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DEPARTAMENTO DE CIÊNCIAS DA VIDA

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UNIVERSIDADE DE COIMBRA

Presynaptic CB_1 cannabinoid receptors control serotonin release in the rodent frontal cortex

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor Attila Köfalvi (Universidade de Coimbra) e do Professor Doutor Rui Carvalho(Universidade de Coimbra)

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“Go confidently in the direction of your dreams.
Live the life you have imagined.”

Henry David Thoreau

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RESUMO

Os CB_1Rs desempenham um papel preponderante na plasticidade sináptica, metabolismo cerebral, neurogénese, e morte celular; fazendo com que o sistema endocanabinoide seja um alvo atrativo em doenças neurológicas e psiquiátricas. Os CB_1Rs estão implicados na patogénese de uma série de desordens de humor e psicoses, sendo então um dos nossos objectivos mapear a possível presença e papel fisiológico dos CB_1R em terminais nervosos frontocorticais, noradrenérgicos (positivos para dopamina-β-hidroxilase), serotonergicos (positivos para SERT), e glutamatergicos (positivos para VGLUT1 e VGLUT2), no rato e em ratinho wild-type e CB_1R knockout. Resultados: Análise em microscópio confocal em fatias de cérebro revelou que os CB_1Rs estão presentes num subconjunto de todos os tipos de terminais nervosos investigados. A ativação dos CB_1R pelo seu agonista WIN55212-2 (1 μM) inibiu a libertação provocada por estímulo e dependente de cálcio do [^{14}C]glutamato e da [^{3}H]serotonina, medidos simultaneamente em sinaptoneurossomas isolados, de certa forma sensíveis a antagonistas dos CB_1R, O-2050 (1 μM) e LY320135 (5 μM). Não foi detectada imunoreactividade para CB_1 e inibição de libertação induzida pelo WIN55212-2 no córtex frontal de ratos KO para CB_1R.

Este é o primeiro relatório sobre CB_1R presinapticos funcionais em terminais serotonergicos, noradrenergicos e glutamatergicos, no córtex frontal de roedores. Como neuromoduladores/ neurotransmissores estão envolvidos na patogénese de algumas desordens de humor e psicoses (as quais são normalmente acompanhadas por uma sinalização alterada de endocanabinoides), é possível então assumir que um controlo deficiente de endocanabinoides em terminais nervosos frontocorticais, poderá contribuir para a patofisiologia de algumas doenças psiquiátricas.

ABSTRACT

CB_1 cannabinoid receptors (CB_1Rs) are the metabotropic 7-transmembrane spanning G protein-coupled receptors of highest density in the mammalian brain. CB_1Rs play a paramount role in synaptic plasticity, brain metabolism, neurogenesis, and cell death, making the endocannabinoid system an attractive target in neurological and psychiatric disorders. CB_1Rs are implicated in the pathogenesis of mood disorders and psychoses, thus here we aimed at mapping the possible distribution and physiological role of presynaptic CB_1Rs in frontocortical noradrenergic (dopamine-β-hydroxylase-positive), serotonergic (SERT-positive) and glutamatergic (VGLUT1- and 2-positive) nerve terminals of the rat, and wild-type / CB_1R knockout mice.

Confocal microscopy analysis in brain slices revealed that CB_1Rs are present in a subset of all the investigated nerve terminal types. CB_1R activation by the CB_1R agonist, WIN55212-2 (1 μM) inhibited the depolarization-evoked release of [^{14}C]glutamate and [^{3}H]serotonin in isolated synaptoneurosomes, in a manner sensitive to selective CB_1R antagonists, O-2050 (1 μM) and LY320135 (5 μM). No CB_1R immunoreactivity and no WIN55212-2-induced inhibition of transmitter release were detected in the frontal cortex of CB_1R knockout mice.

This is the first report on presynaptic functional CB_1Rs in serotonergic, noradrenergic and glutamatergic terminals in the frontal cortex of the rodents. Since all three neuromodulators/ neurotransmitters are involved in the pathogenesis of mood disorders and psychosis (which are typically accompanied with impaired endocannabinoid signaling), it is feasible to assume that an impaired endocannabinoid control on frontocortical nerve endings may contribute to the pathophysiology of psychiatric disorders.

1. INTRODUCTION

1.1 Overview

Brain's serotonergic system plays important roles in synaptic plasticity, perception and the development of neural networks, among many others. Impaired serotonergic signaling is detected in many neurological and psychiatric disorders, among which clinical depression is the most well known. Most therapies involve the control of serotonin reuptake and subtype-specific receptor modulation, but the presynaptic control of serotonin release has also been emerging as a potential therapeutic strategy. Here we aimed at studying the putative presynaptic control of serotonin release in the frontal cortex by a likely candidate, the CB_1 cannabinoid receptor (CB_1R). CB_1Rs have high presynaptic density in the mammalian nervous system, and are involved in the development of mood disorders (Degroot, 2008; Tzavara et al., 2008).

Coincidently with our research aims, a Hungarian research group has discovered that CB_1Rs and $alpha_{2a}$ noradrenergic receptors ($\alpha_{2a}Rs$) control the action of one another in frontocortical noradrenergic terminals of the rat, resulting in a tight regulation of stimulated noradrenaline efflux. Indeed, noradrenaline is another important neuromodulator participating in the preparation of the brain to adverse environmental challenges such as stress and aggression, as well as in mood disorders such as depression.

These data will hopefully contribute to the better understanding of the pathogenesis of depression, but also, of other personality disorders such as aggression, psychoses and anxiety, in which impaired serotonergic, noradrenergic and endocannabinoid signaling are pathogenetic factors.

1.2 Frontal cortex and personality disorders

The frontal part of the cortex (frontal cortex in rodents; prefrontal cortices in man) is the anterior part of the frontal lobes of the brain, standing in front of the motor and premotor areas. It has extensive connections with other cortical regions, as well as with subcortical areas. The dorsal prefrontal cortex in humans is especially interconnected with brain regions involved with attention, cognition and action (Goldman-Rakic et al.,1988), while the ventral prefrontal cortex interconnects with brain regions involved with emotion (Price JL et al. ,1999).

The interaction between the frontal cortex and basal ganglia-thalamocortical circuits underlies mood and emotions in humans (Sackeim et al., 1982; Alexander et al., 1990; George et al., 1997). Neuroimaging and neuropsychological studies suggest that abnormal prefrontal cortex function plays a role in the pathophysiology of affective disorders such as major depression (Baxter et al., 1989; Soares and Mann, 1997; Goodwin, 1997). Mounting evidence indicates structural and functional abnormalities in the prefrontal cortex of antisocial individuals (Davidson et al., 2000; Henry and Moffitt, 1997; Raine, 1993; Raine and Buchsbaum, 1996). However it is important to notice the existence of some inconsistencies in several studies (e.g. significantly increased rather than decreased activation). There are many studies devoted to understanding the role of the prefrontal cortex in neurological disorders such as schizophrenia, bipolar disorder and ADHD. Therefore, I have focused on the frontal cortex of the rat, a putative analogue of the human prefrontal cortex, during my studies.

1.3 The serotonergic system

Serotonin, otherwise known as 5-hydroxytryptamine (5-HT), was isolated and characterized in 1948 by Maurice Rapport and Irvin Page (Rapport et al., 1948a-c). However, the first findings were by Vittorio Erspamer (1935), after showing that an extract from enterochromaffin cells made intestines contract. The first thoughts believed that it contained adrenaline, but two years later Erspamer was able to show that it was an unknown amine, which he named enteramine (Erspamer and Asero, 1952). Later, this substance was termed serotonin after the Latin word *serum* and the Greek word *tonic*. Subsequent studies found ample amount of serotonin in many tissues such as brain, lung, kidney, platelets, and the gastrointestinal tract.

Brodie and Shore (1957) suggested the role of serotonin as a neuromodulator. This hypothesis was based in studies that demonstrated the localization of 5-HT receptors to specific areas of the vertebrate brain (Twarog and Page, 1953; Amin et al., 1954). Later it was elucidated that serotonin was principally located in the nerve endings of neurons in isolated portions of the mammalian brain (Michaelson and Whittaker, 1963; Zieher and DeRobertis, 1963). In 1964, Dahlström and Fuxe mapped the specific nuclei in the brain that contained serotonin, and made this clusters being known as the raphe nuclei. After that several findings linked serotonin to a variety of central nervous system functions such as mood, behavior, sleep cycles and appetite.

Serotonin is a biogenic monoamine structurally similar to epinephrine, norepinephrine, dopamine and histamine. Serotonin production has two steps: the essential amino acid tryptophan is hydroxlyated to 5-hydroxytrytophan (5-HTP) by tryptophan hydroxylase; then 5-HT is decarboxylated to form 5-HT (Clark et al. 1954).

Within the central nervous system (CNS), serotonin is synthesized and stored in the nerve terminals. The raphe nuclei represent the major nuclei with both ascending serotonergic fibers projecting to the forebrain and descending fibers that extend to the medulla and spinal cord (Dahlström and Fuxe, 1964). Ninety-nine percent of total body serotonin is located intracellularly implying thus a tight regulation of serotonin release. Metabolism by the monoamine oxidases (MAO-A and to a smaller extent, by MAO-B) is the primary metabolic pathway for serotonin (McIsaac and Page, 1959).

Serotonergic fibers contain hundreds of release sites (so-called varicosities), which do not form synaptic contact with post-synaptic targets. Therefore, the vast majority of serotonin is released into the extracellular space acting as a volume neuromodulator (Vizi et al., 2004). Serotonin then can bind to autoreceptors or to specific serotonin receptors (5-HT receptors) of surrounding cells (Cerrito and Raiteri, 1979). Binding of serotonin to the autoreceptor acts as a negative feedback against further release into the synaptic cleft (Cerrito and Raiteri, 1979). The main responsible for removing serotonin from the synaptic cleft is the serotonin transporter (SERT), located on the presynaptic membrane.

After being transported into the presynaptic neuron, serotonin is recycled back into presynaptic vesicles where it is protected from being metabolized by MAO-B within the cytosol of the neuron. The two mechanisms directly involved in controlling the availability of serotonin in the synaptic cleft and the extracellular space are the binding of serotonin to its autoreceptor (most typically of the 5-HT_1 subfamily) and the activity of the SERT. Stimulation of the 5-HT_1 autoreceptor decreases further release of serotonin creating a negative feedback, while the SERT actually removes the "excess" of serotonin from the synaptic cleft. We should note that the transporters for noradrenalin and dopamine are also effective to take up serotonin (Vanhatalo and Soinila; 1994; Zhou et al., 2002), thus it is necessary to block these other uptake systems if one aims at studying serotonergic terminals in the brain.

The serotonergic system is rather complex, although many of serotonin's functions in the CNS are already described: Projections from the rostral nuclei of the raphe help regulate temperature,

appetite, sleep cycles, emesis, and sexual behavior. Projections from the caudal nuclei participate in nociception and motor tone. One of the most important serotonin's functions and the one to which in the last years, has been paid more attention to, is its role in psychological disorders. The consensus that depression, mania and anxiety disorders are associated with altered bioavailability of serotonin in the CNS (Kandel, 2001) has grown in the last few years. We need to keep in mind that there is still much to understand about this neuromodulator.

Although the earliest antidepressants such as the MAO inhibitors and tricyclic antidepressants (TCA), aimed specifically at altering serotonin levels, the most specific and potent antidepressant called selective serotonin reuptake inhibitors (SSRIs) came only later. These drugs bind specifically to the SERT, increasing the extracellular half-life of serotonin.

1.4 The noradrenergic system of the brain

Noradrenalin is synthesized from dopamine by the enzyme, dopamine-β-hydroxylase (DβH) which is a selective for noradrenergic neurons. The majority of the brain (limbic system, cortex) is innervated by the ascending noradrenergic bundle from the locus coeroleus, while the hypothalamus receives noradrenergic innervation from the lateral tegmental area. The two functionally different noradrenergic systems in the brain can be divided as the dorsal bundle and the ventral bundle. It is possible that dorsal bundle inhibits serotonergic neurons whilst tonically stimulates dopaminergic neurons. On the other hand, the ventral noradrenergic bundle seems to exert opposite influences (Kostowski, 1979).

Altered noradrenergic signaling in the frontal cortex has been associated with the executive dysfunction component of addiction with its core deficits represented by loss of control, impulsivity and impaired decision making. Stress, experienced during food- or drug-restriction, lead to increased noradrenaline release from varicosities of locus coeruleus noradrenergic neurons in the frontal cortex, through the activation of hypothalamo-pituitary axis, and thereby contribute to the transition from reward to addiction. α_{2A}Rs in the frontal cortex are also instrumental for spatial working memory (Wang et al., 2007) as well as emotional behavior (Zhang et al., 2009).

Moreover, as revealed by the STRADIVARIUS trial, the inhibition of CB_1R receptors can also lead to an increased incidence of depression and suicide (Nissen et al., 2008), which are pathological conditions involving impaired noradrenergic signaling (Pandey and Dwivedi, 2007).

Despite the abundance of data on the action of cannabinoids on complex behavioral changes mediated by the monoaminergic system, relatively few and conflicting investigations concentrated on the interaction of cannabinoids and noradrenalin on the cellular level: In vivo, both cannabinoid agonists and antagonists increase the efflux of noradrenalin from prefrontal cortex (Tzavara et al., 2003; Oropeza et al., 2005; Page et al., 2008), whilst in other regions of the brain such as the hippocampus the direct effect activation of CB_1Rs on noradrenalin release, is inhibition (Kathmann et al., 1999; Schlicker et al., 1997).

It is also well established that central noradrenergic nerve terminals are equipped with presynaptic α_{2A}Rs, another G protein-coupled receptor (GPCR) the activation of which inhibits the release of noradrenalin (Starke, 2001). Moreover, these receptors are also activated by noradrenalin released upon neuronal activity, thereby executing an important fine-tuning mechanism, i.e. the autoinhibition of transmitter release. In the primate prefrontal cortex, α_{2A}Rs are immunolocalized to both pre- and post-synaptic sites, and presynaptic α_{2A}Rs are also expressed by DβH positive varicosities (Aoki et al., 1998; Wang et al., 2007). As both CB_1Rs and α_{2A}Rs use $G_{i/o}$ downstream signaling cascades, the possibility arises that there might be interplay between them.

Interestingly, one of the most known and heavily debated roles of noradrenalin in the brain is its involvement in clinical depression. Although clinical depression is mostly addressed to alteration of serotonin levels, the noradrenalin transporter knockout mice rather than the serotonin transporter knockout mice exhibit antidepressive phenotype (Haenisch and Bönisch, 2011).

1.5 Cannabis and the endocannabinoid system

The following introduction focuses only on the constituents of the endocannabinoid system most relevant to my thesis. Note that there are several more receptors, endogenous ligands and enzymes implicated in the endocannabinoid system whose illustration is beyond the scope of this thesis. Further readings can be found in the book Cannabinoids and the Brain, ed.: Attila Köfalvi (2008, Springer).

Cannabis has been used for more than 8000 years for its recreational effects, and the first medical use of cannabis is dated back to 2727 B.C. in China. Texts related to Hinduism, also mention the use of cannabis for medical proposes in India between 1200 and 800 B.C. The psychotropic properties of cannabis appeared for the first time in a Chinese medical book around

100 B.C., and it is believed that the Scythians first introduced Cannabis sativa in Europe. Cannabis medical uses are widely described and registered along history, from the Roman Empire history, till the late England with Queen Victoria. However, in 1925 the Geneva Convention included cannabis in the list of illicit drugs. Being a symbol of revolution and terrible sins due to its psychotropic effects, in the USA, it was also prohibited and proclaimed as an illegal drug. With heavy weight supporters like President Kennedy and President Johnson, cannabis popularity increased and 200-250 million cannabis users were reported by the UN, worldwide till 1970.

Though Eastern cultures using marijuana as medicine for centuries, Western civilization started the use of marijuana for therapeutic purpose therapeutically only recently. Marijuana revealed to be an effective anesthetic, antihypertensive, and eye pressure reducing (in glaucoma).

In the 60's when hippies became interested in cannabis preparations, the structure and stereochemistry of the naturally occurring trans-^{9}-tetrahydrocannabinol (^{9}-THC), the main psychoactive constituent o marijuana and hashish has been elucidated (Gaoni and Mechoulam, 1964). Later, 65 more biologically active molecules were identified in the plant cannabis. Several other plants have been reported to produce biologically active but hardly or not psychotropic cannabinoid molecules which can provide further starting points for the development of new medicines (Gertsch et al., 2010).

^{9}-THC is responsible for the vast majority of cannabis effects such as antinociception, decreased body temperature, increased hunger and thirst, decreased metabolic rate, motor disturbances and catalepsy, euphoria and dysphoria, anxiety, drowsiness, altered time and audiovisual perceptions, panic attacks and impaired memory (Haagen-Smit et al., 1940; Lowed, 1946; Paton and Pertwee, 1973; Howlett et al., 2004).

Whether how ^{9}-THC exerts these effects was unknown till 1984, when it was found that it inhibits cAMP accumulation (Howlett and Fleming, 1984). Four years later, specific cannabinoid binding sites were reported in the brain (Devane et al., 1988). This made clear that membrane receptors should exist for ^{9}-THC. Indeed, in 1990 both the rat and human CB1Rs (CB_1Rs) were finally cloned and characterized (Gérard et al., 1990, 1991; Matsuda et al., 1990), and the first studies on its distribution found the receptor at an unexpectedly high density in the brain, mostly in the basal ganglia (substantia nigra and globus pallidus), in the hippocampus, in the neocortex and cerebellar cortex, as well as in the hypothalamus (Herkenham et al., 1991). The virtual lack of

CB_1Rs in the brain stem and the medulla oblongata explains why cannabis intoxication can not compromise vegetative functions such as breathing.

Meanwhile, the first endogenous cannabinoid ligand, arachidonoylethanolamine or anandamide (AEA), was found in the porcine brain (Devane et al. 1992), followed by 2-arachidonoylglycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al., 1995). In 1994, the first selective CB_1R antagonist, SR141716A or Rimonabant, was reported (Rinaldi-Carmona et al., 1994), and it was finally been marketed in Europe in 2006 under the name of Acomplia™ to treat cardiometabolic risk factors such as hypercholesterolemia and obesity (Matias et al. 2008). Unfortunately, in 2008, Acomplia has been banned from the market due to rare but serious side effects such as the induction of suicide thoughts and suicide itself (McPartland, 2009). This already prognosticates that the endocannabinoid system is a major player in mood control.

1.6 The endocannabinoid system in mood disorders and anxiety

Indeed, deregulation of the endocannabinoid system has been associated with several neuropsychiatric disorders. The high level of expression of CB_1Rs in brain areas involved in the regulation of cognition and mood functions implies that the endocannabinoid system is probably involved in emotional processing, mood and anxiety regulation. It is also known from recent studies, that endocannabinoid signaling and the consequences on neuronal activity might be important in the etiology of schizophrenia and may help explain the impact of cannabis abuse in psychotic users (Köfalvi and Fritzsche, 2008). Studies with the global CB_1R KO mice revealed that these mice became anhedonic during chronic mild stress sooner than wild type (WT) mice, suggesting that they are more vulnerable to the anhedonic effect of chronic stress (Martin et al. 2002). Recently it was reported that long-lasting impairment of CB_1R function led to the development of a depressive phenotype characterized by anhedonic state, passive coping behavior in the forced swim test and cognitive deficits (Rubino et al., 2008c, 2009). These behaviors were supported by well-known biochemical parameters of depression such as changes in CREB function in specific brain areas, lower levels of markers of neuroplasticity and less active synapses with reduced ability to maintain normal synaptic efficiency in the frontal cortex (Rubino et al., 2008c, 2009).

The most interesting and important findings are related to changes found in CB_1Rs and other elements belonging to the endocannabinoid system, reported in animal models of depression. In

the chronic mild stress paradigm, there was a significant increase in CB_1R density or mRNA in the frontal cortex (Bortolato et al., 2007; Hill et al., 2008). The same increase was seen in another animal model of depression, bilateral olfactory bulbectomy in the rat (Rodriguez-Gaztelumendi et al., 2009). Hungund et al., (2004), also reported a significant increase in CB_1R density and efficiency in the dorso-lateral frontal cortex of depressed suicide victims. We can then assume that either a compensatory upregulation of the CB_1Rs try to counteract a decreased endocannabinoid functioning or the increase in CB_1R density itself contribute to the depressed phenotype.

Most antidepressants increase the level of serotonin and/ or norepinephrine (Berton and Nestler, 2006). Agents that act facilitating endocannabinoid neurotransmission facilitate adaptive stress coping behaviors and attenuate the neuroendocrine response to psychological stressors (Patel et al., 2004). Interestingly, both CB_1R agonists and inhibitors of AEA hydrolysis increase the firing activity of neurons in the dorsal raphe, the major source of serotonin neurons, thus enhancing serotonin release in the hippocampus while exhibiting antidepressant activity (Bambico et al., 2007; Gobbi et al., 2005). Stimulation of CB_1R activity has been shown to increase the release of norepinephrine in the frontal cortex (Gobbi et al., 2005; Oropeza et al., 2005; Bambico et al., 2007). The probable reason for this is the disinhibition of GABAergic input into monoaminergic cell bodies.

Decrease in neurogenesis and neuronal migration has been also put forward as a major pathomechanism for depression. It is well known that factors that predispose to depression, suppress neurogenesis, whereas interventions that reduce depression stimulate neuron formation. We can assume that modulation of hippocampal neurogenesis is crucial to both the onset and treatment of depression (Perera et al., 2008; Drew and Hen, 2007). In various models, administration of cannabinoid agonists as well as pharmacological increase in endocannabinoid levels have been shown to increase neurogenesis and migration in the subventricular zone (Jiang et al., 2005; Hill et al., 2006; Goncalves et al., 2008; Marchalant et al., 2009; Oudin et al., 2011).

Altogether, this data suggest that increasing endocannabinoid exerts antidepressant proprieties by increasing serotonin release (Parolaro et al., 2010). However, this question is more complex than what it appears (for the comparison of the efficacy of the activation and the inhibition of the endocannabinoid system as an antidepressant see Tzavara and Witkin, 2008). In fact, many found that the activation of the endocannabinoid system decreases serotonin release: Acute (Egashira et al., 2002) or 8-day (Sagredo et al. 2005) injection of rats with Δ^9-THC induces a decrease in extracellular serotonin levels or in serotonin release in the frontal cortex. In agreement with these,

Dharmani et al. (2003), Tzavara et al. (2003) and Aso et al. (2009) reported that CB_1R blockade augments frontocortical serotonin release. Furthermore, Mato and colleagues (2007) reported impaired functionality for 5-HT_{1A} and 5-$HT_{2A/C}$ receptors in the CB_1R knockout mouse.

In vitro, CB_1R activation also appears to decrease serotonin release: Nakazi et al. (2000) in cortical slices and Balázsa et al. (2008) in hippocampal slices found that CB_1R activation inhibits evoked serotonin release. Since CB_1R mRNA is present in raphe nuclei cell bodies (Häring et al., 2007) it is feasible that presynaptic CB_1Rs inhibited the evoked release of serotonin, although, polysynaptic processes such as the inhibition of putative glutamatergic drive onto serotonergic terminals might have also participated in this.

Another alternative explanation can be that effects are dose-/concentration-dependent. Indeed, cannabinoid receptor agonists are known to produce bell-shaped dose-response curves in vivo. Though cannabis preparations are used recreationally mainly for their euphoric effects, often accompanied by a reduction in anxiety and a boost in sociability, some cannabis users sometimes experience dysphoric reactions with feelings of anxiety and panic.

Rubino et al. (2008b) reported that in the frontal cortex, tonic activation of CB_1R by low doses of its endogenous agonist, anandamide (AEA) and of another cannabinoid receptor, the $TRPV_1R$ by higher doses of AEA affected anxiety in opposite ways. It was also reported by Parolaro et al. (2010), that if the tissue levels of AEA become too low or too high, leading either to lack of CB_1R activation or to $TRPV_1R$ stimulation, anxiogenic responses are observed. Physiological increases of AEA, on the other hand, promote an anxiolytic response through activation of CB_1Rs.

As mentioned above, deletion of the CB_1R in mice, gave anxiety-like responses in different tests of anxiety such as the elevated plus-maze, the light-dark box, the open-field and the social interaction test (Haller et al., 2002, 2004b; Maccarrone et al., 2002; Martin et al., 2002; Uriguen et al., 2004). In intra-cerebral microinjection studies, activation of CB_1Rs in the frontal cortex, ventral hippocampus and periaqueductal gray, induced an anxiolytic response (Moreira et al., 2009 Rubino et al., 2008a), whereas their activation in the amygdala and dorsal hippocampus gave rise to an anxiogenic one (Roohbakhsh et al., 2007; Rubino et al., 2008a). Therefore, the overall effect might depend on the amount of receptors saturated in each brain region, as already suggested by Hill and Gorzalka (2009).

1.7 Modulation of frontocortical glutamate release by CB_1Rs

Functional presynaptic CB_1Rs have been first described by our laboratory both in rodents and human, and at high density in GABAergic nerve terminals of CCK-positive interneurons (Katona et al., 1999, 2000). Later, functional CB_1Rs were also identified in striatal GABAergic terminals (Köfalvi et al., 2005), and in frontocortical GABAergic terminals (Fortin and Levine, 2007; Chiu et al., 2010). Still, the most interesting finding is that functional CB_1Rs are present in many glutamatergic terminals either in the striatum (Köfalvi et al., 2005), or in the frontal cortex (Auclair et al., 2000; Barbara et al., 2003; Lafourcade et al., 2007). Results obtained from a variety of forebrain regions consistently indicate that cannabinoid agonists reduce glutamatergic synaptic transmission through CB_1R activation (Domenici et al., 2006).

1.8 Endocannabinoids: from where, to where?

In the post-synaptic dendritic compartments apposing CB_1R-positive terminals, in most cases the enzyme diacylglycerol lipase synthesizes the endocannabinoid, 2-arachidonoyl-glycerol (2-AG), and the enzyme phospholipase D cleaves AEA from its precursor upon Ca^{2+} entry and/ or metabotropic receptor stimulation. Then the released endocannabinoid molecules quickly traverse the synaptic cleft to activate presynaptic CB_1Rs (Harkany et al., 2008; Haj-Dahmane and Shen, 2011). Interestingly, one of the post-synaptic receptors capable of inducing endocannabinoid synthesis and the subsequent presynaptic inhibition of glutamate release is the 5-HT_2-type, while 5-HT_{1B} receptors directly inhibit glutamate release acting presynaptically in the same synapse of the inferior olive (Best and Regehr, 2008). Indeed, the wide variety of different metabotropic serotonin receptor genes and their splice variants and RNA-edited products permit the serotonergic system to modulate synaptic transmission in diverse ways, depending on the subtype and the cellular and subcellular distribution of the receptors (Hoyer and Martin, 1996; Jensen et al., 2010This information highlight the importance of the endocannabinoid and the serotonergic systems in the control of synaptic plasticity and memory formation (González-Burgos and Feria-Velasco, 2008; Alger, 2009).

2. AIMS

As understood from the previous section, neurological and psychiatric disorders commonly involve long-term impairment of serotonergic and cannabinoid control of synaptic plasticity in the prefrontal cortex (Otani, 2003; Köfalvi and Fritzsche, 2008; Goto et al., 2010; López-Gil et al., 2010). Yet, little is known about the putative interaction between these two neuromodulators, for example, how do CB_1R modulate serotonergic terminals? Thus, in this study we have mapped the distribution of CB_1Rs in serotonin transporter- (SERT) positive terminals using fluorescent and confocal microscopy in the frontal cortex of the rat and the mouse. We also investigated the consequence of CB_1R activation on stimulation-evoked, Ca^{2+}-dependent serotonin release from frontocortical synaptoneurosomes. For the possible case of negative results in serotonergic terminals, we used glutamatergic terminals and glutamate release as a positive control (Auclair et al., 2000; Barbara et al., 2003; Lafourcade et al., 2007). Finally, the involvement of CB_1R was confirmed by validating key experiments in CB_1R knockout mice.

We have been also invited to participate in collaboration with the laboratory of Dr. Beáta Sperlágh, Institute of Experimental Medicine, Budapest, Hungary. In this study, the Hungarian partner identified by pharmacological tools that putative presynaptic CB_1Rs interacting with $_{2A}$ adrenergic autoreceptors in noradrenergic terminals of the frontal cortex control noradrenalin release. Thus, our task was to bolster those pharmacological findings with immunohistochemistry.

3. MATERIALS AND METHODS

3.1 Subjects

All studies were conducted in accordance with the principles and procedures outlined in the EU guidelines (86/609/EEC) and by FELASA. Animals were housed in an SPF facility, with 12 h light on/off cycles and ad libitum access to food and water. Male Wistar rats (180-240 g, 8-10-week old) were purchased from Charles-River (Barcelona, Spain). CB_1R null-mutant (knockout) male mice (Ledent et al., 1999) and their wild-type littermates on CD-1 background were genotyped from the tail, housed as detailed above and sacrificed daily in pairs (one WT and one KO), until 16 weeks of age.

3.2 Microscopy sections

Under deep sodium pentobarbital anesthesia (100 mg/kg body weight, i.p.), male Wistar rats, CB_1R null-mutant mice of the CD-1 strain and their wild-type littermates were transcardially perfused with fixative (4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4). The brains were removed and immersed in the same fixative overnight and then kept in 30% sucrose in physiological saline (0.9% NaCl) for at least 48 h before sectioning. Forty micron-thick sections from the mouse brains and 30-µm-thick sections from the rat brains were cut using a Cryostat microtome (Leica) and collected into 0.1 M PBS containing 0.1% sodium azide.

3.3 Immunohistochemistry

Sagittal brain sections containing the frontal cortex of the animals were selected. Whether the rat and mouse have frontal cortex and if yes, what are its borders is contentious (Preuss, 1995; Seamans et al., 2008). Thus, we focused our study on the area called frontal associative cortex in the rat and the mouse (Paxinos and Franklin, 1997; Paxinos and Watson, 1998), and we will use the term frontal cortex hereafter. Free floating sections were blocked in 10% normal goat serum (Vector Laboratories, CA, USA)/ 5% BSA/ 0.3% Triton X-100 for 40 min and incubated overnight in a primary antibody cocktail of L-15 rabbit anti-CB_1R (1:1000; raised by Dr. Ken Mackie), and mouse monoclonal anti-SERT (1:250; Abcam, UK), or guinea pig polyclonal anti-VGLUT1 (1:200; Synaptic Systems, Germany), or guinea pig polyclonal anti-VGLUT2 (1:200; Synaptic Systems). Sections were then washed in phosphate buffer (PB; 0.1 M) and incubated with a secondary

antibody cocktail containing DyLight 594 goat anti-guinea pig or anti-rabbit as well as and DyLight 488 goat anti-mouse (all at 1:200; Kirkegaard and Perry Laboratories, Inc, USA), for 2 h. After washing in PB 0.1 M, the sections were mounted and coverslipped using Vectashield Hardest Mounting Medium (Vector Laboratories). Low magnification images where taken on a Zeiss axiovert 200 microscope equipped with AxioVision software and MosaiX module. Confocal images were taken using a Zeiss LSM510 META confocal microscope.

3.4 Quality control for CB_1R immunoreactivity

Before acquiring high-resolution laser scanning images, settings were tested for possible auto-fluorescence in a control slice without primary and secondary antibody treatment. Parameters were set to obtain the representative image seen in figure 1. Next, we observed that slices treated solely with secondary antibodies (DyLight 488 with DyLight 594) also failed to produce detectable staining. In conventional Western blotting, the L-15 antibody recognized a band at ~55 kDa in the RIPA-buffer protein extract from the wild-type but not from the CB_1R knockout mouse cortex figure 1. CB_1R staining in the rat and in the wild-type mouse was virtually the same as with the guinea pig anti-CB_1R (1:1000; Frontier Science, Hokkaido, Japan; licensed by Dr. Masahiko Watanabe) at any resolution tested (MS in preparation). No immunostaining was detected in the CB_1R knockout mouse brain sections at any resolution (**Figure 1**).

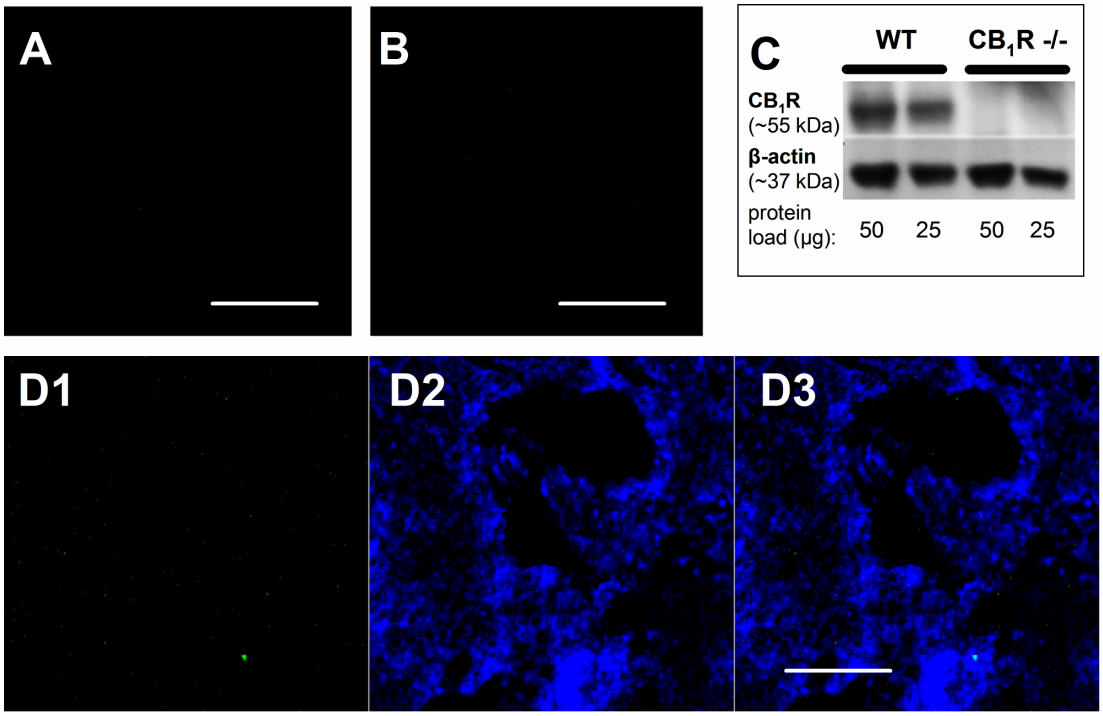

Figure 1. A) Confocal image of a slice without primary and secondary antibodies to control for autofluorescence. B) Secondary antibodies DyLight 488 and 594 without primary antibodies. C) Western blotting with two different protein loads of samples prepared with RIPA buffer from the cortex of WT and CB1R KO mice. D) CB1R (D1) and VGLUT1 (D2) immunoreactivity and merged image (D3) in CB1R knockout mouse frontal cortex. Horizontal bars represent 10 m.

3.5 Dual-label [^{3}H]serotonin/[^{14}C]glutamate release assay from frontocortical nerve terminals

Experiments were carried out as before (Ferreira et al., 2009). Frontal cortices were quickly removed into 2 ml ice-cold sucrose solution (0.32 M, containing 5 mM HEPES, pH 7.4) and were homogenized instantly with a Teflon homogenizer, and centrifuged at 5,000 g for 5 min. The supernatant was centrifuged at 13,000 g for 10 min to obtain the P2 crude synaptosomal fraction. Synaptosomes then were diluted to 0.5 ml with Krebs' solution (in mM: NaCl 113, KCl 3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 10, HEPES 15, pH 7.4, 37°C), containing the noradrenalin transporter inhibitor, reboxetine (30 nM; Tocris Bioscience, UK) and the dopamin transporter inhibitor, GBR12783 (100 nM; Tocris Bioscience) to prevent the non-specific uptake of [^{3}H]serotonin into the respective terminals. All assay medium also contained the MAO-A/B inhibitor, pargyline (10 µM) to prevent [^{3}H]serotonin degradation, and the glutamate decarboxylase inhibitor,

aminooxyacetic acid (100 μM) to prevent [^{14}C]glutamate metabolism. Under this condition, synaptosomes were incubated with both hydroxytryptamine 5-[1,2-^{3}H] creatinine sulfate (American Radiolabeled Chemicals, Inc; final concentration, 300 nM) and [^{14}C]-U-glutamate (PerkinElmer, USA; 20 μM) for 10 min. A 16-microvolume chamber perfusion setup was filled with the preloaded synaptosomes which were trapped by layers of Whatman GF/C filters and superfused continuously at a rate of 0.8 ml/min until the end of the experiment at 37°C. Upon termination of the 10-min washout, 2-min samples were collected for liquid scintillation assay, and the filters were also harvested to obtain the total radioactivity content. After collecting four 2-min samples as baseline, the evoked release of the transmitters was stimulated with 4-aminopyridine (4-AP) for 2 min. Vehicle (0.1% DMSO if agonist was tested alone and 0.2% DMSO when agonist and antagonist were combined) and drugs were added 4 min before the stimulation with 4-AP. In each experiment, treatments were applied in duplicate (i.e. eight conditions/ treatments in duplicate, averaged as n = 1). For all conditions there was an unstimulated vehicle/ drug baseline control, which was then subtracted from the results, obtained with stimulation (**Figure 6A,B**). In this way we measured pure drug effects on the evoked release free from both the putative drug/ vehicle effect on the baseline and the putative vehicle effect on the evoked release. In fact, there was neither significant drug/ vehicle effect per se on the baseline or vehicle DMSO effect on the evoked release of either [^{3}H]serotonin or [^{14}C]glutamate (data not shown).

When the wild-type (WT) and CB_1R KO mice were tested, one mouse of each strain was used simultaneously in the same experiment, i.e. 1 WT for 8 of the 16 channels and 1 KO for the other 8 channels.

The [^{14}C] and [^{3}H] content of each samples were counted by a dual-label protocol using a Tricarb β-counter (PerkinElmer), and DPM values were expressed as fractional release (FR%), i.e. the percent of actual content in the effluent as a function of the total synaptosomal content.

3.6 Chemicals not listed above

WIN55212-2 and 4-aminopyridine were purchased from AscentScientific (UK). HEPES, sucrose, aminooxyacetic acid, and pargyline were obtained from Sigma (Saint Louis, Missouri, USA). O-2050, GBR12783, LY320135, and reboxetine mesylate were bought from Tocris Bioscience, UK. Non-water soluble substances were dissolved or reconstituted in DMSO and stored at -20 ºC until use.

3.7 Data treatment

All data represent mean ± SEM of $n \geq 6$ observations (at least 6 animals) in the release experiments. Statistical significance was calculated on the raw data using repeated measures ANOVA with Bonferroni's post-test for selected groups of data. Data then was normalized to everyday controls to visually enhance effect amplitudes. A $p < 0.05$ was accepted as significant difference.

4. RESULTS

4.1 Immunohistochemistry

CB_1R staining varied from weak to very strong across the sagittal slice of the rat (**Figure 2A1, B1, C1**) and the mouse (**Figure 3A1, B1, C1**). Immunoreactivity was the strongest in the substantia nigra, in the globus pallidus, in the pyramidal cell layer of the hippocampus and in the cortex, including the frontal associative cortex and the pyriform cortex, while was modest in the caudate-putamen and was absent in the corpus callosum. Although not documented with images, the cerebellar cortex also showed strong CB_1R immunoreactivity. For the quality controls for the antibody specificity and the immunohistochemical approaches, see **Figure 1** as well as **Figure 3D**.

In the neocortex, CB_1R staining was laminar. The layers are marked in Figure 6D. Cortical layers II-III, Va and VI stained strongly with the CB_1R antisera, intercalated with low (layers IV and Vb) or no CB_1R staining (layer I) (**Figures 2A4, B4, C4; 3A4, B4, C4, 6B**). The serotonin transporter SERT and the vesicular glutamate transporter type-1 (VGLUT1) homogenously stained all layers of the cortex, while VGLUT2 staining was complementary to CB_1R-positive layers, i.e. in layers I, IV and Vb (**Figures 2A4, B4, C4; 3A4, B4, C4**).

At the highest resolution of confocal microscopy, CB_1R immunoreactivity revealed fiber-like and punctate-like structures, i.e. probable axons and nerve terminals. VGLUT1 immunoreactivity, marking putative glutamatergic terminals was very dense throughout the frontal cortex, and frequently co-localized with CB_1R immunoreactivity (**Figure 4A1-3[mouse], C1-3[rat]**). As mentioned above, VGLUT2 immunoreactivity marking other putative glutamatergic synapses was very sparse in CB_1R-rich areas, in comparison to VGLUT1. Occasionally, CB_1R immunoreactivity confined to VGLUT2-positive terminals as verified in the orthogonal projection of the optical slices (**Figure 4B1-3, D1-3**). SERT immunoreactivity was very strong throughout the neocortex. At the highest resolution of confocal microscopy, SERT staining appeared as an extremely dense meshwork of little dots, but sometimes fiber-like structures could also be detected. CB_1R immunoreactivity gave high frequency co-localization with SERT as confirmed in the orthogonal projection of the optical slices (**Figure 5A1-3[mouse], B1-3[rat]**).

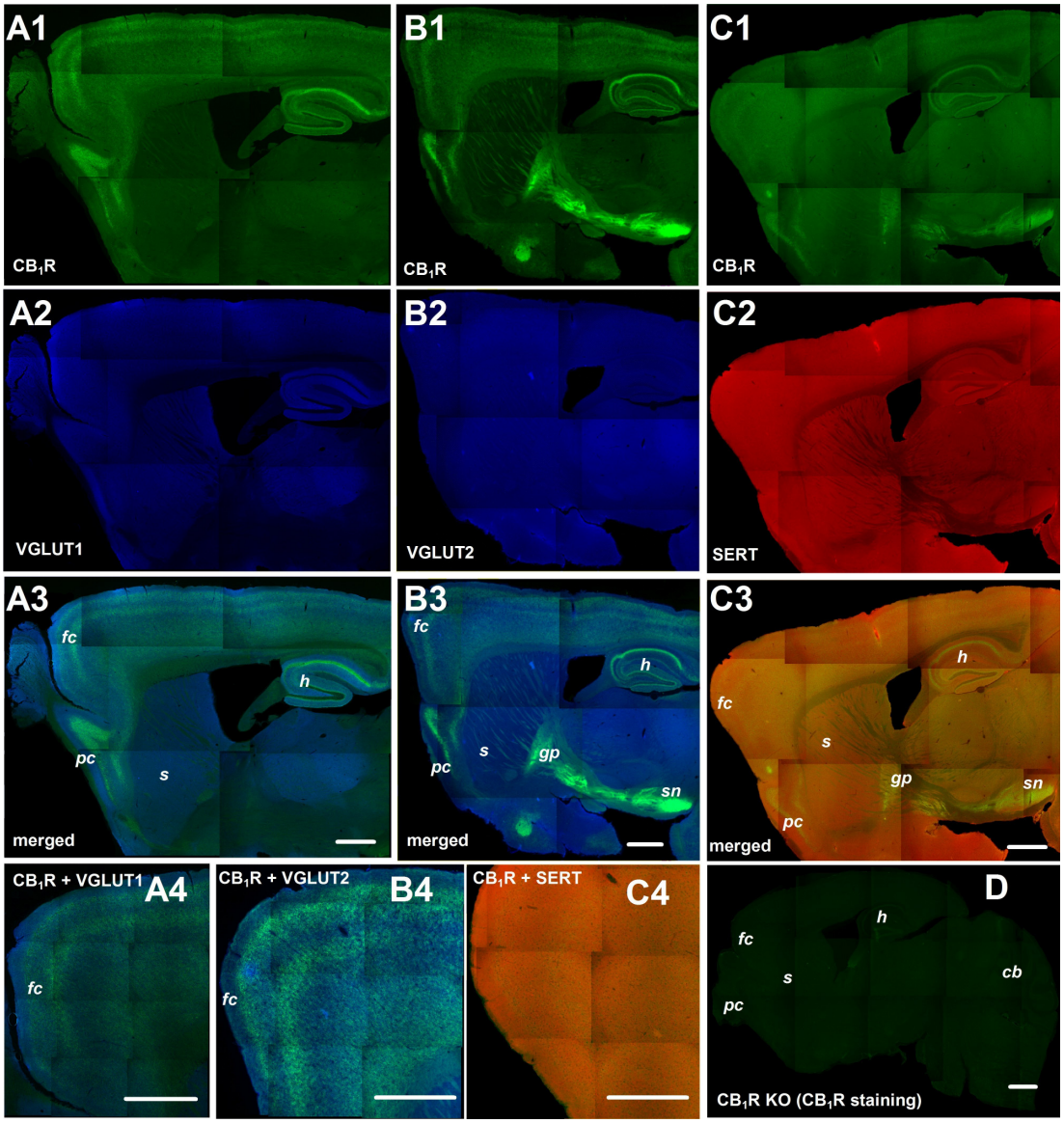

Figure 2. (A1-3, B1-3, C1-3) Low magnification (5 ×) fluorescent microscope images showing the distribution of the CB_1R immunoreactivity in mouse sagittal brain sections and (A4, B4, C4) at 20 × magnification in the frontal cortex. CB_1R staining is overt in the substantia nigra (sn), in the globus pallidus (gp), in the pyramidal cell layer of the hippocampus (hip) and in the cortex, including the frontal associative cortex (fac) and the pyriform cortex (pc), while is modest in the caudate-putamen (s) and is absent in the corpus callosum (cc). (C) No immunostaining was detected in the CB_1R knockout mouse brain slice (Figure 1 D4; cb: cerebellum). Among the cortical layers, layers II-III, Va and VI stained strongly with CB1Rs. Horizontal bars represent 1 mm.

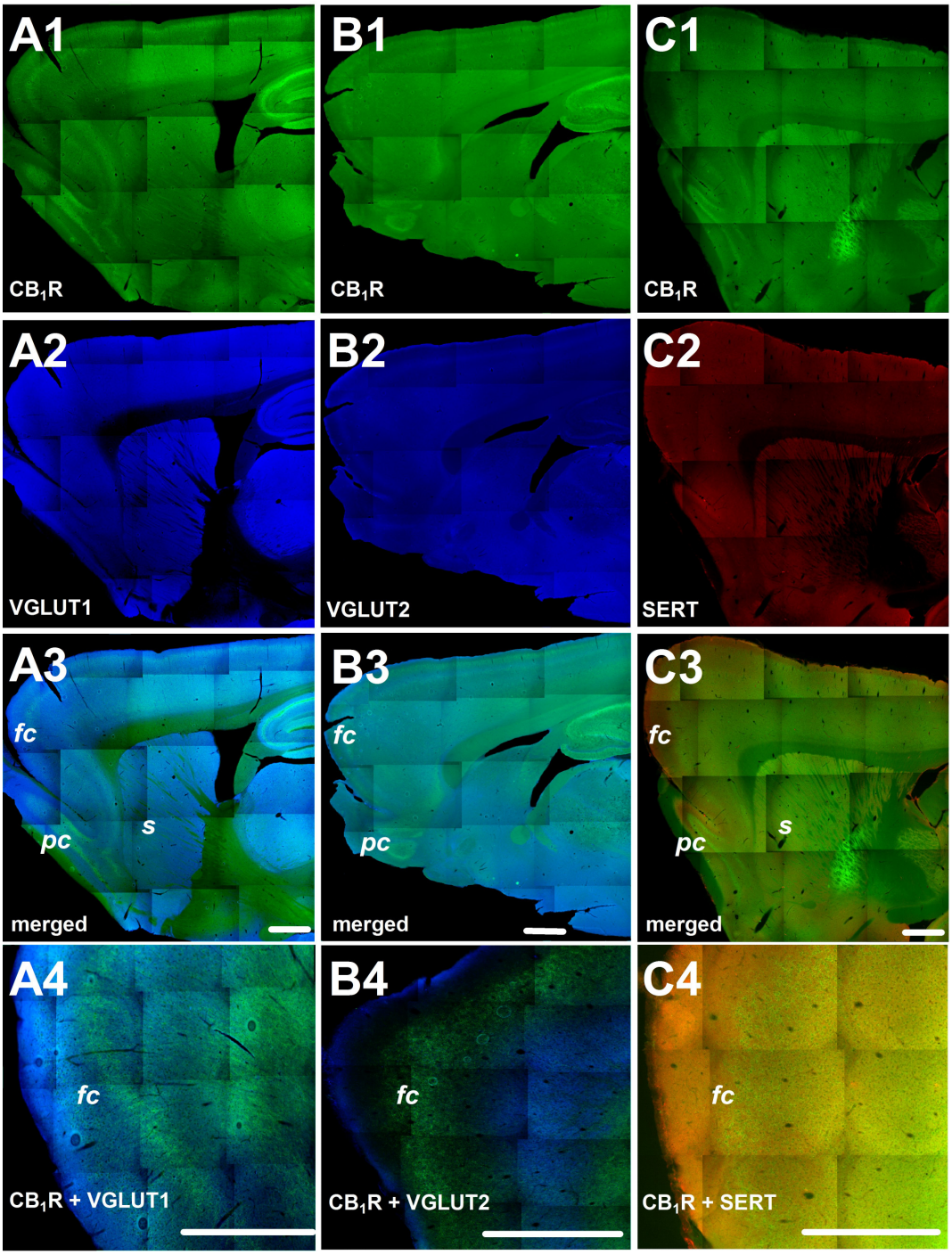

Figure 3. (A1-3, B1-3, C1-3) Low magnification (5 ×) fluorescent microscope images showing the distribution of the CB_1R immunoreactivity in rat sagittal brain sections and (A4, B4, C4) at 20 × magnification in the frontal cortex. CB_1R staining is essentially the same as in the mouse. For the brain region labels see the legend of Figure 1. Horizontal bars represent 1 mm.

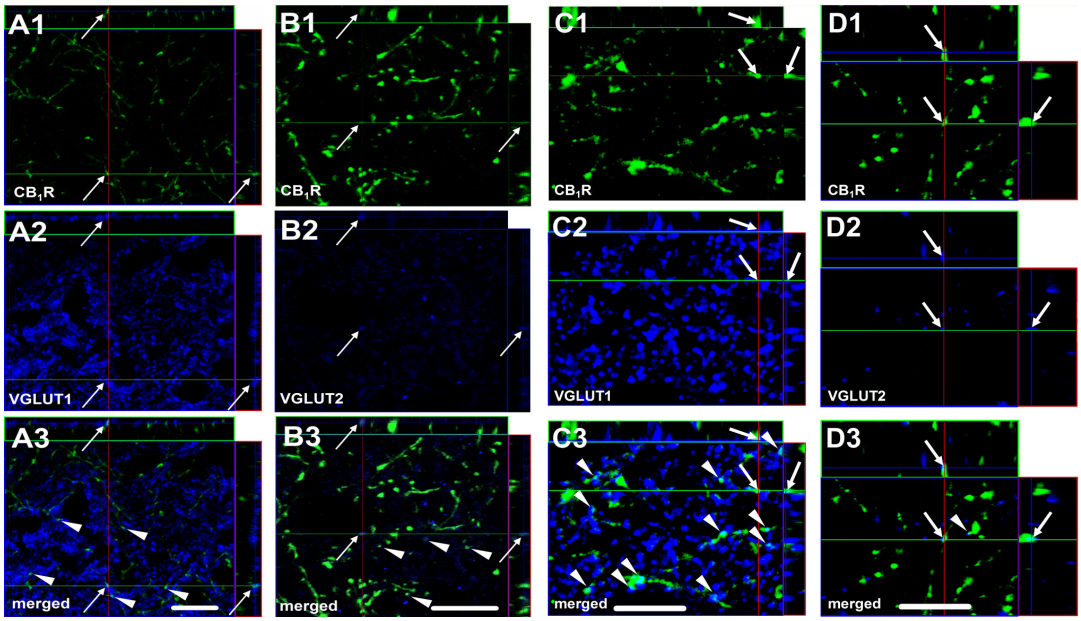

Figure 4. CB_1R is present in VGLUT1-positive terminals, and to a smaller extent, in VGLUT2-positive terminals in the mouse (A1-3, B1-3) and rat (C1-3, D1-3) frontal cortex (confocal microscopy images; 63x/1.40 oil DIC M27, 1 × zoom, at 1024 dpi resolution). Co-localization is detected from all the three directions in the focus of the cross hair of the orthogonal projection of the 380 nm-thin optical section (marked with small arrows). Full arrowheads point to other co-localizations in the same field (A3-D3). Horizontal bars represent 10 µm.

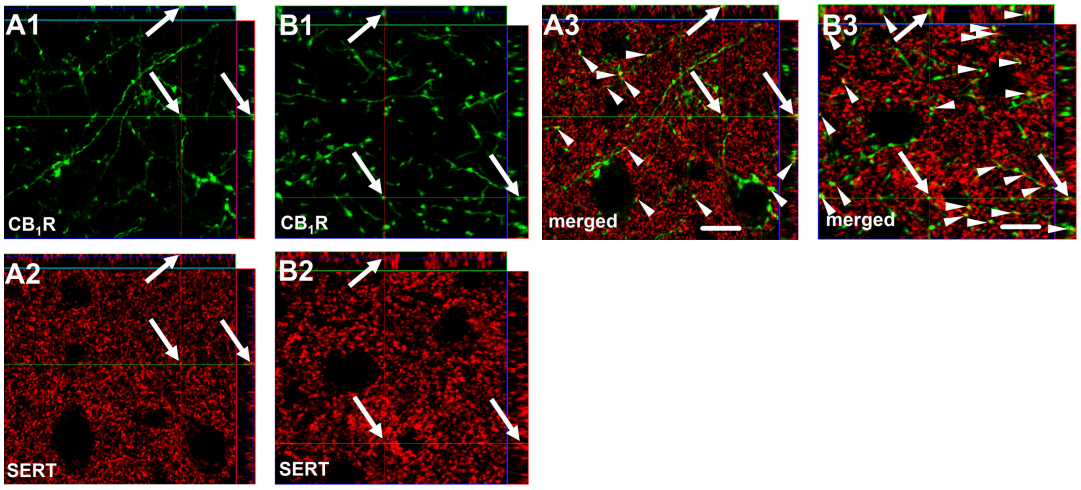

Figure 5. CB_1R is present in SERT-positive terminals in the mouse (A1-3) and rat (B1-3) frontal cortex (confocal microscopy images; 63x/1.40 oil DIC M27, 1 × zoom, at 1024 dpi resolution). Co-localization is detected from all the three directions in the focus of the cross hair of the orthogonal projection of the 380

nm-thin optical section (marked with small arrows). Full arrowheads point to other co-localizations in the same field (A3-D3). Horizontal bars represent 10 µm.

The layered CB_1R staining is somewhat mimicked by $\alpha_{2A}R$-stanining regarding that $\alpha_{2A}R$-positive neurons (possible pyramidal cells; Figure 6C) appear in the layers II-III together with the CB_1R, but less in layer IV, and starting again from layer Va through Vb and VI. This in clear contrast with DβH staining at low resolution which appears almost as background staining but at higher resolution, a meshwork of sometimes convoluted DβH-positive varicose fibers can be detected, such as the one in panel 6E. CB_1R-positive elements sparsely co-localized with $\alpha_{2A}R$ staining, forming likely presynapses connected to layer VI cells (**Figure 7B**). Although double or triple co-localizations were not evident (see e.g. Figure 6E), confocal microscopy analysis could detect triple co-localization, i.e. $\alpha_{2A}R$ staining in CB_1R-positive noradrenergic terminals (**Figure 7C4**).

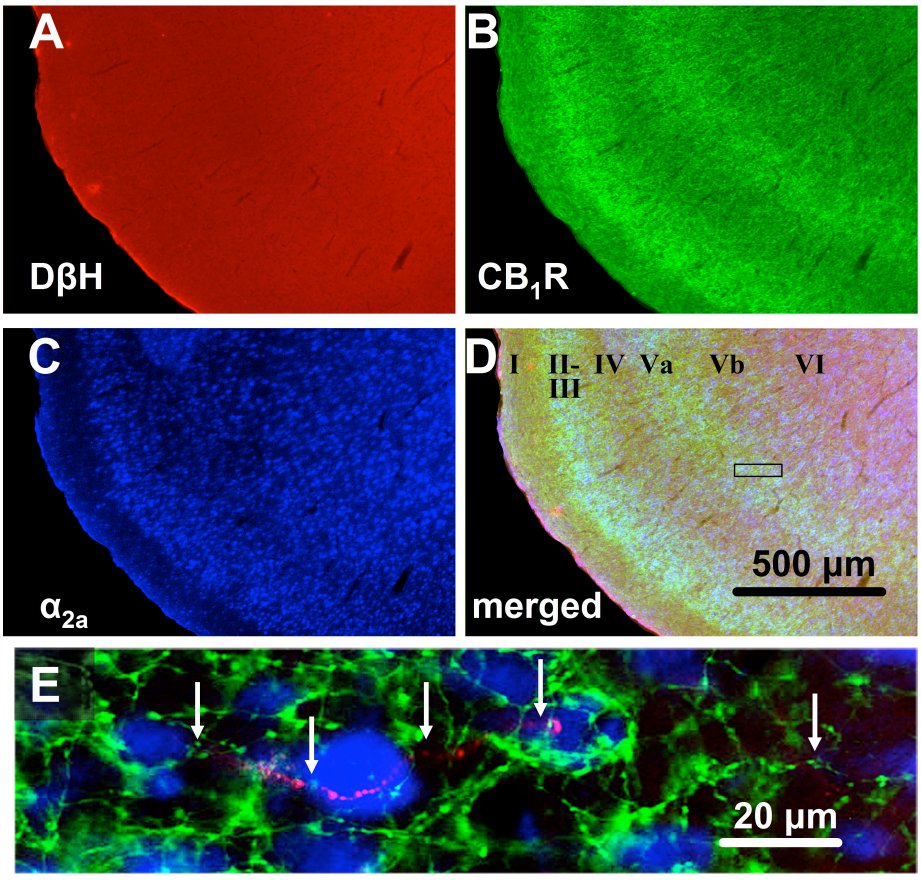

Figure 6. Immunostaining in the rat frontal cortex. DβH (A, red), CB_1R (B, green), $\alpha_{2A}R$ (C, blue) and merged signal in the frontal cortex of the rat (30 µm-thick sagittal slice; 5 × magnification). In panel D, the cortical layers are marked with roman numbers. The little black area in the center of panel D is amplified in panel E. In this panel, a DβH-positive varicose axon (red) is followed by vertical arrows, among $\alpha_{2A}R$-positive cell bodies (putative pyramidal cells, blue), and CB_1R-positive axons (green)

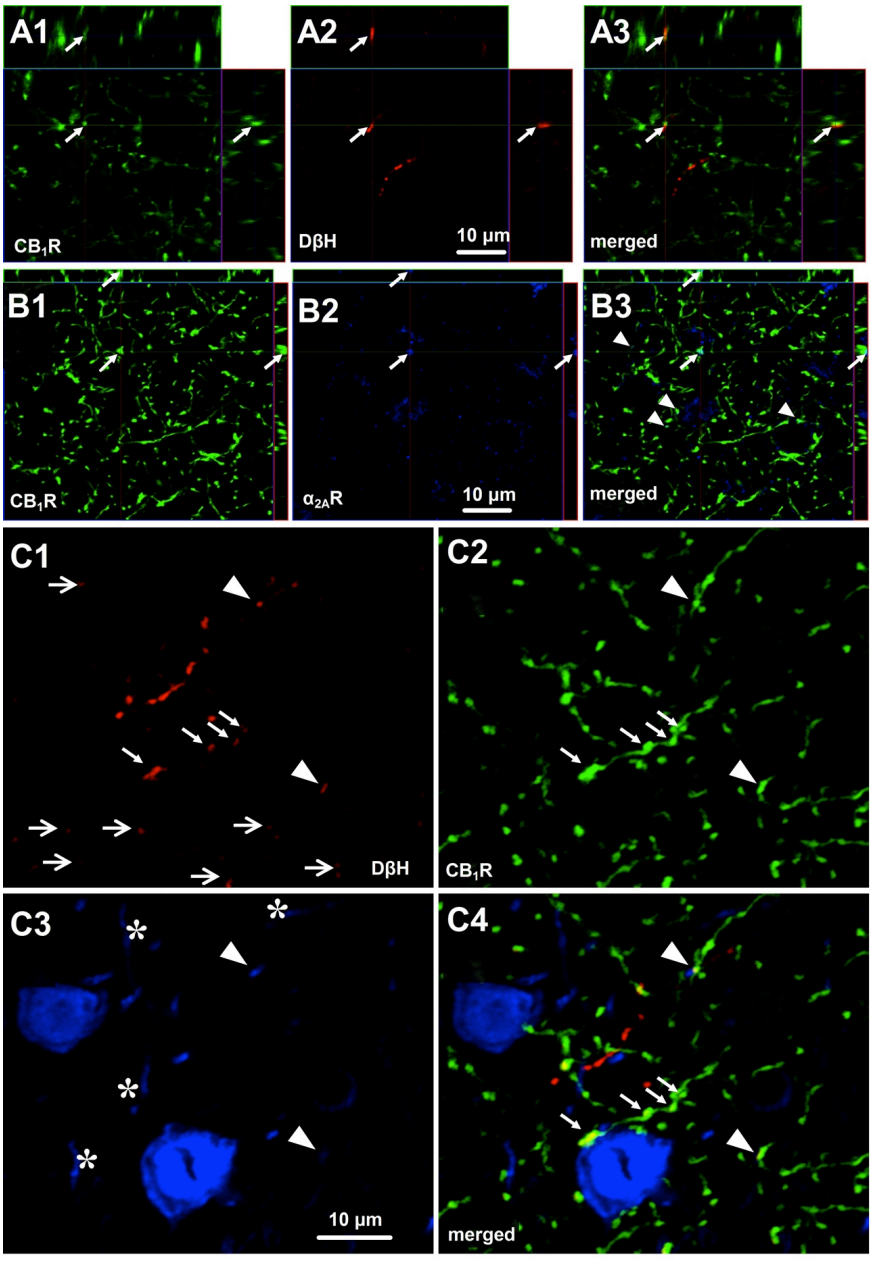

Figure 7. CB_1R, DβH and $\alpha_{2A}R$ co-localization revealed by confocal microscopy in the rat frontal cortex. (A1-3) CB_1R (green; A1), DβH (red; A2) and merged signal (A3) in the layer VI of the frontal cortex of the rat. The yellow composite color is detected from all the three directions in the focus of the cross hair of the orthogonal projection of the 380 nm-thin optical section (marked with small arrows). (B1-3) CB_1R (green; B1), $\alpha_{2A}R$ (blue; B2) and merged signal (B3) in the layer II of the frontal cortex of the rat. Co-localization appears in turquoise from all the three directions in the focus of the cross hair, and is marked with small arrows. Full arrowheads point to other co-localizations in the same field (B3). (C1-4) Triple co-localization among DβH (red; C1) CB_1R (green; C2), and $\alpha_{2A}R$ (blue; C3). In panels (C1, 2, 4), the inclined small arrows point to CB_1R-positive varicosities of a DβH-positive fiber innervating an $\alpha_{2A}R$-positive cell. In this region, several other varicosities were detected with CB_1R staining (marked with horizontal, open arrows in C1). In panel (C3), asterisks label $\alpha_{2A}R$-positive, fiber-like structures (see also Figure 2C). Throughout the four panels, full arrowheads point to sparsely appearing triple colocalizations. All images represent 380 nm-thin optical sections, photographed with a confocal microscope in a sagittal slice (63x/1.40 oil DIC M27, 1 × zoom, at 1024 dpi resolution).

4.2 Functional assay

CB_1R activation decreases presynaptic transmitter release by inhibiting various Ca^{2+} channels (Twitchell et al., 1997), thus one needs a robust Ca^{2+}-dependent release assay to reliably assess CB_1R function. Usually, the stronger the release stimulus the greater and the more reliable the release, however, with increasing release the Ca^{2+}-dependency decreases. Thus first, we optimized the simultaneously measured 4-AP-evoked [^{14}C]glutamate (**Figure 8A**) and [^{3}H]serotonin (**Figure 8B**) release from rat frontocortical synaptosomes for the size of the stimulus versus Ca^{2+}-dependency. We chose stimulation with 4-AP at 300 μM concentration based on the peak amplitude (**Figure 8C**) and reasonable Ca^{2+}-dependency (**Figure 8D**). Notably, we used 100 nM Ca^{2+} instead of the omission of Ca^{2+} to preserve membrane integrity and to diminish Na+ entry through open voltage-gated Ca^{2+} channels in the “calcium-free” condition.

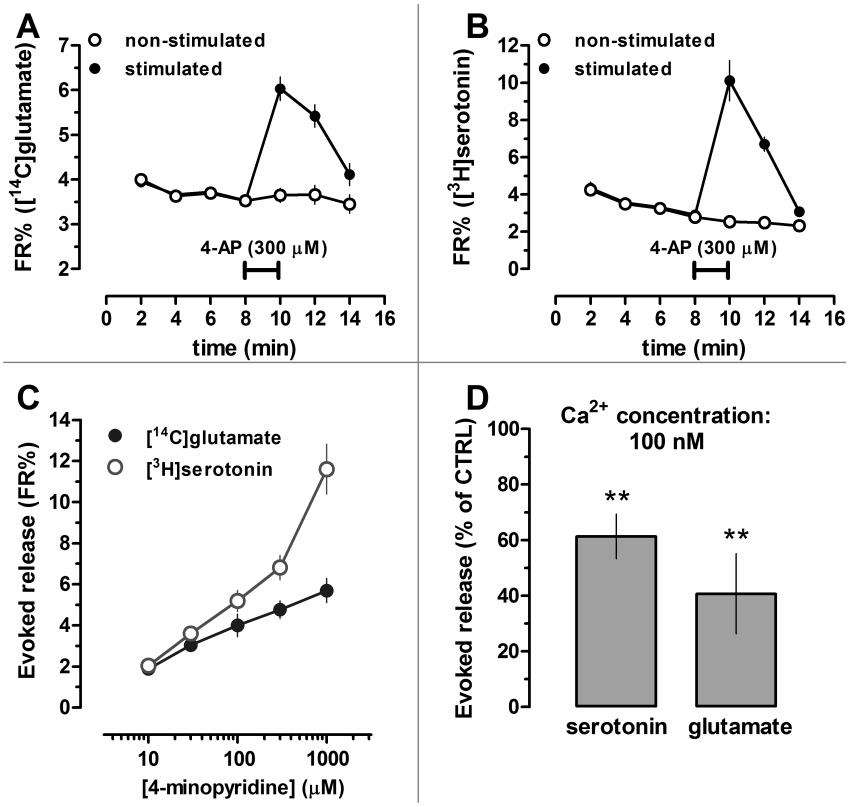

Figure 8. Optimization of the 4-aminopyridine (4-AP)-evoked and simultaneously measured [^{14}C]glutamate/ [^{3}H]serotonin release from rat frontocortical synaptosomes. (A,B) Release diagrams showing the time-course of unstimulated and stimulated (with 300 μM 4-AP for 2 min) release of both transmitters. (C) The concentration-dependent effect of 4-AP on the release of [^{14}C]glutamate and [^{3}H]serotonin. (D) Ca^{2+}-dependency of the 4-AP- (300 μM) evoked release of [^{14}C]glutamate and [^{3}H]serotonin. N ≥ 6, * $p < 0.05$.

The synthetic CB_1R agonist, WIN55212-2 (1 μM) inhibited the evoked release of [^{14}C]glutamate by 35.0 ± 4.7% (n = 8; $p < 0.05$) and [^{3}H]serotonin by 17.4 ± 5.3% (n = 8; $p < 0.05$) (**Figure 9**). The amplitude of the inhibition depends on the size of the Ca^{2+} dependent fraction. Since 38.7 ± 8.0% (n = 6) was the Ca^{2+} dependent fraction for [^{3}H]serotonin and 49.7 ± 17.8% for [^{14}C]glutamate, the altogether inhibitory action of WIN55212-2 on the Ca^{2+}-dependent release is ~44% and ~70% for serotonin and glutamate, respectively.

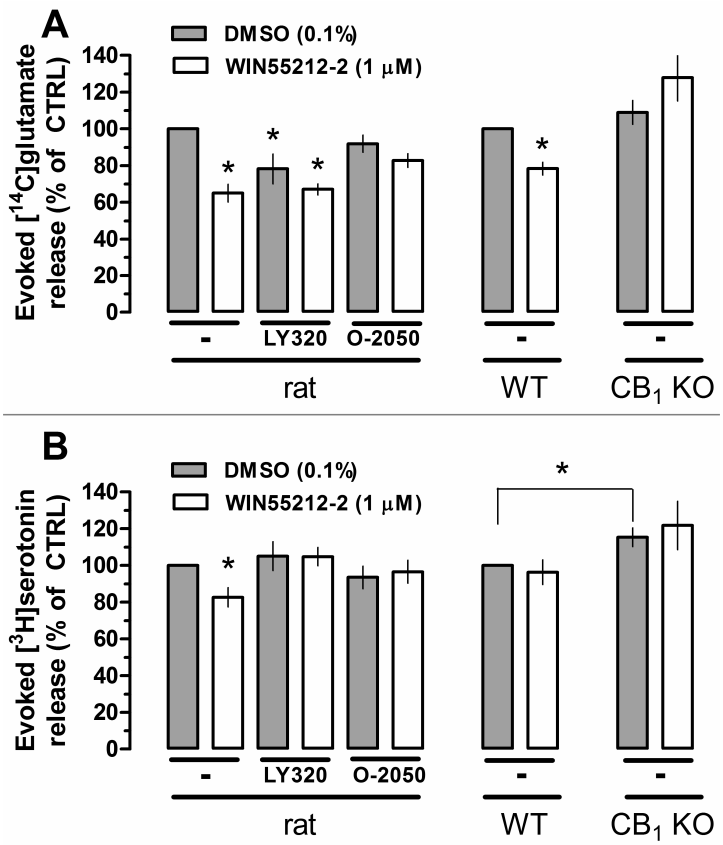

Figure 9. The synthetic CB_1R agonist, WIN55212-2 (1 μM) diminishes the evoked release of both [^{14}C]glutamate (A) and [^{3}H]serotonin (B) in the rat, in a manner sensitive to selective CB_1R antagonists, LY320135 (5 μM) and O-2050 (1 μM). Interestingly, the control evoked release of [^{3}H]serotonin but not that of [^{14}C]glutamate was greater in the CB_1R global knockout mouse. $N \geq 6$, * $p < 0.05$.

Both the low-potency CB_1R inverse agonist, LY320135 (5 μM) and the highly potent and selective neutral CB_1R antagonist, O-2050 (1 μM) abolished the effect of WIN55212-2 on the evoked release of [^{3}H]serotonin (**Figure 9B**). O-2050 in the same experiments also prevented the inhibitory action of WIN55212-2 on the evoked release of [^{14}C]glutamate (**Figure 9A**). However, LY320135 itself had a 21.8 ± 8.2% inhibitory action alone. Although the inhibitory action of WIN55212-2 was largely decreased by LY320135 it still remained statistically significant versus LY320135 alone.

The parameters of the resting and evoked [^{14}C]glutamate and [^{3}H]serotonin releases were not significantly different in the CD-1 mouse form those in the Wistar rat. The amplitude of the evoked [^{14}C]glutamate release was not different between the wild-type (WT) and the CB_1R KO mouse (n = 9, n.s.; **Figure 9A**). However, the inhibitory action of WIN55212-2 (1 μM) was totally absent in the

CB_1R KO mouse (n = 9). In contrast, and to our surprise, CB_1Rs in the serotonergic terminals of the mouse were endogenously active because WIN55212-2 could not induce further inhibition of evoked release of [^{3}H]serotonin, and the evoked release of [^{3}H]serotonin was 15.3 ± 4.8% (n = 10; $p < 0.05$) greater in the CB_1R KO mouse. This difference is actually similar to the size of inhibition by WIN5512-2 of evoked [^{3}H]serotonin release.

5. DISCUSSION

Previous studies reported interaction between the endocannabinoid and the serotonergic systems in the brain. This interaction is bidirectional at different levels, involving various direct and indirect mechanisms and brain areas (see below). To our knowledge, this study is the first showing the presence of presynaptic functional CB_1Rs in serotonergic nerve terminals of the frontal cortex. We showed here that CB_1R activation decreases the Ca^{2+}-dependent release of [^{3}H]serotonin from frontocortical nerve terminals. Previous electrophysiological studies have shown that CB_1R activation inhibits some glutamatergic transmission in the frontal cortex of the rat and mouse (Auclair et al., 2000; Barbara et al., 2003; Lafourcade et al., 2007). Interestingly, it was never mapped in the frontal cortex if CB_1Rs are present in VGLUT1- or VGLUT2-positive terminals, or both. This question has been answered now by our study.

Importantly, the distribution of CB_1R immunoreactivity in the sagittal slices both at low and high resolution was essentially the same as in previous reports using either autoradiography (Herkenham et al., 1991), or immunohistochemistry with light microscopy (Tsou et al., 1998) and fluorescent/ confocal microscopy (Bodor et al., 2005). Although the anatomical description of the endocannabinoid system follows a generally consistent pattern throughout studies and assays, this is not quite true for the physiology and pharmacology. In fact, it is more the exception than the rule when a cannabinoid pharmacology study runs by the book; due to the increasing number of discovered and cloned cannabinoid receptors and the question of ligand selectivity (Köfalvi, 2008). The neutral CB_1R antagonist, O-2050 abolished the effect of WIN55212-2 without having effect per se on the release of serotonin and glutamate. The CB_1R inverse agonist LY320135 did the same on the release of serotonin, but already inhibited per se the release of glutamate and competitively antagonized the effect of WIN55212-2. This latter might be the result of that LY320135 is capable of binding to muscarinic and serotonin receptors in the low micromolar range (Felder et al., 1998). The other, actually more intriguing, finding was that while on both nerve terminal types in the rat and in the glutamatergic terminals in the mouse the CB_1Rs were free from an endogenous tone – as one would expect in superfused synaptosomes – the CB_1Rs appeared to be endogenously active in the serotonergic terminals of the mouse. This would not be the first report on constitutively active CB_1Rs in the brain (see e.g. Hentges et al., 2005), but it is unexpected in superfused nerve

terminals. But then again, understanding that the majority of serotonergic terminals are actually varicosities and the serotonergic communication is rather non-synaptic, the origin and identity of endocannabinoids acting at these terminals/ varicosities are not expected to be the same as for a glutamatergic synapse. We assume that it well might be an autocrine regulation, i.e. these terminals themselves may release endocannabinoids locally. If it is true then it could be partially understood how CB_1Rs can be kept activated in the synaptosomes. The overt difference between the rat and the mouse is also not surprising knowing that there are serious differences even among mouse substrains in those behavioral assays which involve the serotonergic system of the mouse brain (Matsuo et al., 2011). Importantly, *in vivo* there is evidence for that constitutively active CB_1Rs inhibit medial frontocortical serotonin release, as assessed by microdialysis (Tzavara et al., 2003). Additionally, the serotonergic neurons of the raphe nuclei (the origin of the serotonergic fibers of the whole brain) indeed express CB_1R mRNA (Häring et al. 2007) and endocannabinoids release machinery (Haj-Dahmane and Shen, 2009). Thus, theoretically everything is given for a serotonergic neuron to release endocannabinoids to activate its own CB_1Rs.

Interestingly, endocannabinoids do not necessarily need to activate putative CB_1Rs in raphe serotonergic neurons to inhibit serotonergic activity: endocannabinoids acting at presynaptic CB_1Rs in excitatory afferents of other neurons can indirectly inhibit the activity of those neurons. For instance, Haj-Dahmane and Shen (2009) showed that glutamate induces endocannabinoid release from dorsal raphe serotonergic neurons, which in turn decreases further glutamate release. This process is termed depolarization-induced inhibition of excitation or DSE. Thus, CB_1R activation decreases the excitatory input of serotonergic neurons and thereby indirectly decreases serotonin release in the brain. Notably, local glutamate release onto serotonergic fibers (Balázsa et al., 2008) can be also suppressed by CB_1R activation, which further contributes to indirect inhibition of serotonergic activity by endocannabinoids. Such an indirect mechanism could have also contributed to the inhibition by CB_1Rs of the electrically stimulated [^{3}H]serotonin release from cortical slices (Nakazi et al., 2000). Balázsa et al. (2008) elegantly showed that NMDA channel blockade is necessary to reveal CB_1R-mediated direct inhibition of depolarization-induced [^{3}H]serotonin release in the hippocampal slice.

The interaction between the two neuromodulator systems can occur at other levels as well: Cannabinoids are capable of directly inhibiting the serotonin 5-HT_3 channel (Barann et al., 2002; Xiong et al., 2007; Köfalvi, 2008). This together with the direct inhibition of serotonin release by

presynaptic CB_1Rs can be a candidate mechanism for the antiemetic action of cannabinoids (Parker and Limebeer; 2008). The receptor antagonism stretches beyond channel blockade: the natural cannabis constituent cannabigerol is a weak 5-HT_{1A} antagonist (Cascio et al., 2010), while the other cannabis constituent, cannabidiol acts as a 5-HT_{1A} agonist (Ledgerwood et al., 2010). These can contribute to certain extra effects of cannabis beyond the CB_1R and CB_2R. The "non-selective interaction" between the two neuromodulador systems extends to the direct inhibition of the serotonin transporter by cannabinoids (Steffens and Feuerstein, 2003).

Altogether, the endocannabinoid and serotonergic neuromodulator systems anatomically are highly overlapping, and they control the release and the function of each other in a multilayered fashion. This explains why the two systems are so intricately involved in most physiological and pathological mechanisms of the body. To better understand sociopathies and psychiatric disorders, a greater effort is required to study the complex interaction between serotonin and endocannabinoids.

Our Hungarian collaborators examined whether cannabinoid receptors are involved in the modulation of electrical field stimulation evoked [^{3}H]noradrenalin release from rat frontocortical slices, in an experimental paradigm previously described by us (Köfalvi et al., 2005). Drs. Hardy Richter, Ágnes Kittel and Beáta Sperlágh (Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest) observed that WIN55212-2 (1 μM), inhibited the electrical stimulation-evoked efflux of [^{3}H]noradrenalin in a concentration-dependent fashion, which was sensitive to the CB_1R antagonist/ inverse agonist, AM251 (1 μM). In contrast, idazoxan, a selective α_{2A}R antagonist, augmented the evoked [^{3}H]noradrenalin release. Interestingly, the combined effect of WIN55212-2 and idazoxan was supraadditive both *in vitro*, on the release of [^{3}H]noradrenalin, and *in vivo*, on the forced swim behavior test. Furthermore, idazoxan triggered a decrease in CB_1Rs density in the frontal cortex, suggesting that high extracellular level of norepinephrine downregulates CB_1Rs. These all predict that CB_1Rs might interact with α_{2A}Rs in noradrenergic terminals. Indeed, I found with the help of fluorescent and confocal microscopy analysis that CB_1R immunoreactivity co-localizes in a subset of α_{2A}Rs in dopamine-β-hydroxilase-positive (i.e. noradrenergic) fibers in the frontal cortex. α_{2A}Rs are generally known to be presynaptic autoreceptors for noradrenergic fibers

6. CONCLUSIONS AND OUTLOOK

In my studies I successfully concluded the aimed investigations. I established both an immunochemical (i.e. static) and a functional (dynamic) approach to study frontocortical CB_1Rs with clearly positive controls, i.e. the presence of functional CB_1Rs in glutamatergic nerve terminals.

I thus describe here for the first time functional CB_1Rs in monoaminergic nerve terminals, underpinning the role of the endocannabinoid system in mood/ personality disorders. My data also corroborate the undesired side effect - suicide - of long-term Acomplia-intake; which resulted in the removal of the selective CB_1R antagonist, Acomplia from the market of antiobesity medicines (McPartland, 2009).

I found that there has surprisingly little been done to better understand the role of the monoaminergic systems in the (patho)physiology of the lower- and higher-order brain functions such as neurogenesis, metabolism or love. The enormous density of serotonergic fibers exceeding that of the CB_1R-positive fibers make me wonder if my research priorities has been set correctly before.

I intend to further pursue the role of serotonin in the brain, and to render more complex experimental approaches to my previous findings, including more sophisticated immunochemical approaches, selective CB_1R knockout animals in serotonergic neurons (a fresh collaboration between our laboratory and the group of Prof. Beat Lutz, Mainz, Germany), as well as in vivo test, respectively.

I believe that understanding the neuromodulation of neuromodulators (for instance, understanding how CB_1Rs control noradrenalin release) cannot be the objective of a follow-up study. Rather, I would aim at understanding personality disorders, and using my current knowledge on neuromodulator interactions as a tool.

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