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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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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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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

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-

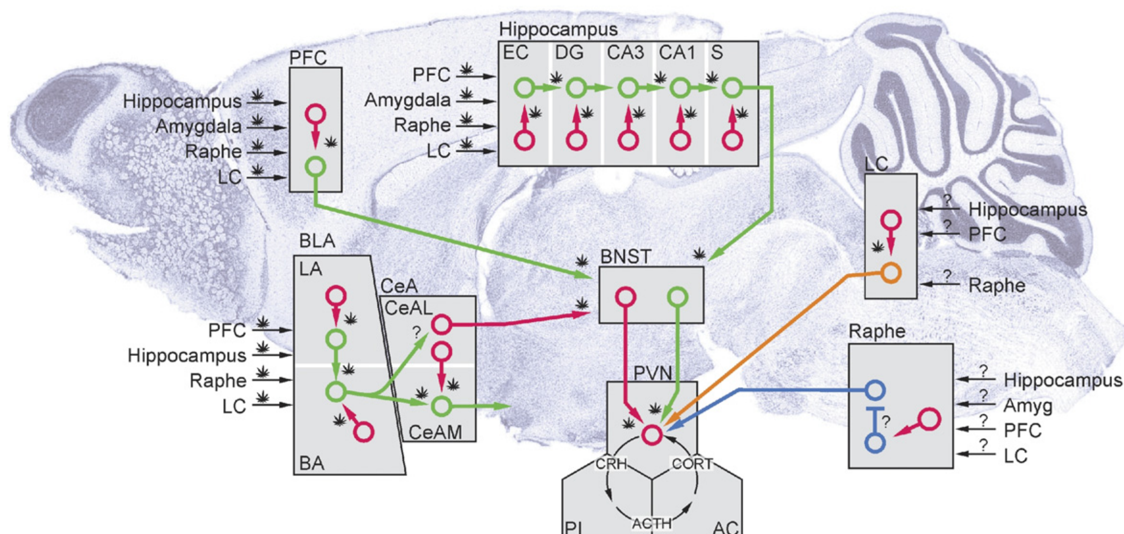

Fig. 1. Schematic illustration of the CB1 receptor distribution within the main stress circuits. The CB1 receptor, indicated as a *Cannabis sativa* leaf, is found at a majority of synaptic connections within and between each major brain region related to the activity of the hypothalamic-pituitary-adrenal (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,

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).

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

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 "bi-directional" 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-

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).

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 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

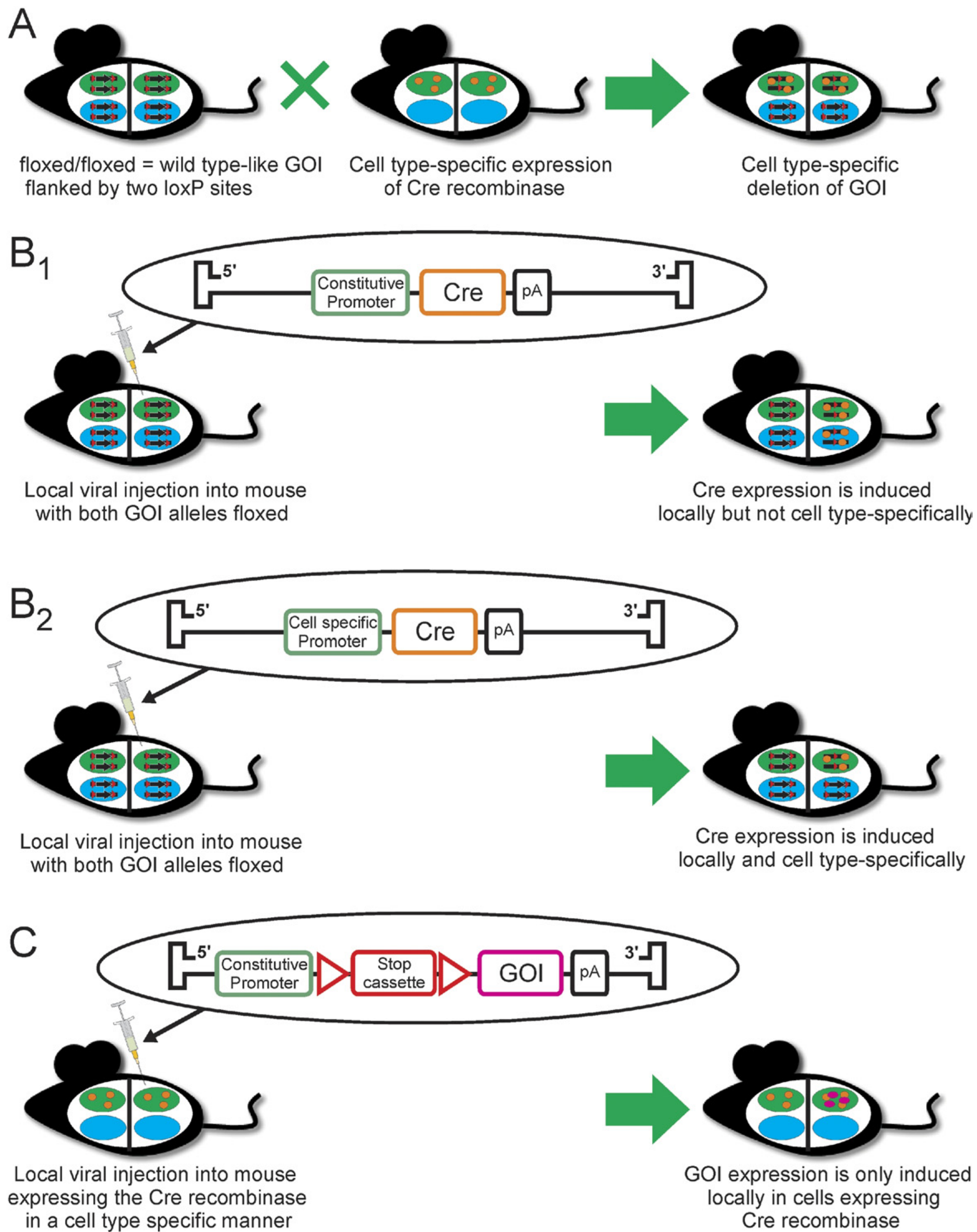

Fig. 2. Schematic illustration of genetic and viral approaches using the Cre/loxP system. Four cells are depicted in each mouse comprising two different cell types (green and blue color) and two different brain regions (the two cells on the left and right part, respectively, separated by a black line). (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.

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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