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Chronic Stress Induces Anxiety via an Amygdalar Intracellular Cascade that Impairs Endocannabinoid Signaling

Highlights

- Mice with ablation of an endogenous PTP1B inhibitor LMO4 have anxiety
- PTP1B dephosphorylates mGluR5 and impairs mGluR5-mediated eCB production
- Glucocorticoids impair LMO4 palmitoylation and increase PTP1B activity
- PTP1B inhibition in the amygdala restores eCBs and relieves stress-induced anxiety

Authors

Zhaohong Qin, Xun Zhou, ..., Matthew N. Hill, Hsiao-Huei Chen

Correspondence

hchen@uottawa.ca

In Brief

Collapse of endocannabinoid signaling in the amygdala underlies stress-induced anxiety. Qin et al. present a mechanism whereby stress (glucocorticoid) causes loss of LMO4-dependent inhibition of tyrosine phosphatase PTP1B that impairs mGluR5-mediated endocannabinoid production. Suppressing amygdalar PTP1B rescues stress-induced anxiety.

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Chronic Stress Induces Anxiety via an Amygdalar Intracellular Cascade that Impairs Endocannabinoid Signaling

Zhaohong Qin,[1] Xun Zhou,[1] Nihar R. Pandey,[1] Haley A. Vecchiarelli,[2] Chloe A. Stewart,[1] Xia Zhang,[3,4] Diane C. Lagace,[4] Jean Michel Brunel,[5] Jean-Claude Béïque,[4] Alexandre F.R. Stewart,[6,7,8] Matthew N. Hill,[2] and Hsiao-Huei Chen[1,4,8,*]
[1]Ottawa Hospital Research Institute, Ottawa, ON K1H8M5, Canada
[2]Hotchkiss Brain Institute and Mathison Centre for Mental Health Research and Education, Departments of Cell Biology and Anatomy & Psychiatry, University of Calgary, Calgary, AB T2N4N1, Canada
[3]Royal Ottawa Mental Health Centre, Ottawa, ON K1Z7K4, Canada
[4]Department of Cellular and Molecular Medicine, Ottawa, ON K1H8M5, Canada
[5]Centre de Recherche en Cancérologie de Marseille, Laboratory of Integrative Structural & Chemical Biology (iSCB), Aix-Marseille Université, 13385 Marseille Cedex 5, France
[6]Department of Biochemistry, Microbiology and Immunology, Ottawa, ON K1H8M5, Canada
[7]University of Ottawa Heart Institute, Ottawa, ON K1Y4W7, Canada
[8]Department of Medicine, University of Ottawa, Ottawa, ON K1H8M5, Canada
*Correspondence: hchen@uottawa.ca
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SUMMARY

Collapse of endocannabinoid (eCB) signaling in the amygdala contributes to stress-induced anxiety, but the mechanisms of this effect remain unclear. eCB production is tied to the function of the glutamate receptor mGluR5, itself dependent on tyrosine phosphorylation. Herein, we identify a novel pathway linking eCB regulation of anxiety through phosphorylation of mGluR5. Mice lacking LMO4, an endogenous inhibitor of the tyrosine phosphatase PTP1B, display reduced mGluR5 phosphorylation, eCB signaling, and profound anxiety that is reversed by genetic or pharmacological suppression of amygdalar PTP1B. Chronically stressed mice exhibited elevated plasma corticosterone, decreased LMO4 palmitoylation, elevated PTP1B activity, reduced amygdalar eCB levels, and anxiety behaviors that were restored by PTP1B inhibition or by glucocorticoid receptor antagonism. Consistently, corticosterone decreased palmitoylation of LMO4 and its inhibition of PTP1B in neuronal cells. Collectively, these data reveal a stress-responsive corticosterone-LMO4-PTP1B-mGluR5 cascade that impairs amygdalar eCB signaling and contributes to the development of anxiety.

INTRODUCTION

Mood and anxiety disorders represent one of the largest health burdens on society today, yet few novel therapeutics have emerged in the past two decades. Neuroimaging has delineated disturbances in the limbic circuit composed of the amygdala, prefrontal cortex (PFC) and hippocampus underlying the pathophysiology of these conditions (Etkin et al., 2009). The current evidence points to hyperexcitability of the amygdala, due in part to impairments in the inhibitory influence of the hippocampus and PFC, as germane to the manifestation of these diseases (Etkin et al., 2009; Kim et al., 2011). While most research has focused on how afferent regulation to the amygdala is disturbed in anxiety, few studies have examined intrinsic changes within amygdalar neurons that could account for hyperexcitability of this structure. As such, characterizing signaling mechanisms in the amygdala that regulate anxiety behavior is key to identifying putative substrates dysregulated in pathological conditions.

In this regard, the endocannabinoid (eCB) system has recently become an interesting player in the regulation of stress and anxiety. Cannabis consumption in humans can have profound effects on anxiety, and similarly, preclinical studies have found that modulation of eCB signaling alters anxiety (Hill and Patel, 2013). Genetic or pharmacological disruption of eCB signaling produces an array of neurobehavioral effects that mirror those produced by exposure to stress, such as increased activation of the hypothalamic-pituitary-adrenal (HPA) axis, elevated levels of anxiety, altered stress-coping behaviors, and impairments in fear extinction (Marsicano et al., 2002; Patel et al., 2004; Shonesy et al., 2014; Steiner et al., 2008). Most, if not all, of these effects are replicated by local disruption of eCB signaling directly within the amygdala (Gunduz-Cinar et al., 2013). Specifically, administration of a CB1 receptor antagonist directly into the basolateral nucleus of the amygdala (BLA) results in an increase in HPA axis activity, elevated levels of anxiety and a disruption of fear extinction (Dono and Currie, 2012; Ganon-Elazar and Akirav, 2009; Hill et al., 2009). This suggests that eCB signaling within the BLA constrains activation of the stress response and anxiety.

Consistent with this stress-buffering role of eCB signaling, several reports indicate that facilitation of eCB signaling can reverse the adverse effects of chronic stress (Bortolato et al., 2007; Hill et al., 2013; Lomazzo et al., 2015; Sumislawski et al.,

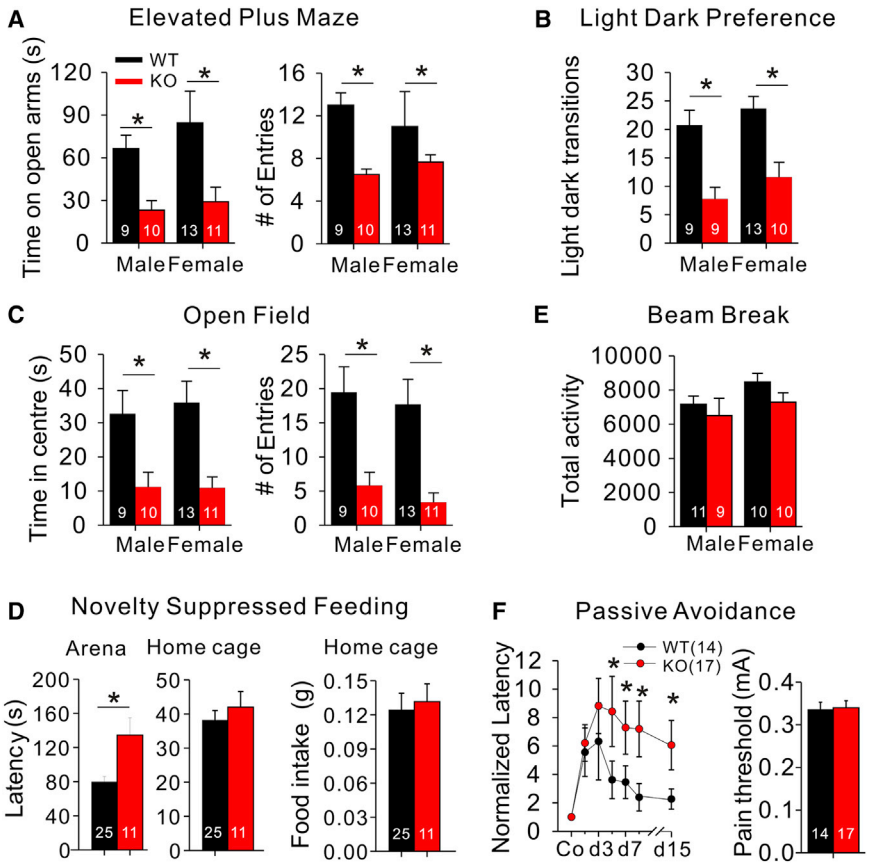

Figure 1. *LMO4*KO Mice Have a Stresslike Anxiety Phenotype with Impaired Fear Extinction

(A–D) EPM (A), LDP test (B), OF test (C), and novelty suppressed feeding (D) revealed a marked anxiety phenotype in *LMO4*KO (*CamK2α*$^{Cre/-}$/*LMO4*$^{flox/flox}$) mice compared with littermate control "WT" mice (*LMO4*$^{flox/flox}$).

(E) Locomotor activity, measured by beam break over a 2 hr period, was similar between littermate KO and WT mice.

(F) *LMO4*KO mice showed impaired fear extinction in the passive avoidance task but no difference at the level of pain threshold compared with WT mice.

Co, conditioning training day. *$p < 0.05$. Error bars represent SEM. Number of mice used indicated in bars.

2011; Zhang et al., 2015). More specifically, exposure to stress results in a sustained collapse of eCB signaling within the amygdala and leads to pathological states of anxiety (Hill et al., 2013). Conversely, extrinsic modalities such as environmental enrichment, dietary polyunsaturated fatty acids, and pharmacological agents (such as salvinorin) that reduce anxiety alter eCB metabolism or signaling within the BLA (Braida et al., 2009; El Rawas et al., 2011; Yamada et al., 2014). Together, these studies indicate that amygdalar eCB signaling is an important regulator of anxiety.

It is well established that the production of eCB is tied to the activity of the metabotropic glutamate receptors (mGluR), particularly mGluR5 (Maccarrone et al., 2008; Maejima et al., 2005; Zhu and Lovinger, 2005). In the case of mGluR5, its phosphorylation at tyrosine residues is required for maximal signaling (Orlando et al., 2002; Tozzi et al., 2001), suggesting that a reduction in phosphorylation could result in consequential effects on eCB production and signaling. Recently, we demonstrated that the LIM domain only 4 (LMO4) protein is an endogenous inhibitor of the protein tyrosine phosphatase PTP1B in the hypothalamus (Pandey et al., 2013; Pandey et al., 2014); however, the impact of PTP1B activity on mGluR5 phosphorylation, and its downstream effects on eCB signaling, have yet to be determined. Of note, 90% of neurons within the BLA are glutamatergic (Carlsen, 1988) and express high levels of LMO4 (Maiya et al., 2012). Interestingly, a partial deletion of LMO4 within the BLA has recently been linked to the regulation of fear learning (Maiya et al., 2012). Together, these data bring forth the previously untested hypothesis that intracellular signaling cascades that regulate mGluR5 signaling could affect anxiety and fear behavior through regulation of eCB signaling.

Using a multidisciplinary approach, catalyzed by the discovery of a profound anxiety phenotype in mice deficient in LMO4 within glutamatergic neurons, our studies elucidate an intracellular signaling cascade whereby chronic stress, through a glucocorticoid-mediated pathway, impairs LMO4-mediated inhibition of PTP1B activity, leading to collapse of eCB production (through a loss of mGluR5 phosphorylation) within the amygdala and causing anxiety behavior. Accordingly, this cascade may represent an entirely novel target for the development of anxiolytic pharmacotherapeutic agents.

RESULTS

LMO4 Knockout Mice Have Increased Anxiety and Impaired Fear Extinction

Deletion of LMO4 within glutamatergic neurons (*CamK2α*$^{Cre/-}$/*LMO4*$^{flox/flox}$; *LMO4* knockout [*LMO4*KO]; Figures S1A and S1B) resulted in an anxiety phenotype in four behavioral tests, including (1) a reduced number of entrances and time spent in the open arms during the elevated plus maze (EPM) test (Figure 1A), (2) a reduced number of transitions between the dark and light chambers in the light/dark preference (LDP) test (Figure 1B), (3) less time spent in the center during the open-field (OF) test (Figure 1C), and (4) increased latency to feed in an OF, but not in the home cage, in the novelty suppressed feeding test (Figure 1D). The anxious phenotype in *LMO4*KO mice is not due to altered basal locomotor activity (Figure 1E). Impaired fear extinction was also present in *LMO4KO* mice, tested by the passive avoidance task (Figure 1F), and this was not due to a difference in pain threshold. Collectively, these data suggest that *LMO4*KO mice have an emotional phenotype that parallels the

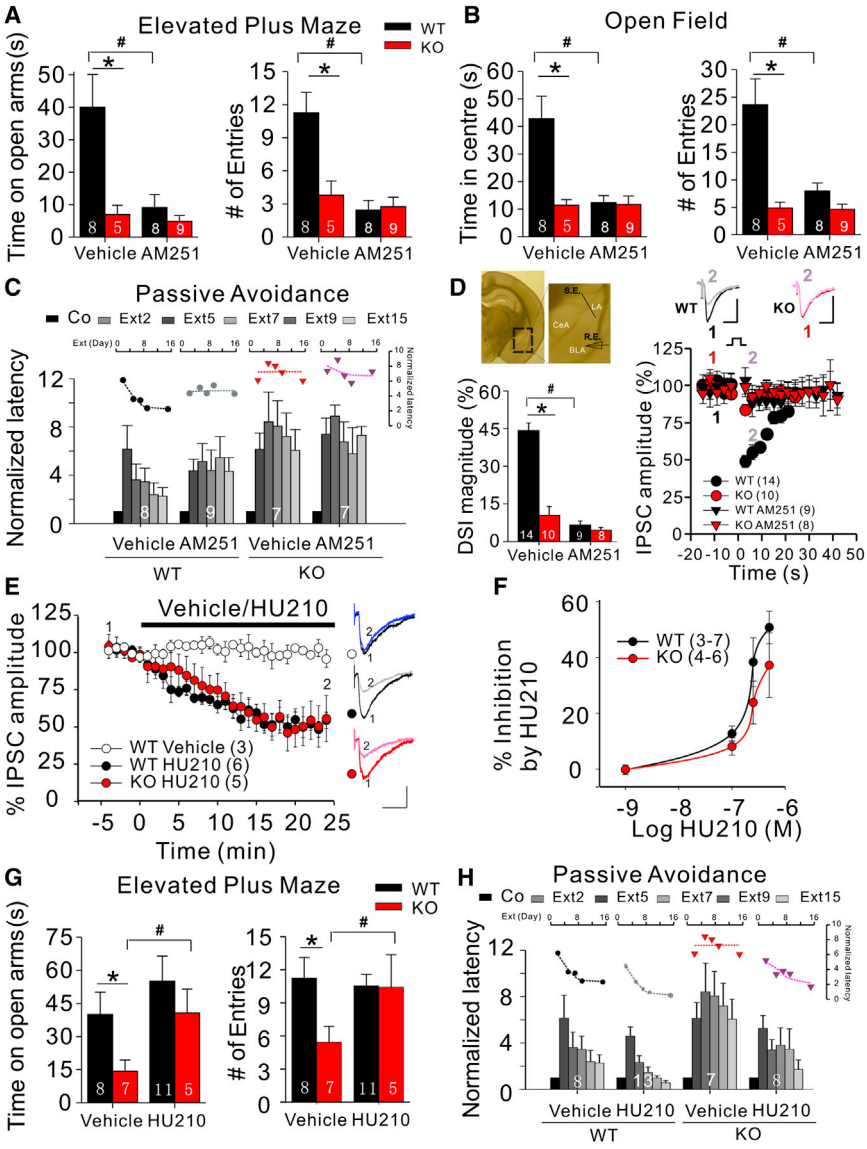

Figure 2. Impaired eCB Signaling Is Associated with Anxiety Phenotype in *LMO4*KO Mice

(A–C) Anxiety and fear extinction phenotype in *LMO4*KO mice look strikingly similar to WT mice treated with the CB1cannabinoid receptor antagonist AM251 (3 mg/kg), and the anxiogenic effect of AM251 in WT mice was occluded in *LMO4*KO mice. Mice were subjected to EPM test 20 min after intra-peritoneal (ip) injection of either vehicle or AM251, followed by OF test 48 hr later. The fear extinction test was carried out in a separate group of mice. Vehicle or AM251 was injected (ip) 20 min before the first extinction trial. Exponential curve fits of latencies are presented above the bars. Co, conditioning training day. Ext2-15, extinction testing days 2 to 15.

(D) DSI was reduced in BLA neurons of *LMO4*KO mice. The reduction was also observed in WT neurons but occluded in LMO4KO neurons treated with the CB1 cannabinoid receptor antagonist AM251. (Top left) Positions of recording and stimulating electrodes for patch-clamp recording. BLA, basolateral amygdala; CeA, central amygdala; LA, lateral amygdala; RE, recording electrode; SE, stimulating electrode. (Top right) Sample traces of IPSCs.

(E) Treatment of BLA neurons with CB1 agonist HU210 (500 nM) reduced eIPSC amplitude to a similar degree.

(F) WT and *LMO4*KO BLA neurons have similar dose-dependent responses to HU210.

(G and H) Ip treatment with the CB1 agonist HU210 (10 μg/kg) abolished the anxiety phenotype (G) or fear extinction deficit (H) of *LMO4*KO mice. Vehicle or drug was delivered (ip) 20 min before EPM test or each extinction trial.

*&#p < 0.05. Error bars represent SEM.

behavioral changes that emerge following stress exposure, such as increased anxiety and impaired fear extinction.

The Anxiety and Fear Extinction Phenotypes in *LMO4*KO Mice Are due to Reduced mGluR-Mediated eCB Signaling

Interestingly, the anxiety and fear extinction phenotypes in *LMO4*KO mice look strikingly similar to those observed in CB1 receptor knockout (KO) mice (Haller et al., 2004; Marsicano et al., 2002), so we sought to determine whether eCB function was altered in *LMO4*KO mice. The CB1cannabinoid receptor antagonist AM251 had a profound anxiogenic effect in wild-type (WT) mice, but this effect was occluded in *LMO4*KO mice in both the EPM and OF tests (Figures 2A–2C). As eCB signaling in the BLA has been linked to both anxiety and fear extinction (Hill et al., 2013; Marsicano et al., 2002), we examined if changes in eCB signaling were present in the amygdalae of *LMO4*KO mice. Whole-cell recordings of BLA neurons ex vivo in brain slices showed that depolarization-induced suppression of inhibition (DSI), an electrophysiological signature of eCB signaling that is blocked by the CB1 receptor antagonist AM251, was reduced in *LMO4*KO mice (Figure 2D). Similarly, another measure of eCB signaling, depolarization-induced suppression of excitation (DSE) was also impaired in LMO4KO mice (Figure S2). These results indicate that amygdalar eCB function is impaired in LMO4KO mice.

To address whether the impairments in eCB signaling were mediated by altered CB1 receptor signaling capacity versus eCB biosynthesis/mobilization, we examined each of these variables separately. To assess the functionality of CB1 receptors, we administered an exogenous CB1 receptor agonist, HU210, to BLA slices. *LMO4*KO and WT BLA neurons showed a similar reduction of amplitude of inhibitory postsynaptic currents (IPSCs) (Figure 2E) and a similar dose response to HU210 (Figure 2F), indicating that CB1 receptor function is not impaired in *LMO4*KO BLA neurons. Consistent with this finding, administration of HU210 rescued the anxiety phenotype (Figure 2G) and restored fear extinction (Figures 2H and S3) in *LMO4*KO mice.

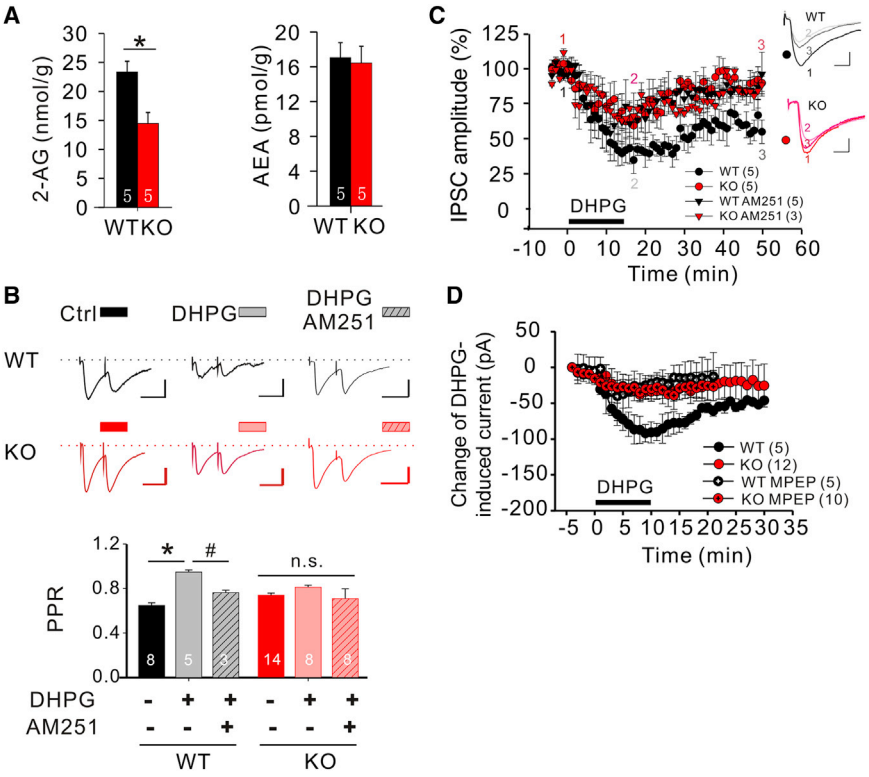

Figure 3. mGluR-Dependent eCB Mobilization Is Impaired in *LMO4*KO Amygdala

(A) 2-AG but not AEA levels in amygdala in *LMO4*KO mice were reduced. n = 5 per group.

(B) DHPG increased the PPR (P2/P1) in WT but not in *LMO4*KO BLA neurons, and this effect was blocked by pre-treatment with CB1R antagonist AM251. Current traces depicting GABA-mediated eIPSCs induced by paired pulses before and 15 min after DHPG application from WT and *LMO4*KO BLA neurons with or without AM251 are shown above the graph; the scale bar represents 200 pA/50 ms.

(C) DHPG-induced long-term depression of IPSC (LTDi) was markedly attenuated in *LMO4*KO compared with littermate control (WT) BLA neurons. LTDi was blocked by the AM251 in WT BLA neurons. Representative traces before (trace 1: black for WT and red for KO) and 15 min after DHPG treatment (trace 2: light gray for WT and light pink for KO) or recovery after DHPG-wash (trace 3: dark gray for WT and dark pink for KO) are shown above the graph; the scale bar represents 100 pA/10 ms.

(D) Change of holding current following administration of the mGluR1/5 agonist DHPG was markedly attenuated in *LMO4*KO compared with control BLA neurons. The mGluR5 antagonist MPEP blocked the DHPG-induced change of holding current. Number of cells recorded in parentheses or indicated in bars.

*$p < 0.05$. Error bars represent SEM.

Given that the function of CB1 receptors appears intact in *LMO4*KO mice, the deficit in eCB function may arise from reduced eCB levels. To that end, we carried out liquid chromatography-mass spectrometry analysis of amygdala samples and found that tissue levels of 2-arachidonoylglycerol (2-AG), but not anandamide (AEA), were reduced in *LMO4*KO mice (Figure 3A). Because 2-AG production is tied to the activity of mGluR5 (Maccarrone et al., 2008), we then examined if mGluR5-mediated eCB signaling was altered in *LMO4*KO mice. Consistent with previous reports (Maccarrone et al., 2008; Maejima et al., 2005; Zhu and Lovinger, 2005), DHPG increased the paired pulse ratio (PPR) and induced inhibitory long-term depression (LTDi) in WT mice. Both responses reflect mGluR5-dependent eCB-mediated alteration in synaptic GABA release (Azad et al., 2004; Marsicano et al., 2002) and were blocked by pretreatment with the CB1R antagonist AM251 (Figures 3B and 3C). These effects of DHPG on the PPR and LTDi were markedly reduced in BLA neurons of *LMO4*KO mice (Figures 3B and 3C), indicating that mGluR5-mediated eCB signaling within the BLA is impaired following ablation of LMO4.

Activation of mGluR5 with DHPG is known to induce inward currents, likely mediated by opening of non-selective cationic channels (Fagni et al., 2000; Gee et al., 2003; Guérineau et al., 1994). Consistent with our findings of impaired mGluR5 signaling in *LMO4*KO mice, the DHPG-induced inward current was markedly reduced in *LMO4*KO BLA neurons compared with WT (Figure 3D). Together, these findings lead us to conclude that impaired eCB signaling in BLA neurons of *LMO4*KO mice could be due to impairments in mGluR5 signaling.

LMO4 Inhibition of PTP1B Activity Regulates mGluR5-Mediated eCB Signaling

Given that lower 2-AG levels were associated with reduced mGluR5 mediated eCB signaling in *LMO4*KO BLA neurons, we examined how mGluR5 function is regulated by LMO4. It has been reported that activation of mGluR5 signaling involves phosphorylation of its tyrosine residues (Orlando et al., 2002; Tozzi et al., 2001). Interestingly, we recently demonstrated that LMO4 is required to suppress the activity of the protein tyrosine phosphatase PTP1B in the hypothalamus (Pandey et al., 2013). Thus, we postulated that elevated PTP1B activity resulting from *LMO4* ablation would lead to impaired mGluR5 function through a dephosphorylation of tyrosine residues, thus reducing mGluR5 signaling. Consistent with our previous study, an in vitro assay revealed a 3-fold elevation of PTP1B tyrosine phosphatase activity in amygdala lysates from *LMO4*KO mice (Figure 4A). This change in PTP1B activity was not due to elevated PTP1B protein levels (Figure 4B). Instead, it reflected lower levels of the oxidized, inactive form of PTP1B in *LMO4*KO amygdala compared with littermate controls (Figure 4B), consistent with our notion that LMO4 promotes the inactivation of PTP1B through oxidation of a cysteine residue in the catalytic domain (Pandey et al., 2013).

We confirmed that mGluR5 is phosphorylated at tyrosine residues (Figure 4C). Furthermore, we showed that PTP1B targets and dephosphorylates mGluR5 and that LMO4 modulates this process (Figure 4C). Endogenous mGluR5 was immunoprecipitated from F11 neuronal cells and shown to be phosphorylated at tyrosine residues using a phosphotyrosine-specific antibody

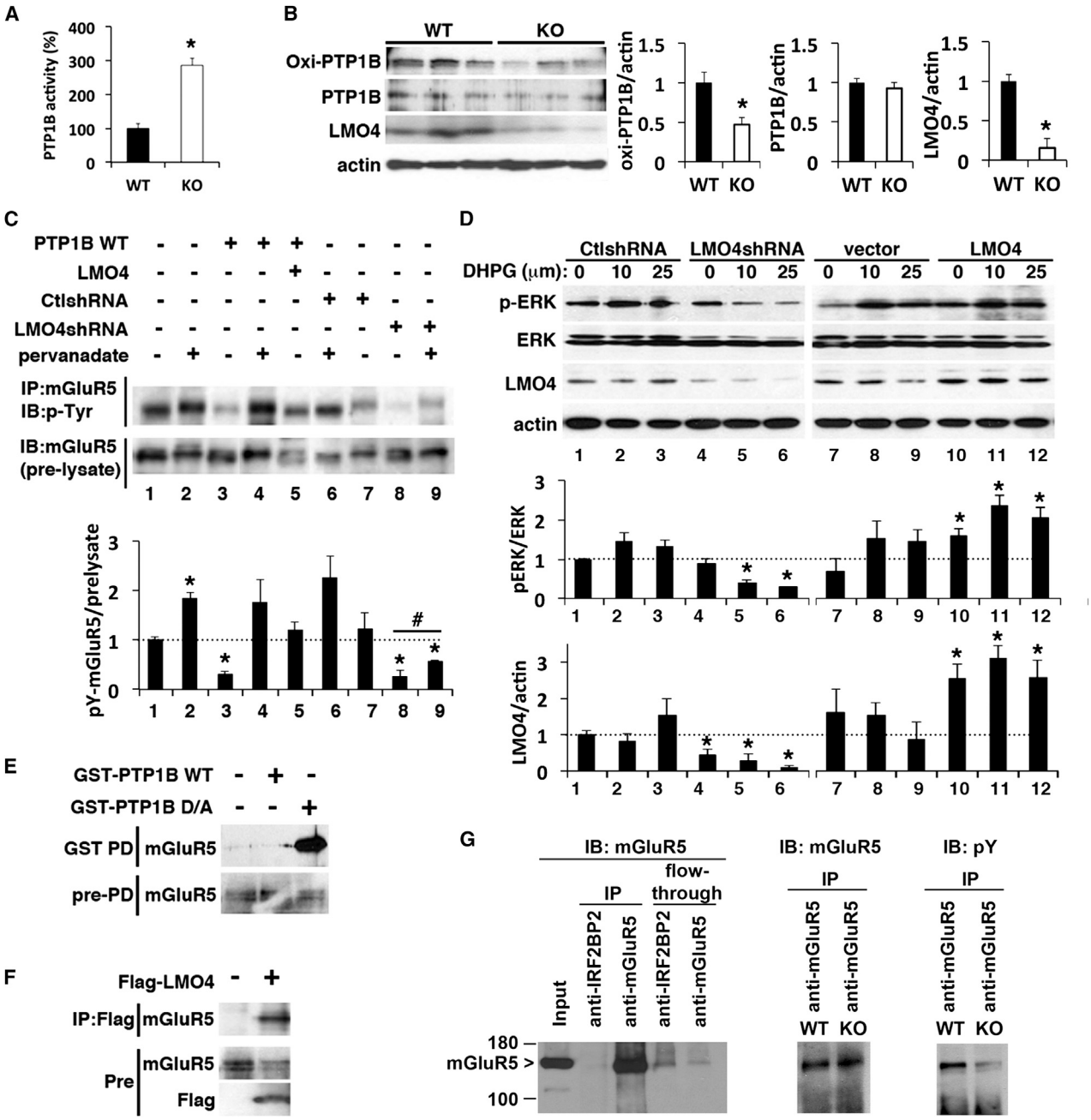

Figure 4. Knockdown of LMO4 Causes Elevated PTP1B Activity, Reduced Phosphorylation of mGluR5 at Tyrosine Residues, and Impaired mGluR5-Dependent eCB Signaling

(A) PTP1B activity is elevated in *LMO4*KO amygdala. n = 8 per group. *p < 0.05. Error bars represent SEM.

(B) PTP1B protein levels are not changed. Rather, there was less oxidized PTP1B (oxi-pTP1B) in the amygdala of *LMO4*KO compared with littermate controls. n = 3 per genotype.

(C) F11 neuronal cells transfected with PTP1B expression vector (lane 3) or *LMO4*shRNA (lane 8) caused reduced tyrosine phosphorylation of mGluR5.

(D) Transient transfection of F11 neuronal cells with *LMO4*shRNA reduced (lanes 4–6), whereas LMO4 overexpression (lanes 10–12) increased DHPG-induced ERK phosphorylation. For (C) and (D), n = 3 experiments. *p < 0.05 compared with lane 1 after Bonferroni correction for multiple testing. #p < 0.05.

(E) Endogenous mGluR5 in F11 neuronal cells was pulled down (PD) with a substrate-trapping mutant (D/A) of GST-tagged PTP1B protein.

(F) Flag-tagged LMO4 is co-immunoprecipitated (IP) with mGluR5 in F11 cells. The pre-immunoprecipitation cell extracts (Pre) were probed with mGluR5 and Flag antibodies. In (B), (C), and (F), blots are quantified on the right.

(G) mGluR5 tyrosine phosphorylation is reduced in LMO4KO amygdala. Amygdala extracts pooled from three littermate WT and three pooled LMO4KO (KO) mice were IP with mGluR5 antibody and immunoblots (IB) were probed with antibodies to p-Tyr or mGluR5. An unrelated antibody (anti-IRF2BP2; Teng et al., 2010) was used as an IP control. Input represents 1/10 of amygdala extracts directly loaded without IP.

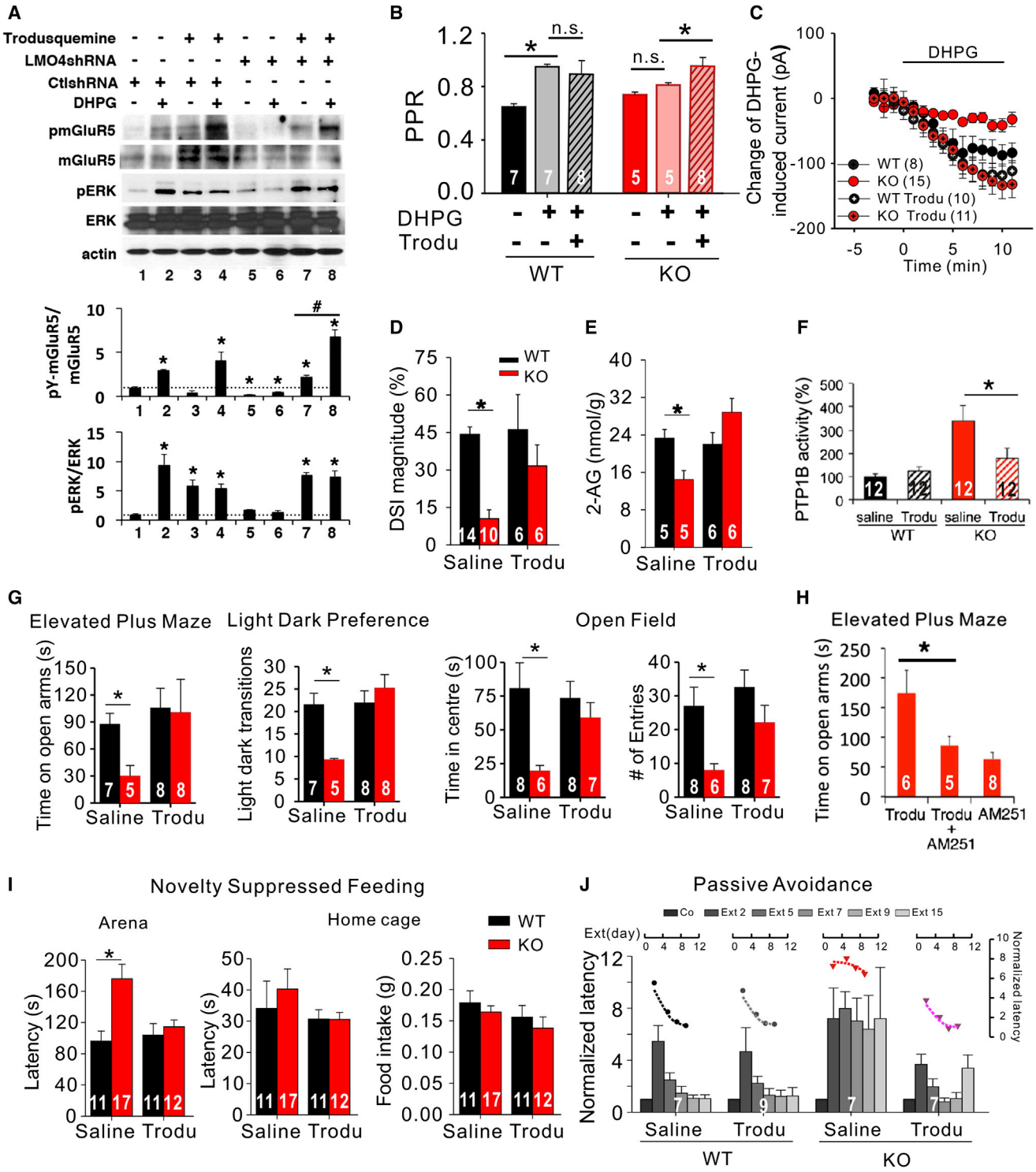

Figure 5. The PTP1B Inhibitor Trodusquemine Normalizes PTP1B Activity, Restores mGluR Function, and Alleviates the Anxiety Phenotype of *LMO4*KO Mice

(A) Pre-treatment with trodusquemine (10 μM) for 30 min restored ERK phosphorylation in response to mGluR1/5 agonist DHPG in F11 neuronal cells. Blots are quantified on the right. n = 3 experiments. *p < 0.05 compared with lane 1 after Bonferroni correction for multiple testing. #p < 0.05.

(B) Trodusquemine restored the facilitation of PPR in response to 10 min of bath applied DHPG in *LMO4*KO BLA neurons.

(C and D) Addition of trodusquemine (10 uM) in the intracellular recording electrode rescued DHPG-induced holding currents (C) and restored DSI (D) in *LMO4*KO BLA neurons.

(legend continued on next page)

(lane 1). mGluR5 tyrosine phosphorylation was markedly reduced by PTP1B overexpression (lane 3), and this effect was blocked by pervanadate, a tyrosine phosphatase inhibitor (lane 4). Importantly, overexpression of LMO4 increased (lane 5) whereas knockdown of *LMO4* reduced mGluR5 tyrosine phosphorylation (compare lanes 8 and 7). Thus, modulation of LMO4 affects mGluR5 phosphorylation. As a functional assay of mGluR5 activity, we also found that DHPG-induced ERK phosphorylation (Kim et al., 2008) was blunted following *LMO4* knockdown by small hairpin RNA (shRNA) (Figure 4D, compare lanes 5 and 6 with lanes 2 and 3) and augmented by overexpression of LMO4 in neuronal cell lines (Figure 4D, compare lanes 10–12 with lanes 7–9). Pull-down assays demonstrated that both LMO4 and PTP1B associate with mGluR5 (Figures 4E and 4F). Using a catalytically inactivated mutant of PTP1B that binds, but does not dephosphorylate or release its substrate (i.e., a substrate-trapping mutant), we observed a strong interaction of this mutant PTP1B with mGluR5 in a glutathione S-transferase (GST) pull-down assay (Figure 4E). This demonstrates that mGluR5 is a bona fide substrate of PTP1B and argues that PTP1B is an endogenous regulator of mGluR5 signaling. We also demonstrated through co-immunoprecipitation that mGluR5 interacts with LMO4 (Figure 4F), suggesting that LMO4 may regulate mGluR5 function in a complex with PTP1B. Consistent with the elevated PTP1B activity (Figure 4A), we also observed reduced tyrosine phosphorylation of mGluR5 in the amygdala of LMO4KO mice (Figure 4G). Collectively, these results suggest that LMO4 affects mGluR5 signaling through PTP1B activity.

The PTP1B Inhibitor Trodusquemine Normalizes PTP1B Activity, Restores mGluR5 Function, and Alleviates the Anxiety Phenotype of *LMO4*KO Mice

Because ablation of *LMO4* is associated with elevated PTP1B activity and reduced mGluR5 phosphorylation and function, we next determined the effect of pharmacological ablation of PTP1B activity on the effects of *LMO4* deletion. Using a small-molecule PTP1B-specific inhibitor, trodusquemine, we demonstrated that inhibition of PTP1B restored tyrosine phosphorylation of mGluR5 in *LMO4* knockdown cells (Figure 5A, compare lanes 7 and 8 with lanes 5 and 6). Moreover, trodusquemine restored DHPG-induced ERK phosphorylation (compare lanes 8 and 6). These results further demonstrated that PTP1B activity affects mGluR5 phosphorylation and associated signaling.

Consistent with this, trodusquemine restored DHPG-induced facilitation of the PPR (Figure 5B), neuronal depolarization (Figure 5C), and DSI (Figure 5D) in *LMO4*KO BLA neurons and amygdalar 2-AG content in *LMO4*KO mice (Figure 5E). Continuing with this line of reasoning, we next investigated whether local PTP1B inhibition within the amygdala might relieve the anxiety phenotype of *LMO4*KO mice. Trodusquemine was delivered by bilateral stereotactic injection to the amygdala and this effectively blocked excess PTP1B activity in *LMO4*KO amygdala (Figure 5F). Similarly, intra-amygdalar trodusquemine completely abolished the anxiety phenotype of *LMO4*KO mice, as assessed in the EPM, LDP, and OF tests (Figure 5G). Of note, this dose of trodusquemine had no effect on PTP1B activity or behavior in littermate controls (Figure 5G). Importantly, the anxiolytic effect of trodusquemine was blocked by the CB1 antagonist AM251 (Figure 5H), demonstrating that the anxiolytic effect of trodusquemine works through a restoration of eCB signaling within the amygdala. Because trodusquemine can pass the blood-brain barrier (Ahima et al., 2002; Lantz et al., 2010), the anxiolytic effect of trodusquemine was also reproducibly observed in *LMO4*KO mice following systemic intra-peritoneal injection, as shown by three anxiety tests (Figure S5), as well as by novelty suppressed feeding test (Figure 5I) and by the passive avoidance test (Figures 5J and S6). Consistent with our model, the anxiolytic effect of trodusquemine was also blocked by a low dose of the mGluR5 antagonist MPEP (Figure S7). Of note, this low dose of MPEP on its own did not affect behavior (Figure S7). Taken together, these results indicate that by inhibiting the phosphatase activity of PTP1B, trodusquemine restores tyrosine phosphorylation of mGluR5 and its activity, which in turn elevates 2-AG signaling within the amygdala and alleviates the anxiety phenotype of *LMO4*KO mice.

shRNA Knockdown of PTP1B in the Amygdala Relieves the Anxiety Phenotype of *LMO4*KO Mice

To further demonstrate that PTP1B in the BLA participates in this process, we examined the effects of genetic PTP1B knockdown in *LMO4*KO mice. Twenty-one days after a bilateral stereotactic injection of lentiviral vectors expressing PTP1B shRNA and GFP into the amygdala (Figure 6A), there was markedly reduced PTP1B protein expression (Figure 6B) and PTP1B activity (Figure 6C) in amygdala extracts. PTP1B shRNA extinguished the anxiety phenotypes of *LMO4*KO mice in the EPM (Figure 6D), the LDP (Figure 6E), and the OF (Figure 6F) tests. In contrast, the control lentiviral vector-injected *LMO4*KO mice displayed an anxiety phenotype in all tests (Figures 6D–6F). Through convergent pharmacological and genetic approaches, these studies confirm the importance of PTP1B activity within the amygdala in mediating the emotional changes evoked by ablation of LMO4.

(E) 2-AG levels in amygdala of *LMO4*KO mice increased 1hr after ip injection of trodusquemine.

(F and G) A single bilateral stereotaxic injection of trodusquemine to the amygdala (see Figure S5) (F) normalized PTP1B activity in *LMO4*KO amygdala 1 week after intra-amygdalar injection and (G) extinguished anxiety phenotypes of *LMO4*KO mice. Behavior tests were conducted 1 day (EPM), 3 days (LDP), and 5 days (OF) after treatment.

(H) The anxiolytic effect of trodusquemine was abolished by the CB1 antagonist AM251. The EPM test was conducted 24 hr after intra-amygdalar coinjection of trodusquemine with vehicle or AM251. Error bars represent SEM.

(I) Seven days after ip injection of trodusquemine (Qin et al., 2015), *LMO4*KO mice have normalized latency spent in the OF arena before first feeding episode in the novelty suppressed feeding test. Error bars represent SEM.

(J) Trodusquemine's effect to rescue fear extinction in *LMO4*KO mice lasts up to 7 days. Saline or trodusquemine was injected (ip) 1hr before the first extinction trial. Exponential curve fits of latencies are presented above the bars.

*$p < 0.05$. Error bars represent SEM. Number of mice used indicated in bars.

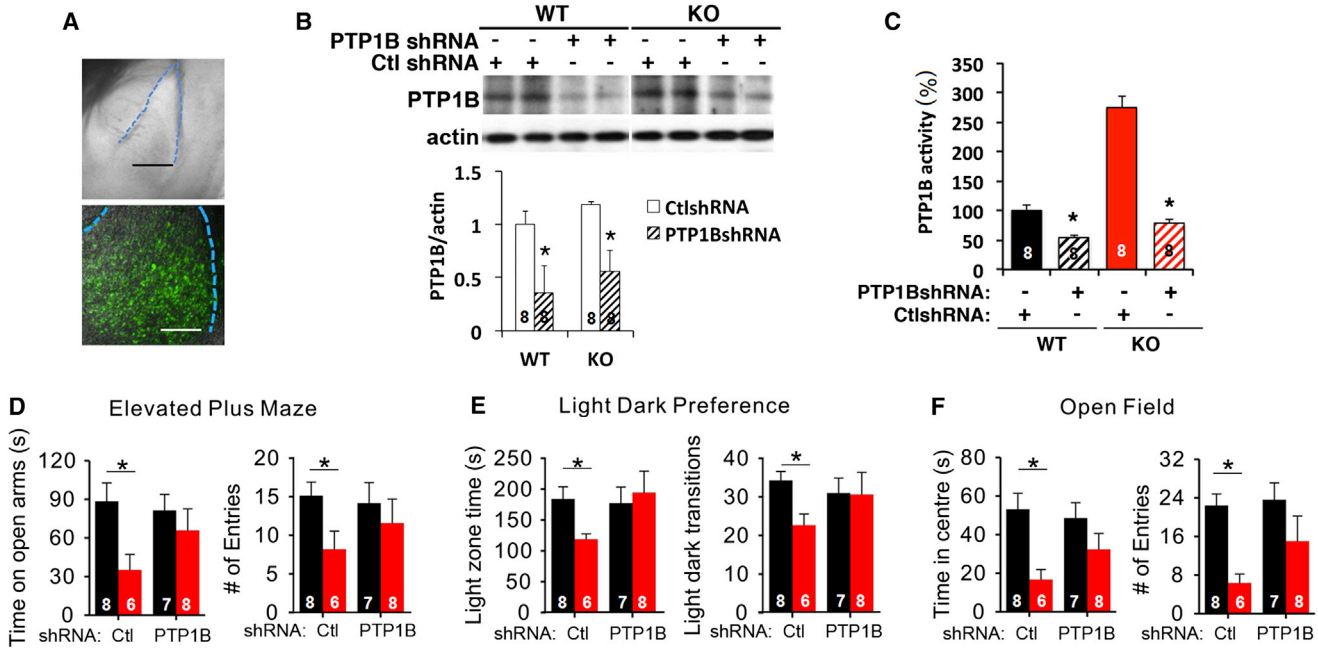

Figure 6. Intra-Amygdalar Injection of Lentiviral PTP1B shRNA Alleviates the Anxiety Phenotype of *LMO4*KO Mice

(A) Representative live images of GFP-positive BLA neurons 21 days after intra-amygdalar injection of lentiviral vectors expressing GFP reporter and *PTP1B* shRNA. (Top) Bright field; (bottom) merged differential interference contrast and fluorescence. The scale bars represent 1 mm.

(B) Western blot of amygdalar extract revealed a reduction in PTP1B protein expression 21 days after intra-amygdalar injection of lentiviral vector expressing *PTP1B* shRNA compared with control shRNA (CtlshRNA). Blots are quantified in the lower panel. n = 3 experiments. *p < 0.05 relative to ctlshRNA.

(C) PTP1B activity assay 21 days after lentiviral vector injection.

(D–F) Bilateral stereotaxic injection of *PTP1B* shRNA (1×10^9 TU/ml, 0.4 μl/side) to the amygdala extinguished anxiety phenotypes of *LMO4*KO mice (red bars) and had no effect in WT mice (black bars). Behavior tests were conducted 12 days (D, EPM), 14 days (E, LDP), and 16 days (F, OF) after treatment.

*p < 0.05. Error bars represent SEM.

Stress-Induced Corticosterone Disrupts the LMO4-PTP1B Cascade to Impair eCB Signaling and Increase Anxiety

The above experiments demonstrate that the loss of LMO4 increases PTP1B activity, which dephosphorylates mGluR5 and consequently reduces eCB signaling within the amygdala. This impairment in mGluR5 and eCB signaling, in turn, results in an increase in anxiety and impairments in fear extinction. This phenotype of the *LMO4*KO mouse is reminiscent of the effects seen following chronic stress or a loss of eCB signaling (Hill et al., 2013; Marsicano et al., 2002; McEwen, 2005). We, and others, have previously found that the ability of chronic stress to modulate emotional behavior is mediated through a breakdown in eCB signaling (Bortolato et al., 2007; Hill et al., 2013; Lomazzo et al., 2015; Sumislawski et al., 2011; Zhong et al., 2014). Having identified LMO4 and PTP1B as regulators of eCB signaling, we further sought to determine if LMO4 and/or PTP1B levels were altered by stress. To that end, WT mice were subjected to a daily 30 min regimen of restraint stress for 8 days that caused a significant increase in the levels of the blood stress hormone corticosterone (CORT; Figure 7A). Interestingly, this chronic stress regimen was also found to elevate PTP1B activity in the amygdala (Figure 7B), not by altering PTP1B expression but rather by preventing the formation of the oxidized inactive form of PTP1B, that is, increasing the levels of active PTP1B enzyme (Figure 7C). Previously, we demonstrated that LMO4 inhibits PTP1B activity by maintaining the oxidized inactive form of PTP1B. This inhibition depends on LMO4 retention at the endoplasmic reticulum through palmitoylation of its C-terminal cysteine residue (Pandey et al., 2013). Consistent with activation of PTP1B, LMO4 palmitoylation was markedly reduced after chronic stress (Figure 7C).

To address whether glucocorticoids are involved in elevating PTP1B activity from stress exposure, cultured neuronal cells were exposed to CORT for 6 hr. This acute treatment increased PTP1B activity (Figure 7D) by reducing oxi-PTP1B levels and LMO4 palmitoylation (Figure 7E). Reduced palmitoylation caused LMO4 to translocate from the cytosol to the nucleus, preventing LMO4 from interacting with PTP1B at the cytoplasm (Figure 7F). Collectively, these data demonstrate that glucocorticoids reduce palmitoylation of LMO4, impairing its ability to interact with and maintain oxidized PTP1B, which in turn results in an increase in PTP1B activity.

To determine if this increase in PTP1B activity contributed to stress-induced changes in eCB signaling and anxiety behavior, we examined the effects of PTP1B inhibition on eCB levels and found that it attenuated the stress induced reductions in the amygdalar tissue content of both AEA and 2-AG levels (Figure 7G). Consistent with this reversal of impaired eCB signaling, we also found that inhibition of PTP1B reversed stress-induced anxiety as measured by the novelty suppressed

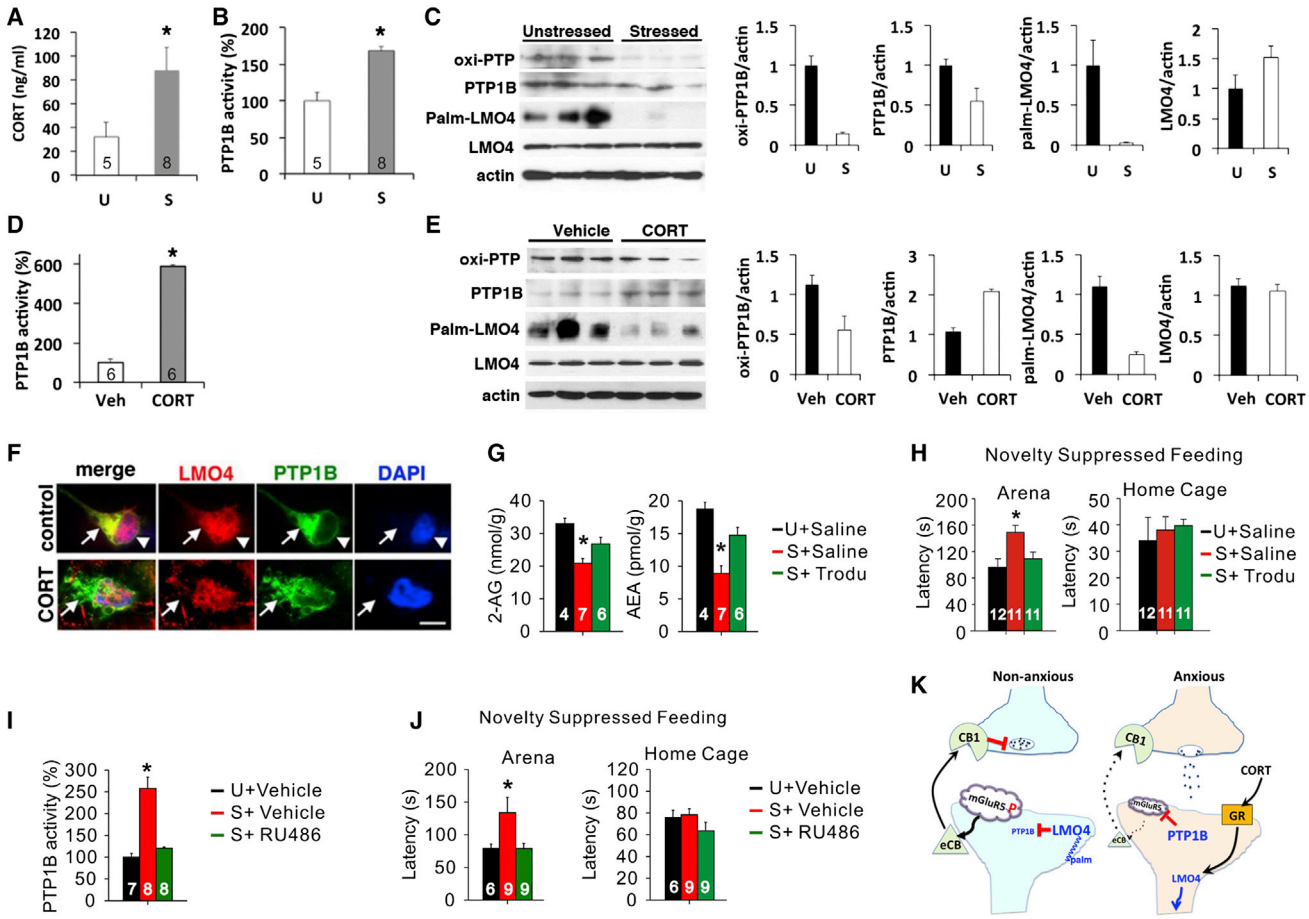

Figure 7. PTP1B Activity Is Elevated in Repeatedly Stressed WT Mice and Trodusquemine Alleviates Stress-Induced Anxiety Phenotype

(A) Elevated corticosteroid (CORT) in repeatedly (8 days) immobilization stressed (s) WT male mice compared to unstressed (u) mice.

(B) Elevated PTP1B activity in amygdala of repeatedly (8 days) immobilization stressed WT male mice, compared with unstressed mice. For (A) and (B), n = 5 to 8 per group.

(C) Immunoblots show lower levels of oxi-PTP and palmitoylated LMO4 (Palm-LMO4) in amygdalae of stressed mice. n = 3 per genotype. *$p < 0.05$.

(D) PTP1B activity is elevated in F11 neuronal cells after 6 hr treatment with CORT (1 μM = 346 ng/ml). n = 6 per group.

(E) Immunoblots show lower levels of oxi-PTP and Palm-LMO4 in CORT-treated F11 cells. n = 3 experiments per condition. Blots are quantified on the right in (D) and (E).

(F) CORT treatment causes LMO4 to translocate from the cytoplasm to the nucleus, thereby preventing its interaction with yellow fluorescent protein-tagged PTP1B at the cytoplasm. The scale bar represents 15 μm.

(G) 2-AG levels are restored in amygdala 1hr after trodusquemine treatment via ip injection.

(H) Repeated restraint stress (8 days) increased latency to feeding in the open arena of the novelty suppressed feeding test. Trodusquemine normalized the latency. Latency in the home cage was not different. *$p < 0.05$. Error bars represent SEM.

(I and J) RU486 (30mg/kg) administered by intraperitoneal injection every other day (4 doses) normalized amygdalar PTP1B activity (I) and ameliorated stress-induced anxiety behavior (J).

(K) A working model of CORT-LMO4-PTP1B-mGluR5-eCB cascade in stress-induced anxiety disorders. (Left) In non-anxious mice, membrane-anchored palmitoylated LMO4 inhibits PTP1B activity and maintains mGluR5 phosphorylation and function. Activation of mGluR5 mobilizes eCB production and release. eCB binds to CB1 receptors at the presynaptic site and inhibits neurotransmitter release. (Right) Chronic stress induces glucocorticoids (CORT) that block LMO4 palmitoylation, prevent LMO4 from interacting with PTP1B and unleash the activity of PTP1B. PTP1B dephosphorylates mGluR5 and impairs mGluR5-dependent eCB production and signaling. GR, membrane-bound glucocorticoid receptor.

feeding test (Figure 7H). To confirm the role of corticosterone in this process in vivo, we tested whether this cascade is dependent on a glucocorticoid mechanism. Consistent with our model, treatment with the glucocorticoid receptor antagonist RU486 during chronic stress normalized PTP1B activity within the amygdala (Figure 7I) and reversed stress-induced anxiety as measured by the novelty suppressed feeding test (Figure 7J). Collectively this study has identified a novel glucocorticoid-responsive LMO4-PTP1B signaling pathway within the amygdala that regulates anxiety through an eCB mechanism. This pathway is potentially relevant for pathological changes in anxiety occurring in response to chronic stress (Figure 7K).

DISCUSSION

Herein, we have demonstrated a novel intracellular signaling cascade within the amygdala that regulates anxiety behavior (see Figure 7K for model). Our data indicate that within the BLA, the intracellular protein LMO4 constrains the activity of PTP1B. Loss of LMO4 results in an increase in PTP1B activity (through a reduction in oxidation of this phosphatase), which subsequently results in dephosphorylation of mGluR5. The dephosphorylation of mGluR5 impairs mGluR5 signaling, which subsequently results in a suppression of 2-AG mediated eCB signaling. In turn, the suppression of eCB signaling within the amygdala results in the manifestation of a stresslike state characterized by elevated anxiety and impairments in fear extinction. Because a collapse of eCB signaling within the amygdala following chronic stress has already been associated with the development of anxiety (Hill et al., 2013), we subsequently demonstrated the importance of the LMO4-PTP1B cascade in this process. Specifically, our data reveal that a glucocorticoid-dependent mechanism reduces LMO4 function following chronic stress, resulting in elevated PTP1B activity that caused a suppression of eCB signaling and the emergence of anxiety. Importantly, inhibition of PTP1B either by a small-molecule antagonist or by an shRNA knockdown within the amygdala was capable of rescuing this behavioral deficit. Collectively, these data shed new light on intracellular signaling pathways within the amygdala that regulate anxiety and propose a potential novel mechanism by which chronic stress modulates eCB signaling and anxiety.

Several lines of evidence have clearly identified that eCB signaling within the amygdala regulates stress, anxiety, and emotional behavior (Gunduz-Cinar et al., 2013). Impairments in amygdalar eCB signaling can increase anxiety, HPA axis activity and impair fear extinction (Ganon-Elazar and Akirav, 2009; Hill et al., 2009; Shonesy et al., 2014), all of which are hallmark indices of stress. The rather serendipitous discovery that mice lacking LMO4 within glutamatergic neurons exhibit a behavioral phenotype that parallels the effects of stress, as well as the phenotype of CB_1 receptor deficient mice, prompted us to explore whether eCB signaling was the mechanism underlying this effect. Consistent with these parallels, we discovered that the loss of LMO4 from glutamatergic neurons resulted in a reduction in both the tissue level of the eCB molecule 2-AG, as well as synaptic plasticity driven by eCB signaling within the amygdala. The fact that this deficit appeared to be mediated by alterations in the presumed synthesis of 2-AG, and not compromised CB_1 receptor function, led us to test whether heightened anxiety could be overcome by exogenous administration of a CB_1 receptor agonist in these mice. Indeed, we found that CB_1 receptor agonism in *LMO4*-deficient mice completely alleviated the anxiety behavior, indicating that this phenotype was driven by impairments in eCB biosynthesis and/or mobilization. This is consistent with a recent report demonstrating that genetic ablation of 2-AG synthesis, which reduced 2-AG levels in the amygdala among other brain regions, resulted in an impairment in DSE within the amygdala as well as resulted in elevated anxiety (Shonesy et al., 2014). Together, these studies would suggest that tonic 2-AG signaling within the amygdala may be necessary to maintain synaptic plasticity and govern anxiety-like behavior; a disruption of this signal could result in aberrant excitability of this structure and the emergence of a state of heightened anxiety.

Our findings contrast that of a recent paper (Maiya et al., 2012) in which a partial knockdown of *LMO4* was found to have no effect on unconditioned anxiety as assessed by many of the same tests used herein. This difference is likely due to the degree or cell-type-specific loss of LMO4. In our *LMO4*-deficient mice, *LMO4* deletion was specific to forebrain glutamatergic neurons: there is more than a 95% loss of *LMO4* mRNA (Figure S1B) and over 80% loss of LMO4 protein within the amygdala (Figure 4B; the remaining 20% of LMO4 protein was likely due to expression in non-glutamatergic neurons). The robust differences in anxiety behavior we found in our mice, compared with that seen in the hemizygous *LMO4* deleted mice (Maiya et al., 2012), suggests one of two possibilities: first, a near complete loss of LMO4 may be required to cause the changes in unconditioned anxiety and amygdala function, and second, LMO4 in non-glutamatergic neurons may exert opposite effects on anxiety behavior, and so a non-specific deletion of this protein could result in a null phenotype due to contrasting effects within restricted cell types. Further research is required to determine if these possibilities explain the discrepancies between these studies.

Capitalizing on this newly determined relationship between LMO4 and eCB signaling, we sought to determine the mechanism by which these two systems interact. Consistent with our previous findings within the hypothalamus (Pandey et al., 2013), we found that the loss of LMO4 increased PTP1B activity within the amygdala. Here, we showed for the first time that increased PTP1B activity resulted in a constitutive decrease in mGluR5 phosphorylation. This dephosphorylation impaired mGluR5 function, as evidenced by a reduction in DHPG-stimulated inward currents in BLA neurons, ERK phosphorylation, and eCB-mediated LTDi, all of which were reversed by inhibition of PTP1B. Importantly, this reversal was recapitulated by pharmacological or genetic ablation of PTP1B exclusively within the amygdala. The fact that PTP1B inhibition alleviated anxiety in *LMO4*-deficient mice through a CB_1 receptor-dependent mechanism further supported the hypothesis that impaired eCB signaling was the mediator of these changes. Collectively, these data expand on the growing body of data indicating the importance of eCB signaling for emotional homeostasis, as well as identify an entirely novel intracellular cascade by which eCB signaling is regulated within the amygdala.

It has been recently demonstrated that elevations in eCB metabolism, and a consequential impairment in eCB signaling, drives changes in amygdala plasticity and emotional behavior following chronic stress (Gunduz-Cinar et al., 2013). Building on these data, we found that chronic stress, through a glucocorticoid-dependent pathway, caused a reduction in palmitoylated LMO4 within the amygdala. Cell culture studies similarly showed that prolonged exposure to corticosterone to neuronal cells similarly caused a reduction in palmitoylation, and a subsequent translocation of LMO4 from the cytosol to the nucleus, where LMO4 functions as a transcription cofactor (Chen et al., 2002, 2007a, 2007b; Gomez-Smith et al., 2010; Schock et al., 2008) but no longer interacts with PTP1B. In line with this finding, PTP1B activity was elevated within the amygdala following chronic stress, and inhibition of PTP1B during chronic stress

attenuated stress-induced suppression of eCB signaling within the amygdala and normalized changes in anxiety from chronic stress. As such, these data identify a novel intracellular mechanism by which chronic stress can impair eCB signaling and promote anxiety.

Interestingly, trodusquemine had no effect on PTP1B activity or anxiety in non-manipulated animals but was capable of reversing the increased anxiety seen in the *LMO4*-deficient mice and following exposure to chronic stress. The specificity of this drug to selectively modulate increased anxiety suggests a potential therapeutic advantage over current anxiolytics, such as benzodiazepines, that possess addictive potential and are general CNS depressants that exert robust effects on behavior regardless of the state of stress or arousal (Schnabel, 1987). More so, this selective effect on states of heightened anxiety parallels what is seen with drugs that boost eCB signaling (Hill et al., 2013; Kathuria et al., 2003) whereby they exhibit few detectable effects on resting behavior but modulate changes in anxiety in response to arousal or stress. That the ability of trodusquemine to suppress anxiety was sensitive to CB_1 receptor blockade suggests an alternate means to afford eCB-mediated relief of stress-induced emotionality, that is, indirectly through inhibition of PTP1B, as opposed to direct inhibition of eCB metabolism. This is similar to a recent indirect approach using substrate-selective inhibitors of COX-2, which also engages an eCB mechanism to reduce anxiety (Hermanson et al., 2013). The fact that inhibition of PTP1B in WT, non-stressed animals had no effect on eCB content suggests that this is not a canonical mechanism of eCB metabolism but a secondary pathway that can be engaged, by chronic stress for example, to suppress eCB signaling.

Taken together, the current data highlight a novel LMO4-PTP1B-eCB relationship within the amygdala that regulates anxiety and is engaged by chronic stress. These data reinforce the importance of eCB signaling within the amygdala in the regulation of emotional homeostasis, but also identify a novel putative target for the regulation of stress-related mood and anxiety disorders. Interestingly, inhibition of PTP1B has recently been shown to reduce diet-induced obesity, and trodusquemine has undergone phase I trials for obesity. Metabolic syndrome and other obesity-related diseases are typically comorbid with mood and anxiety disorders (Lin et al., 2008; Pouwer, 2009), and co-treatment of these conditions has remained a therapeutic challenge. In this regard, the exploration of whether PTP1B inhibition is capable of modulating changes in emotional behavior related to obesity remains to be determined, but a recent report has identified that eCB signaling within the amygdala limits changes in anxiety in obese states (Blasio et al., 2013), highlighting the potential utility of PTP1B inhibition given its ability to regulate eCB signaling. As such, the relationship between LMO4, PTP1B and eCB signaling may prove to be relevant to our understanding of the relationship between stress, metabolism, and emotional behavior.

EXPERIMENTAL PROCEDURES

Animals

CamK2α$^{Cre/-}$/*LMO4*$^{flox/flox}$ (*LMO4*KO) and *LMO4*$^{flox/flox}$ (WT) mice were bred on a CD-1 background as described previously (Qin et al., 2012; Schock et al., 2008; Zhou et al., 2012). All animal use was approved by the University of Ottawa Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care.

Drugs and Anxiety Behavior Tests

See Supplemental Experimental Procedures for details.

Lipid Extraction from Tissue and Mass Spectrometric Detection of eCBs

These procedures have been described previously (Dincheva et al., 2015) and are addressed in detail in Supplemental Experimental Procedures.

Stereotactic Injection of Lentivirus

Lentiviral vectors (0.4 μl, 1 × 10^9 transduction units/ml at a rate of 0.05 μl/min) expressing GFP reporter and PTP1B shRNA or control shRNA were stereotactically injected into the amygdala on both sides. See Supplemental Experimental Procedures for viral vector preparation and stereotactic coordinates. Twenty-one days later, amygdalae were isolated to examine the expression of GFP by fluorescence imaging and PTP1B by immunoblot.

Stress Protocol

Male mice aged 6 to 7 weeks were subjected daily to 30 min of tube restraint in 50 ml conical tubes with ventilation holes for 8 consecutive days (between 10 a.m. and noon). Control mice were left undisturbed in their home cages except for necessary handling to maintain tail markings throughout 8 days.

Corticosterone Radioimmunoassay

Mice were decapitated in the morning, and plasma corticosterone was measured by radioimmunoassay following the manufacturer's protocol using the Corticosterone ^{125}I RIA kits (MP Biomedicals).

Cell Culture, Plasmids, and Transfections

See Supplemental Experimental Procedures for details of the use of F11 neuronal cells, transfection protocol, and expression vectors of LMO4 cDNA, control scrambled shRNA, or LMO4-specific shRNA.

PTP1B Phosphatase Activity Assay

PTP1B phosphatase activity in extracts from amygdalar wedges or F11 cells was measured with the PhosphoSeek PTP1B Assay Kit (BioVision), with appropriate controls (Pandey et al., 2013). As a control, F11 cells were treated with the tyrosine phosphatase inhibitor pervanadate at 100 μM for 30 min.

RNA Extraction and Quantitative RT-PCR

Total mouse amygdalar RNA was extracted and purified from 1-month-old mutant and control mice and analyzed as previously described (Qin et al., 2012).

In Situ Hybridization

Tissues were prepared for in situ hybridization with digoxigenin-labeled antisense or sense riboprobes, as previously described (Duquette et al., 2010).

GST Pull-Down, Immunoprecipitation, and Immunoblot Analysis

F11 cells were cotransfected with Flag-LMO4 and WT or a substrate-trapping mutant (D181A, D/A) GST-PTP1B (Stuible et al., 2008). Lysates were pulled down with glutathione beads (Invitrogen). To detect oxi-PTP1B, PTP1B was first immunoprecipitated with protein G-Sepharose-conjugated rabbit anti-PTP1B antibody (Cell Signaling) and immunoblotted with a mouse antibody specific to oxidized PTP active site (R&D Systems), as previously described (Almontashiri et al., 2014; Pandey et al., 2013). Immunoprecipitation of mGluR5 was carried out as detailed in Supplemental Experimental Procedures. Antibodies were specific to phosphorylated (T202/Y204)-ERK1/2, ERK1/2 (Cell Signaling), phosphorylated tyrosine (Invitrogen), and LMO4 (Pandey et al., 2013). See Supplemental Experimental Procedures for details. An average of 3 immunoblots were quantitated using ImageJ (NIH).

Palmitoylation Assay

Palmitoylation was assessed by a biotin exchange protocol, as we described previously (Pandey et al., 2013).

Electrophysiology

Brain sections (300 μm) containing amygdala were prepared from 3- to 5-week-old mice as described previously (Qin et al., 2012; Zaman et al., 2014) and in detail in Supplemental Experimental Procedures.

Statistical Analysis

Data were acquired and analyzed using pClamp10.1, Sigma Stat 3.5, and SPSS. Differences between treatments were analyzed using Student's t test for paired data or ANOVA followed by the Bonferroni post hoc test for multiple comparisons. Significance was accepted at $p < 0.05$. See Tables S1 and S2 for F values of main effects.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.02.015.

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