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Increased glutamate and decreased glycine release in the RVM during induction of a pre-clinical model of chronic widespread muscle pain

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Abstract

Two injections of acidic saline into the gastrocnemius muscle produce long-lasting hyperalgesia that is initiated and maintained by changes in the rostroventromedial medulla (RVM). Potential underlying mechanisms could be increased release of excitatory neurotransmitters and/or reduced release of inhibitory neurotransmitters, in the RVM. We tested this hypothesis by measuring concentrations of aspartate, glutamate and glycine in response to the first and second injection of acidic saline and compared to intramuscular injections of normal saline using microdialysis with HPLC analysis. We show a significant increase in aspartate and glutamate during the second acid-saline injection compared to normal-saline injections or the first injection of acid-saline. There were also long-lasting decreases in glycine concentrations in the RVM in response to both the first and second injection of acidic saline. It is possible that disinhibition after the first injection leads to long-lasting neuronal changes that allow a greater release of excitatory neurotransmitters after the second injection. We hypothesize that increased release of excitatory neurotransmitters in the RVM drives the release of excitatory neurotransmitters in the spinal cord, central sensitization and the consequent hyperalgesia.

Keywords

pain; hyperalgesia; aspartate; microdialysis; HPLC

Chronic widespread pain, such as fibromyalgia, occurs in about 7% of the population in developed countries and results in significant disability [24]. Basic drug discovery research in this area has been hampered by the lack of appropriate pre-clinical models. Our laboratory developed a rodent model (acidic-saline model) where chronic hyperalgesia is centrally mediated [27]. In this model, two injections of acidic saline, 5 days apart, in the gastrocnemius muscle produce hyperalgesia within hours after the second injection that lasts for weeks [27; 32;41]. A unique feature of this model is that the hyperalgesia is bilateral, widespread and includes sensitivity of the paw, muscle and viscera [19;27;41]. Further the hyperalgesia mediated mainly by central mechanisms and there is no peripheral tissue damage [11;25;27; 41]. Increased central excitability has been shown spinally with sensitization of nociceptive

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neurons, increased glutamate release, and increased activity of the cAMP pathway [11;25; 26;28], and supraspinally local anesthetics administered into rostral ventromedial medullary (RVM) prevent the hyperalgesia [32]. All these data suggest increased neuronal activity (central sensitization) in the spinal cord and medullary nuclei during the second injection of acidic saline that does not occur during the first injection.

Descending facilitatory and inhibitory influences from the RVM influence peripheral nociception [21;35] and persistent pain conditions [5;15;36]. In the acid saline model, local anesthetic administered in the RVM during the second injection of acidic saline prevents the onset of hyperalgesia [32] suggesting that increased excitation in the RVM is important for development of hyperalgesia. Further, low doses of glutamate injected in the RVM facilitate pain [42]. Inhibitory neurotransmitters such as GABA and glycine, are also involved in modulating descending influences from the RVM [14], and loss of inhibition, at the spinal cord level, is critical for development of central sensitization [17]. We therefore hypothesized that release of the excitatory neurotransmitters glutamate and aspartate will increase, and the inhibitory neurotransmitter glycine will decrease in the RVM during the second injection of acidic saline.

All experiments were approved by the Animal Care and Use Committee at the University of Iowa. Male Sprague-Dawley rats (300–350g, n=28), anesthetized with sodium pentobarbital (50mg/kg, i.p.), were placed in a stereotaxic frame for implantation of guide cannulae. The skull was exposed, a small whole drilled and the guide cannulae placed 1mm above the NRM [intra-aural: -2.0mm; mediolateral: 0.0mm; dorsoventral: -8.5mm from the surface]. The cannula was secured to the skull with dental cement and animals were allowed to recover prior to testing.

On the day of the experiment, animals were placed in a small Lucite cubicle and provided free access to food and water. A microdialysis probe (CMA Microdialysis Inc., CMA11/14/02; 2mm membrane length, 0.24mm OD, 6 KD cutoff) was inserted into the guide cannulae and artificial cerebrospinal fluid (ACSF) infused (5µl/min) throughout the experiment. After 1h washout, dialysate samples were collected in 15min increments on ice, frozen on dry ice, and stored at -70°C until analysis.

After 1h of baseline sample collection, animals were anesthetized with 4% halothane for 15min, and the gastrocnemius muscle was injected with 100µl of either pH 4.0(n=12) or pH 7.2(n=14) saline. In half of these animals, the gastrocnemius muscle had been injected with pH 4.0(n=6) or pH 7.2(n=7), 5 days prior. We previously show that intramuscular injection of pH 4.0 results in an average decrease to approximately pH 6.5 in muscle for 5–6 minutes [27], and thus, injection of pH 4.0 represents a relatively small, physiological and short term decrease in pH. After muscle injection, halothane was removed and the animal allowed to recover. At the end of the experiment, rats were euthanized, the brain removed, fixed in 10% formalin and cut on a cryostat at 50µm for analysis of probe placement.

Samples were analyzed for aspartate, glutamate, glycine, serine, asparagine, and glutamine using fluorescent detection with pre-column o-phthalaldehyde (OPT; Sigma; 50µl) derivatization [26]. A 20µl aliquot of sample was diluted in 180µl deionized water containing 1ng/ml of the internal standard homoserine. After derivatization 200µl of each sample was injected onto the column (Supelcosil LC-18; 5µm particle diameter, 4.6mm i.d., 15cm long). Mobile phase consisted of 17% methanol and 0.05M sodium acetate flowing at 1ml/min. The fluorescence detector was set at 330nm for excitation and 420nm for emission. Concentrations were calculated based on a standard curve.

For comparison, data were converted to a percent of baseline with baseline set at 100%. A two-way repeated measures ANOVA examined for effects across time within groups and compared

differences between groups for the day of injection (injection 1 vs. injection 2) and for pH of injection (pH 7.2 vs. pH 4.0). Differences during injection and area under the curve were analyzed separately with a two-way ANOVA for day of injection and pH of injection. Post-hoc testing with a Duncan test compared differences between individual groups. Area under the curve (AUC) was calculated so that no change would be 0, an increased concentration would be positive and a decreased concentration would be negative.

Baseline concentrations for all amino acids were similar between groups and averaged: Glycine: 230 $\pm$ 80ng/ml (3.06 μ moles/L); Glutamate: 287 $\pm$ 56ng/ml (1.95 μ moles/L); Aspartate: 111 $\pm$ 37ng/ml (0.83 μ moles/L); Serine: 230 $\pm$ 80ng/ml (2.19 μ moles/L); Asparagine: 48 $\pm$ 10ng/ml (0.36 μ moles/L); Glutamine 572 $\pm$ 147ng/ml (1.01 μ moles/L). All the concentrations stated above are the concentrations of amino acids in the dialysate, and not corrected for recovery.

Injections of acidic saline resulted in changes in aspartate, glutamate and glycine in the RVM between groups. No significant changes in asparagine, glutamine, or serine in the RVM occurred in response to injection of acidic saline (data not shown). Microdialysis probes were located in the nucleus raphe magnus or the nucleus gigantocellularis pars alpha of the RVM (Figure 1). In 5 animals, probes were misplaced into the cerebellum, ventral midbrain, ventromedial pons, facial nucleus, or the predorsal bundle in the medulla. No amino acid changes were observed in these animals and AUC was not different from those obtained following injection of pH 7.2 saline (AUC:aspartate -2030 ± 1266 ; glutamate -474 ± 750 ; glycine 472 ± 750).

Overall there was a within subjects effect for changes in glutamate across time ($F_{8,14} = 2.7$, $p=0.048$). During injection of acidic saline, there were differences between groups for changes in aspartate ($F_{3,24}=4.8$, $p=0.01$) and glutamate ($F_{3,24}=3.1$, $p=0.04$) in the RVM. Significant increases occurred for aspartate ($p<0.05$) and glutamate ($p<0.05$) during the second injection of acidic saline when compared to the second injection of pH 7.2 (aspartate) or to the first injection of pH 4.0 or pH 7.2 (aspartate, glutamate)(Figures 2, 3). The high level of variance during the second injection of acidic saline is likely due to a very large increase in one of the animals ($>350\%$) when compared to the average response of the other animals in the group (approx. 140%). There were no changes for aspartate or glutamate at any other time. AUC showed there were no changes in amino acid concentrations across the entire 2h sampling period in response to the first or the second injection of acidic saline when compared to injections of pH 7.2(Figures 2, 3). These lack of changes for AUC likely reflects the fact that changes in aspartate and glutamate are occurring during the injection of acidic saline and do not persist throughout the sampling period. Halothane alone did not change the concentrations of aspartate and glutamate when compared to baseline values (aspartate: $104 \pm 7.2\%$; glutamate: $98 \pm 4.2\%$). Thus, increases in glutamate and aspartate occurred in the RVM during the injection of acidic saline but were transient only lasting during the injection.

A significant effect for pH of injection (pH 4.0 vs. pH 7.2)($F_{1,21}=5.0$, $p=0.036$) occurred for changes in glycine concentrations (Figure 4). The effects show a significant decrease in glycine for animals injected with pH 4.0 when compared to those injected with pH 7.2; there was no difference for day of injection. Analysis of the AUC revealed a similar difference for pH of injection ($F_{1,27}=5.0$, $p=0.04$) but not for day of injection. There were significant decreases in glycine concentrations when comparing groups injected with pH 4.0 after the first or second injection (AUC: 2026 ± 733) to those injected with pH 7.2 after the first or second injection (AUC: 248 ± 652) (Figure 2). Halothane alone had no effect on the glycine concentrations when compared to baseline ($97 \pm 5.3\%$). Thus, injection of acidic saline decrease glycine in the RVM, regardless of timing of injection.

Glutamate and aspartate, and their receptors, are localized to the RVM and play a role in facilitation of nociception [1;23]. Glutamate microinjected into the RVM facilitates nociception [42], while blockade of NMDA receptors reverses hyperalgesia in models of persistent pain [3;4;34;38]. Further, the dose-response function for activation of NMDA receptors in the NRM shifts leftward, NR1, NR2A and NR2B subunits of the NMDA increases, and phosphorylation of GluR1 increases after hindpaw inflammation [6;7;18;30]. Together these data suggest that increased release of excitatory neurotransmitters in the RVM, as observed in the current study, is important for the generation and maintenance of hyperalgesia.

Since the RVM both facilitates and inhibits nociception, it is proposed that there is a balance between inhibition and facilitation; after tissue injury this balance shifts to increased facilitation. Indeed RVM facilitation mediates hyperalgesia in several animal models of pain including the repeated acid model [3;21;33;34;38;40]. In fact, blockade of synaptic transmission in the RVM with local anesthetic during the second injection, but not the first injection, prevents the development of hyperalgesia induced by repeated acid injections [32]. This is consistent with an increase in excitatory neurotransmission during the second but not the first injection of acidic saline.

In the current study, the increase in excitatory amino acids was transient and therefore does not correlate with the time course of hyperalgesia observed in this model. However, transient increases in neurotransmitters can cause prolonged changes in postsynaptic neurons and on hyperalgesia [39]. Phosphorylation or increased expression of neurotransmitter receptors could be initiated by transient increases in glutamate; and there are increases in RVM phosphorylation and expression of both AMPA and NMDA receptors after peripheral tissue injury [6;7;18;30]. These RVM increases could drive changes in the spinal cord to result in increased excitability of dorsal horn neurons. In this model, spinally, there is sensitization of dorsal horn neurons, increased glutamate release, increased phosphorylation of the NMDA receptor, and increased phosphorylation of the transcription factor CREB [2;11;26]. Further, the increased release of glutamate spinally occurs after the injection, rather than during, and lasts for up to one week [26].

The inhibitory amino acid glycine showed an overall reduction following the first and second injection of acidic saline. This model requires the second injection of acidic saline to produce hyperalgesia [27] suggesting that the nervous system is changed in some way so the second injection of acid produces a greater response than the first to result in central sensitization. It is also possible that disinhibition after the first injection leads to long-lasting neuronal changes that allow a greater release of excitatory neurotransmitters after the second injection. Our prior study shows that blockade of neuronal activity, with local anesthetic, during the first injection has no effect on the hyperalgesia [32], consistent with the fact that changes occurring immediately after the first injection are unrelated to increased neurotransmitter release, which would be blocked by local anesthetic, but rather decreased neurotransmitter release, which would be unaffected by local anesthetic. However, the hyperalgesia is prevented by blockade of the RVM during the second injection [32]; consistent with the current study showing increases in release of excitatory neurotransmitters during the second injection.

Glycine is an inhibitory neurotransmitter in the RVM that behaves differently from GABA in terms of 'off-cell' activity; GABA inhibits 'off-cells' [8;10;20], glycine does not [9] making it difficult to predict the effects of reduced glycine levels in the RVM. Activation of inhibitory GABA_B receptors in the NRM produces a biphasic effect on nociception; low doses being inhibitory and high doses being facilitatory [31]. It is possible that the role of inhibitory amino acids, like glycine, in the RVM is to inhibit nociception under normal conditions by activating descending inhibitory systems. Since glycine is also a partial-agonist at the NMDA receptors and is required for the activation of NMDA receptors by glutamate [16], a reduction in glycine

concentration could theoretically diminish the NMDA receptor activity and thereby inhibit nociception. However, at the concentrations observed in the current study, the glycine site on the NMDA receptor is likely saturated.

It is recognized that the RVM is involved in several other physiological and pathophysiological processes apart from nociception such as cardiorespiratory or motor system function [12;13;22;37], which were not monitored in the current study. However, we do not believe these factors are the underlying reasons for the pain behavior observed in this model. Prior studies in this model show no deficits in motor performance on a Rota-Rod test, placement test, or gait, and no damage to the muscle itself [27]. Also, there is no visible difference in spontaneous behaviors (grooming etc.), respiratory rates, or urination frequency in the acid saline-injected animals. In addition, behavioral measures are reversed by opioids, pregabalin and NMDA antagonists supporting the conclusion that behavioral responses are related to nociception [25;29;41]. There is also evidence that blockade of NMDA receptors in the RVM results in a reversal of hyperalgesia in pain models [3;4;34;38;40] and that application of glutamate into the RVM facilitates pain behaviors [42].

In conclusion, the transient elevation of EAAs glutamate and aspartate, and reduction in glycine in the RVM could be, at least in part, responsible for induction of hyperalgesia in the acidic-saline model of widespread pain. Specifically, it is possible that loss of inhibition, along with increased excitation in the RVM, leads to increased facilitation of nociception. Shifting the balance to excitation, could lead to increase spinal release of excitatory neurotransmitters, sensitization of dorsal horn neurons, and widespread hyperalgesia observed in this model.

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Figure 1.

Upper panel shows the probe placement sites in the RVM of pH 4.0 group animals and lower panel shows the probe placement sites in the pH 7.2 group. Probes were placed in the nucleus raphe magnus or the paragigantocellularis pars alpha in all groups.

Figure 2.

A. Line graphs showing aspartate concentrations in the RVM in response to the first injection of acidic saline (arrow), in animals receiving pH 4.0 (n=6, open circles) or pH 7.2 (n=7, closed circles). B. Line graphs showing aspartate concentrations in the RVM in response to the second injection of acidic saline (arrow), in animals receiving pH 4.0 (n=6, open circles) or pH 7.2 (n=7, closed circles). *, significantly increased from pH 7.2 or injection 1. C. Area under the curve for the changes in aspartate concentration in response to the first or second injection of pH 4.0 or pH 7.2. No significant differences were observed between groups. There were separate groups were used for single and double injections of saline. Data are the mean $\pm$ S.E.M.

Figure 3.

A. Line graphs showing glutamate concentrations in the RVM in response to the first injection of acidic saline (arrow), in animals receiving pH 4.0 (n=6, open circles) or pH 7.2 (n=7, closed circles). B. Line graphs showing glutamate concentrations in the RVM in response to the second injection of acidic saline (arrow), in animals receiving pH 4.0 (n=6, open circles) or pH 7.2 (n=7, closed circles). *, significantly increased from pH 7.2 or injection 1. C. Area under the curve for the changes in glutamate concentration in response to the first or second injection of pH 4.0 or pH 7.2. No significant differences were observed between groups. There separate groups were used for single and double injections. Data are the mean $\pm$ S.E.M.

Figure 4.

A, B. Line graphs depicting glycine concentrations in response to intramuscular injection of saline on Day 0, first injection, and again on Day 5, second injection. Glycine concentrations in the RVM are significantly reduced in response to the injection of acidic saline when compared to pH 7.2 saline injections, but unrelated to the time of injection, i.e. injection 1 vs. injection 2 (A,B). C. Area under the curve for the changes in glycine concentration. The decrease in glycine that occurs in response to the either the first or second injection of pH 4.0, and is significantly different than injections of pH 7.2 (*, $p < 0.05$). There separate groups were used for single and double injections. Data are the mean $\pm$ S.E.M.