Calcium-Permeable α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid/Kainate Receptors Mediate Development, but Not Maintenance, of Secondary Allodynia Evoked by First-Degree Burn in the Rat

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ABSTRACT

Intrathecal pretreatment with N-methyl-D-aspartate (NMDA) receptor antagonists blocks development of spinal sensitization in a number of pain models. In contrast, secondary mechanical allodynia evoked by thermal injury (52.5°C for 45 s) applied to the hind paw of the rat is not blocked by intrathecal pretreatment with NMDA receptor antagonists. It is, however, blocked by antagonists to the non-NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate (AMPA/KA) and calcium-permeable AMPA/KA receptors. These findings suggest a role for these receptors in the development of spinal sensitization. The present study used the same thermal injury model to assess the effects of the AMPA/KA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and specific calcium-permeable AMPA/KA receptor antagonists philanthotoxin (PHTx) and joro spider toxin (JST) when given as postinjury treatments. Intrathecal saline injection at 5 and 30 min postinjury had no effect on thermal injury-evoked allodynia as measured by calibrated von Frey filaments. In contrast, 36 nmol of CNQX given at either time point reversed allodynia. Intrathecal 13 nmol of PHTx or 9 nmol of JST (higher doses than that required for pretreatment) reversed allodynia at the 5-min time point, but neither drug was antiallodynic at the 30-min time point. Thus, secondary mechanical allodynia in this model is not maintained by calcium-permeable AMPA/KA receptors, but instead requires activation of calcium-impermeable AMPA/KA receptors. This finding supports a role for AMPA/KA receptor function in responses occurring during spinal sensitization.

Peripheral inflammation and tissue injury induce sensitization of spinal cord neurons and enhance spinal nociceptive transmission (Dickenson and Sullivan, 1987; Abram and Yaksh, 1994; Traub, 1997). Behavioral correlates of spinal sensitization include secondary mechanical allodynia, an increased sensitivity to innocuous stimuli in a region adjacent to or distinct from the site of injury. Activation of N-methyl-D-aspartate (NMDA) receptors and subsequent calcium influx are thought to be early and necessary steps in the induction of spinal sensitization and resultant enhanced pain states (Murray et al., 1991; Mao et al., 1992; Yamamoto and Yaksh, 1992b). Accordingly, intrathecal administration of NMDA receptor antagonists has been shown to block both electrophysiological and behavioral manifestations of spinal sensitization (Woolf and Thompson, 1991; Dougherty et al., 1992). It is now apparent, however, that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or kainate (AMPA/KA) receptors can also play a significant role in this stage of nociceptive processing.

The AMPA receptor is composed of GLUR1-GLUR4 subunits, whereas the kainite receptor is composed of GLUR5-GLUR7, KA1, and K2 subunits. Both are permeable to monovalent sodium and potassium ions (Keinanen et al., 1990) and mediate the majority of monosynaptic current produced by glutamate release from primary afferent terminals. Recent studies show that activation of AMPA/KA, and not NMDA, receptors is required for the development of spinal sensitization and secondary hyperalgesia that occurs in models of postincision pain (Pogatzki et al., 2000) and first-degree burn (thermal injury) (Nozaki-Taguchi and Yaksh, 2002). In both models, preinjury treatment with an AMPA/KA receptor antagonist, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo/fquinoxaline-7-sulfonamide (PHTx) and joro spider toxin (JST) when given as postinjury treatments. Intrathecal saline injection at 5 and 30 min postinjury had no effect on thermal injury-evoked allodynia as measured by calibrated von Frey filaments. In contrast, 36 nmol of CNQX given at either time point reversed allodynia. Intrathecal 13 nmol of PHTx or 9 nmol of JST (higher doses than that required for pretreatment) reversed allodynia at the 5-min time point, but neither drug was antiallodynic at the 30-min time point. Thus, secondary mechanical allodynia in this model is not maintained by calcium-permeable AMPA/KA receptors, but instead requires activation of calcium-impermeable AMPA/KA receptors. This finding supports a role for AMPA/KA receptor function in responses occurring during spinal sensitization.

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ABBREVIATIONS: NMDA, N-methyl-D-aspartate; AMPA/KA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate; GLU, glutamate; CNQX, 6-cyano-7-nitroquinoxaline; Ca²⁺-perm-AMPA/KA, calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate; JST, joro spider toxin; PHTx, philanthotoxin; AUC, area under the curve; ANOVA, analysis of variance; CaM-kinase II, calcium/calmodulin-dependent kinase II.
(NBQX) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), blocks development of increased spinal nociceptive responses, whereas NMDA receptor antagonists have little or no effect on enhanced pain behavior.

Calcium-permeable AMPA/KA (Ca\(^{2+}\)-perm-AMPA/KA) receptors substantially influence synaptic activity occurring throughout the central nervous system. Activation of these receptors increases intracellular calcium levels during states of synaptic strengthening (Gu et al., 1996), ischemia (Gorter et al., 1997), and excitotoxicity (Lu et al., 1996; Carriedo et al., 1998). The Ca\(^{2+}\)-perm-AMPA/KA receptors also mediate spinal sensitization. Intrathecal pretreatment with selective antagonists joro spider toxin (JST) or philanthotoxin (PHTx) attenuates development of secondary mechanical allodynia evoked in the thermal injury model (Sorkin et al., 1999, 2001). In addition, joro spider toxin reverses secondary mechanical allodynia in the postinjury pain model (Pogatzki et al., 2003). The present study examined the effects of AMPA/KA and Ca\(^{2+}\)-perm-AMPA/KA receptor antagonists on thermal injury-evoked secondary mechanical allodynia when given as postinjury treatments.

### Materials and Methods

#### Animals

Male Holtzman rats (300–350 g; Harlan, Indianapolis, IN) were housed in 12:12-h light:dark cycle. Food and water were made available ad libitum, except during recovery from surgery and mechanical threshold testing. Effort was made to minimize animal discomfort and reduce number of animals used. All experiments were approved by the Animal Care Committee of the University of California, San Diego.

#### Intrathecal Catheter Implantation

Animals were anesthetized with 3% isoflurane (Halocarbon Laboratories, River Edge, NJ) and catheters (PE-5; Baxter Healthcare Corporation, Deerfield, IL) were implanted into the subarachnoid space and ended over the lumbar enlargement (Yaksh and Rudy, 1976). Animals received 5 ml of intraperitoneal lactated Ringer’s solution (Baxter Healthcare Corporation) immediately after surgery and again at 1 and 2 days postsurgery. Animals were housed individually after intrathecal catheters were implanted. Paw withdrawal threshold and motor function testing occurred 5 days or more after catheter implantation.

#### Assessment of Mechanical Allodynia

Animals were given 30 min to acclimate to their individual testing compartments (26 × 11 × 20 cm) that were comprised of a wire-mesh bottom and clear (Plexiglas) walls and cover, before obtaining two baseline withdrawal threshold measures. Calibrated von Frey filaments (Stoelting, Wood Dale, IL) with buckling forces between 4.7 and 147.05 mN were applied sequentially to a central area of the plantar hind paw at a perpendicular angle until paw withdrawal occurred. The up-down paradigm was used to determine 50% probability of paw withdrawal thresholds (Chaplan et al., 1994).

#### First-Degree Burn and Secondary Mechanical Allodynia

After baseline responses were measured, animals were lightly anesthetized with 2% isoflurane while the left plantar hind paw was placed and held on a 52.5°C metal surface for 45 s at constant pressure by a 10-g sand pouch (Nozaki-Taguchi and Yaksh, 1998). This first-degree burn results in transient redness in the skin and evokes reduced withdrawal thresholds to innocuous mechanical stimuli applied to the previously mentioned central area of the hind paw that is distinct from the injury site. This same area does not display thermal sensitization. After the first-degree burn was applied, animals were returned to individual testing compartments where they recovered from anesthesia within 2 to 3 min.

#### Drugs

Drugs were administered i.t. in 10 μl of sterile saline (Abbott Laboratories, North Chicago, IL) vehicle and included the AMPA/KA receptor antagonist CNQX (mol. wt. 232.2) (Sigma-Aldrich, St. Louis, MO) and the Ca\(^{2+}\)-perm-AMPA/KA receptor antagonists philanthotoxin and joro spider toxin (mol. wt. 777.7 and 585.7, respectively) (Sigma-RBI, Natick, MA).

### Behavioral Experiment 1: Preinjury Treatments

Intrathecal injections were administered 5 min before thermal injury to assess their effect on subsequent withdrawal responses. Control animals received 20 μl of sterile saline flush 5 min before thermal injury. In other groups, 36 nmol of CNQX, 6 nmol of PHTx, or 5 nmol of JST was administered followed by a 10 μl of sterile saline flush. These are the lowest doses found to be effective as preinjury treatments (Sorkin et al., 2001; Nozaki-Taguchi and Yaksh, 2002).

Testing for withdrawal responses began 30 min after thermal injury and continued at 30-min intervals for 2.5 h. The person conducting behavioral testing did not know which agent was administered to each animal.

#### Behavioral Experiment 2: Postinjury Treatments

Intrathecal injections were administered 5 or 30 min after thermal injury to assess their effect on subsequent withdrawal responses. Control animal groups received 20 μl of sterile saline, similar to the preinjury treatments. In other groups, 36 nmol of CNQX, 6 or 13 nmol of PHTx, or 5 or 9 nmol of JST was administered and followed by a 10-μl sterile saline flush.

Testing for withdrawal responses began 30 min after thermal injury for the 5-min post-treatment group and 60 min after thermal injury for the 30-min post-treatment group. Testing continued at 30-min intervals until 2.5 h postinjury. The person conducting behavioral threshold testing did not know which agent was administered to each animal.

#### Behavioral Experiment 3: Effects of Intrathecal Agents on Motor Function

Intrathecal administration of many antagonists of facilitatory spinal cord mechanisms elicits depressive effects on central nervous system activity and motor function. An accelerating rotarod apparatus (Columbus Instruments, Columbus, OH) was used to assess whether the effects of agents on withdrawal threshold responses were due to nonspecific motor deficits versus specific antiallodynic effects.

Animals were trained on the rotarod for 1 to 2 days. Training consisted of at least two 1-min trials at 4 rpm. On the test day, animals were placed on the rotating rod for several seconds at 4 rpm, before the rod began accelerating at 1 rpm/s. The duration of time until the animal fell from the moving rotarod was measured (maximum 180 s). Two measures from each animal were averaged and served as baseline (preintrathecal injection). Animals were restested 30 and 90 min after intrathecal injection of saline or highest drug dose used in experiments (13 nmol of PHTx, 9 nmol of JST, and 36 nmol of CNQX). These time points correlated with the average onset and diminution of thermal injury-evoked secondary mechanical allodynia.

### Statistical Analysis

A Friedman’s test was performed to determine difference between withdrawal threshold responses before and those after thermal injury to hind paws (p < 0.05, secondary mechanical allodynia). A post hoc Dunn’s multiple comparisons analysis followed this statistic. The Mann-Whitney U test determined differences in mean baseline responses between treatment groups, and p < 0.05 was considered significant.

Area under the curve (AUC) values were calculated from the withdrawal threshold values of each animal across time (GraphPad Prism 3.02; GraphPad Software Inc., San Diego, CA). Increase in AUC correlated with a decrease in allodynia. One-way analysis of variance (ANOVA) test was performed to determine difference between the AUC for intrathecal saline versus drugs. This statistic was performed for time points 30 through 180 min after thermal injury for all groups except for the 30-min postinjury treatment group. ANOVA for the latter was performed for time points 60 through 180 min. A post hoc Dunnett’s multiple comparisons analysis followed the ANOVA test.

### Results

Mean baseline withdrawal thresholds did not differ across the pre-, 5-, or 30-min postinjury saline treatments (140.0 ±
4.5, 141.9 ± 3.6, and 142.8 ± 2.2 mN, respectively). All three groups of control animals displayed reduced withdrawal threshold responses after thermal injury, indicating secondary mechanical allodynia (Figs. 1A, 2A, and 3A).

**Preinjury Treatment.** Peak secondary mechanical allodynia after pretreatment with saline occurred 60 min after thermal injury (22.0 ± 4.2 mN) (Fig. 1A). In contrast, allodynia was blocked by preinjury treatment with 6

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**Fig. 1.** A, effects of preinjury treatments on secondary mechanical allodynia. Results are expressed as box and whisker plots: the horizontal line is the median, whereas the top and bottom of the box are 75th and 25th percentiles. Whiskers indicate 90th and 10th percentiles. Baseline (B) withdrawal thresholds were measured before thermal injury and intrathecal injection (arrow). Preinjury treatments were administered 5 min before injury. Preinjury time course of withdrawal thresholds is shown for saline (\( p = 0.0001, n = 7 \)), 6 nmol of PHTx (\( p = 0.0570, n = 8 \)), 5 nmol of JST (\( p = 0.4954, n = 8 \)), and 36 nmol of CNQX (\( p = 0.4439, n = 7 \)) treatments (\( p < 0.05 = \text{antiallodynia} \)). *, \( p < 0.05 \) and ***, \( p < 0.001 \), compared with baseline. ** indicates the median that overlaps the 25th and/or 75th percentile. B, AUC of withdrawal thresholds for preinjury treatments. The AUC for all groups that received intrathecal drugs are greater than saline control. *, \( p < 0.05 \) and **, \( p < 0.001 \), compared with saline control. Values are expressed as mean ± S.E.M.
Fig. 2. A, effects of 5-min postinjury treatments on secondary mechanical allodynia. Results are expressed as box and whisker plots (described in the Fig. 1A legend). Baseline (B) withdrawal thresholds were measured before thermal injury (arrow). Intrathecal post-treatments were administered 5 min after injury. Time course of withdrawal thresholds is shown for saline ($p = 0.0001, n = 7$), 6 nmol of PHTx ($p = 0.0090, n = 7$), 5 nmol of JST ($p = 0.0004, n = 9$), 13 nmol of PHTx ($p = 0.1915, n = 6$), 9 nmol of JST ($p = 0.2317, n = 9$), and 36 nmol of CNQX ($p = 0.2317, n = 6$) treatments ($p > 0.05 = \text{antiallodynia}$). *,$p < 0.05$ and **,$p < 0.01$, compared with baseline. † indicates the median that overlaps the 25th and/or 75th percentile.

B, AUC of withdrawal thresholds for 5-min postinjury treatments. The AUC for low-dose PHTx (6 nmol) or JST (5 nmol) do not differ from saline control. **,$p = 0.001$, compared with saline control. Values are expressed as mean ± S.E.M.
nmol of PHTx, 5 nmol of JST, or 36 nmol of CNQX (Fig. 1A).

The AUC of withdrawal thresholds measured from 30 min to 2.5 h after thermal injury for each treatment group is also shown in Fig. 1B. Values for all preinjury drug treatments are greater than saline control, indicating decreased allodynia.

**Five-Minute Postinjury Treatment.** Peak secondary mechanical allodynia after 5-min post-treatment with saline also occurred 60 min after thermal injury (31.9 ± 5.1 mN).
and differed from baseline (Fig. 2A). Although 6 nmol of PHTx or 5 nmol of JST was effective as a preinjury treatment, neither was sufficient to block allodynia when given 5 min postinjury (Fig. 2A). However, increased doses (13 nmol of PHTx and 9 nmol of JST) were antiallodynic when administered as 5 min postinjury treatments. The CNQX dose, which was effective as a pretreatment, was also able to reverse mechanical allodynia when given 5 min after injury.

The AUC of withdrawal thresholds from 30 min to 2.5 h after thermal injury for saline control and drug groups are also shown in Fig. 2B. There is no difference between areas under the curve for low-dose PHTx (6 nmol) or JST (5 nmol) and saline control.

Thirty-Minute Postinjury Treatment. The peak allodynia after 30 min post-treatment with saline also occurred 60 min after thermal injury (60.73 ± 19.03 mN) (Fig. 3A). When PHTx and JST treatments were delayed until 30 min postinjury they were no longer antiallodynic. In contrast, 30-min post-treatment with 36 nmol of CNQX reversed secondary mechanical allodynia just as it had when given at the two earlier time points.

The AUC of withdrawal thresholds measured from 60 min to 2.5 h after thermal injury for each group are also shown in Fig. 3B. Only the CNQX area under the curve differs from saline control, indicating an antiallodynic effect.

Effects of Intrathecal Injections on Motor Function. Effects of the largest doses of intrathecal PHTx, JST, and CNQX on motor function were assessed by measuring the ability of rats to remain on an accelerating rotarod 30 and 90 min after drugs were administered. Before intrathecal drug injections, mean duration on the rotarod was 113.9 ± 14.6 min and did not differ from saline control (105.9 ± 11.2). Figure 4 shows change from baseline time for rats that received intrathecal saline or drugs. Spinally administered AMPA/KA and Ca2+-permeable AMPA/KA receptor antagonists at antiallodynic doses did not alter the ability of rats to remain on the rotarod, indicating that effects of these drugs on mechanical allodynia were specific and not due to side effects such as sedation or motor deficits.

Discussion

Innocuous or low-frequency noxious stimulation results in excitatory amino acid release that activates postsynaptic non-NMDA receptors. Although these receptors are responsible for fast monosynaptic transmission leading to neuronal depolarization, they also have a role in nociceptive transmission. Intrathecal administration of AMPA/KA receptor antagonists blocks development of acute (Nishiyama et al., 1998) and inflammatory (Stanfa and Dickenson, 1999) pain. A subset of AMPA/KA receptors that lack the GLUR2 receptor subunit and are calcium-permeable (Burnashev et al., 1992) mediate secondary mechanical allodynia occurring in models of postoperative pain (Pogatzki et al., 2003) and first-degree burn (Sorkin et al., 1999, 2001).

The present study confirms that calcium-permeable AMPA/KA receptors are involved in the development of secondary mechanical allodynia evoked by first-degree burn and that their activity parallels NMDA receptor activity in other pain models. For example, NMDA receptors mediate increases in intracellular calcium levels that subsequently induce various second-messenger systems involved in spinal sensitization and resultant hyperalgesia and/or allodynia. Preinjury treatment with NMDA receptor antagonists blocks development of these phenomena (Dougherty et al., 1992; Mao et al., 1992), whereas postinjury treatment has little or no effect (Yamamoto and Yaksh, 1992a). In the present study, pretreatment doses of calcium-permeable AMPA/KA receptor antagonists that were antiallodynic did not reverse secondary mechanical allodynia when administered 5 min after thermal injury. Even increased doses of these antagonists had no effect on allodynia when administered 30 min post-treatment. This suggests that the roles of both NMDA and calcium-permeable non-NMDA receptors in different pain states are similar and that both receptor subtypes may induce some shared or common intracellular mechanisms as a consequence of calcium influx and subsequent calcium-dependent processes.

Because secondary mechanical allodynia occurring in the first-degree burn model has fast onset and dissipates within a couple of hours, it must be mediated by rapid changes in synaptic or intracellular activity, as opposed to prolonged gene transcription or de novo protein synthesis. Previous studies demonstrate calcium-dependent protein kinase activity and increased phosphorylation of the AMPA GLUR1 receptor subunit in the spinal cord dorsal horn as early as 5 min after intradermal capsaicin-evoked hyperalgesia in the rat (Fang et al., 2002, 2003). Phosphorylation of the AMPA GLUR1 subunit by calcium-dependent protein kinases, such as calcium/calmodulin-dependent kinase IIα (CaM-kinase IIα), increases AMPA receptor-channel conductance (Derkach et al., 1998).
1999) and thus, AMPA receptor function. Activated CaM-kinase IIα also promotes AMPA receptor insertion into the postsynaptic membrane (Liao et al., 2001). Thus, CaM-kinase IIα can influence spinal activity by enhancing AMPA receptor function and increasing AMPA receptor density within postsynaptic membranes. Both of these actions could induce development of spinal sensitization occurring in the first-degree burn.

Activation of calcium-permeable AMPA/KAR receptors likely mediates calcium-dependent processes that induce spinal sensitization and allodynia occurring in the first-degree burn. These processes, in turn, may facilitate spinal cord neuronal activity that becomes independent of calcium-permeable AMPA/KAR receptor occupation. The observed lack of calcium-permeable AMPA/KAR receptor involvement in maintenance of secondary mechanical allodynia may be due to a decrease in membrane receptor density caused by clathrin-mediated endocytosis (Lin et al., 2000). Previous electrophysiological studies demonstrate that calcium-permeable AMPA receptors rapidly internalize after their activation (Liu and Cull-Candy, 2000; Liu et al., 2002). Receptor, or receptor subunit, cycling in and out of the postsynaptic membrane (Lissin et al., 1999) may very well shift the level of calcium-permeable AMPA receptor involvement. These mechanisms may correlate with the induction and duration of secondary mechanical allostody observed in the present model of spinal sensitization.

In summary, this study demonstrated that non-NMDA, AMPA/KAR receptors are involved in the development and maintenance of spinal sensitization, whereas specific calcium-permeable AMPA/KAR receptors, such as NMDA receptors, are primarily involved in its development.

References

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