Research Article

GluA1 in central amygdala increases pain but inhibits opioid withdrawal-induced aversion

You-Qing Cai¹, Yuan-Yuan Hou¹, and Zhizhong Z Pan¹

Abstract
The amygdala is important in regulation of emotion-associated behavioral responses both to positive reinforcing stimuli such as addicting opioids and to negative aversive stimuli such as fear and pain. Glutamatergic neurotransmission in amygdala plays a predominant role in amygdala neuronal circuits involved in these emotional responses. However, how specific glutamate receptors act to mediate these amygdala functions remains poorly understood. In this study, we investigated the role of GluA1 subunits of glutamate z-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in central amygdala in modulating behavioral response to aversive stimuli by pain and by opioid withdrawal. We found that the protein level of GluA1 in the central nucleus of amygdala (CeA) was significantly increased in rats under persistent pain and viral upregulation of CeA GluA1 increased pain responses of both hyperalgesia and allodynia in rats. In contrast, the viral upregulation of CeA GluA1 inhibited, while knockdown of CeA GluA1 enhanced, place aversion induced by naloxone-precipitated morphine withdrawal. These results reveal a differential action of CeA GluA1 on the aversive event of sensory pain and opioid withdrawal, likely reflecting two distinct synaptic circuits of GluA1-predominant AMPA receptors within CeA for regulation of pain sensitivity and emotional response to opioid withdrawal.

Keywords
Morphine, conditioned place aversion, AMPA receptors, pain

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Introduction
The amygdala complex, particularly the central nucleus of amygdala (CeA) and the basolateral amygdala (BLA), plays a critical role in mediating emotional responses to both negative events (such as pain and fear) and positive events such as drug reward.¹⁻³ CeA, as the major output of the amygdala complex, receives predominant glutamatergic inputs both from peripheral sites that transmit pain signal and from BLA that functions as a hub conveying processed commanding signal from higher corticolimbic structures.⁴⁻⁸ As such, CeA has been shown to modulate behavioral responses to negative emotion-associated sensory pain and to positive emotion-associated opioid reward.⁸⁻¹⁰ Amygdala mediates these behavioral responses largely by associative leaning and consolidation of the emotional events.¹¹,¹²

Central glutamate receptors, particularly glutamate AMPA receptors (AMPARs), are essential in all neuroplasticity involved in normal brain functions such as learning and memory and in the development of neurological diseases including opioid addiction and chronic pain.²,⁸,¹³,¹⁴ AMPARs are especially crucial for learning and memory of emotional events through activity-dependent synaptic strengthening via recomposition of GluA1 and GluA2 subunits.²,¹⁵⁻²¹ This AMPAR strengthening is achieved by switching from low conductance, GluA2-containing AMPARs to high conductance, homomeric GluA1 AMPARs.¹⁵,²¹⁻²⁵ Thus, increased expression of GluA1 subunits enhances AMPAR signaling and strengthened AMPARs increase

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Pain as an aversive experience is often associated with negative emotion such as aversion and anxiety.\textsuperscript{28–31} While opioid as a rewarding drug produces positive emotion of euphoria, opioid withdrawal induces a series of negative emotional events including dysphoria and aversion.\textsuperscript{32–34} Within CeA, two distinct inputs have been identified: while the input from the parabrachial nucleus (PBN) relays peripheral pain signals,\textsuperscript{8,35,36} the input from BLA carries modulatory signals from corticolumnar structures after evaluating and decision-making processes of emotional events.\textsuperscript{9,11} Importantly, both the PBN-CeA input and the BLA-CeA input are glutamatergic,\textsuperscript{6,19,37,38} and glutamate neurotransmission in CeA has been implicated in both opioid addiction and pain.\textsuperscript{8,13,14} However, it is still largely unknown how activity of the AMPARs in the two CeA glutamate inputs affects pain sensitivity and behavioral responses to addicting opioids.

In this study, we focused on two different aversive events, pain and opioid withdrawal-induced aversion, and determined how activity in GluA1 subunits of AMPARs impacted and altered these two behavioral responses of aversion in rats under persistent pain or after morphine withdrawal.

**Materials and methods**

**Animals**

Male Wistar rats (250–300 g) were used in this study. The rats were housed in groups of three with food and water available ad libitum and in a 12 h light/dark cycle. All behavioral experiments and tests were performed between 8:00 a.m. and 18:00 p.m. To induce a persistent pain condition, a rat received a single intraplantar injection of complete Freund’s adjuvant (CFA, 50 \( \mu l \)) in a hind paw, and experiments were conducted at least one day after the CFA injection. All procedures involving the use of animals conformed to the guidelines set by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center.

**Tests for thresholds of thermal pain and mechanical pain**

All rats were extensively handled and habituated to the test environment and test apparatus for three days before the pain test. In the tail–flick test, latency to a radiant heat stimulus applied to the tail was measured every 10 min. To elicit tail–flick responses within a reliable range without a floor effect or skin damage, the heat intensity was adjusted to obtain stable baseline latencies between 6.5 and 7 s for hyperalgesic response and between 4 and 4.5 s for analgesic response. The cutoff time was 12 s. In the paw-withdrawal test for thermal hyperalgesia, a rat was placed in a Plantar Test Instrument (Model 37370, Ugo Basile, Italy), and paw-withdrawal response to an infrared heat stimulus was measured with a Hargreaves apparatus. Latency in seconds from the onset of the heat stimulus to the paw withdrawal was recorded automatically by the apparatus as threshold and was measured twice with a 5-min interval. In the test for mechanical allodynia, the rat was placed in a plastic box with mesh floor and allowed to acclimate for 20 min. A series of calibrated von Frey filaments were applied perpendicularly to the plantar surface of a hind paw with sufficient force to bend the filament. A brisk movement of the hind paw (withdrawal or flinching) was considered as a positive response. The threshold (g) of the tactile stimulus producing a 50% likelihood of withdrawal was determined by the “up-down” calculating method.\textsuperscript{39} The paw-withdrawal response was measured twice with a 5-min interval.

**Cannula implantation and intracranial microinjection**

General methods for site-specific microinjection were similar to those used in our previous studies.\textsuperscript{35,40–42} Briefly, a rat was anesthetized with isoflurane and restrained in a stereotaxic apparatus. A 26-gauge, single guide cannula (Plastic One, Roanoke, VA) was inserted on each side of the brain, aiming at CeA (anteroposterior, −2.3 mm from the Bregma; lateral, ±4.0 mm; ventral, −8.0 mm from dura).\textsuperscript{43} The guide cannula was then cemented in place to the skull and capped after placement of a solid dummy cannula with the same length as the guide cannula. The implanted rat was housed individually and allowed to recover from the surgery for at least 1 week before experiments. Bilateral microinjection of an agent or viral vector (0.5 or 1 \( \mu l \) each side) into CeA was made through a 33-gauge injector with an infusion pump at a rate of 0.1 \( \mu l \)/min. All cannula placements for bilateral CeA injections were histologically verified afterward.

**Adeno-associated virus vectors**

The construction and their functional validation of adeno-associated virus (AAV) vectors for GluA1 overexpression (AAV-GluA1) and GluA1 knockdown (AAV-GluA1-shRNA) have been described in our previous studies.\textsuperscript{35,41,44} The vector AAV-green fluorescent protein (GFP) was used as control. An animal was injected with 1 \( \mu l \) (\( \sim 5 \times 10^6 \) genome copy (GC)/\( \mu l \)) AAV-GluA1 virus, or 1 \( \mu l \) (\( \sim 2 \times 10^7 \) GC/\( \mu l \)) AAV-GluA1-shRNA virus into CeA on each side of the brain. All vector effects on GluA1 proteins were
confirmed by Western blots. Behavioral experiments were performed 10 days after the virus injection.

Western blot analysis
In separate rat groups one day and three days after the CFA injection, the rat was deeply anesthetized with isoflurane and decapitated. The brain was cut in a vibrotome in cold (4°C) artificial cerebrospinal fluid to obtain brain slices (0.5 mm thick). Both sides of CeA were punched out from the slices with a blunt-end syringe needle (0.8 mm inner diameter), frozen in liquid nitrogen, and stored in a −80°C freezer. CeA tissues were gently homogenized in sucrose buffer and centrifuged at 1000 × g. The supernatant was centrifuged at 10,000 g × 20 min, and the synaptosomal pellet was resuspended in 80 µl radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentration of each sample was determined by the detergent compatible protein assay from Bio-Rad. Equal amounts of protein (25 µg for total protein, 7.5 µg for crude synaptosome) were loaded per lane and separated on an 8% SDS-PAGE gel. The polyvinylidene difluoride membranes with transferred protein were incubated overnight at 4°C with the primary antibody against GluA1 (1:1000, Millipore, Cat. # 05-855 R) and β-actin (1:1000, Santa Cruz Biotechnology, sc-81178). After washes, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch) for 1 h. The blots were developed with ECL plus reagent (GE Healthcare). The densitometric quantification of immunoreactive bands was performed with the AlphaView software (Alpha Innotech Corp.).

Conditioned place aversion
The conditioned place aversion (CPA) procedure for naloxone-precipitated morphine withdrawal was modified from previous reports and has been described in our previous studies. CPA tests were conducted in a standard three-chamber apparatus (MED Associates, St. Albans, VT). The CPA procedure included three phases: (1) pretest (day 1) for baseline of preference/aversion behavior, (2) CPA training (days 2–5), and (3) posttest (day 6) for CPA measurement. In the pretest, after habituation to the test chambers, a rat received an injection of saline (1 ml/kg, s.c.), was placed in the center chamber and was allowed to move freely among the chambers for 15 min. The time the rat spent in each of the two conditioning chambers was recorded automatically. After the pretest, animals were randomly divided into four groups: three control groups and one CPA group. In daily injection procedure, a rat received a first injection in its home cage in the morning and 4 h later in the afternoon, it received a second injection and was immediately confined to a chamber for 40 min. On the first conditioning day (day 2), rats of all groups received an injection of saline for both the first and second injections. On the second conditioning day (day 3), the following agents were given for the first and second injections: for the three control groups: saline and saline, morphine (5.6 mg/kg, s.c.) and saline, and saline and naloxone (0.3 mg/kg, s.c.); for the CPA group: morphine and naloxone. The injection/conditioning procedures for days 2 and 3 were repeated on day 4 and 5, respectively. On day 6, all rats underwent a posttest for 15 min. The CPA was expressed as percentage of the time a rat spent in the naloxone-paired chamber versus the total test time (15 min) during the pretest and posttest. AMPA (100 ng in 0.5 µl each side) was bilaterally infused into CeA 15 min before the posttest.

Statistical analysis
Comparisons of averages of two groups were performed with the two-tailed, unpaired Student’s t test. One-way and two-way analysis of variance for repeated measures with post hoc analysis of the Bonferroni method were used to determine statistical significance in behavioral experiments for effects of treatment and between-group interactions at each time point. A p value of <0.05 was considered statistically significant. All statistical analyses were performed with the Prism software version 7.0 (GraphPad Software). Data are presented as mean ± standard error of the mean.

Results
AMPARs in CeA potentiate baseline pain response
We first examined how glutamate AMPARs in CeA modulated baseline pain behavior. We found that bilateral microinjection of the AMPAR agonist AMPA (100 ng in 0.5 µl each side) into CeA induced a significant decrease in basal pain threshold measured by the tail-flick test in rats when compared to similar CeA microinjection of saline (Figure 1(a)), suggesting that general activation of AMPARs in CeA is pronociceptive, increasing basal sensitivity of pain response. To confirm this pain-enhancing role of CeA AMPARs, we then determined the effect of blocking CeA AMPARs on the pain behavior. In contrast to the effect of AMPA, bilateral CeA microinjection of the AMPAR antagonist cyanquixaline (6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 1.5 µg in 0.5 µl each side) produced a significant antinociceptive effect, increasing the pain threshold (Figure 1(b)). These findings indicate that activity of AMPARs in CeA promotes pain behavior in normal conditions and the pronociceptive AMPAR
activity is tonically active, as its removal by AMPAR antagonist decreases basal pain responses.

**Persistent pain increases GluA1 in CeA**

Given the pain-promoting role of CeA AMPARs shown above, we determined whether the AMPAR level changes under pain conditions, measuring the protein level of GluA1, the predominant subunits of AMPARs. In rats with persistent pain induced by an intraplantar injection of CFA (50 μl) as we have shown before, we found that the protein level of GluA1 was significantly increased one day and three days after CFA injection (Figure 2). This result is consistent with the pain-promoting role of AMPARs and GluA1 subunits in CeA, indicating the possibility that the increase in the activity of CeA AMPARs contributes to the persistent pain conditions.

**Overexpression of GluA1 in CeA promotes pain**

To further validate the causal role of CeA GluA1 in promoting pain response, we used a viral vector AAV-GluA1, which has been constructed and functionally verified in our previous studies, to overexpress the GluA1 subunits of AMPARs locally in CeA, and examined the behavioral effect of this upregulated function of GluA1 and AMPARs in rats under pain conditions. In rats with bilateral infusion of the control vector AAV-GFP into CeA, an intraplantar injection of CFA (50 μl) induced persistent sensitization of inflammatory pain, which lasted more than a week as measured by the paw-withdrawal test for thermal pain of hyperalgesia and by the von Frey test for mechanical pain of allodynia (Figure 3). This CFA-induced pain sensitization is similar to that we reported previously in normal rats. However, in rats with bilateral infusion of the AAV-GluA1 vector for GluA1 overexpression, the CFA-induced hyperalgesia of thermal pain was significantly potentiated throughout the nine-day period tested when compared to the control vector-injected rats (Figure 3). When mechanical pain was measured, the GluA1 vector also significantly increased the CFA-induced pain response of allodynia although to a less extent \( p = 0.039 \) (Figure 3). These results provide evidence for a causal role of predominant GluA1-containing AMPARs in CeA in mediating heightened pain response under pain conditions.
Overexpression of GluA1 in CeA inhibits morphine withdrawal-induced aversion

We have shown previously that CeA GluA1 promotes response to opioid reward of positive emotional stimulation and maintain opioid use during opioid withdrawal. To determine the function of CeA AMPARs in regulation of opioid-related emotional behavior, we then examined the role of CeA GluA1 in response to the negative emotional state induced by opioid withdrawal. Using the paradigm of conditioned place preference (CPP)/CPA in rats with naloxone-precipitated morphine withdrawal (Figure 4(a)), we first conducted the experiments after general activation of CeA AMPARs with CeA-applied AMPA in rats. We found that bilateral microinjection of AMPA (100 ng in 0.5 μl each side) totally blocked the CPA behavior induced by naloxone-precipitated morphine withdrawal (Figure 4(b)), indicating that activation of AMPARs in CeA may inhibit negative aversion behavior under opioid withdrawal.

To further confirm this role of CeA AMPARs in inhibiting the aversion behavior, we determined the effect of GluA1 overexpression in CeA on morphine withdrawal-induced aversion 10 days after bilateral infusion of AAV-GluA1 or AAV-GFP vector into the CeA of rats. In AAV-GFP-infused control rats, naloxone-precipitated CPA behavior under normal conditions; however, in AAV-GluA1-infused rats, the aversion behavior was significantly reduced when compared to that in the control rats (Figure 4(c)). In another control experiment, CeA infusion of naloxone alone did not alter the preference/aversion behavior; and interestingly, overexpression of CeA GluA1 itself in the absence of morphine or morphine withdrawal had no effect on the preference/aversion behavior (Figure 4(d)). Thus, it appears that enhanced function of GluA1 AMPARs in CeA inhibits opioid withdrawal-induced negative response of place aversion, but unlike pain response shown above, increasing the function of CeA GluA1 AMPARs per se is ineffective in altering the preference or aversion behavior.

Knockdown of GluA1 in CeA potentiates morphine withdrawal-induced aversion

Finally, we further verified the results of this GluA1 role by knocking down the GluA1 expression in CeA with an AAV vector expressing a short-hairpin interfering RNA against the GluA1-encoding gene Gria1 (AAV-GluA1-shRNA) and a control vector with scrambled shRNA sequence, as we described and validated previously. We found that CeA infusion of the AAV-GluA1-shRNA vector for GluA1 knockdown failed to change the preference/aversion behavior under normal conditions; however, the GluA1 knockdown significantly increased the withdrawal-induced CPA behavior (Figure 5), an effect opposite to that of GluA1 overexpression shown above. These results further support the notion that the activity of GluA1 AMPARs in CeA reduces the aversive effect of opioid withdrawal.

Discussion

In this study, we have shown that GluA1, the predominant subunits of AMPARs, in CeA exerts differential actions on pain and opioid withdrawal-induced aversion, increasing pain response while decreasing aversive effect of opioid withdrawal. These results highlight the nonuniform roles of CeA AMPARs in modulation of emotional events and indicate distinct synaptic circuits of AMPARs in CeA in the modulating process.

CeA modulation of emotional event, including pain, anxiety and drug reward, has been accentuated by recent
Figure 4. Overexpression of GluA1 in central amygdala inhibits morphine withdrawal-induced aversion. (a) Schematic illustration of experimental procedures for induction of CPA by naloxone-precipitated morphine withdrawal. S, saline; M, morphine; N, naloxone; Hab, habituation. (b) CPA behavior in rats with naloxone (nlx)-precipitated morphine (mor) withdrawal after bilateral infusion of saline (n = 6 rats) or AMPA (100 ng each side, n = 8 rats) into CeA. (c, d) CPA behavior in rats with naloxone-precipitated morphine withdrawal (c, n = 9 rats each group) and in rats conditioned with saline (sal) and naloxone (d, n = 6 rats each group) after bilateral infusion of AAV-GFP or AAV-GluA1 into CeA. **p < 0.01, ****p < 0.0001 (two-way analysis of variance). CPA: conditioned place aversion; AAV: adeno-associated virus; GFP: green fluorescent protein.

Figure 5. Knockdown of GluA1 in central amygdala potentiates morphine withdrawal-induced aversion. CPA behavior in rats with naloxone-precipitated morphine withdrawal (a, n = 8 rats each group) and in rats conditioned with saline and naloxone (b, n = 6 rats each group) after bilateral infusion of AAV-GluA1-shRNA or AAV-GluA1-scrambled (scr) shRNA into CeA. **p < 0.01, ****p < 0.0001 (two-way analysis of variance).
identification of two functionally distinct glutamatergic inputs onto CeA neurons: the PBN-CeA pathway and BLA-CeA pathway. The PBN-CeA pathway relays pain signals from the spinal cord to CeA and conveys an affective pain signal that induces a threat memory. Pain is known as an aversive stimulus and is often associated with negative emotion such as aversion and anxiety. We have shown that CFA-induced persistent pain causes strong and long-lasting (> a month) place aversion in rats. Consistent with this function of the PBN-CeA input, we have shown in this study that upregulation of CeA GluA1 directly increases pain sensitivity of thermal hyperalgesia and mechanical allodynia. Thus, it appears that GluA1 in the AMPAR synapses of the PBN-CeA input functions to transmit signals of pain and associated negative emotion of aversion and anxiety. On the other hand, the BLA-CeA pathway carries processed signals from higher corticolimbic structures about evaluation and decision information on emotional events and its activation inhibits negative emotion of anxiety. We have shown in this study that upregulation of CeA GluA1 reduces opioid withdrawal-induced aversion. Thus, it is likely that GluA1 in the AMPAR synapses of the BLA-CeA input functions to inhibit negative emotion of anxiety and opioid withdrawal. While the viral upregulation of CeA GluA1 in this study is not pathway-specific, the notion of distinct functions of the PBN-CeA and BLA-CeA glutamate pathways in CeA modulation of emotional events is demonstrated by our recent pathway-specific study in which we show that, while selective optogenetic activation of the PBN-CeA pathway causes behaviors of negative emotion including aversion, anxiety and depression in normal rats, optogenetic activation of the BLA-CeA pathway opposes these behaviors of negative emotion, inhibiting anxiety and depression and promoting positive emotion of opioid reward. Therefore, the distinct functions of the two glutamatergic CeA inputs likely underlie the current results of increasing aversive pain but reducing aversive opioid withdrawal by nonselective viral upregulation of GluA1 in CeA.

Adaptive change in GluA1 of AMPARs is an integrative part of central synaptic plasticity involved in both normal brain functions such as learning and memory and emotion-related pathological conditions including pain and drug addiction. Under those conditions, synaptic signaling of AMPARs is strengthened by adaptive upregulation of GluA1 subunits relative to GluA2 subunits through subunit recomposition of AMPARs in glutamate neurotransmission. Thus, pain conditions have been shown to elevate the GluA1 level and associated AMPAR function in central pain-processing sites. Our present results show that upregulation of CeA GluA1 is sufficient to increase pain, suggesting a causal role of CeA GluA1 and AMPARs in promoting pain response. GluA1 expression is also increased by addicting drugs of abuse such as morphine and cocaine in the amygdala and other structures of the brain’s reward circuits. Upregulation of GluA1 in the ventral tegmental area potentiates the rewarding effect of morphine and as we have shown before, CeA-applied AMPA is rewarding by inducing CPP and GluA1 upregulation in CeA facilitates associative learning and acquisition of morphine reward. Opposite to opioid reward, opioid withdrawal is associated with aversion and other negative emotions, nevertheless, GluA1 level in CeA, as we reported recently, is also increased during morphine withdrawal in rats. While this increase might be related to associative learning of the withdrawal condition, knockdown of CeA GluA1 inhibits morphine-seeking behavior after morphine withdrawal, suggesting that CeA GluA1 maintains morphine-seeking behavior after opioid withdrawal, which is consistent with a general, reward-promoting role of CeA GluA1. Thus, it appears that GluA1 in CeA promotes opioid use after opioid withdrawal as reflected in its effects of both maintaining opioid seeking and reducing withdrawal-induced aversion.

A quite interesting question is how the two input-specific circuits of GluA1 AMPARs in CeA that differentially regulate pain and opioid-related emotional responses interact and influence each other. The synaptic connections between the two AMPAR circuits or the two CeA glutamate inputs are still unknown. Nevertheless, we have recently shown that the direct interaction most likely occurs within CeA, as activating the BLA-CeA pathway inhibits various negative emotions induced by activation of the PBN-CeA pathway, suggesting two parallel and interacting AMPAR synaptic circuits and neuronal populations within CeA. Previous studies also suggest circuit-specific and neuron-specific encoding of negative and positive behavioral outcomes in amygdala. Thus, these AMPAR circuits and neurons in CeA, the major output of the amygdala complex, receive and process the signal of peripheral pain and associated emotion, which is then integrated with the modulating signal from higher corticolimbic structures via BLA for the ultimate output signal that regulates both pain sensitivity and emotional responses to emotion-affecting events such as opioid use.

In summary, we have shown in this study that, while GluA1 upregulation in CeA is sufficient to increase the aversive response of pain, it decreases the aversion of opioid withdrawal, revealing two differential modulating effects of CeA GluA1 on pain sensitivity and on emotional aspect of opioid withdrawal. These results highlight the diverse functions of GluA1-dominant AMPARs in CeA in regulation of different aspects of emotion-associated stimuli and behavioral conditions.
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