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Injection of Adjuvant but not Acidic Saline into Craniofacial Muscle Evokes Nociceptive Behaviors and Neuropeptide Expression

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Abstract

Craniofacial muscle pain including muscular temporomandibular disorders accounts for a substantial portion of all pain perceived in the head and neck region. In spite of its high clinical prevalence, the mechanisms of chronic craniofacial muscle pain are not well understood. Injection of acidic saline into rodent hindlimb muscles produces pathologies which resemble muscular pathologies in chronic pain patients. Here we investigated whether analogous transformations occur following repeated injections of acidic saline into the rat masseter muscle. Injection of acidic saline (pH 4) into the masseter muscle transiently lowered intramuscular pH to levels comparable to those reported for rodent hindlimb muscles. Nevertheless, repeated unilateral or bilateral injections of acidic saline (pH 4) into the masseter muscle failed to alter nociceptive behavioral responses as occurs in the hindlimb. Changing the pH of injected saline to pH 3.0 or 5.0 also did not evoke nocifensive behavior. ASIC3 receptors, which are implicated in transformations following acidification of hindlimb muscles, were found on trigeminal ganglion muscle afferent neurons via combined neuronal tracing and immunocytochemistry. In contrast to the acidic saline, injection of complete Freund's adjuvant (CFA) into the masseter muscle induced mechanical allodynia for three weeks, thermal hyperalgesia for one week and an increase in the number of CGRP-immunoreactive muscle afferent neurons in the trigeminal ganglion. Although pH may alter CGRP release in primary afferent neurons, the number of CGRP-muscle afferent neurons did not change following intramuscular injection of acidic saline. Further, there was no change in ganglionic iCGRP levels at one, four or twelve days after intramuscular injection of acidic saline. While these findings extend our earlier reports that CFA-induced muscle inflammation results in behavioral and neuropeptide changes they further suggest that intramuscular acidification in craniofacial muscle evokes different responses than in hindlimb muscle and imply that disparate proton sensing mechanisms underlie these discrepancies.

Keywords

pain; trigeminal; pH; chronic; deep tissue; TMD

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Fifteen percent of the American population suffers from chronic musculoskeletal pain, placing a substantial burden on the health care system (Magni et al., 1993; Weir et al., 2006). In the craniofacial region, muscle pain including muscular temporomandibular disorders (TMD) accounts for a substantial portion of all pain perceived in the head and neck region (McNeill et al., 1990; Epker et al., 1999; Aaron et al., 2000). In spite of their prevalence, the pathophysiological mechanisms of these disorders are poorly understood. Since the central mechanisms of craniofacial pain may differ from those of spinal regions (Bereiter et al., 2000), it is important to investigate chronic pain mechanisms specific to the craniofacial region.

Mechanisms of hindlimb muscle nociception have been investigated using an animal model in which acidic saline is injected repeatedly into hindlimb muscles (Sluka et al., 2001; Skyba et al., 2002; Sluka et al., 2002; Sluka et al., 2003; Hoeger-Bement and Sluka, 2003; Nielsen et al., 2004; Sluka and Audette, 2006; Bement and Sluka, 2007; Yokoyama et al., 2007). Intramuscular injection of acidic saline into the hind limb muscle of rodents evokes central sensitization and long-lasting reductions in paw withdrawal thresholds (Sluka et al., 2001; Skyba et al., 2002). It is unclear whether similar mechanisms are involved in craniofacial muscle pain.

Masticatory muscle pain has often been ascribed to bruxism and other oral habits involving muscle contraction (Clifford et al., 1996). During ischemic muscle contraction intramuscular pH drops (Issberner et al., 1996; Reeh and Steen, 1996), probably due to impaired exchange of gases and metabolic products between capillaries and surrounding tissues (Larsson et al., 1998; Larsson et al., 1999; Eriksen, 2004). We hypothesize that acidification occurs within the masseter muscle producing pain in oral parafunctions such as bruxism.

Activation of acid sensing ion channels (ASICs) at primary afferent terminals may at least be partly responsible for the sensitization of neurons during hyperalgesia since dorsal rhizotomy abolishes ASIC immunoreactivity in the spinal dorsal horn (Olson et al., 1998) and ASIC3 knockout mice do not develop hyperalgesia following repeated acid injection into the gastrocnemius muscle (Sluka et al., 2003). In the trigeminal ganglion ASIC3 receptors have been reported in neurons that innervate tooth pulp and facial skin (Ichikawa and Sugimoto, 2002). Further, electrophysiological recordings indicate the presence of ASIC3 channels in trigeminal ganglion muscle afferent neurons (Connor et al., 2005). In the present study we combined neuronal labeling and immunocytochemistry to investigate the possible presence of ASIC3 receptors in trigeminal ganglion muscle afferent neurons.

Previous studies demonstrate that muscle hyperalgesia is related to increased synthesis and/or release of calcitonin gene-related peptide (CGRP) (Carleson et al., 1997; Reinert et al., 1998; Schafers et al., 2003; Ambalavanar et al., 2006a; Ambalavanar et al., 2006b). Further, the release of CGRP is induced by low pH in trigeminal ganglion primary afferent neurons (Goodis et al., 2006) and that ASIC3 receptors are co-localized with CGRP (Ichikawa and Sugimoto, 2002). Thus, we hypothesized that acidification of the masseter muscle would alter CGRP expression in primary afferent neurons as occurs following CFA-induced muscle inflammation (Carleson et al., 2004; Ambalavanar et al., 2006a; Ambalavanar et al., 2006b). Using behavioral, immunohistochemical and radioimmunoassay techniques we investigated whether CFA or repeated injections of acidic saline into the masseter muscle change mechanical nociceptive withdrawal thresholds and/or neuropeptide expression in trigeminal ganglion muscle afferent neurons.

EXPERIMENTAL PROCEDURES

Adult male Sprague-Dawley rats (275–300g, n = 122) were used in this study. Animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH

publication No. 86-23, revised 1985) and maximum effort was made to minimize the number of animals used and animal suffering. All animal procedures were reviewed and approved by the University of Maryland Animal Care and Use Committee. The number of animals used in each experiment is summarized in Table 1.

Intramuscular pH measurements

Animals (n=16) were anesthetized with isofluorane (2–3%) and a needle electrode (World Precision Instruments, Sarasota, FL) was inserted deep into the superficial masseter muscle, midway between the origin and insertion and between the rostral and caudal border for measuring intramuscular pH. After reading the muscle pH for three minutes, saline either at pH 7.2 (n=6), pH 4.0 (n=6) or CFA (n=4) was injected into the same muscle at approximately 1–2 mm away from the pH electrode tip in separate animals. Tissue pH readings were recorded from the time of injection until 2–3 minutes after the pH reading reached control levels (up to 10–12 minutes).

Masseter muscle injections

Since acidic saline (pH 4.0) injection into the masseter muscle reduced intramuscular pH, we investigated the behavioral responses following intramuscular injection of saline and CFA into the masseter muscle. For injection of substances into the masseter muscle animals were anesthetized with isofluorane (2–3%). Using aseptic technique, the masseter muscle complex was injected either with 150 μl saline at different pH levels (Table 1) or complete Freund's adjuvant (CFA, Sigma F5881, 0.5 mg/ml heat killed *Mycobacterium tuberculosis* suspended in oil:saline = 1:1 emulsion). For retrograde labeling of trigeminal ganglion muscle afferent neurons, a small incision was made in the skin overlying the masseter muscle as previously described (Ambalavanar et al., 2003;Ambalavanar et al., 2005;Ambalavanar et al., 2006b). Six to eight microliters of rhodamine-dextran (10,000 kDa, D-1817, Molecular Probes, Oregon, USA) was injected into multiple portions of the masseter muscle through a single penetration of the fascia overlying the muscle. The muscle was then covered with petroleum jelly to avoid leakage of the tracer. Animals were closely monitored for evidence of distress or pain, and weight gain following the injections.

Behavioral testing

For behavioral experiments, rats were divided into ten groups (Table 1) and injections were made as described above. Animals in Group 1 received an injection of 150 μl of saline at pH 7.2 (n=10) into the right masseter muscle while animals in Group 2 received an injection of 150 μl of complete Freund's adjuvant (CFA, n = 8) into the right masseter muscle. Animals in Group 3 received an injection of 150 μl of saline at pH 4.0 (n=8) into the right masseter muscle. Animals in Group 4 received a single unilateral intramuscular injection of 150 μl of saline at pH 4.0 (n=12) on day 1 and day 5, while animals in Group 5 received injections of 150 μl of saline at pH 4.0 (n=6) into both the right and left masseter muscle on day 1 and day 5. The right masseter muscles of animals in Group 6 were injected with 150 μl of saline at pH 3.0 on days 1, 5 and 16. An additional group of animals, Group 7 received a single unilateral injection of 150 μl of saline at pH 5.0 (n=6) into the masseter muscle on day 1 and day 5. In order to test whether the interval between the two injection plays a role in the development of nociceptive behavior, another group (Group 8) of animals (n=6) received unilateral injection of 150 μl saline at pH 4.0 into the masseter muscle on day 1 and day 2.

Prior to and following saline injections, animals were tested for their response to mechanical stimulation of the masseter muscle region using a rigid von Frey filament coupled with a continuously variable force transducer (Electrovonfrey, model no: 2290, IITC Inc. Woodland Hills, CA.) as described previously (Ambalavanar et al., 2006b; Ambalavanar et al., 2006c). The investigator was unaware of the experimental history of the animals tested. The force

needed to elicit a withdrawal of either the head or hindpaw was recorded following five stimulus presentations at approximately one minute intervals and the mean values of the five readings were used for analysis.

To examine the effect of CFA or acidic saline injections on heat hyperalgesia, animals in Group 9 (n=4) received an injection of 150 μl CFA and Group 10 received an injection of 150 μl of acidic saline at pH 4.0 (n=4) into the masseter muscle on day 1 and day 5 (Sluka et al., 2001). Animals were tested for nocifensive responsiveness to radiant heat stimulation before and after the injections using a modified version of the Hargreaves test (Hargreaves et al., 1988). Each animal was placed in a Plexiglas cubicle that allows minimal movement, and left to habituate for 20 minutes before each test. The animal's head was then positioned gently against the wall until the animal voluntarily kept the head in this position. A radiant heat stimulus was then applied continuously to the masseter muscle region with a projector lamp bulb (CXL/CXR, 7V, 50W) until the animal withdrew the head from the heat source. The time elapsed between the onset of the stimulus and the head withdrawal was recorded automatically with a movement sensor. Prior to the injections, animals were habituated to the testing chamber and handling for two days for at least 30 min each day. Head withdrawal latency measurements were repeated at least five times on each occasion at five minute intervals and a mean value was obtained from these readings. A cut-off time of 20s was adopted to avoid possible tissue damage. The experimenter was blinded to the experimental history of the animals.

Tissue Processing and Immunohistochemistry

Animals that received tracer injection into the masseter muscle (Table 1) were allowed to survive for four days. At the end of four days the animals were deeply anesthetized and perfused transcardially with 0.9% saline and a mixture of 4% paraformaldehyde and 7.5% picric acid in phosphate buffer (0.1M PB, pH 7.4). Trigeminal ganglia and brainstems were dissected, post-fixed for two hours in the same fixative and then immersed in 30% sucrose in PB at 4°C for 24–48 hours.

In order to avoid loss of ganglion tissue during immunohistochemical processing, trigeminal ganglia were embedded in egg-gelatin before cutting. Frozen sections (20–30 μm) were cut and immunostained for CGRP or ASIC3 receptor as described below. Free floating sections were collected in 0.1M phosphate buffered saline (PBS, pH 7.4) and washed in 4 changes of PBS. Sections were then incubated in PBS with 0.4% Triton X-100 (PBST) for 48 hours at 4° C with a rabbit polyclonal antibody against α-CGRP (1:5,000, Penninsula Labs, USA) or guinea pig polyclonal antibody against ASIC3 (1:1000, Neuromics, USA). After rinsing in PBS, the sections were incubated in biotinylated anti rabbit or guinea pig anti-serum (1:200, Vector Laboratories, USA) for one hour at room temperature. To visualize the bound primary antibody, the sections were washed in PBS and incubated in fluorescein isothiocyanate (FITC) conjugated to avidin (1:400, Vector) for 1 hour at room temperature. The sections were then rinsed, mounted onto chrom-alum coated slides, and coverslipped with an aqueous mounting medium (PermaFluor, Shandon, USA). No evidence of non-specific labeling was observed following omission of primary antibodies or preadsorption of the antibody with the appropriate peptide antigen (Ambalavanar et al., 2003). Brainstem sections were cut and examined for rhodamine-dextran-labeled neurons in the facial motor nucleus to evaluate whether tracer had spread to the skin. Muscle tracer injection sites were examined by dissection post-mortem to confirm that tracer injections were confined within the masseter muscle. Tracer injections were confined within the masseter muscle since tracer was not found within the skin neither during post-mortem dissection nor were neurons labeled within the facial motor nucleus.

Quantitative Microscopical Analysis

Trigeminal ganglion sections were examined using wide-field epifluorescent microscopy to identify muscle afferent neurons retrogradely labeled with rhodamine, CGRP- or ASIC3-immunopositive neurons labeled with FITC, and neurons double-labeled with both rhodamine and FITC with appropriate filters (Ambalavanar et al., 2003). Neuronal counts were made by an investigator who was unaware of the experimental history of the animals. Only clearly labeled neurons with a discernable nucleus were included in the counts. Retrogradely labeled muscle afferent neurons are distributed only in the V3 division of the trigeminal ganglion (Arvidsson and Raappana, 1989; Ambalavanar et al., 2003) and not evenly distributed throughout the ganglion. Therefore, every section from each ganglion was examined for labeled neurons in order to avoid potential sampling errors.

Measurement of Immunoreactive CGRP (iCGRP) in the Trigeminal Ganglion using Radioimmunoassay (RIA)

Animals received a single injection of 150 μl of saline at pH 4.0 (n=12) into one masseter muscle and at pH 7.2 into the opposite side masseter muscle of the same animal on day 1 and day 5. Animals were decapitated and their right and left trigeminal ganglia were dissected for radioimmunoassay at one day (n = 4), four days (n = 4), and 12 days (n = 4) following the second saline injection. Trigeminal ganglia were also dissected from uninjected control rats (n = 8). The mandibular (V3) division of each ganglion was identified by following the trajectory of the mandibular branch of the trigeminal nerve as it exits the foramen ovale. The V3 was then separated from V1/V2 as described previously (Ambalavanar et al., 2006b), snap-frozen and stored at −80°C until processed. The wet weight of the dissected V3 division ranged from 7–8 mg while the V1/V2 divisions ranged from 12–14 mg.

Trigeminal ganglia samples were homogenized in an RIA buffer (RK-BUR, Phoenix Pharmaceuticals Inc., Blemont, CA), and the homogenates were centrifuged at 5,000 g at 4°C for 10 minutes. The supernatants were separated and the total protein content in each sample determined by the Bradford's method (protein reagent, Bio-Rad). The superfusate was then used for RIA reactions in duplicate, according to the manufacturer's instructions using an RIA kit for α-CGRP (# RK-015-09, Phoenix Pharmaceuticals Inc., Blemont, CA). CGRP was measured using a rabbit polyclonal antibody against α-CGRP conjugated to bovine serum albumin. The sensitivity was 32pg per tube and the intra-assay and inter-assay variations were 5% and 10% respectively. Cross reactivity for the antibody used was 100% for rat α-CGRP and 35.5% for human α-CGRP.

Statistical Analysis

Data were initially tested for normality (Kolmogorov-Smirnov test) and equal variance (Sigma Stat, Jandel, USA). Differences in central tendency for data which were normally distributed with equal variance were tested using parametric tests (ANOVA followed by a Dunnett's or Bonferroni *post-hoc* test). Behavioral data sets that were not normally distributed and/or had unequal variance were tested for differences in central tendency using either a one-way repeated measures ANOVA or Friedman's repeated measures ANOVA on ranks followed by Dunn's multiple comparisons method for *post-hoc* comparisons. A probability level of 0.05 was chosen for all hypothesis testing.

RESULTS

No morphological signs of inflammation such as edema or redness were observed following intramuscular injection of saline either at neutral or acidic pH (3.0 – 5.0). In contrast to this, inflammatory signs appeared almost immediately in the mandibular region of the head following CFA injection and became more pronounced after 30 minutes. These signs persisted

for two to three days, and subsided four days following the injection of CFA. While animals injected with neutral and acidic saline appeared active and fed normally, those injected with CFA ate very little for the first two days and showed an initial transient weight loss. After this initial period of feeding difficulty, animals fed normally and gained weight over time (Ambalavanar et al., 2006b). In all cases, animals appeared to have fully recovered from isofluorane anesthesia and locomoted normally prior to behavioral testing.

Intramuscular pH Changes Following an Injection of Low pH (pH 4.0) Saline or CFA into the Masseter Muscle

We investigated whether the injection of pH 4.0 saline into the masseter muscle alters tissue pH. Injection of acidic saline (pH 4.0, n=6) but not neutral saline (pH 7.2, n=6) into the masseter muscle caused a reduction in tissue pH for less than ten minutes after the injection (Figure 1). Tissue pH ranged between 6.3–6.8 after the injection of pH 4.0 saline into the masseter muscle. This drop in pH returned to normal pH levels approximately 8–10 minutes after the injection of acidic saline. Injection of CFA (n=4) into the masseter muscle did not cause any change in muscle pH following injection (Figure 1).

Withdrawal Responses to Mechanical Stimulation Following Single Injection of pH 4.0 Saline into the Masseter Muscle

As we found a reduction in tissue pH, our initial experiments examined the head withdrawal thresholds to mechanical stimulation following a single injection of saline (pH 4.0, pH 7.2) or CFA into the masseter muscle. A single injection of saline at pH 4.0 (Figure 2, filled circles, n=8) did not produce any change in head withdrawal thresholds from pre-injection levels (one-way repeated measures ANOVA $p = 0.20$). Following a single injection of saline at pH 7.2 (Figure 2, filled triangles, n=10) into the masseter muscle no reduction in head withdrawal thresholds was observed at any time point from four hours to four weeks following the injection. Withdrawal thresholds did however significantly increase (one-way repeated measures ANOVA $p < 0.001$) at four weeks indicating a possible habituation of animals in experimenter's hands with long-term handling.

Injection of CFA into the masseter muscle (n=8) caused a significant reduction in head withdrawal threshold for mechanical stimulation (Figure 2, filled squares, one-way repeated measures ANOVA $p < 0.001$) from four hours to three weeks after the injection.

Withdrawal Responses to Mechanical Stimulation Following Repeated Injections of pH 4.0 Saline into the Masseter Muscle

Withdrawal thresholds did not differ from baseline values after the first injection of pH 4.0 saline. No significant reductions were observed in head withdrawal thresholds from 4 hours to four weeks after a second injection given on the fifth day. To examine whether the interval between the two injections modulate the behavioral responses, the two injections were separated by two days in an additional group of animals. Withdrawal thresholds did not differ from baseline values when the first and second injections were given two days apart. The withdrawal thresholds were significantly elevated above baseline values at 12 days, three weeks and four weeks after the second injection of acidic saline in both groups. (Figure 3 A, D, one-way repeated measures ANOVA $p < 0.001$).

We also examined the behavioral effects of bilateral repeated injection of pH 4.0 saline by injecting saline into both the right and left masseter muscles (n=6). As observed with the unilateral injection of pH 4.0 saline, bilateral injection of acidic saline at pH 4.0 into the masseter muscle did not produce any reduction in head withdrawal thresholds when either the region of the right or left masseter muscle was mechanically stimulated (Figure 3C). In contrast,

withdrawal thresholds were significantly higher at later time points (Friedman's repeated measures ANOVA on ranks followed by Dunn's multiple comparisons, $p < 0.001$).

Withdrawal Responses to Mechanical Stimulation Repeated Injections of pH 3.0 Saline into the Masseter Muscle

Injection of pH 4.0 saline into the masseter muscle reduces intramuscular pH to nearly pH 6.3 (Figure 1). To examine whether a greater change in tissue pH induces nociceptive behavioral changes, we injected saline at pH 3.0 into the masseter muscle (n=6). The first intramuscular injection of pH 3.0 saline provided a more substantial reduction in head withdrawal thresholds than injection of pH 4.0 saline. A second injection of acidic saline at pH 3.0 into the masseter muscle however, did not produce any reduction in head withdrawal threshold from baseline levels (Figure 3B). A third injection of pH 3.0 saline into the same masseter muscle also did not reduce the withdrawal threshold. In fact, the withdrawal thresholds were higher than the baseline levels at all time points after the third injection (Figure 3B, Friedman's repeated measures ANOVA on ranks followed by Dunn's multiple comparisons, $p \leq 0.001$).

Withdrawal Responses to Mechanical Stimulation Following Repeated Injections of pH 5.0 Saline into the Masseter Muscle

Since the sensitivity of the ASIC3 channels in trigeminal ganglion may be at a tissue pH higher than that resulted from injections of pH 4.0 or pH 3.0, (Connor et al., 2005) we tested behavioral responses after injecting pH 5.0 saline repeatedly into the masseter muscle (n=6). Neither the first (on day 1) nor the second (on day 5) injection evoked any reductions in head withdrawal threshold. To the contrary, the threshold values significantly increased seven days after the second injection (one-way repeated measures ANOVA, $p \leq 0.001$).

Withdrawal Latency to Radiant Heat Stimulation

The latency for head withdrawal evoked by thermal stimulation ranged from 6.5–10s (median 7.4s) before injection. Head withdrawal latencies were significantly reduced from one to seven days after CFA injection into the masseter muscle (one-way repeated measures ANOVA followed by Holm-Sidak multiple comparisons control, $p \leq 0.006$). In contrast to this, the withdrawal latencies did not differ from control baseline values following repeated injections of acidic saline into the masseter muscle (Figure 4, one-way repeated measures ANOVA, $p=0.64$).

ASIC3 Immunoreactivity in Trigeminal Ganglion Muscle Afferent Neurons

As intramuscular acidic saline injections did not evoke nociceptive behavioral responses, we investigated whether the muscle afferent neurons express ASIC3 receptors using retrograde labeling and immunohistochemistry (n=3). Many trigeminal ganglion neurons were immunoreactive for the ASIC3 receptor and were distributed throughout the trigeminal ganglion. ASIC3 immunoreactivity was observed in neurons of different sizes with bright, homogeneous staining throughout the cytoplasm. Muscle afferent neurons retrogradely labeled from the masseter muscle were easily recognized by the accumulation of rhodamine in their cytoplasm. These neurons were found in the mandibular division of the trigeminal ganglion as reported previously (Ambalavanar et al., 2003). Many retrogradely labeled muscle afferent neurons in the trigeminal ganglion were also immunoreactive for ASIC3 (Figure 5). An average of 64 (±9) % of trigeminal ganglion muscle afferent neurons were ASIC3-positive (n=3). The double-labeled neurons included small to medium sized neurons (Figure 5, arrows).

Number of CGRP-immunoreactive Muscle Afferent Neurons in the Trigeminal Ganglion following pH 4.0 saline injection into the masseter muscle

We investigated the number of muscle afferent neurons immunoreactive to CGRP after the injection of saline (pH 4) into the masseter muscle and after the injection of CFA into the masseter muscle. An average of 36 (±8) % of trigeminal ganglion muscle afferent neurons were CGRP-positive in control animals (n=4). The percentage of CGRP-immunoreactive muscle afferent neurons in the trigeminal ganglia from the side of the acid saline-injected masseter muscle did not differ significantly from controls (Figure. 6, one-way ANOVA, p=0.49). In ipsilateral trigeminal ganglion of animals sacrificed 12 days following the second injection of pH 4.0 saline, 32 (±3) % of masseter muscle afferent neurons were immunopositive for CGRP. In animals sacrificed at 32 days after the second injection of acidic saline into the masseter muscle 31.8 (±6) % of muscle afferent neurons were immunoreactive for CGRP. In contrast to this, the percentage of trigeminal ganglion muscle afferent neurons immunopositive for CGRP was significantly higher at 12 and 32 days following CFA injection into the masseter muscle compared to control animals (Figure 6, one-way ANOVA, p=0.001). In ipsilateral ganglia from animals sacrificed 12 days (n=6) following intramuscular CFA injection 55 (±6) % and at 32 days following CFA injection 49 (±4) % of trigeminal ganglion muscle afferent neurons were CGRP-positive.

iCGRP Levels in the Trigeminal Ganglion Following Injection of CFA or pH 4.0 saline into the masseter muscle

Since our previous studies revealed an increase in iCGRP levels in V3 but not in V1/V2 division of the trigeminal ganglion following CFA injection into the masseter muscle, we initially analyzed the V3 division in the present study following acidic saline injection into the masseter muscle. In control animals average iCGRP was 0.78 (±0.26) pg/μg protein in the V3 division of the trigeminal ganglion. In ganglia ipsilateral to the repeated acidic saline injection the iCGRP level was 1.05 (±0.25) pg/μg protein at one day, 1.02 (±0.3) pg/mg protein at four days and 0.85 (±0.2) pg/μg protein at 12 days after the second acid saline injection. Similarly, in ganglia ipsilateral to the injection of pH 7.2, the iCGRP level was 0.87 (±0.1) pg/μg protein at one day, 0.7 (±0.1) pg/μg protein at four days and 0.86 (±0.1) pg/μg protein at 12 days post second acid saline injection. Levels of iCGRP did not differ significantly in the ipsilateral V3 after repeated intramuscular injection of acidic saline (one-way ANOVA, p = 0.237) or pH 7.2 saline (one-way ANOVA, p = 0.56) compared to the controls.

DISCUSSION

This study demonstrates that intramuscular injection of CFA but not repeated injections of acidic saline into the masseter muscle cause chronic behavioral changes as described for hindlimb muscles (Sluka et al., 2001). Further, intramuscular injection of CFA but not acidic saline altered neuropeptide expression in primary afferent neurons innervating the masseter muscle. Our data also show that tissue pH drops to a level comparable to that in limb muscle (Sluka et al., 2001; Gao et al., 2007) following injection of pH 4.0 saline and that ASIC3 receptors are present in trigeminal ganglion muscle afferent neurons. Nevertheless, no behavioral changes were detected with two repeated intramuscular injections on day 1 and day 5 of saline either at pH 4.0, pH 3.0 or pH 5.0. This suggests that regardless of the pH of the solution injected, repeated acidic saline injections into the masseter muscle do not evoke long-lasting muscle hyperalgesia. In fact, an increase in head withdrawal threshold was observed following injection of pH 3.0 saline into the masseter muscle. This may be analogous to the antinociceptive effect reported following mustard oil injection into the jaw joint and mediated via μ opioids (Tambeli et al., 2001).

Additionally, bilateral injections of repeated acidic saline at pH 4.0 or two unilateral injections separated by two days also failed to evoke nociceptive behavioral changes. Taken together these findings imply that differences exist between trigeminal and spinal nociceptive mechanisms. Consistent with this supposition is the fact that experimental muscle pain in humans exhibits different pain thresholds and characteristics for muscles innervated by trigeminal versus cervical spinal nerves (Svensson et al., 2005; Schmidt-Hansen et al., 2006). Human studies also report that central sensitization does not develop following repeated infusions (eight injections within eight hours) of acidic phosphate buffer at pH 5.2 into the forearm (Steen et al., 2001) suggesting that regional or species variability may exist in responses evoked by acidic saline.

Our findings on CFA-induced craniofacial muscle inflammation extends our previous reports (Ambalavanar et al., 2006a; Ambalavanar et al., 2006c). For example, unilateral injection of CFA into the masseter muscle reduces mechanical withdrawal thresholds locally at the site of injection as well as at distant sites (Ambalavanar et al., 2006b; Ambalavanar et al., 2006c). In contrast to this, single or repeated injection of acidic saline into the masseter muscle did not evoke allodynia or thermal hyperalgesia. Injection of CFA into the masseter muscle not only causes a reduction in withdrawal latencies to thermal stimulation but also evokes an increase in the number of CGRP-muscle afferent neurons, and an increase in iCGRP and CGRP mRNA levels in the trigeminal ganglia (Ambalavanar et al., 2006b). Neither the number of CGRP-muscle afferent neurons nor intraganglionic iCGRP levels changed following repeated intramuscular acid injections. These discrepancies indicate that the processes evoked by intramuscular CFA injections differ from those induced by repeated acidic saline injections into the masseter muscle. Our current and previous studies using CFA also show the validity of our behavioral and neurochemical methodologies and demonstrate that these methods are capable of detecting inflammation-induced transformations. In contrast to the data described for repeated saline injections, behavioral responses following a single injection of acidic saline into the masseter muscle did not differ from baseline and are in agreement with that of Sluka et al, (Sluka et al., 2001) in limb muscle. Repeated acidic saline injections into the limb muscle failed to evoke thermal hyperalgesia (Sluka et al., 2001), a finding similar to our observations for craniofacial muscle.

Low tissue pH has been reported in muscle during exhaustive exercise, ischemic muscle contraction and inflammation in humans (Steen et al., 1992; Issberner et al., 1996; Reeh and Steen, 1996). While *in vitro* studies indicate that trigeminal ganglion (Connor et al., 2005) and DRG neurons (Askwith et al., 2000; Price et al., 2001; Sutherland et al., 2001; Immke and McCleskey, 2003; Hoheisel et al., 2004; Molliver et al., 2005; Sluka et al., 2006) respond to very small pH changes, it is important to know whether the tissue pH falls low enough to activate these channels and whether these neurons are sensitive to tissue pH changes *in vivo*. In the present study, following injection of saline at pH 4.0 into the masseter muscle a drop in tissue pH was evident which was comparable to the pH drop reported in hindlimb muscle (Sluka et al., 2001). Despite this pH drop, we did not detect any reduction in withdrawal threshold following repeated injections of pH 4.0 saline. These differences are unlikely to be attributable to methodological differences used to test behavior, since our previous studies (Ambalavanar et al., 2006b; Ambalavanar et al., 2006c) demonstrate that nocifensive behavioral changes can reliably be measured using this methodology. It is important to note that the thermal stimulation was applied to the skin overlying the masseter muscle and therefore it is likely a measure of secondary hyperalgesia as in hindlimb studies (Sluka et al., 2001). The response to mechanical stimulation delivered to the muscle through the skin however, measures a mix of primary and secondary hyperalgesia. Our earlier studies demonstrated that a major portion of the response to mechanical stimulation using a rigid von Frey filament is from muscle stimulation (Ambalavanar et al., 2006b).

In the present study we used retrograde neuronal tracing and immunocytochemistry to demonstrate the presence of ASIC3 receptors in trigeminal ganglion masseter muscle primary afferent neurons. Our results corroborate a previous electrophysiological study which reports the presence of ASIC3 channels on trigeminal ganglion muscle afferent neurons (Connor et al., 2005). Despite the presence of functional ASIC3 channels on these neurons, we failed to detect any behavioral changes following repeated injections of acidic saline into the masseter muscle. These results indicate that either ASIC3 receptors are not activated during injection of acidic saline into craniofacial muscle or ASIC3 activation does not cause long-term neuronal changes and central sensitization in the brainstem as occurs in the spinal cord (Skyba et al., 2002; Sluka et al., 2002; Sluka and Audette, 2006; Bement and Sluka, 2007; Yokoyama et al., 2007).

It is possible that acidic saline injection alone cannot evoke long-term neuronal changes in the trigeminal system and may require a complex chemical environment at nociceptive terminals to induce chronic muscle pain. For example, injection of inflammatory mediators including prostaglandin E2 increases the acid-induced pain ratings in humans (Steen et al., 1996; Birklein et al., 2000; Chen et al., 2006; Rukwied et al., 2007). In addition, acidic solution injected into the forearm skin or muscle resulted in higher pain ratings in patients with complex regional pain syndrome than the control subjects (Birklein et al., 2000) indicating that the presence of inflammation increases responses to tissue acidity. Furthermore, ASICs show persistent activation in the presence of certain neuropeptides (Askwith et al., 2000; Chen et al., 2006). Injection of hypertonic (5%) but not isotonic (0.9%) saline into the craniofacial muscle induces muscle pain in humans (Zhang et al., 1993). The acute pain response in these studies may be due to a transient decrease in pH similar to that observed during the injection of acidic saline in this study.

The role of ASIC receptors in pain processing may be influenced by their subunit composition and the contribution of TRPV1 receptors in proton sensitivity (Tominaga et al., 1998; Ugawa et al., 2002; Poirot et al., 2006; Page et al., 2007). Acidic saline injections into the craniofacial (this study) or hindlimb muscles (Sluka et al., 2001) did not evoke thermal hyperalgesia. The different responses to mechanical stimulation produced by intramuscular acid injections in craniofacial versus hindlimb muscles (Sluka et al., 2001) may therefore represent differences in ASIC receptor subunit composition between DRG and trigeminal ganglion neurons. Studies on ASIC3 channels in dissociated trigeminal and DRG neurons are consistent with this hypothesis. For example, in the DRG, 50% of muscle afferent neurons respond to the application of a pH 5.0 solution and the majority of those neurons are ASIC3/ASIC2b heteromers (Sluka et al., 2003). Specifically, group IV muscle primary afferent neurons in the DRG repond to pH 6.0 (Hoheisel et al., 2004). In addition, inward current was inactivated in trigeminal ganglion neurons with the application of a pH 6.0 solution (Connor et al., 2005) while a sustained current was still evoked by a pH 6.0 solution in DRG neurons (Benson et al., 2002). Trigeminal ganglion masseter muscle afferents however, respond to extracellular pH changes ranging from 7.4 to 6.8 with currents indicative of ASIC3 homomeric channels (Connor et al., 2005). The change in tissue pH following intramuscular injection of pH 4.0 saline was well within the range of the reported sensitivity of ASIC channels in trigeminal ganglion muscle afferent neurons. Despite this no behavioral changes was evoked suggesting that confirming differences in nociceptive mechanisms in craniofacial and hindlimb regions.

The number of CGRP-immunoreactive muscle afferent neurons increased in trigeminal ganglion neurons following CFA-induced muscle inflammation. Repeated acidic saline injections into the masseter muscle however, did not show similar increases in the number of CGRP-immunoreactive muscle afferent neurons in the trigeminal ganglion at 12 or 32 days after the second injection. This suggests that the acidic saline injections did not induce long-term changes in CGRP expression in muscle primary afferent neurons even though ASIC3

receptors co-localize with CGRP in trigeminal ganglion neurons (Ichikawa and Sugimoto, 2002). Our radioimmunoassay results further document that there is no change in iCGRP levels in the V3 division of the trigeminal ganglion one, four, or 12 days following the second intramuscular injection of acidic saline (pH 4.0). Intramuscular injection of saline at pH 7.2 also did not evoke any changes in iCGRP levels confirming our previous observations (Ambalavanar et al., 2006b) that the injection process itself does not cause changes in iCGRP levels within the trigeminal ganglion.

The main findings reported in this study are that repeated acidic saline injections into craniofacial muscle do not evoke chronic pain as occurs in hindlimb muscle nor does acid saline induce neuronal transformations comparable to those which occur in trigeminal ganglion neurons following adjuvant-induced inflammation. These discrepancies could be due to discordanant peripheral mechanisms including variation in channel structure, and/or different central mechanisms. Previous studies demonstrate that central mechanisms of chronic nociception vary between tissue types (Woolf and Wall, 1986; Mense, 1991; Mense and Simons, 2001). Data presented here imply that even within the same tissue type, i.e muscle, pain mechanisms may differ and potentially could allow regional targeting of therapeutics.

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List of Abbreviations

ASIC
: acid sensing ion channel

CFA
: complete Freund’s adjuvant

CGRP
: calcitonin gene-related peptide

FITC
: fluorescein isothiocyanate

iCGRP
: immunoreactive calcitonin gene-related peptide

PB
: phosphate buffer

PBS
: phosphate buffered saline

PBST
: phosphate buffered saline with Triton X-100

RIA
: radioimmunoassay

TMD
: temporomandibular disorders

V3
: mandibular division

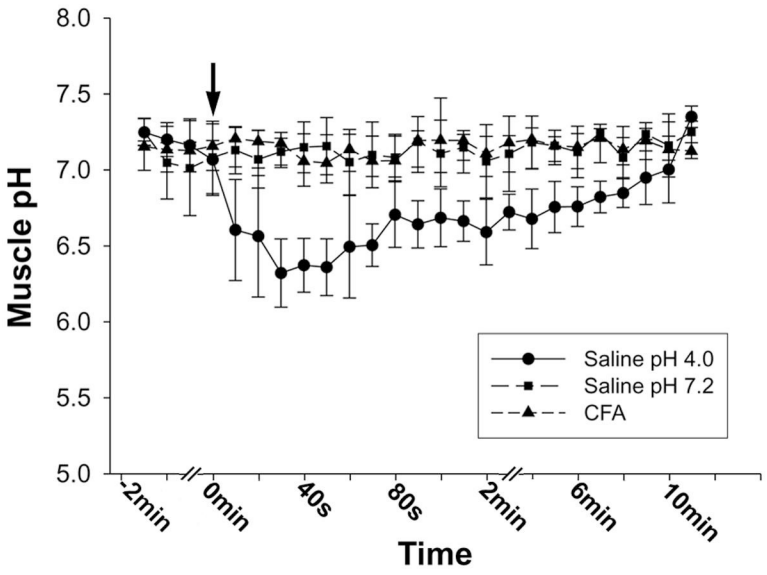

Figure 1.

Intramuscular pH measurements following injection of acidic saline or CFA into the masseter muscle. Injection of acidic saline (pH 4.0, n=6) but not neutral saline (pH 7.2, n=6) or CFA (n=4) into the masseter muscle causes a reduction in tissue pH. The lowest tissue pH measured was 6.3 following the injection of pH 4.0 saline into the masseter muscle. Arrow indicates the time of injection into the masseter muscle.

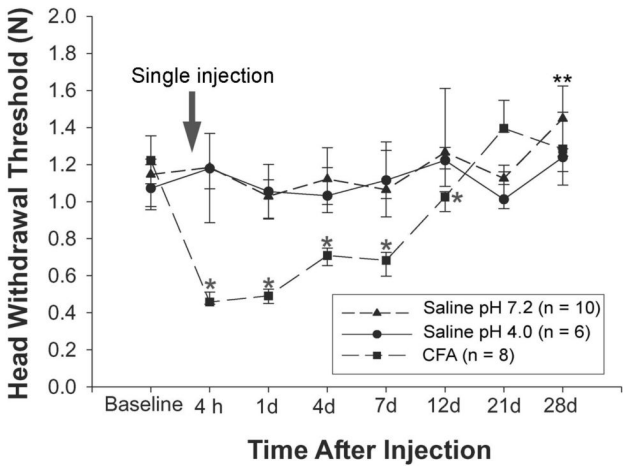

Figure 2.

A single intramuscular injection of acidic or normal saline does not reduce the head withdrawal threshold to mechanical stimulation. One injection of either acidic saline (pH 4.0, filled circles, n=8) or normal neutral saline (pH 7.2, filled triangles, n=10) into the masseter muscle does not reduce the head withdrawal threshold. In both saline injected groups of animals the withdrawal thresholds increased significantly at four weeks (one-way repeated measures ANOVA $p < 0.001$). In contrast to saline injection, injection of CFA into the masseter muscle significantly reduces the threshold for head withdrawal (filled squares, n=8, repeated measures ANOVA $p < 0.001$). For this and all subsequent graphs single asterisks indicate thresholds significantly lower than pre-injection threshold and double asterisks indicate thresholds significantly higher than pre-injection thresholds ($p < 0.001$). Squares, circles and triangles are medians and bars are the 25th and 75th percentiles.

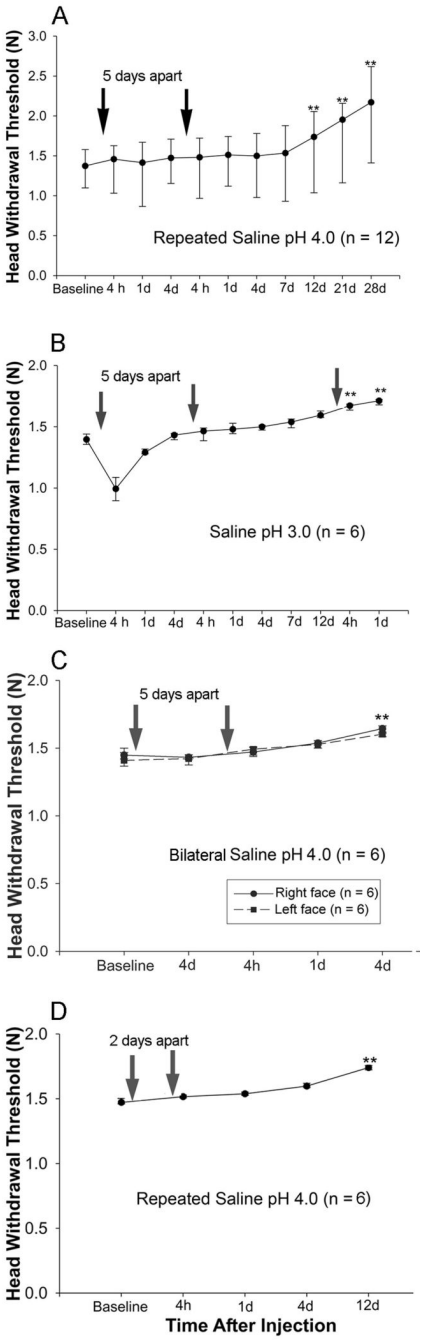

Figure 3.

Repeated injections of acidic saline into one masseter muscle do not reduce the head withdrawal threshold to mechanical stimulation. A) Two unilateral injections of pH 4.0 saline (n=12) separated by five days did not evoke any reduction in head withdrawal. Thresholds were significantly higher 12 days, three weeks and four weeks after the second injection of acidic saline into the masseter muscle (repeated measures ANOVA, $p < 0.0001$). B) Repeated unilateral intramuscular injections of acidic saline at pH 3.0 separated by five days (n=6) did not evoke any reduction in head withdrawal threshold. Withdrawal thresholds were, in fact, higher than the baseline levels at all time points after the third injection (Friedman's repeated measures ANOVA on ranks followed by Dunn's multiple comparisons, $p \leq 0.001$). Arrows

indicate the time of first, second and third injections. C) Repeated bilateral injections of acidic saline (pH 4.0) into both the right and left masseter muscle (n=6) did not reduce the head withdrawal threshold on either side. Withdrawal thresholds were significantly higher four days after the second intramuscular injection of acidic saline (Friedman's repeated measures ANOVA on ranks followed by Dunn's multiple comparisons, $p < 0.001$). D) Two unilateral injections of pH 4.0 saline (n=6) separated by two days did not evoke any reduction in head withdrawal threshold. Single asterisks indicate thresholds significantly lower than pre-injection threshold and double asterisks indicate thresholds significantly higher than pre-injection thresholds ($p < 0.001$). Arrows in A, B, C and D indicate the time of first and second injections of acidic saline.

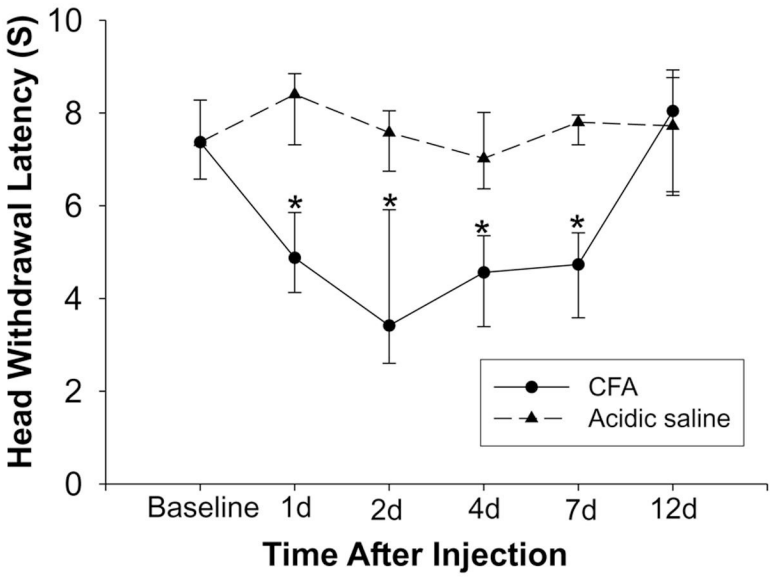

Figure 4.

A single injection of CFA into the masseter muscle causes a reduction from baseline values in head withdrawal latencies for radiant heat stimuli (filled circles). Withdrawal latencies were significantly reduced from one, two, four and seven days after CFA injection into the masseter muscle (one-way repeated measures ANOVA followed by Holm-Sidak multiple comparisons, $p \leq 0.006$). Withdrawal latencies did not differ from control baseline values following repeated injections of acidic saline at pH 4.0 into the masseter muscle (filled triangles, one-way repeated measures ANOVA, p=0.64).

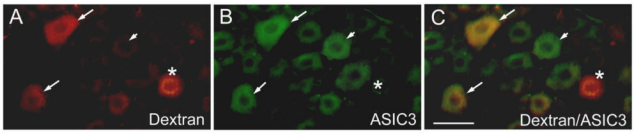

Figure 5.

Trigeminal ganglion muscle primary afferent neurons are immunoreactive for the ASIC3 receptor subunit. Masseter muscle afferent neurons labeled following intramuscular injection of dextran-rhodamine are immunopositive for the ASIC3 receptor (arrows in B and C). Arrowheads identify ASIC3 neurons that are not masseter muscle afferent neurons. Asterisk points to a rhodamine-labeled muscle afferent neuron that is not immunopositive for ASIC3. Scale bar = 50 μm.

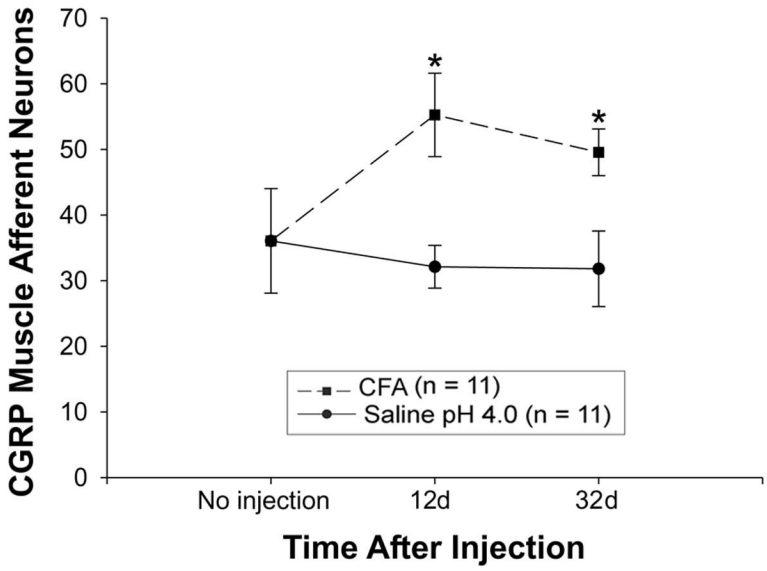

Figure 6.

Injection of intramuscular acidic saline does not alter CGRP immunoreactivity in muscle afferent neurons. Two injections of pH 4.0 saline into the masseter muscle does not change the number of CGRP-immunopositive masseter muscle afferent neurons either 12 days (n=5) or 32 days (n=6) after the second acidic saline injection ($p = 0.559$). In contrast to this, the number of CGRP-immunopositive masseter muscle afferent neurons increases 12 and 32 days following injection of CFA into the masseter muscle ($p = 0.001$). Circles and squares are means and the bars are standard deviations. Asterisks indicate a statistically significant difference from uninjected control animals.

Table 1

Distribution of animals used in each experiment.

Group	n	Masseter Injected with	Time of Intramuscular Injection	Injected Side	Experiment Type
	4	CFA	single	unilateral	tissue pH measurements
	6	saline pH 4.0	single	unilateral	tissue pH measurements
	6	saline pH 7.2	single	unilateral	tissue pH measurements
1	10	saline pH 7.2	single	unilateral	behavior
2	8	CFA	single	unilateral	behavior/ n=5, CGRP immuno (32d)
3	6	saline pH 4.0	single	unilateral	behavior
4	12	saline pH 4.0	day 1, day 5	unilateral	behavior/ n= 6, CGRP immuno (32d)
5	6	saline pH 4.0	day 1, day 5	bilateral	behavior
6	6	saline pH 3.0	day 1,day 5, day 16	unilateral	behavior
7	6	saline pH 5.0	day 1, day 5	unilateral	behavior
8	6	saline pH 4.0	day 1, day 2	unilateral	behavior
9	4	CFA	single	unilateral	behavior (thermal)
10	4	saline pH 4.0	day 1, day 5	unilateral	behavior (thermal)
11	3	dextran tracer	single	unilateral	ASIC3 immuno
12	4	dextran tracer	single	unilateral	CGRP immuno (control)
13	6	CFA	single	unilateral	CGRP immuno (12d)
14	5	saline pH 4.0	day 1, day 5	unilateral	CGRP immuno (12d)
15	8	no injection	-	-	CGRP RIA (control)
16	4	saline pH 4.0, 7.2	day 1, day 5	right pH 4.0 left pH 7.2	CGRP RIA (1d)
17	4	saline pH 4.0, 7.2	day 1, day 5	right pH 4.0 left pH 7.2	CGRP RIA (4d)
18	4	saline pH 4.0, 7.2	day 1, day 5	right pH 4.0 left pH 7.2	CGRP RIA (12d)