

Inhibition of CaMKII α in the Central Nucleus of Amygdala Attenuates Fentanyl-Induced Hyperalgesia in Rats

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ABSTRACT

Opioid-induced hyperalgesia (OIH) is a less-studied phenomenon that has been reported in both preclinical and clinical studies. Although the underlying cause is not entirely understood, OIH is a real-life problem that affects millions of patients on a daily basis. Research has implicated the important contribution of Ca²⁺/calmodulin-dependent protein kinase II α (CaMKII α) to OIH at the level of spinal nociceptors. To expand our understanding of the entire brain circuitry driving OIH, in this study we investigated the role of CaMKII α in the laterocapular division of the central amygdala (CeLC), the conjunctive point between the spinal cord and rostral-ventral medulla. OIH was produced by repeated

fentanyl administration in the rat. Correlating with the development of mechanical allodynia and thermal hyperalgesia, CaMKII α activity was significantly elevated in the CeLC in OIH. In addition, the frequency and amplitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs) in CeLC neurons were significantly increased in OIH. 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]-amino-N-(4-chlorocinnamyl)-N-methylbenzylamine, a CaMKII α inhibitor, dose dependently reversed sensory hypersensitivity, activation of CeLC CaMKII α , and mEPSCs in OIH. Taken together, our data for the first time implicate a critical role of CeLC CaMKII α in OIH.

Introduction

Opioids play an important role in every aspect of modern anesthesia and pain medicine. However, the administration of opioids has sometimes been found to produce opioid-induced hyperalgesia (OIH), defined as a lowered pain threshold caused by opioid exposure (Vanderah et al., 2001; Mao, 2002; Ossipov et al., 2005). Opioids also cause analgesic tolerance that shares similar manifestations of symptoms with OIH since opioid drugs exhibit diminished efficacy with time in both cases (Kim et al., 2014; Lee and Yeomans, 2014). However, OIH differs from opioid tolerance in that increasing opioid dose aggravates pain in OIH, whereas tolerance is generally countered by higher doses (Lee and Yeomans, 2014; Stoicea et al., 2015). The presence of OIH can be a clinical challenge not only in chronic pain management but also in perioperative pain. For the latter, fentanyl is commonly used in routine anesthesia practice given its potent, fast, and short analgesic action. While most OIH studies have focused on the prototype opioid drug morphine, hyperalgesia after fentanyl

exposures needs more and urgent scientific and medical attention.

The neurobiology of OIH is complex and several mechanisms have been proposed (Guignard et al., 2000; Ossipov et al., 2005; Lee and Yeomans, 2014). A number of pronociceptive mechanisms proposed thus far include the activation of central glutamatergic pathways, descending facilitation, alteration of endogenous opioid ligands, and others, for inducing OIH (Célèrier et al., 2000; Vanderah et al., 2001; Lee and Yeomans, 2014). The Ca²⁺/calmodulin-dependent protein kinase II α (CaMKII α), colocalized with the μ -opioid receptor in the spinal cord and central nucleus of amygdala [i.e., laterocapular division of central amygdala (CeLC)] (Brüggemann et al., 2000), is a multifunctional serine/threonine protein kinase that plays a prominent role in glutamate neurotransmission and experience-dependent plasticity at the synaptic level (Lisman et al., 2002; Salling et al., 2016), which makes it a highly significant target in the search for neural mechanisms of OIH. It has been demonstrated that spinal CaMKII α activity is required for the initiation and maintenance of OIH. Intrathecal CaMKII α inhibition can attenuate OIH (Chen et al., 2010) and opioid tolerance and dependence (Tang et al., 2006; Yang et al., 2011). CaMKII α has also been reported to contribute to hyperalgesia priming, a phenomenon

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ABBREVIATIONS: CaMKII α , Ca²⁺/calmodulin-dependent protein kinase II α ; CeLC, laterocapular division of the central amygdala; KN92, 2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; KN93, 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; mEPSC, miniature excitatory postsynaptic current; NMDAR, N-methyl-D-aspartate receptor; OIH, opioid-induced hyperalgesia; p-CaMKII α , phosphorylated Ca²⁺/calmodulin-dependent protein kinase II α ; TRPV1, transient receptor potential vanilloid type 1.

implicated in the transition from acute to chronic pain (Ferrari et al., 2013).

However, neither the peripheral nor the spinal mechanisms can completely explain the cause of OIH (Chu et al., 2011). In this study, we investigated the CaMKII α mechanism in the CeLC, a target of the spino-parabrachio-amygdaloid pain pathway that has been considered as the nociceptive amygdala (Neugebauer et al., 2004; Sarhan et al., 2005, 2013). The CeLC is also the upstream of periaqueductal gray/rostral-ventral medulla/spinal descending pain pathway (Fabry et al., 2003; Neugebauer et al., 2004; Carrasquillo and Gereau, 2007; Hamlin et al., 2007; Tracey and Mantyh, 2007; Martin and Ewan, 2008) that may be involved in OIH (Vanderah et al., 2001; Ossipov et al., 2005). Since spontaneous miniature excitatory postsynaptic currents (mEPSCs), which are observed in the absence of presynaptic action potentials, are frequently used as a parameter to reflect altered synaptic transmission responsible for inflammatory pain (Zhao et al., 2006) and neuropathic pain (Wang et al., 2007; Xu et al., 2008), we determined the changes in CaMKII α activity and mEPSCs in CeLC neurons in a rat model of fentanyl-induced hyperalgesia.

Materials and Methods

Materials

Fentanyl (fentanyl citrate injection) was obtained from Yi Chang Humanwell Pharmaceutical Co., Ltd (Yi Chang, China). 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN93) and 2-[*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN92) monohydrochloride) were purchased from Cayman (Ann Arbor, MI). Other chemicals were obtained from Beyotime (Shanghai, China) and Boster (Shanghai, China).

Animals

Male Sprague-Dawley rats (50–70 g, from the animal laboratory at Tongji Medical College, Huazhong University of Science and Technology) were provided food and water ad libitum prior to the experiments. All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and conducted in accordance with the National Institutes of Health's guide for the care and use of laboratory animals and the policies and recommendations by the International Association for the Study of Pain (Zimmermann, 1983).

OIH Induced by Repeated Subcutaneous Administration of Fentanyl. OIH was induced in rats by four injections of fentanyl (60 μ g/kg per injection, s.c.) at 15-minute intervals, resulting in a cumulative dose of 240 μ g/kg/rat (Célèrier et al., 2000). This is an activity-dependent pain model, i.e., signs of spontaneous pain are typically not observed in the absence of external stimulation or movement (Toyoda et al., 2009). This is a model of intermittent fentanyl injections, rather than chronic administration that can also induce opioid tolerance (Zissen et al., 2007). The nociceptive thresholds were evaluated by mechanical and thermal stimulation at different time points, including 0 (baseline), 1, 5, 6, 6.5, 7, and 8 hours and 1, 2, 3, 4, and 5 days after the last injection of fentanyl (time 0).

Assessment of Mechanical Allodynia

Mechanical sensitivity was assayed by the up-and-down paradigm using von Frey filaments (North Coast, San Jose, CA) according to the Dixon method (Luo et al., 2008; Chen et al., 2009). In brief, rats were individually placed into Plexiglas chambers over a mesh table and acclimated for 30 minutes before the test. Beginning with 1.0 g, each

von Frey filament was applied to the mid-plantar surface of the left hind paw for 5 seconds or until a withdrawal response occurred. Positive response was defined as paw flinching or brisk withdrawal. The time interval between each test was more than 5 minutes. The 50% probability of paw withdrawal threshold was determined by an up-and-down algorithm described previously (Dixon and Mood, 1948; Luo et al., 2008).

Assessment of Thermal Hyperalgesia

Thermal sensitivity was measured using a radiant thermal stimulator (BME-410C; Biomedical Engineering, Boerni science and technology limited company, Guangzhou, China) according to the Hargreaves' method (Hargreaves et al., 1988; Luo et al., 2008; Chen et al., 2009). Rats were placed in a clear plastic enclosure and allowed to acclimatize for 30 minutes before assessment. A radiant heat source was focused onto the plantar surface of the left hind paw through the glass floor. Measurement of thermal sensitivity was started by the activation of the heat source and automatically stopped when paw withdrawal occurred. A cutoff time of 15 seconds was applied to prevent tissue damage.

Western Blotting

Immediately after rats were euthanized, the CeLC was quickly dissected out, frozen, and stored at -80°C before western blotting analysis (Chen et al., 2010). In brief, tissues were homogenized in radio-immuno-precipitation assay buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β -glycerophosphate, EDTA, Na_3VO_4 , and leupeptin] and protein concentration was determined using the bicinchoninic acid method (Beyotime). Samples (20 μ g of total protein) were separated by 10% SDS-PAGE and transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% skim milk for 2 hours at room temperature and incubated overnight in 5% skim milk with a rabbit anti-(T286) phosphorylated CaMKII α (*p*-CaMKII α) antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:500; Boster). After the incubation, blots were washed and incubated at room temperature for 90 minutes with horseradish peroxidase-conjugated anti-rabbit (for *p*-CaMKII α) or anti-mouse (for glyceraldehyde-3-phosphate dehydrogenase) secondary antibody IgG (1:10,000; Boster). The *p*-CaMKII α antibody detected double bands in the experiments, both of which correspond to *p*-CaMKII α (Hu et al., 2016). Signals from both bands were combined for quantification. The immunoreactivity was detected using enhanced chemiluminescence (Thermo Fisher Shanghai, China) and enhanced chemiluminescence signals were detected using a Bio-Rad (Shanghai, China) ChemiDoc system. The *p*-CaMKII α immunoreactivity was expressed as the ratio of the optical densities of *p*-CaMKII α to those of glyceraldehyde-3-phosphate dehydrogenase.

Intra-CeLC Cannulation and Microinjection

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and held in a stereotaxic frame (Zenda, Austin, TX), as previously reported (Han et al., 2010; Sarhan et al., 2013; Zhang et al., 2013). A guide cannula (RWD Life Science, Shenzhen, China) was implanted toward the CeLC in the right hemisphere, using the following coordinates (in mm): 2.2 caudal to bregma, 4.2 lateral to midline, and depth 7.5 according to the Paxinos and Watson flat skull coordinate system (Butler et al., 2011). A 33-gauge dummy cannula was inserted in each guide cannula to avoid clogging. Following cannulation, animals were housed singly and allowed to recover for 5 days prior to the experiment. For drug infusion, the dummy cannula was replaced by an injector that was inserted 0.5 mm beyond the guide cannula to target the CeLC. One end of the tubing was connected to the injector and the other to a 10 μ l Hamilton syringe. The solution was infused with a pump at 0.25 μ l/min for a total of 0.5 μ l on the right

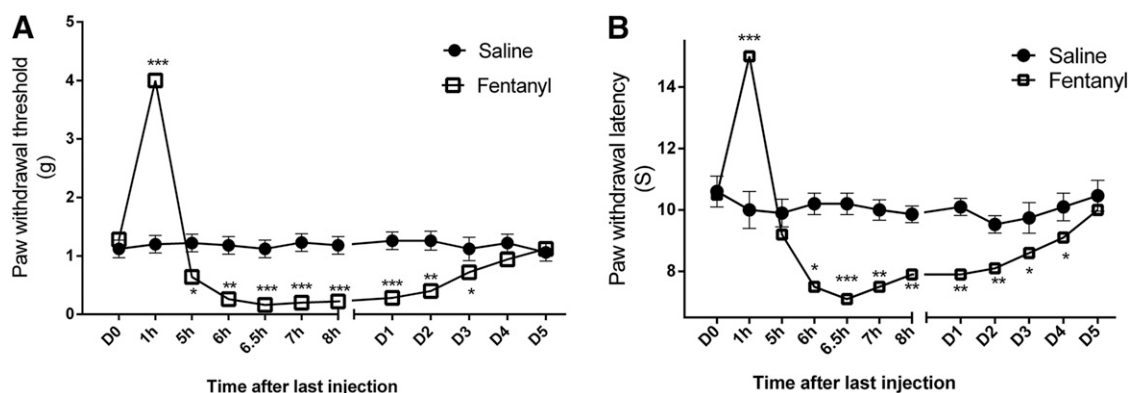


Fig. 1. Repeated fentanyl administration induced OIH including mechanical allodynia (A) and thermal hyperalgesia (B). Rats received saline or fentanyl sulfate on day 0 (60 $\mu\text{g}/\text{kg}$, 4 times, 15-minute intervals, s.c.). The paw withdrawal thresholds to von Frey filament probing and withdrawal latencies to radiant heat were determined. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the saline-treated group, two-way analysis of variance followed by Bonferroni post hoc test. Data represent mean \pm S.E.M. of six rats per group.

CeLC. After infusion, the injector was left in the cannula for another minute to allow the chemicals to diffuse into the injected area. At the end of experiments, the placement of the cannula was verified histologically by injecting 0.5 μl of eosin in the same manner as described previously.

Electrophysiology Experiment

Slice Preparation. Coronal brain slices (350 μm) containing CeLC were obtained from the right hemisphere as described previously (Li et al., 2011) with cutting solution at 4°C. The dissection solution contained (in mM) 213 sucrose, 3 KCl, 1 NaH_2PO_4 , 0.5 CaCl_2 , 5 MgCl_2 , 26 NaHCO_3 , and 10 glucose. After incubation at 25°C for at least 1 hour, a single brain slice was placed in a submerged recording chamber and perfused continuously at a rate of 2 ml/min with artificial cerebrospinal fluid equilibrated with 95% O_2 and 5% CO_2 at 30°C. The artificial cerebrospinal fluid contained (in mM) 125 NaCl, 5 KCl, 1.2 NaH_2PO_4 , 2.6 CaCl_2 , 1.3 MgCl_2 , 26 NaHCO_3 , and 10 glucose. Only 1 to 2 brain slices per animal were used, and only one neuron was recorded in each slice.

Whole-Cell Patch-Clamp Recording. Recording of mEPSCs was performed using whole-cell voltage-clamp methods as previously described (Li et al., 2011). The CeLC neurons were easily discerned under light microscopy and visualized in a transparent circular region adjacent to the basal lateral amygdala (Sah et al., 2003; Fu et al., 2008; Watabe et al., 2013). Electrodes (impedance was 4–6 $\text{M}\Omega$) made from borosilicate glass capillaries (1.5 mm outer diameter, 1.0 mm inner diameter; WPI, Sarasota, FL) were filled with the following internal solution (in mM): 145 KCl, 5 NaCl, 10 HEPES, 5 EGTA, 4 Mg-ATP, and 0.3 $\text{Na}_3\text{-GTP}$. A dual four-pole Bessel filter (Warner Instruments, Hamden, CT), a low-noise Digidata 1322 interface (Molecular Devices, Sunnyvale, CA), HEKA EPC-10 amplifier (HEKA, Lambrecht, Germany), a Pentium PC, and PATCHMASTER software (Molecular Devices) were used for data acquisition and analysis. The head stage voltage was monitored continuously on an oscilloscope to ensure precise performance of the amplifier. High (>2 $\text{G}\Omega$) seal and low (<20 $\text{M}\Omega$) series resistances were checked throughout the experiment [using the pCLAMP10 membrane test function (Molecular Devices)] to ensure high-quality recordings. The mEPSCs were recorded in the presence of 50 μM picrotoxin and 1 μM tetrodotoxin at a holding potential of -70 mV and measured 10 minutes before and 15 minutes after drug application, as described previously (Kiritoshi et al., 2013). A fixed length of traces (5 minutes) was analyzed for frequency and amplitude distributions with Mini Analysis Program 6.0 (Synaptosoft Inc, Fort Lee, NJ) and pCLAMP10 software.

Statistics. The number of rats for behavioral experiments was estimated by power analyses with the aid of the SSize2021 software (National University of Singapore, Singapore) (version 2). With

anticipated $P_1 = 0.95$ and $P_2 = 0.05$, preset analysis power of 0.9, and level of significance of 0.05, the minimal group size was estimate to be four/group. All data are presented as mean \pm S.E.M. Comparisons between groups were analyzed using Student's t test (two groups) or analysis of variance followed by the Bonferroni post hoc test (multiple groups). All graphs and statistical analysis were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). A value of $P < 0.05$ was considered statistically significant.

Results

Fentanyl Induced Mechanical Allodynia and Thermal Hyperalgesia in a Time-Dependent Manner in the Rat. Fentanyl is a commonly used clinical anesthetic agent with significant problems of OIH. To study fentanyl-induced

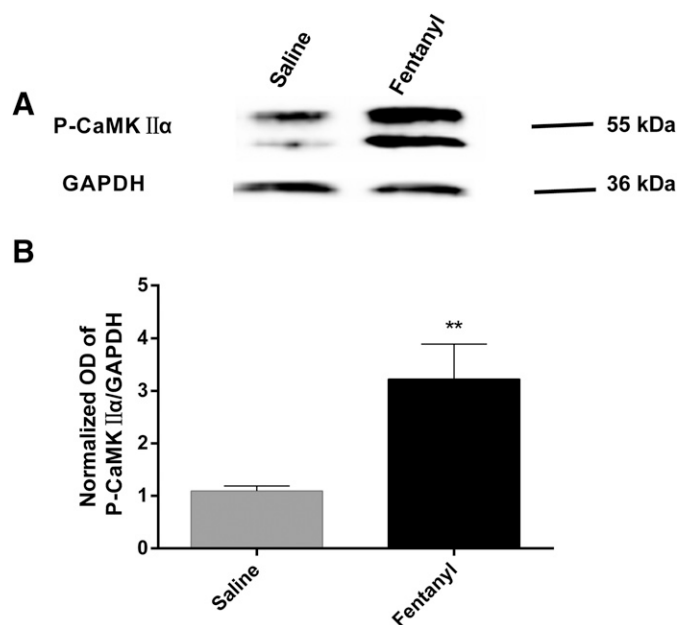


Fig. 2. Repeated fentanyl administration induced CaMKII α activation (p-CaMKII α) in the CeLC. (A) Representative immunoblots of activated CaMKII α (p-CaMKII α) in the CeLC after repeated saline or fentanyl treatment (60 $\mu\text{g}/\text{kg}$, 4 times, 15-minute intervals, s.c.). (B) Histogram of p-CaMKII α , ** $P < 0.01$ compared with the saline group, Student's t test ($t = 3.58$, $n = 12$).

hyperalgesia, we established a preclinical model using four intermittent injections (s.c.) of fentanyl that resulted in significant increases in mechanical and thermal sensitivities compared with saline-treated rats (Fig. 1). Fentanyl initially increased mechanical thresholds and thermal latencies as expected due to the drug's acute analgesic action. Five hours after the last dose of fentanyl, the analgesic effect diminished and detectable mechanical allodynia (Fig. 1A) and thermal hyperalgesia (Fig. 1B) developed, which lasted for 3 days (mechanical allodynia) to 4 days (thermal hyperalgesia).

Fentanyl Increased the Level of *p*-CaMKII α in the CeLC. To evaluate the potential effect of fentanyl on CaMKII α activity, the level of CaMKII α phosphorylation at Thr-286 (*p*-CaMKII α) in the CeLC was determined using the western blotting method 24 hours after induction of OIH (Fig. 2). Compared with saline-treated rats, the content of *p*-CaMKII α in the CeLC was significantly increased in the rat with fentanyl-induced OIH ($P < 0.05$, $n = 12$).

Fentanyl-Induced Hyperalgesia Was Reversed by KN93 Microinjected into the CeLC. To further investigate the functional role of CeLC CaMKII α in OIH, rats were implanted with CeLC cannulas before induction of OIH by fentanyl. After OIH was established, KN93 (a CaMKII

inhibitor) or KN92 (a kinase-inactive chemical analog of KN93) was microinjected into the CeLC via cannulas 6.5 hours after the last dose of fentanyl administration. The established fentanyl OIH was rapidly attenuated by KN93 in a dose-dependent manner (Fig. 3). KN93 (5–7.5 nmol) partially suppressed mechanical allodynia and thermal hyperalgesia. At the highest dose used, KN93 (10 nmol) completely abolished allodynia and hyperalgesia (Fig. 3) ($*P < 0.05$, $***P < 0.001$, compared with the vehicle-treated group, $n = 8–10$ per group). As controls, the same treatment with KN92 (10 nmol) or vehicle (equal volume) did not alter the pain threshold. These data suggested a functional role of CeLC CaMKII α in OIH.

Intra-CeLC KN93 Microinjection Inhibited CeLC *p*-CaMKII α in OIH. To correlate the behavioral effects with biochemical changes, CaMKII α activity (*p*-CaMKII α) in the CeLC was determined by analyzing the level of phosphorylation (*p*-CaMKII α). The KN93 dose dependently reversed fentanyl-induced activation of *p*-CaMKII α . At the highest dose used, KN93 (10 nmol) significantly attenuated *p*-CaMKII α ($P < 0.001$, compared with vehicle group, $n = 6$). Lower doses of KN93 (5 and 7.5 nmol) had a less potent inhibition effect on *p*-CaMKII α . In contrast, fentanyl-induced activation of

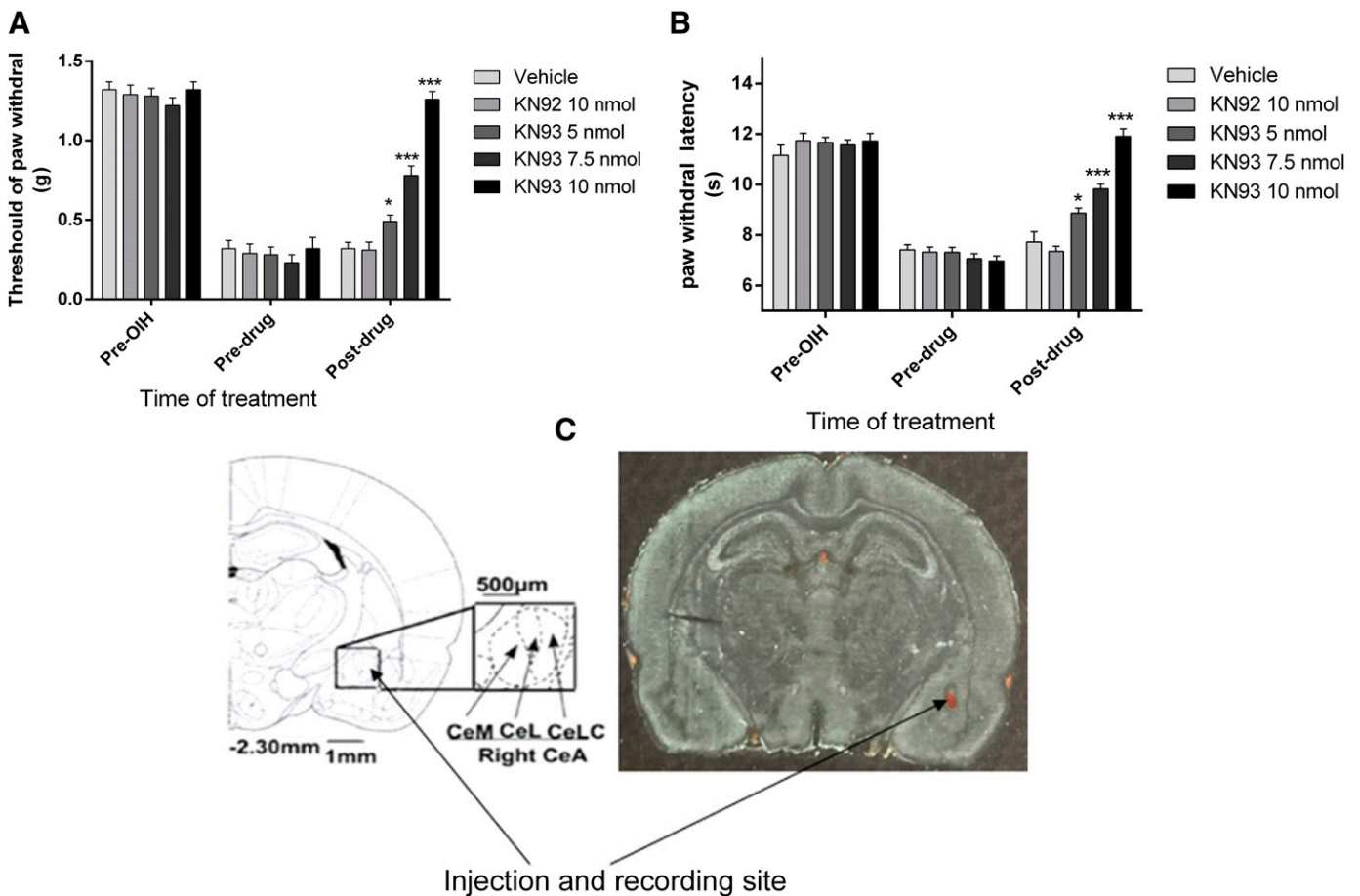


Fig. 3. Dose-dependent reversal of fentanyl-induced mechanical allodynia (A) and thermal hyperalgesia (B) by microinjecting KN93 into the CeLC (C). Rats received saline or fentanyl sulfate to induce OIH (see Fig. 1). KN93 (5–10 nmol), KN92 (10 nmol), or vehicle was administered 6.5 hours after the last dose of fentanyl. Mechanical allodynia and thermal hyperalgesia were tested 30 minutes after injection. KN93, but not KN92, reversed the established fentanyl-induced mechanical allodynia ($F = 70.16$) and thermal hyperalgesia ($F = 50.08$) in a dose-dependent manner. Data are expressed as mean \pm S.E.M. $*P < 0.05$, $***P < 0.001$, compared with the vehicle-treated group, one-way analysis of variance followed by Bonferroni post hoc test, $n = 8–10$ for each group. The cannula placement in the CeLC was verified at the end of the experiments (C).

CaMKII α was not changed by KN92 (10 nmol) (Fig. 4). These data suggested that repeated fentanyl administration induced CaMKII α activation, mechanical allodynia, and thermal hyperalgesia, all of which were attenuated by inhibiting the activity of CeLC CaMKII α with KN93, but not the kinase-inactive chemical analog KN92. Therefore, CeLC CaMKII α is critical for the persistent of OIH.

Suppression of mEPSCs in CeLC Neurons from the OIH Rats by KN93. The analysis of the amplitude and frequency distribution of mEPSCs in the presence of tetrodotoxin can be used to determine pre- versus postsynaptic mechanisms. Presynaptic changes at the transmitter release site affect mEPSC frequency, whereas changes at the postsynaptic membrane alter mEPSC amplitude (quantal size) (Toyoda et al., 2009; Han et al., 2010). We recorded mEPSCs in the CeLC in the presence of 1 μ M tetrodotoxin. KN93 (10 μ M) (Goforth et al., 2004; Shen et al., 2009; Seto et al., 2013) significantly decreased both the frequency (Fig. 5C) and amplitude (Fig. 5D) of mEPSCs recorded from CeLC neurons in slices from OIH rats (12 hours postinduction), but not those from the vehicle-treated rats ($P < 0.05$, $n = 4-7$).

Discussion

In this study, we examined the role of CaMKII α in an experimental model of OIH induced by the anesthetic agent fentanyl. Rats received four injections (s.c) of fentanyl at 15-minute intervals, which were highly reproducible to induce OIH. C  lerier et al. (2000) tested different doses (20–100 μ g/kg) of fentanyl and found that the higher the dose used, the more pronounced was the fentanyl-induced hyperalgesia. In

our pilot experiments, we found that fentanyl at a moderate dose (60 μ g/kg) was the optimum dose because of increased mortality after higher doses.

We found that CaMKII α activity in the CeLC was increased in OIH rats, and this increased CeLC CaMKII α activity correlates well with increased neuronal activity and behavior hyperalgesia. Moreover, inhibition of CeLC CaMKII α by microinjection of KN93 reversed established fentanyl-induced hyperalgesia in a dose-dependent manner, correlating with decreased *p*-CaMKII α . Meanwhile, the frequency and amplitude of mEPSCs in CeLC cells were reduced by KN93 in rats with OIH. In contrast, the kinase-inactive control compound KN92 did not affect *p*-CaMKII α activity or OIH. These data, for the first time, implicated a critical role of CeLC CaMKII α for the maintenance of fentanyl-induced OIH in young rats. Whether this result can be extrapolated to older rats needs to be further studied.

OIH is an activity-dependent pain model, i.e., signs of pain are typically not observed in the absence of external stimulation or movement (Neugebauer et al., 2007). Indeed, lidocaine or clonidine could not induce conditioned place preference in uninjured animals (He et al., 2012) including animals with OIH (He, Hu, and Wang, unpublished data). The present study demonstrates directly that increased amygdala activity in the absence of tissue or nerve injury can exacerbate physiologic pain responses, such as thermal hyperalgesia evoked by noxious heat stimuli and allodynia induced by otherwise in noxious stimulation.

It has reported that the right amygdala develops pain-related plasticity that is coupled to pain facilitation in the arthritis pain model (Han and Neugebauer, 2005). Because of

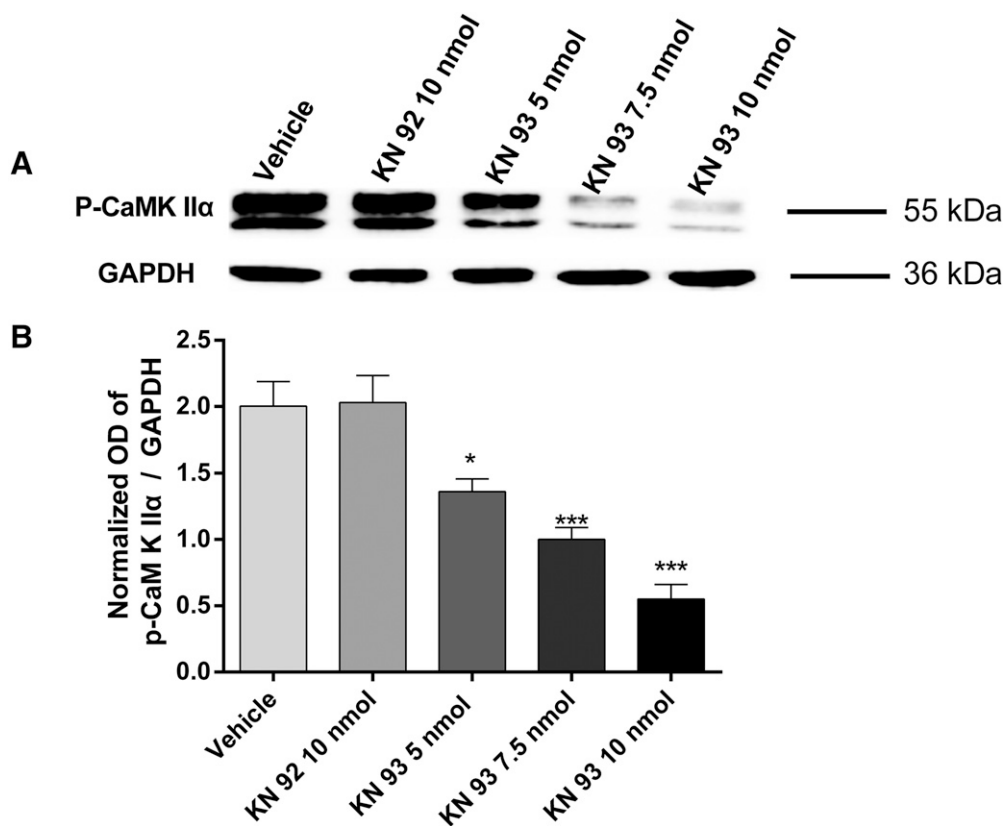


Fig. 4. Suppression of fentanyl-induced CaMKII α activation by KN93 in the CeLC. Rats with fentanyl-induced OIH were treated (intra-CeLC) with KN93 (5–10 nmol), KN92 (10 nmol), or vehicle 6.5 hours after the last dose of fentanyl. One hour later, rats were sacrificed and the central amygdala was taken for the analysis of CaMKII α activation using the immunoblotting method, by determining the degree of CaMKII α T286 autophosphorylation (*p*-CaMKII α). KN93, but not its inactive analog KN92, reversed fentanyl-enhanced CaMKII α activation. (A) Representative immunoblots for *p*-CaMKII α . (B) Histogram of CaMKII α activation. ** $P < 0.01$, *** $P < 0.001$, compared with the vehicle-treated group, $F = 47.07$, one-way analysis of variance followed by Bonferroni post hoc test, $n = 6$ for each group.

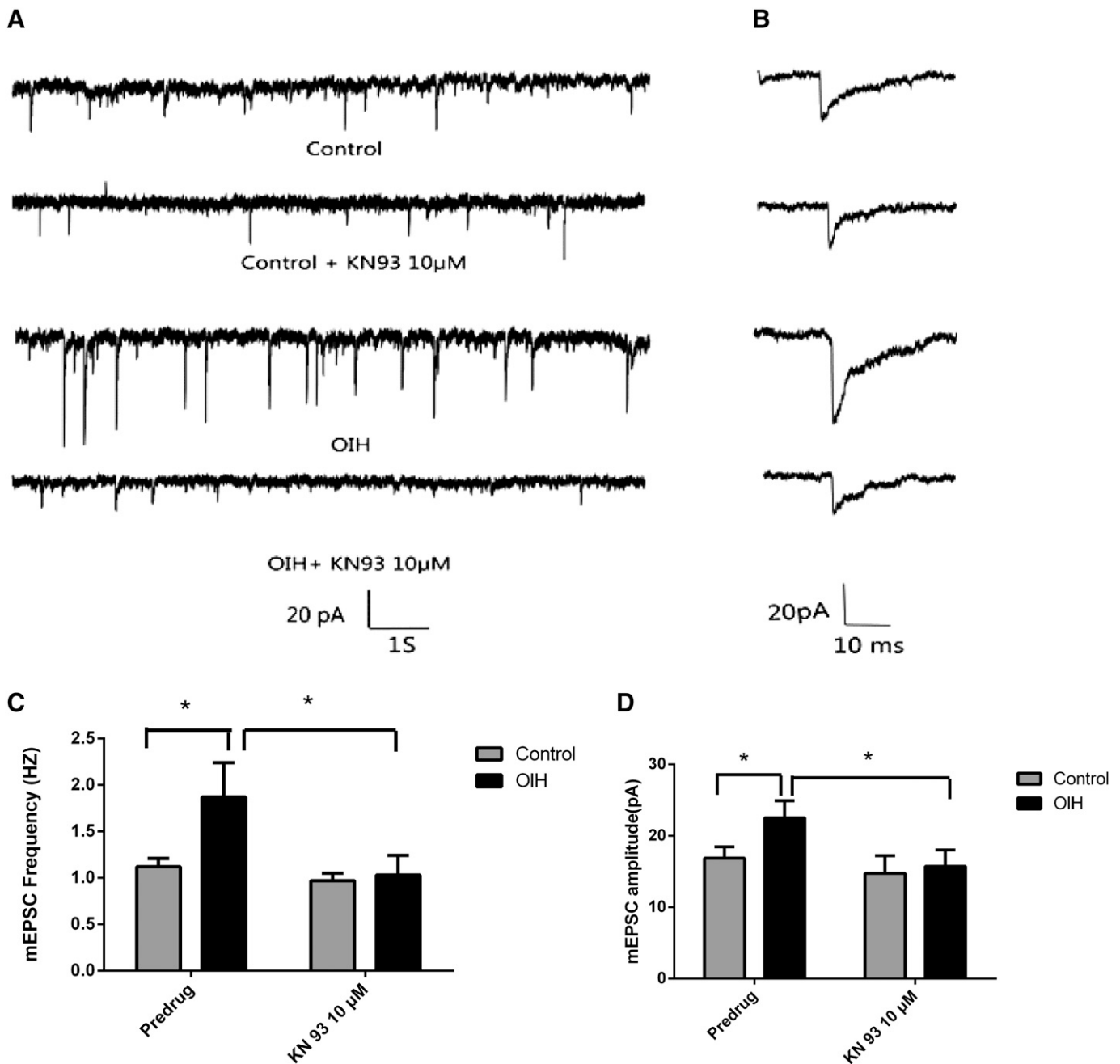


Fig. 5. Suppression of mEPSC frequency and amplitude by KN93 in neurons from OIH rats. (A) mEPSC recordings in control and OIH groups at baseline and after the application of KN93. Calibration: 1 second, 20 pA. (B) Individual mEPSCs obtained from respective recordings. Calibration: 10 ms, 20 pA. (C and D) Bar graphs showing the frequency (C) and amplitude (D) of mEPSCs in the CeLC. Note that the frequency ($t = 2.9$, $P < 0.05$) and amplitude ($t = 3.35$, $P < 0.05$) of mEPSCs were significantly increased in OIH neurons. Also note that the frequency ($t = 3.11$, $P < 0.05$) and amplitude ($t = 3.74$, $P < 0.05$) of mEPSCs were significantly suppressed by KN93. * $P < 0.05$, Student's t test, $n = 4-7$ neurons from each group.

the strong contralateral projection of the spino-parabrachio-amygdaloid pain pathway (Neugebauer et al., 2004), we chose to perform behavioral tests in the rat left plantar, while analyzing the level of p -CaMKII α and recording mEPSCs by whole-cell patch-clamp in the right CeLC.

In addition to CaMKII α , several other potential mechanisms underlying OIH have been suggested (Lee and Yeomans, 2014), such as the β 2-adrenergic receptor (Jordan et al., 2003), spinal cyclooxygenase (Dunbar et al., 2000), as well as local cytokine production (Liang et al., 2008). To date, central sensitization, providing a mechanistic explanation for

how low-threshold A or C fibers can begin to transmit pain, has been generally accepted as an important mechanism in the development of OIH (Chu et al., 2008). Indeed, central sensitization includes both homosynaptic and heterosynaptic facilitations (Sandkühler, 2007), whereas heterosynaptic facilitation alone is responsible for secondary hyperalgesia and allodynia, in which activity in one set of synapses enhances activity in nonactivated synapses, typically by sensitizing the entire neuron (Latremoliere and Woolf, 2009).

The analysis of mEPSCs is a well-established electrophysiological approach to determine pre- versus postsynaptic

mechanisms. Miniature postsynaptic currents are assumed to represent the spontaneous release of individual vesicles or quanta of neurotransmitters from the presynaptic membrane (Kaeser and Regehr, 2014). Presynaptic changes at the transmitter release site affect frequency, whereas changes at the postsynaptic membrane would alter amplitude (quantal size) (Wyllie et al., 1994). Based on the analysis of miniature synaptic events in this study, we found that KN93 decreased both the frequency and amplitude of mEPSCs in neurons from the OIH rats. These data suggest that *p*-CaMKII α regulate synaptic transmission in CeLC neurons through pre- and postsynaptic mechanisms of action, which is consistent with a previous study indicating that both pre- and postsynaptic CaMKII α are necessary for the induction of synaptic plasticity (Ninan and Arancio, 2004). Therefore, reduced excitatory synaptic transmission after KN93 injection is attributable to not only a decrease in probability of presynaptic neurotransmitter release but also to a decrease of postsynaptic responsiveness.

CaMKII α makes up 2% of the total postsynaptic density protein. It has been demonstrated that the *N*-methyl-D-aspartate receptor (NMDAR) is both a trigger and effector of central sensitization (Woolf and Thompson, 1991; Latremoliere and Woolf, 2009). Activation of the NMDAR is an essential step in both initiating and maintaining activity-dependent central sensitization since its blockade by NMDAR antagonists prevent or reverse OIH (Célèrier et al., 2000; Rivat et al., 2002). The postsynaptic membrane of the NMDAR is activated and drives aside the Mg²⁺ ion in the receptor channel. With the opening of the NMDAR, Ca²⁺ ion permeability increases. A large amount of Ca²⁺ enters into the cell and then further activates intracellular Ca²⁺-dependent protein kinases, such as CaMKII α (Lisman et al., 2002). After CaMKII α phosphorylation and binding to NR2B, the excitability of the NMDAR and Ca²⁺ influx increases and further activates CaMKII α , forming a positive feed-forward mechanism (Wang and Wang, 2003; Wilkie et al., 2010).

CaMKII α is also expressed at the presynaptic nerve terminal where it associates with synaptic vesicles (Wang, 2008). Although presynaptic CaMKII α appears to have complex functions in synaptic transmission, studies suggest that presynaptic CaMKII α enhances vesicle motility and facilitates spontaneous neurotransmitter release through promoting synaptic vesicle translocation and increasing Ca²⁺ entry (Wang, 2008). The transient receptor potential vanilloid type 1 (TRPV1) channel, which has been reported to be an essential peripheral mechanism in the expression of morphine-induced hyperalgesia (Vardanyan et al., 2009), is involved in the regulation of synaptic transmission centrally (Shoudai et al., 2010) or peripherally (Sikand and Premkumar, 2007) by enhancing glutamate release from nerve endings (Kaeser and Regehr, 2014; Ramírez-Barrantes et al., 2016). In view of the fact that both TRPV1 mRNA and protein have been found in the central amygdala (Zschenderlein et al., 2011; Ramírez-Barrantes et al., 2016), and that there is evidence for physiologic and pharmacological interactions between CaMKII α and TRPV1 receptors (Price et al., 2005; Nakanishi et al., 2010) forming possible feed-forward loops (Wang et al., 2010), it is therefore possible that presynaptic CaMKII α may also be a regulator of fentanyl-induced hyperalgesia.

In the CeLC, CaMKII α is colocalized with the μ -opioid receptors (Brüggemann et al., 2000; Carlton, 2002). Cellular and biochemical evidence have supported the possibility that CaMKII α and the μ -opioid receptor can directly interact with each other, modulating kinase and receptor activity (Tang et al., 2006; Yang et al., 2011). Therefore, it is plausible that the μ -opioid receptor, CaMKII α , NMDAR, and TRPV1 in the CeLC work in concert to regulate opioid activity and OIH.

Spontaneous activity may adjust synaptic strength by regulating protein synthesis. In cultured hippocampal pyramidal cells, spontaneous glutamate release activates NMDARs and tonically suppresses local protein synthesis in dendrites (Sutton et al., 2004, 2006; Sutton and Schuman, 2006). In this study, the finding of *p*-CaMKII α -induced increases in synaptic transmission and excitability in CeLC neurons indicates it maybe one possible mechanism underlying increased nocifensive responses (mechanical allodynia and thermal hyperalgesia) in the absence of tissue injury.

In conclusion, our findings demonstrate that CaMKII α in the CeLC is involved in the development of fentanyl-induced hyperalgesia that can be disrupted by locally inhibiting CaMKII α in the CeLC through both pre- and postsynaptic mechanisms. The present study provides insights into supraspinal CaMKII α mechanisms promoting OIH, which may facilitate exploring new pharmacological interventions targeting unsolved opioid side effects in the future.

Authorship Contributions

Participated in research design: Z. Li, Wang, Luo.

Conducted experiments: Z. Li, C. Li, Yin.

Performed data analysis: Z. Li, C. Li, Luo.

Wrote or contributed to the writing of the manuscript: Z. Li, Wang, Luo.

References

- Brüggemann I, Schulz S, Wiborny D, and Höllt V (2000) Colocalization of the μ -opioid receptor and calcium/calmodulin-dependent kinase II in distinct pain-processing brain regions. *Brain Res Mol Brain Res* **85**:239–250.
- Butler RK, Nilsson-Todd L, Cleren C, Léna I, Garcia R, and Finn DP (2011) Molecular and electrophysiological changes in the prefrontal cortex-amygdala-dorsal periaqueductal grey pathway during persistent pain state and fear-conditioned analgesia. *Physiol Behav* **104**:1075–1081.
- Carlton SM (2002) Localization of CaMKII α in rat primary sensory neurons: increase in inflammation. *Brain Res* **947**:252–259.
- Carrasquillo Y and Gereau RW, 4th (2007) Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J Neurosci* **27**:1543–1551.
- Célèrier E, Rivat C, Jun Y, Laulin JP, Larcher A, Reynier P, and Simonnet G (2000) Long-lasting hyperalgesia induced by fentanyl in rats: preventive effect of ketamine. *Anesthesiology* **92**:465–472.
- Chen Y, Luo F, Yang C, Kirkmire CM, and Wang ZJ (2009) Acute inhibition of Ca²⁺/calmodulin-dependent protein kinase II reverses experimental neuropathic pain in mice. *J Pharmacol Exp Ther* **330**:650–659.
- Chen Y, Yang C, and Wang ZJ (2010) CaMKII α is required for the initiation and maintenance of opioid-induced hyperalgesia. *J Neurosci* **30**:38–46.
- Chu LF, Angst MS, and Clark D (2008) Opioid-induced hyperalgesia in humans: molecular mechanisms and clinical considerations. *Clin J Pain* **24**:479–496.
- Chu LF, Dairmont J, Zamora AK, Young CA, and Angst MS (2011) The endogenous opioid system is not involved in modulation of opioid-induced hyperalgesia. *J Pain* **12**:108–115.
- Dixon WJ and Mood AM (1948) A method for obtaining and analyzing sensitivity data. *J Am Stat Assoc* **43**:109–126.
- Dunbar SA, Karamov IG, and Buerkle H (2000) The effect of spinal ibuprofen on opioid withdrawal in the rat. *Anesth Analg* **91**:417–422.
- Fabry ME, Bouhassira EE, Suzuka SM, and Nagel RL (2003) Transgenic mice and hemoglobinopathies. *Methods Mol Med* **82**:213–241.
- Ferrari LF, Bogen O, and Levine JD (2013) Role of nociceptor α CaMKII in transition from acute to chronic pain (hyperalgesic priming) in male and female rats. *J Neurosci* **33**:11002–11011.
- Fu Y, Han J, Ishola T, Scerbo M, Adwanikar H, Ramsey C, and Neugebauer V (2008) PKA and ERK, but not PKC, in the amygdala contribute to pain-related synaptic plasticity and behavior. *Mol Pain* **4**:26–45.
- Goforth PB, Ellis EF, and Satin LS (2004) Mechanical injury modulates AMPA receptor kinetics via an NMDA receptor-dependent pathway. *J Neurotrauma* **21**:719–732.

- Guignard B, Bossard AE, Coste C, Sessler DI, Lebrault C, Alfonsi P, Fletcher D, and Chauvin M (2000) Acute opioid tolerance: intraoperative remifentanyl increases postoperative pain and morphine requirement. *Anesthesiology* **93**:409–417.
- Hamlin AS, McNally GP, and Osborne PB (2007) Induction of c-Fos and zif268 in the nociceptive amygdala parallel abstinence hyperalgesia in rats briefly exposed to morphine. *Neuropharmacology* **53**:330–343.
- Han JS, Adwanikar H, Li Z, Ji G, and Neugebauer V (2010) Facilitation of synaptic transmission and pain responses by CGRP in the amygdala of normal rats. *Mol Pain* **6**:10–23.
- Han JS and Neugebauer V (2005) mGluR1 and mGluR5 antagonists in the amygdala inhibit different components of audible and ultrasonic vocalizations in a model of arthritic pain. *Pain* **113**:211–222.
- Hargreaves K, Dubner R, Brown F, Flores C, and Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**:77–88.
- He Y, Tian X, Hu X, Porreca F, and Wang ZJ (2012) Negative reinforcement reveals non-evoked ongoing pain in mice with tissue or nerve injury. *J Pain* **13**:598–607.
- Hu X, Huang F, Szymusiak M, Tian X, Liu Y, and Wang ZJ (2016) PLGA-curcumin attenuates opioid-induced hyperalgesia and inhibits spinal CaMKII α . *PLoS One* **11**:e0146393.
- Jordan BA, Gomes I, Rios C, Filipovska J, and Devi LA (2003) Functional interactions between μ opioid and α_2 -adrenergic receptors. *Mol Pharmacol* **64**:1317–1324.
- Kaesser PS and Regehr WG (2014) Molecular mechanisms for synchronous, asynchronous, and spontaneous neurotransmitter release. *Annu Rev Physiol* **76**:333–363.
- Kim SH, Stoicea N, Soghomonyan S, and Bergese SD (2014) Intraoperative use of remifentanyl and opioid induced hyperalgesia/acute opioid tolerance: systematic review. *Front Pharmacol* **5**:108–126.
- Kiritoshi T, Sun H, Ren W, Stauffer SR, Lindsley CW, Conn PJ, and Neugebauer V (2013) Modulation of pyramidal cell output in the medial prefrontal cortex by mGluR5 interacting with CB1. *Neuropharmacology* **66**:170–178.
- Latreoliere A and Woolf CJ (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* **10**:895–926.
- Lee HJ and Yeomans DC (2014) Opioid induced hyperalgesia in anesthetized settings. *Korean J Anesthesiol* **67**:299–304.
- Li Z, Ji G, and Neugebauer V (2011) Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior. *J Neurosci* **31**:1114–1127.
- Liang D, Shi X, Qiao Y, Angst MS, Yeomans DC, and Clark JD (2008) Chronic morphine administration enhances nociceptive sensitivity and local cytokine production after incision. *Mol Pain* **4**:7.
- Lisman J, Schulman H, and Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci* **3**:175–190.
- Luo F, Yang C, Chen Y, Shukla P, Tang L, Wang LX, and Wang ZJ (2008) Reversal of chronic inflammatory pain by acute inhibition of Ca²⁺/calmodulin-dependent protein kinase II. *J Pharmacol Exp Ther* **325**:267–275.
- Mao J (2002) Opioid-induced abnormal pain sensitivity: implications in clinical opioid therapy. *Pain* **100**:213–217.
- Martin TJ and Ewan E (2008) Chronic pain alters drug self-administration: implications for addiction and pain mechanisms. *Exp Clin Psychopharmacol* **16**:357–366.
- Nakanishi M, Hata K, Nagayama T, Sakurai T, Nishisho T, Wakabayashi H, Hiraga T, Ebisu S, and Yoneda T (2010) Acid activation of Trpv1 leads to an up-regulation of calcitonin gene-related peptide expression in dorsal root ganglion neurons via the CaMK-CREB cascade: a potential mechanism of inflammatory pain. *Mol Biol Cell* **21**:2568–2577.
- Neugebauer V, Han JS, Adwanikar H, Fu Y, and Ji G (2007) Techniques for assessing knee joint pain in arthritis. *Mol Pain* **3**:8.
- Neugebauer V, Li W, Bird GC, and Han JS (2004) The amygdala and persistent pain. *Neuroscientist* **10**:221–234.
- Ninan I and Arancio O (2004) Presynaptic CaMKII is necessary for synaptic plasticity in cultured hippocampal neurons. *Neuron* **42**:129–141.
- Ossipov MH, Lai J, King T, Vanderah TW, and Porreca F (2005) Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. *Biopolymers* **80**:319–324.
- Price TJ, Jeske NA, Flores CM, and Hargreaves KM (2005) Pharmacological interactions between calcium/calmodulin-dependent kinase II α and TRPV1 receptors in rat trigeminal sensory neurons. *Neurosci Lett* **389**:94–98.
- Ramirez-Barrantes R, Cordova C, Poblete H, Muñoz P, Marchant I, Wianny F, and Olivero P (2016) Perspectives of TRPV1 function on the neurogenesis and neural plasticity. *Neural Plast* **2016**:1568145.
- Rivat C, Laulin JP, Corcuff JB, Célérier E, Pain L, and Simonnet G (2002) Fentanyl enhancement of carrageenan-induced long-lasting hyperalgesia in rats: prevention by the N-methyl-D-aspartate receptor antagonist ketamine. *Anesthesiology* **96**:381–391.
- Sah P, Faber ES, Lopez De Armentia M, and Power J (2003) The amygdaloid complex: anatomy and physiology. *Physiol Rev* **83**:803–834.
- Salling MC, Faccidomo SP, Li C, Psilos K, Galunas C, Spanos M, Agoglia AE, Kash TL, and Hodge CW (2016) Moderate alcohol drinking and the amygdala proteome: identification and validation of calcium/calmodulin dependent kinase II and AMPA receptor activity as novel molecular mechanisms of the positive reinforcing effects of alcohol. *Biol Psychiatry* **79**:430–442.
- Sandkühler J (2007) Understanding LTP in pain pathways. *Mol Pain* **3**:9–17.
- Sarhan M, Freund-Mercier MJ, and Veinante P (2005) Branching patterns of parabrachial neurons projecting to the central extended amygdala: single axonal reconstructions. *J Comp Neurol* **491**:418–442.
- Sarhan M, Pawlowski SA, Barthas F, Yalcin I, Kaufling J, Dardente H, Zachariou V, Dileone RJ, Barrot M, and Veinante P (2013) BDNF parabrachio-amygdaloid pathway in morphine-induced analgesia. *Int J Neuropsychopharmacol* **16**:1649–1660.
- Seto SW, Au ALS, Poon CCW, Zhang Q, Li RWS, Yeung JHK, Kong SK, Ngai SM, Wan S, Ho HP, et al. (2013) Acute simvastatin inhibits K_{ATP} channels of porcine coronary artery myocytes. *PLoS One* **8**:e66404.
- Shen G, Mohamed MS, Das P, and Tietz EI (2009) Positive allosteric activation of GABA_A receptors bi-directionally modulates hippocampal glutamate plasticity and behaviour. *Biochem Soc Trans* **37**:1394–1398.
- Shoudai K, Peters JH, McDougall SJ, Fawley JA, and Andresen MC (2010) Thermally active TRPV1 tonically drives central spontaneous glutamate release. *J Neurosci* **30**:14470–14475.
- Sikand P and Premkumar LS (2007) Potentiation of glutamatergic synaptic transmission by protein kinase C-mediated sensitization of TRPV1 at the first sensory synapse. *J Physiol* **581**:631–647.
- Stoicea N, Russell D, Weidner G, Durda M, Joseph NC, Yu J, and Bergese SD (2015) Opioid-induced hyperalgesia in chronic pain patients and the mitigating effects of gabapentin. *Front Pharmacol* **6**:104–109.
- Sutton MA, Ito HT, Cressy P, Kempf C, Woo JC, and Schuman EM (2006) Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell* **125**:785–799.
- Sutton MA and Schuman EM (2006) Dendritic protein synthesis, synaptic plasticity, and memory. *Cell* **127**:49–58.
- Sutton MA, Wall NR, Aakalu GN, and Schuman EM (2004) Regulation of dendritic protein synthesis by miniature synaptic events. *Science* **304**:1979–1983.
- Tang L, Shukla PK, Wang LX, and Wang ZJ (2006) Reversal of morphine antinociceptive tolerance and dependence by the acute supraspinal inhibition of Ca²⁺/calmodulin-dependent protein kinase II. *J Pharmacol Exp Ther* **317**:901–909.
- Toyoda H, Zhao MG, and Zhuo M (2009) Enhanced quantal release of excitatory transmitter in anterior cingulate cortex of adult mice with chronic pain. *Mol Pain* **5**:4–12.
- Tracey I and Mantyh PW (2007) The cerebral signature for pain perception and its modulation. *Neuron* **55**:377–391.
- Vanderah TW, Suenaga NM, Ossipov MH, Malan TP, Jr, Lai J, and Porreca F (2001) Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. *J Neurosci* **21**:279–286.
- Vardanyan A, Wang R, Vanderah TW, Ossipov MH, Lai J, Porreca F, and King T (2009) TRPV1 receptor in expression of opioid-induced hyperalgesia. *J Pain* **10**:243–252.
- Wang LX and Wang ZJ (2003) Animal and cellular models of chronic pain. *Adv Drug Deliv Rev* **55**:949–965.
- Wang XL, Zhang HM, Chen SR, and Pan HL (2007) Altered synaptic input and GABA_B receptor function in spinal superficial dorsal horn neurons in rats with diabetic neuropathy. *J Physiol* **579**:849–861.
- Wang ZJ, Wilkie DJ, and Molokie R (2010) Neurobiological mechanisms of pain in sickle cell disease. *Hematology (Am Soc Hematol Educ Program)* **2010**:403–408.
- Wang ZW (2008) Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol Neurobiol* **38**:153–166.
- Watabe AM, Ochiai T, Nagase M, Takahashi Y, Sato M, and Kato F (2013) Synaptic potentiation in the nociceptive amygdala following fear learning in mice. *Mol Brain* **6**:11.
- Wilkie DJ, Molokie R, Boyd-Seal D, Suarez ML, Kim YO, Zong S, Wittert H, Zhao Z, Saunthararajah Y, and Wang ZJ (2010) Patient-reported outcomes: descriptors of nociceptive and neuropathic pain and barriers to effective pain management in adult outpatients with sickle cell disease. *J Natl Med Assoc* **102**:18–27.
- Woolf CJ and Thompson SW (1991) The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartate receptor activation; implications for the treatment of post-injury pain hypersensitivity states. *Pain* **44**:293–299.
- Wyllie DJ, Manabe T, and Nicoll RA (1994) A rise in postsynaptic Ca²⁺ potentiates miniature excitatory postsynaptic currents and AMPA responses in hippocampal neurons. *Neuron* **12**:127–138.
- Xu H, Wu LJ, Wang H, Zhang X, Vadakkan KI, Kim SS, Steenland HW, and Zhuo M (2008) Presynaptic and postsynaptic amplifications of neuropathic pain in the anterior cingulate cortex. *J Neurosci* **28**:7445–7453.
- Yang C, Chen Y, Tang L, and Wang ZJ (2011) Haloperidol disrupts opioid-antinociceptive tolerance and physical dependence. *J Pharmacol Exp Ther* **338**:164–172.
- Zhang RX, Zhang M, Li A, Pan L, Berman BM, Ren K, and Lao L (2013) DAMGO in the central amygdala alleviates the affective dimension of pain in a rat model of inflammatory hyperalgesia. *Neuroscience* **252**:359–366.
- Zhao MG, Ko SW, Wu LJ, Toyoda H, Xu H, Quan J, Li J, Jia Y, Ren M, Xu ZC, et al. (2006) Enhanced presynaptic neurotransmitter release in the anterior cingulate cortex of mice with chronic pain. *J Neurosci* **26**:8923–8930.
- Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **16**:109–110.
- Zissen MH, Zhang G, McKelvey A, Propst JT, Kendig JJ, and Sweitzer SM (2007) Tolerance, opioid-induced allodynia and withdrawal associated allodynia in infant and young rats. *Neuroscience* **144**:247–262.
- Zschenderlein C, Gebhardt C, von Bohlen und Halbach O, Kulisch C, and Albrecht D (2011) Capsaicin-induced changes in LTP in the lateral amygdala are mediated by TRPV1. *PLoS One* **6**:e16116.

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