Reversible Gating of Endocannabinoid Plasticity in the Amygdala by Chronic Stress: A Potential Role for Monoacylglycerol Lipase Inhibition in the Prevention of Stress-Induced Behavioral Adaptation

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Chronic stress is the primary environmental risk factor for the development and exacerbation of affective disorders, thus understanding the neuroadaptations that occur in response to stress is a critical step in the development of novel therapeutics for depressive and anxiety disorders. Brain endocannabinoid (eCB) signaling is known to modulate emotional behavior and stress responses, and levels of the eCB 2-arachidonoylglycerol (2-AG) are elevated in response to chronic homotypic stress exposure. However, the role of 2-AG in the synaptic and behavioral adaptations to chronic stress is poorly understood. Here, we show that stress-induced development of anxiety-like behavior is paralleled by a transient appearance of low-frequency stimulation-induced, 2-AG-mediated long-term depression at GABAergic synapses in the basolateral amygdala, a key region involved in motivation, affective regulation, and emotional learning. This enhancement of 2-AG signaling is mediated, in part, via downregulation of the primary 2-AG-degrading enzyme monoacylglycerol lipase (MAGL). Acute *in vivo* inhibition of MAGL had little effect on anxiety-related behaviors. However, chronic stress-induced anxiety-like behavior and emergence of long-term depression of GABAergic transmission was prevented by chronic MAGL inhibition, likely via an occlusive mechanism. These data indicate that chronic stress reversibly gates eCB synaptic plasticity at inhibitory synapses in the amygdala, and *in vivo* augmentation of 2-AG levels prevents both behavioral and synaptic adaptations to chronic stress.
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INTRODUCTION

Chronic stress is a major risk factor for the development and exacerbation of many forms of mental illness (Vanitallie, 2002). Cumulative life stressors strongly predict lifetime prevalence of affective disorders, including major depressive disorder and anxiety disorders (Caspi *et al*, 2003; McEwen, 2004). Chronic stress is also a causative agent for the development of posttraumatic stress disorder (Jovanovic and Ressler, 2010). In addition, stress is a risk factor for relapse to drug use (Goeders, 2003), psychotic relapse (van Winkel *et al*, 2008), and completed suicide (Hintikka *et al*, 1998). Given the far-reaching implications of stress on mental health, understanding neuroadaptations to chronic stress could uncover novel molecular targets for the treatment and prevention of a wide variety of psychiatric disorders (McEwen, 2007).

Endocannabinoids (eCBs) are a class of bioactive lipids that have been demonstrated to have important roles in stress-response physiology (Lutz, 2009; Patel and Hillard, 2008; Viveros *et al*, 2005). eCBs such as 2-arachidonoylglycerol (2-AG) are produced by neurons in response to increased activity, calcium influx, and activation of some G-protein-coupled receptors (Kano *et al*, 2009). 2-AG can be released from postsynaptic neurons and function as a retrograde messenger via activation of CB1 receptors located on presynaptic axon terminals (Heifets and Castillo, 2009). 2-AG is degraded primarily by monoacylglycerol lipase (MAGL), also located on axonal terminals (Dinh *et al*, 2002; Yoshida *et al*, 2011), as well as by ABHD6 located postsynaptically (Marrs *et al*, 2010).

We have previously shown that levels of 2-AG are increased in response to chronic homotypic stress in mice

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(Patel et al, 2005, 2009; Rademacher et al, 2008). This effect exhibits sensitization, as the increase becomes progressively larger after increasing numbers of stress exposures (Patel and Hillard, 2008), and shows rapid normalization toward the end of a 1-hour restraint exposure (Patel et al, 2009). We have also shown that stress activation of CB1 receptors, likely by endogenous 2-AG, acts to inhibit stress-induced neuronal activation and corticosterone release (Patel et al, 2004), and the expression of active behavioral coping responses to stress (Patel et al, 2005). Thus, we have hypothesized that eCBs serve as an endogenous anti-stress system.

Several recent studies have demonstrated dynamic changes in eCB signaling in response to chronic stress, with the most consistent finding being a downregulation of CB1 receptor function (Patel et al, 2009; Wamsteeker et al, 2010; Wang et al, 2010). In line with these findings, eCB-mediated synaptic suppression is reduced after chronic stress in the hypothalamus and nucleus accumbens (Wamsteeker et al, 2010; Wang et al, 2010). In stark contrast to these data, we now demonstrate that chronic restraint stress exposure gates the induction of eCB long-term synaptic depression at inhibitory synapses (LTDi) in the basolateral amygdala (BLA), a key brain region involved in emotional learning and stress-response adaptation (Pape and Pare, 2010). This increase is paralleled by the development of anxiety-like behavior, which is prevented by chronic pharmacological augmentation of 2-AG levels. These data support the hypothesis that pharmacological augmentation of 2-AG levels could represent a novel approach to the treatment of stress-related neuropsychiatric disorders.

MATERIALS AND METHODS

Animals and Stress Exposure

Male ICR mice 5–7 weeks of age were used for all experiments (Harlan, Indianapolis, IN). Mice were housed on a 12:12 light–dark cycle (lights on at 0600 in the morning), with food and water available *ad libitum*. All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Mice were housed 4–5 per cage during most experiments. Mice were brought into the restraint room daily and subjected to 1 h of tube restraint in modified 50 ml conical tubes for 10 consecutive days (between 0900–1100 hours). During this time, mice were placed in ventilated animal housing cabinet. Upon termination of the stressor, mice were placed back in their home cage and returned to the animal care facility housing room. Mice were weighed daily before restraint stress. Control mice were left undisturbed in their home cages, except for tail marking at the beginning of the experiment and as needed to maintain identifying marks throughout the 10-day protocol. After each stress episode, plastic tubes were washed with soap and water, and then rinsed in 70% ethanol.

In some experiments, chronic daily JZL184 was administered 1 h before each restraint session (stress), controls were injected, and placed back in their home cage. On the day of electrophysiological recording, control mice were injected with JZL184 and killed 2 h later, whereas stressed mice were injected, followed 1 h later by a 1-h restraint stress exposure, thus controlling for JZL184 injection time between groups.

Ex Vivo Electrophysiology

Animals were retrieved from the colony and allowed to rest in sound attenuating boxes for a minimum of 30 min after which they were killed. Mice were killed by cervical dislocation and decapitated immediately following termination of the last 1-h restraint exposure. A 3-mm coronal block containing the amygdala was cut using a coronal brain matrix kept on ice. Coronal slices (300 μm) were made on a Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL) in a 1–4 °C, oxygenated (95% O_2, 5% CO_2), low-Na^+ artificial cerebral spinal fluid (ACSF) containing in mM: 194 sucrose, 20 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1.2 NaH_2PO_4, 10 glucose, and 26 $NaHCO_3$. Once cut, sections were transferred to a holding chamber containing oxygenated ACSF in mM: 124 NaCl, 2.5 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10 glucose, and 26 $NaHCO_3$ at 24 °C. After 1–4 h, sections were placed in the recording chamber superfused with oxygenated ACSF at a flow rate of 2 ml/min. To pharmacologically isolate IPSCs, ACSF was supplemented with a combination of 20 μM CNQX and 50 μM D/L AP-5. All experiments were carried out at 23–25 °C. For experiments involving lipophilic drugs, ACSF was supplemented with 0.2 g/l fatty acid-free bovine serum albumin.

Patch electrodes (2–3 ΩM) were pulled on a Flaming/Brown microelectrode puller (Sutter Instruments) and filled with internal solution containing in mM: $CsMeSO_3$ 119, TEA-Cl 10, NaCl 5, EGTA 1.1, HEPES 10, Na–ATP 4, Na–GTP 0.3, and QX-314 5, with osmolarity adjusted to 275–285 mOsm, pH 7.25–7.35. In Supplementary Figure S1, 60 mM $CsMeSO_3$ was replaced with 60 mM $CsCl_2$. For current-clamp recordings, we used an internal solution consisting of K-gluconate 120 mM, NaCl 4 mM, HEPES 10 mM, Mg–ATP 4 mM, Na–GTP 0.3 mM, KCl 20 mM, and Na–phosphocreatine 10 mM, with osmolarity adjusted to 275–285 mOsm, pH 7.25–7.35. Visually identified pyramidal neurons within the BLA were used for electrophysiological studies as described previously (Patel et al, 2009). Recordings were made using an Axopatch 700B amplifier (Axon instruments). Voltage-clamp recordings were made at a holding potential of +10 mV (except Supplementary Figure S1, which was conducted at −70 mV). Monosynaptically evoked inhibitory postsynaptic currents (eIPSCs) were elicited by constant current stimulation via a concentric bipolar stimulating electrode placed in the BLA just medial to the external capsule and just ventral to the border between the lateral and basolateral nucleus. eIPSC amplitudes were typically adjusted to 300–1200 pA, with stimulation intensities ranging from 10–40 μA. Test pulse simulations were elicited at 0.1 Hz, with six stimulations being averaged to obtain one data point per minute. LTDi was induced by eliciting 100 pulses at a frequency of 1 Hz at twice the stimulation intensity as the test pulse. Access resistance (Ra) was monitored online, cells that showed changes in Ra of >20% during an experiment or had an Ra >20 mΩ upon break-in were excluded from the analysis.

For analysis of basal cellular properties, current-clamp recordings were conduced. We measured resting membrane

potential upon initial break-in. Intrinsic excitability was determined by a series of 20 pA current injections from a resting potential of −70 mV, to control for differences in resting potential between cells and groups; all other basal properties were also determined from a resting potential of −70 maintained by manual current injection as required. After hyperpolarization amplitude was determined after a 100-ms, 500-pA depolarizing current injection that generated a burst action potential. Current–voltage relationship was determined by a series of 20 pA steps from −100 to + 100 pA. Input resistance was determined by measuring the potential change in response to a 200-ms, −20-pA current injection. Membrane time constant is shown as the first time constant of a double-exponential fit of the decay phase of a brief −100 pA hyperpolarizing current injection. All data was recorded using PClamp 10 (Molecular Devices).

Detection of eCBs

Mice were killed 6 h after the last JZL184 or vehicle treatment, and brains rapidly removed and frozen on dry ice. Lipid extraction was conducted as previously described (Patel *et al*, 2003). eCB analysis was conducted as previously described (Patel *et al*, 2009). Briefly, chromatographic separation of the analytes was achieved on a C-18 column (7.5×0.2 cm, 4 μm held at 40 °C) using the following gradient: 85%B for 0.5 min, %B increased to 99% in 3 min and held at 99% for an additional 2 min. The column was re-equilibrated at initial conditions for 3 min before each injection. The flow rate was 0.5 ml/min. Component A was water and B was methanol, and each component contained 80 μM silver acetate and 0.5% acetic acid (v : v). The analytes were detected via selective reaction monitoring (as $[M + Ag]^+$ complexes except AA, which is ionized as $[(M-H) + 2Ag]^+$) in the positive ion mode using the following reactions (the mass in parentheses represents the mass of the deuterated internal standard): AA (*m/z* 519(527)→409(417)); 2–AG (*m/z* 485(493)→411(419)); and AEA (*m/z* 454(462)→432(440)). Quantification was achieved via stable isotope dilution for AA, 2-AG, and AEA. Levels of analytes are given in pmols of analyte per mg wet tissue weight.

Western Blot

Mice were decapitated without anesthesia, and brain punches were taken from the amygdala and stored as previously described (Patel *et al*, 2009). Two frozen BLA punches, one from each hemisphere, were pooled and Dounce homogenized in a buffer containing 150 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 10 μM pepstatin, 1 mM Na_3VO_4, 1 mM NaF, 10 μg/ml leupeptin, and 1 μM microcystin but no detergent. Samples were incubated at 4 °C for 30 min and then centrifuged at 9000 *g* at 4 °C for 10 min. Supernatants (S1) were adjusted to 1 mg/ml of total protein and saved for immunoblotting, and pellets were resuspended in homogenization buffer containing 1% Triton-X-100. Samples were incubated at 4 °C for 30 min and then centrifuged at 9000 *g* at 4 °C for 10 min. Supernatants (S2) were adjusted to 1 mg/ml of total protein and saved for immunoblotting.

Rabbit anti-MAGL primary antibody was provided by Dr Ken Mackie and used at 1 : 12 500. This antibody has been extensively characterized and shows lack of staining in MAGL knockout mice (Mulder *et al*, 2011). A goat anti-rabbit HRP secondary antibody was purchased from Promega and used at 1 : 2000. Samples (20 μg of protein per lane) were resolved by SDS-PAGE and transferred to nitrocellulose membranes, which were stained with Ponceau-S (Sigma, St Louis, MO) and then digitally scanned. After blocking, membranes were incubated with the indicated primary antibodies overnight at 4 °C. Membranes were washed and incubated with the indicated secondary antibodies for 1 h at room temperature. Secondary antibodies were detected by enhanced chemiluminescence (PerkinElmer) and exposed on X-ray film. NIH Image was used to measure the optical densities of specific immunoblotted proteins and total protein loaded into each lane (Ponceau-S-stained membranes). Densities of immunoblotted proteins were normalized to densities of total protein loaded in the corresponding lane.

Behavioral Assays

Novel open field. To test for open-field locomotor activity in a novel environment, mice were tested for 30 min using automated experimental chambers (27.9×27.9 cm; MED-OFA-510; MED Associates, St Albans, Vermont) under constant illumination within a sound-attenuated room. Activity Monitor v5.10 (MED Associates) was used to analyze open-field activity.

Novelty-induced feeding suppression. Individually housed mice were habituated to a novel, palatable food (Ensure Homemade Vanilla Shake) in their home cages for 30 min/day for 3 days before testing. On the day after habituation, mice were presented with the shake in either their home cages or a novel cage without bedding. For each mouse, we measured the latency to begin feeding. On the following day, mice that had been tested in their home cage were tested in a novel cage and vice versa. Latency to feed in novel cage is reported.

Elevated plus maze. Elevated plus maze is used to assess anxiety-like behavior in rodents. An EPM consisted of two closed arms ($30 \times 10 \times 5$ cm) and two open arms ($30 \times 10 \times 5$ cm) that met at a junction (5×5 cm). The walls of the closed arms were 20 cm high, and a 0.25-cm edge provided grip for animals in the open arms. The entire apparatus was elevated 50 cm above the floor. Light distribution was measured throughout the apparatus (2–6 lx in the closed arms and 20–25 lx in the open arms). For testing, mice were initially placed in the center of the maze and allowed to explore the maze for 5 min. ANY-Maze (San Diego Instruments, San Diego, California) was used to track the location of the mice on the apparatus.

Marble-burying assay. To access marble-burying behavior, we placed polycarbonate mouse cages filled with 4 cm of Diamond Soft Bedding (Harlan Teklad, Madison, Wisconsin) in a sound-attenuated room lit with white light at 75 lx. A total of 15 blue, glass marbles (10 mm diameter)

were arranged evenly in a grid-like manner over the bedding. Individual mice were placed into the cage for 30 min. After testing, mice were carefully removed, and the number of marbles buried (50% or more of the marble covered by bedding) was recorded.

Drugs

SR141716, CNQX, and D/L AP-5 were generous gifts from the National Institute on Mental Health Drug Supply Program. THL and JZL184 were purchased from Cayman Chemical (Ann Arbor, MI). For some experiments, JZL184 from National Institute on Drug Abuse Drug Supply Program was used. MTEP and LY456236 were purchased from Tocris (Ellisville, MO). For *in vivo* studies, JZL184 and SR141716 were administered in a vehicle consisting of 1:1:18 ethanol, emulphor, saline via i.p. injection at 10 μl/g body weight. All other chemicals were purchased form Sigma.

Statistical Analyses

LTDi magnitude was determined by averaging the percent depression in IPSC amplitude between 30–40 min post low-frequency stimulation (LFS). Differences between groups were determined by paired *t*-test, unpaired *t*-test, or one-way ANOVA, followed by Bonferroni's *post hoc* analysis as indicated in the legend. For behavioral analyses, data were analyzed by *t*-test or one-way ANOVA, followed by either Dunnett's or Bonferroni's *post hoc* tests as noted in the figure legends. eCB levels were analyzed by one-way ANOVA, followed by either Dunnett's or Bonferroni's *post hoc* tests as noted in the figure legends. $P < 0.05$ considered significant throughout.

RESULTS

Stress Enhances 2-AG/CB1-Mediated Synaptic Suppression in the BLA

We have previously demonstrated that levels of 2-AG are elevated in response to chronic homotypic stress exposure; however, the degree to which this increase represents a synaptically available pool is not clear (Patel *et al*, 2009). To address this question, we utilized *ex vivo* whole-cell electrophysiology to measure eCB-mediated synaptic plasticity at inhibitory GABAergic synapses onto the BLA pyramidal neurons. To this end, we recorded pharmacologically isolated GABAergic currents from visually identified BLA pyramidal neurons. These eIPSCs were monosynaptic based on the high failure rate, observed at low stimulation intensities, and the short response latencies (2.6 ± 0.36 ms, $n = 5$). CB1 receptors are highly expressed on axon terminals of GABAergic interneurons in the BLA and modulate GABA release via a presynaptic mechanism (Katona *et al*, 2001). CB1 receptor activation is also known to mediate LTDi in this region (Azad *et al*, 2004). However, we found that application of LFS (100 stimuli at 1 Hz) within the BLA just medial to the external capsule had no lasting effect on eIPSC amplitude in control mice (Figure 1a and b). To rule out the possibility that LTDi may be occluded in control mice because of the use of depolarized holding
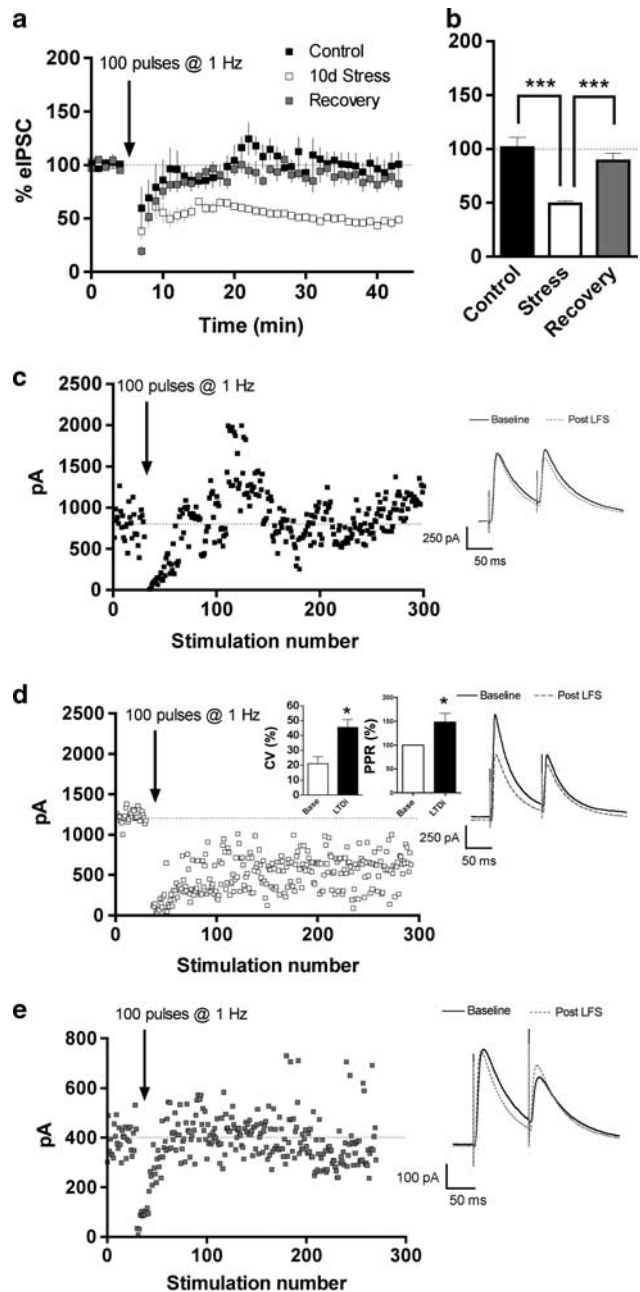

Figure 1 Restraint stress gates induction of LTDi in the BLA. (a) Effects of LFS in control mice ($n = 10$), mice exposed to 10 days of restraint stress ($n = 9$), or 10 days of restraint stress, followed by 7 days of recovery ($n = 8$) on eIPSC amplitude. (b) Summary data indicating the magnitude of LTDi in control, stress, and recovery groups. Individual representative cells from control (c), stress (d), and recovery (e) cells. (d, inset) Effects of LFS-LTDi on CV and PPR in stressed mice. *$P < 0.05$ compared with baseline by paired *t*-test (d, inset) and ***$P < 0.001$ relative to indicated group by ANOVA and Bonferroni's test *post hoc* (b).

potentials for our recordings, we determined the effect of LFS on control cells held at −70 mV using a high chloride-based internal patch solution (see Materials And Methods). However, under these conditions, we were also unable to detect LTDi (Supplementary Figure S1). Furthermore, application of the CB1 antagonist SR141716 did not reveal tonic eCB-mediated suppression of GABA release in control mice when recorded at +10 mV (Supplementary Figure S1),

providing additional evidence against occlusion of LTDi under control conditions at depolarized holding potentials.

In contrast to control mice, in mice exposed to 10 days of repeated restraint stress, LTDi was robustly and consistently induced (control (101.2 ± 9.5%) baseline vs stress (48.8 ± 2.7%) baseline, $P < 0.001$; Figure 1a and b). We also determined the effect of 10 days of restraint stress on intrinsic membrane properties of the BLA neurons, and found only small changes in the input resistance and membrane time constant, with no changes in resting potential or intrinsic excitability (Supplementary Figure S2). Thus, the robust appearance of LTDi in stressed mice is not associated with significant alterations in passive membrane properties of the BLA neurons.

To determine the relative durability of this effect, a separate group of animals were allowed to recover for 7 days after 10 days of chronic restraint stress. After 7 days of recovery, no LTDi was observed after LFS (control (101.2 ± 9.5%) baseline vs recovery (88.9 ± 6.9%) baseline, $P > 0.05$; Figure 1a and b). Data from individual control, stress, and recovery cells are shown in Figure 1c–e.

As eCBs are well known to act at presynaptic CB1 receptors (Kano et al, 2009), and LTDi previously has been shown to be presynaptically expressed (Azad et al, 2004), we

sought to confirm the synaptic site of action of LTDi in stressed mice. LTDi in stressed mice was accompanied by an increase in the paired-pulse ratio (PPR; post-LFS 148.1 ± 19.1% of baseline, $P < 0.05$; Figure 1d, inset); this increase was evident immediately after LFS induction and present throughout the post LFS period (Supplementary Figure S3). As an additional measure of changes in presynaptic release probability, we measured the coefficient of variation (CV) before and after LFS, and found a clear increase in this measure also (baseline (21.2 ± 5.6%) vs post-LFS (45.2 ± 4.2%), $P < 0.05$; Figure 1d, inset). Moreover, no consistent changes in the amplitude-normalized eIPSC decay tau were observed after LFS compared with baseline currents in stressed mice (Supplementary Figure S3), suggesting a lack of postsynaptic change. Consistent with extensive previous data, these data strongly support a presynaptic expression mechanism for LTDi in stressed mice.

To determine the role of CB1 receptors in stress LTDi, we preincubated slices from stressed mice in the CB1 receptor antagonist SR141716 (5 μM) and assayed for LTDi. LFS failed to produce LTDi in slices preincubated in SR141716 (vehicle-stress (48.8 ± 2.7%) baseline vs SR141716-stress (107.3 ± 19.5%) baseline, $P < 0.01$; Figure 2a). As 2-AG is
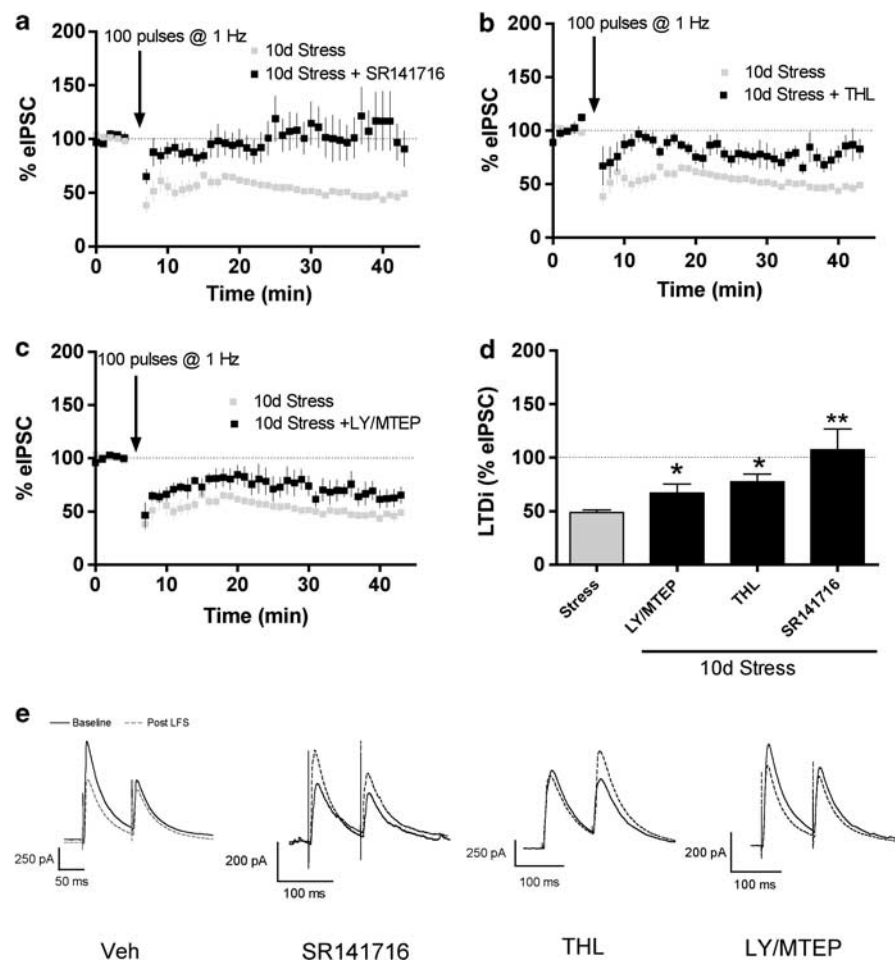

Figure 2 Pharmacological characterization of stress LTDi in the BLA. Effects of the CB1 receptor antagonist SR141716 (5 μM; $n = 10$; a), the DAGL inhibitor THL (10 μM; $n = 8$; b), and a combination of mGluR1 antagonists LY456236/MTEP (10 μM each; $n = 5$; c) on LTDi magnitude in mice exposed to 10 days of stress. Effects of stress alone are shown in gray for comparison. Summary data are shown in d, with representative traces for each group shown in e. *$P < 0.05$ and **$P < 0.01$ by t-test compared with stress alone.

increased by chronic stress (Patel and Hillard, 2008), and LTDi in other brain regions is mediated by 2-AG (Tanimura et al, 2010), we hypothesized that inhibition of the 2-AG synthetic enzyme diacylglycerol lipase (DAGL) would attenuate LTDi in stressed mice. Indeed, preincubation of slices from stressed mice in the DAGL inhibitor THL 10 μM attenuated LTDi magnitude (vehicle-stress (48.8 ± 2.7%) baseline vs THL-stress (77.6 ± 7.1%) baseline, $P < 0.05$; Figure 2b). As LTDi in the BLA has been demonstrated to involve group-1 metabotropic glutamate receptors (mGluR; Azad et al, 2004), we pretreated slices with a combination of mGluR1 and mGluR5 antagonists (LY456236 (10 μM) and MTEP (10 μM), respectively). This combination attenuated LTDi in stressed mice (vehicle-stress (48.8 ± 2.7%) baseline vs LY/MTEP-stress (67.1 ± 8.4%) baseline, $P < 0.05$; Figure 2c). Summary data of inhibitor effects are shown in Figure 2d. Taken together, these data indicate that chronic stress exposure gates the induction of LTDi in the BLA, and that this effect is relatively rapidly reversible with 1 week of recovery.

Downregulation of MAGL Contributes to Enhanced 2-AG Signaling after Chronic Stress

As 2-AG is primarily degraded by MAGL (Long et al, 2009) in the brain, we next determined whether the expression of MAGL was altered within the BLA after chronic stress exposure. We observed a selective decrease in the membrane-associated MAGL in stressed mice compared with controls ($P < 0.05$; Figure 3a). No difference in the cytosolic fraction was observed ($P > 0.05$; Figure 3b). These data suggest that stress-induced downregulation of MAGL could contribute to the expression of LTDi in stressed mice.

Acute Effects of MAGL Inhibition on eCB Levels and Anxiety Behaviors

On the basis of a volume of existing data, we hypothesized that eCB signaling serves anxiolytic functions. Consistent with many previous reports showing anxiogenic effects of CB1 receptor blockade, we found that the CB1 antagonist

Figure 3 Chronic stress decreased MAGL expression in the BLA. Quantification of western blots probes for MAGL in control and stressed mice, in both cytosolic and membrane fractions of BLA tissue ($n = 9$–10 per group). *$P < 0.05$ significantly different from control by t-test. Bands are at ~33 kD.

SR141716 (10 mg/kg) increased feeding latency in the novelty-induced feeding suppression (NIFS) assay (vehicle (220.9 ± 32.2 s) vs SR141716 (1374 ± 161.4 s), $P < 0.0001$ by t-test, $n = 10$/group). To begin to test the role of the eCB 2-AG in the acute modulation of anxiety, we determined whether the pharmacological augmentation of 2-AG produces anxiolytic effects in control mice. To test this hypothesis, we utilized the MAGL inhibitor JZL184 (Long et al, 2009). At 4–6 h after administration, JZL184 dose-dependently increased 2-AG levels ($P < 0.01$, vehicle vs JZL184 (16 mg/kg)), decreased free arachidonic acid levels ($P < 0.01$, vehicle vs JZL184 (16 mg/kg)), and decreased anandamide levels ($P < 0.01$, vehicle vs JZL184 (8 and 16 mg/kg)) in whole-brain extract (Figure 4a–c). As high doses of JZL184 cause locomotor impairments (Long et al, 2009), we first showed that JZL184 between 4 and 16 mg/kg did not affect ambulatory distance in the open field (Figure 4d). Surprisingly, acute administration of JZL184 (8 or 16 mg/kg) had no effect on feeding latency in the NIFS assay in control mice ($P > 0.05$; Figure 4e). However, JZL184 at 16 mg/kg did reduce anxiety as measured in the marble-burying assay ($P < 0.01$, vehicle vs JZL184 (16 mg/kg); Figure 4f). Acute administration of JZL184 (16 mg/kg) had no effect on behavior in the elevated plus maze ($P > 0.05$, for both percent (%) open-arm time and closed-arm entries; Figure 4g–h). Taken together, these data indicate that acute blockade of CB1 receptors produced anxiogenic effects, whereas acute administration of JZL184 increased brain 2-AG levels, had no effect of locomotor activity, and little effect on anxiety measures under control conditions.

Effects of Chronic MAGL Inhibition on eCB Levels and Stress-Induced Anxiety Behaviors

Given the relatively small role of 2-AG in the acute modulation of anxiety behavior, we next considered that 2-AG may be more relevant in the modulation of behavioral adaptations to chronic stress, as we found robust increases in 2-AG-mediated synaptic signaling after chronic stress exposure. We first determined whether the emergence and recovery of LTDi induced by chronic stress was associated with behavioral changes in stressed mice. After 10 days of restraint stress, latency to feed in the NIFS assay was significantly increased relative to control mice (Figure 5a; $P < 0.05$). In mice allowed to recover for 7 days after 10 days of stress, no difference in feeding latency was observed between control and previously stressed mice (Figure 5b; $P > 0.05$). These data indicate that the emergence and recovery of LTDi parallels stress-induced behavioral adaptations. Chronic restraint stress did not induce behavioral changes in the elevated plus maze (Supplementary Figure S4).

We next wanted to determine the effects of chronic JZL184 treatment on stress-induced behavioral adaptations. For these studies, control and stressed mice were pretreated with JZL184 (16 mg/kg) daily for 10 days. To ensure this treatment protocol was effective in increasing 2-AG levels without altering general locomotor behavior, we assayed whole-brain 2-AG levels and locomotor activity in control and stressed mice after 10 days of JZL184 administration. At 6 h after the tenth injection of JZL184, levels of 2-AG were
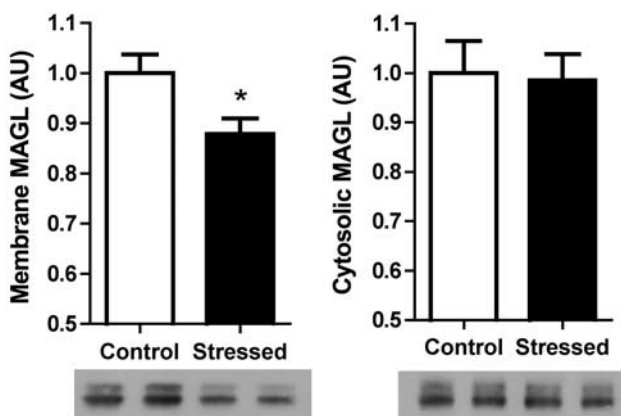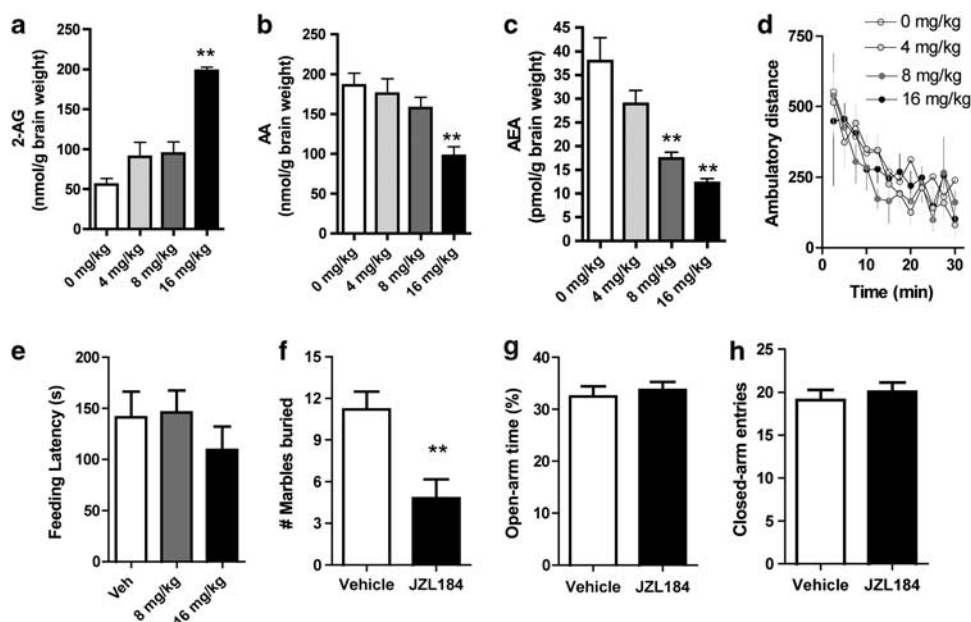
Figure 4 Effects of acute JZL184 treatment on eCB levels and behavior. Effects of JZL184 (4–16 mg/kg) on whole-brain 2-AG (a), AA (b), and AEA (c) levels 6 h after administration. Effects of JZL184 on locomotor activity in the open-field assay (d). Effects of JZL184 (16 mg/kg) on percent (%) open-arm time and closed-arm entries of the elevated plus maze (g–h). Effects of JZL184 (16 mg/kg) on NIFS (e). Effects of JZL184 (16 mg/kg) in the marble-burying assay (f). $n = 8$–10 per group, **$P < 0.01$, significantly different from vehicle treatment by ANOVA, followed by Dunnett's post hoc analysis (a–c) or t-test (f).
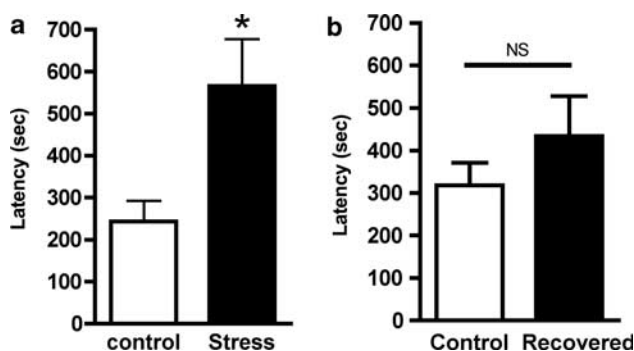

Figure 5 Effects of chronic restraint stress and recovery on feeding latency in the NIFS assay. Ten days of consecutive restraint stress increase feeding latency in the NIFS assay (a). In mice exposed to 10 days or restraint stress, followed by a 7-day recovery period, no changes in feeding latency were observed (b). $n = 8$–10 per group, *$P < 0.05$ by t-test. NS, non significant.

robustly elevated in both control (vehicle vs JZL184, $P < 0.001$; Figure 6a) and stressed (vehicle vs JZL184, $P < 0.001$; Figure 6a) mice. However, arachidonic acid levels were only reduced in stressed mice (vehicle vs JZL184, $P < 0.001$; Figure 6b), and in contrast to acute JZL184 treatment, anandamide levels were not altered in any group (Figure 6c). No effects on ambulatory distance in the novel open-field test were observed after chronic JZL184 treatment (Figure 6d).

Chronic treatment with JZL184 (16 mg/kg) for 10 days did not significantly affect feeding latency in control, non-stressed mice the NIFS assay (vehicle 508.2 ± 101.4 s, $n = 10$ vs chronic JZL184 440.3 ± 70.94 s, $n = 8$, $P = 0.30$). We next determined the effects of chronic JZL184 (16 mg/kg) treatment on stress-induced anxiety using the NIFS assay.

Stressed mice chronically treated with JZL184 showed significantly lower feeding latency than vehicle-treated stressed mice ($P < 0.05$; Figure 6e). Taken together, these data indicate that repeated treatment with low-dose JZL184 causes sustained elevations in 2-AG levels, without signs of tolerance or locomotor impairments, and selectively prevents the development of stress-induced anxiety, as measured using the NIFS assay.

Ex Vivo Synaptic Correlates of Chronic JZL184 Treatment in Stressed Mice

As LTDi expression parallels the appearance of stress-induced anxiety-like behaviors, and chronic treatment with JZL184 prevents the emergence of stress-induced anxiety behaviors, we wanted to determine the effects of chronic JZL184 administration on LFS-LTDi in control and stressed mice. If LTDi at GABAergic synapses in the BLA and stress-induced anxiety behaviors are related, chronic JZL184 treatment should impair LTDi expression in stressed mice. Indeed, in brain slices prepared from mice treated with JZL184 (16 mg/kg) daily, 1 h before each exposure to restraint stress, LFS failed to elicit LTDi ($P > 0.05$; Figure 7a). In fact, there was a trend toward long-term potentiation of GABAergic transmission in the group ($P = 0.9$, baseline vs post-LFS, by t-test; 4/9 cells showed potentiation, whereas the rest showed no effect). Similarly, control mice chronically treated with JZL184 did not show LTDi in response to LFS ($P > 0.05$; Figure 7a). No differences in eIPSC amplitude after LFS between control and stressed mice were observed (Figure 7b; $P > 0.05$).

One possible mechanism that could contribute to the lack of LTDi in stressed mice chronically treated with JZL184 is occlusion of LTDi by endogenous 2-AG, which is robustly
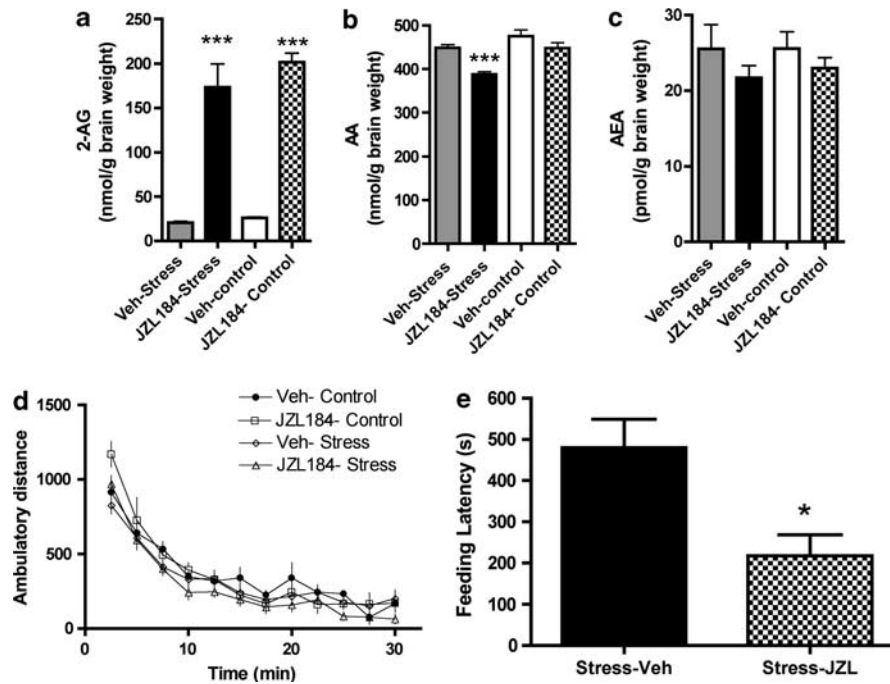
Figure 6 Effects of chronic JZL184 (16 mg/kg) on eCB levels and feeding latency in the NIFS assay. Chronic administration of JZL184 (16 mg/kg) daily 1 h before restraint stress exposure continues to be effective in increasing 2-AG levels in both control and stressed mice (a). Arachidonic acid was only reduced in stressed mice treated with JZL184 (b), and AEA was unchanged in any group (c; $n = 3–5$ per group). Locomotor activity assayed 4 h after the last injection and 2 h after the last stress exposure in the control and stress group, respectively, was unchanged in any group (d). Feeding latency in the NIFS assay was decreased by chronic JZL184 treatment (e; $n = 11–20$ per group). ***$P < 0.001$ significantly different from vehicle treatment by ANOVA, followed by Bonferroni's multiple comparisons test *post hoc*. *$P < 0.05$ significantly different from stress veh by *t*-test.
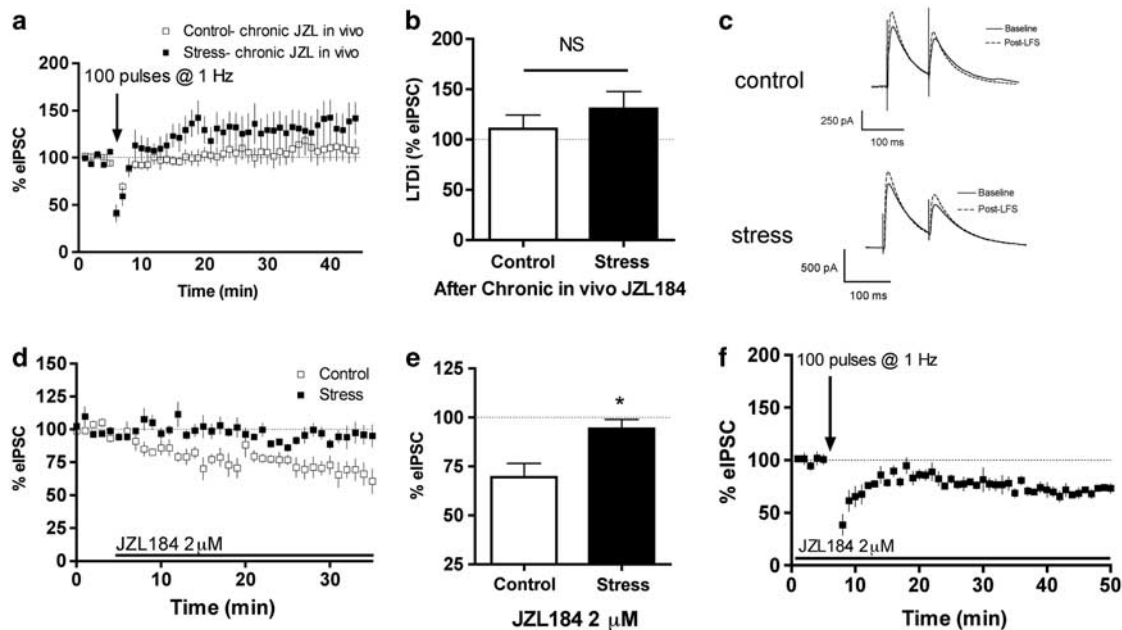

Figure 7 Effects of chronic *in vivo* JZL184 treatment on LTDi expression in control and chronically stressed mice. LFS did not induce LTDi in either control mice or stressed mice chronically treated with 16 mg/kg JZL184 *in vivo* ($n = 8$ per group; a–c). JZL184 application to slices from control mice produced depression of eIPSC amplitude in control, but not in chronically stressed mice ($n = 5–6$ per group; d–e). Bath application of JZL184 to control slices allowed LFS to induce LTDi ($n = 6$; f). *$P < 0.05$ significantly different from control by *t*-test.

elevated by JZL184 treatment (see above). To begin to test this possibility, we wanted to determine the acute effect of JZL184 on eIPSC amplitude in control mice. Bath application of JZL184 decreased eIPSC amplitude relative to

baseline; however, JZL184-induced depression was significantly less in slices from stressed mice relative to control mice (Figure 7d–e; control ($69.4 \pm 7.0\%$) *vs* stress ($94.2 \pm 4.8\%$), $P < 0.05$). Furthermore, incubation of slices

with the MAGL inhibitor JZL184 ($2\,\mu M$) for $>20\,min$ allowed for the induction of LTDi in control mice (Figure 7f; $74.7 \pm 1.2\%$, baseline, $P < 0.05$). These data indicate that JZL184 not only decreases eIPSC amplitude but also allows for the induction of LFS-LTDi in control mice, suggesting that 2-AG degradation may be a limiting factor in LTDi induction in control mice. These data also suggest that chronic *in vivo* administration of JZL184 could indeed occlude *ex vivo* LFS-LTDi, providing a potential explanation for the lack of LTDi in stressed mice chronically treated with JZL184.

DISCUSSION

The main findings of the present work are that chronic restraint stress reversibly gates the induction of LTDi at GABAergic synapses in the BLA. This LTDi was mediated, in part, by DAGL activity, and was dependent partially on mGluR1/5 signaling. One potential molecular mechanism regulating LTDi induction after stress includes downregulation of 2-AG catabolism. We also found that this stress paradigm produced an anxiety-like phenotype, as measured using the NIFS assay. Although acute augmentation of brain 2-AG levels with JZL184 had minimal effects on anxiety-like behaviors, chronic JZL184 administration was able to selectively reverse the anxiety-like phenotype induced by repeated restraint stress. *Ex vivo*, this reversal of stress-induced anxiety was associated with occlusion of LTDi in stressed mice.

Chronic Homotypic Stress Gates LTDi Induction in the BLA

We have shown that 10 days of restraint stress results in the ability of the BLA neurons to express LTDi. This LTDi is presynaptic as indicated by an increase in the CV and PPR, and lack of change in eIPSC decay kinetics, after LTDi induction. LTDi is mediated by CB1 receptors, as it was prevented by preincubation of slices with the CB1 receptor antagonist SR141716. We also demonstrated a partial role for DAGL and mGLUR1/5 receptors in the expression of LTDi. These data suggest that the well-studied mGluR1/5-DAGL-2-AG-CB1 pathway contributes to the LTDi observed in our stressed animals (Kano *et al*, 2009). This enhancement of eCB synaptic signaling is consistent with the prolongation of DSI in the BLA after repeated restraint stress that we previously reported (Patel *et al*, 2009). We also found that the appearance of LTDi is reversible after 7 days of recovery, which is consistent with data showing that desensitization of CB1 receptors after 5 days of restraint stress is reversible after a 3-day recovery period (Wamsteeker *et al*, 2010). We have previously shown that CB1 receptors on GABAergic terminals in the BLA are also partially desensitized by 10 days of restraint stress, thus the enhanced LTDi occurs even in the face of reduced CB1 receptor function (Patel *et al*, 2009).

LTDi at GABAergic synapses onto the BLA neurons of naïve mice has been shown to be dependent on CB1 and mGluR1 receptors, but not dependent on DAGL activity (Azad *et al*, 2004). Our data are partly consistent with these findings, with two exceptions. First, in our study, mice were

required to be exposed to chronic stress to observe LTDi. This discrepancy may be related to the fact that control mice in the study by Azad *et al* (2004) were socially isolated for 2 weeks before experimentation; it is possible that this isolation constituted a stressor. If true, our data are more in line with these findings than they initially appeared. Alternatively, strain differences, or subtle discrepancies in stimulator placement (and thus type/number of afferent fibers recruited during LFS), could contribute to the differential results. It is unlikely that methodological artifacts occluded LTDi in our control mice, as we were also unable to observe LTDi in control mice when recording at hyperpolarized potentials, and no tonic eCB suppression of GABA release was observed at our holding potential of $+10\,mV$. Second, we found that the DAGL inhibitor THL partially prevented LTDi, suggesting at least partial mediation via 2-AG. Our data are consistent with pharmacological, and recent genetic studies that indicate LTDi in many brain regions is dependent on DAGL activity (Tanimura *et al*, 2010). It is possible that the THL-resistant component of LTDi observed in this study is mediated via anandamide as previously suggested (Azad *et al*, 2004).

We have begun to examine the biochemical mechanisms by which stress could gate LTDi induction. One of the primary regulators of 2-AG levels is MAGL (Long *et al*, 2009). MAGL inhibition causes robust increases in 2-AG levels, and is expressed in GABAergic nerve terminals in the BLA (Yoshida *et al*, 2011). We show that inhibition of MAGL by JZL184 allows for LTDi induction in naïve mice, suggesting that 2-AG degradation is a major limiting factor in LTDi induction under control conditions. Thus, we tested the hypothesis that decreased MAGL activity could contribute to the differential expression of LTDi in control *vs* stressed mice. Indeed, membrane-associated MAGL is selectively reduced in the BLA of stressed mice compared with controls. It has been suggested that membrane-associated eCB degrading enzymes are more likely than cytosolic enzyme to regulate 'neurotransmitter' pools of 2-AG, given that they are more likely to have access to released 2-AG at the presynaptic terminal (Blankman *et al*, 2007). A caveat to this conclusion is that enzymatic activity of MAGL was not measured directly, only protein concentration. These data suggest that stress-induced downregulation of MAGL could contribute to the induction of LTDi in the BLA. Other mechanisms including increased synthetic capacity remain open possibilities.

Acute MAGL Inhibition has Little Effect on Anxiety-Like Behaviors

Although acute JZL184 produced large increases in 2-AG levels and reductions in brain anandamide levels, we observed little effect on the elevated plus maze, or NIFS assay, when administered acutely. It has recently been shown in rats that increased environmental aversiveness unmasks an anxiolytic effect of JZL184, thus our conditions may not have been sufficiently aversive to allow detection of an anxiolytic effect (Sciolino *et al*, 2011). We were able to observe an anxiolytic-like response using the marble-burying assay as previously reported (Kinsey *et al*, 2010).

Chronic MAGL Inhibition Prevents the Development of Stress-Induced Anxiety

To begin to examine the role of 2-AG in the behavioral adaptations to chronic stress, we administered JZL184 chronically for 10 days in control and stressed mice. We found that JZL184 continues to produce large increases in 2-AG levels in both control and stressed mice. There were no differences in 2-AG levels between control and chronically stressed mice, likely because of the long duration (~6 h) after stress termination at which measurements were taken. Specifically, the increase in BLA 2-AG levels after chronic restraint stress surge early and begin to decline by the end of the 1-h stress exposure (Patel et al, 2009). Also, the current measurements were global rather than region specific. No effects on general locomotor activity were seen with chronic JZL184 treatment. As chronic stress produced a significant anxiety-like phenotype in the NIFS assay, we determined the effects of chronic JZL184 on NIFS in control and chronically stressed mice. Although chronic JZL184 treatment did not affect feeding latency in control mice, we found that chronic JZL184 treatment attenuated the anxiety-like phenotype induced by chronic restraint stress. It is important to note that we did not observe an effect of stress on the elevated plus maze, likely because of the relatively mild nature of the stress. For example, in this strain of mice, restraint for 6 h per day for 21 days was required to see an anxiogenic effect in the elevated plus maze (Hill et al, 2011). Therefore, it is possible that increased feeding latency observed in the NIFS assay in stressed mice may reflect only mild anxiety or only one particular dimension of anxiety not well captured by the elevated plus maze.

Functional Implications

Our data show a clear association between the emergence of LTDi in the BLA and the expression of stress-induced anxiety-like behaviors. Specifically, in stressed mice LTDi and an anxiety-like phenotype are expressed, after recovery, neither is observed. Furthermore, after chronic JZL184 treatment in stressed mice, neither LTDi nor anxiety-like behaviors were observed. On the basis of this strong association, we attempted to experimentally link the observed stress-induced synaptic adaptations to the behavioral phenotype observed in stressed mice. In brain slices taken from stressed mice chronically treated with JZL184, LFS was unable to induce LTDi. That JZL184 alone depresses eIPSC amplitude, and permits LTDi induction when applied to control slices, suggests that in vivo JZL184 treatment could occlude ex vivo LTDi after chronic treatment. If chronic in vivo JZL184 induces LTDi in vivo, it may suggest that expression of LTDi contributes to the anxiolytic actions of the drug, and that 2-AG/CB1 signaling in the BLA acts to buffer against the development of anxiety. This overall contention is supported by a large body of evidence, indicating that activation of eCB signaling has anxiolytic and antidepressant effects, whereas genetic or pharmacological blockade of CB1 receptors produces anxious and depressive behaviors in animals and humans (Christensen et al, 2007; Hill et al, 2009; Lutz, 2009; Patel and Hillard, 2008; Viveros et al, 2005).

Alternatively, it has been shown that both stress and chronic JZL184 treatment desensitize CB1 receptors (Patel et al, 2009; Schlosburg et al, 2010; Wamsteeker et al, 2010; Wang et al, 2010). If these effects are mediated by different mechanisms, the desensitization could be additive or synergistic, and thus in stressed mice treated with JZL184 the impaired LTDi could be due to more robust receptor desensitization than stress alone. If this is the case, JZL184 could produce anxiolytic effects by blocking LTDi, suggesting that LTDi could contribute to the anxiogenic phenotype, rather than buffer against it. This notion is consistent with data indicating impaired GABAergic transmission in the BLA (as could be expected after LTDi induction) contributes to affective pathology (Quirk and Gehlert, 2003).

It is also well known that CB1 receptors modulate glutamatergic inputs to the BLA (Azad et al, 2003; Kodirov et al, 2009), and the effects of stress on these receptors remain to be determined. Clearly, the net effect of changes in CB1 signaling on both GABAergic and glutamatergic synapses will determine the overall effect on BLA activation. Furthermore, stress can certainly alter glutamatergic and GABAergic transmission in the BLA independently of eCB signaling (Braga et al, 2004; Isoardi et al, 2007; Reznikov et al, 2007; Rodriguez Manzanares et al, 2005), further contributing to the complex effects of stress on amygdala neurocircuitry.

DISCLOSURE

The authors declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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