



Published in final edited form as:

*Neuron*. 2007 June 7; 54(5): 801–812.

## ENDOCANNABINOID-MEDIATED LONG-TERM PLASTICITY REQUIRES cAMP/PKA SIGNALING AND RIM1 $\alpha$

Vivien Chevalyre<sup>\*,1</sup>, Boris D. Heifets<sup>\*,1</sup>, Pascal S. Kaeser<sup>2</sup>, Thomas C. Südhof<sup>2</sup>, and Pablo E. Castillo<sup>#,1</sup>

*1*Dept. of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 104612

*2*Center for Basic Neuroscience, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas 75390

### SUMMARY

Endocannabinoids (eCB) have emerged as key activity-dependent signals that, by activating presynaptic cannabinoid receptors (CB1) coupled to G<sub>i/o</sub> protein, can mediate short-term and long-term synaptic depression (LTD). While the presynaptic mechanisms underlying eCB-dependent short-term depression have been identified, the molecular events linking CB1 receptors to LTD are unknown. Here we show in the hippocampus that long-term, but not short-term, eCB-dependent depression of inhibitory transmission requires presynaptic cAMP/PKA signaling. We further identify the active zone protein RIM1 $\alpha$  as a key mediator of both CB1 receptor effects on the release machinery, as well as eCB-dependent LTD in the hippocampus. Moreover, we show that eCB-dependent LTD in the amygdala and hippocampus shares major mechanistic features. These findings reveal for the first time the signaling pathway by which CB1 receptors mediate long-term effects of eCBs in two crucial brain structures. Further, our results highlight a conserved mechanism of presynaptic plasticity in the brain.

### INTRODUCTION

The endogenous ligands of cannabinoid receptors (endocannabinoids or eCBs) are important regulators of synaptic function (Alger, 2002; Freund et al., 2003) mediating short- and long-term plasticity at both excitatory and inhibitory synapses in a number of brain structures (for a recent review, see Chevalyre et al., 2006). These lipid signaling molecules are generally synthesized “on demand” from membrane precursors in the postsynaptic cell, and diffuse retrogradely to the presynaptic terminal (Piomelli, 2003). Once bound to the presynaptic cannabinoid receptor CB1, eCBs suppress transmitter release either transiently (<1 min) (Diana and Marty, 2004; Kreitzer and Regehr, 2002; Wilson and Nicoll, 2002), or in a consolidated long-term form (>1 hour) (Chevalyre et al., 2006; Gerdeman and Lovinger, 2003) that persists long after CB1 receptor activation has ceased (Chevalyre and Castillo, 2003; Robbe et al., 2002; Ronesi et al., 2004; Sjöström et al., 2003). How activation of the same presynaptic receptor leads to transient versus long-lasting depression remains unknown.

#Correspondence should be addressed to P.E.C. (pcastill@aecom.yu.edu); Pablo E. Castillo, Dominick P. Purpura Dept. of Neuroscience, Albert Einstein College of Medicine, Kennedy Center Rm. 703, 1410 Pelham Parkway South, Bronx, NY 10461, (718) 430 3263, (718) 430 8821

\*These authors contributed equally to this work

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

At present, eCB-mediated long-term depression (eCB-LTD) probably constitutes the best documented and most widely distributed form of activity-dependent, long-term presynaptic plasticity in the brain (Chevalleyre et al., 2006; Gerdeman and Lovinger, 2003). Examples of presynaptic eCB-LTD have been reported at excitatory synapses in dorsal striatum (Gerdeman et al., 2002; Kreitzer and Malenka, 2005), nucleus accumbens (Robbe et al., 2002), neocortex (Bender et al., 2006; Sjöström et al., 2003) and cerebellum (Soler-Llavina and Sabatini, 2006), as well as at inhibitory synapses in hippocampus (Chevalleyre and Castillo, 2003; Chevalleyre and Castillo, 2004) and amygdala (Azad et al., 2004; Marsicano et al., 2002). This prevalence suggests that eCB-LTD may be a fundamental mechanism for making long-term modifications to neural circuits and behavior. On the other hand, transient, eCB-mediated depression of synaptic transmission is exemplified by the phenomenon known as *Depolarization-induced Suppression of Inhibition or Excitation (DSI or DSE)*, a phenomenon well-characterized in the hippocampus and cerebellum, but also observed in other brain areas (reviewed in: Alger, 2002; Diana and Marty, 2004; Kreitzer and Regehr, 2002; Wilson and Nicoll, 2002). It is worth noting that by regulating GABA release, eCBs can also modify the inducibility of non-eCB-mediated forms of synaptic plasticity such as long-term potentiation (LTP) (Azad et al., 2004; Carlson et al., 2002; Chevalleyre and Castillo, 2004). Therefore, whether eCBs engage transient or long-lasting depression may profoundly impact neural circuit processing.

A mechanism for the transient suppression of release in DSI/DSE has been suggested by studies in hippocampus (Lenz et al., 1998; Varma et al., 2002; Wilson et al., 2001) and in cerebellum (Kreitzer and Regehr, 2001). Namely, CB1 receptor activation transiently inhibits presynaptic voltage-gated calcium channels (VGCC) for a period of seconds to minutes, a process likely mediated by the  $\beta\gamma$  G protein subunits (Wilson et al., 2001). One of the most striking requirements distinguishing I-LTD induction from that of DSI is the extended duration of CB1 receptor activity (Chevalleyre and Castillo, 2003). This increased time requirement for I-LTD induction could reflect the recruitment of a distinct signaling pathway by CB1 receptors. A likely candidate pathway for I-LTD is the  $\alpha_{i/o}$  effector limb of the CB1 receptor G protein signaling cascade, i.e. the cAMP/Protein Kinase A (PKA) pathway (Childers and Deadwyler, 1996; Howlett et al., 1986). Indeed, modulation of the cAMP/PKA pathway has been implicated in other presynaptic forms of plasticity in both hippocampus (Huang et al., 1994; Tzounopoulos et al., 1998; Villacres et al., 1998; Weisskopf et al., 1994) and cerebellum (Linden and Ahn, 1999; Salin et al., 1996; Storm et al., 1998). A common theme of these long-term forms of plasticity is that their expression mechanism relies on changes in the transmitter release machinery (Castillo et al., 1997; Castillo et al., 2002; Lonart et al., 2003; Tzounopoulos et al., 1998). In particular, the active zone protein RIM1 $\alpha$  (Wang et al., 1997) has been shown to be necessary for PKA-dependent LTP at several excitatory synapses including the mossy fiber to CA3 pyramidal cell synapse (Castillo et al., 2002), the parallel fiber to Purkinje cell synapse (Castillo et al., 2002; Lonart et al., 2003), and the Schaffer collateral to CA1 pyramidal synapse (Huang et al., 2005). These previous studies prompted us to ask whether eCB-LTD also involves downregulation of the cAMP/PKA pathway and/or a RIM1 $\alpha$ -dependent modification of the release machinery.

In this study, we compared the dependence of DSI and I-LTD on cAMP/PKA signaling, and on the active zone protein RIM1 $\alpha$ . We found that hippocampal I-LTD, but not DSI, requires cAMP/PKA signaling and RIM1 $\alpha$ . In addition, we report that CB1 receptors, via PKA, regulate GABA release downstream of VGCC-mediated calcium influx, and that both CB1 receptors and PKA can target the release machinery in a RIM1 $\alpha$ -dependent manner. Furthermore, we tested the generality of these mechanisms in the basolateral amygdala (BLA) where eCBs mediate I-LTD (Azad et al., 2004; Marsicano et al., 2002). At inhibitory synapses in the BLA, we also found that CB1 receptors target the release machinery via PKA and that RIM1 $\alpha$  is necessary for I-LTD. Taken together, these findings raise the intriguing prospect of a core

mechanism of presynaptic plasticity spanning multiple brain areas and synapse types (i.e. excitatory and inhibitory), and also provide a potential substrate for the hippocampus- and amygdala-dependent memory deficits reported in RIM1 $\alpha$  KO mice (Powell et al., 2004).

## RESULTS

During hippocampal DSI, CB1 receptor-mediated block of the N-type VGCC is believed to depress GABA release (Varma et al., 2002; Wilson et al., 2001). On the other hand, the CB1 receptors' effectors during I-LTD are completely unknown. Because CB1 receptors are G<sub>i/o</sub> coupled (Howlett et al., 2002) and have a well documented inhibitory effect on adenylyl cyclase and PKA activity (Childers and Deadwyler, 1996; Howlett et al., 1986), we tested whether the cAMP/PKA pathway might be involved in eCB-mediated long-term plasticity in slices of mouse hippocampus. First, cAMP levels were raised by continuous activation of adenylyl cyclase with forskolin (FSK), a manipulation that also increased the amplitude of evoked inhibitory postsynaptic currents (IPSCs) by  $25.0 \pm 5.4\%$  ( $n = 6$ ). Theta-burst stimulation (TBS) of presynaptic inputs, which triggered I-LTD under control conditions (Fig. 1A;  $23.8 \pm 2.0\%$ ,  $n = 4$ ), failed in the presence of FSK (Fig. 1A;  $3.8 \pm 3.1\%$ ,  $n = 5$ ,  $p = 0.0014$ ). Changes in cAMP level can affect synaptic transmission through both PKA-dependent and -independent actions (Seino and Shibasaki, 2005), so we next tested the involvement of PKA with two different blockers, the cell-permeable myristoylated form of Protein Kinase A inhibitor 14-22 amide (PKI), and H89. I-LTD was markedly reduced after incubating slices >30 min with either of these compounds (Fig. 1B, PKA blocker  $5.2 \pm 1.8\%$ ,  $n = 11$ , 7 cells in PKI and 4 cells in H89, data were combined since no differential effect was observed by these blockers, vs.  $20.8 \pm 2.0\%$ ,  $n = 6$  in interleaved control slice,  $p < 0.0001$ ). This result likely represents an occlusion of I-LTD, given that CB1 receptors can downregulate PKA activity and PKA inhibition acutely depresses synaptic transmission (see below, Fig. 5B). In order to rule out a postsynaptic role for PKA in I-LTD induction, I-LTD was also tested with the non-myristoylated, membrane impermeant form of PKI, delivered to the postsynaptic cell via the recording pipette. Blocking postsynaptic PKA did not affect I-LTD (Fig. 1B), indicating that the PKA requirement is presynaptic. In marked contrast to these effects on long term plasticity, neither FSK nor PKA blockers altered DSI in the same CA1 pyramidal cells (Fig. 1C), in accordance with previous findings (Morishita et al., 1998; Wilson et al., 2001). We obtained results similar to these with FSK and PKA inhibitors for I-LTD and DSI in brain slices from rats (data not shown). We considered the possibility that interfering with cAMP/PKA signaling blocked I-LTD by reducing glutamate release, which is the initial trigger for I-LTD (Chevalleyre and Castillo, 2003). However, neither PKA blockers nor FSK depressed excitatory synaptic transmission (i.e. CA1 field potentials) after 50 min bath application (PKI:  $1.3 \pm 2.1\%$ ,  $n = 6$ ; H89:  $-0.5 \pm 3.2\%$ ,  $n = 5$ ; FSK:  $24.4 \pm 5.3\%$ ,  $n = 4$ , data not shown). Taken together, these results show that the presynaptic cAMP/PKA pathway is required downstream of CB1 receptor activation for long-term, but not short-term eCB-mediated plasticity.

If CB1 receptors can regulate GABA release, as shown above, by two divergent signalling cascades, it stands to reason that this receptor may also independently target distinct steps of neurotransmitter release. In the hippocampus, previous studies have suggested that CB1 receptors suppress GABA release mainly by targeting VGCCs rather than the vesicle release machinery (Hoffman and Lupica, 2000; Varma et al., 2002; Wilson et al., 2001; Wilson and Nicoll, 2001). In the cerebellum however, CB1 receptors can target the release machinery in a manner that presumably depends on basal calcium levels in the presynaptic terminal (Yamasaki et al., 2006). For this reason, we chose to examine miniature IPSCs (mIPSCs) in CA1 in the presence of 5 mM extracellular Ca<sup>2+</sup>, blocking action potentials and VGCCs with 500 nM TTX and 100  $\mu$ M CdCl<sub>2</sub>, respectively. Application of 5  $\mu$ M WIN 55,212-2 (WIN), a CB1 receptor agonist, significantly decreased mIPSC frequency in the hippocampus (Fig. 2A,  $17.6 \pm 1.2\%$ ,  $n = 5$ ,  $p = 0.0002$ ). This effect was blocked by a CB1 receptor antagonist, AM

251 or SR 141716 (Fig. 2A). In addition, WIN had no effect on mIPSC amplitude (Fig. 2B), indicating a presynaptic action of CB1 receptors on GABA release. As the cAMP/PKA pathway is required for I-LTD induction, we tested whether it is also involved in CB1 receptor-mediated depression of mIPSC frequency. In presence of FSK, mIPSC frequency was increased (by 58.1 %,  $n = 6$ ,  $p = 0.003$ ) and WIN was unable to depress mIPSC frequency (Fig. 2C). Furthermore, blocking PKA with H89 reduced mIPSC frequency to the same extent as WIN alone, and the effects of WIN and H89 did not summate, again suggesting occlusion (Fig. 2C). These results show that in addition to presynaptic VGCCs, CB1 receptors can also target the release machinery via the cAMP/PKA pathway.

What lies downstream PKA in I-LTD? To address this question, we tested whether RIM1 $\alpha$ , a presynaptic active zone protein (Schoch et al., 2002; Wang et al., 1997) which is phosphorylated by PKA (Lonart et al., 2003) and is required for presynaptic forms of LTP at excitatory synapses (Castillo et al., 2002; Lonart et al., 2003), could also be involved in I-LTD. Notably, I-LTD induced by a TBS was abolished in RIM1 $\alpha$  KO mice (Fig. 3A,  $3.9 \pm 1.3$  %,  $n = 18$  cells, 10 KO mice, vs.  $19.1 \pm 1.8$  %,  $n = 14$  cells, 9 WT mice,  $p < 0.001$ ). This effect does not seem to result from deficient CB1 receptor function, as DSI remained unaffected in RIM1 $\alpha$  KO mice (Fig. 3B). The differential sensitivity of I-LTD and DSI to RIM1 $\alpha$  deletion parallels the differential involvement of the cAMP/PKA pathway in these processes and supports the idea that different targets downstream of the CB1 receptor mediate short- and long-term eCB-plasticity.

I-LTD is a heterosynaptic form of plasticity triggered by glutamate release from Schaffer collaterals, and postsynaptic activation of group I metabotropic glutamate receptors (mGluR-I) which stimulates eCB production (Chevaleyre and Castillo, 2003). Because the probability of glutamate release in area CA1 of the hippocampus is reduced in RIM1 $\alpha$  KO mice (Schoch et al., 2002), the loss of I-LTD could simply be due to a subthreshold activation of mGluRs. We addressed this possibility in several ways. First, we found that I-LTD was normal using  $10 \mu\text{M Cd}^{2+}$ , a non-selective VGCC blocker, that depressed excitatory transmission in WT mice to a level similar to that of KO mice ( $\sim 50\%$ , data not shown) (Schoch et al., 2002). Second, a 10 min bath application of the mGluR-I agonist DHPG ( $50 \mu\text{M}$ ), which bypasses glutamate release and also triggers I-LTD upon DHPG washout (Chevaleyre and Castillo, 2003; Edwards et al., 2006), failed to elicit I-LTD in KO mice (Fig. 4A,  $4.9 \pm 3.3$  %,  $n = 5$  cells, 4 KO mice, vs.  $21.7 \pm 1.5$  %,  $n = 5$  cells, 4 WT mice,  $p = 0.0002$ ). Finally, we directly tested the effect of CB1 receptor activation with the CB1 receptor agonist WIN. Because CB1 receptor agonists are lipophilic compounds with slow diffusion kinetics in acute brain slices, for these experiments we monitored extracellular field IPSPs (fIPSPs) (Arai et al., 1995; Lambert et al., 1991) that allow for stable long-term recordings (see methods). In WT mice, bath application of WIN, subsequently chased with the CB1 receptor antagonist SR 141716, led to a lasting depression of fIPSPs (Fig. 4B,  $23.9 \pm 2.8$  %,  $n = 4$ , 3 mice,  $p = 0.0006$ ). In RIM1 $\alpha$  KO mice, WIN acutely depressed the fIPSP to the same extent as observed in WT mice, but fIPSP amplitude subsequently recovered to baseline ( $5.5 \pm 1.5$  %,  $n = 3$ , 2 mice,  $p = 0.0001$  compared to WT). The similar transient depression, but differential long-term effect of WIN at inhibitory synapses strongly echoes the dissociation of DSI and I-LTD in these mice. Together, these results show that impaired glutamate or eCB release cannot account for the lack of I-LTD in RIM1 $\alpha$  KO animals.

Perhaps in slices from RIM1 $\alpha$  KO mice, CB1 receptor-expressing fibers (CB1R $^{+}$ ) are already depressed, thereby occluding attempts to induce I-LTD. Because not all inhibitory inputs express CB1 receptors, the bulk stimulation used in previous experiments (see Methods), by activating a mixture of CB1R $^{+}$  and CB1R $^{-}$  fibers, may have led us to underestimate potential changes specific to CB1R $^{+}$  fibers. To isolate CB1R $^{+}$  synaptic inputs, we lowered the stimulation intensity and selected those inputs that exhibited complete block of transmission

during DSI (Chevaleyre and Castillo, 2004) (Fig. 4C). We found that the IPSC amplitude (excluding failures), failure rate, and paired-pulse ratio of two successive IPSCs were identical in WT and KO mice (Fig. 4D). Taken together, these results show that the lack of I-LTD in RIM1 $\alpha$  KO is not due to an initial change in release probability at CB1R<sup>+</sup> inhibitory synapses.

Our results so far show that PKA and RIM1 $\alpha$  are required for CB1 receptor-mediated LTD. To confirm that RIM1 $\alpha$  is indeed required for both CB1 receptor and PKA action on GABA release, we performed two additional sets of experiments. First, we tested the acute effect of a PKA blocker on GABA release in both WT and KO mice. Because PKA may differentially control GABA release at synapses that do not express the CB1 receptor, we performed this experiment after isolating CB1R<sup>+</sup> fibers with focal stimulation. As shown in figure 5, blockade of PKA by H89 depressed GABAergic synaptic transmission at CB1R<sup>+</sup> inputs, a depression (including failures) that was significantly attenuated in RIM1 $\alpha$  KO mice (Fig. 5A,B;  $35.3 \pm 5.8\%$ ,  $n = 8$  cells, 8 WT mice vs.  $15.2 \pm 4.8\%$ ,  $n = 9$  cells, 8 KO mice,  $p < 0.01$ ). In the absence of PKA blocker, transmission remained stable throughout the experiment (Fig. 5B,  $95.8 \pm 4.7\%$ ,  $n = 7$ ), suggesting that the remaining depression in KO mice probably reflects an action of PKA on targets other than RIM1 $\alpha$ . A major component of the H89-mediated depression of GABA release is likely RIM1 $\alpha$ -dependent, as indicated by the increase in failure rate and paired-pulse ratio in WT, but not KO mice (Fig. 5C,D).

To investigate whether the CB1 receptor-mediated effect on GABAergic release machinery (Fig. 2A) similarly requires RIM1 $\alpha$ , in addition to cAMP/PKA signaling, we tested the effect of CB1 receptor activation on mIPSC activity in WT and KO mice. Again, in order to rule out a contribution of VGCC inhibition to a CB1 receptor-mediated effect, these experiments were performed in presence of 100  $\mu$ M Cd<sup>2+</sup>. As shown in figure 6, WIN-induced depression of mIPSC activity was absent in RIM1 $\alpha$  KO mice (Fig. 6). Together, these results indicate that the PKA/RIM1 $\alpha$  pathway is required for CB1 receptor-mediated effects on the release machinery and long-term, but not short-term, synaptic plasticity.

Given the widespread prevalence of eCB-dependent LTD, we tested whether the RIM1 $\alpha$ -dependent mechanism of long-term plasticity is conserved in other brain structures. In the basolateral amygdala (BLA), where eCBs also mediate a form of I-LTD (Azad et al., 2004; Marsicano et al., 2002), we first investigated whether CB1 receptors could suppress GABA release by targeting the release machinery. Indeed, activation of CB1 receptors by WIN decreased mIPSC frequency in BLA neurons in 5 mM Ca<sup>2+</sup>, an effect blocked by CB1 receptor antagonists (Fig. 7A). WIN induced no significant effect on mIPSC amplitude (data not shown), indicating a presynaptic action of CB1 receptors. Importantly, H89 blocked the WIN-mediated effect on mIPSC frequency (Fig. 7B). These results strongly suggest that at BLA inhibitory synapses, CB1 receptors target the release machinery via PKA to depress GABA release.

The coupling of CB1 receptors to GABA release in BLA parallels our findings in hippocampus, and suggests that CB1 receptor mediated plasticity in these two structures may share important features. We first verified that low frequency stimulation (LFS, 100 stimuli, 1 Hz) within the amygdala, close to the external capsule, triggers a CB1 receptor-dependent LTD of IPSCs in the BLA (data not shown) (Azad et al., 2004; Marsicano et al., 2002). As in the hippocampus (Fig. 3A), BLA I-LTD was abolished in RIM1 $\alpha$  KO mice (Fig. 7C,  $-1.7 \pm 6.3\%$ ,  $n = 11$  cells, 5 KO mice, vs.  $25.3 \pm 2.4\%$ ,  $n = 9$  cells, 6 WT mice,  $p < 0.0016$ ). In addition, to rule out a potential decrease in glutamate release during I-LTD induction in KO mice, we tested whether direct activation of group I mGluRs with an agonist could trigger I-LTD in these mice. Similar to the results observed with LFS, we found that DHPG-evoked LTD was also abolished in RIM1 $\alpha$  KO mice (Fig. 7D,  $27.6 \pm 8.0\%$ ,  $n = 7$ , 3 WT mice, vs.  $3.4 \pm 4.0\%$ ,  $n = 6$ , 4 KO mice,  $p = 0.02$ ). Interestingly, as observed in the hippocampus (Fig. 4A), the IPSC depression during DHPG application was identical in both WT and RIM1 $\alpha$  KO mice, indicating that the acute



effect of CB1 receptor activation on GABA release (Azad et al., 2004) is also independent of RIM1 $\alpha$  in the amygdala. In conclusion, our results show that RIM1 $\alpha$  is necessary for the long-term reduction of GABA release induced by CB1 receptors not only in the hippocampus, but also in the amygdala, and that in both of these structures, the CB1 receptor-mediated effect on release machinery requires PKA.

## DISCUSSION

In this study we investigated the mechanism by which activation of CB1 receptors triggers a long-lasting reduction of transmitter release. First, we report in the hippocampus that eCB-mediated long-term, but not short-term depression, of inhibitory transmission requires cAMP/PKA signaling. Second, we identify the active zone protein RIM1 $\alpha$  as a critical player in CB1 receptor-mediated suppression of GABA release. Finally, we show that RIM1 $\alpha$  is necessary for eCB-dependent LTD in both the hippocampus and amygdala. Our findings support the notion that enduring changes in the release machinery via cAMP/PKA signaling and RIM1 $\alpha$  represent a general mechanism underlying presynaptic forms of long-term plasticity.

### CB1 receptors engage different pathways during short and long term eCB-plasticity

One of the main conclusions of our study is that the signaling pathways downstream of the CB1 receptor that trigger short- and long-term plasticity differ (Fig. 8). The mechanism underlying transient suppression of transmitter release by eCBs, i.e. DSI/DSE, has been extensively studied in several brain areas (reviewed in Alger, 2002; Chevaleyre et al., 2006; Diana and Marty, 2004; Freund et al., 2003; Kreitzer and Regehr, 2002; Wilson and Nicoll, 2002). Most experimental evidence indicates that eCB-mediated short-term plasticity, at least in the hippocampus and cerebellum, is mainly due to a CB1 receptor-mediated reduction of presynaptic Ca<sup>2+</sup> influx through VGCCs. Indeed, at climbing fiber inputs to Purkinje cells, action potential-induced Ca<sup>2+</sup> influx into presynaptic terminals was directly visualized and decreased during DSE to an extent that fully accounted for the decrease in EPSC amplitude (Kreitzer and Regehr, 2001). In the hippocampus, inhibition of presynaptic N-type VGCCs reportedly mediates DSI, and this inhibition likely occurs as a result of the direct action of the G<sub>i/o</sub>  $\beta\gamma$  subunits (Wilson et al., 2001). In support of this mechanism, early studies in expression systems showed that G $\beta\gamma$  dimers, but not the G $\alpha$  subunit are responsible for the modulation of N-type VGCCs (Herlitze et al., 1996; Ikeda, 1996), and that cannabinoids inhibit N-type VGCC (Mackie and Hille, 1992). In addition, the fast onset of DSI (~1 s) (Heinbockel et al., 2005; Wilson et al., 2001), is more consistent with a fast, membrane-delimited pathway of presynaptic inhibition than a phosphorylation-induced change in channel activity which typically requires many seconds and may involve soluble second messengers (Dolphin, 2003; Hescheler and Schultz, 1993; Hille, 1992). Consistent with this idea, DSI was found to be unaffected by broad spectrum blockers of protein kinases and phosphatases (Wilson et al., 2001) and by manipulation of the cAMP pathway (Morishita et al., 1998; Wilson et al., 2001). Our study confirms these observations, thereby supporting the notion that DSI is unlikely to be mediated by adenylyl cyclase inhibition. Furthermore, we demonstrate that both cAMP/PKA signaling and RIM1 $\alpha$  are necessary for I-LTD but not for DSI.

Previous studies at both inhibitory (Chevaleyre and Castillo, 2003) and excitatory (Ronesi et al., 2004) synapses have shown that the induction of eCB-mediated LTD requires CB1 receptor activation for several minutes, and that once this form of plasticity is induced, it becomes independent of CB1 receptor activation. The relatively long induction period suggests that CB1 receptors may engage a different signaling process than the one mediating transient suppression of transmitter release. Indeed, the fast time course of DSI (<1 min) likely reflects the transient profile of eCB production and CB1 receptor engagement since CB1 receptor desensitization does not occur within this time window. Therefore, it is tempting to speculate that DSI requires

neither cAMP/PKA signaling nor RIM1 $\alpha$ , because CB1 receptor activation is too brief to significantly engage the  $\alpha_{i/o}$  effector limb of the CB1 receptor G protein signaling cascade. Our findings are consistent with a model (Fig. 8) whereby brief activation of CB1 receptors, presumably by blocking presynaptic VGCCs via G $\beta\gamma$  subunits (Wilson et al., 2001), leads to short-term depression of transmitter release. In contrast, a more sustained, but still transient activation of CB1 receptors (Chevalleyre and Castillo, 2003;Ronesi et al., 2004), downregulates the cAMP/PKA signaling pathway (Childers and Deadwyler, 1996;Howlett et al., 1986) perhaps via the G $\alpha$  subunit, thereby triggering a RIM1 $\alpha$ -dependent modification in the release machinery to suppress transmitter release in a long-lasting manner.

### **CB1 receptors can control synaptic transmission by targeting the release machinery in a PKA- and RIM1 $\alpha$ -dependent manner**

Most studies have shown that CB1 receptor-mediated suppression of GABA release mainly occurs as a result of presynaptic VGCC modulation (Diana and Marty, 2003;Hajos et al., 2000;Hoffman and Lupica, 2000;Varma et al., 2002;Wilson et al., 2001). Consistent with this mechanism, activation of CB1 receptors depressed the frequency of spontaneous IPSCs (action potential-driven events), but not mIPSC frequency monitored under normal extracellular Ca<sup>2+</sup> concentration not only in the hippocampus (Alger et al., 1996;Hajos et al., 2000;Hoffman and Lupica, 2000;Pitler and Alger, 1994;Varma et al., 2002;Wilson et al., 2001) but also in the amygdala (Azad et al., 2003;Katona et al., 2001). Taken together, these observations could be interpreted to mean that CB1 receptors do not target the release machinery. In the cerebellum however, it has been reported that CB1 receptors can act downstream of Ca<sup>2+</sup> influx (Diana and Marty, 2003;Takahashi and Linden, 2000). A recent report demonstrated that the CB1 receptor-mediated effect on miniature synaptic events depends on basal Ca<sup>2+</sup> in the presynaptic terminal (Yamasaki et al., 2006). A similar observation was also reported in the amygdala (Azad et al., 2003). Thus, in the absence of action potentials -i.e. in presence of TTX- and in blockers of presynaptic Ca<sup>2+</sup> influx, quantal release may still depend on basal Ca<sup>2+</sup> in the terminal (Yamasaki et al., 2006), and it is likely that Ca<sup>2+</sup> released from intracellular stores play an important role in this process (Collin et al., 2005). It is therefore conceivable that decreasing presynaptic Ca<sup>2+</sup>, a likely consequence of continuous action potential blockade with TTX, may mask any CB1 receptor-mediated (Ca<sup>2+</sup>-dependent) effect on the release machinery in normal extracellular Ca<sup>2+</sup>.

We now show in the CA1 area of the hippocampus that after blocking action potentials and Ca<sup>2+</sup> influx through VGCCs, but in 5 mM extracellular Ca<sup>2+</sup>, CB1 receptors suppress mIPSC frequency. Early studies have demonstrated that presynaptic G $\beta\gamma$  protein-coupled receptors can suppress transmitter release downstream Ca<sup>2+</sup>-influx, presumably by targeting the release machinery (Cohen et al., 1992;Dittman and Regehr, 1996;Gereau and Conn, 1995;Jarolimek and Misgeld, 1997;Poncer et al., 1995;Scanziani et al., 1992;Scanziani et al., 1995;Scholz and Miller, 1992;Umehiya and Berger, 1995). While a similar mechanism may contribute to the CB1 receptor-mediated suppression of transmitter release at some synapses (Szabo and Schlicker, 2005), the precise signaling pathway and identity of CB1 receptor targets have been largely unexplored. Our results demonstrate that CB1 receptors can target the release machinery via the cAMP/PKA pathway. Moreover, we provide evidence that both PKA and CB1 receptor action on GABA release depends on the presence of the active zone protein RIM1 $\alpha$ .

Previous studies have suggested that CB1 receptor agonists suppress action-potential driven synaptic responses independently of PKA (Azad et al., 2003;Daniel et al., 2004;Huang et al., 2002;Robbe et al., 2001). While these data seem to conflict with our model (Fig. 8), agonist application likely engages multiple pathways downstream of CB1 receptors. Any one of these mechanisms could suppress transmitter release to such an extent that a concurrent PKA-

dependent change at the level of the release machinery could go undetected. Finally, our findings are consistent with previous reports showing that cAMP/PKA signaling enhances inhibitory synaptic transmission by increasing release probability independently of presynaptic  $Ca^{2+}$  influx not only in the hippocampus (Capogna et al., 1995; Sciancalepore and Cherubini, 1995; Trudeau et al., 1996) but also in cerebellum (Kondo and Marty, 1997; Llano and Gerschenfeld, 1993; Saitow et al., 2005).

It is likely that PKA-dependent modulation of transmitter release occurs as a result of the phosphorylation of presynaptic proteins involved in exocytosis (for a recent review see Seino and Shibasaki, 2005). RIM1 $\alpha$  emerges as a unique candidate as this presynaptic protein is phosphorylated by PKA and its phosphorylation reportedly enhances glutamate release in cerebellum (Lonart et al., 2003). RIM1 $\alpha$  has been exclusively localized to presynaptic nerve terminals in rodents (Wang et al., 1997) and in *C. elegans* (Koushika et al., 2001; Weimer et al., 2006). In situ hybridization studies suggest that RIM1 $\alpha$  is expressed in all neurons (Schoch et al., 2006). Furthermore, immunohistochemistry evidence in the hippocampus shows that RIM1 $\alpha$  is co-expressed with GAD67 (S. Schoch, personal communication), indicating that RIM1 $\alpha$  is present in GABAergic interneurons. In this context, it is tempting to speculate that activation of CB1 receptors, presumably by reducing PKA activity, may lead to a decrease in RIM1 $\alpha$  phosphorylation. However, it is formally possible that some other RIM1 $\alpha$ -partner, also phosphorylated by PKA, may mediate the CB1 receptor-induced effects on the release machinery.

### **PKA and RIM1 $\alpha$ as a general mechanism for presynaptic plasticity**

RIM1 $\alpha$  is a key protein in the active zone (Wang et al., 1997) that regulates neurotransmitter release (Calakos et al., 2004; Koushika et al., 2001; Schoch et al., 2002) and is thought to scaffold and organize the synaptic release machinery (Sudhof, 2004). Its selective localization to active zones and its interactions with multiple presynaptic molecules places RIM1 $\alpha$  in a strategic position to modulate presynaptic release and plasticity (Kaeser and Sudhof, 2005). In addition, RIM1 $\alpha$  knockout mice show significant cognitive deficits (Powell et al., 2004). Previous studies reported that RIM1 $\alpha$  is critical for the long-term enhancement of glutamate release associated with PKA-dependent, NMDA receptor-independent presynaptic forms of LTP at excitatory synapses in both the hippocampus (Castillo et al., 2002) and cerebellum (Castillo et al., 2002; Lonart et al., 2003). More recently, Huang et al (2005) have described a role for RIM1 $\alpha$  in the PKA-dependent late phase of LTP at Schaffer collateral to CA1 pyramidal cell synapses. It is worth noting that all these studies identified a role for RIM1 $\alpha$  exclusively in excitatory LTP. We now demonstrate that CB1 receptor-dependent LTD at inhibitory synapses requires RIM1 $\alpha$ . In addition, we show that cAMP/PKA signaling is necessary for hippocampal I-LTD. Enduring changes in the release machinery mediated by PKA signaling and RIM1 $\alpha$  represent a widespread mechanism underlying presynaptic regulation of long-term plasticity (LTP and LTD) at both excitatory and inhibitory synapses.

### **I-LTD as a substrate for learning and memory**

Disrupting eCB-CB1 receptor signaling has a variety of effects on both hippocampus- and amygdala-dependent memory tasks (Chhatwal et al., 2005; Kamprath et al., 2006; Marsicano et al., 2002; Mikics et al., 2006; Suzuki et al., 2004; Varvel et al., 2005; Varvel and Lichtman, 2002; Varvel et al., 2006). However, the precise role of this pathway, let alone I-LTD, in learning and memory still remains to be fully understood. Unlike manipulations involving the CB1 receptor, the RIM1 $\alpha$  KO maintains short term eCB mediated plasticity (DSI) without the long term form (I-LTD). These mice have striking deficits in acquiring both hippocampus- and amygdala-dependent memories (Powell et al., 2004). I-LTD strongly modulates excitability and induction of LTP in both brain areas (Azad et al., 2004; Chevaleyre and Castillo, 2004), so it is possible that this process, together with other deficits in synaptic function (Huang et al.,



2005;Kaeser and Sudhof, 2005) could contribute to the RIM1 $\alpha$  KO phenotype. Selective disruption of the RIM1 $\alpha$  pathway in a subset of synaptic inputs (e.g. those expressing CB1 receptors) may help to establish the contribution of eCB-mediated long-term plasticity to learning and memory.

## METHODS

Experiments were performed either in C57Bl/6 mice (3-6 weeks; Charles River), paired RIM1 $\alpha$  KO and WT littermates (3-6 weeks), or in Wistar rats (3-5 weeks; Charles River). RIM1 $\alpha$  KO mice were generated as described previously (Schoch et al., 2002). Transverse hippocampal slices were prepared as described elsewhere (Chevalleyre and Castillo, 2003). Animals were killed by decapitation in accordance with institutional regulations. Slices (400  $\mu$ m thickness) were cut on a vibrating slicer (Dosaka, Kyoto, Japan) in ice-cold extracellular solution containing (in mM): 215 Sucrose, 2.5 KCl, 20 glucose, 26 NaHCO<sub>3</sub>, 1.6 NaH<sub>2</sub>PO<sub>4</sub>, 1 CaCl<sub>2</sub>, 4 MgSO<sub>4</sub> and 4 MgCl<sub>2</sub>. The cutting medium was gradually switched to the recording solution (ACSF) that contained (in mM): 124 NaCl, 2.5 KCl, 10 glucose, 26 NaHCO<sub>3</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 2.5 CaCl<sub>2</sub> and 1.3 MgSO<sub>4</sub>. Slices were kept at room temperature for at least 1.5 hr before recording. Cutting and recording solutions were saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.4. Experiments were performed at 25.0  $\pm$  0.1  $^{\circ}$ C except for mIPSC recordings (28.0  $^{\circ}$ C).

CA1 pyramidal cells were blind-patched and recorded in whole-cell voltage clamp mode. For recording IPSCs, hippocampal slices were completely submerged and continuously superfused at a flow rate of 2 ml/min with ACSF containing NMDA and AMPA/Kainate receptor antagonists (25  $\mu$ M d-APV and 10  $\mu$ M NBQX). Constant-voltage, synaptic stimulation (200  $\mu$ s pulse width), was delivered in pairs (100 ms inter-stimulus interval) via a patch-type pipette filled with ACSF and placed in the middle third of *s. radiatum*. For experiments with “bulk” stimulation, stimulation pipette tips were broken to  $\sim$ 20  $\mu$ m and IPSCs were recorded at -60 mV for DSI or +10 mV for I-LTD with an internal solution containing (in mM): 123 cesium gluconate, 1 CaCl<sub>2</sub>, 10 EGTA, 10 Hepes, 10 glucose, 5 ATP, 0.4 GTP (pH 7.2; 280-290 mOsm). We adjusted stimulus intensity to evoke IPSCs of comparable amplitudes across experiments (0.5-1.4 nA). All mIPSCs were recorded with the same internal solution at +20 mV, and ACSF-containing 500 nM TTX, 100  $\mu$ M CdCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, 25  $\mu$ M d-APV and 10  $\mu$ M NBQX. For focal stimulation experiments, IPSCs were recorded at -60 mV with an internal solution containing CsCl instead of CsGluconate. The stimulus strength for these experiments was reduced to elicit small amplitude IPSCs ( $\sim$ 30 pA) and a  $\sim$ 10 % failure rate over  $>$ 10 min baseline. Series resistance (Rs, typically 8-15 M $\Omega$ ) was continuously monitored throughout each experiment with a -5 mV, 80 ms command pulse delivered prior to each stimulus pair; cells with more than 10 % change in Rs were excluded from analysis. I-LTD was induced after a stable baseline by theta-burst stimulation (TBS) consisting of a series of 10 bursts of 5 stimuli (100 Hz within each burst, 200 ms inter-burst interval, repeated 4 times, 5 s apart). I-LTD magnitude was quantified by averaging IPSC amplitudes, for 10 min periods right before and 20 min after TBS. DSI was evoked by a 5 second voltage step from -60 to 0 mV. Typically 2-3 DSIs per cell were averaged prior to group statistical analysis. The peak inhibition during DSI was quantified by averaging IPSC amplitudes 6 and 12 seconds after depolarization. IPSCs were monitored every 6 seconds for DSI and every 20 seconds for I-LTD. 5-10 s epochs were obtained every 15 s for mIPSC analysis. Extracellular field recordings were performed using recording pipettes filled with 1M NaCl. For field EPSPs (fEPSPs) stimulating and recording pipettes were placed in the middle third of CA1 *s. radiatum*. Field IPSP (fIPSP) experiments in CA1 were performed under the same recording conditions as whole-cell IPSC experiments, except that stimulation and recording pipette tips were both placed in *s. pyramidale*  $\sim$ 150  $\mu$ m apart. Stimulating and recording pipettes were placed at a similar depth within the tissue for each experiment (80-100  $\mu$ m). Stimulation intensity was adjusted to produce 0.2-0.4 mV fIPSP. In each experiment, we confirmed that these potentials were completely blocked by the GABA-

A receptor antagonist picrotoxin (100  $\mu$ M), in accordance with previously published reports (Lambert et al, 1991;Arai et al, 1995).

For experiments performed in basolateral amygdala (BLA), coronal slices were prepared using similar methods as described above from 4-8 week old male C57Bl/6 mice. Principal cells in the BLA were identified by their morphological and electrophysiological characteristics (Washburn and Moises, 1992). IPSCs were recorded from principal cells in the basal nucleus using the same internal solution as for focal stimulation in hippocampus, voltage clamped between -60 and -70 mV. Synaptic responses were evoked as paired pulse stimuli (200  $\mu$ s pulse width, 50 ms apart) via monopolar ACSF-filled patch-type pipettes with broken tips (~20  $\mu$ m) placed just medial to the external capsule. I-LTD was triggered with low frequency stimulation (100 stimuli at 1 Hz, intensity twice that of test pulses). Recording conditions for mIPSCs were identical to those for hippocampus.

Whole-cell and extracellular recordings were performed with a MultiClamp 700A amplifier (Axon Instruments Inc., Union City, CA) whose output signals were filtered at 3 kHz, acquired at 5 kHz and analyzed on-line using customized software for IgorPro (Wavemetrics Inc., Lake Oswego, OR). Results are reported as mean  $\pm$  SEM. Statistical analyses were performed using Student's t-test. Unless otherwise stated, drugs were bath applied following dilution into the ACSF from concentrated stock solutions. SR 141716 (NIMH), H-89 (Sigma-Aldrich), forskolin (Calbiochem), NBQX, AM 251, WIN 55,212-2 and CP 55,940 (Tocris) were dissolved in DMSO. d-APV, DHPG (Tocris), Protein Kinase A Inhibitor 14-22 amide (cell permeable, myristoylated) and Protein Kinase A Inhibitor 6-22 Amide (Calbiochem) were dissolved in water. For experiments involving drug treatment of slices, interleaved controls were performed in the same concentration of the respective solvent. Stock solutions of drugs were stored at -20  $^{\circ}$ C prior to use. RIM1 $\alpha$  KO and WT littermates were shipped to Albert Einstein College of Medicine (Bronx, NY) unidentified, and data were acquired and analyzed in a blind fashion; the KO mice exhibit no obvious phenotype (Schoch et al., 2002). Genotype was confirmed afterward by PCR using tail DNA.

#### Acknowledgements

We thank Donald S. Faber, Michael V.L. Bennett and Kanji Takahashi for their constructive comments on the manuscript. We are also grateful to Pankaj Sah for his advice on BLA recordings. This work was supported by the US National Institutes of Health/NIDA (P.E.C.) and the Howard Hughes Medical Institutes (T.C.S.). V.C. was partially supported by the Epilepsy Foundation. P.E.C. is a Pew Biomedical Scholar.

#### References

- Alger BE. Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. *Prog Neurobiol* 2002;68:247–286. [PubMed: 12498988]
- Alger BE, Pitler TA, Wagner JJ, Martin LA, Morishita W, Kirov SA, Lenz RA. Retrograde signalling in depolarization-induced suppression of inhibition in rat hippocampal CA1 cells. *J Physiol* 1996;496(Pt 1):197–209. [PubMed: 8910208]
- Arai A, Silberg J, Lynch G. Differences in the refractory properties of two distinct inhibitory circuitries in field CA1 of the hippocampus. *Brain Res* 1995;704:298–306. [PubMed: 8788926]
- Azad SC, Eder M, Marsicano G, Lutz B, Zieglgansberger W, Rammes G. Activation of the cannabinoid receptor type 1 decreases glutamatergic and GABAergic synaptic transmission in the lateral amygdala of the mouse. *Learn Mem* 2003;10:116–128. [PubMed: 12663750]
- Azad SC, Monory K, Marsicano G, Cravatt BF, Lutz B, Zieglgansberger W, Rammes G. Circuitry for associative plasticity in the amygdala involves endocannabinoid signaling. *J Neurosci* 2004;24:9953–9961. [PubMed: 15525780]
- Bender VA, Bender KJ, Brasier DJ, Feldman DE. Two coincidence detectors for spike timing-dependent plasticity in somatosensory cortex. *J Neurosci* 2006;26:4166–4177. [PubMed: 16624937]

- Calakos N, Schoch S, Sudhof TC, Malenka RC. Multiple roles for the active zone protein RIM1alpha in late stages of neurotransmitter release. *Neuron* 2004;42:889–896. [PubMed: 15207234]
- Capogna M, Gähwiler BH, Thompson SM. Presynaptic enhancement of inhibitory synaptic transmission by protein kinases A and C in the rat hippocampus in vitro. *J Neurosci* 1995;15:1249–1260. [PubMed: 7869096]
- Carlson G, Wang Y, Alger BE. Endocannabinoids facilitate the induction of LTP in the hippocampus. *Nat Neurosci* 2002;5:723–724. [PubMed: 12080342]
- Castillo PE, Janz R, Sudhof TC, Tzounopoulos T, Malenka RC, Nicoll RA. Rab3A is essential for mossy fibre long-term potentiation in the hippocampus. *Nature* 1997;388:590–593. [PubMed: 9252190]
- Castillo PE, Schoch S, Schmitz F, Sudhof TC, Malenka RC. RIM1alpha is required for presynaptic long-term potentiation. *Nature* 2002;415:327–330. [PubMed: 11797010]
- Chevalleyre V, Castillo PE. Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability. *Neuron* 2003;38:461–472. [PubMed: 12741992]
- Chevalleyre V, Castillo PE. Endocannabinoid-mediated metaplasticity in the hippocampus. *Neuron* 2004;43:871–881. [PubMed: 15363397]
- Chevalleyre V, Takahashi KA, Castillo PE. Endocannabinoid-Mediated Synaptic Plasticity in the CNS. *Annu Rev Neurosci* 2006;29:37–75. [PubMed: 16776579]
- Chhatwal JP, Davis M, Maguschak KA, Ressler KJ. Enhancing cannabinoid neurotransmission augments the extinction of conditioned fear. *Neuropsychopharmacology* 2005;30:516–524. [PubMed: 15637635]
- Childers SR, Deadwyler SA. Role of cyclic AMP in the actions of cannabinoid receptors. *Biochem Pharmacol* 1996;52:819–827. [PubMed: 8781498]
- Cohen GA, Doze VA, Madison DV. Opioid inhibition of GABA release from presynaptic terminals of rat hippocampal interneurons. *Neuron* 1992;9:325–335. [PubMed: 1497896]
- Collin T, Marty A, Llano I. Presynaptic calcium stores and synaptic transmission. *Curr Opin Neurobiol* 2005;15:275–281. [PubMed: 15919193]
- Daniel H, Rancillac A, Crepel F. Mechanisms underlying cannabinoid inhibition of presynaptic Ca<sup>2+</sup> influx at parallel fibre synapses of the rat cerebellum. *J Physiol* 2004;557:159–174. [PubMed: 15034129]
- Diana MA, Marty A. Characterization of depolarization-induced suppression of inhibition using paired interneuron–Purkinje cell recordings. *J Neurosci* 2003;23:5906–5918. [PubMed: 12843295]
- Diana MA, Marty A. Endocannabinoid-mediated short-term synaptic plasticity: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE). *Br J Pharmacol* 2004;142:9–19. [PubMed: 15100161]
- Dittman JS, Regehr WG. Contributions of calcium-dependent and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. *J Neurosci* 1996;16:1623–1633. [PubMed: 8774431]
- Dolphin AC. G protein modulation of voltage-gated calcium channels. *Pharmacol Rev* 2003;55:607–627. [PubMed: 14657419]
- Edwards DA, Kim J, Alger BE. Multiple mechanisms of endocannabinoid response initiation in hippocampus. *J Neurophysiol* 2006;95:67–75. [PubMed: 16207781]
- Freund TF, Katona I, Piomelli D. Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev* 2003;83:1017–1066. [PubMed: 12843414]
- Gerdeman GL, Lovinger DM. Emerging roles for endocannabinoids in long-term synaptic plasticity. *Br J Pharmacol* 2003;140:781–789. [PubMed: 14504143]
- Gerdeman GL, Ronesi J, Lovinger DM. Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. *Nat Neurosci* 2002;5:446–451. [PubMed: 11976704]
- Gereau RW, Conn PJ. Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. *J Neurosci* 1995;15:6879–6889. [PubMed: 7472445]
- Hajos N, Katona I, Naiem SS, MacKie K, Ledent C, Mody I, Freund TF. Cannabinoids inhibit hippocampal GABAergic transmission and network oscillations. *Eur J Neurosci* 2000;12:3239–3249. [PubMed: 10998107]

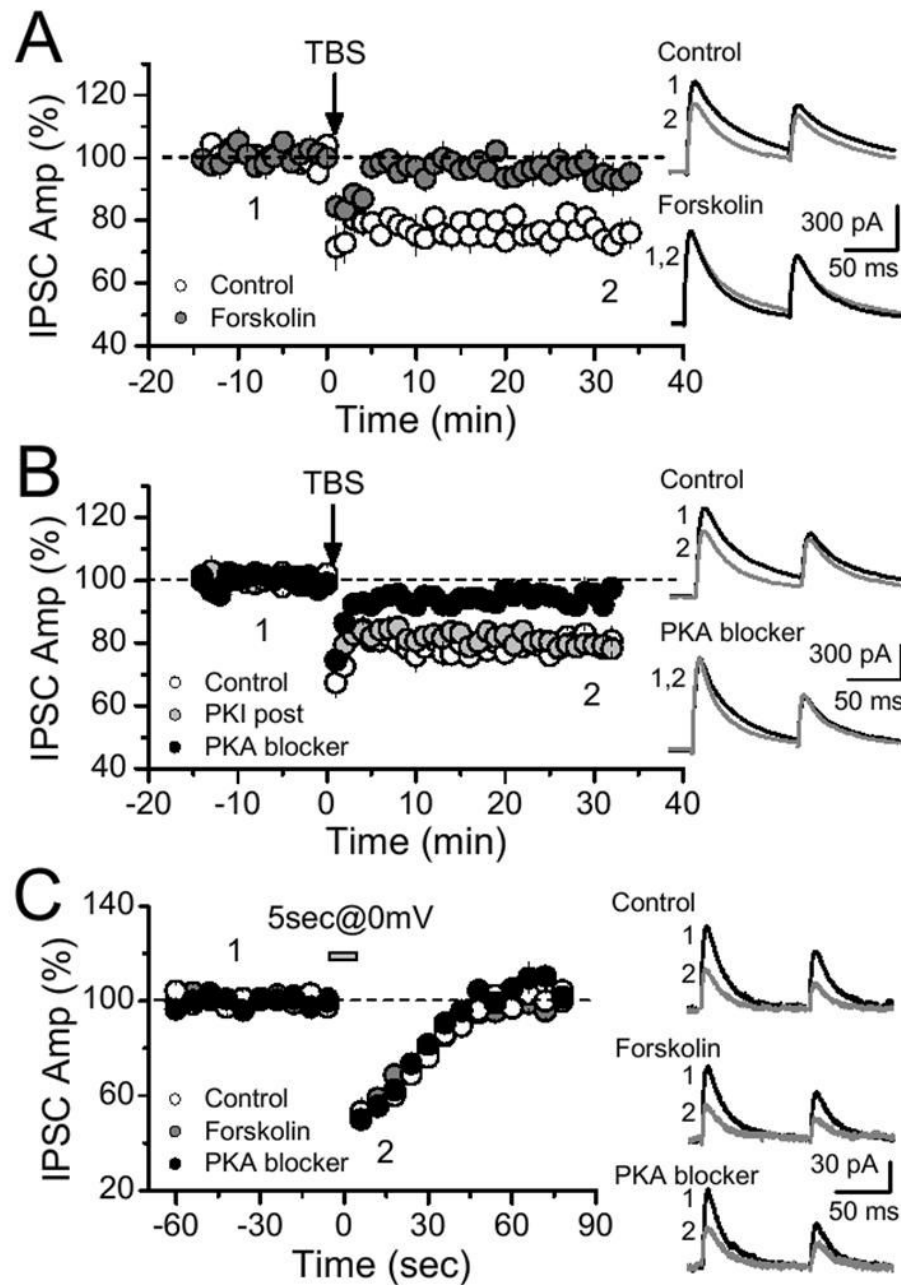
- Heinbockel T, Brager DH, Reich CG, Zhao J, Muralidharan S, Alger BE, Kao JP. Endocannabinoid signaling dynamics probed with optical tools. *J Neurosci* 2005;25:9449–9459. [PubMed: 16221855]
- Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA. Modulation of Ca<sup>2+</sup> channels by G-protein beta gamma subunits. *Nature* 1996;380:258–262. [PubMed: 8637576]
- Hescheler J, Schultz G. G-proteins involved in the calcium channel signalling system. *Curr Opin Neurobiol* 1993;3:360–367. [PubMed: 8396476]
- Hille B. G protein-coupled mechanisms and nervous signaling. *Neuron* 1992;9:187–195. [PubMed: 1353972]
- Hoffman AF, Lupica CR. Mechanisms of cannabinoid inhibition of GABA(A) synaptic transmission in the hippocampus. *J Neurosci* 2000;20:2470–2479. [PubMed: 10729327]
- Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 2002;54:161–202. [PubMed: 12037135]
- Howlett AC, Qualy JM, Khachatrian LL. Involvement of Gi in the inhibition of adenylate cyclase by cannabimimetic drugs. *Mol Pharmacol* 1986;29:307–313. [PubMed: 2869405]
- Huang CC, Chen YL, Lo SW, Hsu KS. Activation of cAMP-dependent protein kinase suppresses the presynaptic cannabinoid inhibition of glutamatergic transmission at corticostriatal synapses. *Mol Pharmacol* 2002;61:578–585. [PubMed: 11854438]
- Huang YY, Li XC, Kandel ER. cAMP contributes to mossy fiber LTP by initiating both a covalently mediated early phase and macromolecular synthesis-dependent late phase. *Cell* 1994;79:69–79. [PubMed: 7923379]
- Huang YY, Zakharenko SS, Schoch S, Kaeser PS, Janz R, Sudhof TC, Siegelbaum SA, Kandel ER. Genetic evidence for a protein-kinase-A-mediated presynaptic component in NMDA-receptor-dependent forms of long-term synaptic potentiation. *Proc Natl Acad Sci U S A* 2005;102:9365–9370. [PubMed: 15967982]
- Ikeda SR. Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. *Nature* 1996;380:255–258. [PubMed: 8637575]
- Jarolimek W, Misgeld U. GABAB receptor-mediated inhibition of tetrodotoxin-resistant GABA release in rodent hippocampal CA1 pyramidal cells. *J Neurosci* 1997;17:1025–1032. [PubMed: 8994057]
- Kaeser PS, Sudhof TC. RIM function in short- and long-term synaptic plasticity. *Biochem Soc Trans* 2005;33:1345–1349. [PubMed: 16246115]
- Kamprath K, Marsicano G, Tang J, Monory K, Bisogno T, Di Marzo V, Lutz B, Wotjak CT. Cannabinoid CB1 receptor mediates fear extinction via habituation-like processes. *J Neurosci* 2006;26:6677–6686. [PubMed: 16793875]
- Katona I, Rancz EA, Acsady L, Ledent C, Mackie K, Hajos N, Freund TF. Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. *J Neurosci* 2001;21:9506–9518. [PubMed: 11717385]
- Kondo S, Marty A. Protein kinase A-mediated enhancement of miniature IPSC frequency by noradrenaline in rat cerebellar stellate cells. *J Physiol* 1997;498:165–176. [PubMed: 9023776]
- Koushika SP, Richmond JE, Hadwiger G, Weimer RM, Jorgensen EM, Nonet ML. A post-docking role for active zone protein Rim. *Nat Neurosci* 2001;4:997–1005. [PubMed: 11559854]
- Kreitzer AC, Malenka RC. Dopamine modulation of state-dependent endocannabinoid release and long-term depression in the striatum. *J Neurosci* 2005;25:10537–10545. [PubMed: 16280591]
- Kreitzer AC, Regehr WG. Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron* 2001;29:717–727. [PubMed: 11301030]
- Kreitzer AC, Regehr WG. Retrograde signaling by endocannabinoids. *Curr Opin Neurobiol* 2002;12:324–330. [PubMed: 12049940]
- Lambert NA, Borroni AM, Grover LM, Teyler TJ. Hyperpolarizing and depolarizing GABAA receptor-mediated dendritic inhibition in area CA1 of the rat hippocampus. *J Neurophysiol* 1991;66:1538–1548. [PubMed: 1684989]
- Lenz RA, Wagner JJ, Alger BE. N- and L-type calcium channel involvement in depolarization-induced suppression of inhibition in rat hippocampal CA1 cells. *J Physiol* 1998;512(Pt 1):61–73. [PubMed: 9729617]

- Linden DJ, Ahn S. Activation of presynaptic cAMP-dependent protein kinase is required for induction of cerebellar long-term potentiation. *J Neurosci* 1999;19:10221–10227. [PubMed: 10575019]
- Llano I, Gerschenfeld HM. Beta-adrenergic enhancement of inhibitory synaptic activity in rat cerebellar stellate and Purkinje cells. *J Physiol* 1993;468:201–224. [PubMed: 8254506]
- Lonart G, Schoch S, Kaeser PS, Larkin CJ, Sudhof TC, Linden DJ. Phosphorylation of RIM1alpha by PKA triggers presynaptic long-term potentiation at cerebellar parallel fiber synapses. *Cell* 2003;115:49–60. [PubMed: 14532002]
- Mackie K, Hille B. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc Natl Acad Sci U S A* 1992;89:3825–3829. [PubMed: 1315042]
- Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglansberger W, et al. The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 2002;418:530–534. [PubMed: 12152079]
- Mikics E, Dombi T, Barsvari B, Varga B, Ledent C, Freund TF, Haller J. The effects of cannabinoids on contextual conditioned fear in CB1 knockout and CD1 mice. *Behav Pharmacol* 2006;17:223–230. [PubMed: 16572000]
- Morishita W, Kirov SA, Alger BE. Evidence for metabotropic glutamate receptor activation in the induction of depolarization-induced suppression of inhibition in hippocampal CA1. *J Neurosci* 1998;18:4870–4882. [PubMed: 9634553]
- Piomelli D. The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci* 2003;4:873–884. [PubMed: 14595399]
- Pitler TA, Alger BE. Depolarization-induced suppression of GABAergic inhibition in rat hippocampal pyramidal cells: G protein involvement in a presynaptic mechanism. *Neuron* 1994;13:1447–1455. [PubMed: 7993636]
- Poncer JC, Shinozaki H, Miles R. Dual modulation of synaptic inhibition by distinct metabotropic glutamate receptors in the rat hippocampus. *J Physiol* 1995;485(Pt 1):121–134. [PubMed: 7658367]
- Powell CM, Schoch S, Monteggia L, Barrot M, Matos MF, Feldmann N, Sudhof TC, Nestler EJ. The presynaptic active zone protein RIM1alpha is critical for normal learning and memory. *Neuron* 2004;42:143–153. [PubMed: 15066271]
- Robbe D, Alonso G, Duchamp F, Bockaert J, Manzoni OJ. Localization and mechanisms of action of cannabinoid receptors at the glutamatergic synapses of the mouse nucleus accumbens. *J Neurosci* 2001;21:109–116. [PubMed: 11150326]
- Robbe D, Kopf M, Remaury A, Bockaert J, Manzoni OJ. Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. *Proc Natl Acad Sci U S A* 2002;99:8384–8388. [PubMed: 12060781]
- Ronesi J, Gerdeman GL, Lovinger DM. Disruption of endocannabinoid release and striatal long-term depression by postsynaptic blockade of endocannabinoid membrane transport. *J Neurosci* 2004;24:1673–1679. [PubMed: 14973237]
- Saitow F, Suzuki H, Konishi S. beta-Adrenoceptor-mediated long-term up-regulation of the release machinery at rat cerebellar GABAergic synapses. *J Physiol* 2005;565:487–502. [PubMed: 15790662]
- Salin PA, Malenka RC, Nicoll RA. Cyclic AMP mediates a presynaptic form of LTP at cerebellar parallel fiber synapses. *Neuron* 1996;16:797–803. [PubMed: 8607997]
- Scanziani M, Capogna M, Gähwiler BH, Thompson SM. Presynaptic inhibition of miniature excitatory synaptic currents by baclofen and adenosine in the hippocampus. *Neuron* 1992;9:919–927. [PubMed: 1358131]
- Scanziani M, Gähwiler BH, Thompson SM. Presynaptic inhibition of excitatory synaptic transmission by muscarinic and metabotropic glutamate receptor activation in the hippocampus: are Ca<sup>2+</sup> channels involved? *Neuropharmacology* 1995;34:1549–1557. [PubMed: 8606802]
- Schoch S, Castillo PE, Jo T, Mukherjee K, Geppert M, Wang Y, Schmitz F, Malenka RC, Sudhof TC. RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature* 2002;415:321–326. [PubMed: 11797009]
- Schoch S, Mittelstaedt T, Kaeser PS, Padgett D, Feldmann N, Chevalleyre V, Castillo PE, Hammer RE, Han W, Schmitz F, et al. Redundant functions of RIM1alpha and RIM2alpha in Ca<sup>2+</sup>-triggered neurotransmitter release. *Embo J* 2006;25:5852–5863. [PubMed: 17124501]



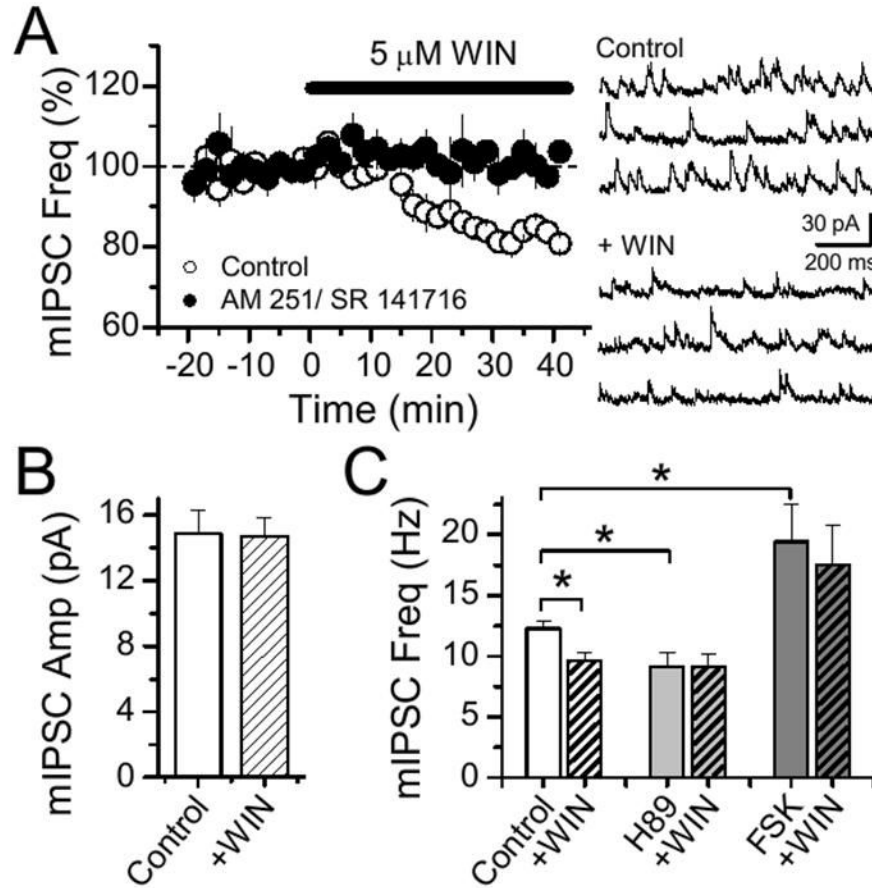
- Scholz KP, Miller RJ. Inhibition of quantal transmitter release in the absence of calcium influx by a G protein-linked adenosine receptor at hippocampal synapses. *Neuron* 1992;8:1139–1150. [PubMed: 1351733]
- Sciancalepore M, Cherubini E. Protein kinase A-dependent increase in frequency of miniature GABAergic currents in rat CA3 hippocampal neurons. *Neurosci Lett* 1995;187:91–94. [PubMed: 7783966]
- Seino S, Shibasaki T. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol Rev* 2005;85:1303–1342. [PubMed: 16183914]
- Sjostrom PJ, Turrigiano GG, Nelson SB. Neocortical LTD via coincident activation of presynaptic NMDA and cannabinoid receptors. *Neuron* 2003;39:641–654. [PubMed: 12925278]
- Soler-Llavina GJ, Sabatini BL. Synapse-specific plasticity and compartmentalized signaling in cerebellar stellate cells. *Nat Neurosci* 2006;9:798–806. [PubMed: 16680164]
- Storm DR, Hansel C, Hacker B, Parent A, Linden DJ. Impaired cerebellar long-term potentiation in type I adenylyl cyclase mutant mice. *Neuron* 1998;20:1199–1210. [PubMed: 9655507]
- Sudhof TC. The synaptic vesicle cycle. *Annu Rev Neurosci* 2004;27:509–547. [PubMed: 15217342]
- Suzuki A, Josselyn SA, Frankland PW, Masushige S, Silva AJ, Kida S. Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* 2004;24:4787–4795. [PubMed: 15152039]
- Szabo B, Schlicker E. Effects of cannabinoids on neurotransmission. *Handb Exp Pharmacol* 2005:327–365. [PubMed: 16596780]
- Takahashi KA, Linden DJ. Cannabinoid receptor modulation of synapses received by cerebellar Purkinje cells. *J Neurophysiol* 2000;83:1167–1180. [PubMed: 10712447]
- Trudeau LE, Emery DG, Haydon PG. Direct modulation of the secretory machinery underlies PKA-dependent synaptic facilitation in hippocampal neurons. *Neuron* 1996;17:789–797. [PubMed: 8893035]
- Tzounopoulos T, Janz R, Sudhof TC, Nicoll RA, Malenka RC. A role for cAMP in long-term depression at hippocampal mossy fiber synapses. *Neuron* 1998;21:837–845. [PubMed: 9808469]
- Umemiya M, Berger AJ. Presynaptic inhibition by serotonin of glycinergic inhibitory synaptic currents in the rat brain stem. *J Neurophysiol* 1995;73:1192–1201. [PubMed: 7608765]
- Varma N, Brager D, Morishita W, Lenz RA, London B, Alger B. Presynaptic factors in the regulation of DSI expression in hippocampus. *Neuropharmacology* 2002;43:550–562. [PubMed: 12367601]
- Varvel SA, Anum EA, Lichtman AH. Disruption of CB(1) receptor signaling impairs extinction of spatial memory in mice. *Psychopharmacology (Berl)* 2005;179:863–872. [PubMed: 15619104]
- Varvel SA, Lichtman AH. Evaluation of CB1 receptor knockout mice in the Morris water maze. *J Pharmacol Exp Ther* 2002;301:915–924. [PubMed: 12023519]
- Varvel SA, Wise LE, Niyuhire F, Cravatt BF, Lichtman AH. Inhibition of Fatty-Acid Amide Hydrolase Accelerates Acquisition and Extinction Rates in a Spatial Memory Task. *Neuropsychopharmacology*. 2006
- Villacres EC, Wong ST, Chavkin C, Storm DR. Type I adenylyl cyclase mutant mice have impaired mossy fiber long-term potentiation. *J Neurosci* 1998;18:3186–3194. [PubMed: 9547227]
- Wang Y, Okamoto M, Schmitz F, Hofmann K, Sudhof TC. Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. *Nature* 1997;388:593–598. [PubMed: 9252191]
- Washburn MS, Moises HC. Electrophysiological and morphological properties of rat basolateral amygdaloid neurons in vitro. *J Neurosci* 1992;12:4066–4079. [PubMed: 1403101]
- Weimer RM, Gracheva EO, Meyrignac O, Miller KG, Richmond JE, Bessereau JL. UNC-13 and UNC-10/rim localize synaptic vesicles to specific membrane domains. *J Neurosci* 2006;26:8040–8047. [PubMed: 16885217]
- Weisskopf MG, Castillo PE, Zalutsky RA, Nicoll RA. Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. *Science* 1994;265:1878–1882. [PubMed: 7916482]
- Wilson RI, Kunos G, Nicoll RA. Presynaptic specificity of endocannabinoid signaling in the hippocampus. *Neuron* 2001;31:453–462. [PubMed: 11516401]
- Wilson RI, Nicoll RA. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* 2001;410:588–592. [PubMed: 11279497]

- Wilson RI, Nicoll RA. Endocannabinoid signaling in the brain. *Science* 2002;296:678–682. [PubMed: 11976437]
- Yamasaki M, Hashimoto K, Kano M. Miniature synaptic events elicited by presynaptic Ca<sup>2+</sup> rise are selectively suppressed by cannabinoid receptor activation in cerebellar Purkinje cells. *J Neurosci* 2006;26:86–95. [PubMed: 16399675]



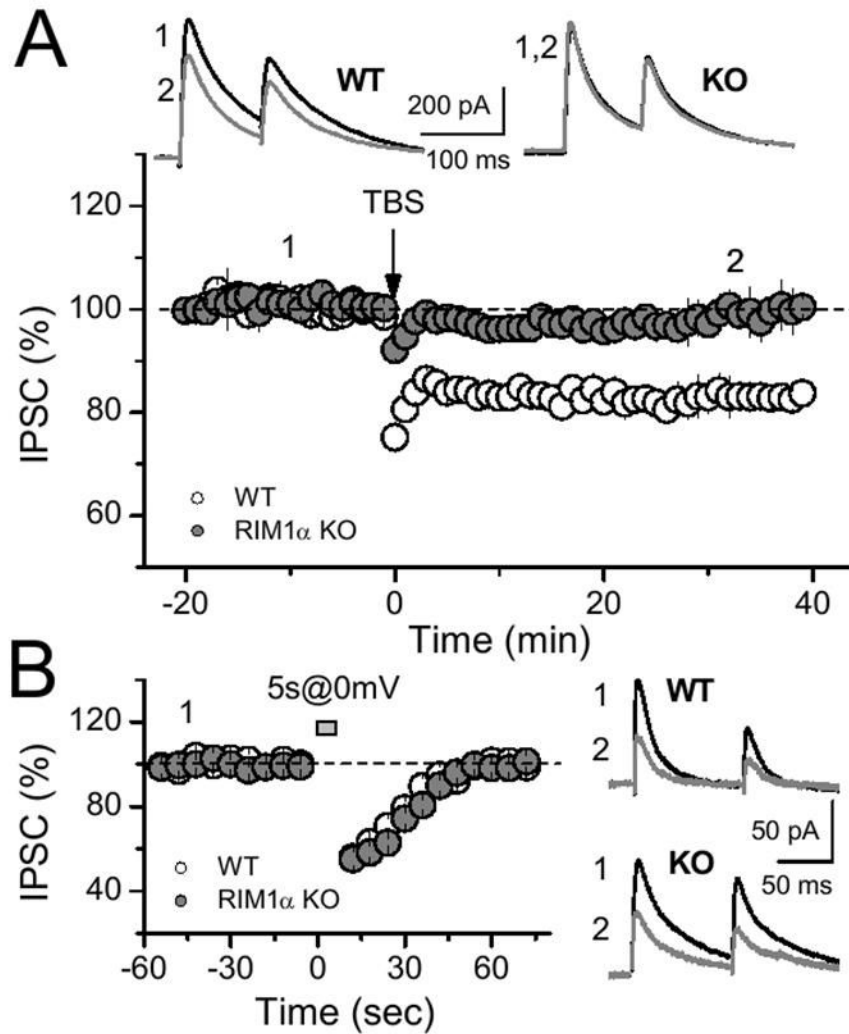
**Figure 1.**

Endocannabinoid-mediated long-term, but not short-term, plasticity requires cAMP/PKA signaling. (A) A persistent increase in cAMP level by incubation (>30 min) and continuous bath application of the adenylyl cyclase activator forskolin (10  $\mu$ M) blocked I-LTD. (B) Incubation and continuous application of either of two different PKA inhibitors (10  $\mu$ M H-89,  $n = 4$ ; 1  $\mu$ M PKI 14-22 amide,  $n = 7$ ) also impaired I-LTD induction. Direct application of a membrane impermeant PKA blocker (1  $\mu$ M PKI 6-22 amide,  $n = 7$  cells, 5 mice) to the postsynaptic pyramidal cell, via recording pipette, did not affect I-LTD. (C) None of these manipulations interfered with DSI. Averaged sample traces taken at times indicated by numbers are shown on the right.



**Figure 2.**

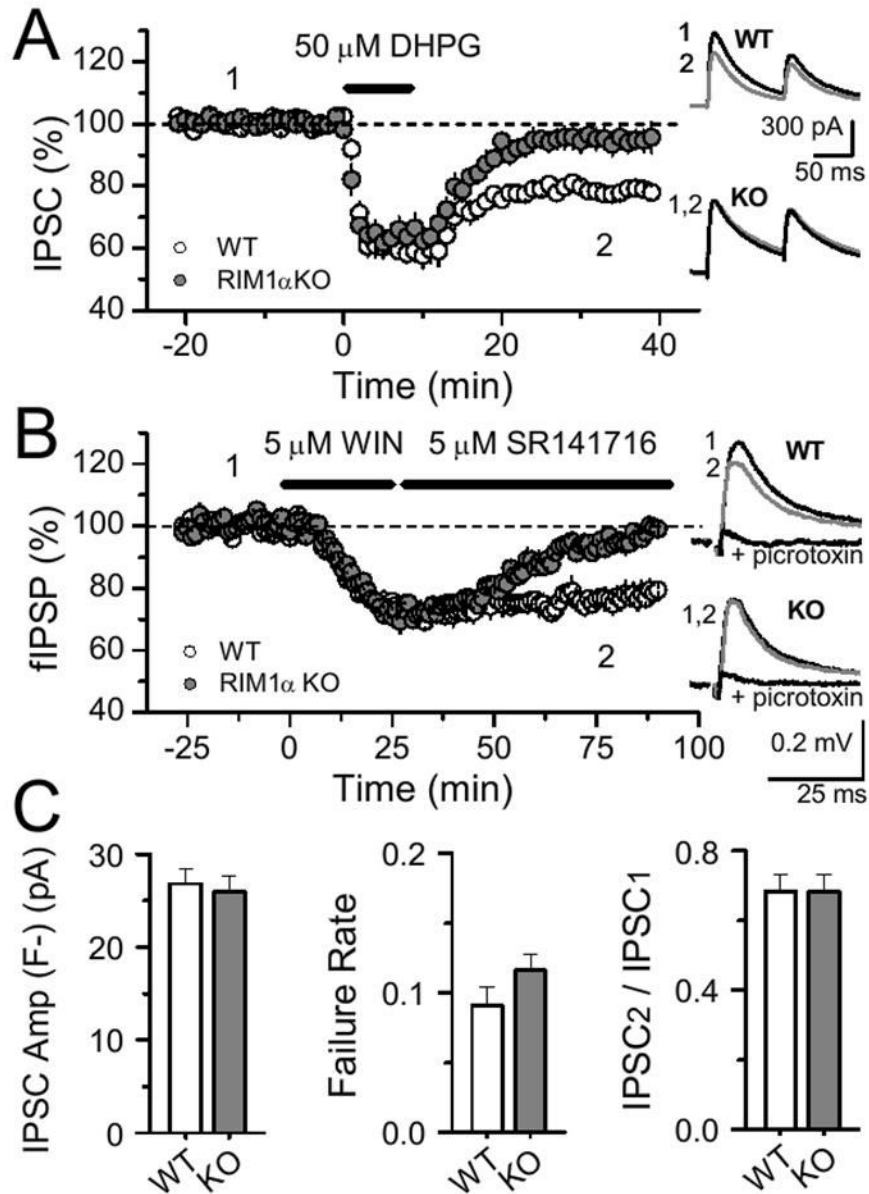
CB1 receptors target the release machinery via the cAMP/PKA pathway. (A) WIN application depressed mIPSC frequency, an effect blocked by CB1 receptor antagonists (2  $\mu$ M AM 251,  $n = 2$ ; 10  $\mu$ M SR141716,  $n = 3$ ). Sample traces before and during WIN application are shown at right. (B) WIN had no effect on mIPSC amplitude. (C) The WIN-mediated depression of mIPSC frequency is blocked in the continuous presence of forskolin ( $n = 6$ ) or H89 ( $n = 5$ ).



**Figure 3.**

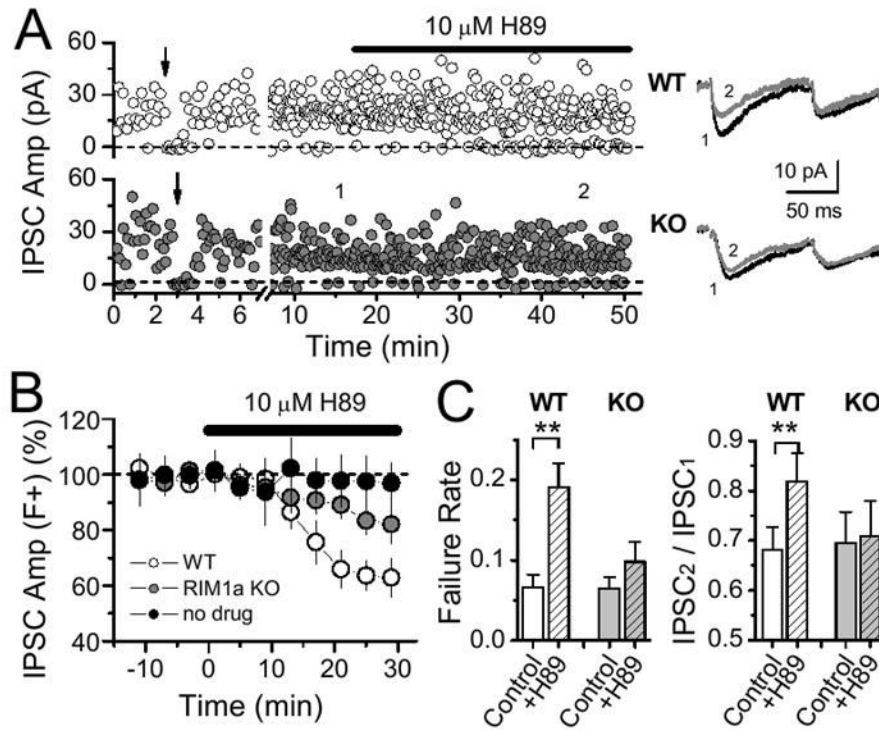
Long-term, but not short-term, eCB-mediated plasticity requires the presynaptic active zone protein RIM1 $\alpha$ . (A) TBS delivered in slices from RIM1 $\alpha$  KO mice triggered no depression compared to the I-LTD obtained in WT littermates ( $n = 18$ , 10 mice and  $n = 14$ , 9 mice respectively). (B) In contrast, DSI was identical in both WT and KO animals. Averaged sample traces are shown above (A) or on the right (B).



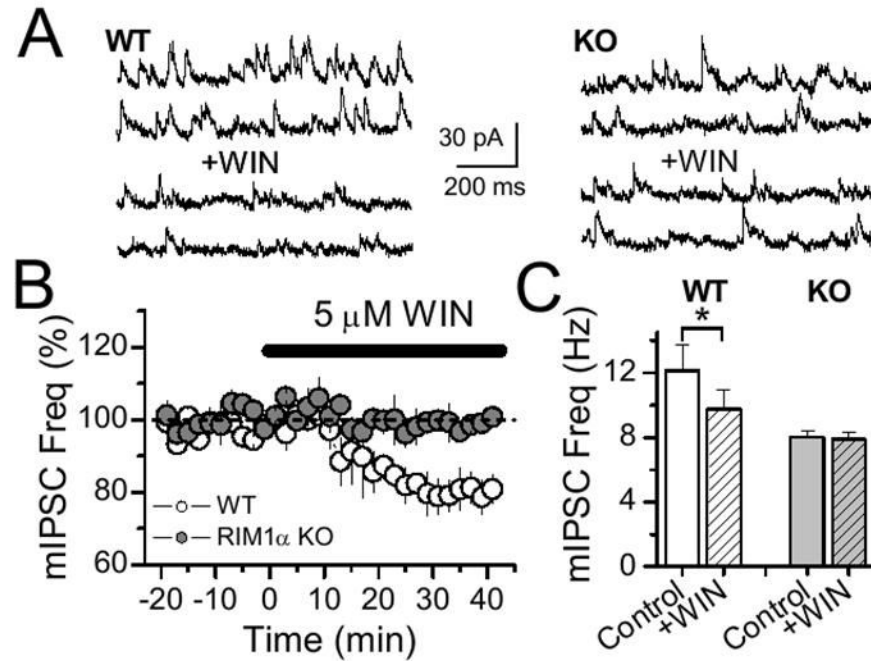


**Figure 4.**

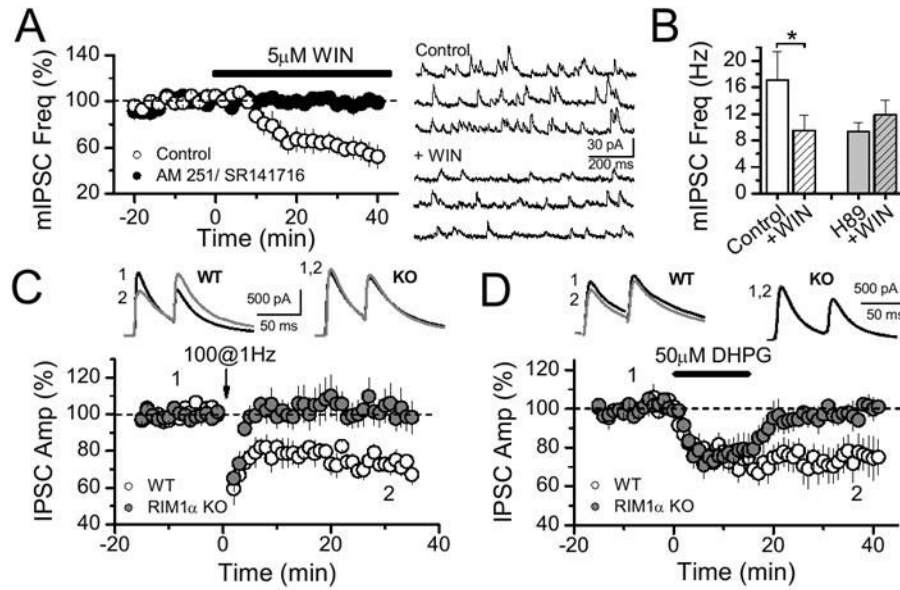
Altered basal transmission at excitatory or inhibitory synapses cannot explain the lack of I-LTD in RIM1 $\alpha$  KO mice. (A) Pharmacological induction of I-LTD by DHPG (50  $\mu$ M, 10 min) was normal in WT mice (n = 5 cells, 4 mice) but impaired in RIM1 $\alpha$  KO mice (n = 5 cells, 4 mice). (B) Similarly, application of the CB1 receptor agonist WIN (5  $\mu$ M, 25 min) subsequently chased with the CB1 receptor antagonist SR 141716 (5  $\mu$ M) induced a lasting depression of field IPSPs in WT (n = 4, 3 mice) but not in RIM1 $\alpha$  KO mice (n = 5, 3 mice). Sample traces before, following WIN application, and in 100  $\mu$ M picrotoxin are shown at right. (C) The average IPSC amplitude (excluding transmission failures), the failure rate and the paired-pulse ratio (IPSC<sub>2</sub>/IPSC<sub>1</sub>) at CB1R<sup>+</sup> fibers isolated by focal stimulation were identical in WT (10 cells, 10 mice) and RIM1 $\alpha$  KO mice (13 cells, 11 mice).

**Figure 5.**

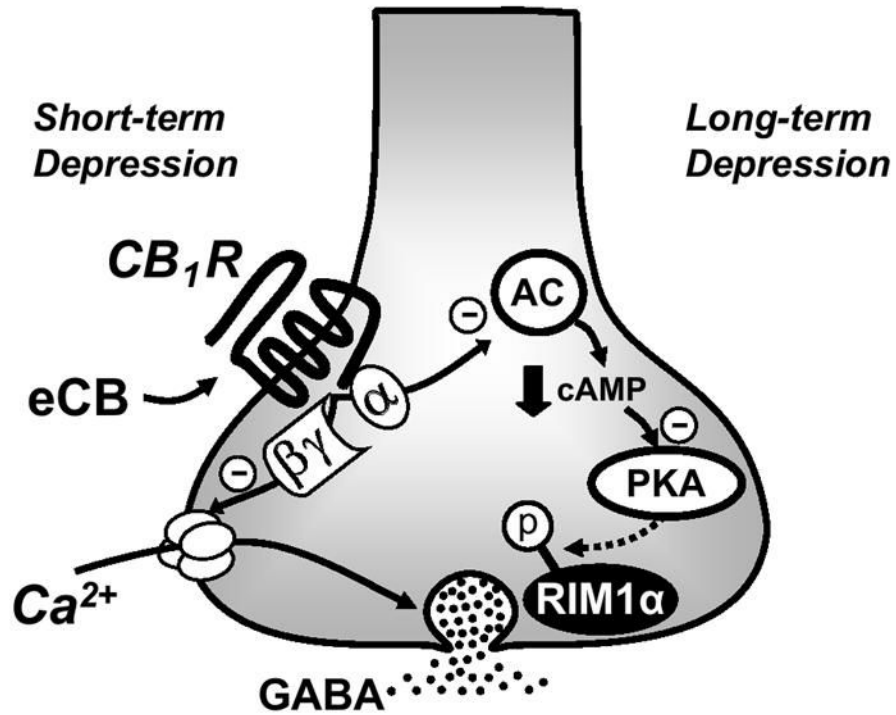
PKA regulation of GABA release at CB1<sup>+</sup> fibers requires RIM1 $\alpha$ . (A) Representative experiments showing the effect of H-89 in WT and RIM1 $\alpha$  KO mice using focal stimulation of CB1R<sup>+</sup> fibers. Averaged sample traces at times indicated by numbers are shown on the right. (B) Summary graph showing significantly greater depression of synaptic transmission (IPSC amplitude, including failures) by H-89 in WT vs. RIM1 $\alpha$  KO mice (8 cells, 8 mice; 9 cells, 8 mice respectively). Without drug application, no significant depression was observed over time. (C) H-89 significantly increased failure rate and aired-pulse ratio (IPSC<sub>2</sub>/IPSC<sub>1</sub>) in WT, but not RIM1 $\alpha$  KO mice (same cells as B).



**Figure 6.** RIM1 $\alpha$  mediates CB1 receptor action on the release machinery. (A) Sample trace before and after WIN application in WT and RIM1 $\alpha$  KO mice. (B) Averaged mIPSC frequency plot before and after WIN application in WT (4 cells, 3 animals) and RIM1 $\alpha$  KO mice (5 cells, 3 animals). (C) Histogram of mIPSC frequency showing the lack of effect of WIN in RIM1 $\alpha$  KO mice.



**Figure 7.** RIM1 $\alpha$  and PKA control GABA release and inhibitory synaptic plasticity in basolateral amygdala. (A) WIN depressed mIPSC frequency in BLA ( $n = 5$ ), an effect blocked by CB1 receptor antagonists ( $5 \mu\text{M}$  AM 251,  $n = 2$ ;  $5 \mu\text{M}$  SR131716,  $n = 2$ ). Sample traces are shown on the right. (B) WIN-mediated depression was blocked by H-89 ( $n = 5$ ). (C) LTD induction was blocked in RIM1 $\alpha$  KO mice (11 cells, 5 mice), compared to their WT littermates (9 cells, 6 mice). (D) LTD induced pharmacologically by DHPG ( $50 \mu\text{M}$ , 15 min) was also abolished in RIM1 $\alpha$  KO mice compared to WT mice ( $n = 7$ , 3 WT mice and  $n = 6$ , 4 KO mice).



**Figure 8.** Model of the divergent signaling pathways downstream of the CB1 receptor that triggers eCB-mediated short- or long-term depression. During eCB-mediated short-term depression, brief activation of CB1 receptors reversibly depresses neurotransmitter release mainly by blocking presynaptic VGCC, presumably via G<sub>β/γ</sub> subunits. In contrast, during the induction of eCB-mediated LTD, a longer-lasting (i.e., few minutes) (Chevalleyre and Castillo, 2003;Ronesi et al., 2004) activation of CB1 receptors inhibits adenylyl cyclase (AC) activity via G<sub>α</sub> subunits, thereby reducing presynaptic cAMP levels and PKA activity. This modification “gates” an enduring change of the release machinery that requires the active zone protein RIM1α. PKA may directly phosphorylate RIM1α and/or other associated proteins.