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Inhibition of temporomandibular joint input to medullary dorsal horn neurons by 5HT3 receptor antagonist in female rats

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Abstract

Repeated forced swim (FS) conditioning enhances nociceptive responses to temporomandibular joint (TMJ) stimulation in male and female rats. The basis for FS-induced TMJ hyperalgesia remains unclear. To test the hypothesis that serotonin 3 receptor (5HT3R) mechanisms contribute to enhanced TMJ nociception after FS, ovariectomized female rats were treated with estradiol and subjected to FS for three days. On day 4, rats were anesthetized with isoflurane and TMJ-responsive neurons were recorded from superficial and deep laminae at the trigeminal subnucleus caudalis/upper cervical ($Vc/C_{1–2}$) region and electromyographic (EMG) activity was recorded from the masseter muscle. Only $Vc/C_{1–2}$ neurons activated by intra-TMJ injections of ATP were included for further analysis. Although neurons in both superficial and deep laminae were activated by ATP, only neurons in deep laminae displayed enhanced responses after FS. Local application of the 5HT3R antagonist, ondansetron (OND), at the $Vc/C_{1–2}$ region reduced the ATP-evoked responses of neurons in superficial and deep laminae and reduced the EMG response in both sham and FS rats. OND also decreased the spontaneous firing rate of neurons in deep laminae and reduced the high threshold convergent cutaneous receptive field area of neurons in superficial and deep laminae in both sham and FS rats. These results revealed that central application of a 5HT3R antagonist, had widespread effects on the properties of TMJ-responsive neurons at the $Vc/C_{1–2}$ region and on jaw muscle reflexes under sham and FS conditions. It is concluded that 5HT3R does not play a unique role in mediating stress-induced hyperalgesia related to TMJ nociception.

Keywords

5-HT3 receptor; nociception; temporomandibular joint; trigeminal subnucleus caudalis

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Introduction

Temporomandibular joint disorders (TMD) represent a family of conditions associated with pain in the temporomandibular joint (TMJ) and masticatory muscles and shares several features with other idiopathic pain conditions such as fibromyalgia and irritable bowel syndrome (Yunus, 2007, Maixner, 2009, Bereiter and Okamoto, 2011). Notably, pain expression often correlates poorly with signs of peripheral tissue damage (Ohrbach and Dworkin, 1998) and TMD patients often display evidence of altered endogenous pain controls (Bragdon et al., 2002, King et al., 2009, Oono et al., 2014). These features suggest a central nervous system (CNS) dysfunction in persistent TMD pain (Sarlani and Greenspan, 2005, Fernandez-de-las-Penas et al., 2009, Pfau et al., 2009, Slade et al., 2014). However, the mechanisms of altered CNS processing of TMJ-related sensory signals remain uncertain. The central projections of sensory nerves that supply the TMJ region terminate mainly in the trigeminal subnucleus caudalis (Vc)/upper cervical cord ($Vc/C_{1–2}$) region (Jacquin et al., 1983, Takemura et al., 1987, Shigenaga et al., 1988). Previously, we reported that estrogen status (Okamoto et al., 2003, Tashiro et al., 2007) and psychophysical stress influenced the encoding properties of TMJ-responsive neurons at $Vc/C_{1–2}$ region in a lamina-specific manner (Okamoto et al., 2012, Okamoto et al., 2013). Female gender and psychological distress are risk factors for persistent TMJ pain (LeResche, 1997, Bereiter and Okamoto, 2011, Maixner et al., 2011, Slade et al., 2014).

The rostral ventromedial medulla (RVM) is a key brainstem region for control of nociceptive input to dorsal horn neurons (Millan, 2002, Porreca et al., 2002, Vanegas and Schaible, 2004, Heinricher et al., 2009) and is the major source of serotonergic (5HT) input to the spinal cord (Bowker et al., 1982; Wei et al. 2010). The family of 5HT receptors consists of seven groups and 15 receptor subtypes (Viguier et al., 2013). The 5HT3 receptor (5HT3R) is the only ligand-gated 5HT receptor subtype and has been linked to pain facilitation in animal models for spinal (Suzuki et al., 2002, Zeitz et al., 2002, Rygh et al., 2006, Svensson et al., 2006) and craniofacial pain (Okamoto et al., 2004, Okamoto et al., 2005, Okubo et al., 2013, Kim et al., 2014). However, the role of 5HT3R in clinical pain management remains uncertain (Faerber et al. 2007; Machu 2011). The $Vc/C_{1–2}$ region receives a dense 5HT nerve fiber input (Pearson and Jennes, 1988, Li et al., 1997) and binds ligands selective for the 5HT3R (Gehlert et al., 1991, Laporte et al., 1992). The 5HT3R is associated with behavioral distress (see Rajkumar and Mahesh, 2010) and musculoskeletal pain in fibromyalgia patients (Seidel and Muller, 2011). Previously, we reported that repeated forced swim (FS) conditioning enhanced the TMJ-evoked activity of neurons in deep laminae at the $Vc/C_{1–2}$ region and jaw muscle activity in male (Okamoto et al., 2012) and female rats (Okamoto et al., 2013). However, the underlying mechanisms by which FS increases TMJ nociception at the level of the $Vc/C_{1–2}$ region are not known. In this study, ondansetron, a 5HT3R antagonist, was applied locally at the $Vc/C_{1–2}$ region and the effects on TMJ-responsive neurons at the $Vc/C_{1–2}$ region and on masseter muscle EMG activity were assessed in female rats under high estrogen conditions.

Experimental procedures

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.

Estradiol treatment and forced swim (FS) conditioning

Ovariectomized (OVX) female rats (250–300 g, Sprague-Dawley, Harlan, Indianapolis, IN, n = 50) were injected with high dose (HE, 30μg/kg) 17-beta estradiol-3-benzoate (E2, dissolved in sesame seed oil (Sigma) for three days. This E2 replacement regimen produces plasma levels of E2 similar to those seen in proestrus rats (Okamoto et al., 2013). Estrogen status was confirmed on the day of the experiment by vaginal smear cytology; HE rats had mostly large nucleated epithelial cells. One hour after E2 injection, rats were exposed to repeated forced swim (FS) conditioning by placement in a plastic cylinder (diameter 30 cm, height 50 cm) containing 20 cm water (24–26°C) for 10 min per day between 09:00 and 11:00 for three days (Okamoto et al., 2012, Okamoto et al., 2013) and experiments were performed on day 4. Sham rats were placed in an empty swim chamber using the same schedule.

Neural recording at the Vc/$C_{1–2}$ region

Animals were sedated with pentobarbital sodium (60 mg/kg, ip) and catheters were positioned in the right femoral artery and jugular vein to monitor blood pressure and for drug infusion, respectively. After tracheotomy, the animals were artificially respired with oxygen-enriched room air and anesthesia was maintained with isoflurane (1–1.5%). The depth of anesthesia was determined by the loss of corneal and hindpaw withdrawal reflexes. Rats received an infusion of the short-acting paralytic agent, gallamine triethiodide (25 mg/kg/h), at the time of neural recording. Expiratory end-tidal CO_2 (3.5%–4.5%), mean arterial pressure (90–120 mm Hg), and body temperature (38°C) were monitored continuously and maintained within the normal range. Rats were placed in a stereotaxic device and portions of the C1 and C2 vertebrae were removed to expose the dorsal surface of the Vc/$C_{1–2}$ region. All TMJ neurons were identified by deep probing of the TMJ region and responded to mechanical stimulation of the condyle surface. The high threshold cutaneous RF area of each TMJ unit was determined using a small blunt forceps (3 mm^2) and mapped onto a standardized series of rat face drawings. After completion of cutaneous RF mapping, a guide cannula (26 gauge) was inserted into the TMJ joint space (approximately 3 mm deep) by a dorsal approach directed toward the posterior aspect of the mandibular condyle to allow repeated delivery of chemical stimuli. Test solutions were injected manually from a microsyringe attached to an inner cannula (33 gauge) that protruded approximately 0.5 mm from the end of the guide cannula. Test solutions of phosphate buffered saline (PBS, pH 7.4) and adenosine triphosphate (ATP, 1 mM, 20 μl in PBS) were injected slowly over 30 s to prevent tachyphylaxis. This concentration of ATP evokes pain sensation in humans (Hamilton and McMahon, 2000, Mork et al., 2003). Previously, we determined that repeated intra-TMJ injections of 1 mM ATP delivered at 20

min intervals evoked consistent responses in Vc/$C_{1–2}$ neurons with no sign of desensitization (Tashiro et al., 2008). TMJ units were recorded from superficial laminae (< 300 μM from brainstem surface) and deep laminae (>800 μM from surface) at the Vc/$C_{1–2}$ region. Only neurons excited by an intra-TMJ injection of ATP (>50% above PBS) were included in this study. All neurons included in this study and recorded in superficial laminae were classified as nociceptive specific (NS), whereas all neurons recorded in deep laminae were classified as wide dynamic range (WDR) based on convergent cutaneous RF properties (see Hu, 1990). The experimental design to test for 5HT3R involvement consisted of: 1) initial intra-TMJ injection of PBS, 2) intra-TMJ injection of ATP alone (1 mM), 3) ondansetron (OND, 0.1 mM, 30 μl, pH = 7.4 in PBS, Tocris, Ellisville, MO) applied to the Vc/$C_{1–2}$ surface 10 min prior to subsequent intra-TMJ injection of ATP, 4) OND (1 mM) 10 min prior to intra-TMJ injection of ATP, and 5) intra-TMJ injection of ATP alone. Intra-TMJ injections of ATP were separated by 20 min intervals after washing the brainstem. Neurons from the following experimental groups were assessed: a) sham superficial laminae (n = 7), b) sham deep laminae (n = 7), c) FS superficial laminae (n = 6), and FS deep laminae (n = 7).

Masseter muscle electromyography (EMG)

Animals were prepared surgically as noted above for neural recording. Masseter muscle EMG activity was recorded from paired wire electrodes (0.12 mm diameter, 5 mm interpolar distance) implanted ~1 mm into the central portion of the masseter muscle ipsilateral to the TMJ cannula. The experimental design to test for 5HT3R involvement consisted of: 1) initial intra-TMJ injection of PBS, 2) intra-TMJ injection of ATP alone, 3) ondansetron (OND, 1 mM, 30 μl, pH = 7.4 in PBS, Tocris, Ellisville, MO) applied topically to the Vc/$C_{1–2}$ surface 10 min prior to subsequent intra-TMJ injection of ATP, 4) intra-TMJ injection of ATP 30 min after OND. Intra-TMJ injections of ATP were separated by 20 min intervals. Masseter muscle EMG activity was sampled at 1000 Hz, amplified, filtered (300–3000 Hz), displayed and stored for analyses offline. EMG activity was assessed in sham rats (n = 12) and in FS-conditioned rats (n = 11).

Data analysis

Neural and EMG activity was amplified, discriminated, stored on a computer and analyzed offline using a PowerLab interface board and LabChart software (AD Instruments, Colorado Springs, CO). Neural recording data were quantified as a response magnitude (Rmag), equivalent to the area under curve, defined as the mean plus 2 times the standard deviation (SD) minus background activity (1 min) subtracted from the total spike count for each 1-second bin. All neurons included in this study displayed a total Rmag after the first 1mM ATP that exceeded the response to PBS > 50%. Note that all PBS-evoked neural responses were minor and not different from baseline firing rates (not shown). The response duration was defined as the time interval after stimulus onset until three consecutive bins with a positive spike count occurred above background and until the value of three consecutive bins no longer exceeded the mean + 2 SD above background activity. The high-threshold convergent cutaneous receptive field area was mapped with a small forceps (~ 3 mm^2) onto a standardized series of rat face drawings and measured by planimetry with NIH Image J software. EMG activity was sampled for 6 min, beginning 3 min before each TMJ stimulus

and for 3 min after stimulation. 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 3 min epoch (μV per 3 min) sampled immediately prior to stimulation. TMJ-evoked EMG activity was calculated as AUC post-ATP injection minus baseline AUC. The latency for TMJ-evoked EMG activity was defined as the time point when AUC for 1 s exceeded the average baseline. Results for neural and EMG activities were assessed statistically by ANOVA, corrected for repeated measures and individual comparisons were made by Newman-Keuls after ANOVA. Power analyses, calculated from similar Rmag and EMG data sets, indicated that a sample size of n = 6 was sufficent for 0.80 power. Values are expressed as mean ± SEM and $p < 0.05$ was considered significant.

Results

OND and TMJ-evoked neural activity

A total of 13 neurons were recorded from superficial laminae of sham (n = 7) and FS rats (n = 6). Figure 1 presents examples of TMJ-evoked activity of units from sham (Fig 1A) and FS rats (Fig 1B) recorded in superficial laminae. As summarized in Fig 2A, FS conditioning had no effect on the ATP-evoked Rmag prior to drug administration ($F_{1,11} = 1.98$, $p > 0.1$). Topical administration of OND 10 min prior to the ATP stimulus caused a significant decrease in Rmag in sham and FS rats ($F_{3,33} = 74.4$, $p < 0.001$) and partial recovery following washout. This was a consistent finding as 6/6 units in FS rats and 5/7 units in sham rats displayed a reduction in Rmag after 1 mM OND of >70% compared to pre-drug values. Response duration (Fig 2B) and response latency (Fig 2C) evoked by ATP were not different between sham and FS groups prior to drug application. OND caused a significant reduction in response duration ($F_{3,33} = 20.9$, $p < 0.001$) in sham and FS rats. By contrast, OND increased the ATP-evoked response latency of units only in FS rats ($F_{3,33} = 5.25$, $p < 0.01$), whereas latency in sham rats was not affected ($F_{3,33} = 1.51$, $p > 0.1$). These confirmed earlier results (Okamoto et al. 2013) that FS conditioning had little effect on TMJ-evoked Rmag values of superficial laminae neurons. However, OND caused a significant decrease in TMJ-evoked Rmag in both sham and FS rats.

Fourteen TMJ-responsive neurons were recorded from deep laminae in sham (n = 7) and FS rats (n = 7). Figure 3 presents histogram examples of TMJ-evoked activity of units from sham (Fig 3A) and FS rats (Fig 3B) recorded in deep laminae. Note that, in contrast to ATP-evoked responses by superficial laminae units, units in deep laminae of FS rats displayed marked increases in firing rate compared to units in sham rats prior to drug application. As summarized in Fig 4A, FS conditioning enhanced the ATP-evoked Rmag compared to sham rats prior to drug administration ($F_{1,12} = 6.44$, $p < 0.025$).

Application of OND 10 min prior to the ATP stimulus significantly reduced Rmag in sham and FS rats in a dose-related manner ($F_{3,36} = 109.4$, $p < 0.001$) with partial recovery following washout. This was a consistent finding as 7/7 units in FS rats and 4/7 units in sham rats displayed a reduction in ATP-evoked Rmag after 1 mM OND of >70% compared to pre-drug values. Prior to OND administration, FS rats displayed an increase in the ATP-evoked response duration (Fig 4B) compared to units from sham rats ($F_{1,45} = 4.4$, $p < 0.05$). OND caused a marked dose-related reduction in response duration ($F_{3,36} = 35.4$, $p < 0.001$)

in FS rats and lesser effects in sham rats. Individual comparisons revealed significantly greater reductions in response duration in FS rats after 0.1 mM ($F_{1,45} = 25.5$, $p < 0.001$) and 1 mM ($F_{1,45} = 4.59$, $p < 0.05$) than that seen in deep units from sham rats. Response latency (Fig 4C) evoked by ATP from deep units in FS and sham rats were not different prior to drug application. Although OND caused an overall increase in the ATP-evoked response latency of units in FS and sham rats ($F_{3,36} = 7.94$, $p < 0.001$), individual comparisons revealed no significant group differences. These data confirmed earlier results (Okamoto et al., 2013) that FS greatly enhanced the TMJ-evoked Rmag values of units in deep laminae.

All TMJ units displayed low (superficial laminae) to moderate (deep laminae) ongoing discharge rates prior to ATP stimulation and drug administration. Group comparisons revealed higher rates of SA for units in deep laminae than units in superficial laminae prior to ATP stimulation and OND application (Fig 5, $F_{3,31} = 7.4$, $p < 0.001$). Individual comparisons indicated that FS conditioning alone caused a small reduction in the SA of units in superficial laminae (Fig 5A, $p < 0.05$), whereas units in deep laminae of FS rats had higher firing rates than units of sham rats (Fig 5B, $p < 0.01$). Although OND caused an overall reduction in the SA of units in FS and sham rats ($F_{3,69} = 18.2$, $p < 0.001$), individual comparisons indicated that OND caused a small decrease in SA for superficial laminae units of FS rats ($F_{3,69} = 4.8$, $p < 0.01$) and no significant effect on SA of units in sham rats ($F_{3,69} = 2.5$, $p < 0.1$). By contrast, OND caused significant reductions in SA of units in deep laminae in FS ($F_{3,69} = 7.3$, $p < 0.01$) and sham rats ($F_{3,69} = 7.4$, $p < 0.01$). The percentage decrease in SA of units in deep laminae to 1 mM OND averaged 48.1 ± 17.4% and 69.5 ± 6.2% for FS and sham rats, respectively. These results suggested that 5HT3R input had a tonic influence on TMJ unit activity in deep laminae under both FS and sham conditions.

All TMJ units received convergent cutaneous input from the ipsilateral face, generally located anterior and ventral to the TMJ (Fig 6A). The high threshold cutaneous RF was mapped onto standardized drawings of the rat face before ATP injections or topical OND application and again after high dose OND (1 mM, Fig 6A). Comparisons across all four groups revealed that TMJ units in deep laminae had significantly larger RF areas than those of superficial laminae units ($F_{3,23} = 14.7$, $p < 0.001$) and that OND reduced the RF area of units in all groups ($F_{1,23} = 241.3$, $p < 0.001$; Fig 6B). FS conditioning increased the RF area of TMJ units in deep laminae ($F_{3,33} = 9.3$, $p < 0.001$), but not of units in superficial laminae. These data further supported the notion of a tonic, centrally mediated, influence of 5HT3R on the properties of TMJ units at the $Vc/C_{1–2}$ region. Resting MAP in FS (n =12) and sham (n =12) rats averaged 98 ± 3 and 98 ± 2 mmHg, respectively, and was not affected by OND ($F_{3, 66} = 2.7$, $P > 0.05$).

OND and TMJ-evoked masseter muscle activity

Masseter muscle EMG activity was sampled over 3 min prior to each intra-TMJ injection of ATP and was similar in FS and sham animals ($F_{3,19} = 1.5$, $p > 0.1$). The ATP-evoked EMG responses in FS were significantly greater than in sham rats prior to OND (Fig 7A, $F_{3,30} = 9.4$, $p < 0.001$) and consistent with previous results (Okamoto et al., 2013). OND alone (1 mM) or vehicle application to the brainstem surface had no effect on resting EMG activity ($F_{2, 38} = 0.9$, $P > 0.1$). However, OND reduced the ATP-evoked AUC responses of FS ($F_{2,38}$

= 40.8, $p < 0.001$) and sham rats ($F_{2,38} = 10.3$, $p < 0.01$) at 10 min post-application (Fig 7). The magnitude of the reduction by OND on ATP-evoked AUC in FS (−86.9 ± 2.5%) and sham rats (−61.5 ± 2.4%) was similar ($p > 0.1$). The latency for TMJ-evoked EMG activity was not different for sham and FS rats prior to OND (Fig 7B, range = 26–13 s, $F_{3,50} = 0.5$, $p > 0.1$). OND increased the EMG latency in FS rats (pre-OND versus post-OND = 13.9 ± 1.9 s and 48.6 ± 21 s, respectively, $F_{2,38} = 11.4$, $p < 0.01$), whereas OND had no effect on response latency in sham rats. These data suggested that resting masseter muscle EMG activity was not under tonic 5HT3 control, whereas TMJ-evoked muscle activity of FS and sham rats was markedly influenced by excitatory 5HT3R-related mechanisms.

Discussion

The present study demonstrated that 5HT3R, acting locally at the Vc/$C_{1–2}$ region, significantly reduced TMJ nociception in female rats. Topical application of the 5HT3R antagonist, ondansetron (OND), to the dorsal brainstem surface had widespread, and marked, effects on TMJ-related nociception: 1) reduced the TMJ-evoked activation of neurons in superficial and deep laminae under sham and FS conditions, 2) reduced ongoing firing rates of TMJ units in deep laminae in sham and FS rats, 3) reduced the convergent cutaneous RF area of TMJ units in superficial and deep laminae in sham and FS rats, and 4) reduced the TMJ-evoked masseter muscle EMG activity under sham and FS conditions. These results indicated that 5HT3R-dependent mechanisms likely play a key role in modulation of neurons at the Vc/$C_{1–2}$ region that process deep craniofacial input and contribute to enhanced sensory and muscle reflex activity after sham and psychophysical stress conditionings.

Stress-induced hyperalgesia (SIH)

Stress-induced hyperalgesia (SIH) has been well documented in several animal models (Imbe et al., 2006, Jennings et al., 2014) and in clinical studies (Crettaz et al., 2013). The RVM, and more specifically, 5HT3R-related mechanisms have been implicated in descending facilitation of nociception in several neuropathic (Wei et al., 2010, Okubo et al., 2013) and inflammatory pain models (Okamoto et al., 2004, Zhao et al., 2007). However, the relationship between the RVM, descending pain facilitatory pathways and psychophysical stress is less well defined. The present study used repeated FS as a model for persistent psychophysical stress (Quintero et al., 2000). This model produced persistent cutaneous and muscle hyperalgesia that lasted for 8–9 days (Suarez-Roca et al., 2006). In the formalin test, FS increased nocifensive behavior, depended on an intact RVM (Imbe et al., 2010) and was reported to involve 5HT3R-related mechanisms (Oyama et al., 1996, Okamoto et al., 2005). Previously, we found that FS increased the expression of phospho-CREB in superficial and deep laminae at the Vc/$C_{1–2}$ region, and of downstream regulated genes, independent of estrogen status (Duenes et al., 2010), suggesting widespread changes in the excitability of trigeminal brainstem neurons associated with TMJ nociception. More recently, we determined that single TMJ-responsive neurons in deep laminae, but not in superficial laminae, at the Vc/$C_{1–2}$ region of male (Okamoto et al., 2012) and female rats (Okamoto et al., 2013) had markedly greater responses to intra-TMJ injections of ATP after

FS than in sham controls. However, the basis for enhanced responsiveness of second-order TMJ neurons in a lamina-specific manner is not known.

Considerable evidence supports the notion of a facilitatory 5HT3R-dependent influence on nociception (Riering et al., 2004, Suzuki et al., 2004b, Viguier et al., 2013). The present results indicated that OND inhibited the input from cutaneous and deep craniofacial tissues to TMJ neurons in superficial and deep laminae at the $Vc/C_{1–2}$ region of sham and FS animals. These data agreed, generally, with results from previous studies in which 5HT3R mechanisms were shown to modify nociception in models of inflammatory and neuropathic pain. For example, spinal application of OND reduced the enhanced mechanical and heat-evoked responses of deep dorsal neurons after carrageenan inflammation and in sham animals (Rahman et al., 2004). Similarly, formalin-evoked responses of superficial laminae neurons at the $Vc/C_{1–2}$ region, recorded 7 days after Complete Freund's Adjuvant (CFA) injection into the TMJ, were enhanced compared to units from sham rats; however, local application of the selective 5HT3R antagonist, tropisetron, reduced the evoked unit activity in both groups (Okamoto et al., 2005). Two weeks after spinal nerve ligation, mechanical-evoked deep dorsal neural activity was enhanced compared to units in sham rats and, although spinal application of OND had a greater inhibitory effect on units from nerve-injured rats, evoked responses also were reduced in sham animals (Suzuki et al., 2004a). Our results and several previous studies have suggested that 5HT3R activation facilitates nociceptive processing under normal conditions and after nerve injury, tissue inflammation or psychophysical stress. However, others have suggested that 5HT3R contributes to nociceptive behavior only after nerve injury and only after a time delay of at least 2 weeks (Okubo et al., 2013). The reasons for these differences were not clear since Okubo et al. (2013) only tested the effects of 5HT3R antagonist on reflex withdrawal behavior and not on the response properties of dorsal horn neurons. We cannot exclude that methodological issues contributed to these differences. For example, we assessed the effects of OND only on TMJ-responsive neurons and in female rats, whereas Okubo et al. (2013) and most previous studies assessed 5HT3R drug effects on cutaneous-evoked behavior in male animals. Earlier studies suggested that descending control of input from deep tissues onto spinal dorsal horn neurons was affected more than input from cutaneous tissues (Yu and Mense, 1990, Chiang et al., 1994). Our results support that conclusion, since the doses of OND (0.9 and 8.8 μg in 30 μl) that blocked the evoked responses of TMJ neurons at the $Vc/C_{1–2}$ region were at or less than those needed to block cutaneous input to spinal dorsal horn neurons (10–100 μg; Suzuki et al., 2002, Rahman et al., 2004, Suzuki et al., 2004a) or the dose of Y25130, a related 5HT3R antagonist, needed to block cutaneous-evoked withdrawal behavior after Vc microinjection (50 μg, Okubo et al., 2013). The role of 5HT3R in pain processing is further confounded by reports that electrical stimulation-evoked dorsal horn neural activity in naïve and inflamed rats was not reduced by spinal application of 5HT3R antagonists, whereas responses to formalin (Green et al., 2000) and natural mechanical stimuli (Suzuki et al., 2002) were inhibited. Thus, the available data from animal studies suggest that 5HT3R involvement in modulation of nociceptive responses depends on multiple factors such as the past history of the preparation (e.g., acute versus chronic injury, inflammation or stress), test stimulus modality and stimulus duration as well as the route and dose of drug administration.

Stress and circuitry for jaw muscle reflexes

A key finding in this study was the pronounced inhibition by OND on TMJ-evoked jaw muscle EMG activity in both sham and FS groups. Previously, we reported that lidocaine blockade of the Vc/$C_{1–2}$ region greatly reduced TMJ-evoked masseter muscle EMG activity in male rats (Okamoto et al., 2012). The present results extend that finding to suggest that 5HT3R activation at the Vc/$C_{1–2}$ region is a key factor in TMJ-evoked jaw muscle reflexes. The circuitry for a TMJ-Vc/$C_{1–2}$ -masseter muscle reflex response is not well defined. At the level of the Vc/$C_{1–2}$ region, only neurons in deep laminae displayed enhanced TMJ-evoked responses after FS to match the increase seen in evoked jaw muscle activity. This suggested that neurons in deep laminae were more critical for TMJ-evoked EMG activity than neurons in superficial laminae; however, other interpretations are possible. For example, OND reduced the TMJ-evoked Rmag of neurons in superficial and deep laminae suggesting that some effects of 5HT3R activation were shared by TMJ nociceptive neurons in both regions. However, only neurons in deep laminae displayed enhanced TMJ-evoked responses after FS, suggesting that neurons in superficial and deep laminae serve different functions in TMJ-related nociception. Suzuki et al. (2004b) have proposed that lamina I neurons receive the majority of direct input from C nociceptors and are necessary to recruit descending controls from the RVM, whereas only neurons in deep laminae are enhanced in a 5HT3R-dependent manner. The present study tested this hypothesis indirectly by recording from neurons in both superficial and deep laminae, whereas most previous studies reported only 5HT3R effects on dorsal horn neurons in deep laminae. Anatomical studies indicated that 5HT-positive terminals in both superficial and deep laminae at the Vc/$C_{1–2}$ region and cervical dorsal horn (Pearson and Jennes, 1988, Li et al., 1997). Similarly, the density of 5HT3R was highest in superficial laminae of spinal dorsal horn and punctate staining in deeper laminae (Maxwell et al., 2003, Conte et al., 2005). Local dorsal microcircuitry also may have contributed to the apparent differential effects of OND on TMJ neurons in superficial and deep laminae. Thus, OND could have acted directly on 5HT3R-positive neurons in each region to alter properties of TMJ units, or alternatively, via 5HT3R on interneurons in superficial laminae that, in turn, projected to TMJ neurons in deep laminae. This notion was supported by anatomical studies indicating a subpopulation of 5HT3R-positive neurons in superficial laminae were GABAergic or enkephalinergic (Huang et al., 2008). It is estimated that more than 80% of 5HT3R-positive axon terminals in superficial laminae are on intrinsic cells and not on terminals of primary afferent fibers (Maxwell et al., 2003). It has long been proposed that neurons in superficial and deep laminae serve different functions in nociception (McMahon and Wall, 1988, Braz et al., 2005) and that local communication between neurons in superficial and deep laminae plays a significant role in pain processing (see Todd, 2010). This may help explain why only neurons in deep laminae had enhanced responses to TMJ stimulation, whereas local application of OND was able to inhibit TMJ-evoked responses in both regions. Alternatively, we cannot exclude that FS conditioning engages additional neurotransmitter systems that preferentially modify the encoding properties of TMJ units in deep laminae.

Rostral ventromedial medulla and stress

Although we cannot exclude that 5HT fiber projections to the Vc/$C_{1–2}$ region originated from regions outside the RVM; however, this seems unlikely based on findings in spinal

dorsal horn of marked depletion of 5HT after selective lesion in RVM or depletion of 5HT (Bowker et al. 1982; Wei et al. 2010). Considerable evidence suggests that the RVM is necessary for the development of SIH (see Imbe et al., 2006, Jennings et al., 2014). For example, nearly 75% of pERK-positive neurons in RVM produced after chronic restraint stress were serotonergic (Imbe et al., 2004). Psychological distress is a risk factor for persistent TMJ pain (Slade et al., 2007, Maixner et al., 2011) and conditions that are often comorbid with TMD such as fibromyalgia (Yunus, 2007, Maixner, 2009). Although 5HT3R antagonists have shown some benefit for patients with fibromyalgia (Seidel and Muller, 2011), the effectiveness in managing TMD pain is not known. The current study revealed significant effects of OND on TMJ-responsive neurons at the Vc/$C_{1–2}$ region and on TMJ-evoked jaw muscle reflexes and suggested that 5HT3R-dependent pharmacotherapy deserves further investigation. It is interesting to note that several antidepressant drugs have been reported to act as functional antagonists at the 5HT3R (Eisensamer et al., 2003) and to colocalize with 5HT3R in raft-like domains in cell membranes (Eisensamer et al., 2005). Thus, other classes of drugs used to treat mood and behavioral disorders may act, in part, through 5HT3R-dependent pathways.

Conclusions

These results suggested that 5HT3R-dependent mechanisms play a key role in processing TMJ-related signals by modifying the properties of neurons at the Vc/$C_{1–2}$ region and altering TMJ-evoked jaw muscle activity. Psychophysical stress markedly enhanced the TMJ-evoked evoked responses of neurons in deep laminae that was prevented by OND. Since local application of OND inhibited the TMJ-evoked activity of neurons in superficial laminae in both FS and sham animals, these data support the hypothesis that 5HT3R-dependent mechanisms have widepread effects on TMJ nociception are are active under stress as well as non-stress conditions.

Acknowledgments

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Abbreviations

5HT3R	serotonin type 3 receptor
FS	repeated forced swim conditioning
OND	ondansetron
RVM	rostral ventromedial medulla
TMD	temporomandibular disorders
TMJ	temporomandibular joint
Vc/$C_{1–2}$	trigeminal subnucleus caudalis/upper cervical spinal cord region

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Highlights

- Blockade of 5HT3R at Vc/$C_{1–2}$ region with ondansetron inhibits TMJ-evoked jaw muscle activity.
- Ondansetron reduced TMJ-evoked unit activity in superficial and deep laminae at Vc/$C_{1–2}$ region.
- Ondansetron reduced TMJ nociception in repeated psychophysical stressed and sham female rats.

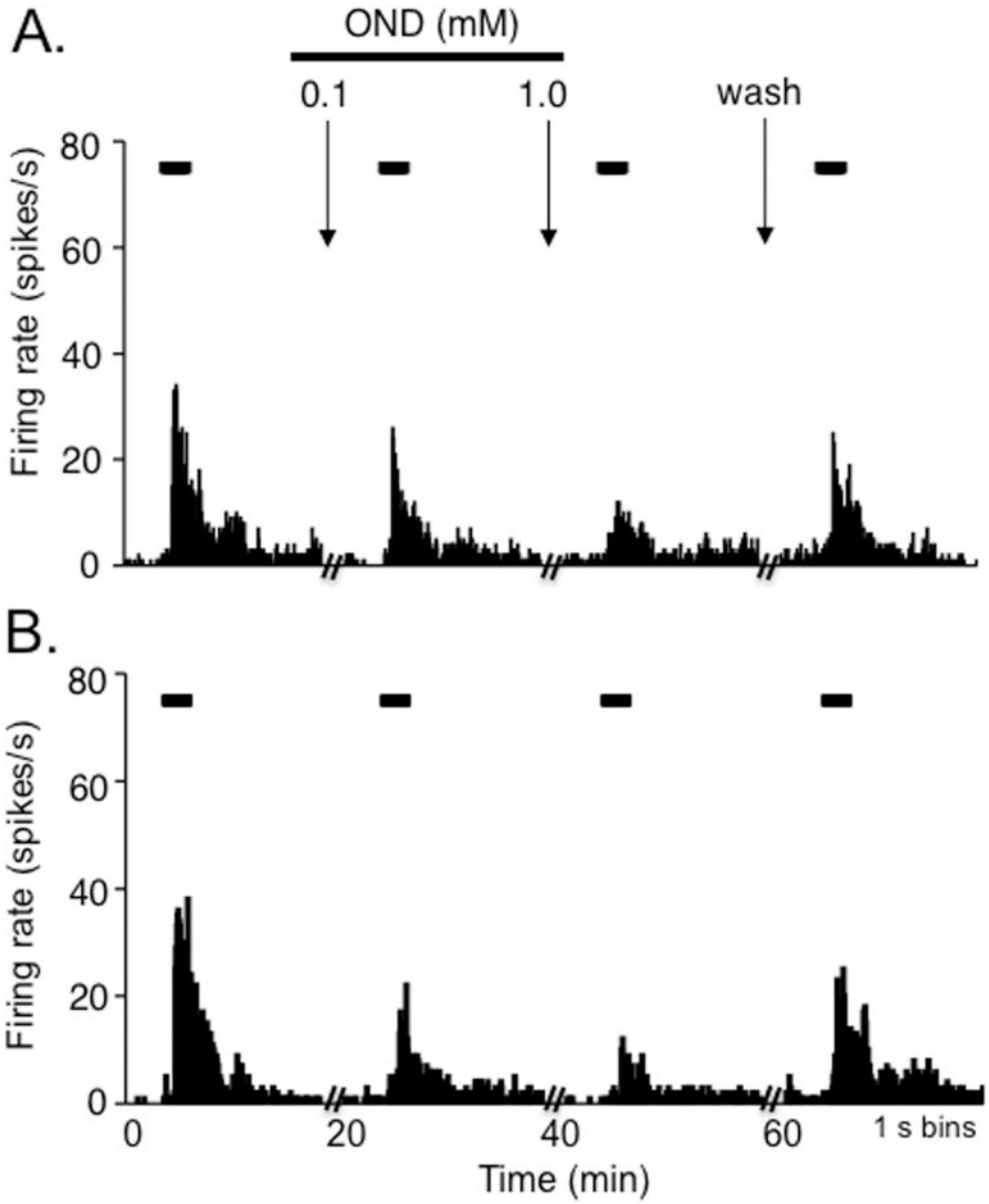

Figure 1.

Peristimulus time histogram examples of the effects of ondansetron on ATP-evoked responses of TMJ neurons recorded in superficial laminae at the Vc/$C_{1–2}$ region of A) sham and B) FS conditioned rats. OND, ondansetron (0.1mM and 1mM, 30μl); arrows indicate OND application; horizontal bars indicate time of ATP injections (30 s).

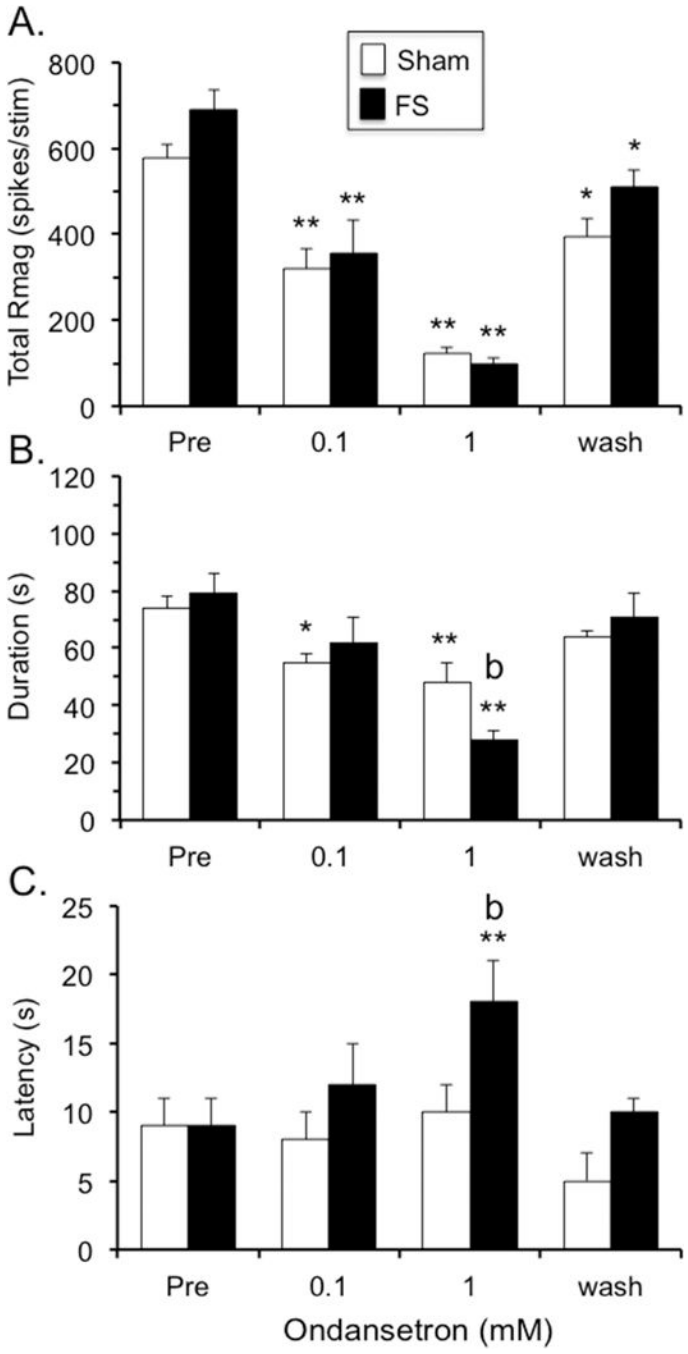

Figure 2.

Effects of ondansetron on TMJ-evoked A) total Rmag, B) response duration and C) response latency of neurons recorded in superficial laminae at the Vc/$C_{1–2}$ region of sham (open bars) and FS conditioned rats (black bars). *p < 0.05, **p < 0.01 versus pre-drug response, b = p < 0.01 versus sham group. Results shown as mean ± sem.

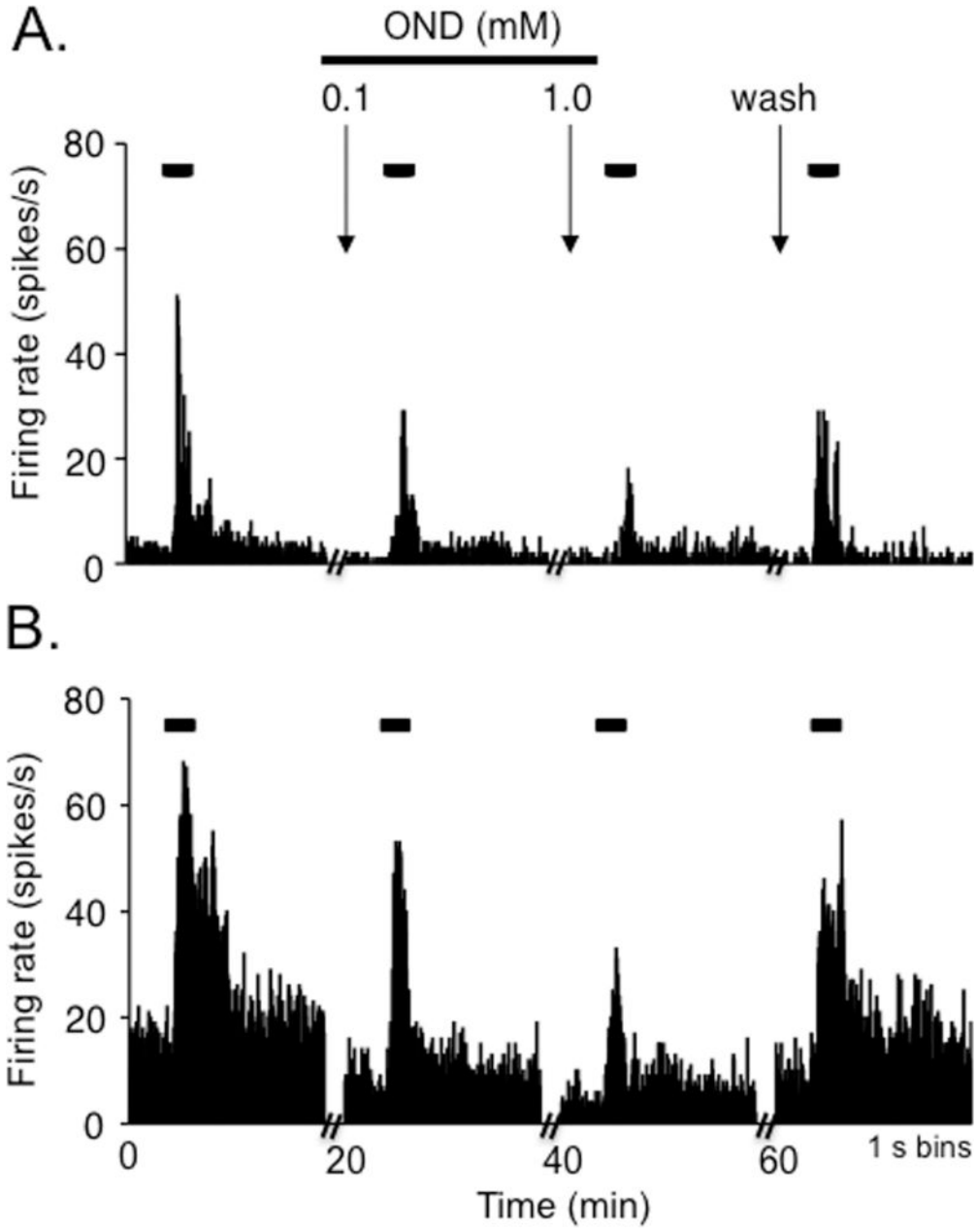

Figure 3.

Peristimulus time histogram examples of the effects of ondansetron on ATP-evoked responses of TMJ neurons recorded in deep laminae at the Vc/$C_{1–2}$ region of A) sham and B) FS conditioned rats. OND, ondansetron (0.1mM and 1mM, 30μl); arrows indicate OND application; horizontal bars indicate time of ATP injections (30 s).

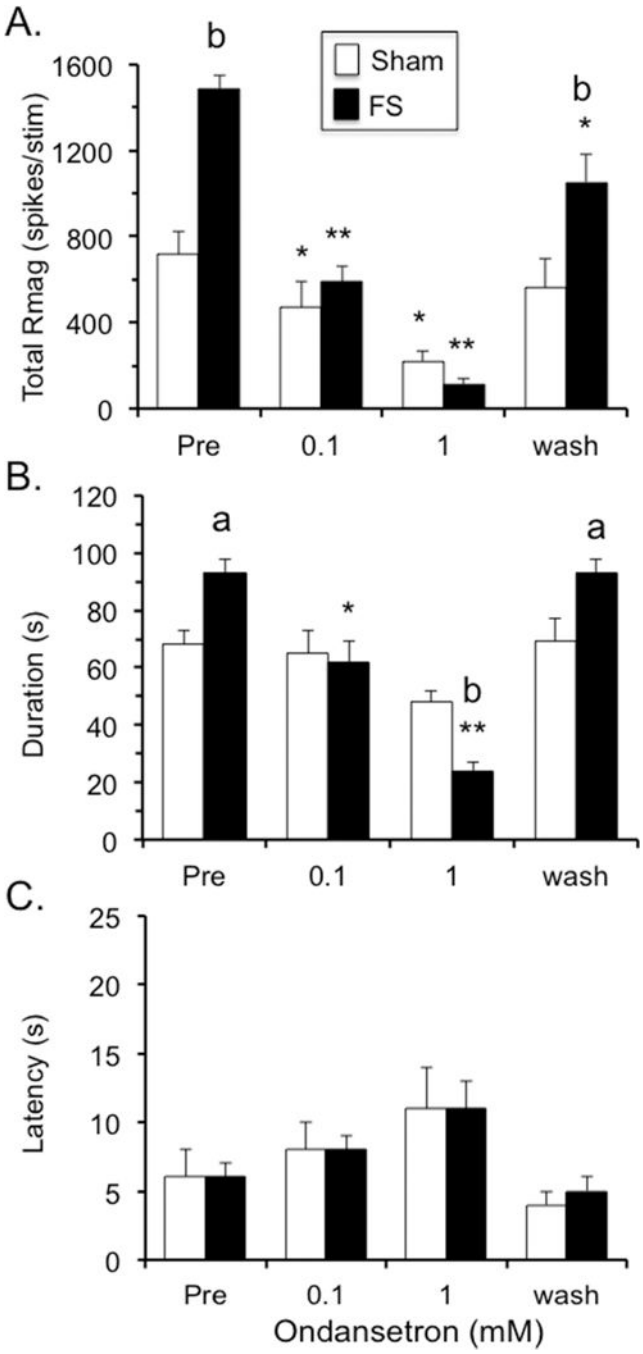

Figure 4.

Effects of ondansetron on TMJ-evoked A) total Rmag, B) response duration and C) response latency of neurons recorded in deep laminae at the $Vc/C_{1–2}$ region of sham (open bars) and FS conditioned rats (black bars). *p < 0.05, **p < 0.01 versus pre-drug response; a = p < 0.05, b = p < 0.01 versus sham group. Results shown as mean ± sem.

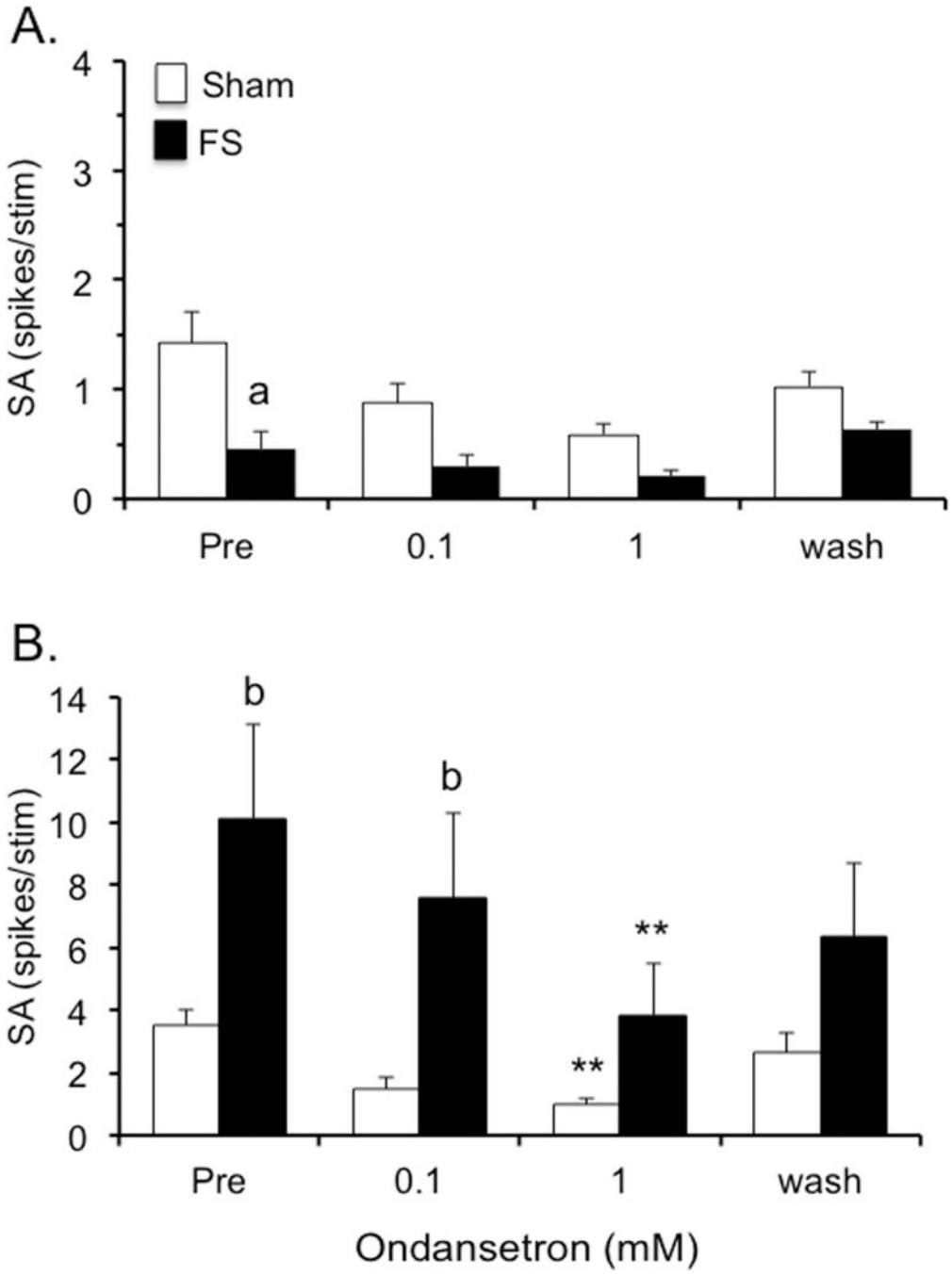

Figure 5.

Effects of ondansetron on spontaneous firing rates (spikes/s) of TMJ neurons recorded from A) superficial laminae and B) deep laminae at the Vc/$C_{1–2}$ region of sham (open bars) and FS conditioned rats (black bars). **$p < 0.01$ versus pre-drug firing rate; a = $p < 0.05$, b = $p < 0.01$ versus sham group. Results shown as mean ± sem.

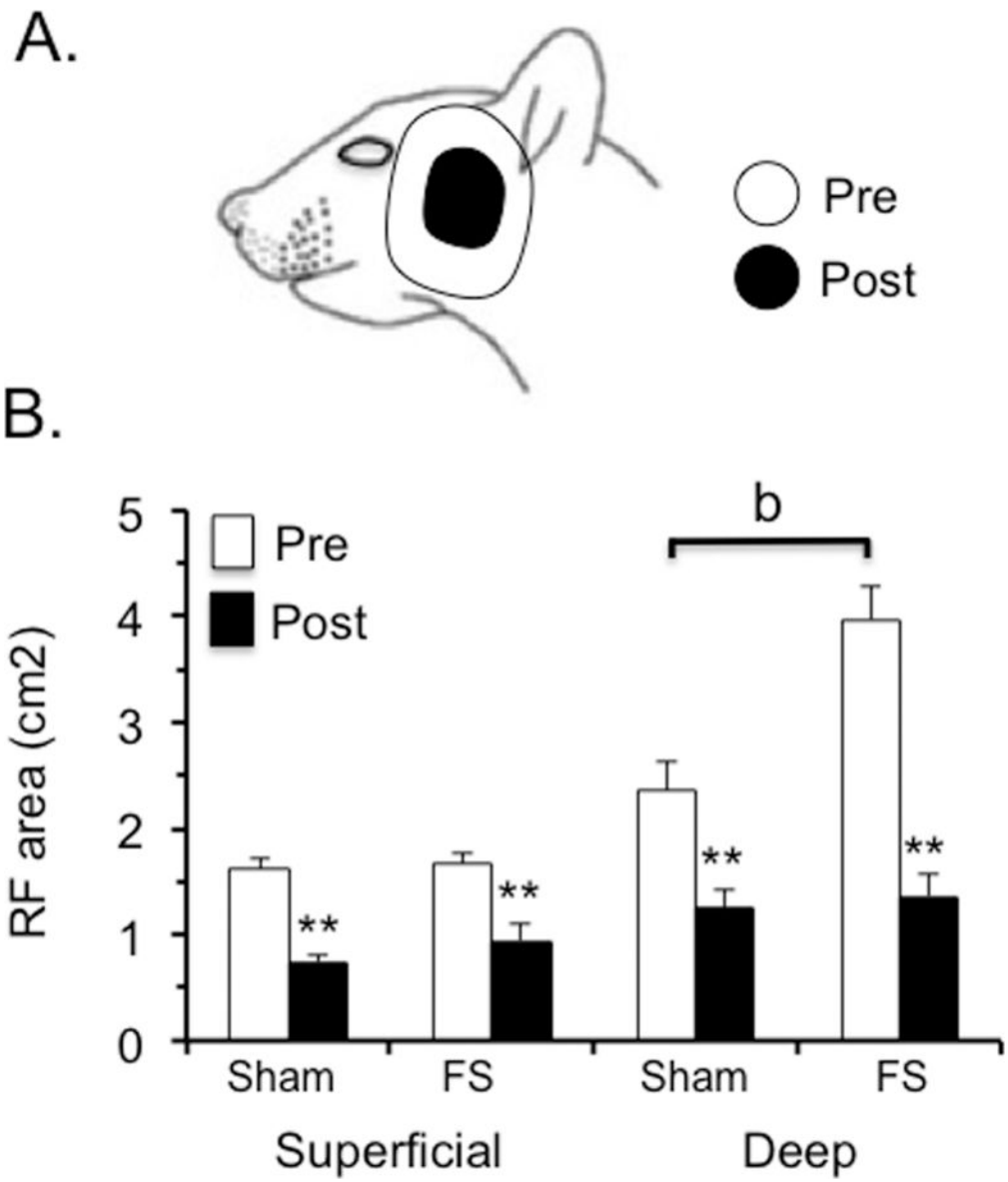

Figure 6.

A) Example of convergent RF area of deep unit in FS rats pre and post OND treatment. B) Effects of ondansetron on high threshold convergent cutaneous RF area of TMJ neurons recorded from superficial laminae and deep laminae at the Vc/C_{1-2} region of sham and FS conditioned rats. Open bars = pre-drug RF area, black bars = post-OND (1 mM) RF areas; **$p < 0.01$ versus pre-drug RF area; b = $p < 0.01$ versus sham group. Results shown as mean ± sem.

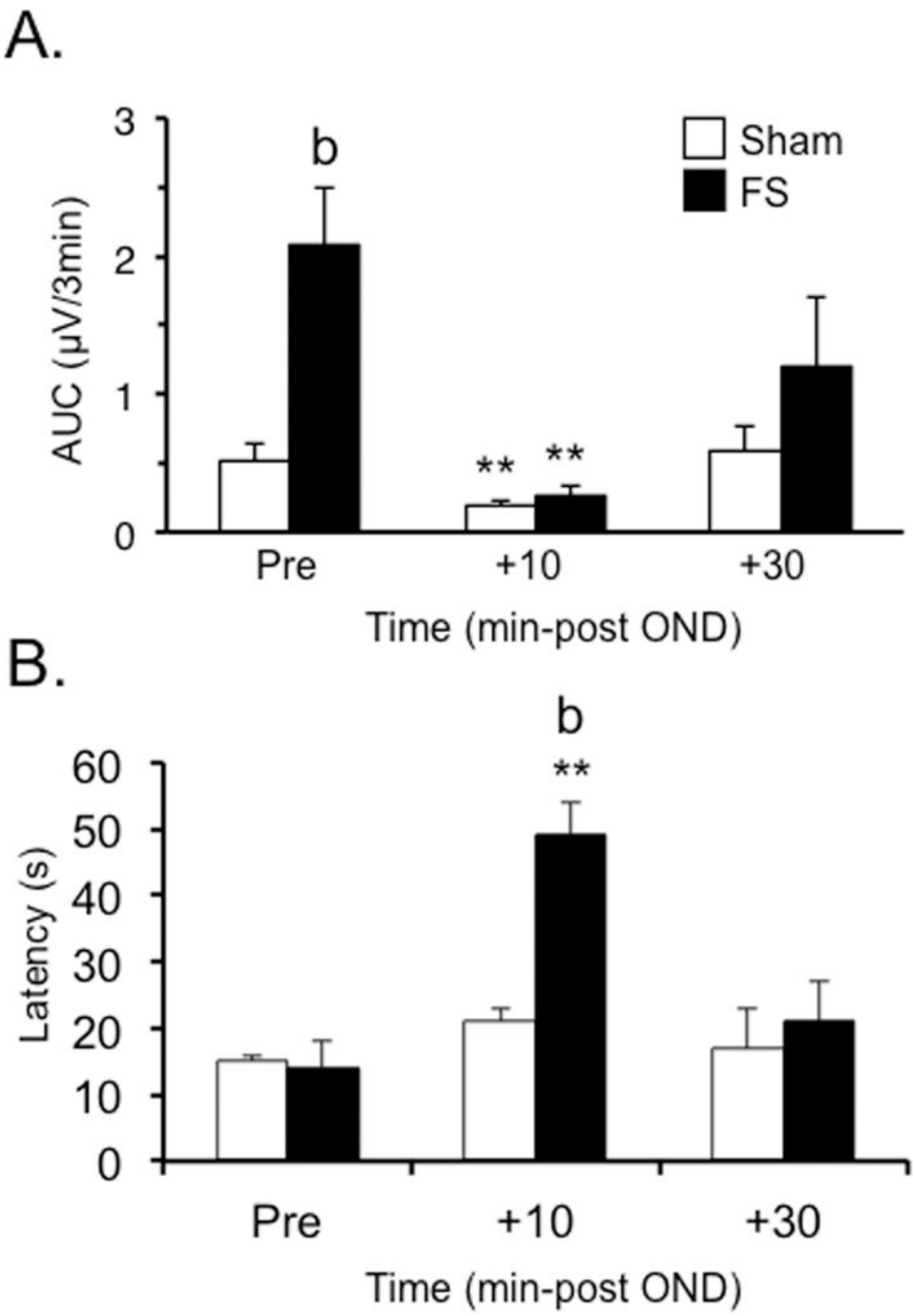

Figure 7.

Effects of ondansetron on TMJ-evoked jaw muscle activity in sham (open bars) and FS rats (black bars). A) ATP-evoked AUC (μV/3 min) and B) response latency. Symbols: **p < 0.01 versus pre-drug, b = p < 0.01 versus sham group. Results shown as mean ± sem.