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Calcium-permeable AMPA/kainate receptors mediate development, but not maintenance, of secondary allodynia evoked by first-degree burn in the rat

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Ca^{2+}-permeable AMPA/KA receptors and allodynia

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Abstract

Intrathecal pre-treatment 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 seconds) applied to the hind paw of the rat is not blocked by intrathecal pre-treatment with NMDA receptor antagonists. It is, however, blocked by antagonists to the non-NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic 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, CNQX, and specific calcium-permeable AMPA/KA receptor antagonists, philanthotoxin (PHTx) and joro spider toxin (JST) when given as post-injury treatments. Intrathecal saline injection at 5 and 30 minutes post injury had no effect on thermal injury-evoked allodynia as measured by calibrated von Frey filaments. In contrast, 36nmol CNQX given at both time points reversed allodynia. Intrathecal 13nmol PHTx or 9nmol JST (higher doses than that required for pre-treatment) reversed allodynia at the 5-minute time point, but neither drug was anti-allodynic at the 30-minute 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 is thought to be an early and necessary step 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-isoxazole-proprionic acid or kainite (AMPA/KA) receptors can also play a significant role in this stage of nociceptive processing.

The AMPA receptor is composed of GLUR1-GLUR4 subunits, while 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 post-incision pain (Pogatzki et al., 2000) and first-degree burn (thermal injury) (Nozaki-Taguchi and Yaksh, 2002). In both models, pre-injury treatment with an AMPA/KA receptor antagonist, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo[f]quinoxaline-7-sulfonamide (NBQX) or 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), blocks

development of increased spinal nociceptive responses, while 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 pre-treatment 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; Sorkin et al., 2001). In addition, joro spider toxin reverses secondary mechanical allodynia in the post-incision 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 post-injury treatments.

Methods

Animals

Male Holtzman rats (300-350g, Harlan Industries, Indianapolis, IN) were housed in 12:12 hour 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% isofluorane (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, 1976). Animals received 5mL of intraperitoneal Lactated Ringer's solution (Baxter Healthcare Corporation, Deerfield, IL) immediately after surgery and again at one and two days post surgery. 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 minutes to acclimate to their individual testing compartments (26 x 11 x 20cm) that were comprised of a wire-mesh bottom and clear

(plexi-glass) walls and cover, prior to obtaining two baseline withdrawal threshold measures. Calibrated von Frey filaments (Stoelting, Wood Dale, IL) with buckling forces between 4.7 and 147.05mN 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% isofluorane while the left plantar hind paw was placed and held on a 52.5°C metal surface for 45 seconds at constant pressure by a 10-gram 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 2-3 minutes.

Drugs

Drugs were administered intrathecally (i.t.) in 10μL of sterile saline (Abbott Laboratories, North Chicago, IL) vehicle and included the AMPA/KA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, molecular weight (M.W.) 232.2) (Sigma, St. Louis, MO) and the Ca^{2+}-perm-AMPA/KA receptor antagonists philanthotoxin and

joro spider toxin (M.W. 777.7 and 565.7, respectively) (Research Biochemicals International, Natick, MA).

Behavioral Experiment 1: Pre-injury treatments

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

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

Behavioral Experiment 2: Post-injury treatments

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

Testing for withdrawal responses began 30 minutes after thermal injury for the 5-minute post-treatment group and 60 minutes after thermal injury for the 30-minute post-treatment group. Testing continued at 30-minute intervals until 2.5 hours post-injury.

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 non-specific motor deficits versus specific anti-allodynic effects.

Animals were trained on the rotarod for 1 to 2 days. Training consisted of at least two 1-minute trials at 4 rotations per minute (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/second. The duration of time until the animal fell from the moving rotarod was measured (maximum 180 seconds). Two measures from each animal were averaged and served as baseline (pre-intrathecal injection). Animals were re-tested 30 and 90 minutes after intrathecal injection of saline or highest drug dose used in experiments (13nmol PHTx, 9nmol JST, and 36nmol 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 test determined differences in mean baseline responses between treatment groups and $p<0.05$ was considered significant.

Areas under the curve (AUC) were calculated from the withdrawal threshold values of each animal across time (GraphPad Prism, 3.02, 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 minutes after thermal injury for all groups except for the 30-minute post-injury treatment group. ANOVA for the latter was performed for time points 60 through 180 minutes. 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-minute post-injury saline treatments (140.0 ± 4.5, 141.9 ± 3.6 and 142.8 ± 2.2mN, respectively). All three groups of control animals displayed reduced withdrawal threshold responses after thermal injury, indicating secondary mechanical allodynia. (Figures 1A, 2A and 3A).

Pre-injury treatment

Peak secondary mechanical allodynia after pre-treatment with saline occurred 60 minutes after thermal injury (22.0 ± 4.2mN) (Figure 1A). In contrast, allodynia was blocked by pre-injury treatment with 6nmol PHTx, 5nmol JST or 36nmol CNQX (Figure 1A).

The areas under the curve (AUC) of withdrawal thresholds measured from thirty minutes to 2.5 hours after thermal injury for each treatment group are also shown in Figure 1B. Values for all pre-injury drug treatments are greater than saline control, indicating decreased allodynia.

5-minute post-injury treatment

Peak secondary mechanical allodynia after 5-minute post-treatment with saline also occurred 60 minutes after thermal injury (31.9 ± 5.1mN) and differed from baseline (Figure 2A). Although 6nmol PHTx or 5nmol JST was effective as a pre-injury treatment, neither was sufficient to block allodynia when given 5 minutes post-injury

(Figure 2A). However, increased doses (13nmol PHTx and 9nmol JST) were anti-allodynic when administered as 5-minute post-injury treatments. The CNQX dose, which was effective as a pre-treatment, was also able to reverse mechanical allodynia when given 5 minutes after injury.

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

30-minute post-injury treatment

The peak allodynia after 30-minute post-treatment with saline also occurred 60 minutes after thermal injury (60.73 ± 19.03mN) (Figure 3A). When PHTx and JST treatments were delayed until 30 minutes post-injury they were no longer anti-allodynic. In contrast, 30-minute post-treatment with 36nmol 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 minutes to 2.5 hours after thermal injury for each group are also shown in Figure 3B. Only the CNQX area under the curve differs from saline control, indicating an anti-allodynic 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 minutes after drugs were administered. Prior to intrathecal drug injections, mean duration on the rotarod was 113.9 ± 14.6 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 Ca^{2+}-permeable AMPA/KA receptor antagonists at anti-allodynic 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 post-operative pain (Pogatzki et al., 2003) and first-degree burn (Sorkin et al., 1999; Sorkin et al., 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. Pre-injury treatment with NMDA receptor antagonists blocks development of these phenomena (Dougherty et al., 1992; Mao et al., 1992), while post-injury treatment has little or no effect (Yamamoto and Yaksh, 1992a). In the present study, pre-treatment doses of calcium-permeable AMPA/KA receptor antagonists that were anti-allodynic did not reverse secondary mechanical allodynia when administered 5 minutes after thermal injury. Even increased doses of these antagonists had no effect on allodynia when administered as 30-minute post-treatments. 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.

As 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 minutes after intradermal capsaicin-evoked hyperalgesia in the rat (Fang et al., 2002; Fang et al., 2003). Phosphorylation of the AMPA GLUR1 subunit by calcium-dependent protein kinases, like calcium/calmodulin-dependent kinase IIα (CaM-Kinase IIα), increases AMPA receptor-channel conductance (Derkach et al., 1999) and thus, AMPA receptor function. Activated CaM-Kinase IIα also promotes AMPA receptor insertion into the post-synaptic membrane (Liao et al., 2001). Thus, CaM-Kinase IIα can influence spinal activity by enhancing AMPA receptor function and increasing AMPA receptor density within post-synaptic membranes. Both of these actions could induce development of spinal sensitization occurring in the first-degree burn.

Activation of calcium-permeable AMPA/KA receptors likely mediates calcium-dependent processes that induce spinal sensitization and allodynia occurring in the first-degree burn. These processes, in turn, appear to facilitate spinal cord neuronal activity that becomes independent of calcium-permeable AMPA/KA receptor occupation. The observed lack of calcium-permeable AMPA/KA 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 post-synaptic 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 allodynia observed in the present model of spinal sensitization.

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

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Footnotes

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Legends for figures

Figure 1

A. Effects of pre-injury treatments on secondary mechanical allodynia. Results are expressed as box and whisker plots: the horizontal line is the median, while the top and bottom are 75^{th} and 25^{th} percentiles. Error bars indicate 90^{th} and 10^{th} percentiles. Baseline (B) withdrawal thresholds were measured before thermal injury and intrathecal injection (arrow). Pre-injury treatments were administered 5 minutes before injury. Pre-injury time course of withdrawal thresholds is shown for saline ($p=0.0001$, n=7), 6nmol PHTx ($p=0.0570$, n=8), 5nmol JST ($p=0.4954$, n=8) and 36nmol CNQX ($p=0.4439$, n=7) treatments ($p>0.05$=anti-allodynia). *$p<0.05$, ***$p<0.001$, compared to baseline. ✓, median=75^{th} percentile. **B. Areas under the curve (AUC) of withdrawal thresholds for pre-injury treatments**. The AUC for all groups that received intrathecal drugs are greater than saline control. *$p<0.05$, **$p<0.001$, compared to saline control. Values are expressed as mean ± S.E.M.

Figure 2

A. Effects of 5-minute post-injury treatments on secondary mechanical allodynia. Results are expressed as box and whisker plots (described in the Figure 1A legend). Baseline (B) withdrawal thresholds were measured before thermal injury (arrow). Intrathecal post-treatments were administered 5 minutes after injury. Time course of withdrawal thresholds is shown for saline ($p=0.0001$, n=7), 6nmol PHTx ($p=0.0090$, n=7), 5nmol JST ($p=0.0004$, n=9), 13nmol PHTx ($p=0.1915$, n= 6), 9nmol JST ($p=0.2317$, n=9) and 36nmol CNQX ($p=0.2317$, n=6) treatments ($p>0.05$=anti-

allodynia). *p<0.05, **p<0.01, compared to baseline. ✓, median=75th percentile. **B. Areas under the curve (AUC) of withdrawal thresholds for 5-minute post-injury treatments**. The AUC for low-dose PHTx (6nmol) or JST (5nmol) do not differ from saline control. **p=0.001, compared to saline control. Values are expressed as mean ± S.E.M.

Figure 3

A. Effects of 30-minute post-injury treatments on secondary mechanical allodynia. Results are expressed as box and whisker plots (described in the Figure 1A legend). Baseline (B) withdrawal thresholds were measured before thermal injury (arrow). Intrathecal post-treatments were administered 30 minutes after injury. Time course of withdrawal thresholds from 60-180 minutes after thermal injury is shown for saline (p=0.0001, n=7), 13nmol PHTx (p=0.0012, n=7), 9mol JST (p=0.0128, n=8) and 36nmol CNQX (p=0.7280, n=9) treatments (p>0.05=anti-allodynia). *p<0.05, **p<0.01, compared to baseline. ✓, median=75th percentile. **B. Areas under the curve (AUC) of withdrawal thresholds for 30-minute post-injury treatments**. Only the CNQX AUC is greater than saline control. *p>0.05, compared to saline control. Values are expressed as mean ± S.E.M.

Figure 4

Effects of intrathecal injections on motor function. Figure shows percent of baseline time (before injection) rats walked on an accelerating rotarod 30 and 90 minutes post intrathecal saline, 13nmol PHTx, 9nmol JST or 36nmol CNQX. Intrathecal treatments with saline or drugs produced no apparent motor deficits. Values are shown as mean ± S.E.M., n=10/group.

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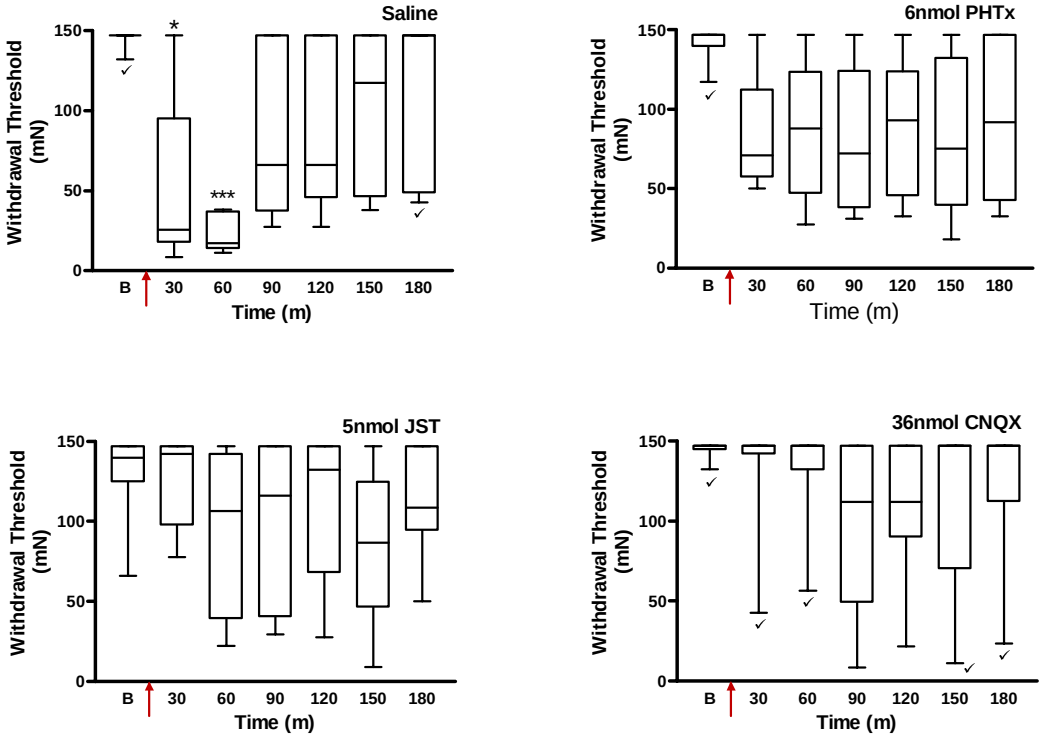
Figure 1

A.



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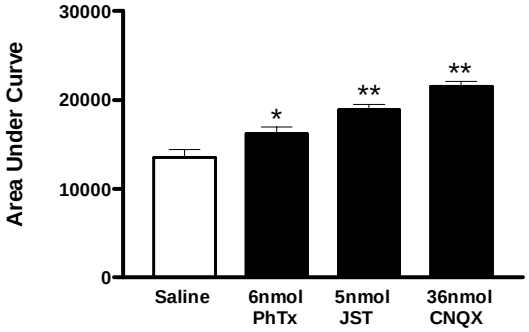

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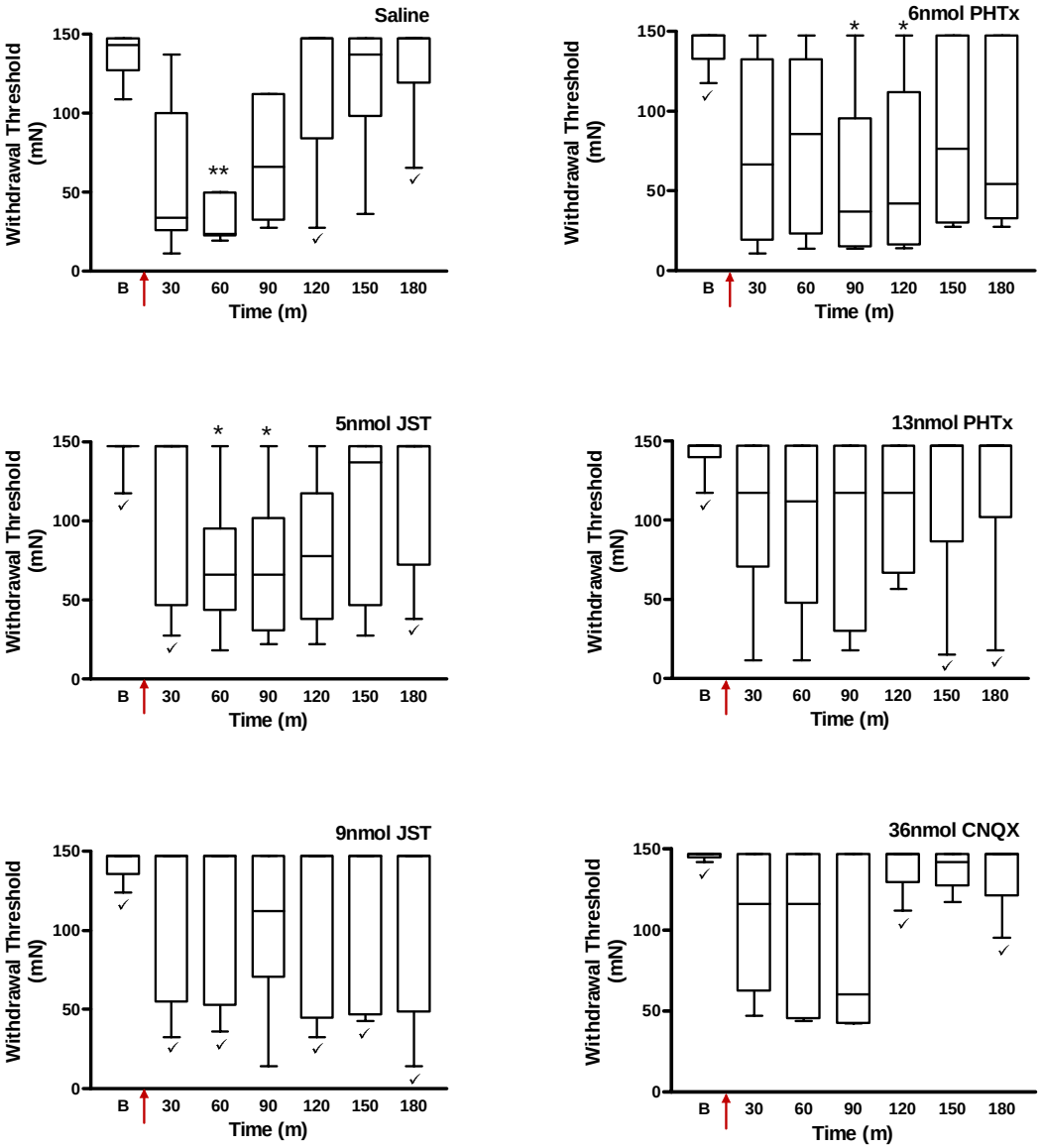
Figure 2

A.



B.

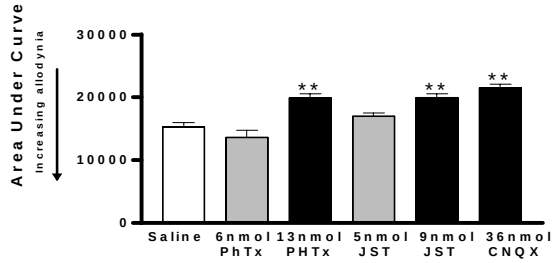


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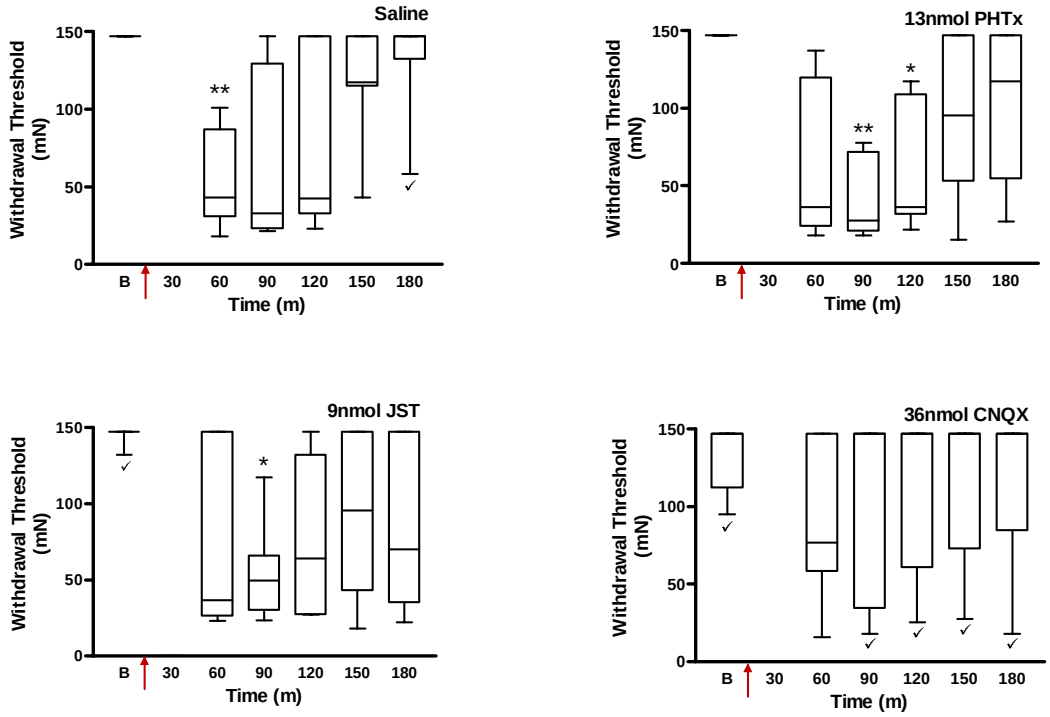
Figure 3

A.



B.

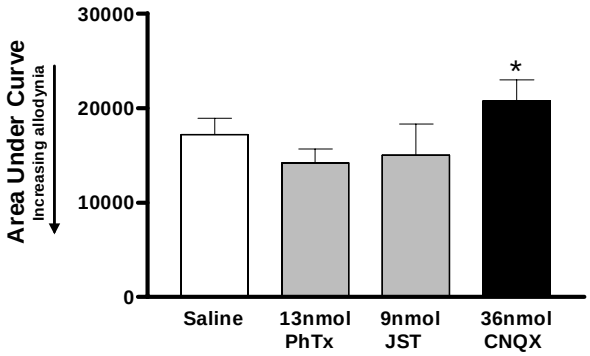


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Figure 4