AMPAkines Target the Nucleus Accumbens to Relieve Postoperative Pain

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ABSTRACT

Background: AMPAkines augment the function of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the brain to increase excitatory outputs. These drugs are known to relieve persistent pain. However, their role in acute pain is unknown. Furthermore, a specific molecular and anatomic target for these novel analgesics remains elusive.

Methods: The authors studied the analgesic role of an AMPAkine, CX546, in a rat paw incision (PI) model of acute postoperative pain. The authors measured the effect of AMPAkines on sensory and depressive symptoms of pain using mechanical hypersensitivity and forced swim tests. The authors asked whether AMPA receptors in the nucleus accumbens (NAc), a key node in the brain's reward and pain circuitry, can be a target for AMPAkine analgesia.

Results: Systemic administration of CX546 (n = 13), compared with control (n = 13), reduced mechanical hypersensitivity (50% withdrawal threshold of 6.05 ± 1.30 g [mean ± SEM] *vs.* 0.62 ± 0.13 g), and it reduced depressive features of pain by decreasing immobility on the forced swim test in PI-treated rats (89.0 ± 15.5 *vs.* 156.7 ± 18.5 s). Meanwhile, CX546 delivered locally into the NAc provided pain-relieving effects in both PI (50% withdrawal threshold of 6.81 ± 1.91 *vs.* 0.50 ± 0.03 g; control, n = 6; CX546, n = 8) and persistent postoperative pain (spared nerve injury) models (50% withdrawal threshold of 3.85 ± 1.23 *vs.* 0.45 ± 0.00 g; control, n = 7; CX546, n = 11). Blocking AMPA receptors in the NAc with 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione inhibited these pain-relieving effects (50% withdrawal threshold of 7.18 ± 1.52 *vs.* 1.59 ± 0.66 g; n = 8 for PI groups; 10.70 ± 3.45 *vs.* 1.39 ± 0.88 g; n = 4 for spared nerve injury groups).

Conclusions: AMPAkines relieve postoperative pain by acting through AMPA receptors in the NAc. **(ANESTHESIOLOGY 2016; 125:1030-43)**

POSTOPERATIVE pain impairs rehabilitation, and 30% of postoperative patients develop persistent or chronic pain.[1] Respiratory depression caused by opioids and other sedatives remains a serious postoperative complication, and common affective pain symptoms such as depressed mood further delay postsurgical recovery.[2–7] Newer and safer analgesics that can treat both sensory and affective pain symptoms are urgently needed.

Glutamate signaling in the central nervous system (CNS) plays an important role in regulating pain sensitivity and mood. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are the primary glutamate receptors in the brain.[8] AMPA receptor signaling is crucial for the function of nucleus accumbens (NAc), a key region for the regulation of both reward- and aversion-driven behaviors.[9–11] Human imaging studies reveal that acute and chronic pain activate the NAc,[12–14] and signaling through AMPA receptors in the NAc generates pain-induced analgesia in animal studies.[15,16] More importantly, recent studies indicate that persistent pain alters AMPA receptor composition and function in the NAc and that increased AMPA receptor activities can relieve sensory and affective symptoms of postoperative pain.[17–19]

What We Already Know about This Topic

- AMPAkines enhance glutamatergic neurotransmission mediated by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor and stimulate respiratory drive in the setting of opiate-induced hypoventilation
- AMPAkines relieve sensory and affective symptoms of persistent pain; whether they can relieve acute postoperative pain is not known
- In rodent models of acute postoperative pain produced by paw incision, the efficacy of AMPAkines in reducing sensory and affective symptoms of acute pain was evaluated

What This Article Tells Us That Is New

- Systemic administration of an AMPAkine reduced pain and depression-like behavior
- AMPAkine administration directly into the nucleus accumbens similarly reduced acute pain
- The data suggest that AMPAkines are novel analgesics for postoperative pain whose effect is mediated in part by activity in the nucleus accumbens

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AMPAkines enhance glutamate transmission by binding to an allosteric site on the AMPA receptor to slow the kinetics of channel deactivation.[20,21] AMPAkines have been investigated in schizophrenia, depression, and Huntington and Alzheimer diseases.[20,22–25] Interestingly, recent studies have shown that AMPAkines can stimulate the respiratory drive in the context of hypoventilation caused by opioids or other sedatives, thus making these drugs tantalizing options in the postoperative setting.[26–28] A previous study has shown that AMPAkines can relieve both sensory and affective symptoms of persistent pain.[29] However, it is not known whether AMPAkines can also relieve acute postoperative pain. If so, such drugs can be ideal analgesics in the postoperative setting to relieve pain and improve mood and at the same time to enhance the safety profile of sedatives commonly administered during or after surgery. From a mechanistic standpoint, AMPAkines are known to have high affinity for neurons in the NAc and brain stem.[30] Given the crucial role AMPA receptors in the brain play in pain regulation, these receptors may form an important target for AMPAkine analgesia.

To investigate the potential analgesic effects of AMPAkines in the postoperative setting, we tested CX546, an established AMPAkine that has been studied in hypoventilation, Rett syndrome, anxiety, and autism,[27,31–34] in a classic acute postoperative pain model—paw incision (PI) model.[35] We examined whether this AMPAkine is able to relieve both mechanical hypersensitivity and depressive symptoms of pain in this model. We delivered CX546 specifically into the NAc to see if AMPA receptors in the NAc could mediate its pain-relieving effects. Furthermore, we tested the analgesic effects of systemic administration of CX546 after blocking AMPA receptors in the NAc locally with 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX). We confirmed the role of the NAc AMPA receptors in AMPAkine analgesia using a persistent postoperative pain (spared nerve injury [SNI]) model. Finally, as glutamate signaling in the NAc may play a role in drug craving and addiction,[36–38] we performed conditioned place preference (CPP) test to show that a short-term use of AMPAkines did not result in craving.

Materials and Methods

Animals

All procedures in this study were approved by the New York University School of Medicine Institutional Animal Care and Use Committee as consistent with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (publication 85-23) to ensure minimal animal use and discomfort. Male Sprague–Dawley rats were purchased from Taconic Farms, USA, and kept at Mispro Biotech Services Facility in Alexandria Center for Life Science, New York, New York, with controlled humidity, room temperature, and 12-h (6:00 AM to 6:00 PM) light–dark cycle. Food and water were available *ad libitum*. Animals arrived to the animal facility at 250 to 300 g and were given on average 7 days to adjust to the new environment before the onset of any experiments. Rats were randomly assigned to each experimental condition. Blinding was attempted whenever possible for all behavior experiments. The person who performed and analyzed the test was blinded to the condition of the animals.

Animal Surgeries and Procedures

PI Procedure. The PI surgery was performed as previously described.[18,35,39] Briefly, rats were anesthetized with isoflurane anesthesia (1.5 to 2%), and the plantar surface of the right hind paw was sterilized and prepared. A 1.5-cm longitudinal incision was made with a number 10 scalpel, through skin and fascia of the right plantar aspect of the paw. The incision started 0.5 cm from the proximal end of the heel and extended to the middle of the paw. The plantaris muscle was elevated and incised longitudinally. Gentle pressure was applied in order to cease bleeding, and the superficial wound was opposed with three single sutures using 5-0 nylon. The animals were allowed to recover in their home cages. Control rats only received isoflurane anesthesia.

SNI Surgery. The SNI surgery has been previously described in detail.[17,40,41] Briefly, under isoflurane anesthesia (1.5 to 2%), the skin on the lateral surface of the right thigh of the rat was incised, and the biceps femoris muscle was dissected in order to expose three branches of the sciatic nerve: sural, common peroneal, and tibial. The common peroneal and tibial nerves were tied with nonabsorbent 5.0 silk sutures at the point of trifurcation. The nerves were then cut distal to the knot, and about 3 to 5 mm of the distal ends were removed. In sham surgeries (control), the nerves mentioned above were dissected but not cut. Muscle and skin layers were then sutured closed in distinct layers.

Cannula Implantation and Intracranial Injections. For cannula implantation, as described previously,[17,42] rats were anesthetized with isoflurane (1.5 to 2%). Rats were stereotaxically implanted with two 26-gauge guide cannulas (PlasticsOne, USA) bilaterally in the NAc core with coordinates as follows: 1.6 mm anterior to bregma; 2.9 mm lateral to the sagittal suture, tips angled 8° toward the midline; and 5.6 mm ventral to skull surface. Cannulas were held in place by dental acrylic, and patency was maintained with occlusion stylets. For intracranial injections, solutions were loaded into two 30-cm lengths of PE-50 tubing attached at one end to 10-μl Hamilton syringes filled with distilled water and at the other end to 33-gauge injector cannula, which extended 2.0 mm beyond the implanted guides. Injection of solution then delivered bilaterally 0.5 μl of injection volume over a period of 100 s. Injector cannulas were kept in place for another 60 s before removal from guides to allow diffusion of solution into the brain. After the removal of injector cannulas from cannula guides, stylets were replaced, and animals were subjected to behavior tests. Behavior tests were done 15 min after intracranial injections. After animal euthanization,

cryogenic brain sections were collected with thickness of 20 μm using Microm HM525 Cryostat (Thermo Fisher Scientific, USA) and analyzed for cannula localization with histologic staining; animals with improper cannula placements were excluded from the study.

Drugs

CX546 (Sigma-Aldrich, USA) was suspended in dimethyl sulfoxide (DMSO) to different concentrations (2.5, 5, 10 mg/kg) for systemic administration; CX546 was resuspended in 0.9% saline to concentrations of 400 and 800 μM/μl for intra-NAc infusions in PI- and SNI-treated rats. NBQX (Tocris Bioscience, USA) was resuspended in 0.9% saline to a concentration of 0.55 nM. CX546 was applied by systemic administration (intraperitoneally) in PI- or SNI-treated rats, while same volumes of DMSO were applied in the control group. Systemic administrations were given 4 h after PI or 14 days after SNI and were followed by behavioral tests. In addition, CX546, NBQX, or saline was locally infused into the NAc of PI-treated and SNI-treated rats. For local infusions, 0.5 μl of drug or control was injected in each side of the brain. Intracranial administrations were given at least 7 days after cannula implantation and were followed by behavioral tests. Intra-NAc infusions were given 4 h and 1, 4, and 7 days after PI or 14 days after SNI.

Animal Behavioral Tests

Mechanical Hypersensitivity Test. A traditional Dixon up-down method with von Frey filaments was used to measure mechanical hypersensitivity as described previously.[41,43,44] In brief, rats were individually placed into plexiglass chambers over a mesh table and acclimated for 20 min before the onset of examination. Beginning with 2.55 g, von Frey filaments in a set with logarithmically incremental stiffness (0.45, 0.75, 1.20, 2.55, 4.40, 6.10, 10.50, and 15.10 g) were applied vertically to the plantar surface of the right paw, adjacent to the wound of rats after PI. For SNI and sham groups, von Frey filaments were applied to the lateral one third of right paws (in the distribution of the sural nerve) of rats after SNI or sham surgery. Fifty percent withdrawal threshold was calculated as described previously.[41]

Forced Swim Test. On the first session of the test, each animal was placed for 15 min into a standard clear Porsolt chamber (Lafayette Instrument Co., USA) with water at 25°C filled to 25 cm. Afterward, the animal was taken out of the chamber, dried, and put back in its home cage. After 24 h, the animal was placed into the Porsolt chamber again under the same conditions for 5 min. Only the second session was videotaped and analyzed. Immobility was defined as a lack of movement of the hind paws lasting greater than 1 s. An independent observer, blinded to the test conditions, examined and graded the total time of immobility for each rat, and the average grade was presented for each animal. The forced swim test (FST) was conducted 4 h after PI or 14 days after SNI.

Locomotion Activities. Rats were placed in a 0.5- × 0.5-m chamber with an overhead camera. Using a video analysis software (ANY-maze, USA), animal movements were tracked during a 30-min test. The total distance traveled was computed for 10 bins of 3 min. Later, videos were visually examined to correlate with automatic results in order to verify tracking accuracy.

CPP. CPP experiments were conducted in a standard three-compartment apparatus (Stoelting Co., USA) consisting of two large compartments of equal size (45 × 40 × 35 cm) joined by a tunnel (40 × 9 × 35 cm). The movements of rats in each chamber were automatically recorded by a camera and analyzed with the ANY-maze software. The CPP protocol was modified from King *et al.*[45] and included preconditioning, conditioning, and testing phases. Preconditioning was performed across 3 days. During preconditioning, all animals were exposed to the environment with full access to all chambers for 30 min each day. On day 3, the movement of each rat was recorded for 15 min and analyzed to verify the absence of any preconditioning chamber preference. Animals spending more than 720 s or less than 120 s of the total time in any chamber were eliminated from further testing or analysis (approximately 15% of total animals). After the preconditioning phase, the rats underwent conditioning for 4 days with alternating intraperitoneal injection of CX546 (10 mg/kg) or control treatment-chamber pairings in the morning and afternoon. During conditioning, rats were placed in the paired chamber without access to the other compartments for 30 min. Drug treatments and chamber pairings were counterbalanced, and at least 4 h separated morning and afternoon sessions. On the test day, the animals were placed into the neutral chamber and had access to all chambers for a total of 15 min. There were no drug treatments on test days. During these 15 min, animal movements in each of the chambers were recorded, and the time spent in either of the treatment chambers was analyzed by the ANY-maze software. Increased time spent in a chamber associated with drug or control treatment indicates preference for that chamber. This test was repeated with morphine (4 mg/kg, subcutaneous injection) instead of CX546 for comparison.

Subcellular Fractionation and Western Blotting

PI-treated rats were anesthetized with isoflurane (1.5 to 2%) and decapitated immediately. Brains were quickly removed, and NAc were collected on ice. Synaptoneurosome fractions were prepared as described previously.[46,47] To prepare synaptoneurosome fractions, NAc samples were homogenized in ice-cold solution A (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM phenylmethane sulfonyl fluoride, and 1× complete protease inhibitors [Roche Applied Science, Germany]). Homogenates were centrifuged at 4,000 rpm for 10 min. The supernatant was collected and the pellet rehomogenized in solution A and centrifuged again at 3,000 rpm for 10 min. Combined supernatants were subjected to a second centrifugation at 3,000 rpm for 10 min. Supernatants were then spun at 14,000 rpm for 30 min. Pellet was

resuspended in solution B (0.32 M sucrose, 1 mM $NaHCO_3$) and homogenized. Homogenate was layered on top of a 5-mL 1 M sucrose and 1.2 M sucrose gradient and centrifuged at 30,000 rpm for 2 h. Purified synaptosomes were collected at the 1 M and 1.2 M sucrose interface, suspended in solution B, and centrifuged at 40,000 rpm for 45 min. Synaptosomal pellets were resuspended in 25 mM Tris with 4% SDS. Fractions were analyzed by Western blot on SDS-PAGE gels as described previously.[46,47] The following antibodies were used: GluA1 (1:1,000; Millipore, USA), GluA2 (1:1,000; Millipore), and tubulin (1:30,000; Sigma, USA).

Data Analysis and Statistics

The results of behavioral experiments were given as mean ± SEM. A two-way ANOVA with repeated measures, followed by *post hoc* multiple pair-wise comparison Bonferroni test, was used to compare the 50% withdrawal threshold of PI *versus* control rats and SNI *versus* sham rats. For the FST, an unpaired two-tailed Student's *t* test was used to compare the performances of PI *versus* control groups, CX546 *versus* DMSO groups, and CX546 *versus* saline groups. For dose–response experiments, a one-way ANOVA was used to compare the analgesia effects of CX546 with DMSO as control. For the experiment testing the duration of analgesic effects, a two-way ANOVA with repeated measures and *post hoc* multiple pair-wise comparison Bonferroni tests were used to compare mechanical hypersensitivity after CX546 *versus* DMSO (control) treatments. A two-way ANOVA with repeated measures, followed by *post hoc* multiple pair-wise comparison Bonferroni test, was also used to compare the 50% withdrawal threshold of CX546 *versus* saline local infusion treatments at multiple time points. An unpaired two-tailed Student's *t* test was used to compare the 50% withdrawal threshold of NBQX *versus* saline in both PI and SNI rats, as well as intracranial CX546 *versus* saline treatments in SNI rats. For locomotion, a two-way ANOVA with repeated measures and *post hoc* multiple pair-wise comparison Bonferroni tests were used to compare the performances of PI *versus* control groups, CX546 *versus* DMSO in PI rats, and CX546 *versus* saline in both PI and SNI rats. For CPP tests, differences in time spent in each chamber before conditioning (preconditioning) and after conditioning (test) were analyzed using a two-way ANOVA with repeated measures followed by *post hoc* Bonferroni tests. An unpaired Student's *t* test was used to compare the Western blot results. For all tests, a *P* value less than 0.05 was considered statistically significant. For intracranial injections, rats with improperly implanted cannulas were excluded from further analysis. Sample sizes were estimated based on previous experience with the same experimental designs.[17–19,29] All data were analyzed using GraphPad Prism Version 6 software (GraphPad, USA).

Results

PI Produces Acute Postoperative Pain

We applied the PI (Brennan) model to mimic acute postincisional pain in rats.[35,39] Here, we incised the right hind paw and measured mechanical hypersensitivity over the next 7 days. As reported previously, mechanical hypersensitivity, which indicates the sensory component of pain, developed quickly after PI (less than 4 h; fig. 1A).[35,39] This sensory hypersensitivity lasted up to 4 days after incision, and it resolved by the seventh day postincision[18,39] (fig. 1A). In contrast, control rats that had only undergone isoflurane anesthesia treatment without surgical incision did not display any mechanical hypersensitivity.

Studies in various animal models have shown that pain can lead to depression-like behaviors in rats.[41,48,49] Decreased motivation is a key feature of depression, especially in the context of pain.[2,3,7] Increased immobility on the FST has been used as a standard measure for decreased motivation or behavioral despair in rodents,[50] and a number of studies

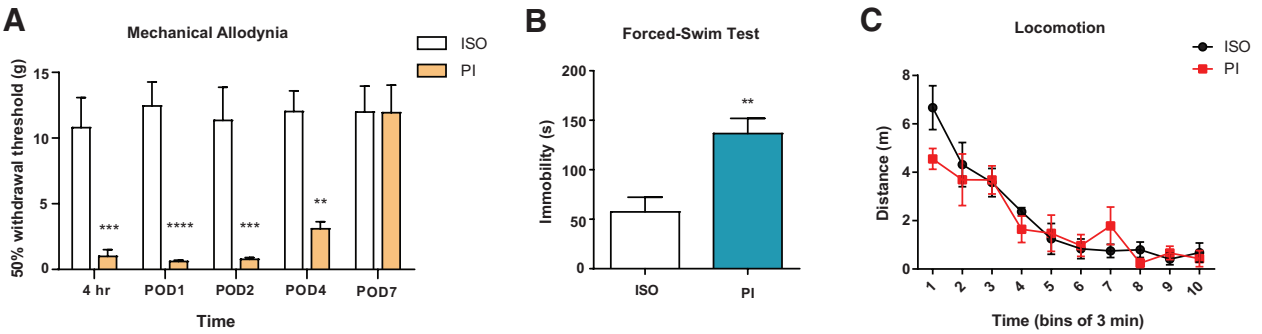

Fig. 1. Paw incision (PI) leads to acute postoperative pain and depression-like behaviors in rats. (*A*) Rats that underwent PI surgery developed mechanical hypersensitivity as early as 4 h and up to 4 days after surgery, compared with control rats that underwent isoflurane (ISO) treatment without surgery. Two-way ANOVA with repeated measures and *post hoc* Bonferroni multiple comparison tests: control group, n = 6; PI group, n = 6; ***P = 0.0004 at 4 h, ****P < 0.0001 at postoperative day (POD) 1, ***P = 0.0001 at POD2, **P = 0.0015 at POD4, and P > 0.9999 at POD7. (*B*) PI-treated rats developed depression-like behaviors compared with control rats 4 h after surgery, as shown by an increase in immobility during the force swim test. Unpaired two-tailed Student's *t* test: control group, n = 5; PI group, n = 4; *P = 0.0081. (*C*) Locomotion was not affected by the PI surgery. Two-way ANOVA: control group, n = 6; PI group, n = 6; P = 0.6096. *Error bars* represent SEM.

have shown clinically relevant pharmacologic validity of this measure.[51] We and others have reported previously that increased immobility on FST was found in various rodent models of pain.[17,19,41,48,49,52,53] Here, we applied FST in our study to assess depression-like behaviors in the PI model. As expected, we found PI-treated rats, compared with control, displayed increased immobility after PI (fig. 1B; $P = 0.0081$). To exclude the possibility that this increased immobility was caused by a deficit in locomotor abilities resulting from pain or incision, we directly measured locomotion in PI-treated and control groups. We found no statistically significant difference in locomotion between these two group of rats (fig. 1C; $P = 0.6096$). Thus, results on the FST suggest that some features of depression-like behaviors can accompany acute postoperative pain.

Systemic Administration of CX546 Relieves Sensory Hypersensitivity and Depression-like Behaviors Associated with Acute Postoperative Pain

CX546 is a well-established AMPAkine that has been found to oppose sedative-induced respiratory depression, and it has been studied in a variety of CNS diseases.[26,27,32,33,54,55] We have previously shown that systemic administration of CX546 can relieve both sensory hypersensitivity and depressive behaviors in response to chronic neuropathic pain and inflammatory pain in rats.[29] The pathogenesis and maintenance of acute postoperative and chronic pain, however, are different mechanistically at synaptic and circuit levels.[56] Hence, we wanted to test whether CX546 could also relieve acute postoperative pain in the PI model. First, we applied CX546 at different doses to test its effect on mechanical hypersensitivity after PI. Compared with control (DMSO), CX546 improved mechanical hypersensitivity at 5 and 10 mg/kg doses (fig. 2A; $P = 0.0404$ and $P < 0.0001$, respectively), with the 10 mg/kg dose providing a greater antihypersensitivity effect. Next, we evaluated the timing and duration of the antiallodynic effect after a single systemic administration. We found that the antinociceptive effects of CX546 (10 mg/kg) began within 1 h after administration (fig. 2B; $P < 0.0001$) and lasted more than 4 h (fig. 2B; $P = 0.03$). This antinociceptive effect diminished after 8 h.

AMPA receptor signaling is known to regulate both sensory and affective components of pain, and we have previously shown that AMPAkines can relieve depressive symptoms of chronic neuropathic pain and inflammatory pain.[29] Thus, we used the FST to assess the effects of CX546 on the depressive symptoms associated with acute incisional pain (fig. 1B). We found that systemic administration of CX546 (10 mg/kg) also alleviated depressive symptoms by decreasing immobility in PI-treated rats (fig. 3A; $P = 0.0486$). Lastly, CX546 did not alter locomotion (fig. 3B).

AMPAkines Target AMPA Receptors in the NAc to Relieve Acute Postoperative Pain

Recent human imaging and animal studies suggested that glutamate signaling in the NAc, the brain's reward center,[9,10] plays a critical role in pain regulation.[12–14] This pain-relieving role for NAc is not surprising, as it receives input from a number of regions important for pain regulation including the prefrontal cortex (PFC), amygdala, and hippocampus,[48,49,57–60] and is known to project to the rostral ventromedial medulla (RVM) to provide descending inhibition.[16] Meanwhile, AMPAkines are known to have high affinity for AMPA receptors in the NAc.[30] Thus, AMPA receptors in the NAc are ideal molecular targets for these drugs.

To test the hypothesis that the pain-relieving properties of AMPAkines act through AMPA receptors in the NAc, we injected CX546 locally into the NAc of PI-treated rats (figs. 4A and 5). Compared with saline control, local infusion of CX546 relieved mechanical hypersensitivity after PI (fig. 4B). Furthermore, the degree of pain relief (as indicated by improvement in the threshold for mechanical hypersensitivity) provided by the intra-NAc infusion of CX546 is

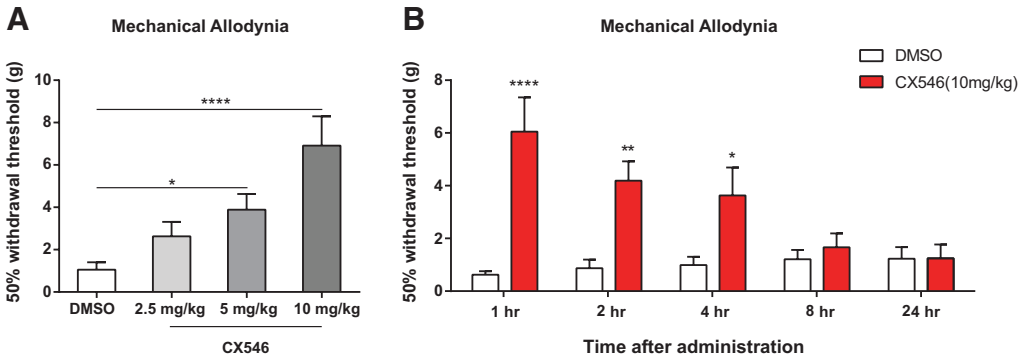

Fig. 2. Systemic administration of CX546 relieves mechanical hypersensitivity in paw incision (PI)–treated rats. (*A*) Intraperitoneal administration of CX546 relieved mechanical hypersensitivity in PI-treated rats at doses of 5 and 10 mg/kg, compared with dimethyl sulfoxide (DMSO). One-way ANOVA with *post hoc* Bonferroni multiple comparison tests: DMSO group, n = 12; CX546 2.5 mg/kg group, n = 7; CX546 5 mg/kg group, n = 7; CX546 10 mg/kg group, n = 6; $^{*}P = 0.0404$, $^{****}P < 0.0001$. (*B*) A single dose of CX546 (10 mg/kg) improved mechanical hypersensitivity in PI-treated rats at 1, 2, and 4 h after intraperitoneal injection, compared with the DMSO control group. Two-way ANOVA with repeated measures: DMSO group, n = 13; CX546 group, n = 13; $^{*}P = 0.0300$, $^{**}P = 0.0031$, $^{****}P < 0.0001$.

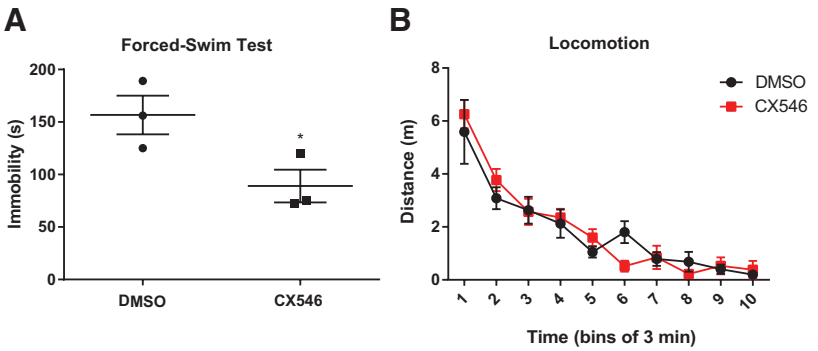

Fig. 3. Systematic administration of CX546 relieves depression-like behaviors in paw incision (PI)–treated rats. (*A*) Intraperitoneal administration of CX546 (10 mg/kg) relieved depression-like behaviors in PI-treated rats, as shown by a decrease of immobility during the force swim test, compared with control group. Unpaired two-tailed Student's *t* test: dimethyl sulfoxide (DMSO) group, n = 3; CX546 group, n = 3; **P* = 0.0486. (*B*) Systemic administration of CX546 (10 mg/kg) did not change locomotion in PI-treated rats, compared with DMSO injection. Two-way ANOVA with repeated measures: DMSO group, n = 7; CX546 group, n = 7; *P* = 0.8364.

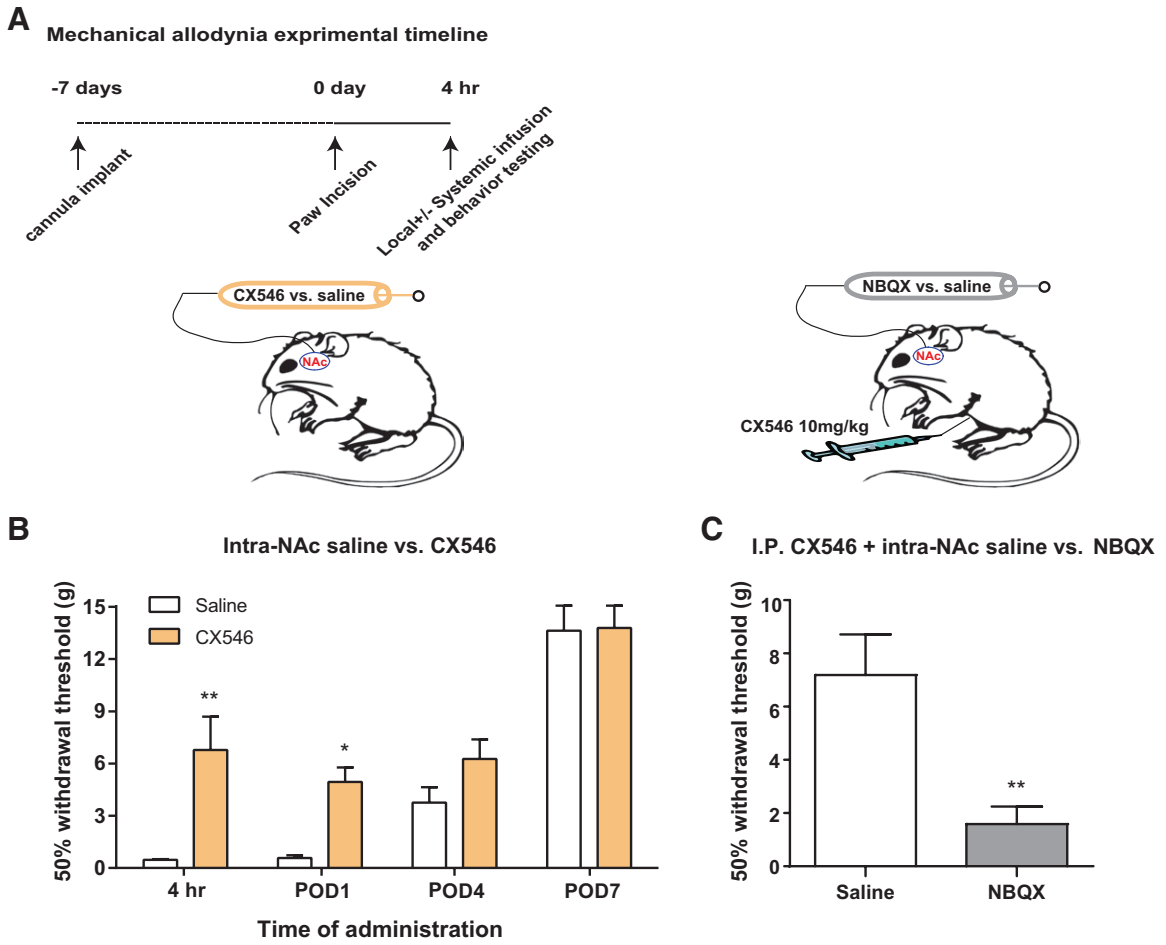

Fig. 4. Local infusion of CX546 into the nucleus accumbens (NAc) relieves mechanical hypersensitivity in paw incision (PI)–treated rats. (*A*) Schematic showing the timeline of pharmacologic experiments. (*B*) Intra-NAc infusion of CX546 (400 μM/μl) 4 h and 1 day after PI reduced mechanical hypersensitivity in PI-treated rats. Two-way ANOVA with Bonferroni post-test: saline group, n = 6; CX546 group, n = 8; **P* = 0.0248, ***P* = 0.0012. (*C*) Intra-NAc infusion of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2, 3-dione (NBQX) blocked the analgesic effects of systemic administration of CX546 (10 mg/kg) in PI-treated rats. Unpaired two-tailed Student's *t* test: saline group, n = 8; NBQX group, n = 8; ***P* = 0.0046. I.P. = intraperitoneal; POD = postoperative day.

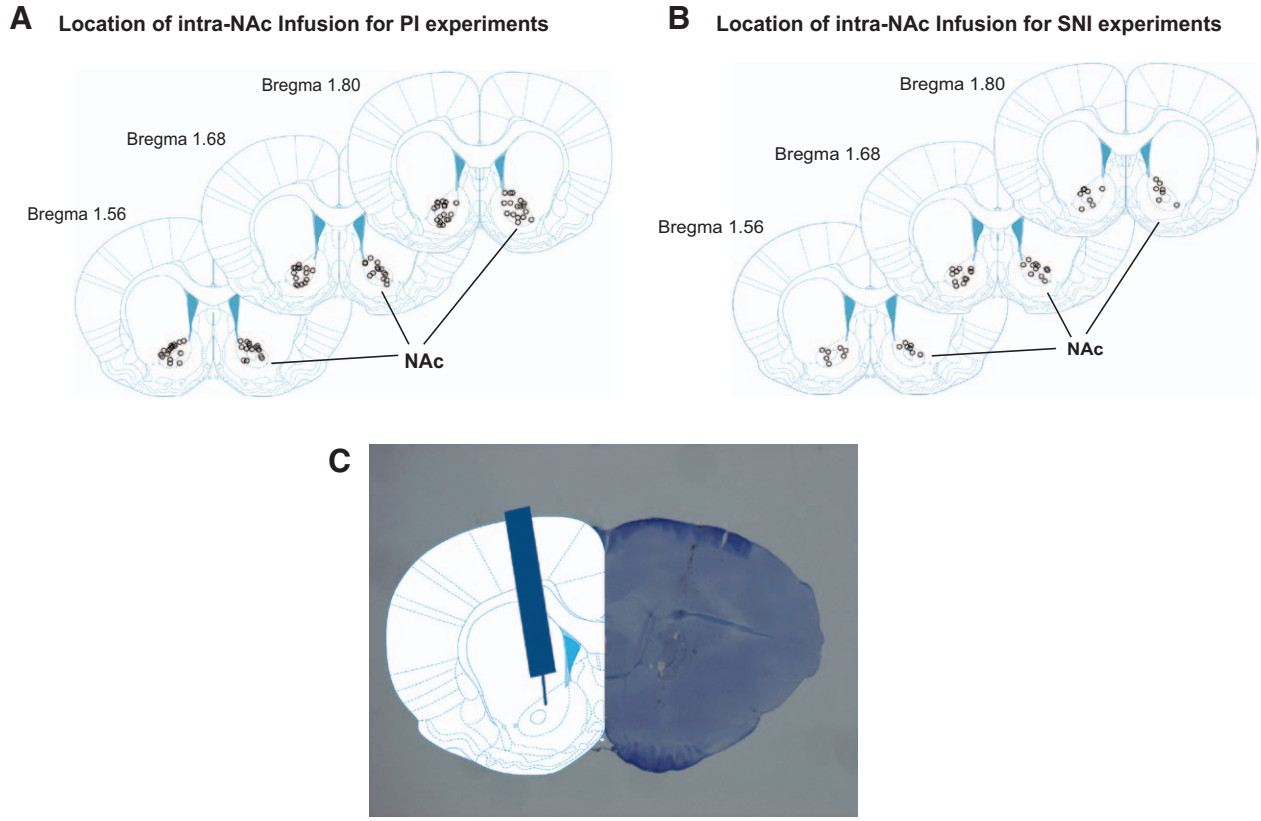

Fig. 5. Site of intracranial pharmacologic delivery. (*A*) Schematic showing the placement of intracranial injectors in paw incision (PI)–treated rats. (*B*) Schematic showing the placement of intracranial injectors in spared nerve injury (SNI)–treated rats. (*C*) Histology of a brain slice containing the nucleus accumbens (NAc).

quantitatively similar to the degree of pain relief provided by the systemic infusion of this drug. These results strongly suggest a prominent role of the NAc in mediating the analgesic effect of AMPAkines.

To further confirm the importance of the NAc AMPA receptors in AMPAkine analgesia, we applied NBQX, a highly specific antagonist of AMPA receptors, locally in the NAc after the systemic infusion of AMPAkines (fig. 4C). Not surprisingly, NBQX, by inhibiting AMPA receptor transmission in the NAc, blocked the antinociceptive effect of AMPAkines (fig. 4C; $P = 0.0046$). It is, of course, conceivable that NBQX inhibits the NAc core function in descending pain control in parallel to the analgesic pathway provided by CX546. Nevertheless, these data, combined with the data indicating that direct intra-NAc infusion of CX546 confers analgesic effects, provide evidence that AMPA receptors in the NAc are an important molecular target for AMPAkines.

Next, we tested whether AMPA receptors in the NAc can also be modified by AMPAkines to relieve the depressive symptoms of pain. We infused CX546 locally into the NAc of PI-treated rats and performed the FST. We found that local infusion of CX546, compared with saline, resulted in a statistically significant decrease of immobility on the FST (fig. 6A; $P = 0.033$), and the degree of improvement in the immobility index is similar to the degree of improvement after systemic infusion of CX546. In contrast, CX546 in the NAc did not affect locomotion (fig. 6B). Thus, by potentiating AMPA receptors in the NAc, AMPAkines can relieve acute postoperative pain.

AMPAkines Target AMPA Receptors in the NAc to Relieve Persistent Postoperative Neuropathic Pain

To further confirm the role of NAc AMPA receptors as a pharmacologic target for AMPAkines, we sought to repeat our pharmacologic experiments in another postoperative model. Persistent neuropathic pain can occur after inadvertent resections of peripheral nerves.[61,62] The SNI model is typically used to model chronic neuropathic pain, but it can also be used to model persistent postoperative neuropathic pain, since the nerve injury and pain symptoms (hypersensitivity and hyperalgesia) in this model resemble the kind of injury and resulting neuropathic pain that can sometimes occur after intraoperative nerve resections.[61,62] Similar to PI, SNI has also been shown to lead to pain and depression-like behaviors in rats.[17] We have previously shown that systemic infusion of CX546 can relieve both mechanical hypersensitivity and depression-like behaviors in this pain model.[29] Here we tested whether AMPA receptors in the NAc function as a pharmacologic target of AMPAkines in this model of persistent postoperative pain (fig. 7A).

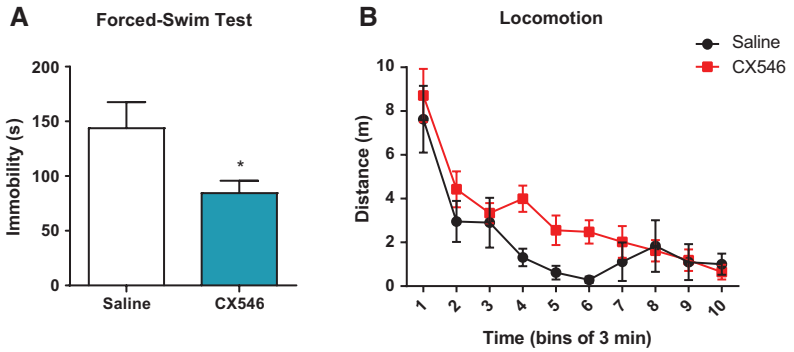

Fig. 6. Local infusion of CX546 into the nucleus accumbens (NAc) relieves depression-like behaviors in paw incision (PI)–treated rats. (*A*) Intra-NAc infusion of CX546 (400 μM/μl) relieved depression-like behaviors in PI rats, as shown by a decrease in immobility during the force swim test, compared with control (saline) group. Unpaired two-tailed Student's *t* test: saline group, n = 10; CX546 group, n = 10; **P* = 0.033. (*B*) Intra-NAc infusion of CX546 (400 μM/μl) did not change locomotion in PI rats. Two-way ANOVA with repeated measures and *post hoc* Bonferroni multiple comparison tests: saline group, n = 6; CX546 group, n = 6; *P* = 0.2027.

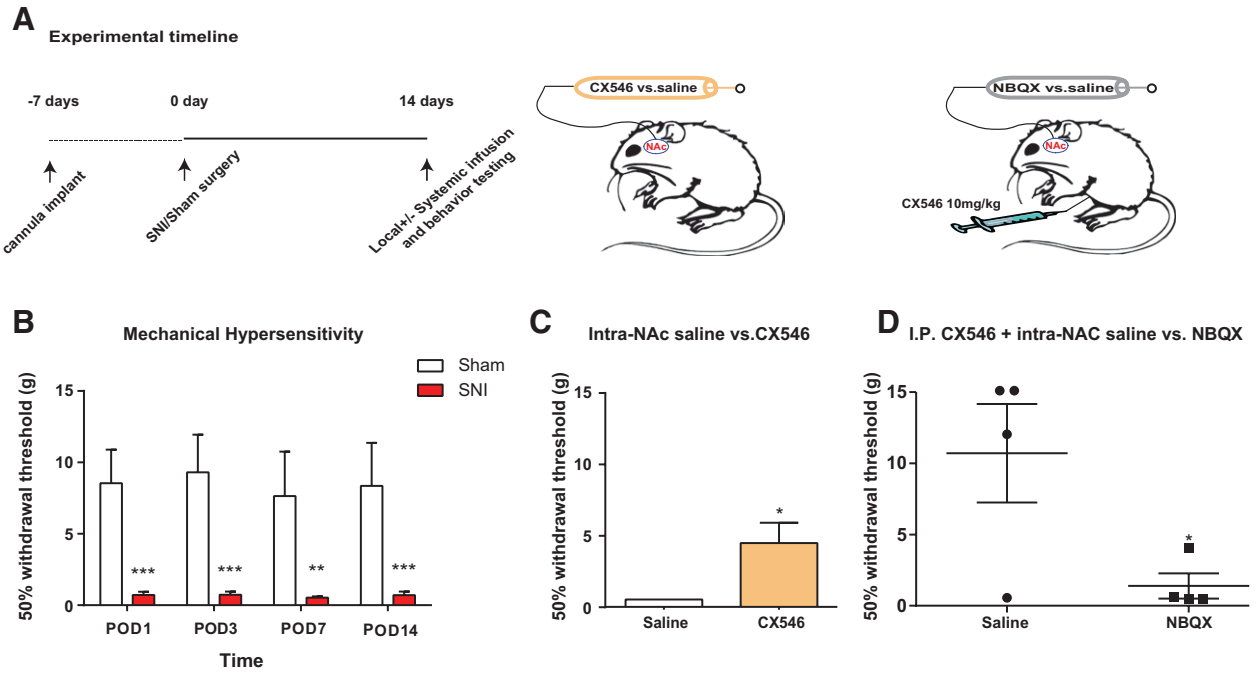

Fig. 7. Local infusion of CX546 into the nucleus accumbens (NAc) decreases mechanical hypersensitivity in rats after spared nerve injury (SNI) procedure. (*A*) Schematic showing the timeline of pharmacologic experiment in SNI-treated rats. (*B*) SNI-treated rats developed mechanical hypersensitivity up to 14 days after surgery, compared with sham-treated rats. Two-way ANOVA with repeated measures and Bonferroni post-tests: sham group, n = 6; SNI group, n= 11; ****P* = 0.0006 for postoperative day (POD) 1, ****P* = 0.0002 for POD3, ***P* = 0.0042 for POD7, ****P* = 0.0009 for POD14. (*C*) Intra-NAc infusion of CX546 (800 μM/μl) relieved mechanical hypersensitivity in SNI-treated rats. Unpaired two-tailed Student's *t* test: saline group, n = 7; CX546 group, n = 11; **P* = 0.0437. (*D*) Intra-NAc infusion of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2, 3-dione (NBQX) blocked the analgesic effects of systemic administration of CX546 (10 mg/kg) in SNI-treated rats. Unpaired two-tailed Student's *t* test: saline group, n = 4; NBQX group, n = 4; **P* = 0.0401.

As shown previously, SNI resulted in persistent mechanical hypersensitivity (fig. 7B).[17,40] When we infused CX546 locally into the NAc of SNI-treated rats, we found that CX546 relieved mechanical hypersensitivity in these rats (fig. 7C; *P* = 0.0437). Next, we infused NBQX to block these AMPA receptors in the NAc. We found that NBQX blocked the antinociceptive effects of systemic administration of CX546 (fig. 7D; *P* = 0.0401). These results suggest that AMPA receptors in the NAc can be targeted by AMPAkines to relieve persistent postoperative pain. We then used FST to assess whether AMPAkines can potentiate these receptors to treat the depressive symptoms of persistent postoperative pain as well. Indeed, intra-NAc infusion of CX546 decreased immobility on the FST without changing locomotion

(fig. 8A, $P = 0.0199$; fig. 8B, $P = 0.9782$). These data suggest that AMPAkines can target the NAc AMPA receptors to relieve persistent postoperative pain.

Short-term Use of AMPAkines Lacks Intrinsic Rewarding Properties

In addition to its ability to provide descending pain inhibition,[12,13,16,17,63–67] the NAc is also known as the brain's reward center. Through its projection to the ventral pallidum and substantia nigra, the NAc can influence the function of ventral anterior, dorsal, and lateral thalamus, which in turn projects to the motor cortex and parts of the PFC. Through this striato-thalamo-cortical circuit, the NAc can regulate behavioral outcomes in the presence of rewards.[68] Furthermore, activation of glutamatergic signaling in the NAc has been involved in the rewarding potential of drugs of addiction.[36–38] Thus, we analyzed the rewarding potential of AMPAkine administration in rats. We used a classic CPP test to assess drug craving (fig. 9A). Since we anticipated that the use of AMPAkines would be limited to the acute postoperative period, we used a 4-day conditioning protocol in naive rats. After 4 consecutive days of conditioning, rats did not develop place preference for the chamber associated with AMPAkine treatment (fig. 9A; $P = 0.9046$). In contrast, 4 days of conditioning resulted in a statistically significantly preference for the chamber associated with morphine, a commonly used analgesic that is known to carry intrinsic rewarding properties (fig. 9B; $P = 0.0349$). These results suggest that while AMPAkines can augment excitatory AMPA receptor transmission in the NAc, its short-term use does not necessarily have intrinsic rewarding properties in wild-type rats. All intracranial drug infusion sites for PI and SNI experiments are shown in fig. 5.

AMPAkines Do Not Alter AMPA Receptor Subunit Levels

Subunit trafficking is important for providing long-term plasticity of excitatory neurotransmission through AMPA receptors.[69] A previous study has shown that persistent postoperative pain, but not acute postoperative

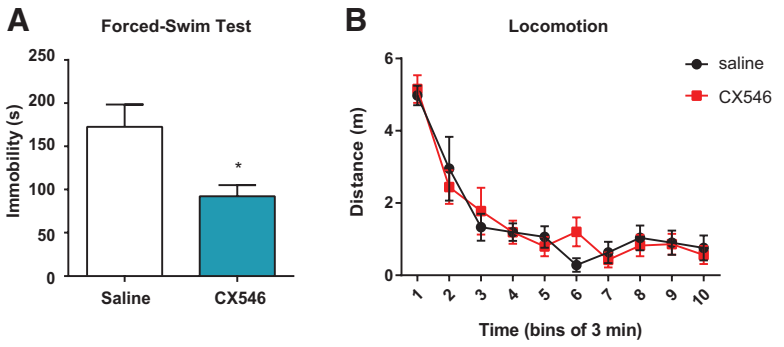

Fig. 8. Local infusion of CX546 into the nucleus accumbens (NAc) relieves depression-like behaviors in spared nerve injury (SNI)–treated rats. (*A*) Intra-NAc infusion of CX546 (800 μM/μl) diminished depression-like behaviors in SNI-treated rats, as shown by a decrease of immobility during the force swim test. Unpaired two-tailed Student's *t* test: saline group, n = 8; CX546 group, n = 7; **P* = 0.0199. (*B*) Intra-NAc infusion of CX546 (800 μM/μl) did not change the locomotion of SNI rats, compared with saline infusion. Two-way ANOVA with repeated measures and Bonferroni post-test: saline group, n = 4; CX546 group, n = 7; *P* = 0.9782.

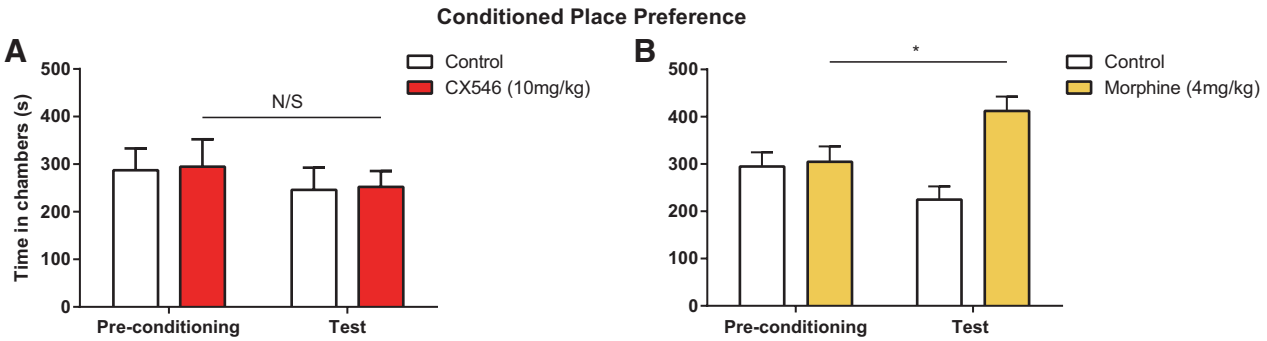

Fig. 9. Short-term use of AMPAkines does not lead to conditioned place preference. (*A*) Rats were conditioned for 4 days with alternating CX546 (10 mg/kg) and control treatments in the morning and afternoon, and then they were tested on the following day for place preference. Rats did not develop place preference for the chamber paired with AMPAkine treatment. Two-way ANOVA with repeated measures and Bonferroni post-test was used to compare preconditioning with test values: n = 11; *P* = 0.9046; N/S = nonsignificant. (*B*) Rats developed place preference for the chamber paired with morphine treatment (subcutaneous injection, 4 mg/kg): n = 10; *P* = 0.0349. **P* < 0.05.

pain, can increase GluA1 subunit levels at the synapse of the NAc core.[18] Here we investigated whether AMPAkines can further increase AMPA receptor trafficking in the NAc. We isolated synaptoneurosome fractions of NAc and measured the GluA1 and GluA2 subunit levels after AMPAkine treatment in PI-operated rats. We did not observe an increase in these subunit levels (fig. 10; P = 0.9972 for GluA1 and P = 0.6249 for GluA2). These results indicate that AMPAkines do not necessarily alter the synaptic levels of AMPA receptors over time. Thus, the pharmacologic effects of AMPAkines are more likely to be restricted to the biophysical function of the AMPA receptors, rather than to the long-term changes in receptor composition. These biochemical results are consistent with our pharmacologic results, demonstrating that the analgesic effects of AMPAkines do not last for a prolonged period of time.

Discussion

In the current study, we have investigated the role of AMPAkines in postoperative pain. We have found that CX546, a well-known AMPAkine, reduces sensory hypersensitivity and depression-like behaviors associated with acute postoperative pain. Furthermore, we have identified AMPA receptors in the NAc as a potential molecular target for these novel analgesics.

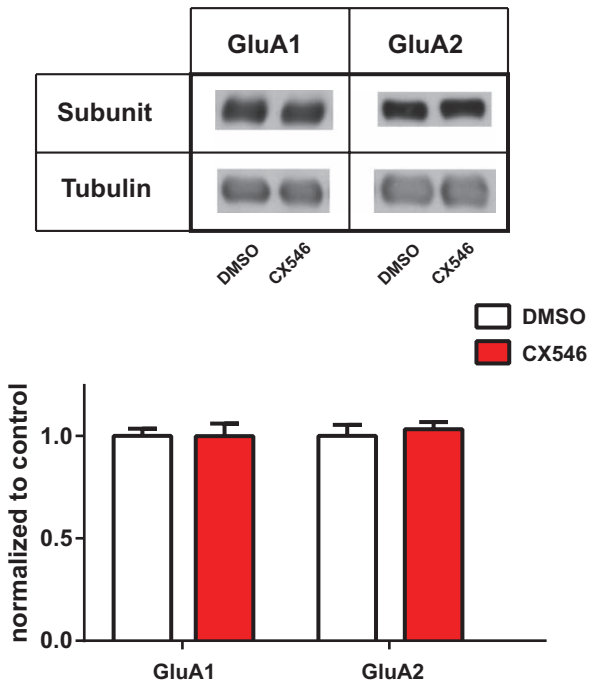

Fig. 10. AMPAkines do not increase α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking. CX546 (10 mg/kg) did not cause changes in levels of GluA1 or GluA2 subunits in the synaptoneurosome fractions of nucleus accumbens on postoperative day 1 after paw incision. Student's t test: n = 12 rats (GluA1); P = 0.9972 and n = 12 rats (GluA2); P = 0.6249. DMSO = dimethyl sulfoxide.

New analgesics that do not suppress the respiratory drive remain urgently needed, especially in the postoperative period. The acute postoperative period is also a high-risk time for the development of depressed mood that leads to worse surgical outcome.[4,5] AMPAkines slow the kinetics of AMPA receptor deactivation to enhance the inward excitatory synaptic current.[20,21] By increasing excitatory inputs in neurons of the pre-Botzinger complex in the medulla, AMPAkines can stimulate the respiratory drive[26,27,70–72] to treat or prevent hypoventilation caused by sedatives often used intra- or postoperatively.[26,28,73,74] A previous study has shown the effect of AMPAkines in persistent or chronic pain. Our current study demonstrates that these drugs can also effectively relieve the incisional pain commonly associated with surgery. Furthermore, the analgesic dose for CX546 found in our study is comparable to the dose tested in rats to treat respiratory depression,[27] and the time course of analgesia after a single administration (4 h) is similar to the duration of respiratory stimulation provided by these drugs.[26,27] Thus, from a pharmacologic and pharmacokinetic standpoint, AMPAkines can relieve postoperative pain, improve mood, and stimulate the respiratory drive at the same time. These properties suggest that AMPAkines can be potentially very useful in the acute postoperative period.

In addition to the canonical periaqueductal gray (PAG)-RVM spinal projection,[75–77] the NAc can also use AMPA receptor signaling to provide descending pain inhibition through its projection to the RVM.[16] Intra-NAc administration of AMPA receptor antagonists, for example, has been shown to specifically disrupt this pain-induced analgesic circuit.[15] Previous work has demonstrated, furthermore, that in both acute and chronic pain states, increasing AMPA receptor signaling in the NAc improves sensory hypersensitivity and associated depressive symptoms, whereas antagonism of AMPA receptor activities has the opposite effect.[17–19] Interestingly, persistent pain can cause a homeostatic increase in AMPA receptor number and function in the NAc, likely as an innate adaptive response.[17,18] Thus, given their role in pain and pain-induced mood changes, AMPA receptors in the NAc are potentially ideal targets for AMPAkines.

Depressed mood has been well described in postoperative pain patients,[2–6] and our finding that AMPAkines can treat pain-induced depression in a rat model of acute postoperative pain is consistent with the role of central AMPA receptors in depression.[78] Other pharmacologic examples include ketamine, which has been shown to elevate the levels of GluA1 AMPA receptor subunits in the brain[79] and effectively treat pain-induced depression.[41] By directly amplifying postsynaptic currents through AMPA receptors, AMPAkines have also been found to treat depression in animal models.[80] Our results demonstrate that this antidepressant effect of AMPAkines is preserved in the acute postoperative pain state. An alternative explanation is that AMPAkines, by relieving pain, also relieve depression associated with pain. This is a strong possibility. However, given the pharmacologic role

of AMPAkines in depression and the importance of AMPA receptors in the NAc in mood disorders,[17,20,23,81] AMPAkines could also be expected to potentially relieve sensory and depressive symptoms of pain independently.

Results on withdrawal tests and the FST can be confounded by locomotor deficits. For example, a worse performance on the FST may reflect a decrease in locomotion due to movement-induced pain or neuropathy. Our locomotion tests, however, do not reveal any deficits, compatible with earlier findings.[17–19] If rats do not demonstrate locomotor deficits over 30 min, they are unlikely to have deficits while swimming for 5 min during the FST. Thus, results on the FST likely reflect the phenotype of depression, rather than deficiencies in locomotion. In addition, other studies on AMPAkines have also demonstrated no effects on locomotion.[54]

A limitation of our study is that we did not sufficiently differentiate the cellular composition within the NAc. The NAc is composed of the core and shell regions anatomically. While core and shell are both primarily composed of γ-aminobutyric acid–mediated medium spiny neurons (MSNs), they differ in precise cellular morphology, neurochemistry, and afferent and efferent projections.[82] Functionally, the core has been proposed to mediate cue-conditioned behavioral activation, such as seeking of rewards or avoidance of noxious stimuli. The shell, meanwhile, is thought to code the salience of a particular behavioral condition.[82] Our pharmacologic and behavior experiments were conducted in the core because of its established role in nociception and its ability to induce behavioral modification reflected in behavioral despair. However, it is conceivable that some of the drug may have spread to the shell subregion, despite being infused directly in the NAc core and limited in volume. It is, therefore, possible that the shell subregion may have contributed to the analgesic effects of the AMPAkines in addition to the core subregion. The shell has been known to mediate the affective responsiveness to rewards and stress[83–85] and thus it has been suggested to code the aversive quality of pain.[86] Furthermore, MSNs can be subdivided into D1 or D2 neurons based on the specific subtypes of dopamine receptors expressed on their surfaces. D1 and D2 neurons have distinct projections and may be involved in different behavioral modifications. Therefore, future studies are needed to further elucidate the roles of D1 *versus* D2 subtypes of MSNs and the role of the NAc shell in AMPAkine analgesia.

AMPA receptor signaling can have both pronociceptive and antinociceptive roles, depending on the target CNS region. In the PFC, PAG, and NAc, augmentation of AMPA receptor signaling results in the activation of descending inhibition through projection to the RVM.[16,87–89] In contrast, in neurons of the spinal dorsal horn, anterior cingulate cortex (ACC), and amygdala, AMPA receptor activities can have pronociceptive effects. For example, chronic inflammatory pain increases membrane targeting of GluA1 AMPA receptor subunits in the spinal dorsal horn,[90–92] leading to the formation of GluA2-lacking receptors to augment pain transmission.[93] Similarly, AMPA receptor signaling in the ACC and amygdala has also been suggested to confer hyperalgesia.[94–98] An interesting pharmacologic property of the AMPAkines is that it has uniquely high affinity for neurons in the NAc and brain stem.[30] Thus, it is plausible that AMPAkines, when given systemically, selectively bind to AMPA receptors in the NAc to activate a descending inhibitory circuit. Nevertheless, the NAc is unlikely to be the only target for AMPAkines, and future studies examining other regions in the brain including the PAG and PFC are needed to elucidate the full spectrum of analgesic mechanisms of these drugs.

Activation of glutamatergic signaling in the NAc is a determinant of a drug's rewarding potential, particularly in craving and relapse.[36–38] Hence, one concern for drugs such as AMPAkines that can activate the glutamatergic signaling in the NAc is whether such drugs can have addictive potential in the clinical setting. Our CPP results suggested that a short-term use of these drugs does not carry intrinsic rewarding or addictive properties. Furthermore, three additional factors lead us to believe that the addictive risk of AMPAkines is minimized in the postoperative setting. First, AMPAkines only exert effects on AMPA receptors that are already open.[21,99] Thus, these drugs have a unique use-dependent profile. Studies have shown that activation of the corticostriatal pathway can exert analgesic effects.[19] Therefore, it is likely that AMPA receptors in the NAc carry out endogenous pain-inhibiting activities, and that AMPAkines function to potentiate this analgesic effect. In this way, AMPAkines may have behavioral specificity for pain conditions. Second, AMPAkines, including CX546, have been studied in clinical trials, but no report of addiction and abuse has been found.[27,28,74] Third, drugs of abuse not only increase glutamatergic signaling, but a vast majority of them also activate either the dopamine or opioid signaling pathways. However, AMPAkines do not activate either of these pathways. Thus, we argue for a relatively short-term use of AMPAkines, as we believe that their use in the acute postoperative period can complement opioid analgesia, and at the same time, their respiratory stimulatory activity increases the margin of safety for opioids. Prudent use in the acute postoperative period is less likely to lead to addiction and abuse.

In summary, we show that AMPAkines have novel analgesic properties in rat models of postoperative pain. A combination of analgesic and respiratory stimulatory properties can make AMPAkines ideal drugs for the acute postoperative period.

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Competing Interests

Dr. Wang has a patent on the use of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor modulators in pain regulation. The other authors declare no competing interests.

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