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Activation of NMDA receptors in the brainstem, RVM and NGC, mediates mechanical hyperalgesia produced by repeated intramuscular injections of acidic saline in rats

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Abstract

Repeated injections of acidic saline into the gastrocnemius muscle induced both muscle and cutaneous hypersensitivity. We have previously shown that microinjection of local anesthetic into either the rostral ventromedial medulla (RVM) or the nucleus reticularis gigantocellularis (NGC) reverses this muscle and cutaneous hypersensitivity. Although prior studies show that NMDA receptors in the RVM play a clear role in mediating visceral and inflammatory hypersensitivity, the role of NMDA receptors in the NGC, or in non-inflammatory muscle pain is unclear. Therefore, the present study evaluated involvement of the NMDA receptors in the RVM and NGC in muscle and cutaneous hypersensitivity induced by repeated intramuscular injections of acidic saline. Repeated intramuscular injections of acidic saline, 5 days apart, resulted in a bilateral decrease in the withdrawal thresholds of the paw and muscle in all groups 24 h after the second injection. Microinjection of NMDA receptor antagonists into the RVM reversed both the muscle and cutaneous hypersensitivity. However, microinjection of NMDA receptor antagonists into the NGC only reversed cutaneous, but not muscle hypersensitivity. These results suggest that NMDA receptors in the RVM mediate both muscle and cutaneous hypersensitivity, but those in the NGC mediated only cutaneous hypersensitivity after muscle insult.

Keywords

muscle pain; RVM; NGC; AP5; MK-801

Introduction

Pain originating from the musculoskeletal system is common and costs billions of dollars in healthcare and lost wages (45). Chronic musculoskeletal pain syndromes such as fibromyalgia and myofascial pain syndrome can be disabling and difficult to treat (19). To address these

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issues, our laboratory developed a model of long-lasting, mechanical hypersensitivity induced by two intramuscular injections of acidic (pH 4.0) saline (31). This procedure produces a bilateral muscle and cutaneous mechanical hypersensitivity, and increased visceral sensitivity (21,31,46). Once the hypersensitivity has developed, dorsal rhizotomy or local anesthetic blockade of afferent input has no effect on the contralateral hypersensitivity(31). In addition, histological analysis of the injection site reveals no significant changes in the muscle when compared to controls that receive intramuscular injection of pH 7.2 saline (31). Together these data suggest that the mechanisms underlying this long-lasting mechanical hypersensitivity reside in the central nervous system and that this hypersensitivity is not dependent on continued nociceptive input from the periphery.

The rostral ventromedial medulla (RVM), which includes the nucleus raphe magnus and nucleus gigantocellularis pars alpha, is involved in pain modulation, both inhibitory and facilitatory (3,11,38). In addition, the nucleus reticularis gigantocellularis (NGC) is also involved with facilitatory modulation of pain in models of visceral, neuropathic and inflammatory nociception (24,34,40,48–50). Recently, we showed that anesthetic blockade of either the RVM or the NGC during the second intramuscular injection of acidic saline prevents the development of cutaneous and muscle hypersensitivity in response to the two intramuscular injections of acidic saline (36). Further, local anesthetic blockade after the development of hyperalgesia reverses both cutaneous and muscle hypersensitivity(36) suggesting continued activation of supraspinal neurons to maintain the hypersensitivity. Thus, brainstem sites in the medulla, RVM and NGC, facilitate both cutaneous and deep tissue hyperalgesia associated with repeated intramuscular injection of acidic saline.

Previous data from our laboratory show that activation of both NMDA and non-NMDA glutamate receptors in the spinal cord are involved in the maintenance of hypersensitivity in this model of chronic, muscle-induced hyperalgesia (28). Pain modulation by the RVM and NGC is also regulated by glutamate. Specifically, microinjections of low doses of glutamate into the RVM or NGC induce hypersensitivity, while applications of higher doses into the same sites produce analgesia (50,51). Furthermore, microinjections of NMDA antagonists into the RVM reverse visceral hypersensitivity produced by colon inflammation (8,37) hypersensitivity produced by nerve injury hyperalgesia (42), and increase the intensity of electrical stimulation necessary for inhibiting responses to transient stimuli in non-inflamed animals (2). In addition, we have shown that there is an increased release of glutamate and aspartate in the RVM in response to the second injection of acidic saline, but not the first injection (26). However, little is known about the central mechanisms and neurotransmitter receptors involved in the modulation of hypersensitivity after muscle insult.

The goal of our study was to characterize the involvement of NMDA receptors in the RVM and NGC in the maintenance of mechanical hypersensitivity from repeated intramuscular injections of pH 4.0 saline. Thus, we tested the hypothesis that blockade of NMDA receptors by a potent and selective competitive NMDA receptor antagonist (AP5) or by a non-competitive NMDA receptor antagonist (MK-801) would reverse the mechanical hypersensitivity induced by repeated injections of acidic saline.

Materials and Methods

All experiments were approved by Animal Care and Use Committee at the University of Iowa and are in accordance with the guidelines of National Institute of Health and the International Association for the Study of Pain on use of laboratory animals. Adult male Sprague-Dawley rats (n = 79; 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 in the light cycle. Food and water were available to the animals *ad libitum*.

Muscle-induced hypersensitivity

Immediately after baseline behavioral measurements, described below, rats were anesthetized with isoflurane (2–5%) and injected with 100 μ l of pH 4.0 sterile saline into gastrocnemius muscle of left hind limb on Day 0 (injection 1) and again on Day 5 (injection 2). This procedure produces bilateral mechanical hypersensitivity of the muscle and paw that lasts up to 4 weeks (31,46).

Placement of guide cannula

An intracerebral guide cannula was stereotactically implanted in the RVM or bilaterally in the NGC 3 days before to first intramuscular injection of vehicle or acidic saline. The rats were anesthetized with an injection of pentobarbital sodium (Nembutal, 50mg/kg, i.p.) and secured in a stereotaxic head holder to implant the 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 mm. For the NGC we used a double cannulae that allowed application of antagonists to bilaterally according to the following coordinates: intra-aural: –2.0 mm; mediolateral: $\pm$ 0.5 mm; and dorsoventral: –7.2 mm for the NGC (22). The cannula was secured to the skull by stainless-steel screws and dental acrylic. A dummy cannula (33 gauge, Plastics One) was inserted into the guide cannula to maintain its patency. All rats were allowed 3 to 5 days to recover before testing began.

To examine placement of the cannula into RVM or NGC, an equivalent volume of methylene blue dye was injected at the end of the experiment. Rats were then euthanized and transcardially perfused with 4% paraformaldehyde. After this, the brain was removed and stored in 30% sucrose. The brain was cut in cross-section at 40 μ m on a cryostat and examined under a light microscope for placement of the cannula.

Drug administration

For the RVM studies, 30 fmol (n = 6), 100 fmol (n = 4), 300 fmol (n = 4), and 1.0 nmol (n=5) of AP5, 0.9 nmol (n = 5), 3.0 nmol (n = 5), 9.0 nmol (n = 5) of MK-801 or vehicle (0.9% sterile saline, n = 7) were administered through the guide cannula 24 h after the second intramuscular injection. For studies the NGC studies, 30 fmol (n = 4), 100 fmol (n = 4) and 300 fmol of AP5 (n = 8) or 0.9 nmol (n = 5), 3.0 nmol (n = 5) and 9.0 nmol of MK-801 (n = 5) or vehicle (n = 7) were used.

For microinjection, a 33-gauge injection cannula was connected to a 10- μ l Hamilton syringe through PE10 tubing backfilled with sterile saline. Microinjection (0.5 μ l) was performed over a 2-min period. The RVM was injected with 0.5 μ l and the NGC was injected with 0.5 μ l on each side. The travel of an air bubble in the tubing was carefully observed to ensure that the drug solution entered the injection cannula. The behavioral tests were conducted immediately before and for up to 4 h after microinjection.

Behavioral measurements

The paw withdrawal threshold (PWT) and the muscle withdrawal threshold (MWT) were tested for all groups of rats, and both tests were performed in the same animals. For the RVM, the PWT was performed before the first and second intramuscular injection, 24 h after the second intramuscular injection, and 30 min, 1 h, 2 h and 4 h following drug administration. MWT was performed prior to the first and second intramuscular injection, 24 h after the second intramuscular injection, and 1 h, 2 h and 4 h after drug administration. For the NGC, the same protocol was performed except the last time period at 4 h following drug injection since

maximal effects were observed within 2h in the RVM. Since the muscle test is a stronger stimulus, we tested it hourly to avoid development of hypersensitivity in response to repeated testing. Preliminary experiments show that testing every 30 minutes increases muscle sensitivity (Sluka, unpublished).

Rats were tested for PWT with Von Frey filaments applied to the paw. Initially, the animals were acclimated within their home cages in the behavior room for 30 minutes. The animals were then placed in transparent Lucite cubicles on a wire mesh elevated plate and acclimated for another 30 minutes before testing. A series of filaments with increasing bending forces (9.4 to 495.8 mN) were applied twice on the plantar surface of the hind paw until the rat withdrew from the stimulus (12). The lowest force at which the rat withdrew its paw from one of two applications was recorded as the paw withdrawal threshold. A decrease in paw withdrawal threshold was interpreted as cutaneous hypersensitivity. This testing method has shown significant test-retest reliability (30).

Rats were also tested for MWT with a pair of forceps applied to the gastrocnemius muscle as previously described (29). Rats were acclimated for 5 min per session in a restraining device, a glove, 3 times a day 1 h apart for 2 days (two days immediately before the first intramuscular injection). The forceps were equipped with two strain gauges to measure force. To measure the MWT, animals were placed in the restrainer, and the experimenter compressed the gastrocnemius muscle with the tip of the forceps while the hind limb was extended. Compression was continued until the animal withdrew the leg or vocalized. The maximum force applied at withdrawal was recorded as the muscle withdrawal threshold. Three trials five minutes apart at each time period were performed and averaged to obtain one reading per time period, and threshold measurements are consistent when repeated within animals. A decrease in withdrawal threshold of the muscle was interpreted as muscle hypersensitivity.

Statistical analysis

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). Values of compression withdrawal thresholds for muscle were represented as percentage of the first baseline (Day 0) and tested with a two-way analysis of variance (two-way ANOVA) with repeated measures, for time (repeated factor) and for treatment as variable followed by a Tukey's pos-hoc test for differences between groups. $P < 0.05$ was considered statistically significant. Data are represented for the muscle withdrawal thresholds as the mean $\pm$ S.E.M. and for the paw withdrawal thresholds as the median with the 25th and 75th percentiles.

Results

Repeated intramuscular injections of acidic saline (Day 0 and Day 5) induced a bilateral decrease in the withdrawal thresholds of the paw and muscle in all groups, 24 h after the second injection, replicating previous findings (31,46) (Figures 1, 2, 3 and, 4). Specifically, the ipsilateral withdrawal threshold of the muscle was $21,320.4 \pm 336.1$ mN before the first injection, $21,090.9 \pm 326.5$ mN before the second injection, and significantly decreased to $16,160.5 \pm 279.6$ mN 24 h after the second intramuscular acid injection. Similarly, the ipsilateral withdrawal threshold of the paw was 215.2 ($210.7 - 495.8$) mN before the first injection, 215.2 ($110.7 - 495.8$) mN before the second injection, and decreased to 61.2 ($36.2 - 69.5$) mN 24 h after the second intramuscular acid injection. These decreases in muscle and paw withdrawal thresholds were similar on the contralateral side and were similar between groups.

Microinjection of AP5 into the RVM 24 h after the second intramuscular injection of pH 4.0 saline reversed the bilateral decrease in PWT and MWT (Figure 1A – D). Statistical analysis showed significant effects for the treatments, time of testing, and interaction between treatment and time of testing for both ipsilateral and contralateral muscle (two-way ANOVA, $p < 0.01$). Microinjections of lower concentrations of the competitive antagonist AP5 (30, 100 and 300 fmol) into the RVM significantly reversed the decrease in PWT after 1 h, 2 h and 4 h ipsilaterally and 2 h and 4 h contralaterally when compared with the vehicle-treated group (Figure 1A – B). For MWT, the 300 fmol concentration of AP5 was statistically different from the vehicle group 2 h after microinjection for the contralateral muscle; and concentrations of 30 fmol, 100 fmol and 300 fmol of AP5 were statistically different from vehicle treatment 4 h after microinjection for the ipsilateral and contralateral muscle (Figure 1C – D). Since microinjection of lower concentrations of AP5 showed a delayed effect we also tested a higher concentration of AP5 (1 nmol) in the RVM. The 1 nmol dose of AP5 showed a shorter onset of effect compared to the prior doses tested with increases in the PWT 30 minutes, 1 h, 2 h and 4 h bilaterally; and increases in the MWT 1 h and 4 h ipsilaterally and 1 h, 2 h and 4 h contralaterally (Figure 1A – D).

We also tested the effect of microinjecting the non-competitive antagonist MK-801 into the RVM. Statistical analysis showed significant effects for the treatments, time of testing, and interaction between treatment and time of testing for both ipsilateral and contralateral muscle (two-way ANOVA, $p < 0.01$). Microinjection of MK-801 into the RVM 24 h after the second intramuscular injection of pH 4.0 saline reversed the bilateral decrease in PWT and MWT (Figure 2A – D). MK-801 reversed the bilateral decrease in PWT induced by repeated intramuscular acid injections 30 min, 1 h, 2 h and 4 h after drug microinjection. Likewise the decrease in MWT was significantly reversed 1 h, 2 h and 4 h after application of MK-801. The reversal of the decreased PWT with the two higher concentrations of MK-801 (3.0 nmol and 9.0 nmol) was significant 30 min, 1 h, 2 h and 4 h after microinjection into the RVM compared with vehicle group ($p < 0.05$). The lower concentration of MK-801 (0.9 nmol) reversed the decreased PWT and MWT 1 h, 2 h and 4 h after administration (Figure 2A – B). For MWT all concentrations of MK-801 were statistically different from vehicle group on 1 h, 2 h and 4h after microinjection ($p < 0.05$; Figure 2C – D).

To test the role of NMDA receptors in the NGC, we administered the 30, 100 or 300 fmol dose of AP5 or 0.9, 3.0 or 9.0 nmol dose of MK-801 bilaterally into the NGC 24 h after second injection of acidic saline. Microinjection of 30, 100 or 300 fmol dose of AP5 into the NGC significantly reversed the bilateral decreased in PWT after 30 min and 1h compared with group that received vehicle intra-NGC ($p < 0.05$; Figure 3A – B). However, all 3 doses of AP5 altered MWT when microinjected into the NGC ($p > 0.05$; Figure 3C – D). Microinjection of 3.0 or 9.0 nmol dose of MK-801 into the NGC reversed the bilateral decreased in PWT after 30 min, 1h and 2h compared with group that received vehicle intra-NGC ($p < 0.05$; Figure 4A – B). However, MK-801 was ineffective in reversing the MWT ($p > 0.05$; Figure 4C – D).

Figure 5 shows coronal sections of the medulla for the microinjection sites for the groups microinjected with vehicle, AP5 or MK-801 into the RVM or NGC.

Discussion

In the current study we evaluated the role of the NMDA receptors in the RVM and NGC on the muscle and cutaneous hypersensitivity induced by repeated intramuscular injections of acidic saline. Microinjection of either a competitive (AP5) or non-competitive (MK-801) NMDA receptor antagonist into the RVM reversed both muscle and cutaneous hypersensitivity. On the other hand, microinjection of NMDA receptor antagonists into NGC reversed only the cutaneous hypersensitivity. This suggests that NMDA receptors in the RVM

are involved in modulation of both muscle and cutaneous hypersensitivity in acidic saline model, while in NGC the NMDA receptors show a specific role in modulation of cutaneous hypersensitivity. To our knowledge this is the first time that differentiation between central modulation of cutaneous and muscle hypersensitivity has been demonstrated.

This model is unique in that it produces a bilateral and widespread hypersensitivity that takes two injections of acid to induce. In rats, there is no hyperalgesia after the first injection. This model is both initiated and maintained by processes within the central nervous system including the RVM and the NGC (28,36). For example anesthetic blockade of the RVM or the NGC during the second injection, but not the first injection, of acidic saline prevents the onset of hypersensitivity 24h later, suggesting a role for the RVM in the induction of hypersensitivity (36). Further anesthetic blockade in the RVM or the NGC 24h after induction of hypersensitivity reverses the hypersensitivity, which is consistent with the current study showing blockade of NMDA receptors in the RVM and the NGC reverse the hypersensitivity at this time period.

We suggest there is a shift in the balance between facilitation and inhibition after muscle insult so that facilitation predominates in this model of chronic widespread pain. Clinically, chronic widespread pain such as fibromyalgia results in a reduction in descending inhibition (16,25, 33), which could be interpreted as an increase in facilitation. In animal studies different models show different time courses for facilitation. After cutaneous inflammation, lesions of the NGC reduce the intensity of stimulating current in the NRM needed to inhibit nociception 3 h, but not 24 h after inflammation. Similarly, NMDA receptor antagonists also reduce the stimulating current needed to produce inhibition at 3 h suggesting that NMDA receptors mediate descending facilitation in the early stages of the development of hypersensitivity (13,34). In contrast, in a neuropathic pain model descending facilitation is not detectable until 6 days after injury (6). Thus, different models in a different pattern of descending facilitation that may be unique to the site of the injury (e.g., nerve, viscera, muscle, skin/subcutaneous).

RVM and Hypersensitivity

The RVM, which includes the nucleus raphe magnus and nucleus gigantocellularis pars alpha, is an essential site of the brainstem nociceptive modulatory system and functionally inhibits or facilitates nociceptive transmission (3,11,17). For example, stimulation of the RVM with high doses of glutamate produces antinociceptive while lower doses of glutamate facilitate nociception (32,37,50,51). During persistent pain conditions there are alterations in both facilitatory and inhibitory outflow from the RVM, which contribute to the hyperalgesia (7, 15,43). Either decreasing inhibition or increasing facilitation in RVM could lead to a hyperalgesia. Previously, we show that microinjection of the local anesthetic into the RVM or the NGC reverses both cutaneous and muscle hypersensitivity induced by acidic saline injection (36), suggesting that descending facilitation predominates over descending inhibition in this model.

Several studies suggest a role for glutamate and activation of NMDA receptors in nociceptive facilitation mechanisms within the RVM (8,37,42). Specifically, microinjection of NMDA receptor antagonists into the RVM blocks visceral hypersensitivity (37) mustard oil-induced hypersensitivity (8), and neuropathic hypersensitivity (42), paralleling the effect in the current study. Interestingly, our results show a delayed reversal of the hypersensitivity by the lower concentrations of the competitive (AP5) antagonist compared to higher concentration of AP5 or non-competitive (MK-801) receptors antagonist. This delayed response may be a result of the 30fmol, 100fmol and 300fmol doses were too small to overcome synaptic glutamate release that is increased in this model during the second injection of acidic saline (26). It should be noted, however, that a previous study shows microinjection of 30fmol or 100fmol of AP5 into the RVM reversed visceral hypersensitivity (8), and in current study 300fmol dose of AP5

reverses cutaneous hypersensitivity in the NGC. On the other hand mustard oil-induced secondary hypersensitivity is reduced by microinjection of 1–2 nmol, but not 500fmol, AP5 into the RVM (43); however, all these data were collected within 45 minutes of drug administration. Thus, different pain conditions, different drug doses, and different sites of injection may reveal differential effects of NMDA receptor blockade.

Classically, three distinct classes of neurons are described in RVM based on changes in activity during nocifensor reflexes (9,10). The “off-cells” are characterized by a pause in discharge before the nocifensive reflex, the “on-cells” are defined by increasing activity just before the withdrawal reflexes and neutral cells do not show changes in firing associated with nociceptive stimuli (9–11,17). Functionally, off-cells and on-cells are implicated in inhibition and facilitation of pain, respectively. Changes in on-cells and off-cells after inflammation could underlie the observed behavioral changes (15,43). Mustard oil-induced inflammation increases activity of on-cells and decreases responses of off-cells (15); while Complete Freund’s Adjuvant (CFA) inflammation appears to result in a phenotypic switch of neutral cells to either on- or off-cells(20). Thus, repeated intramuscular acid injections could increase the responsiveness of on-cells to glutamate by alterations in NMDA receptor expression or phosphorylation of NMDA receptor subunits. Supporting this suggestion, in the RVM, microinjection of AP5 blocks enhanced on-cells activity (43), and there is an upregulation in mRNAs encoding NR1, NR2A and NR2B NMDA receptor subunits after inflammation (20). Together these data suggest an increased in number of NMDA receptors in the RVM could enhance on-cell activity and produce hyperalgesia.

NGC and Hypersensitivity

Early studies show that the NGC facilitates nociception in several models of tissue insult that result in hypersensitivity (49,50). In animals with inflammation, lesions of the NGC block secondary hypersensitivity (39), reduce the current intensity to inhibit nociception (34,42), and spinal dorsal horn Fos expression (41). In relation to acidic saline model, microinjection of the local anesthetic ropivacaine into the NGC reverses both muscle and cutaneous hypersensitivity mediated by acidic saline injection (36). Surprisingly, the current study shows that blockade of NMDA receptors in the NGC only reverses the cutaneous hypersensitivity and has no effect on the muscle hypersensitivity. The difference between our previous results and the current findings may be due to the use of the non-specific blocker (ropivacaine) and specific NMDA antagonists (AP5 and MK-801). During local anesthetic administration, both local neurons and fibers passing through the NGC are blocked. It is thus possible that ropivacaine blocks fibers of passage that mediate muscle hypersensitivity coursing through the NGC. Alternatively, ropivacaine could block neurons responsible for the muscle hypersensitivity that express receptors other than NMDA. In support, prior studies demonstrate that microinjection of the neurotensin, high dose of GABA_B agonists, or orphanin into the NGC facilitate nociception and dorsal horn neuron activity (35,44,49,50). It is also possible that the NGC is somatotopically organized so that our injections are given to a region linked with the plantar surface of the paw instead of the muscle. This is unlikely since prior studies show most neurons in the NGC have large, discontinuous and bilaterally symmetrical receptive fields (23,27), and local anesthetic injected with the same volume and at the same coordinates in the NGC reverses both cutaneous and muscle hypersensitivity (36). Another possibility is that the NMDA antagonists diffuse to the RVM to produce their effect. However, the time course of the effects do not support this conclusion, and one would expect that both cutaneous and muscle hypersensitivity would be reversed by diffusion.

The NGC receives afferents from the spinal cord (1) and projects predominantly to laminae VII, VIII and IX of spinal cord (18). These projections suggest a function of NGC is to modulate autonomic and motor responses related to peripheral stimuli. However, the NGC also sends

direct projections to the RVM (5,18,47), which in turn project densely to laminae I, II and V of spinal cord (4,14). Further, nucleus raphe magnus lesions prevent facilitatory effects produced by the NGC (41). Thus, the NGC could interact with RVM to produce the hypersensitivity after muscle insult. However further studies need to be made to test if NGC and RVM interact or if they represent distinct mechanisms during hypersensitivity after muscle insult.

Summary

In summary, our findings suggest involvement of NMDA receptors of the RVM in the maintenance of both cutaneous and muscle hypersensitivity after muscle insult, while NMDA receptors of the NGC are associated with cutaneous hypersensitivity. Together these data suggest that repeated muscle insult could induce a sensitization of the RVM and/or NGC by one of several potential mechanisms: increasing glutamate release, increasing responsiveness of NMDA receptors, or increasing expression NMDA receptors.

Perspective

The current study shows that NMDA receptors in supraspinal facilitatory sites maintain non-inflammatory muscle pain. Clinical studies in people with chronic widespread, non-inflammatory pain, similarly, show alterations in central excitability. Thus, understanding mechanisms in an animal model could lead to improved treatment for patients with chronic muscle pain.

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Figure 1.

Graphs representing paw (A and B) and muscle (C and D) withdrawal threshold, before 1st, 2nd and 24 h after intramuscular injection of acidic saline and after microinjection of AP5 (30, 100, 300 fmol or 1 nmol) or vehicle into the RVM. The bilateral reduction in paw and muscle withdrawal threshold were reversed by microinjection of AP5 into the RVM. Paw withdrawal thresholds are represented as the median with the 25th and 75th percentiles and muscle withdrawal threshold were represented as means $\pm$ SEM. *, 30, 100, 300 fmol and 1 nmol groups were statistically different from the vehicle group. #, 300 fmol and 1 nmol were statistically different from the vehicle group. +, 1 nmol group was statistically different from the vehicle group.

Figure 2.

Graphs representing paw (A and B) and muscle (C and D) withdrawal threshold, before 1st, 2nd and 24 h after intramuscular injection of acidic saline and after microinjection of MK-801 (0.9, 3.0 or 9.0 nmol) or vehicle into the RVM. The bilateral reduction in paw and muscle withdrawal threshold were reversed by microinjection of MK-801 into the RVM. Paw withdrawal thresholds are represented as the median with the 25th and 75th percentiles and muscle withdrawal threshold were represented as means $\pm$ SEM. *, 0.9, 3.0 and 9.0 nmol groups were statistically different from the vehicle group. +, 3.0 and 9.0 nmol groups were statistically different from the vehicle group.

Figure 3.

Graphs representing paw (A and B) and muscle (C and D) withdrawal threshold, before 1st, 2nd and 24 h after intramuscular injection of acidic saline and after microinjection of AP5 (30, 100 or 300 fmol) or vehicle into the NGC. The bilateral reduction in paw and muscle withdrawal threshold were reversed by microinjection of AP5 into the NGC. Paw withdrawal thresholds are represented as the median with the 25th and 75th percentiles and muscle withdrawal threshold were represented as means $\pm$ SEM. * 30, 100 and 300 fmol groups were statistically different from the vehicle group.

Figure 4.

Graphs representing paw (A and B) and muscle (C and D) withdrawal threshold, before 1st, 2nd and 24 h after intramuscular injection of acidic saline and after microinjection of MK-801 (0.9, 3.0 or 9.0 nmol) or vehicle into the NGC. The bilateral reduction in paw and muscle withdrawal threshold were reversed by microinjection of MK-801 into the RVM. Paw withdrawal thresholds are represented as the median with the 25th and 75th percentiles and muscle withdrawal threshold were represented as means $\pm$ SEM. *, 3.0 and 9.0 nmol groups were statistically different from the vehicle group. +, 3.0 nmol group was statistically different from the vehicle group.

Figure 5.
Schematic coronal sections of the rat brain illustrating sites of microinjections vehicle, AP5 or MK-801 into the RVM or NGC.