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Changes in expression of NMDA-NR1 receptor subunits in the rostral ventromedial medulla modulates pain behaviors

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Summary

Increasing the NR1 subunit of the NMDA receptor in the RVM produces hyperalgesia, while decreasing NR1 in the RVM produces hypoalgesia and prevents development of chronic muscle hyperalgesia.

Keywords

NR1 subunit; NMDA receptors; rostral ventromedial medulla; muscle pain; hyperalgesia; FIV virus

1. Introduction

Glutamate and its receptors in the brainstem play an important role in both inhibitory and facilitatory pathways of pain modulation. Injection of low doses of glutamate into the rostral ventromedial medulla (RVM) facilitates behavioral responses to noxious stimuli while high doses of glutamate inhibit behavioral responses to noxious stimuli^{48,49}. After injury, changes in the balance between facilitation and inhibition occur with enhanced facilitation thought to depend on glutamate binding to NMDA receptors. In animals with tissue injury, there is an enhanced release of glutamate in the RVM and enhanced excitatory transmission of RVM neurons^{27,47}, which can be reversed by pharmacological blockade of NMDA receptors⁴⁷. Pharmacological blockade of NMDA receptors in the RVM also reverses hyperalgesia produced by colon inflammation, nerve injury, or muscle pain induced by repeated acid injections^{8,9,40,43}. Thus, current data support a role for NMDA receptors in the facilitation of nociception within the RVM.

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Conflict of Interest Statement

There are no conflicts of interest.

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NMDA receptors are ionotropic channels, heteromers composed of NR1 and one or more of the 4 NMDA2 (NR2A-D) or 2 NMDA3 (NR3A and B) subunits⁴¹. The NR1 subunit is essential for function of the NMDA receptors and phosphorylation of NR1 is a major mechanism of modulating channel activity and trafficking to the neuronal surface^{5,6}. In the spinal cord, downregulation of NR1 subunits in the dorsal horn has no effect on baseline nociceptive responses in uninjured animals but prevents formalin or complete Freund's adjuvant (CFA)-induced nociceptive behaviors in inflammatory pain models^{7,14,30,35}. In the RVM, gene expression of NR1 is increased for 7 days after CFA-induced hindpaw inflammation²³. Together, these data suggest an important role for the NR1 subunit in nociceptive processing particularly in the brain and spinal cord.

Our recent studies of muscle pain suggest the RVM is a critical site to modulate both cutaneous and muscle hypersensitivity induced by repeated intramuscular acid injections. Anesthetic blockade in the RVM during the intramuscular injections prevents the development of both cutaneous and muscle hypersensitivity while both anesthetic blockade and NMDA receptor blockade in the RVM reverses the hypersensitivity once developed^{9,37}. Our model of deep tissue muscle pain is unique in that once developed, central mechanisms, not peripheral mechanisms, maintain the hyperalgesia^{34,37}. We propose that the NR1 subunit in the RVM is critical for full development of the hypersensitivity after muscle insult. To test this hypothesis we used recombinant feline immunodeficiency virus (FIV) based vectors to overexpress the NR1 subunit in the RVM and measured nociceptive sensitivity to cutaneous and muscle stimuli. We also down-regulated expression of NR1 in the RVM and measured the hyperalgesia produced by repeated acid injections.

2. Materials and Methods

All experiments with laboratory animals were approved by the Animal Care and Use Committee at the University of Iowa and were conducted in accordance with National Institute of Health guidelines. Adult male Sprague-Dawley rats (250–350 g, Harlan, Indianapolis, IN) were used for this study. The animals were housed in a 12-h dark/light cycle, and the testing was done only during the light cycle. Food and water were available to the animals *ad libitum*.

2.1. Placement of guide cannula

An intracerebral guide cannula was stereotactically implanted in the RVM. The rats were anesthetized with pentobarbital sodium (Nembutal, 50mg/kg, i.p.), secured in a stereotactic head holder and implanted with a guide cannula (18.0 mm in length, 26 gauge; Plastics One, Roanoke, VA). After the midline incision, the skull was exposed, and a small hole drilled for placement of the guide cannula. The cannula was placed 3.0 mm dorsal to the RVM using the following coordinates: intra-aural: –2.0 mm; mediolateral: 0.0 mm; and dorsoventral: –7.5 mm²⁵. The cannula was secured to the skull by two stainless-steel screws and dental acrylic cement. An obturator (33 gauge, Plastics One) was inserted into the guide cannula to maintain its patency. All rats were allowed 3 days to recover before testing began.

At the end of the experiment the rats were euthanized and transcardially perfused with 4% paraformaldehyde. The brain was removed and stored in 30% sucrose until processing for immunofluorescence for NR1, described below. The placement of the guide cannula was determined during immunostaining analysis.

2.2. Virus construction

FIV expressing NR1 (FIV-NR1): The pRK5 plasmid containing the cDNA for rat NR1-1a was generously supplied by Dr. Ehlers (Duke University). The full-length cDNA was inserted into the FIV cloning vector, pVETLC SKh10, and verified by restriction mapping. The FIV shuttle

vector expressing GFP (FIV-GFP; control), pVETLeGFP, was obtained from the Gene Transfer Vector Core at the University of Iowa. FIV expressing miRNA to NR1: Five potential miRNA sequences against rat NR1 (NR1)(GenBank Accession NM_017010) were tested. N1540 miRNA, which matches the target sequence, AGUCA AUGGUGACCCAGUGA in rat NR1 at position 1540–1549, showed the greatest percentage knockdown of NR1 expression (68%) of the 5 miRNA sequences in an *in vitro* HEK293T cell assay (unpublished results). N1540 miRNA was ligated into pVETLeGFP at the MfeI site, and sequenced to verify the cloned insert. The N1540 miRNA target sequence is found in all of the 8 known splice variants of NR1, and can inhibit expression of all NR1 subunits. FIV expressing GFP: FIV expressing GFP was used as a control (FIV-GFP).

High titer stocks of FIV viruses were generated by transfecting the FIV cloning vectors with the packaging vector, and the envelope (VSV-G) plasmid at the Gene Transfer Vector Core at the University of Iowa. The VSV-G envelope mediates gene transfer of the FIV viruses predominantly to neurons, rather than to non-neuronal cells ^{2,31}. FIV viruses infect local and not distant neurons ³¹.

2.3. Virus administration

One of three recombinant FIV viruses was microinjected into the RVM 3 days after cannulae implantation surgery: FIV expressing GFP (FIV-GFP; control), FIV expressing NR1 (FIV-NR1), or FIV expressing miRNA to NR1 and GFP (FIV-miRNA). Microinjection (0.5 μ l, 10^{10} TU/ml) was performed over a 2-min period.

2.4. Muscle-induced hypersensitivity

Two weeks after microinjection of FIV recombinant viruses, rats were anesthetized with isoflurane (2–5%) and received the first injection of pH 4.0 sterile saline (100 μ l) into the left gastrocnemius muscle. This was repeated 5 days later for a total of two acidic saline intramuscular injections. This procedure produces bilateral mechanical hypersensitivity of the muscle and paw that lasts up to 4 weeks ^{34,46}.

2.5. Behavioral measurements

The paw withdrawal threshold to mechanical stimuli (PWT) and the muscle withdrawal threshold (MWT) were tested for all groups of rats, and both tests were performed in the same animals. Both PWT and MWT were performed before and 2 weeks after the virus microinjections. Testing was also performed before (2 weeks after virus) and 5 days after the first injection of acidic saline and 24h after the 2nd injection of acidic saline. Rats were tested for PWT with von Frey filaments applied to the paw. Filaments with increasing bending forces (9.4 to 211 mN) were applied twice on the plantar surface of the hind paw until the rat withdrew from the stimulus. PWT is defined as the lowest force at which the withdrawal response from one of two applications was obtained. A decrease in PWT was interpreted as cutaneous hypersensitivity. This testing method has shown significance in test-retest reliability ³³. We were unable to test if there was an increase in cutaneous mechanical sensitivity due to the sensitivity of the assay at higher forces. The maximal force presented to the animal is generally the baseline values (211mN). The next von Frey filament above this force is 427 mN making us unable to detect accurately increases in force.

Rats were tested bilaterally for MWT with a pair of forceps applied to the gastrocnemius muscles as previously described ³². Rats were acclimated for 5 min per session in a restraining device, 3 times a day 1 h apart for 2 days (two days immediately before the first intramuscular injection of acidic saline). The forceps were equipped with two strain gauges to measure force. To measure the MWT, animals were placed in the restrainer, the hindlimb was extended, and the gastrocnemius muscle was compressed with the tip of the forceps until the animal withdrew

the leg. The MWT is defined as the maximum force applied during compression to elicit muscle withdrawal. Three trials five minutes apart at each time period were performed and averaged to obtain one reading per time period. A decrease in withdrawal threshold of the muscle was interpreted as muscle hypersensitivity, and an increase in withdrawal thresholds interpreted as muscle hypoalgesia.

2.6. Immunofluorescence staining of NR1

Three weeks after virus microinjection into the RVM, rats were transcardially perfused with 4% paraformaldehyde. Brain tissue was removed, cryoprotected in 30% sucrose, sectioned at 20 μm thickness, and placed on slides for subsequent analysis. Tissue sections were stained simultaneously with anti-NR1 (1:300, MAB1570, Millipore Corporate Headquarters, Billerica, MA) followed by anti-mouse-IgG2b conjugated to Alexa568, (1:500, Invitrogen). Sections were coverslipped with Vectashield Images were collected on an Olympus BX51 fluorescent microscope. Importantly, all images stained and analyzed at the same time were taken under the same conditions. Sections in which GFP was expressed (miRNA or control) or with visible cannula placement (NR1) were assessed for the number of cells stained for NR1. Cells were counted in 24,549 μm^2 area ($167 \times 147 \mu\text{m}$), encompassing the nucleus raphe magnus, in 5 sections per animal. This area was kept constant across slides and between animals. Cells from all experimental groups were stained simultaneously, the number of cells was counted, and percent of control calculated. Only cells with a visible nucleus were counted.

2.7. Statistical analysis

The changes in expression of NR1 were presented as percentage of change from controls immunostained simultaneously. Comparisons were made with a one-way analysis of variance (ANOVA) followed by a Tukey's pos-hoc test. As mechanical withdrawal thresholds of the paw were not normally distributed, a non-parametric Kruskal–Wallis ANOVA at each time period followed by a Mann-Whitney post-hoc test for testing differences between groups (vehicle and drugs) was done. Values of MWT and PWT after acid injection were represented as percentage of the first baseline (Day 0, two weeks after virus injection) and tested with a two-way analysis of variance (two-way ANOVA) with repeated measures, for time (repeated factor) and for treatment as variables, followed by a Tukey's pos-hoc test for differences between groups. $P < 0.05$ was considered statistically significant. Data for the MWT and PWT are represented as the mean $\pm$ S.E.M.

3. Results

3.1. Viral delivery of vectors to modulate NR1 in the RVM alters expression of NR1

Microinjection of either FIV-NR1 or FIV-miNR1 into the RVM induced changes in expression of the NR1 subunit after microinjection (Figure 1) ($F_{2,13}=60.7$, $p=0.0001$). Quantification of the immunofluorescence following immunohistochemical staining for NR1 showed an average of 27 ± 1.5 cells. The number of cells significantly decreased to 16 ± 1.9 cells in those treated with FIV-miNR1 ($p=0.03$) and increased to 70 ± 8 cells in those treated with FIV-NR1 ($p=0.0001$). Fluorescence microscopy of brain tissue sections for GFP expression revealed that these viruses predominantly infected neuronal cells, and were expressed in the RVM and not in distal neurons.

3.2. Modulation of NR1 in the RVM alters nociceptive sensitivity

To test if overexpression of NR1 affected nociceptive behaviors we tested withdrawal thresholds of the muscle and paw 1 and 2 weeks after injection of the virus into the RVM. Microinjection of FIV-NR1 reduced the thresholds to mechanical stimulation of the paw both 1 and 2 weeks after injection ($P < 0.05$) when compared to injection of FIV-GFP (Figure 2A).

The muscle withdrawal threshold was significantly decreased 2 weeks after microinjection of FIV-NR1 when compared to FIV-GFP controls ($P < 0.05$) (Figure 2B).

To test if downregulation of NR1 affected baseline withdrawal thresholds, we tested MWT at 1 and 2 weeks after injection of the virus. Microinjection of FIV-miNR1 into the RVM significantly increased the MWT when compared to microinjection of FIV-GFP both 1 and 2 weeks after injection ($p < 0.05$, Figure 3).

3.3. Downregulation of NR1 in the RVM prevents the development of injury-induced hypersensitivity

To test if NR1 contributes to the development of hypersensitivity to muscle insult, we tested if downregulation of NR1 in the RVM contributes to the development of cutaneous and muscle hyperalgesia after repeated intramuscular acid injections. Because we show (see Figures 2 and 3) there is a difference in withdrawal thresholds 2 weeks after microinjection of the FIV-miNR1, we calculated a percent change on Day 5 before the second injection, and again on Day 6 24h after the second injection, in comparison to values immediately before the first injection and 2 weeks after FIV-miRNA. In agreement with previous studies^{34,45} repeated intramuscular injections of acidic saline into the left gastrocnemius muscle reduced the cutaneous and muscle withdrawal thresholds in both the ipsilateral and contralateral hindlimbs of rats 24h after the second acidic saline injection in control animals injected with FIV-GFP in the RVM (Figure 4). These decreases in withdrawal thresholds for the paw and muscle 24h after the second acidic saline injection did not occur in the group that was injected with FIV-miNR1. The FIV-miNR1 injected animals displayed PWT and MWT that are significantly increased from controls injected with FIV-GFP 24h after the second intramuscular acidic saline injection (Figure 4). The animals microinjected with FIV-NR1 into the RVM showed decreases in thresholds at 2 weeks after injection of the virus (Figure 2A and B) and showed no further reductions in PWT and MWT after repeated intramuscular acid injections (data not shown).

4. Discussion

In the current study we show for the first time a functional role for the NR1 subunit of the NMDA receptor in the RVM in modulation of pain. Increasing expression of NR1 in the RVM enhances behavioral responses to noxious stimuli, and decreasing NR1 in the RVM reduces behavioral responses to noxious stimuli and prevents development of hyperalgesia in an animal model of muscle pain. These data suggest that regulation of NR1 in the RVM can regulate sensitivity to noxious stimuli and development of hyperalgesia.

There is substantial evidence that NMDA receptors are important for neural plasticity, including that associated with pain^{12,16,23,50}. Pharmacological blockade of NMDA receptors in the spinal cord or RVM, reverses hyperalgesia associated with a variety of noxious stimuli. NMDA receptors are heteromers of 4 subunits that contain at least one NR1 subunit, and NR2 or NR3 subunits. The NR1 subunit is expressed as several splice variants that regulate trafficking and function of NMDA receptors^{5,6}. In the current study we overexpressed NR1-1a, a splice variant that is located predominantly in NMDA receptors of synaptic cell membranes¹⁰. The experimental design of the NR1 overexpression virus (FIV-NR1) was aimed at increasing the cell-surface expression of NR1-1a containing NMDA receptors at the synapse to enhance neuronal activity. The FIV-miRNA virus expresses a miRNA targeted to a conserved sequence for all of the known splice variants of NR1, and could potentially inhibit the expression of all NMDA receptors within the RVM.

Prior studies show that site selective reduction in NR1 impairs neuronal function associated with neuronal plasticity. Antisense or siRNA knockdown of the NR1 subunit in the CA1 region of the hippocampus, NR1 conditional CA1-NR1 knockout mice, or conditional CA3-NR1

knockout mice have deficits in learning, impaired responses to spatial, temporal, olfactory, visual, emotional stimuli and reduced LTP^{18,20,22,24,28,38}. Further, siRNA-mediated knockdown of NR1 in the hippocampus delays the onset for seizures and fear memory²⁰. Decreasing NR1 expression in a small number of hippocampal neurons causes learning deficits³. Interestingly, in CA1-NR1 knockout mice, NR2 expression on CA1 dendrites is absent, and NR2 is retained intracellularly within the endoplasmic reticulum, confirming a role for NR1 in cell surface expression of NMDA receptors¹¹. Thus, in the hippocampus, selective reduction of NR1 reduces learning, long-term potentiation, neuronal responses to NMDA, and distribution of NR2 subunits.

For nociceptive behavior, localized deletion of NR1 in the dorsal horn of adult mice prevented injury-induced nociceptive responses to mechanical and thermal stimuli as well as spontaneous pain-like behaviors^{13,14,30,35}. However, baseline nociceptive latencies to heat are unchanged in cortex-specific NR1-knockout mice^{14,26}, or following blockade of NMDA receptors in the RVM in naïve, uninjured animals^{15,39,44}. In contrast, the present study shows that selective deletion of NR1 in the RVM increases baseline threshold to mechanical stimulation of the muscle. These differences between our data and previous data could result from the type of stimuli (thermal or cutaneous vs. mechanical or muscle) and the site of NR1 down regulation (cortex or spinal cord vs. RVM).

Neurons within the RVM both facilitate and inhibit nociceptive stimuli, and it is generally thought that there is a balance between facilitation and inhibition. Glutamate in the RVM modulates both facilitatory and inhibitory pain pathways^{48,49}. Prior work from our laboratory and others show that NMDA receptors in the RVM play a critical role in both facilitation and inhibition of nociception after tissue insult^{9,17,42,44}. In fact there is an increase in NR1 mRNA in the RVM after peripheral inflammation²³, and pharmacological blockade of NMDA receptors in the RVM prevents development of hyperalgesia after tissue injury^{8,9,39,42}. Further, in this model of acid-induced hyperalgesia the RVM is critical for both development and maintenance of hyperalgesia^{9,37}.

In the spinal cord, conditional deletion of the NR1 subunit mRNA reduced the NMDA currents, but not AMPA currents, by 86–88% in lamina II neurons³⁵. This earlier spinal cord data prompted us to hypothesize that downregulation of NR1 in the RVM would decrease NMDA activity and prevent development of muscle and cutaneous hypersensitivity. In agreement with this hypothesis, microinjection of NMDA receptor antagonists into the RVM blocks visceral hypersensitivity, mustard oil-induced hypersensitivity and neuropathic hypersensitivity^{8,39,42}, as well as muscle and cutaneous hypersensitivity after repeated-acid injections⁹. The current study shows that knockdown of NR1 in the RVM prevents development of hyperalgesia to repeated intramuscular acid injections, a model of neuronal plasticity that requires activation of NMDA receptors in the RVM. However, since the downregulation of NR1 also affected baseline nociceptive responses, the lack of development of hyperalgesia could be from a separate hypoalgesic mechanism. In agreement with the notion that NR1 mediates hypoalgesia in the absence of tissue injury, inhibition of nociceptive responses by electrical stimulation of the NRM in naïve animals is increased by approximately 200% after NMDA receptor blockade in the RVM suggesting tonic NMDA receptor activity mediates hypoalgesia³⁶.

The current study further supports a critical role of the NMDA receptors, and the NR1 subunit, in mediating this balance between inhibition and facilitation of pain since over-expression of NR1-1a in the RVM enhances nociceptive behaviors to cutaneous and muscle mechanical stimuli in animals without tissue injury. The NR1-1a splice variant of NR1 localizes to cell membranes in discrete patches with very little NR1-1a located in the interior regions of the cell¹⁰. Thus, over-expression of the NR1-1a subunit, as in this study, would suggest more membrane-bound NMDA receptors resulting in an enhanced response to endogenous ligands.

This would increase cell excitability and thus enhance facilitation. Enhanced facilitation would be manifested behaviorally as decreased thresholds to nociceptive stimuli, i.e. hyperalgesia. These studies are the first to overexpress NR1 in regions involved in nociception, and show that regulation of NR1 in the RVM may be a critical factor for sensitivity to nociceptive stimuli.

Prior studies have demonstrated the association between development of hypersensitivity and changes in expression of NR1 in the spinal cord where nociceptive afferents terminate. For example, injection of CFA in the temporomandibular joint induces an upregulation of NR1 within trigeminal subnucleus caudalis⁴². Chronic constriction of the sciatic nerve increases the number of NR1-immunoreactive neurons in the dorsal horn of rats²⁹. We suggest that increasing NR1 expression in the RVM is sufficient to induce hyperalgesia since the animals developed hypersensitivity without tissue injury two weeks after injection with FIV-NR1.

In parallel to our studies on pain and NMDA receptor overexpression in the RVM, Kaley-Zylinska et al. overexpressed mouse NR1-1a in the rat hippocampus with an adeno-associated viral vector 1/2^{19,20}. Overexpression of NR1-1a in the rat hippocampus increased fear memory behavior, neurogenesis, facilitated learning and delayed the onset of kainate induced seizures²⁰. This suggests that the neuronal alterations of NMDA receptor expression in the hippocampus can be affected by NR1 expression. There are other studies, however, in which overexpression of NR1 in the rat hippocampus had no effect on learning and memory behaviors^{1,3,4}. In these studies NR1 subunits were over-expressed with HSV-1 vectors and examined the effects of overexpression after only 5 days after introduction of the virus. This short-term overexpression of NR1 occurred in a small number of neurons and the fact that a different splice variant of NR1 may have been overexpressed in the rat hippocampus, may explain the lack of effect on learning and memory in these other studies. The current study expressed NR1 or miNR1 using an FIV vector with a VSV-G envelope to specifically mediate stable gene transfer of the FIV viruses predominantly to neurons, rather than to non-neuronal cells^{2,31}. We show a significant increase in expression of NR1 when using this FIV vector expressing NR1-1a, and a significant decrease in expression of NR1 when using an FIV expressing a miRNA to NR1. Although our changes in expression are significantly different from control, it should be noted that we determined this increase and decrease using profile counting, not with stereology. This limitation using profile counting would likely result in overcounting large cells²¹ and thus we can make conclusions about relative differences and not absolute differences. Behaviorally, we also show, in the current study, a decrease in withdrawal thresholds of the paw, but not the muscle 1 week after infection and decreases in both paw and muscle 2 weeks after infection with the FIV-NR1. It may be that there is less expression of NR1 at 1 week when compared to 2 weeks. Thus we might observe differences in some pain measures and not all pain measures at 1 week. In agreement, prior studies show stable expression between 2 and 8 weeks after infection with FIV-expression vectors³¹.

In summary, we show that selective modulation of NR1 in the RVM modulates pain behaviors in rats. Specifically, increasing expression of NR1 produces hyperalgesia, an enhanced responsiveness to noxious stimuli, while decreasing expression of NR1 produces analgesia, a decreased responsiveness to noxious stimuli. Further decreasing expression of NR1 in the RVM prevents the development of hyperalgesia induced by tissue insult. We thus suggest that NR1 subunits in the RVM are critical for modulating NMDA receptor function, which in turn sets the 'tone' of the nervous system's response to noxious stimuli and tissue injury.

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References

- Adrover MF, Guyot-Revol V, Cheli VT, Blanco C, Vidal R, Alche L, Kornisiuk E, Epstein AL, Jerusalinsky D. Hippocampal infection with HSV-1-derived vectors expressing an NMDAR1 antisense modifies behavior. *Genes Brain Behav* 2003;2:103–113. [PubMed: 12884967]
- Alisky JM, Hughes SM, Sauter SL, Jolly D, Dubensky TW Jr, Staber PD, Chiorini JA, Davidson BL. Transduction of murine cerebellar neurons with recombinant FIV and AAV5 vectors. *Neuroreport* 2000;11:2669–2673. [PubMed: 10976941]
- Cheli V, Adrover M, Blanco C, Ferrari C, Cornea A, Pitossi F, Epstein AL, Jerusalinsky D. Knocking-down the NMDAR1 subunit in a limited amount of neurons in the rat hippocampus impairs learning. *J Neurochem* 2006;97 (Suppl 1):68–73. [PubMed: 16635252]
- Cheli VT, Adrover MF, Blanco C, Rial VE, Guyot-Revol V, Vidal R, Martin E, Alche L, Sanchez G, Acerbo M, Epstein AL, Jerusalinsky D. Gene transfer of NMDAR1 subunit sequences to the rat CNS using herpes simplex virus vectors interfered with habituation. *Cell Mol Neurobiol* 2002;22:303–314. [PubMed: 12469872]
- Chen BS, Braud S, Badger JD, Isaac JT, Roche KW. Regulation of NR1/NR2C N-methyl-D-aspartate (NMDA) receptors by phosphorylation. *J Biol Chem* 2006;281:16583–16590. [PubMed: 16606616]
- Chen BS, Roche KW. Regulation of NMDA receptors by phosphorylation. *Neuropharm* 2007;53:362–368.
- Cheng HT, Suzuki M, Hegarty DM, Xu Q, Weyerbacher AR, South SM, Ohata M, Inturrisi CE. Inflammatory pain-induced signaling events following a conditional deletion of the N-methyl-D-aspartate receptor in spinal cord dorsal horn. *Neuroscience* 2008;155:948–958. [PubMed: 18621103]
- Coutinho SV, Urban MO, Gebhart GF. Role of glutamate receptors and nitric oxide in the rostral ventromedial medulla in visceral hyperalgesia. *Pain* 1998;78:59–69. [PubMed: 9822212]
- daSilva LFS, DeSantana JM, Sluka KA. Activation of NMDA receptors in the brainstem, RVM and Gi mediate hyperalgesia produced by repeated intramuscular injections of acidic saline. *Pain* 2010;11:378–387.
- Ehlers MD, Tingley WG, Huganir RL. Regulated subcellular distribution of the NR1 subunit of the NMDA receptor. *Science* 1995;269:1734–1737. [PubMed: 7569904]
- Fukaya M, Kato A, Lovett C, Tonegawa S, Watanabe M. Retention of NMDA receptor NR2 subunits in the lumen of endoplasmic reticulum in targeted NR1 knockout mice. *Proc Natl Acad Sci U S A* 2003;100:4855–4860. [PubMed: 12676993]
- Gao X, Kim HK, Chung JM, Chung K. Enhancement of NMDA receptor phosphorylation of the spinal dorsal horn and nucleus gracilis neurons in neuropathic rats. *Pain* 2005;116:62–72. [PubMed: 15936881]
- Garraway SM, Xu Q, Inturrisi CE. Design and evaluation of small interfering RNAs that target expression of the N-methyl-D-aspartate receptor NR1 subunit gene in the spinal cord dorsal horn. *J Pharmacol Exp Ther* 2007;322:982–988. [PubMed: 17551091]
- Garraway SM, Xu Q, Inturrisi CE. siRNA-mediated knockdown of the NR1 subunit gene of the NMDA receptor attenuates formalin-induced pain behaviors in adult rats. *J Pain* 2009;10:380–390. [PubMed: 19185544]
- Guan Y, Terayama R, Dubner R, Ren K. Plasticity in excitatory amino acid receptor-mediated descending pain modulation after inflammation. *J Pharmacol Exp Ther* 2002;300:513–520. [PubMed: 11805211]
- Guo W, Zou S, Guan Y, Ikeda T, Tal M, Dubner R, Ren K. Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia. *J NEUROSCI* 2002;22:6208–6217. [PubMed: 12122079]
- Heinricher MM, Schouten JC, Jobst EE. Activation of brainstem N-methyl-D-aspartate receptors is required for the analgesic actions of morphine given systemically. *Pain* 2001;92:129–138. [PubMed: 11323134]
- Huerta PT, Sun LD, Wilson MA, Tonegawa S. Formation of temporal memory requires NMDA receptors within CA1 pyramidal neurons. *Neuron* 2000;25:473–480. [PubMed: 10719900]
- Kalev-Zylinska ML, During MJ. Paradoxical facilitatory effect of low-dose alcohol consumption on memory mediated by NMDA receptors. *J Neurosci* 2007;27:10456–10467. [PubMed: 17898217]

20. Kalev-Zylinska ML, Symes W, Young D, During MJ. Knockdown and overexpression of NR1 modulates NMDA receptor function. *Mol Cell Neurosci* 2009;41:383–396. [PubMed: 19394426]
21. Lekan HA, Coggeshall RE, Lekan. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J Comp Neurol* 1996;364:6–15. [PubMed: 8789272]
22. McHugh TJ, Blum KI, Tsien JZ, Tonegawa S, Wilson MA. Impaired hippocampal representation of space in CA1-specific NMDAR1 knockout mice. *Cell* 1996;87:1339–1349. [PubMed: 8980239]
23. Miki K, Zhou QQ, Guo W, Guan Y, Terayama R, Dubner R, Ren K. Changes in gene expression and neuronal phenotype in brain stem pain modulatory circuitry after inflammation. *J Neurophysiol* 2002;87:750–760. [PubMed: 11826044]
24. Nakazawa K, Quirk MC, Chitwood RA, Watanabe M, Yeckel MF, Sun LD, Kato A, Carr CA, Johnston D, Wilson MA, Tonegawa S. Requirement for hippocampal CA3 NMDA receptors in associative memory recall. *Science* 2002;297:211–218. [PubMed: 12040087]
25. Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Vol. 2. 1986.
26. Quintero GC, Erzurumlu RS, Vaccarino AL. Evaluation of morphine analgesia and motor coordination in mice following cortex-specific knockout of the N-methyl-D-aspartate NR1-subunit. *Neurosci Lett* 2008;437:55–58. [PubMed: 18423864]
27. Radhakrishnan R, Sluka KA. Increased glutamate and decreased glycine release in the rostral ventromedial medulla during induction of a pre-clinical model of chronic widespread muscle pain. *Neurosci Lett* 2009;457:141–145. [PubMed: 19429181]
28. Rampon C, Tang YP, Goodhouse J, Shimizu E, Kyn M, Tsien JZ. Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. *Nat Neurosci* 2000;3:238–244. [PubMed: 10700255]
29. Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Han HJ, Beitz AJ, Lee JH. Depletion of capsaicin-sensitive afferents prevents lamina-dependent increases in spinal N-methyl-D-aspartate receptor subunit 1 expression and phosphorylation associated with thermal hyperalgesia in neuropathic rats. *Eur J Pain* 2008;12:552–563. [PubMed: 17933570]
30. Shimoyama N, Shimoyama M, Davis AM, Monaghan DT, Inturrisi CE. An antisense oligonucleotide to the N-methyl-D-aspartate (NMDA) subunit NMDAR1 attenuates NMDA-induced nociception, hyperalgesia, and morphine tolerance. *J Pharmacol Exp Ther* 2005;312:834–840. [PubMed: 15388787]
31. Sinnayah P, Lindley TE, Staber PD, Cassell MD, Davidson BL, Davisson RL. Selective gene transfer to key cardiovascular regions of the brain: comparison of two viral vector systems. *Hypertension* 2002;39:603–608. [PubMed: 11882616]
32. Skyba DA, Radhakrishnan R, Sluka KA. Characterization of a method for measuring primary hyperalgesia of deep somatic tissue. *Journal of Pain* 2005;6:41–47. [PubMed: 15629417]
33. Sluka KA, Christy MR, Peterson WL, Rudd SL, Troy SM. Reduction of pain-related behaviors with either cold or heat treatment in an animal model of acute arthritis. *Arch Phys Med Rehabil* 1999;80:313–317. [PubMed: 10084440]
34. Sluka KA, Kalra A, Moore SA. Unilateral intramuscular injections of acidic saline produce a bilateral, long-lasting hyperalgesia. *Muscle & Nerve* 2001;24:37–46. [PubMed: 11150964]
35. South SM, Kohno T, Kaspar BK, Hegarty D, Vissel B, Drake CT, Ohata M, Jenab S, Sailer AW, Malkmus S, Masuyama T, Horner P, Bogulavsky J, Gage FH, Yaksh TL, Woolf CJ, Heinemann SF, Inturrisi CE. A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain. *J Neurosci* 2003;23:5031–5040. [PubMed: 12832526]
36. Terayama R, Dubner R, Ren K. The roles of NMDA receptor activation and nucleus reticularis gigantocellularis in the time-dependent changes in descending inhibition after inflammation. *Pain* 2002;97:171–181. [PubMed: 12031790]
37. Tillu DV, Gebhart GF, Sluka KA. Descending facilitatory pathways from the RVM initiate and maintain bilateral hyperalgesia after muscle insult. *Pain* 2008;136:331–339. [PubMed: 17764841]
38. Tsien JZ, Huerta PT, Tonegawa S. The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell* 1996;87:1327–1338. [PubMed: 8980238]

39. Urban MO, Coutinho SV, Gebhart GF. Involvement of excitatory amino acid receptors and nitric oxide in the rostral ventromedial medulla in modulating secondary hyperalgesia produced by mustard oil. *Pain* 1999;81:45–55. [PubMed: 10353492]
40. Urban MO, Zahn PK, Gebhart GF. Descending facilitatory influences from the rostral medial medulla mediate secondary, but not primary hyperalgesia in the rat. *Neurosci* 1999;90:349–352.
41. Villmann C, Becker CM. On the hypes and falls in neuroprotection: targeting the NMDA receptor. *Neuroscientist* 2007;13:594–615. [PubMed: 17911221]
42. Wang S, Lim G, Mao J, Sung B, Mao J. Regulation of the trigeminal NR1 subunit expression induced by inflammation of the temporomandibular joint region in rats. *Pain* 2009;141:97–103. [PubMed: 19058915]
43. Wei H, Pertovaara A. MK-801, and NMDA receptor antagonist, in the rostroventromedial medulla attenuates development of neuropathic symptoms in the rat. *Neuroreport* 1999;10:2933–2937. [PubMed: 10549800]
44. Xu M, Kim CJ, Neubert MJ, Heinricher MM. NMDA receptor-mediated activation of medullary pronociceptive neurons is required for secondary thermal hyperalgesia. *Pain* 2007;127:253–262. [PubMed: 16997469]
45. Yokoyama T, Audette KM, Sluka KA. Pregabalin reduces muscle and cutaneous hyperalgesia in two models of chronic muscle pain in rats. *J Pain*. 2007
46. Yokoyama T, Lisi TL, Moore SA, Sluka KA. Muscle Fatigue Increases the Probability of Developing Hyperalgesia in Mice. *J Pain* 2007;8:692–699. [PubMed: 17627895]
47. Zhang L, Hammond DL. Substance P enhances excitatory synaptic transmission on spinally projecting neurons in the rostral ventromedial medulla after inflammatory injury. *J Neurophysiol* 2009;102:1139–1151. [PubMed: 19494188]
48. Zhuo M, Gebhart GF. Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. *J Neurophysiol* 1997;78:746–758. [PubMed: 9307109]
49. Zhuo M, Sengupta JN, Gebhart GF. Biphasic modulation of spinal visceral nociceptive transmission from the rostroventral medial medulla in the rat. *J Neurophysiol* 2002;87:2225–2236. [PubMed: 11976363]
50. Zou X, Lin Q, Willis WD. Role of protein kinase A in phosphorylation of NMDA receptor 1 subunits in dorsal horn and spinothalamic tract neurons after intradermal injection of capsaicin in rats. *Neuroscience* 2002;115:775–786. [PubMed: 12435416]

Figure 1.

Fluorescent photomicrographs of neurons in RVM immunostained for NR1 in animals microinjected with (A,B) FIV-GFP, (C,D) FIV-miRNA to NR1 or (E,F) FIV-NR1. The white box in A, C and E represents the representative area that is shown at higher magnification in B, D, and F, respectively. The blue box shows the 24,549 m² box that was counted in each section. G. Bars graphs showing the percent change from controls (FIV-GFP, n=4) for animals injected with FIV-NR1 (n=4) and FIV-miNR1 (n=6). Dotted line represents the controls set at 100%. *, p<0.05

Figure 2.

Graphs representing cutaneous (A) and muscle (B) withdrawal thresholds before and after microinjection of FIV-NR1 (n=8) compared to controls injected with FIV-GFP (n=6) into the RVM. Microinjection of FIV-NR1 significantly reduced both (A) cutaneous and (B) muscle withdrawal thresholds. Plot represents mean values $\pm$ S.E.M. * significantly less than controls.

Figure 3.

Graphs representing muscle withdrawal thresholds before and after microinjection of FIV-miNR1 (n=7) compared to controls injected with FIV-GFP (n=6) into the RVM. Microinjection of FIV-miNR1 significantly increased muscle withdrawal threshold. Plot represents mean values $\pm$ S.E.M. * significantly more than controls.

Figure 4.

Graphs representing the percent change (from 2 weeks post-FIV injection) in (A) paw and muscle (B) withdrawal thresholds after acidic saline injections on Day 5 before the second acid injection and again on Day 6 24h after the second acid injection in the muscle for animals injected with FIV-miNR1 (n=7) when compared to those injected with FIV-GFP (n=6). Two injections of acidic saline in muscle (indicated by arrows) induced both cutaneous and muscle hyperalgesia bilaterally in animals microinjected with FIV-GFP into the RVM (A and B). Microinjection of FIV-miNR1 into the RVM prevents both cutaneous (A) and muscle (B) hyperalgesia induced by muscle insult. Plot represents mean values $\pm$ S.E.M. (A and B). * FIV-miNR1 is statistically different from FIV-GFP ($p < 0.05$).