Temporomandibular joint-evoked responses by spinomedullary neurons and masseter muscle are enhanced after repeated psychophysical stress

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Abstract

Psychological stress is a risk factor to develop musculoskeletal pain of the head and neck; however, the basis for this relationship remains uncertain. This study tested the hypothesis that psychophysical stress alone was sufficient to alter the encoding properties of spinomedullary dorsal horn neurons and masseter muscle activity in male rats. Repeated forced swim conditioning increased markedly both the background firing rate and temporomandibular joint (TMJ)-evoked activity of neurons in deep dorsal horn, while neurons in superficial laminae were less affected. Stress also increased the responses to stimulation of facial skin overlying the TMJ of neurons in deep and superficial dorsal horn. TMJ-evoked masseter muscle activity was enhanced significantly in stressed rats, an effect that was reduced by prior blockade of the spinomedullary junction region. These data indicated that repeated psychophysical stress induced widespread effects on the properties of medullary dorsal horn neurons and masseter muscle activity. The effects of stress were seen preferentially on neurons in deep dorsal horn and included enhanced responses to chemosensory input from the TMJ and mechanical input from overlying facial skin. The stress-induced elevation in TMJ-evoked masseter muscle activity matched well the changes seen in dorsal horn neurons. It is concluded that the spinomedullary junction region plays a critical role in the integration of psychophysical stress and sensory information relevant for nociception involving deep craniofacial tissues.

Keywords

pain; forced swim stress; trigeminal subnucleus caudalis; temporomandibular joint; masseter muscle

Introduction

A link between psychological state and chronic pain in deep tissues has long been appreciated; however, the mechanisms that underlie this relationship remain uncertain (Merskey et al., 1996: Yunus, 2007; Keefe & Somers, 2010). Psychological factors influence orofacial pain expression (Aggarwal et al., 2010; Benoliel et al., 2011) and appear to have a prominent effect on temporomandibular joint/muscle disorders (TMJD), a family of conditions involving the temporomandibular joint (TMJ) and masticatory muscles (Huang et al., 2002; Korszun, 2002, Slade et al., 2007; Kanehira et al., 2008; Maixner, 2009).

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Several aspects of TMJD pain suggest a central neural origin: TMJD pain patients often present with no overt signs of injury or inflammation, pain expression is often described as fluctuating and non-progressive (Ohrbach & Dworkin, 1998; Rammelsberg et al., 2003) and behavioral therapies rather than pharmacological treatments often provide the most successful approaches to pain management (List & Axelsson, 2010). Although chronic pain and psychological distress share features consistent with a central origin such as altered stress hormone secretion and autonomic nervous system dysfunction (Yunus, 2007; Maixner, 2009; McEwen & Kalia 2010), the neural substrate that underlies the integration of psychological stress with pain processing remains poorly defined.

Several models have been used to demonstrate that repeated stress modifies nociceptive processing and aversive behavior in animals (see Imbe, 2006); however, few studies have tested the effects of stress against measures of TMJ nociception and jaw-specific function (Huang et al., 2011). The present study used an established model for psychophysical stress, repeated forced swim conditioning (FS) that produces persistent muscle and cutaneous hyperalgesia at spinal levels (Quintero et al., 2000, 2011, Suarez-Roca et al., 2006a,b). The effects of FS were assessed on the encoding properties of spinomedullary dorsal horn neurons excited by TMJ stimulation and on masseter muscle electrical activity in the rat. The spinomedullary region, the junction of the trigeminal subnucleus caudalis (Vc) and the upper cervical cord junction (Vc/C_{1-2}), is the main termination site for sensory fibers supplying the lower jaw (Hathaway et al., 1995; Takeshita et al., 2001; Shigenaga et al., 1986, 1988; Dessem et al., 2007). Particular emphasis was directed at the effects of FS on spinomedullary neurons in superficial versus deep laminae since previous studies have reported distinct differences suggesting that neurons in each region contribute to different aspects of nociceptive processing in trigeminal (Tashiro et al., 2007) and spinal systems (Suzuki et al., 2002; Eckert et al., 2006). Masseter muscle electromyographic (EMG) activity was monitored as an index of peripheral jaw muscle reflex behavior. Altered levels of EMG activity have been linked to orofacial pain conditions in humans (Wang et al., 2004; Bodere et al., 2005; Peck et al. 2008), while jaw muscle EMG has been used extensively as an index of jaw-related behavior in animal models of craniofacial pain (Yu et al. 1995; Cairns et al. 1998).

Materials and methods

The protocols were approved by the Institutional Animal Care and Use Committee of University of Minnesota and conformed to established guidelines set by The National Institutes of Health guide for the care and use of laboratory animals (PHS Law 99-158, revised 2002). All efforts were made to minimize the number of animals used for experiments and their suffering.

General design and conditioning protocol-neurophysiology

Male rats (250-400 g, Sprague-Dawley, Harlan, Indianapolis) were maintained on a 12:12 h light/dark cycle with lights on at 08:00 and given free access to food and water. Rats were exposed to repeated forced swim conditioning (FS) by placement in a plastic cylinder (diameter 30 cm, height 50 cm) containing 20 cm water (24-26°C) for 10 min between 09:00 and 11:00 for three days (Days -3, -2 and -1) as described previously (Quintero et al., 2000, 2003; Duenes et al., 2010). Sham rats served as controls and were placed in an empty swim chamber using the same schedule. During the swim session "immobility" and "struggling" times were recorded (Porsolt et al., 1977). Immobility was defined as time spent in minimal bodily movement to maintain its head above water, whereas struggling was defined as active diving, jumping, or vigorously moving its limbs to break the surface of the water and attempting to escape the container. Rats were dried in a warm environment (30-33°C) after

each swim session and fresh water was used for each rat. On Day 0, rats were anesthetized and prepared for neural or muscle recording.

Measurement of grip force-neurophysiology

Forelimb grip force was measured each day before the swim session and on the day of recording (Kehl et al., 2000). Briefly, the rat was held by the tail and passed gently (~10 cm/s) three times over a wire mesh grid attached to a strain gauge. The maximum forelimb grip force (GF) observed during the 3 trials was determined for each of the 4 days and averaged daily for each rat.

Extracellular recordings

Rats were anesthetized initially with pentobarbital sodium (70 mg/kg, i.p.) and respired artificially with isoflurane (1.5~2.0%) and oxygen-enriched room air after tracheotomy. Catheters were placed in the right femoral artery (blood pressure monitor) and right jugular vein (infusion of gallamine triethiodide). Anesthesia was maintained after surgery with isoflurane (1.2-2.0%) and a paralytic agent, gallamine triethiodide (15-20 mg/kg/h) was infused after completion of all surgical procedures and immediately prior to the recording session. Adequate depth of anesthesia was confirmed by the absence of corneal and hind limb withdrawal reflexes prior to gallamine, fully constricted pupils and constant arterial blood pressure and heart rate. Expiratory end-tidal CO_2 (3.5-4.5%) and mean arterial pressure (MAP, 90-120mmHg) were monitored throughout the experiment and body temperature was maintained 38°C with a heating blanket and thermal probe.

Animals were placed in a stereotaxic frame and portions of the C_1 and C_2 vertebrae were removed to expose the spinomedullary junction Vc/C_{1-2} region and bathed in warm mineral oil. The Vc/C_{1-2} region, located approximately 5 to 7 mm caudal to the obex and ipsilateral to the exposed mandibular condyle, was explored for TMJ-responsive units using the entrance of the C_2 rootlet as a landmark. Extracellular unit activity was recorded with tungsten microelectrodes (5~9 Mohm, Frederick Haer Inc., Bowdoinham, ME) that penetrated the brainstem tangential (~43° off vertical, 60° off midline for laminae I-II, ~36° off vertical, 45° off midline for lamina V) to the surface. Unit activity was amplified, discriminated, stored and analyzed offline on a computer (Apple G4) using a PowerLab interface board and LabChart software (AD Instruments, Colorado Springs, CO). Spike amplitude and shape were monitored continuously and stored on digital tape to confirm unit isolation in off-line analyses. All units included in this study were identified by a vigorous response to gentle mechanical probing of the exposed dorsal surface of the posterior mandibular condyle (see Okamoto et al., 2003, Fig 1). Units were further classified on the basis of cutaneous RF properties as either wide dynamic range (WDR) and activated by brush, press and pinch stimulation of facial skin or as nociceptive specific (NS) and activated only by press or pinch of the skin.

Experimental design-neural recording

A single TMJ-responsive unit was recorded in each animal preparation. Units were recorded in superficial laminae (I-II, see Fig 1A, left) or deeper lamina (V, Fig 1A, right) and within 1.5 mm rostral to the level of entrance of the C_2 rootlets. After confirming the response to posterior condyle stimulation, the face and neck were explored for cutaneous input. The cutaneous receptive field (RF) was then tested for responses to "brushing" (camel hair), "press" (arterial clip, ~20 mm^2) and "pinch" (stiff arterial clip, ~15 mm^2) stimuli applied for 10s. The high threshold RF area was mapped using a small blunt forceps (~3 mm^2) onto a standardized series of rat face drawings. After cutaneous RF testing, a guide cannula (26 gauge) was positioned in the TMJ joint space (~3 mm deep) by a dorsal approach directed at the posterior aspect of the mandibular condyle to allow delivery of chemical stimuli. TMJ

units were activated by injection of adenosine triphosphate (ATP) into the joint space (Tashiro et al., 2007, 2008). Test solutions were delivered from a microsyringe attached by polyethylene tubing to an inner cannula (33 gauge) that protruded ~0.5 mm from the end of the guide cannula. Test solutions consisted of phosphate buffered saline (PBS) and ATP and were delivered slowly over 30 s (total volume = 20 μl) with an inter-injection interval of 20 min to reduce the likelihood of tachyphylaxis. Previously we demonstrated that five consecutive injections of 1 mM ATP at 20 min intervals produced consistent responses (Tashiro et al., 2008). The protocol for chemical injections into the TMJ was: PBS (pH 7.4) followed by four successive doses of ATP (0.001, 0.01, 0.1, and 1.0 mM, pH 7.4, disodium salt, Sigma, St Louis, MO).

Data analysis-neural recording

Neural data were acquired and displayed as peristimulus time histograms (PSTHs) of spikes per 1 s bins, exported to a spreadsheet and analyzed off-line. Spontaneous activity (spikes/s) was calculated as the average spike count over a 1 min epoch immediately preceding each stimulus. The evoked responses were assessed by calculating the response magnitude (Rmag), determined by subtracting the mean plus 2 times the standard deviation (SD) of background activity from the total spike count for each bin. The total Rmag for each stimulus was defined as the cumulative sum of spikes over contiguous bins in which the spike count minus the background was a positive value. The total Rmag is equivalent to the "area under the curve" for each stimulus period. The response duration was defined as the time interval after stimulus onset until three consecutive bins with a positive spike count occurred above background (initial latency) and until the value of three consecutive bins no longer exceeded the mean + 2 SD above background activity as described previously (Hirata et al. 1999). Units that failed to show three consecutive bins with positive Rmag values within 100 s after stimulus onset were considered unresponsive to that condition. Units were defined as ATP-responsive if the total Rmag exceeded the response to PBS by >50%, independent of ATP dose. The threshold dose of ATP was defined as the lowest concentration that produced a total Rmag exceeding that to PBS by >50%. The total Rmag to mechanical stimulation of the skin overlying the TMJ (e.g., brush, press, pinch) was determined over a 10 s stimulus period. Total Rmag, response duration and latency to chemical and mechanical stimuli were assessed statistically by analysis of variance (ANOVA) corrected for repeated measures and individual comparisons were made by Newman-Keuls after ANOVA. Fisher's Exact Probability test determined if the number of units responding to the lowest concentration of ATP (> 50% versus Rmag response to intra-TMJ injection of PBS) was different for FS and Sham groups. The cutaneous high threshold RF areas for units were digitized and quantified by a planimetric method using NIH ImageJ software and compared by ANOVA. Values in the text were expressed as mean ± SEM and $p < 0.05$ was regarded as significant.

Electromyographic (EMG) recording

Animals were anesthetized with urethane (1.2 g/kg ip) and a catheter was positioned in the right femoral artery for monitoring MAP. After tracheotomy, animals were respired artificially with oxygen-enriched room air. End-tidal CO_2, MAP and body temperature were monitored throughout the experiment and kept within normal range. Animals were placed in a stereotaxic frame and the TMJ region was exposed for cannula implantation and ATP injections as described above. A small skin incision was made to expose the surface of the left masseter muscle and paired wire electrodes (0.12 mm diameter, 5 mm interpolar distance) were implanted ~1 mm into the central portion of the muscle. EMG activity was sampled at 1000 Hz, amplified (x10k), filtered (bandwidth 300-3000 Hz), displayed and stored online for analyses.

EMG activity and the role of the Vc/C_{1-2} region

EMG activity was assessed in two separate series of experiments. In the first series EMG activity was measured in FS and sham groups in response to PBS followed by cumulative doses of ATP (0.01, 0.1, 1 mM, 20 µl, pH = 7.4) delivered to the left TMJ region at 20 min intervals. In a second group of rats, lidocaine (2%, 100 nl, pH 7) was injected into **the dorsal horn at the Vc/C_{1-2} region** via a glass micropipette to assess the influence of the Vc/C_{1-2} region on TMJ-evoked EMG activity. In this series EMG activity was sampled in response to an intra-TMJ injection of ATP (1 mM) followed 10 min later by lidocaine injection and then after 10 min a second ATP (1 mM) stimulus was delivered. Additional naive male rats served as controls to assess if repeated ATP injection to the TMJ region evoked consistent EMG responses in 20 min intervals (n = 11).

Data analysis

EMG activity was sampled continuously for 4 min, beginning 2 min before each TMJ stimulus and for 2 min after stimulation. EMG activity was rectified and stored as 1 s bins for off-line analyses. Baseline activity was quantified as the area under the curve (AUC) for the 2 min epoch (µV per 2 min) sampled immediately prior to stimulation. TMJ-evoked EMG activity was calculated as AUC post-ATP injection minus baseline AUC. The response latency (onset) was defined as the first time point when AUC for 1s exceeded the average baseline activity/s. Results were assessed statistically by ANOVA, corrected for repeated measures and individual comparisons were made by Newman-Keuls after ANOVA. The threshold dose of ATP was defined as the lowest concentration that produced an AUC exceeding that to PBS by > 50%. Fisher's Exact Probability test determined if the number of rats responding to the lowest concentration of ATP (> 50% versus AUC to intra-TMJ injection of PBS) was different for FST and Sham groups.

Histology

At the end of each neural recording experiment, rats were given a bolus dose of pentobarbital (100 mg/kg, iv) and perfused through the heart with 10% formalin. The recording site was marked electrolytically (5 µA, 20 s). Recording and injections sites were confirmed from transverse sections (40 µm) and drawn onto a standard series of rat brainstem outlines (Takeshita et al., 2001).

Results

General and behavioral effects of FS conditioning

A total 75 rats were used for neural (n = 37) and EMG (n = 38) recording experiments and subjected to either FS or sham conditioning. Body weights were similar for FS and sham rats before conditioning (FS = 331.2 ± 11.5 g; sham = 323.9 ± 8.7 g). Three days of FS caused a significant reduction in body weight (-8.4 ± 2.3 %, $F_{3, 207}$ = 11.7, P < 0.01), while sham conditioning had no effect (0.1 ± 0.6 %). Immobility time (IT), as a percentage of the 10 min swim session, increased significantly after 2 days of FS compared to the initial session (94.6 ± 0.8% versus 81.8 ± 1.0%, $F_{2, 72}$ = 127, P < 0.001). Grip force (GF) was similar for FS and sham rats prior to conditioning (FS = 1084 ± 22 g; sham = 1074 ± 20 g). After 3 days of FS GF was reduced significantly compared to sham rats (-16.5 ± 3.1 % versus 2.6 ± 1.8%, $F_{3, 207}$ = 15.6, P < 0.01). These results indicated that the FS protocol used here was sufficient to cause significant changes in general behavior (decreased body weight), depression-like behavior (increased IT duration) and forelimb muscle weakness and/or hyperalgesia (decreased GF).

TMJ-responsive neurons: general properties

TMJ-responsive neurons were recorded in superficial laminae (147 ± 17 µm, n = 25) and deeper laminae (1148 ± 55 µm, n = 12) after penetration of the dorsal surface and within 1.5 mm rostral to the level of entrance of the C_2 rootlets. However, since an acute angle of penetration was used, the exact vertical distance from the dorsal brainstem surface could not be determined. All units were spontaneously active. Units in superficial laminae from FS and sham rats displayed similar levels of background activity (FS = 0.9 ± 0.3 spikes/s, n = 12; sham = 1.2 ± 0.3 spikes/s, n = 13, $F_{1,24} = 0.05$, P > 0.1). By contrast, units in deep laminae displayed significantly higher background firing rate after FS than units from sham rats (FS = 9.6 ± 3.3, n = 6; sham = 0.7 ± 0.5 spikes/s, n = 6, $F_{1,11} = 12.7$, P < 0.01). Second, all TMJ-responsive units received convergent input from facial skin overlying the TMJ. TMJ units in superficial laminae were all classified as NS, while units in deep laminae lamina were classified as WDR. The cutaneous RF was positioned over the TMJ and extended anterior and ventral to the joint within the territories for the maxillary and mandibular branches of the trigeminal nerve (see Okamoto et al. 2003, Fig 4C). The high threshold RF areas of NS units in superficial laminae were similar for FS and sham rats; however, WDR units in deep laminae displayed enlarged RF areas ($F_{3,31} = 3.65$, P < 0.05) after FS compared to units in sham rats (Fig 1B). As seen in Fig 1, among NS units in superficial laminae FS also increased the Rmag to pinch stimulation of the cutaneous RF compared to the sham group ($F_{1,18} = 6.87$, P < 0.025, Fig 1C), while among WDR units in deep laminae, FS increased the Rmag to both press and pinch compared to the sham group ($F_{2,18} = 4.35$, P < 0.05, Fig. 1D). These results indicated that FS enhanced both the spontaneous activity and the response to convergent mechanical input from facial skin and that this enhancement occurred preferentially in TMJ units from deep laminae.

TMJ-responsive neurons and ATP-evoked responses

The examples in Fig 2 illustrate that intra-TMJ injections caused a significant ATP dose-related increase in total Rmag after FS and sham conditioning for units in both superficial (Fig 2A and 2B) and deep laminae (Fig 2C and 2D). In superficial laminae, FS increased the total Rmag at each ATP dose tested compared to units from sham rats (Fig 3A, left panel). At the lowest ATP dose tested (0.001 mM), 10 of 12 units in FS rats displayed an increase in Rmag of > 50% above that after PBS injection, while in sham rats only 4 of 13 units were activated. This difference was significant (Fisher's Exact Probability = 0.0011) and suggested that FS reduced the threshold dose of ATP necessary to activate TMJ-responsive units in superficial laminae. Among units in deep laminae, FS markedly increased the total Rmag to each dose of ATP tested compared to responses in sham rats (Fig 3A, right panel). Although the lowest dose of ATP was sufficient to activate 6 of 6 units in deep laminae from FS rats, 4 of 6 units from sham rats also were activated (Fisher's Exact Probability = 0.22) and suggested that FS did not affect the threshold dose of ATP necessary to activate TMJ units in deep laminae. As seen in Fig 3A, the ATP-evoked total Rmag for units in lamina V were significantly greater ($F_{3,36} = 64.4$, P < 0.001) than that seen for units in superficial laminae. In FS rats, deep units showed a significantly greater total Rmag at all ATP concentrations compared to superficial units ($F_{1,17} = 74.8$, P < 0.001, Fig 3A), while in sham rats, deep units showed a greater response at the dose of 0.1 and 1.0 mM ATP ($F_{1,18} = 4.51$, P < 0.01, Fig 3A).

Response duration increased significantly after intra-TMJ injections of ATP for all groups in a dose-dependent manner (Fig 3B). In superficial laminae, FS caused a small, but significantly greater ($F_{1,24} = 9.0$, P < 0.025) increase in response duration versus the sham group, whereas in deep laminae FS markedly increased response duration at each dose of ATP compared to sham rats ($F_{1,11} = 36.9$, P < 0.001). The effect of FS on ATP-evoked response duration was significantly greater for units in deep laminae ($F_{1,17} = 12.9$, P <

0.005), whereas no laminar differences were seen for units from sham rats ($F_{1, 18} = 0.5$, $P > 0.1$). Response latency also was reduced following increasing doses of intra-TMJ injections of ATP in FS and sham groups (Fig 3C). FS significantly reduced the ATP-evoked response latency for units in superficial and deep laminae such that the lowest dose of ATP (0.001 mM) now resulted in the shortest latency ($F_{4, 146} = 18.5$, $P < 0.01$). The effect of FS on response latency was similar for units in superficial and deep laminae.

Electromyographic (EMG) activity ATP dose-effect

Baseline EMG activity was sampled for 2 min prior to intra-TMJ injections of test solutions and was similar for FS and sham rats (AUC: FS = 1.38 ± 0.23 μV/2 min, Sham = 1.24 ± 0.21 μV/2 min, n = 7 rats/group, $F_{1, 49} = 0.8$, $P > 0.1$). Intra-TMJ injections of ATP evoked an increase in EMG activity (AUC minus baseline AUC) in a dose-dependent manner for both groups (Fig 4A, B, $F_{3, 42} = 5.9$, $p < 0.01$), and group comparisons indicated a significantly greater response after FS ($F_{1, 13} = 5.1$ $P < 0.05$, Fig. 4B). By contrast, the lowest dose of ATP (0.01 mM) tested evoked a significant increase in the EMG response in a similar the number of rats after FS (5 of 7 rats) and sham conditioning (4 of 7 rats) and suggested little or no effect on threshold (Fisher's exact probability, $P = 0.37$). Increasing doses of ATP also evoked significant and similar decreases in EMG response latency for FS and sham groups ($F_{3, 49} = 13.3$, $P < 0.01$, Fig. 4B). The minimum latency following intra-TMJ injection of 1 mM ATP averaged 13.3 ± 2.1 s and 17.4 ± 6.5 s for FS and sham groups, respectively. These results indicated that ATP-evoked EMG responses, but not threshold doses of ATP, were significantly affected by FS, consistent with a central neural mechanism.

Effect of lidocaine on EMG responses

To determine if the spinomedullary junction plays a significant role in mediating the EMG response to TMJ stimulation, lidocaine (2%, 100 nl) was microinjected into **the dorsal horn** at the Vc/C$_{1-2}$ region in FS and sham rats. Prior to lidocaine, the ATP-evoked EMG response was significantly greater ($F_{1, 53} = 35.1$, $P < 0.001$) in FS (2.79 ± 0.2 μV/2 min, n = 11) than sham rats (1.32 ± 0.22 μV/2 min, n = 13). As seen in Fig 5 lidocaine blockade of the spinomedullary junction caused a marked reduction in the ATP-evoked EMG response. By 10 min after lidocaine, ATP-evoked responses were reduced significantly ($F_{2, 47} = 56.1$, $P < 0.001$) and similarly in FS and sham rats; however, by 30 min, ATP-evoked EMG responses in FS rats returned to pre-lidocaine levels while responses in sham rats remained reduced (Fig 5, $F_{3, 45} = 20.9$, $P < 0.01$, FS versus sham). In control experiments, the average AUC evoked by the initial ATP injection in naïve animals (n = 11, AUC = 1.81) was similar to that in sham animals (n = 13, AUC = 1.32, $F_{1, 23} = 1.1$, $P > 0.1$) suggesting that sham conditioning did not alter TMJ-evoked EMG responses. The average AUC for the initial ATP injection and spontaneous activity prior to ATP injections were not significantly different from each of 3 subsequent injections ($F_{3, 32} = 1.87$, $P > 0.1$) in naïve rats. These results suggested TMJ-evoked EMG responses depended on a critical relay through the spinomedullary junction and that FS conditioning reduced but did not prevent dependency on this relay.

Discussion

The present study revealed that repeated psychophysical stress alone was sufficient to alter the encoding properties of TMJ-responsive neurons at spinomedullary (Vc/C$_{1-2}$) junction. These results revealed three novel findings. Repeated FS enhanced the responses to sensory input of neurons in deep laminae significantly more than neurons in superficial laminae. FS-induced enhancement of neural responses was widespread and affected inputs from the TMJ

region and facial skin. FS also enhanced TMJ-evoked masseter muscle EMG activity, an effect that depended on a relay through the spinomedullary junction.

TMJD is included among a group of comorbid pain conditions such as fibromyalgia, inflammatory bowel syndrome and chronic headache that share common features (Diatchenko et al. 2006; Yunus 2007). Psychological stress expressed either as anxiety and/ or depression is a common feature of these idiopathic pain conditions and is recognized as a risk factor for TMJD (Huang et al., 2002; Gameiro et al. 2006; Slade et al., 2007; Kanehira et al., 2008) and orofacial pain (Korszun, 2002; Sipila et al., 2006; Benoliel et al., 2011). Although neuroimaging studies in TMJD patients have suggested that brain areas that process cognitive and emotional aspects of pain display structural abnormalities (Younger et al., 2010; Moayedi et al., 2011), the neural basis for the integrative relationship between psychological stress and pain expression remains poorly defined.

It has long been known that acute exposure to a noxious stimulus or psychophysical stress can lead to analgesia, whereas repeated presentation of these same stressors can induce behavioral hyperalgesia in animals (see Imbe et al., 2006). Several studies have suggested that repeated stress also increased orofacial pain-like behavior. For example, three days of non-noxious tooth movement increased aversive behavior (Yozgatian et al., 2008), and several days' exposure to psychological stress decreased the threshold for head withdrawal to jaw muscle mechanical probing (Huang et al., 2011). Repeated restraint stress for several weeks increased jaw-related aversive behavior to intra-TMJ injection of formalin in male rats (Gameiro et al., 2005). However, little is known about the sites and/or mechanisms within the brain that mediate stress-induced facilitation of orofacial pain-like behavior. To address this issue in the context of TMJ nociceptive processing, we adapted the well established model for spinal pain of repeated FS developed by Quintero et al. (2000). Several lines of evidence suggest that this repeated FS protocol recruits central neural mechanisms to produce long lasting cutaneous and muscle hyperalgesia in rodents (Suarez-Roca et al., 2006a). First, repeated FS increased c-fos expression in spinal dorsal horn after formalin injection into the hind paw (Quintero et al., 2003) and increased c-fos and pCREB expression at the spinomedullary junction after mustard oil injection into the TMJ region (Duenes et al., 2010). Second, repeated FS enhanced nocifensive behavior to formalin injection into hindpaw and depended on descending pathways from the rostroventromendial medulla (RVM, Imbe et al. 2010) and monoaminergic transmitter availability (Suraez-Roca et al., 2006a). Third, neurochemical features consistent with mechanisms of central sensitization (Suzuki et al., 2004; Kuner, 2010) such as increased post-synaptic NMDA receptor activity (Quintero et al., 2011), reduced inward-rectifying potassium channel activity in dorsal horn (Ippolito et al., 2005) were involved in FS-induced enhancement of nocifensive behavior. Fourth, an initial reduced release of GABA followed by a delayed increased release of glutamate in spinal dorsal horn was consistent with initiation and maintenance of FS-induced enhanced nocifensive behavior (Quintero et al. 2011). These studies indicate that the repeated FS protocol used in the present study has widespread effects on somatosensory pathways that likely modify trigeminal as well as spinal nociceptive processing.

Repeated FS enhanced the TMJ-evoked responses of neurons in superficial and deep laminae over a wide physiological range of ATP concentration and was markedly greater for neurons in deep versus superficial laminae. FS also increased the responses to mechanical stimulation of facial skin and enlarged the convergent cutaneous RF area of individual TMJ neurons. Although it is well established that neurons in superficial and deep dorsal horn display significant differences in afferent inputs, efferent projection targets and neurochemical markers (see Hunt & Mantyh, 2001; Basbaum et al., 2009), their relative roles in nociceptive processing remain controversial (Craig 2003; Price et al. 2003). Both the

superficial and deep laminae at the Vc/C_{1-2} junction region receive significant direct input from primary afferents that supply the TMJ and masticatory muscles (Jaquin 1983; Shigenaga et al., 1986; Takemura et al. 1987; Shigenaga et al. 1988: Dessem et al. 2007). Neural recording studies have confirmed that TMJ units in superficial and deep laminae at the Vc/C_{1-2} junction encoded the intensity of intra-TMJ stimuli (Takeshita et al., 2001; Okamoto et al., 2003: Tashiro et al., 2007).

The basis for laminae-preferred effects on dorsal horn neurons after FS is not known; however, stimulus modality and specific neurochemical pathways may be involved. In spinal cord mechanical and thermal-evoked central sensitization was greater for neurons in deep than superficial laminae and windup-like responses of deep dorsal horn neurons were more dependent on NMDA mechanisms (Seagrove et al., 2004). Mutant mice lacking the preprotachykinin gene, and thus deficient in substance P, displayed differential deficits in thermal and mechanical sensitization among neurons in superficial and deep dorsal horn (Mazario & Basbaum, 2007). Similarly, in TRPV1 mutant mice spinal dorsal horn neurons in superficial and deep laminae displayed differential responses to noxious heat and thermal sensitization after mustard oil (Eckert et al., 2006). However, it is not known if selective antagonists for TRPV1 or NK1 differentially alter the properties of TMJ-responsive neurons in superficial and deep laminae at the Vc/C_{1-2} region.

The present data cannot exclude that peripheral mechanisms also contributed to FS-induced enhancement of neural activity. Reduced response latency coupled with increased background neural activity often is ascribed to peripheral mechanisms of nociception (Treede et al., 1992). Indeed, response latencies of TMJ neurons in superficial and deep laminae to ATP were reduced after FS and a greater percentage of neurons in both laminae were activated by the lowest dose of ATP. We also cannot exclude that effects on non-neural peripheral tissues could have contributed to these results since repeated psychological stress caused structural and metabolic changes in masseter muscle of rats (Chen et al., 2011), while in humans, acute mental stress significantly affected hemodynamic variables and EMG activity in jaw muscles (Hidaka et al. 2004). An elevation in peripheral nerve activity has been proposed as a critical factor in chronic pain and after exposure to psychophysical stress (Reichling and Levine, 2009).

The nature of pain modulation by psychological stressors in idiopathic conditions such as TMJD suggests a role for endogenous pain control systems (Keay & Bandler, 2001; Bragdon et al., 2002; Gameiro et al., 2006; King et al., 2009) and several features of the repeated FS model in animals support this notion. FS-induced behavioral hyperalgesia was reduced by prior blockade of the RVM (Imbe et al., 2010) or by administration of monoamine reuptake inhibitors (Suarez-Roca et al., 2006b), while brain levels of 5HT were significantly altered after repeated FS (Kirby and Lucki, 1998; Baganz et al., 2010). The periaqueductal grey (PAG) – RVM system is critical for coordinating cognitive, emotional and sensory aspects of nociception (Bandler & Shipley, 1994; Keay et al., 2001) and may contribute to stress-induced hyperalgesia (Martenseon et al., 2009). Stimulation of the PAG had differential effects on A- versus C-fiber induced activation of nociceptive neurons in superficial and deep laminae in spinal dorsal horn (Koutsikou et al., 2007). Reversible spinalization increased both resting and articular-evoked neural activity with significantly greater effects on neurons located in deep than superficial dorsal horn (Cervero et al., 1991). Considerable evidence indicates that nociceptive neurons in superficial and deep laminae contribute differentially to nociception (Suzuki et al., 2002, 2004).

The present results demonstrated that intra-TMJ injections of ATP increased masseter muscle EMG activity in a dose-related manner that depended on a relay through the spinomedullary junction. The EMG response to intra-TMJ injections of algesic agents has

been used extensively as a model for TMJ nociception (Broton and Sessle 1988; Yu et al., 1995; Cairns et al., 1998, 2001). The EMG response to intra-TMJ injections of ATP was enhanced after FS compared to sham rats at all doses of ATP tested The EMG response latency to intra-TMJ injections of ATP was significantly longer than the latency for TMJ unit activity at each ATP dose tested. This suggested that the relationship between EMG and TMJ-responsive neurons likely is complex and involves neurons at the Vc/C_{1-2} region. The finding that acute blockade of the spinomedullary region greatly reduced the evoked EMG response was consistent with previous studies indicating that chronic neurochemical lesion of Vc prevented the increase in masticatory muscle EMG activity evoked by mustard oil injection into the TMJ (Tsai et al., 1999). It remains to be determined if FS-induced modulation of EMG activity and TMJ-responsive spinomedullary neurons share common mechanisms and/or central pathways. This possibility was supported by evidence that stimulation of the PAG reduced the C-fiber, but not A-fiber evoked EMG activity (McMullan and Lumb, 2006a) similar to the preferential reduction of C-fiber input to deep dorsal horn neurons (McMullan and Lumb 2006b).

In conclusion, FS enhanced TMJ-evoked neural activity at the Vc/C_{1-2} junction and masseter muscle EMG activity. The facilitatory effects of FS on Vc/C_{1-2} units were notable in that neurons in deep laminae displayed markedly greater changes in response properties to TMJ and facial skin stimulation than neurons in superficial laminae. The Vc/C_{1-2} junction region also likely was critical for mediating the TMJ-evoked increases in EMG activity since lidocaine blockade of the spinomedullary region reversibly reduced by >60% the evoked EMG response in FS and sham groups. These findings indicated that neural signals evoked by psychological stress and TMJ stimulation were integrated by central mechanisms that included the Vc/C_{1-2} region and may underlie the induction and maintenance of TMJD pain in humans. This would suggest the need to treat the sensory and emotional components of TMJD in order to provide efficient pain relief for this complex condition. In addition, given the high prevalence of persistent TMJD in women compared to men (Maixner 2009; Bereiter and Okamoto 2011) and the marked effects of estrogen status on TMJ-responsive Vc/C_{1-2} neurons (Tashiro et el. 2007), it will be important to determine the relationship between sex hormones and FS in future experiments. These results supported the hypothesis that psychophysical stress alone was sufficient to significantly modify the encoding properties of TMJ-responsive neurons and that the spinomedullary junction was critical for the integration of reflexes relevant for TMJ nociception.

Acknowledgments

Abbreviations

ATP	adenosine triphosphate
AUC	area under curve
EMG	electromyography
FS	forced swim conditioning
GF	grip force
IT	immobility time
NS	nociceptive-specific

RF	receptive field
Rmag	response magnitude
RVM	rostroventromendial medulla
TMJ	temporomandibular joint
TMJD	temporomandibular joint disorder
Vc	trigeminal subnucleus caudalis
Vc/C$_{1-2}$	trigeminal subnucleus caudalis and upper cervical spinal cord junction
WDR	wide dynamic range

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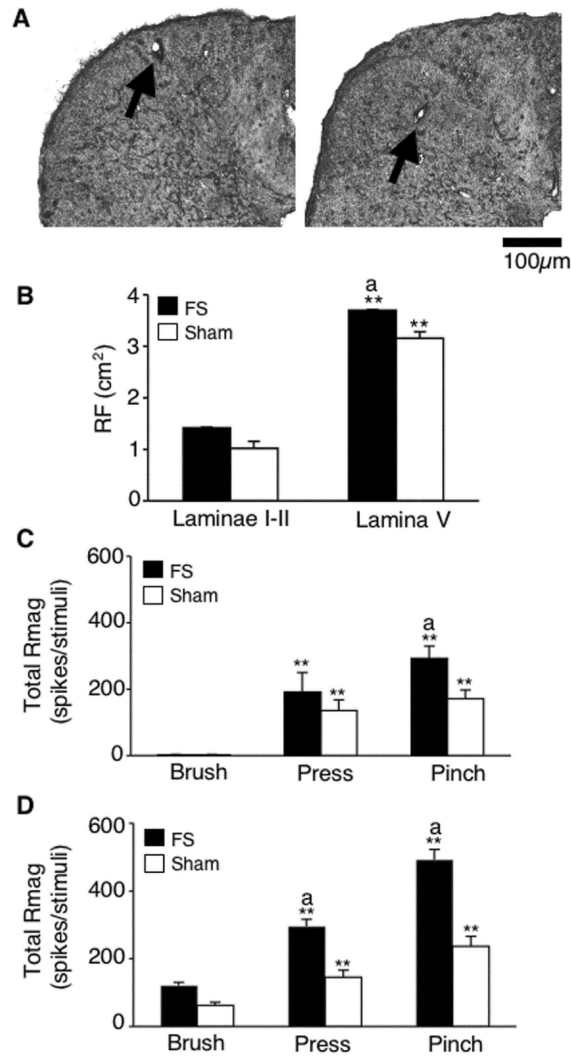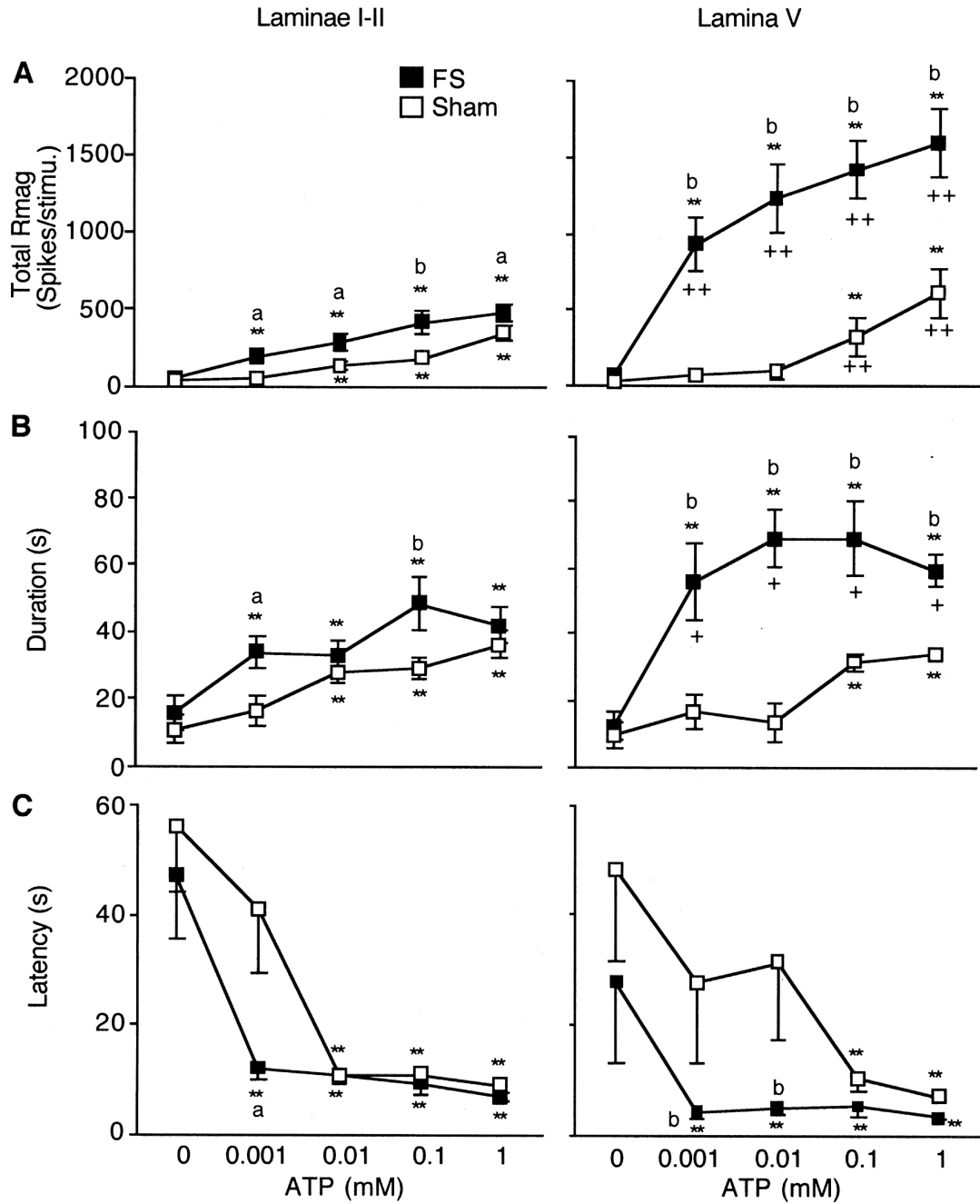
Figure 1.
A. Examples of recording locations of TMJ units in superficial (left) and deep (right) laminae for FS rats. **B**. Convergent cutaneous RF sizes of TMJ units in superficial and deep laminae. * * p < 0.01 vs. superficial laminae units. a = P <0.05 vs. sham group. **C**. Response magnitude of TMJ units in superficial laminae to mechanical stimulation of facial skin for 10sec. **D**. Response magnitude of TMJ units in deep laminae to mechanical stimulation of facial skin for 10sec. ** P < 0.01 vs. response to innocuous brush. a = P < 0.05 vs. sham rats.
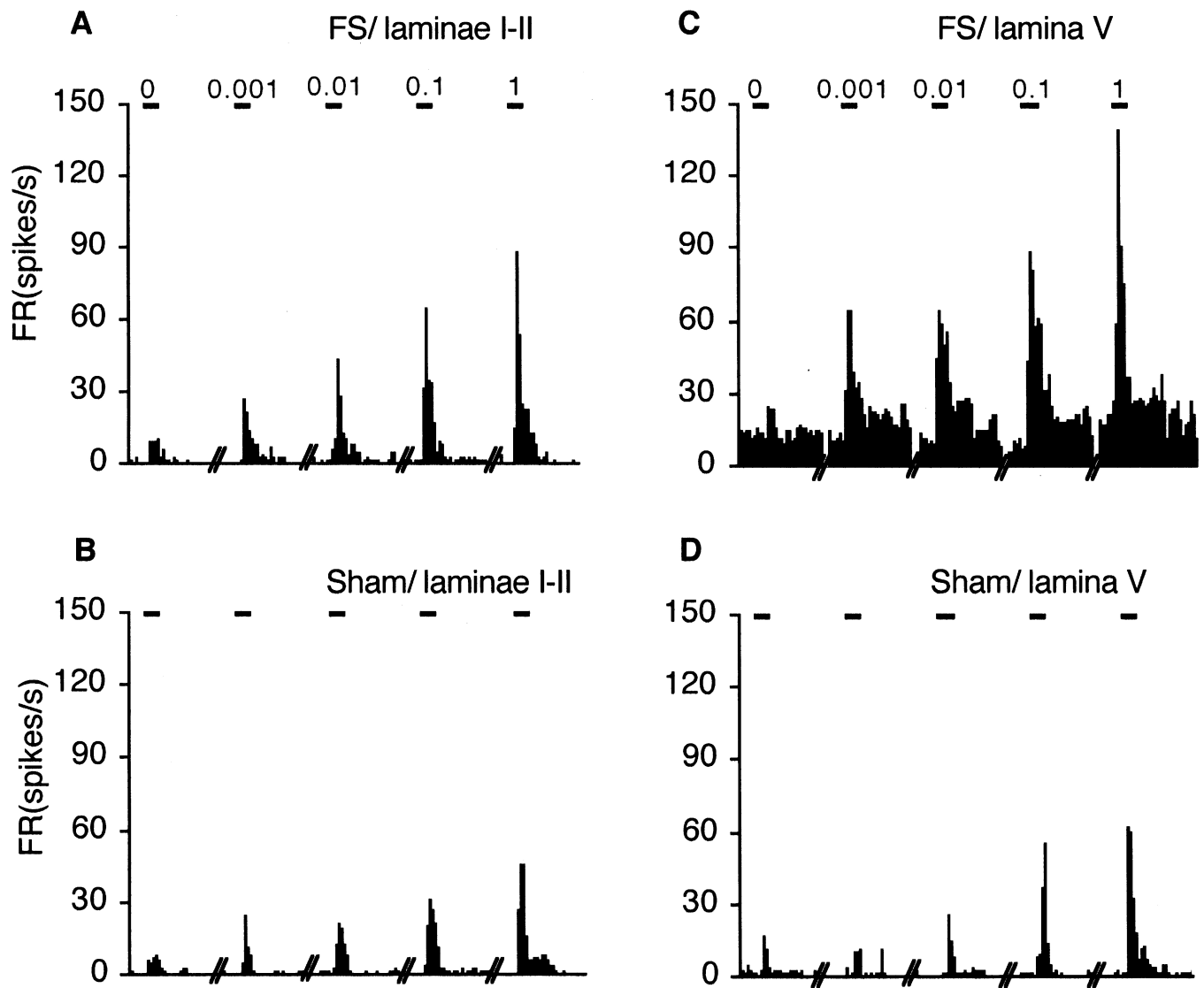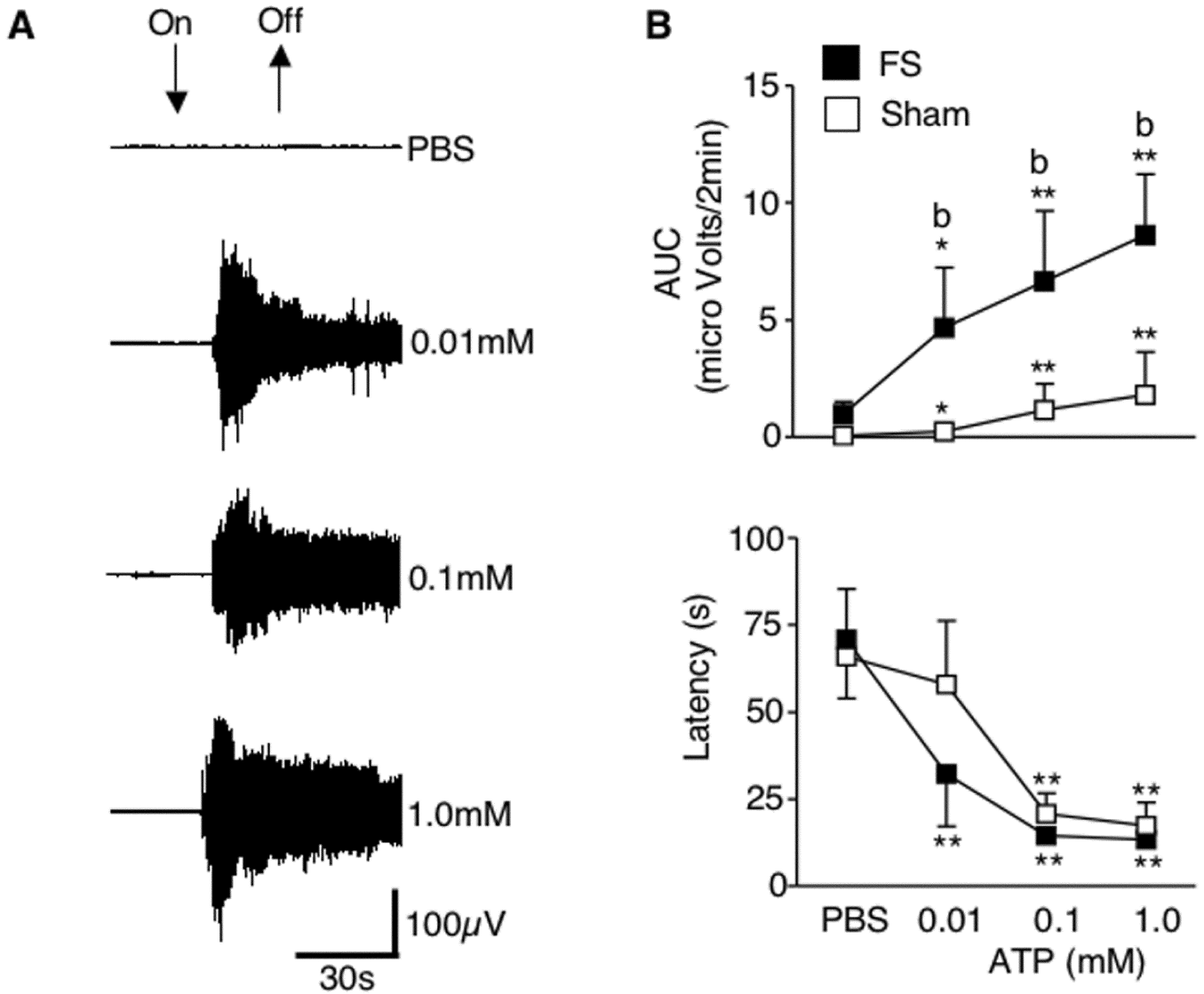
Figure 2.
Peristimulus time histograms of the response to ATP injected into the TMJ on superficial
(A, B) and deep laminae (C, D) from FS and sham rats. Calibration bar above each
histogram = 30 s and indicate stimulus periods for PBS, 0.001, 0.01, 0.1 and 1 mM ATP
injections into the TMJ.

Figure 3.
Effect of ATP on total Rmag (A), response duration (B) and latency (C) for TMJ units
recorded in superficial (left) and deep (right) laminae defined as ATP responsiveness from
FS and sham rats. ** $P < 0.01$ vs. response to PBS (0 mM ATP). a = $P < 0.05$, b = $P < 0.01$
vs. sham group. ++ = $P < 0.01$ vs. superficial laminae units.

Figure 4.
Dose-response relationship for EMG activity in the masseter muscle evoked by ATP injection (0.01, 0.1 and 1 mM) into the TMJ. **A**. An example of EMG activity of the left masseter muscle evoked by the injection of ATP into the left TMJ region from FS rats. **B**. TMJ-evoked masseter muscle activity was significantly increased (upper panel) and response latency was decreased (lower panel) in a dose dependent manner in FS and sham rats. Compared with sham group, FS rats display greater EMG activity indicated by AUC. * $P < 0.05$, ** $P < 0.01$ vs. response to PBS. b = $P < 0.01$ vs. sham group.
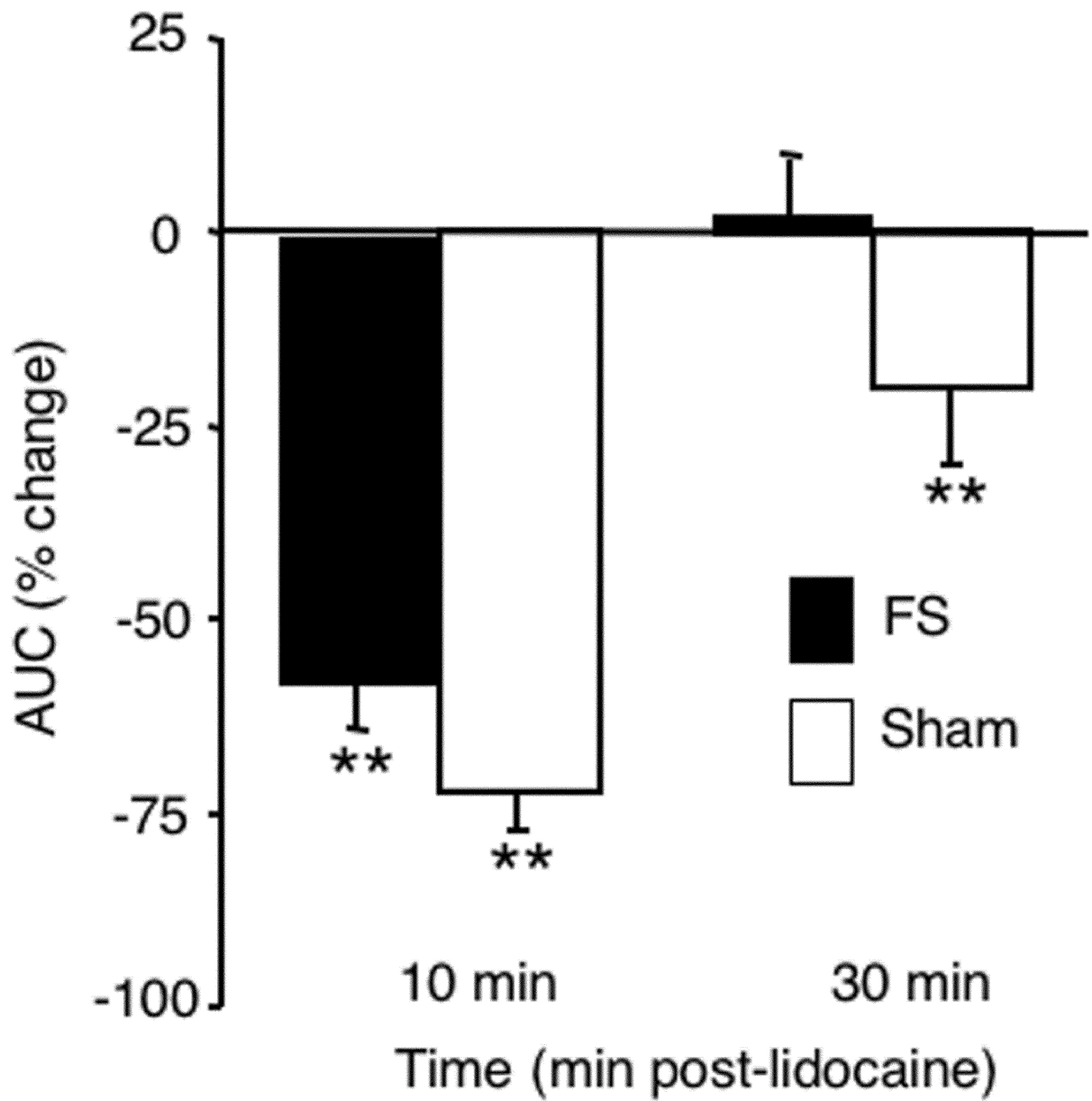
Figure 5.
Blockade of neural activity at the Vc/C_{1-2} junction reduces TMJ-evoked EMG activity in the masseter muscle. Microinjection of lidocaine (2% 100nl) into the Vc/C_{1-2} region decreases TMJ-evoked EMG activity in FS and sham rats. ** $P < 0.01$ vs. pre-lidocaine.