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Differential ascending projections of temporomandibular joint-responsive brainstem neurons to periaqueductal gray and posterior thalamus of male and female rats

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Abstract

Several craniofacial pain conditions including temporomandibular joint disorders (TMJD) are more prevalent in women than men. The basis for sex differences in deep craniofacial pain is not known. The present study compared the magnitude of ascending projections from TMJ-responsive neurons in trigeminal brainstem to the ventrolateral periaqueductal gray (vIPAG) or posterior nucleus of the thalamus (Po) in males and female rats. Fluorogold (FG) was injected into vIPAG or Po and TMJ-responsive neurons were identified by Fos-like immunoreactivity (Fos-LI) after mustard oil injection. TMJ-evoked Fos-LI was similar in males and females; however, significant differences in cell counts were seen for FG single-labeled and Fos/FG double-labeled neurons in trigeminal brainstem. After vIPAG injections, the number of FG-labeled neurons in trigeminal subnucleus interpolaris (Vi), ventral interpolaris/caudalis transition (vl-Vi/Vc) and dorsal paratrigeminal region (dPa5) was greater in females than males. The percentage of Fos/FG double-labeled neurons in vl-Vi/Vc and dPa5 after vIPAG injection also were greater in females than males. By contrast after Po injections, males displayed a greater number of FG-labeled neurons in superficial laminae of Vc and C₁₋₂ and deeper laminae at C₁₋₂ than females. The percentage of Fos/FG double-labeled neurons in superficial laminae of Vc after Po injection also was greater in males than females.

These data revealed significant sex differences in ascending projections from TMJ-responsive neurons in trigeminal brainstem. Such differences may influence the ability of males and females to recruit autonomic reflexes and endogenous pain control circuits relevant for TMJ nociception.

Keywords

sex difference; temporomandibular joint; trigeminal brainstem; periaqueductal gray; posterior thalamus

Temporomandibular joint disorders (TMJD) represent a heterogeneous family of conditions that present with pain in the jaw joint and muscles of mastication. The signs and symptoms of TMJD suggest a central neural dysfunction resulting in pain amplification; however, the

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central neural pathways that underlie TMJD pain are not well defined (Diatchenko et al. 2006; Maixner 2009). Although women are more likely to develop TMJD than men (Bereiter and Okamoto 2011), little is known about brain pathways that process noxious sensory information from deep craniofacial tissues in males and females. Sensory afferent nerves that supply the TMJ and muscles of mastication terminate at multiple rostrocaudal levels of the trigeminal brainstem nuclear complex (TBNC) and upper cervical dorsal horn (Shigenaga et al. 1988; Dessem et al. 2007). C-fos immunohistochemical studies have confirmed that select regions of the TBNC that receive direct input from the TMJ, e.g., dorsal paratrigeminal region (dPa5) and superficial laminae of Vc and upper cervical dorsal horn (C₁₋₂), encode the intensity of an intra-TMJ stimulus in an estrogen-dependent manner (Bereiter 2001; Okamoto et al. 2008). Anatomical tract-tracing combined with c-fos methods have been used to report that TBNC neurons responsive to jaw muscle (Ikeda et al. 2003; Sugiyo et al. 2005) or TMJ injury (Yamazaki et al. 2008) projected to higher brain centers associated with nociceptive processing; however, only male animals were included in these studies.

The aim of the present study was to determine if ascending projections from TMJ-responsive neurons in the TBNC displayed sex differences. Cell counts were made after Fluorogold (FG) injections into the ventrolateral periaqueductal gray (vlPAG) or posterior thalamus (Po) in adult male and female rats. The PAG-rostroventromedial medulla (RVM) complex plays a pivotal role in pain modulation (Tracey and Mantyh 2007), including pain evoked by stimulation of jaw muscles (Kupers et al. 2004) and facial skin (Mainero et al. 2007). The vlPAG preferentially integrates noxious sensory input from deep somatic and visceral tissues (Clement et al. 2000) and, more recently, the anatomical and functional organization of vlPAG-RVM pathways was shown to display sex-related differences that may underlie sexually dimorphic responses to opioid analgesics in spinal pain models (Lloyd and Murphy 2006). The Po receives significant direct input from the TBNC (Iwata et al. 1992; Gauriau and Bernard 2004a). Spinothalamic input to Po derives mainly from nociceptive neurons in superficial and deep laminae (Zhang and Giesler 2005), while Po neurons that encode noxious sensory information project strongly to cortical regions that integrate affective and sensory aspects of pain (Gauriau and Bernard 2004b). Stimulation of specialized craniofacial tissues such as tooth pulp (Zhang et al. 2006) and dura (Burstein et al. 2010) activate Po neurons, while cornea- (Hirata et al. 2000) and TMJ-responsive neurons (Takeshita et al. 2001) recorded from superficial laminae of trigeminal subnucleus caudalis (Vc) are driven antidromically by stimulation sites in the Po, but not from sites in the ventral posterior medial thalamic nucleus (VPM). Although considerable evidence suggests that the vlPAG and Po contribute to the proposed “TMJ pain matrix” in the brain (Bereiter and Okamoto 2011), it is not known if ascending projections from TMJ-responsive neurons in the lower brainstem to these regions are similar in males and females.

EXPERIMENTAL PROCEDURES

The animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota and conformed to the established guidelines set by The National Institutes of Health guide for the care the use of laboratory animals (PHS Law 99–158, revised 2002).

Estradiol (E2) treatment in females

Ovariectomized (OvX) female rats were injected daily with 17 β -estradiol-3-benzoate (Sigma, St. Louis, MO, USA), (E2, 3 μ g/kg) dissolved in 200 μ l sesame oil for 2 days prior to the day of perfusion. Our rationale for comparing males to low E2-treated females was two-fold. First, we were interested in sex differences in projections of TMJ-responsive neurons rather than the effect of estrogen status per se. Females under low estrogen

conditions and males produced similar Fos-like immunoreactivity (Fos-LI) responses in TBNC regions after TMJ stimulation (Bereiter 2001). Second, E2 was given as a low maintenance dose to normalize the elevation of hypothalamic and pituitary hormones resulting from OvX surgery (Ajika et al. 1972). Recent studies in humans indicated that elevated levels of pituitary hormones significantly influenced pain sensation (Tajar et al. 2011). In some cases blood samples were collected at the time of perfusion and measured for E2 by immunoassay (Invitrogen, Camarillo, CA). Plasma E2 levels averaged 6.3 ± 0.9 pg/ml for males ($n = 4$) and 7.6 ± 1.2 pg/ml for OvX E2-treated females ($n = 7$). Vaginal smears were assessed from all female rats and a low E2 status (>90% small nucleated leukocytes) was confirmed on the day of perfusion.

Animal preparation and Fluorogold tracer injection

Weight-matched (280–390 g) female ($n = 16$) and male rats ($n = 18$, Sprague-Dawley, Harlan, Indianapolis, IN) were anesthetized with pentobarbital sodium (55 mg/kg, ip), placed in a stereotaxic frame and the skull positioned so that the bregma and lambda landmarks had equal dorsoventral coordinates. Lidocaine (2% 0.1 ml) was injected into the scalp prior to exposing the skull by dorsal incision and performing a craniotomy over the right hemisphere (2×2 mm). A small opening was cut in the dura and a glass micropipette (40–80 μ m tip diameter) filled with 4% Fluorogold (FG, Fluorochrome, LLC, Denver, CO), dissolved in sterile saline, was lowered into position to the posterior thalamus/ triangular area (Po/PoT; male, $n = 8$; female, $n = 8$) or the caudal ventrolateral/lateral periaqueductal gray area (vlPAG; male, $n = 10$; female, $n = 8$). The stereotaxic coordinates according to Paxinos and Watson (1997) were: Po, 3.2–4.8 mm caudal to bregma, 2.1–2.4 mm lateral to the midline and depth of 5.0–5.4 mm; and PAG, 7.3–8.0 mm caudal to bregma and 0.8–1.0 mm lateral to midline, depth 5.1–5.7 mm. A single FG (50–80 nl) injection was delivered slowly (~10 nl/min) into the target area from a 1 μ l microsyringe. The micropipette was left in the place for 30 min after injection and the scalp wound was washed with sterile saline and closed with nylon suture.

Temporomandibular joint (TMJ) stimulation

Seven days after FG injection, rats were anesthetized with pentobarbital sodium (60 mg/kg) and half of the animals from each FG injection group was prepared for local stimulation of the TMJ region, while the remaining half remained anesthetized for 2 h without further treatment (referred to as “controls” in the text). For rats receiving TMJ stimulation, the preauricular skin overlying the left TMJ was shaved and a small skin incision was made. The selective small fiber excitant, mustard oil (MO, 20% allyl isothiocyanate in mineral oil, 25 μ l), was injected into the TMJ via a 26-gauge needle. Two hours after MO injection, rats were given a bolus injection of pentobarbital sodium (100 mg/kg, i.p.) and perfused transcardially with 100 ml saline, followed by 4% paraformaldehyde in cold 0.01M phosphate buffered saline (PBS, pH 7.4). The brain and brainstem including upper cervical spinal cord were removed, post-fixed for 2 h and transferred to 30% sucrose (w/v) in 0.01M PBS for 2–3 days at 4°C.

Fos protein/FG double labeling

Transverse frozen sections (50 μ m) were cut at the level of the posterior diencephalon and midbrain to identify injection sites. At the lower brainstem, alternate sections from 2 mm rostral to the obex through the C2 spinal segment were saved, transferred serially to multiwell tissue culture plates containing 0.01M PBS and processed first for the protein product of the immediate early gene, *c-fos* (Fos-LI), and then for FG by immunohistochemistry. Sections were incubated in 1% hydrogen peroxide with 0.75% Triton X-100 (Sigma, St. Louis, MO) in PBS for 90 min, 3% normal goat serum (Sigma) in PBS for 30 min, and reacted with polyclonal rabbit anti-Fos antibody (Ab-5; 1:15,000

dilution, Calbiochem, Gibbstown, NJ) overnight at room temperature with gentle agitation. To visualize Fos-LI, sections were incubated with goat anti-rabbit biotinylated secondary IgG (1:400, 90 min, Vector, Burlingame, CA), peroxidase-conjugated avidin-biotin complex (1:100, 60 min, Vectastain ABC Kit; Vector), 0.04% diaminobenzidine-tetra (DAB substrate Kit DAB-Ni; Vector), 0.2% nickel ammonium sulfate (DAB substrate Kit DAB-Ni; Vector) and 0.006% hydrogen peroxide for 10 min. After Fos-LI, sections were rinsed in PBS followed by 3% normal goat serum in PBS for 30 min, and incubated for overnight with rabbit polyclonal anti-FG antibody (1:20,000, Chemicon, Temecula, CA) at 4 °C. Sections then were washed in PBS (3x), incubated with goat anti-rabbit biotinylated secondary IgG (1:400, 90 min, Vector), peroxidase-conjugated avidin-biotin complex (1:100, 60 min, Vectastain ABC Kit; Vector) at room temperature, 0.04% diaminobenzidine-tetra (DAB substrate Kit DAB-Ni; Vector) and 0.006% hydrogen peroxide without nickel ammonium sulfate for 10 minutes. Sections were rinsed in PBS, mounted on gelatin-coated slides, dehydrated and coverslipped. Fos-LI appeared as darkly stained regularly shaped nuclei, while FG appeared as a brown cytoplasmic reaction product. Omission of primary antisera eliminated specific staining for Fos-LI and FG. Photomicrograph images were captured (Olympus model BX51, Central Valley, PA) and adjusted in Adobe Photoshop 7.0 for brightness and contrast balance. Microinjection sites were reconstructed from fluorescent-labeled tissue (DAPI filter, U-MWU2, Olympus) using an X-Cite epifluorescent attachment (EXFO, Mississauga, Ont).

Data analysis

The sections were mounted serially on slides and cell counts for Fos-LI and FG-positive neurons were made without prior knowledge of treatment. Transverse sections (60–70 per rat) were categorized according to the rostrocaudal level, from +2 mm to –6 mm, relative to the obex. The obex is a surface landmark defined by the caudal end of the fourth ventricle approximately 500 µm rostral to the most caudal tip of the trigeminal subnucleus interpolaris (Yoshida et al. 1991). Rostrocaudal levels were determined based on well-established structural landmarks for the lower brainstem and upper cervical spinal cord of adult rats (Molander et al. 1989; Yoshida et al. 1991; Strassman and Vos, 1993).

Fos- and FG-positive cells were counted at X 40 magnification. Cells counted as Fos-positive had a black regularly shaped nucleus, whereas cells counted as FG-positive had a well defined nucleus, that may or may not have expressed Fos-LI, surrounded by a brown-stained peri-nuclear cytoplasmic region with dendritic processes. Since section thickness (50 µm) exceeded by more than 1.5 the estimated soma size for neurons in the brain regions of interest, no correction was made for cell counts (Clarke 1992). The average number of Fos- and FG-positive cells per section was compared for the following trigeminal brainstem regions: 1) subnucleus interpolaris (Vi, +2 to +1 mm relative to obex); 2) dorsal paratrigeminal region (dPa5, +0.5 to –0.5 mm relative to obex), comprised of cells along the dorsolateral edge of the main body of TBNC plus a nuclear grouping within the spinal trigeminal tract; 3) dorsal half at the subnucleus interpolaris/caudalis transition region (dm-Vi/Vc, +0.5 to –0.5 mm to obex), the most rostral pole of Vc (Yoshida et al. 1991); 4) ventral Vi/Vc transition region (vl-Vi/Vc, +0.5 to –0.5 mm); 5) main portion of Vc (mid-Vc, –1 to –4 mm); and 6) upper cervical dorsal horn (C_{1–2}, –5 to –6 mm). At the Vc and C_{1–2} regions separate counts were made for cells in superficial (I–II) and deeper laminae (III–V) according to established landmarks (Molander et al. 1989). Separate counts were made for cells in nucleus tractus solitarius (NTS, +0.5 to –0.5 mm to obex) and caudal ventrolateral medulla (CVLM, +0.5 to –0.5 mm to obex). Total cell counts were assessed per 1 mm (average cell count per 50 µm section × 20, see Figs 1 and 4) or per brain region (cell counts per 50 µm section × 20 × rostrocaudal dimension in mm, see Tables 1, 2 and 3).

Statistical comparisons were made by ANOVA and individual comparisons by Newman-Keuls after ANOVA.

Results

Fos-like immunoreactivity (Fos-LI) produced by TMJ stimulation

Injection of the small fiber excitant, MO, into the left TMJ produced a distinct rostrocaudal distribution of Fos-positive neurons within the ipsilateral (Fig 1, upper panel) and contralateral TBNC (Fig 1, lower panel) and was equivalent for males and females ($F_{1,16} = 0.11$, $P > 0.10$). This pattern of Fos expression was consistent with earlier results (Bereiter 2001; Okamoto et al. 2008) and confirmed that the low dose E2 regimen used here was not sufficient to enhance TMJ-evoked Fos-LI in the TBNC. Analyses of TMJ-evoked Fos-LI within individual brainstem regions found only minor sex differences (Tables 1, 2 and 3). Controls, i.e., animals that received only FG injections into vIPAG or Po, but no TMJ stimulus, had only scattered Fos-positive neurons in the TBNC, mainly at vl-Vi/Vc, and only rarely were any of these neurons also FG-positive (data not shown).

FG-labeled neurons after vIPAG injections

Microinjections of FG were directed at the vIPAG of the right hemisphere; however, as seen in Fig 2A (see example in Fig 2B), FG spread to regions immediately dorsal to the vIPAG in most cases. The greatest number of FG neurons was seen at TBNC regions near the level of the obex, especially at caudal Vi, and although higher counts were found contralateral to the injection, substantial numbers of FG neurons were seen ipsilaterally (Fig 3A). Analyses of cell counts within the TBNC across all rostrocaudal levels revealed significantly more FG neurons in females than males contralateral ($F_{1,8} = 7.04$, $P < 0.05$), but not ipsilateral ($F_{1,8} = 0.58$, $P > 0.10$) to the vIPAG injection. Within specific TBNC regions contralateral to FG injections females displayed greater numbers in Vi ($F_{1,14} = 9.53$, $P < 0.01$), vl-Vi/Vc ($F_{1,14} = 7.24$, $P < 0.025$, Fig 5A), dPa5 ($F_{1,10} = 9.55$, $P < 0.025$, Fig 4A) and in deep laminae of Vc ($F_{1,8} = 15.08$, $P < 0.005$) than males. No sex differences were seen for FG-labeled neurons in superficial laminae at the Vc and C₁₋₂ regions after vIPAG injections (Table 1, see examples in Figs 5B, 5C). Many FG neurons were found bilaterally in the CVLM and NTS (see examples in Figs 4B, 4C) and in similar numbers for males and females (Table 3). Numerous FG-labeled neurons also were found medial to the TBNC in the lower brainstem after vIPAG injections but were not quantified (Figs 6A1 6B1). The micrographic examples shown in Figures 4 and 5 were from males, however, no qualitative differences in the appearance or general distribution of FG- and/or Fos-labeled neurons were seen in either the vIPAG or Po injection series between males and females.

FG-labeled neurons after Po injections

Microinjections of FG directed at Po also included the posterior thalamic triangular area (PoT) in most cases (see Fig 8A and example in Fig 8B). The greatest number of FG-labeled neurons was found at peribex regions of the TBNC, especially Vi, with the vast majority being contralateral to the Po injection (Fig 3B). Comparison of FG cell counts across all rostrocaudal levels of the TBNC revealed no significant sex differences contralateral ($F_{1,6} = 0.74$, $P > 0.10$) or ipsilateral ($F_{1,6} = 0.48$, $P > 0.10$) relative to the Po injection. However, within specific TBNC regions contralateral to FG injections males had significantly more FG neurons in superficial laminae of Vc ($F_{1,12} = 13.7$, $P < 0.005$), superficial laminae at C₁₋₂ ($F_{1,12} = 5.1$, $P < 0.05$) and deep laminae at C₁₋₂ ($F_{1,12} = 7.25$, $P < 0.025$) than females. No sex differences were seen for FG-labeled neurons in peribex regions of the TBNC (Table 2). Many FG neurons were found bilaterally in the CVLM and NTS after Po injections and in equal numbers for males and females (Table 3).

The overall results of single-labeled FG neurons after vIPAG versus Po injections revealed several notable features. First, the average total number of FG-labeled neurons found bilaterally within the TBNC after Po injection (3245 ± 296 cells per rat, $n = 16$) was significantly greater than after vIPAG injection (2053 ± 149 cells per rat, $n = 18$; $F_{1,32} = 13.8$, $P < 0.001$). Second, the pattern of FG-labeled neurons within the TBNC indicated a predominantly contralateral projection to the Po, while the projection to the vIPAG was more bilateral. Third, the average total number of FG-labeled neurons found bilaterally within the CVLM after vIPAG injection (414 ± 23 cells per rat, $n = 18$) was more than twice that seen after Po injection (168 ± 13 cells per rat, $n = 16$, $F_{1,32} = 81.1$, $P < 0.001$). Similarly, the average total number of FG-labeled neurons found bilaterally within the NTS after vIPAG injection (796 ± 78 cells per rat, $n = 18$) was more than twice that seen after Po injections (225 ± 34 cells per rat, $n = 16$, $F_{1,32} = 43.5$, $P < 0.001$). As seen in Table 3 projections from the CVLM and NTS to either the vIPAG or Po were bilateral. Lastly, numerous FG-labeled neurons were seen in reticular areas medial to the TBNC after vIPAG injection (Fig 6A1, B1), whereas very few were seen in these areas after Po injection (Fig 9A1, B1).

Fos/FG double-labeled neurons after vIPAG injections

The number of Fos/FG double-labeled neurons after TMJ stimulation and vIPAG injection was greater in females than males at the vl-Vi/Vc transition ($F_{1,14} = 6.59$, $P < 0.05$) and the dPa5 region ($F_{1,14} = 10.4$, $P < 0.01$) ipsilateral to the TMJ stimulus, but not at other regions of the lower brainstem (Tables 1 and 3). However, when expressed as a percentage of FG neurons that also were Fos-positive there were no sex differences at any region (Fig 7A, 7B). Conversely, when Fos-positive neurons were expressed as a percentage of FG-labeled cells, the percentage found at the dPa5 was significantly greater in females than males ($F_{1,14} = 5.59$, $P < 0.05$; Fig 7C), despite a similar number of Fos-positive neurons alone. Numerous Fos/FG double-labeled neurons also were found at the CVLM and NTS (Figs 6A2, 6B2); however, no sex differences were noted for these regions (Table 3, upper panels). These data suggested that TMJ-responsive neurons at periobex regions of the TBNC were more likely to project to the vIPAG in females than males, whereas those at Vc and C₁₋₂ were equally likely to project to vIPAG.

Fos/FG double-labeled neurons after Po injections

The number of Fos/FG double-labeled neurons seen after TMJ stimulation and Po injection was greater for males than females at superficial laminae of Vc ($F_{1,12} = 24.7$, $P < 0.001$) ipsilateral to the TMJ stimulus, but not at other regions of the lower brainstem (Tables 2 and 3). However, when expressed as a percentage of FG neurons that also were Fos-positive there were no sex differences at any region (Fig 10A, 10B). By contrast, the percentage of Fos-positive neurons at superficial laminae of Vc that also were FG-labeled cells was significantly higher in males than females ($F_{1,12} = 24.7$, $P < 0.001$; Fig 10C, 10D). As noted above for vIPAG injections, nearly all Fos/FG double-labeled neurons after Po injections located outside the borders of the TBNC were within the CVLM and NTS (Figs 9A2, 9B2). The number of Fos/FG double-labeled cells in CVLM and NTS was similar numbers for males and females (Table 3, lower panels). It was further noted that the number of Fos/FG double-labeled neurons in CVLM ($F_{3,14} = 11.6$, $P < 0.001$) and NTS ($F_{3,14} = 9.05$, $P < 0.005$) after Po injection was less than that seen after vIPAG injections. These data suggested that TMJ-responsive neurons in superficial laminae at Vc were more likely to project to Po in males than females, while projections from periobex regions of the TBNC were equally likely in males and females.

Discussion

These data indicated significant sex differences in number of neurons projecting to the vlPAG and Po from different levels of the TBNC. Projections to vlPAG from peribex regions of the TBNC (i.e., dPa5, vl-Vi/Vc) were significantly greater in females than males, whereas those projecting to the Po from more caudal portions of the TBNC (i.e., Vc and C₁₋₂), were greater in males. Results from Fos/FG double labeling indicated TMJ-responsive neurons at the dPa5 were more likely to project to vlPAG in females than males, whereas TMJ-responsive neurons in superficial laminae at Vc were more likely to project to Po in males than females.

TMJD is the most common form of non-dental pain and cognitive, psychophysical and autonomic factors likely contribute to sensory dysfunction (Diatchenko et al. 2006; Maixner 2009). Animal experiments designed to investigate the basis for TMJ nociceptive processing in the lower brainstem have been limited by two main factors. First, the innervation of the TMJ region is complex consisting of trigeminal, vagal and upper cervical sensory fibers (Uddman et al. 1998; Casatti et al. 1999) that converge and terminate at multiple levels of the TBNC (Nishimori et al. 1986; Capra 1987; Shigenaga et al. 1988; Dessem et al. 2007). Fos-LI coupled with anatomical tracing is well suited for this analysis since it allows quantification and functional mapping of complex networks at the single neuron level (Hunt 1987; Menetrey et al. 1989). Second, despite evidence that persistent TMJD is more prevalent in females than males (see Bereiter and Okamoto 2011), the vast majority of studies have used only male animals. Sex-specific differences in modulatory pathways for trigeminal (Nag and Mokha 2006) and spinal nociception (Tershner et al. 2000; Mitrovic et al. 2003; Mogil et al. 2003) are well documented. However, few studies have compared ascending spinal pathways related to nociceptive processing for possible sex differences (Murphy et al. 2009) and none have involved trigeminal pathways.

The organization of afferent input to the TBNC is quite different from spinal cord in that trigeminal afferents project to multiple, and spatially discrete, regions of the TBNC, while spinal afferents terminate in contiguous spinal segments (Takemura et al. 2006; Bereiter et al. 2009). Several studies using Fos-LI as a neuronal activity marker found that acute stimulation of the TMJ region (Hathaway et al. 1995; Zhou et al. 1999) or masticatory muscles (Imbe et al. 1999; Ikeda et al. 2003; Ro et al. 2004) produced a distinct labeling pattern in the TBNC of male rats characterized by high density labeling in superficial laminae of caudal Vc and C₁₋₂ ipsilateral to the stimulus, bilateral labeling at the vl-Vi/Vc transition and lesser, though significant, labeling in the ipsilateral dPa5. Although the functional significance of Fos-LI produced at these different TBNC regions is not yet certain, converging lines of evidence based on stimulus-effect curves, response to analgesic drugs and efferent projection targets have greatly aided data interpretation. Stimulus intensity encoding, seen as increased Fos-LI, was most pronounced in superficial laminae of Vc and C₁₋₂, to a lesser extent at dPa5, but was not seen at the vl-Vi/Vc transition region (Bereiter 2001; Okamoto et al. 2008). Analgesic drugs markedly reduced Fos-LI in superficial laminae of Vc and C₁₋₂ after TMJ or jaw muscle stimulation, while only weak or no reduction was seen at vl-Vi/Vc and dPa5 (Bereiter and Bereiter 2000; Ro et al. 2004). The present study found that ~12% of Fos-positive neurons in superficial laminae of Vc projected to the contralateral Po in males compared to < 4% of neurons in females. Previously, TMJ input to neurons in superficial but not deeper laminae of Vc/C₁₋₂ was significantly affected by estrogen status based on Fos-LI (Bereiter 2001) and single unit recording data (Tashiro et al. 2007). Since we held estrogen status low in this study, a sex-specific difference in the magnitude of ascending projections from caudal Vc to Po was indicated. These data also revealed nearly twice the number of FG-labeled neurons in superficial compared to deep laminae at Vc and C₁₋₂ in males, whereas FG-labeled neurons

were more equally distributed across superficial and deeper laminae in females (see Table 2). Thus, one interpretation of these data was that ascending projections to Po from Vc were more widely distributed across laminae in females than males. Anterograde tracing studies also found that medullary or spinal dorsal horn projections to Po were more dense after injection into superficial versus deeper laminae in male rats (Iwata et al. 1992; Gauriau and Bernard 2004a). Similarly, antidromic stimulation of Po activated a higher percentage of spinal dorsal horn neurons in superficial than deep laminae in male rats (Zhang and Giesler 2005). Although neurons in superficial and deep laminae likely serve different aspects of nociception (Braz et al. 2005; Eckert et al. 2006), it is not yet known if sex differences impact this contribution. A notable finding was the dense projection to Po from Vc and C₁₋₂ in this study versus the sparse projection to VPM seen after masseter muscle inflammation (Ikeda et al. 2003). This agreed well with previous recording studies in which antidromic stimulation in Po, but not in the ventral posterior medial thalamic nucleus, was able to activate TMJ-responsive neurons in superficial laminae of caudal Vc in male rats (Takeshita et al. 2001).

In contrast to results after Po injection in which projections from caudal laminated portions of the TBNC were generally greater in males, vIPAG injects resulted in greater FG-labeling of TBNC neurons, mainly at peribex levels, in females than males. Females displayed more single labeled FG neurons in caudal Vi, vl-Vi/Vc and dPa5 and more double-labeled Fos/FG neurons in vl-Vi/Vc and dPa5 than males. The present study also confirmed previous results that the marginal zone of caudal Vc projected substantially to the vIPAG in male rats (Menetrey et al. 1982; Clement et al. 2000; however, see Beitz 1982). The role of the vl-Vi/Vc in deep craniofacial pain remains uncertain. The Fos-LI response at the vl-Vi/Vc and caudal Vc after TMJ and masseter muscle stimulation differed markedly and suggests different contributions to nociception. First, intensity coding, as seen by enhanced Fos-LI with increasing TMJ stimulus intensity, was weak at the vl-Vi/Vc transition, while proportional increases were seen at the caudal Vc (Bereiter 2001; Okamoto et al. 2008). Second, analgesic drug treatment reduced slightly (Bereiter and Bereiter 2000) or not at all the Fos-LI response to noxious stimuli at vl-Vi/Vc, while Fos-LI at caudal Vc was markedly reduced (Ro et al. 2004; Okamoto et al. 2007). Third, TMJ-evoked Fos-LI at the vl-Vi/Vc transition was similar for males and females, while high estrogen states greatly enhanced Fos-LI at the caudal Vc (Bereiter 2001). Similarly, among OvX females treated with high or low dose E2, Fos-LI produced at caudal Vc after TMJ stimulation was enhanced compared low dose E2 treatment, while Fos-LI at the vl-Vi/Vc was not affected (Okamoto et al. 2008). Fourth, Fos-LI was produced bilaterally at the vl-Vi/Vc in the absence of overt TMJ or muscle stimulation by different anesthetic regimens (Strassman and Vos 1993; Zhou et al. 1999). Although a few Fos-positive neurons were produced in the absence of somatic stimulation, none were found to project to higher brain centers (Ikeda et al. 2003; present study). Collectively, these results suggested that TMJ-responsive neurons at the vl-Vi/Vc do not contribute directly to sensory-discriminative aspects of TMJ nociceptive processing.

Possible alternative roles for TMJ-responsive neurons at vl-Vi/Vc that project to vIPAG include recruitment endogenous pain controls and control of autonomic and homeostatic reflexes. Dysfunction in endogenous pain controls has been proposed as a contributing factor to pain expression in TMJD (King et al. 2009). Both the total number of single-labeled FG neurons and Fos/FG double-labeled neurons after vIPAG injection at the vl-Vi/Vc were greater in females than males (Table 1). Loyd and Murphy (2006) found that the magnitude of direct projections between the vIPAG and RVM was greater in females than males; however, interestingly, the magnitude of Fos/FG double-labeled PAG-RVM projection neurons after noxious hindlimb stimulation was greater in males. Jaw muscle inflammation produced Fos-LI at vl-Vi/Vc neurons, some of which projected directly to the RVM (Sugiyo et al. 2005) and thalamic nucleus submedius in male rats (Ikeda et al. 2003),

consistent with the notion that the vl-Vi/Vc is involved in pain and/or autonomic modulation. Indeed, direct inhibition of vl-Vi/Vc reduced secondary hyperalgesia in male rats after jaw muscle inflammation (Shimizu et al. 2009), while blockade of the vl-Vi/Vc inhibited cardiorespiratory reflexes to nasal stimulation (Panneton and Yavari 1995) and prevented ocular surface stimulus-evoked lacrimation (Hirata et al. 2004). These studies suggested that the vl-Vi/Vc transition likely contributes to trigeminal nociception by recruitment of endogenous pain and autonomic control circuits.

The number of FG single-labeled and Fos/FG double-labeled neurons after vlPAG injection at the dPa5 was greater in females than males (Table 1). The afferent and efferent organization of the dPa5 region differs considerably from the vl-Vi/Vc transition in that it receives direct sensory input from trigeminal, upper cervical and vagus nerve fibers and projects to multiple medullary and pontine regions that control autonomic outflow including the NTS and CVLM (Menetrey and Basbaum 1987; Saxon and Hopkins 1998; Caous et al. 2001). Similar to the response at caudal Vc, TMJ-evoked Fos-LI at the dPa5 increased with greater stimulus intensity, was mainly ipsilateral to the stimulus and was enhanced by high estrogen conditions (Bereiter 2001; Okamoto et al. 2008). Lesion of the dPa5 prevented cardiovascular reflex responses to noxious stimuli (Yu et al. 2002), while recording studies found that dPa5 neurons encoded facial skin stimulus intensity and became sensitized after TMJ injury (Yamazaki et al. 2008). Although the dPa5 likely is critical for somatic autonomic integration in the trigeminal system (Saxon and Hopkins 1998; Caous et al. 2001), these studies were performed in male animals, and thus, it is not known if sex differences influence the contribution of dPa5 to TMJ nociceptive processing.

The significance of greater projections from periobex regions of the TBNC to the vlPAG in females compared to males is not known. However, control of endogenous pain regulatory systems and cardiovascular function are closely linked (Lovick 1993; Bruehl and Chung 2004) and altered control of autonomic function is a feature of several chronic pain conditions that are more prevalent in women than men such as Raynaud's disease (Heidrich 2010) and complex regional pain syndrome (Schwartzman et al. 2009) in addition to TMJD (Bragdon et al. 2002; Mohn et al. 2008). In animal studies, trigeminal nerve-evoked changes in masseter muscle blood flow were significantly different in females and male rats (Ishii et al. 2009); however, the basis for this difference is not certain.

In summary, sex differences in FG-labeled neurons observed after vlPAG injections were mainly at periobex regions (Vi, vl-Vi/Vc, dPa5), while sex differences after Po injections were seen only in the laminated portions of the TBNC (Vc and C₁₋₂). It is well established that women are more likely than men to develop several craniofacial pain conditions including TMJD (Bereiter and Okamoto 2011). Although the basis for this female prevalence is not known, the present study suggests that differences in ascending projections from second-order neurons in TBNC to higher brain centers that integrate nociceptive information may contribute.

Highlights

Projections of TMJ-responsive brainstem neurons were different in males and females.

Projections to PAG were greater in females than males.

Projections to posterior thalamus were greater in males than females.

Abbreviations

CVLM	caudal ventrolateral medulla
C₁₋₂	upper cervical spinal cord
dPa5	dorsal paratrigeminal region
E2	estradiol
FG	Fluorogold
Lam I/II	superficial laminae
Lam III/V	deep laminae
MO	mustard oil (allyl isothiocyanate)
NTS	nucleus tractus solitarii
OvX	ovariectomized
Po	posterior thalamic nucleus
RVM	rostroventromedial medulla
TBNC	trigeminal brainstem nuclear complex
TMJ	temporomandibular joint
Vc	trigeminal subnucleus caudalis
Vi	trigeminal subnucleus interpolaris
Vi/Vc	trigeminal interpolaris/caudalis transition region
vIPAG	ventrolateral periaqueductal gray

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Figure 1.

Average number of Fos-positive neurons produced in the caudal trigeminal brainstem nuclear complex (TBNC) and upper cervical dorsal horn after unilateral injection of the small fiber excitant, mustard oil (20% solution, 25 μ l), into the temporomandibular joint (TMJ) region of male and female rats. Results expressed as average total number of cells per mm ($\pm$ SEM) as a function of distance from the obex. * $P < 0.05$, ** $P < 0.01$ ipsilateral versus contralateral. $N = 8-10$ rats per group.

Figure 2.

Fluorogold (FG) injection sites in the periaqueductal gray (PAG). A. Drawings display the spread of FG (shaded regions) for injections in males (PAGM1–6) and female rats (PAGF1–4). Each vertical column represents the results for one experimental animal. Numbers to the left of the first column indicate the rostrocaudal distance from bregma according to the rat atlas of Paxinos and Watson (1997). B. Micrograph example of an injection site in the PAG. Abbreviations: AQ, aqueduct; DR, dorsal raphe; DLPAG, dorsolateral PAG; LPAG, lateral PAG; SCP, superior cerebellar peduncle; VLPAG, ventrolateral PAG. Calibration = 200 μm .

Figure 3.

Average total number of FG-positive neurons per mm within the TBNC and upper cervical dorsal horn of male and female rats. A. Total number of FG neurons after vIPAG injections of FG. B. Total number of FG neurons after PO injections of FG. Abbreviations: contra, contralateral to injection; ipsi, ipsilateral to injection. * $P < 0.05$, ** $P < 0.01$ ipsilateral versus contralateral; a = $P < 0.05$, b = $P < 0.01$ male versus female. N = 8–10 rats per group.

Figure 4.

Examples of Fos, FG and Fos/FG double-labeled neurons from a male rat after vIPAG injection of FG and TMJ stimulation with mustard oil. A. Field view of the dorsal paratrigeminal region (dPa5). A1–3: enlarged images of numbered regions in A. B. Field view of the commissural nucleus tractus solitarius (NTS). B1–3: enlarged images of numbered regions in B. C. Field view of the caudal ventrolateral medulla (CVLM). C1–3: enlarged images of numbered regions in C. Symbols: + = Fos, # = FG, and * = Fos/FG double-labeled neuron. All examples are from the side ipsilateral to the TMJ stimulus. Calibration: low magnification (A, B, C) = 100 μ m; high magnification (A1, A2, and A3) = 50 μ m.

Figure 5.

Examples of Fos, FG and Fos/FG double-labeled neurons from a male rat after vIPAG injection of FG and TMJ stimulation with mustard oil. A. Field view of the ventral interparietal/caudalis transition (vl-Vi/Vc) transition region. A1–3: enlarged images of numbered regions in A. B. Field view of superficial laminae of Vc. B1–3: enlarged images of numbered regions in B. C. Field view of the upper cervical dorsal horn. C1–3: enlarged images of numbered regions in C. Symbols: + = Fos, # = FG, and * = Fos/FG double-labeled neuron. All examples are from the side ipsilateral to the TMJ stimulus. Calibration: low magnification (A, B, C) = 100 μ m; high magnification (A1, A2, and A3) = 50 μ m.

Figure 6. Camera lucida drawing of an example of Fos, FG and Fos/FG double-labeled neurons in the caudal brainstem and upper cervical spinal cord after FG injection into the vlPAG. A. Example from a male rat. A1, Fos (●) and FG (Δ) labeling; A2, Fos/FG double-labeled neurons (*). B. Example from a female rat. B1, Fos (●) and FG (Δ) labeling; B2, Fos/FG double-labeled neurons (*). Numbers to the side of the right most column refer to the distance in mm relative to obex. All drawings are from the side ipsilateral to the TMJ stimulus.

Figure 7. Double-labeled cells after vlPAG injection and TMJ stimulation in males (M) and females (F). A. Percentage of FG-labeled neurons also labeled for Fos in vl-Vi/Vc and dPa5. B. Percentage of FG-labeled neurons also labeled for Fos in superficial laminae of Vc and C₁₋₂. C. Percentage of Fos-positive neurons also labeled for FG in vl-Vi/Vc and dPa5. D. Percentage of Fos-positive neurons also labeled for FG in superficial laminae of Vc and C₁₋₂. *P < 0.05, **P < 0.01 ipsilateral versus contralateral; a = P < 0.05 male versus female. Ipsi and contra refer to ipsilateral and contralateral to the TMJ stimulus.

Figure 8.

Fluorogold injection sites in posterior nucleus of the thalamus (Po). A. Drawings display the spread of FG (shaded regions) for injections in males (PoM1–4) and E2-treated female rats (PoF1–4). Each vertical column represents the results for one experimental animal. Numbers to the left of the first column indicate the rostrocaudal distance from bregma according to the rat atlas of Paxinos and Watson (1997). B. Micrograph example of an injection site in the Po. Abbreviations: CL, centrolateral thalamic n.; LD, lateral dorsal thalamic n.; Po, posterior thalamic n.; VAL, ventral anterolateral n.; VPM, ventral posterior medial thalamic n.; VPL, ventral posterior lateral thalamic n. Calibration = 200 μ m.

Figure 9.

Camera lucida drawing of an example of Fos, FG and Fos/FG double-labeled neurons in the caudal brainstem and upper cervical spinal cord after FG injection into the PO. A. Example from a male rat. A1, Fos (●) and FG (Δ) labeling; A2, Fos/FG double-labeled neurons (*). B. Example from a female rat. B1, Fos (●) and FG (Δ) labeling; B2, Fos/FG double-labeled neurons (*). Numbers to the side of the right most column refer to the distance in mm relative to obex. All drawings are from the side ipsilateral to the TMJ stimulus.

Figure 10.

Double-labeled cells after Po injection and TMJ stimulation in males (M) and females (F).

A. Percentage of FG-labeled neurons also labeled for Fos in vl-Vi/Vc and dPa5. B.

Percentage of FG-labeled neurons also labeled for Fos in superficial laminae of Vc and

C₁₋₂. C. Percentage of Fos-positive neurons also labeled for FG in vl-Vi/Vc and dPa5. D.

Percentage of Fos-positive neurons also labeled for FG in superficial laminae of Vc and

C₁₋₂. *P < 0.05, **P < 0.01 ipsilateral versus contralateral. Ipsi and contra refer to ipsilateral and contralateral to the TMJ stimulus.

Table 1

Total cell counts for Fos/FG double-labeled, Fos-positive, and FG-positive neurons in rats that received Fluorogold injection into ventrolateral PAG and TMJ stimulation.

	Vi	dm-Vi/Vc	vl-Vi/Vc	dPa5
Male-ipsi				
Fos/FG	6.2 ± 2.6	2.1 ± 1.0	8.0 ± 1.7	2.8 ± 1.3
Fos	356 ± 93.1	200.3 ± 35.3	193 ± 47.8	254 ± 56.8
FG	472 ± 51.8	105.7 ± 11.5	69.3 ± 6.0	9.33 ± 3.0
Female-ipsi				
Fos/FG	8.0 ± 2.8	1.1 ± 0.6	19.0 ± 5.5 ^a	12.0 ± 4.3 ^b
Fos	199 ± 116.5	117 ± 18.7	277 ± 18.7	290 ± 56.7
FG	764 ± 50.3 ^b	131 ± 10.4	93.0 ± 10.7 ^a	41.0 ± 14.2 ^a
Male-contra				
Fos/FG	0.0 ± 0.0	0.8 ± 1.0	3.1 ± 2.2	0.0 ± 0.0
Fos	172 ± 65.2 ^{**}	94.1 ± 21.4 ^{**}	74.3 ± 17.4 [*]	156 ± 37.3
FG	370 ± 70.6	79.3 ± 16.2	48.7 ± 3.0 [*]	5.3 ± 1.7
Female-contra				
Fos/FG	1.3 ± 1.0	0.7 ± 0.5	5.1 ± 1.3 ^{**}	3.1 ± 1.9 ^{**}
Fos	64.0 ± 32.1	92.5 ± 42.1	65.5 ± 5.7 ^{**}	179 ± 23.6
FG	355 ± 81.4 ^{**}	92.0 ± 36.7 [*]	58.5 ± 10.8 ^{**}	27.0 ± 10.3 [*]
	Vc I-II	Vc III-V	C ₁₋₂ I-II	C ₁₋₂ III-V
Male-ipsi				
Fos/FG	62.3 ± 24.3	2.0 ± 0.1	48.7 ± 10.2	0.7 ± 0.7
Fos	814 ± 252	149 ± 7.3	1415 ± 116	194.7 ± 23.0
FG	412 ± 45.4	165 ± 22.0	103 ± 23.7	173.3 ± 21.6
Female-ipsi				
Fos/FG	84.0 ± 34.7	0.0 ± 0.0	46.0 ± 7.4	0.0 ± 0.0
Fos	1082 ± 323	255 ± 27.2	1572 ± 23.4	205 ± 23.8
FG	433 ± 23.4	298 ± 29.5 ^b	130 ± 20.8	165 ± 11.2
Male-contra				
Fos/FG	18.0 ± 10.9 [*]	0.0 ± 0.0	2.2 ± 0.9 ^{**}	0.0 ± 0.0
Fos	335 ± 46.4 [*]	64.0 ± 9.8	138 ± 24.5 ^{**}	48.7 ± 5.4
FG	210 ± 18.8 ^{**}	64.1 ± 9.6 ^{**}	18.7 ± 9.6 ^{**}	63.3 ± 3.2 ^{**}
Female-contra				
Fos/FG	7.4 ± 5.7 ^{**}	0.0 ± 0.0	6.1 ± 3.5 ^{**}	0.0 ± 0.0
Fos	208 ± 52.6 ^{**}	84.0 ± 6.6	168 ± 27.7 ^{**}	60.0 ± 2.6
FG	227 ± 89.1 [*]	93.0 ± 10.1 ^{**}	40.0 ± 14.9 ^{**}	66.0 ± 5.5 ^{**}

Total cell counts per brain region, mean ± SEM. See Methods for number of sections/ CNS region. N = 4–6 rats per treatment group. Ipsi = ipsilateral to TMJ stimulus (left side); contra = contralateral to TMJ stimulus (right side). FG injected into PAG contralateral to TMJ stimulus.

* $P < 0.05$,

** $P < 0.01$ ipsilateral versus contralateral;

^a $= P < 0.05$,

^b $= P < 0.01$ male versus female.

Table 2

Total cell counts for Fos/FG double-labeled, Fos-positive, and FG-positive neurons for rats that received Fluorogold injection into posterior thalamus and TMJ stimulation.

	Vi	dm-Vi/Vc	vl-Vi/Vc	dPa5
Male-ipsi				
Fos/FG	12.1 ± 5.9	5.6 ± 4.3	6.6 ± 3.0	6.5 ± 4.0
Fos	330 ± 146	178 ± 43.4	225 ± 49.3	182 ± 22.8
FG	1987 ± 354	551 ± 105	171 ± 13.1	64.0 ± 25.5
Female-ipsi				
Fos/FG	0.0 ± 0.0	0.6 ± 0.5	6.0 ± 1.8	10.1 ± 4.2
Fos	49.0 ± 31.7	65.5 ± 21.5	160.0 ± 45.3	211 ± 9.0 ^a
FG	1631 ± 629	536 ± 185	124.0 ± 45.8	56.0 ± 7.5
Male-contra				
Fos/FG	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.1	0.0 ± 0.0 [*]
Fos	146 ± 104	105 ± 43.7 [*]	103 ± 65.5 [*]	32.0 ± 16.6 ^{**}
FG	160 ± 84.9 [*]	72.9 ± 16.5 [*]	24.5 ± 10.8 ^{**}	8.1 ± 3.3 ^{**}
Female-contra				
Fos/FG	1.3 ± 1.0	0.6 ± 0.5	2.2 ± 2.0	3.1 ± 1.2 [*]
Fos	83.0 ± 69.8	56.5 ± 48.6	108 ± 34.7	69.1 ± 37.7 ^{**}
FG	250 ± 76.9 [*]	57.0 ± 17.4 [*]	42.0 ± 16.3 [*]	26.0 ± 9.6 [*]
	Vc I-II	Vc III-V	C ₁₋₂ I-II	C ₁₋₂ III-V
Male-ipsi				
Fos/FG	84.0 ± 13.1 ^b	0.0 ± 0.0	53.0 ± 16.4	0.0 ± 0.0
Fos	830 ± 250	221 ± 22.7	1704 ± 252	269 ± 44.1
FG	693 ± 35.2 ^b	393 ± 37.7	118 ± 17.9 ^a	163 ± 20.1 ^a
Female-ipsi				
Fos/FG	26.0 ± 3.5	0.0 ± 0.0	19.0 ± 5.9	0.0 ± 0.0
Fos	699 ± 72.8	197 ± 29.5	1591 ± 121	120 ± 10.7
FG	335 ± 112	295 ± 71.2	88.0 ± 2.3	106 ± 19.2
Male-contra				
Fos/FG	1.6 ± 1.6 ^{**}	0.0 ± 0.0	0.0 ± 0.0 ^{**}	0.0 ± 0.0
Fos	213 ± 60.9 ^{**}	86.0 ± 3.4	68.0 ± 42.0 ^{**}	47.0 ± 2.8
FG	90.0 ± 11.6 ^{**}	50.0 ± 3.4 ^{**}	9.1 ± 5.9 ^{**}	42.0 ± 5.0 ^{**}
Female-contra				
Fos/FG	1.6 ± 1.0 ^{**}	0.0 ± 0.0	2.0 ± 1.2 ^{**}	0.0 ± 0.0
Fos	180 ± 54.5 ^{**}	82.0 ± 8.1	129 ± 16.7 ^{**}	55.1 ± 2.6
FG	122 ± 22.9 [*]	75.1 ± 11.1 ^{**}	8.2 ± 3.4 ^{**}	64.8 ± 10.0

Total cell counts per brain region, mean ± SEM. See Methods for number of sections/CNS region. N = 4–6 rats per treatment group. Ipsi = ipsilateral to TMJ stimulus (left side); contra = contralateral to TMJ stimulus (right side). FG injected into PO contralateral to TMJ stimulus.

* $P < 0.05$,

** $P < 0.01$ ipsilateral versus contralateral;

^a $= P < 0.05$,

^b $= P < 0.01$ male versus female.

Table 3

Total cell counts for Fos/FG double-labeled, Fos-positive, and FG-positive neurons in the CVLM and NTS after Fluorogold injections into vIPAG or PO and TMJ stimulation.

PAG	CVLM	NTS
Male-ipsi		
Fos/FG	98.0 ± 12.1 ^b	48.0 ± 11.4 ^a
Fos	369 ± 31.5	515 ± 57.3
FG	211 ± 23.7	197 ± 47.3
Female-ipsi		
Fos/FG	86.0 ± 12.4 ^a	60.0 ± 10.2 ^a
Fos	350 ± 11.9	600 ± 119
FG	228 ± 25.9	282 ± 84.7
Male-contra		
Fos/FG	82.7 ± 12.6 ^a	107 ± 28.3 ^{b **}
Fos	293 ± 27.5	595 ± 89.6
FG	157 ± 16.1 [*]	493 ± 118 ^{**}
Female-contra		
Fos/FG	83.0 ± 22.2 ^a	152 ± 9.4 ^{b **}
Fos	329 ± 16.7	615 ± 98.6
FG	202 ± 25.8	644 ± 116 ^{**}
PO	CVLM	NTS
Male-ipsi		
Fos/FG	37.0 ± 9.2	12.0 ± 2.3
Fos	406 ± 19.4	437 ± 18.4
FG	100 ± 25.1	73.0 ± 13.4
Female-ipsi		
Fos/FG	32.0 ± 1.6	12.0 ± 1.6
Fos	410 ± 29.1	424 ± 142
FG	92.4 ± 6.6	61.0 ± 7.7
Male-contra		
Fos/FG	21.0 ± 5.7 [*]	24.0 ± 8.2 [*]
Fos	355 ± 41.6	415 ± 46.0
FG	66.0 ± 15.7	103 ± 35.2
Female-contra		
Fos/FG	22.0 ± 2.6 [*]	24.0 ± 4.3
Fos	416 ± 63.3	420 ± 115
FG	69.0 ± 2.5	80.0 ± 21.5

Total cell counts per brain region, mean ± SEM. See Methods for number of sections/CNS region. N = 4–6 rats per treatment group. Ipsi = ipsilateral to TMJ stimulus (left side); contra = contralateral to TMJ stimulus (right side). FG injected into PAG and PO on the right side.

**
P < 0.01 ipsilateral versus contralateral;

^a = $P < 0.05$,

^b = $P < 0.01$ versus similar group in Po injection series.