ROLE OF CANNABINOID TYPE 1 RECEPTOR IN LOCUS COERULEUS ACTIVITY: IMPLICATIONS FOR THERAPEUTIC INTERVENTION IN STRESS-INDUCED PSYCHIATRIC DISORDERS

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LIST OF SYMBOLS and ABBREVIATIONS

μg	micrograms
μm	micrometers
2-AG	2-arachidonylglycerol
4V	fourth ventricle
ABC	avidin-biotin complex
aCSF	artificial cerebrospinal fluid
AEA	N-arachidonylethanolamide/anandamide
AHP	afterhyperpolarization potential
ANOVA	analysis of variance
AP	action potential
AR	adrenoceptor
Bar	Barrington's nucleus
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
C	Celsius
Ca^{2+}	calcium
cAMP	3',5'-cyclic adenosine monophosphate
CB	cannabinoid
CBD	cannabidiol
CBr	cannabinoid receptor
CB1r	cannabinoid type 1 receptor
CB2r	cannabinoid type 2 receptor
CeA	central nucleus of the amygdala
CNS	central nervous system
CRF	corticotropin releasing factor
CRFr	corticotropin releasing factor receptor
CRFr1	corticotropin releasing factor receptor type 1
DA	dopamine
DAB	3,3'-diaminobenzidine tetrahydrochloride dihydrate
DAPI	4',6-diamibino-2-phenylindole

DAT	dopamine transporter
eCB	endocannabinoid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FAAH	fatty acid amide hydrolase
FITC	fluorescein isothiocyanate
FST	forced swim test
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hours
HPA	hypothalamic-pituitary adrenal (axis)
IF	immunofluorescence
k^+	potassium
kDa	kilodaltons
KO	knockout
KOR	κ-opioid receptor
LC	locus coeruleus
M	molar
MAO	monoamine oxidase
MGL	monoacylglycerol
min	minutes
mL	milliliters
MΩ	megaohms
MOR	μ-opioid receptor
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
ms	milliseconds
mV	millivolts
NAPE-PLD	n-acyl-phosphatidylethanolamine-hydrolyzing phospholipase-D
NE	norepinephrine
NET	norepinephrine transporter
NIH	National Institutes of Health
nm	nanometers
nM	nanomolar
NPY	neuropeptide Y
pA	picoamps
PAG	periaqueductal gray
PB	phosphate buffer
PBS	phosphate buffer saline
PC	Pearson's coefficient
PFC	prefrontal cortex
pg	picograms
PGi	paragigantocellularis
PHAL	phaseolus vulgaris-leucoagglutinin

PLCb	phospholipase C b
POD	peroxidase
PrH	nucleus prepositus hypoglossi
PTSD	posttraumatic stress disorder
PVDF	polyvinylidene
PVN	paraventricular nucleus
rpm	rotations per minute
sec (s)	seconds
SEM	standard error of means
SN	substantia nigra
SNARE	soluble NSF attachment protein receptor
syn	synaptophysin
TBS	tris buffered saline
TH	tyrosine hydroxylase
THC	Δ-9 tetrahydrocannabinol
TMB	tetramethylbenzidine
TRITC	tetramethylrhodamine
TRPV1	transient receptor potential vanilloid receptor type 1
TST	tail suspension test
VGlut	vesicular glutamate transporter
WB	Western blot
WT	wild type
Y1	neuropeptide Y receptor type 1
Y2	neuropeptide Y receptor type 2

ABSTRACT

Role of cannabinoid type 1 receptor in locus coeruleus activity:
Implications for therapeutic intervention in stress-induced psychiatric disorders

Ryan Rudolph Wyrofsky
Elisabeth Van Bockstaele

Cannabinoids have profound effects on mood and behavior, in part through their modulation of the stress-integrative locus coeruleus (LC)-noradrenergic system. Cannabinoid type 1 receptor (CB1r) agonists are capable of increasing noradrenergic activity and anxiety-like behaviors; however, they can also decrease stress-induced anxiety. In order to more closely examine the role of CB1r in regulating LC-norepinephrine (NE) activity, whole-cell patch clamp electrophysiology was performed on LC-NE neurons from CB1r-knockout (KO) mice. Since sex differences are found within the endocannabinoid (eCB) system, stress signaling, and the prevalence of psychiatric disorders, both males and females were examined. CB1r deletion caused an increase in LC-NE excitability, input resistance, and NE levels in the prefrontal cortex in male mice, but not females. Additionally, stress peptide corticotropin releasing factor (CRF)-induced increases in LC-NE excitability are lost in CB1r-KO mice. Western blot analysis revealed an increase in CRF and tyrosine hydroxylase expression levels, and decrease in norepinephrine transporter expression in male CB1r-KO compared to WT in

the LC, and an increase in α2-adrenoceptor expression in female CB1r-KO compared to WT. Next, immunoelectron and immunofluorescence microscopy determined the cellular localization of CB1r with respect to the CRF in the LC, showing co-localization of CB1r to CRF-containing amygdalar afferents. Finally social stress, which leads to anxiety-like behaviors, differentially alters eCB system protein levels in the LC in resilient and non-resilient populations of rats across sexes. These results expand the understanding of cannabinoid-CRF-adrenergic interactions, and how targeting CB1r could provide therapeutic relief for anxiety disorders.

INTRODUCTION

The Endocannabinoid System

Cannabis sativa, or marijuana, is one of the most widely used illicit drugs, known to promote relaxation, euphoria, and a feeling of contentment (Velez et al., 1989, Green et al., 2003, Di Marzo et al., 2004). Chronic use, however, can be accompanied by dysphoria, depressive mood, and increased anxiety (Reilly et al., 1998), with the severity of symptoms being exacerbated by exposure to greater concentrations and increased frequency of usage (Lee et al., 2009). The first pharmacologically active compound identified in C. sativa, D-9 tetrahydrocannabinol (THC), was characterized in 1964 and has since been established as its primary psychoactive component (Gaoni and Mechoulam, 1964, Micale et al., 2013). Since its discovery, over 100 additional active components of C. sativa have been identified (Micale et al., 2013).

The first cannabinoid receptor, cannabinoid type 1 receptor (CB1r), was identified in 1988 and a second receptor, cannabinoid type 2 receptor (CB2r), was characterized in 1993 (Devane et al., 1988, Di Marzo et al., 2004). Both are Gi/o protein-coupled receptors with distinct distributions in the body (Castillo et al., 2012). CB1r are one of the most abundant G-protein-coupled receptors in the brain and their activation most commonly results in the inhibition of neurotransmitter release (Herkenham et al., 1990, Castillo et al., 2012). CB2r are most prevalent in the immune system (Van Sickle et al., 2005, Castillo et al., 2012); however, recent studies suggest a presence in the CNS as well, showing CB2r localization in the hippocampus, substantia nigra (SN), periaqueductal gray matter, and parvocellular reticular nucleus (Brusco et al., 2008, Onaivi et al., 2008). Following the identification of the cannabinoid receptors, endogenous cannabinoid ligands, or eCBs, were discovered. The first was N-

arachidonylethanolamide (AEA), which was named ‘anandamide’ after the Sanskrit word meaning ‘bliss’ (Devane et al., 1992, Di Marzo et al., 2004). Another well-characterized eCB is 2-arachidonoylglycerol (2-AG), first isolated from canine intestines by Mechoulam et al. in 1995 (Mechoulam et al., 1995). It is now generally accepted that 2-AG is a full CB1r and CB2r agonist, whereas AEA, which is less potent, is a partial agonist (Sugiura, 2009). Initially, the mechanism proposed for eCB release involved a depolarization-induced event followed by retrograde signaling and binding of the endogenous ligand to presynaptically distributed receptors (Castillo et al., 2012, Wang and Lupica, 2014). New evidence suggests that eCB can regulate synaptic transmission via non-retrograde and autocrine mechanisms, with CB1r having been discovered postsynaptically (Bacci et al., 2004). Furthermore, eCBs can bind and activate transient receptor potential vanilloid receptor type 1 (TRPV1) receptors (Castillo et al., 2012).

The rate of eCB synthesis and degradation determines their signaling profiles. Two primary mechanisms are known to be responsible for 2-AG synthesis: increases in intracellular Ca^{2+} via postsynaptic depolarization and activation of phospholipase C β (PLCβ) via stimulation of group I metabotropic glutamate receptors. PLCβ forms diacylglycerol from the hydrolysis of phosphatidylinositol, which diacylglycerol lipase α (DGL) then converts to 2-AG (Blankman et al., 2007, Castillo et al., 2012). Monoacylglycerol lipase (MGL) is the main enzyme responsible for breaking down 2-AG, rendering it inactive and thus controlling the strength and duration of its modulatory activity (Craft et al., 2013). The synthesis and degradation of AEA is more complex. Though it is known that increases in intracellular Ca^{2+} and postsynaptic depolarization stimulate AEA formation, the mechanism underlying this process has yet to be elucidated

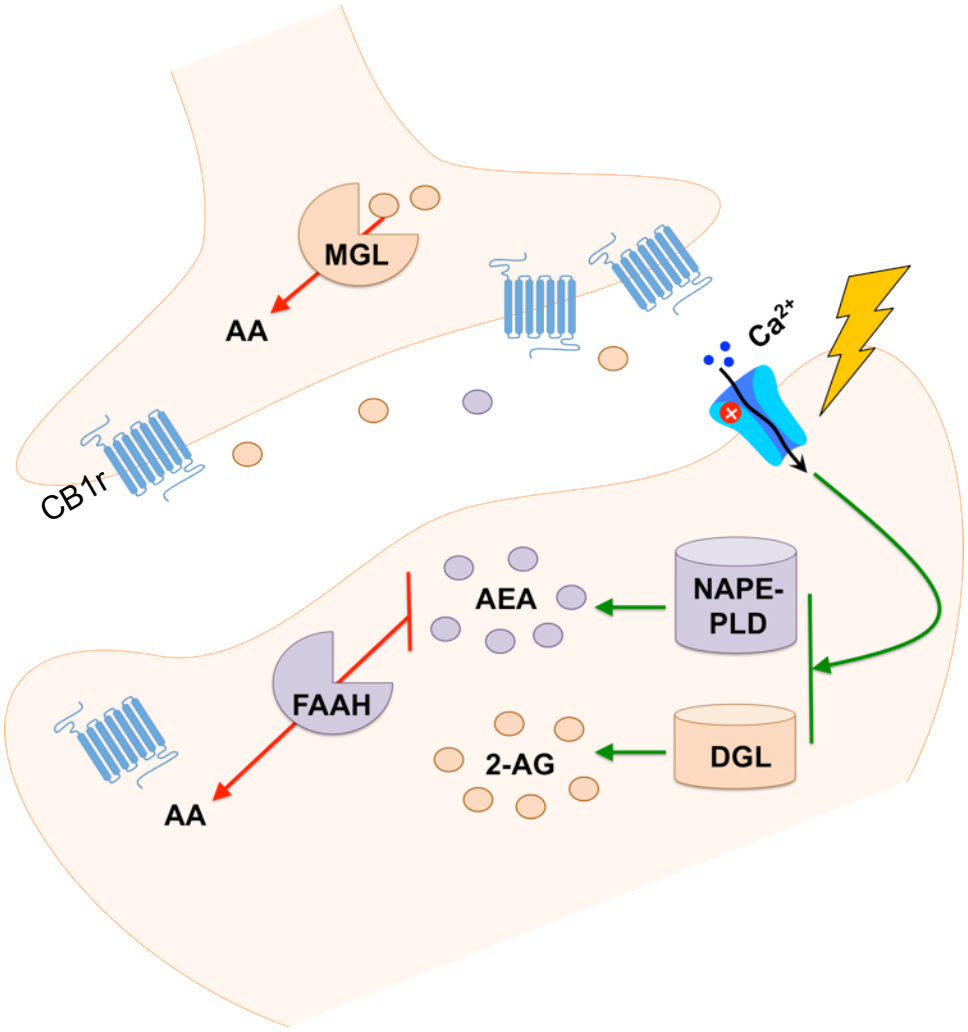


Figure 1
The endocannabinoid system.

Figure 1: The endocannabinoid system. This schematic illustrates the basic components of the endocannbinoid system. Postsynaptic depolarization and influx of Ca^{2+} stimulates eCB synthesis. N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase-D (NAPE-PLD) is the main enzyme responsible for synthesizing anandamide (AEA), while diacylglycerol lipase (DGL) synthesizes 2-arachidonylglycerol (2-AG). These eCBs can then cross through the membrane and diffuse across the synapse, where they retrogradely activate presynaptic cannabinoid type 1 receptors (CB1r). Presynaptic monoacylglycerol (MGL) then metabolizes 2-AG, and fatty acid amide hydrolysis (FAAH) breaks down AEA.

(Di Marzo,2011). N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase-D (NAPE-PLD) has been identified as a contributor to AEA synthesis, but other synthetic pathways have also been reported (Okamoto et al., 2007). AEA is primarily degraded by fatty acid amide hydrolase (FAAH) and, similar to MGL for 2-AG, FAAH controls the spatiotemporal profile for AEA signaling (Castillo et al., 2012). In contrast to the activity-dependent classical eCB signaling, tonic eCB release has been observed in several brain regions (Castillo et al., 2012). A schematic summary of eCB synthesis and degradation can be found in figure 1.

eCB, Mood, and Psychiatric Disorders

According to the National Institute of Mental Health, within a given year, approximately 9.5% of the adult population within the United States (US) suffers from a form of depression and 18.1% from anxiety disorders. The third largest cause of hospitalization for individuals aged 18-44 are Major Depressive Disorder and other mood disorders (Department of Health & Human Services, 2009), and the US spends circa $193.2 billion per year on serious mental illnesses (Insel, 2008). These statistics clearly show the significant impact psychiatric disorders have on the US population.

Recognition of the involvement of the eCB system in the regulation of mood and specifically its role in depression and anxiety arose, in part, from observations obtained from symptomatic individuals (Parolaro et al., 2010, Hauer et al., 2013). A significant increase in CB1r density and efficacy was reported in the dorsolateral prefrontal cortex (PFC) of depressed suicide victims, suggesting that altered functioning of the eCB system in the PFC could contribute to depression (Hungund et al., 2004, Parolaro et al., 2010).

Several other studies examined dysregulation of the eCB system in individuals suffering from PTSD and discovered that PTSD patients had greater CB1r availability throughout the brain as well as a significant decrease in AEA plasma concentrations (Hauer et al., 2013, Neumeister et al., 2013).

Genetic manipulations of the eCB system in animal models, particularly CB1r knockout (KO) mice, have provided insight into how eCB signaling affects behavior. CB1r KO mice exhibit an increase in passive behaviors compared to wild-type (WT) mice in the forced swim test (FST), which is typically interpreted as a depressive phenotype (Steiner et al., 2008). They also show an increase in immobility time compared to WT mice in another animal model of depression, the tail suspension test (TST) (Aso et al., 2008). Additionally, when exposed to chronic mild stress, KO mice develop anhedonia at a faster rate than WT mice, suggesting an increase in vulnerability to chronic stress (Martin et al., 2002). In behavioral paradigms measuring anxiety-like behaviors, such as the elevated plus-maze, open-field test and light-dark box, CB1r KO mice exhibited increased anxiety-like behaviors (Parolaro et al., 2010). CB1r KO mice display hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis and higher levels of circulating corticosterone following stressor exposure, a response that is also commonly observed in depressed patients (Uriguen et al., 2004).

Pharmacological approaches also support a role for the eCB system in mediating depression and anxiety. Acute administration of CB1r agonists decreases the amount of behavioral despair observed in the FST, and similar antidepressant-like effects are observed in the FST and TST following chronic administration (Gobbi et al., 2005, Bambico et al., 2007). In support of this, injection of CB1r agonists directly into brain

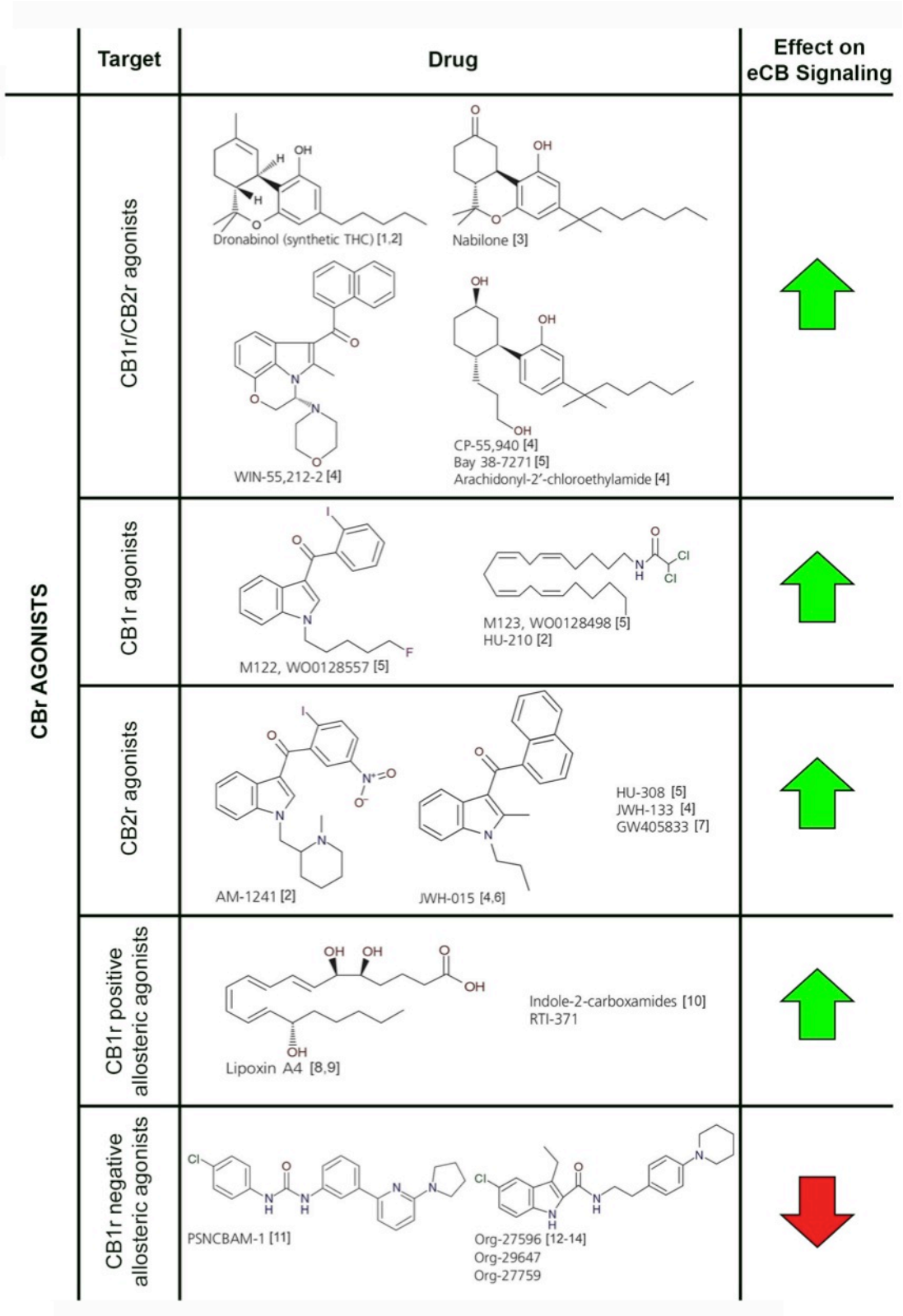
regions that are known to be involved with emotion reduces the depressive phenotype (Parolaro et al., 2010). Consistent with these preclinical findings, Rimonabant, a CB1r antagonist originally intended as an antiobesity drug, was withdrawn from clinical trials due to significant undesirable side effects including depression and suicidal ideation (Nissen et al., 2008). Concerns about mood-altering side effects resulted in the withdrawal of several other CBr antagonists from clinical trials, including Taranabant and Otenabant from Phase III trials and Ibipinabant and Surinabant from Phase II trials (Le Foll et al., 2009). These results provide evidence for a potential protective role of the eCB system in the development and treatment of depression and anxiety.

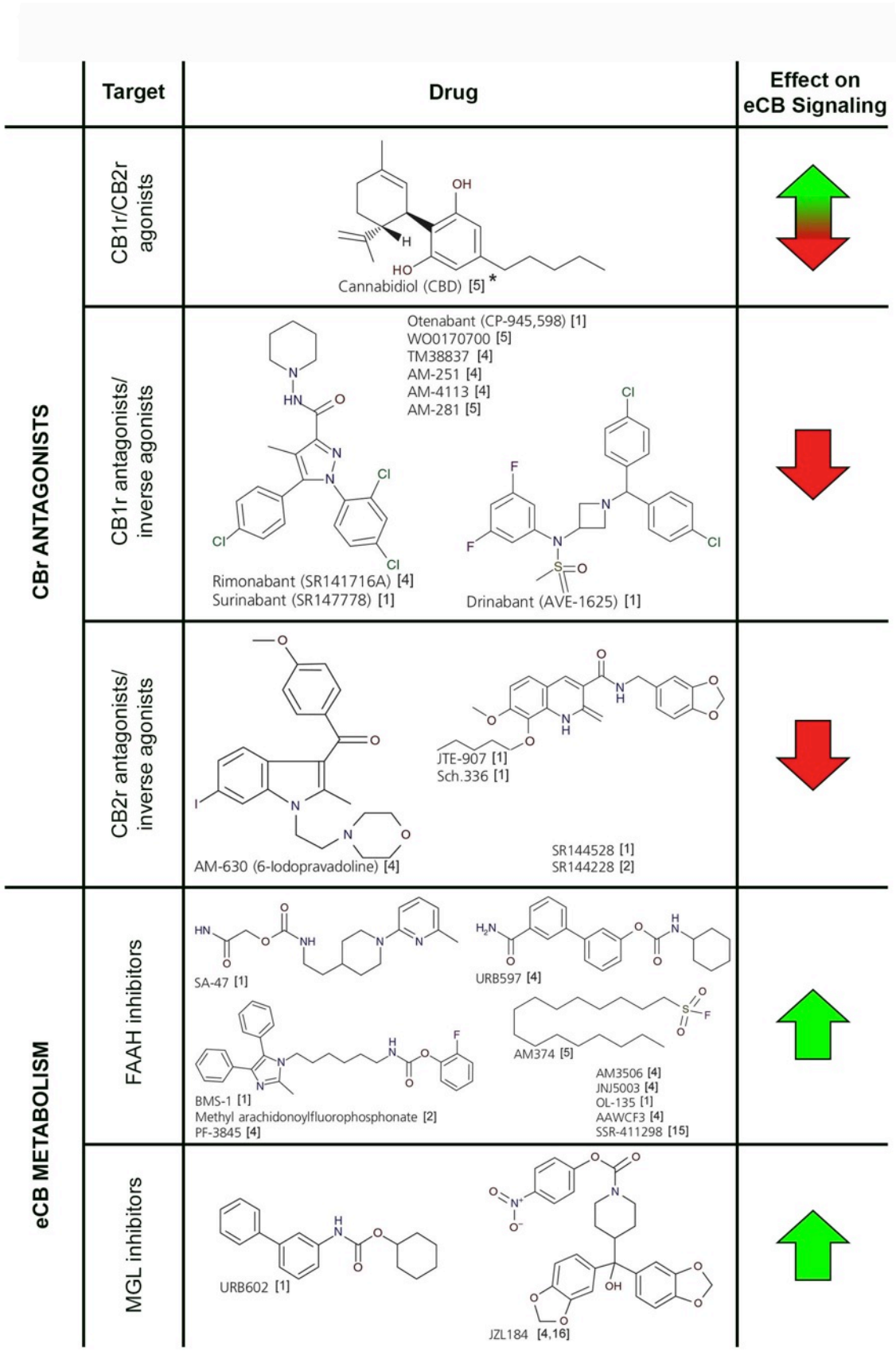
Moreover, the ability of the eCB system to more broadly affect monoaminergic neurotransmission may underlie, in part, cannabinoids' effects on mood. For example, FAAH inhibitors and CB1r agonists enhance serotonergic neurotransmission (Gobbi et al., 2005, Bambico et al., 2007), CB1r activation can stimulate the release of norepinephrine (NE), and cannabinoid receptor agonists stimulate dopamine (DA) efflux in the cortex (Gobbi et al., 2005). Many traditional antidepressants function via targeting monoamines (Wyrofsky et al., 2015); therefore, these findings indicate that eCBs may be neuroprotectective against the development of psychiatric disorders.

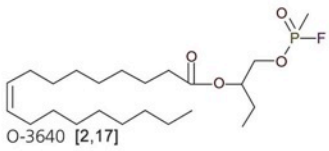
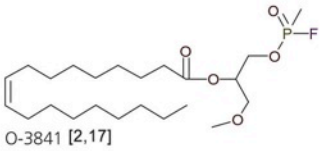
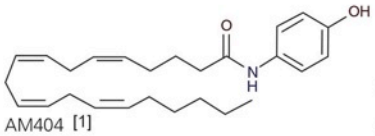
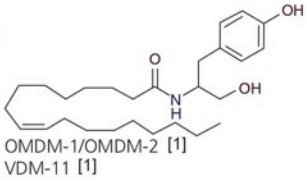
Targeting the eCB System

There are a wide variety of ways in which the eCB system can be targeted and modulated (Table I). The most direct method is by utilizing CB1r or CB2r agonists and antagonists to increase or decrease eCB signaling, respectively. Numerous selective and

Table I: Summary of drugs and compounds that are known to target the eCB system.




Target
Drug
Effect on eCB Signaling
CBr ANTAGONISTS
eCB METABOLISM
CB1r/CB2r agonists
CB1r antagonists/ inverse agonists
CB2r antagonists/ inverse agonists
FAAH inhibitors
MGL inhibitors
Cannabidiol (CBD) [5] *
Otenabant (CP-945,598) [1]
WO0170700 [5]
TM38837 [4]
AM-251 [4]
AM-4113 [4]
AM-281 [5]
Rimonabant (SR141716A) [4]
Surinabant (SR147778) [1]
Drinabant (AVE-1625) [1]
AM-630 (6-Iodopravadoline) [4]
JTE-907 [1]
Sch.336 [1]
SR144528 [1]
SR144228 [2]
SA-47 [1]
URB597 [4]
BMS-1 [1]
Methyl arachidonoylfluorophosphonate [2]
PF-3845 [4]
AM374 [5]
AM3506 [4]
JNJ5003 [4]
OL-135 [1]
AAWCF3 [4]
SSR-411298 [15]
URB602 [1]
JZL184 [4,16]

	Target	Drug	Effect on eCB Signaling
eCB CATABOLISM	DGL Inhibitors	 O-3640 [2,17]  O-3841 [2,17]	
eCB REUPTAKE INHIBITORS	eCBRIs	 AM404 [1]  OMDM-1/OMDM-2 [1] VDM-11 [1] AM1172 [1] O-3246/O-3262 [1]	

Each class of drugs is described with respect to its putative target and effect on eCB signaling.
*While CBD acts as a CB1r antagonist and CB2r inverse agonist, it can increase the eCB response by upregulating CB1r levels and by inhibiting FAAH [18-20].

References

[1] Saito et al., 2010
[2] Murataeva et al., 2012
[3] Fraser, 2009.
[4] Micale et al., 2013
[5] Di Marzo et al., 2004.
[6] Ross et al., 1999.
[7] US NIH, 2014.
[8] Pertwee, 2012.
[9] Navarro et al., 2009.
[10] Pamplona et al., 2012.
[11] Piscitelli et al., 2012.
[12] Ahn et al., 2012.
[13] Gamage et al., 2014.
[14] Horswill et al., 2007.
[15] Long et al., 2009.
[16] Bisogno et al., 2006.
[17] Rabinak et al., 2013.
[18] Thomas et al., 2007.
[19] Hayakawa et al., 2008.
[20] Bisogno et al., 2001.

nonselective agonists and antagonists have been synthesized and characterized (Table I) and have been useful tools in elucidating the role of the eCB system. Several of these compounds have advanced to clinical trials over the past decade or two, though predominantly they have been tested for the treatment of pain, inflammation, neurodegenerative disorders, nausea, obesity, and nicotine and alcohol dependence (Okamoto et al., 2007, Wyrofsky et al., 2015).

eCB signaling can also be modified by targeting the synthetic and metabolic enzymes of AEA and 2-AG (Table I). By inhibiting FAAH and MGL, eCB levels can be increased, allowing for greater signaling to occur. Conversely, by inhibiting eCB synthesis, eCB levels are decreased resulting in less signaling. Finally, the eCB degradative enzymes are located intracellularly, so by blocking eCB uptake into the pre- or postsynaptic cell, eCB levels will remain high and signaling will be increased (Di Marzo et al., 2004). These methods allow more fine-tuning of the eCB system as opposed to the CB1r agonists and antagonists, and many of these approaches have also been utilized to test the effects of altered eCB signaling in preclinical models of psychiatric disorders. More recently, eCB-based drugs have begun clinical testing for the treatment of various psychiatric disorders, including schizophrenia, post-traumatic stress disorder (PTSD), and depression (Table II).

Recently, allosteric CB1r agonists have been identified, which has important implications for drug discovery, as allosteric compounds allow for the modulation of signaling without completely inducing or blocking receptor responses as traditional agonists and antagonists would do (May et al., 2007). Price et al. identified the first allosteric CB1r modulators in 2005. They discovered three Organon compounds that all

Table II: Summary of the clinical progression of endocannabinoid-based drugs tested for the treatment of psychiatric disorders.

TARGET	DRUG	FOR THE TREATMENT OF	CLINICAL PROGRESSION
CB1r/CB2r AGONISTS	GWP42003	Schizophrenia [1]	Phase II
	Dronabinol	Schizophrenia [2]	Phase IIa
		Fear extinction [3]	Phase II (completed)
	THC	PTSD [4]	Phase IV
	Nabilone	PTSD (nightmares) [5]	Phase II
	Sativex (CBD & THC)	Cannabis dependence w/ anxiety [6]	Phase I
		Multiple Sclerosis (cognitive function & mood) [7]	Phase IV
CB1r/CB2r ANTAGONIST	Cannabidiol (CBD)	Schizophrenia (psychotic symptoms) [8]	Phase II (completed)
CB1r ANTAGONIST	AVE-1625	Schizophrenia (cotreatment w/ anti-psychotics) [9]	Phase II
FAAH INHIBITORS	URB597	Schizophrenia [10]	Phase I
	SSR411298	Major Depressive Disorder [11]	Phase II

References
[1] US NIH, 2014a
[2] Schwarcz et al., 2009
[3] Rabinak et al., 2013
[4] US NIH, 2012
[5] Fraser, 2009
[6] US NIH, 2014b
[7] US NIH, 2014c
[8] US NIH, 2008
[9] US NIH, 2010
[10] US NIH, 2013a
[11] US NIH, 2013b

enhanced agonist affinity for the CB1r; however, they function as insurmountable antagonists, decreasing the Emax value for CB1r agonists and increasing the length of time it takes for the agonists to dissociate from the receptor (Price et al., 2005). Subsequent research has shown that Org-27569 might in fact function as a biased ligand, decreasing coupling to cAMP and β-arrestin signaling while increasing Gα-independent ERK phosphorylation and stimulating receptor internalization (Ahn et al., 2012). This compound was tested in several in vivo rodent studies but failed to alter CB1-mediated effects of AEA, CP 55,940 (a CB1r agonist), and THC in anti-nociception, catalepsy, and hypothermia (Gamage et al., 2014); however, other eCBs were not tested in conjunction with Org-27569, nor were psychiatric effects evaluated. PSNCBAM-1 appears to have a similar profile to Org-27596, functioning as a negative allosteric CB1r modulator. *In vivo* studies, though, have shown it to be effective in an acute food-intake model (Horswill et al., 2007). Additionally, several positive allosteric modulators of CB1r activity have been identified and in particular, carbozamides have been found to selectively enhance CB1r activity (Piscitelli et al., 2012). Lipoxin A4 enhances AEA-induced nociception and catalepsy in various mouse models (Pamplona et al., 2012, Pertwee, 2012). Finally, RTI-371, a selective DAT inhibitor, has also been found to increase CP 55940 signaling in vitro in a concentration-dependent fashion (Navarro et al., 2009).

eCB and the Locus Coeruleus

It is known that the noradrenergic system plays a key role in the modulation of emotional states, mood, and arousal (Carvalho and Van Bockstaele, 2012). The locus

coeruleus (LC), a dense region of noradrenergic neurons located off of the fourth ventricle in the brainstem, innervates many regions of the neuraxis and provides the sole source of NE to the medial prefrontal cortex (mPFC) (Sara, 2009). Dysregulation of NE in the mPFC and other terminal regions can result in the development of many psychiatric disorders (Carvalho and Van Bockstaele, 2012). High levels of NE have been correlated with an increased duration of remission in previously depressed patients, implicating a potentially protective role of NE (Johnston et al., 1999, Carvalho and Van Bockstaele, 2012). It is well accepted that NE signaling is important in the pathophysiology of depression (Carvalho and Van Bockstaele, 2012) and compounds that increase NE levels, such as serotonin-norepinephrine reuptake inhibitors, tricyclic antidepressants, and monoamine oxidase (MAO) inhibitors, are effective antidepressants, suggesting that low levels of NE can cause depressive symptoms (Carvalho and Van Bockstaele, 2012). Conversely, other studies have shown that increased noradrenergic signaling following stressors plays a role in the pathophysiology of anxiety, PTSD, and cognitive deficits associated with depression (Birnbaum et al., 1999, Southwick et al., 1999, Goddard et al., 2010). This biphasic effect of NE is not only found in its ability to regulate mood, but also alertness and arousal. Low tonic-low phasic firing of LC-NE neurons results in disengagement while high tonic-low phasic firing results in extreme arousal but difficulty sustaining attention on a given task. The optimal LC discharge rate for focused attention is in between the two, where phasic and coupled firing occurs (Berridge and Waterhouse, 2003). Interestingly, stress is one factor that can push the LC-NE discharge rate into the high tonic-low phasic state resulting in both increased arousal and increased pathophysiology of psychological disorders.

High-resolution neuroanatomical studies have demonstrated coexistence of CB1r with noradrenergic axon terminals in this brain region (Carvalho and Van Bockstaele, 2012). CB1r are localized both pre- and postsynaptically in the LC (Scavone et al., 2010). Most of the presynaptic CB1r were localized to symmetric synapses, indicating that they are most likely regulating GABAergic transmission (Carvalho and Van Bockstaele, 2012). The presence of CB1r in noradrenergic neurons (Scavone et al., 2010) further suggests that the eCB system may modulate noradrenergic activity directly without presynaptic modulation of amino acid signaling (Carvalho and Van Bockstaele, 2012), potentially acting as a subsequent brake on LC activation. The opposing effects of CB1r on noradrenergic terminals decreasing NE release versus CB1r on GABAergic and serotonergic terminals increasing NE release also demonstrate the importance of local eCB levels in alteration of monoamine neurotransmission (Kirilly et al., 2013).

Additional studies support eCB regulation of NE signaling. CB1r agonists CP55940 and WIN 55,212-2 increase spontaneous firing and stimulate immediate early gene c-Fos expression in LC-NE neurons (Patel and Hillard, 2003, Muntoni et al., 2006). Additionally, an increase in Fos expression was observed in dopaminergic neurons following treatment with CBr agonists; however, this increase was blocked by co-treatment with either an α1-adrenoceptor (AR) antagonist or an a2-AR agonist, indicating that the CB agonist-induced increase in dopaminergic activation is likely due to LC-NE activation (Patel and Hillard, 2003, Carvalho and Van Bockstaele, 2012). The dose-dependent increase in LC-NE firing observed after both systemic and central CB agonist administration is blocked by co-administration with SR141716A, a CB1r antagonist (Mendiguren and Pineda, 2006, Muntoni et al., 2006). Interestingly, SR141716A

administration by itself results in a decrease in LC activity, suggesting that tonic eCB production controls the LC under basal conditions (Carvalho and Van Bockstaele, 2012). Also, administration of a FAAH inhibitor increases the spontaneous firing rate of LC-NE neurons, supporting the notion of tonic eCB regulation of LC neurons (Gobbi et al., 2005).

Following CB exposure, increases in NE levels have been observed and may involve mechanisms other than disinhibition of LC noradrenergic neurons (Jentsch et al., 1997). For example, in vitro studies have shown that CBs can inhibit MAO (Fisar, 2010). MAO metabolizes monoamine neurotransmitters, so inhibition would produce increased NE levels. CB-induced decreases in α2-AR expression in the LC have been observed, which would result in an increase in NE release at postsynaptic targets (Carvalho and Van Bockstaele, 2012). Increases in NE efflux in the PFC have been observed following acute and chronic CB administration and pretreatment with SR141716A blocks CB-induced increases in NE levels (Oropeza et al., 2005, Page et al., 2007). Taken together, these data illustrate alterations in NE signaling following CB administration in regions where dysregulation is associated with stress and depressive- and anxiety-like effects.

Effect of eCB on Stress Response

HPA axis hyperactivity is very common in individuals suffering from depression and anxiety (Patel et al., 2004, Uriguen et al., 2004). While glucocorticoid release is initially beneficial, priming the body physiologically and metabolically to deal with threats, long-term secretion can result in maladaptive cardiovascular, metabolic, and even

neurological conditions (McEwen, 2008). There are negative feedback mechanisms in place, allowing glucocorticoids to attenuate HPA axis activity, and studies have shown the PFC to be the critical site for this termination (Hill et al., 2011). Immunohistochemical data and electron microscopy provide evidence that CB1r in layer V of the prelimbic PFC region are found on GABAergic terminals (Hill et al., 2011). Genetic deletion of CB1r and injection of CB1r antagonists directly into the PFC produce an increase in the stress response and corticosterone levels, further implicating the importance of the eCB system in the negative feedback mechanisms on the HPA axis (Hill et al., 2011). Activation of the GABAergic circuits located in either the prelimbic PFC or the bed nucleus of the stria terminalis (BNST) results in a decrease in CRF release from the PVN (Herman et al., 2005).

In vivo rat studies have demonstrated that peripheral injection of corticosterone causes a swift escalation of eCB levels in the PVN, indicating that stress up-regulates hypothalamic eCB levels via a glucocorticoid-mediated mechanism (Hill and McEwen, 2009). This has led Hill et al. to propose a model for the influence of the eCB system on the temporal phases observed in glucocorticoid feedback. It is known that stress causes the production and release of glucocorticoids into the circulation. According to the model, rapid attenuation of the HPA axis occurs via an increase in eCB synthesis and release in the PVN, resulting in the suppression of glutamatergic signaling on CRF-releasing neurons (Hill et al., 2011). A longer, time-delayed feedback loop centers on eCB production in the mPFC. Circulating glucocorticoids stimulate 2-AG synthesis and release in the prelimbic PFC, which then binds to CB1r on GABAergic neurons. This results in the disinhibition of projection neurons that synapse with GABAergic neurons in

the BNST, ultimately causing a decrease in signals projecting to the PVN and a subsequent decrease in CRF release (Hill et al., 2011). While it appears that eCBs are produced on demand in the above pathways, it has been proposed that tonic AEA signaling in the basolateral amygdala (BLA) occurs (Hill and McEwen, 2009). This is the basis for a gatekeeper function, in which tonic eCB tone results in basal inhibition of the HPA axis (Hill et al., 2010b). During acute stress, there is an increase in FAAH activity, resulting in a decrease in AEA levels (Hill and McEwen, 2009). This causes a disinhibition of the principal neurons located in the BLA, subsequently leading to an increased amygdalar output to various regions including the PVN, stimulating the HPA axis (Hill and McEwen, 2009).

The amygdala is a key structure involved in the regulation of fear and emotional memory, and the eCB system plays a role in regulating the amygdala's response to stress. As previously mentioned, stress causes a rapid decrease in AEA levels within the amygdala; however, when stress is absent and corticosterone is administered, an increase in AEA levels within the amygdala is observed (Hill and McEwen, 2009, Hill et al., 2010b). While this might seem contradictory, such a mechanism may be adaptive. Activation of the HPA axis is important for escaping and managing an acute stress; however, problems arise from overactivation of the HPA axis. Therefore, though stress initially causes a decrease in amygdalar AEA levels via a glucocorticoid-independent pathway, the subsequent glucocorticoid release caused by HPA axis stimulation feeds back to increase amygdalar AEA levels, attenuating the HPA axis activity (Hill et al., 2010b). While acute stressors only affect the production of AEA in the amygdala, repeated chronic stressors can increase amygdalar 2-AG signaling temporarily, with

levels beginning to return to normal 1 h after the stressful event (Patel et al., 2009). Therefore, elevated levels of 2-AG following repeated stressors represent another mechanism by which eCBs protect from HPA axis overactivation. Consistent with this hypothesis, injection of a CB1r antagonist locally into the BLA reverses this stress habituation (Hill et al., 2010a).

The LC plays a key role in the cognitive limb of the stress response, which is initiated in parallel with the HPA axis via CRF release from amygdalar afferents (Fig. 2) (Van Bockstaele et al., 1998, Valentino and Van Bockstaele, 2008). NE-containing LC neurons express CRF receptor 1 (CRFr1), and chronic stress and the resulting release of CRF can lead to inappropriate increases in the firing of these neurons (Curtis et al., 1996, Reyes et al., 2008b). Again, this dysregulated NE release in limbic and cortical areas contributes to the debilitating symptoms of depression and anxiety (Morilak and Frazer, 2004, Leonard and Myint, 2009). Just as the eCB system has been shown to modulate regions involved in the HPA axis and stress response, this anti-stress system also affects the cognitive limb and LC directly.

Sex Differences

Considering the known bias in susceptibility to psychiatric disorders between the sexes, understanding interactions between the eCB system, stress, and the LC-NE system across the sexes is of paramount importance. Males are more prone to drug abuse, while females are about twice as likely to develop stress-induced disorders such as depression and anxiety (Kendler et al., 1995, Marcus et al., 2005). Stress also affects both sexes differently; females are more sensitive to low levels of CRF due to both augmented CRF

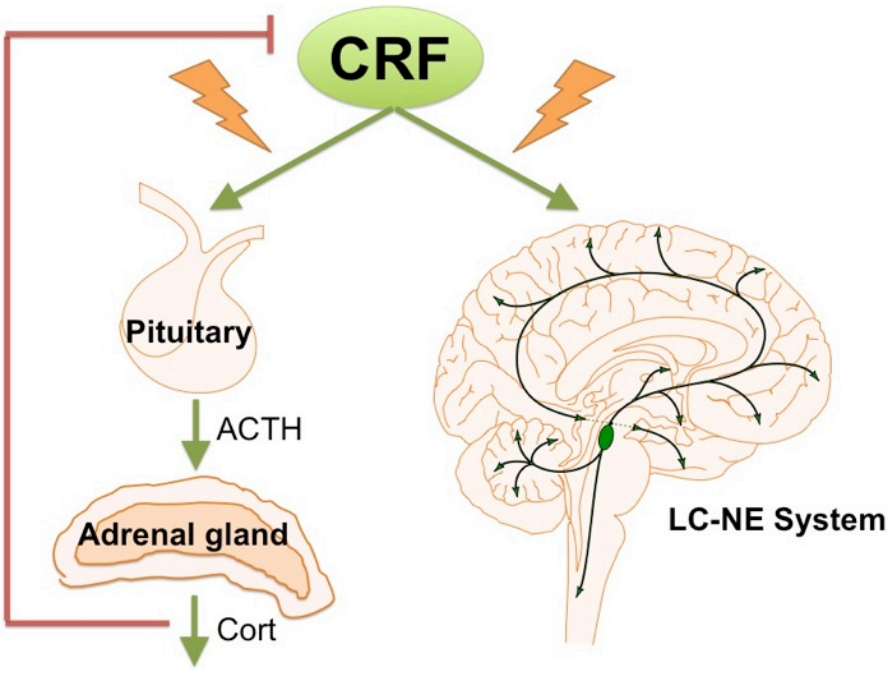


Figure 2
Parallel engagement of stress responses.

Figure 2: Parallel engagement of stress responses. Corticotropin releasing factor (CRF) is responsible for coordinative activation of both the hypothalamic-pituitary-adrenal axis (on the left) and the locus coeruleus (LC)-norepinephrine (NE) system (on the right). CRF from the hypothalamus stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which then causes the adrenal cortex to release corticosterone (cort). Cort then plays a role in the negative feedback loop for the HPA axis, with the help of endocannabinoids. LC-NE activation is a key component in the cognitive limb of the stress response, causing a robust increase in NE in the medial prefrontal cortex and all throughout the neuraxis.

receptor (CRFr) signaling and diminished CRFr internalization after exposure to stress when compared to males (Bangasser et al., 2010). In males, CRF binding to its receptor shows biased signaling towards the recruitment of β-arrestin and receptor internalization, but in females, CRF binding causes a biased response for Gs signaling (Valentino et al., 2013). Additionally, following stressors, female rats have increased dendritic extension into the peri-LC, the region surrounding the LC nucleus where a majority of limbic CRF afferents terminate (Bangasser and Valentino, 2012, Valentino et al., 2013). Females have heightened HPA axis activity, coupled with slower negative feedback of the HPA axis (Handa et al., 1994, Handa and Weiser, 2014). All of these discoveries lead to the generalized conclusion that females have heightened stress signaling within the LC compared to males.

Also, differences across sexes are observed in the eCB system, both anatomically and behaviorally. Females have a greater sensitivity to cannabinoid abuse, dependence, withdrawal, and relapse (Craft et al., 2013). Females also have decreased CB1r density in certain brain regions, including the amygdala and cingulate areas 1 and 3 (Castelli et al., 2014). Additionally, in human depressed patients, while both sexes show a rise in serum AEA, only females show a decrease in 2-AG (Reich et al., 2009). Therefore, it is important to look at both males and females when designing experiments investigating stress and endocannabinoid systems, since sex differences exist in both systems.

Goals of Thesis

While the effect of exogenous cannabinoids on LC-NE activity has been examined, less is known about how the endogenous cannabinoid system and CB1r

deletion affects LC-NE activity. Additionally, the eCB system is capable of modulating stress responses in many brain regions; however, its direct effect on the stress response within the LC is unknown. Therefore, the goals of this project are as follows: 1) to examine the functional consequences of CB1r deletion on LC-NE activity and noradrenergic indices across sexes via whole-cell patch-clamp electrophysiology and Western blotting, 2) to determine how CB1r deletion alters CRF-induced effects on LC-NE neurons via whole-cell patch-clamp electrophysiology, and 3) to test the hypothesis that CB1r are positioned to modulate CRF-containing afferents within the LC by using immunofluorescence and electron microscopy. Taken together, the proposed work will elucidate mechanisms of action of eCB signaling and CRF on the stress-integrative NE-mPFC circuitry and how they converge to regulate behavioral responses to stress across the sexes.

CHAPTER 1

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Effect of cannabinoid type 1 receptor deletion on locus coeruleus-norepinephrine neurons and corticotropin releasing factor-mediated responses

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Abstract

Cannabinoids are capable of modulating mood, arousal, cognition, and behavior, in part via their effects on the noradrenergic nucleus locus coeruleus (LC). Dysregulation of LC signaling and norepinephrine (NE) efflux in the medial prefrontal cortex (mPFC) can lead to the development of psychiatric disorders, and CB1r deletion results in alterations of α2- and β1-adrenoceptors in the mPFC, suggestive of increased LC activity. To determine how CB1r deletion alters LC signaling, whole-cell patch-clamp electrophysiology was conducted in LC-NE neurons of male and female wild type (WT) and CB1r-knock out (KO) mice. CB1r deletion caused a significant increase in LC-NE excitability and input resistance in male but not female mice when compared to WT. CB1r deletion also caused adaptations in several indices of noradrenergic function. CB1r/CB2r-KO male mice had a significant increase in cortical NE levels and tyrosine hydroxylase and CRF levels in the LC compared to WT males. CB1r/CB2r-KO female mice showed a significant increase in LC α2-AR levels compared to WT females. To further probe actions of the endocannabinoid system as an anti-stress neuromediator, the effect of CB1r deletion on CRF-induced responses in the LC was investigated. The increase in LC-NE excitability observed in male and female WT mice following CRF (300 nM) bath application was not observed in CB1r-KO mice. These results indicate that cellular adaptations following CB1r deletion cause a disruption in LC-NE signaling in males but not females, suggesting underlying sex differences in compensation mechanisms in KO mice as well as basal endocannabinoid regulation of LC-NE activity.

Introduction

The brainstem nucleus locus coeruleus (LC) plays an important role in regulating mood, arousal, and emotional states (Aston-Jones and Cohen, 2005; Valentino and Van Bockstaele, 2008), and is the sole provider of norepinephrine (NE) to the medial prefrontal cortex (mPFC) (Sara, 2009). Dysregulation of the LC-NE system resulting in subsequent alterations in mPFC NE levels has been shown to precipitate the development of psychiatric disorders (Mueller et al., 2008; Mueller and Cahill, 2010). One of the most widely used recreational drugs in the world, cannabis, often causes feelings of relaxation and euphoria (Velez et al., 1989; Green et al., 2003; Di Marzo et al., 2004); however, chronic usage can be anxiogenic and produce dysphoria (Reilly et al., 1998). Via activation of cannabinoid type 1 receptors (CB1r), cannabinoids are known to affect attention and anxiety (Witkin et al., 2005; Pattij et al., 2008; Hill and Gorzalka, 2009), in part via actions on noradrenergic circuitry (Carvalho et al., 2010; Carvalho and Van Bockstaele, 2012; Wyrofsky et al., 2017). There are, however, some discrepancies within the cannabinoid field. While some studies find anxiolytic effects and therapeutic promise in cannabinoid treatments, others show that CB1r agonists can have a negative impact and promote anxiogenesis, and the exact dosage and experimental conditions can have a profound effect on whether targeting the endocannabinoid system could be beneficial (Di Marzo, 2008; Wyrofsky et al., 2015). Further investigation of how the eCB system targets neurotransmitter systems, like norepinephrine, can provide further insight into the differential effects of cannabinoid agonism.

Immunofluorescence and immunoelectron microscopy studies have provided direct anatomical evidence for CB1r co-localization to noradrenergic terminals in the

mPFC and localization to TH-containing neurons in the LC (Carvalho et al., 2010; Wyrofsky et al., 2017). Several studies have also shown that CB1r activation increases NE release. CB1r agonists WIN 55,212-2, tetrahydrocannabinol (THC), and CP 55940 dose-dependently increase the spontaneous firing rate of LC neurons (Mendiguren and Pineda, 2006; Muntoni et al., 2006). Both systemic and local administration of CB1r agonist WIN 55,212-2 increases c-Fos expression in LC-NE neurons and NE efflux in the rat mPFC (Oropeza et al., 2005; Page et al., 2008), as does inhibition of eCB catabolism with a fatty acid amide hydrolase (FAAH) inhibitor (Gobbi et al., 2005). Additionally, administration of the CB1r antagonist SR141716A causes a decrease in LC activity, suggesting that during basal conditions (Muntoni et al., 2006), the LC is under tonic eCB regulation (Carvalho and Van Bockstaele, 2012; Wyrofsky et al., 2015). Conversely, systemic administration of a CB1r antagonist rimonabant causes an increase in mPFC and hypothalamic NE levels (Tzavara et al., 2001; Tzavara et al., 2003), and low levels of THC can reduce NE release from synaptosomes (Poddar and Dewey, 1980). These anatomical and functional studies provide evidence that the eCB system can modulate the LC-NE system.

The eCB system is considered an “anti-stress” neuromediator (Viveros et al., 2007; Cota, 2008), playing a role in the hypothalamic pituitary adrenal (HPA) axis negative feedback loop as well as initiating the stress response in the amygdala (Hill and McEwen, 2009; Hill et al., 2010a; Hill et al., 2010b). The LC-NE system is involved in the cognitive limb of the stress response, and is activated in parallel with the HPA axis via corticotropin releasing factor (CRF), the pro-stress neuropeptide (Valentino and Van Bockstaele, 2008). Following a stressor, CRF is released from limbic and autonomic

afferent sources such as the paraventricular nucleus of the hypothalamus, central nucleus of the amygdala, and bed nucleus of the stria terminalis (Van Bockstaele et al., 1996; Van Bockstaele et al., 1999; Van Bockstaele et al., 2001; Valentino and Van Bockstaele, 2008), and can lead to increases in LC-NE firing and dysregulation of NE release in target regions, including the mPFC (Curtis et al., 1996). We have recently shown that CB1r are positioned both pre- and post-synaptically with respect to CRF-containing afferents within the LC, providing a neural substrate for eCB modulation of CRF in this noradrenergic nucleus (Wyrofsky et al., 2017).

Research examining male CB1r-knockout (KO) mice show that CB1r deletion increases anxiety and depressive-like behaviors in CB1r KO mice compared to wild type (WT) controls (Aso et al., 2008; Steiner et al., 2008; Parolaro et al., 2010; Wyrofsky et al., 2015). Additionally, CB1r-KO mice have increased plasma levels of adrenocorticotropin and corticosterone (Uriguen et al., 2004), and increased CRF mRNA expression in the hypothalamus, suggesting heightened HPA activity (Cota, 2008). We have also demonstrated that CB1r-KO mice have reduced basal mPFC neuronal excitability due to desensitization of the normally excitatory mPFC α2-adrenoceptors (ARs) (Reyes et al., 2017). These KO studies suggest that CB1r-KO mice have increased LC-NE release, which desensitized mPFC α2-ARs, resulting in decreased mPFC output (Reyes et al., 2017).

In the present study, we used two strains of cannabinoid receptor KO mice to define electrophysiological properties of LC-NE neurons as well as effects on CRF-mediated responses, and cellular adaptions that occur in absence of the cannabinoid receptors: CB1r-KO mice for *in vitro* slice electrophysiology studies and dual

CB1r/CB2r-KO mice for Western and ELISA experiments. First, whole-cell patch clamp electrophysiological recordings were conducted in WT and CB1r-KO mice to measure basal properties of LC-NE neurons and their excitability. Next, we used Western blot analysis to measure expression levels of the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) in the LC and assessed cortical NE levels via ELISA in CB1r/CB2r-KO mice. We also investigated expression levels of CRF, α2-AR, and NET in the coeruleo-cortical pathway. Finally, the effect of CRF administration on LC-NE activity in CB1r-KO mice was assessed using whole-cell patch-clamp recordings. Considering previous reports showing female rodents are more sensitive to the reinforcing effects of cannabinoids and are more likely to self-administer WIN 55,212-2 than males (Barna et al., 2004; Fattore et al., 2007; Roberts et al., 2014) and sex differences in CRF signaling within the LC, with female rodents having increased sensitivity to CRF following a stressor compared to males (Bangasser et al., 2010; Valentino et al., 2013), we included both male and female mice in the study. These experiments help address the current gap in our understanding of how CB1r-KO differentially affects the LC-NE and stress systems across sexes.

Methods

Animals

For all electrophysiology experiments, male and female wild-type (WT) and CB1r KO mice (9-12 weeks old) were housed four per cage in a controlled environment (12-hour light schedule, temperature at 20^{o}C). Data was obtained from N=5 LC cells from 5 WT male mice, N=4 cells from 4 WT female mice, N=6 cells from 5 KO male mice, and

N=7 cells from 4 KO female mice. For Western blot and ELISA analysis, male and female WT and CB1r/CB2r dual KO mice (9-18 weeks old) were used. Both CB1r and CB1r/CB2r KO mice were originally generated on a C57Bl/6 background by Zimmer et al. (Zimmer et al., 1999) at the National Institutes of Health. Heterozygous breeding pairs were generously donated by Dr. Carl Lupica at the National Institutes of Health and were bred and genotyped at Temple University to obtain CB1r and CB1r/CB2r KO mice and WT littermates. Food and water were provided *ad libitum*. For Western blot analysis, data represents N=3 WT male mice, N=6 KO male mice, N=3 WT female mice, and N=6 KO female mice. For ELISA analysis, data represents N=3 WT male mice, N=5 KO male mice, N=3 WT female mice, and N=5 KO female mice.

The care and use of animals were approved by the Institutional Animal Care and Use Committee of both Drexel University and Temple University, and were conducted in accordance with the *National Institutes of Health's Guide for the Care and Use of Laboratory Animals* (1996), the Health Research Extension Act (1985), and the PHS Policy on Humane Care and Use of Laboratory Animals (1986). All efforts were made to utilize only the minimum number of animals necessary to produce reliable scientific data, and experiments were designed to minimize any animal distress.

Drug preparation and administration

Ovine CRF, generously provided by Dr. Jean Rivier (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA), was dissolved in water to make a 1 mg/mL solution and separated into 10 μL aliquots, which were concentrated using a Savant Speed Vac concentrator. Aliquots were stored at -80^{o}C until

the day of the experiment, when they were then reconstituted in aCSF and added to the perfusion bath at a final concentration of 300 nM CRF. 300 nM CRF was established as the optimal concentration based on a concentration-response curve (100-400 nM) tested in brain slices from male WT mice (data not shown). Additionally, this dose matches with previous sources confirming that 300 nM CRF produces maximal increases in LC-NE excitability (Jedema and Grace, 2004).

Electrophysiology

All electrophysiology procedures were conducted as described previously (Reyes et al., 2012; Cathel et al., 2014). Male and female CB1r KO and WT mice were rapidly decapitated and brains rapidly extracted and placed in ice-cold artificial cerebrospinal fluid (aCSF), in which sucrose (248 mM) was substituted for NaCl. The brains were trimmed down to isolate the brainstem, and 250μm horizontal slices containing the LC were cut on a Vibratome 3000 Plus (Vibratome, St. Louis, MO, USA). Slices were then incubated for 1h in aCSF at 35^{o}C, and bubbled with 95% O_2/5% CO_2. Slices were then maintained at aCSF at room temperature and bubbled with 95% O_2/5% CO_2. The aCSF was composed of the following: 124 mM NaCl, 2.5 mM KCl, 2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM dextrose, and 26 mM $NaHCO_3$.

Slices were transferred to a recording chamber (Warner Instruments, Hamden, CT, USA) and continuously perfused with aCSF at a rate of 1.5-2.0 mL/min at a temperature of 34^{o}C, maintained by an inline solution heater (TC-324; Warner Instruments, Hamden, CT, USA). Data were obtained from one to two neurons per mouse; however, only one neuron was recorded per brain slice. Neurons in the LC were

visualized using a Nikon E600 upright microscope fitted with a 40x water immersion objective, differential interference contrast and infrared filter (Optical Apparatus, Ardmore, PA, USA). The microscope was connected to a CCD camera and computer monitor. LC-NE cells were tentatively identified by their morphology and electrophysiological characteristics (Williams et al., 1984), using the fourth ventricle as a marker for the location of the LC nucleus. Whole-cell recording pipettes were made with borosilicate glass capillary tubing (1.2 mm outer diameter, 0.69 mm inner diameter; Warner Instruments) on a P-97 micropipette puller (Sutter Instruments, Novato, CA, USA). Electrodes were pulled to a resistance of 4-8 MΩ when filled with an intracellular solution containing 120 mM K-gluconate, 10 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 2 mM MgATP, 0.5 mM Na_2GTP, 10 mM Na phosphocreatinine, and 0.1% biocytin, pH 7.3. Cells were approached with the electrode until a GΩ seal was established, and the cell membrane ruptured to obtain a whole-cell recording using HEKA patch-clamp EPC-10 amplifier (HEKA Elecktronik, Pfalz, Germany) under current clamp conditions (I = 0 pA). The series resistance was monitored throughout the experiment, and if it appeared unstable or exceeded four times the electrode resistance, the cell was discarded. Signals were stored on-line using Pulse software, filtered at 1 kHz and digitized at 10 kHz. The liquid junction potential was approximately 9 mV between the pipette solution and the aCSF, and was not subtracted from the data obtained.

At baseline, membrane potential was recorded and input resistance calculated by averaging the voltage change that occurred during a 300pA current pulse and using the current/voltage relationship. Neuronal excitability was assessed in each cell by injecting

a series of current pulses (0-180 pA, 30 pA increments), and the number of action potential per pulse was determined. Additionally membrane characteristics were recorded, including action potential (AP) amplitude, AP duration, AP threshold, afterhyperpolarization potential (AHP) duration, and AHP amplitude. After baseline recordings were performed, 300 nM CRF was bath applied, and membrane potential, input resistance, neuronal excitability, and membrane characteristics were re-assessed 6 minutes after drug application.

Following electrophysiological experiments, dual fluorescence immunohistochemistry techniques were used to confirm that recordings were performed in LC-NE cells. Slices that were used for recordings were post-fixed in 4% formaldehyde solution on 0.1 M phosphate buffer (PB; pH 7.4) for 72 hours. Biocytin-filled (recorded) neurons were visualized using an Alexa Fluor 488-conjugated streptavidin antibody (1:500, ThermoFisher Scientific, Waltham, MA, USA). Tyrosine hydroxylase (TH) was visualized using a primary TH antibody raised in mouse (1:1000, 48 h incubation, Immunostar, Hudson, WI) followed by Alexa Fluor 647-conjugated mouse secondary antibody (1:400, Jackson ImmunoResearch, West Grove, PA, USA). Images were examined using an Olympus IX81 inverted microscope (Olympus, Hatagaya, Shibuya-Ku, Tokyo, Japan) equipped with lasers (Helium Neon laser and Argon laser; models GLG 7000; GLS 5414A and GLG 3135, Showa Optronics Co., Tokyo, Japan) with the excitation wavelength of 488, 543, and 635. Data from recorded cells that were not co-stained with TH were excluded.

Data analysis for electrophysiology

Electrophysiological recordings were analyzed using Clampfit 9.2 (Axon Instruments, Foster City, CA, USA). The effect of genotype on input resistance and membrane characteristics across males and females was tested using two-way ANOVA (sex vs. genotype) followed by post-hoc Tukey's multiple comparison adjustments. These statistics were performed using GraphPad Prism 7.03 (GraphPad Software, San Diego, CA, USA). The effect of sex and genotype on neuronal excitability was tested using a three-way repeated measure ANOVA/mixed-effects regression model (with sex, genotype, and injected current as covariates, along with a random effect for repeated measures from injected current), while the effect of CRF on neuronal excitability was tested by using a second mixed-effects regression model (with sex, genotype, injected current, and drug treatment as covariates, along with a random effect for repeated measures from injected current and drug treatment), both followed by post-hoc Tukey's multiple comparison adjustments when appropriate. Statistical analyses for neuronal excitability were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). Results are presented as mean +/- SEM. P-values less than 0.05 were considered statistically significant.

Protein extraction

Brain tissue from male and female WT and CB1r/CB2r KO mice was rapidly removed from each animal on ice. Using a trephine, the LC and mPFC brain regions were microdissected from each animal. Tissue punches were homogenized with a pestle, sonicated, and extracted in radioimmunoprecipitation assay lysis buffer with a protease

inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice for 20 min. Lysates were cleared by centrifugation at 13,000 rpm for 12 min at 4^{o}C, and supernatants were extracted. Protein concentrations were quantified using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA).

Western blot analysis

Protein extracts were diluted with an equal volume of Novex 2© tris glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA) containing dithiothreitol (Sigma-Aldrich Inc., St. Louis, MO, USA). Cell lysates containing equal amounts of protein (30 µg per condition) were separated on 10% tris-glysine polyacrylamide gels and then electrophoretically transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with Odyssey buffer (1 h, diluted in 0.01M PBS 1:1) and incubated in the various combinations of the following primary antibodies overnight at room temperature (Table I): mouse anti-tyrosine hydroxylase (TH; 1:1000, Immunostar Inc., Hudson, WI), rabbit anti-α2-adrenoceptor (α2-AR; 1:500; Millipore Sigma, Billerica, MA, USA), guinea-pig anti-CRF (1:2000, Peninsula Laboratories, San Carlos, CA, USA), mouse anti-norepinephrine transporter (NET; 1:1000, Millipore Sigma, Billerica, MA, USA). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000, ProteinTech Group, Inc., Rosemont, IL, USA) was used as a loading control, to account for potential variability in amount of sample loaded. Membranes were then rinsed and incubated with infrared fluorescent secondary antibodies (Licor, Lincoln, NE, USA) for 1 h: donkey anti-rabbit IRDye 800CW (1:15,000), donkey anti-guinea-pig IRDye800 CW (1:15000),

Table I.I: Characterization of the primary antibodies used for immunofluorescence microscopy (IF) and Western blotting (WB).

Antigen	Immunogen	Host	Source	Catalog #	Dilution	References
Biocytin	A biotin-binding protein (streptavidin) covalently attached to a fluorescent label (Alexafluor 488)	n/a	ThermoFisher Scientific	S32354	1:500 (IF)	Li & Kirby (2016)
TH	TH purified from rat PC12 cells	Mouse	Immunostar Inc	22941	1:1000 (IF & WB)	Van Bockstaele & Pickel (1993), Oropeza et al. (2005)
α2-AR	Synthetic peptide, amino acids 218-235 of human, rat, and pig	Rabbit	Millipore Sigma	SAB4500548	1:500 (WB)	Reyes et al. (2009),Reyes et al. (2017)
CRF	Synthetic CRF peptide	Guinea-pig	Peninsula Laboratories	T-5007	1:2000 (WB)	Rudoy et al. (2009)
NET	Sodium-dependent NET recombinant protein epitope signature tag (PrEST)	Mouse	Millipore Sigma	MAB5620	1:1000 (WB)	Reyes et al. (2009)
GAPDH	GAPDH fusion protein Ag0766	Mouse	ProteinTech	60004-I-Ig	1:2000 (WB)	Ross et al. (2017)

goat anti-mouse IRDye680LT (1:20,000). Membranes were scanned using the Odyssey Infrared Imaging System (Licor, Lincoln, NE, USA), and protein quantification was determined using Odyssey Infrared Imaging software. Protein quantification was normalized to the loading control, and all data is presented as a ratio of sample protein level to GAPDH level, to allow for comparison between groups.

Additionally, Chameleon Duo Pre-stained Protein Ladder (Licor, Lincoln, NE, USA) was used to determine the molecular weights of protein bands observed: GAPDH – ~37 kDa, CRF – ~25 kDa, α2-AR - ~45 kDa, TH - ~60 kDa, NET - ~80 kDa. Only proteins that did not fall around the same molecular weight were probed for at the same time. After imaging, membranes were stripped with NewBlot PVDF 5x Stripping Buffer (Licor, Lincold, NE, USA) mixed with four parts DiH_2O for 20 min at room temperature. Blots were then rinsed and imaged to ensure removal of antibodies. Then, the blot could be incubated again with other primary antibodies to detect additional proteins.

ELISA

Sandwich ELISA was conducted in accordance with the instructions provided in the High Sensitivity NE Kit (Eagle, Nashau, NH). Tissue lysates containing equal amounts of protein were dispensed into an extraction plate and incubated for 60 min in 100 µl extraction buffer at room temperature on an orbital shaker. The solution was then discarded and the extraction plates were washed before 200 µl acylation buffer was dispensed into each well of the extraction plate and left rotating at room temperature for 20 min. Liquid was then decanted and washed 3 times prior to the dispense of 125µl 0.025 M hydrochloric acid into each well for an additional 20 min for elution. Next, 100

µl eluent was transferred to the enzyme plate with 20 µl of freshly prepared enzyme mix (2 ml Catechol-O-methyltransferase with 0.3 ml S-adenosyl-L-methionine in 0.7 ml enzyme buffer) into all wells of the enzyme plate and left at room temperature for 90 min. Finally, 100 µl of the supernatant of each well was transferred to the NE ELISA with 20 µl of rabbit NE-antiserum and left overnight at 4°C. The following day all wells were decanted and washed 3 times before incubation with 100 µl anti-rabbit IgG-POD-conjugate for 60 min at room temperature on an orbital shaker. The wells were subsequently washed 4 times and incubated with 100 µl of TMB solution for 40 min before 100 µl of stop solution was dispensed into the wells and the plate was read at 450 nm within 15 min. A standard curve was run for each replicate and was used to estimate the concentration of NE in each sample.

Data analysis for Western blot and ELISA

Differences in protein and NE levels were tested using two-way ANOVA/mixed-effects regression model (sex vs. genotype) followed by post-hoc Tukey's multiple comparison adjustments. Statistics for the Western blot and ELISA experiments were performed using GraphPad Prism 7.03 (GraphPad Software, San Diego, CA, USA). Results are presented as mean +/- SEM. P-values less than 0.05 were considered statistically significant.

Results

Heightened LC-NE activity in male CB1r-KO mice

As previously stated, exogenous cannabinoids are known to increase LC-NE activity (Patel and Hillard, 2003; Muntoni et al., 2006). Conversely, others studies have shown that the LC is under tonic eCB regulation and male CB1r-KO mice have decreased α2- and β1-adrenoceptor levels in the mPFC, suggestive of compensatory responses to heightened LC-NE activity (Reyes et al., 2017). In order to elucidate the effect of CB1r-KO on LC-NE signaling across sexes, whole-cell patch-clamp recordings from LC-NE neurons in 250um thick horizontal brain slices from CB1r-knock out (KO) mice and their wild type (WT) C57/Bl6 mice littermates were analyzed (Fig. 1.1). There was a significant effect of sex on input resistance ($F_{1,15}$=5.865, $p<0.05$), genotype on input resistance ($F_{1,15}$=14.54, $p<0.01$), and interaction between sex*genotype on input resistance ($F_{1,15}$=4.771, $p<0.05$). Tukey's post-hoc analysis revealed that male CB1r-KO mice showed a significant increase in input resistance (393 ± 31 MΩ) when compared to male WT mice (225 ± 17 MΩ; $p<0.01$), female WT mice (213 ± 39 MΩ; $p<0.01$), and female KO mice (267 ± 25 MΩ; $p<0.05$), while there was no significant change in input resistance between female WT and KO mice (Fig. 1.1B). A three-way repeated measures ANOVA/mixed effects regression model (genotype effect by sex*current pulse (repeated measure); sex effect by genotype*current pulse (repeated measure)) showed a significant effect of the following in the collected electrophysiology data: genotype ($F_{1,18}$=11.41, $p<0.01$), current pulse ($F_{6,104}$=75.78, $p<0.0001$), interaction between sex*genotype ($F_{1,18}$=7.23, $p<0.05$), interaction between sex*genotype*current pulse ($F_{5,104}$=2.72, $p<0.05$), indicating all three factors were statistically significant in the regression model.

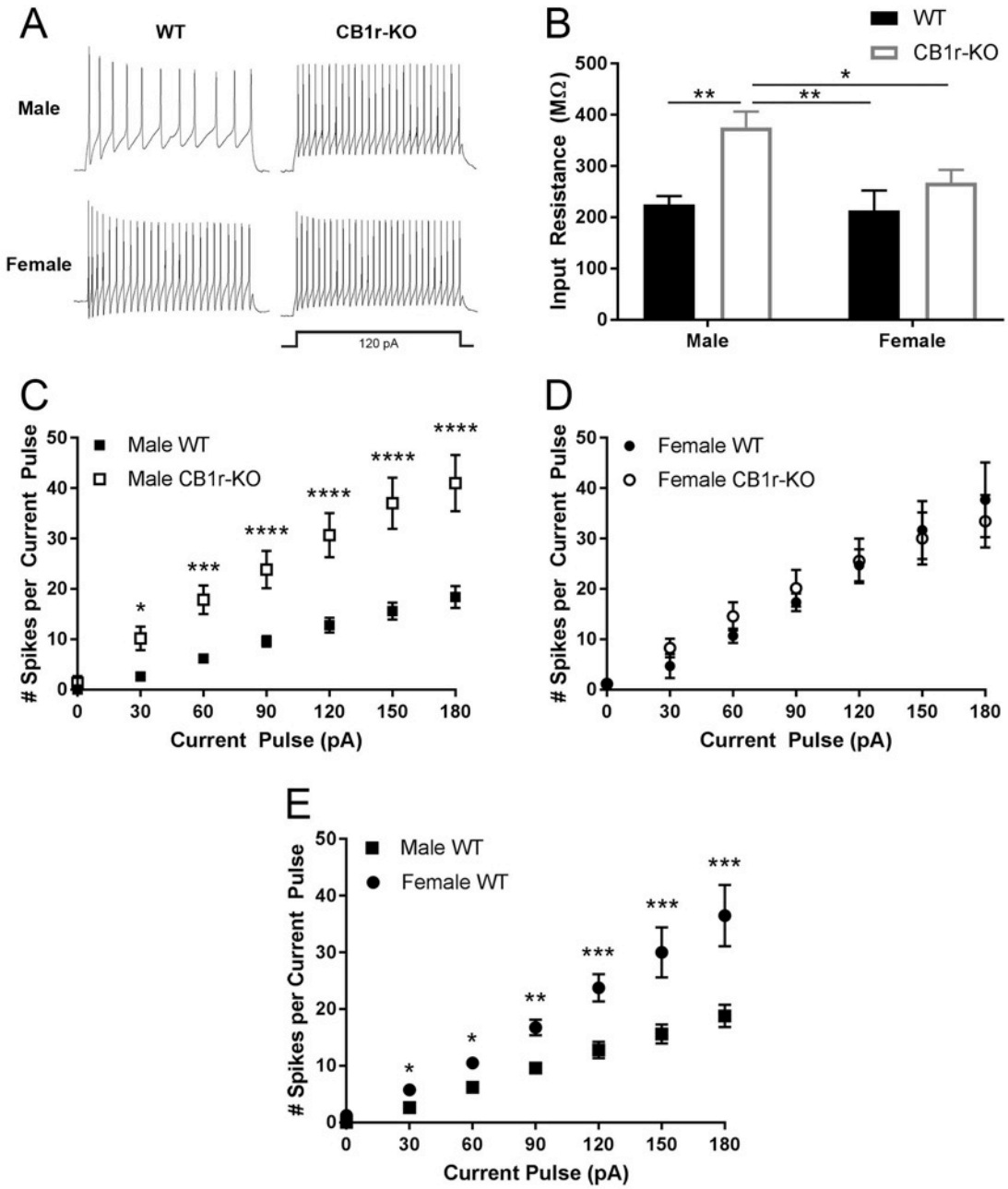

Figure 1.1
CB1r deletion causes an increase in LC-NE excitability in male mice.

Figure 1.1: CB1r deletion causes an increase in LC-NE excitability in male mice. a. Voltage responses to a 120 pA current pulse from representative WT and CB1r-KO male and female mice. **b.** LC-NE neurons from male CB1r-KO mice have a significantly larger input resistance compared to WT males and WT females. CB1r deletion does not cause a change in input resistance in female LC-NE cells. Panels **c**, **d,** and **e** summarize excitability data in the form of stimulus-response curves to a range of current pulses (0-150 pA in 30 pA increments) in male and female WT and CB1r-KO mice. CB1r deletion results in a significant increase in excitability of LC-NE cells in male mice (leftward shift in **c**), which it does not affect LC-NE excitability in female mice (no shift in **d)**. **e.** Female WT mice have a significant increase in LC-NE excitability compared to male WT mice. Data represent mean ± SEM. Astrisks indicate a significant difference between groups as determined by two-way and three-way repeated measure ANOVAs/mixed-effects regression model (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Baseline membrane voltage was not different between CB1r KO and WT for male and female mice. Further statistical examination of the data via Tukey post-hoc analysis revealed that genotype had a significant effect across all current pulses in male mice ($p \leq 0.016$), but not females ($p \geq 0.24$). Male CB1r-KO mice exhibited an increase in LC-NE cell excitability compared to male WT mice [leftward shift of the stimulus-response curve; significantly increased excitability responses to 30 ($p<0.05$), 60 ($p<0.001$), 90 ($p=0.0001$), 120 ($p<0.0001$), 150 ($p<0.0001$), and 180 pA ($p<0.0001$) current pulses] (Fig. 1.1C). However, female CB1r-KO mice did not show a significant increase in excitability compared to female WT mice (Fig. 1.1D). Additionally, a significant effect of sex on WT mice across all current pulses was also observed. When comparing sexes, female WT mice showed a significant increase in LC-NE excitability compared to male WT mice [significantly increased excitability responses to 30 ($p<0.05$), 60 ($p<0.05$), 90 ($p<0.001$), 120 ($p<0.001$), 150 ($p<0.001$), and 180 pA ($p<0.001$). No significant effect of sex was observed between CB1r-KO mice at any of the current pulses. Representative traces from each group showing the number of spikes caused by 120pA current pulse: male WT – 11 spikes, male KO – 25, female WT – 25, female KO – 28 (Fig. 1.1A). Slices containing patched cells were processed for immunohistochemistry, to confirm that the data was collected from NE producing cells within the LC. Only cells that were labeled with both tyrosine hydroxylase, a marker for NE production in the LC, and biocytin were included in the analyses (Fig. 1.2). These data provide evidence that genetic deletion of CB1r increases LC-NE neuron excitability in males, increasing the ability of these male CB1r-KO LC-NE cells to respond to excitatory synaptic inputs.

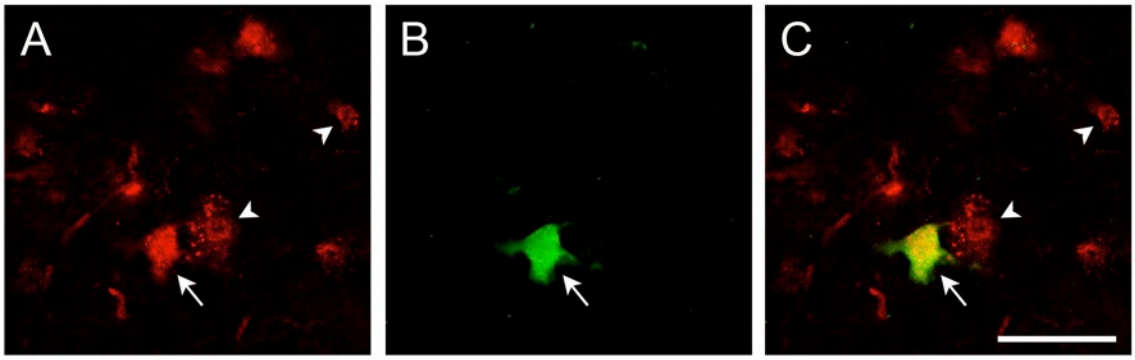

Figure 1.2
Confirming electrophysiological data is obtained from NE producing cells within the LC.

Figure 1.2: Confirming electrophysiological data is obtained from NE producing cells within the LC. Confocal fluorescence micrographs showing TH (red), biocytin (green), and co-localization (yellow) in the LC. **a.** TH was detected using an Alexa Fluor 647-conjugated secondary antibody. **b.** Biocytin was detected using an Alexa Fluor 488-conjugated streptavidin antibody. **c.** *Arrow* depicts co-localization between biocytin and TH, confirming that the patched cell was an LC-NE neuron. *Single arrowheads* indicate TH labeled cell bodies in the LC that were not patched. Scale bar = 25μm.

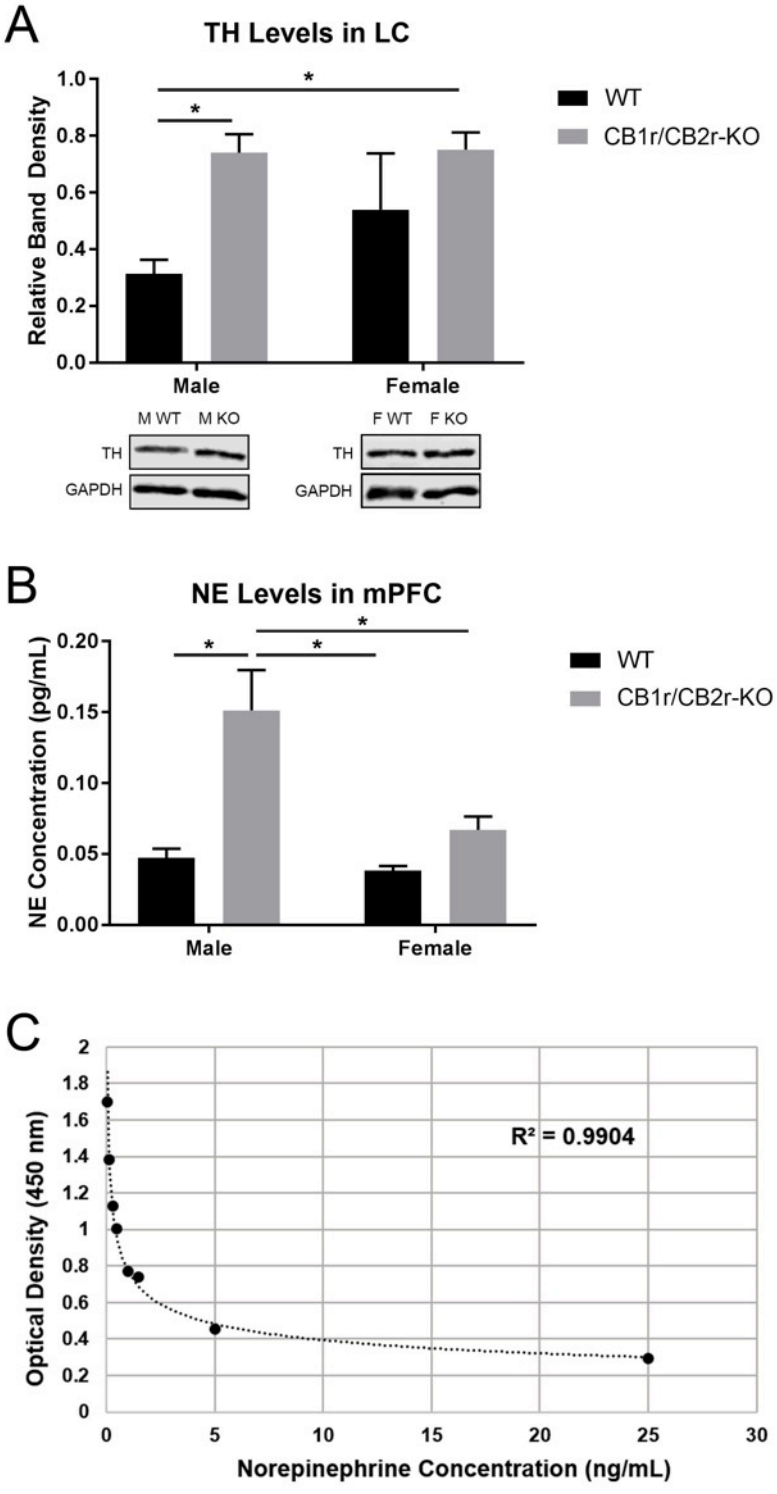

Figure 1.3
Western blot and ELISA analyses of NE indices in the LC and mPFC of male and female WT and CB1r/CB2r-KO mice.

Figure 1.3: Western blot and ELISA analyses of NE indices in the LC and mPFC of male and female WT and CB1r/CB2r-KO mice. Bands shown are representative of one sample obtained from one animal per group. **a.** Western blot analysis for TH in protein extracts from the LC of male and female WT and CB1r/CB2r-KO mice. TH expression is significantly higher in male CB1r/CB2r-KO mice compared to male WT mice; however, there is no significant change in TH expression between female WT and CB1r/CB2r-KO. **b.** ELISA for NE in protein extracts from the mPFC of male and female WT and CB1r/CB2r-KO mice. NE levels are significantly increased in male CB1r-KO mice compared male WT, female WT, and female CB1r/CB2r-KO mice. CB1r deletion does not cause an increase in mPFC NE levels in female mice. **c.** Standard curve used to determine the NE concentration in LC protein extracts from each sample animal. Standards were run on the same plate as samples to avoid variability, and the R^2 value for the curve equals 0.9904. Data represent mean ± SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVAs/mixed-effects regression model (* $p < 0.05$).

Another way to assess alterations in LC-NE activity is to look at changes in NE production (Fig. 1.3). Tyrosine hydroxylase (TH) is involved in the rate-limiting step in NE synthesis, and is used as a marker for NE production within the LC. There was a significant effect of genotype on TH expression levels ($F_{1,14}$=11.76, $p<0.01$). Tukey's post-hoc analysis revealed that WT male mice have significantly lower normalized levels of TH (0.314 ± 0.049) compared to CB1r/CB2r-KO males (0.741 ± 0.064; $p<0.05$) and KO females (0.751 ± 0.061; $p<0.05$), while no significant increase is observed between WT (0.539 ± 0.209) and KO females (0.751 ± 0.061; $p>0.4$) (Fig. 1.3A). By using an ELISA, it is possible to more precisely determine changes in NE levels in the mPFC, representing a concrete endpoint for the effect of cannabinoid receptor deletion on LC-NE activity. Two-way ANOVA indicated a significant effect of genotype on NE levels ($F_{1,11}$=9.752, $p<0.01$). Male CB1r/CB2r-KO mice have significantly increased levels of NE in the mPFC (0.151 ± 0.032 pg/mL; $p<0.05$) compared to all other groups (Fig. 1.3B): WT males (0.049 ± 0.008 pg/mL), WT females (0.039 ± 0.003 pg/mL; $p<0.05$), and KO females (0.071 ± 0.011 pg/mL; $p<0.05$). The Western and ELISA data confirms that deletion of the CB1r causes a significant increase in NE indices in male but not female mice.

Sex differences in cellular adaptations following cannabinoid receptor deletion

In order to better understand what might be causing the increase in LC-NE activity in male CB1r-KO mice but not females, Western blot analyses were performed to determine what other changes in protein levels might be occurring (Fig. 1.4). Within the LC, the α2-AR functions to auto-inhibit LC-NE neurons. When examining changes in

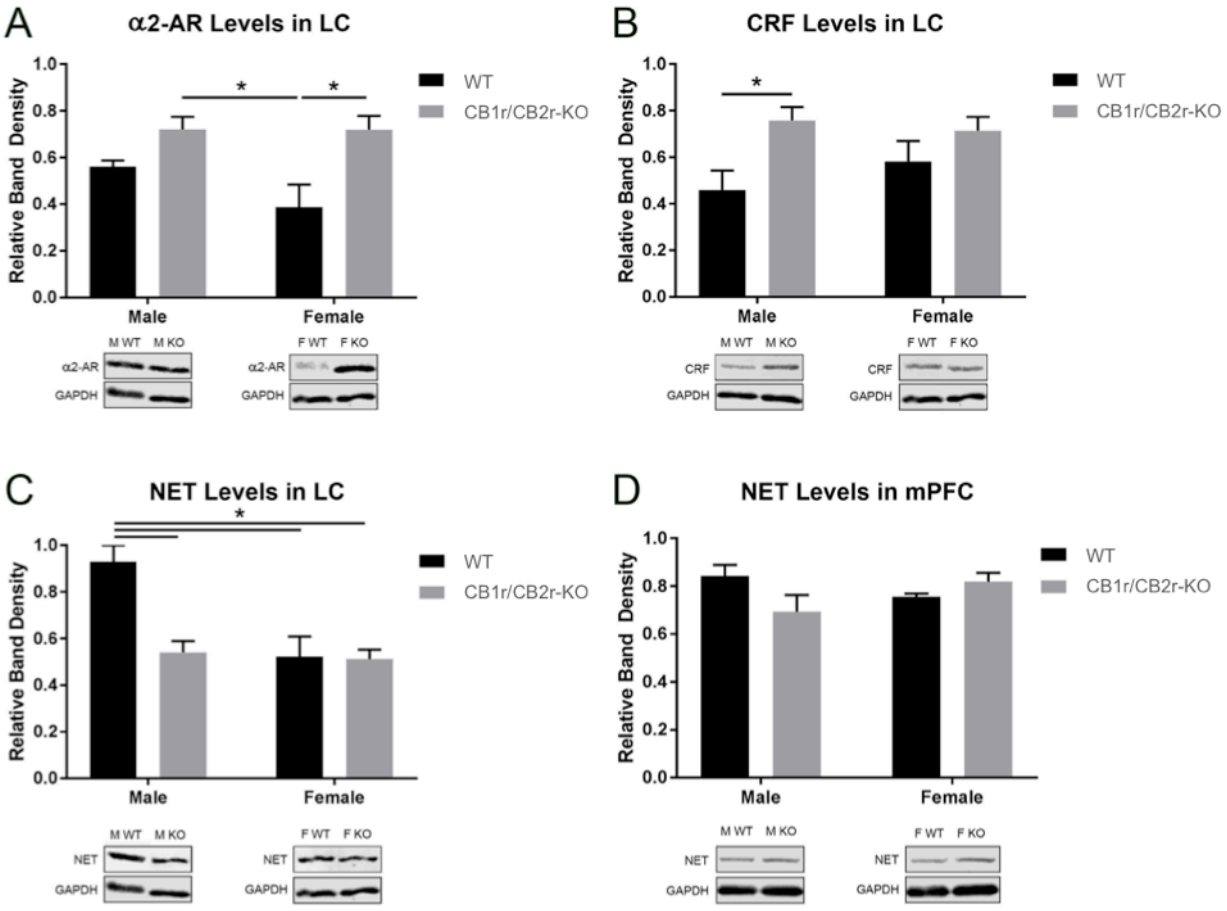

Figure 1.4
Western blot analysis of α2-adrenoreceptor (α2-AR), corticotropin-releasing factor (CRF), and norepinephrine transporter (NET) expression in the LC and mPFC in male and female WT and CB1r/CB2r-KO mice.

Figure 1.4: Western blot analysis of α2-adrenoreceptor (α2-AR), corticotropin-releasing factor (CRF), and norepinephrine transporter (NET) expression in the LC and mPFC in male and female WT and CB1r/CB2r-KO mice. Bands shown are representative of one sample obtained from one animal per group. **a.** Western blot analysis for α2-AR expression in protein extracts from the LC of male and female WT and CB1r/CB2r-KO mice. CB1r/CB2r deletion causes a significant increase in α2-AR expression compared to WT mice in females, but no change in males. **b.** Western blot analysis for CRF expression in protein extracts from the LC of male and female WT and CB1r/CB2r-KO mice. There is a significant increase in CRF levels in the LC of male CB1r/CB2r-KO mice compared to male WT mice, while no change occurs in female mice. **c.** Western blot analysis for NET expression in protein extracts from the LC of male and female WT and CB1r/CB2r-KO mice. Male WT mice have significantly greater NET levels in the LC compared to male CB1r/CB2r-KO, female WT, and female CB1r/CB2r-KO mice. **d.** Western blot analysis for NET expression in protein extracts from the mPFC of male and female WT and CB1r/CB2r-KO mice. No significant change in NET expression is observed across sexes or genotype. Data represent mean ± SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVAs/mixed-effects regression model (* $p < 0.05$).

normalized α2-AR protein levels following CB1r/CB2r deletion (Fig. 1.4A), a two-way ANOVA determined a significant effect of genotype on α2-AR expression ($F_{1,14}$=13.51, p<0.01). Tukey's post-hoc analysis showed no significant change between WT males (0.560 ± 0.027) and KO males (0.721 ± 0.054; p=0.365); however, KO females (0.484 ± 0.009) showed a significant increase compared to WT females (0.720 ± 0.059; p<0.05). CB1r-KO mice have heightened HPA-activity and the LC is involved in the cognitive limb of the stress response; therefore, changes in CRF levels within the LC were also determined (Fig. 1.4B). Two-way ANOVA revealed a significant effect of genotype on CRF expression ($F_{1,14}$=8.888, p<0.01). Tukey's post-hoc analysis determined that CB1r/CB2r deletion resulted in a significant increase in normalized CRF levels in KO males (0.467 ± 0.088) compared to WT males (0.752 ± 0.062; p<0.05), but no significant change was observed between WT (0.581 ± 0.139) and KO females (0.715 ± 0.059; p=0.576). Finally, changes in LC expression of the norepinephrine transporter (NET) were assessed (Fig. 1.4C). Two-way ANOVA revealed a significant effect of sex ($F_{1,13}$=12.8, p<0.01), genotype ($F_{1,13}$=10.66, p<0.01), and interaction between sex* genotype ($F_{1,13}$=9.595, p<0.01) on LC NET expression. Tukey's post-hoc analysis showed significantly greater expression of NET in WT male mice (0.893 ± 0.088) compared to all other groups: KO males (0.541 ± 0.048; p<0.01), WT females (0.522 ± 0.071; p<0.01), and KO females (0.512 ± 0.041; p<0.001). Within the mPFC, there were no significant changes in NET expression (Fig. 1.4D): WT males (0.842 ± 0.047), KO males (0.693 ± 0.070), WT females (0.756 ± 0.013), and KO females (0.819 ± 0.036). The increase in CRF expression in male KO mice compared to WT and α2-AR

expression in female KO mice compared to WT might provide some insight into why CB1r-deletion causes an increase in LC-NE excitability in males but not females.

Loss of CRF-induced increases in LC-NE excitability in CB1r-KO mice

We determined how CRF affects LC-NE excitability under conditions of CB1r deletion. After baseline measurements were recorded from male and female WT neurons, CRF (300 nM) was bath applied and the effect of drug treatment was then recorded. This dose of CRF did not cause a significant increase in the input resistance of male (Fig. 1.5B) or female (Fig. 1.5B') WT and CB1r-KO mice when compared to baseline: male WT CRF (274 ± 29 MΩ), male KO CRF (417 ± 52 MΩ), female WT CRF (259 ± 43 MΩ), and female KO CRF (282 ± 48 MΩ). Data for neuronal excitability was analyzed using a four-way repeated measure ANOVA/mixed-effects regression model (sex effect by genotype*current pulse, genotype effect by sex*current pulse, genotype effect by drug treatment*current pulse, drug effect by genotype*current pulse) and the following effects were found significant: drug treatment ($F_{1,20}$=6.92, $p<0.05$), current pulse ($F_{6,108}$=159.27, $p<0.0001$), interaction between sex*genotype ($F_{1,18}$=10.55, $p<0.01$), interaction between genotype*current pulse ($F_{6,108}$=4.85, $p<0.001$), interaction between sex*genotype*current pulse ($F_{6,108}$=4.80, $p<0.001$), and interaction between genotype*drug treatment ($F_{1,20}$=5.48, $p<0.05$), indicating all four factors were important predictors of neuronal excitability. CRF (300 nM) treatment caused a significant increase in LC-NE cell excitability in both male WT (Fig. 1.5C) and female WT (Fig. 1.5C') mice, as expected. Tukey post-hoc analyses showed a significant drug treatment effect across WT male and female mice ($p<0.0001$ for 30, 60, 90, 120, and 150 pA current pulses; $p<0.01$ for 180 pA

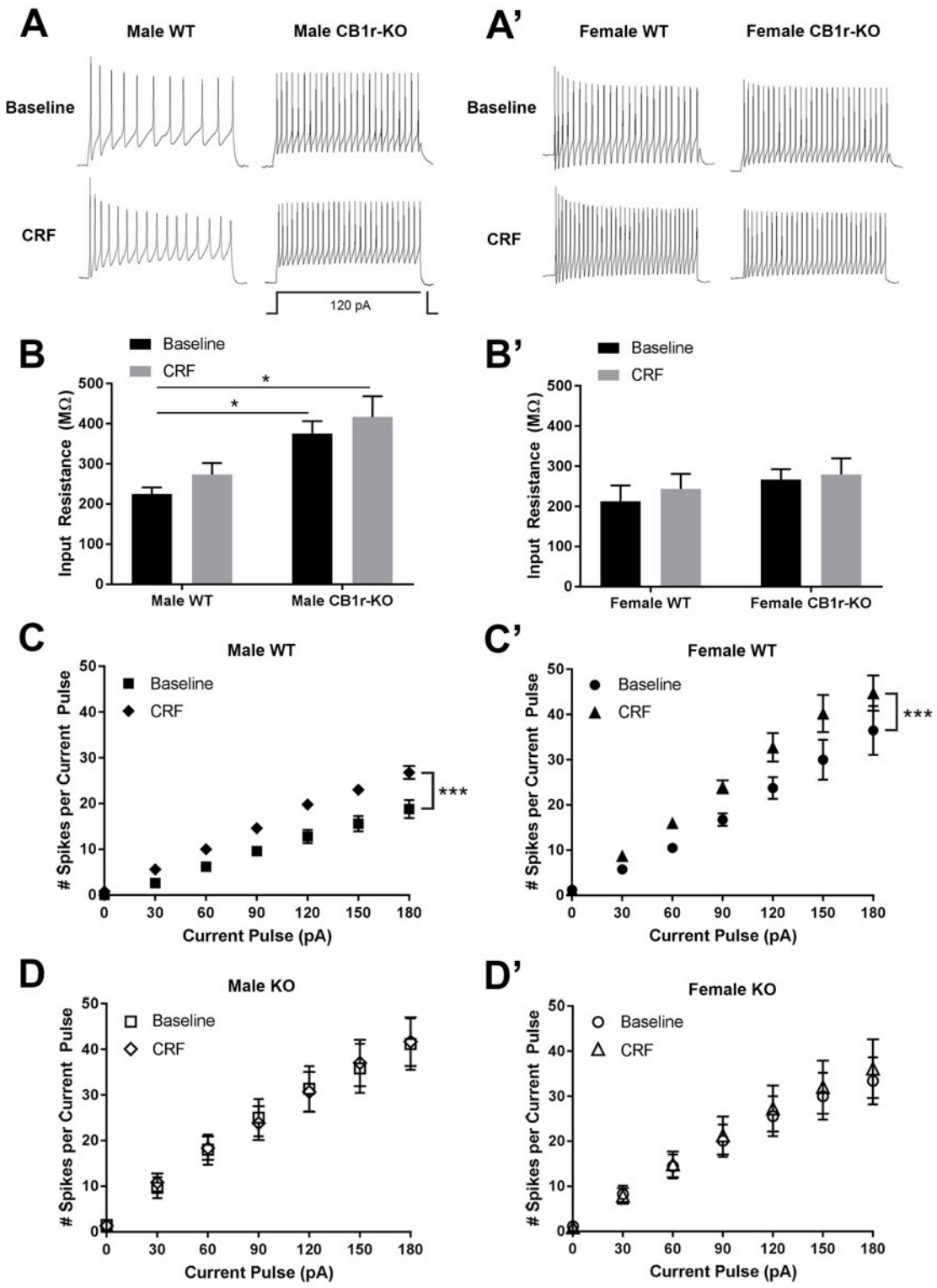

Figure 1.5
CB1r deletion disrupts CRF-induced increases in LC-NE excitability in male and female mice.

Figure 1.5: CB1r deletion disrupts CRF-induced increases in LC-NE excitability in male and female mice. Voltage responses to a 120 pA current pulse are shown from representative WT and CB1r-KO male (**a**) and female (**a'**) mice both at baseline and following CRF (300 nM) treatment. **b.** Bath application of CRF (300 nM) does not cause in increase in WT or CB1r-KO neuronal excitability in male or female (**b'**) mice. Panels **c, c', d, and d'** summarize excitability data in the form of stimulus-response curves to a range of current pulses (0-150 pA in 30 pA increments). Bath application of CRF (300 nM) results in a significant increase in excitability of LC-NE cells in male WT mice (leftward shift in **c**) and female WT mice (leftward shift in **c'**); however, 300 nM CRF does not alter the excitability of LC-NE cells in male CB1r-KO mice (no shift in **d**) and female CB1r-KO mice (no shift in **d'**). Data represent mean ± SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVA (**c** and **c')** and four-way repeated measure ANOVAs/mixed-effects regression model (**d, d', e,** and **e'**; * $p < 0.05$; *** $p < 0.001$).

current pulse). Interestingly, CRF did not cause a significant increase in LC-NE cell excitability in male (Fig. 1.5D) and female (Fig. 1.5D') CB1r-KO mice, as the post-hoc analyses showed no significant effect of drug treatment across KO male and female mice ($p \geq 0.63$). Representative traces from each group showing the number of spikes caused by 120pA current pulse are provided in figure 5: male WT baseline – 11 spikes, male WT CRF – 16 spikes, male KO baseline – 25, male KO CRF – 28 (Fig. 1.5A), female WT baseline – 25 baseline, female WT CRF – 33, female KO baseline – 28, female KO CRF – 30 (Fig. 1.5A'). These electrophysiological results suggest that while 300 nM CRF causes a significant increase in LC-NE cell excitability in WT male and female mice, it is not capable of producing the same effect in CB1r-KO mice.

When examining single action potentials, CRF administration, sex, and genotype did not affect many of the analyzed characteristics (Table II). Resting membrane potential remained constant among all groups, as did action potential threshold, duration, and after-hyperpolarization amplitude. CRF bath application did cause a significant increase in action potential amplitude in WT females compared to baseline ($p<0.05$). Additionally, after-hyperpolarization duration was significantly reduced following CRF administration in WT males, KO males, and WT females ($p<0.01$; Fig. 1.6). CB1r deletion also caused a significant reduction in after- hyperpolarization duration in males, but not in females.

Table II: Action potential and after-hyperpolarization characteristics of LC-NE neurons before and after 300 nM CRF bath application in wild type (WT) and CB1r-knock out (KO) male and female mice. Data represent mean ± SEM. Asterisks indicate a significant difference ($p<0.05$) between groups as determined by two-way ANOVAs/mixed-effects regression models (* : significant difference between CRF treatment and baseline; + : significant difference between sex; ^ : significant difference between genotype).

	Male				Female			
	WT		CB1r KO		WT		CB1r KO	
	Baseline	CRF	Baseline	CRF	Baseline	CRF	Baseline	CRF
Resting Membrane Potential (mV)	-51.50 ± 4.99	-50.25 ± 5.17	-55.40 ± 2.32	-53.60 ± 2.50	-50.67 ± 0.67	-54.00 ± 2.08	-52.00 ± 8.55	-51.25 ± 6.88
Action Potential Threshold (mV)	-25.72 ± 5.20	-24.67 ± 5.62	-32.76 ± 2.60	-34.77 ± 3.21	-31.99 ± 2.72	-34.98 ± 4.98	-26.62 ± 2.08	-33.52 ± 5.83
Action Potential Amplitude (mV)	68.08 ± 2.65	61.48 ± 3.37	63.87 ± 4.78	58.40 ± 5.29	68.82 ± 4.93	67.58 ± 5.86	42.72 ± 10.76	25.58 ± 18.84
Action Potential Duration (ms)	0.88 ± 0.13	0.88 ± 0.17	0.77 ± 0.05	0.77 ± 0.06	0.67 ± 0.09	0.74 ± 0.10	0.98 ± 0.27	0.94 ± 0.32
Afterhyperpolarization Amplitude (mV)	-18.68 ± 2.63	-18.11 ± 2.93	-16.06 ± 1.14	-15.83 ± 1.23	-18.91 ± 1.38	-17.15 ± 2.41	-15.57 ± 0.88	-11.97 ± 2.45
Afterhyperpolarization Duration (ms)	117.4 ± 8.7	80.9 ± 10.9*	85.1 ± 5.2^	55.1 ± 4.3*	73.3 ± 9.4+	45.6 ± 4.6*	60.8 ± 4.3^	48.7 ± 2.5

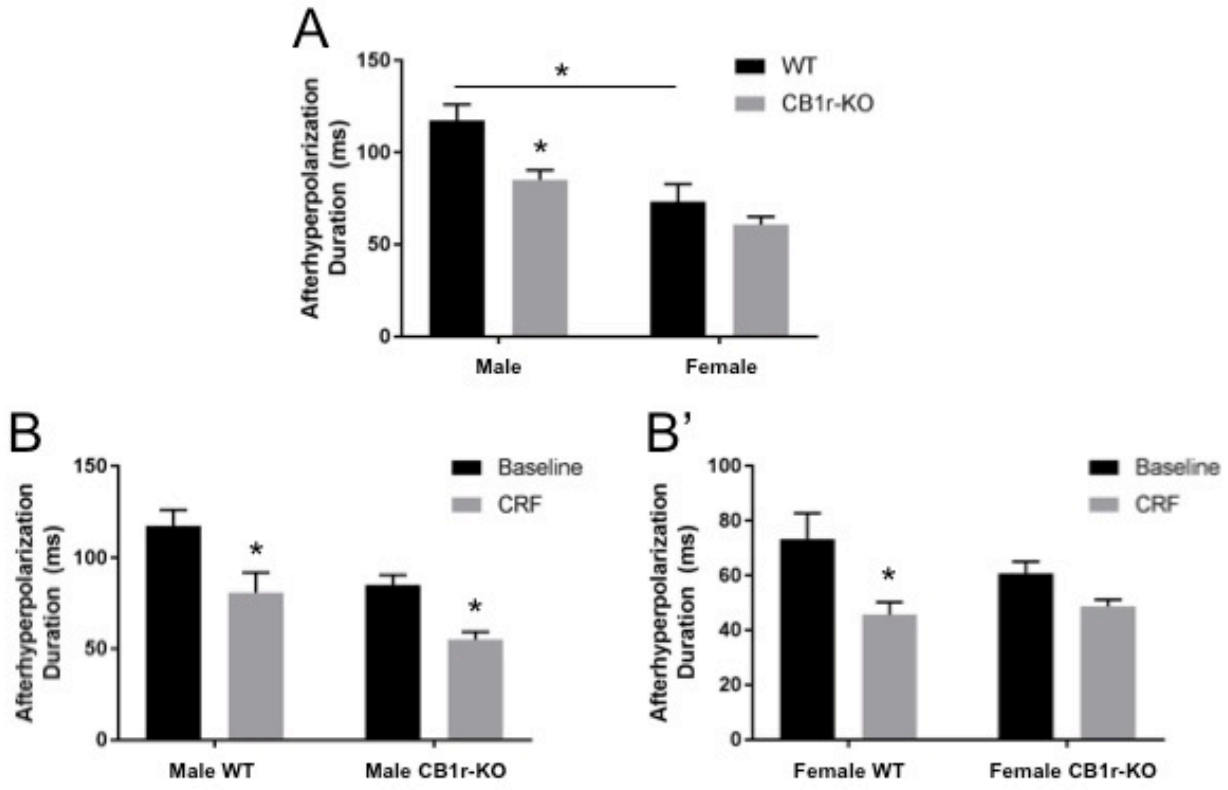

Figure 1.6
Sex, genotype, and CRF treatment affect the after-hyperpolarization (AHP) duration of LC-NE neurons.

Figure 1.6: Sex, genotype, and CRF treatment affect the after-hyperpolarization (AHP) duration of LC-NE neurons. **a.** LC-NE cells from female WT mice have a significantly shorter AHP duration compared to cells from WT males ($p<0.05$). Additionally, there is a sex difference in the effect of CB1r deletion, with neurons from male CB1r-KO mice having a shorter AHP duration compared to neurons male WT mice ($p<0.05$), but no difference between female CB1r-KO and WT cells. **b** and **b'** show the effect of 300 nM CRF bath application on the AHP duration of LC-NE cells, separated by sex. **b.** CRF causes a significant reduction in AHP duration in both male WT and male CB1r-KO neurons ($p<0.05$). **b'.** CRF causes a significant

Discussion

The present study highlights interesting sex differences in LC neurons following cannabinoid receptor deletion. Electrophysiological studies conducted using an in vitro slice preparation show that CB1r deletion results in a significant increase in LC-NE excitability in male mice, but not in females. Additionally, male CB1r-KO mice have a significant increase in TH expression in the LC and NE levels in the mPFC compared to WT males, which is not observed in females. Via Western blot analysis, changes in protein expression across genotype and sex were observed. Male CB1r/CB2r-KO mice exhibited an increase in CRF expression and a decrease in NET expression in the LC compared to male WT mice, and female CB1/CB2r-KO mice had an increase in α2-AR levels in the LC compared to female WT mice. Finally, CB1r deletion affected CRF-induced increases in LC-NE activity. Bath application of CRF caused an increase in LC-NE excitability in male and female WT mice; however, the effect of CRF was lost in CB1r-KO mice. When examining individual membrane characteristics, LC-NE cells from male CB1r-KO mice showed a decrease in after-hyperpolarization duration. Additionally, CRF treatment caused a further decrease in after-hyperpolarization duration in LC-NE neurons from WT males and CB1r-KO males, and also a decrease in WT females. These data further highlight the importance of the endocannabinoid system in maintaining normal brain adrenergic function, especially in male mice, where CB1r deletion had the most profound effect.

Methodological considerations

While electrophysiology experiments were carried out in CB1r-KO mice, Western blot and ELISA experiments were carried out in CB1r/CB2r-KO mice due to tissue availability. Many immunohistochemical studies have identified CB1r as an abundant protein in the LC, both directly on the noradrenergic neurons as well as on presynaptic afferents synapsing onto LC-NE dendrites and cell bodies (Scavone et al., 2010; Wyrofsky et al., 2017). While CB2r is traditionally thought to play a role in the periphery and immune responses (Castillo et al., 2012), growing accumulating evidence suggests that it does in fact play a role in neuronal signaling in some select brain regions (Van Sickle et al., 2005; Gong et al., 2006). However, direct evidence of CB2r on LC-NE neurons has not been shown. Additionally, mixed CB1r/CB2r agonists such as THC, WIN 55,212-2, and CP 55,940 all dose-dependently increase LC-NE firing, and their effects are completely blocked by CB1r-selective antagonist SR141716A (Mendiguren & Pineda, 2006; Muntoni et al., 2006). These studies suggest that there is a significantly greater influence of CB1r than CB2r on LC-NE neurons, providing indirect evidence for a minimal role of CB2r signaling in the LC. Therefore, the effects of CB2r deletion in the LC should be less substantial than that of CB1r deletion, and the differences between CB1r-KO mice and CB1r/CB2r-KO mice should be minimal. However, the caveat exists that findings reported here are due to deletion of both receptor phenotypes.

Several studies have reported fluctuations in CB1r mRNA expression and eCB levels across the estrus cycle in several brain regions, including the anterior pituitary (Gonzales et al., 2000) and hypothalamus (Rodriguez de Fonseca et al., 1994), however the effect of ovarian hormones on CB1r and eCB levels in the LC has not been identified.

While estrogen might alter the endocannabinoid system, no effect of the estrus cycle was observed in preliminary studies examining the anti-depressant like behavioral effects of CB1r antagonism (Steiner et al., 2008). Additionally, a study specifically examining the LC region controlled for the estrus cycle and found no notable effect of phase on female LC-NE excitability, firing rate, or CRFr1 compartmentalization in both WT and CRF-overexpressing mice (Bangasser et al., 2013). Since no direct effect of estrus cycle was has been found on baseline WT LC-NE excitability, and since the effect of circulating sex hormones on the eCB system is less relevant when CB1r are genetically deleted, we did not note the estrus cycle prior to our experiments, though the caveat exists that controlling for the estrus cycle could alter our results.

Some experimental caveats exist with Western blot and ELISA analysis, including the accuracy of tissue punches and equal protein quantities across various animals and groups. In order to ensure that regions of interest were sampled accurately, one investigator preformed the micropunches for each animal. Additionally, Western blots were probed with GAPDH as an internal standard to ensure equal protein loading for each sample. Results were normalized to GAPDH expression, which was comparable across each animal group examined. Finally, while Western blot analysis allows us to examine changes in protein expression levels, subsequent studies examining changes in mRNA levels could be conducted to further elucidate the effects of CB1r deletion on noradrenergic indices in the LC.

CB1r-KO increases LC-NE excitability in males but not females

One of the most interesting and surprising findings from this study is that CB1r deletion affected LC-NE excitability selectively in male subjects. The changes observed in input resistance and after-hyperpolarization (AHP) duration across sex and genotype are consistent with the changes observed in LC-NE excitability. All groups that had heightened LC-NE excitability also showed a decrease in AHP duration: CB1r-KO males and WT females compared to WT males, CRF treated WT males and females compared to baseline. Additionally, CB1r-KO male and CRF treated WT males and females all had an increase in input resistance, suggesting a decrease in ion channel conductance. Neuronal increases in 3',5'-cyclic adenosine monophosphate (cAMP) cause increased Ca^{2+} efflux. Ca^{2+}-mediated increases in K^{+} conductance occur in LC-NE cells, leading to more rapid repolarization (Adams et al., 1982; Osmanovic and Shefner, 1993; Nestler et al., 1999). An increased repolarization rate corresponds with a shorter AHP duration, and suggests a general decrease in overall K^{+} ion conductance (Adams et al., 1982). Results from the Western blot analysis provide further insight into why only certain groups had increased excitability.

In males, a potential mechanism underlying increased LC-NE excitability involves differences in CRF expression. Interestingly, only male CB1r/CB2r-KO mice showed an increase in CRF expression in the LC when compared to WT and female subjects. This was surprising initially, since many studies have shown that CB1-KO mice have heightened HPA axis activity and increased vulnerability to chronic stress (Martin et al., 2002; Uriguen et al., 2004), and female rodents are known to have increased susceptibility to stressors compared to males (Bangasser and Valentino, 2012;

Handa and Weiser, 2014). However, more recent studies examining mice of both sexes report that females do not show HPA axis hyperactivity to the same degree as males following CB1r deletion or antagonism (Atkinson et al., 2010; Roberts et al., 2014). Thus, since the LC-NE cognitive limb of the stress response is engaged in parallel with the HPA axis (Van Bockstaele et al., 1998, Valentino and Van Bockstaele, 2008), it is tempting to speculate that female CB1r-KO mice might show less of an increase in CRF release in the LC compared to males. CRF binds to its receptor, CRFr1, on LC-NE neurons and increases LC-NE firing rate (Curtis et al., 1996). Both acute and chronic stress up-regulate the cAMP pathway (Nestler and Aghajanian, 1997), which, through downstream effects, increases neuronal re-polarization and decreases LC-NE AHP duration (Adams et al., 1982; Nestler et al., 1999). Therefore, increased CRF expression represents one mechanism by which CB1r-KO males might have increased LC-NE excitability while female CB1r-KO mice do not (Fig. 1.7B).

Another potential mechanism underlying the observed sex differences in this study involves alterations in α2-AR expression between female CB1r/CB2r-KO and WT mice. NE released from recurrent collaterals of LC neurons can bind to postsynaptic α2-AR on the LC-NE dendrites resulting in auto-inhibition (Lee et al., 1998a, b). In response, an increase in α2-AR would result in a decrease in cAMP and inhibition of LC-NE neurons, which is characterized by an increase in AHP and decrease in input resistance (Korf et al., 1973). While CB1r/CB2r deletion does not affect α2-AR levels in males, it does cause a significant increase in α2-AR expression in females. This could represent a compensatory mechanism in females, where another inhibitory receptor, α2-AR, is up-regulated to compensate for the loss of another inhibitory receptor, CB1r (Fig.

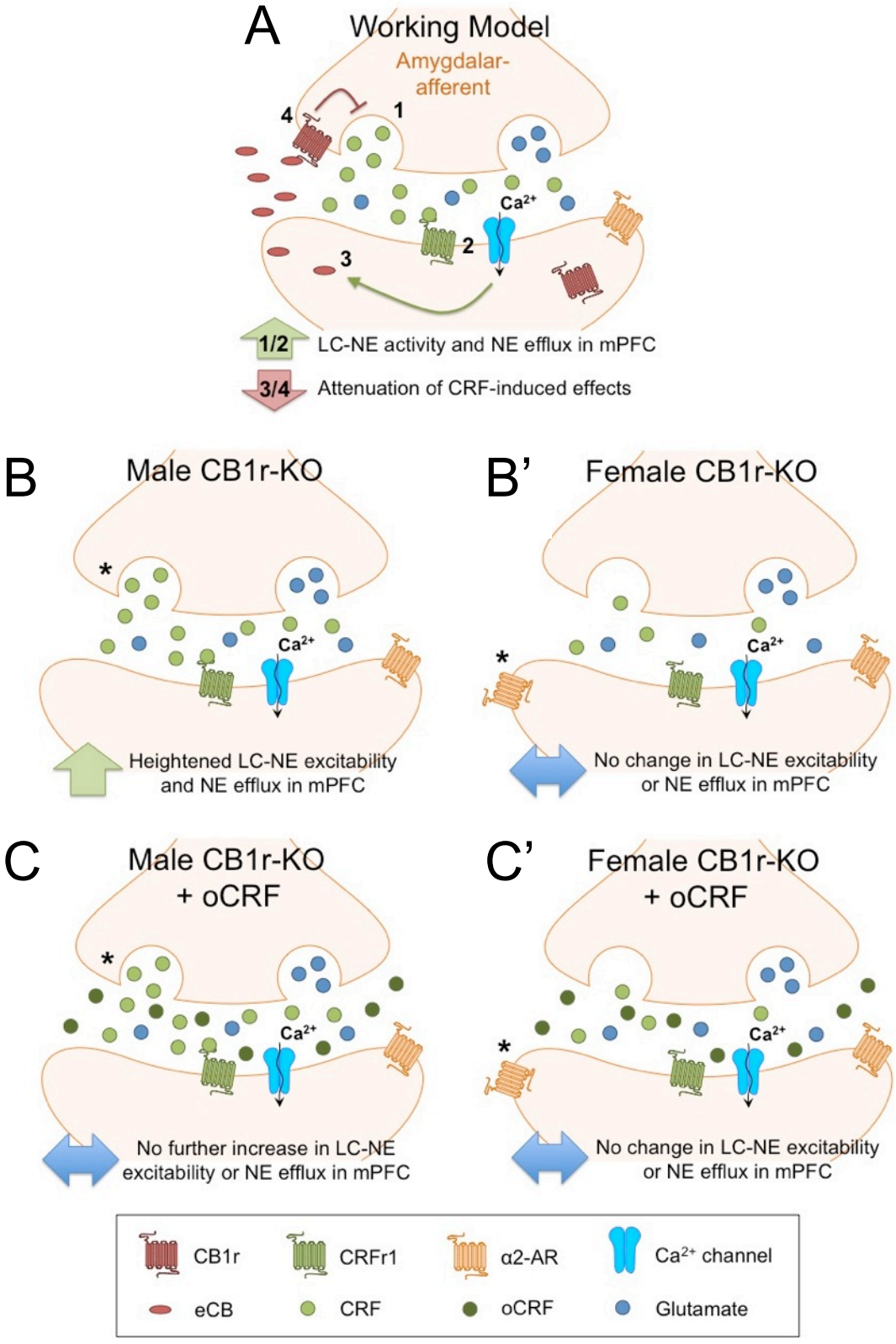

Figure 1.7
Schematic depicting the effects of CB1r deletion on LC-NE indices.

Figure 1.7: Schematic depicting the effects of CB1r deletion on LC-NE indices. This schematic is contains a combination of results from electrophysiology studies performed in CB1r-KO mice with Western blot and ELISA analysis performed on CB1r/CB2r-KO tissue. **a.** Our working model depicting how the endocannabinoid (eCB) system might modulate CRF-mediated responses within the LC. (1) Stressors cause CRF to be released from excitatory amygdalar afferents into the LC. (2) CRF binds to its postsynaptic CRF type 1 receptor (CRFr1), and subsequent post-synaptic depolarization and Ca^{2+} influx leads to increased LC-NE excitability and NE efflux in the mPFC. (3) Increased intracellular Ca^{2+} levels stimulate eCB production, which then cross the synapse in a retrograde fashion to bind to their cannabinoid type 1 receptors (CB1r). (4) CB1r activation leads to inhibition of pre-synaptic CRF and glutamate release, attenuating CRF-induced increases in LC-NE excitability and NE efflux in the mPFC. **b.** Male CB1r/CB2r-KO mice have increased CRF expression within the LC, which could be responsible for the heightened LC-NE excitability and subsequent NE efflux in the mPFC. **b'.** Female CB1r/CB2r-KO mice do not have a significant increase in CRF levels, which corresponds with the lack of change in LC-NE excitability and NE efflux in the mPFC of CB1r-KO mice compared to wild type mice. However, there is a significant increase in α2-adrenoceptor (α2-AR) expression in female CB1r/CB2r-KO mice compared to WT, while there is no significant change in α2-AR expression in males. **c.** Bath application of ovine CRF (oCRF) causes a significant increase in male and female WT mice, but not in CB1r-KO mice. In males, this might be due to saturation of CRFr1 by the increased endogenous CRF levels. **c'.** In females, the lack of oCRF-induced increases in LC-NE excitability could be attributed to the significant increase in α2-AR

expression. oCRF could induce local NE release and subsequent auto-inhibition, preventing oCRF-induced increases in LC-NE activity.

1.7B'). This results in comparable excitability levels across LC-NE neurons, input resistance, and AHP duration in CB1r-KO and WT females.

It is important to note that while WT females have heightened excitability, this does not correlate to a heightened basal firing rate. Previous studies by Bangasser et al. similarly found that female WT mice have increased LC-NE excitability compared to males, but the basal firing rate of these neurons is not significantly different across sexes (Bangasser et al., 2013). In corroboration with Bangasser's findings that male and female LC-NE neurons have similar firing rates, our study shows that there is no significant difference in NE levels in the mPFC across sexes – another endpoint for LC-NE firing. While we observe an increase in LC-NE excitability in male CB1r-KO mice, we did not examine the basal firing rates between male and female WT and CB1r-KO mice. It would be tempting to postulate that CB1r-KO males would also have an increase in LC-NE firing rate compared to the other groups, as they are the only group with increased mPFC NE levels. Subsequent electrophysiology studies could further elucidate this hypothesis.

Loss of CRF-mediated responses in CB1r-KO mice

Although CRF is known to modulate LC-NE neurons and increase their firing rate (Curtis et al., 1996; Valentino et al., 1997; Jedema and Grace, 2004), and this study confirmed that 300 nM CRF bath application is capable of doing so in LC neurons from WT males and females, this effect was not observed in CB1r-KO mice. This loss of CRF-mediated increases in LC-NE excitability in male CB1r-KO mice could be due to the increased endogenous CRF levels saturating CRFr1, rendering the exogenous

application of oCRF ineffective (Fig. 1.7C). In female CB1r-KO mice, the lack of CRF-induced responses might be attributed to the increased α2-AR expression, causing local auto-inhibition and attenuation of LC-NE activity following oCRF administration (Fig. 1.7C').

In addition to the eCB system, there are other endogenous anti-stress peptides that are known to suppress the stress response – several of which have been localized to the LC (Van Bockstaele et al., 2000; Tjoumakaris et al., 2003; Reyes et al., 2008). The opioid system is capable of reducing the effects of stress throughout the brain, predominantly through the G_i-coupled μ-opioid receptor (MOR), which is abundantly expressed in the LC (Williams and North, 1984). Within the LC, the endogenous opioid enkephalin is co-released with CRF, and by itself, enkephalin is capable of inhibiting LC-NE activity (Curtis et al., 2001; Curtis et al., 2012). Compensatory changes to the opioid system in CB1r-KO mice have been established in the striatum and other regions (Steiner et al., 1999; Befort, 2015), and CB1r-KO mice are less likely to self-administer morphine, illustrating the role of CB1r in the reinforcing effects of morphine (Cossu et al., 2001; Navarro et al., 2001). Previous studies from our group have reported that MOR and CB1r co-exist in cellular profiles within the LC, providing a direct anatomical substrate for putative interactions (Scavone et al., 2010). Therefore, it is tempting to speculate that CB1r deletion may modulate the endogenous opioid system in the LC. If MOR were to be up-regulated as a compensation mechanism for the lack of CB1r inhibition, it could contribute to the lack of CRF-induced increases in LC-NE excitability in CB1r-KO neurons. More recently, neuropeptide Y (NPY) has emerged as an anti-stress peptide, capable of attenuating stress-induced anxiety and PTSD symptoms (Heilig,

2004; Cohen et al., 2012; Serova et al., 2013). NPY and its two receptors, Y1 and Y2, are localized in the LC, and are also co-localized to synapses containing CRF-afferents (Warner et al., 2016). Microinjection of NPY into the LC results in anxiolysis (Kask et al., 1998). This anatomical positioning makes it possible for the NPY system to be altered to compensate for CB1r deletion. Future studies and Western blot analyses could be performed to assess whether either the endogenous opioid or NPY systems are dysregulated in CB1r-KO mice.

Finally, chronic stress has been shown to alter CRFr1 trafficking in a sex dependent manner, with males exhibiting increased stress-induced internalization while females exhibit stress-induced recruitment to the plasma membrane (Bangasser et al., 2010; Valentino et al., 2013). It is possible that increased HPA hyperactivity (Uriguen et al., 2004) and a chronic increase in CRF levels in the LC of male CB1r-KO mice could lead to sustained de-sensitization of CRF receptors. Immunoelectron microscopy experiments examining CRF receptor trafficking in LC-NE neurons of male and female CB1r-KO mice could provide further insight into changes in the stress circuitry following CB1r deletion. Additionally, basal differences in CB1r expression and eCB signaling across sexes might be contributing to the findings observed in these studies. Subsequent Western blot analyses investigating CB1r, FAAH, and other eCB metabolic proteins should be performed to determine their contribution to changes in LC-NE excitability across sexes and genotype.

Implications for pharmacotherapies

Dysregulation of NE is a key component in the development of anxiety and other stress-induced psychiatric disorders (Carvalho and Van Bockstaele, 2012). Furthermore, reduction in NET expression in the LC has been identified in individuals suffering from major depression and PTSD (Klimek et al., 1997; Pietrzak et al., 2013). While acute stress does not affect NET levels, chronic and repeated stress exposure can lead to downregulation (Zafar et al., 1997). Increased turnover and NE depletion, which can occur following a stressor (Korf et al., 1973), also leads to the downregulation of NET and upregulation of TH, and increased TH levels in the LC have also been correlated with the development of depression (Klimek et al., 1997). Male CB1r/CB2r-KO mice exhibit a decrease in NET and an increase in TH expression, while no change was observed between female CB1r/CB2r-KO and WT mice. These findings suggest that CB1r deletion or chronic antagonism may be more detrimental to males than females, creating a microenvironment in the LC of male KO mice that mimics conditions of chronic stress.

In addition to cellular adaptations that occur following cannabinoid receptor deletion, the lack of increase in LC-NE excitability in female CB1r-KO mice could be due to less basal eCB signaling in females. If the female LC is under less tonic regulation by eCBs, then removal of CB1r might have less profound of an effect. In the amygdala, basal differences in the eCB system have been observed across sex, with males having greater levels of 2-AG and AEA, while females have increased expression of the enzymes responsible for their degradation (Krebs-Kraft et al., 2010; Craft et al., 2013). A similar phenomenon might exist in the LC, and future Western blot and ELISA analyses of 2-AG, AEA, and their metabolic and synthesizing enzymes would provide further

insight into potential basal sex differences.

We have demonstrated in previous studies that CB1r are localized to CRF-containing afferents from the amygdala, and their presynaptic distribution in the peri-LC suggests that they might be capable of attenuating CRF release via activation by endogenous or exogenous cannabinoids. A working model (Fig. 7A) is that CRF released from amygdalar afferents binds to postsynaptic CRFr1 on LC-NE neurons, causing membrane depolarization and increase in LC-NE excitability. However, the influx of Ca^{2+} then stimulates the synthesis and release of eCBs, which in turn retrogradely traverse the synapse. They then bind to presynaptic CB1r on CRF-containing amygdalar axon terminals, aiding in the suppression of subsequent CRF release and helping return LC-NE activity back to baseline.

CB1r antagonists and inverse agonists have been investigated for a variety of disorders ranging from obesity to schizophrenia (Wyrofsky et al., 2015). Rimonabant, a CB1r antagonist originally used for the treatment of obesity, was discontinued due to adverse psychological side effects (Nissen et al., 2008). Based on the results obtained in this study, CB1r antagonism might not cause an increase in LC-NE excitability to the same degree in females that it would in males, and the effects from CB1r antagonist treatment might be less significant in females, highlighting the importance of using both genders when performing pre-clinical trials. Indeed, this would be in line with analyses performed on the adverse effects of rimonabant, which suggest that the odds ratio for developing depression after taking this CB1r antagonist was greatest in males aged 35-38 (Pi-Sunyer et al., 2006; Nissen et al., 2008). This study adds to the growing literature that dysregulation of the cannabinoid system can lead to the dysregulation of

noradrenergic signaling, especially in males, helping to advance our understanding of how these systems could be targeted for more effective treatment of psychological disorders.

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CHAPTER 2

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Co-localization of the cannabinoid type 1 receptor with corticotropin releasing factor-containing afferents in the noradrenergic nucleus locus coeruleus: implications for the cognitive limb of the stress response

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Abstract

The noradrenergic system has been shown to play a key role in the regulation of stress responses, arousal, mood, and emotional states. Corticotropin releasing factor (CRF) is a primary mediator of stress-induced activation of noradrenergic neurons in the nucleus locus coeruleus (LC). The endocannabinoid (eCB) system also plays a key role in modulating stress responses, acting as an "anti-stress" neuro-mediator. In the present study, we investigated the cellular sites for interactions between the cannabinoid receptor type 1 (CB1r) and CRF in the LC. Immunofluorescence and high-resolution immunoelectron microscopy showed co-localization of CB1r and CRF in both the core and peri-LC areas. Semi-quantitative analysis revealed that, 44% (208/468) of CRF-containing axon terminals in the core and 35% (104/294) in the peri-LC expressed CB1r, while 18% (85/468) of CRF containing axon terminals in the core and 6.5% (19/294) in the peri-LC were presynaptic to CB1r-containing dendrites. In the LC core, CB1r + CRF axon terminals were more frequently of the symmetric (inhibitory) type; while in the peri-LC, a majority were of the asymmetric (excitatory) type. Triple label immunofluorescence results supported the ultrastructural analysis indicating that CB1r + CRF axon terminals contained either gamma amino butyric acid or glutamate. Finally, anterograde transport from the central nucleus of the amygdala (CeA) revealed that CRF-amygdalar afferents projecting to the LC contain CB1r. Taken together, these results indicate that the eCB system is poised to directly modulate stress-integrative heterogeneous CRF afferents in the LC, some of which arise from limbic sources.

Introduction

The stress response is characterized by a coordinated set of endocrine, physiological and cognitive responses to perceived threats in the environment (Ulrich-Lai and Herman 2009). A critical aspect of the endocrine stress response is the tight feedback regulation that serves to restrain and terminate the response (Keller-Wood and Dallman 1984), which when dysregulated, contributes to the etiology of many stress-induced neuropsychiatric disorders (Plotsky et al. 1998; Wingenfeld and Wolf 2011). Feedback inhibition of the hypothalamic-pituitary-adrenal (HPA) axis by glucocorticoids is critical in terminating the endocrine limb of the stress response (Abou-Samra et al. 1986; Keller-Wood and Dallman 1984). However, other neural circuits involved in the stress response are differentially regulated (Herman and Cullinan 1997; Ulrich-Lai and Herman 2009).

Stressors that initiate the HPA response to stress also activate the brainstem locus coeruleus (LC)-norepinephrine (NE) system via the pro-stress neuropeptide, corticotropin releasing factor (CRF) (Vale et al. 1981; Valentino 1988). CRF-immunoreactive axon terminals synapse onto LC-NE dendrites and arise from multiple limbic-related and autonomic-related brain areas (Aston-Jones et al. 1991; Van Bockstaele et al. 1996a, b, 1999). Stress-induced increases in CRF from these afferent sources can lead to inappropriate increases in the firing of LC-NE neurons and subsequent dysregulation of NE release in limbic and cortical areas (Curtis et al. 1996; Valentino et al. 2006; Van Bockstaele et al. 2010). The parallel engagement of the HPA and LC-NE systems serves to coordinate both endocrine and cognitive limbs of the stress response (Valentino and Van Bockstaele 2008a). One mechanism for counteracting stress responses in these neural circuits is through stress-elicited engagement of neuromodulators that act in

opposition to pro-stress systems, such as engagement of the endogenous opioid system (Heilig 2004; Reyes et al. 2008a, 2011; Tjoumakaris et al. 2003; Torner et al. 2001; Valentino and Van Bockstaele 2001; Van Bockstaele et al. 2000). Identifying mechanisms underlying counter-regulation of the stress response may better inform therapeutic strategies to prevent or treat stress-related neuropsychiatric diseases.

The endocannabinoid (eCB) system is considered as an "anti-stress" neuromodulator that modulates pro-stress responses through effects on synaptic activity (Cota 2008; Viveros et al. 2007). Extracts of *cannabis* have been used as stress-reducing medicinals throughout history and by many cultures to reduce anxiety, pain, seizures, mania, and muscle spasms (Zuardi 2006). Modern research confirms certain benefits, with constituents of *cannabis,* Δ^9-tetrahydrocannabinol (THC) and cannabidiol, being reported as effective anti-anxiety agents and stress-reducers (Bergamaschi et al. 2011; Tournier et al. 2003). Emerging evidence also supports a role for the eCB system in the modulation of stress responses through effects on synaptic activity. The eCB ligands, *N*-arachidonoylethanolamine/anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are primarily synthesized postsynaptically in response to increases in intracellular Ca^{2+} or activation of phospholipase C β (Castillo et al. 2012; Di Marzo et al. 2004). Degradation of AEA and 2-AG occurs through the catabolic action of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase respectively (Castillo et al. 2012; Di Marzo et al. 2004). Acting as retrograde messengers, AEA and 2-AG cross the synapse, where they primarily act through G_i-coupled cannabinoid CB1r localized to axon terminals (Castillo et al. 2012; Herkenham et al. 1990; Van Sickle et al. 2005), thereby inhibiting neurotransmitter release. By modulating glutamatergic and gamma amino butyric acid

(GABA) ergic release, CB1r exert a profound effect on post-synaptic neuronal activity (Freund et al. 2003).

CB1r protein and mRNA have been localized to the LC (Derbenev et al. 2004; Herkenham et al. 1991; Mailleux and Vanderhaeghen 1992; Matsuda et al. 1993). At the ultrastructural level, CB1rs in the LC have been shown to be localized both pre-synaptically in axon terminals targeting NE-containing neurons as well as post-synaptically in somatodendritic processes (Scavone et al. 2010). Electron microscopy studies have shown that pre-synaptically distributed CB1r are localized to both excitatory and inhibitory type synapses (Scavone et al. 2010), which is consistent with electrophysiological studies. Systemic administration of CB1r agonists (Muntoni et al. 2006) and fatty acid amide hydrolase (FAAH) inhibitors (Gobbi et al. 2005) increase the firing rate of un-stimulated noradrenergic neurons in the LC in a CB1r-dependent manner. CB1r agonists also increase cFos expression in the LC (Oropeza et al. 2005; Patel and Hillard 2003), enhance *N*-Methyl-D-aspartate (NMDA)-induced firing of LC neurons (Mendiguren and Pineda 2004), and increase NE synthesis (Moranta et al. 2009) and release (Oropeza et al. 2005) in terminal regions. WIN 55212-2 suppresses the inhibition of LC firing induced by activation of GABAergic afferents to the LC (Muntoni et al. 2006). Taken together, these results are consistent with a mechanism by which activation of CB1r on excitatory or inhibitory type terminals in the LC result in increases in the firing of noradrenergic neurons. However, local administration of CB1r agonists into the LC does not alter the spontaneous firing of LC neurons (Mendiguren and Pineda 2006) suggesting an indirect effect of CB1r agonists on LC firing, perhaps through increased afferent activity into the LC.

Convergent lines of evidence support a suppressive CB1r mechanism on CRF. CRF-induced activation of the sympathetic nervous system is inhibited by CB1r agonist administration and is potentiated by CB1r antagonists (Shimizu et al. 2010). Activation of glucocorticoid receptors by cortisol causes an increase in eCB production, which then activates CB1r on presynaptic glutamatergic neurons within the paraventricular nucleus of the hypothalamus (PVN) resulting in a decrease in hypothalamic release of CRF (Hill et al. 2010). Additionally, a longer feedback loop exists, where activation of CB1r on GABA neurons within the prelimbic medial prefrontal cortex (mPFC) causes a disinhibition of GABAergic neurons in the bed nucleus of the stria terminalis (BNST) which then project to the PVN, ultimately leading to a decrease in CRF release (Hill and McEwen 2009; Hill et al. 2010). Because of the complex interaction of the eCB system on stress-related circuitry and the localization of both CB1r and CRF-afferents within the LC, we sought to examine anatomical substrates for putative interactions between CB1r and CRF in the LC. Therefore, the present study used light microscopy, confocal fluorescence microscopy, and high-resolution immunoelectron microscopy to define how CB1r may be positioned to regulate CRF afferents in the LC.

Methods

Animals

For all experiments, male Sprague-Dawley rats between 200 and 300 g (Jackson Laboratory, Sacramento, CA) were used. They were housed two per cage, under standard conditions (25^{o}C temperatures) and a 12 hour light/dark cycle (lights turned on at 7:00am). *Ad libitum* access to food and water was provided, and animal protocols were

approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee in accordance with the revised *Guide for the Care and Use of Laboratory Animals* (1996). All efforts were made to utilize only the minimum number of animals necessary to produce reliable scientific data.

Immunofluorescence

Rats were deeply anesthetized via isoflurane exposure (Vedco, Inc., St. Joseph, MO) in a holding cage. Once a sufficient level of anesthesia was achieved, rats were then transcardially perfused via the ascending aorta with heparin followed by a 4% formaldehyde solution in 0.1 M phosphate buffer (PB; pH 7.4). Brains were then dissected, post-fixed in the formaldehyde solution for 24 h, and placed in 30% sucrose and 0.1 M PB solution before sectioning. Forty-micrometer sections through the rostrocaudal extent of each brain were collected using a cryostat (Microm HM 50, Microm International GmbH, Walldorf, Germany). Serial sections through the LC were placed in 1% sodium borohydride in 0.1 M PB for 30 min to remove any aldehydes remaining from the perfusion, followed by a 30min incubation in 0.5% bovine serum albumin (BSA) in 0.1 M Tris buffered saline (TBS; pH 7.6). Following extensive rinsing in 0.1 M TBS, tissues were incubated overnight in a mixture of primary antibodies including (Table II.I): CRF peptide raised in guinea-pig (1:7000, Peninsula Laboratories, San Carlos, CA), CB1r raised in rabbit (1:1000, kindly provided by Dr. Ken Mackie, Indiana University, IN), vesicular glutamate transporter (VGlut) raised in mouse (1:4000, Synaptic Systems, Gottingen, Germany), glutamate decarboxylase (GAD) raised in goat

Table II.I: Characterization of the primary antibodies used for immunofluorescence microscopy (IF) and electron microscopy (EM).

Antigen	Immunogen	Host	Source	Catalog #	Dilution	References
TH	TH purified from rat PC12 cells	Mouse	Immunostar Inc	22941	1:5000 (IF)	(Van Bockstaele et al., 1993; Oropeza et al., 2005)
CB1r	Last 15 aa of the C terminal of the rat CB1r	Rabbit	Dr. Ken Mackie	n/a	1:1000 (IF & EM)	(Carvalho et al., 2010; Scavone et al., 2010)
CRF	Synthetic CRF peptide	Guinea-pig	Peninsula Laboratories	T-5007	1:7000 (IF) I:2000 (EM)	(Rudoy et al., 2009)
VGlut1	aa 456-560 of Strep-Tag fusion protein of rat VGlut1	Mouse	Synaptic Systems	135 311	1:4000 (IF)	(Javadi et al., 2015)
GAD-65/67	C-terminus of human GAD-67	Goat	Santa Cruz	sc-7513	1:700 (IF)	(Papay et al., 2006; Rubio-Aliaga et al., 2004)
Syn	Rat retina synaptophysin	Mouse	Millipore	MAB368	1:500 (IF)	(Yamanaka et al., 2011)
PHAL	Red kidney bean lectin receptor specific	Goat	Vector Laboratories	AS-2224	1:5,000 (IF)	(Van Bockstaele et al., 2001)

(1:700, Santa Cruz, Santa Cruz, CA), synaptophysin (Syn) raised in mouse (1:500, Merck Millipore, Billerica, MA), tyrosine hydroxylase (TH) raised in mouse (1:5000, Immunostar, Hudson, WI), unconjugated Phaseolus Vulgaris Leucoagglutinin (PHAL) raised in goat (1:5,000, Vector Laboratories, Burlingame, CA). For the primary antibodies that have not been previously characterized by our laboratory (VGlut, GAD, PHAL, and Syn), serial dilutions were performed to determine the optimal antibody concentration for the experiments. To visualize proteins, the following secondary antibodies were used, all at a concentration of 1:400 (Jackson ImmunoResearch): rhodamine isothiocyanate (TRITC) conjugated donkey anti-rabbit, fluorescein isothiocyanate (FITC) conjugated donkey anti-mouse, FITC conjugated donkey anti-goat, Alexafluor 647 conjugated donkey anti-guinea-pig, and Alexafluor 647 conjugated donkey anti-mouse. Additionally, some tissue sections were also incubated with 4',6-diamidino-2-phenylindole (DAPI; EMD Millipore, Billerica, MA) at 1:10,000 for 5 minutes and washed 3 times with 0.05 M PB. The tissue sections were then examined using a Olympus IX81 inverted microscope (Olympus, Hatagaya, Shibuya-Ku, Tokyo, Japan) equipped with lasers (Helium Neon laser and Argon laser; models GLG 7000; GLS 5414A and GLG 3135, Showa Optronics Co., Tokyo, Japan) with the excitation wavelength of 488, 543 and 635. The microscope is also equipped with filters (DM 405-44; BA 505-605; and BA 560-660) and with Olympus Fluoview ASW FV1000 program (Olympus, Hatagaya, Shibuya-Ku, Tokyo, Japan). Analysis of co-localization of profiles were obtained from dually labeled immunofluorescence images of CB1r and CRF taken from alternate LC sections of three rats (n=3) via the Coloc2 plug-in on FIJI ImageJ software. CRF (green) was set to channel 1 and CB1r (red) was set to channel 2, so the

Pearson's coefficients obtained are representative of the likelihood that CB1r is co-localized with respect to CRF. In order to best visualize co-localization in fluorescence micrographs, CB1r was always pseudocolored red, CRF and Syn, pseudocolored green, and glutamic acid decarboxylase (GAD) and vesicular glutamate transporter (VGlut) were pseudocolored cyan. Two sets of control tissues were processed in parallel, one with the omission of primary antibodies and the other with the omission of secondary antibodies. As an additional control, rabbit anti-CB1r was processed with both TRITC conjugated donkey anti-rabbit and Alexafluor 647 conjugated donkey anti-guinea-pig, and guinea-pig anti-CRF was also processed with both TRITC conjugated donkey anti-rabbit and Alexafluor 647 conjugated donkey anti-guinea-pig (Figure 2.1). Since secondary antibody fluorescence was only observed when the corresponding primary antibody was used, there is no detectable cross-reactivity between the antibodies.

Anterograde transport

Surgery was performed on male Sprague-Dawley rats (n=3). Animals injected with PHAL into the central nucleus of the amygdala (CeA) were initially anesthetized with a cocktail of ketamine hydroxide (100mg/kg; Phoenix Pharmaceutical, Inc., St. Joseph, MO) and xylazine (2 mg/kg; Phoenix Pharmaceutical, Inc., St. Joseph, MO) in saline intraperitoneally (i.p.) and placed in a stereotaxic apparatus for surgery. Anesthesia was supplemented with isoflurane (Abbott Laboratories, North Chicago, IL; 0.5-1.0%, in air) via a specialized nose cone affixed to the incisor bar of the stereotaxic frame (Stoelting Corp., Wood Dale, IL). Glass micropipettes (Kwik-Fil, 1.2mm outer diameter; World

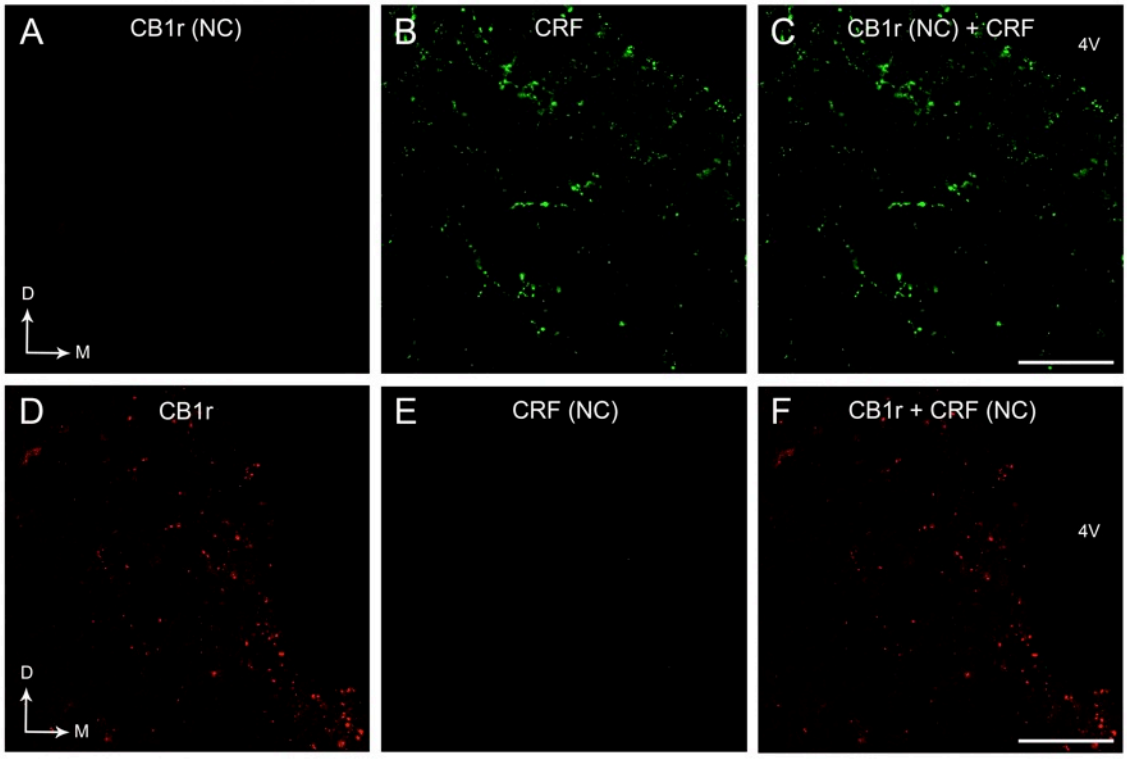

Figure 2.1
Secondary antibodies show no cross-reactivity.

Figure 2.1: Secondary antibodies show no cross-reactivity. Confocal fluorescence micrographs of control experiments that were performed to examine rhodamine isothiocyanate (TRITC)- and Alexafluor 647- conjugated secondary antibody specificity. **a-c.** Tissue was processed with guinea-pig anti-CRF primary antibody, then both Alexafluor 647 conjugated donkey anti-guinea-pig and TRITC conjugated donkey anti-rabbit secondary antibodies. **d-f.** Tissue was processed with rabbit anti-CB1r primary antibody, then both TRITC conjugated donkey anti-rabbit and Alexafluor 647 conjugated donkey anti-guinea-pig secondary antibodies. **a.** With the absence of rabbit anti-CB1r primary antibody, TRITC does not fluoresce. **b.** CRF (green) peptide is visualized by Alexafluor 647 fluorescence. **c.** Merging of TRITC and Alexafluor 647 channels. **d.** CB1r (red) is visualized by TRITC fluorescence. **e.** With the absence of guinea-pig anti-CRF primary antibody, Alexafluor 647 does not fluoresce. **f.** Merging of TRITC and Alexafluor 647 channels. In **a** and **e,** minimal non-specific background labeling is observed**.** This demonstrates the specificity of both TRITC and Alexafluor secondary antibodies for their respective primary antibodies, and do not show any cross-reactivity.

Precision Instruments, Inc., Sarasota, FL) with tip diameters of 15-20µm were filled with 2.5% PHAL (Vector Laboratories, Burlingame, CA). The tips of the glass micropipettes were positioned in the CeA using the following coordinates; 2.3 mm posterior from Bregma and 4.2mm medial/lateral based on the rat brain atlas of Paxinos and Watson (1997). The glass micropipettes were lowered targeting the appropriate coordinates for placement of PHAL into the CeA (6.7mm ventral from the top of the skull), and PHAL was injected using a Picospritzer (General Valve Corporation, Fairfield, NJ) at 24-26 psi, 10ms duration and 0.2 Hz. Injection of PHAL was done unilaterally into the CeA of each animal. Pipettes were left at the site of injection for 5 min after tracer deposit to limit leakage of the tracer along the pipette track. After 10 days, rats were anesthetized and perfused as described above, and tissue was processed for immunohistochemical detection of PHAL, CB1r, and CRF.

Electron microscopy

Rats were anesthetized and perfused as described above, using a 2% formaldehyde and 3.75% acrolein (from Electron Microscopy Sciences) solution. Brains were post-fixed in the formaldehyde and acrolein solution for 24 h, and 40 um sections were cut on a vibratome (Pelco EasiSlicer, Ted Pella, Inc., Redding, CA). Tissues were processed as we previously described (Reyes et al. 2006a, 2008b; Scavone et al. 2011). Briefly, alternate sections through the LC were processed for CRF and CB1r (n=4). Tissues were placed in 1% sodium borohydride in 0.1 M PB (pH 7.4) for 30 min to remove any aldehydes remaining from the perfusion, followed by a 30- min incubation in 0.5% BSA in 0.01 M TBS. They were then rinsed with TBS and incubated overnight with

CRF peptide antibody raised in guinea-pig (1:2000, Peninsula Laboratories) and CB1r antibody raised in rabbit (1:1000). CRF was then visualized with immunoperoxidase labeling via biotinylated donkey anti-guinea-pig antibodies (1:400) for 30 min, followed by an avidin–biotin incubation for 30 min (ABC kit, Vector Laboratories, Burlingame, CA), and visualization with a 5-min reaction in 3,3′-diaminobenzidine (DAB; Sigma-Aldrich Inc., St. Louis, MO) and hydrogen peroxide in 0.1 TBS.

CB1r was visualized through immunogold silver enhancement. Tissues were first washed extensively, then incubated in goat anti-rabbit IgG, conjugated to 1nm gold particles (Amersham Bioscience Corp., Piscataway, NJ) for 2 h. Next, tissues were washed in 0.2% gelatin-phosphate buffered saline (PBS) and 0.8% BSA buffer followed by 0.1M PBS, then incubated for 10 mins in 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.01M PBS. After washing with 0.01M PBS and 0.2M sodium citrate buffer (pH 7.4) sequentially, silver enhancement of the gold particles was done using a silver enhancement kit (Amersham Bioscience Corp.). This process was optimized empirically to determine the optimal enhancement time, which ranged between 5-8 mins. Tissues were then washed again in 0.2M citrate buffer and 0.1M PB, then incubated in 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1M PB. After a 1 h incubation, tissues were washed in 0.1M PB, dehydrated in ascending series of ethanol then propylene oxide, and flat embedded in Epon 812 between two sheets of aclar (Electron Microscopy Sciences). Sections were cut at 70nm on a Leica Ultracut (Leica Microsystems, Wien, Vienna, Austria) with a diamond knife (Diatome-US, Fort Washington, PA), collected on copper mesh grids, and examined with an electron microscope (Morgagni Fei Company, Hillsboro, OR), with digital images captured by an

AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques Corp, Danvers, MA). Tissue was processed with the reverse immunolabels for each primary antibody, with CRF immunolabeled with silver-intensified gold particles and CB1r with peroxidase.

Controls and data analysis

Tissue sections for electron microscopy were obtained from rats with the best immunohistochemical labeling and preservation of ultrastructural morphology. The semi-quantitative approach used in the present study is well established and has been described previously (Reyes et al. 2006b, 2007; Van Bockstaele et al. 1996a, b). While acrolein fixation optimizes the preservation of ultrastructural morphology, the caveat of limited and or differential penetration of immunoreagents in thick tissue sections exists (Chan et al. 1990; Leranth and Pickel 1989). Consequently, the limited penetration of CB1r and CRF may result in an underestimation of the relative frequencies of their distribution. We mitigated this limitation by collecting the tissue sections exclusively near the tissue-Epon interface where penetration is maximal and profile were sampled only when all the markers were present in the surrounding neuropil included in the analysis. The cellular elements were identified based on the description of Peters and colleagues (Peters and Palay 1996). Somata contained a nucleus, Golgi apparatus and smooth endoplasmic reticulum. Proximal dendrites contained endoplasmic reticulum, were postsynaptic to axon terminals and were larger than 0.7 μm in diameter. A terminal was considered to form a synapse if it showed a junctional complex, a restricted zone of parallel membranes with slight enlargement of the intercellular space, and/or associated with postsynaptic

thickening. A synaptic specialization was only limited to the profiles that form clear morphological characteristics of either Type I or Type II (Gray 1959). Asymmetric synapses were identified by thick postsynaptic densities (Gray's type I; Gray 1959). In contrast, symmetric synapses had thin densities (Gray's type II; Gray 1959) both pre- and post-synaptically. An undefined synapse was defined as an axon terminal plasma membrane juxtaposed to that of a dendrite or soma devoid of recognizable membrane specializations and no intervening glial processes. Two individuals quantified the synapse distributions in all profiles analyzed, both reaching the same percentages.

Identification of immunogold-silver labeling in profiles

Selective immunogold-silver labeled profiles were identified by the presence, in single thin sections, of at least two immunogold-silver particles within a cellular compartment. As we previously reported (Reyes et al. 2006b, 2007; Van Bockstaele et al. 1996a, b), single spurious immunogold-silver labeling can contribute to false positive labeling and can be detected on blood vessels, myelin or nuclei. Although minimal spurious labeling was identified in the present study, the criterion for considering an axon or dendrite immunogold-silver labeled was defined by the presence of at least two silver particles in a profile. Whenever possible, the more lightly labeled dendritic labeling for CRF was confirmed by detection in at least two adjacent sections. Profiles containing CRF-labeled axon terminals were counted and their association with CB1r receptors was determined.

Results

CB1r localization in LC: co-existence with CRF

The LC is a compact cluster of NE neurons in the dorsal pons that serves as the primary source of NE in forebrain regions such as the hippocampus and cortex that govern cognition, memory and complex behaviors. To examine the relationship of CB1r with pre-synaptic neural profiles, CB1r immunoreactivity was combined with immunolabeling of an axonal marker, synaptophysin (Syn). Syn is a SNARE protein that is localized to the plasma membrane of axonal terminals (Edelmann et al. 1995). Immunofluorescence microscopy was performed for CB1r and Syn in the LC and DAPI was used to denote the nuclei in the LC region (Figure 2.2). Consistent with its known localization, Syn appeared in varicose processes, some of which were co-localized with CB1r (Figure 2.2d) suggesting that CB1r is located pre-synaptically in axon terminals. There also existed areas of CB1r immunoreactivity lacking Syn immunoreactivity, suggesting that CB1r is associated with profiles other than axon terminals in the LC.

Considering the pre-synaptic distribution of CB1r, we sought to test the hypothesis that the eCB system is positioned to directly modulate CRF-containing afferents within the LC using immunofluorescence detection of CRF and CB1r (Figure 2.3a-c). As previously described in independent studies (Scavone et al. 2010; Valentino et al. 1992; Van Bockstaele et al. 1996a, 1999), CB1r and CRF appeared in punctate varicose processes that were distributed in the LC. Triple immunofluorescence revealed co-localization of CB1r and CRF immunoreactive processes adjacent to TH-immunoreactive neurons (Figure 2.3d-g). These data also show the presence of CB1r in localization while arrowhead and thick arrow point to singly labeled CB1r or Syn,

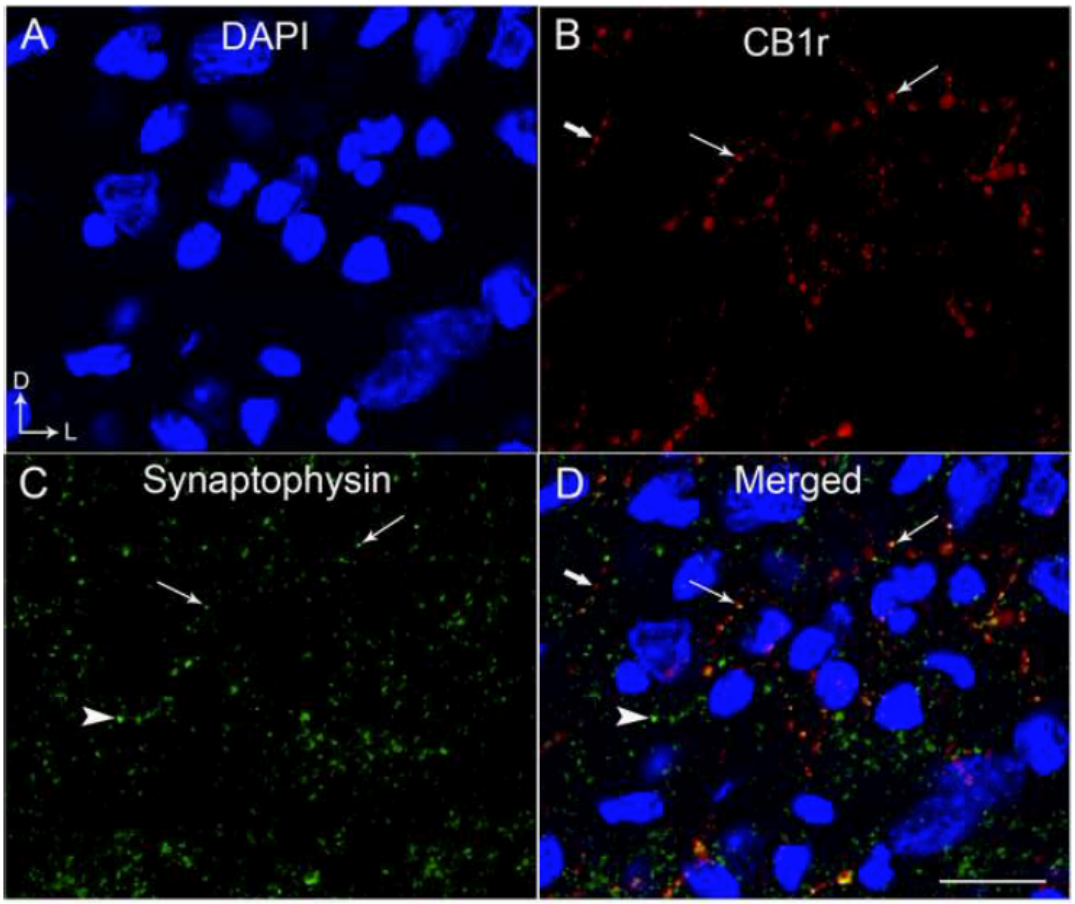

Figure 2.2
CB1r is localized presynaptically in the LC.

Figure 2.2: CB1r is localized presynaptically in the LC. Confocal fluorescence micrographs showing that CB1r (red) and synaptophysin (Syn; green) are co-localized within the LC. **a.** DAPI was used to detect nuclei in LC cell bodies, **b-c.** CB1r was detected using a rhodamine isothiocyanate-conjugated secondary antibody and Syn, an axonal marker, was detected using an Alexafluor 647-conjugated secondary antibody (pseudocolored in green). **d**. CB1r and Syn appear punctate throughout. Co-localization of CB1r and Syn (yellow) can be seen in panel **d**. Arrows point to CB1r and Syn co-respectively. Arrows indicate dorsal (D) and lateral (L) orientation. 4V, fourth ventricle. Scale bar = 25μm.

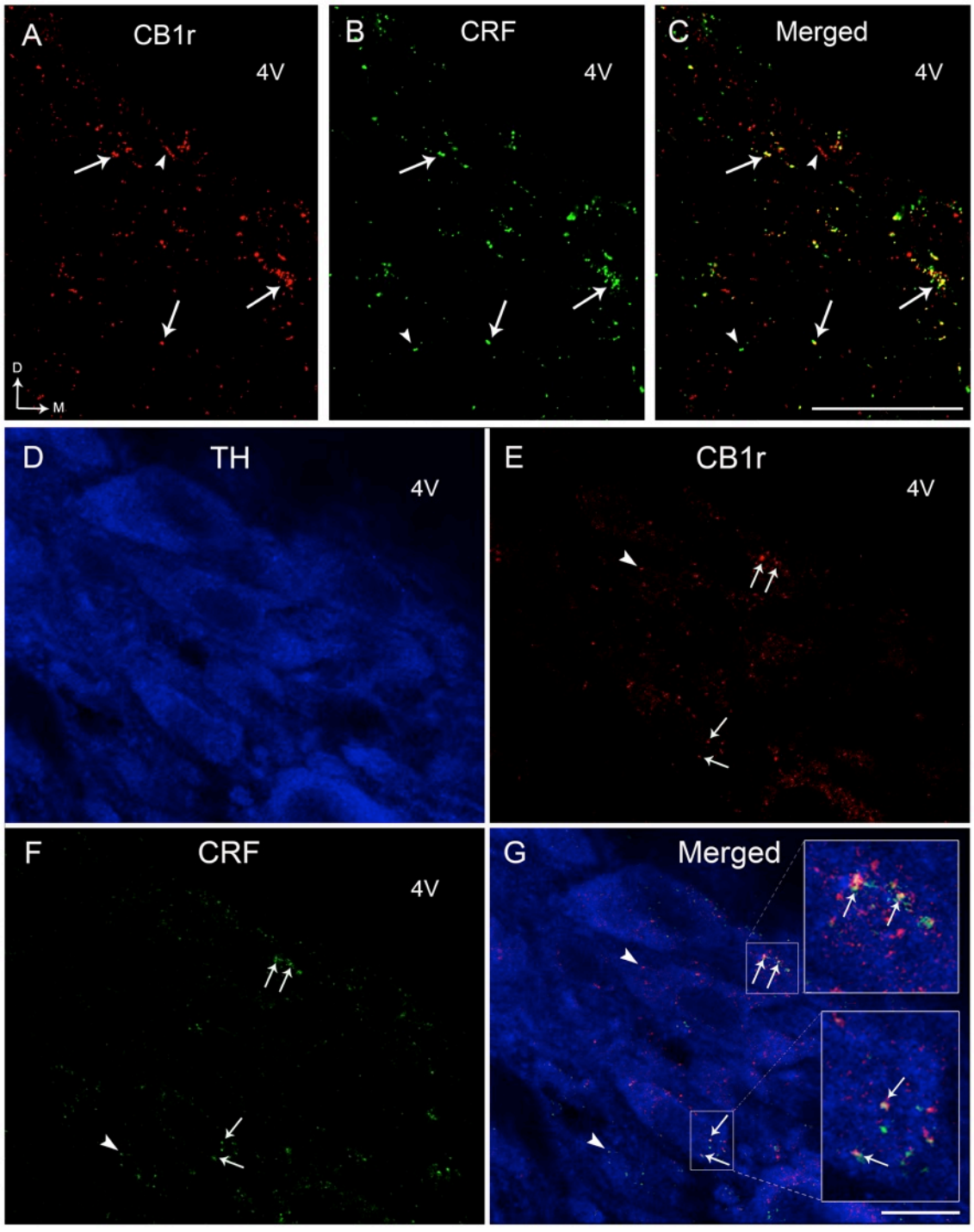

Figure 2.3
CB1r is co-localized with CRF in the LC.

Figure 2.3: CB1r is co-localized with CRF in the LC. a-c: Confocal fluorescence micrographs showing that CB1r (red) and CRF (green) are co-localized in the LC. CB1r was detected using a rhodamine isothiocyanate-conjugated secondary antibody (**a**) and CRF was detected using an Alexafluor 647-conjugated secondary antibody (pseudocolored in green) (**b**). Co-localization of CB1r and CRF (yellow) is shown in a merged image in panel C Arrows denote CB1r and CRF co-localization while arrowhead and thick arrow point to singly labeled CB1r and CRF,respectively. **d-g**: TH, a marker for noradrenergic neurons, was detected using fluorescein isothiocyanate-conjugated secondary antibody (pseudocolored in blue) and was used to show that co-existing CB1r and CRF axon terminals are present within the core of the LC. Also note that CB1r are localized to TH-containing neurons suggesting that CB1r are localized both pre- and post-synaptically in the LC. **g**. Insets show co-localization of CB1r and CRF, and are shown at a higher magnification. Arrows depict triple co-localization of CB1r, CRF, and TH. 4V, fourth ventricle. Scale bar = 25μm.

TH-containing neurons suggesting that CB1r is also found post-synaptically, confirming our previous studies demonstrating that CB1r is localized both pre- and post-synaptically, in the LC (Scavone et al. 2010).

The core of the LC consists of a dense cluster of noradrenergic neurons, with dendrites that extend into the surrounding area, known as the peri-LC (Shipley et al. 1996). CRF afferent nuclei are known to topographically innervate the LC (Van Bockstaele et al. 2001). CRF afferents from limbic regions, such as the amygdala and bed nucleus of the stria terminalis, have been shown to provide topographic innervation of the rostrolateral peri-LC while medullary afferents have been shown to project primarily to the core (Valentino and Van Bockstaele 2008a; Van Bockstaele et al. 1996a, 1999). In order to determine if there is differential distribution between the eCB regulation of CRF afferents in the core vs. peri-LC, confocal images of CB1r and CRF immunoreactivity were analyzed using the imageJ plug-in coloc2, and the average Pearson's coefficient (PC) was determined: for the core, PC = 48.4 +/- 3.12; for the peri-LC, PC = 31.6 +/- 3.78. PC values represent the linear correlation of CB1r (red) signal intensity with respect to CRF (green) signal intensity at each pixel, and a PC > 1 signifies that the signal co-localization is greater than it would be at random, with a PC = 1 indicating perfect correlation (Adler & Parmryd 2010). These values suggest that there is a correlation between CB1r and CRF in both the core and the peri-LC. Analysis of co-localization was further carried out using immunoelectron microscopy.

Ultrastructural localization of CRF and CB1r in the LC

Immunoelectron microscopy was used to further determine the precise subcellular co-localization of CB1r in relation to CRF afferents in the LC (Figure 2.4). Immunoperoxidase labeling was used for the detection of CRF and immunogold-silver labeling was used for the detection of CB1r. These markers are routinely reversed and results showed a similar distribution irrespective of the seconday immunolabel of the primary antibody. The core of the LC consists of a dense cluster of noradrenergic neurons, with dendrites that extend into the surrounding area, known as the peri-LC (Shipley et al. 1996). CRF afferent nuclei are known to topographically innervate the LC (Van Bockstaele et al. 2001). CRF afferents from limbic regions, such as the amygdala and bed nucleus of the stria terminalis, have been shown to provide topographic innervation of the rostrolateral peri-LC while medullary afferents have been shown to project primarily to the core (Valentino and Van Bockstaele 2008a; Van Bockstaele et al. 1996a, 1999). In order to determine if there is differential distribution between the eCB regulation of CRF afferents in the core vs. peri-LC, electron micrographs from the core and the peri-LC were quantified separately.

For analysis of the LC core, a total of 468 profiles were analyzed from at least 5 grids per LC section. At least three LC sections were collected from each Sprague-Dawley rat (n=4). Several interactions between CB1r and CRF-containing axon terminals were observed. One type of interaction demonstrated axon terminals containing both CB1r and CRF, suggesting an anatomical substrate for pre-synaptic modulation of CRF by CB1r (Figure 2.4a-d). It was also observed that CRF-containing afferents target dendrites expressing CB1r, providing a cellular substrate for potential post-synaptic

effects (Figure 10e). Of the 468 CRF-labeled axon terminals analyzed, 44.4% (208/468 profiles) also contained CB1r, and of the 208 CRF+CB1r co-labeled axon terminals, 12.5% (26/208 profiles) contacted dendrites that expressed CB1r postsynaptically. Additionally, 18.2% (85/468 profiles) of CRF axon terminals that did not express CB1r synapsed onto dendrites that contained CB1r. The remainder of CRF terminals did not exhibit CB1r or were not adjacent to profiles exhibiting CB1r immunoreactivity (37.4%; 175/468 profiles).

For peri-LC analysis, a total of 294 profiles were analyzed obtained from at least 5 grids per LC section. At least three LC sections were collected from each Sprague-Dawley rat (n=3). Of the 294 axon terminals analyzed that contained CRF, 35.37% (104/294 profiles) also contained CB1r, and of the 104 CRF+CB1r co-labeled axon terminals, 10.2% (30/104 profiles) contacted dendrites that expressed CB1r postsynaptically. Additionally, 6.46% (19/294 profiles) of CRF axon terminals that did not express CB1r synapsed onto dendrites that contained CB1r. The remainder of CRF terminals did not exhibit CB1r or were not adjacent to profiles exhibiting CB1r immunoreactivity (47.96%; 141/294 profiles). This provides compelling evidence for presynaptic regulation of CRF afferents by the eCB system in both the core and peri-LC areas.

CRF and CB1r co-localize at inhibitory and excitatory synapses in LC

The type of synapses formed by CRF-labeled axon terminals that either contain CB1r or apposed to CB1r-containing dendrites were subsequently analyzed. In the LC

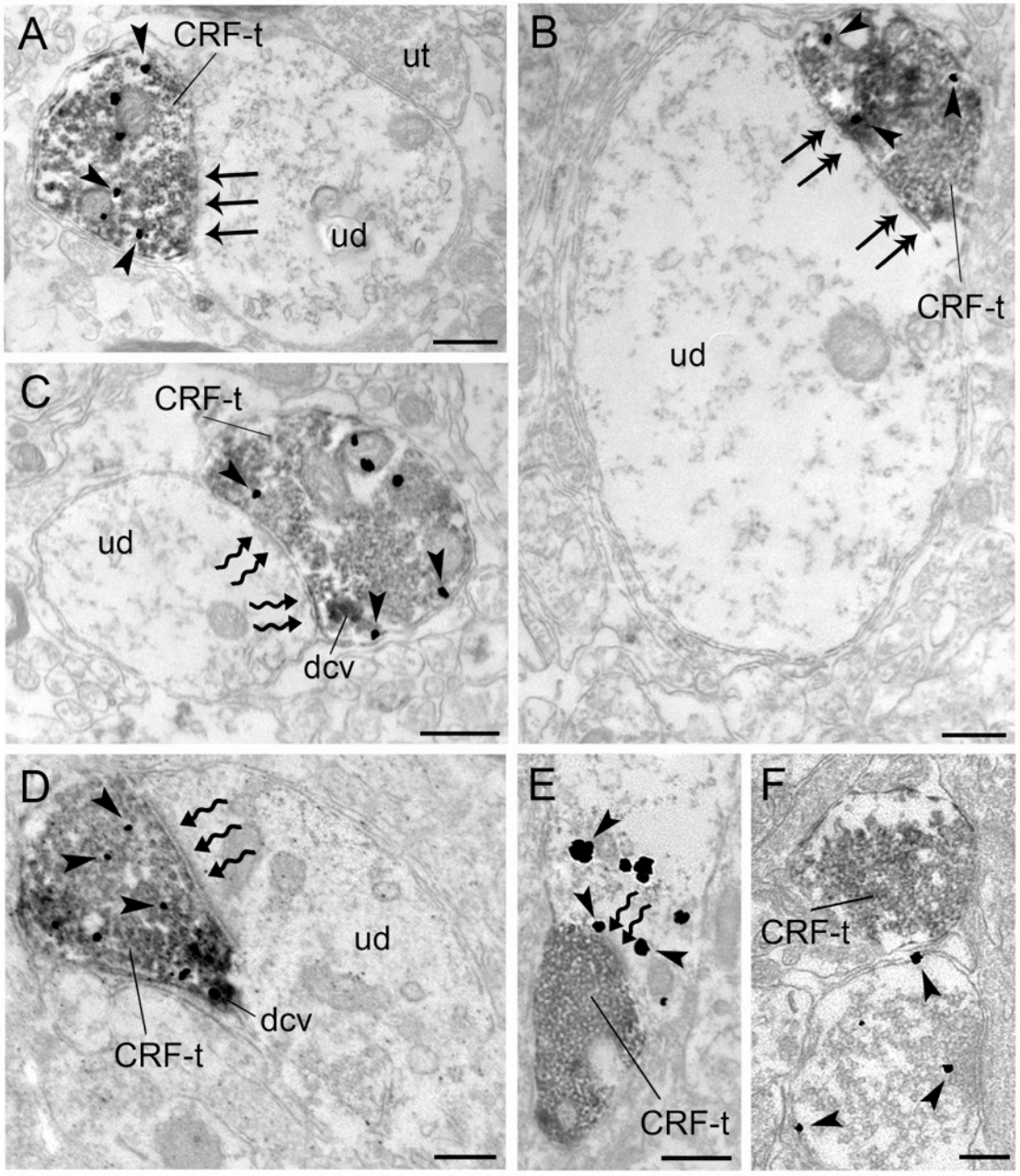

Figure 2.4
CRF-containing afferents co-localize with CB1r in the LC.

Figure 2.4: CRF-containing afferents co-localize with CB1r in the LC. a-f. Representative electron micrographs showing immunoperoxidase labeling for CRF-containing axon terminals (CRF-t) and immunogold-silver labeling for CB1r (arrowheads) in the LC core (A-C) and peri-LC (D-E). **a.** A CRF-labeled axon terminal containing CB1r forms is in direct contact (arrows) with an unlabeled dendrite (ud) in the LC core. **b.** A peroxidase-labeled CRF-t co-localizing CB1r (arrowheads) forms a symmetric type synapse (double arrows) with an unlabeled dendrite (ud) in the LC core. **c.** An axon terminal containing both peroxidase- labeling for CRF and immunogold-silver labeling for CB1r (arrowheads) forms an asymmetric type synapse (zig zag arrows) with an unlabeled dendrite (ud) in the LC core. **d.** A CRF-labeled axon terminal containing CB1r (arrowheads) forms an asymmetric type synapse (zig zag arrows) with an unlabeled dendrite (ud) in the peri-LC. **e.** A peroxidase-labeled CRF axon terminal forming an asymmetric synapse (zig zag arrows) with a dendrite containing immunogold-silver labeled CB1r (arrowheads) **f.** A peroxidase-labeled CRF axon terminal can be seen in close proximity to a separate axon terminal containing immunogold-silver labeled CB1r. dcv, dense core vesicle. Scale bar = 0.5 μm.

core, of the dually labeled CRF- and CB1r axon terminals that formed synapses with unlabeled dendrites (10a-c), 72.0% (131/182 profiles) exhibited symmetric synapses (Figure 2.4b), 17.0% (31/182 profiles) formed asymmetric synapses (Figure 2.4c), and 11.0% (20/182 profiles) formed undefined synapses (Figure 2.4a). For CRF-labeled axon terminals apposed to CB1r-labeled dendrites, 52.9% (45/85 profiles) formed symmetric synapses, 36.5% (31/85 profiles) formed asymmetric synapses, and 10.6% (9/85 profiles) formed undefined synapses. For dually labeled CRF- and CB1r axon terminals apposed to CB1r-labeled dendrites, 50.0% (13/26 profiles) formed symmetric synapses, 38.5% (10/26 profiles) formed asymmetric synapses, and 11.5% (3/26 profiles) formed undefined synapses.

In the peri-LC, of the dually labeled CRF- and CB1r axon terminals that formed synapses with unlabeled dendrites (Figure 2.4d), 21.15% (22/104 profiles) formed symmetric synapses, 53.84% (56/104 profiles) formed asymmetric synapses (Figure 2.4d), and 28.85% (30/104 profiles) formed undefined synapses. For CRF-labeled axon terminals apposed to CB1r-labeled dendrites, 21.05% (4/19 profiles) formed symmetric synapses, 57.89% (11/19 profiles) formed asymmetric synapses, and 21.05% (4/19 profiles) formed undefined synapses. For dual CRF- and CB1r-labeled terminals apposed to CB1r-labeled dendrites, 30.0% (9/30 profiles) formed symmetric synapses, 56.67% (17/30 profiles) formed asymmetric synapses, and 13.33% (4/30 profiles) formed undefined synapses. As compared to the core of the LC, where CB1r and CRF interactions exhibited primarily inhibitory-type synapses, the peri-LC showed a different synaptic organization with dually labeled terminals exhibiting primarily excitatory synapses.

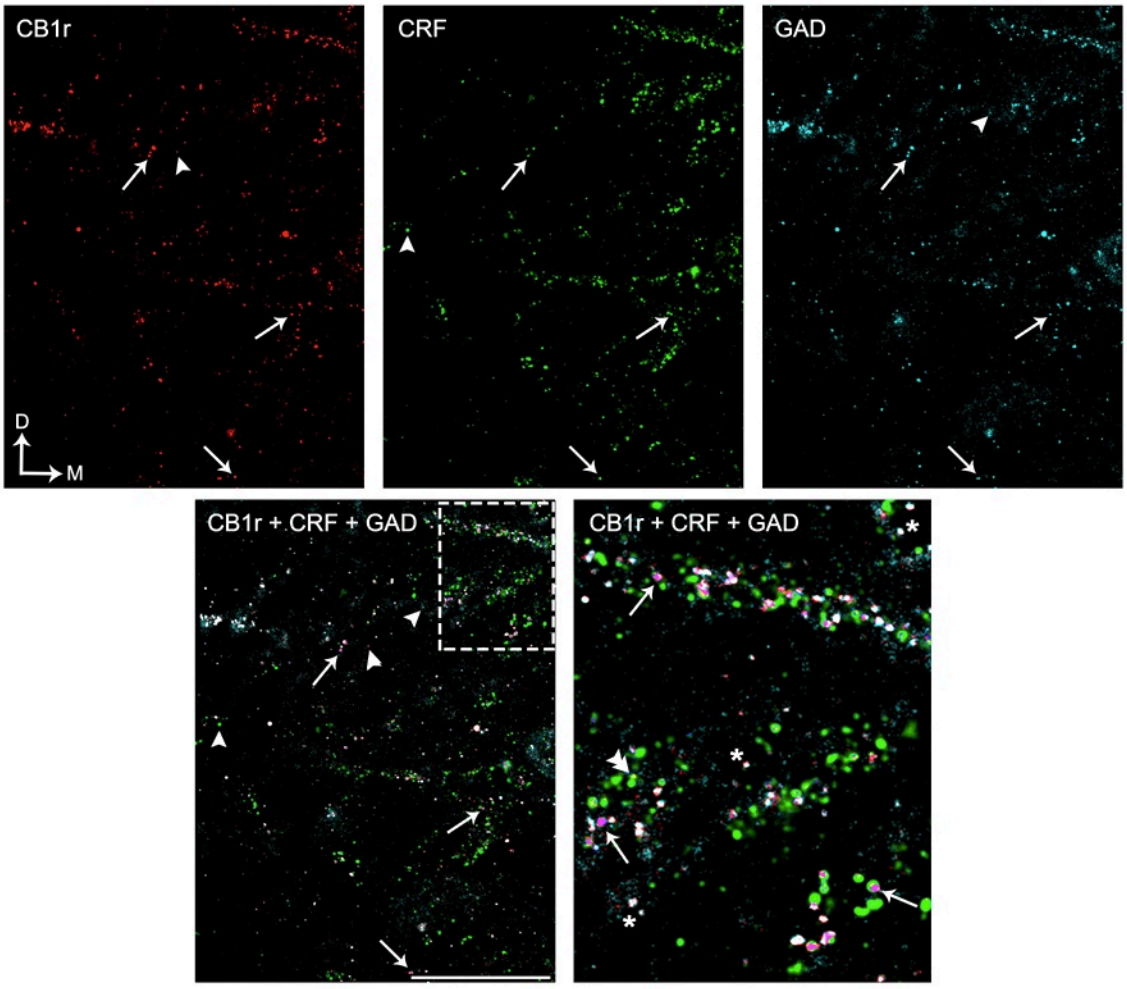

Figure 2.5
CB1r and CRF co-localize with GAD in the LC.

Figure 2.5: CB1r and CRF co-localize with GAD in the LC. Confocal fluorescence micrographs showing CB1r (red), CRF (green), and GAD (cyan) co-localization in the LC. **a.** CB1r was detected using a rhodamine isothiocyanate-conjugated secondary antibody. **b.** CRF was detected using an Alexafluor 647-conjugated secondary antibody (pseudocolored in green). **c.** GAD was detected using a fluorescein isothiocyanate-conjugated secondary antibody (pseudocolored in cyan). **d.** Triple co-localization (pink) can be seen in the bottom row and is depicted by arrows. The inset on the bottom left panel **(d)** is shown at a higher magnification on the bottom right (d'). Additionally, co-localization of CB1r and CRF without GAD (yellow, double arrow heads) and CB1r and GAD without CRF (white, asterisks) is observed. Single arrowheads point to singly labeled CB1r, CRF, and GAD. Scale bar = 30μm.

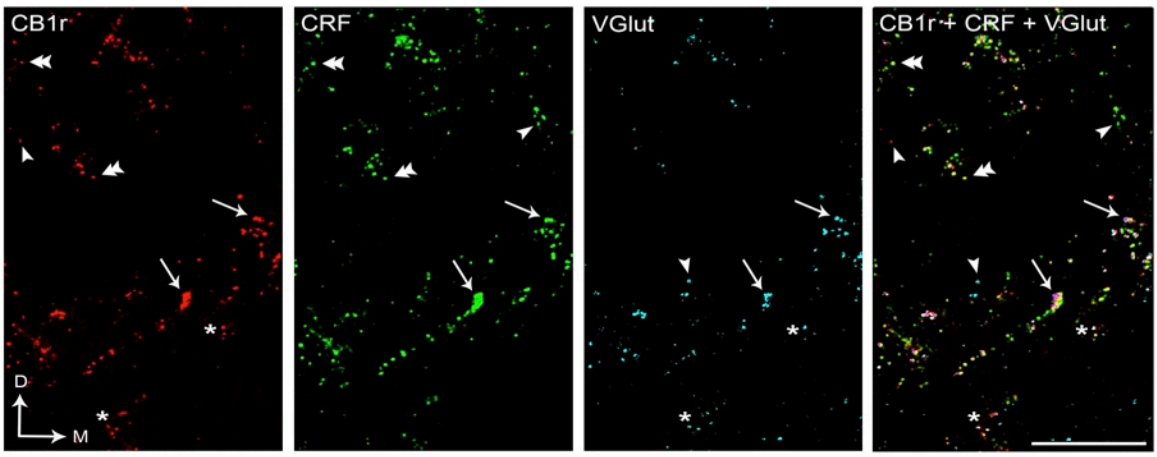

Figure 2.6
CB1r and CRF co-localize with VGlut in the LC.

Figure 2.6: CB1r and CRF co-localize with VGlut in the LC. Confocal fluorescence micrographs showing CB1r (red), CRF (green), and VGlut (cyan) co-localization in the LC. **a.** CB1r was detected using a rhodamine isothiocyanate-conjugated secondary antibody. **b.** CRF was detected using an Alexafluor 647-conjugated secondary antibody (pseudocolored in green). **c.** VGlut was detected using a fluorescein isothiocyanate-conjugated secondary antibody (pseudocolored in cyan). **d.** Triple co-localization (pink) can be seen in the right panels and is depicted by arrows. Additionally, co-localization of CB1r and CRF without VGlut (yellow, double arrow heads) and CB1r and VGlut without CRF (white, asterisks) is observed. Single arrowheads point to singly labeled CB1r, CRF, and GAD. Scale bar = 30μm.

The different morphological characteristics of dually labeled CRF and CB1r synaptic specializations in the core vs peri-LC suggested that CB1r modulation of either inhibitory or excitatory CRF afferents. To further explicate the neurochemical signature of dually labeled CRF and CB1r synapses, triple labeling immunofluorescence was performed. In addition to staining for CRF and CB1r, GAD, the enzyme responsible for GABA synthesis in axon terminals (Fonnum et al. 1970), was used as a marker for GABAergic neurons (Figure 2.5) and VGlut, a protein responsible for filling synaptic vesicles with glutamate (Fremeau et al. 2004), was used as a marker for glutamatergic neurons (Figure 2.6). Figures 2.5 and 2.6 demonstrate immunocytochemical evidence that CB1r, CRF, and GAD or VGlut are co-localized, suggesting that CB1r and CRF are expressed at both excitatory and inhibitory synapses. In addition, Figures 2.5 and 2.6 show co-localization between CB1r and CRF in axon terminals lacking GAD or VGlut, respectively, as well as evidence for CB1r and GAD or VGlut in axon terminals lacking CRF.

CB1r and CRF co-localize in amygdalar projections to the LC

CRF-afferents from both autonomic and limbic regions project to the LC, and the central nucleus of the amygdala (CeA) is one of the key limbic inputs involved in stress signaling (Aston-Jones et al. 1991; Van Bockstaele et al. 1996a, b, 1999). Previous electron microscopy tracing studies have shown that within the rostrolateral peri-LC, approximately 35% of axon terminals from the amygdala co-localize with CRF, and 22% of CRF-labeled profiles originate from the amygdala (Van Bockstaele et al., 1998).

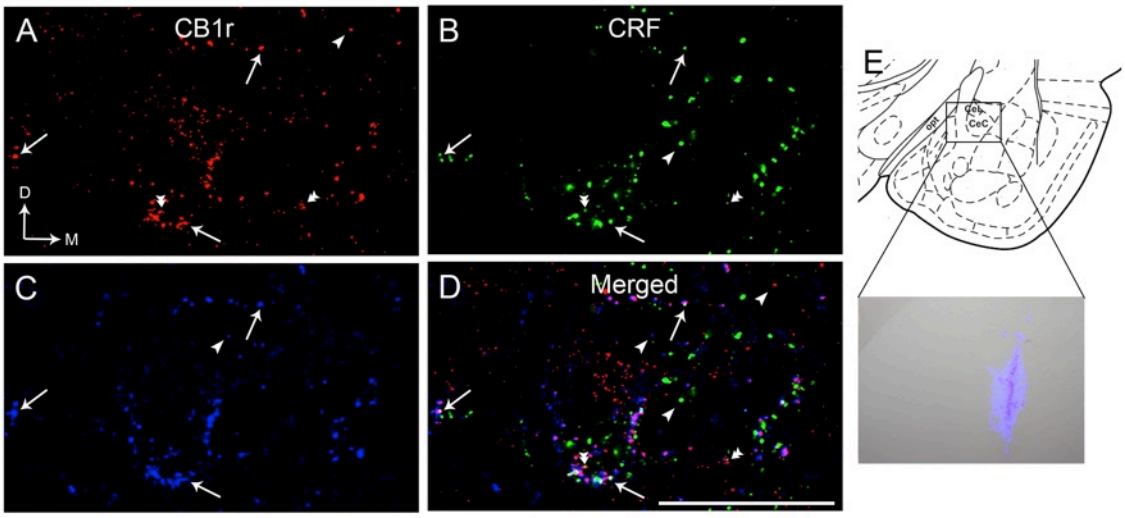

Figure 2.7
CB1r and CRF co-localize in PHAL-labeled amygdalar afferents to the LC.

Figure 2.7: CB1r and CRF co-localize in PHAL-labeled amygdalar afferents to the LC. The anterograde tracer PHAL was injected into the central nucleus of the amygdala (CeA) and immunofluorescence labeling was conducted for PHAL, CB1r and CRF in LC sections. **a-d.** Confocal fluorescence micrographs demonstrate triple co-colocalization of CB1r, CRF, and PHAL in the peri-LC. CB1r was detected using a rhodamine isothiocyanate-conjugated secondary antibody **(a)** and CRF was detected using an Alexafluor 647-conjugated secondary antibody (pseudocolored in green) (**b**) and PHAL was detected using fluorescein isothiocyanate-conjugated secondary antibody (pseudocolored in blue) (**c**). **d.** Triple co-localization (white) can be observed, and is depicted by arrows. Double arrowheads point to dual co-localization of CB1r and CRF (yellow). Single arrowheads point to singly labeled CB1r, CRF, and PHAL. Scale bar = 25μm. **e.** A schematic diagram adapted from the rat brain atlas of Paxinos and Watson (Paxinos and Watson 1997) depicting the location of the CeA. The box illustrates the region in which the lower image was taken. This image showing an overlay of fluorescein isothiocyanate-labeled PHAL injection site with the same section stained with NIssl shows that the injection was positioned in the CeA. Opt: optic tract. Scale bar = .5mm.

Anterograde transport of PHAL from the CeA (Figure 2.7e) revealed that amygdalar projections to the LC that contain CRF also express CB1r (Figure 2.7a-d), suggesting that CB1r are positioned to modulate amygdalar CRF-release.

Discussion

While it is known that CRF and the eCB system independently regulate noradrenergic neurons in the LC, the present results demonstrate a direct interaction between the two by providing ultrastructural evidence for CB1r localization to CRF-containing axon terminals in the LC. To our knowledge, these findings provide the first anatomical evidence that the eCB system is positioned to directly modulate CRF stress-integrative circuitry within the LC-NE system. In addition, morphological analyses at the electron microscopic level revealed that dually labeled CB1r + CRF axon terminals exhibited Gray's Type I (asymmetric or excitatory-type) and Gray's Type II (symmetric or inhibitory type) synapses. Interestingly, to our knowledge, this is the first subcellular evidence that CB1r and CRF are co-localized within the LC. Type I synapses were more frequently found in the peri-LC, a known source of CRF limbic afferents, while Type II synapses were more frequently localized in the core of the LC, a known source or autonomic and visceroreceptive afferents. The ultrastructural data were confirmed by a triple immunofluorescence labeling approach showing that dually labeled CRF and CB1r afferents contain markers for either excitatory or inhibitory type amino acids. These results suggest that eCB modulation of CRF afferents will produce differential consequences on LC neuronal activity depending on whether distinct CRF afferents that contain co-existing excitatory or inhibitory amino acid transmitters are engaged, and

provide the first evidence that topographic distinctions occur between CB1r and CRF co-localization with inhibitory and excitatory amino acids in the core and peri-LC, respectively. Finally, co-localization of CB1r, CRF, and PHAL in the LC demonstrates that CB1r are localized in CRF-containing afferents that arise from the amygdala.

Methodological considerations

Dual labeling immunocytochemistry with peroxidase detection and immunogold-silver labeling combined with electron microscopy makes it possible to identify the subcellular localization of receptors within a defined neuronal population. However, some limitations need to be considered when interpreting results from pre-embedding immunoelectron microscopy experiments. Often, there is limited and/or differential penetration of the primary and secondary antibodies, especially in thicker tissue sections (Chan et al. 1990; Leranth and Pickel 1989). For example, antibodies directed against CRF or CB1r may not have penetrated the tissue section sufficiently, resulting in an underestimation of the number of CRF-containing afferents or CB1r in the LC. In order to minimize this caveat, only tissue sections where both markers could be detected near the tissue-Epon interface were analyzed (Leranth and Pickel 1989). Additionally, while classifying synapses as symmetric or asymmetric at the electron microscopic level is suggestive of inhibitory or excitatory type synapses (Gray 1959; Harris and Weinberg 2012), it is not definitive. Therefore, triple immunofluorescence using GAD as a marker for GABAergic synapses and VGlut as a marker for glutamatergic synapses was used to unequivocally establish the presence of inhibitory or excitatory amino acids in dually labeled CRF + CB1r afferents.

CRF regulation of LC neurons: implications for modulation by CB1r

The LC is a stress-integrative system that consists of a dense cluster of noradrenergic somata, defined as the core, with extensive dendritic processes extending from the core into surrounding portions of the neuropil, known as the peri-LC (Shipley et al. 1996). CRF fibers have been shown to prominently innervate peri-LC areas when compared to the core (Valentino et al. 2001; Van Bockstaele et al. 1996a, 1999). CRF-containing afferents originating from the central nucleus of the amygdala (CeA; Van Bockstaele et al. 1998), Barrington's nucleus (Bar; Valentino et al. 1996), the paraventricular nucleus of the hypothalamus (PVN; Reyes et al. 2005), and the nucleus paragigantocellularis (PGi; Van Bockstaele et al. 2001) form primarily asymmetric or excitatory-type synapses with LC dendrites. Additional CRF afferents arise from the BNST (Van Bockstaele et al. 1999), ventrolateral periaqueductal gray (PAG; Van Bockstaele et al. 2001), and the nucleus prepositus hypoglossi (PrH; Van Bockstaele et al. 2001) and form largely symmetric or inhibitory-type synapses (Figure 2.8a). CRF afferents also exhibit topographic innervation of the LC core and peri-LC areas, with the CeA and BNST projecting to the peri-LC, while Bar, the PVN, PGi, PAG, and PrH project to the core (Figure 2.8b) (Van Bockstaele et al. 2001). CRF exerts a primarily postsynaptic regulation of LC neurons where it acts upon CRF type 1 receptors that are prominently distributed within the LC (Curtis et al. 1999; Reyes et al. 2006a, 2008b).

During stress, CRF is released to shift the activity of LC neurons to a high tonic state that promotes scanning of the environment and behavioral flexibility (Curtis et al. 2001, 2002, 2012; Kreibich et al. 2008; Valentino et al. 2001; Valentino and Van

A

Circuit	Co-localizing Amino Acid	Function	Effect of eCB System on LC Activity
CNA → LC	EAA	Emotionally-charged stimuli; fear	↓
PGi → LC	EAA	Autonomic and visceroceptive processing	↓
PrH → LC	GABA	Oculomotor functioning; REM sleep	↑
PVN → LC	EAA	HPA-axis activation; autonomic responses	↓

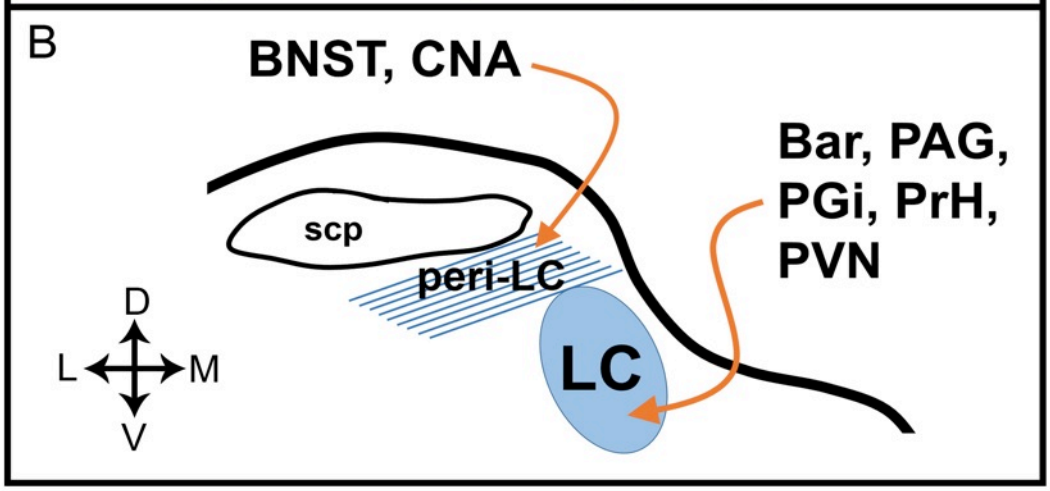

Figure 2.8
Functional consequences of eCB modulation of CRF afferents

Figure 2.8: Functional consequences of eCB modulation of CRF afferents. a. Table showing known CRF projections to the LC, their putative co-localizing amino acid, and function. **b.** Schematic depicting the topographic innervation of the LC by CRF-afferents. Bar, PAG, PGi, PrH, and PVN are all known to project to the core of the LC, while the BNST and CNA project to the peri-LC.

Bockstaele 2005; Van Bockstaele et al. 2010; Xu et al. 2004). Previous neuroanatomical and electrophysiological studies demonstrated selective presynaptic inhibition of CRF afferent input by selective KOR agonists (Kreibich et al. 2008; Reyes et al. 2007). By allowing LC neurons to fire spontaneously, but attenuating information from excitatory afferents, pre-synaptic regulation of CRF by KOR may serve to protect the LC from over-activation (Kreibich et al. 2008). The present study reveals an additional component involved in the presynaptic regulation of CRF afferents in the LC, the CB1r. CB1r are known to be present in stress responsive circuits that are essential to the expression of stress-related behaviors (Hill et al. 2010; Shimizu et al. 2010). For example, the eCB system plays a critical role in glucocorticoid-mediated fast feedback inhibition of the HPA axis (Hill and McEwen 2009; Hill et al. 2010), and acute restraint stress has been shown to increase the synthesis of endogenous eCB in limbic forebrain areas (Haller et al. 2002; Martin et al. 2002; Patel et al. 2005). CB1r agonist administration has been shown to alter LC-neuronal discharge and NE release in target regions during basal and stress conditions (Herkenham et al. 1990; Oropeza et al. 2005; Page et al. 2007, 2008; Reyes et al. 2012).

Ultrastructural analysis in the present study reveals that a majority of CRF and CB1r dual labeled afferents in the peri-LC form Type I or asymmetric synapses, suggesting that the eCB system may modulate release of CRF from limbic afferents, such as the amygdala, which was confirmed by combining anterograde labeling from the CeA with immunofluorescence detection of CRF and CB1r. eCB signaling within the amygdala is necessary for habituation and adaptation of fear related behaviors (Kamprath et al. 2006; Marsicano et al. 2002; Wyrofsky et al. 2015). It is tempting to speculate that

eCB modulation of the amygdalar CRF-afferents in the LC could also play a role in attenuating emotionally-charged stimuli. LC activation causes an increase in NE release in the mPFC, which plays a critical role in aversive memory extinction, and NE dysregulation can lead to the development of anxiety disorders (Wyrofsky et al. 2015; Mueller & Cahill 2010; Mueller et al. 2008). CRF release from the amygdala is known to increase LC activity. The co-localization of CB1r on amygdalar CRF-afferents provides a potential mechanism for the eCB system to modulate the stress response and attenuate stress-induced dysregulation of frontal cortical activity, which may result in enhancing traumatic memory extinction and diminish anxiety-like behaviors.

A smaller percentage of, CRF-afferents co-expressing CB1r in the peri-LC formed Type II or symmetric synapses; therefore, the eCB system could also have an effect on CRF projections from the BNST. Unlike the peri-LC, a large majority of CB1r and CRF dual labeled synapses in the core region were of the inhibitory type (Type II synapses). GABA + CRF afferents originate in regions responsible for providing sensory and autonomic stimuli to the LC (Aston-Jones et al. 1991; Samuels and Szabadi 2008; Van Bockstaele et al. 2001). LC neuronal activity has a biphasic effect on arousal and attention: low tonic activity via involvement of GABA is associated with disengagement from the environment while phasic activity is optimal for sustained focused attention (Aston-Jones 1985; Aston-Jones and Cohen 2005). High tonic activity correlates with a shift towards scanning the environment and heightened arousal (Aston-Jones and Cohen 2005; Berridge and Waterhouse 2003; Valentino and Van Bockstaele 2008b). While an initial shift to high tonic activity results in CRF-induced increases in behavioral engagement and scanning and is beneficial for adaptive responses to a stressor, chronic

high tonic activity disrupts focused attention (Aston-Jones and Cohen 2005; Valentino and Van Bockstaele 2008b). In this regard, eCB modulation of CRF could act to return LC activity to optimal phasic levels.

In other brain regions, such as the hippocampus and cerebellum, it has been shown that CB1r, can be located in the peri-synaptic region of both excitatory and GABAergic synapses (Kawamura et al. 2006; Nyiri et al. 2005). It is possible that further studies examining the regions adjacent to CRF afferents would reveal CB1r localization. Moreover, while CB1r is the predominant cannabinoid receptor in the brain (Scavone et al. 2010; Wyrofsky et al. 2015), eCBs can act at other receptors. Specifically, AEA has been shown to bind and activate transient receptor potential vanilloid type 1 receptors (TRPV1), resulting in long term depression within the dentate gyrus in a CB1r-independent manner (Chavez et al. 2010; Ryskamp et al. 2014). TRPV1 expression has been reported in the LC (Caterina 2003; Toth et al. 2005). Future immunoelectron microscopy studies could examine the exact location of TRPV1 receptors, and if they are localized to excitatory CRF-containing terminals, they could represent another manner in which the eCB system could affect stress input from the PVN, Bar, and PGi.

Additionally, our data demonstrate CB1r labeling in somatodendritic processes, consistent with our previous reports (Scavone et al. 2010). It is not clear whether these CB1r are functional within the LC or whether these are CB1r being transported to noradrenergic axon terminals in the frontal cortex. We have previously demonstrated that noradrenergic axon terminals in the prefrontal cortex exhibit CB1r (Oropeza et al. 2007) and LC neurons express CB1r mRNA (Tsou et al. 1998; Matsuda et al. 1993). Interestingly, there is evidence for functional postsynaptically distributed CB1 receptors

in other brain regions. Cytoplasmic CB1r distribution has been observed within the rat caudate putamen nucleus (Rodriguez et al. 2001). Also, in HEK-293 cells transfected with CB1r, ~85% of CB1r are localized in intracellular vesicles (Leterrier et al. 2004), and the changes in subcellular localization seems to be attributed to activation-dependent internalization via endosomes during steady state conditions (Thibault et al. 2013). Ongoing slice physiology studies within the LC in our laboratory are exploring the functional significance of postsynaptically distributed CB1r (Wyrofsky et al. 2016). Therefore, future studies will provide critical information on the functional significance of pre- and post-synaptically distributed CB1r in the LC.

Functional implications

Targeting the eCB regulation of the LC-NE stress-integrative circuit could provide therapeutic relief for various stress-induced anxiety disorders (Wyrofsky et al. 2015). For example, the inability to extinguish aversive and fearful memories coupled with repeated re-consolidation of these memories in limbic circuits underlies the pathophysiology of post-traumatic stress disorder (PTSD) and other anxiety disorders (Jovanovic and Ressler 2010; Lehner et al. 2009), and NE is involved in both processes. Consolidation of emotional memories involves LC-NE inputs to the amygdala (Ferry et al. 1999; McGaugh et al. 1996), while extinction of these memories involves LC-NE signaling in the mPFC (Mueller and Cahill 2010; Mueller et al. 2008). Several cannabinoid receptor ligands including THC (an active component in cannabis) and nabilone (a synthetic cannabinoid ligand) have shown promise in clinical studies at reducing the symptoms and flashbacks associated with PTSD (Fraser 2009; U.S. National

Institutes of Health 2012), and many individuals suffering from PTSD self-medicate with cannabis (Passie et al. 2012).

Interestingly, cannabinoids are known to affect anxiety in a bi-directional and dose-dependent manner, with lower doses generally producing anxiolytic effects while higher doses result in anxiogenesis (Rey et al. 2012; Trezza and Vanderschuren 2008; Viveros et al. 2005). A recent study using CB1r conditional knock out mice showed that CB1r activation on GABAergic neurons in the forebrain is necessary for the anxiogenic effects of cannabinoids, while CB1r activation on cortical glutamatergic neurons is necessary for the anxiolytic effects (Rey et al. 2012). It is tempting to speculate that a similar mechanism applies to eCB modulation of CRF afferents in the LC. We have previously shown that CB1r is positioned to modulate at symmetric and asymmetric synapses (Scavone et al. 2010). Moreover, using single-unit extracellular recordings have demonstrated that CB1r activation can modulate synaptic transmission within the LC via the glutamatergic and GABAergic systems (Muntoni et al., 2006; Mendiguren & Pineda 2004). While these data provide evidence of CB1r activation of LC through the excitatory and inhibitory neurotransmission, our present results is the first report illustrating the distribution and topography of CB1r modulation of glutamatergic and GABAergic CRF-afferents not only at the immunofluorescence level but more importantly and interestingly at the ultrastructural level. In addition, this is the first report showing differential topography in synaptic signature of CB1r and CRF co-localization where asymmetric synapses indicative of excitatory transmission predominate in the peri-LC and symmetric synapse predominates in LC core indicative of inhibitory transmission. CRF-afferents co-localizing CB1r in the peri-LC and forming asymmetric synapses

suggest co-localization with glutamate (Van Bockstaele et al. 1996a, 1999), and we have shown CB1r and CRF co-localization within afferents originating from the amygdala, a brain region responsible for providing fear-related stimuli and emotional input (Davis 1992; Kamprath et al. 2006; Walker et al. 2003). Blocking signaling from the amygdala via CB1r activation in the peri-LC could contribute to cannabinoid-induced anxiolytic effects. Because dysregulation of NE in the mPFC is known to contribute to the development of anxiety disorders (Anand and Charney 2000; Carvalho and Van Bockstaele 2012; Itoi and Sugimoto 2010; Nutt 2006; Southwick et al. 1999), targeting the eCB modulation of CRF afferents in the LC during stress may underlie the efficacy of nabilone in PTSD patients.

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CHAPTER 3

EFFECT OF SOCIAL STRESS ON THE ENDOCANNABINOID SYSTEM IN THE LOCUS COERULEUS

Abstract

Social stress is a major contributor to the development of anxiety and psychological disorders, largely through the excitatory effects of corticotropin releasing factor (CRF) on locus coeruleus (LC)-norepinephrine (NE) system. Increased NE efflux in the medial prefrontal cortex (mPFC) and hyperactivity of noradrenergic LC neurons contributes to the pathology of anxiety and depression. However, not all exposed to social stress will develop such disorders, as different coping strategies and cellular adaptations exist between resilient and vulnerable individuals. Rodents exposed to the resident-intruder model of social stress developed two distinct phenotypes based on the latency to assume a defeated posture: the resilient long latency rats and the vulnerable short latency rats. Previous studies have found that alterations to the opioid system underlie differences observed between both groups. Given that the endocannabinoid (eCB) system modulates noradrenergic transmission and functions as an anti-stress neuromediator, it is possible that cellular adaptations to the eCB system might also contribute to the different coping strategies. Therefore, the levels of diacylglycerol lipase (DGL), responsible for synthesizing eCBs, and fatty acid amide hydrolase (FAAH), responsible for degrading eCBs, were determined across sexes via Western blot analysis of LC from male and female following social defeat. While no change in FAAH expression was observed, long latency rats had lower levels of DGL and short latency rats had heightened DGL expression. Additionally, a significant decrease in CRF type 1 receptor expression was found in both males and females. These cellular adaptations suggest that the eCB system might play a role in the different coping strategies.

Introduction

Chronic stress is known to contribute to the pathophysiology of psychological disorders (McEwen, 2008, Carvalho and Van Bockstaele, 2012). In our current society, people experience social stressors – death of a family member, divorce, bullying, psychological abuse – more often than any other type (Kendler et al., 1995, Bjorkqvist, 2001). Each of these types of stressors has been correlated to an increased likelihood of developing depression or anxiety disorders (Kessler et al., 1994, Kendler et al., 1995). One of the most well established animal models for social stress is the resident-intruder paradigm, in which a smaller intruder rat is exposed to a more aggressive resident rat. Rats exposed to repeated social defeat by the larger resident rat develop increased depressive- and anxiety-like phenotypes as well as long lasting hyperactivation of stress signaling and the hypothalamic-pituitary-adrenal (HPA) axis (Wood et al., 2010, Wood and Bhatnagar, 2015).

While the type and severity of stressor bears importance on the development of psychiatric disorders, so does the individual's ability to cope with the stress. Active coping strategies center around behavioral responses in an attempt to minimize harm and reduce stress, and often lead to resilience from the anxiogenic effects of the social stressor (Veenema et al., 2003, Wood and Bhatnagar, 2015). Passive coping strategies involve feelings of helplessness and immobility, which are associated with an increased susceptibility to depression and anxiety disorders (Billings and Moos, 1984, Wood and Bhatnagar, 2015). These two coping strategies can be observed in the resident-intruder model by separating rats based on their average latency to assume a defeated posture. Rats belonging to the short latency group exhibit HPA axis dysregulation and depressive-

like behaviors, while those in the long latency group show decreased efficacy of CRF and appear to be more resilient to the development of depressive-like behaviors (Wood et al., 2010, Wood and Bhatnagar, 2015).

The stress-integrative locus coeruleus (LC) is responsible for providing norepinephrine (NE) throughout the entire neuraxis and medial prefrontal cortex (mPFC), where dysregulation in NE levels can lead to depression and anxiety (Valentino and Van Bockstaele, 2005, Carvalho and Van Bockstaele, 2012). The LC is sensitive to social stressors (Chaijale et al., 2013), and the resident-intruder paradigm robustly increases sympathetic activation acutely compared to non-social stress paradigms (Sgoifo et al., 1999). Corticotropin releasing factor (CRF) is responsible for the increased LC-NE activity following a stressor via corticotropin releasing factor type 1 receptor (CRFr1) (Curtis et al., 1996, Curtis et al., 2012). The endocannabionid (eCB) system modulates the stress response in many brain regions (Wyrofsky et al., 2015), and recent studies have localized the cannabinoid type 1 receptor (CB1r) to CRF-containing afferents in the LC, suggesting that the eCB system is positioned to modulate the stress response within this noradrenergic nucleus (Wyrofsky et al., 2017). Previous studies investigating the effect of social stress on the LC in male rats have found a decrease in LC-NE activity following repeated stress, compared to an increase in activity after an acute stressor (Chaijale et al., 2013). Additionally, differences in cellular adaptations in the LC occur across phenotypes, with an increase in opioid signaling in long latency rats compared to short latency and control groups, suggesting one mechanism for combating chronic stress in the LC (Chaijale et al., 2013, Reyes et al., 2015).

In order to determine whether differences in another anti-stress system - the eCB system - exist between the long and short latency groups, Western blot analysis was performed on LC micropunches from control, short latency, and long latency groups. Both male and female rats were tested, as sex differences in stress circuitry, the eCB system, and the prevalence of psychiatric disorders exist (Kendler et al., 1995, Marcus et al., 2005, Reich et al., 2009, Bangasser and Valentino, 2012, Craft et al., 2013). The two most common eCBs, 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide (AEA), are often up- and down-regulated following acute and chronic stress via their synthesis and degradation (Hill et al., 2010, Morena et al., 2016). Fatty acid amide hydrolase (FAAH) is responsible for the degradation of AEA, and diacylglycerol lipase α (DGL) synthesizes 2-AG (Castillo et al., 2012). Therefore, changes in both FAAH and DGL expression levels across phenotypes and sexes were examined.

Methods

Protein sample extracts from the LC were generously procured from Dr. Seema Bhatnagar's laboratory at the Children's Hospital of Pennsylvania. The resident-intruder paradigm and protein extraction and quantification were performed by members of the Bhatnagar and Valentino Laboratories.

Resident-intruder paradigm

Male and female Sprague-Dawley rats (275-300g) were used as controls and intruders. Larger male Long-Evans retired breeders (650-850g) and lactating female Long-Evans breeders were used as residents (Charles River, Wilmington, MA). The

paradigm was based on the model established originally by Miczek (Miczek, 1979), and has been previously described (Wood et al., 2010). For each social stress episode, the intruder rat was placed in the home cage of a novel aggressive resident rat. Once the intruder rat assumed a subordinate defeated position for approximately 3 sec, the rats were separated by a wire mesh to prevent further physical contact between the two rodents. Intruder rats remained in the wire mesh enclosure until the 30-minute session was complete, at which time they were returned to their own home cage. Control rats were placed in a novel cage for 30 mins, in a wire mesh enclosure. This procedure occurred for 5 consecutive days, and after the final session, rats were rapidly decapitated, brains extracted, and tissue was flash frozen for later use. Intruder rats were separated into short or long latency groups based on the average time it took for them to reach subordination: short latency < 300s, long latency > 300s.

Protein extraction and quantification

The LC region was microdissected from male and female control, short latency, and long latency rats. Tissue punches were homogenized with a pestle, sonicated, and extracted in radioimmunoprecipitation assay lysis buffer with a protease inhibitor cocktail on ice for 20 min. Lysates were cleared by centrifugation at 13,000 rpm for 12 min at 4^{o}C, and supernatants were extracted. Protein concentrations were quantified using the bicinchoninic acid protein assay reagent.

Western blot analysis

Protein extracts were diluted with an equal volume of Novex 2© tris glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA) containing dithiothreitol (Sigma-Aldrich Inc., St. Louis, MO, USA). Cell lysates containing equal amounts of protein (30 μg per condition) were separated on 10% tris-glysine polyacrylamide gels and then electrophoretically transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with Odyssey buffer (1 h, diluted in 0.01M PBS 1:1) and incubated in the various combinations of the following primary antibodies overnight at room temperature rabbit anti-diacylglycerol lipase α (DGL; 1:500) rabbit anti-fatty acid amide hydrolase (FAAH; 1:250), and rabbit anti-CRF receptor type 1 (CRFr1; 1:500). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000) was used as a loading control, to account for potential variability in amount of sample loaded. Membranes were then rinsed and incubated with infrared fluorescent secondary antibodies (Licor, Lincoln, NE, USA) for 1 h: donkey anti-rabbit IRDye 800CW (1:15000), goat anti-mouse IRDye680LT (1:20000). Membranes were scanned using the Odyssey Infrared Imaging System (Licor, Lincoln, NE, USA), and protein quantification was determined using Odyssey Infrared Imaging software.

Protein quantification was normalized to the loading control, and all data is presented as a ratio of sample protein level to GAPDH level, to allow for comparison between groups. Additionally, Chameleon Duo Pre-stained Protein Ladder (Licor, Lincoln, NE, USA) was used to determine the molecular weights of protein bands observed: GAPDH – ~37 kDa, CRFr1 – ~50 kDa, FAAH - ~63 kDa, DGL - ~110 kDa.

Data represents N=6 male control rats, N=7 male long latency rats, N=5 male short latency rats, N=6 female control rats, N=5 female long latency rats, N=6 female short latency rats.

Data analysis

Differences in protein expression levels were tested using two-way ANOVAs (sex vs. phenotype) followed by post-hoc Tukey's multiple comparison adjustments. Statistics were performed using GraphPad Prism 7.03 (GraphPad Software, San Diego, CA, USA). Results are presented as mean +/- SEM.

Results

Effect of social stress on eCB protein expression in the LC

Western blot analysis of LC tissue from various social defeat groups across sexes revealed differential expression of DGL levels (Fig. 3.1A). Analysis of control rats showed that under basal conditions, there is a sex difference in DGL levels in the LC: females (0.489 ± 0.092) had significantly lower expression compared to males (0.748 ± 0.094; $p<0.05$). Regarding change in expression across phenotypes, males had a significant reduction in DGL expression in the long latency group (0.457 ± 0.046) compared to control ($p<0.05$), while no significant change between control and short latency groups (0.581 ± 0.033) was observed. In females, no change between control and long latency groups (0.514 ± 0.063) was found; however, there was a significant increase in short latency females (0.807 ± 0.082; $p<0.05$) compared to control.

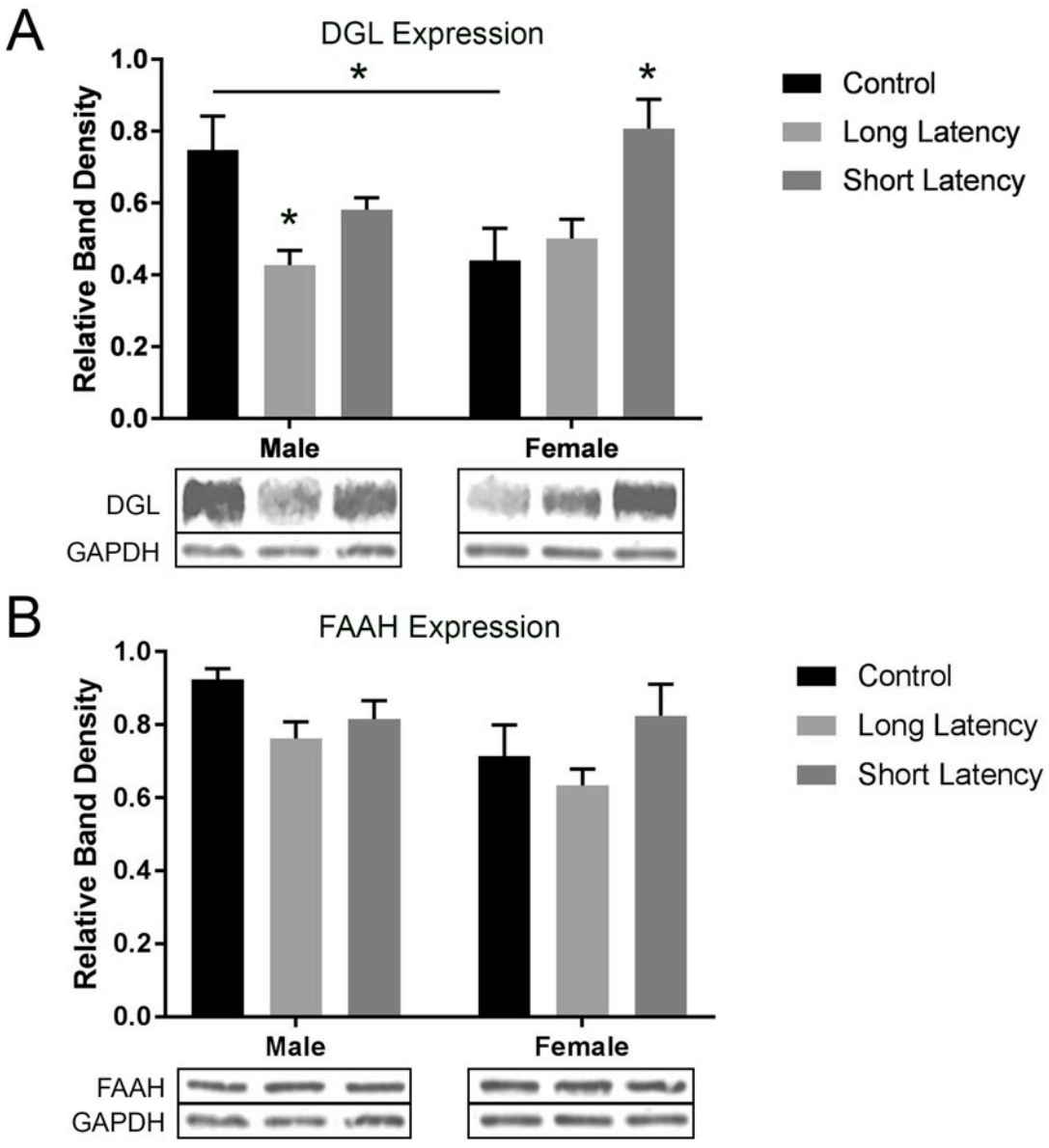

Figure 3.1
Western blot analysis of diacylglycerol lipase α (DGL), fatty acid amide hydrolysis (FAAH) in male and female rats following 5 days of social stress

Figure 3.1: Western blot analysis of diacylglycerol lipase α (DGL), fatty acid amide hydrolysis (FAAH) in male and female rats following 5 days of social stress. Bands shown are representative of one sample obtained from one animal per group. **a.** Western blot analysis for DGL expression in protein extracts from the LC showed that DGL expression is decreased in male long latency rats compared to control, while DGL expression is increased in female short latency rats compared to control. Additionally, female control rats showed significantly less DGL expression compared to males. **b.** Western blot analysis for FAAH expression in protein extracts from the LC showed no significant differences between males and females across phenotypes. Data represent mean ± SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVA/mixed-effects regression model (* $p<0.05$).

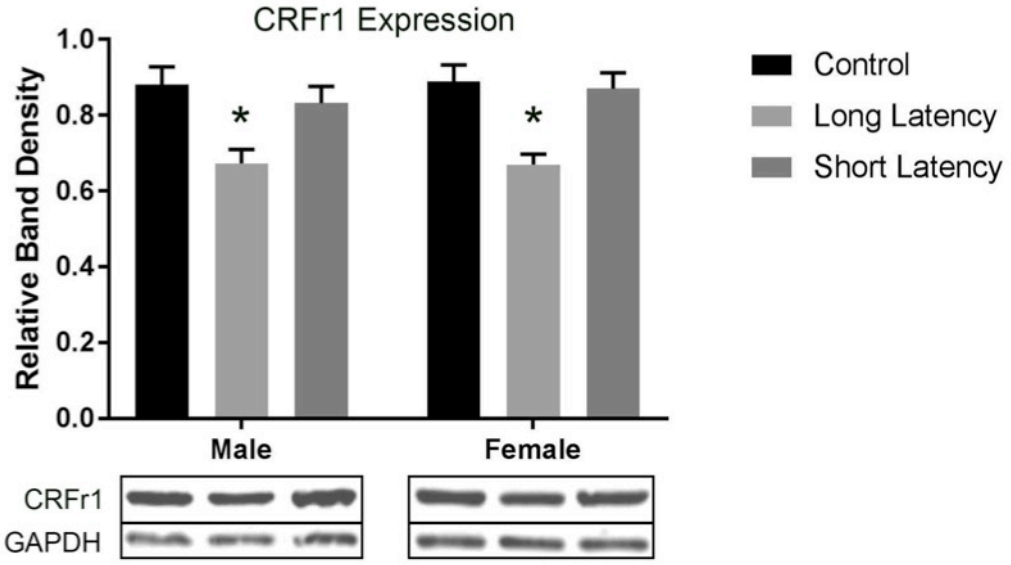

Figure 3.2
Western blot analysis of corticotropin releasing factor type 1 receptor (CRFr1) in male and female rats following 5 days of social stress.

Figure 3.2: Western blot analysis of corticotropin releasing factor type 1 receptor (CRFr1) in male and female rats following 5 days of social stress. Bands shown are representative of one sample obtained from one animal per group. Western blot analysis for CRFr1 expression in protein extracts from the LC revealed a significant decrease in levels in male and female long latency groups compared to control, and no change between short latency groups and control. Data represent mean ± SEM. Asterisks indicate a significant difference between groups as determined by two-way ANOVA/ mixed-effects regression model (* $p<0.05$).

When examining FAAH (Fig. 3.1B), no significant changes in expression levels were detected between sexes or phenotypes. FAAH protein expression across sex and phenotype was as follows: male control – 0.923 ± 0.030, male long latency 0.762 ± 0.040, male short latency – 0.815 ± 0.052, female control – 0.714 ± 0.085, female long latency – 0.634 ± 0.045, female short latency – 0.825 ± 0.087.

Effects of social stress on CRFr1 expression in the LC

CRF exerts its effects on LC-NE neurons via CRFr1, which are expressed throughout the LC and are very prominent in the peri-LC region (Valentino and Van Bockstaele, 2005, Reyes et al., 2008). Western blot analysis revealed that, in addition to altering the eCB system, changes in CRFr1 receptor expression were identified across phenotypes (Fig. 3.2). CRFr1 levels in LC tissue from both long latency males and females were significantly decreased compared to male and female controls ($p<0.05$), and no significant difference between short latency and control groups was detected. Relative protein expression for CRFr1 across sex and phenotype was as follows: male controls – 0.880 ± 0.047, male long latency – 0.684 ± 0.042, male short latency – 0.817 ± 0.052, female control – 0.888 ± 0.036, female long latency – 0.678 ± 0.018, female short latency 0.087 ± 0.042. Decreased CRFr1 expression in the LC of long latency male rats was previously identified (Chaijale et al., 2013). A similar trend was found in females, confirming that the resilient long latency rats have decreased responsiveness to CRF following chronic social stress across both sexes, suggesting one mechanism by which long latency rats are protecting themselves from social stress and a resulting increase in CRF levels.

Discussion

Chronic social stress has been shown to differentially alter opioid signaling in the LC across long and short latency phenotypes in male rats. The present study demonstrates that the eCB system is also affected following social defeat stress in both male and female rats. Via Western blot analysis, DGL expression was significantly decreased in the LC of long latency social defeat male rats compared to control, while females showed a significant increase in DGL expression in short latency rats compared to control. Additionally, basal sex differences in DGL levels exist, with females having lower expression than males. No significant change in LC FAAH expression was observed across sex or phenotype. When investigating CRFr1 expression in the LC following social stress, males and females showed the same trend: a significant decrease in long latency rats compared to control, and no significant change in short latency rats. These data confirm that the differences in the eCB system exist between the resilient long latency and more vulnerable short latency rats.

Differences in DGL expression across phenotypes

DGL is one of the main proteins responsible for the synthesis of eCB 2-AG (Castillo et al., 2012). A decrease in DGL expression would suggest a decrease in 2-AG synthesis while, conversely, an increase in DGL expression would suggest an increase in 2-AG levels. Based on the data above, male long latency rats have a decrease in DGL levels compared to control, and female short latency rats have an increase in DGL levels compared to control. DGL expression across sexes for short latency and long latency groups show no significant difference, with long latency rats having higher DGL

expression compared to short latency. This implies short latency rats have more 2-AG in the LC compared to long latency, suggesting there is increased eCB signaling in the short latency groups. In regards to the basal sex difference observed in DGL levels in control rats, greater expression was observed in males compared to females. This might suggest that the eCB system is primed and ready to combat stress in males, and that females have less tonic 2-AG regulation of LC-NE excitability. Indeed, Krebs-Craft et al. (2010) have discovered that males have higher levels of AEA and 2-AG in the amygdala compared to males (Craft et al., 2013), confirming region specific sex differences within the eCB system.

One hallmark characteristic of the short latency group is an inability to attenuate HPA axis hyperactivity, while the rats in the long latency group have both decreased HPA axis activity as well as a decreased efficacy of CRF in the paraventricular nucleus (PVN) of the hypothalamus (Wood et al., 2010). Studies have found that elevated corticosterone levels are responsible for increasing 2-AG (Morena et al., 2016), thus HPA axis hyperactivity in short latency rats could be responsible for the heightened DGL expression. Since short latency rats have heightened stress signaling, increased DGL and 2-AG levels could be an attempt to counteract increased CRF levels and aberrant LC-NE activity. Conversely, long latency rats have developed adaptations in brain regions such as the PVN to keep HPA axis and stress levels in balance (Wood et al., 2010); therefore, an increase in eCB synthesis within the LC is not necessary to maintain normal LC-NE activity.

Functional consequences

Active coping strategies often involve high sympathetic and noradrenergic reactivity in response to a stressful stimulus (Fokkema et al., 1995, Walker et al., 2009). While hyperactive stress signaling and CRF release in the LC would cause an increase in LC-NE activity, this is maladaptive chronically, and can lead to the development of psychiatric disorders (Valentino and Van Bockstaele, 2008). By having a decrease in CRFr1 levels and an increase in opioid signaling, resilient long latency rats might be protecting themselves from LC-NE hyperactivity (Reyes et al., 2015). Coupled with lower DGL expression, long latency rats exposed to a stressor could have a more moderate and appropriate increase in LC-NE activity. This would allow resilient rats to engage in active coping strategies that involve successful cognitive functioning, as a robust increase in high phasic low tonic LC-NE activity via CRF can cause problems with focused attention and hyperarousal (Valentino and Van Bockstaele, 2008).

In humans, when chronic stress is not attenuated, pathological changes can lead to the development of PTSD and anxiety disorders (Valentino and Van Bockstaele, 2008, Wood and Bhatnagar, 2015). Indeed, HPA hyperactivity and CRF release is often found in individuals suffering from major depression (Swaab et al., 2005). These clinical findings correspond with the above observations from social defeat rats, and changes in eCB signaling between long latency and short latency groups might be an additional mechanism responsible for resilience, further implicating the eCB system as a viable target for the treatment of psychological disorders.

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DISCUSSION

Overall Conclusions

Through numerous techniques, the experiments presented in this thesis greatly improve our understanding of the CB1r role in the LC. First, the functional consequences of CB1r deletion on LC-NE activity across sexes were determined. CB1r-KO caused a significant increase in noradrenergic indices in male mice compared to WT: KO mice had an increase in LC-NE excitability, input resistance, TH expression within the LC, and NE levels in the mPFC. These noradrenergic indices were not significantly different following CB1r deletion in female mice. Western blot analyses of LC tissue from male and female CB1r/CB2r-KO mice also highlighted several sex differences. Male CB1r/CB2r-KO mice showed a significant increase in CRF expression and in NET expression compared to male WT mice, while female CB1r/CB2r-KO mice showed a significant increase in α2-AR expression compared to female WT mice, and these adaptations might play a role in the resulting dysregulation of LC-NE activity that occurs in male but not female CB1r-KO mice. Second, while 300 nM CRF was capable of increasing LC-NE excitability in WT brain slices from both male and female mice, LC-NE neurons from CB1r-KO mice were not affected by 300 nM CRF bath application, which could be due to other cellular adaptations that have occurred in the CB1r-KO mice. Third, direct anatomical evidence has been provided for CB1r modulation of CRF-containing afferents. Via immunofluorescence and immunoelectron microscopy, co-localization between CB1r and the CRF peptide in both the core and peri-LC was detected, suggesting that CB1r activation by endogenous and exogenous cannabinoids could produce an effect on CRF release in the LC and subsequent LC-NE neuronal activity. Finally, Western blot analysis of LC tissue from social defeat animals showed

male long latency rats had decreased expression of the eCB synthesizing enzyme DGL compared to control, while female short latency rats had higher levels compared to control, highlighting differences in the eCB system between resilient and vulnerable rats across sexes. Additionally, control males had higher DGL expression compared to control females, suggesting a sex difference in basal eCB tone in the LC. These studies combined demonstrate the importance of proper CB1r signaling in the LC, especially in males where deletion results in aberrant LC-NE excitability, and provide another mechanism by which the eCB system can modulate stress circuitry.

Research Implications Across Studies

Studies examining CB1r in other brain regions have demonstrated the importance of the eCB system in regulating various aspects of the stress response. From CB1r in the paraventricular nucleus of the hypothalamus and eCB involvement in the negative feedback loop of the HPA axis (Patel et al., 2004, Hill and McEwen, 2009, Hill and Tasker, 2012), to activation of CB1r in the amygdala via fluctuating levels of eCB to help control the fear response when a stressor is not present (Hill et al., 2009, Gunduz-Cinar et al., 2013), this system has repeatedly been identified as a key modulator of stress signaling. The new data presented above provide further evidence for the importance of the eCB system in modulating the stress response, specifically within the LC. Global lifetime deletion of CB1r in males causes an increase in CRF within the LC, which supports previous findings that CB1r-KO mice have heightened HPA axis activity. However, an increase in LC-NE excitability and a subsequent increase in NE efflux in the

mPFC demonstrate that CB1r deletion also significantly affects the cognitive limb of the stress response.

Role of eCB System in LC-NE Activity

CB1r mRNA and protein expression have been localized within the LC (Herkenham et al., 1991, Mailleux and Vanderhaeghen, 1992, Matsuda et al., 1993, Derbenev et al., 2004). Additionally, electron microscopy studies have shown that CB1r are found on presynaptic axon terminals that synapse with NE-producing LC neurons as well as post-synaptically in somatodendritic processes of LC cells (Scavone et al., 2010). The presence of CB1r on LC-NE neurons is functional, as indicated by electrophysiological studies showing that CB1r agonists and FAAH inhibitors increase the basal firing rate of LC-NE cells, c-Fos expression of LC neurons, and NE efflux in the mPFC (Gobbi et al., 2005, Oropeza et al., 2005, Mendiguren and Pineda, 2006, Muntoni et al., 2006, Page et al., 2008). Additionally, there is tonic eCB production in the LC, as sole application of a CB1r antagonist is capable of decreasing LC-NE activity (Muntoni et al., 2006, Carvalho and Van Bockstaele, 2012). Interestingly, the LC appears to be under biphasic regulation of the eCB system, as other studies have found that systemic administration of rimonabant, a CB1r antagonist, increases NE levels in the mPFC and PVN (Tzavara et al., 2001, Tzavara et al., 2003), and low levels of THC can cause a decrease in NE release from synaptosomes (Poddar and Dewey, 1980). Therefore, it appears that the eCB system serves to modulate the LC-NE system to maintain an optimal level of activity. In support of this, our laboratory has recently demonstrated a reduction in basal mPFC neuronal excitability in CB1r-KO mice, caused

by desensitization of the normally excitatory mPFC α2-adrenoceptors (ARs) (Reyes et al., 2017). This indicates that without a functioning eCB system, aberrant LC-NE activity is observed, where CB1r-KO mice have increased LC-NE activity, resulting in mPFC α2-AR desensitization, and subsequent decreased mPFC output (Fig. 3). Additionally, when LC-NE neurons are excited via potassium chloride (KCl) bath application, CB1r agonist pre-treatment is capable of attenuating the KCl-induced increases in LC-NE firing (Mendiguren and Pineda, 2007). These data suggest that the eCB system might function to prevent over-activation of LC-NE neurons.

Since CRF release in the LC increases LC-NE excitability and activity, it is tempting to speculate that the eCB system can serve to attenuate these CRF-induced increases as well (Curtis et al., 1996). Indeed, the immunofluorescence studies performed in chapter 2 demonstrated that CB1r are co-localized with CRF in the rat LC. Additionally, using Phaseolus Vulgaris Leucoagglutinin (PHAL) as an anterograde tracer injected into the amygdala, CB1r are directly positioned to regulate CRF release from amygdalar limbic afferents. Immunoelectron quantification has confirmed that CB1r are localized both pre- and post-synaptically with respect to CRF in both the core and peri-LC and in both excitatory and inhibitory synapses. These findings provide an anatomical substrate for direct eCB modulation of CRF. Because aberrant NE release in the mPFC contributes to the development of stress-induced psychiatric disorders, eCB modulation of CRF release in the LC during stress could play a role in the anxiolytic effects of CB1r agonists.

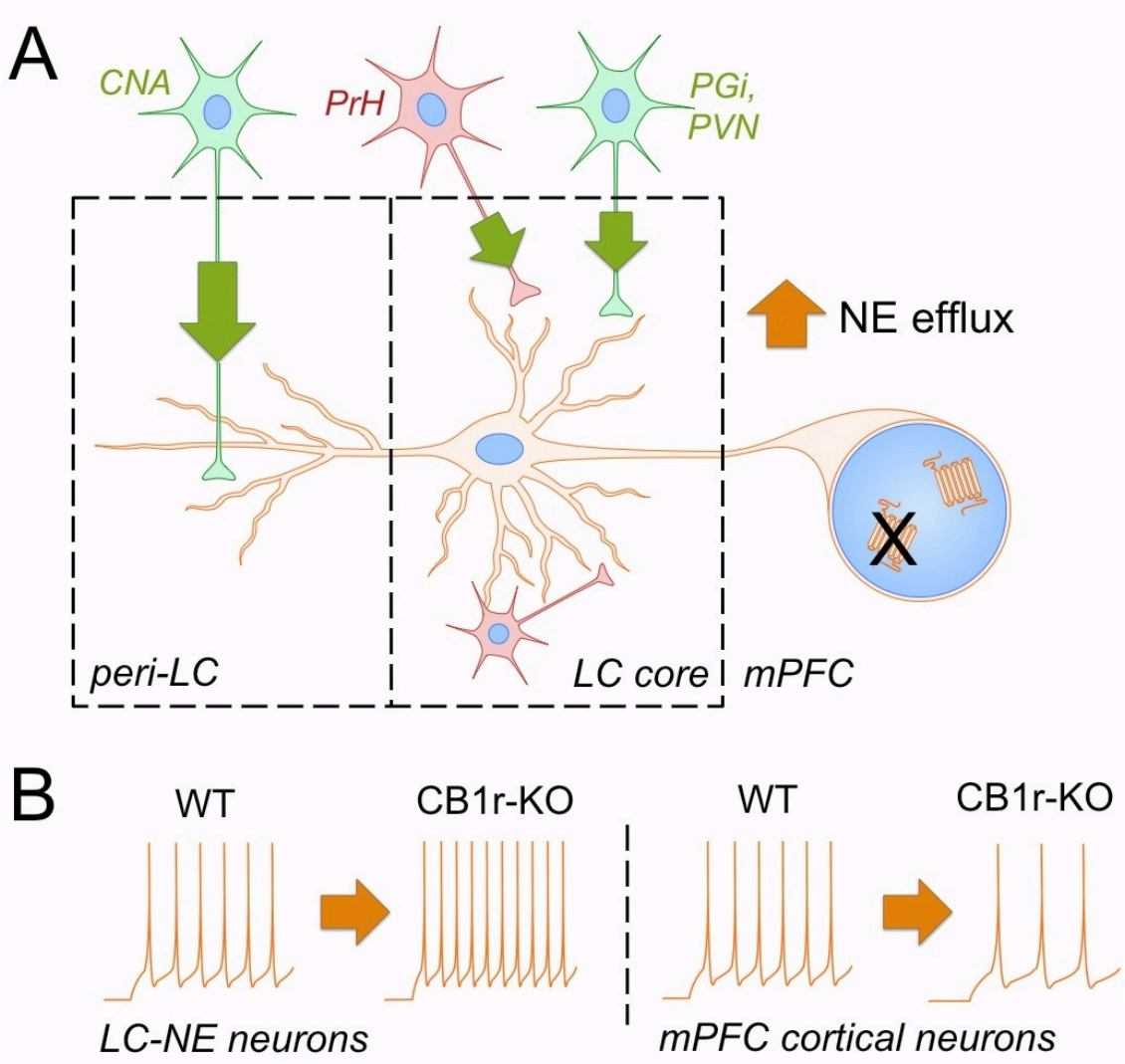

Figure 3
Schematic depicting alterations to LC-mPFC microcircuit in male CB1r-KO mice.

Figure 3: Schematic depicting alterations to LC-mPFC microcircuit in male CB1r-KO mice. A. The nucleus paragigantocellularis (PGi) and paraventricular nucleus of the hypothalamus (PVN) provide excitatory/glutamatergic (green neurons) input and the nucleus prepositus hypoglossi (PrH) provides inhibitory/GABAergic (red neurons) to the locus coeruleus (LC) core, while the amygdala (CNA) provides excitatory/glutamatergic input to the peri-LC. These afferents can also release corticotropin-releasing factor (CRF), as indicated by the green arrows. Additionally, GABAergic interneurons are present in the LC. Cannabinoid type 1 receptors (CB1r) have been localized to excitatory and inhibitory synapses in the core and peri-LC, providing a mechanism for eCB modulation of limbic and autonomic projections, suggesting that deletion of CB1r could result in dysregulation of neurotransmitter and CRF release in the LC. Indeed, male CB1r-knock out (KO) mice have a significant increase in NE levels in the medial prefrontal cortex (mPFC) compared to wild type (WT), resulting in the desensitization and decreased expression of α2-adrenoceptors. **B.** *In vitro* whole-cell patch clamp recordings reveal that male CB1r-KO mice have increased LC-NE excitability compared to WT, which corresponds with the increase in mPFC NE levels. Additionally, male CB1r-KO mice have a decrease in mPFC cortical neuron excitability, likely resulting from desensitization of excitatory mPFC α2-adrenoceptors. Traces are examples of neuronal excitability, as would be measured by counting the number of action potentials that occur during application of increasing current pulses.

Working Model of eCB Regulation of LC-NE Activity

CB1r have been localized to CRF-containing afferents from the amygdala, and their presynaptic distribution in the peri-LC suggests that they might be capable of attenuating CRF release via activation by endogenous or exogenous cannabinoids. Based on our collective data, we propose the following model for eCB regulation of LC-NE neuronal activity. Initially, acute stress exposure causes release of CRF into the LC, from a variety of brain regions, but predominantly from amygdalar afferents (Fig. 4A). CRF then binds to CRFr1 on LC-NE somatodendritic processes, causing postsynaptic depolarization and an increase in LC-NE activity and NE release in the mPFC. This depolarization and influx in intracellular calcium levels stimulates an increase in DGL activity, causing the synthesis and release of the eCB 2-AG (Fig. 4B). 2-AG retrogradely crosses the synaptic cleft to bind to presynaptic CB1r, which have been localized to amygdalar-CRF afferents, where its activation leads to a decrease in CRF release and subsequent attenuation of LC-NE activity. However, under conditions of chronic stress in vulnerable subpopulations, such as short latency females in our social stress model, we have found an increase in DGL expression. This could present a problem when stressors are no longer present, as the increased DGL levels could lead to an increase in 2-AG synthesis, even when a stressor is not present. While eCBs are capable of attenuating LC-NE excitability, overexpression of eCBs have been shown to also increase basal LC-NE activity. Therefore, hyperactivity of the eCB system following chronic stress might lead to activation of CB1r at other neighboring synapses in the LC, such as inhibitory interneurons, where CB1r have also been localized (Fig. 4C). This disinhibition onto LC dendrites would cause an increase in LC-NE activity, and aberrant NE release in the

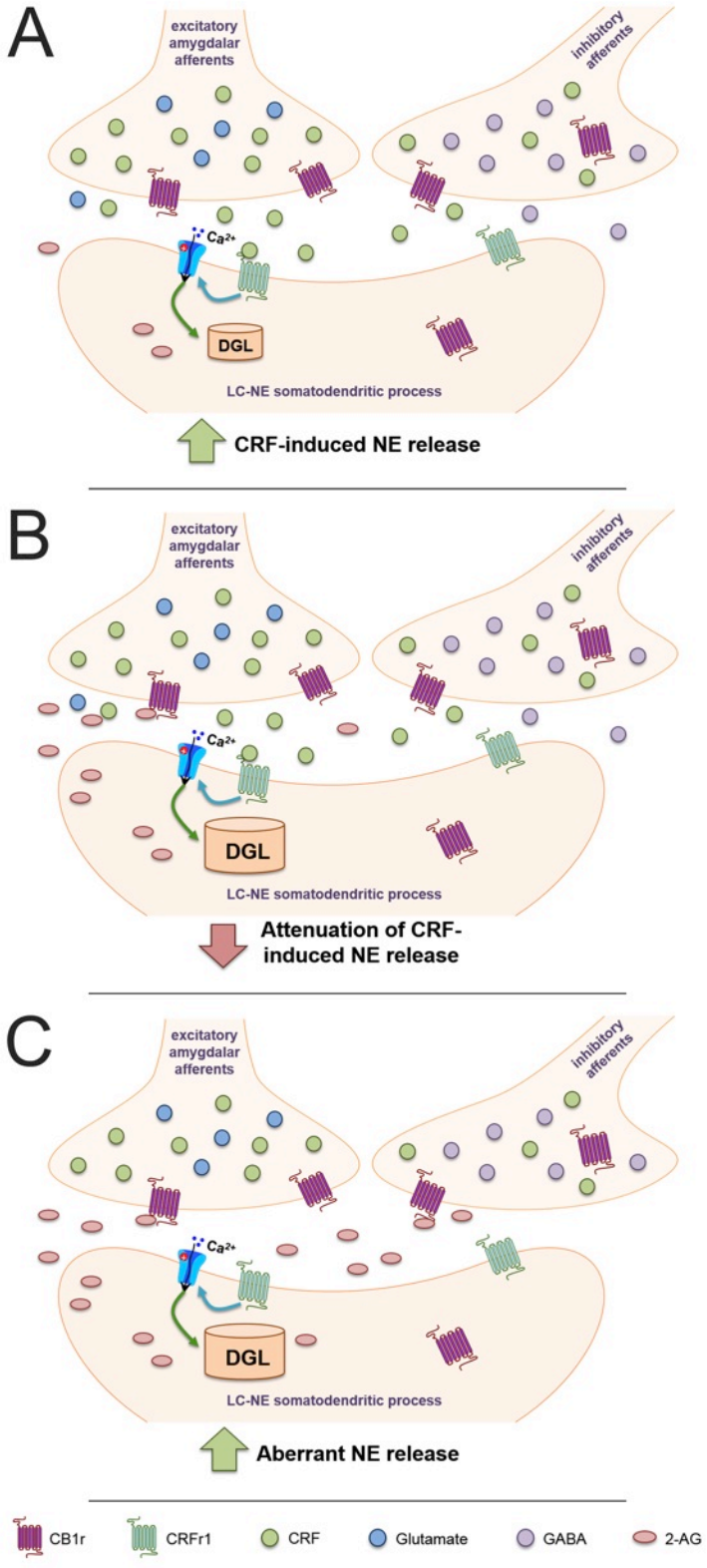

Figure 4
Working model for eCB modulation of LC following chronic stress.

Figure 4: Working model for eCB modulation of LC following chronic stress. Cannabinoid type 1 receptor (CB1r) has been localized to excitatory and inhibitory corticotropin-releasing factor (CRF) afferents in the locus coeruleus (LC), and anterograde tract tracing has found CB1r specifically on excitatory amygdalar-CRF afferents, the main source of CRF to the LC. **A.** Following a stressor, CRF is released in the LC, where it binds to corticotropin-releasing factor receptor type 1 (CRFr1). This causes postsynaptic depolarization of LC-norepinephrine (NE) neurons, leading to an increase in activity and NE efflux in the medial prefrontal cortex (mPFC). **B.** CRF-induced depolarization and influx in intracellular calcium (Ca^{2+}) stimulates diacylglycerol lipase (DGL) to synthesize and release 2-arachidonlyglycerol (2-AG) into the synaptic cleft. 2-AG then crosses the synapse and binds to CB1r. This inhibits the continued release of CRF, attenuating the CRF-induced increases in LC-NE activity and NE efflux, and helping to diminish the stress response. **C.** Chronic stress, especially in vulnerable female subpopulations, results in high DGL expression. Increased DGL would suggest greater production of 2-AG, which could bind to CB1r on neighboring synapses, causing inhibition of GABAergic interneurons and non-CRF releasing afferents. This dysregulation synaptic activity and disinhibition could further excite LC-NE neurons can cause aberrant NE release in the mPFC, and could contribute to the increased propensity for females to develop stress-induced psychiatric disorders.

mPFC could contribute to anxiety and depressive-like behaviors. As females are known to be more susceptible to stress-induced psychiatric disorders, a significant increase in LC DGL expression following chronic stress in vulnerable female populations might be contributing to the disparity in prevalence of PTSD and depression.

Sex Differences in eCB Regulation of LC-NE Activity

The studies performed in chapter 1 demonstrate that CB1r deletion differentially affects male and female LC neurons. Using *in vitro* slice electrophysiology, Western blotting, and ELISA analysis, we discovered that CB1r-KO caused a significant increase in noradrenergic indices in male mice compared to WT: male KO mice had an increase in LC-NE excitability, input resistance, tyrosine hydroxylase (TH) expression within the LC, and NE levels in the mPFC (Fig. 3). These noradrenergic indices were not altered following CB1r deletion in female mice. Western blot analyses of LC tissue from male and female CB1r/CB2r-KO mice also highlighted several sex differences. Male CB1r/CB2r-KO mice showed a significant increase in CRF expression and in NET expression compared to male WT mice, while female CB1r/CB2r-KO mice showed a significant increase in α2-AR expression compared to female WT mice, and these adaptations might play a role in the resulting dysregulation of LC-NE activity that occurs in male but not female CB1r-KO mice. Additionally, we tested LC-NE activity in response to CRF under conditions of CB1r deletion. While 300 nM CRF was capable of increasing LC-NE excitability in WT brain slices from both male and female mice, LC-NE neurons from CB1r-KO mice were not affected by 300 nM CRF bath application. This could be attributed to cellular adaptations observed in the CB1r-KO mice, such as

increased α2-AR signaling in female KO mice, saturation of CRFr1 in male KO mice resulting from their increased endogenous CRF levels, or alterations to CRFr1 trafficking or synthesis. This shows the importance of the eCB system in maintaining normal LC-NE excitability and responsiveness to stress signaling.

At first, the lack of change in LC-NE activity of CB1r-KO females compared to WT females was surprising, given that females have heightened stress signaling and HPA axis activity following stressors such as restraint stress compared to males (Buynitsky and Mostofsky, 2009). However, further exploration into existing literature supports the notion that CB1r-KO might have less of an effect on stress circuitry in females. Roberts et al. (2014) have discovered that female CB1r-KO mice do not show the same increase in circulating corticosterone levels 30 minutes after restraint stress that male CB1r-KO mice show, in part due to an increase in capacity for corticosteroid-binding globulin to bind corticosterone and reduce free circulating levels (Roberts et al., 2014). Another study shows that acute administration of a CB1r antagonist produces a more robust increase in HPA activity in male rats, while females show significantly less of an increase following treatment (Atkinson et al., 2010). Additionally, sex differences in the eCB system, such as an increased cannabinoid self-administration rate (Fattore et al., 2007, Fattore et al., 2009) and lower CB1r density in several brain regions in female mice compared to males (Rodriguez de Fonseca et al., 1994), implicate a reduced eCB tone in females. In regards to the amygdala, studies have found a decrease in 2-AG and AEA levels that correspond with decreased expression of their metabolizing enzymes (Krebs-Craft et al., 2010, Craft et al., 2013). The Western blot analysis in chapter 3 provides evidence basal differences in LC eCB levels across sex, with female rats have

significantly less DGL expression in the LC than males. Less DGL would correspond with a decrease in 2-AG synthesis, suggesting the female LC is under less tonic regulation by the eCB system. Taken together, discovering that female CB1r-KO mice do not show an increase in LC-NE excitability does in fact corroborate with other studies suggesting that female mice undergo compensatory changes to counteract the effect of long-term global CB1r deletion on stress responses.

Future directions

Perhaps one of the most intriguing findings from these experiments is that CRF-induced increases in LC-NE excitability are lost in CB1r-KO mice. As mentioned in chapter 2, Western blot analyses should be performed in order to further investigate if a possible up-regulation of other anti-stress systems, such as the opioid or NPY systems, could be responsible for the lack of response to CRF in the LC. Changes in CRFr1 levels in CB1r-KO mice should also be determined, as a significant decrease in male CB1r-KO mice could be responsible for a decreased effect of CRF on LC-NE excitability. Furthermore, we know that stressors cause differential trafficking of CRFr1 in males versus females, with females having an increase in membrane bound CRFr1 while males promote internalization (Bangasser et al., 2010, Valentino et al., 2013). Immunoelectron microscopy studies would show whether CB1r-KO males, facing a chronic increase in CRF levels in the LC, have continued internalization of CRFr1, and whether female CB1r-KO mice have altered CRFr1 trafficking compared to WT females.

While chapter 2 was successful in providing anatomical evidence for CB1r localization to CRF axon terminals in the LC, these studies were only performed in

males. Given the known sex differences in both stress circuitry and the eCB system (Craft et al., 2013, Valentino et al., 2013, Castelli et al., 2014), it would be useful to investigate whether females have a different pattern of CB1r localization than males. Since CB1r-KO females did not show an increase in LC-NE excitability, perhaps they have less CB1r localized to CRF-containing afferents or greater receptor internalization compared to males. Future immunoelectron microscopy studies could shed light on this possibility. Additionally, data from chapter 3 shows that, within the LC, females have a significantly lower DGL expression compared to males. Subsequent ELISA studies could be performed on LC tissue to confirm basal sex differences in eCB levels.

Finally, the working model proposed above for eCB modulation of CRF-induced activation of LC-NE neurons could be tested via whole-cell patch clamp electrophysiology experiments. Brain sections from male and female WT mice could be excised as described in chapter 1 methods. Before CRF bath application, pretreatment with a CB1r agonist or FAAH inhibitor would demonstrate whether the eCB system could attenuate the effects of CRF on LC-NE cells.

Practical applications

Effects of cannabinoids on fear and PTSD

In addition to dysregulation of cannabinoid-adrenergic signaling, problems with cannabinoid-amygdalar signaling also plays a key part in the development of PTSD (Jovanovic and Ressler, 2010, Wyrofsky et al., 2015). Understanding the effects of cannabinoids in the amygdala is of paramount importance when investigating the LC, as the LC is heavily targeted by both glutamatergic and CRF-containing afferents from

amygdala (Van Bockstaele et al., 1996, Reyes et al., 2011, Wyrofsky et al., 2017).

A direct role for eCB modulation of the emotional components of amygdalar function has been observed in animal studies. Classical associative fear conditioning and extinction behavioral models show that eCB levels are increased in the amygdala during the extinction session; however, these increases were not observed during the initial fear condition, and fear behaviors persisted longer in the extinction sessions in CB1r KO mice compared to WT mice (Marsicano et al., 2002). Rats exposed to a footshock followed by situational reminders, which is a potential model for PTSD, exhibit impaired extinction of the traumatic memory and increased CB1r levels in the hippocampus (CA1) and PFC, and these alterations were prevented by WIN 55,212-2 administration following exposure to the traumatic event (Korem and Akirav, 2014). Combined with the results from other studies, it has been concluded that amygdalar-eCB signaling is critical for both within- and between-session habituation and adaption of fear-related behaviors (Marsicano et al., 2002, Kamprath et al., 2006). These data suggest that the eCB system is essential for regulating amygdalar function and that the amygdala is a nucleus where eCB signaling can affect both neuroendocrine and stress adaptation behaviors (Hill et al., 2010b).

In addition to affecting fear-related behaviors, the eCB system is also involved in the consolidation, retrieval, and extinction of emotionally charged and distressing memories (Fig. 5) (Atsak et al., 2012). As previously mentioned, glucocorticoids that are released following a stressor can stimulate eCB synthesis, which in turn inhibits GABAergic neurotransmission. This disinhibition of GABAergic projections from the BLA to the LC results in increased NE release and its subsequent binding to β-ARs, causing the consolidation of stressful and potentially traumatic memories (Hill et al.,

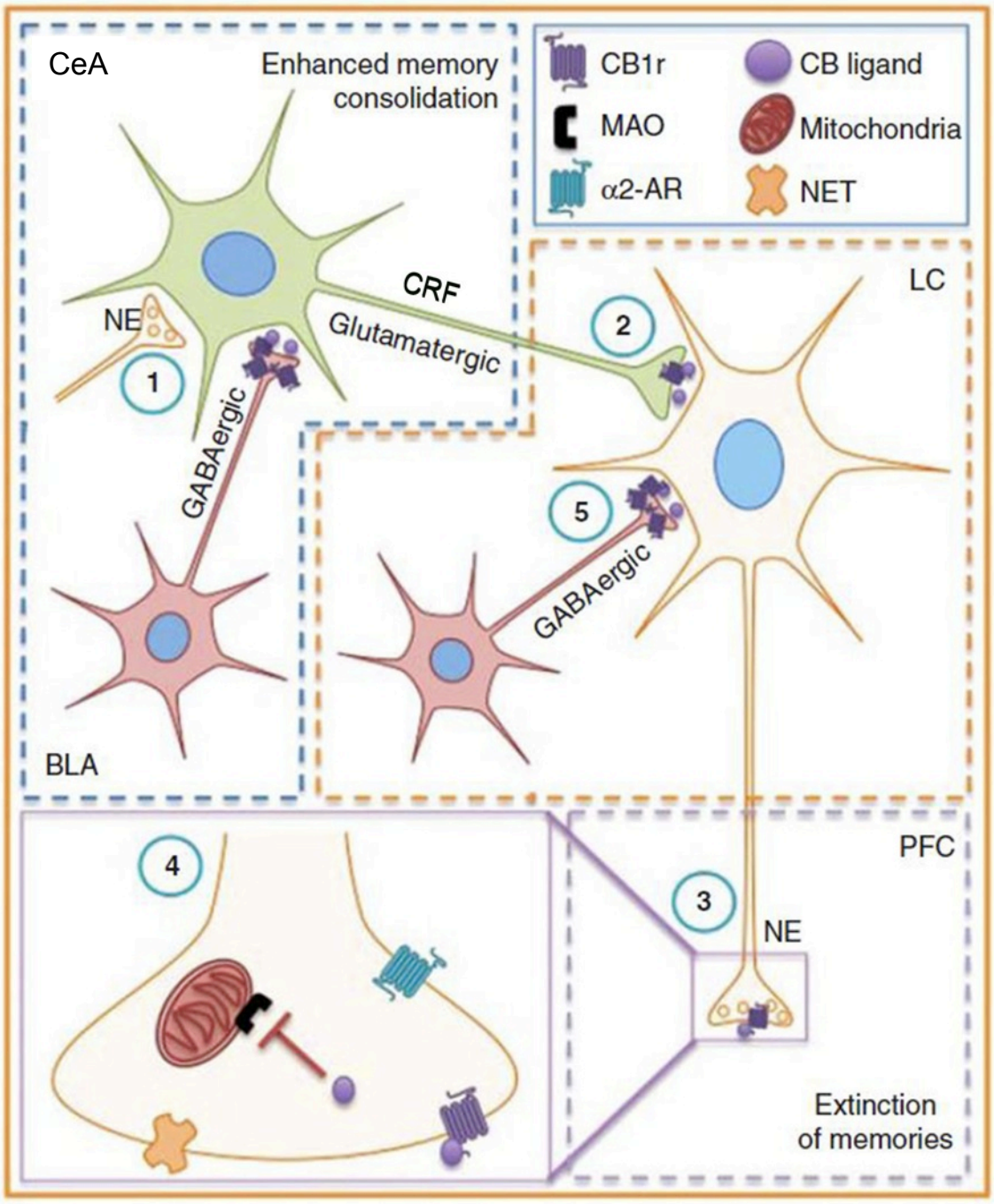

Figure 5
Schematic diagram depicting cannabinoid-adrenergic interactions in stress-integrative circuitry.

Figure 5: Schematic diagram depicting cannabinoid-adrenergic interactions in stress-integrative circuitry. The basolateral complex of the amygdala (BLA) has been implicated in the consolidation of emotionally arousing experiences and involves glucocorticoid-mediated increases in eCB release and interactions with norepinephrine (NE) (Campolongo et al., 2009). (1) eCBs are posited to increase BLA activity by decreasing GABAergic neurotransmission (Duvarci and Pare, 2007). (2) Disinhibition of GABAergic interneurons results in an increase of glutamatergic signaling in the central nucleus of the amygdala (CeA), a source of excitatory and CRF-containing afferents to the LC (Valentino and Van Bockstaele, 2008). (3) Activation of the LC via amygdalar CRF and glutamate causes an increase in noradrenergic signaling and NE release in postsynaptic targets, such as the prefrontal cortex (PFC). Given that the PFC represents a critical region in mediating the extinction of traumatic/aversive memories, treatments involving the eCB system that target this region may help alleviate symptoms of anxiety disorders by increasing extinction of such memories. For example, (4) CBs have been shown to inhibit monoamine oxidase (MAO), representing another mechanism in which CB signaling can regulate NE levels. (5) Targeting GABAergic projections to the L with CB ligands can potentially modulate LC afferent activity to the PFC. Achieving the proper balance in frontal cortical activity by targeting cannabinoid-adrenergic interactions may result in enhancing extinction of aversive memories and diminish anxiety-like behaviors that are precipitated by stress.

2010b). Administration of WIN 55,212-2 or glucocorticoid receptor antagonist RU-486 into the BLA before exposure to a stressful stimuli prevented the enhancement in memory consolidation that is normally observed (Ramot and Akirav, 2012). Since stress also leads to rapid increases in FAAH levels, FAAH inhibitors prevent the degradation of AEA, which in turn promotes long-term fear extinction in animal models via CB1r binding in the BLA and provides protection against stress-induced alterations to eCB signaling (Gunduz-Cinar et al., 2013). It has been suggested that since eCBs are released within the BLA during fear extinction, the resulting eCB-dependent negative feedback on the HPA axis is critical for the extinction of traumatic memories (Marsicano et al., 2002, de Bitencourt et al., 2013). Therefore, compounds that enhance the eCB system could serve as therapeutics for PTSD.

Therapeutic potential for the treatment of psychiatric disorders

The ability of the eCB system to modulate both noradrenergic neurotransmission and the HPA axis makes it a potentially attractive therapeutic target for the treatment of numerous psychiatric disorders, which involve abnormal function of these systems. As described earlier in this review, there is an extensive body of preclinical data demonstrating that various compounds and manipulations that increase CB signaling produce effects in behavioral assays that are predictive of therapeutic efficacy. Although several CB compounds have been evaluated in clinical trials for non-psychiatric disorders such as obesity and pain, it is only recently that some of these compounds have begun to be tested for psychiatric disorders including schizophrenia, PTSD, and depression (Table 2) (Fraser, 2009, Leweke et al., 2012). While only a limited number of studies have

released information on the results of their trials, some of them seem particularly promising. For example, CBD resulted in relief from psychotic symptoms in acute schizophrenic patients that was comparable to a potent antipsychotic while resulting in fewer side effects. In a study of PTSD, nabilone, a synthetic cannabinoid, greatly improved the quality of sleep and decreased the number of daytime flashbacks in treatment-resistant patients (Fraser, 2009). Nabilone also significantly improved PTSD-associated insomnia, chronic pain, and nightmares in a retrospective study of 104 mentally ill men (Cameron et al., 2014). A second study found that THC treatment, twice a day over the course of 3 weeks, decreased the number of nightmares and increased sleep quality in 10 patients suffering from chronic PTSD (Shalev et al., 2013). PTSD patients are plagued with debilitating flashbacks of a horrific event, potentially due to dysfunctional retrieval and extinction of emotional memories (Nemeroff et al., 2006, Akirav, 2013). These results are consistent with clinical studies showing that many individuals afflicted with PTSD self-medicate with cannabis to help alleviate their symptoms (Passie et al., 2012). Cannabis use is correlated with both the onset and severity of PTSD symptoms (Cougle et al., 2011, Potter et al., 2011). Since it is known that the eCB system is involved in these processes and that people suffering from PTSD often self-medicate with cannabis, other compounds that increase eCB signaling could prove to be therapeutic as well.

The eCB and noradrenergic systems are significantly and dynamically impacted by stress (Cassens et al., 1980, Flugge et al., 2004, Gorzalka et al., 2008, Hill and McEwen, 2010, Shinba et al., 2010) and noradrenergic transmission is responsible for cannabinoid-induced activation of the HPA axis (McLaughlin et al., 2009). Under

conditions of acute stress, NE is increased centrally and peripherally (Cassens et al., 1980, Abercrombie and Jacobs, 1987, Page and Valentino, 1994, Valentino et al., 1997, Ferry et al., 1999, Nestler et al., 1999, Sands et al., 2000) while the eCB system tonically constrains activation of neural circuits, including the HPA axis (Gorzalka et al., 2008, Steiner and Wotjak, 2008). However, disrupted noradrenergic and eCB signaling is associated with an inability to adapt to chronic stress (Nestler et al., 1999, Wong et al., 2000, Flugge et al., 2004, Hill and Gorzalka, 2004, Gorzalka et al., 2008, Hill et al., 2008). Previous studies from our group indicate a different consequence to the regulation of NE by cannabinoids under stress conditions. Specifically, stress-induced increases in cortical NE levels are significantly attenuated by prior treatment with a cannabinoid receptor agonist suggesting complex actions of cannabinoids on noradrenergic circuitry that vary under basal versus stress conditions. One working model posits that, under basal conditions, decreased signaling of presynaptically distributed CB1r localized to noradrenergic afferents contribute to local increases in cortical NE and AR desensitization. Under conditions of stress where NE levels are elevated, increased release of eCB from cortical neurons attenuates presynaptic release of NE potentially leading to AR sensitization.

There is significant potential for establishing cannabinoid-adrenergic interactions as a novel target in the development of improved treatment strategies for stress-induced anxiety. The pathophysiology underlying anxiety disorders, and specifically PTSD, may be related to an inability to extinguish aversive memories (Lehner et al., 2009). Increased salience of aversive memories due to activation of limbic circuits and poor cognitive inhibition/flexibility due to decreased cortical activity may contribute to the behavioral

expression of anxiety. Understanding the cellular mechanism responsible for extinction of fear memories may provide the basis for more effective forms of clinical treatment of anxiety. Patients with PTSD suffer from recurrent retrieval of traumatic memories in the form of context-induced flashbacks and repeated nightmares. Repeated re-consolidation of fear memories in limbic circuits and inability to extinguish fear memories (Jovanovic and Ressler, 2010) are thought to underlie the pathophysiology of PTSD. Consolidation of emotionally arousing memories involves, in part, noradrenergic circuits targeting the amygdala (McGaugh et al., 1996, Ferry et al., 1999), while extinction of memory is dependent on the mPFC (Mueller et al., 2008). Pharmacological manipulation of AR systems has provided symptomatic relief in PTSD patients (Taylor et al., 2008, Byers et al., 2010), suggesting that therapeutic improvement may result, in part, from attenuation of signaling of sensitized ARs. Moreover, the cannabinoid receptor agonist, nabilone, has recently been reported to be effective in management of symptoms of PTSD (Fraser, 2009). Taken with recent evidence that the eCB and noradrenergic systems interact in stress-related memory consolidation (Fig. 5) (Campolongo et al., 2009, Hill and McEwen, 2010), targeting interactions between these two systems may represent a novel approach for the treatment of stress-induced anxiety disorders. Elucidating reciprocal interactions between the cannabinoid-adrenergic systems in stress-integrative circuits is vital for demonstrating that interaction of the two is important in modulating stress-induced anxiety and extinction of conditioned fear. Given that the PFC represents a critical region in mediating the extinction of traumatic/aversive memories, treatments that target this region may help alleviate symptoms of anxiety disorders by increasing extinction of such memories. Achieving the proper balance in frontal cortical activity by

targeting cannabinoid-adrenergic interactions may result in enhancing extinction of aversive memories and diminish anxiety-like behaviors that are precipitated by stress.

The increasing availability of different classes of compounds that target discrete aspects of the eCB system provides a unique opportunity to more thoroughly evaluate the importance of cannabinoid-adrenergic interactions on anxious behaviors in both preclinical and clinical studies. As mentioned earlier, the preliminary results obtained with the synthetic cannabinoid nabilone as well as the natural cannabinoid THC and cannabis itself for the treatment of PTSD have been very promising. As females have a higher propensity to develop PTSD and anxiety disorders, understanding sex differences in the cellular mechanisms that underlie cannabinoid and noradrenergic dysregulation following stressors is of great importance. Chapters 1 and 3 have revealed important sex differences in the eCB system within the LC. Electrophysiology results suggest that CB1r deletion has less of an effect in female rodents compared to males, as male CB1r-KO mice showed a significant increase in LC-NE excitability compared to WT, but no change was observed in females. Western analysis of social defeat tissue show a significant reduction in basal DGL levels in female rats compared to males, suggesting that females have less tonic eCB signaling than males. Therefore, removal of eCB signaling might have less profound of an effect on female LC-NE activity, and eCB-targeting therapeutics might affect males and females differently. This is in line with analyses performed on the adverse effects of CB1r antagonist rimonabant, which suggested that the odds ratio for developing anxiety and depression following treatment was the largest in males aged 35-38 (Pi-Sunyer et al., 2006, Nissen et al., 2008). Future studies should be aimed at investigating the effectiveness of eCB-targeting compounds at

treating stress-induced psychiatric disorders across sexes, in hopes of finding better therapeutic interventions for those suffering from anxiety, depression, and PTSD.

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