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Role of the basolateral nucleus of the amygdala in endocannabinoid-mediated stress-induced analgesia

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Abstract

Recent work in our laboratories has demonstrated that an opioid-independent form of stress-induced analgesia (SIA) is mediated by endogenous ligands for cannabinoid receptors–anandamide and 2-arachidonoylglycerol (2-AG) [A.G. Hohmann, R.L. Suplita, N.M. Bolton, M.H. Neely, D. Fegley, R. Mangieri, J.F. Krey, J.M. Walker, P.V. Holmes, J.D. Crystal, A. Duranti, A. Tontini, M. Mor, G. Tarzia, D. Piomelli, An endocannabinoid mechanism for stress-induced analgesia, Nature 435 (2005) 1108–1112]. The present study was conducted to examine the contribution of cannabinoid CB_1 receptors in the basolateral nucleus of the amygdala (BLA) and central nucleus of the amygdala (CeA) to nonopioid SIA. SIA was induced by continuous footshock (3 min 0.9 mA) and quantified behaviorally using the tail-flick test. Microinjection of the CB_1 antagonist/inverse agonist rimonabant (SR141716A) into the BLA, a limbic forebrain region with high densities of CB_1 receptors, suppressed SIA relative to control conditions. By contrast, the same dose administered into the CeA, where CB_1 immunoreactivity is largely absent, or outside the amygdala did not alter SIA. To examine the contribution of endocannabinoids in the BLA to SIA, we used selective pharmacological inhibitors of the anandamide-degrading enzyme fatty-acid amide hydrolase (FAAH) and the 2-arachidonoylglycerol-degrading enzyme monoacylglycerol lipase (MGL). The FAAH inhibitor URB597 and MGL inhibitor URB602, at doses that enhanced SIA following microinjection in the midbrain periaqueductal gray, did not alter SIA relative to control conditions. Our findings suggest that CB_1 receptors in the BLA but not the CeA contribute to SIA, but pharmacological inhibition of endocannabinoid degradation at these sites does not affect the expression of stress antinociception.
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Keywords: 2-AG; Anandamide; Antinociception; FAAH; MGL; Periaqueductal gray

Environmental stressors activate descending pain inhibitory systems, which suppress pain by inhibiting the transmission of impulses from nociceptors to the central nervous system. This antinociceptive response, termed stress-induced analgesia (SIA), is mediated, in part, by the release of opioid peptides. However, opioid-dependent and opioid-independent forms of SIA can be differentially activated based upon stressor parameters and duration [18]. Recent research in our laboratories has demonstrated that an endocannabinoid signaling system mediates nonopioid SIA induced by continuous footshock [14]. A role for cannabinoid CB_1 receptors in SIA was demonstrated by our observations that competitive CB_1 antagonists, administered systemically or locally in the dorsolateral periaqueduc-tal gray (dPAG), block nonopioid SIA. Furthermore, SIA is attenuated in rats rendered tolerant to cannabinoids, but not in rats rendered tolerant to morphine [14]. In the midbrain PAG, a key structure implicated in the descending control of pain, stress triggers the rapid mobilization of two endocannabinoid lipids—2-arachidonoyl glycerol and anandamide [14]. These compounds are hydrolyzed in vivo by distinct serine hydrolases. Anandamide is degraded by fatty-acid amide hydrolase (FAAH) [6,7], whereas 2-AG is hydrolyzed by monoacylglycerol lipase (MGL) [9]. Inhibition of either FAAH or MGL [14,29] in the PAG also enhances SIA in a CB_1-dependent manner, further supporting a role for endocannabinoids in regulating expression of SIA at the supraspinal level.

The distribution of CB_1 receptors in the brain suggests several anatomical regions where endocannabinoid actions could modulate SIA. One such region is the amygdala, an area of the limbic forebrain implicated in both fear conditioning [5] and

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affective dimensions of pain [3,19]. An ascending spino-ponto-amygdaloid circuit has been postulated as an "affective" nociceptive pathway [3,19]. CB_1 immunoreactivity is dense in the basolateral nucleus of the amygdala (BLA) [12,16], but is reportedly absent in the central nucleus of the amygdala (CeA) [16]. CB_1 immunoreactivity is associated with a distinct subpopulation of GABAergic interneurons in the BLA [16], corresponding to large cholecystokinin-positive cells [23]. The distribution of FAAH and MGL at this site also correlates well with the distribution of CB_1 receptors [11].

The anatomical localization of CB_1 in the BLA is consistent with electrophysiological data demonstrating that activation of these receptors presynaptically modulates GABAergic transmission [16]. Endocannabinoids may act as retrograde messengers to control neuronal signaling in the BLA. For example, a form of short-term synaptic plasticity – depolarization-induced suppression of inhibition (DSI) – in the BLA is blocked by CB_1 antagonists [30]. BLA efferents innervate the CeA, the main amygdaloid output nucleus, which sends projections to the PAG and other regions. Thus, an endocannabinoid-mediated reduction of GABA release would disinhibit principal neurons innervating the CeA, to control information processing in the amygdala [2,17]. Unilateral microinjection of cannabinoid agonists into the amygdala also induces antinociception in the tail-flick test [22], supporting a role for this structure in modulation of pain sensitivity. Furthermore, microinjections of the GABA agonist muscimol in the CeA attenuates cannabinoid antinociception [19]. Unilateral or bilateral lesions of the CeA also suppress the antinociceptive effects elicited by both systemic cannabinoids [19,20] and diverse environmental challenges [10]. Endocannabinoid signaling in the BLA also mediates extinction of aversive memories [21], suggesting that endocannabinoids modulate multiple responses to stress via actions in the amygdala.

In the present study, we investigated the role of cannabinoid CB_1 receptors in the BLA and CeA in nonopioid SIA in rats. First, the CB_1-selective antagonist/inverse agonist rimonabant was microinjected into the BLA and the CeA to examine the contribution of these sites to endocannabinoid-mediated SIA. Based upon the distributions of CB_1 receptors in these sites, we hypothesized that pharmacological blockade of CB_1 receptors in the BLA, but not the CeA, would suppress nonopioid SIA. To examine the contribution of endocannabinoids in the amygdala to SIA, we administered selective pharmacological inhibitors of FAAH and MGL locally in the BLA, at doses that enhanced nonopioid SIA following microinjection into the midbrain PAG. To this end, we used two pharmacological inhibitors that selectively target either FAAH or MGL. The FAAH inhibitor URB597 increases brain accumulation of anandamide but not 2-AG [15] and enhances SIA in a CB_1-dependent manner [14]. On the other hand, the MGL inhibitor URB602 increases levels of 2-AG, but not anandamide, in the midbrain PAG and enhances CB_1-mediated SIA when microinjected into this structure [14].

Sixty-three male Sprague–Dawley rats (250–400 g) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee, and followed the guidelines of the International Association of the Study of Pain [31] and the National Institutes of Health. Rimonabant was obtained from NIDA. URB597 was purchased from Cayman (Ann Arbor, MI). URB602 (biphenyl-3-yl carbamic acid cyclohexyl ester) was synthesized by reacting diimidazole-1-ylmethanone with biphenyl-3-yl amine in acetonitrile in the presence of 4-dimethylaminopyradine and subsequently with cyclohexanol as described previously [14]. Animals were anesthetized with a mixture of sodium pentobarbital and ketamine. Stainless steel guide cannulae (24 g, Small Parts, Miami Lakes, FL) were unilaterally implanted above either the BLA (AP −2.8 mm AP, +4.8 mm LAT, −8.8 mm DV) or CeA (−2.5 mm AP, +4.0 mm LAT, and −7.8 DV) using zero points from bregma, the midline suture and the surface of the skull, respectively [26]. Cannulae were fixed to the skull using skull screws and dental acrylic.

Five to seven days after surgery, rats were habituated to restraining tubes prior to testing. The latency to remove the tail from a radiant heat source (11TC Model 336 Tail-flick Analgesia Meter) was measured using the tail-flick test. Drug or vehicle (DMSO, 0.5 μl) was microinjected using a microinfusion pump over 60 s into either the BLA, CeA or deliberately off-site. In experiment 1, rimonabant (2 nmol) or vehicle was microinjected into either the BLA or CeA ($n = 6$–9/group, respectively) 5 min prior to foot shock. In experiment 2, URB597 (0.1 nmol), URB602 (0.1 nmol), or vehicle ($n = 6$–10/group) was administered to the BLA 32 min prior to foot shock. Doses and delays were selected based upon previous studies demonstrating efficacy of the identical drug treatments following microinjection into the midbrain PAG [14]. SIA was induced by exposing rats to continuous foot shock (0.9 mA, ac current, 3 min) using a Lafayette grid-shock apparatus and quantified behaviorally using the radiant heat tail-flick test. Removal of the tail from the heat source terminated application of thermal stimulation. Tail-flick latencies were monitored over 4 min immediately prior to exposure to the stressor to evaluate changes in basal nociceptive thresholds induced by pharmacological manipulations. Tail withdrawal latencies were measured at 2-min intervals before (baseline) and after foot shock. A ceiling tail-flick latency of 10 s was employed to prevent tissue damage. In all studies, the experimenter was blinded to the experimental condition.

Following testing, rats were euthanized with sodium pentobarbital and perfused with saline followed by formalin. Brains were removed, cryoprotected overnight, cryostat-cut (40 μm thickness), and mounted onto gelatin-subbed slides. Sections were dried and stained with cresyl violet. Injection sites were confirmed histologically using a light microscope. Microinjection sites were confirmed for thirty-seven animals in the BLA (Fig. 1c) and fifteen animals in the CeA (Fig. 1d). Eleven animals were used as off-site controls. Only animals with histologically confirmed microinjection sites were included in data for analysis. Tail flick data were blocked for each subject by averaging every two adjacent tail-flick latencies into a single mean, as described previously [14]. Means of two-trial blocks, calculated for each subject, were subjected to repeated measures analysis of variance (ANOVA) and ANOVA, as appropriate. Post hoc comparisons were performed using the Fisher's protected least-squares difference (PLSD), with $P < 0.05$ considered significant.
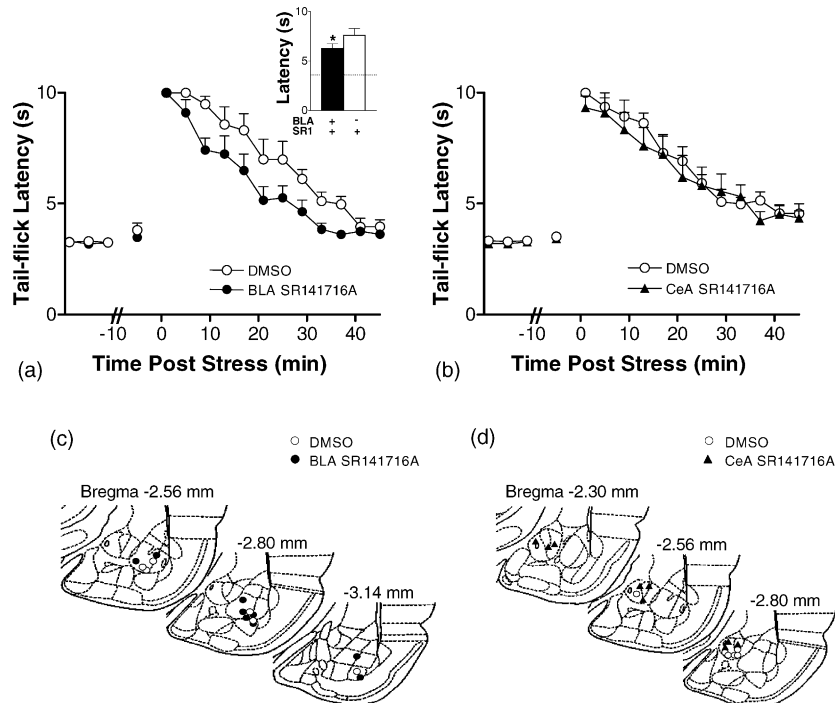
Fig. 1. Microinjection of the CB$_1$ antagonist rimonabant (2 nmol) into the BLA but not the CeA attenuates nonopioid SIA. Rimonabant suppressed stress antinociception in the tail-flick test following microinjection in the (a) BLA ($P < 0.04$) but not the (b) CeA relative to vehicle-treated controls. Inset: Intra-BLA microinjection of rimonabant suppressed stress antinociception relative to off-site controls ($P < 0.04$). Data are expressed as mean ± S.E.M. (c and d) Coronal reconstruction of microinjection sites for drug (closed symbols) and vehicle (open symbols) groups in the (c) BLA and (d) CeA.

In all studies, baseline tail-flick latencies did not differ between groups prior to administration of drug or vehicle. Moreover, latencies recorded just prior to foot shock, following injection of drug or vehicle, were similar between groups, indicating that the injection alone was not sufficient to induce antinociception. In all studies, foot shock increased tail-flick latencies ($P < 0.002$ for all experiments).

Post-shock tail-flick latencies were attenuated in rats receiving intra-BLA microinjection of rimonabant relative to vehicle ($F_{(1,13)}$ 5.764; $P < 0.04$) (Fig. 1a and c). Intra-BLA microinjection of rimonabant also decreased stress antinociception compared with off-site controls receiving the same dose ($F_{(1,14)}$ 5.181; $P < 0.04$) (Fig. 1a inset). By contrast, intra-CeA microinjection of rimonabant did not alter tail-flick latencies relative to vehicle (Fig. 1b and d). Neither the FAAH inhibitor URB597 nor the MGL inhibitor URB602 altered SIA (Fig. 2a and b), at doses that markedly enhanced SIA following microinjection into the midbrain PAG [14].

The present study demonstrates that pharmacological blockade of CB$_1$ receptors in the BLA attenuates nonopioid SIA induced by continuous foot shock. Microinjection of rimonabant into the BLA, where CB$_1$ receptors are dense, suppressed stress-induced antinociception relative to control conditions. By contrast, microinjection of rimonabant into the CeA, where CB$_1$ receptors are largely absent, failed to suppress nonopioid SIA. Microinjection of rimonabant into regions outside the amygdala also failed to suppress SIA, suggesting that the actions of the cannabinoid antagonist were not due to diffusion to distal sites. Our data are consistent with the observation that CB$_1$ agonists depress monosynaptic evoked inhibitory post-synaptic potentials (IPSCs) in the BLA but not in the CeA [16]. Our results, therefore, suggest that CB$_1$ receptors in the BLA modulate local inhibitory networks in the BLA to ultimately regulate expression of SIA. Nonetheless, neither the FAAH inhibitor URB597 nor the MGL inhibitor URB602 enhanced SIA following microinjection into the BLA, at doses that markedly potentiated SIA following microinjection into the midbrain dPAG [14]. These differences likely reflect differential modulatory roles of distinct endocannabinoids in the ascending "affective" pain pathway compared to descending pain modulatory systems.

Anatomical studies suggest that CB$_1$ is preferentially associated with GABAergic, as opposed to glutamatergic, synapses in the BLA [16]. Nonetheless, in the lateral amygdala, endocannabinoids mediate reductions in both local inhibitory inputs as well as excitatory transmission, whose actions could exert opposing effects [1,27]. Low frequency stimulation of the lateral amygdala also mobilizes endocannabinoids from BLA neurons to presynaptically induce a long-term depression of inhibitory GABAergic transmission (LTDi) [2,21]. Endocannabinoid LTDi in the BLA in turn, enhances excitatory synaptic transmission in the CeA [2]. A specific role for anandamide, but not 2-AG, in this form of neuronal plasticity is suggested by two complementary observations. First, LTDi is enhanced in FAAH$^{-/-}$ mice [2], which are impaired in their ability to metabolize anandamide [6]. Second, endocannabinoid mobilization in LTDi apparently requires the activation of the adenylyl-cyclase-protein kinase A pathway in the BLA, but not the phospholipase C-diacylglycerol lipase pathway [2] that is implicated in 2-AG formation [28].
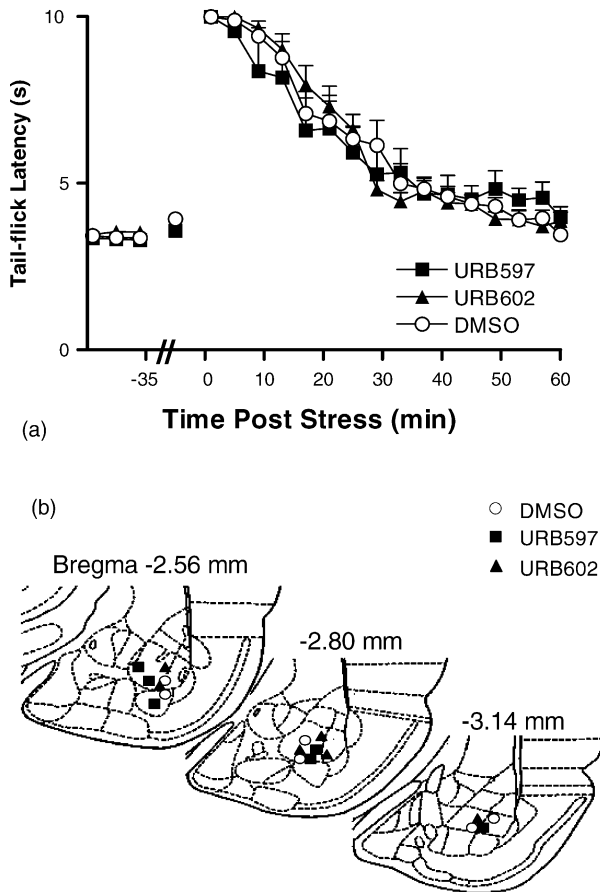
Fig. 2. Pharmacological inhibition of FAAH or MGL in the BLA does not alter nonopioid SIA in the tail-flick test. (a) Microinjection of the FAAH inhibitor URB597 (0.1 nmol) and the MGL inhibitor URB602 (0.1 nmol) into the BLA did not alter SIA, at doses that enhanced SIA following microinjection into the PAG. Data are expressed as mean ± S.E.M. (b) Coronal reconstruction of microinjection sites for groups receiving URB597 (closed squares), URB602 (closed triangles) or vehicle (open circles).

This latter finding is in contrast to our observations that 2-AG appears to be the primary endocannabinoid implicated in nonopioid SIA [14]; a strong temporal correspondence exists between cannabinoid SIA and the accumulation of 2-AG, but not anandamide, in the PAG. Liquid chromatography mass spectrometric studies are required to determine how foot shock-induced endocannabinoid mobilization in the BLA differs from that observed previously in the PAG. It is also possible that changes in SIA could be more prominent when supraspinally-mediated measures of antinociception are employed that are more sensitive to the affective dimensions of pain (formalin versus tail-flick test; see [13]). Our data do not preclude the possibility that the doses of FAAH and MGL inhibitors employed here were unable to surmount high levels of FAAH and MGL activity at this site relative to the PAG. It is also possible that enzymes other than FAAH or MGL may participate in endocannabinoid deactivation at these sites [4].

We propose that CB_1 receptor activation in the BLA removes inhibitory control over projection neurons innervating the CeA. The CeA thus receives multimodal sensory information, and in turn, coordinates appropriate behavioral, hormonal, and auto-nomic responses to stress via efferent projections [8]. The lack of effect of the FAAH inhibitor in our study is consistent with previous work demonstrating that $FAAH^{-/-}$ mice and mice treated with URB597, administered systemically, show similar amygdalar activation in response to restraint stress compared to control mice [24]. In the BLA, restraint stress produced a low level of Fos induction, which was unaffected by cannabinoid treatment, whereas, the combination of restraint stress and CB_1 agonist administration produced robust Fos induction within the CeA. These data support a synergistic interaction between environmental stress and CB_1 receptor activation in the amygdala [24] that could contribute to the behavioral phenotype observed here. CB_1 as well as FAAH and MGL immunoreactivity are abundant in the BLA, suggesting that incoming stimuli may trigger the on-demand formation of endocannabinoids to activate CB_1 receptors prior to undergoing enzymatic hydrolysis. Activation of CB_1 receptors in the BLA, in turn, reduce the inhibitory tone exerted on principal neurons, eventually propagating this signal to brain regions implicated in antinociception, most notably the PAG [10].

In the dPAG, foot shock stress stimulates mobilization of the endocannabinoids, 2-AG and anandamide [14], and microinjection of CB_1 antagonists into this site virtually abolishes nonopioid SIA [14]. Our results suggest that endocannabinoids may be differentially modulated by stress in the amygdala. For example, in the amygdala, anandamide levels are decreased after the first exposure to restraint stress whereas 2-AG levels are unchanged [25]. Our results collectively suggest that the facilitatory effects of endocannabinoids in specifically enhancing antinociceptive responses to foot shock stress occur downstream of receptor interactions in the BLA.

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