The cannabinoid CB1 receptor system is functionally involved in the processing and encoding of emotionally salient sensory information, learning and memory. The CB1 receptor is found in high concentrations in brain structures that are critical for emotional processing, including the basolateral amygdala (BLA) and the medial prefrontal cortex (mPFC). In addition, synaptic plasticity in the form of long-term potentiation (LTP) within the BLA > mPFC pathway is an established correlate of exposure to emotionally salient events. We performed a series of in vivo LTP studies by applying tetanic stimulation to the BLA combined with recordings of local field potentials within prelimbic cortical (PLC) region of the rat mPFC. Systemic pretreatment with AM-251 dose dependently blocked LTP along the BLA–PLC pathway and also the behavioral acquisition of conditioned fear memories. We next performed a series of microinfusion experiments wherein CB1 receptor transmission within the BLA > PLC circuit was pharmacologically blocked. Asymmetrical, interhemispheric blockade of CB1 receptor transmission along the BLA > PLC pathway prevented the acquisition of emotionally salient associative memory. Our results indicate that coordinated CB1 receptor transmission within the BLA > PLC pathway is critically involved in the encoding of emotional fear memories and modulates neural plasticity related to the encoding of emotionally salient associative learning.

Keywords: amygdala, cannabinoids, emotional learning, long-term potentiation, memory, prefrontal cortex

Introduction

Considerable evidence demonstrates that the endocannabinoid neurotransmitter system is critical for the processing of emotionally salient sensory information and for the encoding and expression of emotional memory (Marsicano et al. 2002; Milad and Quirk 2002; Laviolette and Grace 2006a, 2006b). Furthermore, there is increasing clinical evidence linking disturbances in endocannabinoid transmission in the etiology of disorders such as schizophrenia (Cohen et al. 2008; Schneider 2008). The CB1 receptor system is ubiquitously distributed within the mammalian brain and is found in relatively high concentrations in neural regions important for emotional learning and memory processing, including the amygdala and prefrontal cortex (PFC; Tsou et al. 1998; McDonald and Mascagni 2001). In terms of emotional learning and memory processing, 2 regions of particular importance are the basolateral amygdala (BLA) nucleus and medial prefrontal cortex (mPFC), which are functionally connected and can regulate neuronal plasticity processes associated with emotional learning and memory (Aggleton 2000; Rosenkranz and Grace 2002, 2003; Laviolette et al. 2005; Laviolette and Grace 2006a, 2006b). With the BLA, CB1 receptors are highly expressed on inhibitory local γ-aminobutyric acidergic (GABAergic) inter-neurons but are absent in the adjacent central nucleus (Herkenham et al. 1990; Tsou et al. 1998; Katona et al. 2001). Activation of these BLA CB1 receptors has been shown to decrease the activity of inhibitory interneurons that in turn increase output from BLA pyramidal projection neurons (Pistis et al. 2004). Furthermore, both the expression and extinction of conditioned fear have been correlated with increased release of endocannabinoids within the BLA (Marsicano et al. 2002), suggesting functional involvement of the BLA CB1 receptor system during emotional learning and memory formation.

Neuronal plasticity in the form of long-term potentiation (LTP) is a well-established correlate of learning and memory processing, and LTP between the BLA and mPFC is readily demonstrable and strongly modulated by exposure to emotionally salient events (Maroun and Richter-Levin 2003; Maroun 2006). Cannabinoid CB1 receptor transmission has been reported to strongly modify synaptic plasticity in a variety of brain regions, including the amygdala (Azad et al. 2003, 2004). However, the potential role of CB1 receptor signaling within the BLA > prelimbic cortex (PLC) pathway has not previously been demonstrated in the context of LTP formation within this circuit. In the present study, we examined the potential role of cannabinoid CB1 transmission during the formation of LTP measured in vivo, along the BLA > PLC circuit. In vivo field potential recordings within the PLC revealed that tetanic stimulation of the BLA resulted in the formation of strong LTP within this circuit. We found that pharmacological blockade of CB1 transmission was sufficient to block the formation of BLA > PLC LTP and the behavioral encoding of emotional memory formation within this pathway. In addition, asymmetrical pharmacological blockade CB1 receptor signaling between the BLA and PLC completely blocked the encoding of emotionally salient fear memory demonstrating an important role for coordinated CB1 signaling in both BLA and PLC during the formation of emotional associative memory.

Materials and Methods

Animals and Surgery

For in vivo electrophysiological recording experiments, male Sprague Dawley rats (300–350 g) were anesthetized with urethane (Sigma, St. Louis, MO; 1.4–1.5 gm/kg, intraperitoneal [i.p.]) and placed in a stereotaxic device. Body temperature was monitored with a rectal temperature probe and maintained at 37 °C. Incisions were made in the scalp to expose the skull, burl holes were drilled, and the dura overlying the BLA and/or mPFC was removed. Coordinates for these areas were determined using the stereotaxic atlas of Paxinos and Watson (1997) with the following coordinates from bregma (in mm): BLA: anteroposterior (AP), -3.0; lateral (L), ±5.0; ventral (V), from -7.4 to -7.6.
to 8.0 from the dural surface; PLC: AP, +3.0 rostral; L, ±0.8-1.0; V, -3.4 from the dural surface. For in vivo electrophysiological experiments, a bipolar, concentric stimulating electrode (NEX, Torrance, CA) was slowly lowered unilaterally into the BLA, following that a glass recording microelectrode (WPI, Sarasota, FL) was slowly lowered into the ipsilateral PLC. For all surgical procedures for behavioral pharmacology experiments, animals were anesthetized with a ketamine (80 mg/mL)-xylazine (6 mg/mL) mixture and placed in a stereotactic frame. Cannulae were secured with jeweler’s screws and dental acrylic. Coordinates for the BLA or PLC were determined using the stereotactic atlas of Paxinos and Watson (1997) with the following coordinates from bregma (in mm): BLA AP, -3.0; L, ±5.0; V, -7.4 from the dural surface; PLC (at a 15° angle): AP, +3.0 rostral; L, ±19.0; V, -3.0 from the dural surface.

Electrophysiology
In order to measure extracellular field potentials evoked by BLA stimulation, microelectrodes were pulled from borosilicate glass (WPI) by a vertical puller (Narishige, Japan) to a resistance between 1 and 4 MΩ for extracellular field potential recordings. The microelectrode was filled with a 2% Pontamine sky blue solution in 0.5 M sodium acetate and was lowered stereotactically into the PLC using the above described stereotactic coordinates. Concentric bipolar stimulation electrodes were slowly lowered into BLA, ipsilateral to the PLC recording electrode position. Once the recording and stimulating electrodes were stereotactically positioned, anesthetized rats were left undisturbed for at least 4–5 min prior to beginning the LTP experiments. Extracellular signals within the PLC (local field potentials) were amplified using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and recorded through a Digidata 1440a acquisition system (Molecular Devices) using pClamp 10 software. Extracellular recordings were typically filtered at low pass at 1 kHz, high pass at 20 Hz, and sampled at 10 kHz. Recording electrodes were slowly lowered into the mPFC via a hydraulic micropositioner until suitable recording locations with stable BLA-responsive field potentials were isolated. Once stable field potentials were localized within the PLC, preliminary off-line measurements were made of the excitatory post-synaptic potential (EPSP) amplitude using the averages of 5-10 repetitive responses to a given stimulation intensity applied at 0.1 Hz. Baseline field potential responses within the PLC were determined by delivering BLA stimulation at 20–200 μA, sufficient to evoke ~30–50% of the maximal amplitude of the evoked field potentials.

In Vivo LTP Procedure
LTP was induced ipsilaterally in the BLA > PLC pathway using a modified version of a previously reported protocol (Maroun and Richter-Levin 2003). Briefly, concentric bipolar stimulation electrodes were placed in the BLA according to the above described coordinates. Burst stimulation was applied to the BLA in 3 sets of 10 trains at 100 Hz, with an interstimulus interval of 5 min. The amplitude of the negative ongoing potential was determined using the analysis procedure of Maroun and Richter-Levin (2003). LTP was measured as an increase in EPSP amplitude. Following the induction of LTP, field potentials were then recorded at 30, 60, and 90 min. The competitive and specific cannabinoid CB1 receptor antagonist, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide trifluoroacetate salt (AM-251; Tocris, Bristol, United Kingdom; 0.01-1.0 mg/kg; i.p.) was injected 30 min prior to the commencement of LTP induction experiments. We selected the time of 30 min postinjection for electrophysiological LTP studies based on previous reports indicating that AM-251 produces both a slow and an enduring potentiation of CB1 receptors. Thus, the competitive and specific CB1 receptor antagonist AM-251 (1-5-50 ng/0.5 μL) was injected through a 28-gage microinfusion injector (Plastics One, Roanoke, VA). For intra-BLA AM-251 behavioral conditioning experiments, AM-251 (5-50 ng/0.5 μL; Sigma) was dissolved in dimethyl sulfoxide then diluted in phosphate-buffered saline (PBS; pH adjusted to 7.4) and microinfused bilaterally into the BLA immediately before the start of the fear-conditioning procedure. For systemic administration experiments during electrophysiological recordings, doses of AM-251 (0.01-1.0 mg/kg) were selected based on previous reports demonstrating that doses within this range effectively block neuronal emotional associative fear conditioning within the mPFC neither influencing baseline neuronal activity levels within the BLA > mPFC circuit nor causing any reduction in neuronal sensitivity to footshock administration (Laviolette and Grace 2006b).

Histological Analysis
At the conclusion of experiments, extracellular recording sites in the mPFC, electrical stimulation sites in the BLA, and bilateral intra-mPFC guide cannula placements were all verified histologically. For electrophysiological studies, the electrode position was marked with an iontophoretic deposit of Pontamine sky blue (~20 μA; continuous negative current for 10-15 min), after which rats were transcardially perfused with 10% formalin in 0.05% PBS. The brains were placed into 25% sucrose in 10% formalin prior to sectioning and mounting. The mounted sections were passed through graded alcohols and distilled water and stained with neutral red for 2–5 min, then briefly rinsed in double-distilled water. Recording sites were identified with Pontamine sky blue spots. The stimulation site was determined from the ventral most point of the stimulating electrode track identified under light microscopy. For the analysis of bilateral intra-BLA and intra-PLC microinfusion studies, rats were deeply anesthetized and perfused with a 10% formalin solution. Brains were removed and fixed in 10% formalin solution for a minimum of 24 h. Brains were cryoprotected with 25% sucrose in 0.1 M phosphate buffer, frozen, and sectioned with a cryostat at ±20 microns. Mounted sections were then stained with Cresyl violet. Bilateral mPFC microinfusion locations were determined by the location of the injector tip.

Statistics
Behavioral and/or electrophysiological data were analyzed with 1-, 2-, or 3-way analysis of variance (ANOVA) or Student’s t-tests where appropriate. Post hoc analyses were performed with Fisher’s least significant difference tests or Newman-Keuls tests.

Olfactory Fear-Conditioning Procedure
Rats were taken from their home cages, received sham microinfusions into the BLA, and were habituated for 30 min in a ventilated conditioning chamber with an electric grid floor inside a sound-attenuated room. Olfactory fear conditioning took place in 1 of 2 distinct environments, counterbalanced within groups: “shock” environment A had white walls with vertical black stripes and a metallic grid shock floor, whereas shock environment B had white walls with black dots with a grid shock floor. Testing 24 h later took place in 1 of 2 alternate environments, where animals had not previously received electric shock, counterbalanced within groups: “test” environment A had white walls with vertical black stripes and a gray Plexiglas floor, whereas test environment B had white walls with black dots with a gray Plexiglas floor. On day 1 (habitation phase), animals were habituated to a random combination of shock environment A or B and test environment A or B in a counterbalanced order for 30 min in each environment. On day 2 (conditioning phase), animals were returned to the conditioning room. Bilateral intra-BLA microinfusions of saline vehicle or the CB1 receptor antagonist AM-251 (2.5-50 ng/0.5 μL) were performed, and the rat was placed in the previously assigned shock environment for olfactory associative conditioning. During the conditioning phase, one of the odors (almond or peppermint) was presented to the animal for 19 s, and a footshock was then delivered (0.8 mA) through an electric grid floor (Colbourn Instruments, Lehigh Valley, PA) for 1 s. Two min later, the alternate odor was presented for 20 s (CS−) in the absence of footshock. This conditioning cycle was
repeated 5 times. On the following day (test phase), rats were returned to the test room and placed in the previously assigned test environment. Before odor presentation, the rat was allowed to explore the environment for 1 min during which baseline levels of freezing and exploratory behavior were observed. Odors (CS+ or CS−) were then presented for 5 min each to the animal in a counterbalanced order, and the amount of time freezing was recorded. Freezing activity was scored by an observer blind to the experimental condition. Rat "freezing behavior" was defined as complete immobility, with the exception of respiratory-related movement. We also analyzed exploratory behavior in response to presentations of CS+ or CS- odors, as described previously (Rosenkrantz and Grace 2003; Laviolette et al. 2005). Exploratory behavior was scored as follows: 0 = no locomotion; 1 = ambulation across one side of the testing chamber; 2 = ambulation across 2 sides; 3 = exploration of the full perimeter of the testing chamber; 4 = exploration of the center and entire perimeter of the test chamber; a score was assigned for every minute of each of the 5 min during the odor presentations, and the mean exploratory score was tabulated for the entire test session.

Blockade of CB1 Receptor Signaling within the BLA > PLC Pathway

In order to examine potential functional interactions between the PFC and amygdala in the context of CB1 receptor transmission during emotional memory formation, we performed a series of microinfusion studies wherein CB1 receptor transmission was simultaneously blocked either interhemispherically (via asymmetric microinfusions of AM-251 into the BLA of one hemisphere and the PLC of the contralateral hemisphere), relative to control groups who had unitary blockade of CB1 receptor transmission in either the BLA or PLC within a single hemisphere. The experimental design for these studies is summarized in Figure 1. Experimental groups were as follows: Group 1 received simultaneous asymmetric intra-BLA and intra-PLC AM-251 microinfusions at a concentration of either 50 ng/0.5 μL (n = 7) or a lower dose of 5 ng/0.5 μL (n = 7). Group 2 received only intra-BLA AM-251 (50 ng/0.5 μL) in one hemisphere and received intra-PLC saline vehicle in the contralateral hemisphere. Group 3 received only intra-PLC AM-251 (50 ng/0.5 μL; n = 7) in one hemisphere and received intra-BLA saline vehicle in the contralateral hemisphere. Group 4 (pseudoasymmetric blockade) received simultaneous intra-BLA saline vehicle in one hemisphere and received intra-PLC saline vehicle in the contralateral hemisphere (n = 7). This concentration range for AM-251 is based upon previously reported behavioral findings demonstrating that intra-PFC microinfusions of AM-251 effectively block the encoding of olfactory associative fear learning within this dose range (Laviolette and Grace 2006a).

Footshock Sensitivity Analysis

To determine whether our pharmacological manipulations (either systemic or intra-BLA/PLC administration of the CB1 receptor antagonist, AM-251) induced alterations in sensitivity to footshock, we rigorously monitored behavioral responses to these manipulations after drug administration. For intra-BLA/PLC disconnection fear-conditioning experiments, we performed a separate control experiment wherein animals received either saline into the BLA/PLC or the CB1 antagonist (AM-251; 50 ng/0.5 μL) into the BLA/PLC and were placed in one of the fear-conditioning chambers in a counterbalanced order. We presented the footshock (0.8 mA; 1 s) 5 times, following the same time course of presentation as in the olfactory fear-conditioning experiments, but with the absence of any olfactory cue delivery. Immediately after each footshock presentation, we measured sensitivity to footshock over 4 separate variables: 1) Percentage of freezing behavior for the 20-s period immediately after the footshock; 2) number of jumps in response to footshock; 3) amount of defecation (in grams) during the entire footshock testing session; and 4) number of "rearing" events during the testing period. For systemic AM-251 footshock sensitivity control experiments, rats received the effective dose of AM-251 (1.0 mg/kg; i.p.) 30 min prior to undergoing the same footshock sensitivity assay described above. All of these behavioral indices of footshock sensitivity have been identified as reliable indicators of fear reactivity to the presentation of a footshock stimulus (Antoniadis and McDonald 1999).

Histological Analysis: In Vivo LTP Studies

In Figure 2, histological analyses of representative PLC recording sites and BLA stimulation sites are presented. In Figure 2A, we present a microphotograph of a representative electrode recording placement in layer V of the PLC. A schematic representation of representative recording sites within the PLC is presented in Figure 2B. Figure 2C shows a microphotograph of a typical intra-BLA stimulation site. Histological analysis revealed stimulation sites to be localized to the anatomical boundaries of the BLA (Fig. 2D). Animals found to have either recording or stimulation sites localized outside the boundaries of either the PLC or BLA were excluded from analysis.

High-Frequency Stimulation of the BLA Induces Robust LTP within the Prelimbic Cortex

We tested 4 groups for BLA > PLC LTP in the present study: A control group received systemic saline administration prior to
LTP induction (n = 23). In addition, we tested 3 systemic doses of the competitive CB1 receptor antagonist, AM-251: 0.01 mg (n = 8); 0.1 mg/kg (n = 7); and 1.0 mg (n = 8). High-frequency stimulation (HFS) of the BLA (see Materials and Methods) produced robust and long-lasting LTP of extracellularly recorded field potentials within the PLC, similar to previous reports (Maroun and Richter-Levin 2003; Maroun 2006). Average EPSPs at +30, +60, and +90 min of experiments from control group (n = 23) were 197.9 ± 13.58, 183 ± 10.99, and 157.7 ± 7.37% of Pre-HFS values, respectively. In Figure 3A, a representative LTP experiment from a saline control animal shows typical, robust induction of LTP as indicated by strongly potentiated EPSP amplitude relative to control at 30, 60, and 90 min post-HFS. In Figure 3B, we present group data comparing average EPSP amplitude measurements pre- and post-HFS application from saline control group (n = 23), again demonstrating robust induction of LTP along the BLA > PLC pathway following tetanic stimulation of the BLA, at all time points tested post-HFS.

Systemic Blockade of CB1 Receptors Prevents the Development of LTP within the BLA > PLC Pathway

Systemic pretreatment with the CB1 receptor antagonist, AM-251 (0.01–1.0 mg/kg; i.p.), dose dependently blocked the in vivo induction of LTP within the BLA > PLC pathway. In Figure 4A, group data showing mean % change in EPSP amplitude from baseline across all AM-251 doses (0.01–1.0 mg/kg) are presented. One-way ANOVA revealed a significant main effect of treatment (F3,42 = 6.1; P < 0.001) and post hoc analysis revealed that only the higher dose of 1 mg/kg; i.p. effectively blocked in vivo LTP induction along the BLA > PLC pathway (P < 0.01). In contrast, pretreatment with a suprathreshold dose of AM-251 (1.0 mg/kg; i.p.) completely blocked the induction of LTP as indicated by strongly attenuated potentiation of EPSP amplitude relative to baseline at all time points tested. A representative LTP experiment from a single animal pretreated with 1.0 mg/kg AM 251 is presented in Figure 4B, showing strong blockade of EPSP potentiation post-BLA HFS presentation, at all times tested. In Figure 4C, we present group data showing the effects of pretreatment with the suprathreshold dose of AM-251 (1.0 mg/kg; i.p.; n = 8) versus animals pretreated with saline vehicle (n = 23). Relative to baseline levels, average EPSP amplitude of the time points tested at +30, +60, and +90 min of experiments from the AM-251 group (1.0 mg/kg; i.p.) were 128.6 ± 7.07, 124.7 ± 6.697, and 113.8 ± 6.76% of pre-HFS values, respectively. AM-251 (1.0 mg/kg; i.p.) significantly inhibited enhancement of EPSP amplitude after HFS as demonstrated by t-tests comparing saline control versus AM-251: at +30 min, t = 2.931, P* < 0.01 (197.9 ± 13.58% vs. 128.6 ± 7.07%); at +60 min, t = 3.036, P < 0.01 (183 ± 10.99% vs. 124.7 ± 6.697%); at +90 min, t = 3.324, P < 0.01 (157.7 ± 7.37 vs. 113.8 ± 6.76%).
Systemic CB1 Receptor Blockade Dose Dependently Blocks the Acquisition of Emotional Fear Memory

We next performed an olfactory fear-conditioning procedure in awake behaving animals, to determine if the same systemic dose range of AM-251 (0.1–1.0 mg/kg; i.p.) may block the encoding of emotional memory, as described for our intra-BLA microinfusion studies, using the same dose range of AM-251 as those used in our LTP studies (0.1–1.0 mg/kg; i.p.). Pretreatment with systemic administration of AM-251 dose dependently blocked the acquisition of differential olfactory fear conditioning at the same dose (1.0 mg/kg; i.p.) and time course as that required to block in vivo LTP along the BLA > PLC pathway (see Materials and Methods; Fig. 5). Two-way ANOVA revealed a significant main effect of treatment on time spent freezing ($F_{2,33} = 20.0; P < 0.001$). Post hoc analysis revealed that animals treated with the higher dose of 1.0 mg/kg AM 251 ($n = 6$) displayed no associative freezing to CS+ relative to CS− presentations ($P > 0.05$), whereas both saline controls ($n = 6$) and animals receiving a lower dose of AM 251 (0.1 mg/kg; $n = 6$) showed significantly greater freezing to CS+ relative to CS− presentations ($P's < 0.01$) In addition, comparisons of CS+ and CS− freezing scores across groups revealed that time spent freezing in response to CS− presentations were significantly higher in animals treated with 1.0 mg/kg AM 251 relative to animals receiving the lower dose (0.1 mg/kg) or saline ($P's < 0.01$), whereas time spent freezing to CS+ presentations were significantly lower in animals receiving the high dose of AM 251 (1.0 mg/kg) relative to animals receiving the lower dose (0.1 mg/kg; $P < 0.01$; Fig. 5A). Analysis of exploratory behavior revealed a significant interaction of group and treatment on time spent freezing (Fig. 5B; $F_{2,33} = 5.53; P < 0.05$). Post hoc analysis revealed that animals treated with the higher dose of 1.0 mg/kg AM-251 ($n = 6$) displayed no differential exploratory behavior CS+ relative to CS− presentations ($P > 0.05$), whereas both saline controls ($n = 6$) and animals receiving a lower dose of AM-251 (0.1 mg/kg; $n = 6$) did show significantly greater suppression of behavior to CS+ relative to CS− presentations ($P's < 0.01$). In addition, comparisons of CS+ and CS− freezing scores across groups revealed that conditioned exploratory behavior was significantly depressed in response to CS− presentations, comparing animals receiving the highest dose of AM 251 (1.0 mg/kg) versus saline controls ($P < 0.01$) or animals receiving the lower dose of AM 251 (0.1 mg/kg; $P < 0.05$; Fig. 5B). To control for any possible nociception-related effects of AM-251, we performed behavioral footshock sensitivity assays (see Material and Methods), comparing saline treated ($n = 6$) versus 1.0 mg/kg; i.p. AM 251 ($n = 6$) treated rats. Statistical analysis revealed no significant behavioral differences between groups in terms of percentage of time spent freezing postfootshock (Fig. 5C; $t_s = 0.07; P > 0.05$); mean number of rears following footshock presentations (Fig. 5D; $t_s = 0.25; P > 0.05$); mean number of jumps following footshock presentations (Fig. 5E; $t_s = 1.58; P > 0.05$); or amount of defecation during the footshock sensitivity test session (Fig. 5F; $t_s = 0.4; P > 0.05$).

Asymmetrical Blockade of CB1 Receptor Transmission in the BLA > PLC Pathway Blocks the Acquisition of Differential Olfactory Fear Memory

Given that systemic blockade of CB1 receptor transmission effectively blocks both neuronal plasticity along the BLA > PLC pathway and prevents the encoding of emotional associative memory (Figs 4 and 5), we next performed a series of microinfusion experiments within the BLA > PLC circuit to determine if CB1 receptor blockade produced its effects on emotional memory encoding in either the BLA, PLC or if integrated CB1 receptor transmission within both regions of
revealing that whereas animals treated with saline (Fig. 7A) showed significantly less freezing to CS− presentations relative to animals receiving the lower dose of intra-BLA/PLC AM 251 (50 ng/0.5 μL) than either the saline control group or animals receiving the higher dose of AM-251 (1.0 mg/kg; i.p.), both of which displayed significantly greater freezing to CS− relative to CS+ presentations. (B) AM-251 also dose dependently blocked the encoding of differential associative olfactory fear memory as demonstrated by a block in the expression of conditioned suppression of exploratory activity following CS+ relative to CS− olfactory cue presentations at the effective dose of AM-251 (1.0 mg/kg; i.p.) relative to saline or a lower dose of AM-251 (0.1 mg/kg; i.p.) pretreatment. Sensitivity tests (see methods) revealed that the effective dose of AM-251 did not alter sensitivity to footshock presentations (panels C−F) as saline versus AM-251 pretreated groups demonstrated no significant differences in measures of freezing, rearing, jumping, or defecation, following footshock presentations.

Analysis of exploratory behavior in experimental group 1 revealed a significant interaction between group and treatment (F_{1,37} = 12.03; P < 0.01) on time spent freezing to CS+ relative to CS− presentations at testing (Fig. 7A) with post hoc analysis revealing that whereas animals treated with saline (n = 6) or a subthreshold dose of intra-BLA/PFC AM-251 (50 ng/0.5 μL; n = 7) displayed significantly higher levels of freezing specifically in response to CS+ presentations (P's < 0.01), this differential associative freezing was blocked in animals receiving a suprathreshold dose of intra-BLA/PFC AM-251 (50 ng/0.5 μL; n = 7) displayed significantly higher levels of conditioned suppression of exploratory behavior in response to CS+ presentations, this effect was blocked in animals receiving a suprathreshold dose of intra-BLA/PFC AM-251 (50 ng/0.5 μL; P > 0.05). In addition, post hoc comparisons between exploratory scores to CS− presentations across groups revealed that animals receiving the lower dose of BLA/PLC AM 251 (50 ng/0.5 μL) showed lower levels of freezing to CS− presentations relative to saline control animals (P < 0.05). Conversely, comparing exploratory scores to CS+ presentations across groups revealed that animals receiving the highest dose of intra-BLA/PFC AM 251 (50 ng/0.5 μL) showed significantly higher exploratory activity to CS+ presentations than either the saline control group or animals receiving the lower dose of intra-BLA/PFC AM 251 (50 ng/0.5 μL; P < 0.05; P < 0.01, respectively).

Analysis of freezing behavior in experimental groups 2 and 3 (unilateral BLA or PLC CB1 receptor blockade) revealed a significant main effect of treatment (F_{1,41} = 25.9; P < 0.001) on time spent freezing to CS+ relative to CS− presentations at testing (Fig. 7C) with post hoc analysis revealing that all groups (saline/saline [n = 6]; saline/BLA AM-251 [n = 7]; saline/PLC AM-251 [n = 7]) demonstrated significantly higher levels of freezing behavior specifically in response to CS+ presentations (P's < 0.01). Analysis of exploratory behavior similarly revealed
a significant main effect of treatment ($F_{1.41} = 29.2; P < 0.001$) on exploratory behavioral scores during CS+ or CS− presentations (Fig. 7D) with post hoc analysis revealing that whereas animals treated with saline ($n = 6$), single hemisphere intra-BLA AM-251 (50 ng/0.5 μL; $n = 7$) or single hemisphere intra-PLC AM-251 (50 ng/0.5 μL; $n = 7$), displayed significantly higher levels of conditioned suppression of exploratory behavior in response to CS+ presentations. Post hoc comparisons between CS+ versus CS− measures of freezing or exploratory behavior scores across groups revealed no significant differences. To control for any possible nociception-related effects of BLA > PLC CB1 receptor transmission disconnection, we again performed behavioral footshock sensitivity assays (see Materials and Methods), comparing saline treated ($n = 6$) versus rats receiving simultaneous intra-BLA/PLC microinfusions of the effective dose of AM-251 (50 ng/0.5 μL). Statistical analysis revealed no significant behavioral differences between groups in terms of percentage of time spent freezing postfootshock (Fig. 8A; $t_s = 1.5; P > 0.05$); mean number of rears following footshock presentations (Fig. 8B; $t_s = 0.44; P > 0.05$); mean number of jumps following footshock presentations (Fig. 8C; $t_s = 2.2; P > 0.05$); or amount of defecation during the footshock sensitivity test session (Fig. 8D; $t_s = 0.76; P > 0.05; P > 0.05$). Thus, similar to our footshock sensitivity tests following systemic AM-251 administration, disconnection blockade of intra-BLA and intra-PLC CB1 receptors with the highest effective dose of AM-251 (50 ng/0.5 μL) did not produce any appreciable attenuation in behavioral indices of footshock sensitivity.

Discussion

The endocannabinoid system is involved importantly in the control of synaptic plasticity related to emotional learning and memory (Marsicano et al. 2002; Laviolette and Grace 2006a, 2006b) and increasing evidence links abnormalities within the CB1 system to the emotional disturbances and psychosis associated with schizophrenia (Cohen et al. 2008; Schneider 2008). Indeed, recent clinical evidence suggests that prefrontal cortical perturbations in CB1 receptor transmission or in levels of endogenous anandamide, may be neuropsychological correlates of schizophrenia (Giuffrida et al. 2004; Zavitsanou et al. 2004; Newell et al. 2006; Bangalore et al. 2008; Koethe et al. 2009). In terms of endocannabinoid-mediated neuronal plasticity mechanisms, previous reports have demonstrated that retrograde endocannabinoid transmission via CB1 receptors can strongly modulate synaptic plasticity specifically within layers V/VI of the rodent PLC (Laforucade et al. 2007), demonstrating an important functional role for endocannabinoid transmission within this specific mPFC subregion. In addition, cannabinoid transmission can strongly modulate neuronal plasticity processes directly within the amygdala (Pistis et al. 2004; Roche et al. 2007). Given the important functional and anatomical relationships between the amygdala and PFC, it is not surprising that various studies have demonstrated a role for CB1 receptor transmission during the processing of neuronal plasticity and emotional associative learning behaviors within this neural circuit (Azad et al. 2004; Laviolette and Grace 2006b; Roche et al. 2007). Indeed, within the rodent mPFC, CB1 receptor activation strongly potentiates emotional associative learning and memory encoding via BLA-dependent inputs (Laviolette and Grace 2006b). However, our results demonstrate that integrated CB1 receptor transmission within both the BLA and PLC may be essential for the encoding of emotional memories and for the induction of synaptic plasticity between the BLA and PLC, a neuronal correlate of learning and memory.

The present series of experiments focused on the PLC subdivision of the mPFC for several reasons. Although the BLA sends projections to most areas of the rodent mPFC, including both infralimbic (IFL) and PLC areas, these amygdala-cortical inputs are arranged in laminar fashion, with the most direct BLA input terminating in layers V and VI of the PLC subregion (Bacon et al. 1996; Orozco-Cabal et al. 2006). Furthermore, BLA inputs to the PLC region have been shown to mediate the integration of incentive value during instrumental learning (Balleine and Dickenson 1998). Anatomically, outputs from PLC target the ventral striatum more strongly than IFL neurons (Vertes 2004), and subpopulations of mPFC neurons that send direct efferents to the ventral striatum have been reported to encode emotional associative memory (McGinty and Grace 2008) suggesting that functional connections between the BLA > PLC regions may be important in the encoding of emotionally salient memory. Finally, considerable evidence implicates the IFL as more critical for the signaling of extinction-related emotional learning and memory processes (Milad and Quirk 2002) rather than the acquisition (encoding)
The present results are consistent with previous work implicating CB1 receptor transmission within the PLC as an important modulator of learning and memory-related neuronal plasticity (Lafourcade et al. 2007).

CB1 Receptor Transmission Modulates Neuronal Plasticity along the Basolateral Amygdala-Prelimbic Cortical Pathway

The measurement of neuronal plasticity in the form of LTP is a well-established neurophysiological correlate of learning and memory processing. The use of in vivo LTP induction is advantageous in being able to directly compare drug effects on synaptic plasticity parameters within the intact brain and compare similarly effective drug concentrations in behaviorally correlative studies. Previous evidence implicates the BLA > PFC pathway as an important circuit for the induction of synaptic plasticity related to emotional experience. Thus, exposure to acute stressors has been shown to block the induction of LTP, measured in vivo, along the BLA > PFC circuit in rodents (Maroun and Richter-Levin 2003). Furthermore, this plasticity is reversed by acute stress exposure in the descending pathway from the mPFC to the BLA (Maroun 2006), suggesting that emotionally salient experiences can strongly modulate neuronal plasticity within this circuit via ascending and descending pathways, and can influence plasticity mechanisms within both

Figure 7. Effects of CB1 receptor blockade within the BLA-PLC pathway on the encoding of olfactory fear memory. (A) Asymmetric blockade of CB1 receptor signaling in the BLA > PLC pathway with microinfusion of AM-251 (see Materials and Methods) dose dependently prevented the encoding of associative fear memory. Although animals receiving saline pretreatment or a lower dose of AM-251 (5 ng/0.5 μL) displayed significant freezing behavior in response to CS+ presentations, this effect was blocked with BLA-PLC microinfusions of a higher dose of AM-251 (50 ng/0.5 μL). (B) Similarly, asymmetric blockade of CB1 receptor signaling in the BLA > PLC pathway with AM-251 dose dependently prevented the encoding of relative associative fear memory in response to CS+ versus CS− presentations expressed as conditioned suppression of exploratory behavior. Although animals receiving saline pretreatment or a lower dose of AM-251 (5 ng/0.5 μL) displayed significant suppression of exploratory behavior in response to CS+ presentations, this effect was completely blocked with BLA-PLC microinfusions of a higher dose of AM-251 (50 ng/0.5 μL). (C) In contrast, unitary blockade of CB1 signaling in either the BLA or PLC was not sufficient to block associative fear learning in the BLA > PLC pathway.

Figure 8. Effects of intra-BLA/PLC CB1 receptor blockade on footshock sensitivity. To control for any potential nociception-related effects of asymmetric BLA > PLC CB1 receptor blockade, separate control groups received either intra-BLA/PFC AM 251 (50 ng/0.5 μL) or saline. Measures of footshock sensitivity (see Materials and Methods) revealed that this effective dose of AM-251 did not alter sensitivity to footshock presentations as saline versus AM-251 pretreated groups demonstrated no significant differences in measures of freezing behavior (A), rearing (B), jumping (C), or defecation (D), following footshock (0.8 mA) presentations.
the amygdalar and prefrontal cortical components of the pathway.

We found that tetanic stimulation of the BLA produced robust plasticity along the BLA > PLC pathway in the form of LTP and that this in vivo neuronal plasticity was dose dependently blocked by the administration of the CB1 receptor antagonist, AM-251. The same systemic dose range of AM-251 effectively blocked the encoding of olfactory associative fear memory in behaving animals. To our knowledge, these results are the first demonstration of BLA > PLC CB1-receptor dependent neuronal plasticity, correlated with a behavioral blockade of associative fear conditioning within the same drug concentration and administration time course range. A previous study using transgenic mice with genetic deletion of the CB1 receptor demonstrated a specific block of “extinction” learning of fear-related associative memory but found no effect on the acquisition of conditioned fear (Marsicano et al. 2002). Although several important procedural differences exist between these studies and the present procedures (e.g., species difference and auditory vs. olfactory sensory modalities), one possibility is that compensatory systems may developmentally take over the role of CB1 receptor transmission during the acquisition phase of emotional learning, because transgenic animals are lacking a functional CB1 receptor system from inception, whereas the present study examined acute, reversible blockade of intra-BLA or global CB1 receptor populations in adult rodents. In addition, the present studies focused on the PLC division of the PFC rather than the more ventrally located IFL cortex, a region that has been implicated as being critical for extinction-related learning processes (Milad and Quirk 2002).

**Emotional Memory Encoding Involves Integrated CB1 Receptor Transmission within the BLA > PLC Circuit**

Our initial experiments demonstrated that systemic blockade of CB1 receptor signaling could strongly block the induction of in vivo LTP within the BLA > PLC circuit and the encoding of emotional associative memory. However, to more closely characterize the neuroanatomical and basis for these systemic effects, our behavioral-anatomical experiments demonstrated that whereas unilateral blockade of CB1 transmission within either the BLA or PLC alone was not sufficient for the observed blockade of emotional memory formation, simultaneously blocking CB1 receptor transmission in the BLA and contralateral PLC, was sufficient to block the encoding of associative emotional memory. Interestingly, behavioral results demonstrated that whereas differential associative learning was abolished by intra-BLA/PLC or systemic CB1 receptor blockade, animals were still capable of olfactory conditioning. For example, although a relative decrease in differential conditioned freezing behavior was observed in response to CS+ versus CS− presentations with increasing doses of AM 251, these behavioral outcomes were concomitant to relative increases in conditioned responses to CS− presentations (Figs 5A and 7A). Conversely, we observed a relative decrease in conditioned exploratory responses to CS− presentations (Figs 5B and 7B) with increasing doses of AM 251, concomitant to a relative increase in responding to CS+ presentations. Although future studies are required to explore this phenomenon, this may suggest a specific role for CB1 transmission during the encoding of associative conditioned stimuli with differential emotional salience, as CB1 receptor blockade prevented the encoding of relative CS− versus CS+ associations, but did not cause a generalized impairment in olfactory conditioning.

The present results are consistent with previous reports that have found correlated increases in endocannabinoid transmission within the BLA during emotionally salient experiences (Marsicano et al. 2002) and with evidence demonstrating a critical role for CB1 receptor transmission within the PFC during the encoding of associative emotional memory (Laviolette and Grace 2006a, 2006b). However, to our knowledge, this is the first evidence to suggest that functionally integrated CB1 receptor transmission within the BLA > PLC pathway may be critical for the encoding of emotional memory.

Although the precise mechanisms by which CB1 receptors modulate neuronal activity within the BLA are not fully understood, various studies have reported that activation of CB1 receptors can decrease GABAergic synaptic transmission in several brain regions important for emotional learning, including the nucleus accumbens (Hoffman and Lupica 2001; Manzoni and Bockaert 2001), hippocampus (Hoffman and Lupica 2001), amygdala (Azad et al. 2003, 2004), and PFC (Lafourcade et al. 2007). Indeed, activation of CB1 receptors within the lateral amygdala has been shown to inhibit the activity of inhibitory feedback onto pyramidal output neurons via GABAergic interneurons and inhibit long-term depression (LTD; Azad et al. 2003). Within the mPFC, activation of CB1 receptors potentiates emotional learning and memory formation to subthreshold fear-conditioning stimuli, both behaviorally and within single neurons (Laviolette and Grace 2006b), suggesting that activation of prefrontal cortical CB1 receptors may amplify emotionally salient associative learning signals. This effect depends upon functional input from the BLA because inactivation of this amygdala input prior to conditioning blocks the potentiating effects of CB1 receptor activation (Laviolette et al. 2005; Laviolette and Grace 2006b).

Within the BLA, high concentrations of CB1 receptors are found localized on a subpopulation of inhibitory interneurons (McDonald and Mascagni 2001) suggesting an important regulatory role for CB1 receptor transmission within the BLA through endocannabinoid signaling. In this case, activation of inhibitory CB1 receptors associated with this BLA neuronal population would be expected to decrease local inhibitory feedback on pyramidal amygdalar outputs neurons, as suggested by other recording studies that have reported strong inhibition of BLA interneurons following application of CB1 receptor agonists (Azad et al. 2004; Pistis et al. 2004). Within the PLC, CB1 receptor activation has been demonstrated to mediate LTD at excitatory synapses within the PLC, however, the precise neurochemical identity of these synapses is not known (Lafourcade et al. 2007). We have reported previously that active output from the BLA to the PFC is required for the encoding of emotionally salient information within neurons of the PFC and also for CB1-receptor mediated modulation of emotional memory processing within single PFC neurons (Laviolette et al. 2005; Laviolette and Grace 2006a). Although future studies are required to more clearly characterize the functional effects of CB1 receptor modulation on distinct neuronal subpopulations within the BLA and PFC, the present results suggest that functional interactions mediated through CB1 receptor signaling between both the BLA and PLC is required for encoding of emotionally salient information within this circuit.
The present study focused specifically on the role of the CB1 receptor system in the BLA > PLC circuit during the acquisition (encoding) phase of emotional associative learning using an olfactory fear-conditioning assay, as previously reported (Laviolette et al. 2005; Laviolette and Grace 2006b). Interestingly, previous evidence has reported that pharmacological blockade of PLC activity failed to block the acquisition of auditory or contextual fear memories, suggesting that neuronal regions extrinsic to the PLC may be able to compensate for associative emotional memory encoding in the absence of PLC neuronal activity (Corcoran and Quirk 2007). Although this report used different fear-conditioning stimuli and modalities, these findings are not necessarily incompatible with the present results because our experiments revealed that integrated CB1 receptor transduction within both the BLA and PLC was required for associative memory encoding of olfactory fear stimuli. Furthermore, the present results are consistent with previous studies demonstrating that neurons within the mPFC that receive direct inputs from the BLA are involved specifically in the encoding and plasticity associated with emotional associative memory formation (Laviolette et al. 2005; Laviolette and Grace 2006).

The BLA endocannabinoid system is involved importantly in the processing of nociceptive sensory information, and activation of CB1 receptors within the BLA has been reported to produce analgesic effects (Manning et al. 2003; Hasanein et al. 2007) and modulates fear-conditioned analgesia (Finn et al. 2004, 2006). To rule out the possibility that intra-BLA CB1 receptor blockade may have interfered with the processing of footshock presentations via nociceptive modulation, we examined the effective doses of intra-BLA AM-251 on the sensitivity to footshock-related fear behaviors. We observed no differences between groups receiving intra-BLA CB1 receptor blockade with control groups, indicating that our observed block of olfactory fear memory acquisition was not related to any unconditioned effects of CB1 receptor blockade on nociceptive processing. Furthermore, similar systemic doses of AM-251 have no effect on footshock or olfactory cue processing and do not affect baseline spontaneous neuronal activity within the mPFC, as reported previously (Laviolette and Grace 2006b).

Conclusions
The present results identify the BLA > PLC pathway as an important neural circuit for cannabinoid-mediated encoding of emotionally salient associative memory. Interestingly, doses of a CB1 receptor antagonist that were effective in blocking the formation of in vivo LTP along the BLA > PLC circuit were equally effective in preventing the acquisition of associative emotional learning and memory. Given the known ability of CB1 receptor substrates within the PFC to modulate emotional learning and memory plasticity mechanisms (Laviolette and Grace 2006a, 2006b; Lafourcade et al. 2007), the present results suggest an integrative network of cortical and subcortical CB1 receptor-mediated emotional learning mechanisms in both the BLA and PLC region, which may act in concert to modulate emotional memory encoding and neuronal associative plasticity learning processes.

Notes
Conflict of Interest: None declared.

Address correspondence to Dr. Steven Laviolette, 468 Medical Science Building, Department of Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada N6A 5C1. Email: steven.laviolette@schulich.uwo.ca.

References


