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CANNABINOID TYPE 1 RECEPTORS LOCATED ON SINGLE-MINDED 1–EXPRESSING NEURONS CONTROL EMOTIONAL BEHAVIORS

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Abstract—This study has investigated the role of hypothalamic and amygdalar type-1 cannabinoid (CB1) receptors in the emotional and neuroendocrine responses to stress. To do so, we used the Cre/loxP system to generate conditional mutant mice lacking the *CB1* gene in neurons expressing the transcription factor single-minded 1 (*Sim1*). This choice was dictated by former evidence for *Sim1*-Cre transgenic mice bearing Cre activity in all areas expressing *Sim1*, which chiefly includes the hypothalamus (especially the paraventricular nucleus, the supraoptic nucleus, and the posterior hypothalamus) and the mediobasal amygdala. Genomic DNA analyses in Sim1-CB1$^{-/-}$ mice indicated that the *CB1* allele was excised from the hypothalamus and the amygdala, but not from the cortex, the striatum, the thalamus, the nucleus accumbens, the brainstem, the hippocampus, the pituitary gland, and the spinal cord. Double-fluorescent *in situ* hybridization experiments further indicated that Sim1-CB1$^{-/-}$ mice displayed a weaker CB1 receptor mRNA expression in the paraventricular nucleus of the hypothalamus and the mediobasal part of the amygdala, compared to wild-type animals. Individually housed Sim1-CB1$^{-/-}$ mice and their Sim1-CB1$^{+/+}$ littermates were exposed to anxiety and fear memory tests under basal conditions as well as after acute/repeated social stress. A principal component analysis of the behaviors of Sim1-CB1$^{-/-}$ and Sim1-CB1$^{+/+}$ mice in anxiety tests (open field, elevated plus-maze, and light/dark box) revealed that CB1 receptors from Sim1-expressing neurons exert tonic, albeit opposite, controls of locomotor and anxiety reactivity to novel environments. No difference between genotypes was observed during the recall of contextual fear conditioning or during active avoidance learning. Sim1-CB1$^{-/-}$, but not Sim1-CB1$^{+/+}$, mice proved sensitive to an acute social stress as this procedure reverted the increased ambulation in the center of the open field. The stimulatory influence of repeated social stress on body and adrenal weights, water intake, and sucrose preference was similar in the two genotypes. On the other hand, repeated social stress abolished the decrease in cued-fear conditioned expression that was observed in Sim1-CB1$^{-/-}$ mice, compared to Sim1-CB1$^{+/+}$ mice. This study suggests that CB1 receptors located on *Sim1*-expressing neurons exert a tonic control on locomotor reactivity, unconditioned anxiety, and cued-fear expression under basal conditions as well as after acute or repeated stress.

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Key words: anxiety, CB1 receptor, conditioned fear, locomotor reactivity, single-minded 1, social defeat stress.

The endocannabinoid system (ECS), formed by cannabinoid receptors (CB1 and CB2), their endogenous lipid ligands (endocannabinoids, ECBs), and the machinery for synthesis and degradation of ECBs plays a key role in the psychoneuroendocrine responses to stress (Patel and Hillard, 2008; Steiner and Wotjak, 2008; Hill et al., 2010). One argument in support of this statement stems from the location of cannabinoid type 1 (CB1) receptors, which is the main cannabinoid receptor type through which the ECS exerts its functions in neurons and glial cells. This receptor is found in a vast array of brain regions/nuclei that are involved in the regulation of psychoneuroendocrine responses to stress, including anxiety, fear, and adrenocortical and sympathetic activation. These regions/nuclei include cortical areas, the basal ganglia, the periaqueductal gray, and the paraventricular nucleus of the hypothalamus (PVN; Herkenham et al., 1990; Glass et al., 1997; Tsou et al., 1998; Katona et al., 1999; Marsicano and Lutz, 1999). This essential role of CB1 receptors is mainly accounted for by their strategic location on the presynaptic terminals of numerous neuronal types where they retrogradely and negatively control transmitter release (Alger, 2002; Piomelli, 2003; Chevaleyre et al., 2006; Marsicano and Lutz, 2006). This holds true for numerous neuronal systems endowed with emotional, cognitive, or neuroendocrine regulatory impacts on stress responses. A second argument for a role of the ECS in the modulation of stress responses derives from pharmacological findings. Direct selective CB1 receptor agonists and antagonists as well as drugs affecting the synthesis or the degradation of endocannabinoids (i.e. indirect CB1 receptor effectors) all exert psychological and/or neuroendocrine effects (Wotjak, 2005; Pagotto et al., 2006; Lafenêtre et al., 2007; Steiner and Wotjak, 2008; Lutz, 2009). However, the systemic use of CB1 receptor ligands and chemicals affecting endocan-

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Abbreviations: BNST, bed nucleus of the stria terminalis; CB1, cannabinoid type 1; CRH, corticotropin-releasing hormone; D-FISH, double-fluorescent *in situ* hybridization; ECS, endocannabinoid system; HPA, hypothalamo-pituitary-adrenal axis; LH, lateral hypothalamus; MBA, mediobasal amygdala; MeCP2, methyl-CpG-binding protein 2; PCR, polymerase chain reaction; PH, posterior hypothalamus; PVN, paraventricular nucleus of the hypothalamus; Sim 1, single-minded one; SON, supraoptic nucleus.

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nabinoid synthesis/degradation does not provide information on which, how, and when particular physiological events stimulate the ECS. Such an uncertainty extends to the identity of the brain regions and/or neuronal networks where CB1 receptors are stimulated during these events.

One means through which these questions may be examined is the use of conditional mutants for the CB1 receptor using the Cre/lox P strategy. Albeit not devoid of limits (e.g. developmental effects of the mutation, surge of compensatory mechanisms to overcome the consequences of the mutation), the use of mutants wherein CB1 receptors are selectively deleted from restricted neuronal populations may help to further dissect the role of central CB1 receptors in the modulation of CNS functions, including emotionality (Marsicano et al., 2003; Monory et al., 2006; Jacob et al., 2009; Lafenêtre et al., 2009; Puighermanal et al., 2009; Bellocchio et al., 2010).

As mentioned, CB1 receptors are present in the PVN, a nucleus of prime importance in the management of the behavioral and neuroendocrine responses to stress. Therein, CB1 receptors, which play a regulatory role on the activity of the hypothalamo–pituitary–adrenal (HPA) axis (Di et al., 2003; Cota et al., 2007), have been shown to be sensitive to repeated stress procedures under conditions excluding adaptation to the stressor (Wamsteeker et al., 2010). However, whether CB1 receptors located in this nucleus play a direct role on emotional processes is unknown. To answer this question, we crossed "floxed" CB1 mutant mice (Marsicano et al., 2003) with transgenic mice bearing Cre expression under the control of the regulatory sequences of the *single-minded 1* (Sim1) gene. *Sim1* is one mammalian homolog of the drosophila transcription factor *sim* that is highly expressed during development and postnatally in the PVN/supraoptic nucleus (SON), where it exerts a key control of the neuroendocrine and metabolic balance (Fan et al., 1996; Michaud et al., 1998; Balthasar et al., 2005). Indeed, mice deficient for *Sim1* die early due to total PVN dysfunction, while *Sim1* heterozygous mice display obesity and hyperphagia (Michaud et al., 2001). In addition to its neuroendocrine and metabolic impact, Sim1 controls emotional behaviors, as a selective deletion of, for eg, methyl-CpG-binding protein 2 (MeCP2) from Sim1-expressing neurons triggers anxiety, as assessed in the open field, and aggressivity (Fyffe et al., 2008). These phenotypic changes could however involve additional brain regions as Sim1 is present in the nucleus of the olfactory tract and the posterior hypothalamus (PH; Balthasar et al., 2005). Although weakly expressed, Sim1 is also present in the lateral hypothalamus (LH), the mediobasal amygdala (MBA), the bed nucleus of the stria terminalis (BNST), and the periaqueductal gray (Balthasar et al., 2005), that is, brain areas involved in the control of innate and/or conditioned behavioral responses to aversive stimuli (Guimarães et al., 2005).

In keeping with all these observations, we aimed at comparing the behavioral consequences of the deletion of CB1 receptors from Sim1-expressing neurons (Sim1-CB1$^{-/-}$), as compared to their wild-type controls (Sim1-CB1$^{+/+}$). To do so, we phenotyped individually housed Sim1-CB1$^{+/+}$ and Sim1-CB1$^{-/-}$ mice with respect to several emotionality dimensions under basal and acute/repeated stress conditions. Thus, we first compared the two genotypes under baseline conditions with respect to (i) unconditioned anxiety in the open field, the elevated plus-maze, and the light/dark box, that is, classic anxiety tests, (ii) contextual fear memory, as assessed by the freezing response to the placement in an environment that was associated 24 h beforehand to a single footshock exposure, and (iii) active coping with a conditioned threat as assessed by means of an active avoidance test. Thereafter, Sim1-CB1$^{+/+}$ and Sim1-CB1$^{-/-}$ mice were compared for their behavioral responses to social defeat stress. Social stress, a model we have used in the past (Berton et al., 1998, 1999), was chosen because it bears high ethological validity (Buwalda et al., 2005; Miczek et al., 2008), including in the human species where it is a major precipitating factor in numerous psychiatric illnesses (Huhman, 2006). To this aim, we monitored body weight, food intake, water intake, sucrose preference, unconditioned anxiety, and cued-fear memory after acute and/or repeated social stress. In addition, we compared the adrenocorticotropic reactivity to repeated social defeats in both genotypes by examining the hyperplasic consequences of such a stress procedure on the adrenal glands of Sim1-CB1$^{+/+}$ and Sim1-CB1$^{-/-}$ mice.

EXPERIMENTAL PROCEDURES

Animals

Sim1-Cre expressing mice (The Jackson Laboratory, Bar Harbor, ME, USA) were crossed with homozygous *CB1*-flox mice (*CB1*$^{f/f}$) (Marsicano et al., 2003) to obtain heterozygous Cre-expressing/*CB1*-flox mice (*CB1*$^{Sim1\text{-}Cre;f/+}$, step 1). These mice were again crossed with *CB1*$^{f/f}$ to obtain homozygous *CB1*$^{Sim1\text{-}Cre;f/f}$ mice (step 2). Male mice from step 2 were finally bred with *CB1*$^{f/f}$ females to generate littermate experimental animals (*CB*$_1^{Sim1\text{-}Cre;f/f}$ and *CB*$_1^{f/f}$, called thereafter Sim1-CB$_1^{-/-}$ and Sim1-CB$_1^{+/+}$, respectively; step 3). Genotyping for the Cre transgene was performed by polymerase chain reaction (PCR) using the following primers: forward: 5′-GATCGCTGCCAGGATATACG; reverse: 5′-CATCGCCATCTTCCAGCAG. Genotyping/regenotyping for the CB1-floxed locus, which was performed at 1 week of age and at the end of the experiments, was assessed as described previously (Marsicano et al., 2003).

Male wild-type (Sim1-CB1$^{+/+}$) and mutant (Sim1-CB1$^{-/-}$) littermate mice were aged 2–4 months at the start of the experiments. All mice were housed individually 1 week prior to the experiments and provided with food and water *ad libitum* under a 12-h light/dark cycle (lights on at 07:00 h). For social stress protocols, individually housed male CD1 mice, purchased from Charles River (L'Arbresle, France), were used. These animals, which arrived in the animal facilities 6–12 months earlier, were housed according to the environmental conditions described for Sim-CB1 mice. All CD1 mice had been selected on their ability to attack an intruder with an initial latency shorter than 20 s.

Pain and discomfort of the animals were reduced to minimum in strict compliance with European directives and French laws on animal experimentation (authorization number 06369).

Biochemical procedures

Genomic DNA analyses. To check for the effective deletion of CB1 receptors from the hippocampus and the amygdala and to