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Glucocorticoid feedback uncovers retrograde opioid signaling at hypothalamic synapses

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Stressful experience initiates a neuroendocrine response culminating in the release of glucocorticoid hormones into the blood. Glucocorticoids feed back to the brain, causing adaptations that prevent excessive hormone responses to subsequent challenges. How these changes occur remains unknown. We found that glucocorticoid receptor activation in rodent hypothalamic neuroendocrine neurons following *in vivo* stress is a metaplastic signal that allows GABA synapses to undergo activity-dependent long-term depression (LTD_{GABA}). LTD_{GABA} was unmasked through glucocorticoid receptor–dependent inhibition of Regulator of G protein Signaling 4 (RGS4), which amplified signaling through postsynaptic metabotropic glutamate receptors. This drove somatodendritic opioid release, resulting in a persistent retrograde suppression of synaptic transmission through presynaptic μ receptors. Together, our data provide new evidence for retrograde opioid signaling at synapses in neuroendocrine circuits and represent a potential mechanism underlying glucocorticoid contributions to stress adaptation.

Exposure to stress results in two prominent neuroendocrine responses: central and peripheral catecholamine release and a surge of glucocorticoids into the blood stream. Through temporally and mechanistically distinct pathways, both mediators are essential for appropriate behavior and mood regulation[1,2]. One unique and critical function of glucocorticoids is feedback at stress circuits, curtailing hormone release in response to subsequent challenges. This serves a self-limiting homeostatic function in the face of diverse and repeated stress challenges[3]. Despite this fundamental role for glucocorticoids in shaping endocrine function with experience, relatively little is known about how it might be accomplished.

Adaptive control of the neuroendocrine response to stress resides with a small cluster of neurons in the paraventricular nucleus of the hypothalamus (PVN). These parvocellular neuroendocrine cells (PNCs) at the head of the hypothalamic-pituitary-adrenal (HPA) stress axis are positioned as the definitive point of neural stress integration; their activity is a function of both synaptic drive and negative feedback by glucocorticoids[3]. The majority of synapses onto PNCs are GABAergic[4]. GABA transmission onto PNCs restrains basal stress axis output[5] and is, itself, sensitive to stress[6,7]. Notably, stress exposure causes diminished chloride extrusion capacity in PNCs, resulting in a situation in which GABA is excitatory during stress[5,8]. Thus, although it is counterintuitive, dampening GABA transmission alleviates the activation of the endocrine response[8].

In addition to corticotropin-releasing hormone (CRH) and vasopressin, PNCs synthesize proenkephalin-derived opioid peptides[9]. Enkephalins have been implicated as putative mediators of adaptive change to stress axis function[9]. Consistent with this idea, mice lacking proenkephalin exhibit prolonged glucocorticoid elevation to stress[10], suggesting that opioids may participate in glucocorticoid negative feedback. The cellular actions of endogenous opioid signaling have not been explored in PNCs; in other systems, they function as retrograde signals to inhibit neurotransmitter release[11,12]. We hypothesized that opioids are intermediaries of glucocorticoid actions in the PVN. Using whole-cell patch-clamp recordings of PNCs from naive and stress-exposed rats, we examined GABA synapse strength and responses to patterned afferent activity. We found that a single stressful experience, followed by a 90-min temporal delay, unmasked activity-dependent, heterosynaptic LTD_{GABA} that was mediated by retrograde opioid signaling.

RESULTS

Glucocorticoid receptor activation unmasks LTD_{GABA}

In response to an acute stress, plasma corticosterone (CORT, the major rodent glucocorticoid) rapidly rises; peak concentrations are reached 15–30 min after stress onset, persist during the stress and subside slowly thereafter[1]. Subsequent access of CORT to the brain is regulated, and time of peak elevation lags that of plasma CORT[13]. To investigate the potential effects of CORT exposure resulting from stress, we examined PNCs in *in vitro* hypothalamic slices prepared from rats exposed to 30 min of immobilization stress followed by incrementally increasing periods of recovery before being killed (**Fig. 1a**). Naive (unstressed) rats served as our age-matched controls. In whole-cell voltage-clamp recordings at −80 mV, we electrically evoked inhibitory postsynaptic currents (eIPSCs; in 10 μM DNQX). eIPSC amplitude was used as an indicator of synaptic strength. We did not observe any appreciable alterations in cellular or synaptic properties between cells obtained from naive ($n = 142$) and stressed ($n = 40$) rats (**Supplementary Fig. 1**). Following 10-min baseline recording, we paired afferent 10-Hz synaptic stimulation with subthreshold depolarization to −40 mV for 5 min; a protocol reminiscent of those used at various synapses to induce activity-dependent plasticity[14,15]. In naive

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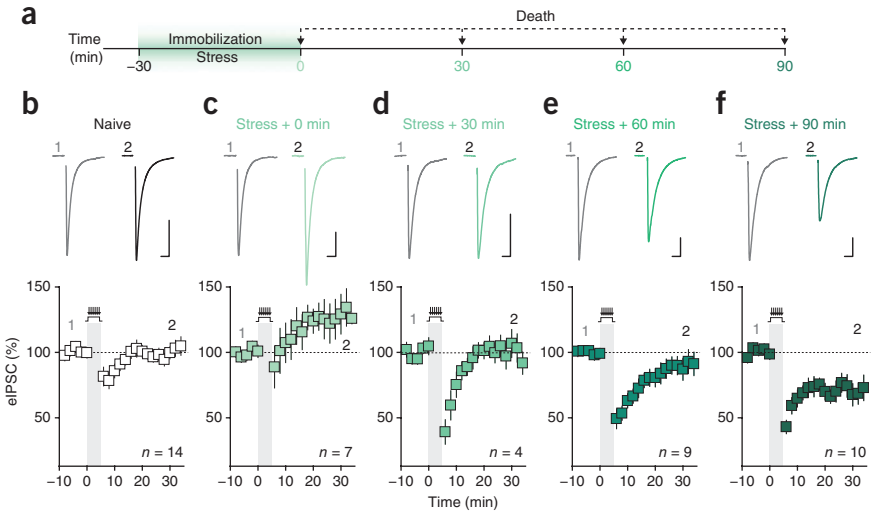
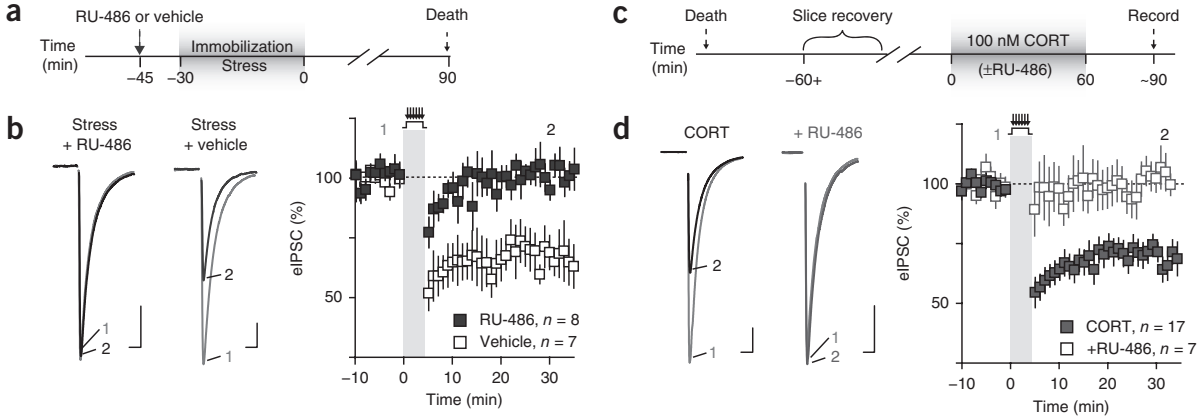


Figure 1 Stress unmasks long–term plasticity of GABA synapses. (**a**) Overview of experimental procedure. (**b**–**f**) Top, sample traces of eIPSCs recorded from individual PNCs either before (1) or 30 min after (2) a pairing stimulation protocol consisting of 5 min of afferent stimulation (10 Hz) and postsynaptic depolarization (to –40 mV). Bottom, summary graphs for each treatment group showing normalized eIPSC amplitudes before and after pairing (naive, $n = 14$ cells, 10 rats; stress + 0 min, $n = 7$ cells, 4 rats; stress + 30 min, $n = 4$ cells, 3 rats; stress + 60 min, $n = 9$ cells, 4 rats; stress + 90 min, $n = 10$ cells, 7 rats). Scale bars represent 50 pA and 10 ms. Data are presented as mean ± s.e.m.

slices, pairing transiently suppressed eIPSC amplitude (84.9 ± 6.6% of baseline amplitude; **Fig. 1b**), which recovered quickly (104.4 ± 4.5% of baseline at 30 min; **Fig. 1b**). Pairing in slices prepared immediately following the stress potentiated eIPSCs (long-term potentiation, LTP; to 126.6 ± 10.2% of baseline at 30 min, one-sample *t* test, $P = 0.039$; **Fig. 1c**), similar to observations by Inoue *et al.*[16] (also in this issue). In slices from rats allowed to recover for 30 or 60 min following the end of stress, we failed to observe any persistent changes to eIPSC amplitude (**Fig. 1d**,**e**). Further extending the post-stress recovery period to 90 min produced both an initial depression (42.4 ± 5.2%, $P < 0.0001$; **Fig. 1f**) and unmasked a long-term depression of eIPSC amplitude (LTD_{GABA}) that persisted for at least 30 min after pairing (69.4 ± 8.3% of baseline, $P = 0.0042$). LTD_{GABA} was not evident in naive slices when pairing protocol duration was increased (92.7 ± 5.6%, $P = 0.24$; **Supplementary Fig. 2f**), suggesting that a threshold change does not underlie differences in responses between naive and stressed rats. These results indicate that acute stress, with varied temporal delay, uncovers both conditional activity-dependent LTP and LTD_{GABA} in PNCs.

The temporal delay in unmasking of LTD_{GABA} following acute immobilization is consistent with exposure to an *in vivo* associative signal, such as CORT, which canonically has a slow onset of action compared with noradrenaline[2]. Consequently, we tested whether activation of glucocorticoid receptors is an obligatory permissive factor for LTD_{GABA}. Rats were given an intraperitoneal injection of either the glucocorticoid receptor antagonist RU-486 (25 mg per kg of body weight) or vehicle (DMSO) 15 min before immobilization and allowed to recover for 90 min afterwards (**Fig. 2a**). *In vivo* RU-486 pre-treatment completely prevented LTD_{GABA} (102.9 ± 5.0% of baseline, $P = 0.58$; **Fig. 2b**). Vehicle injection had no effect (67.8 ± 8.1% of baseline,



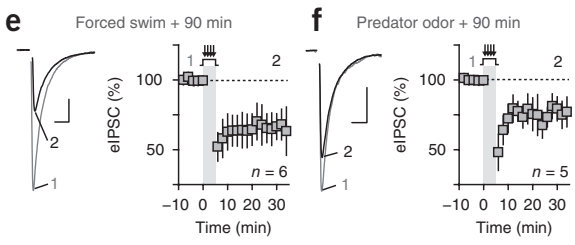


Figure 2 Glucocorticoid receptor activation is necessary and sufficient to unmask LTD_{GABA}. (**a**) Overview of experimental procedure in which either the glucocorticoid receptor antagonist RU-486 (25 mg per kg) or vehicle were administered intraperitoneally 15 min before stress. (**b**) Left, sample eIPSC traces from individual cells of stressed and RU-486–treated or stressed and vehicle-treated rats before and 30 min after pairing. Right, graph summarizes normalized eIPSC amplitudes in these groups (RU-486, $n = 8$ cells, 5 rats; vehicle, $n = 7$ cells, 3 rats). (**c**) Overview of experimental procedure in which hypothalamic slices from naive rats were incubated *in vitro* with CORT (100 nM) in the presence or absence of RU-486 (500 nM). (**d**) Left, sample eIPSC traces from individual cells of CORT- and CORT + RU-486–incubated slices before and 30 min after pairing. Right, graph summarizes normalized eIPSC amplitudes in these groups (CORT, $n = 17$ cells, 16 rats; CORT + RU-486, $n = 7$ cells, 4 rats). (**e**,**f**) Left, sample eIPSC traces before and 30 min after pairing. Right, graphs summarizing normalized eIPSC amplitude in cells from rats exposed to forced swim ($n = 6$ cells, 3 rats) or predator odor ($n = 5$ cells, 5 rats) followed by a 90-min recovery period. Scale bars represent 50 pA and 10 ms. Data are presented as mean ± s.e.m.

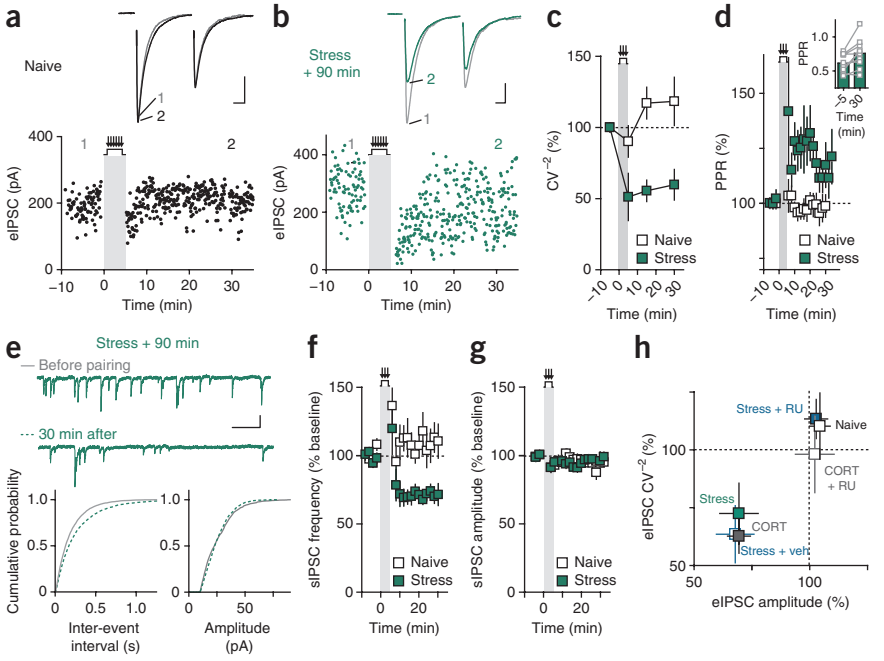


Figure 3 LTD_{GABA} is expressed presynaptically. (**a**,**b**) Sample paired-pulse traces and amplitude time courses of eIPSCs before and after pairing from individual cells in slices prepared from naive or stressed rats. Scale bars represent 50 pA and 10 ms. (**c**,**d**) Summary graphs of normalized eIPSC variability (CV^{-2}) and PPR responses to the pairing protocol in cells from naive (n = 14 cells, 10 rats) and stressed rats (n = 10 cells, 7 rats). (**e**) Sample traces of sIPSCs and corresponding cumulative probability distribution plots of inter-event interval and amplitude before and 30 min following pairing in a cell from a stressed rat. Scale bars represent 20 pA and 0.25 s. (**f**,**g**) Summary graphs of normalized sIPSC frequency and amplitude response in naive and stressed cells. (**h**) Relationship between eIPSC amplitude and CV^{-2} changes at 30 min. Data are presented as mean ± s.e.m.

$P < 0.0001$). These data suggest that glucocorticoid receptor activation is necessary for stress-associated LTD_{GABA}. They do not, however, provide information about anatomical specificity, nor do they indicate whether glucocorticoid receptor activation is sufficient in the absence of stress. Thus, we probed the effects of local CORT administration to *in vitro* hypothalamic slices. Individual slices from naive rats were incubated in CORT (100 nM) either with or without RU-486 (500 nM) for 1 h, followed by an additional 30-min recovery period before recording (**Fig. 2c**). As with stress, we did not observe any changes in basal cellular and synaptic properties in CORT-treated PNCs (**Supplementary Fig. 1**). We did observe LTD_{GABA} in response to pairing in CORT-exposed cells (69.4 ± 4.9% of baseline, $P < 0.0001$; **Fig. 2d**). These changes were prevented by co-incubation with RU-486 (104.3 ± 5.5% of baseline; $P = 0.46$). Next, we asked whether other stressors, which activate the HPA axis and elevate CORT, could also unmask LTD_{GABA}. We observed LTD_{GABA} in response to pairing in slices obtained 90 min following either forced swim or predator odor exposure (swim, 66.6 ± 9.2% of baseline, $P = 0.015$; predator, 73.8 ± 7.0% of baseline, $P = 0.020$; **Fig. 2e**,**f**). Together, these findings indicate that local glucocorticoid receptor activation in PNCs following stressful experience is necessary and sufficient to permit the induction of activity-dependent LTD_{GABA}.

We next probed for a locus (presynaptic versus postsynaptic) of expression for LTD_{GABA} (**Fig. 3**). To assess GABA release probability during these experiments, we examined variability in eIPSC amplitude (inverse of the squared coefficient of variation, CV^{-2}) and the ratio between a pair of eIPSCs delivered in brief succession (paired-pulse ratio, PPR; **Fig. 3a**,**b**). CV^{-2} was reduced in stressed cells 30 min after pairing (to 59.7 ± 10.8% of baseline, $P = 0.004$; **Fig. 3c**), but remained unchanged in naive cells (118.3 ± 15.2% of baseline, $P = 0.26$). PPR was unchanged by pairing in naive cells (99.8 ± 4.7% of baseline, $P = 0.97$; **Fig. 3d**), but it was significantly increased in stressed cells (118.2 ± 6.8% of baseline, $P = 0.021$; from 0.62 ± 0.14 to 0.75 ± 0.21, un-normalized PPR, $P = 0.009$, paired t test). Next, we analyzed the inter-event interval or frequency and amplitude of spontaneous IPSCs (sIPSCs) from these recordings (**Fig. 3e**–**g**). sIPSC frequency decreased in cells from stressed (74.7 ± 4.6% of baseline, $P < 0.0001$; **Fig. 3e**,**f**), but not naive rats (106.9 ± 9.0% of baseline, $P = 0.46$). sIPSC amplitude remained unchanged in both conditions (**Fig. 3g**). These data are consistent with decreased presynaptic release during LTD_{GABA}. Similarly, we noted that LTD_{GABA} in CORT-treated slices was also accompanied by an increase in PPR (to 119.2 ± 6.5% of baseline, $P = 0.010$; **Supplementary Fig. 2a**), a decrease in CV^{-2} (62.7 ± 7.4% of baseline, $P = 0.0002$; **Fig. 3h**) and a reduction in sIPSC frequency, but not amplitude (frequency to 77.5 ± 6.9% of baseline, $P = 0.016$; **Supplementary Fig. 2b**–**e**). Indeed, across *in vivo* and *in vitro* experimental conditions, changes to CV^{-2} were consistently related to changes in eIPSC amplitude (**Fig. 3h**). Taken together, our data strongly indicate that glucocorticoid-associated LTD_{GABA} is a consequence of a decrease in presynaptic GABA release probability.

LTD_{GABA} is induced heterosynaptically

Given that electrical stimulation of synaptic inputs can recruit axons non-specifically, we used an optogenetic tool to test whether exclusive activation of GABA synapses was sufficient for LTD induction. In CORT-treated slices from *vGAT-mhChR2-YFP* mice expressing channelrhodopsin2 (ChR2) under the vesicular GABA transporter (also known as *Slc32a1*) promoter, we found that pairing delivered with light-evoked stimulation did not elicit LTD_{GABA}, whereas electrical stimulation in wild-type mice did (ChR2, 119.6 ± 12.3% of baseline, $P = 0.17$; electrical, 74.5 ± 5.8% of baseline, $P = 0.003$; **Fig. 4a**).

Metabotropic glutamate receptors (mGluRs) are important for GABA synapse plasticity requiring heterosynaptic induction[14–17]. We conducted experiments to examine the mGluR contributions in LTD_{GABA}. In CORT-treated slices, we failed to induce LTD_{GABA} in the presence of the non-selective group I/II mGluR antagonist MCPG (200 μM, 97.9 ± 7.5% of baseline, $P = 0.93$; **Supplementary Fig. 3a**). We next tested group I mGluR subtypes 1 and 5. Treatment with the mGluR5 antagonist MTEP (10 μM) completely abolished LTD_{GABA} in CORT-treated slices (eIPSC, 101.0 ± 5.0% of baseline, $P = 0.84$; **Fig. 4b**). In contrast, inclusion of selective mGluR1 antagonist JNJ-16259685 (750 nM) did not prevent LTD_{GABA} (73.2 ± 7.6% of baseline, $P = 0.017$; **Fig. 4c**). Preventing activation of NMDA receptors (NMDARs) with intracellular MK801 (1 mM) also failed to affect the expression of LTD_{GABA} (73.6 ± 5.0% of baseline, $P = 0.006$; **Supplementary Fig. 3b**). These data indicate that group I mGluRs, particularly mGluR5, are part of a heterosynaptic mechanism involved in LTD_{GABA} following glucocorticoid exposure.

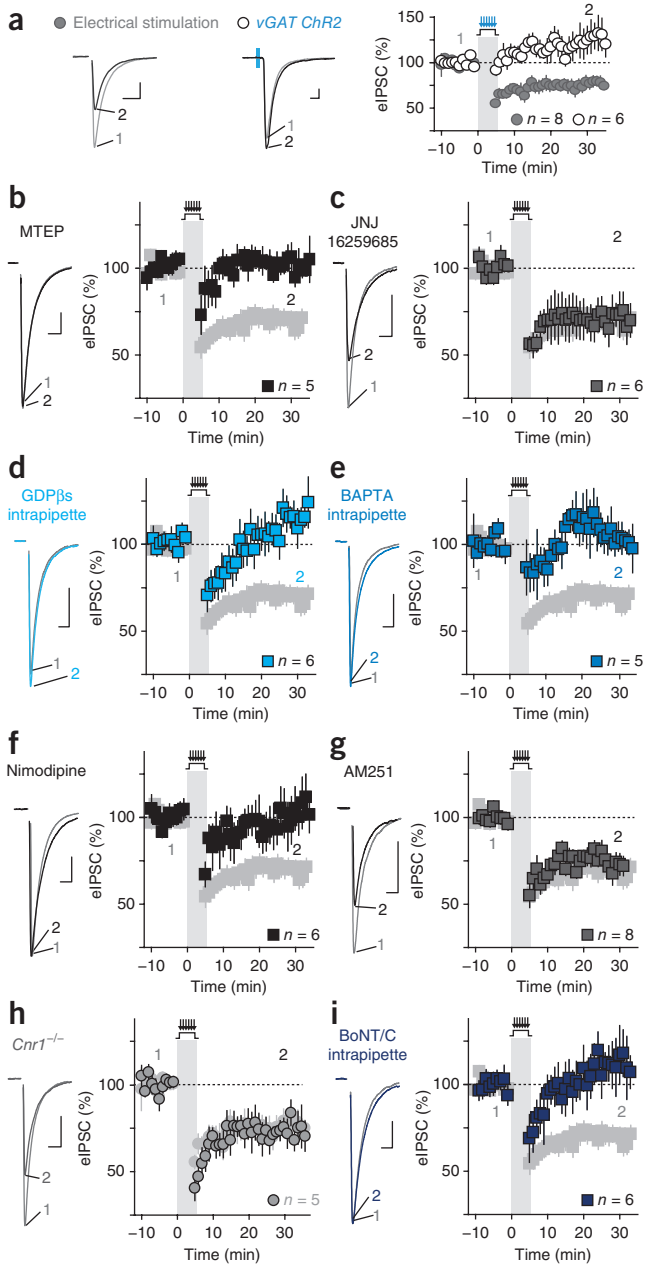


Figure 4 LTD_{GABA} induction is heterosynaptic and requires a retrograde signal. (**a**) Response to pairing in CORT-treated slices with exclusive activation of GABA synapses in *vGAT-ChR2* mice (open circles, $n = 6$ cells, 3 mice). Comparison with electrical stimulation in slices from wild-type mice (filled circles, $n = 8$ cells, 5 mice). Sample eIPSCs from individual PNCs are shown to the left and a summary graph to right. (**b**,**c**) Effects of mGluR5 antagonist MTEP (10 μM, $n = 5$ cells, 3 rats; **b**) or mGluR1 antagonist JNJ16259685 (750 nM, $n = 6$ cells, 5 rats; **c**) on LTD. (**d**,**e**) Prevention of LTD_{GABA} by inclusion in the patch pipette of GTPase inhibitor GDPβs (2 mM, $n = 6$ cells, 3 rats; **d**) or the calcium chelator BAPTA (10 mM, $n = 5$ cells, 4 rats; **e**). (**f**) Effect of bath application of the L-type calcium channel blocker nimodipine on LTD_{GABA} (20 μM, $n = 6$ cells, 4 rats). (**g**,**h**) Effects of the CB1R antagonist AM251 in rats (3 μM; $n = 8$ cells, 5 rats; **g**) or genetic deletion of the CB1R (*Cnr1*$^{-/-}$) in mice ($n = 5$ cells, 3 mice; **h**) on LTD_{GABA} with eIPSC traces (left) and summary time course (right). (**i**) Prevention of LTD_{GABA} by intrapipette inclusion of SNARE-dependent exocytosis inhibitor BoNT/C (5 μg ml^{-1}, $n = 6$ cells, 3 rats). Control LTD_{GABA} replotted in filled gray squares (rat) or circles (mouse). Scale bars represent 50 pA and 10 ms. Data are presented as mean ± s.e.m.

A vesicle-based retrograde signal mediates LTD_{GABA}

We next tested whether the mGluR responsible for induction of LTD_{GABA} is postsynaptic. We interfered with G protein signaling only in the postsynaptic PNC by including the non-hydrolysable GDP analog GDPβs (2 mM) in the intrapipette solution. Under these conditions, we failed to observe LTD_{GABA} in CORT-treated cells (104.8 ± 7.7% of baseline, $P = 0.56$; **Fig. 4d**). Given that mGluR5 is coupled to $G_{\alpha q}$-type intracellular pathways and exerts many effects through elevations in intracellular calcium, we next assessed the effect of adding the fast calcium buffer BAPTA (10 mM) intrapipette. This also prevented expression of LTD_{GABA} (102.0 ± 8.1% of baseline, $P = 0.81$; **Fig. 4e**). As postsynaptic depolarization was necessary LTD_{GABA} induction, we tested the involvement of voltage-dependent calcium channels. Consistent with this idea, the L-type calcium channel antagonist nimodipine prevented LTD_{GABA} (99.5 ± 12.3% of baseline, $P = 0.97$; **Fig. 4f**). These results provide evidence that a postsynaptic mGluR- and calcium-dependent signaling pathway is required for LTD_{GABA} following CORT exposure.

LTD_{GABA} requires heterosynaptic activation of postsynaptic mGluR5, but manifests as a presynaptic decrease in release probability, suggesting the presence of a retrograde signal. One widely described form of mGluR-dependent LTD_{GABA} requires retrograde signaling by endocannabinoids (eCBs)[17–19]. We previously characterized short-term retrograde eCB signaling at GABA synapses onto PNCs[7], which suggests that eCBs are functional at these synapses; given that we found that short-term eCB signaling is enhanced by acute exposure to CORT, we hypothesized that recruitment of eCBs and activation of CB1 receptors (CB1Rs) may contribute to glucocorticoid LTD_{GABA}. Following exposure to CORT, slices were incubated in artificial cerebrospinal fluid (aCSF) containing the CB1R antagonist AM251 (3 μM) for a minimum of 30 min. CB1R blockade, however, failed to prevent LTD_{GABA} (72.6 ± 3.2% of baseline, $P < 0.0001$; **Fig. 4g**). To further test this idea, we assessed LTD_{GABA} in mice lacking CB1Rs (*Cnr1*$^{-/-}$). We found that that LTD_{GABA} persisted in CORT-treated slices from *Cnr1*$^{-/-}$ mice (72.7 ± 5.1% of baseline, $P = 0.0032$; **Fig. 4h**). A transient receptor potential vanilloid channel blocker, capsazepine, also failed to prevent LTD_{GABA} (66.4 ± 8.5% of baseline, $P = 0.016$; **Supplementary Fig. 3c**). On the basis of these data, we conclude that eCBs are not the retrograde signal responsible for expression of LTD_{GABA} at these synapses.

In addition to lipid-derived retrograde messengers, neurons, including PNCs[20], release conventional and peptide transmitters that are packaged in vesicles in the somatodendritic compartment[18]. To test for the contribution of a vesicularly packaged retrograde transmitter, we conducted experiments in which the soluble NSF attachment protein receptor (SNARE)-dependent exocytosis inhibitor botulinum toxin C (BoNT/C, 5 μg ml^{-1}) was included in the patch pipette. Inclusion of BoNT/C prevented LTD_{GABA} following pairing (105.3 ± 7.0% of baseline, $P = 0.49$; **Fig. 4i**). Collectively, these observations indicate that LTD_{GABA} requires the activation of postsynaptic mGluRs, an increase in intracellular calcium and the fusion of neurotransmitter-filled vesicles postsynaptically. Given that these events underlie presynaptic reduction of GABA release, a retrograde signal is likely recruited by this mechanism.

Glucocorticoids alter mGluR signaling via RGS4

We next tested whether pharmacological activation of mGluRs was sufficient to recapitulate suppression of GABA transmission and whether this mechanism was altered by glucocorticoid exposure.

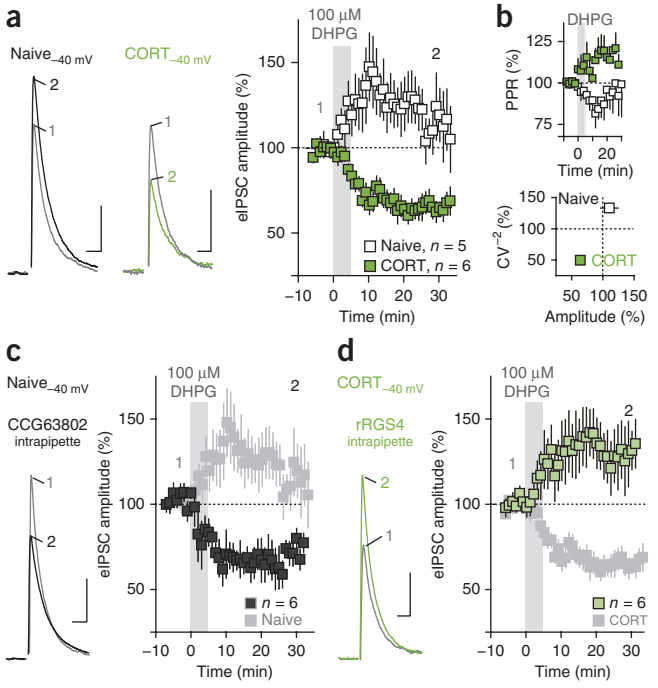


Figure 5 Glucocorticoid receptor activation modifies mGluR signaling via RGS4. (**a**) Suppression of GABA transmission by mGluR agonist after glucocorticoid exposure. Left, sample eIPSC traces at a holding potential of −40 mV from individual naive and CORT-treated cells before and 30 min after bath applied mGluR1 and mGluR5 agonist DHPG (100 µM, 5 min). Right, summary of the effects of DHPG on eIPSC amplitude at −40 mV in naive (n = 5 cells, 3 rats) and CORT-treated (n = 6 cells, 4 rats) slices. (**b**) Top, summary time course graph of normalized PPR in response to DHPG in naive or CORT-treated slices. Bottom, relationship between eIPSC amplitude and CV^{-2} changes 30 min post-DHPG for each treatment group. (**c**) mGluR supression of GABA transmission in naive slices with inhibition of postsynaptic RGS4. eIPSC traces from an individual cell (left) and summary data (right) from cells in a naive slice treated with DHPG when the RGS4 inhibitor CCG63802 (100 µM) was included in the patch pipette (n = 6 cells, 4 rats). (**d**) mGluR modulation of GABA transmission in CORT-treated slices with postsynaptic RGS4. eIPSC traces from an individual cell (left) and summary data (right) from cells of CORT-treated slices in which the pipette solution contained recombinant RGS4 (50 pM, n = 6 cells, 3 rats). Scale bars represent 50 pA and 10 ms. Data are presented as mean ± s.e.m.

Given that LTD_{GABA} requires high voltage–activated L-type calcium channels and is evident only when afferent stimulation and depolarization to −40 mV are paired together (depolarization alone, 118.3 ± 10.8% of baseline, P = 0.15; stimulation alone, 92.6 ± 6.8% of baseline, P = 0.31; **Supplementary Fig. 2g**,**h**), we tested the hypothesis that LTD_{GABA} results from membrane state–dependent activation of mGluRs. We took recordings of eIPSCs at either −40 or −80 mV and bath applied the group I mGluR agonist DHPG (100 µM) for 5 min. At −40 mV, eIPSCs are outward currents; we lowered intracellular chloride (4 mM) to increase anion inward driving force through the $GABA_A$ receptor. We first confirmed that LTD_{GABA} was still readily observed with reversed chloride driving force (70.4 ± 9.2% of baseline, P = 0.02; **Supplementary Fig. 2i**). Notably, DHPG potentiated eIPSC amplitude in naive slices under these conditions (132.3 ± 11.6% of baseline at 10 min, P = 0.049; **Fig. 5a**). In contrast, in CORT-treated slices, DHPG elicited long-lasting depression of eIPSCs (63.0 ± 4.7% of baseline, P = 0.0006; **Fig. 5a**), which was accompanied by increased PPR (119.6 ± 2.7% of baseline, P = 0.0020; **Fig. 5b**) and a decrease in eIPSC CV^{-2} (49.5 ± 7.6% of baseline, P = 0.0012; **Fig. 5b**). Similar results were obtained at −80 mV; following CORT treatment, DHPG no longer enhanced eIPSC amplitude, as it did in naive cells, although no significant depression was observed (76.6 ± 10.6% of baseline, P = 0.069; **Supplementary Fig. 2k**). From these data, we conclude that mGluR activation at a depolarized membrane potential is sufficient to recapitulate LTD_{GABA}. Furthermore, CORT exposure unmasks LTD_{GABA} by functionally altering the outcome of mGluR signaling.

We next sought to examine how glucocorticoids alter mGluR signaling. Regulator of G protein signaling (RGS) proteins, in particular RGS4, associate with group I mGluRs and stifle G_q-mediated signaling through GTPase acceleration[21]. RGS4 is abundantly expressed in the PVN and is potently downregulated by stress and glucocorticoid receptor activation[22,23]; this provides a compelling and testable potential mechanism. To test the hypothesis that RGS4 restrains mGluR signaling in naive PNCs, we included the RGS4 inhibitor CCG63802 (100 µM) in the pipette solution and bath applied DHPG. Postsynaptic inhibition of RGS4 was sufficient to unmask a DHPG-mediated LTD_{GABA} that was similar to that seen with CORT treatment (to 71.2 ± 7.0% of baseline, P = 0.0093; **Fig. 5c**). We next conducted the corollary experiment and included recombinant RGS4 in the pipette when recording from cells in CORT-treated slices. This completely prevented eIPSC depression following DHPG (132.0 ± 11.3%, P = 0.036; **Fig. 5d**). These data suggest that inhibition of RGS4 by glucocortioids is sufficient to enhance mGluR5 signaling and allow for the expression of LTD_{GABA}.

Persistent µ opioid receptor signaling underlies LTD_{GABA}

PVN neuroendocrine cells release neurotransmitters from vesicles in their somatodendritic compartment[20,24]. Opioid peptides released from magnocellular neurosecretory cells (MNCs) cause presynaptic LTD at glutamate synapses[11,25]. PNCs produce many peptides in a stress-dependent manner; this includes pro-enkephalin opioid gene products such as met- and leu-enkephalin[26,27]. We hypothesized that vesicular somatodendritic release of an opioid peptide is responsible for LTD_{GABA} following CORT exposure. In CORT-treated slices, continuous bath application of the broad-spectrum opioid receptor antagonist naloxone (5 µM) prevented pairing-induced depression of eIPSC amplitude (100.7 ± 7.9% of baseline, P = 0.93; **Fig. 6a**). Naloxone also prevented LTD_{GABA}-associated changes to PPR (95.5 ± 4.4% of baseline, P = 0.33), CV^{-2} (131.1 ± 16.4%, P = 0.10) and sIPSC frequency (112.4 ± 13.3%, P = 0.38). Similarly, the µ opioid receptor subtype antagonist CTAP (1 µM) prevented LTD_{GABA} (99.0 ± 9.3% of baseline, P = 0.92; **Fig. 6b**). Neither the δ opioid receptor antagonist naltrindole (1 µM, 71.1 ± 8.2% of baseline, P = 0.017) nor the κ opioid receptor antagonist nor-binaltorphimine (1 µM, 71.5 ± 5.6% of baseline, P = 0.0037) prevented LTD_{GABA} following pairing (**Supplementary Fig. 4c**). We did, however, note suppressive effects of the κ opioid receptor agonist U69593 (1 µM, 21.7 ± 7.6% of baseline, P = 0.0005), but not the δ opioid receptor agonist DPDPE (1 µM, 100.8 ± 5.8% of baseline, P = 0.09) on eIPSC amplitude (**Supplementary Fig. 4a**,**b**). These pharmacological data suggest that µ opioid receptors are necessary for induction of LTD_{GABA} following CORT exposure. Finally, we assessed LTD_{GABA} in *Oprm1*$^{-/-}$ mice[28]. We failed to observe any lasting depression of eIPSCs (106.4 ± 9.3% of baseline, P = 0.52; **Fig. 6c**). These data confirm that µ opioid receptors are necessary for LTD_{GABA}.

µ opioid receptor subtypes are commonly located on GABA neurons and their synaptic terminals[29–31]. If an endogenous opioid were released from PNCs, its actions would likely be spatially restricted

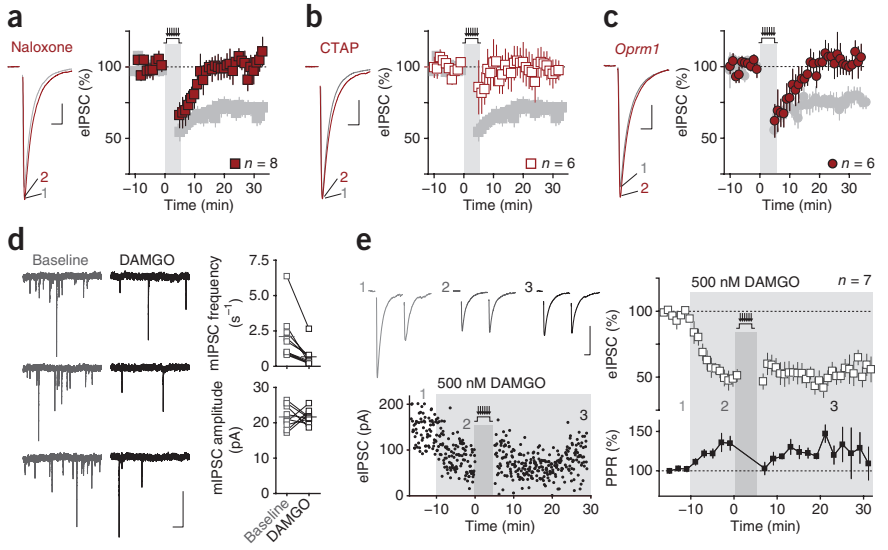


Figure 6 Presynaptic μ opioid receptors mediate LTD_{GABA}. (**a**,**b**) Effect of μ opioid receptor antagonism on LTD_{GABA}. eIPSC traces (left) and summary time course (right) showing the effects of pairing in CORT-treated slices in the presence of the nonspecific opioid receptor antagonist naloxone (5 μM, $n = 8$ cells, 5 rats) or the μ opioid receptor antagonist CTAP (1 μM, $n = 6$ cells, 4 rats). (**c**) Effect of genetic deletion of μ opioid receptors on LTD. eIPSC traces (left) and summary time course (right) showing the effects of pairing in CORT-treated slices from *Oprm1*$^{-/-}$ mice ($n = 6$ cells, 3 mice). (**d**) Sample recording from an individual PNC (left) and summary graphs (right) of the frequency and amplitude of mIPSCs recorded in tetrodotoxin (1 μM) showing the reduction of mIPSC frequency elicited by the μ opioid receptor agonist DAMGO (1 μM, $n = 9$ cells, 7 rats). (**e**) Occlusion of LTD by DAMGO. eIPSC traces (above left) and eIPSC amplitude time course (below left) are shown from a single neuron (CORT treated) during baseline recording, following bath perfusion of DAMGO (500 nM) and 25 min after pairing. Summarized time course graph (right) showing the effects of pairing on normalized eIPSC amplitude (top) and PPR (bottom, $n = 7$ cells, 4 rats) following DAMGO treatment. Control LTD_{GABA} is re-plotted in filled gray squares (rat) or circles (mouse). Scale bars represent 50 pA and 10 ms in **a**–**d** and **g**, and 25 pA and 0.5 s in **e**. Data are presented as mean ± s.e.m.

to local terminals, particularly as cell bodies of afferent GABA neurons reside outside the PVN[4]. Consistent with this hypothesis, we found that the μ opioid receptor agonist DAMGO (1 μM) significantly reduced the frequency of miniature IPSCs (mIPSCs, 1 μM tetrodotoxin; from 2.1 ± 0.6 to 0.7 ± 0.2 events per s, $P = 0.0002$, paired t test; **Fig. 6d**), but not their amplitude (21.6 ± 1.1 pA before, 21.6 ± 0.7 pA after, $P = 0.99$), suggesting that μ opioid receptors on terminals contacting PNCs regulate GABA release probability. We then asked whether activation of presynaptically located μ opioid receptors would occlude the induction and expression of LTD. In CORT-treated cells, DAMGO (500 nM) depressed eIPSC amplitude (48.4 ± 8.4% of baseline; PPR, 136.1 ± 11.5%; **Fig. 6e**). Once eIPSC amplitude had stabilized, we delivered the pairing protocol. This had no additional effect on either eIPSC amplitude or PPR. At 25 min after pairing, eIPSC amplitude was 52.8 ± 4.5% of pre-DAMGO baseline (paired t test, $P = 0.48$; PPR, 133.8 ± 9.8%, paired t test, $P = 0.89$). We did not observe significant changes to PNC holding current during DAMGO treatment (pooled 500 nM to 1 μM DAMGO; I_{hold} before, −17.3 ± 2.1 pA; after, −13.7 ± 2.7 pA; $n = 27$ cells, paired t test, $P = 0.13$; data not shown). Taken together, these results indicate that μ opioid receptors located at synaptic terminals suppress GABA release and that their activation by an exogenous ligand occludes subsequent induction of LTD_{GABA}.

Although necessary for LTD_{GABA} expression, it is not clear whether μ opioid receptor

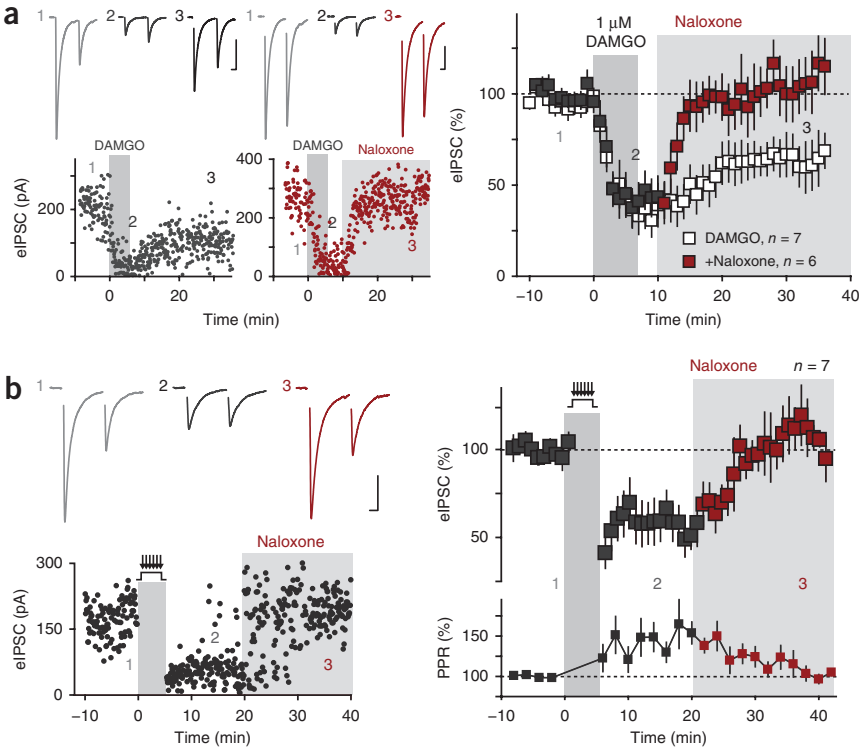


Figure 7 LTD_{GABA} is reversible by opioid receptor antagonism. (**a**) Reversal of μ opioid receptor agonist suppressed transmission by opioid receptor antagonist chase. Left, sample eIPSC traces. Right, plot of eIPSC amplitudes from a neuron treated with 1 μM DAMGO for 7 min and another neuron with DAMGO treatment followed by naloxone (5 μM) at 10 min. Bottom left, summary graphs showing effects of DAMGO on eIPSC amplitude alone ($n = 7$ cells, 6 rats) or followed by naloxone ($n = 6$ cells, 5 rats). (**b**) Reversal of LTD by an opioid receptor antagonist. Left, sample eIPSC traces (top) and plot of eIPSC amplitude (bottom) taken from an individual cell in CORT-treated slices subjected to pairing followed by naloxone 20 min later. Right, summary of the effects of naloxone (5 μM) applied following induction of CORT LTD on eIPSC amplitude (above) and PPR (below) ($n = 7$ cells, 6 rats). Scale bars represent 50 pA and 10 ms. Data are presented as mean ± s.e.m.

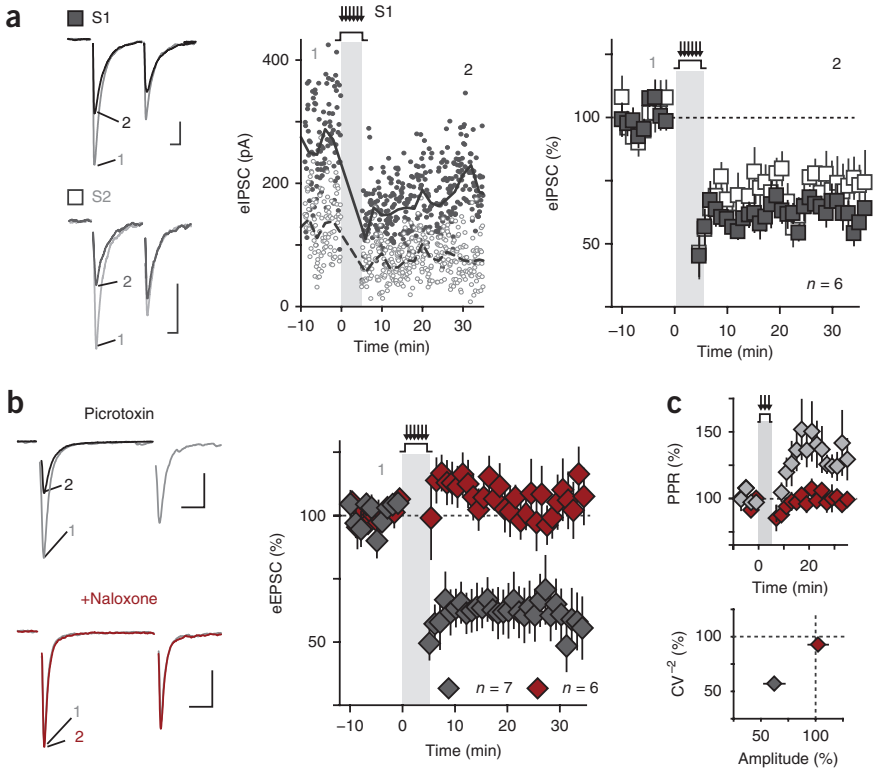


Figure 8 LTD_{GABA} is not synapse specific. (**a**) One PNC was recorded and two independent synaptic inputs (S1 and S2) onto the cell were stimulated. Only S1 was activated during the pairing protocol. eIPSC traces (left) and eIPSC amplitude graph (center) from two inputs onto a single neuron before and after pairing are shown. Right, summary data revealed pairing-induced depression of eIPSC amplitude of S1 and S2 ($n = 6$ cells, 4 rats). (**b**,**c**) Opioid receptor–mediated CORT LTD at excitatory synapses. (**b**) Left, eEPSC traces isolated by picrotoxin (100 μM) before and 30 min after pairing in a cell from a CORT-incubated slice (top) and a cell recorded in the presence of naloxone (bottom). Right, summary time course of the effects of pairing on eEPSC amplitude with ($n = 6$ cells, 5 rats) or without naloxone ($n = 7$ cells, 5 rats). (**c**) Plot of normalized PPR response to pairing (top) and relationship between eEPSC and CV^{-2} (bottom) in CORT-treated cells with or without naloxone. Scale bars represent 50 pA and 10 ms. Data are presented as mean ± s.e.m.

activation is necessary for its maintenance. We applied the opioid receptor antagonist naloxone (5 μM) 20 min following either DAMGO treatment or induction of LTD_{GABA} by the pairing protocol. Transient μ opioid receptor activation by DAMGO (1 μM, 7 min) resulted in a long-lasting depression of eIPSCs (65.6 ± 9.0% of baseline at 35 min, $P = 0.0088$; **Fig. 7a**). This depression was completely reversed by naloxone (111.7 ± 14.1% baseline at 35 min, $P = 0.44$; **Fig. 7a**). These results suggest that transient μ opioid receptor activation is capable of eliciting a long-lasting synaptic change that requires persistent opioid receptor signaling. Next, following pairing, we established that eIPSC amplitude was suppressed (60.9 ± 10.4% of baseline, $P = 0.0093$; **Fig. 7b**). Subsequent application of naloxone caused a recovery of eIPSCs to near-baseline level (108.5 ± 12.9% of baseline at 35 min, $P = 0.53$; **Fig. 7a**,**b**). PPR also returned to baseline (146.1 ± 16.8% at 20 min, $P = 0.033$; 106.4 ± 7.3% at 35 min, $P = 0.42$; **Fig. 7b**). This was not a result of pre-existing opioid receptor tone, as naloxone application to CORT-treated PNCs (in the absence of pairing) had no effect on eIPSC amplitude (108.0 ± 4.0% of baseline, $n = 5$ cells, $P = 0.12$; data not shown). In summary, LTD_{GABA} requires the μ opioid receptor for both expression and maintenance of suppressed GABA release. This could be a result of either persistent effects of μ opioid receptor activation or sustained vesicular release of the opioid peptide.

LTD_{GABA} does not display synapse specificity

Opioid release and signaling may occur across the entire somatodendritic axis or at locally recruited segments of the dendrite. Furthermore, presynaptic activity or μ opioid receptor expression could be restricted to certain inputs. Thus, we probed whether LTD_{GABA} exhibited synapse specificity. Given that mIPSC frequency was sensitive to the μ opioid receptor agonist DAMGO and that sIPSCs were also suppressed during LTD_{GABA}, we hypothesized that release, spread and/or efficacy of endogenously released opioids would not be limited to synapses that were active during pairing. To test this, we electrically activated two distinct GABAergic inputs onto PNCs, S1 and S2, verifying their independence by confirming that the synaptic strength and release probability of one pathway was unaffected by recruiting the other pathway. Delivering the 10-Hz stimulation during pairing through S1 depressed eIPSC amplitudes at both S1 and S2 inputs (S1, 64.7 ± 4.4% of baseline, $P = 0.005$; S2, 71.8 ± 10.2% of baseline, $P = 0.039$; **Fig. 8a**).

Finally, given this finding, and that GABA and glutamate synapses on PNCs are intermingled[32], we hypothesized that somatodendritically released opioids may also depress glutamate synapses. First, we tested for the presence of functional μ opioid receptors at glutamate synapses. In slices incubated *in vitro* with CORT, evoked excitatory postsynaptic currents (eEPSCs) were suppressed by DAMGO (40.8 ± 6.2% of baseline, $P < 0.0001$; **Supplementary Fig. 4d**). Next, we applied the pairing protocol used above and observed a long-lasting depression of glutamate transmission. eEPSC amplitude at 30 min was suppressed to 62.3 ± 9.4% of baseline ($P = 0.0073$; **Fig. 8b**), which was accompanied by an increased PPR (125.6 ± 6.6% of baseline, $P = 0.0081$) and a decrease in CV^{-2} (57.0 ± 15.0% of baseline, $P = 0.028$), suggesting a presynaptic locus of expression. Naloxone completely prevented expression of LTD (102.4 ± 9.0% of baseline, $P = 0.802$; **Fig. 8b**), changes in PPR (100.2 ± 9.9% of baseline, $P = 0.99$) and changes in CV^{-2} (92.8 ± 12.6% of baseline, $P = 0.59$). These results indicate that LTD mediated by opioid receptors in PNCs following glucocorticoid exposure occurs in a synapse-independent fashion.

DISCUSSION

We found that glucocorticoids, elevated in response to a stress experience, are instructive signals in the hypothalamus that allow for subsequent correlated synaptic and cellular activity to suppress GABA release probability. By suppressing RGS4 in PNCs, glucocorticoids functionally altered the outcome of postsynaptic mGluR signaling during synaptic stimulation, culminating in calcium-dependent vesicle exocytosis and the liberation of a retrograde opioid signal from the somatodendritic compartment. Activation of presynaptic μ opioid receptors was necessary for the expression and maintenance

of decreased neurotransmitter release, implicating an endogenous opioid as the most likely candidate for this retrograde signal.

Glucocorticoid-associated LTD_{GABA} requires heterosynaptic recruitment of mGluR5 located on PNCs themselves. This finding is consistent with reports of enhanced mGluR1 and mGluR5 signaling following stress or CORT exposure[33]. Notably, pairing of afferent stimulation with a postsynaptic depolarization was necessary for LTD_{GABA} suggesting that G_q-linked mGluRs in our system may behave as voltage-dependent 'coincidence detectors'[34,35]. Membrane depolarization has been shown to amplify mGluR signaling by enhancing contributions of voltage-gated calcium channels[36], which can synergize with and sustain calcium sourced by mGluRs from IP_3 receptor–gated stores[34]. Although the mechanisms regulating somatodendritic exocytosis are not well defined[24], neuronal activity and G_q-coupled receptors cooperatively drive calcium-dependent dendritic peptide release[37]. For example, synaptic mGluR activation during burst firing in MNCs[25] and L-type channels in dentate granule cells are important for dendritic release of the opioid dynorphin[38]. Consistent with these previous studies, our findings indicate that calcium entry through L-type voltage-gated calcium channels is obligatory for LTD_{GABA}. Although somatodendritic vesicular release from PNCs can also occur following calcium influx through NMDARs[20], we found that LTD_{GABA} persisted after NMDAR blockade.

Our data suggest that μ opioid receptor activation is necessary for expression of LTD_{GABA}. Notably, we also found that ongoing opioid receptor activation is required for LTD maintenance, which is unconventional as an expression mechanism for long-term plasticity. μ opioid receptors are functionally expressed in the PVN, and influence PNC activity and HPA function in a stress state–dependent manner[10,39,40]. In other brain regions, μ opioid receptors are widely expressed on GABAergic neurons and terminals[31]. μ opioid receptor agonists hyperpolarize inhibitory neurons[29–31] and interfere with inhibitory synapse plasticity[41]. Agonist activation of μ opioid receptors locally expressed at synaptic terminals also suppresses GABA release probability[42] and can induce LTD at both GABA and glutamate synapses[43,44]. In spite of this, there are only a few demonstrations of functional synaptic actions of endogenously produced and retrograde acting opioids[11,12]. One might conjecture that a likely candidate for the endogenous μ opioid receptor ligand produced by PNCs and mediating LTD_{GABA} is an enkephalin-like peptide. PNC enkephalins are a compelling candidate for experience-dependent control of neuroendocrine function and adaptation. Proenkephalin transcripts are incrementally upregulated by acute and repeated stress[45] in a glucocorticoid-dependent manner[46,47]. Proenkephalin is also increasingly colocalized with c-Fos and/or CRH following stressful conditions[9,48], suggesting that enkephalin-containing neurons may be relevant to stress-related PNC plasticity and that enkephalin-derived peptides may exist in PNCs as adaptogenic signaling molecules.

Although the LTD_{GABA} that we observed was not mediated by eCBs, it shares many similarities with eCB LTD, which also occurs at synapses throughout the brain[17,19]. G_q-coupled metabotropic receptor activation is a strong stimulus for eCB production and is required for eCB LTD[14,15,17,19]. In addition, glucocorticoids enhance both eCB-mediated short- and long-term plasticity at GABA synapses[7,49]. We found that the switch in mGluR signaling necessary for LTD_{GABA} following CORT exposure is likely RGS4, a molecule that has recently been shown to regulate eCB LTD through gating mGluR signaling in the striatum[50]. Despite these common features, our results indicate that LTD_{GABA} occurs independently of CB1Rs and, to the best of our knowledge, is the first demonstration of an eCB-independent presynaptic LTD at mature GABAergic synapses.

PNC activity is known to be a function of both synaptic drive and circulating glucocorticoid levels. The CORT actions that we observed emerged in the time period classically defined as the delayed domain of glucocorticoid feedback[3]. During this time, endocrine responses to any subsequent stressors are blunted in proportion to the levels of CORT produced by the first exposure[3]. This period conforms to the time estimated for both the entry of CORT into the brain[13] and slow emergence of genomic glucocorticoid receptor–dependent actions[3]. Given that GABA transmission onto PNCs during stress is excitatory[5,8], we propose that a retrograde opioid suppression of both GABA and glutamate release during a sustained period of PNC activity represents a synaptic correlate of the glucocorticoid-induced refractory period imposed onto PNCs[3]. This mechanism may act to mask or compete with the priming mechanisms imparted to PNCs during stress[3,20]. One such mechanism, set in place by the metaplastic actions of the other major stress mediator noradrenaline, is detailed in the accompanying study by Inoue *et al.*[16]. Together, our findings provide mechanistic underpinnings for bidirectional synaptic adaptations that can occur during different temporal windows after a single stress experience. We observed that these two forms of plasticity also exhibit different thresholds for induction. For example, unlike the LTP_{GABA} reported here at 0 min after stress, and extensively detailed by Inoue *et al.*[16], LTD_{GABA} was only evident following a relatively longer period of sustained synaptic and postsynaptic activity. Although speculative, given the paucity of data regarding firing patterns of PNCs or their afferents during *in vivo* stress, this induction requirement suggests that LTD_{GABA} may preferentially serve a homeostatic function, imposing a ceiling on HPA activation and limiting systemic exposure to pathological levels of glucocorticoids during prolonged periods of stress. Our findings suggest that polarity of synaptic metaplasticity on PNCs is a function of the time domain over which the body's two principal stress mediators elicit their actions and hint at the complex dynamics that allow stress circuits to respond and evolve with experience.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.I.W.C. designed and conducted experiments, analyzed the data, and wrote the manuscript. T.F. and W.I. conducted experiments, analyzed data and contributed to manuscript preparation. J.S.B. designed experiments, prepared the manuscript and supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animal handling and stress procedure. All protocols were approved by the University of Calgary Animal Care and Use Committee, in accordance with the Canadian Council for Animal Care. Group-housed juvenile male Sprague-Dawley rats (postnatal day 22–31, Charles River), and wild-type C57BL6/J (Jackson Laboratories), *Oprm1*$^{-/-}$ (Jackson Stock #007559), *vGAT-mhChR2-YFP* BAC transgenic (Jackson Stock #014548), and *Cnr1*$^{-/-}$ (from K. Sharkey) mice (bred to C57BL6/J background, postnatal day 28–50) were kept on a 12-h:12-h light-dark cycle with *ad libitum* access to food and water. Stress was carried out 2–3 h after the onset of light during the trough of circadian fluctuation in plasma CORT. Immobilization stress consisted of cervical and caudal immobilization and confinement in a plastic cylinder for 30 min. Forced swim stress was carried out for 20 min in a plastic bucket (40-cm internal diameter) and 30–32 °C water at a depth where the bottom could not be touched by the rat. For exposure to predator odor, rats were placed in an empty cage for 30 min with a tissue soaked with 2,5-dihydro-2,4,5-trimethylthiazoline (Contech), a compound isolated from fox feces[20]. In some experiments, an intraperitoneal injection of RU-486 (25 mg per kg) or DMSO vehicle preceded stress by 15 min. Following stress, the rat was placed alone in a fresh cage until slice preparation.

Slice preparation and electrophysiology. Animals were anesthetized with isoflurane and decapitated. The brain was quickly removed, submerged and coronally sectioned on a vibratome (Leica) to 300 µM in slicing solution (0 °C, 95% O_2/5% CO_2 saturated) containing 87 mM NaCl, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 25 mM $NaHCO_3$, 25 mM D-glucose, 1.25 mM NaH_2PO_4 and 75 mM sucrose. After placement into aCSF (30 °C, 95% O_2/5% CO_2 saturated) containing 126 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.25 mM NaH_2PO_4 and 10 mM glucose, hypothalamic slices recovered for at least 1 h. Subsequently, some slices were placed for 1 h into aCSF containing 100 nM corticosterone and/or 500 nM RU-486 (Sigma, final DMSO vehicle, <0.0001%). Once transferred to a recording chamber superfused with aCSF (1 ml min^{-1}, 30–32 °C, 95% O_2/5% CO_2), slices were visualized using an AxioskopII FS Plus (Zeiss) upright microscope fitted with infrared differential interference contrast optics. Pulled borosilicate glass pipettes (3–6 MΩ) were filled with a solution containing 108 mM potassium gluconate, 2 mM $MgCl_2$, 8 mM sodium gluconate, 8 mM KCl, 1 mM K_2-EGTA, 4 mM K_2-ATP, 0.3 mM Na_3-GTP and 10 mM HEPES. In the indicated experiments, KCl was reduced to 4 mM or the following were added: 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA, Sigma), 5 µg ml^{-1} BoNT/C (List Biological), 1 mM (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801), 2 mM GDPβs (Na_3GTP-free solution), CCG63802 (Tocris) or recombinant RGS4 (Genway). All other drugs were bath applied by perfusion pump. MCPG, MTEP, JNJ 16259685, capsazepine and DHPG were obtained from Tocris, [D-Pen^{2,5}]Enkephalin, [D-Pen^2,D-Pen^5]Enkephalin (DPDPE) was from Bachem, and nimodipine, picrotoxin, U69593, [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin (DAMGO), CTAP, naltrindole and naloxone were from Sigma.

Whole-cell patch-clamp recordings were performed from PNCs identified by location, morphology and current-clamp fingerprint, as previously described[5,7,20]. Of the 2–4 PVN slices obtained from each animal, one cell was recorded per slice. Slices were randomly assigned to treatment or no treatment groups; a minimum of two cells per litter were used as no treatment control. Each group consisted of data obtained from at a minimum three animals from two different litters. Sample sizes were determined *post hoc* based on those used in previous studies[5,7,20]. Experimenters were not blinded to treatment. PNCs were voltage clamped at −80 mV with constant perfusion of 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 µM, Tocris) or picrotoxin (100 µM, Sigma). Pairs of postsynaptic currents (IPSCs) were evoked 50 ms apart at 0.2-Hz intervals using a monopolar aCSF-filled glass electrode placed about 25–50 µm ventromedially from the recorded cell. To activate ChR2, a fiber optic cable (105-µm core diameter) was placed 1–2 mm from the PVN and a blue light laser (473 nm, OptoGeni 473, IkeCool) delivered 3–5-ms light pulses at 0.2 Hz. The protocol used to elicit LTD consisted of 10-Hz synaptic stimulation paired with a voltage-clamp step to −40 mV for 5 min. Access resistance was continuously monitored; recordings in which values exceeded 20 MΩ or 15% change were excluded from analysis.

Data analysis and statistics. Signals were amplified (Multiclamp 700B, Molecular Devices), low-pass filtered at 1 kHz, digitized at 10 kHz (Digidata 1322, Molecular Devices) and recorded (pClamp 9.2, Molecular Devices) for offline analysis. PSC amplitudes were calculated by subtraction of peak synaptic current from pre-stimulation baseline current. sIPSC events, with eIPSCs and stimulus artifacts removed, were detected using variable thresholds and confirmed by eye (MiniAnalysis, Synaptosoft). For each cell, mean eIPSC/eEPSC amplitude, PPR (second evoke/first evoke) or sIPSC event frequency/amplitude obtained over a 2-min recording interval were normalized and expressed as a percent of baseline recording values. CV^{-2} was analyzed with a 5-min interval and expressed as percent baseline. Gaussian distribution of the data was confirmed by a D'Agostino and Pearson omnibus normality test (GraphPad Prism 4). A one-sample *t* test (versus 100%) was used to assess deviation in normalized values from baseline, and a paired two-tailed Student's *t* test (where stated) to assess deviation in non-normalized values. $P < 0.05$ was considered the level of statistical significance.

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