Mechanistically Distinct Modes of Endocannabinoid Mobilization at Glutamatergic Synapses of the Lateral Division of the Central Amygdala and Its Regulation by Chronic Stress

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To my grandmother, father, mother, Renee, and Zane.

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PREFACE

Stress-related psychiatric disorders are one of the most common mental disorders in the United States, affecting approximately 18% of the U.S. population. Given both its prevalence and debilitating nature, many studies have sought to determine the neurological underpinnings of these disorders in an attempt to identify potential therapeutic targets. Such studies have successfully determined a number of neuroanatomical regions of interest, one of which is the central amygdala (CeA). The CeA is a key brain structure that mediates the processing of physiological and behavioral aspects of the stress response and its activity strongly correlates with the symptomology of stress-related psychiatric illnesses, such as post traumatic stress disorder (PTSD).

Interestingly, the brain's endocannabinoid (eCB) system has also been implicated in stress-related behavior processing as well as the pathophysiology of stress-related psychiatric illnesses. This functional and pathological overlap between the CeA and the eCB system suggest a potential interaction between both systems in stress-related pathologies. A putative locus for this potential interaction may lie at the terminals of excitatory inputs that drive CeA function. However, eCB signaling mechanisms at these terminals remain largely unexplored. To examine this intriguing possibility, we hypothesized that: 1) eCB signaling mechanisms are present at glutamatergic terminals within the lateral division of the CeA (CeAL) and recruit the CeAL cholinergic system to enhance eCB mobilization and 2) repetitive exposure to aversive stimuli modulates eCB signaling at CeAL excitatory synapses to potentially support eCB-mediated adaptive processes that mitigate the pathological consequences of chronic stress exposure.

To test these hypotheses, we first characterized the expression of the endocannabinoid signaling (eCB) machinery within the CeAL. Thereafter, we utilized *ex vivo* electrophysiological techniques to examine functional eCB signaling at excitatory synapses of the CeAL using a combination of pharmacological and genetic tools. These experiments revealed both the expression and subcellular localization of CB1 receptors and the eCB synthetic machinery at CeAL glutamatergic synapses. The functional significance of these findings was subsequently demonstrated by the discovery of short-term eCB signaling mechanisms, in the form of depolarization-induced suppression of excitation (DSE), at the glutamatergic terminals of the CeAL. Furthermore, we also determined that the cholinergic system of the CeAL recruits distinct endocannabinoids using disparate signaling mechanisms via: 1) acute- and persistent- muscarinic receptor (mAChR)-driven eCB release as well as 2) calcium-assisted mAChR-driven eCB mobilization. As such, CeAL excitatory terminals exhibit mechanistically distinct modes of eCB signaling mechanisms that may potentially support diverse synaptic functionalities. Lastly, in an effort to determine these putative functionalities, we assessed the effects of chronic stress on eCB signaling at glutamatergic CeAL synapses. These experimental approaches revealed that homotypic chronic restraint stress enhances 2-AG mobilization and CB1 receptor signaling at CeAL glutamatergic synapses.

Collectively, these results suggest that the eCB machinery is present at CeAL glutamatergic terminals and functionally decreases the efficacy of CeAL excitatory drive through multiple signaling mechanisms. As such, the eCB system is well-positioned to modulate the overall excitatory balance of the CeAL microcircuitry and to decrease the pathological drive for CeAL-mediated physiological and behavioral outputs elicited by stress exposure. As such, eCB

signaling at CeAL excitatory synapses is a potential locus for eCB mediated regulation of physiological and behavioral adaptations to acute and repetitive stress exposure.

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LIST OF ABBREVIATIONS

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid receptor ..... AMPAR

Acetylcholinesterase .....AChE

2-Arachidonoylglycerol 2-AG

(2-R)-amino-5-phosphopentanoate AP5

Anandamide ..... AEA

N-arachidonoylethanolamide ..... AEA

Artificial Cerebrospinal Fluid .....ACSF

D-(-)-2-Amino-5-phosphonopentanoic acid AP-5

Basolateral amygdala .....BLA

Intracellular calcium concentration..... $[Ca^{2+}]_i$

6-cyano-7-nitroquinoxaline-2,3-dione ..... CNQX

Calcium-assisted receptor driven endocannabinoid release ..... Ca^{2+}-RER

Cannabinoid type 1 receptor CB1

Cannabinoid type 1 receptor mediated long term depression CB1-LTD

Central nucleus of the amygdala, capsular ..... CeC

Central nucleus of the amygdala, lateral.....CeAL

Central nucleus of the amygdala, intermediate.....CeI

Central nucleus of the amygdala, medial.....CeAM

CB1-receptor interacting protein .....CRIP

Choline acetyltransferase.....ChAT

Chronic Restraint Stress ..... CRS

CP 55940 ..... CP

Central Nervous System ..... CNS

Corticotropin releasing hormone ..... CRH

cyclic adenosine monophosphate ..... cAMP

cyclic guanosine monophosphate ..... cGMP

Cytoplasmic phospholipase A2 ..... cPLA2

Delta-9-tetrahydrocannabinol ..... Δ^9-THC

Diacylglycerol ..... DAG

Diacylglycerol lipase alpha ..... DAGLα

Dorsal lateral striatum ..... dl striatum

Depolarization-induced suppression of excitation ..... DSE

Endocannabinoid ..... eCB

Fatty Acid Amide Hydrolase ..... FAAH

Field Excitatory Postsynaptic Potentials ..... fEPSP

γ-aminobutyric Acid ..... GABA

Glutamate decarboxylase ..... GAD

G-protein coupled receptor ..... GPCR

Hypothalamic-Pituitary Adrenal Axis ..... HPA Axis

Lateral Amygdala ..... LA

Late Firing ..... LF

Long term depression ..... LTD

Medium spiny neuron ..... MSN

Metabotropic glutamate receptor ..... mGluR

Monoacylglycerol lipase ..... MAGL

Medial Amygdala ..... MeA

Medially arising pathway ..... MP

Muscarinic acetylcholine receptor ..... mAChR

Muscarinic acetylcholine receptor subtype 1 ..... M_1

Muscarinic acetylcholine receptor subtype 2 ..... M_2

Muscarinic acetylcholine receptor subtype 3 ..... M_3

Muscarinic acetylcholine receptor subtype 4 ..... M_4

Muscarinic acetylcholine receptor subtype 5 ..... M_5

N-methyl-D-aspartate Receptor ..... NMDAR

Nitric Oxide ..... NO

Nucleus of the solitary tract ..... NTS

Nucleus Basalis ..... NB

Nucleus Basalis of Meynert ..... NBM

Oxotremorine-M ..... Oxo-M

Parvocellular neuroendocrine cells ..... PNC

Paired pulse facilitation ..... PPF

Paired pulse ratio ..... PPR

Periaqueductal gray ..... PAG

Phosphotidylethanolamine ..... PE

Phospholipase A ..... PLA

Phospholipase C ..... PLC

Phospholipase D.....PLD

Phospholipase C beta.....PLCβ

Paraventricular Nucleus.....PVN

Post Synaptic Density.....PSD

Posttraumatic Stress Disorder.....PTSD

Protein Kinase C delta.....PKCδ

Receptor driven eCB Release.....RER

Regular Firing.....RF

Somatostatin.....SOM

Spontaneous Excitatory Postsynaptic Currents.....sEPSCs

Short-term depression.....STD

Vesicular acetylcholine transporter.....VAChT

Voltage Gated Calcium Channels.....VGCC

Wildtype.....WT

CHAPTER I

INTRODUCTION

Introduction to Endogenous Cannabinoid System

The *Cannabis sativa* plant has been used for centuries for its therapeutic and stress-attenuating properties. Its psychoactive actions such as mood alterations, memory and motor control impairments were later found to be mediated by Δ^9-tetrahydrocannabinol (Δ^9 THC), the major psychoactive component of cannabis (Adams and Martin, 1996; Gaoni and Mechoulam, 1971). Since then, a number of biological analogs of Δ^9 THC have been synthesized and are collectively called cannabinoids, due to their cannabimimetic actions (Howlett, 1995).

The Cannabinoid Type 1 Receptor is the Primary Cannabinoid Receptor in the CNS

The discovery of Δ^9 THC paved the way for the discovery of the two major cannabinoid receptors, CB1 and CB2 (Matsuda et al., 1990; Munro et al., 1993). Though Δ^9 THC binds to both receptors, they have very different distribution patterns, with CB2 receptors shown to be mainly present in the periphery (Munro et al., 1993), although more recent studies have begun to reveal low levels of CB2 receptor expression in microglia and neuronal cells in several brain regions (Gong et al., 2006; Van Sickle et al., 2005). Of the two cannabinoid receptors, CB1 receptors are more abundant in the central nervous system (CNS) and global CB1 knockout mice ($CB1^{-/-}$) as well as pharmacological inhibition of CB1 receptors have determined that CB1, as compared to the CB2, receptors are primarily responsible for the psychoactive actions of exogenous cannabinoids as well as the physiological effects of endogenous cannabinoids within

the CNS (Elphick and Egertova, 2001; Ledent et al., 1999). Additionally, CB1 receptors are one of the most common GPCRs in the CNS (Glass et al., 1997) which potentially explains the broad effects of CB1 activation on CNS-mediated functions.

The Cannabinoid Type 1 Receptor Structure and Functional Signaling

The CB1 receptor is a ~ 473 amino acid $G\alpha_{i/o}$-coupled protein coupled receptor (GPCR) with 97-99% homology between rodents and humans (Chakrabarti et al., 1995; Gerard et al., 1990). Once activated, presynaptically localized CB1 receptors trigger multiple signaling transduction pathways via the $G\alpha_{i/o}$ family of G proteins (Pertwee, 1997), which functions to activate mitogen-activated protein kinases, as well as inhibit adenylate cyclase and calcium influx, via inhibition of voltage gated Ca^{2+} channels. This results in the attenuation of neurotransmitter release, particularly at glutamate and GABAergic synapses (Kano et al., 2009a). Furthermore, CB1-$G\alpha_{i/o}$ activation also modulates a number of ion channels and enzymes in a cAMP-dependent or –independent manner resulting in, for example, the activation of A-type and inwardly rectifying potassium channels (Mackie et al., 1995), as well as, the inhibition of N-and P/Q type calcium channels (Twitchell et al., 1997) and D- and M type potassium channels (Morishita et al., 1998; Schweitzer, 2000). As such, eCBs primarily regulate synaptic strength by attenuating neurotransmitter release (Betke et al., 2012; Freund et al., 2003).

The characteristics of the CB1 receptor ligands, however, play a significant role in determining the signaling transduction mechanism recruited following CB1 receptor activation. $G\alpha_{i/o}$-proteins interact with the C-terminus and/or 3rd intracellular loop of the CB1 receptor (Mukhopadhyay et al., 2000; Nie and Lewis, 2001). However, distinct $G\alpha_{i/o}$-protein types interact with specific regions of the CB1 receptor. For example, $G\alpha_{i1}$ and $G\alpha_{i2}$ interact with the 3rd intracellular loop of the CB1 receptor, while $G\alpha_{i3}$ and $G\alpha_{o}$ interact only with the C-terminus

(Mukhopadhyay and Howlett, 2001). In light of this, orthosteric and allosteric ligand binding (e.g. classical or non-classical cannabinoids, etc.), activate different types of $G\alpha_{i/o}$ proteins, suggesting that multiple active conformations of the CB1 receptor exists each of which can be differentially stabilized by distinct ligands to facilitate disparate forms of CB- $G\alpha_{i/o}$ signaling (e.g. CB1–mediated, calcium independent vesicle release mechanisms) (Hudson et al., 2010a; Mukhopadhyay and Howlett, 2005). Adding to this ligand-directed functional selectivity there is evidence to suggest that: 1) activation of CB1 receptors also results in signaling via $G\alpha_s$ (Sugiura et al., 2002) and $G\alpha_{q/11}$ proteins to activate adenylate cyclase and increase intracellular calcium concentrations $[Ca^{2+}]_i$ (Lauckner et al., 2005) and 2) CB1 receptors may form heterodimers with several other receptors indicating that CB1 receptors not only exhibit ligand-directed but also heterodimer-directed functional selectivity. Collectively, CB1 receptor activation has the potential to recruit a diverse array of signaling pathways that may differentially underlie its actions at synapses within the CNS (Hudson et al., 2010).

The Cannabinoid Type 1 Ligand Binding Sites

Site-directed mutagenesis experiments reveal that the binding sites of cannabinoids are embedded in the transmembrane helices of the CB1 receptor (Song and Bonner, 1996). Additionally, NMR spectroscopy experiments have shown that cannabinoids laterally diffuses within one membrane leaflet and interact with a hydrophobic grove formed by the CB1 receptor helices 3 and 6 (Makriyannis et al., 2005; Tian et al., 2005). It has been proposed that the receptor exists as a homodimer *in vivo* (Wager-Miller et al., 2002) or, as mentioned in the previous paragraph, heterodimerize with a number of other GPCRs such as the type 2 dopamine receptor (D_2) (Kearn et al., 2005) or the orexin 1 receptor (Hilairet et al., 2003). In addition to interactions with other synaptic GPCRS broadening CB1 receptor synaptic functions, more

recent studies by Niehaus and co-workers (2007) have determined that CB1 receptor interactions with synaptically localized proteins, such as the CB1 receptor interacting protein (CRIP), can limit the constitutive activity of the CB1 receptor (Niehaus et al., 2007). Thus, mechanisms for increased diversity as well as increased regulation of CB1 receptor signaling exits at central synapses (Mackie, 2005).

Distribution and Density of CB1 receptors at Central Synapses

Subcellular analyses of CB1 expressing synapses within the CNS, have revealed that these receptors are preferentially targeted to presynaptic elements. As is observed in our study, this selective localization results in the dissociation between the regional distribution of CB1 mRNA and CB1 immunoreactivity particularly under conditions where CB1 receptors are predominantly expressed in projection neurons (Kawamura et al., 2006). Of additional note, CB1 receptors are largely condensed within the perisynaptic element on the synaptic side of the axolemma as compared to the synaptic and extrasynaptic axonal compartments (Kawamura et al., 2006; Nyilas et al., 2008). Thus, CB1 receptors are ideally positioned to bind endocannabinoids (eCBs) that are produced on the perisynaptic and extrasynaptic surface of dendritic shafts, cell bodies, and spines of the postsynaptic neuron (Katona et al., 2006b; Yoshida et al., 2006). Analyses of CB1 receptor sub-cellular localization have also revealed that inhibitory synapses generally have a higher concentration of CB1 receptors relative to excitatory synapses, with the enrichment at inhibitory synapses varying dependent upon the brain region. For example, CB1 labeling on inhibitory synaptic elements are 30 times and 6 times higher for hippocampal CA1 pyramidal and cerebellar Purkinje cells, respectively, as compared to excitatory synapses on similar cell types in these regions (Kawamura et al., 2006; Uchigashima et al., 2007a).

General Features of CB1 Receptor mRNA within the Amygdala

Expression studies have demonstrated that CB1 receptors are found in high concentrations in cortico-limbic brain regions responsible for emotional processing and the coordination of the stress response. Of particular interest for this study is CB1 receptor expression and subcellular localization within the amygdala. In the amygdala, *in situ* hybridization (ISH) studies have separated CB1-mRNA expressing neurons into low expressing and high expressing groups. High CB1 mRNA-expressing cells are largely found only within cortical-like structures of the amygdala, such as the BLA. On the other hand, low CB1-expressing cells are more evenly distributed and found within both the BLA and central amygdala (Berrendero et al., 1998; Berrendero et al., 1999; Chhatwal et al., 2005; Hermann and Lutz, 2005; Mailleux et al., 1992a; Mailleux et al., 1992b; Marsicano and Lutz, 1999; Matsuda et al., 1993; Matsuda et al., 1990; McLaughlin et al., 1994; Wang et al., 2003). As such, a large number of high and low-CB1 mRNA expressing cells are found within the BLA, while low-expressing CB1 mRNA cells are found within the central amygdala (CeA) (Yoshida et al., 2011b).

Within the rodents CeA, CB1 mRNA expression has generally been described as lower, than that observed within the BLA, but yet still present (Chhatwal et al., 2005; Hermann and Lutz, 2005; Marsicano and Lutz, 1999; Matsuda et al., 1993; Yoshida et al., 2011b). Furthermore, these low levels of CeA CB1 mRNA is at levels comparable to other brain regions such as the global pallidus and the lateral hypothalamus (Hermann and Lutz, 2005). Though CB1 mRNA expression appears to be low but present within the CeA, earlier studies have not addressed whether subregional differences in CB1 mRNA expression exist within subdivisions of the CeA.

CB1 Receptor Protein Expression within the Amygdala

Using rodent models, immunohistochemical studies have also revealed high levels of CB1 receptor protein expression within the amygdala (Egertova et al., 2003; Egertova and Elphick, 2000; Eggan and Lewis, 2006; Katona et al., 2001; McDonald and Mascagni, 2001; Ong and Mackie, 1999; Patel et al., 2005b; Pettit et al., 1998; Tsou et al., 1998b). The first examinations of CB1 protein expression was carried out by Tsou and colleagues (1988) using an antibody directed against the CB1 receptor N-terminus which showed that CB1 receptor protein was found in both the BLA and CeA (Tsou et al., 1998b). A subsequent study by Katona and co-workers (2001) utilized a CB1 receptor antibody directed against the C-terminus of the CB1 receptor protein. In parallel with the CB1 mRNA studies discussed in the last paragraph, a high expression of CB1 receptor protein was found in the BLA while CB1 receptor protein expression remained below detection threshold in the CeA (Katona et al., 2001). Consistent with this below threshold CB1 immunoreactivity observed within the CeA, activation of the CB1 receptor using the CB1 receptor agonist, WIN 55,212-2, revealed a lack of CB1 receptor signaling on inhibitory synaptic transmission (Katona et al., 2001). Conversely, later experimental evidence presented by Roberto and colleagues (2008) as well as Kamprath and co-workers (2011) demonstrated the functional presence of CB1 receptors within the CeA (Kamprath et al., 2011; Roberto et al., 2010a). Of particular note, Kamprath and co-workers (2008) also demonstrated that within the CeA, CB1 receptor protein is present but largely localized to the medial (CeAM), but not the lateral subdivision of the CeA (CeAL) (Kamprath et al., 2009)—suggesting that subregional differences in CB1 receptor protein expression may exist within this region. Given the conflicting evidence of CB1 receptor protein expression within the CeA, additional assessments of CB1 receptor expression and function are needed to conclusively characterize CeAL CB1 receptors.

Introduction to Endocannabinoids

Endocannabinoids are a class of bioactive lipids, produced by neurons and glia, that act as endogenous ligands of the cannabinoid receptors (Kano et al., 2009a). The first endocannabinoid was isolated from pig brain and was named N-arachidonoylethanolamide (AEA) or "anandamide" based on the Sanskrit word *anada* which means "bliss". AEA was later found to be a partial agonist of the CB1 and CB_2 receptors and present in the brain at concentrations of picomoles per gram of tissue (Sugiura et al., 2002). Subsequently, the other major endocannabinoid, 2-arachidonoylglycerol (2-AG), was isolated from canine gut and rat brain and found to be present at relatively higher concentrations (nanomoles per gram of tissue). Unlike AEA, 2-AG was also found to act as a full agonist at both the CB1 and CB_2 receptors (Sugiura et al., 2006). Despite these differences, AEA and 2-AG share the lipophilicity of Δ^9 THC and are both structurally similar given that they are arachidonate-derived neuroactive lipid signaling molecules (Hill et al., 2010c). Aside from these structural similarity, AEA is an N-acylethanolamine (NAE) whilst 2-AG is a monoacylglycerol (MAG) and are, thus, regulated by distinct biosynthetic and metabolic pathways that will be discussed further in the subsequent section (Kano et al., 2009a).

Other putative endocannabinoids include *O*-arachidonoylethanolamine (viodhamine) (Porter et al., 2002) and 2-arachidonoyl glycerol ether (noladin ether) (Hanus et al., 1993). The latter, noladin ether, was originally prepared as a metabolically stable analog of 2-AG but was later isolated from porcine brain by Mechoulam and co-workers (2001) (Hanus et al., 2001b) and found to bind to CB1 receptors with higher affinity than that observed at CB_2 receptors. Later studies, however, have reported that noladin ether could not be detected in mammalian brain (Oka et al., 2003). Given these conflicting data as well as the paucity of information regarding

other putative endocannabinoids, it is not clear whether noladin ether or other putative endocannabinoids function as agonists at cannabinoid receptors within the CNS.

Biosynthesis and Metabolism of Endocannabinoids

In response to a variety of cellular stimuli, endocannabinoids and related bioactive lipid signaling molecules are postsynaptically generated from membrane phospholipids by specific hydrolase or a combination of acyltransferases and hydrolases. Unlike classical neurotransmitters, eCBs are not stored in vesicles, but are produced and released on demand at central synapses. Upon release, eCBs retrogradely activate presynaptic CB1 receptors which, in turn, inhibit neurotransmitter release through $G\alpha_{i/o}$ signaling pathways discussed earlier in the Introduction (Wilson and Nicoll, 2002). Significant experimental evidence suggests that the eCBs, AEA and 2-AG, are recruited by distinct neuronal activity patterns and, once mobilized, appear to mediate the unique actions of the eCB system. Following receptor binding, these eCBs are degraded by hydrolases. In general, the synthesizing enzymes for AEA and 2-AG are tightly regulated and expressed at much lower levels than the degrading enzymes, thus enabling their on demand synthesis and fast metabolism (Valenti et al., 2004). Despite this similarity, however, AEA and 2-AG have very distinct biosynthetic and metabolic pathways, with the latter taking place in disparate subcellular localizations with respect to the synapse (Nazzaro et al., 2012). Given these non-overlapping characteristics, it is therefore not surprising that AEA and 2-AG appear to play distinct roles in eCB-mediated functions within the CNS. Collectively, AEA and 2-AG are not merely redundant signaling molecules but perform different roles in eCB functionality in the CNS (Long et al., 2009d).

Biosynthesis of 2-AG

2-arachidonoylglycerol (2-AG) is the primary eCB that mediates retrograde synaptic signaling at central synapses (Castillo et al., 2012). Numerous biochemical studies have revealed that 2-AG is post-synaptically synthesized by a number of pathways. The primary pathway for 2-AG synthesis is PLC-mediated hydrolysis of arachidonic acid containing phospholipids which, in turn, produces an arachidonyl-containing diacylglycerol (DAG). Thereafter, DAG is converted to 2-AG by the actions of diacylglycerol lipase, a membrane associated enzyme that preferentially hydrolyzes DAG at the sn-1 position (Okazaki et al., 1981). During development, DAGL is localized to axon terminals (Brittis et al., 1996), whereas in adulthood, DAGL appears to be predominately localized in the axon terminals (Kano et al., 2009a). Early cloning experiments revealed that two closely related genes encode two forms of DAGL, DAGLα and DAGLβ (Bisogno et al., 2003). Experimental over-expression of DAGLα (Bisogno et al., 2003) and pharmacological blockade or knockdown of endogenous DAGL α/β (Jung et al., 2005) suggest that DAGLα and/or DAGLβ are the primary 2-AG synthetic enzymes. To determine the contributions of the two DAGL isoforms in 2-AG synthesis and retrograde signaling in the brain, Tanimura and colleagues (2010) generated rodent models of global DAGLα and DAGLβ loss. These experimental analyses revealed that DAGLα deficiency decreased total 2-AG levels and eCB-mediated retrograde signaling at central synapses while DAGLβ loss had no effect on these measures. Conversely, similar experimental analyses by Gao and co-worker (2010) showed that DAGLα reduces brain 2-AG levels by 80% while global DAGLβ loss reduced brain 2-AG levels by 50%. Despite this 50% loss of total 2-AG levels in DAGLβ knockout mice, retrograde eCB signaling was only impaired in DAGLα knockout mice. Collectively, these results suggest strongly suggest that DAGLα is the primary enzyme responsible for 2-AG production and retrograde suppression at central synapses.

Expression of DAGLα in the Amygdala

Two studies have examined the expression of DAGLα in the amygdala. Patel and co-workers (2009) demonstrated heterogeneity in DAGLα expression within the amygdala. High levels were observed in the BLA and dLA, whereas much lower levels were observed in the vLA and BMA. Within the CeA, the CeAL had a higher expression of DAGLα as compared to the medial subdivision of the CeA (CeAM). A more recent study by Yoshida *et al.* (2011) demonstrated a similar pattern of DAGLα (Yoshida et al., 2011b). These studies showed that DAGLα protein is more heavily expressed in the BLA as compared to the CeA. Further examination of the BLA determined that DAGLα protein in close apposition to CB1 expressing terminals and is clustered at invaginating inhibitory synapses on pyramidal cells within this region. Unlike the BLA, the CeA do not exhibit these unique nerve terminals despite having robust DAGLα protein expression.

2-AG Synthesis in the Central Synapses

As mentioned in the previous paragraph, 2-AG can be produced by a number of DAGLα mediated biosynthetic pathways (see Figure 1). The first is a calcium-dependent mechanism, prototypically elicited by postsynaptic depolarization. This postsynaptic depolarization activates L-type calcium channels and enhances the conversion of diacylglycerol to 2-AG via the activation of DAGLα (Ohno-Shosaku et al., 2005). This form of eCB-mediated retrograde suppression is otherwise known as Ca^{2+}-dependent endocannabinoid release (Ca^{2+}ER) or depolarization induced suppression of excitation or inhibition (DSE or DSI). The second and third forms of DAGLα- mediated 2-AG synthesis are mediated by Gαq-protein-coupled receptor ($G\alpha_qPCR$) signaling. The second is a Gαq-protein-coupled receptor ($G\alpha_qPCR$) driven pathway mediated via the activation of PLCβ under basal intracellular calcium levels ($[Ca^{2+}]_i$). Under

basal $[Ca^{2+}]_i$, PLCβ activation increases DAG and subsequently drives 2-AG synthesis via a DAGLα-dependent process (Hashimotodani et al., 2007). This second form of DAGLα-mediated 2-AG synthesis is otherwise known as basal receptor driven endocannabinoid release (basal RER). Finally, the third form of 2-AG mediated synthesis occurs as a result of increased PLCβ activity following increased $[Ca^{2+}]_i$ (Hashimotodani et al., 2005). This leads to a combinatory mechanism whereby depolarization-induced calcium influx facilitates Gαq-receptor driven 2-AG release by enhancing PLCβ activity (Hashimotodani et al., 2005; Ohno-Shosaku et al., 2012). This third form of endocannabinoid mobilization is also referred to as Ca^{2+}-assisted receptor driven endocannabinoid release (Ca^{2+}-assisted RER) (Kano et al., 2009b).

Termination of 2-AG Signaling At Central Synapses

Significant experimental evidence shows that monoacylglycerol lipase (MAGL) is the primary enzyme that catalyzes the 2-AG hydrolysis *in vivo* (see Figure 1). MGL is a member of the serine hydrolase family that hydrolyzes 2-AG into arachidonic acid and glycerol (Dinh et al., 2002). Studies by the Cravatt group (2010) showed that MAGL accounts for 85% of 2-AG hydrolysis *in vivo* while additional work from this group and others have found that the remaining 15% of 2-AG hydrolysis is catalyzed by the serine hydrolases, ABHD6 and ABHD12 (Blankman et al., 2007; Schlosburg et al., 2010).

Earlier immunohistochemical studies, using an N-terminal antibody against the rodent MGL, revealed punctuate MAGL protein expression within the BLA, but much weaker MAGL expression within CeA (Gulyas et al., 2004b)—results that were later confirmed by work presented by the Watanabe group (2010). Furthermore, subcellular examinations of MAGL within the amygdala revealed that MAGL is localized presynaptically at a subset of axon terminals that form asymmetrical and symmetrical synapses within the BLA (Gulyas et al.,

2004b). These experimental results are consistent with MAGL subcellular localization in other brain regions (Kano et al., 2009a). Despite subcellular analyses demonstrating MAGL expression at BLA synapses, similar analyses are lacking within the CeA.

Biosynthesis of Anandamide: NAPE-PLD Dependent Pathway

In contrast to 2-AG, the mechanisms regulating synaptic AEA synthesis are not well understood and has been shown to involve the activation of $G\alpha_q$PCRs (Chavez et al., 2010a; Grueter et al., 2010a; Huang and Woolley, 2012)) (see Figure 1). Currently, the canonical pathway for AEA biosynthesis is thought to be composed of two enzymatic reactions, collectively referred to as the 'transacylation-phosphodiesterase pathway'. The first step of this pathway involves the Ca^{2+} dependent- N-acyltransferase catalysis of N-arachidonoylphosphatidylethanolamine (NAPE) production, via the transfer of the *sn-1* acyl group of glycerophospholipids to phosphotidylethanolamine (PE) (Di et al., 2005). Subsequent to its production, NAPE is hydrolyzed to AEA as well as phosphatidic acid in a reaction that is catalyzed by the enzyme, N-acylphophatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD). As such, NAT and NAPE-PLD are considered to critical for AEA biosynthesis, with the latter demonstrating age-dependent increases in activity and expression (Morishita et al., 2005), whilst the former exhibits the opposite trend (Moesgaard et al., 2000). Subsequent *in vivo* examinations of the role of NAPE-PLD in AEA synthesis have, however, produced inconsistent results. Work by Leung and co-workers (2006) showed that NAPE-PLD knockout mice are viable and do not exhibit any change in total brain AEA levels (Leung et al., 2006), suggesting that NAPE-independent AEA synthetic pathways exist *in vivo*. Arguing against this, however, is more recent work by the Ueda group which showed a greater than 60% decrease in brain AEA levels in their global NAPE-PLD knockout mice (Tsuboi et al., 2011).

Expression of NAPE-PLD in the CNS

Analyses of NAPE-PLD presence within the brain reveal that NAPE-PLD mRNA and protein exhibit complementary patterns of expression (Egertova et al., 2008). NAPE-PLD is abundantly expressed in neuronal cell-types within hippocampus, with weaker expression observed in other brain regions such as amygdala. There, NAPE-PLD immunoreactivity is higher in the BLA as compared to the CeA (Egertova et al., 2008). Interestingly, this study also revealed that within the hippocampus, NAPE-PLD immunoreactivity is present in the granule cell bodies and axons. These results suggest that NAPE-PLD catalysis of AEA synthesis occurs presynaptically which is inconsistent the canonical postsynaptic locus of eCB synthesis. Consistent with these findings, analyses of NAPE-PLD localization by Nyilas and colleagues (2008) demonstrate that NAPE-PLD is predominantly localized presynaptically on the smooth endoplasmic reticulum of the hippocampus. In contrast to these reports, work by Cristino and colleagues (2008) argue that NAPE-PLD is postsynaptic expressed within the hippocampus (Cristino et al., 2008) suggesting that NAPE-PLD may be localized pre-and postsynaptically in the CNS. In support of these findings, recent high resolution immunogold EM analyses demonstrate that NAPE-PLD is localized both pre- and post-synaptically in the ventromedial nucleus of the hypothalamus (VMH), with a preferential dendritic localization (Reguero et al., 2014). Collectively, these studies suggest that: 1) NAPE-PLD may facilitate AEA biosynthesis in both synaptic compartments and 2) NAPE-PLD mediated AEA-CB1 receptor signaling may occur in a retrograde and/or anterograde manner within the hippocampus and VMH. However, high resolution analyses of NAPE-PLD subcellular localization in other brain regions that also exhibit NAPE-PLD protein expression, such as the amygdala, will have to be undertaken to determine whether NAPE-PLD expression on both sides of the synapse is a generalizable theme in the CNS.

AEA Biosynthesis: NAPE-PLD Independent Pathways

Though additional studies (e.g. pharmacological, etc.) will have to be undertaken to characterize the role of NAPE-PLD in global and regional AEA synthesis within the brain, NAPE-PLD knockout studies suggest that NAPE-dependent and –independent pathways for AEA exists *in vivo*. Indeed, more recent studies have suggested that NAPE-PLD independent AEA synthetic pathways exist *in vivo* (Ueda et al., 2005). These newly discovered multi-step pathways include: 1) via N-acylated lysophospholipid, 2) members of the PLA/acyltransferase family, and 3) the collective action of PLC and protein phosphatase (Kano et al., 2009a; Ueda et al., 2013).

Termination of AEA Synaptic Signaling at Central Synapses

FAAH is a serine hydrolase that catalyzes the degradation of anandamide into arachidonic acid and ethanolamine (Cravatt et al., 1996) (see Figure 1). It is an important regulator of brain anandamide content (Cravatt et al., 2001a; Kathuria et al., 2003; Patel et al., 2005a) and is an emerging target for drug discovery for a variety of disease states such as stress-related psychiatric disorders (Cravatt and Lichtman, 2003; Kathuria et al., 2003; Lichtman et al., 2004; Patel et al., 2004). Initial ISH studies by Cravatt and co-workers (1997) revealed an intense hybridization signal within the amygdala specifically within the BLA complex, with a weaker signal observed in the CeA (Thomas et al., 1997). These studies were closely followed by immunohistochemical localization of FAAH within the central nervous system using a C-terminal antibody (Tsou et al., 1998c). Within the BLA, FAAH immunoreactivity was described to be moderate to strong. Within the CeA, however, only a few cells were found to express cytoplasmic FAAH immunoreactivity—which is consistent with the expression of other components of the eCB system within this region. Similarly, Elphick and co-workers, using an antibody raised against amino acids 38-579 (Patricelli et al., 1998), describe FAAH

immunoreactivity within the somata of neurons throughout the BLA (Egertova et al., 2003; Egertova et al., 1998). Freund and co-workers, using this same antibody (Patricelli et al., 1998), and another generated against a native 6X-His tagged truncation of FAAH (Bracey et al., 2002), also published detailed light and EM descriptions of FAAH within the rat and mouse amygdala (Gulyas et al., 2004b). At the light microscopic level, strong cellular (cytoplasmic and proximal dendritic) and a granular/reticular neuropil staining was observed within the BLA. In the CeA, only faint and occasional neurons were FAAH immunoreactive in this study (Gulyas et al., 2004b).

Conclusion

Given the effects of the *Cannabis sativa* plant on emotionality and stress responsivity, it is not surprising that CB1 receptors and other biosynthetic and metabolic components of the eCB system are expressed in the amygdala—a critical component of the cortico-limbic circuit that regulates emotionality (Hill et al., 2010c). Though these studies suggest that the BLA may act as an anatomical substrate for eCB-mediated regulation of emotionality, a number of questions remain unanswered. Two such questions include: 1) what is the subcellular localization of the eCB synthetic and metabolic machineries in other key subdivisions of the amygdala, such as the CeA, and 2) what is the functional consequence of the localization of eCB biosynthetic machinery at central synapses within subdivisions of the amygdala? In the remaining sections of the Introduction we will examine the role of the amygdala, with a focus on the CeA, in mediating the behavioral consequences of exogenous and endogenous cannabinoids.

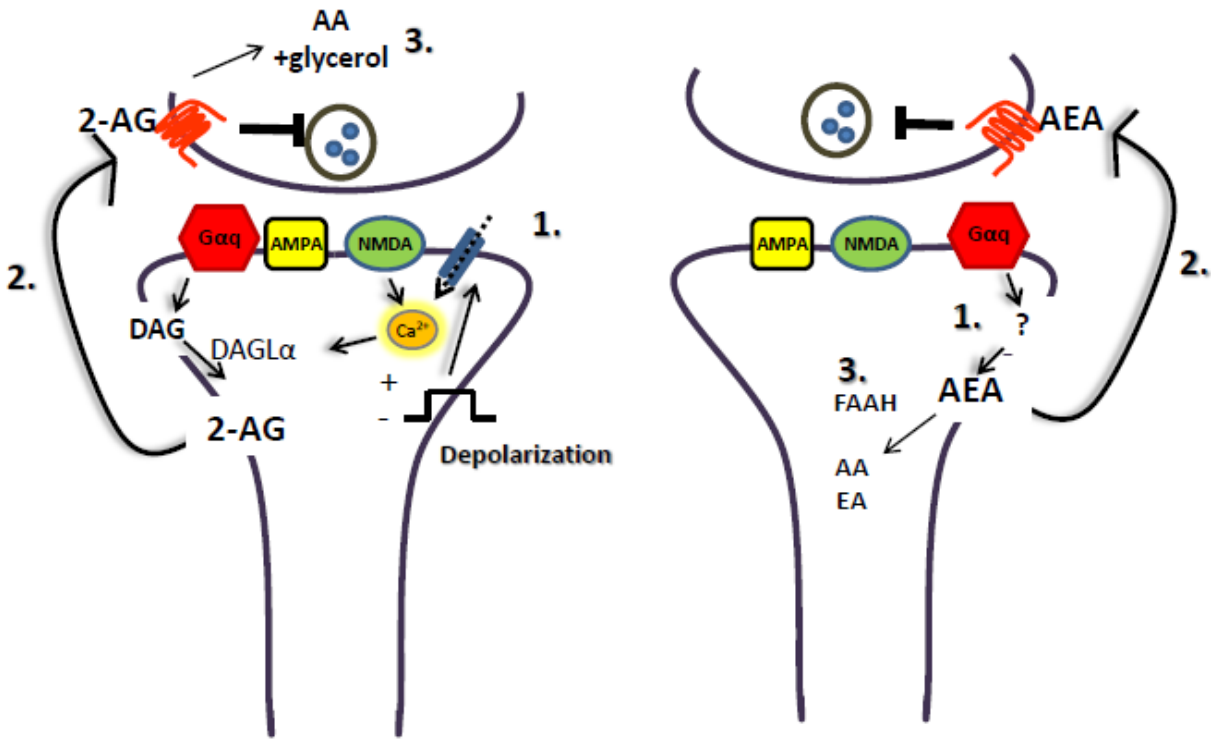

Figure 1. Endocannabinoid Biosynthetic and Metabolic Pathways at Central Synapses. 2-AG and AEA have distinct biosynthetic and metabolic pathways.(1) Though both postsynaptically produced, 2-AG synthesis can be triggered by the activation of $G\alpha_q$-PCR and/or depolarization-induced activation of voltage-dependent calcium channels. Relatively less is known about the biosynthetic pathway for AEA but recent studies reveal $G\alpha_q$-mediated AEA synthetic pathways at central synapses.(2) Once produced, both eCBs activate presynaptically localized CB1 receptors. (3) Thereafter, 2-AG and AEA are metabolized via hydrolysis which is catalyzed by two distinct enzymes that display disparate subcellular localizations. AA, arachidonic acid; EA, ethanolamine.

The Stress Response Represents an Adaptive Mechanism

Building upon Claude Bernard's concept of the "internal milieu", French physiologist Walter Canon, described an organism's *homeostasis* (derived from the Greek words for "the same"-*homeo* and "standing"-*stasis*) as the net result of mechanisms that maintain stable conditions *necessary* for survival (Cannon, 1929). By describing homeostasis as a necessary means for survival, Cannon imbues this state with a sense of priority that all organisms must achieve (Ulrich-Lai and Herman, 2009). Of equal importance, Cannon also noted that homeostasis is a *dynamic* process where "changes [are] …automatically met by increased effectiveness of… factors that resist this change" (Cannon, 1932).

In line with Cannon's earlier work, Hungarian physiologist Hans Selye and colleagues performed seminal characterizations of the "factors that resist [homeostatic]…change" following challenges to or disruptions of homeostasis" (Selye, 1951). The resulting body of work led to the discovery that *physical* or *psychological* perturbations to homeostasis elicited by internal or external threats—otherwise known as *stressors*—evoke biological mechanisms to counter these disturbances and regain homeostasis. These challenges to homeostasis evoked what Selye described as a "General Adaptation Syndrome" (GAS) or *stress response*. Selye's early work and subsequent experimental evidence in this field (Hill and McEwen, 2010; Ulrich-Lai and Herman, 2009) has since characterized the stress response as an *adaptive* process, i.e. a collection of biological and/or behavioral change(s) mounted to counteract stressor-activated homeostatic perturbations with the goal of maintaining physiologic integrity in the most demanding of circumstances (Herman, 2013; McEwen and Wingfield, 2003; Selye, 1951; Ulrich-Lai and Herman, 2009).

Response to Aversive Stimuli Is Composed of Three Stages

As further described by Selye, adaptive processes are supported by three stages of the GAS (See Figure 2). The first, is the *Alarm* stage and is triggered by stressor-related cues that are conveyed to the brain by all primary sensory systems which, in turn, mobilizes the *stress circuitry*— an evolutionary conserved synergism of neuronal and neuroendocrine systems that support physiological and behavioral changes to appropriately cope with the real or perceived threat (Ulrich-Lai and Herman, 2009). At the apex of this rapidly activated stress circuitry is arousal of both the adrenomedullary arm of the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis (Herman, 2013).

Following this initial stress exposure, the body shifts into the second phase of the General Adaptation Syndrome known as the *Resistance* stage (Selye, 1951). This second stage consists of either of two possibilities: the stressor is quickly resolved and ongoing feedback processes are recruited to restore homeostatic balance; consequently the stress response is a short-term adaptation to the original threat. The alternate possibility manifests when the stressor persists, whether continuous or continual, resulting in the protracted recruitment of mechanisms to resist threat impact and maintain a state of arousal. One such mechanism is a learning and memory process engaged to refine the stress response to subsequent homeostatic challenges. This form of adaptation manifests as either *habituation*, decreased responsivity to highly predictable low-intensity stimulus presentation, or *sensitization*, non-specific generalization of a highly aversive stimuli— both of which are largely controlled by cortico-limbic brain regions (Castellucci et al., 1970; Kamprath and Wotjak, 2004) and dependent upon the severity and predictability of the stressor. Overall, such mechanisms maintain a persistent state of vigilance in response to continuous stress exposures (Herman, 2013).

However, the process of adaptation is costly. Chronic stress responses represent attempts at adaptation, but can constitute physiologic challenges themselves (Herman, 2013; Sterling and Edelmann, 1988). If the exposure to the stressor continues for a substantial time or increases in severity /unpredictability, the organism enters the third phase of the GAS, i.e. the *exhaustion* phase. During this period, adaptive processes earlier garnered to resist homeostatic changes begin to approach exhaustion (e.g. depletion of energy stores, etc.). Furthermore, continued efforts of ANS and HPA axis activation to cope with persistent stressor exposure become deleterious to the organism. For example, increased HPA axis activation can impair numerous bodily functions, enhance sympathetic drive leading to cardiovascular disease, and precipitate pathological changes within the neuronal circuitry that regulate emotional processing (Price and Drevets, 2010). Thus, the initially adaptive characteristics of the stress response become *maladaptive*, as biological and behavioral responses to stressors become counterproductive to the interests of the organism (Herman, 2013). Given these deleterious characteristics of chronic stress, it is not surprising that preclinical and human studies have shown that protracted exposure to stress is one of the underlying factors for the generation of stress-related psychiatric disorders, such as anxiety and post traumatic stress disorder (PTSD) (Hill et al., 2009d; McEwen, 2007; Rosenkranz et al., 2010; Taber and Hurley, 2009; Vyas et al., 2002).

As will be discussed in this thesis, the endocannabinoid (eCB) system is strongly implicated in the three stages of the General Adaptation Syndrome and plays a critical role in regulating adaptive processes that attenuate the deleterious consequences of chronic stress exposure. As will be further discussed, significant evidence suggests that the eCB system's stress attenuating properties results from its actions within the cortico-limbic stress circuitry that control the neuroendocrine, physiological, and behavioral components of the stress response.

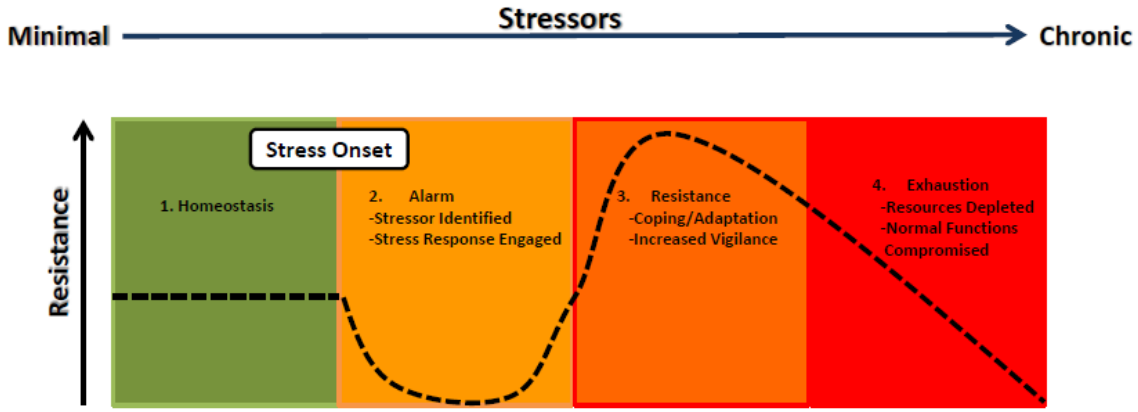

Figure 2. The General Adaptation Syndrome.
The General Adaptation Syndrome, originally described by Dr. Hans Selye (1951), consists of four consecutive stages: (1) *Homeostasis*, (2) *Alarm*, (3) *Resistance*, and (4) *Exhaustion* and describes the biological patterns triggered by stress exposure over time. Adapted from Han Seyle's *General Adaptation Syndrome and Diseases of Adaptation*.

The Alarm Stage is Facilitated by the Initial Activation of the ANS and the HPA axis

The central nervous system (CNS) is pivotal to the stress response as it perceives aversive stimuli and orchestrates appropriate physiological and behavioral responses to threat (Sanacora et al., 2012). Within the CNS, the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenocortical (HPA) axis are the primary mechanisms for reinstating homeostasis in response to homeostatic challenges. The ANS provides the most immediate response to stressors, through its sympathetic and parasympathetic divisions, which incorporate opposing yet complementary functions to mediate rapid changes in physiological and behavioral states via neural innervations of end organs. First, the sympatho-adrenomedullary axis of the autonomic nervous system quickly, i.e. within seconds, change visceral functions (e.g. increased breathing, heart rate, etc.) via adrenergic mediated changes in respiratory and cardiovascular systems, thereby preparing the organisms to cope with the physiological challenge. Subsequently, the activation of the parasympathetic division of the ANS attenuates the activity of the sympatho-adrenomedullary axis, resulting in a short-lived stress response (Ulrich-Lai and Herman, 2009).

Activation of the (HPA) axis represents the principal neural regulation of the endocrine system and supplements the ANS's rapid initiation and termination of the stress response. When faced with a stressor, activation of the HPA axis begins by the stimulation of the parvocellular neuroendocrine cells (PNCs) of the paraventricular nucleus (PVN) of the hypothalamus. These cells are the apex of the HPA axis and drive neuroendocrine responses to stress. This stimulation allows for the secretion of corticotropin releasing hormone (CRH) from parvocellular terminals of the PVN into the portal circulation of the median eminence. PNCs also send axonal projections to autonomic targets in the brainstem and spinal cord (Swanson and Kuypers, 1980; Ulrich-Lai and Herman, 2009) suggestive of additional co-ordination of neuroendocrine and autonomic output at the level of the hypothalamus. Once released, CRH bind to CRH receptors

expressed on corticotroph cells within the anterior pituitary to stimulate the synthesis and secretion of adrenocorticotropic hormone (ACTH). ACTH, in turn, initiates the synthesis and release of glucocorticoids (corticosterone in rodents and cortisol in humans) from the adrenal cortex directly into the peripheral circulation. This facilitates the coordination of brain and bodily functions, via the actions of the glucocorticoid receptors, geared towards coping with stress, recovery, and adaptation (Laryea et al., 2012) (Groeneweg et al., 2011; Roozendaal and McGaugh, 1996a, b; Roozendaal et al., 1996).

Basal HPA Axis Activity

Though the HPA axis is activated by exposure to imminent threat, this neuroendocrine system is also active under basal conditions and this, in tune, supports the homeostatic state of the organisms (Herman, 2013). Under basal conditions, the synthesis and release of glucocorticoids are also dynamically regulated by circadian cues (Biddie et al., 2012). The peak glucocorticoid secretion correlates with the anticipation of an organism's active phase and is supported by an hourly ultradian rhythm of hormone secretion. This pulsivity of circulating glucocorticoid hormone secretion provides the basis of "continuous dynamic equilibrium" which is believed to be critical for optimizing the functional tone of numerous systems (Joels and Vreugdenhil, 1998) and maintaining an organism's stress responsivity, i.e., the flexibility to respond to and recover from stress (Lightman and Conway-Campbell, 2010). As will be discussed later, changes in basal HPA activity act as an adaptive response to repetitive stress exposure (Hill et al., 2009b).

Endocannabinoid Signaling Acts as Stress Buffer During the Alarm and Resistance Stages of Psychosocial Stress

As mentioned above, significant evidence points to the endocannabinoid system (eCB) as a dynamic facilitator of adaptive processes in response to chronic stress exposure. Such evidence

include the critical role of the eCB system in decreasing protracted neuroendocrine, physiological, and behavioral responses elicited by persistent exposure to aversive stimuli (Haller et al., 2004b; Martin et al., 2002; Steiner et al., 2008c; Uriguen et al., 2004), as well as preclinical studies which demonstrate a negative correlation between acute cannabinoid consumption (via the *Cannabis sativa* plant, etc.) and stress responsiveness/ basal anxiety states in humans and rodents (Haller et al 2002; Green et al 2003). Collectively, these data demonstrate that the eCB system acts as an intrinsic stress buffering mechanism by both limiting the deleterious homeostatic changes elicited by the chronic recruitment of the stress response and facilitating positive neuroendocrine and behavioral adaptations (such as, increased vigilance and active stress coping behaviors) (Campolongo et al., 2009; Haller et al., 2004a; Herman, 2013; Marsicano et al., 2002). Though the stress regulating properties of the endocannabinoid system is well established, an outstanding question in this field is what are the neuroanatomical substrates of eCB stress attenuating actions? Given the ubiquitous distribution of the CB1 receptor in the cortico-limbic circuitry (Matsuda et al., 1990; Turner and Herkenham, 1991) as well as the regulatory effects of exogenous and endogenous cannabinoids on stress response mechanisms elicited by limbic pathways (Atkinson et al., 2010; Cota et al., 2007; Patel et al., 2004; Steiner et al., 2008a; Steiner et al., 2008b; Steiner et al., 2008c), most experimental interrogations have focused on the limbic system in an attempt to address this outstanding question.

Stressor Type Influences the Neuroanatomical Regions Recruited by the Stress Response

The stressor type significantly influences the repertoire of neural populations that perceive the stressor and engage in adaptive responses to stress (Joels and Baram, 2009). Physical and psychological threats to homeostasis recruit two distinct domains of stress activation. The first, i.e. physical stressors (e.g. blood loss, respiratory distress, or pain), represents a homeostatic

challenge that is recognized by somatic, visceral, or circumventricular sensory pathways and requires an immediate 'systemic' reaction that is triggered by reflexive mechanisms (Herman et al., 2003).

The second, i.e. psychological stressors (e.g. immobilization or forced swim stress), recruits brain regions involved in higher order processing/ decision-making and represents responses mounted in anticipation of, rather than in reaction, to a homeostatic threat. This type of stress is generated by previous experiences (i.e. associative learning) or species-specific predispositions (e.g. the aversion to snakes in humans or the innate fear of fox feces odor in mice) and allows the organism to detect novel stimuli that predict sources of harm (Ohman and Mineka, 2001). When activated by threat, psychological stressors engage stress mediators in the cortico-limbic circuitry that subserve decision making (e.g. prefrontal cortex), learning and memory, and emotionality (e.g. amygdala).

Though both type of stressors largely recruit different neuronal populations and brain regions, they are not entirely segregated systems as physical stressors may have a psychological facet and vice versa (Joels and Baram, 2009). Furthermore, the environment associated with a physical stressor can itself be conditioned, resulting in an anticipatory response when the conditioned physical stressor is next encountered (Herman et al., 2003). One limbic region that acts as an anatomical and functional convergence point for both systemic and psychological domains of stress activation, as well as a potential substrate for eCB system's role in the stress response, is the amygdala.

The Amygdala is a Key Player in the Adaptive Processes in Response to Physical and Psychological Stressors

The amygdala supports the neural mechanisms underlying the emotional interpretation of and appropriate response to salient environmental stimuli (Davis, 1992; LeDoux, 1995; Sah et al.,

2003; Weiskrantz, 1956). It accomplishes this through its connectivity with visceral, cortical, and subcortical circuits to orchestrate neuroendocrine, such as activation of the HPA axis, as well as autonomic and behavioral responses to aversive stimuli (Hill et al., 2010c). Though there appears to be inter-species differences in the size and organization of the amygdala, there are strong physiological similarities between human and rodent amygdala-mediated functionalities (Darwin, 1872; Davis, 1992; LaBar et al., 1998), particularly as it relates to defense actions and reactions to threat. In support of this, bilateral amygdala lesion studies in humans (Bechara et al., 1995; Feinstein et al., 2011), primates (Mair et al., 1979) and rodents (LeDoux et al., 1990a; LeDoux et al., 1990b; Roozendaal et al., 1990) demonstrate attenuated autonomic and behavioral stress responses to stimuli previously conditioned to be associated with a threatening stimuli.

The Amygdala is Composed of Functionally Integrated Subnuclei

The amygdala is a functionally integrated complex, subcortical to the medial temporal lobe, and consists of 13 anatomically and physiologically heterogeneous nuclei. These nuclei evolved during different phylogenetical stages and possess distinct cytoarchitectural and neurochemical features, as well as specific patterns of connectivity (Berretta et al., 2005). Based on these characteristics, these nuclei can be broadly grouped as: frontotemporal (lateral, basal, and accessory basal nuclei), autonomic (central and medial nuclei), olfactory groups (main olfactory and accessory olfactory nuclei), and a separate set of nuclei that do not separate well with the other nuclei (intercalated cell masses and the amygdalo-hippocampal area) (Sah et al., 2003; Swanson and Petrovich, 1998). Of these nuclei, the lateral, basolateral and the central amygdalas' integral role in the stress response have been investigated extensively and appear to represent an integrated unit in the processing of responses to emotionally relevant stimuli such as

environmental threats (Herman et al., 2003; LeDoux, 2003; Tye et al., 2011; Walker and Davis, 2002a, b).

Focal lesion, anatomical tracing, ontogenetic, and electrophysiological studies have revealed a unique functional and anatomical interconnectivity within the amygdala. Such studies have demonstrated that the lateral amygdala nuclei (LA) act as an early site of convergence for sensory-related information (LeDoux et al., 1990a; LeDoux et al., 1990b; Quirk et al., 1997) from a number of brain regions such as the thalamus and cortex. Following exposure to a stressor, thalamic inputs rapidly deliver sensory information whereas cortical inputs convey delayed information from visual, auditory, or somatosensory cortices (LeDoux, 2000). As such, there are "multiple roads" of sensory processing recruited to initiate stress responses (LeDoux, 1994, 1995). The LA, in turn, projects to the central amygdala (CeA) either directly or indirectly via the basolateral amygdala (BLA), another amygdala nuclei that receives strong inputs from sensory-related brain region and has garnered considerable attention as a stress-regulatory structure that, though the activation of GRs and the eCB system, facilitates stress-related cued learning and emotional memory (Bhatnagar et al., 2004; Roozendaal et al., 1997a; Roozendaal and McGaugh, 1996a). The CeA is considered the output nucleus of the amygdala and, as will be described later, lacks the intra-amygdala reciprocal connectivity characteristic of other amygdala nuclei, exhibits patterns that strongly implicate the CeA as a site of information integration, and mobilizes neuroendocrine, autonomic, and behavioral responses that support an organism's adaptive responses to stress exposure.

Amygdala Exhibits Subregional Stress Response Specialization

Stress-induced amygdala activation is essential for responses to novel stressors and adaptation to persistent stressors (Kim and Jung, 2006)). However, there is a marked subregional

specialization of stress integrative functions within the amygdala. The medial (MeA) and BLA appears to be preferentially activated, as measured by early gene expression changes as an index of neuronal activation, by psychological stressors (Cullinan et al., 1995; Dayas et al., 2001a; Dayas et al., 2001b; Patel et al., 2005c; Patel et al., 2005d). Conversely, the CeA exhibits a more complex activation profile, as similar early gene activation studies suggest that the CeA is not only stimulated by psychological stressors but by systemic stressors as well. Furthermore, it appears that the CeA is preferentially activated by systemic stressors as compared to psychogenic stressors (Cullinan et al., 1995; Ericsson et al., 1994; Xu et al., 1999); suggestive of the CeA's role in the regulation of responses to a broad array of stress responses (Iwata et al., 1987; LeDoux et al., 1988a; Roozendaal et al., 1990, 1991a, b) .

Endocannabinoid Signaling in the Amygdala Modulates Endocrine Responses to Repetitive Homotypic Stressors

As mentioned earlier in the Introduction, the endocannabinoid system exhibits dynamic responses to the changing climate of stress, thus it is well-suited to mediate adaptive mechanisms in response to persistent recruitment of the stress response. *In vitro* chronic corticosterone treatment or repetitive exposure to low aversive homotypic stress paradigms, such as restraint stress, results in the habituation of HPA axis activation (Hill et al., 2008a; Rademacher et al., 2008; Wamsteeker et al., 2010) such that persistent stress exposure induces basal HPA hypersecretion and restraint-stress induced hyposecretion. Respectively, these changes increase vigilance (Kamprath et al., 2006b; Kamprath et al., 2009; Kamprath and Wotjak, 2004), yet decreases energy resources allotted to stressors that prior experience demonstrates as low-aversive. Consistent with advantageous stress adaptations elicited following homotypic stress exposure, habituations in neuroendocrine responses are also accompanied by increases in active escape behaviors (Patel et al., 2005d). Subsequent experimental evidence revealed that these

advantageous adaptations in the HPA axis and stress-elicited behaviors are mediated by the eCB system (Patel et al., 2005c; Rossi et al., 2008b; Steiner et al., 2008a; Steiner et al., 2008b; Steiner et al., 2008c) (Haring et al., 2013). Furthermore, evidence presented by Hill and co-workers (2010b) demonstrate that dynamic changes in both eCB content and eCB signaling *within the BLA* underlies repetitive homotypic stress-induced adaptations of the HPA axis activation (Hill et al., 2010b). Collectively, this evidence strongly suggesting that eCB signaling within the amygdala is a key node in mediating the regulatory actions of the eCB system on the stress response.

2-AG and AEA Signaling Plays Distinct Roles in Adaptations to Chronic Stress Exposure

Chronic homotypic restraint stress exposure (CRS) progressively increases BLA 2-AG content and 2-AG mediated short- and long-term eCB synaptic plasticity at inhibitory BLA synapses. These changes are hypothesized to underlie the CRS-induced HPA axis hyposecretion (Hill et al., 2010c; Ostrander et al., 2006; Patel et al., 2009a) as activity dependent increases in 2-AG-mediated signaling may dampen the BLA-HPA circuitry. In support of this hypothesis, Hill and others (2009) demonstrate that intra-BLA infusions of the CB1 agonist, HU-210, attenuate repetitive stress-induced glucocorticoid release (Hill et al., 2009c). Furthermore, systemic increases in 2-AG levels are associated with decreased indices of stress-induced anxiety suggesting that CRS-induced cellular, synaptic and endocrine adaptations may be the functional consequence of increased BLA-2AG signaling (Patel et al., 2005c; Patel et al., 2005d; Steiner et al., 2008b; Steiner et al., 2008d). Consistent with increased CB1 signaling conferring advantageous adaptations in the stress response, experimental evidence from Campolongo and colleagues (2009) show that BLA infusions of the CB1 receptor agonist, WIN 55, 212-2, promotes enhanced memory consolidation which, as the authors argue, may support learning and

memory processes engaged to refine the stress response to repetitive homeostatic challenges. Collectively, these studies suggest that augmented 2-AG content and 2-AG signaling at central facilitates stress habituation in response to repetitive homotypic stress exposure (Campolongo et al., 2009).

BLA-AEA Tonic Signaling Facilitates Tonic inhibition of Basal HPA Axis Activation

AEA content within the BLA is also affected by chronic homotypic stress exposure but the pattern of AEA content change and, thus, its functional consequences are different than that of 2-AG. Experimental evidence demonstrates that chronic homotypic stress paradigms rapidly decrease BLA-AEA content which most likely results from a progressive increase in amygdalar FAAH V_{max}, the rate of FAAH-catalyzed reactions (Hill et al., 2013b; Rademacher et al., 2008). The functional consequence of decreased BLA-AEA content appears to be basal HPA axis hypersecretion (Hill et al., 2010c), as demonstrated by evidence generated by Hill and colleagues (2005) which show the attenuation of stress-induced corticosterone secretion following pharmacological inhibition of BLA-FAAH (Hill et al., 2009b). These experimental results support the function of BLA-AEA as a "HPA gatekeeper" (Hill et al., 2010b; Hill et al., 2009b; Patel et al., 2004) through tonic basal inhibition.

These data also strongly suggest that CRS-induced decreases in BLA-AEA levels support the adaptive state of heightened vigilance at the neuroendocrine and behavioral levels (Hill et al., 2010b; Hill et al., 2009c). In support of this, HPA axis activity and behavioral responsiveness are more enhanced by novel stressors following prior exposure to a chronic stress paradigm as compared to behaviorally naïve controls exposed to similar novel stressors (Bhatnagar et al., 1998; Bhatnagar and Meaney, 1995; Hauger et al., 1990). Furthermore, following repetitive stress exposure, increased HPA axis secretion is observed during the rising phase of HPA axis

activity and this rise may further contribute to the observed facilitation of HPA secretion (Windle et al., 1998). As such, AEA mediated regulation of HPA axis activity may act as a potential mechanism by which eCBs attenuate stress responsivity. Overall, it appears that both 2-AG and AEA signaling in the amygdala allows an organism to increase its defensive responses to novel aversive stimuli, where the threat value is of an unknown magnitude, whilst decreasing it stress-related responses to stimuli with threat of known magnitude (Bhatnagar et al., 2003).

Repetitive exposure to homotypic stressors also results in neuroendocrine, physiological, and behavioral changes that rely on the activity of extra-BLA regions within cortico-limbic circuitry. For example, chronic homotypic stressors elicit adaptations in autonomic (e.g. heart rate, and blood pressure increases) (Bartolomucci et al., 2003; Moore et al., 2001) and passive behavioral stress responses (decreased freezing/startle response) (Bielajew et al., 2002; Kamprath et al., 2006a; Kamprath and Wotjak, 2004). As alluded to above, significant experimental evidence demonstrates that these components of the stress response are regulated by the eCB system (Hill et al., 2010b). Interestingly, significant evidence also strongly suggest that the central amygdala (CeA), an extra-BLA amygdalar region, plays a significant role in controlling similar neuroendocrine, physiological, and behavioral responses to aversive stimuli via its extensive afferent and efferent connections with other stress responsive brain regions which will be discussed in the next section. Given these qualities, the CeA may potentially be a critical site for eCB-mediated regulation of neuroendocrine, autonomic, and behavioral components of the stress response (Kamprath et al., 2011; LeDoux et al., 1988b).

The Central Amygdala Regulates Physiological and Behavioral Components of Stress Responses

Experimental evidence supports the role of the CeA as a key site for the integration of stress-related information and the subsequent regulation of physiological and behavioral components of

the stress response. Both acute and chronic stress increases *c-fos* gene expression within the CeA, indicating that increased neuronal activity is recruited within this region following exposure to aversive stimuli (Bhatnagar and Dallman, 1998; Bhatnagar et al., 1998; Kollack-Walker et al., 1997). Early studies demonstrate that these changes in CeA neuronal activity are the result of stress-induced adaptations of the intra-CeA circuitry as single-and multi-unit activity measurements of antidromically-identified CeA neurons, which project to cardiovascular regulatory nuclei in the medulla, decrease following repeated presentations of conditioned stimuli (Pascoe and Kapp, 1985). Furthermore, this decrease in CeA neuronal activity is paralleled by decreases in heart rate changes following repeated presentation of conditioned stimuli (Applegate et al., 1982; Pascoe and Kapp, 1985). As such, the CeA appears to be a critical anatomical region for regulating changes in stress responsivity following persistent exposure to stressors.

Consistent with experience-dependent changes in CeA neuronal activity, experimental evidence points to the CeA as a key site for the regulation of physiological and autonomic responses to stress. Early pharmacological and lesion studies show that bilateral CeA inactivation significantly reduced ACTH and glucocorticoid secretion in response to aversive stimuli (Beaulieu et al., 1986; Van de Kar et al., 1991), decreased CRH secretion and medial eminence immunoreactivity following photic or acoustic stimulation (Feldman et al., 1994), impaired both catecholamine release (Roozendaal et al., 1990) and stress-induced heart rate changes (Roozendaal et al., 1991a, b), as well as inhibited startle and freezing behavioral responses following conditioned and unconditioned aversive stimuli exposure (Roozendaal et al., 1990). Furthermore, electrical and chemical CeA stimulation elicits autonomic and behavioral responses that resemble unconditioned and conditioned responses to systemic and psychological

stressors (Ciocchi et al., 2010; Iwata et al., 1987; Li et al., 2013; Reis and Oliphant, 1964) while lesions of brain regions to which the CeA projects attenuate autonomic and behavioral responses previously elicited by aversive environmental stimuli (Iwata et al., 1986a; Iwata et al., 1986b; LeDoux et al., 1988a; van der Kooy et al., 1984). Collectively, these studies support the role of the CeA as an integral neurosubstrate that regulates the physiological and behavioral responses to aversive stimuli exposure. These diverse functions of the CeA are thought to be consequences of its extensive efferent pathways that innervate a number of brain regions implicated in the sympathetic, parasympathetic, neuroendocrine, and passive behavioral responses mobilized following exposure to aversive stimuli (Bouret et al., 2003; Jolkkonen et al., 2002; LeDoux et al., 1988b). Although studies characterizing CeA synapses in efferent target circuitry are lacking (Penzo et al., 2014), extensive tracing and optogenetic studies have identified a number of CeA mono- and multi-synaptic efferent pathways to stress responsive brain regions and these are summarized below in Figure 3.

The Central Amygdala is Largely Composed of Four Subdivision

The central amygdala is largely composed of four subdivisions: the capsular (CeC), the lateral, the intermediate (CeI), collectively known as the lateral nucleus of the central amygdala (CeAL), and the medial subdivision (CeAM) (Cassell et al., 1999a; Jolkkonen and Pitkanen, 1998; McDonald, 1982a). The principal (95%) neuronal type of the CeAL is GABAergic medium-sized densely spinous neurons (MSNs). These MSNs are embryonically striatal in origin (Puelles, 2001; Swanson and Petrovich, 1998) and, thus, are morphologically similar to the medium spiny neurons (MSNs) of the striatum (Sun and Cassell, 1993). Electrophysiological properties of rodent CeAL neurons can be separated into two groups based on firing properties of

the cell: regular (RF) and late firing (LF) and each group represents 50% of the CeAL neuronal population (Dumont et al., 2002; Lopez de Armentia and Sah, 2004a).

Unlike the CeAL, the CeAM demonstrate a different distribution of cell types as RF, LF, and low-threshold bursting (LTB) neurons exhibit an incidence of 27%, 2%, and 71%, respectively (Dumont et al., 2002; Lopez de Armentia and Sah, 2004a). Furthermore, the CeAM is largely composed of pyramiform (fusiform/spindle-shaped), sparsely spiny neurons similar to the principal neurons found in the ventral pallidum, with a smaller portion of cells appearing to be GABAergic (Haubensak et al., 2010; Sun and Cassell, 1993). However, both rodent and primate CeAM display low GABA expression but a high expression of glutamate decarboxylase (GAD), the enzyme responsible for converting glutamate to GABA, which suggests that CeAM GABAergic neurons may be underestimated (Haubensak et al., 2010; Li et al., 2013; Pitkanen and Amaral, 1994; Sun and Cassell, 1993). These features, in addition to the intrinsic neurochemical characteristics and intra- and inter- connectivity of the CeAL and CeAM, suggest a complex architecture that closely parallels that of the multi-compartmentalized organization of the nucleus accumbens and the ventral pallidum (Cassell et al., 1999a).

The CeAL-CeAM Circuitry Controls Central Amygdala Efferent Pathways

The organization of the CeAL and CeAM circuitry closely parallels that of the striatal-pallidum complex. Similar to the striatum, anatomical and functional studies demonstrate that the CeAL exhibit inhibitory control, via locally targeted axon collaterals within the CeAL and a few long-range projections to extra-CeA stress-responsive brain regions such as the bed nucleus of the stria terminalis (Cassell et al., 1986), midbrain periaqueductal gray (PAG) and the paraventricular nucleus of the thalamus (Padilla-Coreano et al., 2012; Sun and Cassell, 1993; Swanson and Petrovich, 1998). CeAL long-range projecting neurons innervate extra-CeA brain

region largely via non-overlapping neurons (Penzo et al., 2014) and act as additional avenues through which the CeAL participates in the autonomic, endocrine, and behavioral regulation of stress responses. Additionally, the CeAL also extensively innervates the CeAM and provides substantial tonic inhibitory control of CeAM neuronal activity (Jolkkonen and Pitkanen, 1998; Tye et al., 2011; Veinante et al., 2003).

The CeAM is the Major Output Region of the CeAL

In comparison, the CeAM does not exhibit reciprocal projections to the CeAL but, rather, has local axonal arborizations that participate in the CeAM intrinsic circuitry (Ciocchi et al., 2010; McDonald, 1982a). Furthermore, the CeAM also exhibits efferent pathways (via the stria terminalis and ventral amygalofugal pathway (Ciocchi et al., 2010; Delaney et al., 2007; Hopkins and Holstege, 1978) that innervate diencephalic, mesencephalic, medulallary regions within the central nervous system which form the anatomical substrate for the CeA-mediated modulation of autonomic, neuroendocrine, and behavioral responses to stress exposure (Ciocchi et al., 2010; Viviani et al., 2011).

Though synaptic contacts between CeA projection neurons and target regions have been less characterized, it is hypothesized that CeA axonal terminals make largely GABAergic contacts onto the dendrites and soma within regions of interest, as is demonstrated in the nucleus of the solitary tract (NTS) (Jolkkonen et al., 2002; Jolkkonen et al., 2001; Saha et al., 2000). Overall, this evidence indicates that the CeAL provides tonic inhibitory control of the CeA output circuitry and is, therefore, well-positioned to powerfully restrain the components of the stress response via signaling mechanisms such as the eCB system.

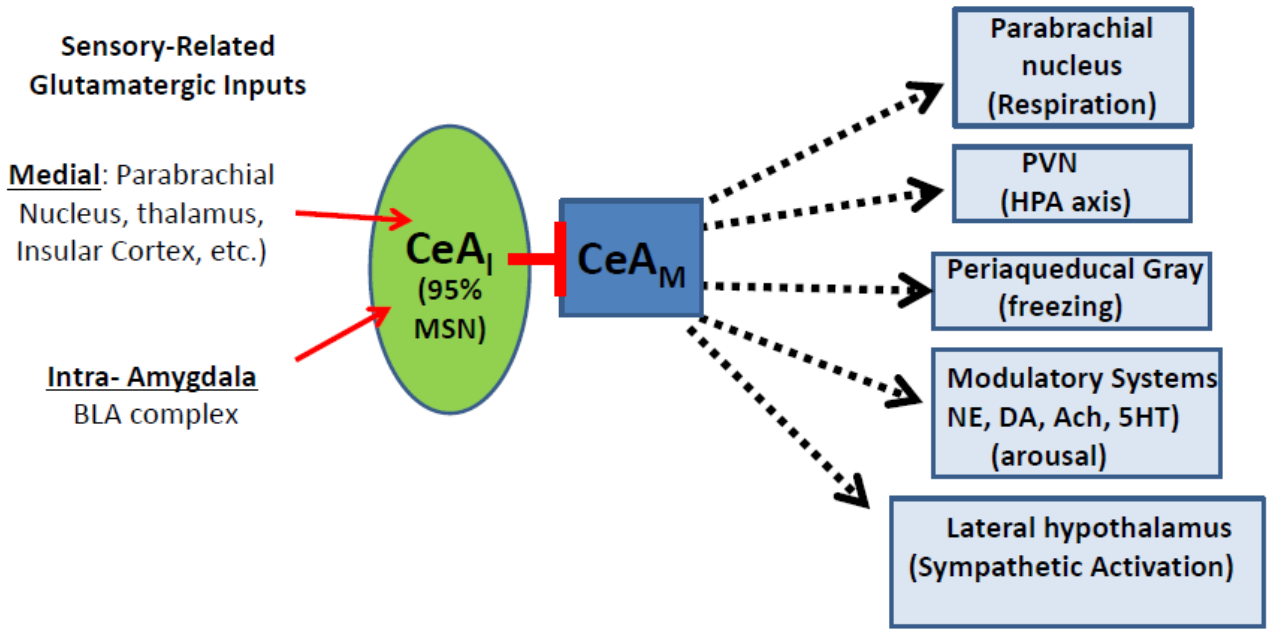

Figure 3. Schematic Representation of Known CeA Afferents and Efferent Pathways.

Aversive Stimuli Exposure Drives Synaptic Changes within the CeAL Microcircuitry

Similar to the branching patterns found in the matrix of the striatum, Golgi staining studies demonstrate that CeAL MSNs can be divided, based on axonal collaboration, into two categories: neurons with extensive local collaterals but a relatively unbranched axon extending out of the CeAL and a second neuronal type with few local collaterals but extensive branching extending out of the CeAL (Cassell et al., 1999b). Recent *in vivo* single unit recordings in awake, behaving animals have also identified two distinct neuronal populations that display inverse directionality in synaptic plasticity following exposure to threat-conditioned stimuli. In response to conditioned aversive stimuli one subpopulation, the CeALon neurons, display rapid and persistent increases in neuronal activity whilst another subpopulation, CeALoff neurons, exhibit a delayed and persistent decrease in neuronal activity that results from feed-forward inhibition by locally targeting collaterals of CeALon neurons (Ciocchi et al 2010). Accompanying and more recent studies have genetically defined these functional neuronal populations as protein kinase C delta negative/somatostatin positive (i.e. CeALon /PKCδ^-/SOM$^+$) and protein kinase C delta positive/ somatostatin negative (CeALoff /PKCδ^+/SOM$^-$) neurons, respectively (Haubensak et al., 2010{Li, 2013 #3984). Work by Li and others (2013) have demonstrated that these disparate neuronal populations form a distinct CeAL-microcircuitry where aversive stimuli exposure differentially elicits persistent changes in the synaptic efficacy of excitatory neurotransmission onto both cell types (Li et al., 2013). These changes, as will be discussed in the subsequent section, drive distinct changes in the CeAL microcircuitry which, subsequently, modifies the expression of CeA-mediated stress-related functions (Gozzi et al., 2010).

Parallel to these findings in the CeAL, work by Viviani and others have also demonstrated that a unique microcircuitry also exists within the CeAM (Viviani et al., 2011). Though relatively unexplored, there appears to be cytochemically and functionally distinct

neuronal populations within the CeAM that are targeted by non-overlapping CeAL neurons and, in turn, facilitate specific behavioral and physiological components of the stress response. Collectively, these data suggest that the CeAL-CeAM circuitry is a powerful locus in the regulation of autonomic, endocrine and behavioral responses to stress.

CeAL Glutamatergic Inputs Drive CeA Function

The lateral division of the central amygdala (CeAL) receives extensive intra-and inter-amygdala glutamatergic inputs carrying sensory information from all modalities. This information is relayed to the CeAL via the fast excitatory neurotransmission of α-amino-3-hydroxy-5-methylisoazole-4-proprionic acid type glutamate receptors (AMPARs) (Li and Neugebauer, 2004; Lopez de Armentia and Sah, 2003) and GluN2B containing N-methyl-D-aspartate (NMDA) receptors. Intra-amygdala glutamatergic inputs arise from amygdala subnuclei such as the LA (Jolkkonen and Pitkanen, 1998; Li et al., 2013) and BLA (Delaney et al., 2007; Sarhan et al., 2005; Tye et al., 2011, regions heavily implicated in components of the stress response. Extra-amygdala regions conveying sensory- related information to the CeAL include brain stem inputs from the parabrachial nucleus ({Krukoff, 1993 #7920) which form asymmetrical perisomatic synapses onto CeAL dendritic shafts, spines, and soma (Dong et al., 2010), 2) the nucleus of the solitary tract (Batten et al., 2002), 3) hypothalamic inputs from ventromedial nucleus (Canteras et al., 1994), 3) thalamic inputs from paraventricular nucleus (Moga et al., 1995; Turner and Herkenham, 1991; Vertes and Hoover, 2008), and 4) cortical brain regions such as the ventral entorhinal (McDonald and Mascagni, 1997), insula (Sun et al., 1994) infralimbic, and prelimbic cortices (McDonald et al., 1996).

Endocannabinoid Signaling in the CeAL: Potential Locus for eCB-Mediated Adaptive Mechanisms Following Chronic Stress Exposure

Although detailed studies examining the subcellular location of the CB1 receptor were previously lacking, early expression studies observed CB1 receptor expression within the CeA (Cota, 2007a; Kamprath et al., 2011; Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1990). Furthermore, systemic application of the CB1 receptor antagonist, SR141716, increased c-fos expression in the CeA of behaviorally naïve Winstar rats, which indicates that tonic CB1 receptor activity dampens inter- or intra-CeA excitatory drive (Singh et al., 2004). More recent studies by Roberto and colleagues (2010) demonstrated that CeAM inhibitory synapses were persistently inhibited by tonic CB1 signaling. Collectively, these studies support the presence of functional CB1 receptors within the CeA (Roberto et al., 2010a). These described studies, however, contradict earlier studies by Katona and colleagues (2001) which found a lack of functional CB1 receptors, as interrogated by WIN 55212-2, at CeA inhibitory synapses. However, based on the data that I will discuss in my thesis, these discrepancies may be explained by potential differences in the CB1 sensitivity of inhibitory synapses within CeA.

eCB-Mediated Synaptic Adaptations within the CeA and its Functional Role in CeA-Mediated Stress Behavior

The CeA exhibits experience-dependent modifications of eCB-mediated synaptic plasticity following exposure to aversive stimuli. For example, recent studies by Kamprath and colleagues (2011) reveal enhanced depolarization-induced suppression of BLA-CeAM as well as CeAL-CeAM synapses (Kamprath et al., 2011) following exposure to aversive stimuli. To determine the functional relevance of experience-dependent adaptations of eCB-signaling within the CeAL, the authors infused the CB1 agonist, AM 251, within the CeA prior to exposure to the conditioned stimuli. The results from this experiment reveal that inhibiting CB1 receptors within

the CeA increased the freezing response of mice during the initial exposure to the conditioned stimuli, suggesting that CeA-CB1 signaling gates short-term adaptations to aversive stimuli (Kamprath et al., 2011). In support of this conclusion, earlier studies by Zarrindast and co-workers (2008) show that intra-CeA infusion of the CB1 agonist, ACPA, decreased measures of anxiety in rats as measured by elevated plus maze (Zarrindast et al., 2008). Collectively, these studies support the role of eCB signaling within the CeA as gating behavioral responses to aversive stimuli. However, with the exception of work by Kamprath and colleagues (2011), there remains a paucity of studies that have examined whether eCB signaling mechanisms within CeA microcircuitry, such as muscarinic receptor driven eCB synthesis observed in other stress responsive brain regions (Narushima et al., 2007b; Uchigashima et al., 2007c), underlie this functional interaction between the CeA and eCB system (Kamprath et al., 2011).

The Cholinergic Synapse within the CNS

Acetylcholine (ACh) is the first neurotransmitter to be discovered and primarily modulates synaptic transmission in the brain (Picciotto et al., 2012). ACh is synthesized in the nerve terminal from acetyl coenzyme A (acetyl-CoA), which is derived from pyruvate generated by glycolysis and choline, which is present within the plasma at a concentration of 10mM. Choline is transported from the plasma into the nerve terminal by a sodium dependent choline transporter. Once within the nerve terminal, the synthesis of ACh via acetyl-CoA and choline condensation is catalyzed by the cytosolic enzyme, choline acetyltransferase, (Fischer et al., 2010a; Fischer et al., 2010b) and packaged into synaptic vesicles by the vesicular ACh transporter. Once released within the synapse, acetylcholine concentration rapidly increases 50,000 fold in milliseconds. Subsequently, cholinergic signaling is rapidly terminated by the hydrolysis of ACh by acetylcholinesterase (AChE) (Nair et al., 1994), a serine hydrolase. AChE

has a high catalytic activity (5000 molecules of ACh/AChE/second) and hydrolyzes ACh to acetate and choline, which, in turn, cycles back into the ACh biosynthetic pathway.

Local and Projection Cholinergic Neurons Target Diverse Brain Regions

ACh is targeted to different brain regions via local cholinergic interneurons (largely found within the striatum, nucleus accumbens, and neocortex of rodents and humans) or long-range projection neurons that innervate distal brain regions (Benagiano et al., 2003; Mesulam, 1995). Cholinergic projections neurons are organized into relatively discrete cell groups, Ch1-Ch6, which innervate distinct brain regions (Bubser et al., 2012). The basal forebrain cholinergic projection neurons (Ch1-Ch4) consist of synaptically interconnected groups of cells located within the medial septum, diagonal band of Broca, and the nucleus basalis of Meynert (NBM). Upon further examination, the NBM was found to constitute an aggregation of discontinuous islands of large multipolar cells, with extensive dendritic trees, and acts as the major source of ACh for the CeA (Bigl and Arendt, 1991; Schwaber et al., 1987).

mAChRs Display Distinct Regional Distributions and Subcellular Localizations

Acetylcholine exerts it physiological actions via the activation of a family of GPCRs known as muscarinic acetylcholine receptors (mAChRs). mAChRs are members of the Family-A G-protein coupled receptors (GPCRs) and consists of five subtypes, M_1-M_5 (numbered in the order of their discovery). These mAChR subtypes are expressed throughout the CNS, but each subtype exhibits different regional distributions and subcellular localizations throughout the brain (Bonner et al., 1987; Bonner et al., 1988; Levey, 1993; Levey et al., 1991b). Of the 5 subtypes, the M_5 subtype has the lowest expression in the CNS and is expressed in select brain regions (the highest receptor density is found within the striatum: ~25fmol/mg) (Vilaro et al., 1990). On the other hand, the M_1, M_2, and M_4 subtypes are the most abundant. Moreover $M_{1/4}$ and M_2 mAChRs

have opposite expression patterns with the $M_{1/4}$ expression being the highest rostral brain regions and decreasing caudally while the M_2 mAChR has the converse expression pattern (Wall et al., 1992a, b) (Weiner et al., 1990). As such, $M_{1/4}$ mAChRs are the most predominant mAChR in the cortex, striatum, and hippocampus and is largely thought to be localized postsynaptically. Conversely, M_2 $mAChR_s$ are the most predominant mAChR in the thalamus, hypothalamus, cerebellum, and brainstem and are largely thought to be localized presynaptically (Levey, 1993; Levey et al., 1991a; Rouse et al., 1998).

Pharmacology of Muscarinic Receptors

All five muscarinic subtypes show high sequence homology among the amino acid residues, within transmembrane domains 3,5-7, which forms the orthosteric ACh-binding pocket. Greater sequence variability is observed at the N-and C-termini as well as the third intracellular loop which displays the largest sequence divergence and length and is often the target for subtype specific antibodies. As a consequence, M_1-M_5 mAChRs range from 460 to 589 amino acids (Bonner et al., 1987; Hulme et al., 2003a; Hulme et al., 2003b; Wess, 1996; Wess et al., 1996). Within the binding domain, a carboxylic acid group from a highly conserved aspartic acid residue in the third transmembrane domain of the mAChR provides the negative charge for binding to the positively charged ACh headgroup (Burgen, 1965; Hanin et al., 1966; Hulme, 1990). A consequence of this high sequence homology, however, is that very few sub-type specific agonists and antagonists have been developed (Hulme et al., 2003a; Hulme et al., 2003b). As such, most muscarinic agonists, such as Oxotremorine-M (Oxo-M), have similar affinities across subtypes (Kukkonen et al., 1996; Thomas et al., 2008) and, thus, have a broad array of *in vivo* side effects, one of the most notable being the SLUD syndrome (salivation, lacrimation, urination, defecation) following systemic Oxo-M application (Karanth et al., 2007).

In addition to the acetylcholine orthosteric binding site, muscarinic receptor subtypes have a number of allosteric sites that can modulate agonist function (Digby et al., 2012). These mAChR allosteric binding sites are the targets of newly developed, subtype specific mAChR ligand such as the M_1 specific agonist, VU0357017 (Digby et al., 2012). Furthermore, non-selective mAChR antagonists ,such as Atropine, are being replaced by more selective antagonists such as Pirenzepine, a M_1 preferring antagonist (Buckley et al., 1989; Wess et al., 1989) and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), a M_3 preferring antagonist (Lambrecht et al., 1989). As their selectivity description suggests, none of the currently available muscarinic antagonists are highly selective with most compounds only exhibiting a 10-fold higher affinity for the mAChR subtypes for which they are most selective.

mAChR Subtypes Couple to Distinct G-proteins

The importance of mAChR subtype-specific pharmacological control is further highlighted by the diversity of signaling cascades recruited by mAChR activation. Based on sequence comparison and the biochemical consequences of mAChR activation, the M_1-M_5 mAChR subtypes can be divided into two functionally distinct groups. One group includes the M_2 and M_4 receptors that selectively activate $G\alpha_{i/o}$-type G proteins that negatively coupled to adenylate cyclase (Migeon and Nathanson, 1994; Migeon et al., 1994; Picciotto et al., 2012). The other group is composed of M_1, M_3, and M_5 receptors which show selectivity for $G\alpha_{q/11}$ G-proteins and diverse PLC-mediated second messenger signaling cascades implicated in eCB biosynthesis (Kano et al., 2009a).

mAChRs Exhibit Dynamic G-protein Coupling in Response to Orthosteric Activation

In addition to these mAChR-specific signaling cascades, evidence of orthosteric agonism-induced changes in mAChR G-protein functional coupling has also been reported. Although 10-

fold right shifted, $M_{1/3}$ mAChRs have been shown to couple to Gαs signaling (Burford and Nahorski, 1996; Thomas et al., 2008) and $G\alpha_{i/o}$ coupled signaling (Akam et al., 2001; Thomas et al., 2008) which suggests that orthosteric agonism of mAChRs can recruit noncanonical downstream signaling pathways. For example, M_1 mAChR stimulation has been shown to stimulate a pertussis-toxin sensitive component of M-current inhibition (Haley et al., 2000; Lechner et al., 2003), PKC/Ca^{2+}-independent, $G\alpha_s$-dependent signaling (Migeon et al., 1994), as well as $M_{1/3}$ mAChR recruitment of PLA2 signaling pathways. To further add to this complexity, though much of the literature has focused $G\alpha_{q/11}$-PLC coupled signaling of $M_{1/3}$ mAChRs some *in vitro* experiments results suggest that brief high dose orthosteric activation of $M_{1/3}$ mAChRs also induces PLD activity in a dose- (Sandmann and Wurtman, 1990) and time-dependent manner which, over time, leads to PLD activity desensitization (~30 minutes) and the concomitant sensitization of PLC activity (McKenzie et al., 1992b; Schmidt et al., 1995b). Given this diversity in mAChRs' functional coupling, dissimilar secondary signaling cascades and their respective effectors may be recruited following orthosteric mAChR activation. This promiscuous signal transduction following orthosteric receptor activation is also observed in other G-protein families such as the calcium receptor (Rey et al 2005), as well as, the A1 adenosine receptor (Cordeaux et al., 2000) and represents additional diversity in GPCR signaling.

$G\alpha_q$-coupled mAChR activation and Endocannabinoid Signaling

One signaling cascade recruited via the activation of mAChR is that which can elicit eCB biosynthesis. The Alger research group (2002) was the first to demonstrate that orthosteric agonism of mAChR activation dose-dependently enhances eCB release. Moreover, this synthesis and release was found to occur via a postsynaptic mechanism, as measured by two forms of

eCB-mediated synaptic plasticity: RER-Ca^{2+} assisted- and RER- eCB release at hippocampal inhibitory synapses (Kim et al., 2002b). In subsequent experiments by additional groups, mAChR-driven eCB mobilization was also identified in other brain regions (namely the striatum and periaqueductal gray) and determined to mediated via $M_{1/3}$ mAChR signaling (Fukudome et al., 2004b; Ohno-Shosaku et al., 2003) and capable of mediating both *in vitro* AEA (van der Stelt et al., 2005) (Stella and Piomelli, 2001) and 2-AG synthesis (Edwards et al., 2006b) (Lau and Vaughan, 2008; Narushima et al., 2007a; Uchigashima et al., 2007a). Despite the collective evidence for mAChR-driven eCB production in a number of brain regions, two discrepancies have arisen: evidence for PLCβ/DAGL Ca^{2+}-dependent and-independent forms of mAChR-eCB release within the striatum, cerebellum, and hippocampus. Though the underlying cause of this inconsistency is unknown, the lack of functional bias exhibited by orthosteric mAChR activation may be a potential reason(Hashimotodani et al., 2005) (Chiu and Castillo, 2008) (Uchigashima et al., 2007a).

Muscarinic Receptor Signaling and Additional Retrograde Signaling

Inconsistencies in eCB biosynthetic pathways recruited by mAChR activation may also be explained by the ability of mAChRs to recruit additional retrograde signaling pathways. Significant experimental evidence demonstrate that activation of mAChRs also drive synthesis of the diffusible retrograde signal molecule, nitric oxide (NO) (Christopoulos and El-Fakahany, 1999; Wang et al., 1994) and NO-mediated synaptic plasticity (Wotta et al., 1998) (Borda et al., 1998; Makara et al., 2007). Interesting, a number of studies have also demonstrated that interactions between the NO and eCB systems can also attenuate synaptic transmission.

In support of this, components of the nitric oxide synthetic pathway have been shown at CB1 containing synapses and, similarly to Ca^{2+} and Ca^{2+}-RER forms of eCB synthesis, synaptic NO

can be mobilized by increases in intracellular calcium (Crosby et al., 2011). Furthermore, NO as been shown to augment eCB mediated synaptic plasticity at both inhibitory and excitatory synapses within a number of brain regions such as the hypothalamus (Crosby et al., 2011) cerebellum (Safo and Regehr, 2005) striatum (Chepkova et al., 2009), and the hippocampus (Makara et al., 2007). Though the exact mechanism for this interaction is unknown, it has been determined that NO can act upstream (Makara et al., 2007) or downstream (Chepkova et al., 2009; Safo and Regehr, 2005) of CB1 receptor signaling. Alternatively, NO can also directly regulate CB1 receptors and prevent agonist induced desensitization and internalization via the nitrosylation of GPCR kinases that mediate GPCR mediated internalization (Kokkola et al., 2005; Whalen et al., 2007). Alternatively, NO can also modulate synaptic transmission, independent of CB1 receptors, via downstream cyclic guanosine monophosphate (cGMP)-mediated processes such as the activation of cGMP-dependent protein kinase G which modulates the transmitter release machinery (Lange et al., 2012).

Muscarinic Receptor Signaling in the Central Amygdala

Though cholinergic signaling at synapses within the lateral division of the central amygdala (CeAL) has yet to be examined, components of cholinergic system are expressed within this region. The CeAL receives strong cholinergic innervations from the nucleus basalis of Meynert, with the majority of VAChT-positive terminal fields forming perisomatic basket-like terminations around the soma of CeAL neurons (Heckers et al., 1994) (Schafer et al., 1998b). Furthermore, other cholinergic markers such as heavy ChAT and weaker AChE immunoreactivity are also present throughout the CeAL in non-human primates (Amaral and Bassett, 1989) and rodents (Saha et al., 2000) (Van der Zee and Keijser, 2011). Pan-mAChR staining in the CeAL, using the M-35 mAChR antibody, also demonstrates robust mAChR

staining which overlaps strongly with GABAergic markers (overlap between mAChR and GAD is 94%) (Van der Zee and Keijser, 2011; van der Zee and Luiten, 1999.). Additionally, subtype specific immunocytochemical analyses reveal that M_1-M_4 subtypes are robustly expressed within the CeAL, with $M_{2/3/4}$ mAChRs displaying the greatest expression levels (Levey et al., 1993; Levey et al., 1991b).

Potential for mAChR-eCB Mediated Synaptic Plasticity in the Lateral Division of the CeA and Its Functional Relevance

Though yet to be explored, the presence of the cholinergic system within the CeAL alludes to the potential for mAChR-mediated synaptic plasticity within the CeAL circuitry. One such mAChR-mediated synaptic plasticity is mAChR-eCB mobilization particularly given the high expression of PLCβ1 mRNA (Watanabe et al 1998), the potential expression of CB1 receptors within this region, (Matsuda et al., 1990) (Tsou et al., 1998a) (Cota, 2007b) as well as evidence of weak FAAH and MAGL expression (Gulyas et al., 2004a) (Yoshida et al., 2011b) within the CeAL. Given the critical role of the CeAL circuitry in controlling the physiological and behavioral responses to stress, a potential locus for this eCB-mediated effect on the stress response may lie at the glutamatergic synapses that drive CeAL synapses. Furthermore, given evidence of $G\alpha_q$-coupled mAChR-mediated eCB mobilization in other brain regions (Edwards et al., 2006b; Kano et al., 2009a; Lau and Vaughan, 2008; Narushima et al., 2007a; Uchigashima et al., 2007a), mAChR-eCB release at CeAL synapses may enhance eCB signaling under basal and even chronic stress conditions. In support of the latter, acute immobilization and footshock stressors increase acetylcholine release in the prefrontal cortex and amygdala, suggesting that acute exposure to aversive stimuli facilitates stress-induced adaptive changes in cholinergic signaling (Mark et al., 1996). In additional support of stress-induced facilitation of amygdalar mAChR signaling, Gilad and colleagues (1984 and 1985) also demonstrate that chronic stress

exposure results in adaptations in cholinergic terminals as measured by post-stress hippocampal synaptosomal preparations (Finkelstein et al., 1985; Gilad et al., 1985; Rabey et al., 1984). These experiments revealed that repetitive immobilization stress decreased cholinergic uptake while progressively increasing acetylcholine release. Taken together, these results suggest that stress exposure enhances cholinergic signaling which, in turn, act as the substrate through eCB synthesis is increased to buffer against adverse synaptic and subsequent physiological, autonomic, and behavioral changes elicited by chronic stress exposure.

Conclusion

The amygdala regulates the processing of autonomic, physiological, and behavioral reactions to aversive stimuli. Furthermore, compelling evidence in limbic brain regions such as the BLA, strongly implicates the eCB system in both the processing of the stress response, as well as neuroadaptations to chronic stress exposure. Interestingly, this eCB-mediated function remains largely unexplored in the CeA: the amygdala's "output" structure that mediates the processing of stress-related behavior and whose activity strongly correlates with the symptomology of stress-related psychiatric illnesses, such as post traumatic stress disorder. The CeA's ability to orchestrate stress-related responses lie, in part, with CeAL glutamatergic inputs that bring sensory-related information from extra-CeAL stress-responsive brain regions.

Given this critical role of excitatory CeAL afferents, it is likely that eCB-mediated modulation, at these terminals, significantly contributes to the eCB system's role regulating stress responsivity. In support of this, the CeAL is highly enriched with the $G\alpha_{q/11}$-coupled mAChR which have been implicated in receptor-driven eCB production in other stress-responsive brain regions. As such, the aim of my thesis work was is three-fold: 1) to explore the expression of the eCB system and 2) to examine mechanisms of eCB- mediated synaptic

plasticity at CeAL glutamatergic synapses, as well as 3) to determine whether eCB-mediated synaptic plasticity at CeAL glutamatergic synapses are affected by repetitive stress exposure.

CHAPTER II

MATERIALS AND METHODS

Animals

Anatomical experiments, with the exception of the double immunofluorescence labeling (ICR mice, Harlan Indianapolis, IN), were carried out in wild type and $CB1^{-/-}$ C57BL/6 mice. All electrophysiology experiments were performed using male ICR mice (Harlan, Indianapolis, IN) 4-6 weeks old. Wild type, $CB1^{-/-}$, and $FAAH^{-/-}$ mice, on the ICR background, were derived from heterozygote breeding pairs (kindly provided by Dr. C.J. Hillard, Medical College of Wisconsin, Milwaukee, WI). Mice were housed on a 12:12 light-dark cycle with food and water available *ad libitum*. All studies were approved by the Vanderbilt University Institutional Animal Care and Use Committee as well as the Committee of the Scientific Ethics of Animal Research (22.1/4027/0033/2009). Furthermore, all studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, as well as the institutional guidelines of ethical code, and the Hungarian Act of Animal Care and Experimentation (1998. XXVIII. Section 243/1998).

Restraint Stress Paradigm

Mice were brought to the restraint room daily and subjected to 1 hour of restraint in modified 50ml conical tubes, for 10 consecutive days between 0900-1100 hours. During restraint, mice were placed in a ventilated animal housing cabinet and immediately upon termination of the restrain stress, the mice were returned to their home cage and animal care facility housing room. Control mice were only handled during tail marking at the beginning of, and as needed

throughout, the 10 day stress paradigm. Following each stress episode, restraint tubes were washed thoroughly with soap and warm water, then rinsed with 70% ethanol.

Drugs and Chemicals

SR141716 (Rimonabant) was a gift from the National Institute on Mental Health Drug Supply Program. JZL 184 was also a gift from the National Institute on Drug Abuse Drug Supply Program. Oxotremorine-M (Oxo-M) and 4-DAMP were purchased from Tocris Bioscience (Ellisville, MO) while Pirenzepine dihydrochloride and Atropine were obtained from Sigma-Aldrich (St. Louis, MO). PF-3845 was kindly provided by Dr. Douglas Johnson (Pfizer). Picrotoxin was purchased from Abcam Biochemicals (Cambridge, MA). All other drugs were acquired from Cayman Chemicals (Ann Arbor, MI). Drugs sparingly soluble in aqueous solutions were first dissolved with DMSO and, thereafter, diluted in artificial cerebral spinal fluid (ACSF). For experiments utilizing lipophilic drugs, the ACSF was supplemented with 0.5g/L fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich) to increase drug solubility and minimize nonspecific binding of lipophilic compounds. Equal amounts of DMSO and BSA were used in control solutions.

Brain Slice Preparation

Mice were anesthetized with isoflurane, then transcardially perfused with ice-cold high sucrose, low Na^+-containing ACSF and sacrificed by decapitation. Following decapitation, the brain was removed and a 3mm coronal block of the amygdala was cut using an ice-chilled, coronal brain matrix. Thereafter, hemisected coronal slices (200-300µm) were made using a Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL) in a 1-4°C oxygenated (95% v/v O_2, 5% v/v CO_2) high sucrose, low Na^+ - containing ACSF comprised of (in mM): 208 sucrose, 2.5 KCl, 1.6 NaH_2PO_4, 1 $CaCl_2 \cdot 2H_2O$, 4 $MgCl_2 \cdot 6H_2O$, 4 $MgSO_4 \cdot 7H_2O$, 26 $NaHCO_3$, 1 ascorbate, 3 Na-

pyruvate, and 20 glucose. Once cut, slices were transferred to a 32°C oxygenated recovery buffer composed of (in mM): 100 sucrose, 60 NaCl, 2.5 KCl, 1.4 NaH_2PO_4, 1.1 $CaCl_2 \cdot 2H2O$, 3.2 $MgCl_2 \cdot 6H_2O$, 2 $MgSO_4 \cdot 7H2O$, 22 $NaHCO_3$, 1 ascorbate, 3 Na-pyruvate, and 20 glucose for 20 minutes followed by a minimum of 30 minutes in 24°C, oxygenated ACSF (in mM): 113 NaCl, 2.5 KCl, 1.2 $MgSO_4 \cdot 7H2O$, 1 NaH_2PO_4,2.5 , 2.5 $CaCl_2 \cdot 2H2O$, 26 $NaHCO_3$, 1 ascorbate, and 3 Na-pyruvate, and 20 glucose. Thereafter, slices were placed in a submerged recording chamber where they were continuously perfused with oxygenated ACSF (30-32°C) at a flow rate of 2-3 milliliters/minute. For all electrophysiology experiments, other than those examining GABAergic currents, the ACSF was supplemented with the $GABA_A$ receptor antagonist, pircotoxin (25-50μM), to isolate excitatory neurotransmission. To isolate eIPSCs, the ACSF was supplemented with AP-5 (50μM) and CNQX (20μM).

Field Potential Recordings

A bipolar stainless-steel stimulating electrode and a borosilicate glass recording electrode filled with ACSF were placed in the CeAL to elicit and record extracellular field responses (fEPSPs), respectively. fEPSPs were elicited at a rate of 0.05 Hz, with stimulation intensities ranging from ~100-200μA and measured with a low resistance (2-3 MΩ) extracellular electrode pulled with borosilicate glass on a Flaming-Brown Micropipette Puller (Sutter) and filled with ACSF. Stimulating electrode was placed medial to the recording electrode in the lateral division of the central amygdala (CeAL). Stable baseline fEPSPs were recorded for 20 min, followed by oxo-M (1μM) bath application for the indicated time period. For atropine + oxo-M (1μM) experiments, 1μM atropine was bath applied prior to (~30 min) and during baseline acquisition, as well as during 1μM oxo-M application. For all experiments, the N1 was monitored online and

experiments that demonstrated a ≥ 20% change in N1 were discarded. Analyses measured the percent change of the N2, following drug application, relative to baseline.

Whole-Cell Voltage-Clamp Recordings

Whole-cell voltage-clamp recordings were performed on CeAL neurons easily identified visually by their medium-sized, spherical somata. Patch electrodes were pulled on a Flaming/Brown microelectrode puller (Sutter Instruments) and filled with solution containing (in mM): 120 K^+-gluconate, 4 NaCl, 10 HEPES, 20 KCl, 4 Mg-ATP, 0.3 Na-GTP, and 10 Na-phosphocreatine (pH 7.25-7.35, adjusted with KOH). For intracellular loading of the calcium chelator, BAPTA (Sigma-Aldrich), 20-40mM K^+-gluconate was replaced with 20-40mM BAPTA sodium salt. For all experiments, access resistance (Ra) was monitored online and cells that demonstrated a >20% change in Ra were excluded from analysis. Additionally, a time period of ≥5 minutes, post break-in, was allowed for internal solution exchange and stabilization of membrane properties prior to initiation of experiments.

Monosynaptic evoked excitatory postsynaptic currents (eEPSCs) were elicited via constant-current stimulation of local glutamatergic fibers via an ACSF-filled glass electrode placed ~100µm from the cell soma. All recordings were carried out at a holding potential of -70mV. eEPSC amplitudes were typically adjusted to 200-1200pA, with stimulation intensities ranging from 10-70µA. For drug application studies, paired stimulations were elicited at a rate of 0.1Hz, with an interstimulus interval of 50ms, and six consecutive responses were averaged to generate one data point per minute. Following a five- six minute baseline, drugs of interest were bath applied. The magnitude of the drug effect was calculated as a percentage of averaged responses relative to baseline. Calculated paired-pulse ratios (PPRs) were defined as the ratio of the second eEPSC amplitude relative to that of the first. PPR changes in response to alterations

in presynaptic release probability and was compared before and after drug application or DSE induction.

Induction and Quantification of DSE

For DSE studies, responses were evoked with a single stimulation pulse delivered every 5 seconds to generate a 50 sec- and 100 sec- epoch prior to and following DSE induction, respectively. To induce DSE, a depolarizing pulse (-70 to 0mV) was applied to the postsynaptic neuron for 2-10 seconds as specified by the experimental conditions. The maximum DSE was classified as the first eEPSC following the depolarizing pulse. Within each DSE trial, eEPSC amplitudes were normalized to the averaged baseline response and data from two DSE trials, per cell, were averaged for analyses.

LTD Induction Protocol

Stimulus induction of MP-CeAL LTD consisted of 1 seconds of 4-Hz alternating with 1 seconds of rest for 180 iterations (total protocol time of 6 minutes). Following LTD induction, eEPSPs recovered to a new baseline which was generally lower than that of the original.

Chronic Stress Paradigm

Daily, mice were brought into the restraint room and subjected to 1 hour of restraint in modified 50 ml conical tubes for 10 consecutive days (between 0900-1100 hours). During the restraint period, mice were placed in a sound attenuating, ventilated animal housing cabinet. Upon termination of restraint, mice were placed in their home cage and returned to the animal care facility housing room. Control mice were left undisturbed in their home cages, except for tail marking at the beginning of the experiment. After each stress episode, plastic tubes were washed with soap and water, rinsed with 70% ethanol and left to air dry.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.01. Statistical significance between the means of two independent groups was assessed using two-tailed paired or unpaired *t*-test unless variance differed significantly (Bartlett's test for equal variances), in which case non-parametric Mann-Whitney (U) tests were used. Statistical comparisons between two or more groups were performed using one or two-way analysis of variance (ANOVA). F and P values for ANOVA are provided above individual figures. Post hoc analyses were conducted by Dunnett's or Sidak's test as indicated in the text. F-test for equality of variances between two independent groups was also used as indicated in the text. Cumulative probability plots were analyzed by Kolmogorov-Smirnov (KS) test. Statistical significance is indicated as follows: *$p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$. Averaged data are presented as means ± S.E.M.

Anatomical Experiments

For *in situ* hybridization, immunoperoxidase labeling, and electron microscopic analyses anesthetized male C57BL/6 mice (n=12) were perfused transcardially with 100 ml of 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB, pH: 7.4). After perfusion, the brain was removed from the skull, cut into blocks, and 50 µm thick coronal sections containing the amygdala were cut with a Leica Vibratome (Leica Microsystems, Weitzlar, Germany) and kept in washing buffer for further treatment. For confocal microscopy, male ICR mice (Harlan, Indianapolis, IN), 4 to 6 weeks old, were used. Mice were anesthetized using isoflurane and transcardially perfused with cold phosphate buffered solution (10ml) followed by ice-cold 20% formaldehyde. Brains were then removed, postfixed for 24 hours in the same fixative, and cryoprotected in 30% sucrose for 4 days. 40µm coronal sections of the amygdala were cut on a

cryostat (Leica, CM 3050 S) and stored in an anti-freeze solution (0.1M phosphate buffer, ethylene glycol, and sucrose), at -20°C, before fluorescent immunohistochemical staining.

Non-radioactive free-floating *in situ* hybridization was carried out following the protocol as previously described in detail (Peterfi et al., 2012). We prepared antisense and sense riboprobes against a 1170 base pair (bp) long section (from position 1967 to 3136 in the open reading frame) of mouse DAGLα coding sequence using the following primers: forward, 5'-TCA TGG AGG GGC TCA ATA AG; reverse, 5'-CTA GCG TGC CGA GAT GAC CA (Katona et al., 2006). The CB1 riboprobe was generated against a 738 bp long region of mouse CB1 coding sequence (from position 548 to 1285 in the open reading frame; forward primer, 5'-CTA ATC AAA GAC TGA GGT TA; reverse primer, 5'- CAC AGA GCC TCG GCA GAC GT). Free-floating immunoperoxidase staining also followed the previously established protocol (Peterfi et al., 2012). The antibody (diluted 1:3000) against an internal segment of the DAGLα protein was described earlier (Katona et al., 2006a), and its specificity was recently confirmed in DAGLα knockout mouse forebrain sections (Ludanyi et al., 2011). The antibody (diluted 1:200) against the C-terminus of CB1 was previously described in (Fukudome et al., 2004a)) and its specificity has been confirmed in many studies, including the present one. The DAGL-α, MAP2, and M_1 immunohistochemistry presented in Figure 5F-H and Figure 5E were generated using the rabbit anti-DAGLα polyclonal antibody (1:500), the mouse anti-MAP2 monoclonal antibody obtained from Millipore (1:2000), and the rabbit anti-M_1 receptor polyclonal antibody purchased from Alamone labs Ltd. (1:200). Thenceforth, brain sections were washed in 3 changes of tris-buffered saline (TBS), incubated in 10mM sodium citrate (pH=9; 80°C) for 30 minutes, followed by (3x10 min) TBS washes. Subsequently, slices were incubated in TBS+ (TBS supplemented with 4% horse serum and 0.2% Triton X-100) for 30 minutes and in primary antibody (room

temperature) overnight. The next day, slices were washed in TBS+ (3x10 min) and incubated with Alexa Fluor 546 donkey anti-rabbit IgG (Life Technologies, 1:1000) and DyLight 488 donkey anti-mouse IgG (Jackson ImmunoResearch, 1:500) for 2.5 hours at room temperature. Stained slices were then washed using TBS (3x10 min), mounted unto slides with 0.15% gelatin solution, and imaged with a Zeiss LSM 710 confocal microscope. Images were analyzed with Zeiss LSM Image Browser software.

For electron microscopic analyses, after development of the immunostaining, the sections were treated with 0.5% OsO_4, dehydrated in an ascending series of ethanol and acetonitrile solutions, and finally embedded into Durcupan™ ACM Fluka (Sigma). During dehydration, sections were also treated with 1% uranyl acetate in 70% ethanol for 20 minutes. After overnight incubation in Durcupan, the sections were mounted onto glass slides and coverslips were sealed by polymerization of Durcupan at 56 ºC for 48 hours. From sections embedded in Durcupan, areas of interest from the CeAL were re-embedded and re-sectioned for electron microscopy. Sections were collected on Formvar-coated single-slot grids, stained with lead citrate, and examined with a Hitachi 7100 electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

CHAPTER III

EXAMINATION OF ENDOCANNABINOID SIGNALING ELEMENTS AT CeAL GLUTAMATERGIC SYNAPSE

Introduction

Endocannabinoids are a class of bioactive lipids produced by neurons and glia (Kano et al., 2009a). 2-arachidonoylglycerol (2-AG) is thought to be the primary eCB that mediates retrograde synaptic signaling at central synapses (Castillo et al., 2012). 2-AG is post-synaptically synthesized by diacylglycerol lipase α (DAGLα) via calcium- and Gαq-protein-coupled receptor ($G\alpha_q$PCR)-dependent mechanisms (Hashimotodani et al., 2007; Hashimotodani et al., 2005; Ohno-Shosaku et al., 2005; Ohno-Shosaku et al., 2012). In contrast to 2-AG, the mechanisms regulating synaptic anandamide (AEA) synthesis are not well understood, but can involve $G\alpha_q$PCR activation (Chavez et al., 2010a; Grueter et al., 2010a; Huang and Woolley, 2012). Once produced, 2-AG and AEA are primarily degraded by monoacylglycerol lipase and fatty acid amide hydrolase, respectively (Cravatt et al., 2001b; Dinh et al., 2002; Long et al., 2009b), both of which were previously demonstrated to exhibit weak expression patterns within the CeAL (Gulyas et al., 2004a; Yoshida et al., 2011b). This expression, albeit weak, alludes to the potential of eCB mediated signaling despite earlier studies demonstrating the lack of expression and CB1 receptor mediated signaling in this region (Kamprath et al., 2011; Katona et al., 2001). In this study, we address this discrepancy by utilizing new reagents to examine the expression of eCB signaling elements at CeAL glutamatergic synapses. Subsequently, we also examine

whether these signaling elements mediate eCB-mediated synaptic plasticity of CeAL glutamatergic neurotransmission.

Results

Localization of CB1 Receptors in the CeAL

Although prior studies suggested a negligible role of eCBs in the modulation of CeA synaptic signaling (Katona et al., 2001), our *in situ* hybridization studies revealed a detectable CB1 receptor (CB1) *in situ* signal within the CeAL and strong expression in the basolateral amygdala (BLA) of wild-type, but not CB1 knockout (KO; $CB1^{-/-}$), mice (Figure 8A-C). The presence of CB1 mRNA in the majority of BLA neurons suggests that BLA-CeAL glutamatergic terminals may express CB1 protein (Figure 8C). If this were true, we would expect to see CB1 immunoreactivity in the CeAL, in spite of its low CB1 *in situ* signal, as CB1 receptors are preferentially targeted to presynaptic elements (Fukudome et al., 2004b; Kawamura et al., 2006). To test this hypothesis, we employed a CB1 antibody previously used to localize CB1 receptors on excitatory terminals in other brain regions (Uchigashima et al., 2007c) (Yoshida et al., 2011a). Using this antibody, CB1 receptors were detected at high levels in both the CeAL and CeAM of wild type, but not $CB1^{-/-}$ mice (Figure 8D-F). Additionally, electron microscopic (EM) examinations revealed CB1 receptor expression in the majority of presynaptic boutons forming asymmetric synapses onto dendritic shafts and spines within the CeAL (Figure $8G_{1\text{-}2}$ and I).

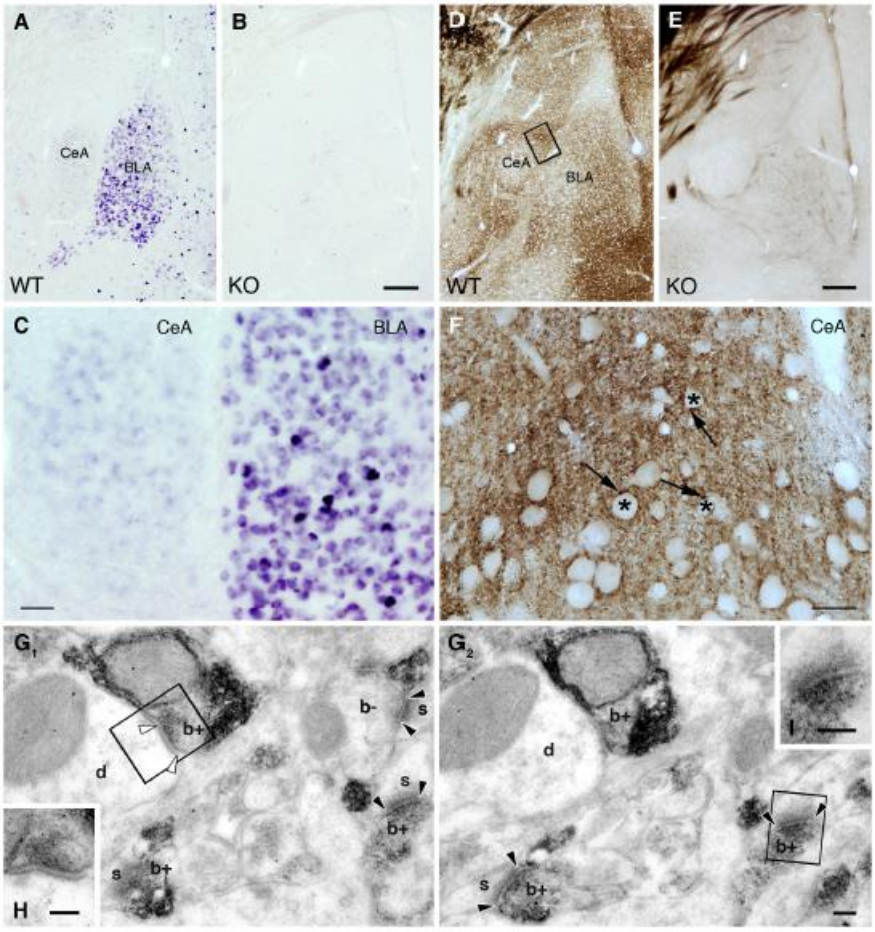

Figure 4. CB1 Receptors are Present on Excitatory Terminals in the CeAL.
(A) In situ hybridization reveals the presence of CB1 mRNA in both the CeA and the BLA of wild type mice. (B) The specificity of the riboprobe is confirmed by using $CB1^{-/-}$ animals. (C) The very high levels of CB1 mRNA observed in a few scattered neurons in the BLA likely correspond to GABAergic interneurons. The vast majority of BLA neurons express moderate levels of CB1 mRNA. In contrast, CB1 mRNA expression in the CeA was only slightly above detection threshold. (D-E) Immunoperoxidase staining demonstrates the presence of the CB1 protein in both the CeA and BLA, which was confirmed in our $CB1^{-/-}$ samples. (F) Higher magnification light micrographs reveal the dense CB1 labeling in the neuropil throughout the CeAL. Asterisks depict CB1-immunonegative cell bodies, whereas CB1-immunopositive labeling appears as punctate staining indicating the compartmentalized distribution of the protein. (G_1-G_2) Serial electron micrographs illustrate the selective presynaptic accumulation of CB1 in boutons (b+), which form mainly asymmetric (flanked by black arrowheads) and sometimes symmetric (white arrowheads) synapses with dendrites (d) and spine heads (s). CB1 staining remained under detection threshold in a few axon terminals (b-), which highlights quantitative differences in CB1 expression between terminal types innervating the CeAL. (H-I) The anatomical nature of the synapse type is illustrated at higher magnification. Scale bars: A, B, D, E are 200 µm; C is 50 µm; F is 20 µm; G_1, G_2, H, I are 100 nm.

Localization of DAGLα Expression in the CeAL

Considering that 2-AG is one of the primary ligands mediating eCB retrograde signaling at central synapses, we next examined the expression of the 2-AG synthesizing enzyme, DAGLα, in the CeA. *In situ* hybridization confirmed the expression of DAGLα mRNA in both the BLA and CeAL (Figure 5A-C). Subsequent immunohistochemical analyses, using an anti-DAGLα antibody whose specificity in the forebrain has been confirmed in DAGLα$^{-/-}$ mice (Ludanyi et al., 2011), uncovered punctate staining patterns throughout the CeAL (Figure 5D-E). Furthermore, double immunofluorescence labeling and subsequent confocal microscopy revealed DAGLα-positive puncta in close apposition to and a few immunoreactive overlap with MAP2 labeled dendritic shafts in the CeAL (Figure 5F-H). These results suggest that DAGLα is localized within and outside of dendrites either in pre- or postsynaptic compartments. To differentiate between these two possibilities, we performed immunoperoxidase labeling and utilized EM to visualize DAGLα at the synaptic level. We found that DAGLα was indeed localized postsynaptically in the majority of dendritic shafts and spine heads forming asymmetric synapses in the CeAL (Figure 5I-L). Taken together these data conclusively demonstrate the presence of eCB signaling elements at glutamatergic synapses in the CeAL.

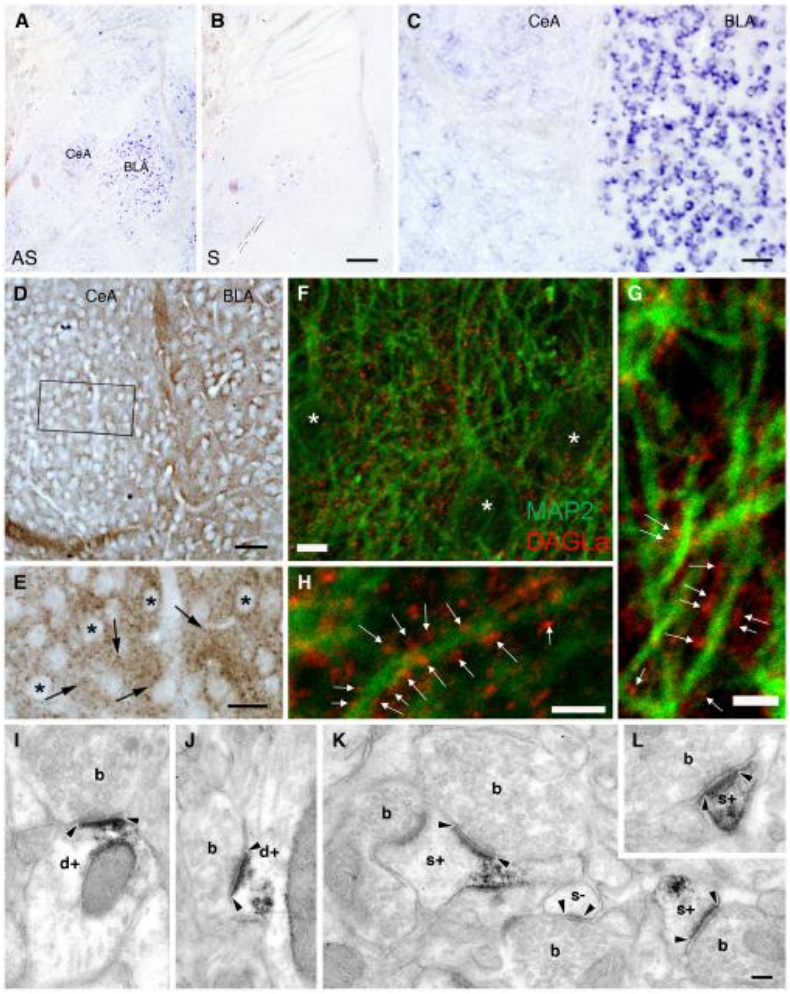

Figure 5. DAGLα is a Postsynaptic Enzyme in the CeAL.
(A-B) *In situ* hybridization demonstrates the expression of DAGLα mRNA in both the BLA and CeA. AS and S depicts experiments performed by antisense and sense riboprobes, respectively. (C) Expression of DAGLα mRNA is notably higher in the BLA compared to the CeA. (D) However, at the protein level there is less difference between the two regions. (E) High magnification of the boxed region in D reveals that granular DAGLα-immunoreactivity (labeled by arrows) is present in the neuropil among cell bodies. (F-H) Confocal immunofluorescence analysis shows that DAGLα-immunoreactivity (red puncta indicated by white arrows) outlines MAP2-positive dendritic profiles (green). Asterisks denote CeAL cell bodies. (I-L) Electron micrographs provide ample evidence for the postsynaptic localization of DAGLα. Immunoreactivity represented by the black diaminobenzidine (DAB) precipitate was often present in dendrites (d+) and spine heads (s+), but never in boutons (b). Black arrowheads indicate the edge of the asymmetric synapses. Black arrowheads highlight asymmetric synapses. Scale bars: A-B are 200 µm; C-D are 50 µm; E is 20 µm; F is 5µm; G-H are 2.5µm; I-L are 100 nm.

CB1 Receptors Modulate Glutamate Release onto CeAL Neurons

To determine the functional significance of CB1 receptor expression in the CeAL, we conducted whole-cell voltage-clamp electrophysiological recordings in the presence of saturating concentrations of the $GABA_A$ receptor antagonist, picrotoxin (25-50μM), to isolate glutamatergic currents. Consistent with the localization of CB1 receptors on excitatory axon terminals in the CeAL, we found that activation of CB1 receptors with the cannabinoid agonist ,CP55940 (5μM), significantly depressed eEPSC amplitude to 52±4% of baseline in CeAL neurons from wild-type (WT) mice (WT 52.47±3.94% vs. $CB1^{-/-}$ 114±8%; $t(8)=7.18$, $p<0.0001$; Figure 6A-B). No significant effect on PPR was observed following 5μM CP55940 application to WT or $CB1^{-/-}$ cells (normalized PPR: WT 1.06±0.06 vs. $CB1^{-/-}$ 0.89±0.06; $t(8)=2$, $p=0.08$; Figure 6C). Additional analyses of spontaneous EPSCs (sEPSCs) revealed a selective effect of 5μM CP55940 to reduce sEPSC frequency (vehicle 4.35±0.92 Hz vs. CP55940 1.59±0.27 Hz; $U=50.00$, $p=0.008$).

Although the selective effect of CP55940 on frequency, but not amplitude, of sEPSCs suggests a presynaptic locus of action, the lack of effect on PPR was surprising. Therefore, we evaluated the effects of 2-AG-ether, a metabolically stable analog of 2-AG (Laine et al., 2002), to better elucidate the mechanisms by which eCB signaling with an endogenous (Hanus et al., 2001a), rather than a synthetic, agonist modulates glutamate release. Indeed, 50 μM 2-AG-ether caused robust synaptic depression (baseline 100.3±1.2% vs. 2-AG-ether 49.1±9.5%; $t(3)=6.13$, $p<0.01$ by paired *t*-test; Figure 6G) that was associated with a significant increase in PPR ($t(3)=3.9$, $p<0.05$ by paired *t*-test; Figure 6G inset). Collectively, these data indicate that CB1 function to suppress glutamate release onto CeAL neurons.

Since CB1 receptors in other brain regions robustly modulate GABAergic transmission (Castillo et al., 2012; Kano et al., 2009a), we also tested the effects of CP55940 (5μM) on GABAergic currents in the CeAL recorded in the presence of CNQX (20μM) and AP-5 (50μM). Generally consistent with our previous report (Katona et al., 2001), and our electron microscopic observation of only a few CeAL GABAergic terminals being CB1-positive (Figure 4G_1-G_2 and H), the effects on GABAergic transmission were small and inconsistent (baseline 100.0±0.0% vs. 76.1±9.3%; $t(7)=2.6$, $p<0.05$; Figure 6H). When compared to the effects of CP55940 (5μM) on glutamatergic transmission (from CeAL cells depicted in Fig. 6B), CP55940-induced depression of GABAergic transmission showed a significantly greater variance compared to effects on glutamate release (F-test to compare variances, $p<0.05$; Figure 6I). These data suggest that the major role of CB1 signaling in the CeAL is to broadly regulate glutamatergic transmission, while synapse- or cell type-specific modulation of GABAergic transmission may also occur.

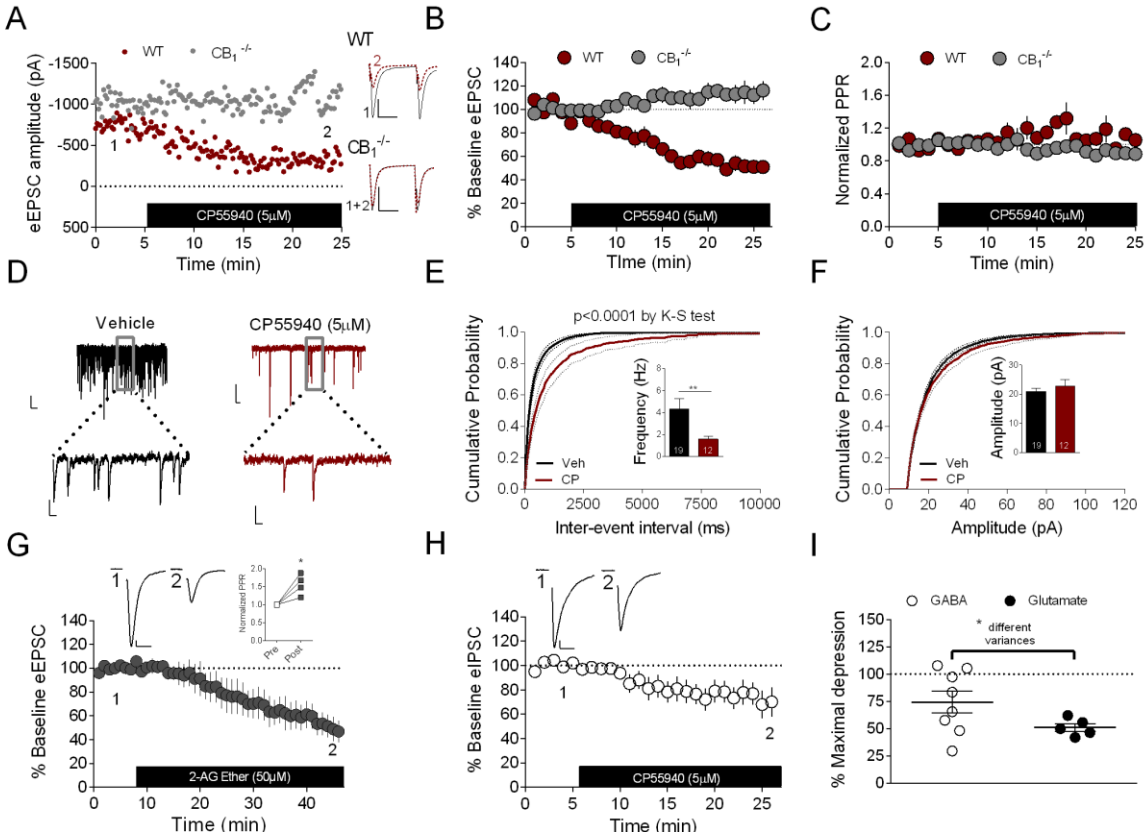

Figure 6. Activation of CB1 Receptors Modulate Glutamate Release in the CeAL.
(A-C) CP55940 depresses eEPSC amplitude in WT but not CB1$^{-/-}$ mice, but does not affect PPR. (D-F) CP55940 reduces sEPSC frequency (E) but not amplitude (F). (G) 2-AG-ether depresses eEPSC amplitude and increases PPR (inset). (H) CP55940 decreases eIPSC amplitude. (I) Comparison of CP55940 effects on eIPSC and eEPSC amplitude. *p<0.05, **p<0.01. Calibration scale bars in (A): 200pA, 25ms. Calibration scale bars for sEPSCs (D) at lower magnification (10pA, 100ms) and higher magnification (10pA, 20ms). All other scale bars: 10ms, 100pA. Data presented as mean ± SEM.

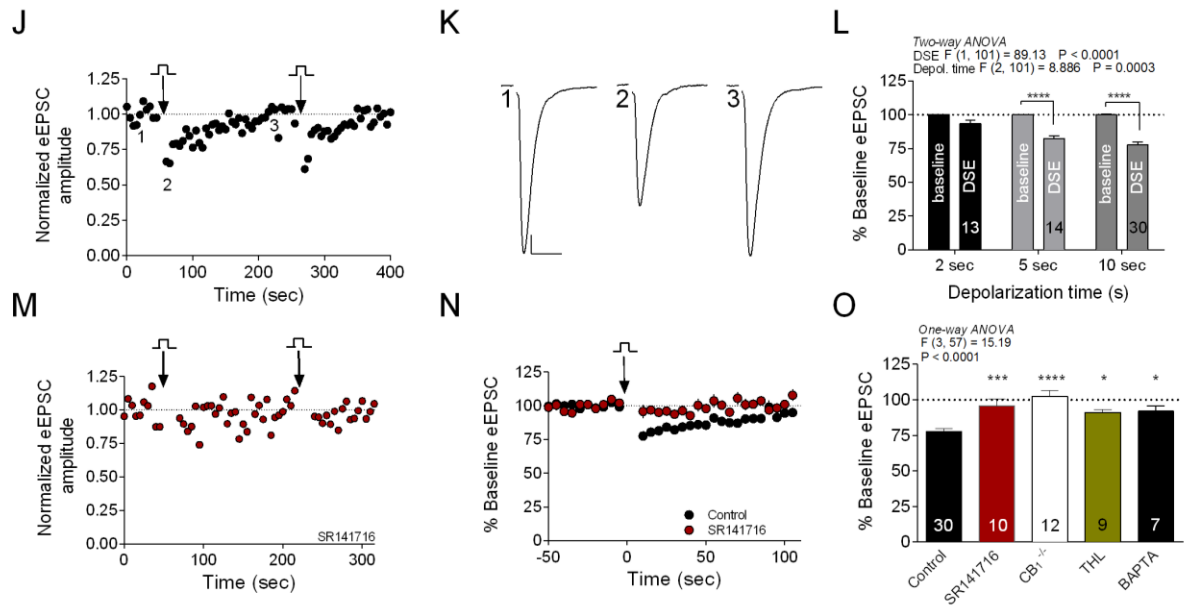

Figure 6 continued (contd.). CeAL Glutamatergic Synapses Exhibit DSE.
(J-L) Effects of postsynaptic depolarization on eEPSC amplitude; DSE in representative cell (J-K), and summary data of DSE after 2, 5 or 10 seconds of postsynaptic depolarization relative to corresponding baseline (L). (M-N) Effects of SR141716 on DSE after 10- second depolarization. (O) Summary data showing effects of SR141716, CB1 deletion, THL, and intracellular BAPTA loading on DSE magnitude relative to control 10 second DSE. Control group in (O) represents the same data set as 10 second depolarization in (L). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Numbers of tested cells are indicated in bars for this and subsequent figures. All scale bars: 10ms, 100pA. Data presented as mean ± SEM. Also see Figure 7.

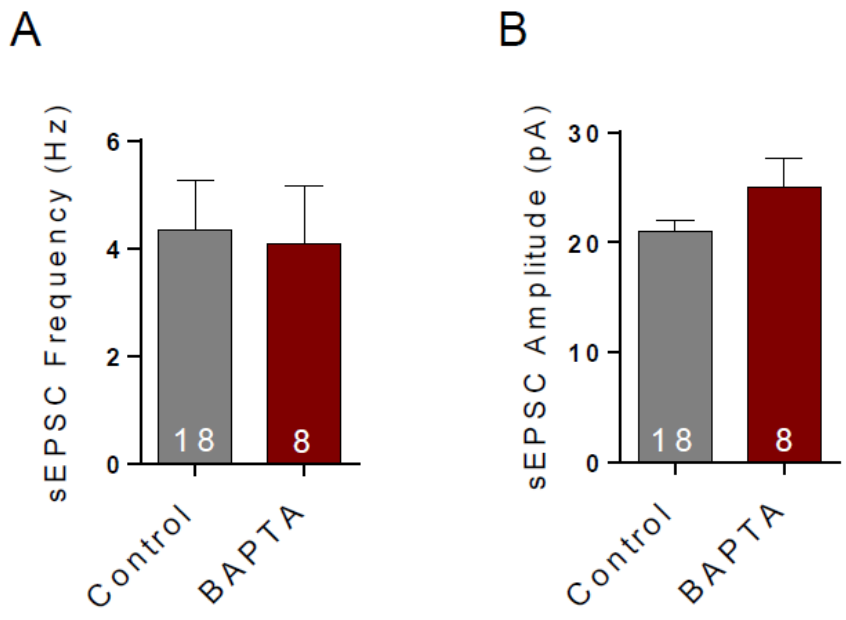

Figure 7. Effects of intracellular BAPTA on baseline glutamatergic transmission in the CeAL, related to Figure 6.

Intracellular BAPTA does not affect CeAL spontaneous excitatory postsynaptic current (sEPSC) (A) frequency or (B) amplitude. (Frequency: control 4.35±0.9 Hz vs. BAPTA 4.1±1.0 Hz, $p>0.05$; Figure 7A); (Amplitude: control 21.0±1.0 pA vs. BAPTA 25.1±2.6 pA, $p>0.05$; Figure 7B). Statistical comparison performed using unpaired *t*-test.

Ca^{2+}-Driven eCB Release in the CeAL

We next examined whether CeAL glutamatergic synapses express depolarization-induced suppression of excitation (DSE), a Ca^{2+}-DAGLα-dependent form of 2-AG-mediated eCB retrograde signaling (Ohno-Shosaku et al., 2012). Two-way ANOVA revealed a significant effect of DSE (depolarization) and postsynaptic depolarization duration (Figure 6J-L) on eEPSC amplitude. Post-hoc Sidak's analysis revealed depolarization of CeAL neurons from -70 to 0 mV resulted in a transient depression of eEPSC amplitude that was significantly different from corresponding baseline values after 5 ($p<0.001$) and 10 seconds ($p<0.001$) of postsynaptic depolarization. One-way ANOVA followed by Dunnett's post hoc analysis revealed that CeAL 10 second DSE was blocked by the CB1 receptor antagonist, SR141716 (control 77.65±2.06% vs. 5μM SR141716 95.84±4.84%, $p<0.001$; Figure 6M-O) and absent in $CB1^{-/-}$ mice ($CB1^{-/-}$ 102.5±3.84%, $p<0.0001$; Figure 6O). DSE was also blocked by the DAGL inhibitor THL (10μM THL 91.20±2.13%, $p<0.05$; Figure 6O) and postsynaptic calcium chelation with 40mM BAPTA (BAPTA 92.07±1.46%, $p<0.05$; Figure 6O), indicating that Ca^{2+}-driven short-term eCB mobilization at CeAL glutamatergic synapses is mediated by 2-AG activation of CB1 receptors. Intracellular loading of BAPTA alone did not affect frequency or amplitude of eEPSCs in CeAL neurons (control frequency 4.35±0.9 Hz vs. BAPTA 4.1±1.0 Hz, $p>0.05$; control amplitude 21.0±1.0 pA vs. BAPTA 25.1±2.6, $p>0.05$; Figure 7).

Discussion

eCB Signaling Components are Present at CeAL Glutamatergic Synapses

Our results demonstrate DAGLα and CB1 receptor are expressed and functional at CeAL glutamatergic synapses. DAGLα protein expression is observed in the CeAL at both the mRNA and protein level, with ultrastructural studies demonstrating clear localization within

postsynaptic dendritic spine heads and dendritic shafts adjacent to asymmetric synapses. This localization of DAGLα to dendritic spine heads and shafts is consistent with earlier studies showing: 1) DAGLα present, but not forming perisomatic clustering, within the CeA (Yoshida et al., 2011b) and 2) a similar pattern of DAGLα synaptic localization at CB1-expressing excitatory terminals in other brain regions such as the hippocampus, cerebellum, and striatum (Uchigashima et al., 2007c; Yoshida et al., 2011b).

In contrast to DAGLα, our high-resolution analyses showed CB1 localized to presynaptic terminals forming asymmetric synapses onto postsynaptic dendrites and dendritic spines (Kano et al., 2009b). Earlier expression studies demonstrate CB1 present within the CeA (Matsuda et al., 1990; Tsou et al., 1998a), yet later studies argued against CB1 receptor expression in this region (Kamprath et al., 2011; Katona et al., 2001). As such, we utilized an antibody previously used to detect CB1 expression at glutamatergic synapses to conclusively address this discrepancy (see (Katona et al., 2006a; Uchigashima et al., 2007b). These experimental results unambiguously demonstrate the presence of CB1 protein at excitatory CeAL terminals.

We hypothesize that the lack of CB1 detection in earlier studies arose as a result of two reasons. The first is potential qualitative differences in CB1 characteristics at glutamatergic and GABAergic terminals that differently affect CB1 antigen recognition. Such differences may include differential masking by anchoring proteins such as CB1-receptor interacting protein (CRIP) (Niehaus et al., 2007) or distinct intracellular conformations that result in disparate levels of access to the CB1 antibody recognition site at the two terminals. In support of this hypothesis, antibodies that successfully detected CB1 receptor expression within the CeA targeted the N termini of CB1 (Matsuda et al., 1990; Tsou et al., 1998a) while antibodies that failed to detect CB1 expression above detection threshold where targeted to the C-termini, which is located

intracellularly (Kamprath et al., 2011; Katona et al., 2001). The antibody used in this study addresses this problem as it is a polyclonal antibody, thus it is a mixture of ~30 individual monoclonal IgGs which increases the probability of having IgGs that are able to recognize a broader scale of CB1 receptor conformations that perhaps include unique receptor conformations unique to glutamatergic terminals. If this hypothesis were true, CB1 expression on glutamatergic terminals of the CeAL would be above threshold using this as compared to previously utilized antibodies.

Alternatively, quantitative differences in CB1 receptor expression in the CeAL may also underlie the lack of previous CB1 detection in this region. Immunohistological experiments in other brain regions have noted a difference of up to 30 fold on GABAergic terminals as compared to glutamatergic terminals (Kawamura et al., 2006). If this pattern of high CB1 density detection is true for all brain regions, CB1 levels at CeAL glutamatergic synapses may be below threshold in density and, thus, remained undetected by previously utilized antibodies. Given that we are able to successfully demonstrate CB1 expression at glutamatergic synapses, the CB1-antibody used in this study likely has anti-CB1 IgGs in a high enough titer to be able to bind to the few CB1 that are expressed in this region. However, a final measurement of IgG concentrations, using spectrophotometry, for this and previously used CB1 antibodies would be needed to provide stronger support for this latter hypothesis.

Functional CB1 Receptors are Present at CeAL Glutamatergic Synapses

Consistent with the anatomical data, our additional analysis of eCB signaling also supports the presence of functional CB1 in the CeAL. We found that direct activation of CB1 reliably reduced eEPSC amplitude, while effects on GABAergic transmission were more variable. The greater CB1 sensitivity of glutamatergic as compared to GABAergic synapses is consistent with our

anatomical data, which shows less CB1 receptors expressed at inhibitory synapses. This data also explains the lack of CB1 sensitivity reported earlier (Katona et al., 2001) and is a potential mechanism for eCB signaling modulation of inhibitory signaling within the CeAL microcircuitry.

This pattern of terminal bias in CB1 density and sensitivity, however, is inconsistent with that which is observed in other brain regions (Kano et al 2009). As alluded to earlier, CB1 receptors display a higher density and greater CB1 sensitivity at inhibitory as compared to excitatory terminals in other regions of the amygdala (Yoshida et al 2011) and extra-amygdalar CB1-sensitive brain regions (Kano et al., 2009a). Utilizing a similar CB1 antibody (Fukudome et al., 2004b), these studies revealed CB1 receptor expression differences that ranges from 3-4 fold in striatum (Uchigashima et al., 2007c) to 30 fold in the hippocampus (Kawamura et al., 2006). Analogous to the terminal-differences in CB1 sensitivity found in our study, the difference in CB1 density in these brain regions corresponds with a similar disparity in the CB1 activation threshold for both terminal types (Tanimura et al., 2012; Tanimura et al., 2010). As such, the CeAL appears to be unique in its terminal preference for CB1 receptor density and sensitivity and, given the role of glutamatergic signaling in driving CeAL activity (Ciocchi et al., 2010), suggests that eCB signaling at CeAL glutamatergic terminals is positioned to strongly modulate central amygdala activation and, therefore, function.

Potential Mechanisms for CB1 Mediated Synaptic Depression at CeAL Glutamatergic Synapses

Consistent with a presynaptic locus of CB1 activity, 2-AG-ether and DSE both induce synaptic depression associated with an increase in PPR, i.e. paired pulse facilitation (PPF). This presynaptic locus of CB1 activity is further supported by changes in the frequency, but not amplitude, of sEPSCs following CP55940 (CP) bath application. However, in contrast to these

data, CP55940 application reduced *evoked* EPSCs (eEPSCs) without having an effect on PPR which suggests that CP-mediated inhibition of eEPSC does not occur through CB1-induced decreases in glutamate release probability. This result is surprising given our other data supporting a presynaptic locus of CB1 receptor activity in this region. Furthermore, previous studies have observed a presynaptic locus of CB1 agonist-mediated glutamatergic depression as indicated by CP-induced PPF in the extended amygdala (Puente et al., 2011a) and decreased coefficient of variation in the prefrontal cortex (Lafourcade et al., 2007). Traditionally, most experimental interrogations of exogenous CB1 agonist-mediated synaptic depression have utilized WIN 55212-2 (WIN), a member of the aminoalkylindole class of cannabinoid agonists (Howlett et al., 2002). These experiments have consistently demonstrated a presynaptic locus, via PPF, of CB1-inhibition of synaptic transmission (Kano et al., 2009a). As such, our CP mediated effects on CeAL eEPSCs is not consistent with our other data or with that of other CB1 exogenous agonists application to eEPSCs in other brain regions.

The inconsistency in CP-mediated effects on CeAL eEPSCs can be explained by the following possibilities. First, WIN 55212-2 and CP55940 are dissimilar structurally and generate different receptor conformations upon agonist binding (Georgieva et al., 2008), with WIN shown to interact with distinct residues than those targeted by classical, non-classical, and eicosanoid agonists (Shim and Howlett, 2006). These differences are thought to be one of the underlying reasons why WIN is the least restrictive of the cannabinoid agonists in its ability to activate all CB1-G protein subtypes (CB1-$G\alpha_i$ = CB1-$G\alpha_s$ > CB1-$G\alpha_{q/11}$= CB1-$G\alpha_o$). Conversely, CP demonstrates greater selectivity in CB1-G protein activation as it preferentially activates CB1-$G\alpha_i$ signaling (Bonhaus et al., 1998; McIntosh et al., 2007) and thus was our agonist of choice. Therefore, CP55940 activation of CB1 may selectively target the synaptic release machinery

primarily via $G\alpha_{i/o}$ coupled mechanisms which, based on the WIN-induced pleiotropic CB1-G protein signaling, may manifest as a different manner of presynaptic inhibition than that observed with WIN or other CB1 agonists (Hudson et al., 2010b).

One such mechanism, which serves as a second possibility for the observed inconsistencies, is Ca^{2+}-independent attenuation of vesicle release. Though $G\alpha_{i/o}$-coupled inhibition of synaptic release via Ca^{2+}-dependent mechanisms, i.e. decreased $[Ca^{2+}]_i$, is well established (Kreitzer and Regehr, 2001) (Herlitze et al., 1996; Ohno-Shosaku et al., 2001; Wu and Saggau, 1995a), Ca^{2+}-independent inhibition is also observed following the activation of $G\alpha_{i/o}$-coupled GPCRs (Blackmer et al., 2001; Delaney et al., 2007; Silinsky, 1984). *In vitro* experiments demonstrate that this mechanism occurs via $\beta\gamma$ interactions with the C-terminus of the SNAP-25 protein which, in turn, occludes the synaptotagmin-SNAP25 interactions needed for calcium dependent vesicular release (Yoon et al., 2007; Zhao et al., 2010) following activity induced increases in $[Ca^{2+}]_i$. This alternative pathway explains receptor-mediated inhibition of spontaneous release events (mEPSCs) that occurs independently of evoked calcium entry in the peripheral and central nervous system (Silinsky, 1984; Stephens, 2009). Additionally, both Ca^{2+}-dependent and -independent forms of $G\beta\gamma$ signaling: 1) co-exist in the same terminal yet are recruited by different types of presynaptic receptors (Hamid et al., 2014) and 2) can be mobilized by the same GPCR localized in different brain regions (Cox et al., 2000; Wu and Saggau, 1995b). Though the mechanisms underlying this diversity is not well understood, agonist-induced functional selectivity of recruited effectors and regional differences in the expression of $G\beta\gamma$ subunits, respectively, are potential reasons underlying this diversity in $G\alpha_{i/o}$-recruited Ca^{2+}-dependent and -independent vesicular release (Betke et al., 2014).

Likewise, noradrenergic-α_{2A} receptor activation inhibit Ca^{2+} channels in sensory neurons (Dunlap and Fischbach, 1978), yet *ex vivo* examinations of noradrenergic-α_{2A} receptors reveal a $G\alpha_{i/o}$-coupled $G\beta\gamma$/SNARE-mediated inhibition of vesicular release at CeAL excitatory terminals (Delaney et al., 2007). Given the lack of $[Ca^{2+}]_i$ dependence on the inhibition of synaptic release, agonist-induced-$G\beta\gamma$/SNARE interactions affected the number of presynaptic release sites (N), with no measurable effects of PPR. Given that these excitatory terminals are also recruited in our population of evoked EPSCs, the potential for CP-induced $G\beta\gamma$/SNARE mediated synaptic inhibition, despite evidence of CP-Ca^{2+} dependent mechanisms in other brain regions, potentially explains the lack of change in PPR.

eCB Mobilization by CeAL Neurons Mediates Short-Term Synaptic Plasticity of Excitatory CeAL Synapses

Consistent with our studies demonstrating eCB signaling elements at CeAL excitatory synapses and CB1 receptor-mediated depression of glutamatergic signaling, we found that CeAL neurons express prototypic 2-AG-mediated eCB signaling, i.e. DSE, mediated via a calcium-dependent, THL-sensitive, and CB1-dependent mechanism. Congruent with the calcium dependency of eCB mobilization at central synapses (Kreitzer and Regehr, 2001), increasing CeAL postsynaptic depolarization time increases DSE magnitude as also observed at excitatory synapses within the striatum (Uchigashima et al., 2007c) and the BLA (Yoshida et al., 2011b). Though not significantly different from $CB1^{-/-}$ conditions, BAPTA and THL DSE conditions exhibit non significant residual DSE which may reflect activity-induced residual DAGLα and Ca^{2+}-independent forms of CB1 receptor signaling (Azad et al., 2004).

Conclusion

Collectively, these data support the well-established anatomical substrate for retrograde eCB signaling at central synapses (Katona and Freund, 2012). Furthermore, these studies adds to the previous demonstration of FAAH and MAGL expression in the CeAL (Gulyas et al., 2004a), recent demonstration of DSE in the CeAM (Kamprath et al., 2011), and tonic eCB release at CeAM GABAergic synapses (Roberto et al., 2010b). As discussed above, there are distinct differences in the density and non-classical agonist induced CB1 signaling that appear to be unique to the CeAL as compared to other regions that exhibit eCB mediated synaptic plasticity. As will be discussed in subsequent chapters, anomalous characteristics of CeAL eCB signaling emerges as a strong theme in our analysis of eCB mobilization mechanisms at CeAL glutamatergic synapses.

CHAPTER IV

CeAL GLUTAMATERGIC SYNAPSES EXHIBIT SHORT-TERM PLASTICITY: ROLE OF MUSCARINIC RECEPTOR ACTIVATION

Introduction

In addition to Ca^{2+}-dependent eCB-STD, pharmacological- and afferent stimulation-induced activation of $G\alpha_{q/11}$-receptors also facilitate 2-AG and AEA-mediated synaptic plasticity (Kano et al., 2009a). These mechanisms of eCB-mediated synaptic modulations are known as: 1) calcium-assisted receptor driven eCB release (Ca^{2+}-RER) produced from the synergistic effect of depolarization induced $[Ca^{2+}]_i$ elevation and $G\alpha_{q/11}$-coupled receptor activation (Hashimotodani et al., 2005) as well as 2) $G\alpha_{q/11}$-receptor-driven eCB release (RER) under basal Ca^{2+} conditions (Maejima et al., 2001)—both of which results in enhanced eCB production at central synapses via the mobilization of PLCβ following $G\alpha_{q/11}$-coupled receptor activation (Kano et al., 2009a).

Of particular relevance to the CeAL is $G\alpha_{q/11}$- coupled muscarinic receptors (mAChRs) given that the CeAL receives dense cholinergic inputs from the basal forebrain (Hecker and Mesulam, 1994) in addition to exhibiting high expression of $G\alpha_{q/11}$- mAChRs, namely M_1/M_3 muscarinic receptor subtypes (Levey, 1993; Roozendaal et al., 1997b; van der Zee et al., 1997) ,as well as, other cholinergic synaptic components (Schafer et al., 1998a). As such, the substrate for $G\alpha_{q/11}$–coupled mAChR mediated Ca^{2+}-RER and RER are present within the CeAL. Indeed, other brain regions with similar expression patterns of cholinergic signaling components, such as

the hippocampus and striatum, exhibit mAChR-mediated enhanced eCB mobilization (Kim et al., 2002b; Narushima et al., 2007b; Uchigashima et al., 2007c). Furthermore, acute and chronic stress exposure enhances cholinergic signaling in limbic brain regions such as the hippocampus (Finkelstein et al., 1985; Gilad et al., 1987; Gonzalez and Pazos, 1992) and amygdala (Mark et al., 1996), thus increased cholinergic signaling in the CeAL may enhance eCB influence on CeAL functionality in the stress response.

To determine whether mAChR-mediated forms of eCB mobilization takes place at CeAL glutamatergic synapses, we first examined whether $G\alpha_{q/11}$-coupled mAChRs are expressed and functional in the CeAL. Subsequently, we determined whether mAChR activation can induce eCB mobilization in the form of Ca^{2+}-assisted- and mAChR receptor-driven eCB release at CeAL glutamatergic synapses.

Results

Depolarization-dependent mACh-Receptor Driven eCB Release Occurs in the CeAL Following Prolonged mAChR Activation

To determine whether activation of mAChRs drives eCB mobilization in the CeAL, we first sought to examine the functional effects of mAChR activation on CeAL glutamatergic transmission. Experimental results from CeAL field potential recordings (fEPSPs) demonstrated that bath application of the mAChR agonist, Oxo-M (1μM), reduced the amplitude of fEPSPs to 44.40±3.69% of baseline (baseline 100.90±1.18% vs. maximal Oxo-M-induced depression 44.40±3.69%, $p<0.0001$ by paired t-test; Figure 8A), an effect that reversed following drug washout (baseline 100.90±1.18% vs. post Oxo-M washout 96.14±7.13%, $p=0.79$ by paired t-test; Figure 4A). To test whether this Oxo-M induced depression was mediated by mAChR activation, we bath applied 1μM atropine, a non-selective mAChR antagonist, prior to and during Oxo-M

(1μM) application. Atropine application completely blocked the effect of 1μM Oxo-M on fEPSPs (baseline 100.3±0.79% vs. atropine+Oxo-M 96.47±4.38%, p=0.87 by paired *t*-test; Figure 8A). Using whole-cell recordings we found that Oxo-M caused robust depression of eEPSC amplitude, an effect reduced by the M_1-preferring antagonist, pirenzepine (1μM; $p<0.0001$) and eliminated by the M_3-preferring antagonist 4-DAMP (500 nM, $p<0.0001$; Figure. 8B). Oxo-M-induced synaptic depression was associated with a large increase in PPR, which was attenuated by pirenzepine ($p<0.001$) and blocked by 4-DAMP ($p<0.0001$; Figure 8C), suggesting Oxo-M induced synaptic depression is mediated by M_1/M_3 receptor activation and expressed presynaptically. Importantly, neither pirenzepine nor 4-DAMP exerted any effects on basal glutamatergic transmission when applied alone to control CeAL slices (Figure 9). Additionally, our immunofluorescence confocal microscopy data revealed a moderate expression of the M_1 receptor subtype throughout the CeAL (Figure 8E1-E2). At high magnification, M_1 staining appears as tiny puncta closely apposed to, but not overlapping with, MAP2-positive dendrites and perikarya. Together, these data demonstrate the presence of functional M_1/M_3 mAChRs in the CeAL.

mAChR Activation Enhances CeAL DSE in a CB1 and M1/M3 Dependent Manner

On account of M_1/M_3 mAChRs functionally present at CeAL glutamatergic synapses, we next evaluated the presence of mAChR-driven eCB release at excitatory synapses within the CeAL. It has been previously reported that DSE is effectively enhanced by the coincidental activation of $G\alpha_{q/11}$-coupled receptors, such as M_1/M_3 receptors, via a mechanism involving Ca^{2+} enhancement of PLCβ activity (Hashimotodani et al., 2005; Kim et al., 2002a; Narushima et al., 2006). Consistent with the presence of Ca^{2+}-assisted $G\alpha_{q/11}$-receptor driven eCB mobilization, our results revealed that pretreatment with Oxo-M (1μM), for at least 30 minutes, significantly

enhanced 10s DSE as compared to DSE examined under control conditions (control DSE 84.82±3.0% vs. Oxo-M DSE 60.72±5.76%; $t(18)=4.1$, $p<0.001$; Figure 8F and H). DSE under control and Oxo-M conditions were both associated with increases in PPR ($p<0.05$ and $p<0.01$ respectively by paired t-test; Oxo-M DSE PPR is significantly greater than control DSE PPR $p<0.05$; Figure 8G). Thereafter, we investigated the concentration and postsynaptic depolarization-time dependency of oxo-M-mediated DSE enhancement. Two-way ANOVA revealed a significant effect of depolarization time ($F_{(2,101)}= 20.11$, $p<0.0001$) and oxo-M treatment ($F_{(3,101)}=6.87$, $p=0.0003$; Figure 8H[1]) on CeAL DSE

Since mAChR activation enhances CeAL DSE, we next investigated the mechanisms by which Oxo-M facilitated DSE enhancement of CeAL glutamatergic transmission. One-way ANOVA revealed that 1μM Oxo-M-mediated DSE enhancement was attenuated in both $CB1^{-/-}$ CeAL cells (Oxo-M-WT 57.16±2.56% vs. Oxo-M-$CB1^{-/-}$ 87.03±3.77%, $p<0.0001$; Figure 8 I and L) and CeAL cells pretreated with 10μM THL for at least 60 minutes (Oxo-M 57.16±2.56% vs. THL+ Oxo-M 86.08± 2.73%, $p<0.0001$; Figure 8I and L). These results suggest that the simultaneous activation of mAChRs and postsynaptic depolarization results in the facilitation of 2-AG release at excitatory synapses within the CeAL. We next examined the muscarinic subtypes involved in the Oxo-M-mediated enhancement of depolarization-induced 2-AG release. Application of the M_1- or the M_3-preferring antagonists, 1μM pirenzepine or 500nM 4-DAMP respectively, significantly reduced the 1μM Oxo-M-dependent DSE enhancement (Oxo-M 57.16%±2.56% vs. Oxo-M+pirenzepine 73.92±3.92%, $p<0.01$; Oxo-M 57.16±2.56% vs. Oxo-M+4-DAMP 75.32±4.75%, $p<0.01$, Figure 8J-L). Collectively, these results suggest that both M_1 and M_3 receptors play a role in the mAChR-mediated enhancement of CeAL DSE. Interestingly, in $CB1^{-/-}$ mice and THL (10μM) pretreatment conditions, 10 second depolarization in the

presence of Oxo-M elicited a small residual DSE ($CB1^{-/-}$ baseline 100.0±0.0% vs. Maximal DSE 87.03±3.77%, THL baseline 100.0%±0.0% vs. Maximal DSE 86.08%±2.73%, $p<0.01$ by paired *t*-test for each condition), suggesting possible CB1- and M_1/M_3- independent residual effects induced by depolarization in the presence of Oxo-M.

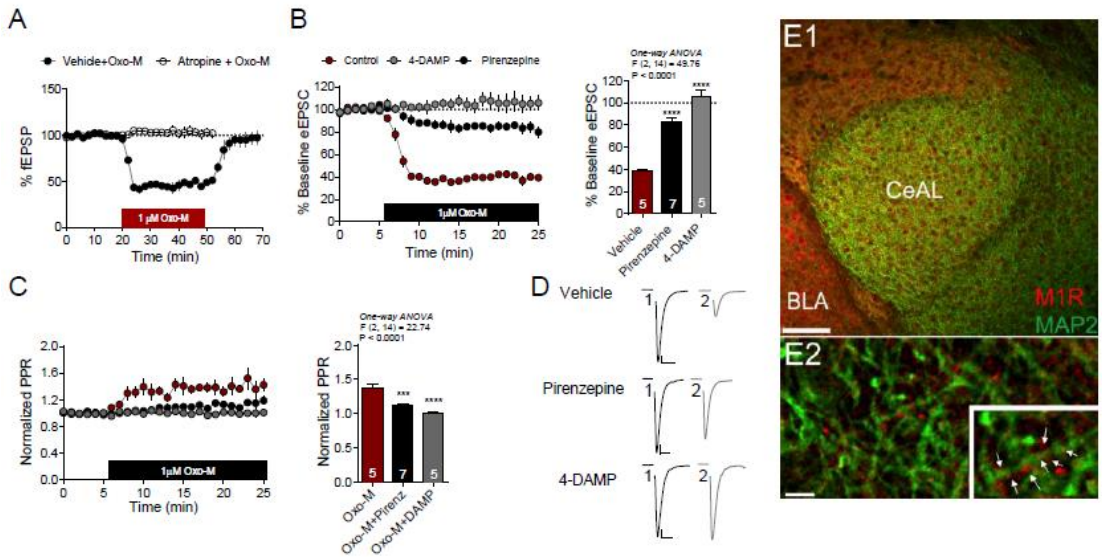

Figure 8. mAChRs Modulate Glutamate Release.
(A) 1 µM Oxo-M depresses fEPSP amplitude, which is blocked in the presence of atropine. (B) Oxo-M-induced eEPSC depression is blocked by pirenzepine and 4-DAMP pretreatment. (C) Oxo-M increases PPR, which is blocked by pirenzepine and 4-DAMP pretreatment. (D) Representative traces of Oxo-M-induced eEPSC depression under vehicle, pirenzepine and 4-DAMP conditions. (E) Distribution of M_1 receptor (red) and the dendritic marker MAP2 (green) in the CeAL at low magnification; higher magnification shows punctate M1 staining in close apposition to MAP2 positive dendritic shafts (arrows in inset) (E1; scale bar 100µm, E2; bar 5µm, inset 7.5µm). **p<0.01, ***p<0.001, ****p<0.0001. Scale bars: 10ms, 100pA. Data presented as mean ± SEM. Also see Figure 9.

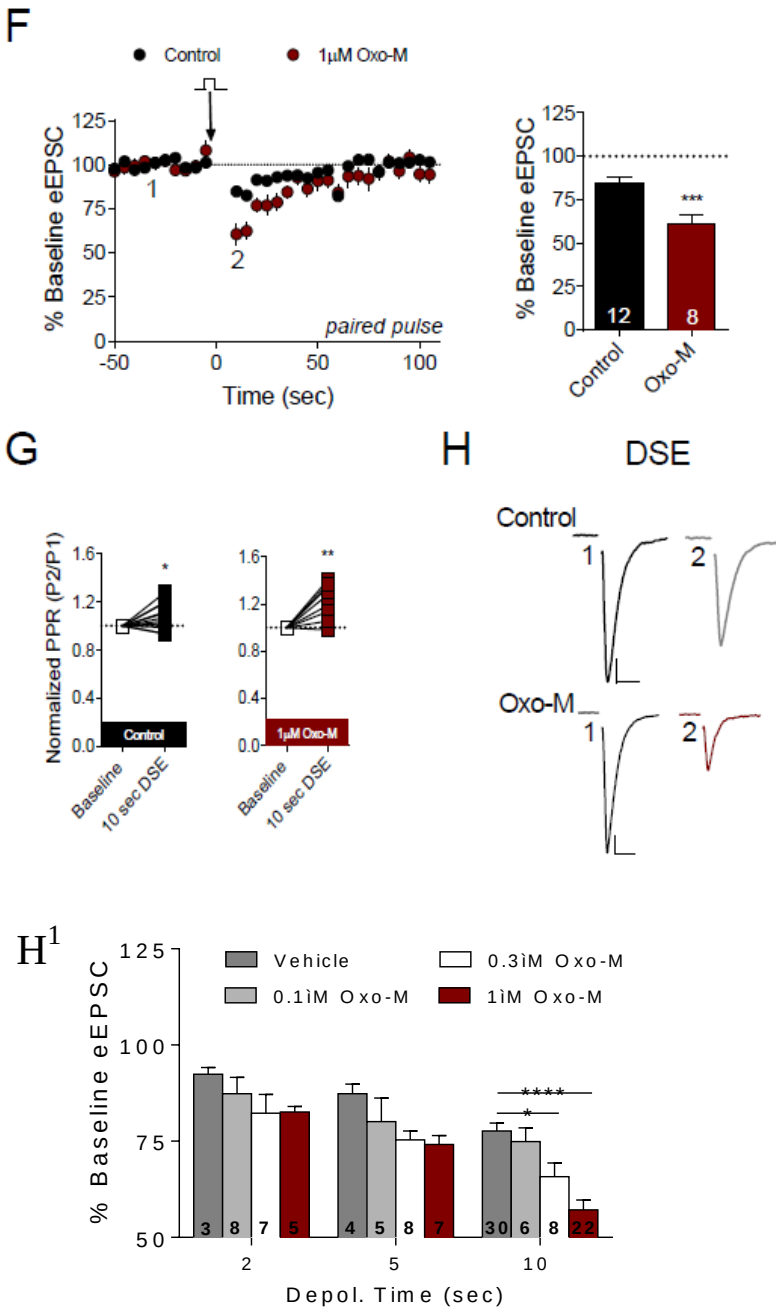

Figure 8 contd. mAChRs Activation Enhance CeAL DSE.
(F) 1μM Oxo-M enhances DSE induced by 10 second depolarization. (G) PPR is increased by 10 second depolarization in both control and Oxo-M conditions. (H) Representative traces of control and Oxo-DSE. * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bars: 10ms, 100pA. Data presented as mean ± SEM.

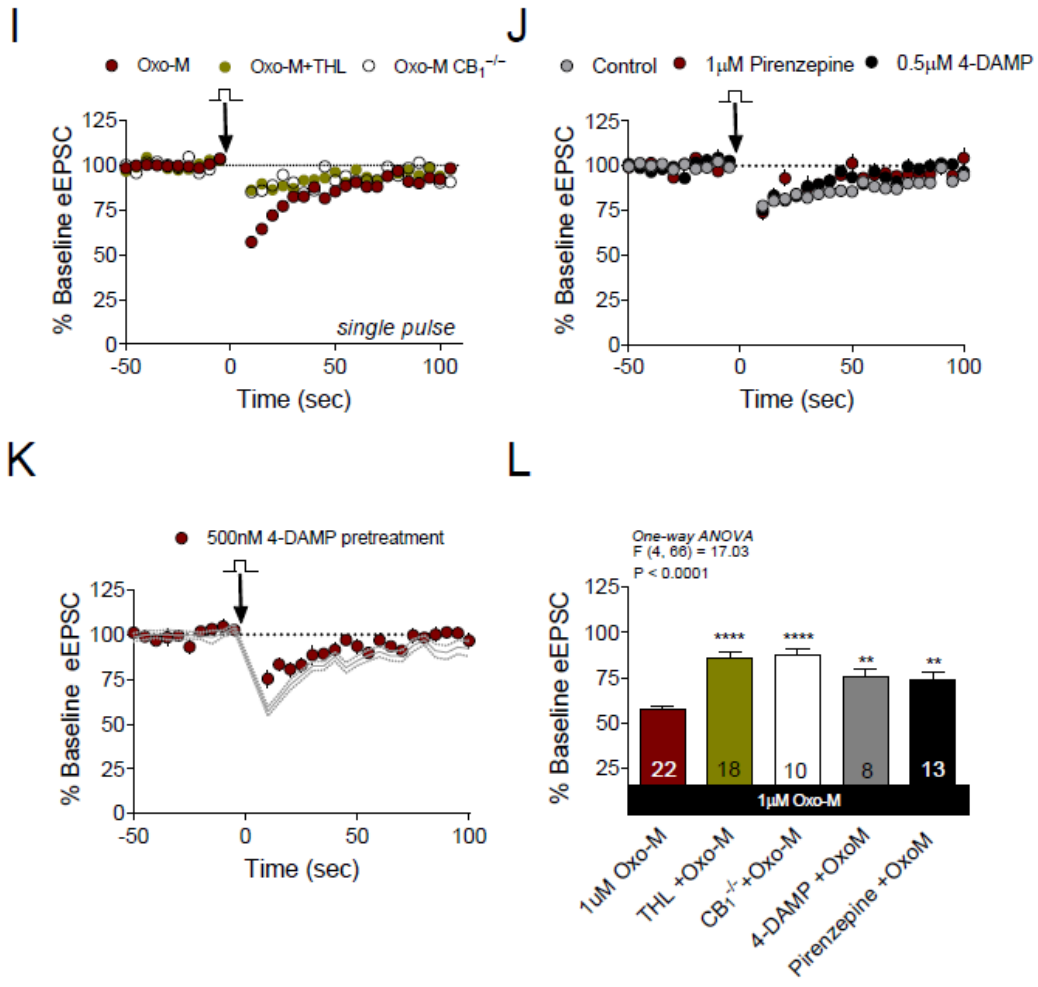

Figure 8 contd. mAChRs-DSE Enhancement is CB1-and M1/M3- Receptor Dependent. (I) DSE in the presence of OXO-M is attenuated by THL and in CB1$^{-/-}$ mice. (J-K) Effects of pirenzepine and 4-DAMP on DSE in the presence of 1μM Oxo-M; grey faded lines represent Oxo-M only DSE condition from (I) for visual comparison purposes. (L) Summary data of the effects of THL, CB1 deletion, pirenzepine, and 4-DAMP on 10 second DSE in the presence of Oxo-M. **p<0.01, ****p<0.0001. Data presented as mean ± SEM. Also see Figure 9.

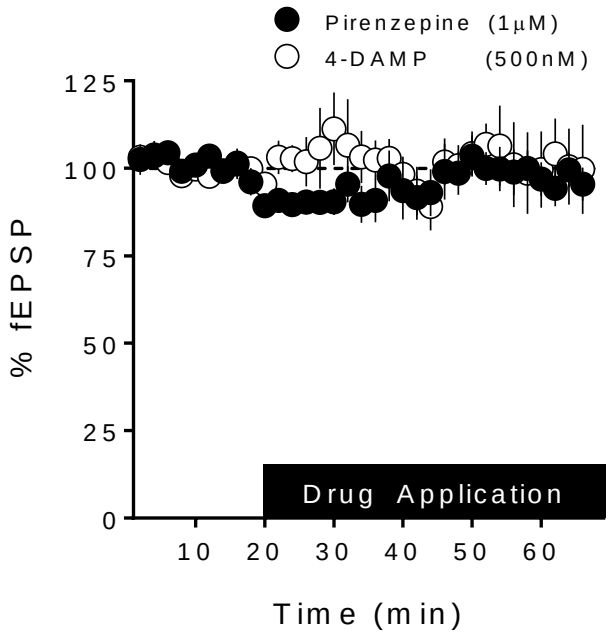

Figure 9. mAChR antagonists do not affect baseline glutamatergic transmission during prolonged drug exposure, related to Figure 8.

Neither pirenzepine nor 4-DAMP affect fEPSPs in the CeAL.

Prolonged mAChR Activation Drives Ca^{2+}- and DAGL-*Dependent* eCB Release

Given the presence of Ca^{2+}-assisted eCB release at CeAL glutamatergic synapses, we next examined mAChR driven endocannabinoid mobilization in the absence of depolarization, i.e., receptor driven eCB release (RER). However, as alluded to earlier, one potential confound of Oxo-M's use is CB1- and M_1/M_3- independent effects of Oxo-M resulting from its high affinity for the largely presynaptic $G\alpha_{i/o}$ mAChRs subtypes, i.e. M_2/M_4 receptors. To exclude the possibility of the CB1-independent component of Oxo-M potentially confounding our analysis, we selectively evaluated CB1-dependent synaptic effects of Oxo-M by examining the effects of CB1 inhibition in the absence and presence of Oxo-M. We reasoned that if prolonged mAChR activation induces tonic eCB release and activation of CB1 that subsequently depresses glutamatergic transmission, bath application of a CB1 receptor antagonist should progressively relieve this tonic eCB inhibition and cause an apparent synaptic potentiation. Thus, this experimental design would allow us to isolate eCB-CB1 mediated synaptic effects induced by prolonged mAChR activation.

To do this we pretreated slices with 1μM Oxo-M for ≥ 60 minutes and subsequently performed whole-cell patch clamp experiments where, after obtaining a stable baseline, we bath applied 5μM SR141716 in the continued presence of 1μM Oxo-M (see Figure10A for experimental design). Consistent with our hypothesis, SR141716 (5μM) wash-on significantly increased eEPSC amplitude in slices pretreated with 1μM Oxo-M relative to control (no Oxo-M) conditions (Figure10B-D). Maximal potentiation induced by SR141716 in the presence of continuous Oxo-M was 143.20±6.59% compared to 113.30±4.09% under control conditions (p<0.001; Figure10B-D). Interestingly, unlike eCB release following brief Oxo-M application, continuous mAChR activation appeared to promote eCB mobilization through a THL- and a

Ca^{2+}-*dependent* mechanism as pretreatment with 10μM THL or 20mM intracellular BAPTA completely abolished SR141716-induced synaptic potentiation ($p<0.0001$ for each condition; Figure10C-E). Maximal SR141716-induced enhancement after 10μM THL pretreatment (vehicle 113.30±4.09% vs. THL + Oxo-M 107.60±7.54%, $p=0.88$; Figure10C-D) or 20mM BAPTA postsynaptic loading (vehicle 113.30±4.09% vs. BAPTA+ Oxo-M 99.25±6.10%, $p=0.34$; Figure10C-D) was not significantly different from SR141716-induced synaptic potentiation under control (no Oxo-M) conditions.

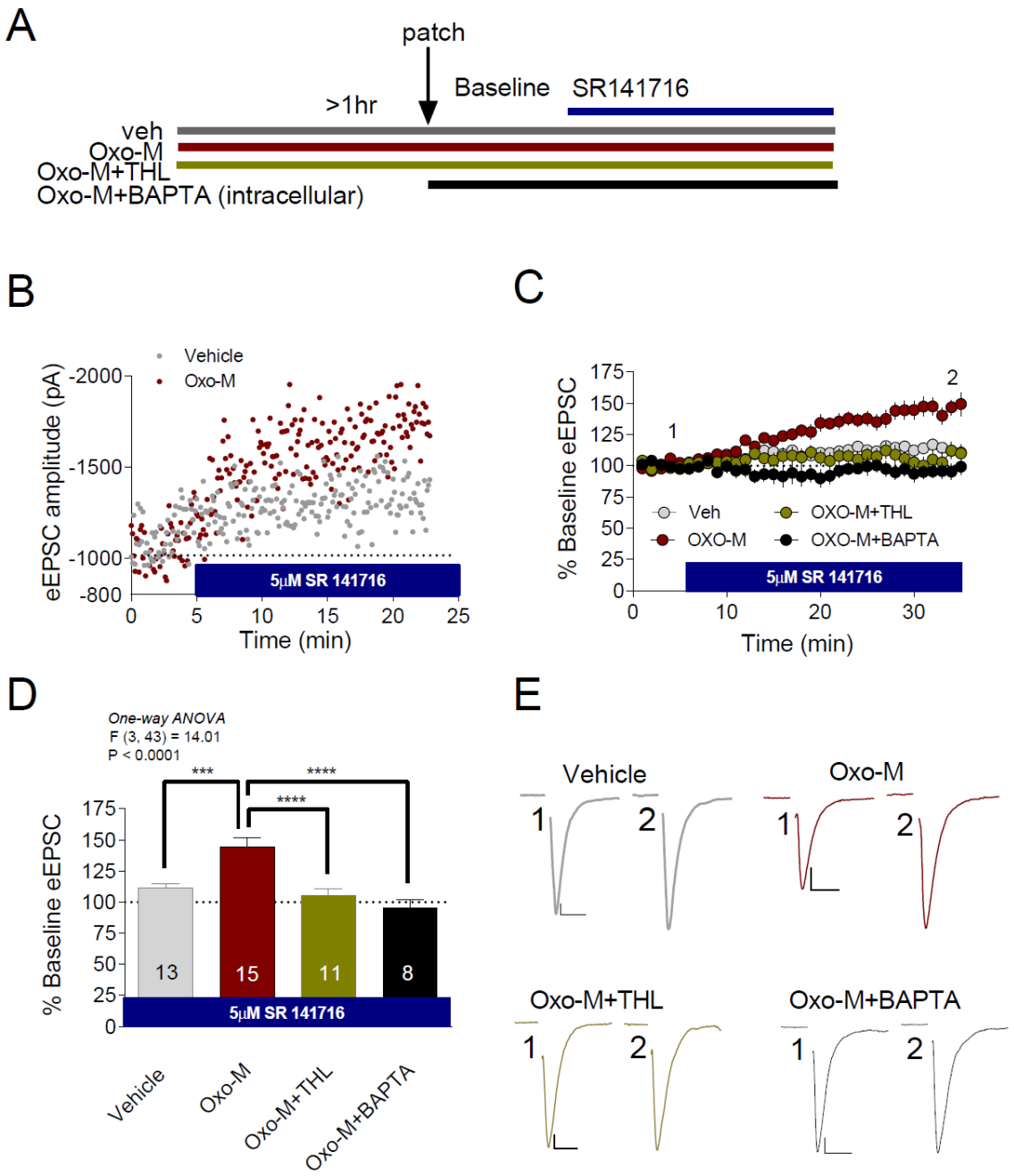

Figure 10. Persistent mAChR Activity Drives Ca2+- and DAGL-Dependent eCB Release. (A) Diagram of experimental design. (B-C) Representative cells and group data showing that in the continuous presence of Oxo-M, SR141716 causes synaptic potentiation relative to vehicle conditions. (C) Co-incubation of THL and Oxo-M prevents SR141716-induced synaptic potentiation, as does intracellular BAPTA loading. (D) Summary data showing the effects of SR141716 under vehicle, Oxo-M, Oxo-M+THL, and Oxo-M+BAPTA pre-treatment conditions. (E) Representative traces of summary data in (D). ***p<0.001, ****p<0.0001. Scale bars: 10ms, 100pA. Data presented as mean ± SEM.

Discussion

Prolonged Activation of $G\alpha_{q/11}$-coupled mAChRs Mobilizes 2-AG to Induce Short-Term Depression of CeAL Glutamatergic Transmission

These experimental results reveal that mAChR activation facilitates eCB-mediated synaptic depression of CeAL excitatory neurotransmission. Though other brain regions exhibit mAChR-LTD via postsynaptic mechanisms downstream of mAChR activation (Huang and Hsu, 2010; Jo et al., 2010; McCoy and McMahon, 2007), our initial experimental studies uncovered a reversible synaptic depression following mAChR agonism. These experimental results are consistent with parallel examinations of inhibitory synaptic transmission (Edwards et al., 2006b; Hashimotodani et al., 2005) within the hippocampus as well as this and earlier studies demonstrating the strong expression of $G\alpha_{q/11}$-coupled mAChRs within the CeAL (Levey et al., 1991b; Yoshida et al., 2011b). Our subsequent experimental results also determined that mAChR activation for ~ 30-60 minutes, which was previously determined to cause limited M_1 mAChR internalization and down-regulation (Thomas et al., 2009), enhances 2-AG-mediated synaptic modulation in the form of Ca^{2+}-assisted-RER and RER at CeAL glutamatergic synapses.

Continuous M_1/M_3 mAChR Activation Enhances CeAL DSE via Increased 2-AG Synthesis

As similarly observed at striatal inhibitory synapses following the activation of $G\alpha_{q/11}$-coupled mGluRs (Uchigashima et al., 2007c), examinations of Ca^{2+}-RER under conditions of continuous mAChR activation reveals a concentration and depolarization-time dependency of CeAL DSE enhancement. Also consistent with other brain regions (Kano et al., 2009a), this mAChR-enhancement of DSE is THL-sensitive, as well as M_1/M_3- and CB1-dependent, thus implicating 2-AG as the eCB mobilized following prolong mAChR activation.

eCB-independent From of Synaptic Depression Present at CeAL Excitatory Synapses

Under these experimental conditions, postsynaptic depolarization also generates a statistically significant THL insensitive, CB1- and M_1/M_3- independent depression of CeAL glutamatergic synapses potentially a consequence of depolarization induced phasic nitric oxide (NO) synthesis as is also observed in the hippocampus following DSE induction subsequent to mAChR activation (Makara et al., 2007). Through the mAChR subtype(s) involved in that study remain uncharacterized, previous studies have noted that M_2/M_4 mAChRs, also found postsynaptically at striatal dendritic shafts and spines (Hersch et al., 1994), couple to the activation of nitric oxide synthase (Waid et al., 2000; Wang et al., 1997), the biosynthetic enzyme of NO (Garthwaite and Boulton, 1995). Of further note, both M_2/M_4 mAChRs and neuronal nitric oxide synthase are strongly and weakly (respectively) expressed in the CeAL (Lange et al., 2012; Levey, 1993), thus the biosynthetic substrates for mAChR-NO synthesis and downstream modulation of synaptic transmission is present within the CeAL and may have been recruited under our experimental conditions.

Prolonged mAChR Activation Induces a DAGL- and Ca^{2+} Mediated eCB Release at CeAL Glutamatergic Synapses

In accordance with enhanced phasic 2-AG mobilization following prolonged mAChR activation, continuous mAChR activation (≥ 60 minutes) also enhanced tonic 2-AG release at CeAL glutamatergic synapses. Under these experimental conditions, our SR141716 wash-on studies revealed strong synaptic potentiation following CB1 blockade in slices incubated with Oxo-M, as compared to vehicle pretreatment conditions. This synaptic potentiation required intracellular calcium and was THL-*sensitive*, strongly suggesting that this Oxo-M-induced tonic eCB signal is mediated by 2-AG synthesized by the canonical calcium-DAGL-dependent pathway (Kano et al.,

2009a). These findings are consistent with recent studies in MAGL knock-out mice, which suggest that 2-AG can act as a tonic eCB retrograde messenger (Pan et al., 2011).

Mechanisms underlying mAChR-driven 2-AG Synthesis at CeAL Glutamatergic Synapses

Collectively, these data argue that prolonged mAChR activation mobilizes 2-AG to modulate afferent glutamatergic transmission within the CeAL. In light of this study and previous examinations of $G\alpha_{q/11}$-coupled eCB mobilization at central synapses (Hashimotodani et al., 2005; Jung et al., 2012; Tanimura et al., 2010), it appears that M_1/M_3 mAChR activation results in the sequential recruitment of PLCβ, which produces DAG, and DAGLα ,which converts DAG to 2-AG, thereby facilitating eCB mediated retrograde suppression of excitation in this region. Our morphological studies described in Chapter 3 have shown that DAGLα is present at asymmetric synapses within the CeAL while earlier work by Watanabe and colleagues (Watanabe et al., 1998) demonstrated that PLCβ1 mRNA is also highly expressed in the CeAL region. Furthermore, earlier studies as well as this study have shown that $G\alpha_{q/11}$-coupled mAChR subtypes are highly expressed in the CeAL (Levey et al., 1991a), thus the molecular substrate for mAChR-2-AG synthesis and mobilization are present at CeAL excitatory synapses; perhaps forming a eCB signalosome: a focal point for the generation of a 2-AG pool specifically recruited under conditions of prolonged $G\alpha_q$ activation (Jung et al., 2012).

Functional Implications of mAChR-mediated eCB Mobilization at CeAL Glutamatergic Synapses

The CeAL receives strong cholinergic input from cells within the nucleus basalis (NB) which, as demonstrated by rodent models, exhibit increased firing rates following exposure to threatening cues (Whalen et al., 1994). Though the NB contains both cholinergic and non-cholinergic cell bodies (Gritti et al., 1997), stress-induced increases in ACh content in both the amygdala and

other NB projecting brain regions (Mark et al., 1996) lends support to increased amygdala ACh content as a result of threat-induced increases in the firing rate of NB cholinergic cells. A consequence of this increased endogenous ACh is persistent activation of $G\alpha_{q/11}$ coupled mAChR which, based on our experimental results, tonically mobilize 2-AG in the form of RER. Alternatively, increased endogenous ACh may also provide a state in which depolarization-induced $[Ca^{2+}]_i$, as is elicited by backpropagating action potentials in the presence of mAChR agonists (Nakamura et al., 2000) or NMDA receptor activation (Ohno-Shosaku et al., 2007), can readily facilitate phasic 2-AG attenuation of afferent input to this region.

Conclusion

In the present study we show, for the first time, crosstalk between the eCB and cholinergic systems to facilitate attenuation of CeAL excitatory transmission. Following mAChR activation, 2-AG mediated synaptic depression in the forms of Ca^{2+} assisted- RER and RER mediated 2-AG signaling are observed at CeA glutamatergic afferents. Given the key role of the CeAL in facilitating physiological and behavioral changes in response to threat exposure (Iwata et al., 1987; LeDoux et al., 1988a), molecular interactions between the cholinergic and eCB system may act as an essential substrate for eCB's stress attenuating physiological and behavioral properties. However, in light of recent insight into the CeA microcircuitry (Ciocchi et al., 2010; Li et al., 2013), additional work will have to be undertaken to determine the cell-type specific effects of mAChR-2-AG mobilization on CeAL circuitry and stress-related functionality.

CHAPTER V

FUNCTIONAL SEGREGATION BETWEEN DAGL-AND-Ca2+- DEPENDENT AND -INDEPENDENT ENDOCANNABINOID MOBILIZATION AT CENTRAL AMYGDALA GLUTAMATERGIC SYNAPSES: CENTRAL ROLE OF TIME-DEPENDENT MUSCARINIC RECEPTOR ACTIVATION

Introduction

As discussed in Chapter 4, *prolonged* muscarinic receptor (mAChR) activation facilitates a Ca^{2+} and DAGLα-dependent form of 2-AG mediated phasic and tonic depression of glutamatergic transmission in the CeAL. However, previous studies have also determined that *acute* activation of $G\alpha_{q/11}$ –coupled GPCRs can induce Ca^{2+}- and/or 2-AG-independent forms of CB1- synaptic plasticity in other brain regions such as the hippocampus (Edwards et al., 2006a; Kim et al., 2002b; Zhang et al., 2011), dorsal raphe nucleus (Haj-Dahmane and Shen, 2005) and cerebellum (Maejima et al., 2001). Therefore, to fully achieve the aim of these studies it is also important to determine whether *both* acute and prolonged mAChR activation mobilize similar eCBs at CeAL glutamatergic synapses. To test this, we acutely activated mAChRs (for ~20 minutes) and used pharmacological and genetic approaches to determine whether acute mAChR activation drives CB1 synaptic depression. After this experimental approach, we next determined which eCB facilitated this form of eCB-mediated synaptic plasticity.

Results

Acute mAChR-driven eCB signaling in the CeAL

To determine whether acute application of Oxo-M can induce eCB release at CeAL glutamatergic synapses in the absence of depolarization, we applied Oxo-M for ~20 minutes and assessed eCB release during this period, using pharmacological and genetic approaches (see Figure 11A for experimental design). Our results revealed that Oxo-M application dose-dependently suppressed eEPSC amplitude with maximal depression observed with 1μM Oxo-M (baseline 99.77±0.54% vs. Oxo-M 34.61±1.36%, $p<0.0001$ by paired *t*-test; Figures 5B-D and K). We next explored the contribution of CB1 activation to Oxo-M-mediated synaptic depression. Maximal Oxo-M-mediated depression was significantly attenuated in the presence of the CB1 receptor antagonist SR141716 following either 0.3μM Oxo-M (Oxo-M 46.24±4.25% vs. Oxo-M+SR141716 59.93±2.81%; $t(8)=2.69$, $p<0.05$) or 1 μM Oxo-M application (Oxo-M 34.61±1.36% vs. Oxo-M+SR141716 53.11±2.73%; $t(17)=5.98$, $p<0.001$; See Figure11C-D and K).

We also examined the effects of SR141716 on Oxo-M induced elevation in PPR and found that SR141716 pretreatment significantly attenuated the 1μM Oxo-M-induced increase in PPR ($p<0.0001$; Figure11E). Importantly, the residual Oxo-M depression in SR141716-treated slices was associated with a residual increase in PPR ($p<0.001$; Figure11E), indicating that the non-CB1 component of Oxo-M-induced depression is also presynaptic in nature. Given these findings following the pharmacological inhibition of CB1 receptors, we next sought to confirm our pharmacological results using $CB1^{-/-}$ mice. In these experiments, Oxo-M induced synaptic depression was significantly attenuated in $CB1^{-/-}$ mice (WT Oxo-M 37.91±2.83% vs. $CB1^{-/-}$ Oxo-M 55.94±5.32%; $t(12)=3.0$, $p<0.05$; Figure11F) and the maximal 1μM Oxo-M-mediated increase

in PPR was also significantly attenuated in $CB1^{-/-}$ mice ($p<0.001$; Figure11G). Collectively, these data indicate that $G\alpha_{q/11}$-receptor driven eCB mobilization can be initiated by mAChR activity in the CeAL, which in turn, contributes to Oxo-M-mediated synaptic depression of CeAL glutamatergic transmission.

Acute mAChR Activation Drives Ca^{2+}- and DAGL-*Independent* eCB Release

In light of previous studies, the roles of intracellular Ca^{2+} and DAGL in $G\alpha_{q/11}$-receptor driven eCB release remain uncertain (Edwards et al., 2006a; Hashimotodani et al., 2005; Kim et al., 2002a; Tanimura et al., 2010; Zhang et al., 2011). Therefore, we next examined the requirement for Ca^{2+} and DAGL activity in acute mAChR-driven eCB mobilization in the CeAL. First, we tested whether Oxo-M-mediated eCB release requires increases in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$. Postsynaptic loading of the fast Ca^{2+} chelator, BAPTA (20mM), did not affect 1μM Oxo-M-mediated synaptic depression and the maximal Oxo-M induced depression did not differ significantly from those observed under control conditions ($p>0.05$; Figure 11H and 11K). Similarly, THL pretreatment (10μM, ≥60 minutes) did not inhibit 1 μM Oxo-M-mediated depression of eEPSC amplitude ($p>0.05$; Figure11I and K). Lastly, since a recent study suggested cytoplasmic phospholipase A_2 ($cPLA_2$) may facilitate 2-AG synaptic signaling in the cerebellum (Wang et al., 2012) and M_1 mAChR orthosteric agonism has also been shown to recruit cPLA2 -mediated signaling (Liu et al., 2006). As such, we tested the involvement of cPLA2 in Oxo-M-mediated synaptic depression as an alternate mechanism by which mAChR activation could release 2-AG. However, the cPLA2 inhibitor, AACOCF3 (10μM), did not significantly affect Oxo-M-mediated synaptic depression ($p>0.05$; Figure11J and K). These data suggest that acute mAChR-driven eCB release within the CeAL occurs independently of increased $[Ca^{2+}]_i$, DAGL, and cPLA2 activity—indicative of a *2-AG-independent* synaptic

depression at CeAL glutamatergic synapses. Collectively, these data suggest a possible time-dependent switch from a BAPTA- and THL-*insensitive* to a BAPTA- and THL-*sensitive* mAChR-receptor-driven eCB release mechanism following mAChR stimulation at CeAL glutamatergic synapses.

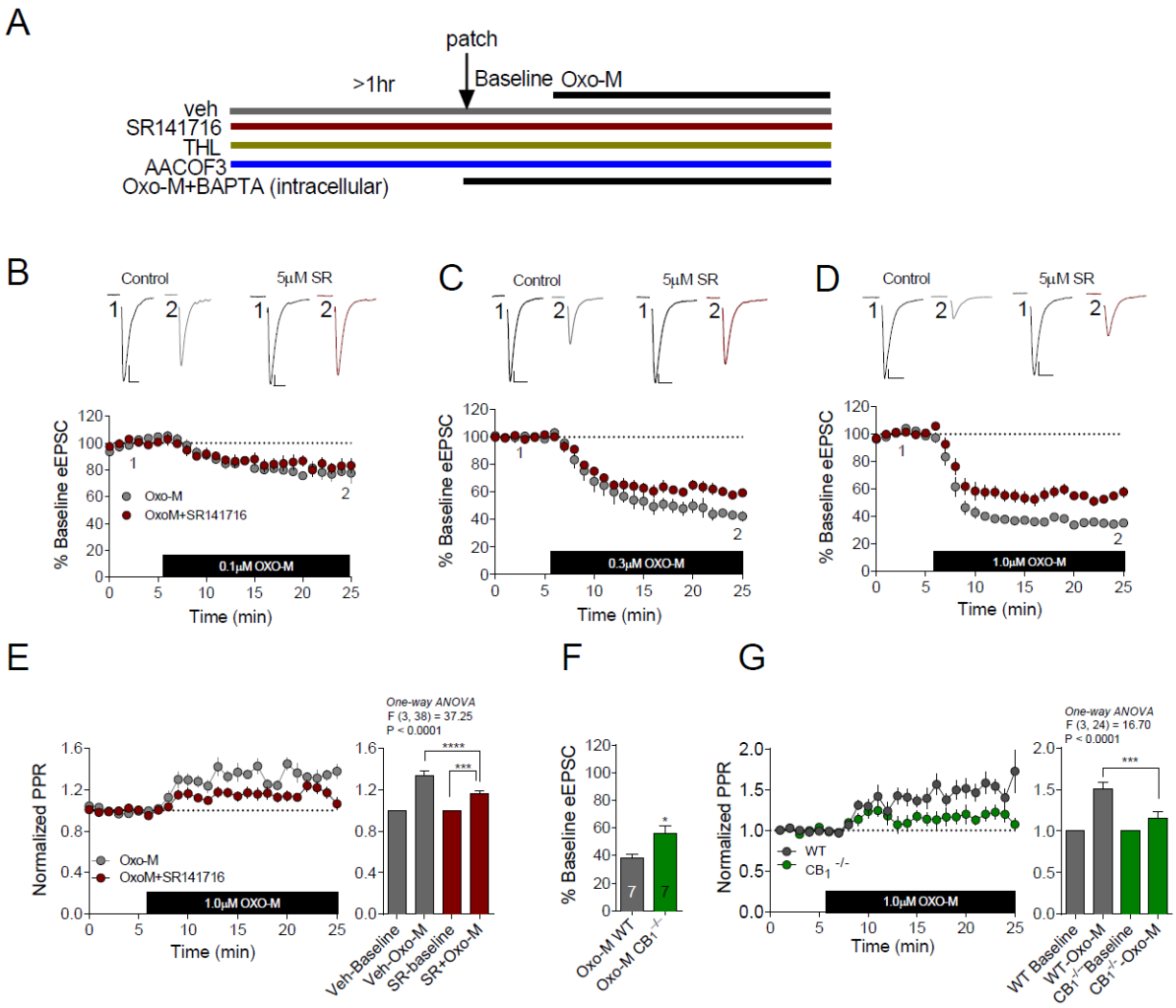

Figure 11. Acute mAChR Activity Drives Ca2+- and DAGL-Independent eCB Release. (A) Diagram of experimental design. (B-D) Oxo-M induced eEPSC depression is partially blocked by SR141716 at 0.3μM and 1 μM Oxo-M conditions. (E) 1 μM Oxo-M-induced increase in PPR is attenuated by SR141716; residual depression in the presence of SR141716 is associated with a residual increase in PPR. (F) 1μM Oxo-M induced eEPSC depression is attenuated in $CB1^{-/-}$ mice. (G) The increase in PPR after Oxo-M application is attenuated in $CB1^{-/-}$ mice. (H-K) Effects of intracellular 40mM BAPTA .*p<0.05, ***p<0.001, ****p<0.0001. Scale bars: 10ms, 100pA. Data presented as mean ± SEM.

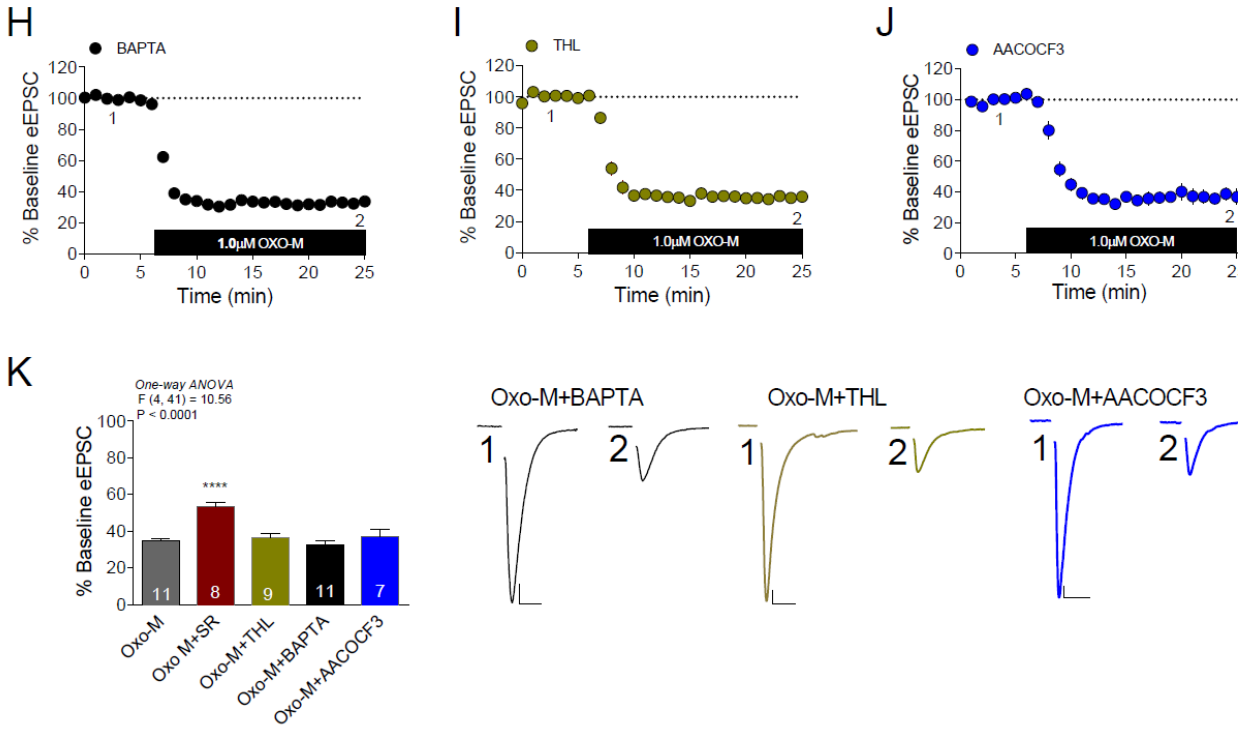

Figure 11 contd. Acute mAChR Activity Drives Ca2+- and DAGL-Independent eCB Release.

(H), THL (I), and the PLA2 inhibitor AACOCF3 (J), on 1μM Oxo-M-induced eEPSC depression. (K) Bar graph and representative traces of summary data depicting the effects of SR141716, THL, BAPTA, and AACOCF3 on 1μM Oxo-M-mediated maximal eEPSC depression. ****p<0.0001. Scale bars: 10ms, 100pA. Data presented as mean ± SEM.

Acute mAChR Activation Drives Synaptic AEA Release

Given that acute mAChR activation promotes a 2-AG independent form of CB1-mediated synaptic depression at CeAL glutamatergic synapses, we next assessed whether the other major eCB in the CNS, AEA, was mediated this depression. Several studies have indicated that pharmacological or afferent stimulation-induced activation of $G\alpha_{q/11}$-coupled receptors can mobilize AEA mediated synaptic plasticity within the amygdala (Azad et al., 2004), hippocampus (Chavez et al., 2010b; Huang and Woolley, 2012), and nucleus accumbens (Grueter et al., 2010b). Given this evidence, we next sought to investigate whether the Ca^{2+}- and DAGL-independent acute Oxo-M-mediated synaptic depression is mediated by AEA, rather than 2-AG, synthesis and mobilization.

In comparison to 2AG, the biosynthetic pathway for AEA is poorly characterized (Leung et al., 2006), as such, assessments of AEA mediated eCB-mediated synaptic plasticity have largely been limited to pharmacologically and genetically inhibiting fatty acid amide hydrolase (FAAH) (Chavez et al., 2010b; Grueter et al., 2010b; Huang and Woolley, 2012), the primary catabolic enzyme of AEA (Cravatt et al., 2001c). To test the involvement of AEA in mediating acute Oxo-M mediated synaptic depression, we assessed the effects of 1uM Oxo-M application on CeAL glutamatergic transmission using mice models with global FAAH loss ($FAAH^{-/-}$). These experiments revealed no differences between age-matched wildtype (WT) controls and $FAAH^{-/-}$ mice (WT+0.3μM Oxo-M 53.76%±5.51% n=6 vs. $FAAH^{-/-}$ + 0.3μM Oxo-M 48.25%±3.0% *n*=8, p>0.05, Figure 12I). Compensatory mechanisms in $FAAH^{-/-}$ conditions could account for the lack of difference between the WT and FAAH-/- conditions (Merritt et al., 2008). To determine whether this is indeed the case, we also determined the effects of pharmacological FAAH inhibition on Oxo-M-mediated acute synaptic depression (see Figure 12A for experimental design). Pretreatment with the selective FAAH inhibitor, PF-3845 (5μM),

attenuated Oxo-M-mediated synaptic depression at both 0.3μM and 1 μM Oxo-M concentrations ($p<0.01$ for each condition) (Vehicle+ 1μM Oxo-M 55.06%±0.01 n=11, PF 3845+ 1μM Oxo-M 77.26% ±2.03%; Figure 12B-D).

We also tested the effects of the monoacylglycerol lipase (MAGL) inhibitor, JZL-184 (2μM), on Oxo-M-induced synaptic depression to further rule out a role for 2-AG in acute Oxo-M-mediated synaptic depression. Consistent with the lack of BAPTA and THL sensitivity, prolonged MAGL blockade did not significantly affect subsequent Oxo-M-mediated acute synaptic depression at either 0.3μM or 1μM Oxo-M concentration ($p>0.05$ for each; Figure 12B-D). Together these data indicate that inhibiting AEA, but not 2-AG, degradation modifies acute Oxo-M-mediated synaptic depression, however the direction of effect was somewhat unexpected. Specifically, if acute Oxo-M application causes release of AEA, blocking AEA degradation would be expected to increase Oxo-M synaptic depression rather than decrease it. Furthermore, the lack of enhancement was not due to a floor effect as both maximal (1μM) and sub-maximal (0.3μM) concentrations of Oxo-M showed reduced efficacy in the presence of FAAH, but not MAGL, inhibition.

An alternate explanation for our results is that PF-3845, but not JZL-184, occludes the effects of Oxo-M. If this were the case, PF-3845 would be expected to cause increased AEA and a CB1- dependent synaptic depression, thereby occluding subsequent AEA-mediated synaptic depression initiated by acute Oxo-M application. Consistent with this hypothesis, 5μM PF-3845 wash-on produced a CB1-dependent synaptic depression of glutamatergic transmission (PF-3845+vehicle 83.31%±5.34% vs. PF-3845+SR141716 102.80%±3.91%; $t(14)=2.79$, $p<0.05$; Figure 12E-F). These data, combined with the lack of occlusion of acute Oxo-M-mediated synaptic depression by the MAGL inhibitor, JZL-184, strongly implicate AEA, rather than 2-

AG, as the eCB ligand subserving synaptic depression induced by acute Oxo-M application. However, it is possible the lack of occlusion by MAGL inhibition could be due to the fact that JZL-184 alone did not significantly increase 2-AG content. To exclude this possibility and strengthen the support for an AEA-mediated process, we tested the ability of JZL-184 to cause synaptic depression of glutamatergic transmission. Consistent with our hypothesis, JZL-184 produced a CB1-dependent synaptic depression of glutamatergic signaling (JZL-184+ vehicle 74.29%±4.24% vs. JZL-184+SR141716 96.38%±2.97%; $t(9)=4.1$, $p< 0.01$; Figure 12G-H). Taken together, these data provide converging evidence that acute Oxo-M-mediated synaptic depression causes synthesis/release of AEA that acts on CB1 receptors to reduce glutamate release. Conversely, prolonged Oxo-M stimulation of mAChRs enhances DSE and elicits tonic CB1-mediated synaptic depression via the release of 2-AG through the canonical calcium-DAGL-dependent biosynthetic pathway (Figure 18).

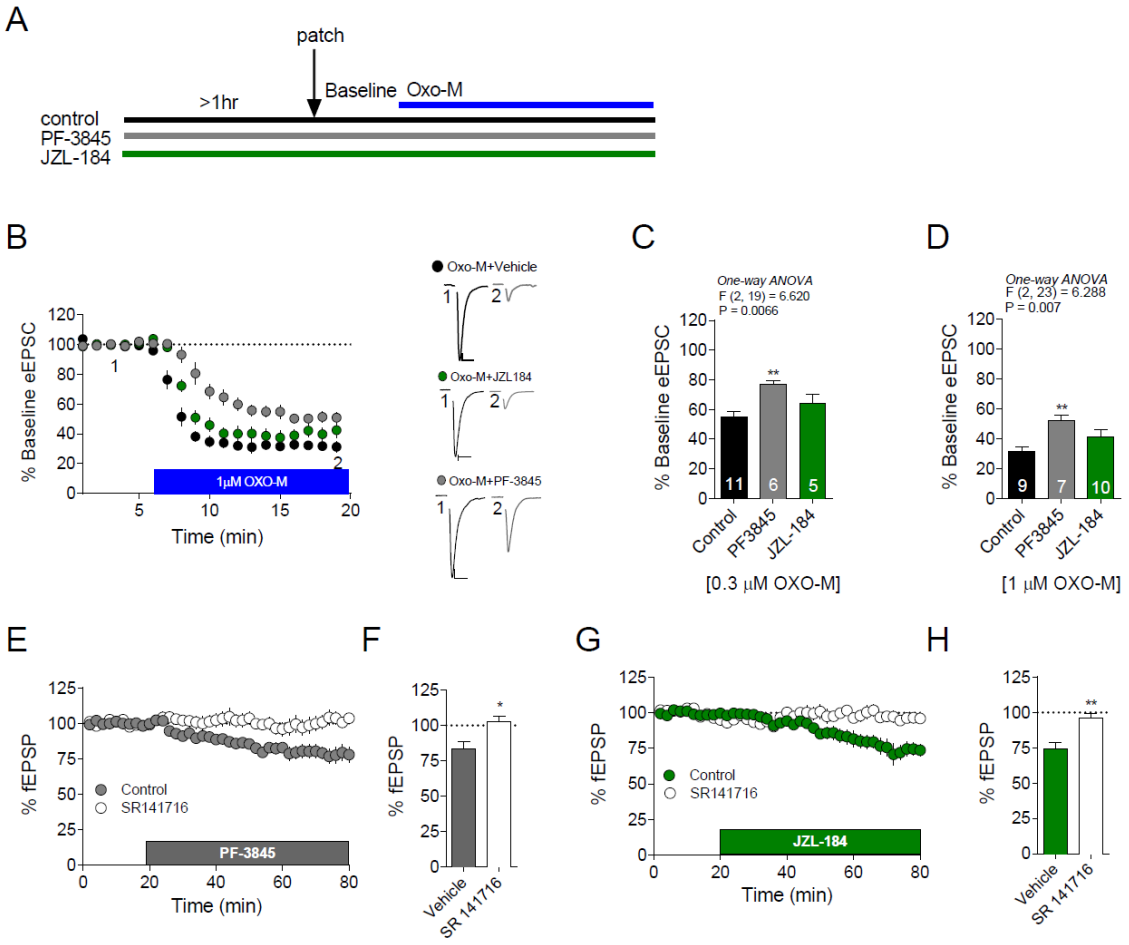

Figure 12. Acute mAChR Receptor Activity Drives Synaptic AEA Signaling.
(A) Experimental design for B-D. (B-D) Oxo-M-induced acute synaptic depression (0.3 μM and 1 μM) is partially occluded by the FAAH inhibitor PF-3845, but not the MAGL inhibitor JZL-184; time-course for 1μM Oxo-M condition shown in (B). (E-F) Effects of PF-3845 on synaptic depression under control or CB1 antagonist, SR141716, pretreatment conditions. (G-H) Effects of JZL-184 on synaptic depression under control or CB1 antagonist, SR141716, pretreatment conditions. (I) Diagrammatic representation of differences between acute vs. prolonged mAChR activation with Oxo-M. Acute Oxo-M application induces a short-lived "burst" of AEA to reduce afferent glutamate release, while prolonged mAChR activation causes a tonic calcium- and DAGL-dependent 2-AG release. (J) During prolonged mAChR activation, tonic 2-AG release continues and calcium-assisted mAChR-driven 2-AG release is induced by co-incident postsynaptic depolarization (i.e. DSE enhancement in the presence of continuous Oxo-M). (+): activate; AEA: anandamide; 2-AG: 2-arachidonoylglycerol; Ca++: calcium; Ca^{2+}R-eCBR: calcium-assisted receptor driven eCB release; CB1: CB1 receptor; DAGL: diacylglycerol lipase; M1/3: $M_{1/3}$ mAChR; PLC: phospholipase C. *p<0.05, **p<0.01. Scale bars: 10ms, 100pA. Data presented as mean ± SEM. Also see Figure 13.

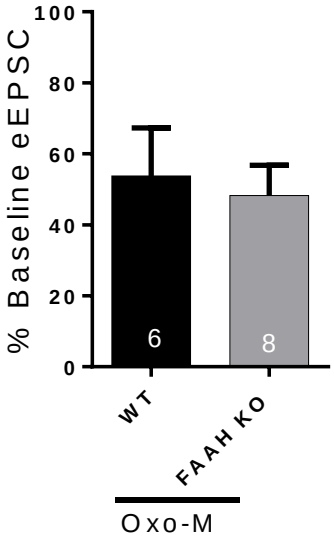

Figure 12 contd. (I) Acute mAChR Receptor Activation Elicits the Same Degree of eEPSC Depression in WT and FAAH -/- mice.

Lack of Oxo-M-Mediated Acute or Tonic eCB Signaling At Excitatory Synapses of the Striatum

Thus far, our data indicate that acute Oxo-M activation of mAChRs drives AEA release and subsequent depression of glutamatergic signaling via CB1 activation. In contrast, prolonged mAChR activity results in 2-AG-mediated tonic CB1 activation and enhancement of DSE. Since this is the first demonstration, to the best of our knowledge, of time-dependent generation of AEA and 2-AG following $G\alpha_{q/11}$ GPCR activation, we wanted to examine whether this was a generalizable phenomenon. Therefore, we tested this phenomenon in the striatum given the strong morphological, hodological, and cytoarchitectural similarities between the striatum and the CeAL (McDonald, 1982b). Acute application of Oxo-M (1μM) caused robust presynaptic depression in the striatum, however, this depression was not affected by SR141716 pretreatment ($p>0.05$; Figure 13A-C). Similarly, SR141716 failed to produce synaptic potentiation in the presence or absence of prolonged Oxo-M (1μM) pre-treatment (Figure 13D). In contrast, prolonged Oxo-M (1μM) application was able to enhance DSE relative to control conditions ($p<0.01$; Figure 13E) and this enhancement was blocked by SR141716 ($p<0.0001$). These data indicate that Oxo-M (1μM) is able to enhance DSE in the striatum, but that mAChRs do not trigger acute AEA or tonic 2-AG release to regulate glutamatergic transmission in this region. Thus, mAChR-driven multimodal eCB release may not be a generalized feature of central synapses.

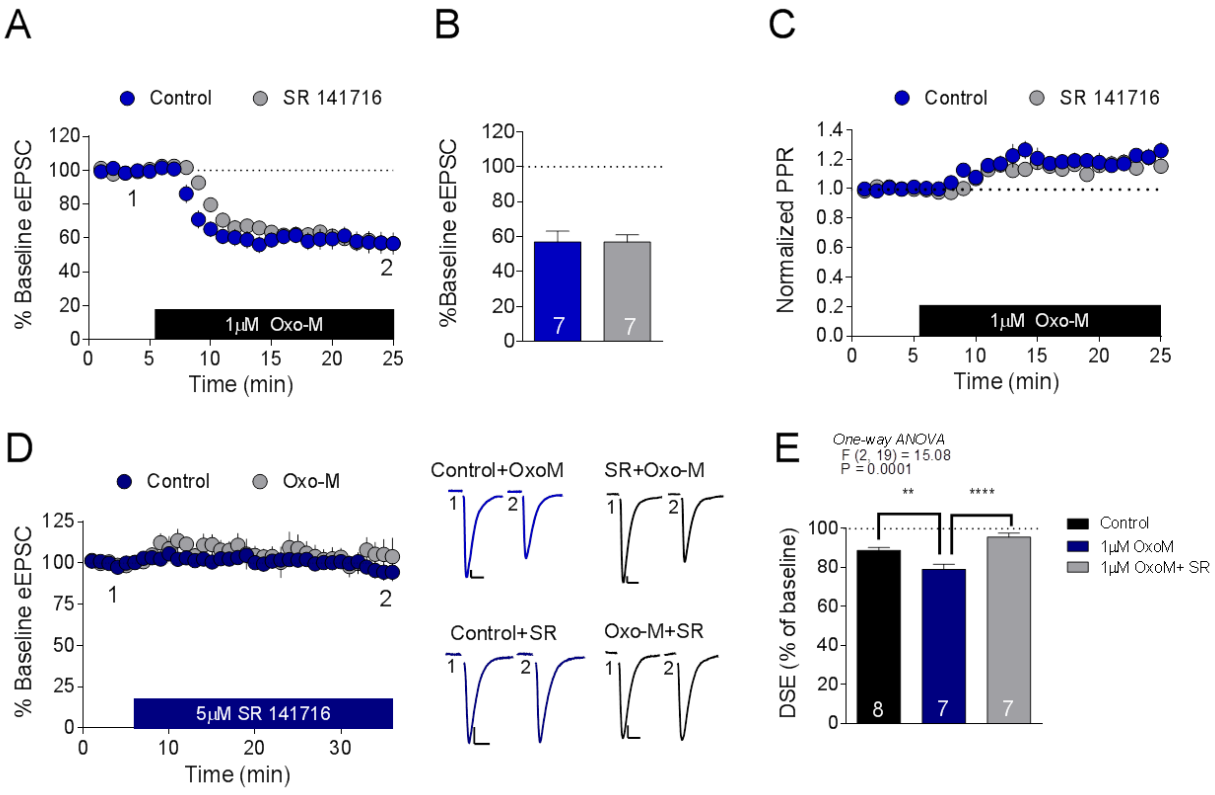

Figure 13. Oxo-M does not elicit acute or tonic eCB release at Excitatory Synapses of the dorsal lateral striatum.

(A-B) Acute application of Oxo-M causes synaptic depression in dorsal striatal neurons, which is not affected by pre-incubation with the CB1 receptor antagonist, SR141716. (C) Oxo-M induced eEPSC depression is associated with an increase in PPR which is not affected by SR141716. (D) After continuous Oxo-M incubation, SR141716 does not induce synaptic potentiation. Top and bottom rows are representative current traces from experiments (A) and (D), respectively. (E) Oxo-M enhanced DSE magnitude in dorsal striatal neurons, which is blocked by the CB1 antagonist, SR141716. **p<0.01, **** p<0.0001 by ANOVA followed by Sidak's multiple comparisons test.

Discussion

Acute mAChR Activation Mediate CB1-Dependent and -Independent Attenuation of CeAL Glutamatergic Transmission

Our data indicate that acute application of Oxo-M causes a robust presynaptically-expressed synaptic depression that is partially mediated by CB1 activation. Similar experimental studies by Lau and Vaughan (2008) in the periaqueductal grey (PAG), suggest that the CB1-independent effect may results from direct suppression of glutamate release via activation of $G\alpha_{i/o}$-coupled M_2 mAChRs. In support of this, previous studies have also shown that M_2 mAChRs are highly expressed in the CeAL(Buckley et al., 1988). Though the functional implications of partial mAChR-CB1 signaling are not entirely clear, cholinergic recruitment of eCB signaling may supplement mAChR-driven synaptic plasticity under conditions of high neuronal activity such as that observed for a subset of CeAL neurons following exposure to threatening cues (Ciocchi et al., 2010; Haubensak et al., 2010)

In contrast to this partial mAChR-CB1 synaptic depression observed at excitatory synapses of the CeAL and PAG, inhibitory synapses in other brain regions such as the hippocampus (Edwards et al., 2006b) and the striatum (Narushima et al., 2007b; Uchigashima et al., 2007c) exhibit complete mAChR-CB1 mediated synaptic depression. As such, it appears that excitatory synapses have a decreased ability to initiate eCB-mobilization via mAChR activation, a claim further supported by the lack of mAChR-eCB/CB1 synaptic depression observed at excitatory synapses within the dl striatum in our study and similar studies performed by Uchigashima and colleagues (Uchigashima et al., 2007c). This difference between excitatory and inhibitory dl striatal synapses is not observed following acute $G\alpha_{q/11}$-mGluR- CB1 mediated synaptic depression (Kreitzer and Malenka, 2005; Uchigashima et al., 2007a), thus excluding the possibility of generalizable differences in $G\alpha_{q/11}$ signaling between excitatory and inhibitory

synapses. This synapse specific distinction may perhaps be explained by greater segregation between M_2- and M_1/M_3-exclusively containing synapses for inhibitory as compared to excitatory synapses (Fukudome et al., 2004b). Alternatively, distinct subcellular localizations of postsynaptic mAChRs at excitatory synapses may result in a relatively less efficient functional coupling of postsynaptic mAChRs to the eCB synthesis machinery as is hypothesized, based on the subcellular localization of M_1 mAChRs, for mAChR-eCB mobilization at striatal glutamatergic terminals (Uchigashima et al., 2007a).

Acute mAChR Activation Results in a THL and Ca^{2+}-insensitive CB1 Synaptic Depression at CeAL Glutamatergic Synapses

Mechanistically, acute mAChR-CB1-mediated synaptic depression does not require elevations in intracellular calcium, is THL-*insensitive*, and does not require cPLA2 activity. Moreover, inhibition of 2-AG degradation with JZL-184 did not significantly attenuate acute Oxo-M-mediated synaptic depression, although a trend towards attenuation was observed possibly resulting from partial FAAH inhibition observed *in vitro* and *in vivo* studies by the Cravatt group following JZL-184 treatment (Long et al., 2009a; Long et al., 2009c; Schlosburg et al., 2010). Collectively, these data appear to exclude 2-AG as the eCB ligand mediating the acute CB1-dependent synaptic depression induced by short-term mAChR activation.

Acute mAChR Activation Mobilizes Anandamide within the CeAL

In addition to 2-AG, $G\alpha_{q/11}$ proteins also appear to couple to AEA biosynthesis as demonstrated by rodent models of $G\alpha_{q/11}$ forebrain specific deletion which exhibit decreased hippocampal AEA content as compared to wildtype controls (Wettschureck et al., 2006). Furthermore, activation of $G\alpha_{q/11}$-coupled mGluRs in other regions of the amygdala also recruits a Ca^{2+}-independent forms of AEA-synaptic depression (Azad et al., 2004), thus Ca^{2+}-independent forms

of $G\alpha_{q/11}$-AEA interactions may also occur within the CeAL as well. Given this evidence, we wondered whether the observed THL and Ca^{2+} insensitive acute mAChR synaptic depression was facilitated by AEA release at CeAL glutamatergic synapses.

We found that FAAH inhibition, which on its own caused a CB1-dependent synaptic depression of glutamatergic transmission, partially occluded acute Oxo-M-mediated synaptic depression to the same degree as similar experiments performed following genetic and pharmacological inhibition of CB1. As such, it appears that AEA may facilitate acute mAChR mediated synaptic plasticity. Huang and Woolley (2012), who showed that estrogen/ $G\alpha_{q/11}$-$mGluR_1$-induced depression of GABAergic transmission in the hippocampus was occluded by FAAH inhibition, but not MAGL inhibition, also reached similar conclusions (Huang and Woolley, 2012). Given that PF 3845 application alone causes a CB1-dependent synaptic depression to the same degree as that which is observed in our occlusion studies, it appears that continuous FAAH inhibition may maximize the AEA synthetic capacity of CeAL cells thereby excluding AEA metabolites from contributing to the recycling, synthesis, and release of new AEA (Placzek et al., 2008). Thus, under these conditions, further $G\alpha_{q/11}$-mAChR mediated AEA synthesis would be occluded as is observed under our experimental conditions. In support of this, *in vitro* studies by McFarland and colleagues (2006) have demonstrated that the AEA metabolites, arachidonic acid and ethanolamine, accumulate in lipid rafts in a FAAH dependent manner (Day et al., 2001) and this act as microdomains for AEA re-synthesis from its metabolites (McFarland et al., 2004; McFarland et al., 2006).

Potential Mechanism Underlying Time-Dependent Dissociation of Multimodal eCB Signaling

Previous studies have demonstrated that cells within the same brain regions can produce both AEA and 2-AG that act as retrograde eCBs signals (Huang and Woolley, 2012; Kim and Alger,

2010; Lerner and Kreitzer, 2012; Mathur et al., 2013; Puente et al., 2011b). Similarly, several studies have also demonstrated that activation of $G\alpha_{q/11}$-coupled receptors can induce 2-AG or AEA release in several brain regions (Chavez et al., 2010a; Grueter et al., 2010a; Hashimotodani et al., 2013; Lerner and Kreitzer, 2012; Maccarrone et al., 2008a). However, to the best of our knowledge, our data are the first to provide experimental evidence that AEA and 2-AG can be released in response to activation of the same $G\alpha_{q/11}$-coupled receptor depending only on the duration of $G\alpha_{q/11}$-receptor stimulation. Specifically, acute mAChR activation causes short-lived AEA-mediated synaptic depression, while as discussed in Chapter 4, prolonged mAChR stimulation causes 2-AG-mediated phasic and tonic depression of CeAL glutamatergic transmission. Thus, in CeAL neurons, mAChR stimulation can initiate multimodal eCB signaling depending only on the duration (e.g., minutes or hours) of mAChR $G\alpha_{q/11}$-receptor stimulation. The mechanistic basis for this temporal "switch" in eCB signaling the CeAL remains to be determined, but may likely be related to time-dependent differences in the coupling of mAChRs to distinct signaling pathways important for AEA and 2-AG synthesis (Mangoura et al., 1995; McKenzie et al., 1992a; Schmidt et al., 1995a) or the functional inhibition of 2-AG biosynthesis via AEA-mediated suppression of $G\alpha_{q/11}$ –glutathione mediated 2-AG biosynthesis, as is observed in the ventral striatum (Cristino et al., 2006; Maccarrone et al., 2008b). However, clear delineation of the biosynthetic pathway for synaptic AEA synthesis and the development of pharmacological tools to probe these claims system will be required to conclusively assign AEA as the eCB ligand mediating acute Oxo-M driven synaptic depression.

Functional Implications of Time-Dependent AEA and 2-AG Recruitment at CeAL Glutamatergic Synapses

Recent studies have begun to highlight the dissociable roles of AEA and 2-AG signaling at central synapses. For example, in the bed nucleus of the stria terminalis, AEA mediates long-

term depression (LTD), while 2-AG mediates short-term depression (STD) in the form of DSE (Puente et al., 2011b). Furthermore, different forms of associative neural activity can elicit both AEA- and 2-AG-mediated LTD (Lerner and Kreitzer, 2012) at excitatory synapses of the striatum. More recently, inhibitory synapses in the striatum have also been shown to release AEA and 2-AG in a synapse specific and state-dependent manner (Mathur et al., 2013). Thus, AEA and 2-AG appear to not be "redundant" eCB signaling molecules but, rather, rely on distinct duration-, activity-, and synapse-dependent mechanisms for biosynthesis and release. In support of the non-overlapping mechanisms for AEA and 2-AG synaptic mobilization, both eCBs have distinct subcellular localizations of molecular pathways relevant for their synthesis and degradation which may provide the anatomical substrate for AEA and 2-AG to be produced and released from defined cellular compartments in response to precisely timed stimuli. Furthermore, more recent work has begun to shed light on the functional relevance for these largely non-overlapping characteristics of AEA and 2-AG. Work by our group (2005) amongst others have revealed that both AEA and 2-AG amygdalar content are differentially regulated (decreased and increased, respectively) by chronic stress exposure. Furthermore, additional evidence strongly suggests that these differential changes in AEA and 2-AG play distinct roles in the physiological and behavioral adaptations to chronic homotypic stress exposure (Hill et al., 2010c; Patel et al., 2005d). Given the enhancing effects of acute and chronic stress exposure on acetylcholine synaptic release and content (Das et al., 2000; Finkelstein et al., 1985; Gilad et al., 1987; Mark et al., 1996), chronic stress may also enhance amygdalar acetylcholine content and, in a time dependent manner, recruit distinct eCBs to facilitate CeAL mediated adaptations of physiological and behavioral responses to chronic homotypic stress exposure. However, additional work will have to be carried out to determine: 1) whether CeAL acetylcholine content

changes in response to chronic stressors of diverse duration and saliency and 2) the impact of inhibiting CeAL muscarinic signaling on measures of stress response adaptations.

Conclusion

Our acute mAChR experiments reveal a partial CB1 synaptic depression at CeAL glutamatergic synapses that appears to be mediated by AEA mobilization which is in contrast to the eCB mobilized following prolonged mAChR activation at these synapses. As such, it appears that mAChR activation causes a time-dependent switch in coupling to AEA and 2-AG release, a mechanism that appears to be unique to excitatory synapses of the CeAL. Though shown to be mediated via afferent activity dependent mechanisms, other brain regions have also demonstrated that the same cell types can mobilize both AEA and 2-AG in response to different stimulation profiles. Here we add to this eCB signaling diversity by demonstrating time dependent-dissociated mobilization of AEA and 2-AG signaling in response to mAChR activation. Continued investigation of multimodal eCB signaling could provide insight into the activity-dependent mechanisms that modulate synaptic efficacy.

CHAPTER VI

EFFECTS OF CHRONIC RESTRAINT STRESS ON ENDOCANNABINOID SIGNALING AT CeAL GLUTAMATERGIC SYNAPSES

Introduction

Stress initiates a collection of adaptive processes that engages physiological, neuroendocrine, and behavioral responses elicited to allow an organism to cope with imminent threat (Herman, 2013; LeDoux, 2014). However, when these mechanisms are persistently recruited the adaptive benefits of the acute stress response diminishes as prolonged or cumulative stress exposure enlists deleterious changes, ranging from structural (Vyas et al., 2002) to behavioral (Hill et al., 2011b; Zhong et al., 2014) that often preclude normal function. Accordingly, chronic exposure to psychosocial stress is a major risk factor for the development of stress-related psychiatric illness, such as post-traumatic stress disorder (PTSD) (Jovanovic and Ressler, 2010). In rodent models, chronic psychosocial stressors recapitulate many of the biochemical, structural, and behavioral aspects of stress-related psychiatric disorders (Pittenger and Duman, 2008). Thus, parallel studies of chronic stress exposure in both rodent models and humans hold significant translational advantages for understanding the pathophysiology of and developing critical treatment paradigms for stress-related psychiatric disorders.

Such studies have identified the eCB system, an important regulator of stress responsivity (Hill et al., 2010c), as a promising target for the treatment of chronic-stress associated

psychiatric illnesses (Fraser, 2009; Leweke et al., 2012). In support of this, genetic (Haller et al., 2002; Haller et al., 2004b; Hill et al., 2011a; Kamprath et al., 2006b; Marsicano et al., 2002) or pharmacological disruption (Hill et al., 2009b; Patel et al., 2004; Steiner et al., 2008c; Varga et al., 1995; Wade et al., 2006) of eCB signaling in humans and rodent models precipitates phenotypic states similar to those elicited by chronic stress exposure (Christensen et al., 2007; Hill et al., 2009d; Moreira et al., 2009). Furthermore, an attractive quality of the eCB system is its role in facilitating stress adaptations: a progressive decrease in the recruitment of stress responses to limit the deleterious actions that accompany prolonged recruitment of the stress response (Herman, 2013). Significant data suggests that the eCB system's highly dynamic nature produces temporally and anatomically distinct changes in eCB signaling components within stress-regulating brain regions following exposure to repetitive stress (Hill et al., 2010c). These changes, in turn, are thought to be critical for the habituation of the stress response to the aversive characteristics of threatening stimuli (Kamprath et al., 2011).

In human studies, structural changes of neurons within the amygdala as well as increased amygdalar activity are associated with PTSD pathology (Price and Drevets, 2010; Shin and Liberzon, 2010)—phenotypic states that are also recapitulated in rodent models of chronic stress (Vyas et al., 2002; Vyas et al., 2004). Given these stress-induced changes, the amygdala is thought to act as a key anatomical substrate in the pathophysiology of stress-related psychiatric disorders. In light of this, more recent studies have begun to focus on the functional intersections between the amygdala and the eCB system in an effort to determine whether eCB-related signaling in this region shapes stress-related pathologies. In doing so, most experimental attentions have focused on the BLA which demonstrate changes in both eCB content (i.e. increases and decreases in 2-AG and AEA, respectively) (Patel et al., 2005d) and eCB- mediated

short- and long-term synaptic plasticity subsequent to repetitive psychosocial stress exposure (Hill et al., 2009b; Patel et al., 2009b; Patel et al., 2005d; Rademacher et al., 2008; Ramikie and Patel, 2011; Sumislawski et al., 2011). Additionally, work by Hill and colleagues (2013) have extended these findings to demonstrate that these stress-engendered changes to the BLA eCB system act to facilitate adaptations in the physiological and behavioral responses to repetitive stress exposure (Hill et al., 2013b).

Though these changes in the eCB system have shed light on BLA-eCB functions in stress adaptations, less is known about whether these stress-induced changes in eCB function also occur at the level of CeAL, the main output nuclei of the amygdala. The CeAL is a key regulator of the physiological, endocrine, behavioral responses to aversive stimuli (Bouret et al., 2003; Ciocchi et al., 2010; Feldman et al., 1994; Iwata et al., 1987; LeDoux et al., 1988b). Critical to the orchestration of these responses is the extensive convergence of excitatory inputs at the level of the CeAL. These excitatory inputs arise from stress-responsive brain regions such as the cortex, thalamus, and amygdala (Delaney et al., 2007; Li et al., 2013; Tye et al., 2011) which, in turn, drive the activity of the CeAL microcircuitry— the output of which activates descending projections to brain regions responsible for initiating outcome measures of the stress response (Haubensak et al., 2010; Li et al., 2013; Tye et al., 2011). As such, excitatory afferents of the CeAL act as crucial custodians of the neural substrates underlying the stress response and are a potential site for the regulation of CeAL-mediated stress responses. Indeed, CeAL glutamatergic synapses express several forms of synaptic plasticity hypothesized to be important for a variety of adaptive and pathological forms of stress-related behaviors (Delaney et al., 2007). In support of this, recent work by Li and colleagues (2013) demonstrate that exposure to threatening cues recruits experience-dependent synaptic plasticity of CeAL glutamatergic transmission in a cell-

type specific and bidirectional manner (Li et al., 2013; Penzo et al., 2014). This experience-dependent synaptic plasticity, the authors argue, ultimately determined the strength and nature of CeAL-mediated stress responses.

As demonstrated in the last three Chapters, activation of the CeAL eCB system is also well positioned to modulate synaptic transmission at this critical locus and, perhaps, this modulation acts as a neural substrate for the eCB system's stress attenuating properties. In support of this functional intersection between the eCB system and CeAL excitatory synapses, *in vivo* activation of CeA CB1 receptors are anxiolytic as measured by elevated plus maze measurements in rodent models (Zarrindast et al., 2008), whilst *in vivo* inhibition of CeA CB1 receptors acutely inhibit measures of behavioral adaptations to conditioned aversive stimuli (Kamprath et al., 2011). In light of this, we examined the effects of repetitive restraint stress exposure on eCB-mediated signaling at CeAL glutamatergic synapses.

Results

Effects of Repetitive Restraint Stress on DSE of Locally Evoked CeAL Glutamatergic Transmission

In earlier studies, Kamprath and colleagues (2011) demonstrated enhanced DSE, in the CeAM, following prior exposure to aversive stimuli (Kamprath et al., 2011). These results were reversible and suggested that excitatory synapses within the CEA are susceptible to transient stress-induced modifications of phasic eCB-mediated synaptic plasticity. To test the validity of this argument, we also assessed whether prior exposure to stressors also sensitized phasic eCB signaling at excitatory CeAL synapses. Given that 10 sec postsynaptic depolarization (10 sec DSE) elicited DSE at CeAL excitatory synapses in our earlier experiments (see Chapters 3), we first examined whether similar experimental paradigms would also promote phasic eCB-

mediated suppression of locally evoked CeAL glutamatergic transmission following repetitive stress exposure. Similarly to our previous experiments, excitatory postsynaptic currents (eEPSCs) were evoked upon non-specific local electrical microstimulation within the CeAL. To induce DSE, CeAL neurons were depolarized from -70mV to 0mV through direct current injections (duration of current injections =10 seconds) and the effects of this depolarization on eEPSCs were examined. Our experimental analyses revealed that subsequent to 10 consecutive days of 1 hour restraint, we observed a significant enhancement ($p<0.05$) of maximal DSE at CeAL glutamatergic synapses (63.37%±3.31% of baseline *n*=8, Figure 14C and 14D) as compared to behaviorally naïve mice (76.67%±3.88% of baseline *n*=10, Figure 14C and 14D). However, our analysis of late DSE (average amplitude of last 10 eEPSCs of the experiment) revealed no difference between our control and chronic stress conditions (control 93.62%±2.23% *n*=10 vs. stress 98.34%±2.24% , n=8; $p>0.05$, Figure 14A).

Furthermore, stress-induced enhancement of maximal CeAL DSE exhibited a postsynaptic depolarization dependency, with significant enhancement of CeAL DSE also observed following shorter postsynaptic depolarization times of 5 sec (control 80.52%±2.53% *n*=10 vs. stress 68.53%±3.52%, n=9; $p<0.05$, Figure 14B, D), while 2 sec postsynaptic depolarization exhibited a non-significant enhancement of CeAL DSE (control 93.86%±3.34% *n*=10 vs. stress 81.25%±6.59%, n=6; $p=0.07$, Figure 14C-D). Collectively, these data demonstrate that chronic stress exposure enhances phasic eCB signaling at CeAL synapses.

Restraint Stress-Enhancement of CeAL DSE is CB1 Receptor Dependent

As enhanced CeAL DSE is observed under our chronic restraint stress conditions, we next examined whether this augmented DSE is CB1 dependent given that other retrograde signaling molecules that mediate depolarization-induced synaptic depression, such as nitric oxide

(Makara et al., 2007), can be increased by chronic restraint stress exposure (Olivenza et al., 2000). To determine whether the CeAL short-term depression is CB1 dependent, we repeated our DSE protocols in the presence of the CB1 receptor antagonist, SR 141716. Following pretreatment with the CB1 receptor antagonist, SR 141716 (SR;5μM), maximal CeAL DSE was abolished in our chronic stress conditions as compared to controls (stress+vehicle: 63.37%±3.31%, n=8 vs. stress+SR: 92.22%±4.15%, n=10, $p<0.0001$, Figure 14E). Thus, consistent with previously reported modulatory effects of stress exposure on the eCB system, chronic restraint stress augments CB1 receptor signaling at CeAL glutamatergic synapses.

Increased 2-AG Biosynthesis at CeAL Glutamatergic Synapses Facilitates Enhanced DSE

Using a similar restraint stress paradigm, earlier studies have demonstrated that amygdalar 2-AG content increases following repetitive exposure to stress (Patel et al., 2005e; Rademacher et al., 2008) (Hill et al., 2010a; Hill et al., 2010c; Malcher-Lopes et al., 2006). In an effort to increase the resolution of these earlier studies, our lab has more recently shown that at the level of the BLA, homotypic stress exposure increases 2-AG content and phasic signaling capacity at inhibitory synapses (Patel et al., 2009a; Sumislawski et al., 2011). This precedence for stress-induced elevations in amygdalar 2-AG signaling led us to hypothesize that CeAL DSE enhancement resulted from stress-induced increases in phasic 2-AG biosynthesis.

To test this hypothesis, we pharmacologically inhibited the 2-AG biosynthetic enzyme, DAGLα (Tanimura et al., 2010), prior to assessing CeAL DSE in our chronic stress conditions. Consistent with stress-induced increases in 2-AG biosynthesis, pretreatment with the DAGL inhibitor, THL (10μM), significantly attenuated the enhanced CeAL DSE following chronic restraint stress (stress+vehicle: 63.37%±3.31%, n=8 vs. stress+THL: 88.63%±2.81%, n=4, $p<0.001$ Figure 14E). Collectively, these data suggests that the enhanced 2-AG phasic

biosynthesis, rather than 2-AG metabolism, is one of the mechanisms by which repetitive-stress enhances phasic 2-AG signaling at CeAL glutamatergic synapses.

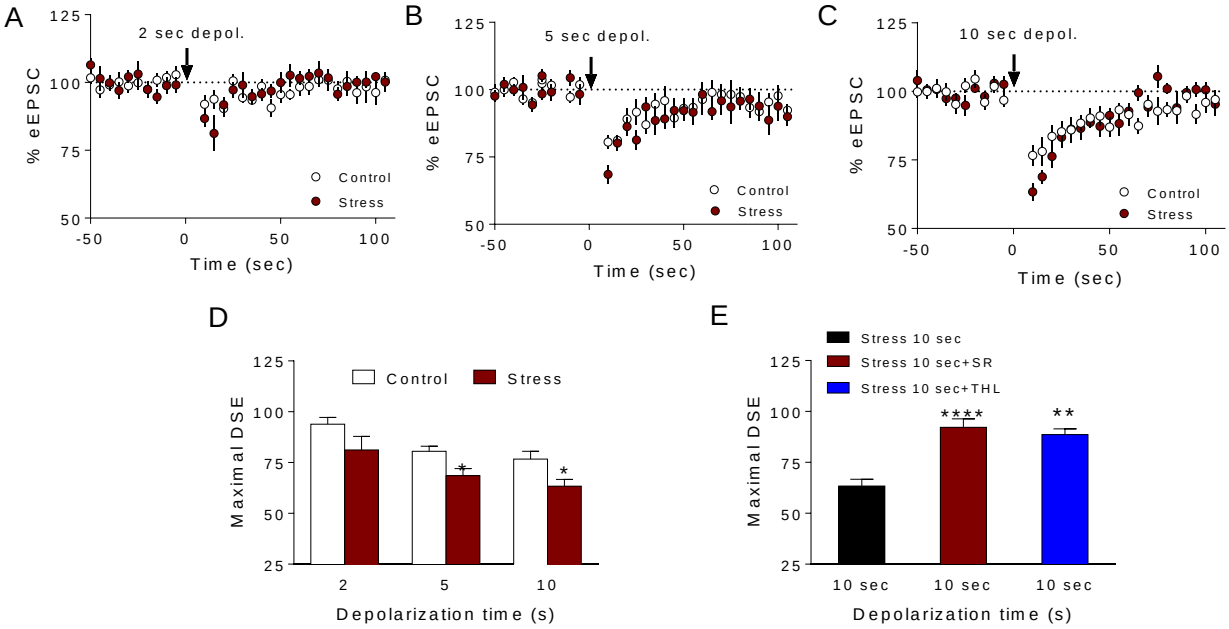

Figure 14. Chronic Restraint Stress Enhances CeAL DSE.
(A-C) Effects of postsynaptic depolarization on CeAL eEPSCs subsequent to chronic restraint stress (CRS). D) Average maximal CeAL DSE under control conditions and subsequent to chronic restraint stress paradigm. E) CRS-CeAL DSE is inhibited in the presence of the CB1 receptor inhibitor, SR 141716 (5μM) and the DAGL inhibitor, THL (10μM) as compared to vehicle conditions. *p<0.05, **p<0.01, ****p<0.0001. Data presented as mean ± SEM.

Afferent Specific Effects of Homotypic Restraint Stress on CB1 Signaling at Amygdalar Glutamatergic Terminals

Though our experimental examinations thus far implicate augmented 2-AG biosynthesis as one of the underlying mechanism for enhanced CeAL DSE, stress-related increases in CB1 receptors signaling may also act in parallel and contribute to phasic inhibition of synaptic transmission. If enhanced CB1 activity were indeed an underlying mechanism for enhanced phasic 2-AG mobilization at these synapses, our current experimental approach of non-specific afferent stimulation, via local electrical microstimulations, may mask afferent specific changes in CB1 receptor signaling following chronic stress exposure. To address this potential confound, we targeted the medial glutamatergic afferent pathway of the CeAL to increase the specificity of our electrical stimulation and directly assessed CB1 receptor signaling using the exogenous CB1 receptor agonist, CP 55940 (10μM).

In our initial experiments, we electrically stimulated glutamatergic fibers arising medial to the CeAL (medial glutamatergic pathway; MP). This pathway consists of afferents originating in the lateral parabrachial nucleus (Bernard et al., 1993; Delaney et al., 2007; Lopez et al., 2004) ,which convey ascending nociceptive information to the CeAL, and thalamic inputs that convey sensory-related information to the CeAL (Ehrlich et al., 2009). To directly assess these glutamatergic afferents, we examined the effects of direct CB1 receptor activation on evoked MP-CeAL postsynaptic potentials. These experiments revealed that 10μM CP 55940 application results in a similarly depressed electrically stimulated MP CeAL glutamatergic inputs under both control and chronically stressed conditions (control+CP 59.32% ±3.93% n=9 vs. stress+CP 52.88%±3.837% n=5, $p>0.05$, Figure 15B). Through the maximal CB1-mediated depression did not differ between the two treatment groups, for our chronic stress experimental conditions, our two-way ANOVA analysis revealed a significant effect of time ($p<0.001$), and repetitive stress

exposure ($p< 0.001$) but no interaction effect ($p>0.05$). Collectively, these data indicate that chronic stress exposure does not enhance CB1-mediated suppression of CeAL glutamatergic afferents.

In an alternative experimental approach, we next examined whether this experimental result also occurred in other regions of the amygdala that implicated in the stress response. As such, we targeting the glutamatergic afferents of the lateral amygdala (LA), as these inputs relay critical stress-related sensory information to the LA allowing the LA to, in turn, relay polymodal sensory information from the thalamus and cortex to the CeAL (Quirk et al., 1995). In doing so the LA-CeAL circuitry modifies CeAL micro-circuitry and functionality in the stress response (Li et al., 2013; Quirk et al., 1997). Following repetitive exposure to restraint stress, we electrically stimulated the external capsule (EC) to evoke field excitatory postsynaptic potentials (fEPSPs) within the LA and subsequently bath applied the CB1 receptor agonist, CP 55940 (10μM;CP). These experiments reveal that 10μM CP 55940 application maximally suppressed fEPSP amplitude to 52.52%±5.69% of baseline (n=5; Figure 15A) in control mice. However, in mice exposed to 10 days of restraint stress CP-induced depression was significantly less (stress+CP 74.79%±6.29% n=6; Figure), with the maximal CP-induced eEPSP depression significantly reduced in our chronic stress conditions as compared to control conditions ($p<0.05$). Two-way ANOVA analysis reveal a significant effect of repeated stress ($p<0.0001$), time ($p<0.0001$), as well as an interaction between the two parameters ($p<0.0001$). These data indicate that repetitive restraint stress decreases the sensitivity of CB1 receptors localized to glutamatergic terminals in the LA but has no effect on MP-CeAL synapses.

Chronic Stress Exposure Enhances 2-AG Biosynthesis at MP-CeAL Synapses

To determine whether increased 2-AG biosynthesis at MP-CeAL synapses also supports CeAL DSE enhancement observed in our earlier experiments, we next examined the effects of inhibiting MAGL, 2-AG's primary catabolic enzyme, on CeAL DSE. We reasoned that if chronic stress were to increase 2-AG biosynthesis at MP-CeAL excitatory synapses, inhibiting tonic MAGL activity would cause greater 2-AG mediated synaptic depression in our chronically stressed mice as compared to controls. Consistent with this hypothesis, our experimental results indeed show that bath application of the MAGL inhibitor, JZL-184 (2μM), caused an enhanced depression of MP evoked excitatory CeAL potentials in chronic stress conditions as compared to non-stressed controls (Figure 15C). Two-way ANOVA analysis showed a significant effect of chronic stress ($p < 0.0001$) and time ($p < 0.0001$), but no interaction ($p>0.05$) between the two treatment groups. Collectively, the results suggest that repetitive stress augments 2-AG biosynthesis at MP-CeAL synapses that enhanced the attenuation of glutamatergic drive to the CeAL.

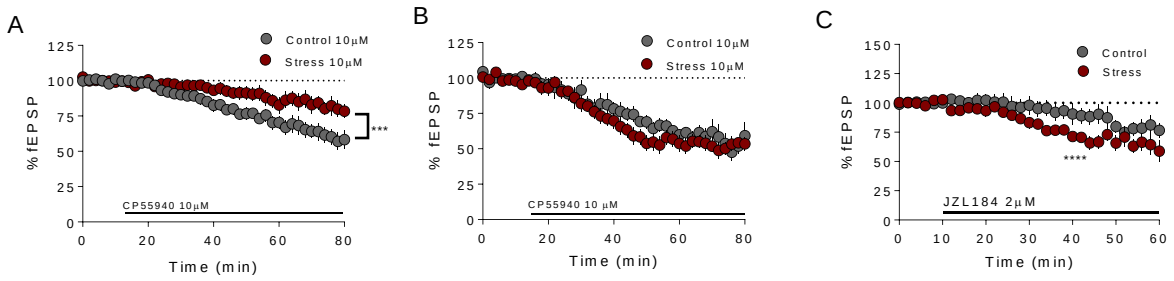

Figure 15. Region Specific Effects of Chronic Stress on eCB Signaling at CeAL Glutamatergic Synapses.
(A) CP 55940 (10μM) depresses LA-CeAL fEPSPs greater in behaviorally naïve controls as compared to chronically restraint mice. (B) CP 55940 (10μM) depresses MP-CeAL fEPSPs to a greater degree in chronically restrained mice as compared to controls. (C) MP-CeAL glutamatergic synapses of chronically restraint mice demonstrate larger JZL-184 (2μM)-induced synaptic depression as compared to behaviorally naïve controls. ***$p<0.001$, ****$p<0.0001$. Data presented as mean ± SEM.

Effects of Repetitive Stress Exposure on MP-CeAL LTD

Our experimental results, thus far, indicate an enhanced capacity for phasic 2-AG synthesis and synaptic plasticity at excitatory synapses arising from glutamatergic inputs medial to the CeAL. Given that eCB-mediated short-term depression is augmented by chronic stress exposure, we next determined whether long-term depression at MP-CeAL synapses was also regulated by repetitive stress. Previous reports by Sumislawski and colleagues (2011) have shown that chronic stress enhances low-frequency stimulation (LFS)- induced LTD of BLA inhibitory synapses through augmented 2-AG signaling (Sumislawski et al., 2011). To determine whether CeAL excitatory synapses were similarly affected by chronic stress, we first assessed whether administration of another low-frequency stimulation (LFS) paradigm (4Hz, 1-ms stimuli alternating with 1 sec of rest for 180 iterations for a total of 6 minutes), previously shown to elicit eCB-mediated LTD of hippocampal excitatory synaptic transmission (Kellogg et al., 2009), also elicited LTD of MP-CeAL synapses. In wildtype conditions, administration of this LTD stimuli paradigm to MP arising glutamatergic afferents resulted in the persistent suppression of MP-CeAL fEPSPs to 77.26%±2.07% (of baseline n=6); an effect largely absent in age-matched global $CB1^{-/-}$ mice (99.06%±3.37% n=11, $p<0.0001$, Figure 16A).

Given that our LTD paradigm induces a persistent CB1-dependent depression of evoked MP-CeAL glutamatergic responses, we next determined whether this LTD is also modulated by prior stress exposure. Following our chronic restraint paradigm, our LTD induction protocol at medially arising glutamatergic afferents resulted in a persistent depression of MP-CeAL glutamatergic synapses that was significantly larger than that elicited under control conditions (control 78.97%±4.06% n=12 vs. stress 58.49%±4.50% n=11, $p<0.01$, Figure 16B). Collectively, this experimental evidence suggested that chronic stress facilitates enhanced CB1-mediated LTD at MP-CeAL excitatory synapses.

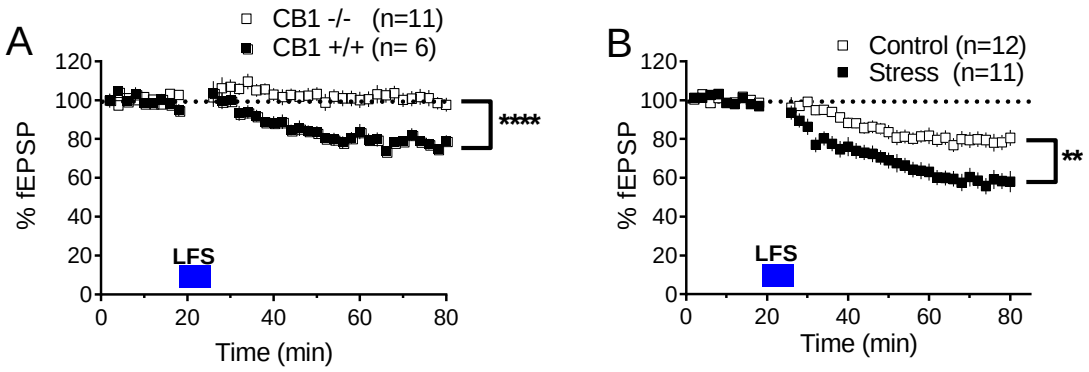

Figure 16. MP-CeAL Synapses Also Exhibit Enhanced LTD following Chronic Restraint Stress.

(A)Low-frequency stimulation (LFS)-induced LTD of MP-CeAL fEPSPs is present in WT mice but absent in $CB1^{-/-}$. (B) LFS-induced LTD of MP-CeAL excitatory synapses is enhanced in chronically stress mice as compared to behaviorally naïve controls. **p<0.01, ****p<0.0001. Data presented as mean ± SEM.

Effects of Augmented 2-AG levels on HPA Axis Activation

Our data thus far suggests that chronic repetitive stress augments phasic 2-AG mobilization and CB1-mediated LTD at CeAL glutamatergic synapses. These stress-dependent changes in the CeAL eCB system, we hypothesize, will decrease glutamatergic-mediated activation of the CeAL and subsequently attenuate CeA-mediated components of the stress response, such as the activity of the HPA axis (2001; Ciocchi et al., 2010; Kolber et al., 2008; LeDoux et al., 1988a). In a proof of principle experiment, we next tested whether pharmacologically enhancing endogenous 2-AG content could attenuate CeA-mediated functions such as basal HPA axis activity. To increase endogenous 2-AG levels, 1 hour before exposure to acute restraint stress we systemically treated mice with the MAGL inhibitor, JZL 184 (16mg/kg), at a concentration we have previously shown increases brain 2-AG levels (Sumislawski et al., 2011). These experiments revealed that acute restraint stress increases basal HPA axis activity, as indicated by stress-induced increases in corticosterone plasma levels. Furthermore, this stress-corticosterone release was significantly attenuated in our JZL-pretreatment conditions as compared to our vehicle treated animals (vehicle+ stress 132ng/ml±29.43 vs. JZL184+stress 88.34±21.79ng/ml n=5/treatment condition, $p<0.05$, Figure 17). Collectively, the data presented thus far suggest that augmented 2-AG signaling at CeAL glutamatergic synapses may serve to attenuate excitatory drive and decrease activation of CeA mediated functions of the stress response.

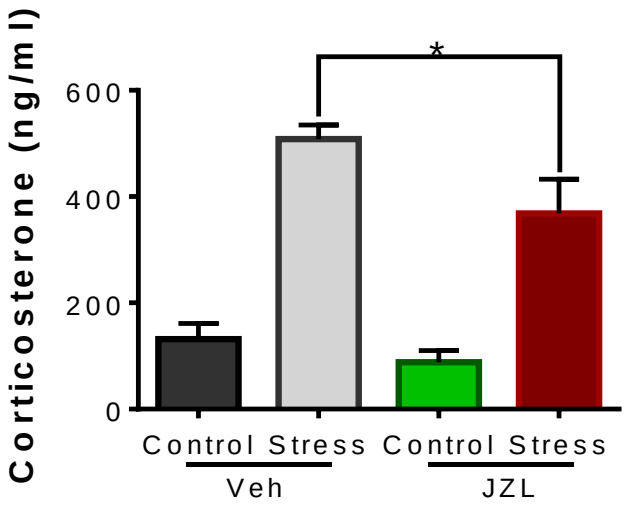

Figure 17. Systemic Increase in 2-AG Attenuates Acute Stress Induced HPA Axis Activation.
Systemic JZL-184 (16mg/kg) treatment significantly attenuated acute stress induced HPA axis activation.*$p<0.05$. Data presented as mean ± SEM.

Discussion

The principal findings from this work are that repetitive restraint stress exposure enhances eCB mediated short-and long-term synaptic depression of CeAL glutamatergic synapses. This conclusion is supported by evidence demonstrating enhanced phasic 2-AG signaling as well as augmented CB1-mediated long-term depression of CeAL excitatory synapses. Furthermore, chronic restraint stress elicits afferent-dependent changes in CB1 receptor signaling at stress-regulating glutamatergic terminals within the amygdala. We reached this conclusion as CB1 signaling at LA, but not MP-CeAL, glutamatergic afferent terminals is attenuated following chronic stress exposure. Lastly, in an effort to determine the functional implications of stress-dependent enhancement of CeAL 2-AG content, we show that pharmacologically increasing systemic 2-AG levels attenuated HPA axis activation—a CeAL-mediated component of the stress response. Similar to other studies in our lab, these data suggests that repetitive stress exposure sensitizes the eCB system at central synapses of the amygdala. Here we have shown that, at the level of CeAL glutamatergic synapses, homotypic chronic restraint stress augments 2-AG, consequently enhancing eCB-mediated synaptic modulation of CeAL excitatory transmission.

Repetitive Restraint Stress-Induced Enhancement of CeAL DSE

Prior biochemical and electrophysiological data suggest that repetitive restraint stress exposure results in adaptations in amygdalar 2-AG content and signaling capacity (Patel et al., 2009a; Rademacher et al., 2008; Sumislawski et al., 2011). In an effort to extend these findings, we tested whether this increased amygdalar 2-AG content correlates with enhanced 2-AG mobilization at CeAL excitatory synapses. Consistent with this hypothesis, our data reveal that repetitive restraint stress exposure enhances the maximal DSE of locally recruited CeAL

glutamatergic transmission in a CB1 and 2-AG dependent manner, while causing no change the late CeAL DSE. This lack of effect on late CeAL DSE implicate enhanced 2-AG biosynthesis as one of the mechanisms underlying enhanced 2-AG phasic synaptic modulation of CeAL glutamatergic synapses following chronic stress exposure.

Interestingly, these outcomes are the converse of experimental observation in the BLA, a neighboring amygdala subnuclei that also exhibits increased 2-AG levels and signaling following repetitive stress exposure (Hill et al., 2010c). At inhibitory synapses of the BLA, a similar restraint stress paradigm did not affect maximal DSE but, on the other hand, enhanced late DSE (Patel et al., 2009a). Though these stress-dependent synaptic changes are different, this discrepancy in the maximal DSE and the time course of 2-AG mediated synaptic plasticity between the two amygdala subnuclei may be explained by morphological differences at inhibitory BLA synapses as compared to excitatory synapses of the CeAL. Recent work by Yoshida and colleagues (2011) have show that 2-AG mediated DSE is more readily induced and saturated at postsynaptic depolarization profiles as low as 0.1 seconds. This, they strongly argue, is a consequence of perisomatic invaginating synapses enriched with the molecular components of the eCB biosynthetic machinery that are uniquely present in the BLA, but absent in the CeAL (Yoshida et al., 2011b). Thus, the 5 seconds postsynaptic depolarization protocol used in our earlier studies (Patel et al., 2009a) may have saturated the maximal 2-AG biosynthetic capacity at BLA inhibitory synapses, thus occluding detectable differences in the 2-AG biosynthetic capacity between behaviorally naïve and repetitively stressed mice.

Alternatively, two mechanistically distinct modes of action may support stress-induced increases in 2-AG levels in the BLA and the CeAL. The enhanced maximal CeAL DSE in our chronic stress conditions, with no difference in late CeAL DSE, suggests that stress-induced

increases in 2-AG biosynthesis perhaps through increased DAGL activity or postsynaptic mechanisms upstream of DAGLα activation, such as $G\alpha_q$ receptor activation (Jung et al., 2012), may potentially be at play. On the other hand, augmented 2-AG content and DSE at BLA inhibitory synapses were found to be a consequence of repetitive stress-induced MAGL down-regulation (Patel et al., 2009b). Collectively, these results suggest that repetitive stress exerts subregional specific regulation of 2-AG levels by differentially targeting 2-AG biosynthesis and metabolism within distinct amygdalar nuclei. However, additional experimental analyses of CeAL DAGL expression and activity will be needed to conclusively determine whether this chronic stress- dependent differential effect is indeed the case.

CeAL Glutamatergic Synapses Exhibit Afferent Specific Regulation by Repetitive Stress Exposure

The CeAL receives intra-and inter-amygdaloid afferent inputs largely thought to relay different sensory modalities to the CeAL, which results in activity dependent changes in the CeAL microcircuitry and stress-related functionalities. CeAL afferents arising medial to the CeAL contain afferents originating in the lateral parabrachial area (Bernard et al., 1993; Neugebauer et al., 2003) (Lopez de Armentia and Sah, 2004b) and thalamus (Pitkanen et al., 1995; Sun et al., 1994; Yasui et al., 1991); each convey nociceptive and sensory related information, respectively. Our experimental results reveal that repetitive stress exposure does not affect maximal CB1 dependent synaptic depression at CeAL glutamatergic synapses. Unlike the CeAL, glutamatergic inputs to the LA exhibit functional downregulation of CB1 receptors signaling following repetitive stress exposure as compared to behaviorally naïve controls. These experimental results are consistent with decreased CB1 receptor signaling in the paraventricular nucleus of the hypothalamus (Wamsteeker et al., 2010), BLA (Patel et al., 2009b), and striatum (Rossi et al., 2008a) subsequent to other chronic stress paradigms (Hill et al., 2005). Given that glutamatergic

terminals within the LA facilitate the relay of polymodal sensory information from the LA to the CeAL, this stress dependent effect on eCB modulation of LA excitatory drive may have an indirect effect on the CeAL microcircuitry. As such, though stress-dependent changes in CeAL glutamatergic synapses are not observed following chronic stress exposure, other chronic-stress mediated changes in synaptic CB1 receptor functions within the amygdala can still modify the information flow through the CeAL microcircuitry and affect the physiological and behavioral functions elicited by CeAL activity.

Conversely, medially arising afferent pathways of the CeAL display enhanced eCB signaling (i.e. increased phasic 2-AG biosynthesis) subsequent to repetitive stress exposure as compared to controls. Furthermore, these synapses are also sensitive to CB1 mediated LTD, which is also enhanced in a chronic stress-dependent manner. Though our initial DSE studies suggests that enhanced 2-AG biosynthesis may facilitate this enhanced LTD in our chronic stress conditions, additional work will have to be done to determine the eCB (i.e. 2-AG or AEA) mediating this persistent depression. As such, MP-CeAL synapses are highly sensitive to eCB-mediated regulation following repetitive restraint stress exposure. Collectively, these experimental results illustrate that chronic restraint stress differentially affects the CB1 regulation of glutamatergic inputs to CeAL as well as other amygdalar nuclei whose activation regulate CeAL outflow.

Functional Implications of Chronic Restraint Stress-Induced Regulation of CeAL Glutamatergic Afferents

In human studies, converging lines of evidence parallel preclinical findings of stress-induced modulation of the eCB system acting as a mechanism for stress adaptation. Work by Hill and colleagues (2009) have shown that acute social stress increases circulating levels of 2-AG while Chouker and authors (2010) have also reported exaggerated stress responses observed in humans

who do not show elevations in 2-AG following exposure to parabolic flight stress (Chouker et al., 2010; Hill and Gorzalka, 2009). Thus, consistent with our rodent studies, stress-induced elevated 2-AG appears to be an adaptive response that constrains stress responsivity by contributing to the termination of the stress response (Evanson et al., 2010; Hill et al., 2011a). Similar studies in chronic-stress related psychiatric disorders, such as depression and PTSD, are however less clear. Reduction in plasma 2-AG or AEA plasma concentrations are seen in individuals with PTSD (Hill et al., 2009b; Hill et al., 2008b). Though future studies will have to be undertaken to determine the variables (e.g. trauma differences, genetic background, duration and acuity of stress exposure, etc.) that may explain the differences between studies, a theme of retained capacity to mount a 2-AG response following exposure to stress is still observed in studies that found reduced basal eCB levels. Consistent with this, sustained elevations of 2-AG and AEA are found in trauma-exposed populations with PTSD, where the magnitude of the stressor is associated with the activation of eCB system (Hill et al., 2013a). Thus, despite the differences observed in the studies, the eCB system is mobilized by threat exposure to buffer against the negative consequence of stress (Hauer et al., 2013).

Conclusion

Overall, these data strongly suggest that eCB signaling mechanisms are modulated by chronic repetitive stress and this enhanced eCB-mediated synaptic plasticity potentially facilitate stress-adaptations through the regulation of excitatory inputs into the CeAL. Though increased anatomical resolution of our *ex vivo* experimental studies will be needed to fully assess the behavioral effects of enhanced afferent specific CB1 signaling, our initial studies suggests that eCB signaling mechanisms under naïve and repetitive stress conditions provides a locus for eCB regulation of CeAL circuitry activation following repetitive stress exposure.

CHAPTER VII

GENERAL CONCLUSION AND FUTURE STUDIES

Mechanistically Distinct Modes of Endocannabinoid Mobilization is Present at CeAL Excitatory Synapses

Collectively, our data demonstrates that eCB signaling mechanisms are present at CeAL glutamatergic synapses. Under control conditions, eCB mediated short-and long-term synaptic plasticity are present at CeAL glutamatergic synapses, suggesting that eCB signaling mechanisms can be recruited under conditions of strong neuronal activity. Consistent with the dynamic nature of the eCB system, diverse experimental contexts such as acute and prolonged mAChR activation, as well as, chronic restraint stress exposure augments the eCB-synthesis capacity of CeAL neurons. These data demonstrate that the eCB synthesis machinery at CeAL glutamatergic synapses is sensitive to the changing contexts within this region and strongly implicates the eCB system in the regulation of CeAL activity as well as CeAL-elicited physiological and behavioral components of the stress response. In support of this claim, our analysis of increased endogenous 2-AG on stress responsivity reveal an attenuation of acute-stress-induced HPA axis activation. This data suggests that increased eCB content may affect CeAL-mediated functions. More detailed analysis will be needed to fully determine the effects of eCB-attenuation of CeAL afferent input on its functionality in the stress response. Such analyses should include genetic and optogenetic tools that will allow for a more acute analysis of whether

eCB-signaling regulate CeAL glutamatergic, or even local inhibitory, synapses in a cell-type or afferent-specific manner.

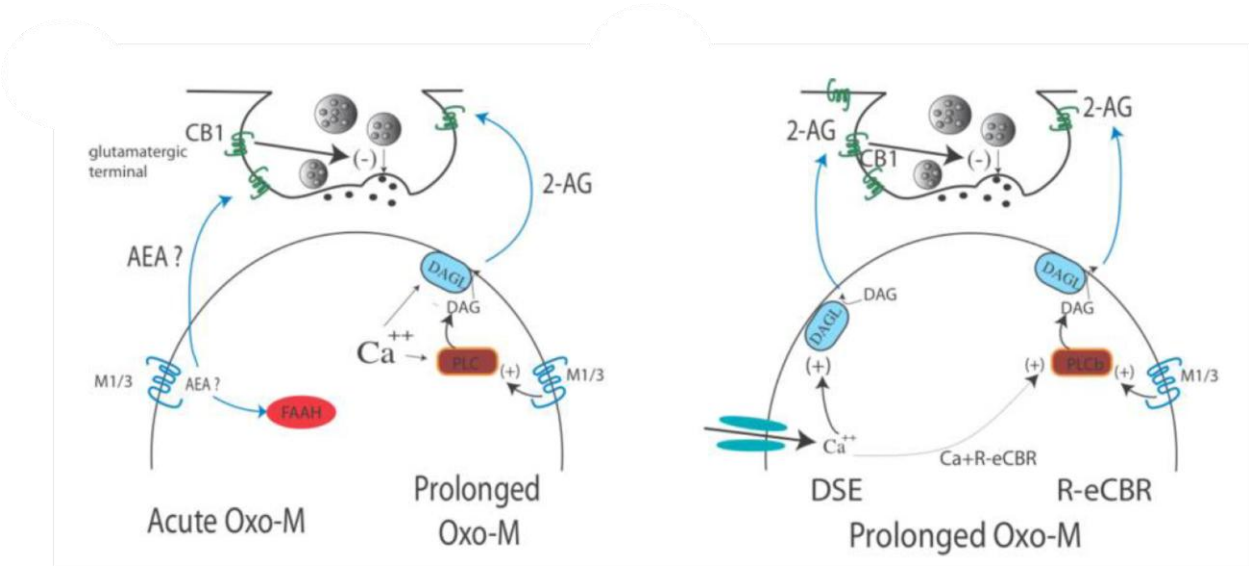

Figure 18. Multiple Mechanistically Distinct Modes of Endocannabinoid Mobilization is Present at CeAL Excitatory Synapses.
Under control conditions, CeAL synapses exhibit DSE. However, under conditions of prolonged mAChR activation, 2-AG is mobilized under conditions of basal Ca^{2+} levels (R-eCBR) and following depolarization-induced increases in $[Ca^{2+}]_i$. Conversely, acute mAChR activation drives AEA , not 2-AG, mobilization suggesting a time-dependent coupling of mAChRs to diverse signaling transduction pathways responsible for the successive synthesis of AEA and 2-AG. Of further note, this phenomenon appears to be unique to the CeAL.

Future Studies

Endocannabinoid Signaling at the Level of the CeAL Micro-circuitry

Here, we show that eCB signaling suppresses afferent glutamatergic transmission onto CeAL neurons, which could represent an important synaptic mechanism regulating stress response physiology and anxiety-like behaviors. As discussed in the Introduction section, the CeAL is composed of heterogeneous neurons that form a functional microcircuitry that gates the expression of fear- and stress-related responses via different glutamatergic afferent inputs. For the experiments discussed in this body of work, we have examined eCB signaling at CeAL excitatory synapses without regard of cell type or glutamatergic afferent. However, to truly determine the effects of eCB signaling on CeAL microcircuitry output and overall function in the stress response, the next steps must involve performing similar studies in a cell-type and afferent specific manner as eCB signaling at specific cell types or afferents will have different effects on the functionality of the CeAL microcircuit. For example, eCB-mediated inhibition of excitatory drive to protein kinase C-δ-expressing GABAergic CeAL projection neurons would disinhibit CeAM neurons and increase fear and anxiety responses (Ciocchi et al., 2010; Haubensak et al., 2010). This model is consistent with well-known behavioral effects of high doses of exogenous cannabinoids (Patel and Hillard, 2006) and, therefore, could represent a synaptic correlate of cannabinoid anxiogenesis. In contrast, inhibition of glutamatergic drive to locally-targeting somatostatin-expressing GABAergic neurons would disinhibit CeAL projection cell activity, inhibit CeAM activity, and ultimately reduce anxiety and fear responses which is one of the physiological function of eCB signaling (Hill et al., 2009a; Lutz, 2007; Riebe et al., 2012). Future studies aimed at elucidating the afferent- and cell type-specific effects of eCB signaling within the CeA microcircuitry, particularly using *in vivo* tools, will be critical to advancing our

understanding of the synaptic mechanisms by which eCB signaling modulates stress-responses, anxiety, and emotional learning.

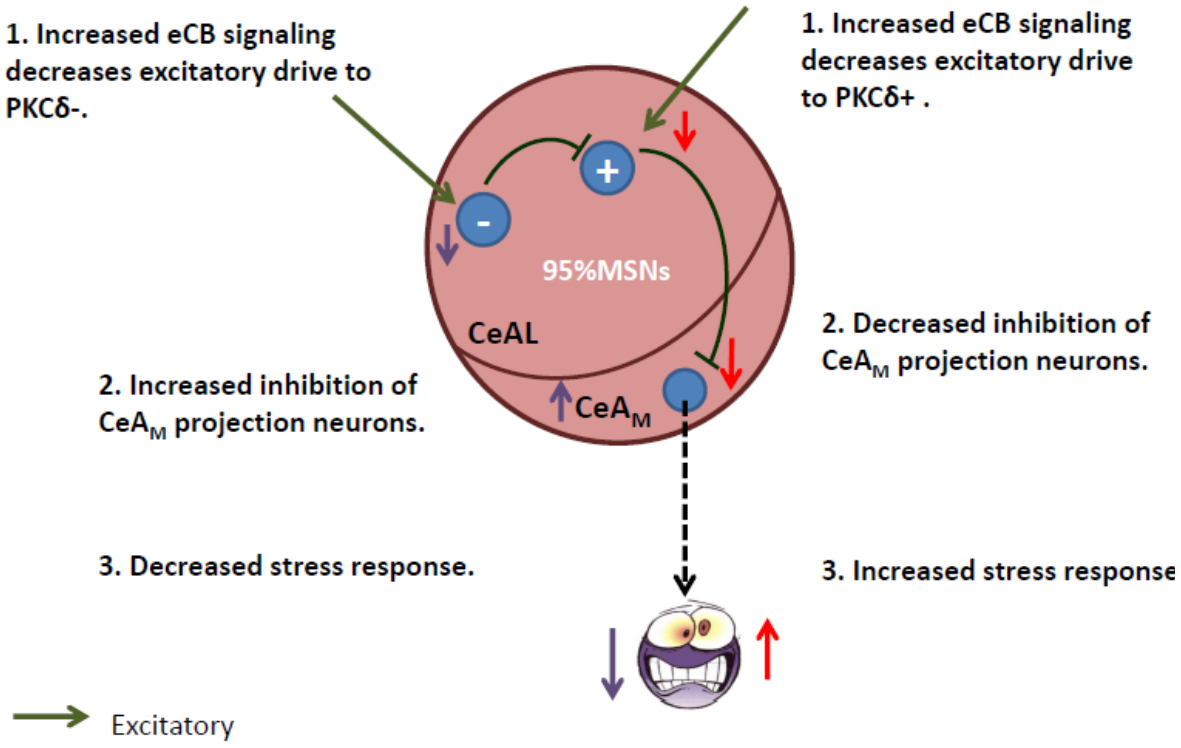

Figure 19. Conclusions: Functional Implications of Experimental Results.
Collectively, our data supports two possibilities: **A)** eCB- mediated attenuation of excitatory transmission at synapses of PKCδ$^-$ CeAL neurons, which subsequently increases inhibition of CeAM neuronal output neurons and attenuates stress response activation. **B)** *Alternatively*, CB1-mediated attenuation of glutamatergic inputs to PKCδ$^+$ CeAL cells disinhibits CeAM projection neurons and increases stress responsivity.

Mechanisms Driving Repetitive Restraint Stress-Enhancement of CeAL DSE

Our data suggests that enhanced 2-AG biosynthesis serves as one of the mechanisms driving enhancement of CeAL DSE following repetitive stress exposure. A potential avenue of support for stress-induced augmentation of 2-AG synthesis is mAChR-mediated increases in 2-AG synthesis. Data presented in Chapters 4 and 5, show that mAChR activation can enhance eCB mobilization at CeAL glutamatergic synapses. Furthermore, activation of stress circuits is well-known to lead to increased synaptic acetylcholine levels, increased mAChR affinity, and amygdalar acetylcholine content in limbic regions (Finkelstein et al., 1985; Gilad et al., 1985; Mark et al., 1996). Therefore, similarly to the eCB system, the cholinergic system also exhibit stress-induced adaptations. As a result, acetylcholine-eCB interactions may potentially act as an additional mechanism through which 2-AG biosynthesis is enhanced under conditions of repetitive stress exposure. In support of this, our previous pharmacological assessments of mAChR-mediated eCB mobilization at CeAL excitatory synapses show an enhancement of CeAL DSE to the same degree as that which is observed following chronic restraint stress. Though not further probed in our study, we also observed enhanced CeAL LTD in our chronic stress conditions, which may potentially be due to augmented 2-AG mobilization as a result of stress-dependent increases in cholinergic signaling. Given the evidence presented in this thesis, the potential for functional intersections between the cholinergic and eCB systems in facilitating stress adaptations at the levels of the CeAL excitatory synapses appears to be very promising and may be a potential therapeutic tool in the treatment of stress-related psychiatric disorders.

Of further note, the CeAL also expresses a number of $G\alpha_{q/11}$-coupled GPCRs that have also been implicated in driving eCB synthesis in other brain regions (Kano et al., 2009a). Such receptors include peptide receptors, such as the orexin receptor , which has been shown to stimulate eCB release using *in vitro* (Turunen et al., 2012) and *ex vivo* studies (Haj-Dahmane and

Shen, 2005). Furthermore, other $G\alpha_{q/11}$-protein coupled receptors such as the group I metabotropic glutamate (Kolber et al., 2010), adrenoceptor (Delaney et al., 2007), and oxytocin receptors (Viviani et al., 2011) are also highly expressed within the CeAL and have been shown to induce eCB release in other stress responsive brain regions (Kano et al., 2009a). As such, other forms of $G\alpha_{q/11}$-coupled eCB release may also be present at glutamatergic synapses of the CeAL and is an interesting avenue to explore in future studies. Interestingly, more recent work in the CeAL by the Stoop research group have shown that intra-CeA application of the oxytocin agonist, TGOT, decreased conditioned freezing (Viviani et al., 2011), which is the opposite result that is observed following intra-CeA application of the CB1 receptor antagonist, AM251(Kamprath et al., 2011). Collectively, these results suggests that $G\alpha_{q/11}$-protein coupled receptors-induced eCB release in the CeAL, such as through oxytocin receptor signaling, is potentially one of the neural substrates through which eCB mediate its fear and stress attenuating properties and may act as a potential therapeutic target for the treatment of fear and stress-related disorders.

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