of the animal and minimal movements to keep the body's balance.

Resident intruder test: The resident-intruder test was performed by placing a novel, group housed intruder into the home cage of the test animal for 10 min. This paradigm allows evaluating social exploration and aggressive behavior (Goyens and Noirot, 1975). To decrease interaction induced by the intruder, younger animals (males, 11-13 weeks of age) were used as intruders. Experiment was video-recorded, and the total interaction time of the animals spent exploring was measured by an experimenter blind to the genotype. Interaction was defined by any type of physical interaction clearly directed towards the partner. Duration and number of fights were evaluated separately. Fighting was defined by physical struggling between the interaction partners initiated by an attack of the resident towards the intruder.

Sociability Test: A modified sociability test was performed, based on a published protocol (Moy et al., 2004). In short, the test chamber (41 cm x 42 cm x 70 cm [H x W x L]) was divided into three compartments (40 cm x 40 cm x 22 cm [H x L x W]), all accessible by openings (7.5 cm x 10 cm [H x W]) in the dividing walls. Chambers and cages were cleaned with 70% ethanol between each trial to avoid olfactory cues. The total time during which the test animals spent in each of the compartments during sociability and social novelty phase was measured. Male C57BL/6N animals (10-12 weeks old) were used as interaction partners for the sociability and social novelty phase.

Habituation Phase: The test animal was placed into the middle compartment for 5 min with entries to the side compartments blocked.

Sociability Phase: After the habituation phase, blockades of the entries were removed, allowing free access to the side compartments. By doing this, the animal tested was exposed to a novel C57BL/6N interaction partner and a novel object (round cage described below),

positioned in the two side compartments. The position of the interaction partner (left vs. right compartment) was alternated between trials to avoid any bias. The interaction partner itself was enclosed in a round cage (10 cm in diameter; 30cm high [upper 20 cm Plexiglass, lower 10 cm covered by metal bars 1 cm apart to allow interaction but prevent fighting]). To minimize stress levels of the animals used as interaction partners, they were habituated to the cages four times for 10 min. To counterbalance individual differences of these interaction partners they were equally used for wild-type and mutant test mice. The novel object control (empty cage, no animal) was always positioned in the opposite compartment to the cage with the interaction partner.

Novel Object Recognition Task: As described in Chapter 3, the novel object recognition task combines a general exploration test with a visual recognition memory paradigm. The test was performed in a white plastic open field chamber (40 cm x 40 cm x 40 cm [H x W x L]). The protocol used was modified from Ennaceur and Delacour (1988), Tang et al. (1999), and Tordera et al. (2007). This experiment was performed by Alejandro Aparisi Rey (Ph.D. student in the Lutz' laboratory).

For habituation, the animals were placed into the empty open field and allowed to explore the box for 10 min once a day for two days. On day 3, two identical objects (O1 left, and O1 right; two metal cubes with 4 cm x 3 cm x 5 cm [H x W x L]) were placed symmetrically 6-7 cm from the walls and separated 16-18 cm from each other. The mouse was placed into the box at an equal distance from both objects and video-recorded for 10 min. After this first exposure to the object, the mouse was returned to its home cage.

2 h and 24 h later, the mouse was placed again into the open field and exposed to the familiar object (O1) and to a novel object (O2 for the 2 h time point, and O3 for the 24 h time point, respectively) each time for 10 min (retention tests). The novel object O2 was a plastic billiard ball (5.72 cm in diameter) fixed on a metal plate (0.2 cm) and O3 was a round glass flask (6 cm x 3 cm [H x W]), filled with sand and closed with a black rubber plug. The familiar object was always positioned on the left side, while the new object was on the right side. Box and objects were cleaned with 70% ethanol after each trial to avoid olfactory cues. Experiment was video-recorded and the total time that the animal spent exploring each of the two objects in training and retention phase was evaluated by an experimenter blind to the genotype. Object exploration was defined as the orientation of the nose directly to the object at a distance <2 cm and/or touching the object with the nose and whiskers. Time spent climbing and sitting on the object were not regarded as exploration, and was therefore excluded from

measurement (Ennaceur and Delacour, 1988), as these activities do not present a form of exploration of the object but rather of the environment. In contrast to chapter 4 we calculated here the recognition index which represents the percentage of time the animals spend with the familiar (F) object [(F)/(Novel Object + F)].

5.2.5. Cotricosterone measurements

Serotonin is known to influence the HPA axis, thus, plasma corticosterone levels were evaluated in non-stressed (directly taken from the home cage) and stressed (killed after FST paradigm) animals. Mice were decapitated, and the blood was collected in EDTA di-Calcium containing collection tubes (KABE Labortechnik, EDTA1000A). Plasma was separated from blood cells by centrifugation of the blood at 13,000 rpm for 5 min at 4°C and was stored until use at -20°C. Corticosterone concentration was analysed using the Corticosterone EIA Kit (IBL, IB79112) and measured by the FLUOstar Galaxy from BMG Labtech.

5.2.6. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of individual data points. Results were considered to be significant at $p < 0.05$. All behavioral endpoints were initially analyzed using unpaired Student's t-test or two-way ANOVA, using genotype and object as variables and Bonferroni post-tests to correct for multiple comparisons.

5.3. Results

5.3.1. Genotyping of CB1$^{flox/flox;\ tph2\text{-}Cre\text{-}ERT2}$

By using the primers G100 (Cre forward primer), G101 (Cre reverse primer), G51 (CB1 forward primer) and G51 (CB1 reverse primer), the respective band for Cre-ER^{T2} (400 bp) and $CB_1^{flox/flox}$ locus (500 bp) was detected, enabling the evaluation of the genotypes (Fig. 5.3).

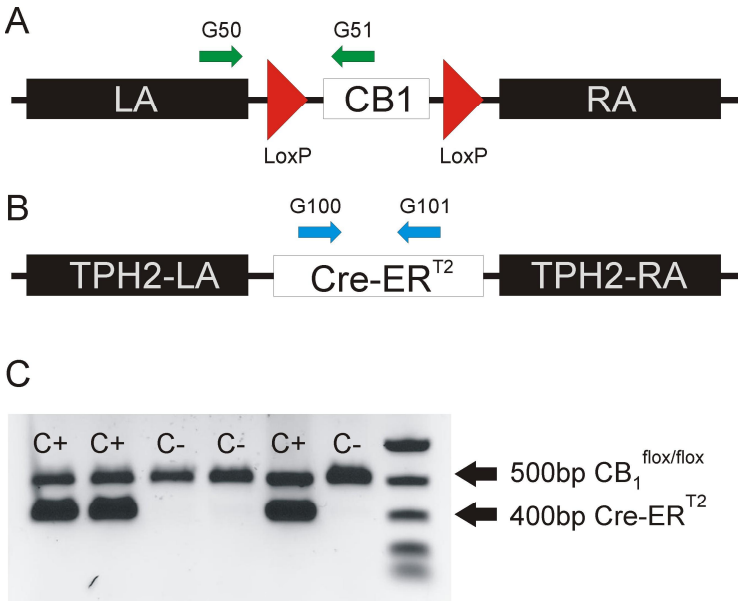

Fig. 5.3: Genotyping results. Schematic illustrations of **(A)** $CB_1^{flox/flox}$ locus and **(B)** TPH2-Cre-ER^{T2} locus. **(C)** Detection of the respective bands for Cre-ER^{T2} (400 bp) and $CB_1^{flox/flox}$ (500 bp). C+ = animals positive for the Cre-ER^{T2} gene and $CB_1^{flox/flox}$. C- = $CB_1^{flox/flox}$ animals lacking the Cre-ER^{T2} gene. LHA = left homology arm; RHA = right homology arm

5.3.2. Cre-ER^{T2} expression is specific for serotonergic neurons

Immunohistochemical detection of the Cre-ER^{T2} revealed an exclusive expression on serotonergic neurons (Fig. 5.4), i.e., Cre-ER^{T2} was only present in the raphe nuclei, but not in other brain regions.

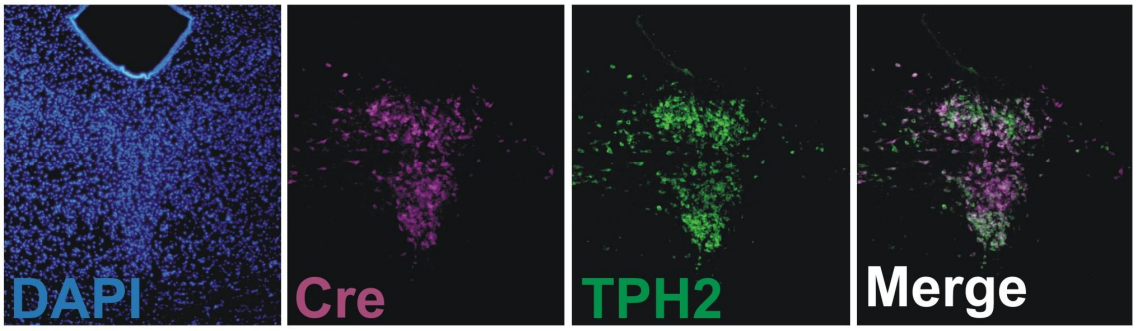

Fig. 5.4: TPH2-specific expression of Cre-ER^{T2} recombinase. False color representative images showing distribution of Cre-ER^{T2} (magenta) in TPH2 (green) specific cells of the dorsal raphe nucleus.

5.3.3. Cre-ER^{T2} localization in the nucleus is induced by tamoxifen

As illustrated in Figure 5.1 tamoxifen enables the Cre-ER^{T2} to translocate into the nucleus. One approach to evaluate the functionality of this inducible system was the visualization of this translocation. In fact, after treatment with tamoxifen the Cre-ER^{T2} staining is detectable in the nucleus and cytoplasm (Fig. 5.5A-A``). If no tamoxifen is present, the Cre-ER$^{T2}$ staining is limited to the cytoplasm of the cell (Fig. 5.5B-B``). No staining is visible in Cre-ER^{T2} deficient mice (Fig. 5.5 C-C``).

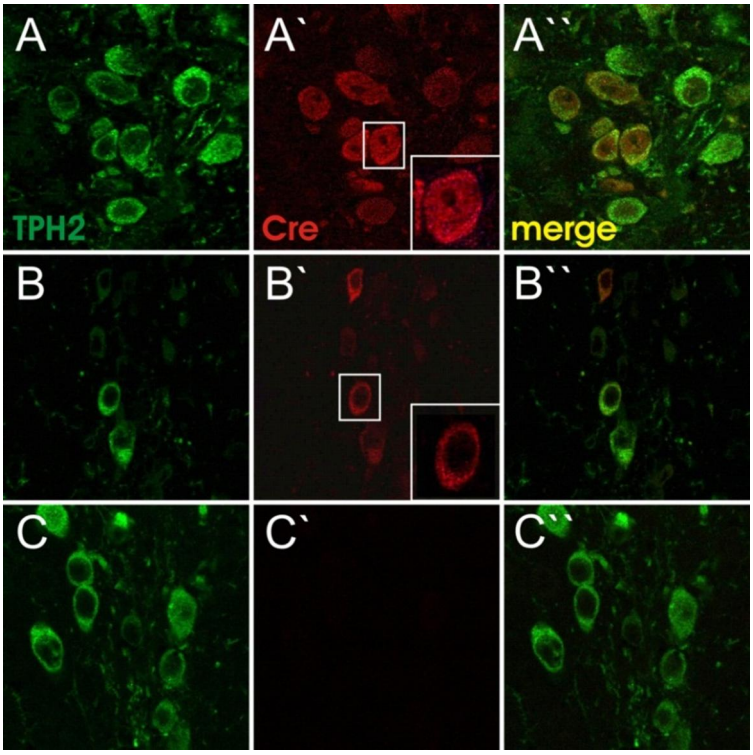

Fig. 5.5: Tamoxifen-dependent transport of Cre-ER^{T2} into the nucleus. Images showing cellular distribution of TPH2 (green, left column) and Cre-ER^{T2} (red, middle column) in the raphe nuclei. Cre-ER^{T2} staining is detectable within the nucleus if tamoxifen is present **(A-A``)**, in absence of tamoxifen Cre-ER$^{T2}$ staining is limited to cytosol **(B-B``)**. No specific staining is detectable on brain sections lacking Cre-ER^{T2} **(C-C``)**.

5.3.4. Recombination event is induced by tamoxifen

The recombination event requires the presence of Cre-ER^{T2} and tamoxifen. As shown above, Cre recombinase can only be found in serotonergic neurons positive for TPH2, which are restricted to the mid- and hindbrain raphe nuclei (Fig. 5.4; Patel et al., 2004). In fact, in animals positive for the Cre-ER^{T2}, the respective band indicating the recombination at the $CB_1^{flox/flox}$ locus (600 bp; Fig. 5.6A) was limited to the raphe region and only detectable after

treatment with tamoxifen (Fig. 5.6B). Thus, no band was detectable in all regions tested for animals lacking the Cre-ER^{T2} as well as in animals possessing the Cre-ER^{T2}, but were not treated with tamoxifen (see Fig. 5.6C).

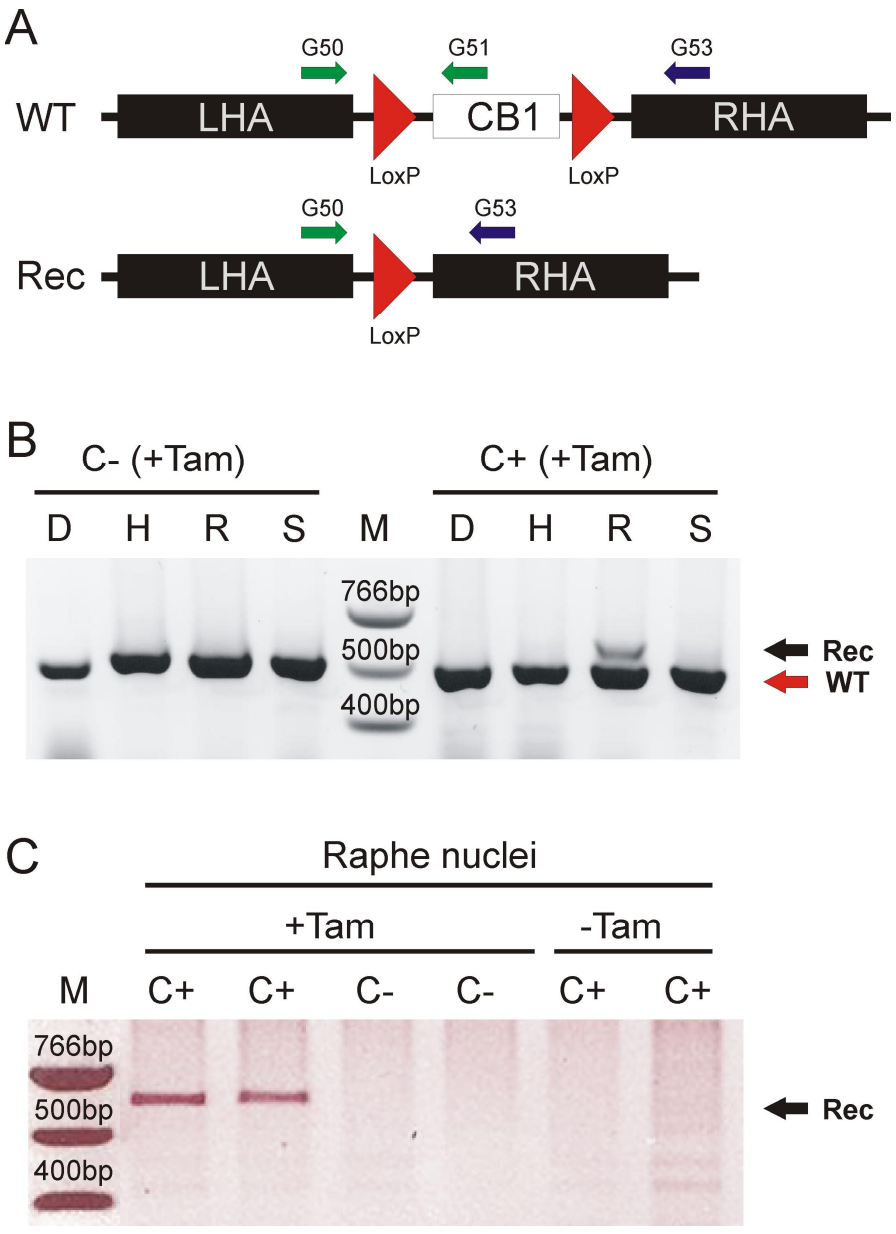

Fig. 5.6: Induction of the recombination event. (A) Schematic illustration of the CB_1 receptor locus before (WT) and after recombination (Rec), including the primer localization. **(B)** Respective band indicating a successful recombination event (600 bp) was limited to the raphe region. **(C)** The recombination band can only be detected in animals positive for Cre-ER^{T2} after treatment with tamoxifen. C+, Cre-ER^{T2} positive; C-, Cre-ER^{T2} negative; +Tam, tamoxifen treated; -Tam, no tamoxifen. D = duodenum; H = hippocampus; R = raphe: S = striatum.

5.3.5. Evaluation of body weight and feeding efficacy

Deletion of the CB_1 receptor in serotonergic neurons leads to an attenuated body weight development. Thus, comparison of body weight and feeding behavior between the mutants and their respective wild-type littermates displayed a significantly lower increase in body weight after tamoxifen injection. In this respect, body weight increase was significant for both groups before tamoxifen injection, but only significant for TPH2-$CB_1^{+/+}$ animals after tamoxifen treatment (TPH2-$CB_1^{+/+}$ before tamoxifen [$F_{(1,22)}$ = 16.44, $p < 0.001$]; TPH2-$CB_1^{-/-}$ before tamoxifen [$F_{(1,22)}$ = 4.7, $p < 0.05$]; TPH2-$CB_1^{+/+}$ after [$F_{(1,31)}$ = 12.97, $p < 0.001$]; TPH2-$CB_1^{-/-}$ after tamoxifen [$F_{(1,34)}$ = 0.822; p = 0.37]; Fig. 5.7A,B).

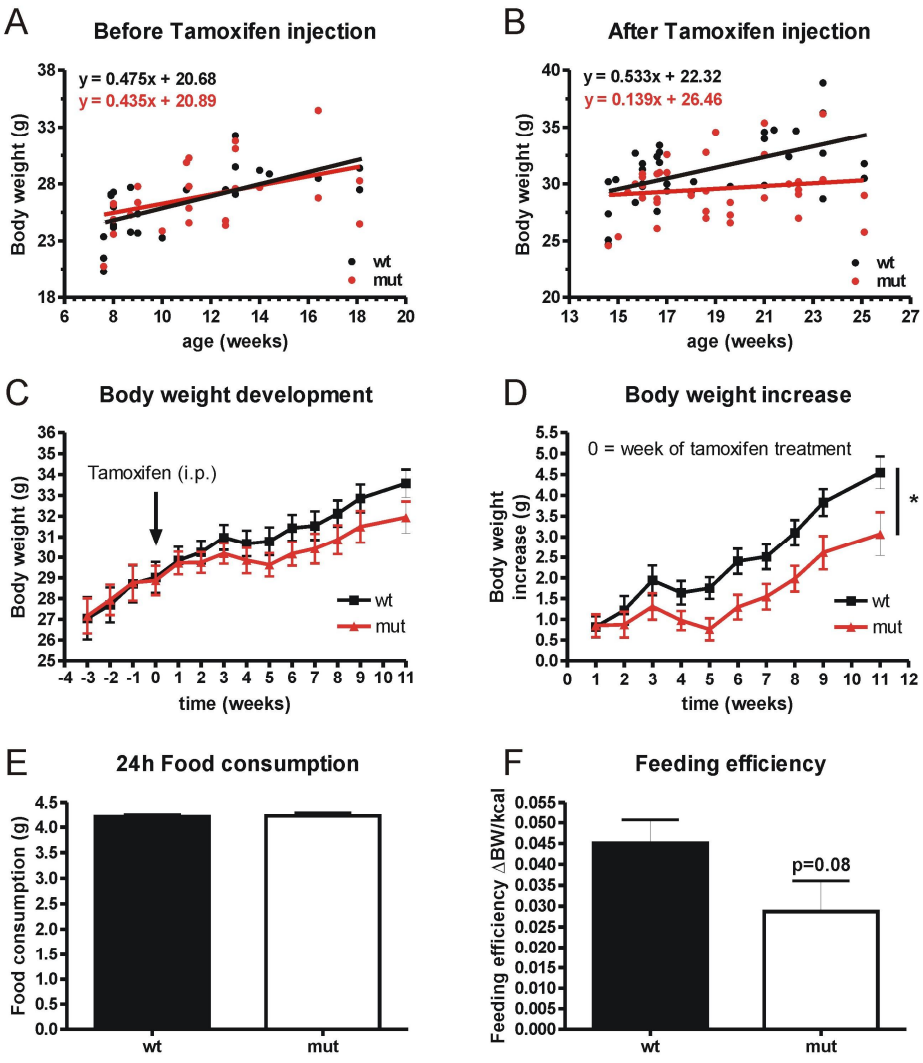

Fig. 5.7: Body weight development and feeding behavior: Body weight development **(A)** before and **(B)** after tamoxifen treatment. **(C)** Body weight and **(D)** body weight increase over time. **(E)** Averaged 24 h food consumption and **(F)** feed efficiency. Mut = TPH2-$CB_1^{-/-}$; wt = TPH2-$CB_1^{+/+}$. 2-way ANOVA (genotype differences), *p<0.05.

This was underlined by the absolute body weight changes (Fig. 5C,D). Even though differences in the overall body weight between the two genotypes did not reach significance (Fig. 5.7C), the differences in body weight development did ([$F_{(1,41)}$ = 5.57; $p < 0.05$]; Fig. 5.7D). No significant difference was seen in the averaged 24 h food consumption as well as in the calculated feed efficiency (24 h food consumption [$T_{(41)}$ = 0.1208, p = 0.9044]; feed efficiency [$T_{(41)}$ = 1.758, p = 0.0863]; Fig. 5.7E,F).

Similar findings were detected as response towards a 24 h food deprivation. However, here, body weight differences between the two genotypes reached significance (Genotype [$F_{(1,252)}$ = 8.51; $p < 0.01$]; Fig. 5.8A). The food consumption and feed efficiency was not altered in the mutant animals as compared to wild-type littermates (24 h food consumption [$T_{(64)}$ = 1.747, p = 0.0854]; feed efficiency [$T_{(62)}$ = 0.9741, p = 0.3338]; Fig. 5.8B,C).

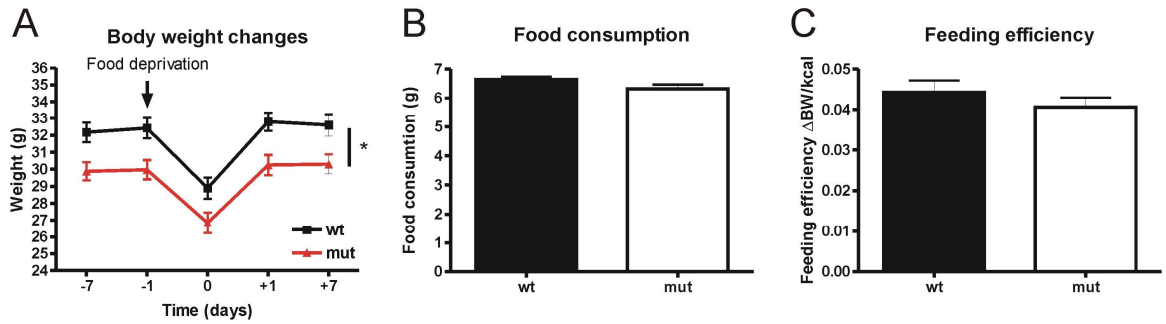

Fig. 5.8: Body weight development and feeding behavior during food deprivation. (A) Body weight changes during food deprivation revealed no alterations in mutants as compared to TPH2-wild-type littermates, except for the overall body weight. **(B)** 24 h food consumption and **(C)** resulting feed efficiency during a 24 h period following food deprivation showed no differences between genotypes. mut =TPH2-$CB_1^{-/-}$; wt = TPH2-$CB_1^{+/+}$; 2-way ANOVA (genotype differences), *p<0.05.

5.3.6. Locomotor activity and motor skill are unaltered

Evaluation of motoric skill in the RotaRod test and locomotor activity in the open field paradigm revealed no performance differences between genotypes (genotype effect motoric skills [$F_{(1,630)}$ = 0.61; p = 0.4384]; time effect motoric skills [$F_{(14,630)}$ = 40.02; $p < 0.001$]; locomotion [$T_{(90)}$ = 0.3604; p = 0.719]; Fig. 5.9A,B).

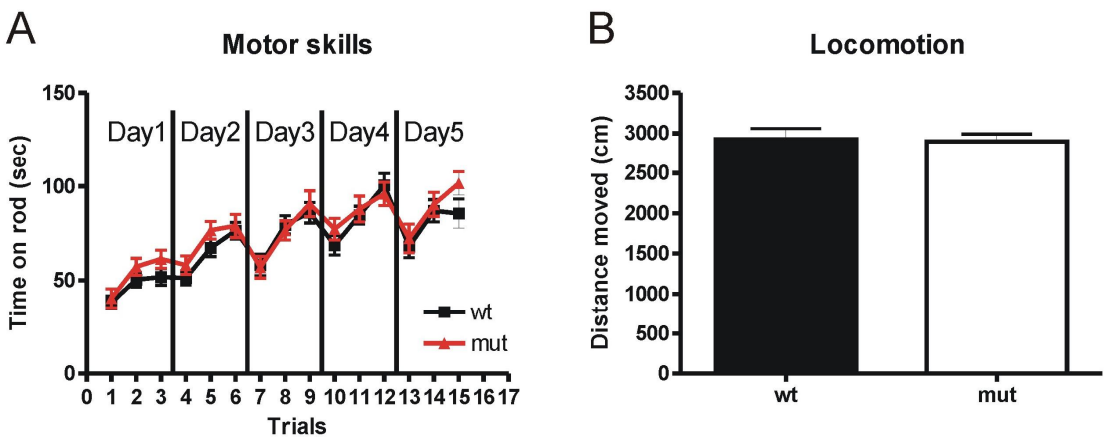

Fig. 5.9: Motor learning and locomotor activity are not altered. (A) No genotype effect was observed in basic motor skills and motor learning (time on rod) on RotaRod. **(B)** Similarly, the locomotor activity (distance moved) during a 10-min interval in the open field was not altered between genotypes. mut =TPH2-$CB_1^{-/-}$; wt = TPH2-$CB_1^{+/+}$

5.3.7. Basic anxiety seems to be unaltered

Basic fear response was evaluated by the light dark test and elevated plus maze paradigm, two established and acknowledged anxiety paradigms. However, none of the tests revealed any genotype differences. Thus, in the light dark test neither risk assessment, entries into the light compartment as well as the time spent in the light compartment was not changed in the TPH2-$CB1^{+/+}$ as compared to TPH2-$CB1^{-/-}$ littermates (entries [$T_{(66)}$ = 0.7156; p = 0.4767]; risk assessment [$T_{(66)}$ = 0.8003; p = 0.4264]; time spent in the light compartment [$T_{(1,66)}$ = 0.8003; p = 0.3953]; Fig. 5.10A). Similarly, no differences between the genotypes were detected in the elevated plus maze regarding the percentage of time spent and entries on the open arm (time [$T_{(88)}$ = 0.2288; p = 0.8195]; entries [$T_{(88)}$ = 0.5616; p = 0.5758]; Fig. 5.10B).

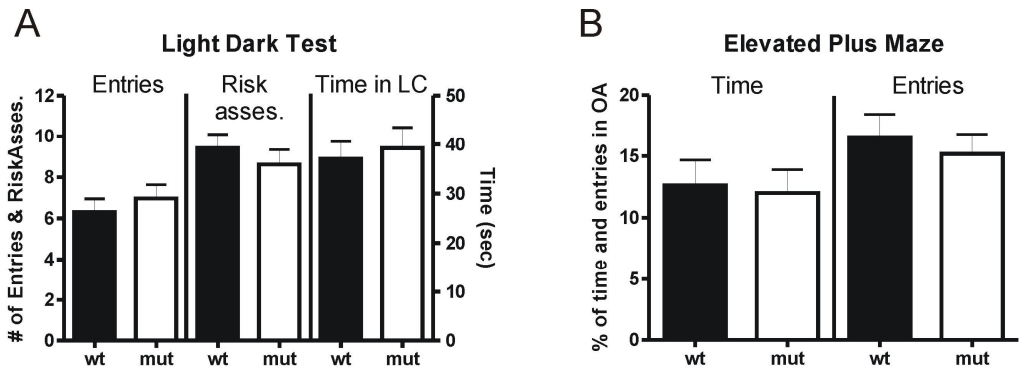

Fig. 5.10: Anxiety response is unaltered. No genotype effect was observed in **(A)** the light dark test and **(B)** the elevated plus maze paradigm. Risk asses = risk assessment; LC = light compartment; wt = TPH2-$CB_1^{+/+}$; mut = TPH2-$CB_1^{-/-}$.

5.3.7. Increased aversive stimuli induce anxiolytic phenotype

In contrast to the basic anxiety paradigms, such as the light dark test or the elevated plus maze paradigm, the introduction of increased aversive environment, namely, bright light or inescapable stress, as well as a social exploratory stimulus, led to the induction of behavioral changes in the TPH2-$CB_1^{-/-}$ animals as compared to the TPH2-$CB_1^{+/+}$ littermates. In contrast, object exploration seemed to be unaltered.

Novelty Suppressed Feeding: Even though the first contact with the food pellet was not altered in the novelty suppressed feeding paradigm, the TPH2-$CB_1^{-/-}$ mutants showed an increased latency to start eating the food pellet (delay first contact [$T_{(44)}$ = 0.5774; p = 0.5666]; delay eating [$T_{(44)}$ = 2.112; p < 0.05]; Fig. 5.11A). Furthermore, an increased number of interactions with the food pellet was observed, even though the interaction frequency was not altered (# of interactions [$T_{(43)}$ = 2.103; p < 0.05]; interaction frequency [$T_{(43)}$ = 0.3810; p = 0.7051]; Fig. 5.11B). However, the TPH2-$CB_1^{-/-}$ animals showed an increase in digging events and time spend digging (digging events [$T_{(43)}$ = 2.774; p < 0.01]; time spend digging [$T_{(43)}$ = 2.465; p < 0.05]; Fig. 5.11C)

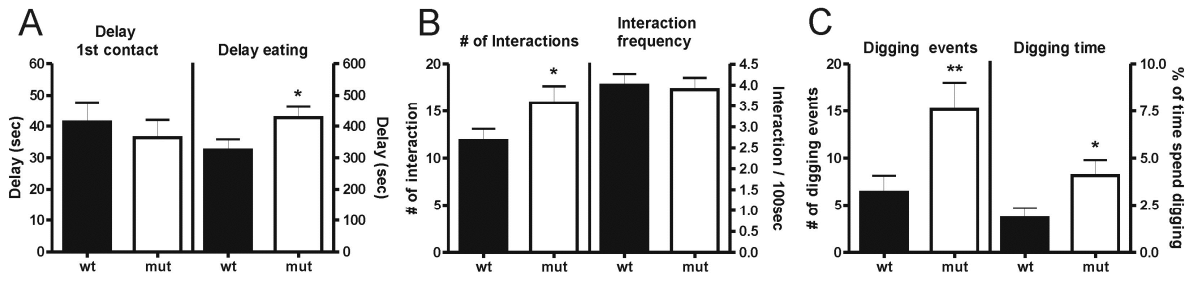

Fig. 5.11: Increased aversive environment in the novelty suppressed feeding paradigm induces anxiety-like behavior. (A) Even though no genotype effect was observed in the time required for the first contact, mutants showed a prolonged time to start eating. **(B)** The number but not frequency of interactions with the food pellet was significantly increased. **(C)** Interestingly, number of digging events and percentage of time spent digging was significantly increased. wt = TPH2-$CB_1^{+/+}$; mut = TPH2-$CB_1^{-/-}$; t-test (discrimination index), *p<0.05.

Sociability test: The introduction of a social interaction partner in the sociability paradigm revealed a decreased time spent in the compartment with the interaction partner for the TPH2-$CB_1^{-/-}$ mice compared to their wild-type littermates ($T_{(39)}$ = 2.144, p < 0.05; Fig. 5.12A). Interestingly, while the time spent in the middle compartment did not differ between the genotypes, the mutant mice spent significantly more time in the compartment with the empty cage (empty cage [$T_{(39)}$ = 2.046, p < 0.05]; middle [$T_{(39)}$ = 0.4578, p = 0.6496]; Fig. 5.12A).

This finding was partially mirrored by the distance moved. Thus, the TPH2-$CB_1^{-/-}$ mice displayed a significant reduction in the distance moved in the compartment with the interaction partner ($T_{(37)}$ = 2.620, $p < 0.05$; Fig. 5.12B). No difference was seen for the locomotor activity in the other two compartments and the complete distance moved (empty cage [$T_{(37)}$ = 0.7431, p = 0.4621]; middle [$T_{(37)}$ = 0.2509, p = 0.833]; [$T_{(37)}$ = 1.367, p = 0.1799]; Fig. 5.12B).

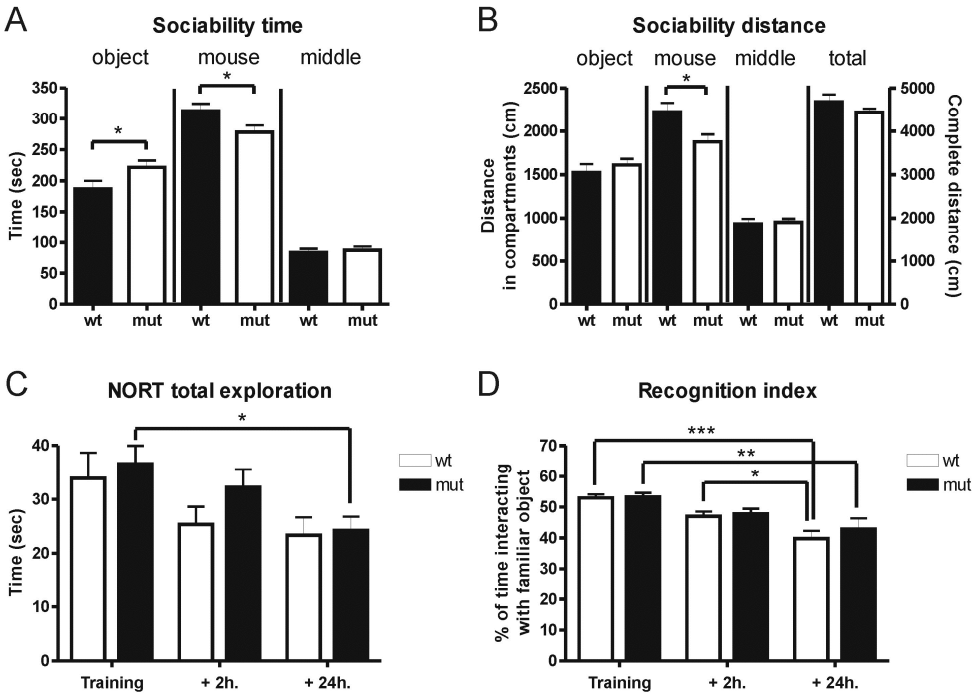

Fig. 5.12: Social, but not object exploration is reduced in mutant animals. (A) Exploratory drive towards a social interaction partner and object stimulus in the sociability test was altered in mutant animals as compared to wild-type littermates. **(B)** Locomotor activity during the sociability test was not altered except for the compartment with the social interaction partner. **(C,D)** Interestingly, no genotype differences were observed in the novel object recognition test (NORT). Both groups spend equal amount of time exploring the objects **(C)** and recognized the familiar object **(F)**. wt = TPH2-$CB_1^{+/+}$; mut = TPH2-$CB_1^{-/-}$. Bonferoni post-test following 2-way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

Novel object recognition test: The analysis of the novel object recognition test revealed no genotype difference, neither in general object exploration nor in object recognition for all three time points (object exploration [$F_{(1,63)}$ = 0.6438; p = 0.4253]; object recognition [$F_{(1,63)}$ = 1.459; p = 0.2316]; Fig. 5.12C,D). There was a significant effect of time on exploration, namely, being decreased over the three phases ($F_{(2,63)}$ = 5.243; $p < 0.01$; Fig. 5.12C). However, Bonferoni post-test analysis revealed only a significant difference for the TPH2-

$CB_1^{-/-}$ mice between training and 24h retention phase ($T_{(63)} = 2.413$; $p < 0.05$; Fig. 5.12C). A similar situation is observed for the object recognition, were time significantly affect the recognition index ($F_{(2,63)} = 15.81$; $p < 0.001$; Fig. 5.12D). Here, Bonferoni post-test analysis showed significant changes for both genotypes, TPH2-$CB_1^{-/-}$ (training vs. 24h [$T_{(63)} = 3.432$; $p < 0.01$; Fig. 5.12D) and TPH2-$CB_1^{+/+}$ (training vs. 24h [$T_{(63)} = 4.545$; $p < 0.001$]; 2h vs. 24h [$T_{(63)} = 2.494$; $p < 0.05$]; Fig. 5.12D).

Resident intruder paradigm: Similarly to the sociability test, TPH2-$CB_1^{-/-}$ animals displayed a significantly decreased time interacting with the social interaction partner as compared to their wild-type littermates in the resident intruder paradigm too (interaction-absolute time [$T_{(56)} = 2.178$; $p < 0.05$]; interaction-percentage of time [$T_{(56)} = 2.639$; $p < 0.05$]; Fig. 5.13A). No difference was observed for the interaction induced by the intruder (interaction-absolute time [$T_{(56)} = 0.9704$; $p = 0.3360$]; interaction-percentage of time [$T_{(56)} = 0.3301$; $p = 0.7425$]; Fig. 5.13A). There was no genotype difference detectable regarding the number of fights ($T_{(56)} = 0.5779$; $p = 0.5657$; Fig. 5.13B).

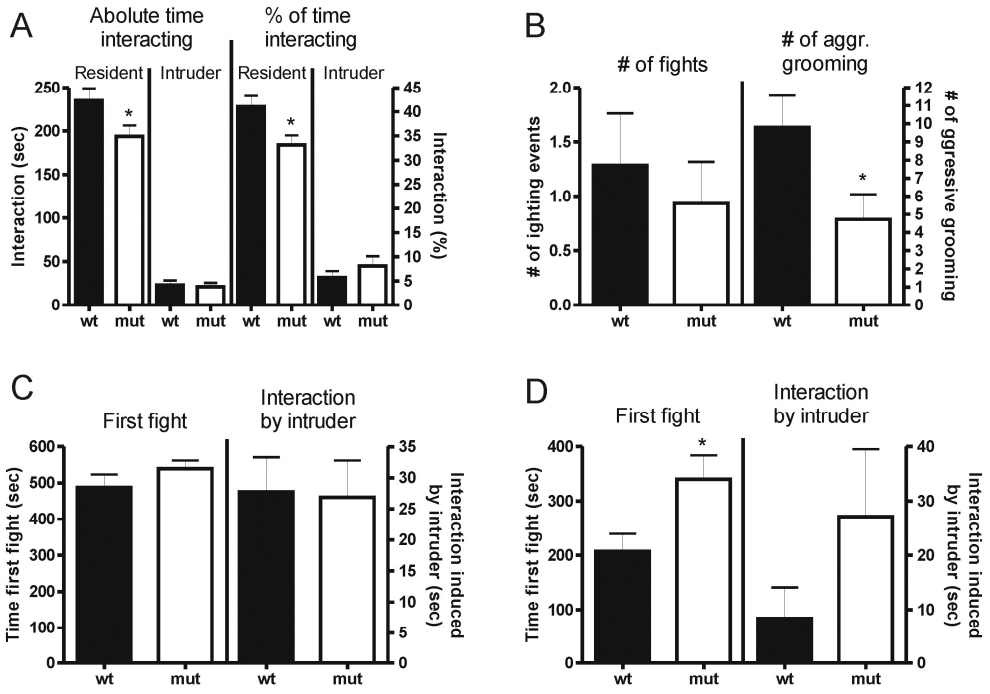

Fig. 5.13: Resident intruder test shows decreased social interaction and aggressive behavior in mutant animals. **(A)** Time spent investigating a social interaction partner was decreased in mutant animals as compared to wild-type littermates. **(B)** Dominant behavior, but not the number of fights during the social interaction was altered in the mutants. **(C,D)** Interestingly, the first fighting event was significantly delayed for mutant animals, but only when non-fighting animals were excluded. wt = TPH2-$CB_1^{+/+}$; mut = TPH2-$CB_1^{-/-}$. Student´s t-test *p<0.05.

However, a decreased number of aggressive grooming events was observed in the mutants ($T_{(19)} = 2.273$; $p < 0.05$; Fig. 5.13B). This finding was underlined by the latency the first fight occurred. Even though the overall latency of the first fight was not different between the genotypes, a significant increased delay for the TPH2-$CB_1^{-/-}$ animals was detectable for those animals which did fight (overall latency [$T_{(56)} = 1.249$; $p = 0.2168$]; Fig. 5.13C; latency fighting animals only [$T_{(13)} = 2.493$; $p < 0.05$]; Fig. 5.13D). In both cases, behavior of the intruders did not differ (overall latency [$T_{(56)} = 0.4937$; $p = 0.6235$]; Fig. 5.13C; latency fighting animals only [$T_{(13)} = 1.438$; $p = 0.1742$]; Fig. 5.13D).

Forced Swim Test: The inescapable stress of the forced swim test induced a significant decrease in time spent immobile, here termed floating behavior ($T_{(42)} = 2.033$; $p < 0.05$; Fig. 5.14A). To complete the assay, we also measured corticosterone levels in stressed (after FST) and unstressed condition (no FST). As shown in Figure 5.14B, no significant differences were observed in either of the two conditions (unstressed [$T_{(17)} = 0.9445$; $p = 0.3581$]; stressed [$T_{(18)} = 1.511$; $p = 0.1482$]; Fig. 5.13B).

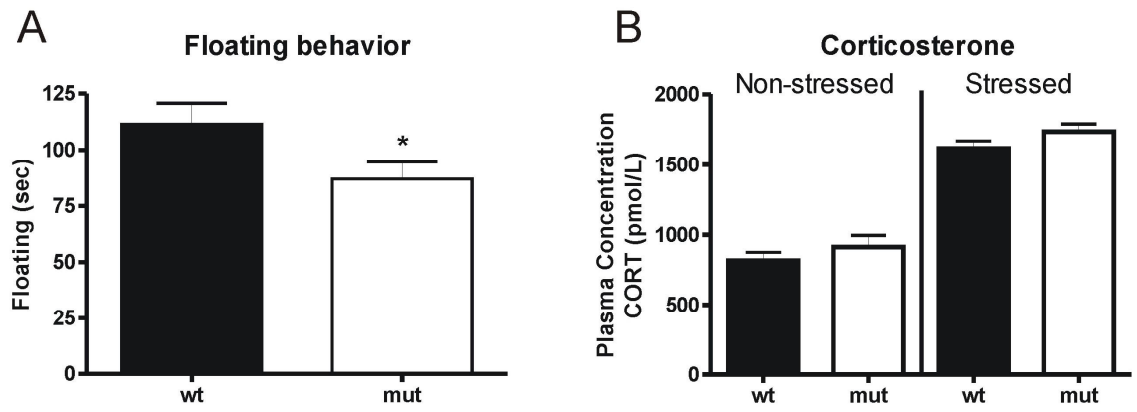

Fig. 5.14: Floating behavior and corticosterone levels during the forced swim test. **(A)** Mutants displayed a decreased floating behavior in the forced swim test as compared to wild-type littermates. **(B)** No genotype differences were observed in either basal (non-stressed) or after forced swim test (stressed) conditions. wt = TPH2-$CB_1^{+/+}$; mut = TPH2-$CB_1^{-/-}$. Student´s t-test: *p < 0.05

5.4. Discussion

In this study, I showed the functionality of the inducible the Cre-ER^{T2} system as described by Weber et al. (2009), and, more importantly, proved for the first time that the CB_1 receptor in serotonergic neurons is of physiological relevance. These data suggest a fine tuning of serotonergic activity via the activation of the CB_1 receptor in this particular neuronal subpopulation. Interestingly, the phenotype appears only under stressful situations, as no behavioral differences were observed in basic locomotion and anxiety paradigms (see Fig. 5.9 and 5.10). However, a strongly aversive environment or inescapable stress, as displayed in the novelty suppressed feeding paradigm and forced swim test, respectively, induced behavioral changes (see Fig. 5.11, 5.14). In the novelty suppressed feeding paradigm, TPH2-$CB_1^{-/-}$ mice required more time to start eating and spend more time digging as compared with control mice. The novelty suppressed feeding paradigm was established as an anxiety test to measure the rodent's aversion to eat in a novel environment. The test is sensitive to acute administration of anxiolytic drugs, such as benzodiazepines, which induce a decreased delay in approaching and eating the food pellet (Belzung et al., 1987). Also, the increased digging behavior observed for the TPH2-$CB_1^{-/-}$ animals might indicate a stereotypic response in this stressful situation to the high aversive and emotional distress (Pietropaolo et al., 2011).

On the other hand, the decreased time spent immobile in the forced swim test is normally regarded to be an antidepressant-like effect, as antidepessant drugs reduce the time spent immobile in this particular paradigm (Borsini and Meli, 1988; Petit-Demouliere et al., 2005). Nevertheless, as already mentioned in the Chapter 4, this particular effect might be induced by multiple underlying mechanisms (Petit-Demouliere et al., 2005). One explanation might be an unbalanced stress coping based on an increased fear level. In fact, the parallel presence of increased anxiety-like and depressive-like response was already seen previously in connection in 5-HT_{1A} receptor deficient mice (Heisler et al., 2003).

Nevertheless, an anxiety-like phenotype would be supported to some extent by the decreased time TPH2-$CB_1^{-/-}$ mice spend with a social interaction partner in the sociability test and resident intruder paradigm (see Fig. 5.12A,B, and 5.13A). Interestingly, the investigatory drive itself seems to be unaltered, as no genotype difference was observed in the time spent in the middle compartment of the sociability box as well as the object exploration in the novel object recognition task (see Fig. 5.12). Thus, the data suggests a decreased interest towards a social stimulus in the TPH2-$CB_1^{-/-}$ mice.

A more detailed analysis of the resident intruder paradigm revealed additional social phenotype differences between TPH2-$CB_1^{-/-}$ and TPH2-$CB_1^{+/+}$ mice. Thus, TPH2-$CB_1^{-/-}$ animals displayed a decreased number of aggressive grooming events as well as a delay in the first fighting event, suggesting a decreased level of aggression or dominance in the mutant animals as compared to their wild-type littermates (see Fig. 5.13B,D). Highly important is the fact that the intruder behavior can be excluded as possible confounding factor, as the intruder behavior was not different between the genotypes (see Fig. 5.13).

Not only social interaction and anxiety seem to be controlled by CB_1 receptor function in serotonergic neurons. CB_1 receptor deletion from serotonergic neurons also led to slightly but significantly attenuated body weight increase (see Fig. 5.7A-D). Food intake and feed efficacy, however, was not significantly altered, neither in long-term nor acutely following a 24 h food deprivation period (see Fig. 5.7E,F, and 5.8B,C), suggesting that deleting CB_1 receptor from serotonergic neurons did not influence cellular metabolism. Recent findings with this mouse line suggest that stress is the driving factor to induce the phenotype differences, as Dubreucq et al. (2012) detected a significantly lower increase of body weight in mutant animals as compared to the wild-type littermates, following one week of social defeat.

Taken together, the deletion of the CB_1 receptor from serotonergic neurons seems to alter the behavioral performance in response to stress, causing an increased awareness of aversive stimuli and social contact. Serotonin is centrally involved in a multitude of behavior responses, most importantly in emotion and social interaction (Cools et al., 2007; Waider et al., 2011). Due to the anti-depressive and anxiolytic properties of serotonin reuptake inhibitors, which increase the serotonergic tone by blocking serotonin transporter function, serotonin has normally been associated with a decrease of fear and stress response (Williams et al., 2010, Haenisch and Boenisch, 2010). However, accumulating evidence suggests more diverse effects of chronic serotonin excess (Haenisch and Boenisch, 2010). Thus, genetic and pharmacological inhibition of the serotonin reuptake transporter leads to an elevated anxiety level as well as induce a reduced aggression in mice (Jansen et al., 2010, 2011; Homberg and Lesch, 2011). In line with this, the deletion of the 5-HT1A autoreceptor leads to an increased fear response as well (Heisler et al., 2003). These findings profoundly change the view on serotonin and its role on emotional and social responses. Furthermore, these novel insights are completely in line with our findings in this study. Similarly to deleting the serotonin reuptake transporter inducing an increased serotonergic tone, the deletion of the CB_1 receptor should potentially also increase serotonergic drive in a constant manner. Both genetic modifications

induced a delay in the time required to start eating the novelty suppressed feeding paradigm, both showed an increased latency to start fighting and a reduced time spent with a social interaction partner (Jansen et al., 2011; Homberg and Lesch, 2011; the present study). Detailed analysis of the serotonin reuptake transporter deficient mice revealed a strong effect of stress, environment and life history on the phenotype (Heiming et al., 2009; Jansen et al., 2010, 2011; Homberg and Lesch, 2011).

Interestingly, the same seems to be true for TPH2-$CB_1^{-/-}$ animals, even though such detailed evaluation as just mentioned for the serotonin reuptake deficient mice, has not been performed yet. Nevertheless, mainly high aversive situations and social stimuli induced the phenotype. Furthermore, one should keep in mind that in our experiments mice were single housed, which can also be regarded as social stress and can have influence on behavioral performance (Arndt et al., 2009; Martin and Brown, 2010). Most striking in this respect, however, was the finding that one week of social defeat induced weight changes (Dubreucq et al., 2012) similar to those we presented in the present study.et al.

In summary, we could show for the first time the physiological relevance of the CB_1 receptor on serotonergic neurons, apparently responsible for fine tuning serotonin activity as suggested by the behavioral changes induced by deleting the receptor specifically in this neuronal population. Our findings are in line with recent insights on serotonin function changing the dogma of serotonin, possessing predominantly positive emotional effects. Thus, apparently a chronic desensitisation of serotonergic signalling appears to induce negative emotional changes (Canli and Lesch, 2007; Homberg and Lesch, 2012). Psychiatric disorders, such as autism spectrum disorders, are often associated with similar behavioral changes, such as decreased social interaction, depression and anxiety. In fact, serotonin reuptake inhibitors are often used to treat the depressive and anxiety symptoms, even though clinical relevance has remained elusive (Williams et al., 2010). Nevertheless, these drug types are highly potent in the treatment of depressive disorder, suggesting that psychiatric diseases might be based on more complex physiological alterations, which require also a complex approach. Our data might further indicate a contributing role of serotonergic CB_1 receptor in the negative effects observed when the CB_1 receptor antagonist rimonabant was applied chronically (Moreira and Crippa, 2009).

Chapter 6

Conclusions

6. Conclusions

6.1. Summary

Our data showed that a functional eCB system is essential for an adequate behavioral performance, in particular regarding exploration and stress coping (see Table 6.1). Thus, we could prove the importance of the CB_1 receptor on specific neuronal subpopulations for object exploration, interaction with a social partner and inescapable stress. Deleting the CB_1 receptor from GABAergic and glutamatergic neurons led to opposite phenotypes, namely an increased or decreased exploratory drive towards an object or social stimuli, respectively (see Chapter 3). In contrast to this general modulation of exploration, the deletion of the CB_1 receptor from serotonergic neurons caused no altered exploratory drive *per se* (see Chapter 5; Fig. 12). Here, the social stimulus is an essential requirement to induce a change in phenotype (see Chapter 5; Fig. 12 and 13). Thus, the missing cannabinoid control mechanism leads to a decreased interest towards a social interaction partner.

	Locomotion	Object Exploration	Social Exploration	Aggression	Immobility	Anxiety response
Wild-type	Normal	Normal	Normal	Normal	Normal	Normal
Glu-CB1$^{-/-}$	Normal ↓	Decreased	Decreased	Increased	Decreased	Normal
GABA-CB1$^{-/-}$	Normal ↑	Increased	Increased	Normal	Normal	Normal
D1-CB1$^{-/-}$	Normal	Normal	Normal	Normal	ns	ns
TPH2-CB1$^{-/-}$	Normal	Normal	Decreased	Decreased	Decreased	Normal *

Table 6.1: Overview on the behavioral performance of mice lacking the CB_1 receptor in specific neuronal populations. Ns = no statement, Normal = wild-type performance, black arrows indicate observed differences only in a single experiment phase. * indicates significant changes under high aversive conditions.

In inescapable stress situations, as shown in the forced swim test, the deletion of the CB_1 receptor from glutamatergic or serotonergic neurons induces a reduction in floating behavior. CB_1 receptor depletion from GABAergic neurons, on the other hand, had no impact on the phenotype, even though the antidepressant-like effect of rimonabant depended on this particular receptor subpopulation (Chapter 4; Fig. 4.5B). As discussed in Chapter 4, this discrepancy might be due to compensatory mechanisms in the GABA-$CB_1^{-/-}$ animals.

Whether the decreased immobility for TPH2-$CB_1^{-/-}$ and Glu-$CB_1^{-/-}$ animals is in fact an antidepressant-like response needs to be questioned. Thus, the increased anxiety-like behavior for these mice in the exploration and under high aversive situations might also suggest an inappropriate stress coping (Chapter 4; Chapter 6).

Taken together, there are two highly interesting findings, one being the opposite function of GABAergic and glutamatergic neurons, and second, being the similar phenotypes detectable for the TPH2-$CB_1^{-/-}$ and Glu-$CB_1^{-/-}$ animals, suggesting that these two transmitter systems might be functionally connected (see Table 6.1). The latter idea was further underlined by the dependence of the antidepressant-like phenotype in Glu-$CB_1^{-/-}$ mice on serotonin transmission (Chapter 4, Fig. 4.6).

6.2. Conclusion and Outlook

The results presented in this study revealed novel insights and support existing data on the physiological importance of specific CB_1 receptor-expressing neuronal subpopulations. First, we could underline the opposing roles of the CB_1 receptor in cortical GABAergic and glutamatergic neurons in terms of exploratory behavior. Even though this has already been suggested previously (Lafenêtre et al., 2009; Bellocchio et al., 2011), our approach combined for the first time animals lacking the CB_1 receptor in both neuronal populations in paradigms evaluating social and object exploration. Thus, these two CB_1 receptor populations seem to control a balanced investigatory drive. These findings together with the results obtained from the forced swim test, further suggest these receptor populations to be responsible for the opposing effects observed by the application of low or high doses of cannabinoids (Moreira and Lutz 2008; Aparisi Rey et al., 2012).

Interestingly, a connection between CB_1 receptors on glutamatergic and serotonergic neurons appears to exist. In fact, both neuronal populations have already been shown to be functionally linked, in particular at the level of the raphe nuclei, the origin of serotonergic neurons. Our results combined with published data indicate two pathways within the raphe nucleus for this connection (see Fig. 6.1). In particular, Celada and colleagues suggested this complex situation where excitatory input to the dorsal raphe nucleus leads simultaneously to an inhibition of serotonergic neuron firing, but at the same time to an activation of a subpopulation of serotonergic neurons (Celada et al., 2001).

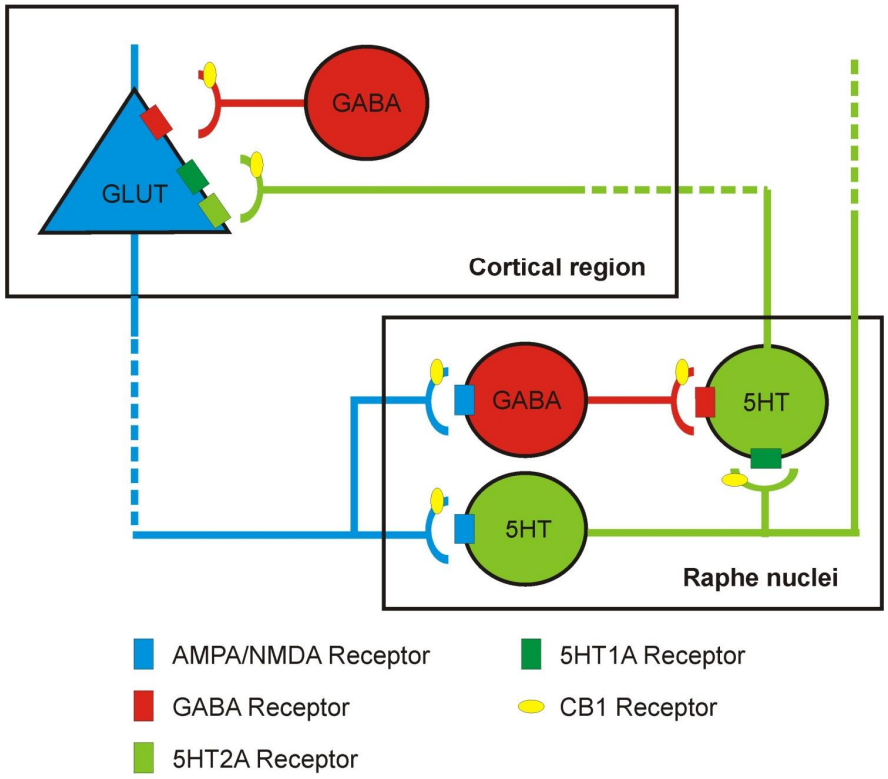

Figure 6.1: Schematic overview on cortical-raphe connections. Cortical glutamatergic input on GABAergic and serotonergic neurons in the raphe region activate these two neuronal population. GABA and serotonin can both inhibit neighbouring serotonergic neurons by activating GABA and 5-HT1A receptors.
Modified illustration from Celada et al. 2001

In our approach, similarities between TPH2-$CB_1^{-/-}$ and Glu-$CB_1^{-/-}$ animals were found at the behavioral level, as both displayed anxiogenic-like profiles depending on the paradigm. This behavior might be due to a chronically enhanced serotonin release, as both the nature of the eCB system as well as our findings suggest that both mutants possess an increased serotonergic drive. For once, CB_1 receptor depletion from serotonergic neurons reduced inhibitory control on 5-HT release in these cells. Furthermore, a direct excitatory glutamatergic input onto serotonergic neurons was previously shown (Celada et al., 2001; Haj-Dahmane and Shen, 2009). However, electrophysiological studies suggested rather a contrasting connection between the two systems (Celada et al., 2001; Bambico et al., 2007; see Fig. 6.1). These studies indicated mainly an indirect connection of glutamatergic and serotonergic neurons, via GABAergic interneurons (Celada et al., 2001; Bambico et al., 2007). In particular, Celada and colleagues suggested a more complex situation (Celada et al., 2001). Here PFC stimulation led mainly, though not exclusively, to an inhibition of

serotonergic neuron firing, however, a subset of serotonergic neurons was activated. The authors argued that excitatory inputs on GABAergic and serotonergic interneurons activate these cells, which in turn have an inhibitory function on neighboring serotonergic neurons, via 5-HT1A and GABA receptors (Celada et al., 2001; see Fig. 6.1). One explanation for our findings in TPH2-$CB_1^{-/-}$ and Glu-$CB_1^{-/-}$ animals might therefore be an attenuated inhibitory function via the desensitization of 5-HT1A autoreceptor on serotonergic neurons (Hervaz et al., 2000; Rozeske et al., 2011). Thus, it might be highly interesting to evaluate the expression profile of the 5-HT1A receptor in the raphe region of TPH2-$CB_1^{-/-}$ and Glu-$CB_1^{-/-}$ animals. Additionally, it would be interesting to anatomically characterize CB_1 receptor presence in the raphe region which has been suggested be electrophysiological findings (Haj-Dahmane and Shen, 2009).

The behavioral changes induced by CB_1 receptor deletion might also be interesting in respect to treat psychiatric disorders. Thus, complex neurological diseases (e.g. autism, attention-deficit and schizophrenic disorders) are often accompanied by an alteration in the investigatory and social skills (Newcorn, 2001; Solanto, 2002; Russel, 2007; Perry *et al*, 2009; Kawa and Pisula, 2010; Patterson, 2011). Inappropriate investigatory drive may be caused by various genetic and environmental factors which lead to a malfunction in different neurotransmitter systems, in particular serotonin, as well as GABA and glutamate (Feng *et al*, 2005; Moon *et al*, 2006; Siesser *et al*, 2006; Russel, 2007; Helms *et al*, 2008; Loos *et al*, 2009; Bevilacqua *et al*, 2010; Kelsoe *et al*, 2010). In fact, direct and indirect connections between these disorders and a dysregulation of serotonergic, GABAergic and glutamatergic transmission have already been suggested (Del Arco *et al*, 2010; Chao *et al*, 2010; Sala *et al*, 201; Amitai *et al*, 2011; Guidali *et al*, 2011; Javitt, 2011).

Another important clinical application could be the treatment of depression. We underlined the antidepressive-like effects of cannabinoids by applying different doses of CB_1 receptor agonist and antagonist in the forced swim test (Bambico et al. 2007, Steiner et al., 2008). Furthermore, testing the conditional mutants, partly also in combination with cannabinoid treatment, revealed the importance of CB_1 receptor subpopulations on serotonergic, GABAergic and glutamatergic neurons to control a balanced stress response.

As mentioned in antidepressant-like effects in animal models cannot always be fully accounted as a “real” antidepressant effect. Moreover, the acute and chronic applications need to be considered. Probably the most famous example is the retraction of rimonabant as anti-obesity drug, because of its psychiatric side effects following chronic use, namely an increase of depression and suicidal imaginations (Moreira and Crippa, 2009).

Taken together, cannabinoids elicit their behavioral effects, among others, via regulating serotonin, glutamate and GABA release (Ruehle et al., 2011; Häring et al., 2011). The existence of multiple targets might account for the opposite effects of low and high doses of cannabinoid drugs. Increasing evidence combined with the results presented in this study confirm that the molecular and behavioral outcome seem to be highly dependent on concentration and spatio-temporal activity of cannabinoids. Therefore, the clinical application of cannabinoid for treatment purposes can be a promising strategy, but needs to be handled with care to avoid unwanted side effects.

References

References

Alger, B.E. (2002). Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. Prog. Neurobiology 68: 247–286.

Amitai, N., Kuczenski, R., Behrens, M.M., Markou, A. (2011). Repeated phencyclidine administration alters glutamate release and decreases GABA markers in the prefrontal cortex of rats. Neuropharmacology 69: 875-82.

Arndt, S.S., Laarakker, M.C., van Lith, H.A., van der Staay, F.J., Gieling, E., Salomons, A.R., van't Klooster, J., Ohl, F. (2009). Individual housing of mice--impact on behaviour and stress responses. Physiol. Behav. 7: 385-93.

Azad, S.C., Kurz, J., Marsicano, G., Lutz, B., Zieglgansberger, W., Rammes, G. (2008). Activation of CB1 specifically located on GABAergic interneurons inhibits LTD in the lateral amygdala. Learn. Mem. 15:143-152.

Baker, K.G., Halliday, G.M., Halasz, P., Hornung, J.P., Geffen, L.B., Cotton, R.G., Törk, I. (1991). Cytoarchitecture of serotonin-synthesizing neurons in the pontine tegmentum of the human brain. Synapse 7(4):301-20.

Bambico, F.R.., Katz, N., Debonnel, G., Gobbi, G., (2007). Cannabinoids elicit antidepressant-like behaviour and activate serotonergic neurons through the medial prefrontal cortex. J. Neurosci. 27: 11700-11711.

Bambico, F., Duranti, A., Tontini, A., Tarzia, G., Gobbi, G., (2009). Endocannabinoids in the treatment of mood disorder: Evidence from animal models. Curr. Pharm. Des. 15:1623-1646.

Bambico, F.R., Cassano, T., Dominguez-Lopez, S., Katz, N., Walker, C.D., Piomelli, D., Gobbi, G., (2010). Genetic deletion of fatty acid amide hydrolase alters emotional behaviour and serotonergic transmission in the dorsal raphe, prefrontal cortex, and hippocampus. Neuropsychopharmacology 35: 2083-2100.

Bambico, F.R., Hattan, P.R., Garant, J.P., Gobbi, G., (2012). Effect of delta-9-tetrahydrocannabinol on behavioral despair and on presynaptic and postsynaptic serotonergic transmission. Prog. Neuropsychopharmacol. In press.

Bellocchio L, Cervino C, Pasquali R, Pagotto U. (2008). The endocannabinoid system and energy metabolism. J. Neuroendocrinol. 20: 850-7.

Bellocchio, L., Lafenêtre, P., Cannich, A., Cota, D., Puente, N., Grandes, P., Chaouloff, F., Piazza, P.V., Marsicano, G. (2010). Bimodal control of stimulated food intake by the endocannabinoid system. Nat. Neurosci. 13: 281-3.

Belzung, C., Misslin, R., Vogel, E., Dodd, R.H., Chapouthier, G. (1987). Anxiogenic effects of methyl-beta-carboline-3-carboxylate in a light/dark choice situation. Pharmacol. Biochem. Behav. 28(1):29-33.

Berrendero, F., Maldonado, R. (2002). Involvement of the opioid system in the anxiolytic-like effects induced by Δ^9-tetrahydrocannabinol. Psychopharmacology (Berl) 163:111-117.

Best, A.R., Regehr, W.G. (2008). Serotonin evokes endocannabinoid release and retrogradely suppresses excitatory synapses. J. Neurosci. 28:6508-15.

Beyer, C.E., Dwyer, J.M., Piesla, M.J., Platt, B.J., Shen, R., Rahman, Z., Chan, K., Manners, M.T., Samad, T.A., Kennedy, J.D., Bingham, B., Whiteside, G.T. (2010). Depression-like phenotype following chronic CB1 receptor antagonism. Neurobiol. Dis. 39: 48-155.

Bevilacqua, L., Doly, S., Kaprio, J., Yuan, Q., Tikkanen, R., Paunio, T., et al (2010). A population-specific HTR2B stop codon predisposes to severe impulsivity. Nature 468: 1061-6.

Borsini, F., Meli, A. (1988). Is the forced swimming test a suitable model for revealing antidepressant activity? Psychopharmacology (Berl) 94: 147-60.

Braida, D., Limonta, V., Malabarba, L., Zani, A., Sala, M. (2007). 5-HT1A receptors are involved in the anxiolytic effect of Δ^9-tetrahydrocannabinol and AM 404, the anandamide transport inhibitor, in Sprague-Dawley rats. Eur. J. Pharmacol. 555: 156-163.

Branda, C.S., Dymecki, S.M. (2004). Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Dev. Cell 6: 7-28.

Brocard, J., Feil, R., Chambon, P., Metzger, D. (1998). A chimeric Cre recombinase inducible by synthetic,but not by natural ligands of the glucocorticoid receptor. Nucleic Acids Res. 26: 4086-90.

Canli, T., Lesch, K.P. (2007). Long story short: the serotonin transporter in emotion regulation and social cognition. Nat. Neurosci. 10: 1103-9.

Carkaci-Salli, N., Salli, U., Kuntz-Melcavage, K.L., Pennock, M.M., Ozgen, H., Tekin, I., Freeman, W.M., Vrana, K.E. (2011). TPH2 in the ventral tegmental area of the male rat brain. Brain Res. Bull. 84: 376-80.

Chevaleyre, V., Takahashi, K.A., Castillo, P.E. (2006). Endocannabinoid-mediated synaptic plasticity in the CNS. Annu. Rev. Neurosci.29: 37-76.

Cristino, L., Starowicz, K., De Petrocellis, L., Morishita, J., Ueda, N., Guglielmotti, V., Di Marzo, V. (2008). Immunohistochemical localization of anabolic and catabolic enzymes for anandamide and other putative endovanilloids in the hippocampus and cerebellar cortex of the mouse brain. Neuroscience 151: 955-968.

Celada, P., Puig, M.V., Casanovas, J.M., Guillazo, G., Artigas, F. (2001). Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: Involvement of serotonin-1A, GABA(A), and glutamate receptors. J. Neurosci. 21: 9917-9929.

Cools, R., Roberts, A.C., Robbins, T.W. (2008). Serotoninergic regulation of emotional and behavioural control processes. Trends Cogn. Sci. 12: 31-40.

Corrodi, H., Hanson L.C. (1966). Central effects of an inhibitor of tyrosine hydroxylation. Psychopharmacologia 10: 116-125.

Cryan, J.F., Mombereau, C. (2004). In search of a depressed mouse: utility of models for studying depression-related behaviour in genetically modified mice. Mol. Psychiatry 9: 326-357.

Dahlstrom, A., Fuxe, K. (1964). Localization of monoamines in the lower brain stem. Experientia, 20: 398-9.

De Petrocellis, L., Di Marzo, V. (2010). Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels. J. Neuroimmune Pharmacol. 5:103-121.

Del Arco, A., Ronzoni, G., Mora, F. (2010). Prefrontal stimulation of $GABA_A$ receptors counteracts the corticolimbic hyperactivity produced by NMDA antagonists in the prefrontal cortex of the rat. Psychopharmacology (Berl) 214: 525-36.

Dubreucq, S., Matias, I., Cardinal, P., Häring, M., Lutz, B., Marsicano, G., Chaouloff, F. (2012). Genetic Dissection of the Role of Cannabinoid Type-1 Receptors in the Emotional Consequences of Repeated Social Stress in Mice. Neuropsychopharmacology. doi: 10.1038/npp.2012.36.

Egashira, N., Matsuda, T., Koushi, E., Higashihara, F., Mishima, K., Chidori, S., Hasebe, N., Iwasaki, K., Nishimura, R., Oishi, R., Fujiwara, M., (2008). Δ^9-tetrahydrocannabinol prolongs the immobility time in the mouse forced swim test: involvement of cannabinoid CB1 receptor and serotonergic system. Eur. J. Pharmacol. 589: 117-121.

Egertova, M., Cravatt, B.F., Elphick, M.R. (2003). Comparative analysis of fatty acid amide hydrolase and CB1 cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. Neuroscience 119: 481-496.

El-Alfy, A.T., Ivey, K., Robinsonm K., Ahmed, S., Radwan, M., Slade, D., Khan, I., ElSohly, M., Ross, S., (2010). Antidepressant-like effect of Δ^9-tetrahydrocannabinol and other cannabinoids isolated from Cannabis sativa L. Pharmacol. Biochem. Behav. 95: 434-442.

Ennaceur, A., Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1" Behavioral data. Behav. Brain Res. 31: 47-59.

Feng, Y., Crosbie, J., Wigg, K., Pathare, T., Ickowicz, A., Schachar, R., et al. (2005). The SNAP25 gene as a susceptibility gene contributing to attention-deficit hyperactivity disorder. Mol. Psychiatry 10: 998-1005, 973.

Fernández-Ruiz, J., Hernández, M., Ramos, J.A. (2010). Cannabinoid-dopamine interaction in the pathophysiology and treatment of CNS disorders. CNS Neurosci. Ther. 16: e72-91.

Forray, M.I., Gysling, K. (2004). Role of noradrenergic projections to the bed nucleus of the stria terminalis in the regulation of the hypothalamic-pituitary-adrenal axis. Brain Res. Rev. 47: 145-160.

Fusar-Poli, P., Crippa, J.A., Bhattacharyya, S., Borgwardt, S.J., Allen, P., Martin-Santos, R., Seal, M., Surguladze, S.A., O'Carrol, C., Atakan, Z., Zuardi, A.W., McGuire, P.K. (2009). Distinct effects of Δ9-tetrahydrocannabinol and cannabidiol on neural activation during emotional processing. Arch. Gen. Psychiatry 66: 95-105.

Gobbi, G., Bambico, F.R., Mangieri, R., Bortolato, M., Campolongo, P., Solinas, M., Cassano, T., Morgese, M.G., Debonnel, G., Duranti, A., Tontini, A., Tarzia, G., Mor, M., Trezza, V., Goldberg, S.R., Cuomo, V., Piomelli, D. (2005). Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc. Natl. Acad. Sci. U S A 102: 18620-18625.

Gorzalka, B.B., Hill, M.N. (2011). Putative role of endocannabinoid signaling in the etiology of depression and actions of antidepressants. Prog. Neuropsychopharmacol. Biol. Psychiatry 35: 1575-1585.

Griebel, G., Stemmelin, J., Scatton, B. (2005). Effects of the cannabinoid CB1 receptor antagonist rimonabant in models of emotional reactivity in rodents. Biol. Psychiatry 57: 261-267.

Goyens, J., Noirot, E. (1975). Effects of cohabitation with females on aggressive behavior between male mice. Dev. Psychobiol. 8: 79-84.

Guidali, C., Viganò, D., Petrosino, S., Zamberletti, E., Realini, N., Binelli, G., et al. (2011). Cannabinoid CB1 receptor antagonism prevents neurochemical and behavioural deficits induced by chronic phencyclidine. Int. J. Neuropsychopharmacol. 14: 17-28.

Gulyas, A.I., Cravatt, B.F., Bracey, M.H., Dinh, T.P., Piomelli, D., Boscia, F., Freund, T.F. (2004). Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. Eur. J. Neurosci. 20: 441-458.

Haenisch, B., Bönisch, H. (2011). Pharmacological characterization of tegaserod at the wild type and 124Cys variant of the human 5-HT1B receptor. Pharmacogenet. Genomics 21(7):432-5. doi: 10.1097/FPC.0b013e3283469f1d.

Hall, W., Solowij, N. (1998). Adverse effects of cannabis. Lancet 352: 1611-1616.

Haller, J., Varga, B., Ledent, C., Barna, I., Freund, T.F. (2004). Context-dependent effects of CB1 cannabinoid gene disruption on anxiety-like and social behaviour in mice. Eur. J. Neurosci. 19: 1906-12.

Haj-Dahmane, S., Shen, R.Y. (2009). Endocannabinoids suppress excitatory synaptic transmission to dorsal raphe serotonin neurons through the activation of presynaptic CB1 receptors. J. Pharmacol. Exp. Ther. 33: 186-96.

Haj-Dahmane, S., Shen, R.Y. (2011). Modulation of the serotonin system by endocannabinoid signaling. Neuropharmacol. 61: 414-420.

Häring, M., Marsicano, G., Lutz, B., Monory, K. (2007). Identification of the cannabinoid receptor type 1 in serotonergic cells of raphe nuclei in mice. Neuroscience 146: 1212-1219.

Häring, M., Kaiser, N., Monory, K., Lutz, B. (2011). Circuit specific functions of cannabinoid CB1 receptor in the balance of investigatory drive and exploration. PLoS One 6:e26617.

Häring, M., Guggenhuber, S., Lutz, B. (2012). Neuronal populations mediating the effects of endocannabinoids on stress and emotionality. Neuroscience 204:145-58.

Helms, C.M., Gubner, N.R., Wilhelm, C.J., Mitchell, S.H., Grandy, D.K. (2008). D4 receptor deficiency in mice has limited effects on impulsivity and novelty seeking. Pharmacol. Biochem. Behav. 90: 387-93.

Heiming, R.S., Jansen, F., Lewejohann, L., Kaiser, S., Schmitt, A., Lesch, K.P., Sachser, N. Living in a dangerous world: the shaping of behavioral profile by early environment and 5-HTT genotype. Front. Behav. Neurosci. 3: 26.

Heisler, L.K., Chu, H.M., Brennan, T.J., Danao, J.A., Bajwa, P., Parsons, L.H., Tecott, L.H. (1998). Elevated anxiety and antidepressant-like responses in serotonin 5-HT1A receptor mutant mice. Proc. Natl. Acad. Sci. 95: 15049-54.

Hill, M.N., Gorzalka, B.B. (2005). Pharmacological enhancement of cannabinoid CB1 receptor activity elicits an antidepressant-like response in the rat forced swim test. Eur Neuropsychopharmacol. 15: 593-9.

Hill, M.N., Hillard, C.J., Bambico, F.R., Patel, S., Gorzalka, B.B., Gobbi, G. (2009). The therapeutic potential of the endocannabinoid system for the development of a novel class of antidepressants. Trends Pharmacol. Sci. 9: 484-493.

Hill, M.N., McLaughlin, R.J., Bingham, B., Shrestha, L., Lee, T.T., Gray, J.M., Hillard, C.J., Gorzalka, B.B., Viau, V. (2010). Endogenous cannabinoid signaling is essential for stress adaptation. Proc. Natl. Acad. Sci. U S A 107: 9406-9411.

Hillard, C.J. (2000). Biochemistry and pharmacology of the endocannabinoids arachidonylethanolamide and 2-arachidonylglycerol. Prostaglandins Other Lipid Mediat 61:c3-18.

Hoffman, A.F., Laaris, N., Kawamura, M., Masino, S.A., Lupica, C.R. (2010). Control of cannabinoid CB1 receptor function on glutamate axon terminals by endogenous adenosine acting at A1 receptors. J. Neurosci. 30: 545-55

Homberg, J.R., Lesch, K.P. (2011). Looking on the bright side of serotonin transporter gene variation. Biol. Psychiatry.69: 513-9.

Höschl, C., Svestka, J. (2008). Escitalopram for the treatment of major depression and anxiety disorders. Expert Rev. Neurother. 8: 537-552.

Howlett, A.C., Blume, L.C., Dalton, G.D. (2010). CB(1) cannabinoid receptors and their associated proteins. Curr. Med. Chem. 17: 1382-93.

Indra, A.K., Warot. X., Brocard, J., Bornert, J.M., Xiao, J.H., Chambon, P., Metzger, D. (1999). Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. Nucleic Acids Res. 27: 4324-7.

Jacob, W., Yassouridis, A., Marsicano, G., Monory, K., Lutz, B., Wotjak, C.T. (2009). Endocannabinoids render exploratory behaviour largely independent of the test aversiveness: role of glutamatergic transmission. Genes Brain Behav. 8: 685-98.

Jankowski, M.P., Sesack, S.R. (2004). Prefrontal cortical projections to the rat dorsal raphe nucleus: ultrastructural features and associations with serotonin and gamma-aminobutyric acid neurons. J. Comp. Neurol. 468: 518-529.

Jansen, F., Heiming, R.S., Lewejohann, L., Touma, C., Palme, R., Schmitt, A., Lesch, K.P., Sachser, N. (2010). Modulation of behavioural profile and stress response by 5-HTT genotype and social experience in adulthood. Behav. Brain Res. 207: 21-9.

Jansen, F., Heiming, R.S., Kloke, V., Kaiser, S., Palme, R., Lesch, K.P., Sachser, N. (2011). Away game or home match: the influence of venue and serotonin transporter genotype on the display of offensive aggression. Behav. Brain Res. 219: 291-301.

Jesse, C.R., Wilhelm, E.A., Bortolatto, C.F., Nogueira, C.W. (2010). Evidence for the involvement of the noradrenergic system, dopaminergic and imidazoline receptors in the antidepressant-like effect of tramadol in mice. Pharmacol. Biochem. Behav. 95, 344-350.

Jiang, W., Zhang, Y., Xiao, L., Van Cleemput, J., Ji, S.P., Bai, G., Zhang, X. (2005). Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic and antidepressant-like effects. J. Clin. Invest. 115: 3104-3116.

Kano, M., Ohno-Shosaku, T., Hashimotodani, Y., Uchigashima, M., Watanabe, M. (2009). Endocannabinoid-mediated control of synaptic transmission. Physiol. Rev. 89: 309-80.

Kaster, M.P., Santos, A.R., Rodrigues, A.L. (2005). Involvement of 5-HT1A receptors in the antidepressant-like effects of adenosine in the forced swim test. Brain Res. Bull. 67: 53-61.

Ludányi, A., Eross, L., Czirják, S., Vajda, J., Halász, P., Watanabe, M., Palkovits, M., Maglóczky, Z., Freund, T.F., Katona, I. (2008). Downregulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus. J. Neurosci. 28: 2976-90.

Kawa, R., Pisula, E. (2010). Locomotor activity, object exploration and space preference in children with autism and Down syndrome. Acta Neurobiol. Exp. 70: 131-40.

Kelsoe, J.R. (2010). Behavioural neuroscience: A gene for impulsivity. Nature 468: 1049-50.

Lafenêtre, P., Chaouloff, F., Marsicano, G. (2009). Bidirectional regulation of novelty-induced behavioral inhibition by the endocannabinoid system. Neuropharmacology 57: 715-21.

Laorden, M.L., Castells, M.T., Martínez, M.D., Martínez, P.J., Milanés, M.V. (2000). Activation of c-fos expression in hypothalamic nuclei by mu- and kappa-receptor agonists: correlation with catecholaminergic activity in the hypothalamic paraventricular nucleus. Endocrinol. 141: 1366-76.

Laaris, N., Good, C.H., Lupica, C.R. (2010). Delta9-tetrahydrocannabinol is a full agonist at CB1 receptors on GABA neuron axon terminals in the hippocampus. Neuropharmacology. 59: 121-7.

Leonard, B.E. (2005). The HPA and immune axes in stress: the involvement of the serotonergic system. Eur. Psychiatry 20 Suppl 3:S302-306.

Lockie, S.H., Czyzyk, T.A., Chaudhary, N., Perez-Tilve, D., Woods, S.C., Oldfield, B.J., Statnick, M.A., Tschöp, M.H. (2011). CNS opioid signaling separates cannabinoid receptor 1-mediated effects on body weight and mood-related behavior in mice. Endocrinol. 152: 3661-7.

Loos, M., van der Sluis, S., Bochdanovits, Z., van Zutphen, I.J., Pattij, T., Stiedl, O. (2009). Activity and impulsive action are controlled by different genetic and environmental factors. Genes Brain. Behav. 8: 817-28.

Lucki, I., Dalvi, A., Mayorga, A.J. (2001). Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. Psychopharmacology (Berl) 155: 315-322.

Malek, Z.S., Dardente, H., Pevet, P., Raison, S. (2005). Tissue-specific expression of tryptophan hydroxylase mRNAs in the rat midbrain: anatomical evidence and daily profiles. Eur. J. Neurosci. 22: 895-901.

Manzanares, J., Corchero, J., Fuentes, J.A. (1999). Opioid and cannabinoid receptor-mediated regulation of the increase in adrenocorticotropin hormone and corticosterone plasma concentrations induced by central administration of delta(9)-tetrahydrocannabinol in rats. Brain Res. 839: 173-9.

Marsicano, G., Lutz, B. (1999). Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur. J. Neurosci. 11: 4213-25.

Marsicano, G., Lutz, B. (2006). Neuromodulatory functions of the endocannabinoid system. J. Endocrinol. Invest. 29: 27-46.

Marsicano, G., Kuner, R. (2008). Anatomical distribution of receptors, ligands and enzymes in the brain and the spinal cord: circuitries and neurochemistry. In Cannabinoids and the brain (ed. A. Kofalvi) 161-202 (Springer, New York, 2008).

Marrs, W.R., Blankman, J.L., Horne, E.A., Thomazeau, A., Lin, Y.H., Coy, J., Bodor, A.L., Muccioli, G.G., Hu, S.S., Woodruff, G., Fung, S., Lafourcade, M., Alexander, J.P., Long, J.Z., Li, W., Xu, C., Moller, T., Mackie, K., Manzoni, O.J., Cravatt, B.F., Stella, N. (2010). The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. Nat. Neurosci. 13: 951-957.

Martin, M., Ledent, C., Parmentier, M., Maldonado, R., Valverde, O. (2002). Involvement of CB1 cannabinoid receptors in emotional behaviour. Psychopharmacology (Berl) 159: 379-87.

Massa, F., Mancini, G., Schmidt, H., Steindel, F., Mackie, K., Angioni, C., Oliet, S.H., Geisslinger, G., Lutz, B. (2010). Alterations in the hippocampal endocannabinoid system in diet-induced obese mice. J. Neurosci. 30: 6273-6281.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346: 561-564.

McLaughlin, R.J., Hill, M.N., Gorzalka, B.B. (2009). Monoaminergic neurotransmission contributes to cannabinoid-induced activation of the hypothalamic-pituitary-adrenal axis. Eur. J. Pharmacol. 624: 71-76.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., et al. (1995).Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. 50: 83-90.

Mendiguren, A., Pineda, J. (2009). Effect of the CB(1) receptor antagonists rimonabant and AM251 on the firing rate of dorsal raphe nucleus neurons in rat brain slices. Br. J. Pharmacol. 158: 1579-87.

Monory, K., Massa, F., Egertova, M., Eder, M., Blaudzun, H., Westenbroek, R., Kelsch, W., Jacob, W., Marsch, R., Ekker, M., Long, J., Rubenstein, J.L., Goebbels, S., Nave, K.A., During, M., Klugmann, M., Wolfel, B., Dodt, H.U., Zieglgansberger, W., Wotjak, C.T., Mackie, K., Elphick, M.R., Marsicano, G., Lutz, B. (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. Neuron 51: 455-466.

Monory, K., Blaudzun, H., Massa, F., Kaiser, N., Lemberger, T., Schütz, G., et al. (2007). Genetic dissection of behavioural and autonomic effects of Δ^9-tetrahydrocannabinol in mice. PLoS Biol. 5: e269.

Moon, J., Beaudin, A.E., Verosky, S., Driscoll, L.L., Weiskopf, M., Levitsky, D.A., et al. (2006). Attentional dysfunction, impulsivity, and resistance to change in a mouse model of fragile X syndrome. Behav. Neurosci. 120: 1367-79.

Moreira, F.A., Lutz, B. (2008). The endocannabinoid system: emotion, learning and addiction. Addict. Biol. 13: 196-212.

Moreira, F.A., Crippa, J.A. (2009). The psychiatric side effects of rimonabant. Rev. Bras. Psiquiatr. 31: 145-153.

Moreira, F.A., Grieb, M., Lutz, B. (2009). Central side effects of therapies based on CB1 cannabinoid receptors agonist and antagonists: focus on anxiety and depression. Best Pract. Res. Clin. Endocrinol. Metab. 23: 133-144.

Moreira, F.A., Wotjak, C.T. (2010). Cannabinoids and anxiety. Curr. Top. Behav. Neurosci. 2, 429-450.

Moy, S.S., Nadler, J.J., Perez, A., Barbaro, R.P., Johns, J.M., Magnuson, T.R., et al. (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav. 3: 287-302.

Moy, S.S., Nadler, J.J., Young, N.B., Nonneman, R.J., Grossman, A.W., Murphy, D.L., et al. (2009). Social approach in genetically engineered mouse lines relevant to autism. Genes Brain. Behav. 8: 129-42.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. (1992). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Biotechnology 24: 17-27.

Muntoni, A.L., Pillolla, G., Melis, M., Perra, S., Gessa, G.L., Pistis, M. (2006). Cannabinoids modulate spontaneous neuronal activity and evoked inhibition of locus coeruleus noradrenergic neurons. Eur. J. Neurosci. 23: 2385-2394.

Nakazi, M., Bauer, U., Nickel, T., Kathmann, M. & Schlicker, E. (2000). Inhibition of serotonin release in the mouse brain via presynaptic cannabinoid CB1 receptors. Naunyn-Schmiedeberg's Archives of Pharmacology. 361: 19-24.

Nayak, S.V., Rondé, P., Spier, A.D., Lummis, S.C., Nichols, R.A. (1999). Calcium changes induced by presynaptic 5-hydroxytryptamine-3 serotonin receptors on isolated terminals from various regions of the rat brain. Neuroscience 91: 107-17.

Newcorn, J.H. (2001). New treatments and approaches for attention deficit hyperactivity disorder. Curr. Psychiatry Rep. 3: 87–91.

Nyberg, J.M., Vekovischeva, O., Sandnabba, N.K. (2003). Anxiety profiles of mice selectively bred for intermale aggression. Behav. Genet. 33: 503-11.

Oliet, S.H., Baimoukhametova, D.V., Piet, R., Bains, J.S. (2007). Retrograde regulation of GABA transmission by the tonic release of oxytocin and endocannabinoids governs postsynaptic firing. J. Neurosci. 27: 1325-33.

Oropeza, V.C., Mackie, K., Van Bockstaele, E.J. (2007). Cannabinoid receptors are localized to noradrenergic axon terminals in the rat frontal cortex. Brain Res. 1127: 36-44.

O'Shea, M., Singh, M.E., McGregor, I.S., Mallet, P.E. (2004). Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. J. Psychopharmacol. 18: 502-8.

Pandey, S., Davis, J. & Pandey, G. (1995). Phosphoinositide system-linked serotonin receptor subtypes and their pharmacological properties and clinical correlates. J. Psychiatry Neurosci. 20: 215–225.

Patel, P.D., Pontrello, C. & Burke, S. (2004). Robust and tissue-specific expression of TPH2 versus TPH1 in rat raphe and pineal gland. Biol. Psychiatry 55: 428-433.

Patel, S., Roelke, C.T., Rademacher, D.J., Cullinan, W.E., Hillard, C.J. (2004). Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. Endocrinol. 145: 5431-5438.

Patel, S., Hillard, C.J. (2006). Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. J. Pharmacol. Exp. Ther. 318: 304-311.

Parrish, J.C., Nichols, D.E. (2006). Serotonin 5-HT(2A) receptor activation induces 2-arachidonoylglycerol release through a phospholipase c-dependent mechanism. J. Neurochem. 99(4):1164-75.

Patel, A., Siegel, A., Zalcman, S.S. (2010). Lack of aggression and anxiolytic-like behavior in TNF receptor (TNF-R1 and TNF-R2) deficient mice. Brain Behav. Immun. 24: 1276-80.

Patterson, P.H. (2011). Modeling autistic features in animals. Pediatr. Res. 69: 34-40

Perry, W., Minassian, A., Paulus, M.P., Young, J.W., Kincaid, M.J., Ferguson, E. J. et al. (2009). A reverse translational study of dysfunctional exploration in psychiatric disorders: from mice to men. Arch. Gen. Psychiatry. 66:1072–1080

Petit-Demouliere, B., Chenu, F., Bourin, M. (2005). Forced swimming test in mice: a review of antidepressant activity. Psychopharmacology (Berl). 177(3):245-55.

Petrosino, S., Di Marzo, V. (2010). FAAH and MAGL inhibitors: therapeutic opportunities from regulating endocannabinoid levels. Curr. Opin. Investig. Drugs. 11:51-62.

Pietropaolo, S., Delage, P., Cayzac, S., Crusio, W.E., Cho, Y.H. Sex-dependent changes in social behaviors in motor pre-symptomatic R6/1 mice. PLoS One 6(5):e19965.

Piomelli, D. (2003). The molecular logic of endocannabinoid signalling. Nat. Rev. Neurosci. 4: 873-84.

Pompili, M., Serafini, G., Innamorati, M., Moller-Leimkuhler, A.M., Giupponi, G., Girardi, P., Tatarelli, R., Lester, D. (2010). The hypothalamic-pituitary-adrenal axis and serotonin abnormalities: a selective overview for the implications of suicide prevention. Eur. Arch. Psychiatry Clin. Neurosci. 260:583-600.

Rapport, M., Green, A. & Page, I. (1948). Serum vasoconstrictor (Serotonin). III. Chem. Inactivation. J. Biol. 6: 1237-1241.

Ruehle, S., Aparisi Rey, A., Remmers, F., Lutz, B. (2011). The endocannabinoid system in anxiety, fear memory and habituation. J. Psychopharmacol. 26(1):23-39.

Russell, V.A. (2007). Neurobiology of animal models of attention-deficit hyperactivity disorder. J. Neurosci. Methods 166: I-XIV.

Sala, M., Braida, D., Lentini, D., Busnelli, M., Bulgheroni, E., Capurro, V., et al. (2011). Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: A neurobehavioral model of autism. Biol. Psychiatry 69: 875-82

Sato, T.L., Jequier, E., Lovenberg, W., Sjoerdsma, A. (1967). Characterization of a tryptophan hydroxylating enzyme from malignant mouse mast cell. Eur. J. Pharmacol. 1: 18-25.

Schneider M, Schömig E, Leweke FM (2008). Acute and chronic cannabinoid treatment differentially affects recognition memory and social behavior in pubertal and adult rats. Addict. Biol. 13: 345-57.

Siesser WB, Zhao J, Miller LR, Cheng SY, McDonald MP (2006). Transgenic mice expressing a human mutant beta1 thyroid receptor are hyperactive, impulsive, and inattentive. Genes Brain Behav. 5: 282-97.

Solanto MV (2002). Dopamine dysfunction in AD/HD: integrating clinical and basic neuroscience research. Behav. Brain Res. 130: 65–71.

Steiner, M.A., Marsicano, G., Nestler, E.J., Holsboer, F., Lutz, B., Wotjak, CT. (2008a). Antidepressant-like behavioral effects of impaired cannabinoid receptor type 1 signaling coincide with exaggerated corticosterone secretion in mice. Psychoneuroendocrinol. 33: 54-67.

Steiner, M.A., Wanisch, K., Monory, K., Marsicano, G., Borroni, E., Bächli, H., Holsboer, F., Lutz B., Wotjak, C.T. (2008b). Impaired cannabinoid receptor type 1 signaling interferes with stress-coping behaviour in mice. Pharmacogenomics J. 8: 196-208.

Steiner, M.A., Marsicano, G., Wotjak, C.T., Lutz, B. (2008c). Conditional cannabinoid receptor type 1 mutants reveal neuron subpopulation-specific effects on behavioral and neuroendocrine stress responses. Psychoneuroendocrinol. 33: 1165-1170.

Steiner, M.A., Wotjak, C.T. (2008). Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis. Prog. Brain. Res. 170: 397-432.

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A. & Waku, K. (1995). 2-Arachidonoylgylcerol: A Possible Endogenous Cannabinoid Receptor Ligand in Brain. Biochemical and Biophysical Research Communications. 215: 89-97.

Tang, Y.P., Shimizu, E., Dube, G.R., Rampon, C., Kerchner, G.A., Zhuo, M., Liu, G., Tsien, J.Z. (1999). Genetic enhancement of learning and memory in mice. Nature 401: 63-69.

Thor, D.H., Flannelly, K.J. (1976). Age of intruder and territorial-elicited aggression in male long-evans rats. Behav. Biol. 17: 237-41.

Tordera, R.M., Totterdell, S., Wojcik, S.M., Brose, N., Elizalde, N., Lasheras, B., Del Rio, J. (2007). Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). Eur. J. Neurosci. 25: 281–290.

Trezza, V., Vanderschuren, L.J. (2008a). Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology (Berl). 197: 217-27.

Trezza, V., Vanderschuren, L.J. (2008b). Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. Eur. Neuropsychopharmacol. 18: 519-30.

Trezza, V., Vanderschuren, L.J. (2009). Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. J. Pharmacol. Exp. Ther. 328: 343-50

Turner, J.H., Gelasco, A.K., Raymond, J.R. (2004). Calmodulin interacts with the third intracellular loop of the serotonin 5-hydroxytryptamine1A receptor at two distinct sites: putative role in receptor phosphorylation by protein kinase C. J. Biol. Chem. 279: 17027-37.

Twarog, B.M. & Page, I.H. (1953). Serotonin Content of Some Mammalian Tissues and Urine and a Method for Its Determination. Am. J. Physiol. 175: 157-161.

Tzavara, E.T., Davis, R.J., Perry, K.W., Li, X., Salhoff, C., Bymaster, F.P., Witkin, J.M., Nomikos, G.G. (2003). The CB1 receptor antagonist SR141716A selectively increases monoaminergic neurotransmission in the medial prefrontal cortex: implications for therapeutic actions. Br. J. Pharmacol. 138: 544-553.

Urigüen, L., Pérez-Rial, S., Ledent, C., Palomo, T., Manzanares, J. (2004). Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors. Neuropharmacology 46: 966-73.

Veenema, A.H., Torner, L., Blume, A., Beiderbeck, D.I., Neumann, I.D. (2007). Low inborn anxiety correlates with high intermale aggression: link to ACTH response and neuronal activation of the hypothalamic paraventricular nucleus. Horm. Behav. 51: 11-9.

Veenstra-VanderWeele, J., Anderson, G.M. & Edwin, H. (2000). Pharmacogenetics and the serotonin system: initial studies and future directions. Eur. J. Pharmacol. 410: 165-181.

Viveros, M.P., Marco, E.M., File, S.E. (2005). Endocannabinoid system and stress and anxiety responses. Pharmacol. Biochem. Behav. 81: 331-342.

Wade, M.R., Degroot, A., Nomikos, G.G. (2006). Cannabinoid CB1 receptor antagonism modulates plasma corticosterone in rodents. Eur. J. Pharmacol. 551: 162-7.

Waider, J., Araragi, N., Gutknecht, L., Lesch, K.P. (2011). Tryptophan hydroxylase-2 (TPH2) in disorders of cognitive control and emotion regulation: a perspective. Psychoneuroendocrinology. 36: 393-405.

Walther, D.J. & Bader, M. (2003). A unique central tryptophan hydroxylase isoform. Biochem. Pharmacol., 66: 1673-1680.

Weber, T., Böhm, G., Hermann, E., Schütz, G., Schönig, K., Bartsch, D. (2009). Inducible gene manipulations in serotonergic neurons. Mol. Neurosci. 2:24.

Wenger, T., Ledent, C., Tramu, G. (2003). The endogenous cannabinoid, anandamide, activates the hypothalamo-pituitary-adrenal axis in CB1 cannabinoid receptor knockout mice. Neuroendocrinol. 78: 294-300.

Williams, K., Wheeler, D.M., Silove, N., Hazell, P. (2010). Selective serotonin reuptake inhibitors (SSRIs) for autism spectrum disorders (ASD).Cochrane Database Syst. Rev. (8):CD004677.

Wotjak, C.T., (2005). Role of endogenous cannabinoids in cognition and emotionality. Mini. Rev. Med. Chem. 5: 659-670.

Wu, W.L., Lin, Y.W., Min, M.Y., Chen, C.C. (2010). Mice lacking Asic3 show reduced anxiety-like behavior on the elevated plus maze and reduced aggression. Genes Brain Behav. 9: 603-14.

Zanelati, T., Biojone, C., Moreira, F.A., Guimaraes, F.S., Joca, S.R. (2010). Antidepressant-like effects of cannabidiol in mice: Possible role of 5-HT1A receptors Br. J. Pharmacol. 159: 122-128.

Zhou, F.C., Tao-Cheng, J.H., Segu, L., Patel, T., Wang, Y. (1998). Serotonin transporters are located on the axons beyond the synaptic junctions: anatomical and functional evidence. Brain Res. 805: 241-54.

Zuardi, A.W., Shirakawa, I., Finkelfarb, E., Karniol, I.G. (1982). Action of cannabidiol on the anxiety and other effects produced by Δ9-THC in normal subjects. Psychopharmacology (Berl) 76: 245-250.

Appendix

I. Abbreviation

Abbreviation	Description
2-AG	2-arachidonoyl glycerol
5-HT	5-hydroxytryptamine
5-HT1-7	5-hydroxytryptamine receptor type 1-7
5-HTP	5-hydroxytryptophan
5-HTT	serotonin reuptake transporter
ABHD6	α,β-hydrolase domain 6
ACTH	adrenocorticotropic hormone
AC	adrenal cortex
AdCy	adenylase cyclase
AEA	anandamide
ANOVA	analysis of variance
BA	basolateral nucleus of BLA
BLA	basolateral amygdala
BNST	bed nuclei of the stria terminalis
CA	cornu ammonis
CB_1 receptor	cannabinoid type 1 receptor
CeA	central amygdala
CeAL	lateral part of CeA;
CeAM	medial part of CeA
CORT	corticosterone
Cre	cyclization or Causes recombinase
Cre-ER^{T2}	cre recombinase fused to a mutated ligand binding domain of the human estrogen receptor
CRH	corticotropin-releasing hormone
D1	dopamine type 1 receptor
DAGL	diacylglycerol lipase
DG	dentate gyrus
DI	discrimination index
DMSO	dimethylsulfoxid
EC	entorhinal cortex
eCB	endocannabinoid
EMT	endocannabinoid membrane transporter
eEPSCs	evoked excitatory postsynaptic currents
F	familiar
FAAH	fatty acid amide hydrolase
FST	forced swim test
GABA	γ-(aminobutyric acid

HPA	hypothalamic-pituitary-adrenal axis
HSP	heat shock protein
i.p.	Intraperitoneal
LA	lateral nucleus of BLA
LC	locus coeruleus
LHA	left homology arm
loxP	locus of crossover (x) in P1
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
NAT	n-acyltransferase
nO	novel object
nA	novel animal
NORT	novel object recognition test
pCPA	parachlorophenylalanine
PFC	prefrontal cortex
PI	pituitary
PLC	phospholipase C
PLD	phospholipase D
PFC	prefrontal cortex
PVN	hypothalamic paraventricular nucleus
RHA	right homology arm
RI	recognition index
Rim	rimonabant (SR141716); CB_1 receptor antagonist
S	subiculum
SEM	standard error of means
THC	Δ^9-tetrahydrocannabinol
TPH1	tryptophan hydroxylase type 1
TPH2	tryptophan hydroxylase type 2
TRPV1	transient receptor potential cation channel subfamily V1
Tween-80	polyoxyethylenesorbitan monooleate
Veh	vehicle
VMAT2	vesicular monoamine transporter type-2
WAY	WAY100635; 5HT1A receptor antagonist

Additional international abbreviations were used for units of measurements

II. Wild-type and transgenic animals

Strain	Description
C57BL/6N mice	Wild-type strain from Charles River, Germany
Glu-$CB_1^{-/-}$ mice	$CB_1^{flox/flox;Nex\text{-}cre}$ mice (Monory et al., 2006); animals with genetic deletion of the CB_1 receptor from glutamatergic neurons
GABA-$CB_1^{-/-}$ mice	$CB_1^{flox/flox;dlx\text{-}cre}$ mice (Massa et al., 2010); animals with genetic deletion of the CB_1 receptor from GABAergic neurons
TPH2-$CB_1^{-/-}$ mice	$CB_1^{flox/flox;tph2\text{-}CreERT2}$ mice (Häring et al., unpublished); animals depleted of the CB1 receptor from serotonergic neurons
D1-$CB_1^{-/-}$ mice	$CB_1^{flox/flox;D1\text{-}cre}$ mice (Monory et al., 2007); animals with genetic deletion of the CB_1 receptor from glutamatergic neurons

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V. Danksagung

VI. Curriculum vitae

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Education

10.2006 - today	**Johannes Gutenberg University, Mainz** Ph.D. education in the group of Prof. Beat Lutz
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10.2003 - 09.2004	**Lund University, Sweden; ERASMUS Exchange** (course language: English)
04.2001 - 09.2003	**Johannes Gutenberg University, Mainz** Biology Basic Studies
07.1996 - 06.1999	**Heimschule St. Landolin, Ettenheim** High School Main subjects: Biology, Economy
07.1990 - 06.1996	**Emil-Dörle Realschule, Herbolzheim** Junior High School
07.1986 - 06.1990	**Grundschule, Herbolzheim** Elementary School

Job activities

11.2000 - 03.2001	**EBM-Papst St. Georgen GmbH & Co. KG** Werk 2, Rheinhausenstraße 17 79336 Herbolzheim Function: internal logistic
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Alternative Service

08.1999 - 07.2000 **Community hospital , Herbolzheim**
Internal medicine and surgery infirmary
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Teaching

During my Ph.D. thesis I have assisted and supervised medical and biology students in the following practical courses:

2006-2009 **Biochemistry and molecular biology for medical students**

2006-2011 **Physiological chemistry and pathobiochemistry for biology students**
(2010-2011: organization of the course for the institute)

2010-2011 **IAK-Neuro; molecular and cellular neurobiology for biology students**

2010 **"Detecting gene expression in the nervous system by *in situ* hybridisation" for Ph.D. students**
(supported by the German Neuroscience Society)

Rewards

10.2010 "**Young investigator award**" received at "Cannabinoids in Medicine Symposium" in Jerusalem, Israel.

Extracurricular activies

10.2002 - 10.2006 Member of dormitory representation

04.2007 – 03.2009 Neurobiology Graduate School of the Medical School of the Johannes Gutenberg University

Since 2009 I joined the Mixed-Volleyball Team of the University, Mainz.

Publication list

First-author

Häring, M., Marsicano, G., Lutz, B., Monory, K. (2007). Identification of the cannabinoid receptor type 1 in serotonergic cells of raphe nuclei in mice. Neuroscience 146:1212-9.

Häring, M., Kaiser, N., Monory, K., Lutz, B. (2011). Circuit specific functions of cannabinoid CB1 receptor in the balance of investigatory drive and exploration. PLoS ONE 6(11):e26617.

Häring, M., Guggenhuber, S., Lutz, B. (2012). Neuronal populations mediating the effects of endocannabinoids on stress and emotionality. Neuroscience 204:145-58. (Review)

Co-authorship

Kamprath, K., Romo-Parra, H., Häring, M., Gaburro, S., Doengi, M., Lutz, B., Pape, H.C. (2011). Short-term adaptation of conditioned fear responses through endocannabinoid signaling in the central amygdala. Neuropsychopharmacology 36(3):652-63.

Dubreucq, S., Matias, I., Cardinal, P., Häring, M., Lutz, B., Marsicano, G., Chaouloff, F. (2012). Genetic dissection of the role of the cannabinoid type -1 receptors in the emotional consequences of repeated social stress in mice. Neuropsychopharmacology doi: 10.1038/npp.2012.36.

Underlined manuscripts are attached at the end of the thesis.

Conference attandence

29.03. – 01.04.2007	**Seventh Göttingen Meeting of the German Neuroscience Conference;** Poster presentation
26.06. – 30.06.2007	**17th ICRS Symposium on the Cannabinoids;** Poster presentation
04.12.2009	**9th Annual IAK Meeting, Mainz;** Oral presentation
17.10. – 21.19.2009	**SFN conference Chicago;** Poster presentation
18.06. – 19.06.2010	**Cannabinoid Workshop Bonn;** Poster presentation
31.10. – 04.11.2010	**Cannabinoid in Biology and Medicine Workshop Jerusalem;** Poster presentation
22.05. – 27.05.2010	**GRC Meeting Les Diablerets, Cannabinoid function in the CNS;** Poster presentation

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Circuit Specific Functions of Cannabinoid CB1 Receptor in the Balance of Investigatory Drive and Exploration

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Abstract

Well balanced novelty seeking and exploration are fundamental behaviours for survival and are found to be dysfunctional in several psychiatric disorders. Recent studies suggest that the endocannabinoid (eCB) system is an important control system for investigatory drive. Pharmacological treatment of rodents with cannabinergic drugs results in altered social and object investigation. Interestingly, contradictory results have been obtained, depending on the treatment, drug concentration and experimental conditions. The cannabinoid type 1 (CB1) receptor, a central component of the eCB system, is predominantly found at the synapses of two opposing neuronal populations, i.e. on inhibitory GABAergic and excitatory glutamatergic neurons. In the present study, using different transgenic mouse lines, we aimed at investigating the impact of CB1 receptor inactivation in glutamatergic or GABAergic neurons on investigatory behaviour. We evaluated animate (interaction partner) and inanimate (object) exploratory behaviour in three different paradigms. We show that exploration was increased when CB1 receptor was deleted from cortical and striatal GABAergic neurons. No effect was observed when CB1 receptor was deleted specifically from dopamine receptor D1-expressing striatal GABAergic medium spiny neurons. In contrast, deletion of CB1 receptor from cortical glutamatergic neurons resulted in a decreased exploration. Thus, our results indicate that exploratory behaviour is accurately balanced in both, the social and non-social context, by the eCB system via CB1 receptor activation on cortical glutamatergic and GABAergic neurons. In addition, the results could explain the contradictory findings of previous pharmacological studies and could further suggest a possibility to readjust an imbalance in exploratory behaviour observed in psychiatric disorders.

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Introduction

Adequate novelty seeking and exploration are fundamental behaviours for survival. Dysfunctional exploratory profiles have been found in several distinct neuronal disorders, such as attention deficit disorder and schizophrenia-like diseases, expressed by modulated social behaviour and novelty seeking [1–5]. Thus, identifying control mechanisms of exploratory behaviour might allow new treatment strategies. Two recent studies indicated that the endocannabinoid (eCB) system might be important for a balanced response to novel situations [6,7], but these studies elucidated only some aspects on the function of the eCB system in exploratory behaviour. Lafenêtre et al. [6] concentrated on object recognition with repeated exposures to a novel object and food pellet, thus, strongly reducing the novelty factor every day. Jacob et al. [7] performed multiple behavioural paradigms, including also social interaction studies. However, the latter study was only performed with animals lacking the cannabinoid type 1 (CB1) receptor completely or specifically in cortical glutamatergic neurons. To this end, the present study aimed at further detailing CB1 receptor functions in investigatory drive and exploration behaviour.

One important factor in exploratory behaviour is how a respective situation is evaluated. Brain regions involved in these evaluation processes, such as amygdala, hippocampus, and prefrontal cortex, show high levels of CB1 receptor mRNA and protein [8,9]. These cortical areas possess two major neuronal subpopulations expressing the CB1 receptor; GABAergic interneurons (with high CB1 receptor levels) and glutamatergic neurons (with low CB1 receptor levels) [8,10–12]. The two neuronal populations represent the two major opposing players regarding the excitation state of the brain, namely GABAergic interneurons being inhibitory and glutamatergic neurons being excitatory. The endogenous ligands of CB1 receptor, the so-called endocannabinoids, are synthesized at the postsynapse and travel retrogradely to the CB1 receptor located at the presynapse [13]. Activated CB1 receptor then suppresses neurotransmitter release. Therefore, a functional eCB system may have a protective role to prevent an imbalance of neuronal activity and inadequate behavioural responses. In accordance with this notion, it was shown that the inactivation of the CB1 receptor gene from glutamatergic neurons leads to an increased vulnerability to kainic acid-induced seizures [10]. Furthermore, other behavioural studies indicated a bidirectional role of the eCB system in anxiety response based on CB1 receptor located on these two neuronal subpopulations [6,7,14].

Anxiety plays a critical role in exploratory and investigatory behaviour, and several pharmacological studies have shown the

importance of the eCB system in social behaviour [15–19]. The results depended strongly on the treatment and experimental conditions, and they seemed to be contradictory at first sight. The acute and chronic administration of CB1 receptor agonists (Δ^9-THC or WIN55,212-2), especially during adolescence, led to a decreased social interaction in rats. Opposing to this, treatment of adolescent rats with URB597, an inhibitor of anandamide degradation, or VDM11, a putative anandamide reuptake inhibitor, resulted in increased social play behaviour [17–19]. These latter findings are supported by studies with transgenic mice lacking the CB1 receptor ubiquitously or specifically in cortical glutamatergic neurons, where a decrease in object exploration and social interaction was shown, depending on the behavioural context [7,20]. Altogether, these data suggest that strong systemic activation of the eCB system has anti-social effects, whereas on-demand enhancement of anandamide signalling and subsequent activation of CB1 receptor has a pro-social effect.

By using several conditional CB1 receptor knock-out mice, we aimed at investigating whether CB1 receptor on different neuronal cell types might explain the contradictory findings in social interaction and object exploration mentioned above. In order to address this question, we applied different behavioural paradigms to analyze inanimate (object) exploration and animate (interaction partner) exploration. Evaluating the results, we could detect a decreased exploratory drive in mice lacking CB1 receptor in cortical glutamatergic neurons. Mice lacking CB1 receptor in GABAergic neurons, including the striatum, displayed opposite results, namely, an increased exploratory drive. No changes in exploration were observed for mice lacking CB1 receptor specifically in striatal dopamine receptor D1-positive GABAergic medium spiny neurons. Thus, we hypothesize that cortical GABAergic interneurons are important for the increased exploratory drive. Altogether, our results suggest that exploratory behaviour (animate and inanimate) is balanced by the eCB system via CB1 receptor activation on the two opposing neuronal subpopulations.

Materials and Methods

Animals

This study was performed on adult (5–7 months old) male mutant mice and their respective wild-type littermates. Animals were housed in a temperature- and humidity-controlled room (22°C±1; 50%±1) with a 12 h light-dark cycle (lights on at 1 am) and had access to food and water *ad libitum*. The experimental protocols were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Ethical Committee on animal care and use of Rhineland-Palatinate, Germany. Generation, breeding and genotyping of the mutant lines were performed according to previous publications: $CB1^{loxP/loxP;Nex\text{-}cre}$ mice (referred to as Glu-$CB1^{-/-}$ mice; [10]), $CB1^{loxP/loxP;Dlx5/6\text{-}cre}$ mice (referred to as GABA-$CB1^{-/-}$ mice; [10,21]), and $CB1^{loxP/loxP;D1\text{-}cre}$ mice (referred to as D1-$CB1^{-/-}$ mice; [11]). While Glu-$CB1^{-/-}$ mice lack the CB1 receptor in cortical glutamatergic neurons, GABA-$CB1^{-/-}$ mice lack the CB1 receptor specifically in GABAergic neurons [10]. In D1-$CB1^{-/-}$ mice, the CB1 receptor inactivation can primarily be found in GABAergic striatal medium spiny neurons, but also in a minor fraction of glutamatergic neurons in layer VI of the neocortex [11]. Wild-type littermates do not possess the respective Cre recombinase transgenic allele, and contain the CB1 floxed allele in a homozygous state. These mice were referred to as Glu-$CB1^{+/+}$, D1-$CB1^{+/+}$ and GABA-$CB1^{+/+}$. All mutant lines were bred for >10 generations on the background of C57BL/6N mice from Charles River, Germany. For detailed information on the anatomical differences in CB1 receptor expression, see Monory et al. [11].

Experimental design

Animals were group-housed (3–5 animals per cage type 2 (26.5×20.5×14.0 cm), EBECO Germany) until one week before behavioural testing. Animals were then separated and single-housed to avoid behavioural differences between dominant and subordinate animals. The same animals were used in each paradigm. Between each experimental paradigm, animals were allowed to rest for one week. All experiments were performed one hour after turning off the lights (2 pm), in the active phase of the animals, with only a minimal red light source in the room (0 lux).

Open Field and Novel Object Recognition Task

The novel object recognition task combines a general exploration test with a visual recognition memory paradigm. Therefore, it is used to evaluate object exploration and object recognition. The test was performed in a white plastic open field chamber (H40 cm×W40 cm×L40 cm). The protocol used was modified from Ennaceur and Delacour , Tang et al., and Tordera et al. [22–24].

For habituation, the animals were placed into the empty open field and allowed to explore the box for 10 min once a day for two days. The first habituation session was analyzed according to a standard open field paradigm, hence, total distance moved and time spent in the center (defined as 20 cm×20 cm) was evaluated using SMART software (PanLab, Spain). On day 3, two identical objects (O1 left, and O1 right; two metal cubes with H4 cm×W3 cm×L5 cm) were placed symmetrically 6–7 cm from the walls and separated 16–18 cm from each other. The mouse was placed into the box at an equal distance from both objects and video-recorded for 10 min. After this first exposure to the object, the mouse was returned to its home cage. 2 h and 24 h later, the mouse was placed again into the open field and exposed to the familiar object (O1) and to a novel object (O2 for the 2 h time point, and O3 for the 24 h time point, respectively) each time for 10 min (retention tests). The novel object O2 was a plastic billiard ball (5.72 cm in diameter) fixed on a metal plate (0.2 cm) and O3 was a round glass flask (H6 cm×W3 cm), filled with sand and closed with a black rubber plug. The familiar object was always positioned on the left side, while the new object was on the right side. Box and objects were cleaned with 70% ethanol after each trial to avoid olfactory cues. Experiment was video-recorded and the total time that the animal spent exploring each of the two objects in training and retention phase was evaluated by an experimenter blind to the genotype. Object exploration was defined as the orientation of the nose directly to the object at a distance <2 cm and/or touching the object with the nose and whiskers. Time spent climbing and sitting on the object were not regarded as exploration, and was therefore excluded from measurement [22], as these activities do not present a form of exploration. The discrimination index (DI) was calculated as the difference between the time spent exploring the new (N) and familiar (F) object, divided by the total time exploring the objects [(N−F)/(N+F)]. A positive DI is considered to reflect increased memory retention for the familiar object [24].

Sociability Test

A modified sociability test was performed, based on a published protocol [25]. In short, the test chamber (H41 cm×W42 cm×L70 cm) was divided into three compartments (H40 cm×W40 cm ×L22 cm), all accessible by openings (H7.5 cm×W10 cm) in the

dividing walls. Chambers and cages were cleaned with 70% ethanol between each trial to avoid olfactory cues. Experiment was video-recorded, and the total time that the test animals spent in each of the compartments during sociability and social novelty phase was measured by SMART software (PanLab, Spain). Male C57BL/6N animals (10–12 weeks old) were used as interaction partners for the sociability and social novelty phase.

Habituation Phase. The test animal was placed into the middle compartment for 5 min with entries to the side compartments blocked.

Sociability Phase. After the habituation phase, blockades of the entries were removed, allowing free access to the side compartments for 10 min. By doing this, the animal tested was exposed to a novel C57BL/6N interaction partner and a novel object (round cage described below), positioned in the two side compartments. The position of the interaction partner (left vs. right compartment) was alternated between trials to avoid any bias. The interaction partner itself was enclosed in a round cage (10 cm in diameter; 30 cm high [upper 20 cm Plexiglass, lower 10 cm covered by metal bars 1 cm apart to allow interaction but prevents fighting]). To minimize stress levels of the animals used as interaction partners, they were habituated to the cages four times for 10 min distributed over two days prior to the actual test days. To counterbalance individual differences of these interaction partners they were equally used for wild-type and mutant test mice. The novel object control (empty cage, no animal) was always positioned in the opposite compartment to the cage with the interaction partner. The discrimination index (DI) was calculated as the difference between the time spent exploring the novel object (nO) and the novel animal (nA), divided by the total time exploring both [(nO−nA)/(nO+nA)]. A positive DI is considered to reflect increased preference for the social interaction partner.

Social Novelty Phase. 2 h after the sociability phase, an additional, unknown interaction partner (novel) was introduced. The interaction partner from the sociability phase (familiar) was again placed into the same cage and same compartment as before. The novel animal was placed into the former empty cage and positioned at the respective side compartment. Openings were unblocked. The test animal was placed into the middle compartment, and the test animal was allowed to freely explore for 10 min. The DI was calculated as the difference between the time spent exploring the new (N) and the familiar (F) animal, divided by the total time exploring both [(N−F)/(N+F)]. A positive DI is considered to reflect increased memory retention for the familiar animal.

Resident-Intruder Test

The resident-intruder test was performed by placing a novel, group-housed intruder into the home cage of the test animal for 10 min. This paradigm allows evaluating social exploration and aggressive behaviour [26]. To decrease interaction induced by the intruder, younger animals (males, 11–13 weeks) were used as intruders. Experiment was video-recorded, and the total interaction time of the animals spent exploring was measured by an experimenter blind to the genotype. Interaction was defined by any type of physical interaction induced by the resident clearly directed towards the partner. Duration, percentage of time and number of fights were evaluated separately. Fighting was defined by physical struggling between the interaction partners initiated by an attack of the resident towards the intruder.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of individual data points. Results were considered to be significant at $p<0.05$. All behavioural endpoints of the novel object recognition task were initially analyzed using two-way ANOVA, using genotype and object as variables and Bonferroni post-tests to correct for multiple comparisons. In some cases, to analyze the locomotion effects in the open field, the sociability in the sociability test and the aggression in the resident-intruder paradigm for each genotype, data were analysed using an unpaired Student's t-test or Kruskal-Wallis statistic. Additionally, in order to evaluate whether the DI of the genotypes deviated significantly from zero, we used the unpaired t-test with Welch's correction. Graphs and statistics were generated by GraphPad Prism 4.03 (GraphPad Software; http://www.graphpad.com).

Results

Open Field

The evaluation of the locomotor activity in the open field revealed that only the GABA-CB1$^{-/-}$ mice showed an alteration ($T_{(18)} = 3.213$, $p = 0.0048$; Table 1). None of the other mutants showed any change in the distance moved compared with their respective wild-type littermates in the open field (Glu-CB1 line [$T_{(34)} = 1.609$, $p = 0.1169$]; D1-CB1 line [$T_{(21)} = 0.5618$, $p = 0.5802$]). In regard to the time spent in the center region of the open field, we could not detect an alteration in any of the mutants (Glu-CB1 line [$T_{(34)} = 0.8168$, $p = 0.4197$]; GABA-CB1 line [$T_{(18)} = 1.418$, $p = 0.1733$]; D1-CB1 line [$T_{(21)} = 0.9048$, $p = 0.3758$]; see Table 1).

Novel Object Recognition Task

The analysis of the novel object recognition task (referred to as NORT in Table 1) revealed a decrease in general object exploration in Glu-CB1$^{-/-}$ mice as compared to wild-type littermate controls (Fig. 1A,D,G). We detected a significant decrease in time spent with the objects O1 in the training session ($F_{(1,62)} = 4.183$, $p = 0.0451$; Fig. 1A), but also in the 2 h ($F_{(1,66)} = 13.68$, $p = 0.0004$; Fig. 1D) and 24 h retention sessions ($F_{(1,66)} = 32.87$, $p<0.0001$; Fig. 1G) for the novel object O2. In contrast, GABA-CB1$^{-/-}$ mice displayed a general increase in exploration in all the sessions as compared to controls (training [$F_{(1,74)} = 17.88$, $p<0.0001$], 2 h retention [$F_{(1,74)} = 8.411$, $p = 0.0049$)], 24 h retention [$F_{(1,74)} = 6.172$, $p = 0.0152$]; Fig. 1B,E,H). In the D1-CB1 mutant line, no genotype differences were observed in the general object exploration (training [$F_{(1,44)} = 1.760$, $p = 0.1915$], 2 h retention [$F_{(1,44)} = 0.08051$, $p = 0.7721$], 24 h retention [$F_{(1,44)} = 3.317$, $p = 0.0754$]; Fig. 1C,F,I).

Evaluation of the discrimination index (DI) revealed that all groups, independent of the line, showed no differences within the training session regarding the exploration of the left and the right object O1, respectively. (Glu-CB1$^{+/+}$ [$T_{(20)} = 0.8230$, $p = 0.4202$]; Glu-CB1$^{-/-}$ [$T_{(11)} = 0.9582$, $p = 0.3585$]; GABA-CB1$^{+/+}$ [$T_{(15)} = 1.118$, $p = 0.2812$]; GABA-CB1$^{-/-}$ [$T_{(22)} = 1.959$, $p = 0.0630$]; D1-CB1$^{+/+}$ [$T_{(11)} = 1.447$, $p = 0.1758$]; D1-CB1$^{-/-}$ [$T_{(11)} = 1.679$, $p = 0.1213$]; Table 1). Furthermore, no discrimination differences compared to their respective wild-type controls were found for all mutants within the training session (Glu-CB1 line [$T_{(31)} = 1.407$, $p = 0.1693$]; GABA-CB1 line [$T_{(37)} = 0.06488$, $p = 0.9486$]; D1-CB1 line [$T_{(22)} = 1.951$, $p = 0.0639$]; Table 1).

In the 2 h retention phase, several groups lacked a significant discrimination between the familiar and the novel object. Only Glu-CB1$^{+/+}$, D1-CB1$^{+/+}$ and D1-CB1$^{-/-}$ animals displayed a significant preference towards the novel stimulus (Glu-CB1$^{+/+}$ [$T_{(21)} = 4.806$, $p<0.0001$]; Glu-CB1$^{-/-}$ [$T_{(12)} = 1.220$, $p = 0.2458$]; GABA-CB1$^{+/+}$ [$T_{(15)} = 0.07097$, $p = 0.9444$]; GABA-CB1$^{-/-}$ [$T_{(22)} = 1.366$, $p = 0.1858$]; D1-CB1$^{+/+}$ [$T_{(10)} = 2.502$, $p = 0.0313$]; D1-CB1$^{-/-}$ [$T_{(10)} = 2.238$, $p = 0.0492$]; Table 1). Comparison

Table 1. Locomotion, anxiety and memory.

	Glu-CB1		GABA-CB1		D1-CB1	
Paradigm	**+/+**	**−/−**	**+/+**	**−/−**	**+/+**	**−/−**
	Distance Moved (cm)					
Open Field	2824±209	2324±182	2621±306	3801±202**	4456±91	4368±131
Sociability						
Habituation	2012±113	1569±162*	1597±59	1730±64	1669±81	1850±118
Sociability	5171±205	4891±312	4945±127	5083±175	5055±162	5079±157
Social Novelty	3973±211	3125±227*	4023±141	4623±175*	3863±292	3942±160
	Time in Center (sec)					
Open Field	63.3 ± 12	80.9±20	156.0±48	82.0±21	65.4±11	82.4±15
	Discrimination Index (DI)					
NORT						
Training	0.01±0.01	−0.08±0.08	−0.03±0.03	−0.03±0.02	−0.08±0.05	0.03±0.02
Retention 2 h	0.16±0.03#	0.06±0.05	0.00±0.06	−0.03±0.03	0.15±0.06#	0.08±0.03#
Retention 24 h	0.25±0.06#	0.06±0.13	0.18±0.06#	0.15±0.05#	0.27±0.08#	0.18±0.05#
Sociability						
Sociability	0.29±0.03#	0.12±0.07*	0.27±0.03#	0.35±0.04#	0.20±0.04#	0.30±0.04#
Social Novelty	0.05±0.03	0.08±0.09	−0.01±0.05	0.09±0.03#	0.03±0.06	0.03±0.06

Evaluation of locomotion (distance moved), anxiety (time in center) and memory (discrimination index) for all mutant lines; $^{+/+}$ (wild-type), $^{-/-}$ (mutant); t-test analysis:
*$p<0.05$;
**$p<0.01$ (significance between genotype);
#$p<0.05$ (significant from 0; positive recognition of novel object).
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between the mutants and their respective wild-type littermates displayed no significant differences in all lines (Glu-CB1 line [$T_{(33)} = 1.775$, $p = 0.0850$]; GABA-CB1 line [$T_{(37)} = 0.6235$, $p = 0.5368$]; D1-CB1 line [$T_{(20)} = 0.9965$, $p = 0.3309$]; Table 1).

In the 24 h retention phase, independently of the genotype, all groups showed a significant preference towards the novel object, with the only exception of the Glu-CB1$^{-/-}$ animals (Glu-CB1$^{+/+}$ [$T_{(21)} = 4.472$, $p = 0.0002$]; Glu-CB1$^{-/-}$ [$T_{(12)} = 0.4328$, $p = 0.6729$]; GABA-CB1$^{+/+}$ [$T_{(15)} = 2.818$, $p = 0.0129$]; GABA-CB1$^{-/-}$ [$T_{(22)} = 3.072$, $p = 0.0056$]; D1-CB1$^{+/+}$ [$T_{(11)} = 3.601$, $p = 0.0042$]; D1-CB1$^{-/-}$ [$T_{(11)} = 3.540$, $p = 0.0046$]; Table 1). Comparison between the mutants and their respective wild-type littermates diplayed no genotype difference (Glu-CB1 line [$T_{(33)} = 1.522$, $p = 0.1374$]; GABA-CB1 line [$T_{(37)} = 0.1255$, $p = 0.9008$]; D1-CB1 line [$T_{(22)} = 1.049$, $p = 0.3055$]; Table 1).

The evaluation of object specific exploration (O1 left or O1-3 right) over the three sessions (training, 2 h retention and 24 h retention), revealed a significant difference for the Glu-CB1$^{-/-}$ as compared to their littermate controls. Thus, the Glu-CB1$^{-/-}$ mutants showed a steadily decreasing investigatory behaviour for both, the left object (increasing familiarity) and the right object (always novel) (Glu-CB1$^{-/-}$ interaction [object/time]: $F_{(2,48)} = 0.1537$, $p = 0.8580$; Bonferroni post-test: training $p>0.05$, 2 h $p>0.05$, 24 h $p>0.05$; Fig. 1A,D,G). This phenomenon was only seen in the Glu-CB1$^{+/+}$ mice for the left object (increasing familiarity), while the time spent investigating the right object (always novel) remained constant (Glu-CB1$^{+/+}$ interaction [object/time]: $F_{(2,84)} = 4.851$, $p = 0.0101$; Bonferroni post-test: training $p>0.05$, 2 h $p>0.05$, 24 h $p<0.01$; Fig. 1A,D,G). It was further possible to detect a significant difference between the genotypes in exploring the right object, but not the left object over the three sessions (left object interaction [genotype/time]: $F_{(2,48)} = 0.2283$, $p = 0.7965$; Bonferroni post-test: training $p>0.05$, 2 h $p>0.05$, 24 h $p>0.05$; right object interaction [genotype/time]: $F_{(2,66)} = 3.522$, $p = 0.0352$; Bonferroni post-test: training $p>0.05$, 2 h $p<0.05$, 24 h $p<0.001$; Fig. 1A,D,G).

Sociability Test

During the sociability phase, the Glu-CB1$^{-/-}$ animals showed a significant increase in time spent in the middle compartment ($T_{(33)} = 2.247$, $p = 0.0314$; Fig. 2A). Accordingly, these mutants displayed a significant decrease in time spent with the interaction partner but not with the object (mouse [$T_{(33)} = 3.734$, $p = 0.0007$]; object [$T_{(33)} = 1.412$, $p = 0.1672$]; Fig. 2A). A similar result was obtained, when the novel interaction partner was introduced during the social novelty test. While the Glu-CB1$^{-/-}$ mice spent more time in the middle compartment, they spent less time with the familiar and novel partner as compared to the wild-type littermates (middle [$T_{(33)} = 3.772$, $p = 0.006$]; familiar [$T_{(33)} = 2.263$, $p = 0.0303$]; unknown [$T_{(33)} = 2.596$, $p = 0.0140$]; Fig. 2D). This phenotype was opposite to the findings with the GABA-CB1 line. In the sociability phase as well as in the social novelty phase, the GABA-CB1$^{-/-}$ mice showed a significant increase in time spent with the novel interaction partner as compared to controls (sociability [$T_{(57)} = 2.099$, $p = 0.0403$]; social novelty [$T_{(35)} = 3.063$, $p = 0.0042$]; Fig. 2B,E). The time spent in the middle compartment was consequently decreased (sociability [$T_{(57)} = 2.740$, $p = 0.0082$]; social novelty [$T_{(35)} = 2.168$, $p = 0.037$]). Interestingly, the time spent in the compartment with the empty cage (i.e. the object only) during the sociability phase as well as the time spent with the familiar animal (social novelty test) were not different between mutants and controls (object [$T_{(57)} = 1.114$, $p = 0.2699$]; familiar [$T_{(35)} = 1.017$, $p = 0.3162$]; Fig. 2B,E). The analysis of the D1-CB1 line did not reveal any significant genotype differences in the 3 phases of the sociability test (Fig. 2C,F). Only a non-significant trend was observed in the

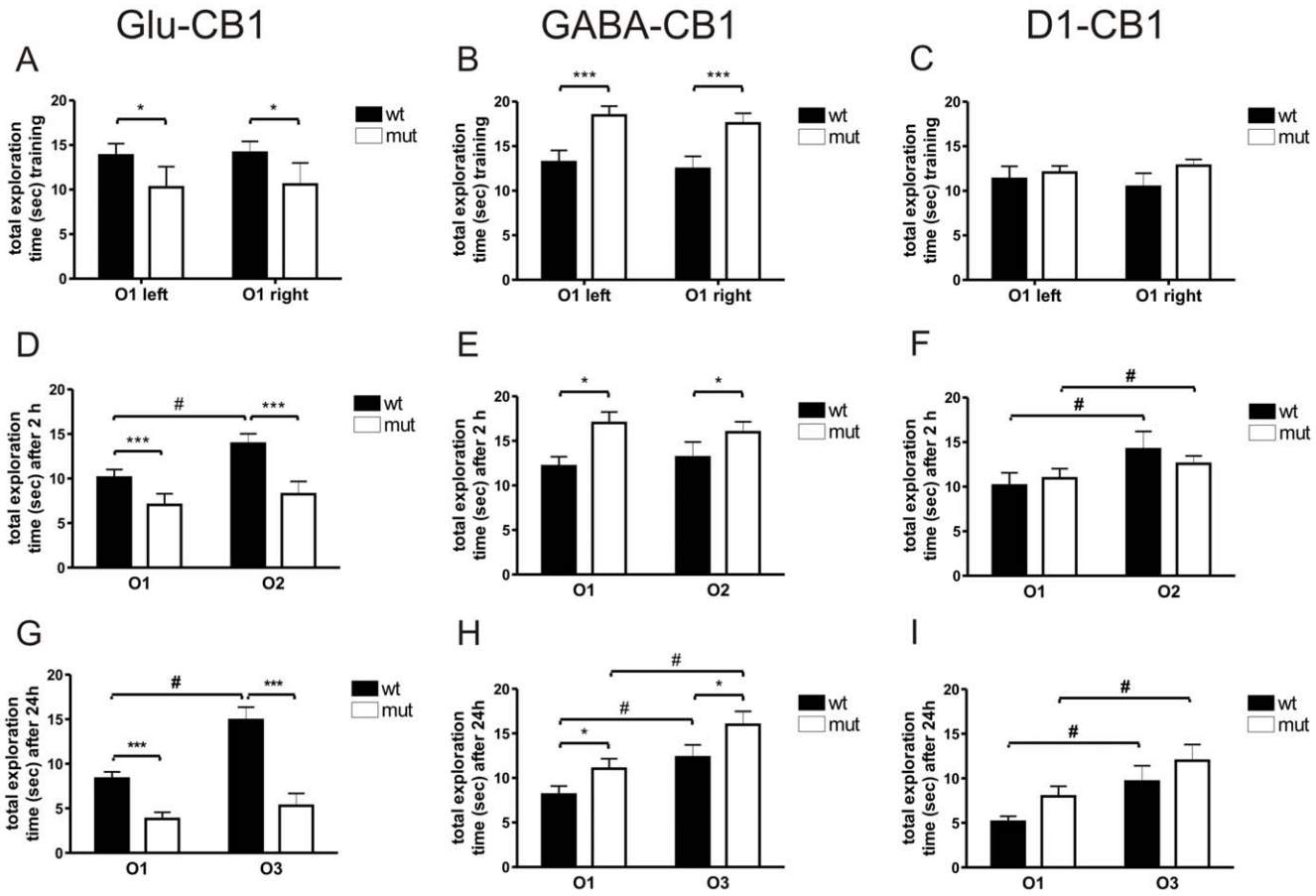

Figure 1. Inanimate exploration in the novel object recognition task. (A–C) Total time of exploration of two identical objects (O1, both on left and right side) during the training session for three conditional CB1 receptor mutant lines (Glu-CB1 [n = 23+13], GABA-CB1 [n = 18+23], D1-CB1 [12+12]) and their wild-type control littermates. (D–F) Total time of exploration of familiar object (O1) and novel object (O2 or O3) during the retention session after 2 h or 24 h (G–I). Glu-CB1$^{-/-}$ mice displayed a reduced exploration, while GABA-CB1$^{-/-}$ mice showed an increased exploration both in the training and retention session when compared to their wild-type littermate controls. No significant genotype differences were observed in the D1-CB1 mutant line. 2-way ANOVA (genotype differences) *p<0.05, ***p<0.001; t-test (discrimination index DI) $^{\#}$p<0.05.
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sociability phase. Even though time spent in the middle area was not altered ($T_{(28)} = 0.9190$, $p = 0.3659$), it seemed that the mutants showed a slight preference to explore the interaction partner ($T_{(28)} = 1.909$, $p = 0.0666$) rather than the empty cage ($T_{(28)} = 1.859$, $p = 0.0736$), i.e. the object (Fig. 2C). In the social novelty phase, not even a trend was detectable (familiar [$T_{(30)} = 0.7636$ $p = 0.4511$]; novel [$T_{(30)} = 0.5840$ $p = 0.5636$]; middle [$T_{(30)} = 0.6112$ $p = 0.5457$]; Fig. 2F).

The evaluation of the DI showed only minimal differences between the genotypes. In the sociability phase, the Glu-CB1$^{-/-}$ animals showed an impaired preference towards the interaction partner as compared to their controls ($T_{(33)} = 2.537$, $p<0.0161$; Table 1). In contrast, the GABA-CB1$^{-/-}$ mice and the D1-CB1$^{-/-}$ mice showed no significant changes in the preference towards the interaction partner (GABA-CB1$^{-/-}$ [$T_{(57)} = 1.507$, $p<0.1373$], D1-CB1$^{-/-}$ [$T_{(28)} = 1.636$, $p<0.1130$]; Table 1). In the social novelty phase, no DI differences were observed in any of the lines (Glu-CB1 line [$T_{(33)} = 0.3977$ $p = 0.6934$]; GABA-CB1 line [$T_{(34)} = 1.794$ $p = 0.0817$]; D1-CB1 line [$T_{(30)} = 0.6126$ $p = 0.547$]).

For all lines and genotypes, except for the Glu-CB1$^{-/-}$ mice, we observed a strong preference towards the social interaction partner over the object in the sociability phase (Glu-CB1$^{+/+}$ [$T_{(21)} = 10.47$, $p<0.0001$]; Glu-CB1$^{-/-}$ [$T_{(12)} = 1.559$, $p = 0.1450$]; GABA-CB1$^{+/+}$ [$T_{(27)} = 8.309$, $p<0.0001$]; GABA-CB1$^{-/-}$ [$T_{(30)} = 8.187$, $p<0.0001$]; D1-CB1$^{+/+}$ [$T_{(16)} = 5.017$, $p = 0.0002$]; D1-CB1$^{-/-}$ [$T_{(13)} = 7.458$, $p<0.0001$]; Table 1). In the social novelty phase, none of the groups, except for the GABA-CB1$^{-/-}$ mice, showed any preference towards the novel over the familiar interaction partner (Glu-CB1$^{+/+}$ [$T_{(21)} = 1.453$, $p<0.1610$]; Glu-CB1$^{-/-}$ [$T_{(12)} = 0.8652$, $p = 0.4039$]; GABA-CB1$^{+/+}$ [$T_{(15)} = 0.2402$, $p = 0.8134$]; GABA-CB1$^{-/-}$ [$T_{(19)} = 2.674$, $p = 0.0150$]; D1-CB1$^{+/+}$ [$T_{(16)} = 0.4262$, $p = 0.6756$]; D1-CB1$^{-/-}$ [$T_{(14)} = 0.4437$, $p = 0.6841$]; see Fig. 1).

The evaluation of the locomotor activity revealed no significant changes in the habituation phase of the sociability test, except for the Glu-CB1$^{-/-}$ mice, which showed a decrease in locomotion (Glu-CB1 line [$T_{(33)} = 2.312$, $p = 0.0271$]; GABA-CB1 line [$T_{(60)} = 1.506$, $p = 0.1374$]; D1-CB1 line [$T_{(29)} = 1.571$, $p = 0.1270$]). In the sociability phase, no alteration in the distance moved was observed in any of the lines (Glu-CB1 line [$T_{(29)} = 0.7833$, $p = 0.4398$]; GABA-CB1 line [$T_{(62)} = 0.6159$, $p = 0.5402$]; D1-CB1 line [$T_{(30)} = 0.1082$, $p = 0.9145$]). However, a significant decrease and increase in the distance moved was detected in the social novelty phase for the Glu-CB1$^{-/-}$ mice and the GABA-CB1$^{-/-}$ mice, respectively (Glu-CB1 line [$T_{(33)} = 2.575$, $p = 0.0146$]; GABA-CB1

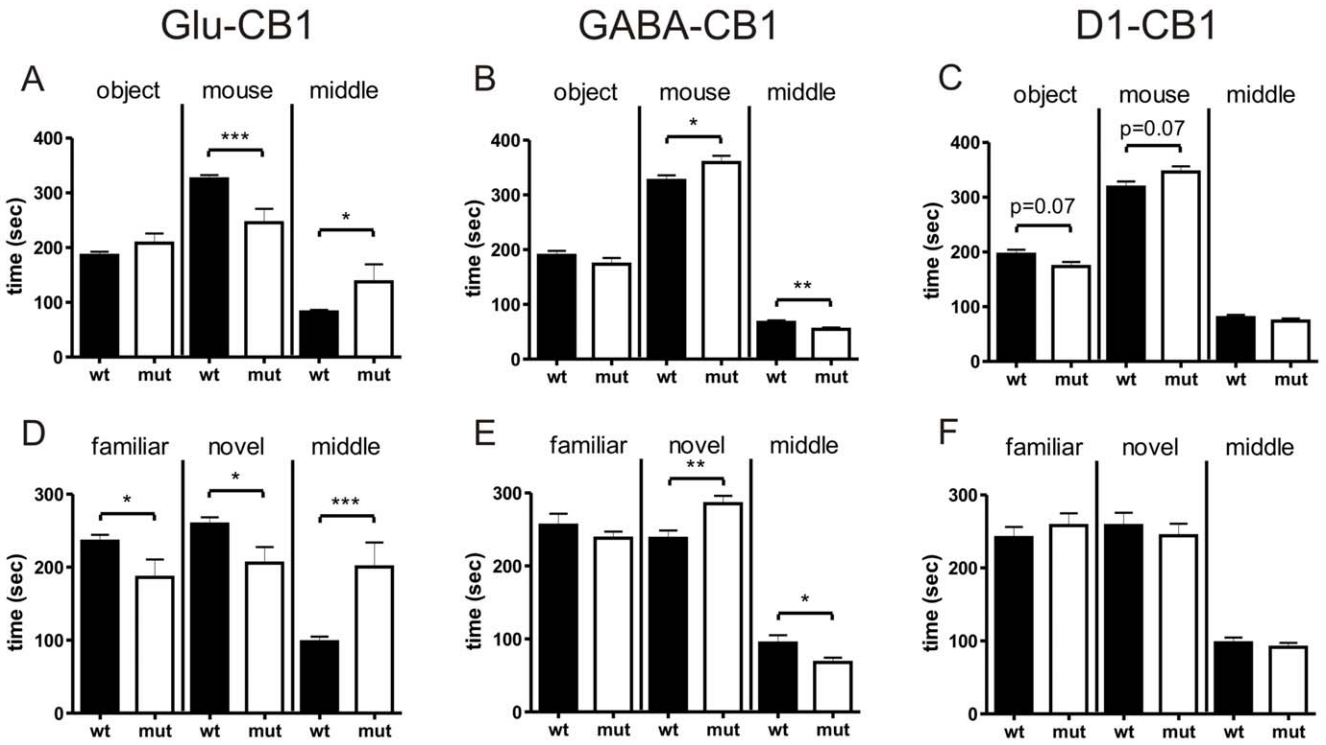

Figure 2. Animate vs. inanimate exploration in the sociability test. (A–C) Comparison of animate (mouse) and inanimate (object, "empty") exploration for the three mutants lines (Glu-CB1 [n = 22+13], GABA-CB1 [n = 18+23], D1-CB1 [16+16]) and their wild-type littermate controls during the sociability phase. (D–F) Exploration of the familiar and the novel interaction partner for during the social novelty phase. Glu-CB1$^{-/-}$ mice displayed no significant change in the exploration session, where there was a choice between the object and the interaction partner. In the social novelty phase, however, the interaction with a novel interaction partner was decreased when compared with their wild-type littermate controls. GABA-CB1$^{-/-}$ mice showed an increased social interaction in both sessions. In the D1-CB1 mutant line, no genotype differences were observed neither in the sociability nor in the social novelty phase. n = 11–20 animals; t-test *p<0.05, **p<0.01.
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line [$T_{(38)} = 2.591$, $p = 0.0135$]). The D1-CB1$^{-/-}$ mice again showed no change in the distance moved as compared to their respective wild-type littermates ($T_{(30)} = 0.2386$, $p = 0.8130$).

Resident-Intruder Test

Glu-CB1$^{-/-}$ mice displayed a significant decrease interacting with the intruder animals for the 10 min interaction phase as compared with wild-types ($T_{(35)} = 2.297$, $p = 0.0277$). Splitting the 10 min period into two 5 min bins revealed that the difference in interaction was mainly visible for the first 5 min bin ($T_{(35)} = 3.106$, $p = 0.0038$) (Fig. 3A). In addition, Glu-CB1$^{-/-}$ mice displayed an altered aggressive behaviour. Even though the number of fights was not different between the genotypes, the time that Glu-CB1$^{-/-}$ mice spent fighting the intruder was increased ($T_{(35)} = 2.249$, $p = 0.0309$) (Fig. 3D). As observed in the previous experiments, we detected an opposite phenotype in the GABA-CB1$^{-/-}$ animals, which showed an increased interaction with the intruder animal ($T_{(29)} = 2.522$, $p = 0.0174$) (Fig. 3B). The overall fighting with the younger intruder did not change as compared to the wild-type littermates ($T_{(26)} = 0.4227$, $p = 0.6760$, $T_{(29)} = 0.6286$, $p = 0.5345$) (Fig. 3E). D1-CB1$^{-/-}$ mice again displayed no phenotype differences, neither in interaction time spent with the intruder ($T_{(20)} = 0.3481$, $p = 0.7314$), nor in fighting behaviour ($T_{(22)} = 0.0000$, $p = 1.0$, $T_{(22)} = 0.8261$, $p = 0.4176$) (Fig. 3C,F).

Additional analysis revealed that Glu-CB1$^{+/+}$ animals displayed a significant increase in aggression as compared to the other control groups, GABA-CB1$^{+/+}$ and D1-CB1$^{+/+}$. Thus, differences were detected in number of fights (Kruskal-Wallis statistic = 7.478, $p = 0.0238$; Dunn's Multiple Comparison Post-Test: Glu-CB1$^{+/+}$ vs GABA-CB1$^{+/+}$ $p<0.05$, Glu-CB1$^{+/+}$ vs D1-CB1$^{+/+}$ $p>0.05$, GABA-CB1$^{+/+}$ vs D1-CB1$^{+/+}$ $p>0.05$), as well as % of time fighting (Kruskal-Wallis statistic = 7.584, $p = 0.0226$; Dunn's Multiple Comparison Post-Test: Glu-CB1$^{+/+}$ vs GABA-CB1$^{+/+}$ $p<0.05$, Glu-CB1$^{+/+}$ vs D1-CB1$^{+/+}$ $p>0.05$, GABA-CB1$^{+/+}$ vs D1-CB1$^{+/+}$ $p>0.05$).

Discussion

Using different conditional CB1 receptor mutant mice, we were able to show that the deletion of the CB1 receptor from forebrain GABAergic or cortical glutamatergic neurons resulted in an opposite behavioural outcome regarding animate and inanimate exploration. On the other hand, deletion of the CB1 receptor from dopamine receptor D1-expressing GABAergic striatal medium spiny neurons did not result in any significant changes. These findings suggest a regulatory function of the eCB system in cortical GABAergic and glutamatergic circuits to prevent neuronal and behavioural imbalance.

Mice lacking the CB1 receptor on glutamatergic neurons displayed a decreased exploratory behaviour, both in animate interaction (the interaction with a partner) and inanimate interaction (the interaction with an object). A similar decrease in object and social exploration was found in earlier studies, which were related with increased fear [6,7]. In our study, the decrease in exploration was seen when the mouse was exposed to a social interaction partner and/or to an object, and seemed to be

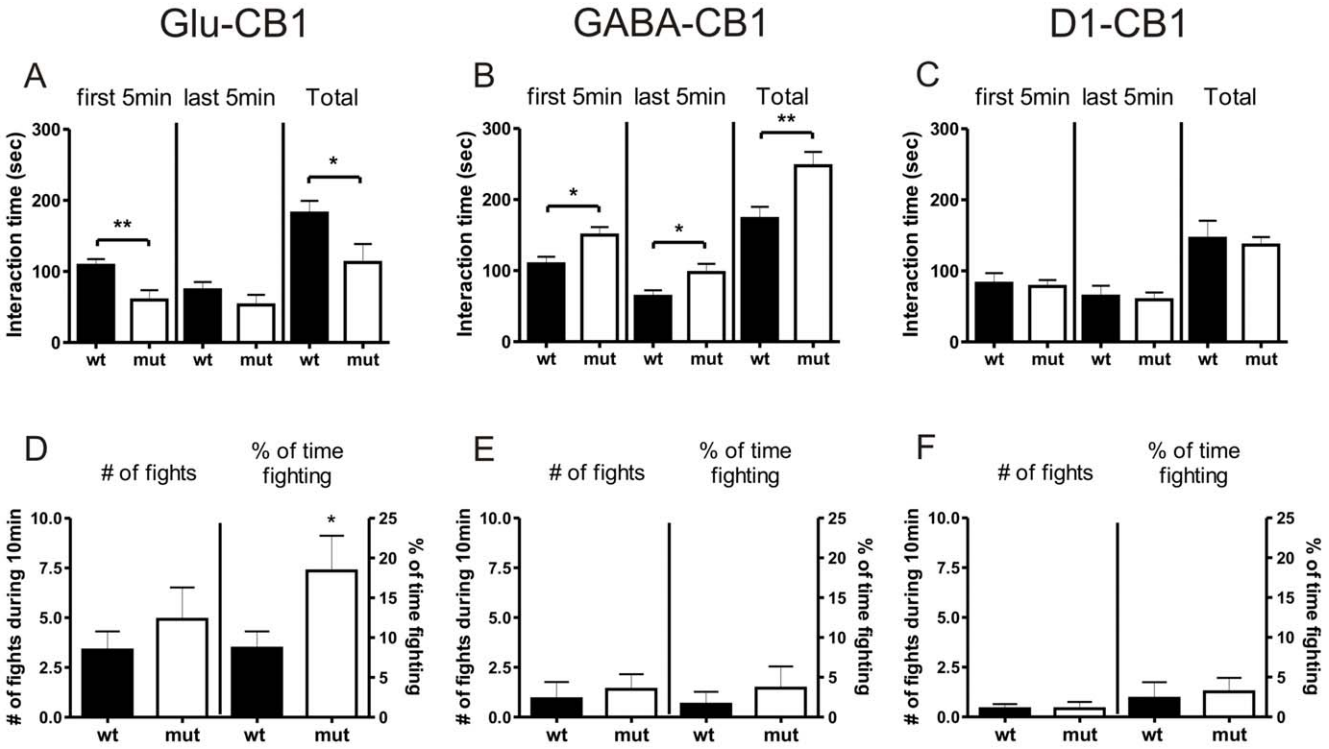

Figure 3. Animate exploration in the resident-intruder test. (A–C) Social interaction with an unknown, younger intruder for all three mutant lines (Glu-CB1 [n = 23+13], GABA-CB1 [n = 18+23], D1-CB1 [n = 16+16]). (D–E) Number of fights induced by the resident is shown for all three mutant lines. Glu-CB1$^{-/-}$ mice showed a significantly reduced exploration during the first 5 min observation period and an increased aggression towards the intruder when compared to wild-type littermate controls. GABA-CB1$^{-/-}$ mice displayed an increased interaction with the intruder, but no difference in aggressive behaviour. D1-CB1$^{-/-}$ mice showed no behavioural changes as compared to their wild-type littermate controls. t-test *$p<0.05$, **$p<0.01$.

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independent of novelty (Figs. 1, 2D). This phenotype was also visible in the resident-intruder test. However, the decreased social investigation was mainly based on a lower exploration during the first 5 min interval, a period important for information gathering (Fig. 3A).

The anxiogenic-like behaviour associated to these mutants can also explain the significantly higher aggression level found in the resident-intruder paradigm (Fig. 3D), a behaviour which was also observed in complete CB1 receptor knock-out animals [27]. The age and strength of the intruder compared to the resident is highly important [28]. In our case, the intruders were weaker and should not be regarded as a threat. We would therefore suggest that the deletion of the CB1 receptor from glutamatergic neurons might result in an inadequate aggressive response, suggesting an important role of CB1 receptor on this neuronal population in aggression. CB1 receptor in cortical GABAergic interneurons appears to mediate an opposite behaviour. While D1-CB1$^{-/-}$ animals (CB1 receptor loss primarily in the striatum), did not reveal any significant difference as compared to wild-type littermates, we observed that GABA-CB1$^{-/-}$ mice (lacking CB1 receptor additionally from cortical GABAergic interneurons), showed an increase in animate and inanimate exploration. Accordingly, increased investigatory behaviour toward novel food or object was previously observed in the GABA-CB1$^{-/-}$ mice [6]. Interestingly, Glu-CB1$^{+/+}$ control animals displayed an increased aggressive behaviour in the resident intruder paradigm as compared to the other wild-type controls, GABA-CB1$^{+/+}$ and D1-CB1$^{+/+}$. This elevated aggression might be explained by the fact that both the wild-type and mutant littermates are group-housed during growth. In case of the Glu-CB1 line, the modulated social behaviour of the Glu-CB1$^{-/-}$ mutants might have an effect on their wild-type littermates.

Taken together, these results suggest an anxiolytic-like function of the CB1 receptor on glutamatergic neurons and an anxiogenic-like function of the CB1 receptor on GABAergic interneurons. However, a generalized conclusion on the involvement CB1 receptor on cortical glutamatergic neurons in anxiety is not yet possible to be drawn, as under our experimental conditions, the open field test was not congruent with this notion. Neither Glu-CB1$^{-/-}$ nor GABA-CB1$^{-/-}$ mutants spend a different period of time in the more aversive center zone as compared with their respective wild-type littermates (Table 1). In addition, studies with these animals on the elevated plus maze, an anxiety test, did not reveal any changes either [7,14]. Also, levels of corticosterone under basal and stressful conditions were found to be similar between mutant and wild-type controls in both mutant lines [29]. Thus, it seems that a respective exploratory stimulus, such as an object or interaction partner, is required to induce a phenotype in these mice.

An alternative explanation for the observed differences can be alterations in spontaneous locomotor activity. In fact, we observed for both the Glu-CB1$^{-/-}$ and GABA-CB1$^{-/-}$ changes in the distance moved, namely a decrease and increase, respectively. It seems unlikely that the difference in locomotion was the driving force underlying the exploration phenotypes, as the mutants, in contrast to the variation in animate and social investigation, did not always display the locomotor alterations (Table 1). We argue that a respective context (e.g. handling threshold, exploratory

stimulus) is required for a detectable locomotion phenotype in our mutant lines. A similar situation seems to be true for the general investigatory drive. Thus, the clear differences in exploring object or interaction partner is not mirrored by the findings in the open field test, where we were not able to detect any alteration in the time spent in the more aversive center region (Table 1). This notion is supported by other studies with these mutant lines, where a behavioural change is only detectable in the presence of a respective stimulus or pharmacological modification of the eCB system [6,7].

A further explanation for the behavioural differences might be memory alterations in the respective mutant. However, this might only account for the Glu-CB1$^{-/-}$ mutants, as all other animals, independently of line and genotype, displayed a similar memory and recognition performance. Especially after 24 hours, mice recognized and distinguished strongly between familiar and novel objects (Table 1). The low discrimination index to the familiar object after a 2 hour interval in several groups, however, is unexpected and cannot be explained at this point. Only Glu-CB1$^{-/-}$ failed to show a clear preference towards the novel object in both retention sessions, indicating a memory deficit. Problematic for the interpretation is the overall low exploration for this mouse line, which is true for all three sessions of the novel object recognition test, as well as the other behavioural paradigms. Of special interest is the altered behaviour of the mutants in response to the novel objects. While wild-type littermates displayed a constant interest for the novel objects (O1–O3), the Glu-CB1$^{-/-}$ animals showed a steadily decreasing exploration over the three sessions (Fig. 1A,D,G). For both genotypes, such a decrease was seen regarding the exploration of the familiar objects (O1), which is not surprising, as novelty of this object strongly decreased with each session. Thus, the Glu-CB1$^{-/-}$ mice appeared to respond to the familiar and novel object in a similar way, suggesting rather a habituation to the context than a memory deficit. Nevertheless, a final conclusion cannot be made.

As mentioned above, all groups, independently of the line and the genotype, showed a stronger preference for the social interaction partner as compared to the object in the sociability test (Table 1). This behaviour was expected, as animals normally prefer social over non-social contacts [25]. Surprisingly, we could not detect a significant preference towards the novel interaction partner in the social novelty phase (Table 1). While this preference was observed in several lines [25], in our hands it was only recognizable in the GABA-CB1$^{-/-}$ mice. This finding could indicate that social discrimination is impaired in these mutants. However, comparable results from other studies suggest that a strong social preference does not necessarily predict a strong preference for social novelty. As a matter of fact, two different components of social behaviour were postulated to underlie sociability and social novelty, respectively. In addition, life history and development are responsible for lower or higher novelty preference [30].

Taken together the strong differences observed in the GABA-CB1$^{-/-}$ and Glu-CB1$^{-/-}$ animals in respect to their wild-type littermates might be explained by anxiolytic and anxiogenic responses to novelty, respectively. Nevertheless, the eCB system has also been shown to be involved in learning and memory function, which should be kept in mind here [31,32]. It may be even likely that both anxiety and memory components function together in our paradigms, but to solve this issue would require further investigations using other behavioural paradigms.

Our results, namely the increase of exploration following the deletion of GABAergic CB1 receptor and the decrease of exploratory behaviour following the deletion of glutamatergic CB1 receptors, may explain the contradictory findings using Δ^9-THC, URB597 and VDM11, as described in above. We suggest that increased or decreased exploratory drive, respectively, as response to cannabinoid treatment depends on the predominant modulation of either GABAergic or glutamatergic CB1 receptor, e.g. the activation of GABAergic CB1 receptor decreases exploration, while the activation of glutamatergic CB1 receptor leads to an increased investigatory drive. Thus, the decreased exploration induced by chronic and systemic activation of the eCB system with Δ^9-THC might be due to the exogenous activation of the CB1 receptor in GABAergic interneurons [15–17]. The increased exploratory profile after inhibition of anandamide degradation or reuptake could be explained by a specific on-demand activation of the CB1 receptor on glutamatergic neurons [17]. On the other hand, the increased animate and inanimate interaction as a result of the complete deletion of the CB1 receptor might be caused by the increased GABAergic drive [7,20]. It seems that the GABAergic drive is the predominant factor for behavioural outcome, when the eCB system is activated or blocked in a chronic manner. This makes the increased social interaction after URB597 treatment even more interesting, as in this case, the glutamatergic drive seems to be the predominant component. To test this hypothesis, Glu-CB1$^{-/-}$ or GABA-CB1$^{-/-}$ have to be injected with the respective drugs in comparable doses and tested in behavioural paradigms. Similar contradictory results were observed in pharmacological studies on anxiety and stress levels after cannabinoid administration, both being strongly involved in investigatory and exploratory drive [33,34]. The opposite effects might also be based on cortical GABAergic or glutamatergic transmission. Therefore, depending on its specific spatiotemporal activation within neuronal circuits, this system can act as a major "bi-directional" neuromodulator [14,34].

Our results might also be interesting in respect to some disorders, which are associated with inappropriate exploratory drive. Thus, a direct and indirect relation between these disorders and a dysregulation of GABAergic and/or glutamatergic transmission can be proposed. In animal models for autism, modulation of GABAergic transmission seems to be important [35,36]. The induction of schizophrenia-like symptoms by administration of the NMDA receptor antagonist phencyclidine revealed an alteration of glutamatergic and GABAergic signalling in the prefrontal cortex [37]. Interestingly, the effects of phencyclidine could be blocked by CB1 receptor antagonist treatment [38]. It was further shown that down-regulation of cortical glutamatergic drive resulted in an increase in dopamine levels and a hyperactive phenotype, which could be blocked by cortical GABA receptor activation [39]. These findings indicate a cortical control in these neuronal disorders, caused also by imbalanced GABAergic and glutamatergic transmission, a mechanism also suggested by our findings. Recent publications even suggest glutamatergic, instead of dopaminergic transmission to be the major factor of schizophrenia [40].

In conclusion, our results indicate a major, but opposite role of the eCB system in cortical GABAergic and glutamatergic neurons in the regulation of exploration (Table 2). Hence, further investigations along this line should be able to detail the diverse effects of cannabinergic drugs on investigatory behaviour. As investigatory drive is often associated with impulsive behaviour, studies using respective paradigms would be of great interest. Lastly, in future studies, the regulatory properties of the eCB system on cortical excitatory and inhibitory drive should be exploited in psychiatric disorders, opening up a therapeutic avenue to restore a possible cortical imbalance pharmacologically.

Table 2. Summary of behavioural changes induced by conditional CB1 receptor deletion.

	Locomotion	Object Exploration	Social Exploration	Aggression
Wild-type	Normal	Normal	Normal	Normal
Glu-CB1$^{-/-}$	Decreased	Decreased	Decreased	Increased
GABA-CB1$^{-/-}$	Increased	Increased	Increased	Normal
D1-CB1$^{-/-}$	Normal	Normal	Normal	Normal

"Normal" refers to similar to the wild-type behaviour on spontaneous locomotor activity (locomotion), investigation of object (object exploration) or of interaction partner (social exploration) and fights initiated (aggression).
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Author Contributions

Conceived and designed the experiments: MH NK KM BL. Performed the experiments: MH NK. Analyzed the data: MH NK KM. Contributed reagents/materials/analysis tools: MH NK KM BL. Wrote the paper: MH NK KM BL.

References

1. Newcorn JH (2001) New treatments and approaches for attention deficit hyperactivity disorder. Curr Psychiatry Rep 3: 87–91.
2. Solanto MV (2002) Dopamine dysfunction in AD/HD: integrating clinical and basic neuroscience research. Behav Brain Res 130: 65–71.
3. Perry W, Minassian A, Paulus MP, Young JW, Kincaid MJ, et al. (2009) A reverse translational study of dysfunctional exploration in psychiatric disorders: from mice to men. Arch Gen Psychiatry 66: 1072–1080.
4. Kawa R, Pisula E (2010) Locomotor activity, object exploration and space preference in children with autism and Down syndrome. Acta Neurobiol Exp 70: 131–140.
5. Patterson PH (2011) Modeling autistic features in animals. Pediatr Res 69: 34–40.
6. Lafenêtre P, Chaouloff F, Marsicano G (2009) Bidirectional regulation of novelty-induced behavioral inhibition by the endocannabinoid system. Neuropharmacology 57: 715–721.
7. Jacob W, Yassouridis A, Marsicano G, Monory K, Lutz B, et al. (2009) Endocannabinoids render exploratory behaviour largely independent of the test aversiveness: role of glutamatergic transmission. Genes Brain Behav 8: 685–698.
8. Marsicano G, Lutz B (1999) Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur J Neurosci 11: 4213–4225.
9. Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83: 393–411.
10. Monory K, Massa F, Egertová M, Eder M, Blaudzun H, et al. (2006) The endocannabinoid system controls key epileptogenic circuits in the hippocampus. Neuron 51: 455–466.
11. Monory K, Blaudzun H, Massa F, Kaiser N, Lemberger T, et al. (2007) Genetic dissection of behavioural and autonomic effects of Δ^9-tetrahydrocannabinol in mice. PLoS Biol 5: e269.
12. Katona I, Urban GM, Wallace M, Ledent C, Jung KM, et al. (2006) Molecular composition of the endocannabinoid system at glutamatergic synapses. J Neurosci 26: 5628–5637.
13. Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M (2009) Endocannabinoid-mediated control of synaptic transmission. Physiol Rev 89: 309–380.
14. Ruehle S, Aparisi Rey A, Remmers F, Lutz B (2011) The endocannabinoid system in anxiety, fear memory and habituation. J Psychopharmacology;print copy in press (DOI: 10.1177/0269881111408958).
15. O'Shea M, Singh ME, McGregor IS, Mallet PE (2004) Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. J Psychopharmacol 18: 502–508.
16. Schneider M, Schömig E, Leweke FM (2008) Acute and chronic cannabinoid treatment differentially affects recognition memory and social behavior in pubertal and adult rats. Addict Biol 13: 345–357.
17. Trezza V, Vanderschuren LJ (2008a) Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology (Berl) 197: 217–227.
18. Trezza V, Vanderschuren LJ (2008b) Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. Eur Neuropsychopharmacol 18: 519–530.
19. Trezza V, Vanderschuren LJ (2009) Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. J Pharmacol Exp Ther 328: 343–350.
20. Haller J, Varga B, Ledent C, Barna I, Freund TF (2004) Context-dependent effects of CB1 cannabinoid gene disruption on anxiety-like and social behaviour in mice. Eur J Neurosci 19: 1906–1912.
21. Massa F, Mancini G, Schmidt H, Steindel F, Mackie K, et al. (2010) Alterations in the hippocampal endocannabinoid system in diet-induced obese mice. J Neurosci 30: 6273–6281.
22. Ennaceur A, Delacour J (1988) A new one-trial test for neurobiological studies of memory in rats. 1″ Behavioral data. Behavioural Brain Research 31: 47–59.
23. Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, et al. (1999) Genetic enhancement of learning and memory in mice. Nature 401: 63–69.
24. Tordera RM, Totterdell S, Wojcik SM, Brose N, Elizalde N, et al. (2007) Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). Eur J Neurosci 25: 281–290.
25. Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, et al. (2004) Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav 3: 287–302.
26. Goyens J, Noirot E (1975) Effects of cohabitation with females on aggressive behavior between male mice. Dev Psychobiol 8: 79–84.
27. Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O (2002) Involvement of CB1 cannabinoid receptors in emotional behaviour. Psychopharmacology (Berl) 159: 379–387.
28. Thor DH, Flannelly KJ (1976) Age of intruder and territorial-elicited aggression in male long-evans rats. Behav Biol 17: 237–241.
29. Steiner MA, Marsicano G, Nestler EJ, Holsboer F, Lutz B, et al. (2008) Antidepressant-like behavioral effects of impaired cannabinoid receptor type 1 signaling coincide with exaggerated corticosterone secretion in mice. Psychoneuroendocrinology 33: 54–67.
30. Moy SS, Nadler JJ, Young NB, Nonneman RJ, Grossman AW, et al. (2009) Social approach in genetically engineered mouse lines relevant to autism. Genes Brain Behav 8: 129–142.
31. Moreira FA, Lutz B (2008) The endocannabinoid system: emotion, learning and addiction. Addict Biol 13: 196–212.
32. Marsicano G, Lafenêtre P (2009) Roles of the endocannabinoid system in learning and memory. Curr Top Behav Neurosci 1: 201–230.
33. Hill MN, Gorzalka BB (2005) Pharmacological enhancement of cannabinoid CB1 receptor activity elicits an antidepressant-like response in the rat forced swim test. Eur Neuropsychopharmacol 15: 593–399.
34. Viveros MP, Marco EM, File SE (2005) Endocannabinoid system and stress and anxiety responses. Pharmacol Biochem Behav 81: 331–342.
35. Chao HT, Chen H, Samaco RC, Xue M, Chahrour M, et al. (2010) Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. Nature 468: 263–269.
36. Sala M, Braida D, Lentini D, Busnelli M, Bulgheroni E, et al. (2011) Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: A neurobehavioral model of autism. Biol Psychiatry 69: 875–882.
37. Amitai N, Kuczenski R, Behrens MM, Markou A (2011) Repeated phencyclidine administration alters glutamate release and decreases GABA markers in the prefrontal cortex of rats. Neuropharmacol 69: 875–882.
38. Del Arco A, Ronzoni G, Mora F (2010) Prefrontal stimulation of GABAA receptors counteracts the corticolimbic hyperactivity produced by NMDA

antagonists in the prefrontal cortex of the rat. Psychopharmacology (Berl) 214: 525–536.
39. Guidali C, Viganò D, Petrosino S, Zamberletti E, Realini N, et al. (2011) Cannabinoid CB1 receptor antagonism prevents neurochemical and behavioural deficits induced by chronic phencyclidine. Int J Neuropsychopharmacol 14: 17–28.
40. Javitt DC (2010) Glutamatergic theories of schizophrenia. Isr J Psychiatry Relat Sci 47: 4–16.

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REVIEW

NEURONAL POPULATIONS MEDIATING THE EFFECTS OF ENDOCANNABINOIDS ON STRESS AND EMOTIONALITY

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Abstract—An adequate emotional response to stress is essential for survival and requires the fine-tuned regulation of several distinct neuronal circuits. Therefore, a precise control of these circuits is necessary to prevent behavioral imbalances. During the last decade, numerous investigations have evidenced that the endocannabinoid (eCB) system is able to crucially control stress coping. Its central component, the cannabinoid type 1 receptor (CB1 receptor), is located at the presynapse, where it is able to attenuate neurotransmitter release after its activation by postsynaptically produced and released eCBs. To date, the eCB system has been found to control the neurotransmitter release from several neuron populations (e.g. GABA, glutamate, catecholamines and monoamines), suggesting a general mechanism for tuning neuronal activity, and thereby regulating emotion and stress responses. In this review, we aim at summarizing the anatomical and functional relation of the eCB system to an adequate response to stressful situations. Of special interest will be neuronal connections to the hypothalamic-pituitary-adrenal axis, but also circuits between cortical structures, such as prefrontal cortex, amygdala and hippocampus, and subcortical regions, such as raphe nuclei and locus coeruleus. We further like to step toward allocating eCB system functions to distinct cellular subpopulations in the brain. It has emerged that the eCB system is spatially well defined, and its detailed knowledge is a prerequisite for understanding the eCB system in the context of controlling behavior. Thus, advanced approaches combining different genetic and pharmacological tools to dissect specific eCB system functions are of particular interest.

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Key words: cannabinoid receptor, endocannabinoid, stress, mouse genetics, adeno-associated virus, Cre/loxP system.

Contents

ENDOCANNABINOIDS, EMOTION AND STRESS

Emotionality describes a highly complex behavior in response to various environmental stimuli. An appropriate emotional outcome requires fine-tuned neurotransmitter release processes and functional neuronal circuits. Therefore, prevention of an imbalanced signaling is highly important, especially in stressful situations. One of the endogenous control mechanisms is constituted by the endocannabinoid (eCB) system, which is named according to its sensitivity to Δ^9-tetrahydrocannabinol (THC), the main psychoactive compound of *Cannabis sativa*. Since the cannabinoid type 1 receptor (CB1 receptor) has been discovered (Matsuda et al., 1990), the members of the eCB system have steadily increased, comprising different ligands, synthesizing and degrading enzymes as well as other cannabinoid receptors (Petrosino and Di Marzo, 2010). There are two major endogenous ligands (named endocannabinoids; eCBs), *N*-arachidonoyl ethanolamine (anandamide, AEA) (Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG) (Sugiura et al., 1995). Unlike "classical" neurotransmitters, the eCBs are not stored in vesicles at the presynapse, but are synthesized from lipid membrane precursor molecules on demand by Ca^{2+} dependent and independent mechanisms in the postsynapse (Kano et al., 2009). The ligands travel by a still unknown

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Abbreviations: AAV, adeno-associated virus; ABHD6, α,β-hydrolase domain 6; ACTH, adrenocorticotropic hormone; AEA, anandamide; BA, basolateral nucleus of BLA; BLA, basolateral amygdala complex; BNST, bed nucleus of the stria terminalis; CB1 receptor, cannabinoid type 1 receptor; CB2, cannabinoid type 2 receptor; CCK, cholecystokinin; CeA, central amygdala; CeAL, lateral part of central amygdala; CeAM, medial part of central amygdala; CRH, corticotropin-releasing hormone; EC, entorhinal cortex; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; GOI, gene of interest; GPR55, G protein-coupled receptor 55; HPA axis, hypothalamic-pituitary-adrenal axis; LA, lateral nucleus of BLA; LC, locus coeruleus; MAGL, monoacylglycerol lipase; NTS, nucleus of the solitary tract; PFC, prefrontal cortex; PVN, paraventricular nucleus; S, subiculum; THC, Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential cation channel vanilloid type 1; 2-AG, 2-arachidonoyl.

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mechanism retrogradely across the synaptic cleft to the presynaptically located CB1 receptor. Activation of the CB1 receptor induces the major feature of the eCB system, namely the inhibition of neurotransmitter release by modulation of several ion channels and kinases (Kano et al., 2009; Turu and Hunyady, 2010). Cannabinergic signaling is limited by a still poorly defined uptake process and rather well-characterized intracellular hydrolysis by fatty acid amide hydrolase (FAAH) for AEA, monoacylglycerol lipase (MAGL), and serine hydrolase α,β-hydrolase domain 6 (ABHD6) for 2-AG (Kano et al., 2009; Marrs et al., 2010). Experimental evidence has suggested that the uptake process is mediated by a transporter mechanism (Hillard et al., 1997). In fact, a truncated FAAH protein lacking the amidase activity has recently been identified as an AEA transporter (Fu et al., 2011). Interestingly, the degrading enzymes for the two major eCBs display distinct subcellular and synaptic localization, suggesting different signaling properties for AEA and 2-AG (Cristino et al., 2008; Kano et al., 2009). Although FAAH is mostly found in the postsynapse, MAGL is primarily colocalized with the CB1 receptor in the presynaptic structure (Egertova et al., 2003; Gulyas et al., 2004; Kano et al., 2009; Keimpema et al., 2010). Moreover, differential functions of AEA and 2-AG in the modulation of neuronal transmission processes have recently been described in the bed nucleus of the stria terminalis (BNST) (Puente et al., 2011). To date, there is clear evidence for other receptors in the CNS that are modulated by eCBs, such as the transient receptor potential cation channel vanilloid type 1 (TRPV1) (Chávez et al., 2010; De Petrocellis and Di Marzo, 2010), G protein-coupled receptor 55 (GPR55) (Baker et al., 2006; Nevalainen and Irving, 2010), peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors (O'Sullivan and Kendall, 2010), and $GABA_A$ receptor (Sigel et al., 2011), making the understanding of distinct eCB system functions even more difficult. The existence of the cannabinoid type 2 receptor (CB2 receptor) in brain tissue has been under discussion for the last years. Accumulating evidence has shown the presence and physiological importance of the CB2 receptor in the CNS (Onaivi et al., 2006; García-Gutiérrez et al., 2010). In the present review, we will focus on CB1 receptor-related functions. Nevertheless, the role of the CB2 receptor as well as the degrading enzymes FAAH and MAGL will also be discussed.

Taken together, the eCB system with its property of modulating neurotransmission is an interesting candidate to control behavior (Kano et al., 2009). In fact, various pharmacological and genetic studies show a variety of behavioral responses, in particular changes in mood and emotionality (Lutz, 2009; Hill and McEwen, 2010; Moreira and Wotjak, 2010). Interestingly, in animal models, cannabinergic drugs have been shown to possess biphasic effects depending on the dose (Kathuria et al., 2003; Gobbi et al., 2005; Hill and Gorzalka, 2005; Viveros et al., 2005; Bambico et al., 2007). This is in accordance with effects in humans, where opposite (depressive or euphoric) experiences after cannabis use were reported (Fusar-Poli et al., 2009).

However, ubiquitous pharmacological and genetic approaches might not be sufficient to precisely dissect the mechanisms underlying this biphasic phenomenon. It is our belief that the CB1 receptor, but also the other components of eCB system, on distinct neuronal populations is responsible for these opposing effects. Hence, a general activation or inhibition of the eCB system might shade specific effects. In addition, some neuronal populations might be differently affected by lower or higher availability of cannabinoids, which is based on an unequal sensitivity and/or availability of the eCB receptors and/or their respective signaling cascades.

A major drawback of a pharmacological approach is the lack of cellular specificity of the applied drug. Similarly, insufficient for the detailed functional analysis of the eCB system is a ubiquitous genetic deletion of a respective eCB system component, as distinct effects might be shaded. Thus, a more local and cell type-specific understanding of the eCB system is necessary to pinpoint particular eCB related effects. To target this problem, more complex approaches combining different state-of-the-art genetic and pharmacological tools are required. In fact, recent publications on mice lacking the CB1 receptor only in GABAergic or glutamatergic neurons do show opposite responses to stressful situations, which was only partially seen in mice with ubiquitous deletion (Lafenêtre et al., 2009; Jacob et al., 2009; Häring et al., 2011). Also viral gene delivery systems combined together with transgenic animals seem to be a promising strategy (Guggenhuber et al., 2010).

NEURONAL CIRCUITS INVOLVED IN STRESS AND EMOTION

The hypothalamic-pituitary-adrenal (HPA) axis is the major circuit involved in the response to a stressful situation (Ulrich-Lai and Herman, 2009; Hill and McEwen, 2010). Upon exposure to stressful stimuli, neurons of the hypothalamic paraventricular nucleus (PVN) secrete corticotropin-releasing hormone (CRH) into the portal vessels of the median eminence. In the pituitary, CRH initiates the secretion of adrenocorticotropic hormone (ACTH), which in turn induces the synthesis and release of glucocorticoid hormones (corticosterone in mice and rats and cortisol in humans) in the inner adrenal cortex into the bloodstream. Besides a fast mobilization of stored energy, released glucocorticoids inhibit HPA axis activity by feedback mechanisms (Steiner et al., 2008a; Ulrich-Lai and Herman, 2009; Hill et al., 2010a).

Despite of its central role, the HPA axis is only appropriately operational in connection with additional networks spanning from brainstem nuclei to specific limbic system structures (Fig. 1). How specific regulatory networks control glucocorticoid release in response to stress is influenced by a number of factors, such as different stressor types (reactive vs. anticipatory stressor, physical vs. psychological stressor) (Dedovic et al., 2009). These brain regions execute their regulatory functions on HPA axis activity by targeting the PVN of the hypothalamus (Herman et al., 2003; Jankord and Herman, 2008). Brainstem struc-

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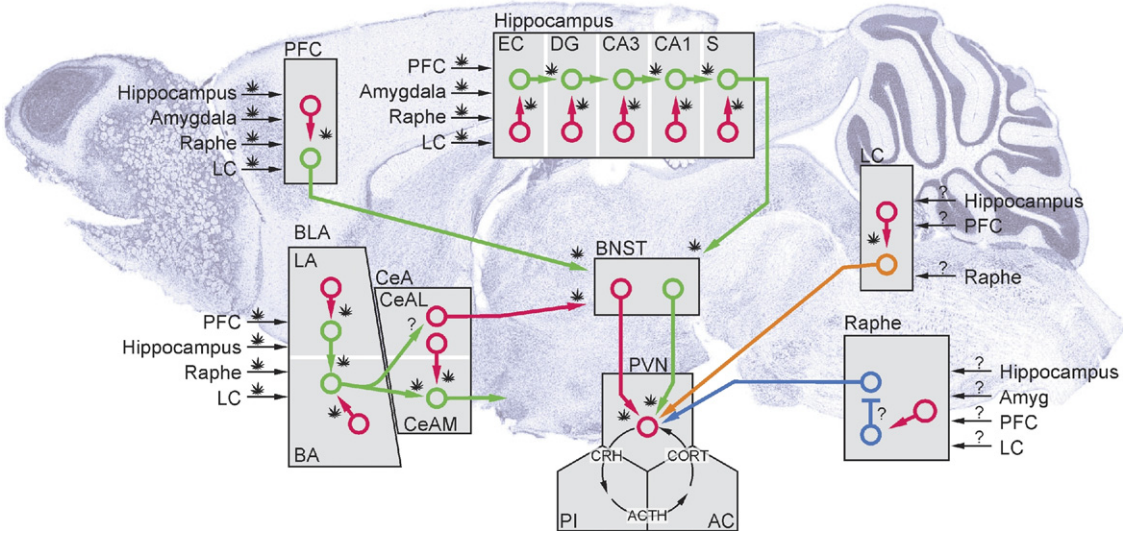

Fig. 1. Schematic illustration of the CB1 receptor distribution within the main stress circuits. The CB1 receptor, indicated as a *Cannabis sativa* leaf, is found at a majority of synaptic connections within and between each major brain region related to the activity of the hypothalamic-pituitary-adrenal (HPA) axis, which is controlled by the subcortical structure paraventricular nucleus (PVN) of the hypothalamus, the pituitary (PI), and the adrenal cortex (AC). A most dominant distribution of CB1 receptor is found in GABAergic (red) and glutamatergic (green) neurons in limbic regions, that is, prefrontal cortex (PFA), amygdala (BLA, CeA), bed nuclei of the stria terminalis (BNST), and hippocampus. Additionally, serotonergic (blue) and noradrenergic (orange) neurons from brainstem nuclei are also involved in the stress response. A projection to a particular brain region is depicted as an arrow with the specification where the projection originates from. Question mark (?) indicates that the presence of CB1 receptor at a given projection has not yet been experimentally and clearly proven. Abbreviations: AC, adrenal cortex; ACTH, adrenocorticotropin; BNST, bed nuclei of the stria terminalis; BA, basolateral nucleus of BLA; BLA, basolateral amygdala complex; CA, cornu ammonis; CeA, central amygdala; CeAL, lateral part of CeA; CeAM, medial part of CeA; CORT, corticosterone; CRH, corticotropin-releasing hormone; DG, dentate gyrus; EC, entorhinal cortex; LA, lateral nucleus of BLA; LC, locus coeruleus; PFC, prefrontal cortex; PI, pituitary gland; PVN, paraventricular nucleus of the hypothalamus; S, subiculum.

tures, such as the locus coeruleus (LC) and the nucleus of the solitary tract (NTS; not depicted in Fig. 1), can activate PVN neurons through direct noradrenergic projections (Cunningham et al., 1990; Ulrich-Lai and Herman, 2009). Also serotonergic afferents from the raphe nuclei directly innervate neurons in the PVN (Lowry, 2002; Zhang et al., 2002). Limbic forebrain regions including the amygdala, the hippocampus, and the prefrontal cortex (PFC) are mainly indirectly connected with the HPA axis (Ulrich-Lai and Herman, 2009). The information is first processed by relay stations, such as the BNST or the preoptic area of hypothalamus. In contrast to the mostly excitatory stimulus from brainstem nuclei (i.e. LC, NTS, raphe nuclei), these relay sites send mainly inhibitory (GABAergic) projections onto PVN neurons. Therefore, excitatory input from the hippocampus and the PFC inhibits HPA axis activity by activating inhibitory projections to PVN neurons (Jankord and Herman, 2008). The amygdala, in contrast, sends primarily inhibitory GABAergic efferents to the relay sites BNST and preoptic area. This blocks the inhibitory input onto the PVN and results in disinhibition of PVN neurons and, therefore, increases HPA activity (Jankord and Herman, 2008). Nevertheless, the PVN also contains glutamatergic terminals that activate the HPA axis. Origin of these excitatory neurons is the PVN itself, but also other hypothalamic subnuclei and to some extent the relay sites BNST and preoptic area (Csáki et al., 2000).

In addition to the multiple inputs onto the HPA axis, these brain regions also show a vast connectivity between these structures (Fig. 1). Thus, the PFC sends glutamatergic projections to the hippocampus, amygdala, LC, and raphe nuclei (Del Arco and Mora, 2009; Vázquez-Borsetti et al., 2011). The hippocampus sends its glutamatergic projections via its output region, the subiculum (S) and possibly the entorhinal cortex (EC), to cortical and subcortical regions, including amygdala, the PFC, the raphe nuclei, and the LC (Rosene and Van Hoesen, 1977; van Groen and Wyss, 1990; Myers and Scharfman, 2011).

The amygdala also sends efferent fibers to cortical and subcortical regions. Connections to the hippocampus and the PFC originate mainly from the basolateral complex of the amygdala (Sah and Lopez De Armentia, 2003). Inhibitory input to subcortical regions are often indirect via the BNST and originate from the central amygdala (CeA) (Dong et al., 2001). However, also direct connections to other hypothalamic and brainstem regions originate from the medial part of the central amygdala (CeAM) (Petrovich et al., 2001). A direct innervation of the LC remains elusive (Luppi et al., 1995; Peyron et al., 1998; Benes, 2010).

The rather small raphe nuclei display a multitude of serotonergic projection areas, among which are the PFC, the limbic regions (hippocampus and amygdala), as well as the LC (Molliver, 1987; Fitzgerald, 2011; Charnay and Leger, 2010). Similarly, noradrenergic efferents from the LC have been found to innervate the PFC, the limbic structures, and the raphe nuclei (Levitt and Moore, 1978; Loy et al., 1980; Fitzgerald, 2011).

Moreover, the PFC, the hippocampus, and the amygdala are connected among each other and represent within each region highly organized processing structures,

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displaying internal circuits and subregional organizations. Thus, the hippocampus possesses a trisynaptic circuit consisting of three glutamatergic connections between its subregions (Amaral and Witter, 1989). Hence, glutamatergic projections from the EC innervate the dendrites from dentate gyrus (DG) granule cells. These cells send efferents, the mossy fibers, onto the dendritic arbors of the pyramidal cells in the cornu ammonis subregion CA3, which in turn innervate, through the so-called Schaffer collaterals, dendrites of pyramidal neurons in the cornu ammonis subregion CA1. Recent studies even indicate that the CA3 region itself might be a main entry point into the hippocampal formation with higher processing function because of additional "back projections" to the EC and connections with GABAergic interneurons (not depicted in Fig. 1; Myers and Scharfman, 2011).

The PFC area also consists of several subregions containing diverse glutamatergic projection neurons and GABAergic interneurons. It is mainly divided into two regions, the orbital and medial PFC, which gives rise to two distinct networks. Both regions are connected with different brain structures, but also send projections toward each other (for review, see Ongür and Price, 2000; Tanji and Hoshi, 2008). Of special interest regarding the contribution of the PFC to the stress response is the medial PFC, which represents its main output region. Glutamatergic neurons from the subregions are known to innervate other limbic regions as well as the hypothalamus and brainstem areas (Petrovich et al., 2005; Bambico et al., 2007).

The amygdala, in contrast, consists of several interconnected nuclei, the most prominent being the central, the basal, and the lateral nucleus (Krettek and Price, 1978; Pape and Pare, 2010). Although the basolateral amygdala complex (BLA) has cortical origin and properties, possessing a majority of glutamatergic neurons and a minority of GABAergic interneurons (McDonald, 1982; Ramikie and Patel, 2012), the CeA nucleus is of striatal origin and properties, consisting mainly of medium spiny-type GABAergic neurons (Ehrlich et al., 2009). Main input region of the amygdaloid structure is the BLA, which sends excitatory projections to the main amygdala output area, the CeA (Krettek and Price, 1978; Ramikie and Patel, 2012). However, this serial pathway described earlier is more complex, as the CeA has also been suggested as an additional processing site (Ehrlich et al., 2009; Pape and Pare, 2010). The CeA can further be subdivided into medial part of central amygdala (CeAM) and lateral part of central amygdala (CeAL). Both subregions receive excitatory input from the BLA, but the CeAM also receives inhibitory input from the CeAL (Ramikie and Patel, 2012). An additional inhibitory source within the amygdala circuits is the intercalating neurons (not depicted in Fig. 1). These GABAergic cells surround the amygdala complex, receive inputs from the PFC, and send inhibitory projections into the different subnuclei, in particular to the CeA (Millhouse, 1986; Ehrlich et al., 2009).

The raphe nuclei and the LC are in comparison rather simple structures, even though several raphe nuclei exist that send serotonergic projections to each other. Like in the limbic structures, there are also internal inhibitory cells within these brainstem regions, which are mostly GABAergic, but also serotonergic (Tao and Auerbach, 2000; Aston-Jones et al., 2004; Celada et al., 2001).

ANATOMICAL CONNECTION OF THE STRESS CIRCUITS WITH THE eCB SYSTEM

General distribution of the eCB system

The overall expression of CB1 receptor mRNA and protein is vastly distributed over the whole CNS with major localization in cortical areas, amygdala, striatum, and cerebellum. Moderate and low expression levels can be seen in thalamic, hypothalamic, and brainstem regions (Marsicano and Lutz, 1999; Mackie, 2005; Marsicano and Kuner, 2008). To date, its presence has been verified directly in GABAergic, cholinergic, glutamatergic, noradrenergic, and serotonergic neuron terminals, with highest levels in GABAergic neurons (Marsicano and Lutz, 1999; Degroot et al., 2006; Monory et al., 2006; Oropeza et al., 2007; Häring et al., 2007).

The presence of eCB-degrading enzymes FAAH and MAGL are often, but not always, associated with the CB1 receptor expression (Basavarajappa, 2007; Marsicano and Kuner, 2008; Ramikie and Patel, 2012). FAAH expression is vastly distributed through the CNS, but displays frequently a complementary pattern with the CB1 receptor, namely, the enzyme being close to CB1 receptor-positive terminals (Thomas et al., 1997; Egertova et al., 2003). Both proteins can be found in cortical regions, although in several subcortical nuclei FAAH distribution seems to be independent of CB1 receptor signaling (e.g. raphe nuclei and several thalamic nuclei). In contrast, FAAH protein is low or even absent in regions known for high CB1 receptor content, such as substantia nigra and globus pallidus. Regarding the presence in stress-related neuronal subpopulations, FAAH can be detected mostly in cortical glutamatergic cells (Basavarajappa, 2007).

MAGL, the 2-AG–degrading enzyme shows in comparison with FAAH a strong colocalization with the CB1 receptor not only at the regional distribution but also at the subcellular level. In general, MAGL expression can be found at high levels in the cortex, hippocampus, amygdala, and cerebellum. In contrast to CB1 expression, MAGL mRNA levels are also prominent in the thalamic anterodorsal nucleus, but only at low levels in the nucleus accumbens (Dinh et al., 2002). At protein level, the enzyme can be found predominantly in axon terminals of granule cells, CA3 pyramidal cells, and partly in interneurons of the hippocampus (Gulyas et al., 2004). CB2 receptor mRNA was described in several brain regions, among which are the limbic areas as well as the median raphe nucleus (Onaivi et al., 2006; García-Gutiérrez et al., 2010). Highest levels of CB2 receptor protein was found in the hippocampus and the cerebral cortex (Onaivi et al., 2006; García-Gutiérrez et al., 2010). In contrast to the synaptic localization of the CB1 receptor, the CB2 receptor appears to be localized in the cell body and dendrites (Onaivi et al., 2006; Suárez et al., 2009).

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The eCB system in specific neuronal circuits

Because of its vast abundance of the CB1 receptor in the mammalian CNS, it is not surprising that the receptor is also found in the major brain structures involved in the stress response (Fig. 1). The most prominent expression of the CB1 receptor can be seen in the hippocampal formation, in the BLA, and in the PFC (Marsicano and Kuner, 2008). Here, CB1 receptor is expressed at very high levels in cholecystokinin (CCK)-positive GABAergic interneurons (Marsicano and Lutz, 1999; Azad et al., 2008; Morozov et al., 2009) and at moderate to low levels in glutamatergic terminals (Monory et al., 2006; Kawamura et al., 2006; Kano et al., 2009).

Even though the vast majority of CB1 receptor in the limbic areas arises from GABAergic and glutamatergic neurons, the receptor has also been detected on serotonergic and noradrenergic terminals, adding an additional way on how endocannabinoids influence limbic circuits (Häring et al., 2007; Oropeza et al., 2007). A most striking feature of the receptor protein localization in the amygdala is its high levels in the basolateral part, but it is almost undetectable in the central nucleus (Mackie, 2005; Marsicano and Kuner, 2008). In fact, CB1 receptor protein has only recently been clearly detected in the central nucleus of the amygdala (Kamprath et al., 2010).

FAAH within all three limbic regions (PFC, hippocampus, amygdala) is expressed at high levels and is located on the soma and dendrites of glutamatergic neurons (Cristino et al., 2008). GABAergic interneurons, which possess the highest amount of the CB1 receptor, lack FAAH completely (Egertova et al., 1998; Dinh et al., 2002; Basavarajappa, 2007). MAGL is expressed by both GABAergic and glutamatergic neurons, but is mostly localized at axon terminals of glutamatergic neurons, for example, hippocampal granule cells and CA3 pyramidal cells, and only partly on interneurons (Gulyas et al., 2004). Similar to the CB1 receptor, FAAH and MAGL are found in high levels in the BLA, whereas only low levels can be found in the CeA (Ramikie and Patel, 2012). CB2 receptor was found to be present mainly in glutamatergic neurons in the pyramidal cell layer of the hippocampus and the cerebral cortex (Onaivi et al., 2006, in press; García-Gutiérrez et al., 2010). A detailed analysis of the hippocampal distribution revealed a staining of putative dendritic fibers and terminals in all major subregions of the hippocampus (Suárez et al., 2009).

The BNST, also called the extended amygdala, is a major relay site, where CB1 receptor is located on both glutamatergic and GABAergic terminals (Puente et al., 2010).

In the brainstem, noradrenergic neurons of the LC and of the NTS express CB1 receptor protein (Jelsing et al., 2009; Carvalho et al., 2010). Using different binding assays, the CB1 receptor protein was detected within the noradrenergic nuclei on catecholaminergic terminals (Herkenham et al., 1991; Scavone et al., 2010). Noradrenergic terminals positive for the CB1 receptor were localized in the frontal cortex (Oropeza et al., 2007; Page et al., 2008). The two eCB degrading enzymes have so far not been anatomically detected within the LC. Only in the NTS, FAAH was found at protein level (Van Sickle et al., 2001).

Within the raphe nuclei, only a subfraction of serotonergic neurons express the CB1 receptor mRNA at very low levels (Häring et al., 2007). Nevertheless, CB1 receptor-positive serotonergic fibers could be detected in the hippocampus and the amygdala (Häring et al., 2007). Even though electrophysiological studies indicated the presence of the CB1 receptor on inhibitory and excitatory synapses within the raphe nuclei, anatomical evidence is still missing (Bambico et al., 2009; Haj-Dahmane and Shen, 2011). Regarding the distribution of the degrading enzymes, only FAAH was detected in the dorsal and median raphe region (Egertova et al., 2003).

Clear evidence for the eCB system could also be found within the HPA axis. The CB1 receptor was detected at mRNA and protein level in the PVN. Immunoreactivity is predominantly located on GABAergic terminals (Castelli et al. (2007), but it was only recently identified on glutamatergic neurons of the PVN (Hrabovszky et al., in press). Another study focusing on thyrothropin-releasing hormone neurons in the PVN also showed clear evidence for CB1 receptor-positive synapses on these cells (Deli et al., 2009). To date, the presence of FAAH and MAGL in the PVN has not been described in detail. Nevertheless, FAAH protein and MAGL mRNA have been identified at low levels in the complete hypothalamic region (Egertova et al., 1998; Dinh et al., 2002).

PHARMACOLOGICAL AND GENETIC MODULATIONS OF eCB SYSTEM ACTIVITY AND THE EFFECTS ON STRESS RESPONSES

There is a large body of data showing that stress, emotionality, and the eCB system are strongly connected with each others. Thus, both changes in eCB signaling after stress as well as changes in stress responses after modulation of eCB system activity have been described.

Effects of stress on eCB system activity

The duration of the stressful stimuli is an important factor in determining the extent of the alterations of the eCB system. Acute stress induces rather short-term effects on eCB signaling. After exposure to acute restraint stress, tissue content of AEA was significantly decreased in the amygdala, presumably as a consequence of increased hydrolysis by FAAH. In contrast, 2-AG was unaltered within the limbic forebrain, medial PFC, amygdala, and cerebellum (Patel et al., 2005; Hill et al., 2009, 2011). Recent data suggested an additional effect of the HPA feedback mechanism in the PVN. Corticosterone application rapidly induced eCB synthesis in hypothalamic slices (Malcher-Lopes et al., 2006), but also *in vivo* in the hypothalamus (Evanson et al., 2010; Hill et al., 2010c), and in the medial PFC (Hill et al., 2011). A similar feature of corticosterone in stimulating the release of eCBs by a nongenomic mechanism was also observed in the basolateral amygdala (Campolongo et al., 2009; Hill and

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McEwen, 2009). Furthermore, corticosterone was found to rapidly suppress glutamate release from excitatory synapses in hypothalamic slices in a CB1 receptor-dependent manner (Di et al., 2003).

Thus, the eCB system seems to be involved in the fast feedback mechanism of the HPA axis. Activation of glucocorticoid receptor in the PVN by corticosterone induces the synthesis of eCBs, which then activate CB1 receptor on glutamatergic terminals. Hence, glutamate release is suppressed, leading to decreased activation of PVN neurons (Hill et al., 2010b), which in turn results in an attenuated stress response.

As persistent stress constitutes a main risk factor for neuropsychiatric diseases, such as depression, chronic stress models in rodents offer an attractive translational research line (Holsboer and Ising, 2010). Using such models, long-lasting changes in eCB signaling, which are based on expression changes of the CB1 receptor and/or eCB-synthesizing or eCB-degrading enzymes, were observed. In this regard, exposure to chronic stress impaired CB1 receptor function in GABAergic neurons of the rat hippocampus (Hu et al., 2011). In line with this finding, chronic stress altered eCB signaling in dorsal root ganglia neurons, which are affected by CRH and involved in stress-induced hyperalgesia (Hong et al., 2009). Furthermore, it was shown that chronic corticosterone treatment or repeated exposure to water avoidance stress resulted in a decrease in CB1 receptor levels and an increase in FAAH activity levels (Hong et al., 2010; Bowles et al., 2012). Changes in CB1 receptor expression levels were also observed in the hippocampus after exposure to chronic stress. Interestingly, the effect was seen predominantly in the dorsal fraction of the hippocampus and showed gender differences (Reich et al., 2009). Although in males, comparably high levels of CB1 receptor are downregulated, in females, rather low levels of CB1 receptor are increased as response to repeated stress (Reich et al., 2009). Moreover, chronic stress resulted in decreased CB1 receptor mRNA levels in both genders (Xing et al., 2011). A downregulation of CB1 receptor protein levels was also seen after maternal deprivation (Suárez et al., 2009). The same study showed a parallel increase in CB2 receptor expression, suggesting a switch in eCB signaling.

Regarding the eCB levels, chronic restraint stress results in a progressive increase in 2-AG content within the medial PFC, limbic forebrain, amygdala, hippocampus, and hypothalamus (Patel et al., 2005; Rademacher et al., 2008; Patel et al., 2009). Patel and colleagues also demonstrated decreased AEA levels in the amygdala after chronic restraint stress (Patel et al., 2005). Furthermore, following chronic corticosterone treatment, reduced AEA levels were observed in the hippocampus and the amygdala, which was caused by an increase in FAAH activity (Bowles et al., 2012). In the striatum, chronic unpredictable stress did not induce any change in the levels of 2-AG and AEA (Wang et al., 2010). Chronic corticosterone treatment also resulted in increased 2-AG contents in the amygdala and dorsal root ganglia (Hill et al., 2005; Hong et al., 2010). Hill and colleagues suggested that the effect of repeated stress on amygdala 2-AG content could be secondary to a persistent increase in glucocorticoid signaling (Hill et al., 2010a).

Effects of pharmacological eCB system activity modulation on stress

Pharmacological modifications of the eCB system can reveal the relationship between eCB system activity and the molecular as well as the behavioral responses to stress. Thus, application of cannabinergic drugs directly influences excitatory and inhibitory inputs, respectively, to PVN neurons, resulting in an altered HPA axis activity. In behavioral paradigms of stress and anxiety, cannabinergic drugs have a strong influence on the behavioral outcome. During the last years, a “dual” role of eCB signaling could be evidenced by agonizing or antagonizing CB1 receptor activation (Moreira and Lutz, 2008). The literature showed that CB1 receptor agonist or CB1 receptor antagonist treatment could induce similar effects at the molecular and the behavioral level. Moreover, depending on the concentration of the cannabinergic drug, opposed effects could be observed. Therefore, eCB signaling can act as a “bidirectional” neuromodulator depending on its specific spatiotemporal modulation within neuronal circuits (Moreira and Lutz, 2008). As it was already pointed out earlier, these discrepancies are often been attributed to differences in dosage and treatment duration, experimental conditions, and species (Bambico et al., 2010; Zanettini et al., 2011). Thus, one possible explanation for this peculiar feature might be changes in the initial baseline stress level of an animal, which is controlled by a multitude of genetic, environmental, and experimental factors. This baseline might modulate the activity of the eCB system and by this, even though induced by opposite pharmacological interventions, results in a similar outcome (Wotjak, 2005; Viveros et al., 2005). Also allosteric modulation and internalization of the CB1 receptor as well as the recruitment of endocannabinoids to other receptors, such as TRPV1, GPR55, and $GABA_A$ receptor, are under discussion, resulting in a modulation of eCB signaling (Bosier et al., 2010; Pamplona and Takahashi, in press; Sigel et al., 2011). Another interesting explanation of the “dual” role of eCBs may be the differential CB1 receptor activation on different neuronal populations. Studies on cell type-specific CB1 receptor mutant mice, which will be discussed later, suggest CB1 receptor on glutamatergic and GABAergic neurons to be key players (Lafenêtre et al., 2009; Jacob et al., 2009; Häring et al., 2011).

At the molecular level, activation of the CB1 receptor with low doses of THC leads to an increase in plasma corticosterone levels, an effect blocked by low doses of the CB1 receptor inverse agonist rimonabant. Surprisingly, high doses of rimonabant failed to attenuate the THC-induced HPA axis activation and were shown to enhance corticosterone levels discretely (Manzanares et al., 1999). Accordingly, Patel et al. (2004) showed that under stressful conditions cannabinoid agonist administration can elicit a dose-dependent biphasic effect on corticosterone secretion. Although low agonist concentrations resulted in de-

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creased plasma corticosterone levels, high agonist levels led to increased corticosterone secretion (Wenger et al., 2003; Patel et al., 2004; Wade et al., 2006). To dissect the involvement of different neurotransmitters on HPA axis activation, McLaughlin et al. (2009) coadministered a high dose of cannabinoid agonist and specific antagonists of the serotonergic, noradrenergic, and glutamatergic neurotransmitter system, respectively. This study revealed that activation of the HPA axis by cannabinoid treatment seemed to be mediated by serotonergic and noradrenergic, but not glutamatergic neurotransmission (McLaughlin et al. (2009), supporting previous results on the roles of monoamines on HPA axis activity. Thus, an increase in serotonergic transmission is followed by an increase in corticosterone levels, whereas blockade of serotonin signaling had the opposite effect (for review, see Leonard, 2005; Pompili et al., 2010). Congruently, the enhancement of noradrenergic transmission had similar effects (for review, see Forray and Gysling, 2004). The fact that the inhibition of ionotropic glutamatergic receptors had no effects is not surprising, as the ubiquitous activation of CB1 receptor will also attenuate glutamatergic transmission (Kano et al., 2009). Two other investigations further observed an increased activity of noradrenergic neurons after enhancing CB1 receptor signaling by application of a FAAH inhibitor or a CB1 receptor agonist (Gobbi et al., 2005; Muntoni et al., 2006).

Interestingly, these data suggest an indirect influence on serotonergic and noradrenergic neurons, hence, inhibitory interneurons within these two brainstem nuclei seem to play important roles. In fact, Muntoni and colleagues could show that CB1 receptor activation attenuated the inhibitory input from the nucleus prepositus hypoglossi, the main GABAergic input to the LC (Muntoni et al., 2006). However, strong evidence exists that also glutamatergic connections can be highly important, at least in the connection of the PFC to the raphe nuclei. The dissection of the PFC-dorsal raphe nucleus projection blocked the increase in serotonergic firing after local administration of a CB1 receptor agonist (low doses) into the PFC (Bambico et al., 2007). It is possible that glutamatergic neurons innervate inhibitory brainstem neurons, which regulate serotonin release, a mechanism already proposed previously (Celada et al., 2001), and which is also plausible for the LC.

Another important influence on the HPA axis activity is mediated by the BLA. Acute exposure to stress enhanced FAAH activity and thereby decreased AEA levels (Rademacher et al., 2008; Hill et al., 2009). Interestingly, local injection of CB1 receptor agonists and FAAH inhibitors into the BLA resulted in a reduction of HPA axis activation after stress (Ganon-Elazar and Akirav, 2009; Hill et al., 2009). Regarding the effect of cannabinoids on glutamate release within the BLA, Hill and colleagues suggested a model whereby stress interrupts a tonic AEA-induced CB1 receptor activation on glutamatergic terminals by increasing FAAH activity. The decrease in AEA levels leads to an increased output and increased HPA axis activity (Hill et al., 2010a). Somewhat contradictory is the finding of increased 2-AG levels after chronic stress in the amygdala (Hill et al., 2010a). Hence, 2-AG and AEA are divergently regulated by chronic stress. A possible explanation for this phenomenon might be a differential regulation of the 2-AG–degrading enzymes MAGL and ABHD6, respectively. Hill and colleagues suggested that increased 2-AG levels are highly important for the adaptation to stress (Hill et al., 2010a). It was proposed that 2-AG, like AEA, functions via CB1 receptor on glutamatergic terminals in the BLA, inhibiting excitatory input onto the amygdala. The subsequent attenuation of amygdala output decreases HPA axis activity.

At the behavioral level, similar biphasic effects of cannabinergic drugs can be observed resulting either in an antidepressant-like/anxiolytic, no effect, or a depressive-like/anxiogenic phenotype. In stress and anxiety paradigms, low doses of CB1 receptor agonist led to an antidepressant-like/anxiolytic effect (Gobbi et al., 2005; Bambico et al., 2007; Marco and Viveros, 2009), whereas high doses showed the opposite effect (Gobbi et al., 2005; Patel and Hillard, 2006; Egashira et al., 2008; Marco and Viveros, 2009). Regarding the CB1 receptor antagonist rimonabant, the situation seems to be more difficult, as similar doses induced both depressive-like/anxiogenic (Patel and Hillard, 2006; Steiner et al, 2008a; Marco et al., 2011) and antidepressant-like/anxiolytic response (Haller et al., 2009; Rodgers et al., 2003; Marco et al., 2011). The depressive-like/anxiogenic effects of rimonabant were also reported in humans, which ultimately led to the withdrawal of this antiobesity drug from the European market (Van Gaal et al., 2008; Doggrell, 2008; Marco et al., 2011).

As mentioned earlier, one hypothesis is the involvement of different neuronal populations in this biphasic effect of cannabinoids, as the CB1 receptor was found in various neuronal subpopulations. Depending on the strength of enhancement or attenuation of eCB signaling, distinct neuronal populations might be affected, leading to this biphasic effect. Nevertheless, solely pharmacological approaches are not able to clarify these issues in a satisfying manner.

Genetic approaches for deciphering eCB-mediated stress circuits

Other studies investigated the impact of the eCB system on the HPA axis by genetic means. Global genetic deletion of the CB1 receptor enhances HPA axis activity, thus, resembling the effect of antagonist treatment. Moreover, CB1 receptor knockout mice exhibit elevated levels of CRH mRNA in the PVN, which indicates a sustained activation of the HPA axis (Cota et al., 2003, 2007; Steiner et al., 2008a). It also signifies an impairment of the fast feedback mechanism described previously (Cota, 2008; Steiner and Wotjak, 2008; Hill et al., 2010b). Thus, the increased HPA axis activity could be explained by the lack of CB1 receptor on glutamatergic terminals in the PVN and the inability of corticosterone-induced eCB synthesis to block glutamatergic drive (Cota et al., 2007; Steiner and Wotjak, 2008).

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Steiner et al. (2008b) tried to dissect the impact of CB1 receptor activation at distinct neuronal populations on HPA axis function by using conditional CB1 receptor knockout mice. Here, CB1 receptor was deleted from GABAergic neurons and glutamatergic neurons of the forebrain and subcortical regions, respectively. After forced swim stress, corticosterone plasma levels were increased in the glutamatergic CB1 receptor knockout mice, but remained unaltered in the GABAergic CB1 receptor knockout mice (Steiner et al., 2008b). These findings support the notion of a direct involvement of the CB1 receptor at glutamatergic terminals on HPA axis activation.

Other studies using the same mutant lines support the hypothesis that different neuronal populations might be involved in the biphasic molecular and behavioral effects (Lafenêtre et al., 2009; Jacob et al., 2009; Häring et al., 2011). In fact, these studies suggested CB1 receptor on glutamatergic and GABAergic neurons to be the key players in this phenomenon. Exposing animals lacking the CB1 receptor specifically either on glutamatergic neurons or GABAergic neurons to a novel stimulus revealed opposite behavioral phenotypes. Thus, Lafenêtre et al. (2009) could show that the deletion of the CB1 receptor from glutamatergic neurons leads to an attenuation of approaching and investigating the novel stimulus. In contrast, animals lacking the CB1 receptor specifically from GABAergic neurons displayed an enhanced novelty seeking (Lafenêtre et al., 2009). The anxiogenic-like behavior of the animals lacking the CB1 receptor on glutamatergic neurons was also seen by Jacob et al. (2009). Interestingly, the behavioral difference between the wild-type littermates and the mutants were mainly observed in object and social investigation paradigms. No difference was observed in classical anxiety paradigms, such as elevated plus maze and light-dark box, suggesting that a respective stimulus is required. At this point, Jacob et al. (2009) did not analyze the GABAergic-specific CB1 receptor deficient mice. An additional approach addressing specifically object and social investigation in the same two mutant lines, lacking the CB1 receptor either in glutamatergic or GABAergic neurons, underlined the opposite role of the receptor in these neuronal subpopulations (Häring et al., 2011). Taken together, these data suggest that the CB1 receptor on glutamatergic neurons mediates anxiolytic and on GABAergic neurons anxiogenic responses, respectively.

Apart from genetic manipulation of CB1 receptor expression, other studies characterized the effects of deleting or overexpressing different components of the eCB system. Especially, the blockade of eCB degradation emerged to be a promising path to target depressive disorders. Hence, pharmacological inhibition of eCB degradation enables to enhance several beneficial effects of direct cannabinoid receptor agonist, but simultaneously reducing side effects (Petrosino and Di Marzo, 2010). This phenomenon is explainable by the mechanism of action of the eCB system. As described earlier, CB1 receptors are activated on demand. Exogenous agonist or antagonist treatment artificially induces or blocks eCB signaling. In contrast, the blockade of eCB degradation only prolongs the eCB signaling, where it was already endogenously activated (e.g. Pan et al., 2009).

The generation of transgenic mice with ubiquitous loss of FAAH underlined the pharmacological findings. Thus, the deletion of FAAH enhances the signaling properties of AEA and was found to produce an antidepressant-like and anxiolytic-like effect (Moreira et al., 2008; Bambico et al., 2010). Interestingly, antidepressant-like effects of cannabinergic drugs seem also to be dependent on serotonin transmission (Bambico et al., 2010). Regarding the CB2 receptor, blockade of receptor function by antisense oligonucleotides as well as by pharmacological approaches induced an anxiolytic response (Onaivi et al., 2008). In line with these findings, overexpression of the CB2 receptor reduced depressive-like behavior (García-Gutiérrez et al., 2010, 2011). The analysis of a nucleotide polymorphism in the CB2 receptor gene locus even suggests a correlation between decreased receptor function and an increased incidence in schizophrenia (Ishiguro et al., 2010).

COMBINING VIRAL INJECTIONS, CONDITIONAL MUTAGENESIS, AND PHARMACOLOGY TO REVEAL DISTINCT eCB SYSTEM FUNCTIONS: A NOVEL APPROACH

Global pharmacological and genetic manipulations of the eCB system have enabled to retrieve its major molecular and cellular properties, also within the context of the entire organism. Local microinjections of drugs modulating eCB system activity in combination with the manipulation of other neurotransmitter systems even revealed subregional functions and the involvement of distinct neuronal subpopulations (Ganon-Elazar and Akirav, 2009; Hill et al., 2009; McLaughlin et al., 2009). Nevertheless, to dissect cell type- and region-specific roles of the eCB system, more sophisticated and neuron-specific analyses are required. First steps have been made using transgenic mice, lacking the CB1 receptor in a specific neuronal population by exploiting the Cre/loxP system (Monory and Lutz, 2009) (Fig. 2A). This powerful genetic tool has established new vista on cell type-specific gene function in the nervous system in the context of neuronal networks (Gavériaux-Ruff and Kieffer, 2007), but possible caveats have to be considered using this approach, such as compensatory processes or disturbances of complex neuronal interactions, leading to possible misinterpretations of the phenotypes observed (Alger, 2006). One step further in these genetic analyses is the combination of transgenic animals and local injections of an adequate viral vector, such as the adeno-associated virus (AAV). Since Kaplitt and colleagues provided proof for a safe and efficient AAV mediated CNS gene transfer (Kaplitt et al., 1994), recombinant AAV vectors have become increasingly popular in CNS gene delivery applications because of their lack of pathogenicity, neurotropism, and ability to establish sustained transgene expression with very little tendency to integrate into the genome of the host cell. The combination of mouse genetics and AAV gene delivery could be used to delete a gene of interest in a specific brain region or even in a

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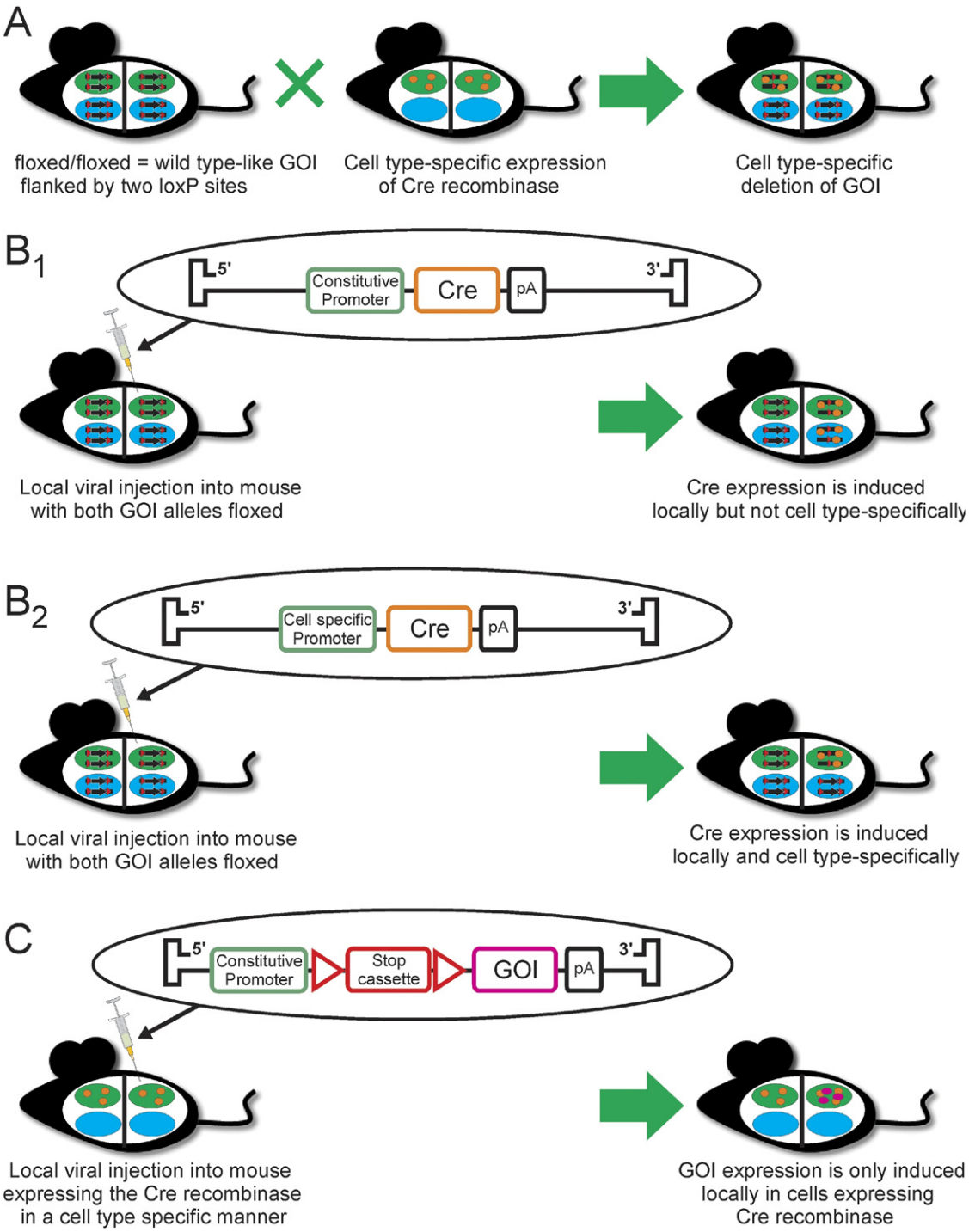

Fig. 2. Schematic illustration of genetic and viral approaches using the Cre/loxP system. Four cells are depicted in each mouse comprising two different cell types (green and blue color) and two different brain regions (the two cells on the left and right part, respectively, separated by a black line). (A) Deletion of GOI in a cell type-specific manner using the Cre/loxP system by using a crossing procedure, starting with a floxed/floxed GOI mouse line and a transgenic mouse line expressing Cre recombinase in a cell type-specific manner. (B_1) Region-specific deletion of a GOI by stereotactic injection of a recombinant virus, expressing Cre recombinase under the control of a constitutive promoter, into a floxed/floxed GOI mouse line. (B_2) Similar approach as in B_1, but because of the cell type-specific promoter driving Cre recombinase expression, the GOI is deleted both in a region- and cell type-specific manner. (C) Complementary approach as described in B_2 to achieve cell type-specific overexpression of GOI. Transgenic mouse line expressing Cre recombinase in a cell type-specific manner is used for viral injection. The viral construct contains a GOI, which is transcriptionally silenced by a Stop cassette flanked by loxP sites. After injection, the Stop cassette is excised in the cells expressing Cre recombinase, transcriptional silencing is abolished, and the GOI will be expressed. Abbreviations: GOI, gene of interest; pA, polyadenylation signal; Stop cassette, sequences containing transcriptional termination signals.

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specific nucleus. In this approach, a transgenic mouse line carries a gene that is flanked by two loxP sites (a floxed/floxed mouse), and AAV expressing Cre recombinase is injected into the brain region of interest (Fig. 2B_1). This approach was already implemented previously and showed that the CB1 receptor deletion from hippocampal neurons led to an increased susceptibility to chemically induced seizures (Monory et al., 2006). An advanced version of this approach will be the use of a cell type-specific promoter driving Cre recombinase expression in the viral construct (Fig. 2B_2). The utilization of this strategy allows a local and cell type-specific gene deletion. Furthermore, this strategy can be used to overexpress a gene of interest (GOI), by exchanging the Cre recombinase gene with the GOI sequence. Although the approach described in Fig. 2B_2 seems to be a very elegant strategy, it also has a major drawback, given by the limited packaging capacity of the AAV and the complexity of promoter regions. In respect to cell type-specific overexpression of a GOI, another strategy is very promising. Here, a transgenic mouse line defines cell type-specificity by driving Cre recombinase under cell type-specific regulatory sequences. Brain region specificity is determined by the stereotaxic injection of AAV, whose transgene expression is depending on the presence of Cre recombinase by excising a transcriptional Stop cassette (Fig. 2C). Guggenhuber and colleagues used this approach and showed that CB1 receptor overexpression in hippocampal pyramidal neurons resulted in enhanced protection against chemically induced excitotoxicity (Guggenhuber et al., 2010).

The eCB system can affect the output of several neurotransmitter systems and numerous brain regions that are involved in ensuring adequate stress responses. Experiments using the genetic approaches described earlier would be highly valuable and necessary to dissect distinct cell type-specific functions of the eCB system. In this context, brain region-specific deletion of the CB1 receptor or brain region- and cell type-specific CB1 receptor overexpression and its effect on stress behavior would be a very promising approach. Furthermore, the AAV gene delivery would not need to be restricted to genes such as the CB1 receptor or Cre recombinase. Focusing on the enzymes involved in the eCB metabolism would also be of great importance, as AEA and 2-AG levels are altered in several brain regions after chronic stress (Patel et al., 2005; Rademacher et al., 2008; Bowles et al., 2012).

SUMMARY: eCB SYSTEM MEDIATES DIFFERENTIAL FUNCTIONS ON DIFFERENT NEURONS

Taken together, HPA axis activity is influenced by distinct neurotransmitter systems present in specific brain regions. Thus, an adequate behavioral response to a stressful situation depends not only on the functionality of the HPA axis, but also on brain regions innervating PVN neurons and on the crosstalk between these structures. The aggregate relay of associational information determines the induction of a stress response and therewith the secretion of glucocorticoids. We described that the eCB system plays a critical role in the synaptic and neuronal organization of these stress circuits, since the CB1 receptor is located within each circuit at multiple sites. Pharmacological and genetic approaches have helped understanding the importance and main functions of these connections in a remarkable manner. Nevertheless, distinct pathways have still to be analyzed in more detail. Of special interest should be the function of the eCB system in neuronal subpopulations in specific brain structures. We propose combinational approaches using genetic and pharmacological tools to specifically interfere with eCB signaling.

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REFERENCES

Alger BE (2006) Not too excited? Thank your endocannabinoids. Neuron 51:393–395.

Amaral DG, Witter MP (1989) The three-dimensional organization of the hippocampal formation: a review of anatomical data. Neuroscience 31:571–591.

Aston-Jones G, Zhu Y, Card JP (2004) Numerous GABAergic afferents to locus ceruleus in the pericerulear dendritic zone: possible interneuronal pool. J Neurosci 24:2313–2321.

Azad SC, Kurz J, Marsicano G, Lutz B, Zieglgänsberger W, Rammes G (2008) Activation of CB1 specifically located on GABAergic interneurons inhibits LTD in the lateral amygdala. Learn Mem 15:143–152.

Baker D, Pryce G, Davies WL, Hiley CR (2006) In silico patent searching reveals a new cannabinoid receptor. Trends Pharmacol Sci 27:1–4.

Bambico FR, Cassano T, Dominguez-Lopez S, Katz N, Walker CD, Piomelli D, Gobbi G (2010) Genetic deletion of fatty acid amide hydrolase alters emotional behavior and serotonergic transmission in the dorsal raphe, prefrontal cortex, and hippocampus. Neuropsychopharmacology 35:2083–2100.

Bambico FR, Katz N, Debonnel G, Gobbi G (2007) Cannabinoids elicit antidepressant-like behavior and activate serotonergic neurons through the medial prefrontal cortex. J Neurosci 27:11700–11711.

Bambico FR, Nguyen NT, Gobbi G (2009) Decline in serotonergic firing activity and desensitization of 5-HT1A autoreceptors after chronic unpredictable stress. Eur Neuropsychopharmacol 19:215–228.

Basavarajappa BS (2007) Critical enzymes involved in endocannabinoid metabolism. Protein Pept Lett 14:237–246.

Benes FM (2010) Amygdalocortical circuitry in schizophrenia: from circuits to molecules. Neuropsychopharmacology 35:239–257.

Bosier B, Muccioli GG, Hermans E, Lambert DM (2010) Functionally selective cannabinoid receptor signalling: therapeutic implications and opportunities. Biochem Pharmacol 80:1–12.

Bowles NP, Hill MN, Bhagat SM, Karatsoreos IN, Hillard CJ, McEwen BS (2012) Chronic, noninvasive glucocorticoid administration suppresses limbic endocannabinoid signaling in mice. Neuroscience.

Campolongo P, Roozendaal B, Trezza V, Hauer D, Schelling G, McGaugh JL, Cuomo V (2009) Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. Proc Natl Acad Sci U S A 106:4888–4893.

Carvalho AF, Mackie K, Van Bockstaele EJ (2010) Cannabinoid modulation of limbic forebrain noradrenergic circuitry. Eur J Neurosci 31:286–301.

Castelli MP, Piras AP, Melis T, Succu S, Sanna F, Melis MR, Collu S, Ennas MG, Diaz G, Mackie K, Argiolas A (2007) Cannabinoid CB1

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receptors in the paraventricular nucleus and central control of penile erection: immunocytochemistry, autoradiography and behavioral studies. Neuroscience 147:197–206.

Celada P, Puig MV, Casanovas JM, Guillazo G, Artigas F (2001) Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: involvement of serotonin-1A, GABA(A), and glutamate receptors. J Neurosci 21:9917–9929.

Charnay Y, Leger L (2010) Brain serotonergic circuitries. Dialogues Clin Neurosci 12:471–487.

Chávez AE, Chiu CQ, Castillo PE (2010) TRPV1 activation by endogenous anandamide triggers postsynaptic long-term depression in dentate gyrus. Nat Neurosci 13:1511–1518.

Cota D (2008) The role of the endocannabinoid system in the regulation of hypothalamic-pituitary-adrenal axis activity. J Neuroendocrinol 20 (Suppl 1):35–38.

Cota D, Marsicano G, Tschöp M, Grübler Y, Flachskamm C, Schubert M, Auer D, Yassouridis A, Thöne-Reineke C, Ortmann S, Tomassoni F, Cervino C, Nisoli E, Linthorst AC, Pasquali R, Lutz B, Stalla GK, Pagotto U (2003) The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. J Clin Invest 112:423–431.

Cota D, Steiner MA, Marsicano G, Cervino C, Herman JP, Grubler Y, Stalla J, Pasquali R, Lutz B, Stalla GK, Pagotto U (2007) Requirement of cannabinoid receptor type 1 for the basal modulation of hypothalamic-pituitary-adrenal axis function. Endocrinology 148: 1574–1581.

Cristino L, Starowicz K, De Petrocellis L, Morishita J, Ueda N, Guglielmotti V, Di Marzo V (2008) Immunohistochemical localization of anabolic and catabolic enzymes for anandamide and other putative endovanilloids in the hippocampus and cerebellar cortex of the mouse brain. Neuroscience 151:955–968.

Csáki A, Kocsis K, Halász B, Kiss J (2000) Localization of glutamatergic/aspartatergic neurons projecting to the hypothalamic paraventricular nucleus studied by retrograde transport of [3H]D-aspartate autoradiography. Neuroscience 101:637–655.

Cunningham ET Jr., Bohn MC, Sawchenko PE (1990) Organization of adrenergic inputs to the paraventricular and supraoptic nuclei of the hypothalamus in the rat. J Comp Neurol 292:651–667.

De Petrocellis L, Di Marzo V (2010) Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels. J Neuroimmune Pharmacol 5:103–121.

Dedovic K, Duchesne A, Andrews J, Engert V, Pruessner JC (2009) The brain and the stress axis: the neural correlates of cortisol regulation in response to stress. Neuroimage 47:864–871.

Degroot A, Köfalvi A, Wade MR, Davis RJ, Rodrigues RJ, Rebola N, Cunha RA, Nomikos GG (2006) CB1 receptor antagonism increases hippocampal acetylcholine release: site and mechanism of action. Mol Pharmacol 70:1236–1245.

Del Arco A, Mora F (2009) Neurotransmitters and prefrontal cortex-limbic system interactions: implications for plasticity and psychiatric disorders. J Neural Transm 116:941–952.

Deli L, Wittmann G, Kallo I, Lechan RM, Watanabe M, Liposits Z, Fekete C (2009) Type 1 cannabinoid receptor-containing axons innervate hypophysiotropic thyrotropin-releasing hormone-synthesizing neurons. Endocrinology 150:98–103.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258:1946–1949.

Di S, Malcher-Lopes R, Halmos KC, Tasker JG (2003) Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. J Neurosci 23:4850–4857.

Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A 99:10819–10824.

Doggrell SA (2008) Is rimonabant efficacious and safe in the treatment of obesity? Expert Opin Pharmacother 9:2727–2731.

Dong HW, Petrovich GD, Swanson LW (2001) Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain Res Brain Res Rev 38:192–246.

Egashira N, Matsuda T, Koushi E, Higashihara F, Mishima K, Chidori S, Hasebe N, Iwasaki K, Nishimura R, Oishi R, Fujiwara M (2008) Delta(9)-tetrahydrocannabinol prolongs the immobility time in the mouse forced swim test: involvement of cannabinoid CB(1) receptor and serotonergic system. Eur J Pharmacol 589:117–121.

Egertova M, Cravatt BF, Elphick MR (2003) Comparative analysis of fatty acid amide hydrolase and cb(1) cannabinoid receptor expression in the mouse brain: evidence of a widespread role for fatty acid amide hydrolase in regulation of endocannabinoid signaling. Neuroscience 119:481–496.

Egertova M, Giang DK, Cravatt BF, Elphick MR (1998) A new perspective on cannabinoid signalling: complementary localization of fatty acid amide hydrolase and the CB1 receptor in rat brain. Proc Biol Sci 265:2081–2085.

Ehrlich I, Humeau Y, Grenier F, Ciocchi S, Herry C, Lüthi A (2009) Amygdala inhibitory circuits and the control of fear memory. Neuron 62:757–771.

Evanson NK, Tasker JG, Hill MN, Hillard CJ, Herman JP (2010) Fast feedback inhibition of the HPA axis by glucocorticoids is mediated by endocannabinoid signaling. Endocrinology 151:4811–4819.

Fitzgerald PJ (2011) A neurochemical yin and yang: does serotonin activate and norepinephrine deactivate the prefrontal cortex? Psychopharmacology (Berl) 213:171–182.

Forray MI, Gysling K (2004) Role of noradrenergic projections to the bed nucleus of the stria terminalis in the regulation of the hypothalamic-pituitary-adrenal axis. Brain Res Brain Res Rev 47:145–160.

Fu J, Bottegoni G, Sasso O, Bertorelli R, Rocchia W, Masetti M, Guijarro A, Lodola A, Armirotti A, Garau G, Bandiera T, Reggiani A, Mor M, Cavalli A, Piomelli D (2011) A catalytically silent FAAH-1 variant drives anandamide transport in neurons. Nat Neurosci 15:64–69.

Fusar-Poli P, Crippa JA, Bhattacharyya S, Borgwardt SJ, Allen P, Martin-Santos R, Seal M, Surguladze SA, O'Carrol C, Atakan Z, Zuardi AW, McGuire PK (2009) Distinct effects of {delta}9-tetrahydrocannabinol and cannabidiol on neural activation during emotional processing. Arch Gen Psychiatry 66:95–105.

Ganon-Elazar E, Akirav I (2009) Cannabinoid receptor activation in the basolateral amygdala blocks the effects of stress on the conditioning and extinction of inhibitory avoidance. J Neurosci 29:11078–11088.

García-Gutiérrez MS, Manzanares J (2011) Overexpression of CB2 cannabinoid receptors decreased vulnerability to anxiety and impaired anxiolytic action of alprazolam in mice. J Psychopharmacol 25:111–120.

García-Gutiérrez MS, Pérez-Ortiz JM, Gutiérrez-Adán A, Manzanares J (2010) Depression-resistant endophenotype in mice overexpressing cannabinoid CB(2) receptors. Br J Pharmacol 160: 1773–1784.

Gavériaux-Ruff C, Kieffer BL (2007) Conditional gene targeting in the mouse nervous system: insights into brain function and diseases. Pharmacol Ther 113:619–634.

Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, Cassano T, Morgese MG, Debonnel G, Duranti A, Tontini A, Tarzia G, Mor M, Trezza V, Goldberg SR, Cuomo V, Piomelli D (2005) Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc Natl Acad Sci U S A 102:18620–18625.

Guggenhuber S, Monory K, Lutz B, Klugmann M (2010) AAV vector-mediated overexpression of CB1 cannabinoid receptor in pyramidal neurons of the hippocampus protects against seizure-induced excitoxicity. PLoS One 5:e15707.

Gulyas AI, Cravatt BF, Bracey MH, Dinh TP, Piomelli D, Boscia F, Freund TF (2004) Segregation of two endocannabinoid-hydrolyz-

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ing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. Eur J Neurosci 20:441–458.

Haj-Dahmane S, Shen RY (2011) Modulation of the serotonin system by endocannabinoid signaling. Neuropharmacology 61:414–420.

Haller J, Barna I, Barsvari B, Gyimesi Pelczer K, Yasar S, Panlilio LV, Goldberg S (2009) Interactions between environmental aversiveness and the anxiolytic effects of enhanced cannabinoid signaling by FAAH inhibition in rats. Psychopharmacology 204:607–616.

Häring M, Kaiser N, Monory K, Lutz B (2011) Circuit specific functions of cannabinoid CB1 receptor in the balance of investigatory drive and exploration. PLoS One 6:e26617.

Häring M, Marsicano G, Lutz B, Monory K (2007) Identification of the cannabinoid receptor type 1 in serotonergic cells of raphe nuclei in mice. Neuroscience 146:1212–1219.

Herkenham M, Lynn AB, de Costa BR, Richfield EK (1991) Neuronal localization of cannabinoid receptors in the basal ganglia of the rat. Brain Res 547:267–274.

Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE (2003) Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24:151–180.

Hill MN, Gorzalka BB (2005) Pharmacological enhancement of cannabinoid CB1 receptor activity elicits an antidepressant-like response in the rat forced swim test. Eur Neuropsychopharmacol 15:593–599.

Hill MN, Karatsoreos IN, Hillard CJ, McEwen BS (2010c) Rapid elevations in limbic endocannabinoid content by glucocorticoid hormones in vivo. Psychoneuroendocrinology 35:1333–1338.

Hill MN, McEwen BS (2009) Endocannabinoids: the silent partner of glucocorticoids in the synapse. Proc Natl Acad Sci U S A 106:4579–4580.

Hill MN, McEwen BS (2010) Involvement of the endocannabinoid system in the neurobehavioural effects of stress and glucocorticoids. Prog Neuropsychopharmacol Biol Psychiatry 34:791–797.

Hill MN, McLaughlin RJ, Bingham B, Shrestha L, Lee TT, Gray JM, Hillard CJ, Gorzalka BB, Viau V (2010a) Endogenous cannabinoid signaling is essential for stress adaptation. Proc Natl Acad Sci U S A 107:9406–9411.

Hill MN, McLaughlin RJ, Morrish AC, Viau V, Floresco SB, Hillard CJ, Gorzalka BB (2009) Suppression of amygdalar endocannabinoid signaling by stress contributes to activation of the hypothalamic-pituitary-adrenal axis. Neuropsychopharmacology 34:2733–2745.

Hill MN, McLaughlin RJ, Pan B, Fitzgerald ML, Roberts CJ, Lee TT, Karatsoreos IN, Mackie K, Viau V, Pickel VM, McEwen BS, Liu QS, Gorzalka BB, Hillard CJ (2011) Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. J Neurosci 31:10506–10515.

Hill MN, Patel S, Campolongo P, Tasker JG, Wotjak CT, Bains JS (2010b) Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. J Neurosci 30:14980–14986.

Hill MN, Patel S, Carrier EJ, Rademacher DJ, Ormerod BK, Hillard CJ, Gorzalka BB (2005) Downregulation of endocannabinoid signaling in the hippocampus following chronic unpredictable stress. Neuropsychopharmacology 30:508–515.

Hillard CJ, Edgemond WS, Jarrahian A, Campbell WB (1997) Accumulation of *N*-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. J Neurochem 69:631–638.

Holsboer F, Ising M (2010) Stress hormone regulation: biological role and translation into therapy. Annu Rev Psychol 61:81–109.

Hong S, Fan J, Kemmerer ES, Evans S, Li Y, Wiley JW (2009) Reciprocal changes in vanilloid (TRPV1) and endocannabinoid (CB1) receptors contribute to visceral hyperalgesia in the water avoidance stressed rat. Gut 58:202–210.

Hong S, Zheng G, Wu X, Snider NT, Owyang C, Wiley JW (2010) Corticosterone mediates reciprocal changes in CB1 and TRPV1 receptors in primary sensory neurons in the chronically stressed rat. Gastroenterology 140:627.e4–637.e4.

Hrabovszky E, Wittmann G, Kallo I, Fuzesi T, Fekete C, Liposits Z (in press) Distribution of type 1 cannabinoid receptor expressing neurons in the septal-hypothalamic region of the mouse. Colocalization with GABAergic and glutamatergic markers. J Comp Neurol, in press.

Hu W, Zhang M, Czéh B, Zhang W, Flügge G (2011) Chronic restraint stress impairs endocannabinoid mediated suppression of GABAergic signaling in the hippocampus of adult male rats. Brain Res Bull 85:374–379.

Ishiguro H, Horiuchi Y, Ishikawa M, Koga M, Imai K, Suzuki Y, Morikawa M, Inada T, Watanabe Y, Takahashi M, Someya T, Ujike H, Iwata N, Ozaki N, Onaivi ES, Kunugi H, Sasaki T, Itokawa M, Arai M, Niizato K, Iritani S, Naka I, Ohashi J, Kakita A, Takahashi H, Nawa H, Arinami T (2010) Brain cannabinoid CB2 receptor in schizophrenia. Biol Psych 67:974–982.

Jacob W, Yassouridis A, Marsicano G, Monory K, Lutz B, Wotjak CT (2009) Endocannabinoids render exploratory behaviour largely independent of the test aversiveness: role of glutamatergic transmission. Genes Brain Behav 8:685–698.

Jankord R, Herman JP (2008) Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. Ann N Y Acad Sci 1148:64–73.

Jelsing J, Galzin AM, Guillot E, Pruniaux MP, Larsen PJ, Vrang N (2009) Localization and phenotypic characterization of brainstem neurons activated by rimonabant and WIN55, 212-2. Brain Res Bull 78:202–210.

Kamprath K, Romo-Parra H, Haring M, Gaburro S, Doengi M, Lutz B, Pape HC (2010) Short-term adaptation of conditioned fear responses through endocannabinoid signaling in the central amygdala. Neuropsychopharmacology 36:652–663.

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

Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ (1994) Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nat Genet 8:148–154.

Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D (2003) Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 9:76–81.

Kawamura Y, Fukaya M, Maejima T, Yoshida T, Miura E, Watanabe M, Ohno-Shosaku T, Kano M (2006) The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum. J Neurosci 26:2991–3001.

Keimpema E, Barabas K, Morozov YM, Tortoriello G, Torii M, Cameron G, Yanagawa Y, Watanabe M, Mackie K, Harkany T (2010) Differential subcellular recruitment of monoacylglycerol lipase generates spatial specificity of 2-arachidonoyl glycerol signaling during axonal pathfinding. J Neurosci 30:13992–14007.

Krettek JE, Price JL (1978) Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat. J Comp Neurol 178:225–254.

Lafenêtre P, Chaouloff F, Marsicano G (2009) Bidirectional regulation of novelty-induced behavioral inhibition by the endocannabinoid system. Neuropharmacology 57:715–721.

Leonard BE (2005) The HPA and immune axes in stress: the involvement of the serotonergic system. Eur Psychiatry 20 (Suppl 3):S302–S306.

Levitt P, Moore RY (1978) Developmental organization of raphe serotonin neuron groups in the rat. Anat Embryol (Berl) 154:241–251.

Lowry CA (2002) Functional subsets of serotonergic neurones: implications for control of the hypothalamic-pituitary-adrenal axis. J Neuroendocrinol 14:911–923.

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Loy R, Koziell DA, Lindsey JD, Moore RY (1980) Noradrenergic innervation of the adult rat hippocampal formation. J Comp Neurol 189:699–710.

Luppi PH, Aston-Jones G, Akaoka H, Chouvet G, Jouvet M (1995) Afferent projections to the rat locus coeruleus demonstrated by retrograde and anterograde tracing with cholera-toxin B subunit and Phaseolus vulgaris leucoagglutinin. Neuroscience 65:119–160.

Lutz B (2009) Endocannabinoid signals in the control of emotion. Curr Opin Pharmacol 9:46–52.

Mackie K (2005) Distribution of cannabinoid receptors in the central and peripheral nervous system. Handb Exp Pharmacol: 299–325.

Malcher-Lopes R, Di S, Marcheselli VS, Weng FJ, Stuart CT, Bazan NG, Tasker JG (2006) Opposing crosstalk between leptin and glucocorticoids rapidly modulates synaptic excitation via endocannabinoid release. J Neurosci 26:6643–6650.

Manzanares J, Corchero J, Fuentes JA (1999) Opioid and cannabinoid receptor-mediated regulation of the increase in adrenocorticotropin hormone and corticosterone plasma concentrations induced by central administration of delta(9)-tetrahydrocannabinol in rats. Brain Res 839:173–179.

Marco EM, Garcia-Gutierrez MS, Bermudez-Silva FJ, Moreira FA, Guimaraes F, Manzanares J, Viveros MP (2011) Endocannabinoid system and psychiatry: in search of a neurobiological basis for detrimental and potential therapeutic effects. Front Behav Neurosci 5:63.

Marco EM, Viveros MP (2009) The critical role of the endocannabinoid system in emotional homeostasis: avoiding excess and deficiencies. Mini Rev Med Chem 9:1407–1415.

Marrs WR, Blankman JL, Horne EA, Thomazeau A, Lin YH, Coy J, Bodor AL, Muccioli GG, Hu SS, Woodruff G, Fung S, Lafourcade M, Alexander JP, Long JZ, Li W, Xu C, Möller T, Mackie K, Manzoni OJ, Cravatt BF, Stella N (2010) The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. Nat Neurosci 13:951–957.

Marsicano G, Kuner R (2008) Anatomical distribution of receptors, ligands and enzymes in the brain and the spinal cord: circuitries and neurochemistry. In: Cannabinoids and the brain (Kofalvi A, ed), pp 161–202. New York: Springer.

Marsicano G, Lutz B (1999) Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur J Neurosci 11:4213–4225.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564.

McDonald AJ (1982) Neurons of the lateral and basolateral amygdaloid nuclei: a Golgi study in the rat. J Comp Neurol 212:293–312.

McLaughlin RJ, Hill MN, Gorzalka BB (2009) Monoaminergic neurotransmission contributes to cannabinoid-induced activation of the hypothalamic-pituitary-adrenal axis. Eur J Pharmacol 624: 71–76.

Millhouse OE (1986) The intercalated cells of the amygdala. J Comp Neurol 247:246–271.

Molliver ME (1987) Serotonergic neuronal systems: what their anatomic organization tells us about function. J Clin Psychopharmacol 7:3S–23S.

Monory K, Lutz B (2009) Genetic models of the endocannabinoid system. Curr Top Behav Neurosci 1:111–139.

Monory K, Massa F, Egertová M, Eder M, Blaudzun H, Westenbroek R, Kelsch W, Jacob W, Marsch R, Ekker M, Long J, Rubenstein JL, Goebbels S, Nave KA, During M, Klugmann M, Wölfel B, Dodt HU, Zieglgänsberger W, Wotjak CT, Mackie K, Elphick MR, Marsicano G, Lutz B (2006) The endocannabinoid system controls key epileptogenic circuits in the hippocampus. Neuron 51:455–466.

Moreira FA, Kaiser N, Monory K, Lutz B (2008) Reduced anxiety-like behaviour induced by genetic and pharmacological inhibition of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) is mediated by CB1 receptors. Neuropharmacology 54:141–150.

Moreira FA, Lutz B (2008) The endocannabinoid system: emotion, learning and addiction. Addict Biol 13:196–212.

Moreira FA, Wotjak CT (2010) Cannabinoids and anxiety. Curr Top Behav Neurosci 2:429–450.

Morozov YM, Torii M, Rakic P (2009) Origin, early commitment, migratory routes, and destination of cannabinoid type 1 receptor-containing interneurons. Cereb Cortex 19 (Suppl 1):i78–i89.

Muntoni AL, Pillolla G, Melis M, Perra S, Gessa GL, Pistis M (2006) Cannabinoids modulate spontaneous neuronal activity and evoked inhibition of locus coeruleus noradrenergic neurons. Eur J Neurosci 23:2385–2394.

Myers CE, Scharfman HE (2011) Pattern separation in the dentate gyrus: a role for the CA3 backprojection. Hippocampus 21: 1190–1215.

Nevalainen T, Irving AJ (2010) GPR55, a lysophosphatidylinositol receptor with cannabinoid sensitivity? Curr Top Med Chem 10:799–813.

O'Sullivan SE, Kendall DA (2010) Cannabinoid activation of peroxisome proliferator-activated receptors: potential for modulation of inflammatory disease. Immunobioloy 215:611–616.

Onaivi ES, Ishiguro H, Gong JP, Patel S, Meozzi PA, Myers L, Perchuk A, Mora Z, Tagliaferro PA, Gardner E, Brusco A, Akinshola BE, Hope B, Lujilde J, Inada T, Iwasaki S, Macharia D, Teasenfitz L, Arinami T, Uhl GR (2008) Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. PLoS One 3:e1640.

Onaivi ES, Ishiguro H, Gong JP, Patel S, Perchuk A, Meozzi PA, Myers L, Mora Z, Tagliaferro P, Gardner E, Brusco A, Akinshola BE, Liu QR, Hope B, Iwasaki S, Arinami T, Teasenfitz L, Uhl GR (2006) Discovery of the presence and functional expression of cannabinoid CB2 receptors in brain. Ann N Y Acad Sci 1074:514–536.

Onaivi ES, Ishiguro H, Gu S, Liu QR (in press) CNS effects of CB2 cannabinoid receptors: beyond neuro-immuno-cannabinoid activity. J Psychopharmacol, in press.

Ongür D, Price JL (2000) The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans. Cereb Cortex 10:206–219.

Oropeza VC, Mackie K, Van Bockstaele EJ (2007) Cannabinoid receptors are localized to noradrenergic axon terminals in the rat frontal cortex. Brain Res 1127:36–44.

Page ME, Oropeza VC, Van Bockstaele EJ (2008) Local administration of a cannabinoid agonist alters norepinephrine efflux in the rat frontal cortex. Neurosci Lett 431:1–5.

Pamplona FA, Takahashi RN (in press) Psychopharmacology of the endocannabinoids: far beyond anandamide. J Psychopharmacol, in press.

Pan B, Wang W, Long JZ, Sun D, Hillard CJ, Cravatt BF, Liu QS (2009) Blockade of 2-arachidonoylglycerol hydrolysis by selective monoacylglycerol lipase inhibitor 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184) enhances retrograde endocannabinoid signaling. J Pharmacol Exp Ther 331:591–597.

Pape HC, Pare D (2010) Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. Physiol Rev 90:419–463.

Patel S, Hillard CJ (2006) Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. J Pharmacol Exp Ther 318:304–311.

Patel S, Kingsley PJ, Mackie K, Marnett LJ, Winder DG (2009) Repeated homotypic stress elevates 2-arachidonoylglycerol levels and enhances short-term endocannabinoid signaling at inhibitory synapses in basolateral amygdala. Neuropsychopharmacology 34:2699–2709.

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Patel S, Roelke CT, Rademacher DJ, Cullinan WE, Hillard CJ (2004) Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. Endocrinology 145:5431–5438.

Patel S, Roelke CT, Rademacher DJ, Hillard CJ (2005) Inhibition of restraint stress-induced neural and behavioural activation by endogenous cannabinoid signalling. Eur J Neurosci 21:1057–1069.

Petrosino S, Di Marzo V (2010) FAAH and MAGL inhibitors: therapeutic opportunities from regulating endocannabinoid levels. Curr Opin Investig Drugs 11:51–62.

Petrovich GD, Canteras NS, Swanson LW (2001) Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. Brain Res Brain Res Rev 38:247–289.

Petrovich GD, Holland PC, Gallagher M (2005) Amygdalar and prefrontal pathways to the lateral hypothalamus are activated by a learned cue that stimulates eating. J Neurosci 25:8295–8302.

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18:9996–10015.

Pompili M, Serafini G, Innamorati M, Möller-Leimkühler AM, Giupponi G, Girardi P, Tatarelli R, Lester D (2010) The hypothalamic-pituitary-adrenal axis and serotonin abnormalities: a selective overview for the implications of suicide prevention. Eur Arch Psychiatry Clin Neurosci 260:583–600.

Puente N, Cui Y, Lassalle O, Lafourcade M, Georges F, Venance L, Grandes P, Manzoni OJ (2011) Polymodal activation of the endocannabinoid system in the extended amygdala. Nat Neurosci 14:1542–1547.

Puente N, Elezgarai I, Lafourcade M, Reguero L, Marsicano G, Georges F, Manzoni OJ, Grandes P (2010) Localization and function of the cannabinoid CB1 receptor in the anterolateral bed nucleus of the stria terminalis. PLoS One 5:e8869.

Rademacher DJ, Meier SE, Shi L, Ho WS, Jarrahian A, Hillard CJ (2008) Effects of acute and repeated restraint stress on endocannabinoid content in the amygdala, ventral striatum, and medial prefrontal cortex in mice. Neuropharmacology 54:108–116.

Ramikie TS, Patel S (2012) Endocannabinoid signaling in the amygdala: anatomy, synaptic signaling, behavior, and adaptations to stress. Neuroscience.

Reich CG, Taylor ME, McCarthy MM (2009) Differential effects of chronic unpredictable stress on hippocampal CB1 receptors in male and female rats. Behav Brain Res 203:264–269.

Rodgers RJ, Haller J, Halasz J, Mikics E (2003). 'One-trial sensitization' to the anxiolytic-like effects of cannabinoid receptor antagonist SR141716A in the mouse elevated plus-maze. Eur J Neurosci 17:1279–1286.

Rosene DL, Van Hoesen GW (1977) Hippocampal efferents reach widespread areas of cerebral cortex and amygdala in the rhesus monkey. Science 198:315–317.

Sah P, Lopez De Armentia M (2003) Excitatory synaptic transmission in the lateral and central amygdala. Ann N Y Acad Sci 985:67–77.

Scavone JL, Mackie K, Van Bockstaele EJ (2010) Characterization of cannabinoid-1 receptors in the locus coeruleus: relationship with mu-opioid receptors. Brain Res 1312:18–31.

Sigel E, Baur R, Rácz I, Marazzi J, Smart TG, Zimmer A, Gertsch J (2011) The major central endocannabinoid directly acts at GABA(A) receptors. Proc Natl Acad Sci U S A 108:18150–18155.

Steiner MA, Marsicano G, Nestler EJ, Holsboer F, Lutz B, Wotjak CT (2008a) Antidepressant-like behavioral effects of impaired cannabinoid receptor type 1 signaling coincide with exaggerated corticosterone secretion in mice. Psychoneuroendocrinology 33:54–67.

Steiner MA, Marsicano G, Wotjak CT, Lutz B (2008b) Conditional cannabinoid receptor type 1 mutants reveal neuron subpopulation-specific effects on behavioral and neuroendocrine stress responses. Psychoneuroendocrinology 33:1165–1170.

Steiner MA, Wotjak CT (2008) Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis. Prog Brain Res 170:397–432.

Suárez J, Llorente R, Romero-Zerbo SY, Mateos B, Bermúdez-Silva FJ, de Fonseca FR, Viveros MP (2009) Early maternal deprivation induces gender-dependent changes on the expression of hippocampal CB(1) and CB(2) cannabinoid receptors of neonatal rats. Hippocampus 19:623–632.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:89–97.

Tanji J, Hoshi E (2008) Role of the lateral prefrontal cortex in executive behavioral control. Physiol Rev 88:37–57.

Tao R, Auerbach SB (2000) Regulation of serotonin release by GABA and excitatory amino acids. J Psychopharmacol 14:100–113.

Thomas EA, Cravatt BF, Danielson PE, Gilula NB, Sutcliffe JG (1997) Fatty acid amide hydrolase, the degradative enzyme for anandamide and oleamide, has selective distribution in neurons within the rat central nervous system. J Neurosci Res 50:1047–1052.

Turu G, Hunyady L (2010) Signal transduction of the CB1 cannabinoid receptor. J Mol Endocrinol 44:75–85.

Ulrich-Lai YM, Herman JP (2009) Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 10:397–409.

Van Gaal L, Pi-Sunyer X, Després JP, McCarthy C, Scheen A (2008) Efficacy and safety of rimonabant for improvement of multiple cardiometabolic risk factors in overweight/obese patients: pooled 1-year data from the Rimonabant in Obesity (RIO) program. Diabetes Care 31 (Suppl 2):S229–S240.

van Groen T, Wyss JM (1990) The connections of presubiculum and parasubiculum in the rat. Brain Res 518:227–243.

Van Sickle MD, Oland LD, Ho W, Hillard CJ, Mackie K, Davison JS, Sharkey KA (2001) Cannabinoids inhibit emesis through CB1 receptors in the brainstem of the ferret. Gastroenterology 121:767–774.

Vázquez-Borsetti P, Celada P, Cortés R, Artigas F (2011) Simultaneous projections from prefrontal cortex to dopaminergic and serotonergic nuclei. Int J Neuropsychopharmacol 14:289–302.

Viveros MP, Marco EM, File SE (2005) Endocannabinoid system and stress and anxiety responses. Pharmacol Biochem Behav 81:331–342.

Wade MR, Degroot A, Nomikos GG (2006) Cannabinoid CB1 receptor antagonism modulates plasma corticosterone in rodents. Eur J Pharmacol 551:162–167.

Wang W, Sun D, Pan B, Roberts CJ, Sun X, Hillard CJ, Liu QS (2010) Deficiency in endocannabinoid signaling in the nucleus accumbens induced by chronic unpredictable stress. Neuropsychopharmacology 35:2249–2261.

Wenger T, Ledent C, Tramu G (2003) The endogenous cannabinoid, anandamide, activates the hypothalamo-pituitary-adrenal axis in CB1 cannabinoid receptor knockout mice. Neuroendocrinology 78:294–300.

Wotjak CT (2005) Role of endogenous cannabinoids in cognition and emotionality. Mini Rev Med Chem 5:659–670.

Xing G, Carlton J, Zhang L, Jiang X, Fullerton C, Li H, Ursano R (2011) Cannabinoid receptor expression and phosphorylation are differentially regulated between male and female cerebellum and brain stem after repeated stress: implication for PTSD and drug abuse. Neurosci Lett 502:5–9.

Zanettini C, Panlilio LV, Alicki M, Goldberg SR, Haller J, Yasar S (2011) Effects of endocannabinoid system modulation on cognitive and emotional behavior. Front Behav Neurosci 5:57.

Zhang Y, Damjanoska KJ, Carrasco GA, Dudas B, D'Souza DN, Tetzlaff J, Garcia F, Hanley NR, Scripathirathan K, Petersen BR, Gray TS, Battaglia G, Muma NA, Van de Kar LD (2002) Evidence that 5-HT2A receptors in the hypothalamic paraventricular nucleus mediate neuroendocrine responses to (-)DOI. J Neurosci 22:9635–9642.

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Genetic Dissection of the Role of Cannabinoid Type-1 Receptors in the Emotional Consequences of Repeated Social Stress in Mice

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The endocannabinoid system (ECS) tightly controls emotional responses to acute aversive stimuli. Repeated stress alters ECS activity but the role played by the ECS in the emotional consequences of repeated stress has not been investigated in detail. This study used social defeat stress, together with pharmacology and genetics to examine the role of cannabinoid type-1 (CB_1) receptors on repeated stress-induced emotional alterations. Seven daily social defeat sessions increased water (but not food) intake, sucrose preference, anxiety, cued fear expression, and adrenal weight in C57BL/6N mice. The first and the last social stress sessions triggered immediate brain region-dependent changes in the concentrations of the principal endocannabinoids anandamide and 2-arachidonoylglycerol. Pretreatment before each of the seven stress sessions with the CB_1 receptor antagonist rimonabant prolonged freezing responses of stressed mice during cued fear recall tests. Repeated social stress abolished the increased fear expression displayed by constitutive CB_1 receptor-deficient mice. The use of mutant mice lacking CB_1 receptors from cortical glutamatergic neurons or from GABAergic neurons indicated that it is the absence of the former CB_1 receptor population that is responsible for the fear responses in socially stressed CB_1 mutant mice. In addition, stress-induced hypolocomotor reactivity was amplified by the absence of CB_1 receptors from GABAergic neurons. Mutant mice lacking CB_1 receptors from serotonergic neurons displayed a higher anxiety but decreased cued fear expression than their wild-type controls. These mutant mice failed to show social stress-elicited increased sucrose preference. This study shows that (i) release of endocannabinoids during stress exposure impedes stress-elicited amplification of cued fear behavior, (ii) social stress opposes the increased fear expression and delayed between-session extinction because of the absence of CB_1 receptors from cortical glutamatergic neurons, and (iii) CB_1 receptors on central serotonergic neurons are involved in the sweet consumption response to repeated stress. *Neuropsychopharmacology* advance online publication, 21 March 2012; doi:10.1038/npp.2012.36

Keywords: social stress; CB_1 receptor; anxiety; fear; sucrose consumption; hypothalamo-pituitary-adrenal axis

INTRODUCTION

The cannabinoid type-1 (CB_1) receptor, which is the predominant endocannabinoid receptor in neurons, is mainly located in the presynaptic compartment where it negatively impacts neurotransmitter release (Alger, 2002; Piomelli, 2003; Chevaleyre *et al*, 2006; Ohno-Shosaku *et al*, 2012). This receptor is found throughout the brain, including in regions/nuclei—such as cortical areas, the basal ganglia, and the hypothalamus—involved in the control of emotional reactivity (Herkenham *et al*, 1990; Glass *et al*, 1997; Tsou *et al*, 1998; Katona *et al*, 1999; Marsicano and Lutz, 1999). Direct evidence for a tonic role of CB_1 receptors in the control of emotionality has been gathered by means of pharmacology and genetics. Thus, the use of CB_1 receptor antagonists and of CB_1 receptor mutant mice has underlined the prominent role of CB_1 receptors on locomotor reactivity, anxiety, and fear responses to the acute exposure to aversive environments (Viveros *et al*, 2005; Wotjak, 2005; Lafenêtre *et al*, 2007; Lutz, 2009). However, the tight interactions between the endocannabinoid system (ECS) and stress circuits are not limited to the acute exposure to aversive stimuli. Repeated exposure to homotypic or heterotypic stressors affect in a brain region-dependent manner all components of the ECS (Patel and Hillard, 2008; Hill *et al*, 2010b; Riebe and Wotjak, 2011). This is true for the concentrations of the major endocannabinoids, namely

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anandamide (AEA) and 2-arachidonoylglycerol (2-AG), the activities of their respective degrading enzymes, and the mRNA and protein expression of CB_1 receptors (Hill *et al*, 2005; Bortolato *et al*, 2007; Rademacher *et al*, 2008; Patel *et al*, 2009; Reich *et al*, 2009; Hill *et al*, 2010a; Zoppi *et al*, 2011). Moreover, repeated homotypic and heterotypic stress affect the agonist-binding properties of CB_1 receptors and/or CB_1 receptor-mediated control of neurotransmitter release (Hill *et al*, 2005; Rossi *et al*, 2008; Patel *et al*, 2009; Wamsteeker *et al*, 2010).

These data indicate that repeated stress alters different components of the ECS, including CB_1 receptor-mediated control of neurotransmission. However, whether these alterations extend to the control of emotionality exerted by CB_1 receptors has been only scarcely addressed. It has been reported that pretreatment with the CB_1 receptor antagonist rimonabant (SR141716) amplifies escape behavior during acute/repeated restraint stress (Patel *et al*, 2005). Nonetheless, how this observation relates to stress coping is not clear as rimonabant pretreatment may facilitate passive behavior during stress (Steiner *et al*, 2008). Besides its influence on escape behavior, CB_1 receptor blockade further increases the anhedonic consequence of repeated restraint stress, as revealed by sucrose preference tests (Rademacher and Hillard, 2007). Lastly, one recent study indicates that the genetic deletion of CB_1 receptors amplifies the anxiogenic consequences of repeated restraint stress, as assessed in the elevated plus-maze (Hill *et al*, 2011). These observations, which suggest that CB_1 receptors have a tonic regulatory role on several aspects of emotionality during repeated stress events, raise three main issues. First, as these data were gathered using one single model of stress, ie, restraint stress, it remains to be investigated whether this link between CB_1 receptors and emotionality is present under the same modalities in other stress models. Although restraint is a useful model for stress studies, other stress procedures, including social defeat (Buwalda *et al*, 2005; Miczek *et al*, 2008; Golden *et al*, 2011), have been proposed to better model stress-related psychopathologies in humans. Second, it is unknown whether the control exerted by CB_1 receptors on stress-induced anhedonia and anxiety extends to other emotional consequences of repeated stress. As an illustration, it is at the present time unknown whether the well-documented inhibitory effects of repeated stress on fear memory extinction (Rau *et al*, 2005; Akirav and Maroun, 2007), a cognitive process tightly controlled by CB_1 receptors (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005; Kamprath *et al*, 2006), is accounted for by stress-induced alterations in the ECS. Lastly, none of the above mentioned studies on the interactions between CB_1 receptors and emotionality in repeatedly stressed animals addressed the key question of the neuronal populations through which CB_1 receptors exert their input. In this context, the use of mutant lines where CB_1 receptors are missing from specific neuronal populations (Marsicano *et al*, 2003; Monory *et al*, 2006; Jacob *et al*, 2009; Lafenêtre *et al*, 2009; Puighermanal *et al*, 2009; Bellocchio *et al*, 2010) can help gathering crucial information on the relationships between the central ECS and stress circuitry.

The goal of this study was to further define the relationships between CB_1 receptors and the emotional consequences of repeated stress by addressing the three issues raised above. With regard to the first issue, repeated social defeat was chosen here as a stress model in light of (i) its high ethological value (Buwalda *et al*, 2005; Miczek *et al*, 2008), (ii) its relevance to the etiology of human mood disorders (Huhman, 2006; Miczek *et al*, 2008), and (iii) past evidence that in our hands the social defeat model triggers a vast array of emotional, metabolic, and endocrine changes (Dubreucq *et al*, 2012). We first ensured that social stress triggered changes in the ECS, as assessed by the analysis of brain tissue levels of AEA and 2-AG in mice acutely or repeatedly submitted to a social defeat protocol. Next, we investigated the respective effects of pharmacological CB_1 receptor blockade by rimonabant and of the constitutive deletion of CB_1 receptors on the consequences of social stress on food and water intake, sucrose preference, unconditioned anxiety, and cued fear memory. In addition, the important role of CB_1 receptors in the control of the activity of the hypothalamo-pituitary-adrenal (HPA) axis in both control and stressed individuals (Steiner and Wotjak, 2008; Hill *et al*, 2010a) led us to measure the weights of the adrenal glands as an index of chronic HPA axis reactivity to repeated stress. In a last series of experiments, we examined whether CB_1 receptors located respectively on cortical glutamatergic neurons, on GABAergic neurons, or on serotonergic neurons exert a control on the aforementioned emotional, metabolic, and endocrine responses to social stress. To achieve this aim, we used conditional mutant lines wherein the *CB_1* receptor gene was selectively deleted from each of these neuronal populations.

MATERIALS AND METHODS

Animals

The experiments were conducted in strict compliance with European directives and French laws on animal experimentation (authorization number 06369). This study involved 2–3-month-old male C57BL/6N mice purchased from Janvier (Le Genest Saint-Isle, France), 3–12-month-old male CD1 mice purchased from Charles Rivers (L'Arbresle, France), and 2–3-month-old constitutive/conditional male CB_1 receptor mutant and wild-type animals bred at the NeuroCentre Magendie. All mice were housed individually 1–2 weeks before experiments with food and water *ad libitum* under a 12-h light/dark cycle (lights on at 07:00 hours). Wild-type and constitutive CB_1 receptor mutant mice (referred to in the text as $CB_1^{+/+}$ and $CB_1^{-/-}$, respectively), wild-type and conditional mutant mice lacking CB_1 receptors from cortical glutamatergic neurons (referred to in the text as Glu-$CB_1^{+/+}$ and Glu-$CB_1^{-/-}$, respectively), and wild-type and conditional mutant mice lacking CB_1 receptors from GABAergic neurons (referred to in the text as GABA-$CB_1^{+/+}$ and GABA-$CB_1^{-/-}$, respectively), were obtained, maintained, and genotyped/regenotyped, as described previously (Marsicano *et al*, 2002; Marsicano *et al*, 2003; Monory *et al*, 2006; Bellocchio *et al*, 2010). Conditional mutant mice lacking CB_1 receptors from central serotonergic neurons (referred to below as TPH2-$CB_1^{-/-}$) and their wild-type controls (referred to below as TPH2-$CB_1^{+/+}$) were generated through a three-step process.

The first step was achieved by crossing homozygous CB_1-floxed ($CB_1^{f/f}$) mice (Marsicano *et al*, 2003) with mice bearing a tamoxifen-inducible Cre-ER^{T2} recombinase expressed under the regulatory sequences of the mouse tryptophan hydroxylase 2 (*Tph2*) gene locus (Weber *et al*, 2009). In a second step, heterozygous Cre-expressing/CB_1-floxed mice ($CB_1^{TPH2-CreERT2;f/+}$) were again crossed with $CB_1^{f/f}$ to obtain homozygous Cre-expressing/CB_1-flox mice ($CB_1^{TPH2-CreERT2;f/f}$). Male mice from step 2 were finally bred with $CB_1^{f/f}$ females to generate littermate experimental animals ($CB_1^{TPH2-CreERT2;f/f}$ and $CB_1^{f/f}$, referred to as TPH2-$CB_1^{-/-}$ and TPH2-$CB_1^{+/+}$, respectively). Genotyping (at 2 weeks of age) and regenotyping (at the end of the experiments) of the *Cre* transgene were performed by PCR using the primers 5′-CCACTGCGGGCTCTACTTC-3′ (forward) and 5′-TGATGATCTTCTGGCACAGCAG-3′ (reverse), whereas genotyping for the CB_1-floxed locus was performed as described (Marsicano *et al*, 2003). Induction of Cre-mediated recombination was performed by injecting i.p. all mice (including TPH2-$CB_1^{+/+}$ mice) daily for 5 days with 10 mg/ml tamoxifen (Sigma-Aldrich, St Quentin Fallavier, France) dissolved in sesame oil and ethanol (10 : 1) (Imai *et al*, 2000). All animals, injected when 5–10-week-old, were used at least 3 weeks after the end of tamoxifen treatment. Note that PCR on genomic DNA have confirmed that tamoxifen treatment leads to a specific deletion of the CB_1 gene in the dorsal raphe nucleus of TPH2-$CB_1^{-/-}$ mice (Bellocchio *et al*, submitted). All lines were in a mixed genetic background, with a predominant C57BL/6N contribution. For each line, the wild-type animals and the constitutive/conditional mutant animals used in this study were littermates. As illustrated above for the generation of the TPH2-*CB1* line, mice from the GABA-*CB1* and the Glu-*CB1* lines were generated from crossings between $CB_1^{Cre;f/f}$ males and $CB_1^{f/f}$ females to avoid (i) differences in maternal behavior and (ii) potential germline transmission of the gene deletion in the GABA-*CB1* line (Massa *et al*, 2010).

Social Stress Protocol

Except for the experiments aimed at comparing the effects of acute and repeated social defeats on brain endocannabinoid levels or on cued fear memory one day after conditioning (see below), all experiments involved a daily stress protocol, which began at 16:00 hours, and that was repeated over 7 consecutive days. This protocol consisted of the following three different periods (Dubreucq *et al*, 2012): (i) placement of the experimental mouse in a wire mesh cylinder inside the home cage of a resident CD1 mouse for 30 min (sensory contacts between mice), (ii) removal of the wire mesh cylinder for 15 min (sensory and physical contacts), a period during which the latency for the first attack and the number of attacks by the resident were scored (note that all mice tested displayed an upright posture, indicating defeat), and (iii) reiteration of (i) for another 30 min, after which the experimental mouse was returned to its home cage (Figure 1a). As already documented, each daily confrontation involved an experimental mouse and a resident mouse that were unknown to each other (Dubreucq *et al*, 2012). During confrontations, water bottles were removed from all cages, including those housing the unstressed animals. All CD1 residents used in this study were selected 2 weeks after their arrival for their ability to attack an intruder within 20–30 s. These mice were then kept and used for social stress protocols up to 1 year of age.

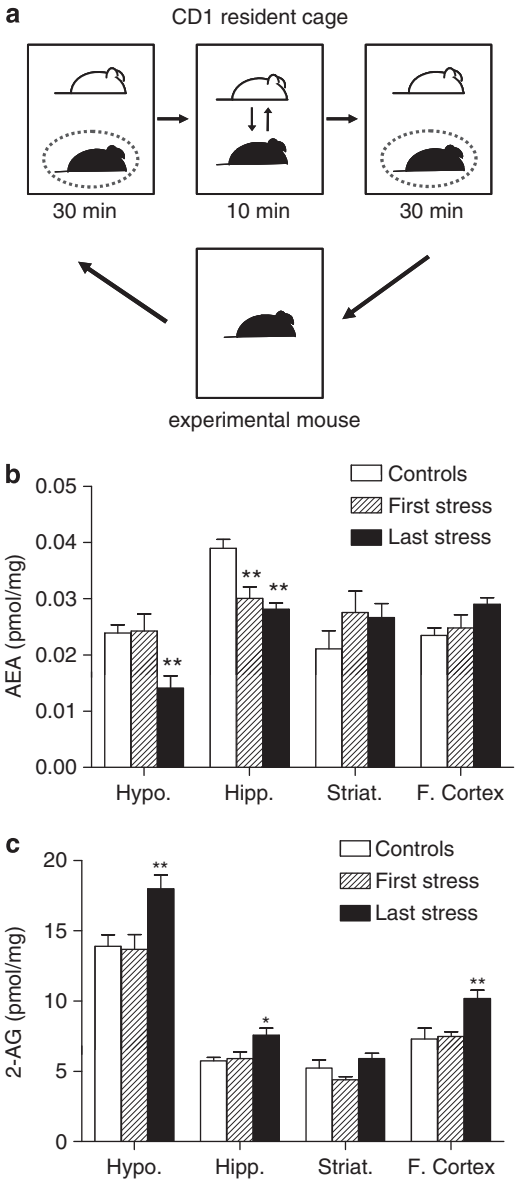

Figure 1 Social stress protocol and effects of acute/repeated social stress on central endocannabinoid concentrations. Each experimental mouse was placed for 70 min in the home cage of a CD1 resident, with one direct physical confrontation following and preceding phases of sensory contacts (a). Respective effects of the first and the last of seven social stress sessions on hypothalamic (Hypo.), hippocampal (Hipp.), striatal (Striat.), and frontocortical (F. Cortex) anandamide (AEA) (b) and 2-arachidonoylglycerol (2-AG) (c) concentrations in C57Bl/6N mice. Values are the mean ± SEM of 5–8 animals. *$p<0.05$ and **$p<0.01$ for the effects of acute/repeated stress.

Endocannabinoid Analyses

In one single series of experiments, control mice were killed by cervical dislocation at the same time as mice that had been exposed for the first time or for the seventh time to

social stress. Note that stressed mice were killed immediately after the second step of the stress procedure detailed above. The hypothalamus, the frontal cortex, the striatum, and the hippocampus were rapidly dissected out on dry ice and stored at −80 °C for the estimation of AEA and 2-AG concentrations. The extraction, purification, and quantification of AEA and 2-AG from brain areas were performed as previously described (Lafourcade *et al*, 2011; Lourenço *et al*, 2011). First, brain areas were homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2 : 1 : 1, *v*/*v*) containing internal deuterated standards. The dried lipid extract was pre-purified if necessary by open bed chromatography on silica gel mini-columns, eluted with increasing concentrations of methanol in chloroform. Samples were then subjected to isotope-dilution liquid chromatography-chemical ionization-tandem mass spectrometric analysis (LC-MS/MS). Mass spectral analyses were performed on a TSQ Quantum triple quadrupole instrument (Thermo-Finnigan, San Jose, CA, USA) equipped with an APCI source (atmospheric pressure chemical ionization) and operating in positive-ion mode. A sensitive and specific LC-MS/MS method was developed and validated for endocannabinoid quantification. The amounts of AEA and 2-AG were determined using a calibration curve and expressed as pmol/mg tissue.

General Procedure

This study examined the respective influences of (i) CB_1 receptor blockade by rimonabant, (ii) CB_1 receptor deletion from the whole body, and (iii) CB_1 receptor deletion from either cortical glutamatergic neurons, GABAergic neurons, or serotonergic neurons on several emotional, metabolic, and endocrine consequences of repeated social stress. All control and stressed animals were handled daily throughout the course of the experiments. Except for one series of experiments (which included mice from the CB_1, the Glu-CB_1, and the GABA-CB_1 lines), individual food and water intakes were measured on a daily basis. Within each experimental series, several individuals were randomly tested during the night that followed the seventh stress session for their sucrose intakes in a free choice paradigm (note that these animals underwent a preliminary 1-week habituation period to water and sucrose before stress; see below). On the morning that followed the seventh stress session, mice were first exposed to an elevated plus-maze test. In the afternoon of that same day, mice were then cued fear-conditioned before being tested in fear recall sessions on each of the three following afternoons (ie, 24–72 h after conditioning). One day after the last of these recall sessions, several mice, taken at random, were killed by cervical dislocation and their adrenals dissected out. In one series of experiments aimed at measuring the respective effects of acute and repeated stress on cued fear memory, several mice were exposed for the first time to social defeat when repeatedly stressed mice underwent their seventh stress session. All mice, including controls, were cued fear-conditioned 1 day after stress and tested in a recall session another day later. Investigations were all conducted without any knowledge of genotypes and/or treatments until final analyses.

Rimonabant Administration

Control and stressed C57Bl6/N were daily injected (30 min before each of the seven stress sessions) with the CB_1 receptor antagonist rimonabant (3 mg/kg; Sigma-Aldrich) or its vehicle (one drop of Tween 80 in 3 ml of 1.25% dimethylsulphoxide and 0.9% NaCl). Control (ie, unstressed) animals were injected at the same time. All animals were injected on the basis of their individual body weights.

Food and Water Intakes

The individual amounts of food and water consumed were measured each morning, beginning 1 day before the first stress session. With respect to water consumption during the night that followed the last stress, the difference between the respective intakes of animals left with water alone and those of animals exposed to a water/sucrose choice (see below) were low, compared with the total amounts measured during the six preceding days in both animal groups. Accordingly, water intakes of the mice that underwent this choice test were taken into account into the total amount of water consumed through the 7-day protocol. Note that food amounts were not corrected for spillage and that water leaks were avoided by providing 50-ml plastic bottles connected to sippers bearing ball-shaped stoppers (Habitrail, Hagen, France).

Elevated Plus-Maze

The apparatus, made of black Perspex, consisted of four elevated arms (height: 66 cm) 45-cm long and 10-cm wide (Letica, Barcelona, Spain). The arms were arranged in a cross-like disposition, with two opposite arms being enclosed by 50 cm high walls made of grey Perspex, and the two other arms being open. The four arms were connected by a squared central platform (10 × 10 cm). Both the central platform and the open arms were under bright illumination (100–120 lux) whereas the closed arms were under weak illumination (30 lux). Each mouse was placed on the central platform, facing an open arm. The number of visits to, and the time spent on, the open arms and the closed arms were recorded for 5 min, using a videocamera placed above the apparatus, as described previously (Dubreucq *et al*, 2012). Note that in several instances, mice (three $CB_1^{+/+}$, two $CB_1^{-/-}$, two GABA-$CB_1^{+/+}$, and two GABA-$CB_1^{-/-}$) had to be excluded from the analysis because of peculiar behaviors (full immobility on the central platform or absence of closed arm visits) or falls from open arms.

Cued Fear-Conditioning and Recall

A conditioning box, made of grey Perspex (length: 26 cm, width: 18 cm,and height: 25 cm) with a metal grid floor, was located in a sound-proof chamber (length: 55 cm, width: 60 cm,and height: 50 cm; Imetronic, Pessac, France) in a room adjacent to the housing room. On the conditioning day, each mouse was placed in the conditioning box and left free to explore for 3 min. A sound (1.5 kHz, 60 dB) was then emitted for 20 s, with the last second of tone emission being

coupled to one single footshock (0.5 mA). The animal was left in the fear-conditioning box for another minute without any stimulus before being removed from the apparatus, and housed back in its home cage. On the 3 consecutive days (recall tests), the top of each home cage was removed to be covered by a grid, allowing full observation of the mouse in its cage. The home cage was then placed into the sound-proof chamber. After a 3-min pre-tone period, the tone used for conditioning was presented again for a 3-min period. The mouse was then left for another minute in the chamber before removal of the home cage, which was returned back to the housing facility room. The presence of freezing (ie, lack of movements excepted those associated with breathing) was monitored every 20 s during the 3-min exposure to sound on each of the three recall tests, as previously reported (Dubreucq *et al*, 2010). Freezing behavior was scored by means of a customized EVENTLOG program.

Sucrose Preference

Two 50-ml bottles (see above) filled respectively with water and 2% sucrose were provided throughout the 6 days that preceded the stress protocol to estimate basal sucrose preferences. Each day, the positions of the bottles in the cages were switched as to avoid preference. Mice were then given only water, except during the night that followed the seventh social defeat session, where these mice had also access to a 2% sucrose solution. Water and sucrose amounts were monitored on the basis of weight differences (Dubreucq *et al*, 2012). When needed, preference ratios were calculated for each individual as the amount of sucrose ingested over the sum of the sucrose and water amounts ingested.

Adrenal Weights

As mentioned previously (Dubreucq *et al*, 2012), the fat surrounding the glands was visualized using an Olympus SZX10 Stereo microscope (Olympus, Bordeaux, France) and removed for subsequent adrenal weight measurements.

Statistics

All analyses were performed with the GB-Stat software (v10; Dynamic Microsystems, Silver Spring, MD, USA). Comparisons were achieved through Student's *t*-tests when assessing two-group comparisons, and by means of ANOVAs with/without repeated factors for multiple-group comparisons. *Post hoc* group comparisons, which were performed using Tukey's multiple comparison test, were achieved only if interactions between main variables were found significant. When necessary, data were log-transformed to reach homogeneity of the variances. In all tests, the significance level was preset to $p<0.05$.

RESULTS

Acute and Repeated Social Stress Target Central Endocannabinoids

The first and/or the seventh stress sessions decreased AEA levels in the hypothalamus ($F_{(2,15)}=6.99$; $p=0.0071$) and hippocampus ($F_{(2,18)}=12.57$; $p=0.0004$), but not in the striatum or the frontal cortex (Figure 1b). On the other hand, the last, but not the first, session of social stress increased hypothalamic ($F_{(2,18)}=6.62$; $p=0.007$), hippocampal ($F_{(2,17)}=3.67$; $p=0.047$), and frontocortical ($F_{(2,18)}=5.83$; $p=0.011$) 2-AG levels (Figure 1c).

Pretreatment With a CB_1 Receptor Antagonist Prolongs the Stimulatory Effect of Repeated Social Stress on Cued Fear Memory

The number of daily attacks during the stress sessions was similar in vehicle- and in rimonabant-pretreated stressed mice (18.20 ± 0.60 and 17.98 ± 0.41, respectively; $n=14$ in each group). Both social stress ($F_{(1,36)}=32.26$; $p<0.0001$) and rimonabant pretreatment ($F_{(1,36)}=7.47$; $p=0.0097$) increased water consumption (Figure 2a), but not food intake (data not shown), throughout the 7-day stress protocol. When offered water and sucrose as drinking solutions after the last stress session, the consumption of sucrose, but not that of water, was found to be increased by stress in both vehicle- and rimonabant-pretreated mice ($F_{(1,15)}=6.29$; $p=0.0241$; Figure 2b). In the elevated plus-maze, social stress bore hypolocomotor influences, as revealed by the analysis of the number of closed arm entries ($F_{(1,51)}=13.92$; $p=0.0005$; Figure 2c). Such an inhibitory influence of stress extended to the percent time spent in the open arms ($F_{(1,51)}=7.55$; $p=0.0084$; Figure 2d) while only a trend for an inhibitory effect of stress on the percent number of open arm visits was observed ($14.18\pm2.52\%$ and $12\pm3.07\%$ in 13 vehicle- and 14 rimonabant-pretreated control mice, respectively, as opposed to $9.13\pm3.37\%$ and $6.53\pm2.38\%$ in 14 stressed vehicle-pretreated mice and 14 stressed rimonabant-pretreated mice, respectively). Freezing behavior during cued fear recall sessions (ie, 24–72 h after fear conditioning) was dependent on the recall day ($F_{(2,102)}=47.56$; $p<0.0001$); however, whatever the recall day, social stress increased freezing behavior in all mice ($F_{(1,51)}=7.47$; $p=0.0086$) (Figure 2e). Moreover, the extent to which the recall session and stress affected freezing behavior was influenced by rimonabant pretreatment ($F_{(2,102)}=3.24$; $p=0.043$ for the stress $\times$ recall session $\times$ pretreatment interaction). Thus, social stress amplified freezing behavior during the first session in vehicle-pretreated mice while leaving unaffected that measured on the following recall sessions (Figure 2e). In contrast, rimonabant pretreatment extended the stimulatory effect of stress on freezing to the last two recall sessions (Figure 2e). These results were confirmed when within-session scores were analyzed; freezing scores were still accounted for by the interaction between stress and the recall session in vehicle-pretreated animals ($F_{(2,50)}=3.45$; $p=0.0395$; Supplementary Figure S1a) whereas stress *per se* amplified freezing scores in rimonabant-pretreated mice ($F_{(1,26)}=6.47$; $p=0.0172$; Supplementary Figure S1b). On the other hand, stress did not affect within-session extinction in vehicle- and rimonabant-pretreated mice (Supplementary Figures S1a and b) but increased the initial freezing responses to the cue whatever the mouse group or the recall session considered ($F_{(1,51)}=3.45$; $p=0.0395$; Supplementary Figure S1c). Lastly, repeated social stress increased

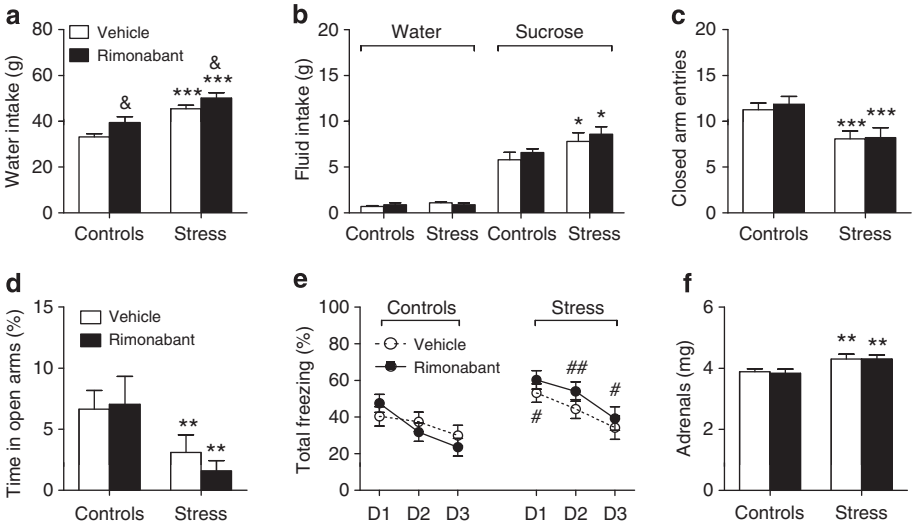

Figure 2 Effects of rimonabant pretreatment before each of the seven social stress sessions on the emotional profiles of control and stressed mice. This profile included 7-day water intakes (a), water and sucrose intakes in a free choice paradigm after the seventh stress session (b), closed arm entries (c) and percent time spent in the open arms (d) of an elevated plus-maze, freezing behavior during cued fear recall sessions 24–72 h after conditioning (e), and adrenal weight (f). D1–D3 stand for days 1–3. Values are the mean ± SEM of 10–14 animals, except for (b), which refer to 4–5 animals per group. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ for the overall effect of social stress in the ANOVA; $^{\&}p<0.05$ for the overall influence of the pretreatment in the ANOVA; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ for the *post hoc* effects of stress. See text for ANOVAs and additional statistics.

adrenal weight in both vehicle- and rimonabant-preteated mice ($F_{(1,48)}=11.61$; $p=0.0013$; Figure 2f).

CB_1 Receptors are Involved in the Potentiating Effect of Repeated Social Stress on Conditioned Freezing

Mice from the $CB_1^{+/+}$ and the $CB_1^{-/-}$ groups received an equivalent number of daily attacks throughout the stress protocol (20.02 ± 1.01 and 20.27 ± 1.00, respectively; $n=13$ in each group). Repeated stress increased water intake in a genotype-independent manner ($F_{(1,43)}=60.2$; $p<0.0005$; Figure 3a), this change occurring without any influence of stress and/or genotype on food intake (data not shown). In the sucrose preference test, the genotype bore no effect on the stimulatory impact of repeated stress on sucrose consumption ($F_{(1,28)}=27.61$; $p<0.0001$; Figure 3b). Repeated stress, but not the genotype, was endowed with a weak, albeit significant, inhibitory impact on elevated plus-maze behaviors, whether the number of closed arm entries ($F_{(1,47)}=4.16$; $p=0.0469$; Figure 3c), the percent time spent on open arms ($F_{(1,47)}=4.51$; $p=0.039$; Figure 3d), or the percent number of open arm visits (12.96 ± 2.83% and 11.99 ± 3.27% in 14 control wild-type and 15 mutant mice, respectively, as opposed to 8.21 ± 2.29% and 4.02 ± 2.16% in 11 stressed wild-type and 11 stressed mutant mice, respectively; $F_{(1,47)}=5.03$; $p=0.030$) were considered. On the other hand, the genotype, alone ($F_{(1,53)}=31.12$; $p<0.0001$) and in combination with either stress ($F_{(1,53)}=7.95$; $p=0.0068$) or the recall session ($F_{(2,106)}=16.51$; $p<0.0001$) influenced freezing behavior during cued fear memory recall sessions (Figure 3e). Thus, control $CB_1^{-/-}$ mice displayed increased freezing behavior, compared with their $CB_1^{+/+}$ littermates, a difference which increased with the number of recall sessions (Figure 3e). Whereas repeated stress amplified the freezing response to the tone in $CB_1^{+/+}$ mice, it weakened that of $CB_1^{-/-}$ mice, especially during the last two recall sessions (Figure 3e). Within-session patterns of freezing confirmed that repeated stress stimulated this behavior in $CB_1^{+/+}$ mice ($F_{(1,27)}=5.98$; $p=0.02$; Supplementary Figure S1d) whereas the influence of stress depended on the recall day in $CB_1^{-/-}$ mice ($F_{(2,52)}=7.11$; $p=0.0019$); thus, a decreased freezing response was observed in stressed $CB_1^{-/-}$ mice during the last two recall sessions (Supplementary Figure S1e). Initial freezing responses to the tone were dictated by the genotype ($F_{(1,53)}=22.43$; $p<0.001$) and by the respective interactions between stress and either the genotype or the recall day ($F_{(1,53)}=5.71$; $p=0.0205$ and $F_{(2,106)}=7.42$; $p=0.001$; Supplementary Figure S1f). At last, prior repeated stress increased adrenal weight in both genotypes ($F_{(1,40)}=19.1$; $p<0.0001$; Figure 3f).

CB_1 Receptors on Cortical Glutamatergic Neurons are Involved in the Potentiating Effect of Repeated Social Stress on Conditioned Freezing

Glu-$CB_1^{+/+}$ and Glu-$CB_1^{-/-}$ mice did not differ either in the number of attacks received (21.83 ± 0.53 and 20.50 ± 0.86, respectively; $n=12$ in each group) or in the amplitude of the dipsogenic effect of repeated stress ($F_{(1,39)}=51.69$; $p<0.0001$; Figure 4a). As opposed to water intakes, food intakes proved insensitive to the stress procedure (data not shown). When tested after the last stress session in a sucrose/water choice paradigm, the two genotypes responded in an identical manner to the stimulatory influence of stress on sucrose ingestion ($F_{(1,14)}=6.91$; $p=0.0198$; Figure 4b). In the elevated plus-maze, the mouse genotype and/or the stress procedure affected neither the closed arm entries (Figure 4c) nor the percent time spent in the open arms of the elevated plus-maze (albeit trends for negative effects of stress and of the mutation could be noted; see Figure 4d). Conversely, Glu-$CB_1^{-/-}$ mice displayed a lower percent number of visits on open arms (11.25 ± 3.41 and 7.18 ± 3.18 in 13 control and 12 stressed

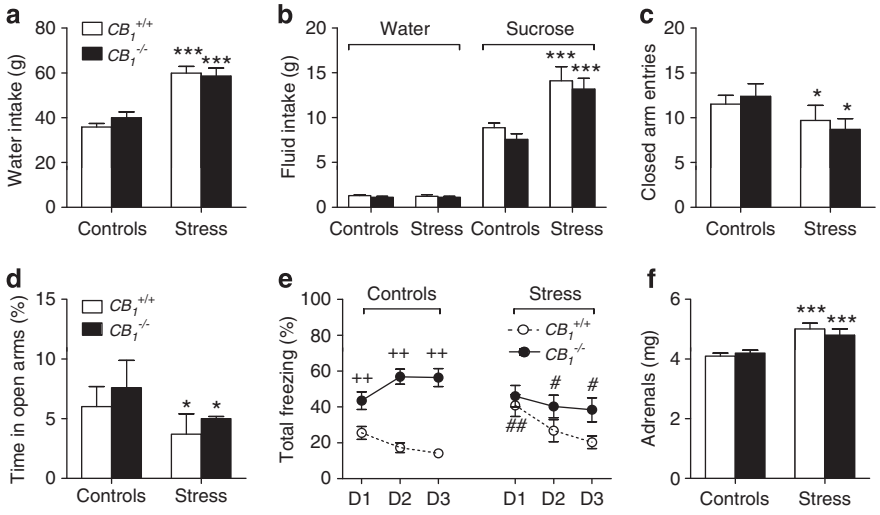

Figure 3 Influence of the constitutive mutation of cannabinoid type-1 (CB_1) receptors on the emotional profiles of control and stressed mice. This profile included 7-day water intakes (a), water and sucrose intakes in a free choice paradigm after the seventh stress session (b), closed arm entries (c) and percent time spent in the open arms (d) of an elevated plus-maze, freezing behavior during cued fear recall sessions 24–72 h after conditioning (e), and adrenal weight (f). D1–D3 stand for days 1–3. Values are the mean ± SEM of 11–16 animals, except for (b), which refer to 7–10 animals per group. $^{*}p<0.05$ and $^{***}p<0.001$ for the overall effect of social stress in the ANOVA; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ for stress influences within each genotype; $^{++}p<0.01$ for the genotype influence in unstressed animals. See text for ANOVAs and additional statistics.

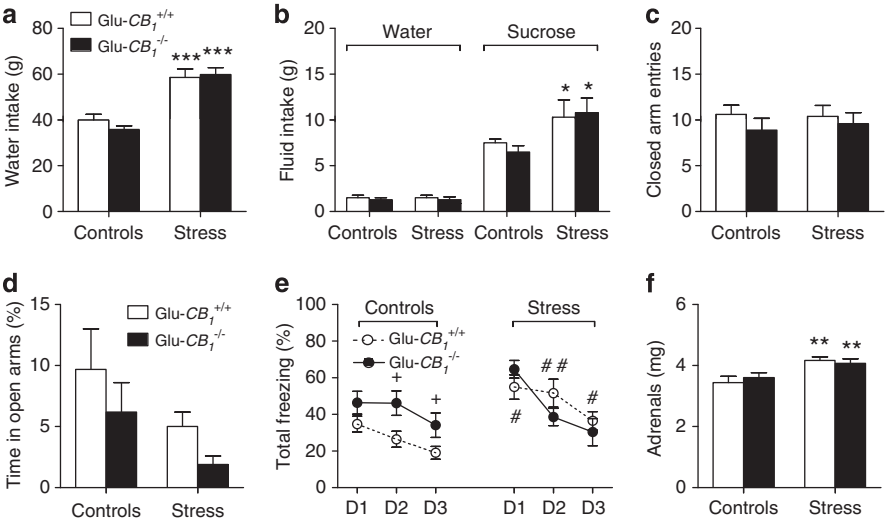

Figure 4 Influence of the conditional mutation of cannabinoid type-1 (CB_1) receptors from cortical glutamatergic neurons on the emotional profiles of control and stressed mice. This profile included 7-day water intakes (a), water and sucrose intakes in a free choice paradigm after the seventh stress session (b), closed arm entries (c) and percent time spent in the open arms (d) of an elevated plus-maze, freezing behavior during cued fear recall sessions 24–72 h after conditioning (e), and adrenal weight (f). D1–D3 stand for days 1–3. Values are the mean ± SEM of 9–14 animals, except for (b) that refers to 4–5 animals per group. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ for the overall effect of social stress in the ANOVA; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ for stress influences within each genotype; $^{+}p<0.05$ for the genotype influence in unstressed animals. See text for ANOVAs and additional statistics.

animals, respectively) than Glu-$CB_1^{+/+}$ littermates (20.12 ± 4.30 and 14.21 ± 2.73 in 12 control and 11 stressed animals, respectively; $F_{(1,44)}=4.71$; $p=0.036$). Stress, alone ($F_{(1,44)}=6.47$; $p=0.0145$) or in interaction with either the genotype ($F_{(1,44)}=4.17$; $p=0.047$), the recall session ($F_{(2,88)}=3.88$; $p=0.0241$) or both ($F_{(2,88)}=5.29$; $p=0.0067$), affected freezing behavior during recall (Figure 4e). Indeed, mutant animals displayed increased freezing behavior, compared with their wild-type controls, but that difference vanished with stress because of an increase in freezing in wild-type animals, but not in mutant animals (Figure 4e). Within-session analyses confirmed these results as stress-increased freezing throughout all recall sessions in Glu-$CB_1^{+/+}$ mice ($F_{(1,23)}=10.21$; $p=0.004$; Supplementary Figure S2a) whereas it increased freezing behavior only during the first recall session in Glu-$CB_1^{-/-}$ mice ($F_{(2,42)}=7.95$; $p=0.0012$ for the stress × recall session interaction; Supplementary Figure S2b). Initial freezing responses to the cue were affected by both social stress ($F_{(1,44)}=4.08$; $p=0.0496$) and the interaction between stress, genotype, and recall session ($F_{(2,88)}=3.56$; $p=0.0325$; Supplementary Figure S2c). Adrenal weight analyses indicated that repeated stress increased that variable in the two genotypes ($F_{(1,37)}=13.60$; $p=0.0007$ for the effect of social stress; Figure 4f).

CB_1 Receptors in GABAergic Neurons Control the Amplitude of Repeated Social Stress-Induced Decreases in Locomotor Reactivity

GABA-$CB_1^{+/+}$ and GABA-$CB_1^{-/-}$ mice did not differ with respect to the number of social attacks (20.05 ± 0.86 and 19.90 ± 0.51, respectively; $n = 13$–14 in each group). The consumption of water throughout the 7-day protocol, but not that of food (data not shown), was increased by social stress in a genotype-independent manner ($F_{(1,39)} = 25.79$; $p < 0.0001$; Figure 5a). Social stress selectively increased sweet consumption in the sucrose test ($F_{(1,23)} = 7.84$; $p = 0.0102$; Figure 5b), and did so in a genotype-independent manner. Locomotor reactivity, as measured by the number of closed arm entries in the elevated plus-maze, was reduced in stressed animals ($F_{(1,46)} = 13.47$; $p = 0.0006$), particularly in GABA-$CB_1^{-/-}$ mice ($F_{(1,46)} = 6.13$; $p = 0.017$ for the stress × genotype interaction; Figure 5c). On the other hand, social stress decreased in both genotypes the percent time spent on the open arms ($F_{(1,46)} = 8.9$; $p = 0.0046$; Figure 5d) and the percent number of open arm visits ($14.23 \pm 2.96\%$ and $10.65 \pm 3.39\%$ in 12 control wild-type and 11 mutant mice, respectively, as opposed to $7.83 \pm 2.05\%$ and $4.80 \pm 2.46\%$ in 13 stressed wild-type and 14 stressed mutant mice, respectively; $F_{(1,46)} = 4.85$; $p = 0.032$ for the influence of stress). Fear recall experiments revealed that either the deletion of CB_1 receptors from GABAergic neurons ($F_{(1,50)} = 5.75$; $p = 0.0203$) or prior exposure to social stress ($F_{(1,50)} = 5.41$; $p = 0.0241$) increased freezing responses to the presentation of the cue (Figure 5e). Within-session analyses of freezing scores within each genotype indicated that the stimulatory influence of social stress on freezing behavior was significant in GABA-$CB_1^{-/-}$ mice ($F_{(1,25)} = 4.34$; $p = 0.0476$; Supplementary Figure S2e), but not in GABA-$CB_1^{+/+}$ mice (Supplementary Figure S2d). Interestingly, the impact of social stress on freezing in GABA-$CB_1^{-/-}$ mice was dependent on the intra-session period of analysis ($F_{(2,50)} = 4.23$; $p = 0.0201$), suggesting that stress delayed within-session extinction of conditioned fear (Supplementary Figure S2d). Social stress, either alone or in association with the genotype, did not affect the initial freezing responses to the cue (Supplementary Figure S2f) but it had a major impact on total freezing behavior in GABA-$CB_1^{-/-}$ mice (Figure 5e; Supplementary Figure S2e). Stress increased adrenal weights to similar extents in both genotypes ($F_{(1,33)} = 2.39$; $p = 0.0111$), although a non-significant trend toward a more pronounced effect in GABA-$CB_1^{-/-}$ mice, compared with GABA-$CB_1^{+/+}$ mice, was apparent (Figure 5f).

CB_1 Receptors on Serotonergic Neurons Mediate the Stimulatory Effects of Repeated Social Stress on Sucrose Preference

The number of social attacks was similar in TPH2-$CB_1^{+/+}$ (21.87 ± 1.19, $n = 10$) and TPH2-$CB_1^{-/-}$ (21 ± 1.40, $n = 11$) mice, as was the dipsogenic consequence of stress ($F_{(1,33)} = 36.31$; $p < 0.0001$; Figure 6a). The increased water consumption in stressed mice was not associated with changes in food intake over the 7-day period of analysis (data not shown). In the sucrose preference test, social stress stimulated sucrose intake ($F_{(1,32)} = 14.00$; $p = 0.0007$), as compared with water intake (Figure 6b). Albeit weaker than its impact on sucrose intake, stress also increased water intake in TPH2-$CB_1^{-/-}$ mice ($F_{(1,32)} = 10.61$; $p = 0.0027$ for the stress × genotype interaction; Figure 6b). Taken with this last observation, the trend for a decreased sucrose intake in stressed TPH2-$CB_1^{-/-}$ mice, compared with stressed TPH2-$CB_1^{+/+}$ (Figure 6b), led us to analyze the respective preference ratios. Hence, the genotype influenced the net impact of stress on preference ratios ($F_{(1,32)} = 6.54$;

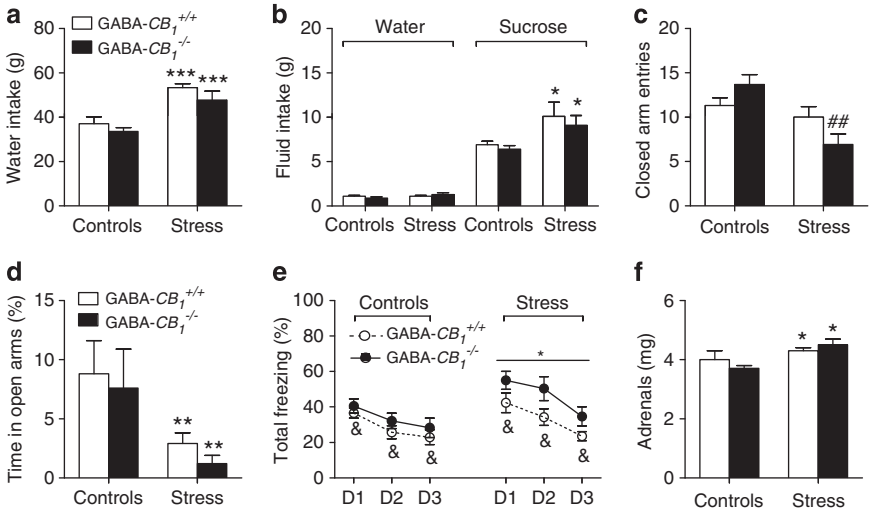

Figure 5 Influence of the conditional mutation of cannabinoid type-1 (CB_1) receptors from GABAergic neurons on the emotional profiles of control and stressed mice. This profile included 7-day water intakes (a), water and sucrose intakes in a free choice paradigm after the seventh stress session (b), closed arm entries (c) and percent time spent in the open arms (d) of an elevated plus-maze, freezing behavior during cued fear recall sessions 24–72 h after conditioning (e), and adrenal weight (f). D1–D3 stand for days 1–3. Values are the mean ± SEM of 9–14 animals, except for (b) that refers to 6–7 animals per group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for the overall effect of social stress in the ANOVA; $^{\&}p < 0.05$ for the overall influence of the genotype in the ANOVA; $^{\#\#}p < 0.01$ for stress influences within each genotype. See text for ANOVAs and additional statistics.

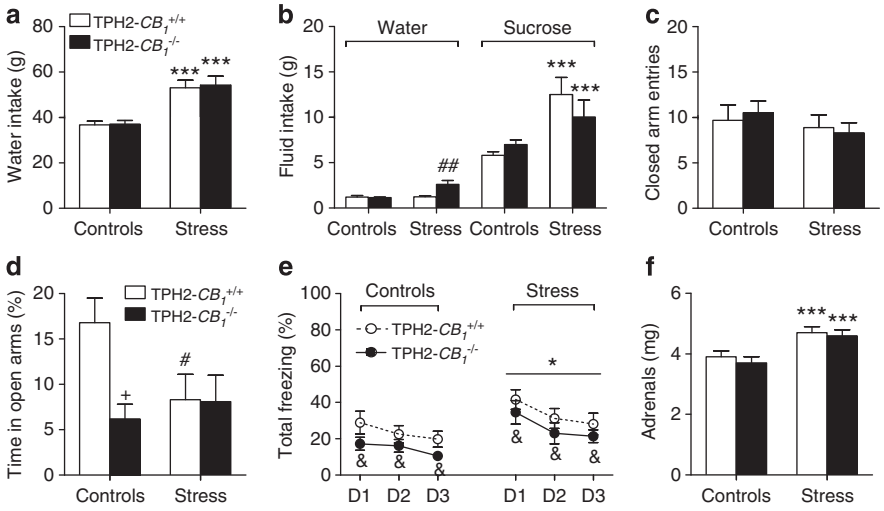

Figure 6 Influence of the conditional mutation of cannabinoid type-1 (CB_1) receptors from serotonergic neurons on the emotional profiles of control and stressed mice. This profile included 7-day water intakes (a), water and sucrose intakes in a free choice paradigm after the seventh stress session (b), closed arm entries (c) and percent time spent in the open arms (d) of an elevated plus-maze, freezing behavior during cued fear recall sessions 24–72 h after conditioning (e), and adrenal weight (f). D1–D3 stand for days 1–3. Values are the mean ± SEM of 9–11 animals, except for (b) that refers to 8–10 animals per group. $^{*}p<0.05$ and $^{***}p<0.001$ for the overall effect of social stress in the ANOVA; $^{\&}p<0.05$ for the overall influence of the genotype in the ANOVA; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ for stress influences within each genotype; $^{+}p<0.05$ for the genotype influence in unstressed or stressed animals. See text for ANOVAs and additional statistics.

$p=0.0155$ for the stress × genotype interaction), with stressed TPH2-$CB_1^{-/-}$ mice (75.97 ± 5.96%, $n=8$) differing from stressed TPH2-$CB_1^{+/+}$ mice (90.22 ± 1.47%, $n=9$; $p<0.05$) whereas controls did not differ (81.7 ± 3.13% and 85.69 ± 0.82% in nine TPH2-$CB_1^{+/+}$ mice and in ten TPH2-$CB_1^{-/-}$ mice, respectively). In the elevated plus-maze, stressed mice did not significantly differ from their control counterparts, both for their closed arm visits (Figure 6c) and for their percent visits in the open arms (17.30 ± 3.63% and 11.05 ± 2.68% in 9 control wild-type mice and 11 control mutant mice, respectively, as opposed to 13.56 ± 3.98% and 9.68 ± 2.44% in 10 stressed wild-type mice and 11 stressed mutant mice, respectively). At the opposite, the percent time spent in the open arms was lower in TPH2-$CB_1^{-/-}$ mice, as compared with TPH2-$CB_1^{+/+}$ mice ($F_{(1,37)}=4.60$; $p=0.0386$), whereas stress bore an inhibitory impact on that variable in TPH2-$CB_1^{+/+}$, but not in TPH2-$CB_1^{-/-}$, mice ($F_{(1,37)}=4.18$; $p=0.048$ for the stress × genotype interaction; Figure 6d). The deletion of CB1 receptors from central serotonergic neurons decreased ($F_{(1,37)}=4.26$; $p=0.046$), whereas social stress increased in a genotype-independent manner ($F_{(1,37)}=6.23$; $p=0.0171$), the freezing responses to the presentation of the cue during recall (Figure 6e). The analyses of within-session freezing behaviors in each genotype further indicated that social stress increased the amplitude of freezing in TPH2-$CB_1^{-/-}$ mice ($F_{(1,20)}=4.42$; $p=0.0484$; Supplementary Figure S2h) whereas its impact in TPH2-$CB_1^{+/+}$ mice did not reach significance because of the heterogeneity of the data (Supplementary Figure S2g). Stress did not affect the initial freezing responses to the cue, although a trend toward a stimulatory impact could be noted in the last recall session (Supplementary Figure S2i). Lastly, a stimulatory effect of social stress on adrenal weight was observed in both genotypes ($F_{(1,18)}=17.77$; $p=0.0005$; Figure 6f).

DISCUSSION

The goal of this study was to examine whether the ECS has a role in the processing of repeated social defeat and in the expression of emotional responses to such a stressor. In addition, this study addressed the question of the neuronal populations through which the ECS modulates these emotional responses to repeated social defeat. We first analyzed the influence of CB_1 receptor blockade before each stress session, and compared it with the impact of the constitutive mutation of CB_1 receptors. Thereafter, we addressed the possibility that distinct CB_1 receptor-expressing neuronal populations exert discrete or even opposite influences in stressed animals that could be masked in constitutive CB_1 receptor mutants. To this aim, we analyzed the respective influences of conditioned deletions of CB_1 receptors from cortical glutamatergic neurons, from GABAergic neurons, or from serotonergic neurons on the emotional profile of repeatedly stressed individuals. The results show that (i) stress-induced endocannabinoid release modulates the expression of cued fear memory, (ii) CB_1 receptors located on serotonergic neurons control unconditioned anxiety and cued fear expression, and mediate the increased sucrose preference observed in repeatedly stressed animals, (iii) CB_1 receptors on GABAergic neurons are involved in the locomotor reactivity profile of stressed animals, and (iv) social stress abolishes the deficits in fear extinction of constitutive CB_1 receptor mutants, an action that might be linked to the absence of CB_1 receptors from cortical glutamatergic neurons.

Treatments with indirect/direct CB_1 receptor agonists in stressed animals have helped to tackle the interactions between the ECS and stress circuitry. As an illustration, indirect/direct CB_1 receptor stimulation may reduce repeated stress-elicited body weight reductions, anhedonia

for sucrose, unconditioned anxiety, and proinflammatory consequences (Bortolato *et al*, 2007; Zoppi *et al*, 2011; but see Hill and Gorzalka, 2004). Although these studies provide new routes of therapeutic interventions in the management of stress-related disorders, they do not address the crucial question of the tonic role, if any, exerted by the ECS in the emotional consequences of repeated stress. Neurochemical, biochemical, and electrophysiological tools have indicated that the actions of the ECS are stress sensitive (Patel and Hillard, 2008; Hill *et al*, 2010b; Riebe and Wotjak, 2011). Whether, in turn, the ECS is involved in the emotional impacts of repeated stress is, however, a matter that has been only sparsely addressed. This issue led us to use the social defeat model of stress and to examine the respective amplitudes of a broad array of responses to repeated stress in mice fully or partly devoid of CB_1 receptor activity. However, one prerequisite for the use of social stress in the present study was the need to gather evidence that the ECS is sensitive to that stressor. Indirect support for such an alteration initially stemmed from the report that repeated social defeat impaired the ability of a CB_1 receptor agonist to decrease GABA release in the striatum (Rossi *et al*, 2008). The present analysis of central AEA and 2-AG levels in control and stressed animals provides a direct proof that the ECS is sensitive to a social defeat paradigm. Thus, AEA and 2-AG levels were modified in a brain region-dependent manner in acutely and repeatedly defeated mice. Acute social defeat decreased hippocampal AEA concentrations, but it did not impact on AEA levels in other brain regions or on 2-AG. Exposure to the last of the seven social defeats decreased also hypothalamic AEA levels whereas it increased 2-AG levels in the hypothalamus, hippocampus, and prefrontal cortex. These brain region- and endocannabinoid-dependent changes display similarity with those, respectively, documented in mice submitted to acute and repeated restraint stress sessions (Patel *et al*, 2005; Rademacher *et al*, 2008). This observation suggests that the mechanisms leading to these stress-elicited changes in AEA and 2-AG levels may be common to social defeat and restraint. Indeed, there is an experimental support to suggest that hyperactivity of the HPA axis could be such a mechanism (Hill *et al*, 2010b; Riebe and Wotjak, 2011). The initial observation that repeated, but not acute, exposure to a homotypic stressor increases 2-AG levels in mouse corticolimbic areas, including the basolateral amygdala (BLA), has raised the hypothesis that these increases may contribute to stress habituation (Patel and Hillard, 2008; Patel *et al*, 2009). Consistently, recent data in repeatedly restrained rats suggested that stress-induced decreases in corticolimbic AEA levels and increases in amygdaloid 2-AG levels are valuable markers of the habituation of the corticotropic axis to restraint (Hill *et al*, 2010a). These series of results open the question of the relationship between the aforementioned changes in endocannabinoid levels in our repeatedly socially defeated mice and habituation to repeated social stress. The answer to that question is not simple; thus, compared with acutely stressed animals, animals exposed to repeated social stress may show habituation, lack of habituation, or even sensitization according to the variable examined. As an illustration, the amplitude of the hyperthermia and the intensity of ultrasonic vocalization during the sensorial phase (see Figure 1a) of the social stress procedure have been shown to be increased in repeatedly stressed animals, compared with acutely stressed animals (Tornatzky and Miczek, 1994; Bhatnagar *et al*, 2006). At the opposite, the amplitudes of both the tachycardia and the rise in circulating corticosterone levels associated to the social stress paradigm were found to desensitize progressively with the number of social stress episodes (Tornatzky and Miczek, 1994; Bhatnagar *et al*, 2006). In our hands, repeatedly defeated mice expressed higher (initial and total) freezing responses to cue presentation 1 day after conditioning, compared with acutely defeated mice and control mice (Supplementary Figure S3). This observation suggests that conditioned freezing behavior sensitized with the number of stress sessions. On the other hand, we have already reported that the dipsogenic response to social stress was not different between the first and the fifth stress session whereas the increased social stress-elicited sucrose preference was observed in repeatedly, but not in acutely, stressed animals (Dubreucq *et al*, 2012). These observations indicate that the relevance of repeated social stress-induced changes in CNS endocannabinoid levels to stress adaptation is at the present time difficult to determine. Actually, the study of the emotional, metabolic, and endocrine consequences of alterations in AEA and 2-AG synthesis/degradation processes in repeatedly defeated animals could help to solve this issue.

Repeated social defeats increased water consumption in all experiments. This dipsogenic impact of repeated stress, which has been documented in the past using mice exposed either to repeated social defeats (Krishnan *et al*, 2007; Dubreucq *et al*, 2012) or to mixed stressors (Strekalova *et al*, 2006), was not accounted for by changes in food intake. The mechanisms underlying repeated stress-induced dipsogeny as well as its significance in the context of adaptation to stress are unknown. The aforementioned observation that the daily increase in water intake in stressed animals is already maximal after the first social defeat episode (Dubreucq *et al*, 2012) indicates that repeated stress-induced dipsogeny is a phenotypic response that is held constant after each stress episode. It has been proposed that dipsogeny may belong to a behavioral repertoire that includes anhedonia for sucrose (Strekalova *et al*, 2006). The present study, wherein social stress in vehicle-injected animals and in wild-type animals triggered both dipsogeny and increased preference for sucrose, does not lend support to this hypothesis. Another finding provided by the present study is that stress-induced dipsogeny may be independent from the ECS as none of the paradigms used to alter CB_1 receptor function, including that aimed to block these receptors during stress only, proved effective on that variable.

It is now 30 years that the sucrose consumption/preference test is used to monitor the consequences of stress on hedonic processes, the dysregulation of which is a core symptom in human depression (Katz, 1981). When effective on sucrose consumption/preference, repeated stress is found to reduce sweet consumption in most, but not all, studies (Willner, 2005). The observation that repeated stress, such as chronic mild stress, may increase, rather than decrease, sweet consumption in a minority of studies has been considered a 'genuine phenomenon' with specific

neurobiological grounds (eg, hyperactivity of the mesolimbic dopaminergic system: Willner, 2005). As for chronic mild stress, social stress has been mainly shown to decrease sucrose preference (Krishnan *et al*, 2007; Becker *et al*, 2008; Covington *et al*, 2009; Miczek *et al*, 2011). However, other reports have concluded that social stress stimulates sucrose preference (Dubreucq *et al*, 2012) or bears no influence on that variable (Croft *et al*, 2005; Hollis *et al*, 2010). That our socially defeated mice increased, rather than decreased, their preference for sucrose, compared with their unstressed counterparts, is noteworthy. Whether the rewarding properties and/or the caloric value of sucrose drive this increase is presently unknown. The use of saccharin, which lacks caloric value, could help to tackle this issue. As indicated above, our observation of an increased preference for sucrose in repeatedly stressed mice has been already reported in animals exposed to chronic mild stress (Willner, 2005) or to other stressors (Dess, 1992; Pecoraro *et al*, 2004; Leigh Gibson, 2006). Several hypotheses can be proposed to understand this peculiar behavior in our stressed mice. The so-called 'comfort food hypothesis' (Dallman *et al*, 2003) postulates that the increased consumption of carbohydrates, due to their rewarding and caloric properties, may help to reduce hyperactivity of the HPA axis and of the sympathetic nervous system in stressed individuals. Stress-induced corticosterone release (in conjunction with insulin release) likely subserves increased carbohydrate consumption in stressed animals (Pecoraro *et al*, 2005), which is consistent with human observations indicating that, when submitted to psychological stress, women with high levels of cortisol (the human equivalent of rodent corticosterone) show increased sweet consumption, as opposed to individuals with low levels of cortisol (Newman *et al*, 2007). The second hypothesis is linked to the observation that mice submitted to a mixture of stressors or to social defeats for 4–5 weeks show respectively no change (Rygula *et al*, 2005) or increased (Strekalova *et al*, 2006) preference for sucrose (compared with their controls) when tested during the first or the second week of stress, but decreased preference for sucrose when tested thereafter. As already reported above, our social stress model triggers a progressive increase in sucrose preference, ie, sucrose preference is not altered after a single social defeat (Dubreucq *et al*, 2012). It is therefore possible that our mice might display decreased preference for sucrose if exposed to a higher number of social stress sessions. Lastly, because genetic factors have a key role in the physiological and emotional responses of the individual when confronted to social stress (Berton *et al*, 1997; Berton *et al*, 1998), we cannot exclude the hypothesis that the genetic background of our stressed mice had an impact on their sucrose preference.

The key role exerted by the ECS in the regulation of reward circuitry (Maldonado *et al*, 2006) led us to dissect the role of the ECS on stress-elicited sucrose overconsumption. Mice pretreated with the CB_1 receptor antagonist rimonabant before each stress session did not behave differently from vehicle-pretreated stressed mice. This result indicates that stress-elicited sucrose overconsumption was not accounted for by CB_1 receptor stimulation during stress. Such a conclusion differs from that gathered in a previous study, where rimonabant pretreatment before each stress session amplified the inhibitory impact of stress on sucrose preference (Rademacher and Hillard, 2007). This discrepancy may be accounted for by numerous methodological differences, compared with the present study, including the stress model (restraint *vs* social defeat), the impact of that stressor on sucrose preference (inhibition *vs* stimulation), the duration of each sucrose preference test (1 h *vs* 12 h), the concentration of sucrose (10% *vs* 2%), and the metabolic state of the animals (20-h food- and water-deprivation before each test *vs* non-deprivation). Except for mice lacking CB_1 receptors on serotonergic neurons, none of the genetic manipulations of the ECS affected stress-induced overconsumption of sucrose. The observation that the behavior of TPH2-$CB_1^{-/-}$ mice did not extend to $CB_1^{-/-}$ mice may be considered paradoxical at first glance. Because social stress depends on the behavior of the resident mice, the possibility that TPH2-$CB_1^{-/-}$ mice underwent less stress than TPH2-$CB_1^{+/+}$ mice on the one hand, and $CB_1^{-/-}$ mice on the other hand, might be considered. However, the observation that our stressed mice all received an equivalent number of daily attacks and displayed upright postures and squealing, which are overt signs of subordination (Miczek *et al*, 2001), renders this hypothesis unlikely. Actually, that conditional CB_1 receptor mutants display phenotypes differing from those measured in $CB_1^{-/-}$ mice is not incongruent. Fasting-induced food intake as well as the consumption of palatable food are diminished in $CB_1^{-/-}$ mice, but amplified in GABA-$CB_1^{-/-}$ mice (Bellocchio *et al*, 2010), although the majority of CNS CB_1 receptors are located on GABAergic neurons (Marsicano and Lutz, 1999; Monory *et al*, 2006; Bellocchio *et al*, 2010). This observation suggests that cell type-specific functions of CB1 receptors might be masked by the constitutive deletion of the receptor gene. Taken with these findings, the present study underlines the need to dissect the ECS at the level of the neuronal phenotype to understand its role in brain functions. Our observation that the stress-induced increase in sucrose preference was absent in TPH2-$CB_1^{-/-}$ mice, as opposed to TPH2-$CB_1^{+/+}$ mice, indicates that the population of CB_1 receptors located on dorsal raphe serotonergic neurons (Häring *et al*, 2007), albeit discrete, is essential for the expression of that phenotypic response to stress. Interestingly, control (ie unstressed) TPH2-$CB_1^{-/-}$ mice did not display any alteration in sucrose preference, compared with their wild-type controls, a finding that was recently replicated using lower (1%) and higher (up to 9%) concentrations of sucrose (data not shown). These results indicate that CB1 receptors on serotonergic neurons are involved in the regulation of sucrose intake under stressful conditions associated with increased sweet consumption, but not under control conditions. The inhibitory role of CB_1 receptors on GABA and glutamate neurotransmission (Alger, 2002; Piomelli, 2003; Chevaleyre *et al*, 2006; Ohno-Shosaku *et al*, 2012) may indeed extend to serotonergic transmission (Nakazi *et al*, 2000; but see Gobbi *et al*, 2005). It is thus expected that under conditions of ECS hyperactivity, the selective lack of CB_1 receptors from serotonergic neurons might result in an increased serotonin (5-HT) release, especially in animals exposed to stress, a situation favoring 5-HT neurotransmission (Chaouloff, 1993; Chaouloff, 2000). To the best of our knowledge, neither direct evidence for such an increased release of 5-HT nor its consequences on 5-HT neurotransmission

have been documented. The sole information available is based on studies performed on $CB_1^{-/-}$ mice, and which reported increased 5-HT release and alterations in pre- and post-synaptic receptor expression and/or function (Mato *et al*, 2007; Aso *et al*, 2009). Dorsal raphe neurons project along three ascending pathways to numerous brain locations (including the nucleus accumbens, the prefrontal cortex, the amygdala, the ventral/dorsal hippocampus, and the lateral septum) involved in the regulation of hedonic processes, but also anxiety- and fear-related behaviors (Michelsen *et al*, 2007). The present observation that CB_1 receptors on dorsal raphe serotonergic neurons control sucrose preference in stressed animals, but also open arm behaviors in the elevated plus-maze and cued fear expression (see below), indicates the need for future experiments to examine the functional role of CB_1 receptors on 5-HT release in these brain regions.

Comparative analyses of open arm behaviors in the elevated plus-maze (ie, anxiety-related indices; Ramos and Mormède, 1998; Crawley, 2008) between $CB_1^{+/+}$ mice and $CB_1^{-/-}$ mice have led to contradictory results (Viveros *et al*, 2005; Wotjak, 2005; Lafenêtre *et al*, 2007). The present study reveals that under our experimental settings (see below), neither repeated CB_1 receptor blockade nor CB_1 receptor mutation in cortical glutamatergic neurons or in GABAergic neurons altered open arm behaviors. Indeed, only trends, such as those observed in Glu-$CB_1^{-/-}$ mice, could be noted. The lack of effect of these mutations could be accounted for by the low aversiveness of the anxiety test used here (ie, an experimental condition chosen on purpose to be able to further observe stress-induced increases in anxiety). Indeed, naive (ie, unhandled) $CB_1^{-/-}$, Glu-$CB_1^{-/-}$ mice, and GABA-$CB_1^{-/-}$ mice all display anxiety when tested in a more aversive context such as a light/dark box (Moustié *et al*, unpublished data). Taken together, these observations support the hypothesis that CB_1 receptors exert a tonic control on anxiety responses when measured under highly aversive conditions (Haller *et al*, 2004). However, the present study indicates also that such a statement may not apply to all CB_1 receptor populations. Thus, under our experimental conditions, we observed that TPH2-$CB_1^{-/-}$ mice spent less time in the open arms and tended to visit less frequently the open arms, but not the closed arms (an index of locomotor reactivity: Ramos and Mormède, 1998), compared with TPH2-$CB_1^{+/+}$ mice.

Repeated social defeat has been documented for its anxiogenic impact (Merlo-Pich *et al*, 1993; Berton *et al*, 1998; Krishnan *et al*, 2007). The present study confirms this statement and shows that neither CB_1 receptor blockade during stress nor CB_1 receptor mutation in the whole body, in cortical glutamatergic neurons, or in GABAergic neurons influenced significantly open arm behaviors in stressed animals. On the other hand, it is unknown whether repeated stress proved inefficient on the percent time spent in the open arms by TPH2-$CB_1^{-/-}$ mice because (i) a floor effect was reached because of the mutation and/or (ii) CB_1 receptors on serotonergic neurons mediate the anxiogenic impact of repeated stress. Future studies will be required to tackle this particular issue. Besides its anxiogenic impact, a 1-week exposure to repeated stress may also alter the initial (ie, first 5 min) locomotor response to the placement into novel environments (but see Strekalova *et al*, 2005). As an illustration, 7 daily restraint sessions increased locomotor reactivity in an open field (Ito *et al*, 2010) whereas 7–10 daily social defeat sessions decreased locomotion in novel activity cages or in an elevated plus-maze (Berton *et al*, 1998; Rygula *et al*, 2005; Krishnan *et al*, 2007). Here, repeated stress decreased locomotor reactivity, as assessed in the elevated plus-maze, and did so only in several mouse groups (eg, vehicle- and rimonabant-injected mice, $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, GABA-$CB_1^{+/+}$ and GABA-$CB_1^{-/-}$ mice). Of particular interest was the finding that the deletion of CB_1 receptors from GABAergic neurons amplified the hypolocomotor effect of stress. This suggests that endocannabinoid release, and then stimulation of these receptors, might help buffering the inhibitory impact of repeated stress on locomotor reactivity.

There is now extensive evidence that CB_1 receptors exert a tonic control on conditioned freezing responses to fearful stimuli (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005; Kamprath *et al*, 2006). Thus, acute CB_1 receptor blockade immediately before the first recall session (Marsicano *et al*, 2002; Suzuki *et al*, 2004; Chhatwal *et al*, 2005) or the genetic deletion of CB_1 receptors (Marsicano *et al*, 2002; Kamprath *et al*, 2006; Dubreucq *et al*, 2010) delays conditioned freezing extinction, possibly as a consequence of a dysregulation of habituation processes (Kamprath *et al*, 2006). The use of conditional CB_1 receptor mutants has further suggested that it is the absence of CB_1 receptors from cortical glutamatergic neurons that may be responsible for the phenotype observed in constitutive CB_1 receptor mutants (Kamprath *et al*, 2009). In the present study, between-session analyses of freezing behaviors and the comparison between the initial freezing responses to the cue in the control (unstressed) groups revealed that $CB_1^{-/-}$ mice displayed increased fear expression and delayed extinction, compared with $CB_1^{+/+}$ mice. On the other hand, within-session analyses of freezing in the two genotypes did not reveal differences in extinction rates, including if analyzed on a daily basis (data not shown). These data, which confirm that between- and within-session extinction processes are independent (Plendl and Wotjak, 2010), differ from previous findings (Marsicano *et al*, 2002; Kamprath *et al*, 2006; Plendl and Wotjak, 2010). It is likely that experimental differences between protocols, including recall environments, sound and shock intensities, and day time, underlie our failure to observe within-session extinction of freezing in $CB_1^{-/-}$ mice. A deregulation of conditioned fear responses has been observed in acutely and/or repeatedly stressed animals. Indeed, repeatedly stressed rats and mice that were cued fear-conditioned 1 or 7 days after the last stress session displayed an increased fear expression and/or impaired recall of extinction memory, compared with unstressed animals (Izquierdo *et al*, 2006; Miracle *et al*, 2006; Garcia *et al*, 2008). Interestingly, these stress-elicited changes in fear memory were more obvious when unstressed animals had reached extinction and were not accounted for by changes in acquisition during the conditioning sessions (Izquierdo *et al*, 2006; Miracle *et al*, 2006; Garcia *et al*, 2008; but see Rau and Fanselow, 2009). In the present study, between-session analyses of freezing in C57BL/6N mice (including vehicle-injected animals) and in wild-type animals (especially in $CB_1^{+/+}$, Glu-$CB_1^{+/+}$, and TPH2-$CB_1^{+/+}$ mice) confirmed that

repeated stress increases fear expression during recall (without changing the immediate freezing response to tone-shock pairing; data not shown). On the other hand, between- and within-session analyses of the effects of social stress in all mice revealed that stress did not affect extinction processes (as indicated by the inability of social stress to alter significantly the daily extinction slopes). Pretreatment with rimonabant before each stress session amplified freezing behavior throughout the three recall sessions, compared with vehicle-pretreated-stressed mice. This result suggests that the release of endocannabinoids that occurs during repeated social stress is involved in the extinction of a fear conditioned by a stimulus different from that used to stress the animals. Interestingly, the absence of CB_1 receptors from GABAergic neurons led to a pattern of freezing behavior that resembled that observed in rimonabant-pretreated mice, ie, an amplification of freezing during recall. These results suggest that rimonabant acted mainly through the blockade of that CB_1 receptor population. Surprisingly, stressed $CB_1^{-/-}$ mice displayed decreased freezing responses to the auditory cue during the last two recall sessions, compared with stressed $CB_1^{+/+}$ mice, and a similar phenotype was observed in stressed Glu-$CB_1^{-/-}$ mice, compared with stressed Glu-$CB_1^{+/+}$ mice. Thus, in both mouse lines, prior repeated stress reduced the difference in freezing behavior between wild-type and mutant littermates. Despite some differences between $CB_1^{-/-}$ mice and Glu-$CB_1^{-/-}$ mice, this observation suggests that the complex recall session-dependent freezing behavior observed in stressed $CB_1^{-/-}$ mice lies on changes in the release of glutamate from cortical neurons. The brain regions involved in the fear memory profiles of stressed $CB_1^{-/-}$ mice and Glu-$CB_1^{-/-}$ mice are unknown at the present time. The BLA might have a key role because CB_1 receptors located therein have been recently shown to exert an inhibitory control over fear expression/extinction during late recall (48–72 h after conditioning, corresponding to our last two recall sessions), but not during early recall (ie, 24 h after conditioning, corresponding to our first recall session) (Kamprath *et al*, 2011).

In summary, this study reveals that (i) CB_1 receptors located on serotonergic neurons exert a control on the increased preference for sucrose triggered by repeated stress, (ii) stress-elicited increases in freezing responses to a cue during fear recall sessions are amplified by prior blockade of CB_1 receptors before each stress session, an effect that could be mediated by CB_1 receptors on GABAergic neurons, (iii) this last population exerts a control on the amplitude of the hypolocomotor reactivity that results from repeated stress, and (iv) repeated stress reverse in a time-dependent manner the increased conditioned freezing behavior observed in animals lacking CB1 receptors from cortical glutamatergic neurons. On the basis of the use of pharmacologically, genetically, and an ethologically relevant model of stress, this study opens new routes of investigation on the role of distinct CB_1 receptor populations in the emotional consequences of repeated stress.

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DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

Akirav I, Maroun M (2007). The role of the medial prefrontal cortex-amygdala circuit in stress effects on the extinction of fear. *Neural Plast* **2007**: 30873.

Alger E (2002). Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. *Prog Neurobiol* **68**: 247–286.

Aso E, Renoir T, Mengod G, Ledent C, Hamon M, Maldonado R *et al* (2009). Lack of CB_1 receptor activity impairs serotonergic negative feedback. *J Neurochem* **109**: 935–944.

Becker C, Zeau B, Rivat C, Blugeot A, Hamon M, Benoliel JJ (2008). Repeated social defeat-induced depression-like behavioral and biological alterations in rats: involvement of cholecystokinin. *Mol Psychiatry* **13**: 1079–1092.

Bellocchio L, Lafenêtre P, Cannich A, Cota D, Puente N, Grandes P *et al* (2010). Bimodal control of stimulated food intake by the endocannabinoid system. *Nature Neurosci* **13**: 281–283.

Berton O, Aguerre S, Sarrieau A, Mormède P, Chaouloff F (1998). Differential effects of social stress on central serotonergic activity and emotional reactivity in Lewis and spontaneously hypertensive rats. *Neuroscience* **82**: 147–159.

Berton O, Ramos A, Chaouloff F, Mormède P (1997). Behavioral reactivity to social and nonsocial stimulations: a multivariate analysis of six inbred rat strains. *Behav Genet* **27**: 155–166.

Bhatnagar S, Vining C, Iyer V, Kinni V (2006). Changes in hypothalamic-pituitary-adrenal function, body temperature, body weight and food intake with repeated social stress exposure in rats. *J Neuroendocrinol* **18**: 13–34.

Bortolato M, Mangieri RA, Fu J, Kim JH, Arguello O, Duranti A *et al* (2007). Antidepressant-like activity of the fatty acid amide hydrolase inhibitor URB597 in a rat model of chronic mild stress. *Biol Psychiatry* **62**: 1103–1110.

Buwalda B, Kole MHP, Veenema AH, Huininga M, de Boer SF, Korte SM *et al* (2005). Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning. *Neurosci Biobehav Rev* **29**: 83–97.

Chaouloff F (1993). Physiopharmacological interactions between stress hormones and central serotonergic systems. *Brain Res Rev* **18**: 1–32.

Chaouloff F (2000). Serotonin, stress and corticoids. *J Psychopharmacol* **14**: 139–151.

Chevaleyre V, Takahashi KA, Castillo PE (2006). Endocannabinoid-mediated synaptic plasticity in the CNS. *Annu Rev Neurosci* **29**: 37–76.

Chhatwal JP, Davis M, Maguschak KA, Ressler KJ (2005). Enhancing cannabinoid neurotransmission augments the extinction of conditioned fear. *Neuropsychopharmacology* **30**: 516–524.

Covington HE, Maze I, LaPlant QC, Vialou VF, Ohnishi YN, Berton O *et al* (2009). Antidepressant actions of histone deacetylase inhibitors. *J Neurosci* **29**: 11451–11460.

Crawley JN (2008). Behavioral phenotyping strategies for mutant mice. *Neuron* **57**: 809–818.

Croft AP, Brooks SP, Cole J, Little HJ (2005). Social defeat increases alcohol preference of C57BL/10 strain mice; effect prevented by a CCKB antagonist. *Psychopharmacology* **183**: 163–170.

Dallman MF, Pecoraro N, Akana SF, La Fleur SE, Gomez F, Houshyar H *et al* (2003). Chronic stress and obesity: a new view of 'comfort food'. *Proc Natl Acad Sci USA* **100**: 11696–11701.

Dess NK (1992). Divergent responses to saccharin vs. sucrose availability after stress in rats. *Physiol Behav* **52**: 115–125.

Dubreucq S, Kambire S, Conforzi M, Metna-Laurent M, Cannich A, Soria-Gomez E *et al* (2012). Cannabinoid type 1 receptors located on single-minded 1-expressing neurons control emotional behaviors. *Neuroscience* **204**: 230–244.

Dubreucq S, Koehl M, Abrous DN, Marsicano G, Chaouloff F (2010). CB1 receptor deficiency decreases wheel-running activity: consequences on emotional behaviours and hippocampal neurogenesis. *Exp Neurol* **224**: 106–113.

Garcia R, Spennato G, Nilsson-Todd L, Moreau JL, Deschaux O (2008). Hippocampal low-frequency stimulation and chronic mild stress similarly disrupt fear extinction memory in rats. *Neurobiol Learn Mem* **89**: 560–566.

Glass M, Dragunow M, Faull R (1997). Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal neonatal and adult human brain. *Neuroscience* **77**: 299–331.

Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M *et al* (2005). Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proc Natl Acad Sci USA* **102**: 18620–18625.

Golden SA, Covington HE, Berton O, Russo SJ (2011). A standardized protocol for repeated social defeat stress in mice. *Nat Protoc* **6**: 1183–1191.

Haller J, Varga B, Ledent C, Barna I, Freund TF (2004). Context-dependent effects of CB1 cannabinoid gene disruption on anxiety-like and social behaviour in mice. *Eur J Neurosci* **19**: 1906–1912.

Häring M, Marsicano G, Lutz B, Monory K (2007). Identification of the cannabinoid receptor type 1 in serotonergic cells of raphe nuclei in mice. *Neuroscience* **146**: 1212–1219.

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR *et al* (1990). Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA* **87**: 1932–1936.

Hill MN, Gorzalka BB (2004). Enhancement of anxiety-like responsiveness to the cannabinoid CB_1 receptor agonist HU-210 following chronic stress. *Eur J Pharmacol* **499**: 291–295.

Hill MN, Hillard CJ, McEwen BS (2011). Alterations in corticolimbic dendritic morphology and emotional behavior in cannabinoid CB_1 receptor-deficient mice parallel the effects of chronic stress. *Cereb Cortex* **21**: 2056–2064.

Hill MN, McLaughlin RJ, Bingham B, Shrestha L, Lee TT, Gray JM *et al* (2010a). Endogenous cannabinoid signaling is essential for stress adaptation. *Proc Natl Acad Sci USA* **107**: 9406–9411.

Hill MN, Patel S, Campolongo P, Tasker JG, Wotjak CT, Bains JS (2010b). Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. *J Neurosci* **30**: 14980–14986.

Hill MN, Patel S, Carrier EJ, Rademacher DJ, Ormerod BK, Hillard CJ *et al* (2005). Downregulation of endocannabinoid signaling in the hippocampus following chronic unpredictable stress. *Neuropsychopharmacology* **30**: 508–515.

Hollis F, Wang H, Dietz D, Gunjan A, Kabbaj M (2010). The effects of repeated social defeat on long-term depressive-like behavior and short-term histone modifications in the hippocampus in male Sprague-Dawley rats. *Psychopharmacology* **211**: 69–77.

Huhman KL (2006). Social conflict models: can they inform us about human psychopathology? *Horm Behav* **50**: 640–646.

Imai T, Chambon P, Metzger D (2000). Inducible site-specific somatic mutagenesis in mouse hepatocytes. *Genesis* **26**: 147–148.

Ito H, Nagano M, Suzuki H, Murakoshi T (2010). Chronic stress enhances synaptic plasticity due to disinhibition in the anterior cingulate cortex and induces hyper-locomotion in mice. *Neuropharmacology* **58**: 746–757.

Izquierdo A, Wellman CL, Holmes A (2006). Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice. *J Neurosci* **26**: 5733–5738.

Jacob W, Yassouridis A, Marsicano G, Monory K, Lutz B, Wotjak CT (2009). Endocannabinoids render exploratory behaviour largely independent of the test aversiveness: role of glutamatergic transmission. *Genes Brain Behav* **8**: 685–698.

Kamprath K, Marsicano G, Tang J, Monory K, Bisogno T, Di Marzo V *et al* (2006). Cannabinoid CB1 receptor mediates fear extinction via habituation-like processes. *J Neurosci* **26**: 6677–6686.

Kamprath K, Plendl W, Marsicano G, Deussing JM, Wurst W, Lutz B *et al* (2009). Endocannabinoids mediate acute fear adaptation via glutamatergic neurons independently of corticotropin-releasing hormone signaling. *Genes Brain Behav* **8**: 203–211.

Kamprath K, Romo-Parra H, Häring M, Gaburro S, Doengi M, Lutz B *et al* (2011). Short-term adaptation of conditioned fear responses through endocannabinoid signaling in the central amygdala. *Neuropsychopharmacology* **36**: 652–663.

Katona I, Sperlagh B, Ski A, Käfalvi A, Vizi ES, Mackie K *et al* (1999). Presynaptically located CB_1 receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J Neurosci* **19**: 4544–4558.

Katz RJ (1981). Animal models and human depressive disorders. *Neurosci Biobehav Rev* **5**: 231–246.

Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ *et al* (2007). Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* **131**: 391–404.

Lafenêtre P, Chaouloff F, Marsicano G (2007). The endocannabinoid system in the processing of anxiety and fear and how CB1 receptors may modulate fear extinction. *Pharmacol Res* **56**: 367–381.

Lafenêtre P, Chaouloff F, Marsicano G (2009). Bidirectional regulation of novelty-induced behavioral inhibition by the endocannabinoid system. *Neuropharmacology* **57**: 715–721.

Lafourcade M, Larrieu T, Mato S, Duffaud A, Sepers M, Matias I *et al* (2011). Nutritional omega-3 deficiency abolishes endocannabinoid-mediated neuronal functions. *Nature Neurosci* **14**: 345–350.

Leigh Gibson E (2006). Emotional influences on food choice: sensory, physiological and psychological pathways. *Physiol Behav* **89**: 53–61.

Lourenço J, Matias I, Marsicano G, Mulle C (2011). Pharmacological activation of kainate receptors drives endocannabinoid mobilization. *J Neurosci* **31**: 3243–3248.

Lutz B (2009). Endocannabinoid signals in the control of emotion. *Curr Opin Pharmacol* **9**: 46–52.

Maldonado R, Valverde O, Berrendero F (2006). Involvement of the endocannabinoid system in drug addiction. *Trends Neurosci* **29**: 225–232.

Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A *et al* (2003). CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* **302**: 84–88.

Marsicano G, Lutz B (1999). Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur J Neurosci* **11**: 4213–4225.

Marsicano G, Wotjak CT, Azad S, Bisogno T, Rammes G, Cascio MG *et al* (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature* **418**: 530–534.

Massa F, Mancini G, Schmidt H, Steindel F, Mackie K, Angioni C *et al* (2010). Alterations in the hippocampal endocannabinoid system in diet-induced obese mice. *J Neurosci* **30**: 6273–6281.

Mato S, Aso E, Castro E, Martín M, Valverde O, Maldonado R *et al* (2007). CB_1 knockout mice display impaired functionality of 5-HT_{1A} and 5-$HT_{2A/C}$ receptors. *J Neurochem* **103**: 2111–2120.

Merlo-Pich E, Heinrichs SC, Rivier C, Miczek KA, Fisher DA, Koob GF (1993). Blockade of pituitary-adrenal axis activation induced by peripheral immunoneutralization of corticotropin-releasing factor does not affect the behavioral response to social defeat stress in rats. *Psychoneuroendocrinology* **18**: 495–507.

Michelsen KA, Schmitz C, Steinbusch HW (2007). The dorsal raphe nucleus – from silver stainings to a role in depression. *Brain Res Rev* **55**: 329–342.

Miczek KA, Maxson SC, Fish EW, Faccidomo S (2001). Aggressive behavioral phenotypes in mice. *Behav Brain Res* **125**: 167–181.

Miczek KA, Nikulina EM, Shimamoto A, Covington HE (2011). Escalated or suppressed cocaine reward, tegmental BDNF, and accumbal dopamine caused by episodic versus continuous social stress in rats. *J Neurosci* **31**: 9848–9857.

Miczek KA, Yap JJ, Covington HE (2008). Social stress, therapeutics and drug abuse: preclinical models of escalated and depressed intake. *Pharmacol Ther* **120**: 102–128.

Miracle AD, Brace MF, Huyck KD, Singler SA, Wellman CL (2006). Chronic stress impairs recall of extinction of conditioned fear. *Neurobiol Learn Mem* **85**: 213–218.

Monory K, Massa F, Egertová M, Eder M, Blaudzun H, Westenbroek R *et al* (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron* **51**: 455–466.

Nakazi M, Bauer U, Nickel T, Kathmann M, Schlicker E (2000). Inhibition of serotonin release in the mouse brain via presynaptic cannabinoid CB1 receptors. *Naunyn Schmiedebergs Arch Pharmacol* **361**: 19–24.

Newman E, O'Connor DB, Conner M (2007). Daily hassles and eating behaviour: the role of cortisol reactivity status. *Psychoneuroendocrinology* **32**: 125–132.

Ohno-Shosaku T, Tanimura A, Hashimotodani Y, Kano M (2012). Endocannabinoids and retrograde modulation of synaptic transmission. *Neuroscientist* (in press).

Patel S, Hillard CJ (2008). Adaptations in endocannabinoid signaling in response to repeated homotypic stress: a novel mechanism for stress habituation. *Eur J Neurosci* **27**: 2821–2829.

Patel S, Kingsley PJ, Mackie K, Marnett LJ, Winder DG (2009). Repeated homotypic stress elevates 2-arachidonoylglycerol levels and enhances short-term endocannabinoid signaling at inhibitory synapses in basolateral amygdala. *Neuropsychopharmacology* **34**: 2699–2709.

Patel S, Roelke CT, Rademacher DJ, Hillard CJ (2005). Inhibition of restraint stress-induced neural and behavioural activation by endogenous cannabinoid signalling. *Eur J Neurosci* **21**: 1057–1069.

Pecoraro N, Gomez F, Dallman MF (2005). Glucocorticoids dose-dependently remodel energy stores and amplify incentive relativity effects. *Psychoneuroendocrinology* **30**: 815–825.

Pecoraro N, Reyes F, Gomez F, Bhargava A, Dallman MF (2004). Chronic stress promotes palatable feeding, which reduces signs of stress: feedforward and feedback effects of chronic stress. *Endocrinology* **145**: 3754–3762.

Piomelli D (2003). The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci* **4**: 873–884.

Plendl W, Wotjak CT (2010). Dissociation of within- and between-session extinction of conditioned fear. *J Neuroscience* **30**: 4990–4998.

Puighermanal E, Marsicano G, Busquets-Garcia A, Lutz B, Maldonado R, Ozaita A (2009). Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nature Neurosci* **12**: 1152–1158.

Rademacher DJ, Hillard CJ (2007). Interactions between endocannabinoids and stress-induced decreased sensitivity to natural reward. *Prog Neuropsychopharmacol Biol Psychiatry* **31**: 633–641.

Rademacher DJ, Meier SE, Shi L, Ho WS, Jarrahian A, Hillard CJ (2008). Effects of acute and repeated restraint stress on endocannabinoid content in the amygdala, ventral striatum, and medial prefrontal cortex in mice. *Neuropharmacology* **54**: 108–116.

Ramos A, Mormède P (1998). Stress and emotionality: a multidimensional and genetic approach. *Neurosci Biobehav Rev* **22**: 33–57.

Rau V, DeCola JP, Fanselow MS (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev* **29**: 1207–1223.

Rau V, Fanselow MS (2009). Exposure to a stressor produces a long lasting enhancement of fear learning in rats. *Stress* **12**: 125–133.

Reich CG, Taylor ME, McCarthy MM (2009). Differential effects of chronic unpredictable stress on hippocampal CB1 receptors in male and female rats. *Behav Brain Res* **203**: 264–269.

Riebe CJ, Wotjak CT (2011). Endocannabinoids and stress. *Stress* **14**: 384–397.

Rossi S, De Chiara V, Musella A, Kusayanagi H, Mataluni G, Bernardi G *et al* (2008). Chronic psychoemotional stress impairs cannabinoid-receptor-mediated control of GABA transmission in the striatum. *J Neurosci* **28**: 7284–7292.

Rygula R, Abumaria N, Flügge G, Fuchs E, Rüther E, Havemann-Reinecke U (2005). Anhedonia and motivational deficits in rats: impact of chronic social stress. *Behav Brain Res* **162**: 127–134.

Steiner MA, Wanisch K, Monory K, Marsicano G, Borroni E, Bächli H *et al* (2008). Impaired cannabinoid receptor type 1 signaling interferes with stress-coping behavior in mice. *Pharmacogenomics J* **8**: 196–208.

Steiner MA, Wotjak CT (2008). Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis. *Prog Brain Res* **170**: 397–432.

Strekalova T, Gorenkova N, Schunk E, Dolgov O, Bartsch D (2006). Selective effects of citalopram in a mouse model of stress-induced anhedonia with a control for chronic stress. *Behav Pharmacol* **17**: 271–287.

Strekalova T, Spanagel R, Dolgov A, Bartsch D (2005). Stress-induced hyperlocomotion as a confounding factor in anxiety and depression models in mice. *Behav Pharmacol* **16**: 171–180.

Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ, Kida S (2004). Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* **24**: 4787–4795.

Tornatzky W, Miczek KA (1994). Behavioral and autonomic responses to intermittent social stress: differential protection by clonidine and metoprolol. *Psychopharmacology* **116**: 346–356.

Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM (1998). Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* **83**: 393–411.

Viveros MP, Marco EM, File SE (2005). Endocannabinoid system and stress and anxiety responses. *Pharmacol Biochem Behav* **81**: 331–342.

Wamsteeker JI, Kuzmiski JB, Bains JS (2010). Repeated stress impairs endocannabinoid signaling in the paraventricular nucleus of the hypothalamus. *J Neurosci* **30**: 11188–11196.

Weber T, Böhm G, Hermann E, Schütz G, Schönig K, Bartsch D (2009). Inducible gene manipulations in serotonergic neurons. *Front Mol Neurosci* **2**: 24.

Willner P (2005). Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology* **52**: 90–110.

Wotjak CT (2005). Role of endogenous cannabinoids in cognition and emotionality. *Mini Rev Med Chem* **5**: 559–570.

Zoppi S, Pérez Nievas BG, Madrigal JL, Manzanares J, Leza JC, García-Bueno B (2011). Regulatory role of cannabinoid receptor 1 in stress-induced excitotoxicity and neuroinflammation. *Neuropsychopharmacology* **36**: 805–818.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)