Multiple Mechanistically Distinct Modes of Endocannabinoid Mobilization at Central Amygdala Glutamatergic Synapses

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SUMMARY

The central amygdala (CeA) is a key structure at the limbic-motor interface regulating stress-responses and emotional learning. Endocannabinoid (eCB) signaling is heavily implicated in the regulation of stress-response physiology and emotional learning processes; however, the role of eCBs in the modulation of synaptic efficacy in the CeA is not well understood. Here we describe the subcellular localization of CB_1 cannabinoid receptors and eCB synthetic machinery at glutamatergic synapses in the CeA, and find that CeA neurons exhibit multiple mechanistically and temporally distinct modes of postsynaptic eCB mobilization. These data identify a prominent role for eCBs in the modulation of excitatory drive to CeA neurons and provide insight into the mechanisms by which eCB signaling could regulate stress-responses and emotional learning processes.

HIGHLIGHTS

1) eCB signaling components are expressed at CeA glutamatergic synapses.

2) Activation of the CB_1 receptor suppresses glutamate release in the CeA.

3) CeA neurons express calcium-dependent and receptor-driven eCB signaling.

4) CeA muscarinic receptors drive temporally distinct multimodal eCB release.

INTRODUCTION

The central amygdala (CeA) plays a key role in emotional learning processes (Ehrlich et al., 2009; Pape and Pare, 2010). Perhaps most well-studied is the role the CeA plays in unconditioned and conditioned fear generation (Ciocchi et al., 2010; Li et al., 2013; Tye et al., 2011), fear extinction and conditioned inhibition (Amano et al., 2010), as well as conditioned orienting responses to emotionally salient stimuli (El-Amamy and Holland, 2007; Groshek et al., 2005). The CeA is a subcortical structure, mainly composed of GABAergic neurons, and can be largely divided into a lateral (CeAL) and medial (CeAM) subdivision (Cassell et al., 1999). CeAL neurons share significant morphological, cytoarchitectural and phenotypic homology to striatal medium-spiny neurons (MSNs) whereas CeAM neurons are considered to be pallidal-like in nature (Cassell et al., 1999). Thus, in analogy to the striato-pallidal circuitry of the basal ganglia, the CeAL-CeAM pathway appears to conform to this fundamental functional motif of forebrain organization. Specifically, the CeAL acts as the primary input nucleus of the CeA and receives strong glutamatergic drive from cortical (McDonald et al., 1999), thalamic (Li and Kirouac, 2008), as well as intra-amygdala and brainstem sources (Dong et al., 2010), and projects GABAergic axon terminals to the CeAM (Sun et al., 1994). The CeAM, in turn, projects to downstream regions involved in the expression of fear and arousal responses to salient stimuli (Davis, 1997). Functional studies have recently shown that activation of the CeAL strongly inhibits CeAM output neurons and reduces behavioral fear and anxiety responses (Ciocchi et al., 2010; Li et al., 2013; Tye et al., 2011). Thus, enhanced inhibitory control of CeAM neurons by elevated activity of certain CeAL inputs may serve to constrain conditioned fear and anxiety (Amano et al., 2010;

Ciocchi et al., 2010). Taken together, these data suggest that understanding the synaptic mechanisms regulating excitatory drive to CeAL neurons could provide significant insight into the mechanisms regulating the expression of fear and anxiety.

Endocannabinoids are a class of bioactive lipids produced by neurons and glia in the central nervous system (Kano et al., 2009). 2-arachidonoylglycerol (2-AG) is the primary eCB that mediates eCB retrograde synaptic signaling at central synapses (Castillo et al., 2012). 2-AG is post-synaptically synthesized via diacylglycerol lipase α (DAGLα) via two primary mechanisms. The first is a calcium-dependent mechanism, prototypically elicited by postsynaptic depolarization, which activates L-type calcium channels and enhances the conversion of diacylglycerol to 2-AG via DAGLα activity (Ohno-Shosaku et al., 2005). The second is a Gq-protein-coupled receptor (GqPCR) driven pathway mediated via the activation of PLCβ under basal intracellular calcium levels ($[Ca^{2+}]_i$). Under these conditions, PLCβ activation increases diacylglycerol and 2-AG levels, the latter by a DAGLα-dependent process. (Hashimotodani et al., 2007). Given that PLCβ activity is increased by a rise in $[Ca^{2+}]_i$ (Hashimotodani et al., 2005), a combinatory mechanism also exists whereby depolarization-induced calcium influx can facilitate Gq-receptor driven 2-AG release by enhancing PLCβ activity (Hashimotodani et al., 2005; Ohno-Shosaku et al., 2012). In contrast to 2-AG, the mechanisms regulating synaptic AEA signaling are not well understood, but can involve GqPCR activation (Chavez et al., 2010; Grueter et al., 2010; Huang and Woolley, 2012).

Despite the prominent role of eCB signaling in the regulation of fear, anxiety and stress responses (Hill et al., 2010; Lutz, 2007; Ramikie and Patel, 2011; Riebe et al., 2012), the role of eCB signaling in the modulation of CeA circuitry has been relatively

under-investigated. This may, in part, be due to previous anatomical studies that demonstrated very weak CB_1 receptor immunoreactivity within the CeA (Kamprath et al., 2011; Katona et al., 2001), especially relative to the high CB_1 receptor levels in the basolateral amygdala (BLA). In contrast to earlier studies, we utilized high affinity antibodies to demonstrate pre- and postsynaptic localization of CB_1 receptors and DAGLα, respectively, at glutamatergic synapses within the CeAL. Subsequently, we elucidated several mechanistically distinct modes of postsynaptic eCB mobilization in CeAL neurons, which underlie temporally dissociated forms of eCB-mediated synaptic depression of CeAL glutamatergic neurotransmission.

RESULTS

Localization of endocannabinoid signaling machinery in the CeAL

The anatomical localization and functional significance of eCB signaling in the BLA has been well studied (Katona et al., 2001; Ramikie and Patel, 2011; Yoshida et al., 2011). In contrast, eCB signaling in the CeA has remained largely unexplored. One reason for the relative lack of experimental attention stems from early anatomical data in which CB_1 receptor expression remained under detection threshold in the CeA (Katona et al., 2001). However, these early studies utilized an anti-CB_1 receptor antibody with relatively lower affinity, and which primarily recognized CB_1 receptors on BLA GABAergic interneurons that express very high levels of CB_1 receptors.

Our detailed examination of CB_1 receptor expression within the CeA began with *in situ* hybridization studies that revealed a weak CB_1 *in situ* signal within the CeA, and much stronger expression levels in the BLA of wild-type, but not CB_1 knockout (KO;

$CB_1^{-/-}$), mice (Figure 1A-C). The presence of CB_1 mRNA in the majority of BLA neurons indicates that BLA cells, which project glutamatergic afferents to the CeAL, might also express CB_1 receptors (Figure 1C). Therefore, we employed a new high affinity anti-CB_1 antibody to probe the localization of CB_1 receptors in the CeA (Yoshida et al., 2011). Using this antibody, CB_1 receptors were clearly detected at high levels in both the CeAL and CeAM of wild type, but not $CB_1^{-/-}$ mice (Figure 1D-F). Additionally, electron microscopic (EM) examination revealed CB_1 receptor expression in presynaptic boutons forming asymmetric synapses onto dendritic shafts and spines within the CeAL (Figure $1G_1$-G_2, I).

Considering that 2-AG is the primary ligand 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 in both the BLA and CeA (Figure 2A-C). Immunohistochemistry, using an anti-DAGLα antibody whose specificity in the forebrain has been confirmed in DAGLα$^{-/-}$ mice (Ludanyi et al., 2011), uncovered a punctate staining pattern throughout the CeAL (Figure 2D-E). Double immunofluorescence labeling and confocal microscopy revealed DAGLα-positive puncta in close apposition to MAP2 labeled dendritic shafts in the CeAL (Figure 2F-H), suggestive of DAGLα localization 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 dendritic shafts and spine heads forming asymmetric synapses in the CeAL (Figure 2I-L). Taken together

these data demonstrate the presence of eCB signaling elements at glutamatergic synapses in the CeAL.

CB_1 receptors modulate glutamate release onto CeAL neurons

To determine the functional significance of CB_1 receptor expression in the CeAL, we conducted whole-cell voltage-clamp electrophysiological recordings in the presence of picrotoxin (25-50μM) to isolate glutamatergic currents. Consistent with the localization of CB_1 receptors on excitatory axon terminals in the CeAL, we found activation of CB_1 receptors with the cannabinoid agonist CP55940 (5μM) significantly depressed eEPSC amplitudes to 52%±4% of baseline in CeAL neurons from wild-type (WT) mice; an effect absent in cells from $CB_1^{-/-}$ mice (WT 52.47±3.94% vs. $CB_1^{-/-}$ 114±8%; $t(8)=7.18$; $p<0.0001$; Figure 3A-B). No significant effect on PPR was observed following 5μM CP55940 application to WT or $CB_1^{-/-}$ cells (WT normalized PPR 1.06±0.06 vs. $CB_1^{-/-}$ 0.89±0.06; $t(8)=2$, $p=0.08$; Figure 3C). However, analysis of spontaneous EPSCs (sEPSCs) revealed a selective effect of 5μM CP55940 to reduce sEPSCs frequency (vehicle 4.35±0.92 Hz vs. CP55940 1.59±0.27 Hz; $U=50.00$, $p=0.008$), but not amplitude (vehicle 20.99±1.00 pA vs. CP55940 22.80±2.20 pA; $U=9$, $p=0.32$; Figure 3D-F), strongly suggesting a presynaptic locus of synaptic depression.

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 the eCB 2-AG and putative eCB with agonist activity at the CB_1 receptor (Hanus et al., 2001), to better elucidate the mechanisms by which eCB signaling, rather than a synthetic agonist, modulates glutamate release. Indeed, 2-AG-ether caused robust synaptic

depression (baseline 100.3±1.2% vs. 2-AG-ether 49.1±9.5%; $t(3)=6.13$ $p<0.01$; Figure 3G) that was associated with a significant increase in PPR ($t(3)=3.9$ $p<0.05$; Figure 3G inset). These data suggest that CB_1 receptors function to suppress glutamate release onto CeAL neurons.

Since CB_1 receptors in other brain regions robustly modulate GABAergic transmission (Castillo et al., 2012; Kano et al., 2009), we 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 with the electron microscopic observation that only a few GABAergic terminals were CB_1-positive in the CeAL (Figure $1G_1$-G_2, H), the effects on GABAergic transmission were small (baseline 100.0±0.0% vs. 76.1±9.3%, $t(7)=2.6$ $p<0.05$; Figure 3H). When compared to the effects of CP55940 (5μM) on glutamatergic transmission (from CeAL cells depicted in Fig. 3B), 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 3I). These data suggest that the major role of CB_1 signaling in the CeAL is to broadly regulate glutamatergic transmission, while synapse- or cell-type specific effects on GABAergic transmission may also occur to a lesser degree.

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 3J-L). Post-hoc Sidak's analysis revealed depolarization of CeAL neurons from

-70 mV 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 CeAL 10 second DSE was blocked by the CB_1 receptor antagonist, SR141716 (control 77.65±2.06% vs. 5μM SR141716 95.84±4.84%, $p<0.001$; Figure 3M-O) and absent in $CB_1^{-/-}$ mice ($CB_1^{-/-}$ 102.5±3.84%, $p<0.0001$; Figure 3O). DSE was also blocked by the DAGL inhibitor THL (10μM THL 91.20±2.13%, $p<0.05$; Figure 3O), and postsynaptic calcium chelation with 40mM BAPTA (BAPTA 92.07±1.46%, $p<0.05$; Figure 3O), indicating that Ca^{2+}-driven short-term eCB mobilization at CeAL glutamatergic synapses is mediated by 2-AG activation of CB_1 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, $p>0.05$ by t-test; control amplitude 21.0±1.0 pA vs. BAPTA 25.1±2.6, $p>0.05$ by t-test).

Ca^{2+}-assisted-mACh-receptor driven eCB release in the CeAL

In addition to Ca^{2+}-dependent eCB release, Gq-receptor-driven eCB mobilization is a common feature of central synapses (Katona and Freund, 2012). For example, in the hippocampus, activation of Gq-coupled M_1/M_3 muscarinic acetylcholine receptors (mAChRs) has been shown to mobilize eCB signaling in a calcium-independent manner (Kim et al., 2002; Straiker and Mackie, 2007). Importantly, mAChRs are also highly expressed in the CeAL (Roozendaal et al., 1997; van der Zee et al., 1997), which is innervated by cholinergic projection neurons originating in the basal forebrain (Heckers et al., 1994). 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 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%, $p<0.0001$; Figure 4A), an effect that reversed following drug washout (baseline 100.90±1.18% vs. post Oxo-M washout 96.14±7.13%, $p=0.79$; 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%; $p=0.87$; Figure 4A). Using whole-cell recordings we found that Oxo-M also caused robust depression of eEPSC amplitude that was 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. 4B). Oxo-M-induced synaptic depression was associated with a large increase in PPR, which was reduced by pirenzepine ($p<0.001$) and blocked by 4-DAMP ($p<0.0001$; Figure 4C), suggesting Oxo-M induced synaptic depression is mediated by $M_{1/3}$ receptor activation and expressed presynaptically. Importantly, neither pirenzepine nor 4-DAMP exerted any effects on glutamatergic transmission when applied alone to control CeAL slices (Supplementary Figure 1). Additionally, our immunofluorescence confocal microscopy data revealed a moderate expression of the M_1 receptor subtype throughout the CeAL (Figure 4E1-E2). At high magnification, M_1 staining appears as tiny puncta closely apposed to, but not overlapping with, MAP2-positve dendrites and perikarya. Together, these data demonstrate the presence of functional $M_{1/3}$ mAChRs at glutamatergic synapses in the CeAL.

Because M_1 mAchRs are primarily somatodendritically located at the ultrastructural level just like DAGLα (Figure 2I-L) (Yamasaki et al., 2010), we next evaluated the presence of mAChR-driven postsynaptic eCB release at excitatory synapses within the CeAL. It has been previously reported that DSE is effectively enhanced by the coincidental activation of Gq-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., 2002; Narushima et al., 2006). Consistent with the presence of Ca^{2+}-assisted-Gq-receptor driven eCB mobilization, our results revealed that Oxo-M (1μM) pre-incubation significantly enhanced 10s DSE as compared to DSE similarly performed under control ACSF conditions (control DSE 84.8±3.0% vs. Oxo-M DSE 57.16±2.56% $t(18)=4.1$ $p<0.001$; Figure 4F 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 $p<0.05$ by unpaired t-test; Figure 4G).

We next investigated the mechanisms of Oxo-M mediated enhancement of DSE in the CeAL. One-way ANOVA revealed that Oxo-M (1μM)-mediated DSE enhancement was reduced in both $CB_1^{-/-}$ CeAL cells (Oxo-M-WT 57.16±2.56% vs. Oxo-M-$CB_1^{-/-}$ 87.03±3.77%, $p<0.0001$; Figure 4 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 4I 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 depolarization-induced enhancement of Oxo-M-mediated 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 4J-K). 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 $CB_1^{-/-}$ mice 10 second depolarization in the presence of Oxo-M elicited a small residual DSE (baseline 100.0±0.0% vs. 87.03±3.77% $p<0.01$), suggesting possible CB_1 independent residual effects induced by depolarization in the presence of Oxo-M.

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 (Figure 5A). 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%, $p<0.0001$; Figures 5B-D and K). We next explored the contribution of CB_1 receptor activation to Oxo-M-mediated synaptic depression in the CeAL. In the presence of the CB_1 receptor antagonist, SR141716 (5μM, ≥ 60 minute pretreatment), Oxo-M-mediated synaptic depression was significantly attenuated at 0.3 μM and 1 μM Oxo-M concentrations relative to Oxo-M+vehicle experimental conditions (Figure 5B-D and K). Maximal Oxo-M-mediated depression was significantly attenuated in the presence of SR141716 following either 0.3 μM 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 Figure 5C-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 ($p<0.0001$; Figure 5E). Importantly, the residual Oxo-M depression in SR141716-treated slices was associated with a residual significant increase in PPR ($p<0.001$; Figure 5E), indicating that the non-CB_1 component of Oxo-M induced depression is also presynaptic in nature. Given that Oxo-M-induced synaptic depression is only partially CB_1-dependent, we sought to confirm these findings using $CB_1^{-/-}$ mice. Oxo-M induced synaptic depression was significantly attenuated in $CB_1^{-/-}$ mice (WT Oxo-M 37.91±2.83% vs. $CB_1^{-/-}$ Oxo-M 55.94±5.32% $t(12)=3.0$ $p<0.05$; Figure 5F). The maximal 1μM Oxo-M-mediated increase in PPR was also significantly attenuated in $CB_1^{-/-}$ mice ($p<0.001$; Figure 5G). Collectively, these data indicate that Gq-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-driven eCB release occurs via a Ca^{2+}-DAGL-PLA2 *independent* mechanism

In light of previous studies, the roles of intracellular Ca^{2+} and DAGL in Gq-receptor driven eCB release remain uncertain (Edwards et al., 2006; Hashimotodani et al., 2005; Kim et al., 2002; 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 5H and K). Similarly, THL pretreatment (10μM, ≥ 60 minutes pretreatment) did not inhibit 1 μM Oxo-M-mediated depression of eEPSC amplitude ($p>0.05$; Figure 5I and K). Lastly, since recent studies have suggested PLA2 may be required for 2-AG synaptic signaling in the cerebellum (Wang et al., 2012), we tested for the involvement of PLA2 in Oxo-M synaptic depression as an alternate mechanism by which Oxo-M could release 2-AG. However, the PLA2 inhibitor, AACOCF3 (10μM), did not significantly affect Oxo-M-mediated synaptic depression ($p>0.05$; Figure 5J and K). These data suggest that acute mAChR-driven eCB release within the CeAL occurs independently of increases in $[Ca^{2+}]_i$ and does not require DAGL or PLA2 activity.

Prolonged mAChR activation drives Ca^{2+}- and DAGL-*dependent* eCB release

The lack of calcium and THL sensitivity of Oxo-M-mediated acute synaptic depression was somewhat surprising in light of recent studies strongly implicating calcium and DAGLα in Gq-receptor driven eCB release (Castillo et al., 2012; Hashimotodani et al., 2013; Katona and Freund, 2012). To exclude the possibility that the non-CB_1-dependent component of Oxo-M induced acute depression was potentially confounding our analysis, we took an alternate approach aimed at selectively evaluating CB_1-dependent synaptic effects of Oxo-M. 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 Figure 6A for experimental design). We reasoned that, if prolonged mAChR

activation induces tonic eCB release and activation of CB_1, which depress glutamatergic transmission, then bath application of a CB_1 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-CB_1 mediated synaptic effects induced by prolonged mAChR activation by Oxo-M. Consistent with this hypothesis, SR141716 (5μM) wash-on significantly increased eEPSC amplitude in slices pretreated with 1μM Oxo-M relative to control no Oxo-M conditions (Figure 6B-D). Maximal potentiation induced by SR141716 in the presence of continuous 1μM Oxo-M was 143.20±6.59% compared to 113.30±4.09% under control conditions (p<0.001; Figure 6B-D). Interestingly, unlike acute Oxo-M-driven eCB release following brief application, continuous mAChR activation appeared to promote eCB mobilization through a THL- and a Ca^{2+}-*dependent* mechanism as pretreatment with 10μM THL for ≥ 60 minutes or 20mM intracellular BAPTA completely abolished SR141716-induced synaptic potentiation (p<0.0001 for each; Figure 6C-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; Figure 6C-D) or 20mM BAPTA postsynaptic loading (vehicle 113.30±4.09% vs. BAPTA+ Oxo-M 99.25±6.10%, p=0.34; Figure 6C-D) was not significantly different from SR141716-induced synaptic potentiation under control (no Oxo-M) conditions. These data suggest a possible temporal switch from a Ca^{2+}- and DAGL-*insensitive* to a Ca^{2+}- and DAGL-*sensitive* mAChR-receptor-driven eCB release following prolonged mAChR stimulation.

Acute mAChR activation drives synaptic AEA release

Several recent studies have indicated that Gq-coupled receptors can mobilize AEA signaling (Chavez et al., 2010; Grueter et al., 2010). As such, we sought to investigate whether the calcium and DAGL independent effects of acute Oxo-M application on eCB-mediated synaptic depression might be mediated via AEA, rather than 2-AG, signaling. To examine this possibility we determined the effects of inhibiting AEA degradation, with the fatty acid amide hydrolase (FAAH) inhibitor PF-3845, on Oxo-M mediated acute synaptic depression (see Figure 7A for experimental design). PF-3845 (5μM) pretreatment partially attenuated Oxo-M mediated synaptic depression at both 0.3μM ($p<0.01$) and 1 μM Oxo-M concentrations ($p<0.01$; Figure 7B-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 0.3 or 1μM Oxo-M concentration ($p>0.05$ for each; Figure 7B-D). Together these data indicate that interfering with 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. The lack of enhancement was not due to a floor effect since both maximal and sub-maximal concentrations of Oxo-M showed reduced efficacy in the presence of FAAH, but not MAGL, inhibition.

An alternate explanation for our results is that the acute effects of Oxo-M are occluded by PF-3845, but not JZL-184. If this were the case PF-3845 would be expected

to cause synaptic depression alone, which would then occlude subsequent AEA-mediated synaptic depression initiated by acute Oxo-M application. Consistent with this hypothesis, PF-3845 produced a CB_1-dependent synaptic depression alone (PF-3845+vehicle 83.31%±5.34% vs. PF-3845+SR141716 102.80%±3.91%, $t(14)=2.79$; $p<0.05$; Figure 7E-F). These data, combined with the lack of occlusion of acute Oxo-M-mediated synaptic depression by the MAGL inhibitor JZL-184 described above, strongly implicate AEA, rather than 2-AG, as the eCB ligand subserving synaptic depression 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 produce synaptic depression. To exclude this possibility and strengthen the support for an AEA-mediated process, we tested the ability of JZL-184 to produce synaptic depression alone. Consistent with our hypothesis, JZL-184 produced a CB_1 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 7G-H). Taken together, these data provide converging evidence that acute Oxo-M mediated synaptic depression causes synthesis/release of AEA that acts on CB_1 receptors to reduce glutamate release, and that prolonged Oxo-M stimulation of mAChRs enhances DSE and causes tonic CB_1-mediated synaptic depression via release of 2-AG through the canonical calcium-DAGL-dependent pathway (Figure 7I-J).

Lack of Oxo-M-mediated acute or tonic eCB signaling in striatum

Thus far, our data indicate that acute Oxo-M activation of mAChRs drives AEA release and subsequent depression of glutamatergic signaling via CB_1 activation. In contrast, prolonged mAChR activity results in 2-AG-mediated tonic CB_1 activation and enhancement of DSE. Since this is the first demonstration, to the best of our knowledge,

of temporally dissociated AEA and 2-AG release by the same stimulus, 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 CeA (McDonald, 1982). Acute application of Oxo-M (1μM) caused robust presynaptic depression in the striatum, however this depression was not affected by SR141716 pretreatment ($p>0.05$; Supplemental Figure 2A-C). Similarly, SR141716 failed to produce synaptic potentiation in the presence or absence of continuous prolonged Oxo-M (1μM) pre-treatment (Supplemental Figure 2D-E). In contrast, prolonged Oxo-M (1μM) application was able to enhance DSE relative to control ACSF ($p<0.01$; Supplemental Figure 2F), which 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 is not likely a generalized feature of central synapses, but is expressed by CeAL neurons.

DISCUSSION

The molecular architecture of eCB signaling at CeAL glutamatergic synapses

Here we report the distribution and subcellular localization of CB_1 receptors and the 2-AG synthetic enzyme, DAGLα, in the CeAL. CB_1 receptors are localized to presynaptic terminals forming asymmetric synapses on postsynaptic dendrites and dendritic spines. In contrast, DAGLα is expressed within postsynaptic CeAL neurons at the mRNA and protein level, with ultrastructural studies demonstrating clear localization within dendritic

spine heads and dendritic shafts adjacent to asymmetric synapses. Overall, these data support the well-established anatomical substrate for retrograde eCB signaling at central synapses (Katona and Freund, 2012). The present findings also extend earlier work in which CB_1 receptor-immunoreactivity level remained under detection threshold in this region (Katona et al., 2001). However, these earlier studies were conducted with anti-CB_1 receptor antibodies that preferentially labeled CB_1 receptors localized on GABAergic axon terminals due to the much higher level of CB_1 receptor expression on GABAergic terminals relative to glutamatergic terminals. Subsequent generation of higher affinity antibodies has now allowed for the detection of CB_1 receptors on glutamatergic axon terminals in several brain regions (Katona et al., 2006; Uchigashima et al., 2007). Using these reagents, we can now provide clear anatomical evidence for strong CB_1 expression on CeAL glutamatergic terminals. It is noteworthy that Kamprath and co-workers recently showed sparse CB_1 labeling in the CeAM using an antibody that recognizes CB_1 primarily on GABAergic terminals (Kamprath et al., 2011). In contrast, we show high levels of CB_1 expression in the CeAM in addition to CeAL, suggesting that a significant fraction of CB_1 expression in the CeAM, as in the CeAL, is likely found on glutamatergic terminals.

Consistent with our anatomical data, we found that activation of CB_1 receptors reliably reduced eEPSC amplitude, while effects on GABAergic transmission were more variable (Katona et al., 2001). Interestingly, the cannabinoid agonist CP55940 reduced eEPSC amplitude without clear effect on PPR. In contrast, the metabolically stable 2-AG analog, 2-AG-ether, and DSE both cause synaptic depression associated with an increase in PPR. Although our data that CP55940 affected the frequency, but not amplitude of

sEPSCs, suggest a presynaptic site of action, the lack of effect on PPR was unexpected and inconsistent with the effects of DSE and 2-AG-ether. Although previous studies have shown presynaptic inhibition of glutamate release in the CeAL in the absence of changes in PPR (Delaney et al., 2007), the discrepancy between different CB_1 ligands is more difficult to explain. One possibility could be related to ligand-directed signaling at the CB_1 receptor (Hudson et al., 2010), which would imply multiple presynaptic mechanisms downstream of receptor activation could result in reduced glutamate transmission. Specifically, eCB ligands such as 2-AG, and its stable analog 2-AG-ether, could activate signaling cascades downstream of CB_1 to reduce glutamate release probability, while CP55940 could cause changes in the number of release sites (Delaney et al., 2007). Further studies will clearly be required to test this hypothesis.

Multiple modes of postsynaptic eCB mobilization by CeAL neurons

Based on our studies demonstrating eCB signaling elements at CeAL excitatory synapses and CB_1 receptor mediated depression of glutamatergic signaling, we next examined the mechanisms regulating postsynaptic eCB release from CeAL neurons. Given substantial evidence that 2-AG acts as the primary eCB mediating retrograde synaptic signaling (Tanimura et al., 2010), as well as the expression of DAGLα at CeAL glutamatergic synapses, we next focused on the elucidation of mechanisms regulating 2-AG mobilization from CeAL neurons. In line with previous data in the hippocampus (Chiu and Castillo, 2007) and cerebellum (Kreitzer and Regehr, 2001), CeAL neurons expressed DSE mediated via a calcium-dependent, THL-sensitive, and CB_1-dependent mechanism. These data are consistent with depolarization-dependent activation of calcium channels initiating synthesis and release of 2-AG, which activates CB_1 receptors

to transiently depress glutamate release. These studies add to a recent demonstration of DSE in the CeAM (Kamprath et al., 2011), and tonic eCB release at CeAM GABAergic synapses (Roberto et al., 2010).

We next examined the mechanisms by which Gq-coupled mAChRs induced eCB signaling at CeAL glutamatergic synapses. Our data indicate that acute application of Oxo-M causes a robust presynaptic depression that is partially mediated via activation of CB_1 receptors. These data are consistent with findings in the periaqueductal grey (Lau and Vaughan, 2008), where Oxo-M-induced depression of glutamatergic transmission is partially eCB-mediated. Mechanistically, this acute CB_1-dependent depression does not require elevations in intracellular calcium, is THL-*insensitive*, and does not require PLA2 activity. Moreover, inhibition of 2-AG degradation with JZL-184 did not affect Oxo-M-mediated acute synaptic depression. Taken together, these data appear to exclude 2-AG as the eCB ligand mediating the acute CB_1-sensitive synaptic depression induced by short-term mAChR activation. We next tested the hypothesis that another eCB ligand, namely AEA, mediates eCB-mediated synaptic depression induced by acute mAChR activation. We found that FAAH inhibition, which caused a CB_1-dependent synaptic depression alone, partially occluded acute Oxo-M-mediated synaptic depression, implicating AEA in this process. Huang and Woolley (2012), who showed that estrogen-induced depression of GABAergic transmission in the hippocampus was occluded by FAAH inhibition, but not MAGL inhibition, reached similar conclusions (Huang and Woolley, 2012). Although, clear delineation of the biosynthetic pathways for synaptic AEA synthesis and the development of pharmacological tools to probe this system will

be required to conclusively assign AEA as the eCB ligand mediating acute Oxo-M driven eCB release.

In contrast to the putative AEA-mediated synaptic depression induced by acute mAChR activation, prolonged activation of mAChR increases 2-AG-mediated signaling processes. First, after prolonged Oxo-M incubation, DSE is enhanced in an $M_1/_3$-dependent, THL-sensitive, and CB_1-dependent manner, consistent with findings in other brain regions (Kano et al., 2009). Interestingly, our SR141716 wash-on studies revealed strong synaptic potentiation by CB_1 blockade in slices incubated with Oxo-M but not vehicle. These data suggest tonic eCB release can be induced by prolonged mAChR activity. This synaptic potentiation required increases in intracellular calcium and was THL-*sensitive* strongly suggesting that this tonic Oxo-M-induced eCB signal is mediated by 2-AG synthesized by the canonical calcium-DAGL-dependent pathway (Kano et al., 2009). These findings are consistent with recent studies in MAGL knock-out mice which suggest that 2-AG can also act as a tonic eCB retrograde messenger (Pan et al., 2011). Overall, these data indicate that CeAL neurons can mobilize multiple forms of eCB signaling to modulate afferent glutamatergic transmission.

Temporally dissociated multimodal eCB release from CeAL neurons

Previous studies have demonstrated that some cells 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., 2011). Similarly, several studies have demonstrated that activation of Gq-coupled receptors can induce 2-AG and also AEA release in several brain regions (Chavez et al., 2010; Grueter et al., 2010; Hashimotodani et al., 2013; Lerner and Kreitzer, 2012; Maccarrone et al., 2008).

However, to the best of our knowledge, our data are the first to provide experimental evidence that AEA and 2-AG can be released by the same cell in response to activation of the same Gq-coupled receptor depending only on the duration of Gq-receptor stimulation. Specifically, acute mAChR activation causes short-lived AEA-mediated synaptic depression, while prolonged mAChR stimulation causes 2-AG-mediated tonic eCB depression of glutamatergic transmission. Thus, in CeAL neurons, but not dorsal striatal neurons, mAChR stimulation can initiate multimodal eCB signaling depending only on the duration of Gq-receptor stimulation.

What possible mechanisms could underlie this temporally dissociated eCB release by CeAL neurons? $M_{1/3}$ receptor activation has been demonstrated to couple to signal transduction pathways important for 2-AG synthesis including PLC and increases in intracellular calcium (Mangoura et al., 1995; Sandmann et al., 1991; Schmidt et al., 1995a). $M_{1/3}$ receptors also couple to PLD (Mangoura et al., 1995; McKenzie et al., 1992; Schmidt et al., 1995b), which could be important for AEA synthesis (Leung et al., 2006). Interestingly, *in vitro* cellular studies show that $M_{1/3}$ coupling to PLD undergoes rapid desensitization within minutes (McKenzie et al., 1992; Schmidt et al., 1995b); in contrast $M_{1/3}$ coupling to PLC does not desensitize in response to prolonged agonist activation, and in fact shows a progressive sensitization (Schmidt et al., 1995b). These findings provide support for the hypothesis that the mechanistic basis for the temporal "switch" from Oxo-M induced AEA release to 2-AG release following prolonged exposure, is facilitated by the rapid desensitization of the PLD signaling and sensitization of the PLC signaling by Oxo-M. Functionally, this model predicts that upon $M_{1/3}$ receptor stimulation there is a burst of AEA release that dampens afferent glutamatergic drive to CeAL

neurons, while prolonged and continuous receptor activation causes a rapid desensitization of this pathway and termination of AEA signaling. In contrast, during prolonged $M_{1/3}$ receptor activation, the sensitizing PLC signaling results in tonic generation of diacylglycerol, which is hydrolyzed by DAGLα to generate 2-AG and subsequent tonic and long lasting CB_1-mediated suppression of glutamatergic drive to CeAL neurons.

Functional implications of multimodal eCB signaling

Recent studies have begun to highlight the dissociable roles of AEA and 2-AG signaling on multiple levels. For example, in the bed nucleus of the stria terminalis, AEA mediates long-term synaptic depression, while 2-AG mediates short-term depression (STD) in the form of DSE (Puente et al., 2011). In contrast, in the striatum different forms of associative neural activity can elicit both AEA- and 2-AG-mediated long-term depression (Lerner and Kreitzer, 2012). More recently, different inhibitory synapses in the striatum have been shown to release AEA and 2-AG in a state-dependent manner (Mathur et al., 2013). Thus, AEA and 2-AG are clearly not "redundant" signaling molecules and have distinct duration-, activity-, and synapse-dependent effects. Here we add to this eCB ligand diversity by demonstrating temporally-dissociated mobilization of AEA and 2-AG signaling in response to Gq-receptor activity. Thus, activity-dependent multimodal eCB signaling could exert powerful modulation of synaptic transmission on different time scales (LTD vs. STD), at distinct synapses, and in response to different patterns and durations of neural stimulation. Continued investigation of multimodal eCB signaling could provide insight into the activity-dependent mechanisms sculpting synaptic efficacy.

Here we show that eCB signaling suppresses afferent glutamatergic transmission onto CeAL neurons. Cannabinoid-mediated inhibition of excitatory drive to the CeAL could represent a previously unrecognized synaptic mechanism contributing to the well-known effects of cannabinoids on stress and anxiety-related behaviors (Hill et al., 2010; Patel et al., 2009), and on emotional learning processes (Lutz, 2007). Further elucidating the cell-type and synapse-specific effects of multimodal eCB signaling in the CeAL could provide insight into the cellular and circuit-level mechanisms by which eCBs modulate motivational states and emotional learning.

EXPERIMENTAL PROCEDURES

Anatomical experiments

In situ hybridization, immunoperoxidase and immuno-electron microscopy experiments were conducted as previously described (Peterfi et al., 2012) and detailed in supplemental experimental procedures.

Electrophysiology

Whole-cell voltage clamp and field potential electrophysiological experiments were carried out in 4-5 week old male ICR mice as described previously (Patel et al., 2009; Sumislawski et al., 2011) and delineated in the supplemental experimental procedures. Briefly, mice were sacrificed by transcardial perfusion with ice-cold high sucrose, low Na+ containing ACSF under isoflurane anesthesia and coronal slices prepared using a vibratome (Leica Microsystems, Bannockburn, IL). For whole-cell voltage clamp electrophysiological experiments, eEPSCs were recorded from CeAL neurons via local microstimulation, ~100μm from the cell soma.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. CB_1 receptors are present on excitatory terminals in the CeAL.

(A) *In situ* hybridization reveals the presence of CB_1 mRNA in both the CeA and the BLA of wild type mice. (B) The specificity of the riboprobe is confirmed by using $CB_1^{-/-}$ animals. (C) The very high levels of CB_1 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 CB_1. In contrast, CB_1 expression in the CeA was only slightly above detection threshold. (D-E) Immunoperoxidase staining demonstrates the presence of the CB_1 protein in both the CeA and BLA, which was confirmed in our $CB_1^{-/-}$ samples. (F) Higher magnification light micrograph reveals the dense CB_1 labeling in the neuropil throughout the CeAL. Asterisks depict CB_1-immunonegative cell bodies, whereas CB_1-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 CB_1 in boutons (b+), which form mainly asymmetric (flanked by black arrowheads) and sometimes symmetric (white arrowheads) synapses with dendrites (d) and spine heads (s). CB_1 staining remained under detection threshold in a few axon terminals (b-), which highlights quantitative differences in CB_1 expression between terminal types innervating the CeAL. (H-I) The anatomical nature of the synapse type is illustrated at higher magnification. Note the lack or presence of postsynaptic density at symmetric (H) or asymmetric (I) connections, respectively. 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.

Figure 2. DAGLαis 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 or sense riboprobes, respectively. (C) Expression of DAGLαmRNA 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). (I-L) Electron micrographs provide ample evidence for the postsynaptic concentration 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 sign the edge of the 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.

Figure 3. CB_1 receptors modulate glutamate release in the CeAL.

(A-C) CP55940 depresses eEPSC amplitude in WT but not $CB_1^{-/-}$ 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. (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, CB_1 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. Calibration scale in (A): 200pA, 25ms. Calibration bars for sEPSCs (D) at lower magnification (10pA, 100ms) and higher magnification (10pA, 20ms). All other scale bars: 10ms, 100pA.

Figure 4. mAChRs modulate glutamate release and enhance DSE

(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. (C) Oxo-M increases PPR, which is blocked by pirenzepine and 4-DAMP. (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; bar 100μm, E2; bar 5μm, inset 7.5μm). (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-M DSE. (I) DSE in the presence of OXO-M is attenuated by THL and in $CB_1^{-/-}$ mice. (J-K) Effects of pirenzepine and 4-DAMP on DSE in the presence of 1μM Oxo-M; grey faded line represents Oxo-M only DSE condition from (I) for visual comparison purposes. (L) Summary data of the effects of THL, CB_1 deletion, pirenzepine, and 4-DAMP on 10 second DSE in the presence of Oxo-M. **p<0.01, ***p<0.001, ****p<0.0001. Scale bars: 10ms, 100pA.

Figure 5. Acute mAChR activity drives Ca^{2+}- and DAGL-*independent* eCB release.

(A) Diagram of experimental design. (B-D) Oxo-M induced eEPSC depression is partially blocked by SR141716 at 0.3 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 $CB_1^{-/-}$ mice. (G) The increase in PPR after Oxo-M

application is attenuated in $CB_1^{-/-}$ mice. (H-K) Effects of intracellular 20mM BAPTA (H), THL (10μM) (I), and the PLA2 inhibitor, AACOCF3 (10μM) (J) on 1μM Oxo-M-induced eEPSC depression. (K) Summary data depicting the effects of SR141716, THL, BAPTA, and AACOCF3 on 1μM Oxo-M-mediated maximal eEPSC depression. *p<0.05, ***p<0.001, ****p<0.0001. Scale bars: 10ms, 100pA.

Figure 6. Persistent mAChR activity drives Ca^{2+}- 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-incubated 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.

Figure 7. 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 CB_1 antagonist pretreatment conditions. (G-H) Effects of JZL-184 on synaptic depression under control or CB_1 antagonist pretreatment conditions. (I) Diagrammatic representation of differences between acute vs. prolonged mAChR stimulation with Oxo-M. Acute Oxo-M application induces a short-lived "burst" of AEA to reduce afferent glutamate release, while prolonged mAChR stimulation causes a tonic calcium- and DAGL-dependent 2-AG release. (J) During

prolonged mAChR stimulation 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). *$p<0.05$, **$p<0.01$. Scale bars: 10ms, 100pA.