RESEARCH ARTICLE

Anatomical Evidence of Pruriceptive Trigeminothalamic and Trigeminoparabrachial Projection Neurons in Mice

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ABSTRACT

Itch is relayed to higher centers by projection neurons in the spinal and medullary dorsal horn. We employed a double-label method to map the ascending projections of pruriceptive and nociceptive trigeminal and spinal neurons. The retrograde tracer fluorogold (FG) was stereotaxically injected into the right thalamus or lateral parabrachial area (LPb) in mice. Seven days later, mice received intradermal (id) microinjection of histamine, chloroquine, capsaicin, or vehicle into the left cheek. Histamine, chloroquine, and capsaicin intradermally elicited similar distributions of Fos-positive neurons in the medial aspect of the superficial medullary and spinal dorsal horn from the trigeminal subnucleus caudalis to C2. Among neurons retrogradely labeled from the thalamus, 43%, 8%, and 22% were Fos-positive following id histamine, chloroquine, or capsaicin. Among the Fos-positive neurons following pruritic or capsaicin stimuli, ~1–2% were retrogradely labeled with FG. Trigeminoparabrachial projection neurons exhibited a higher incidence of double labeling in the superficial dorsal horn. Among the neurons retrogradely labeled from LPb, 36%, 29%, and 33% were Fos positive following id injection of histamine, chloroquine, and capsaicin, respectively. Among Fos-positive neurons elicited by id histamine, chloroquine, and capsaicin, respectively, 3.7%, 4.3%, and 4.1% were retrogradely labeled from LPb. The present results indicate that, overall, relatively small subpopulations of pruriceptive and/or nociceptive neurons innervating the cheek project to thalamus or LPb. These results imply that the vast majority of pruritogen- and algogen-responsive spinal neurons are likely to function as interneurons relaying information to projection neurons and/or participating in segmental nocifensive circuits. J. Comp. Neurol. 000:000–000, 2015.

INDEXING TERMS: itch (pruritus); pain; trigeminal projection neurons; double label; Fos; superficial dorsal horn; interneurons

The molecular biology and neural processing of itch have been a topic of considerable recent interest (Akiyama and Carstens, 2013; Tominaga and Takamori, 2013; Kremer et al., 2014; LaMotte et al., 2014). The sensation of itch is thought to be conveyed together with pain via the spinothalamic tract (Bickford, 1938; Hyndman and Wolkin, 1943; White et al., 1950; Davidson et al., 2014). The spinothalamic tract originates largely, based on retrograde tracing studies, from neurons in lamina I as well as deeper laminae in mice (Davidson et al., 2010), rats (Giesler et al., 1976; Burstein et al., 1990b), and monkeys (Trevino et al., 1973). An early electrophysiological study indicated that a subpopulation of axons in the ventrolateral spinal cord, where spinothalamic tract fibers pass, responded to cutaneous application of cowhage spicules (Wei and Tuckett, 1991), which contain proteases that elicit itch via protease-activated receptors PAR2 and PAR4 (Reddy et al., 2008). A later study identified a small subpopulation of lamina I spinothalamic tract neurons in the cat that responded to cutaneous application of histamine (Andrew and Craig, 2001). More recent studies have identified a more sizable subpopulation of

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primate spinothalamic tract neurons in lamina I and deeper laminae that responded to intradermal injection of histamine or application of cowhage spicules as well as capsaicin (Simone et al., 2004: Davidson et al., 2007, 2012). Neurons in lamina I are of particular interest in the transmission of chemogenic itch and pain because intradermal injection of pruritogens and algogens elicits Fos expression (a marker of neuronal activity (Harris, 1998) mainly in superficial laminae of the spinal dorsal horn (Yao et al., 1992; Carstens et al., 1995; Jinks et al., 2002; Nojima et al., 2003; Nakano et al., 2008; Akiyama et al., 2009).

A recent study used antidromic stimulation in rats to identify a subpopulation of trigeminothalamic neurons in superficial and deeper dorsal horn laminae that responded to intradermal cheek microinjection of the pruritogen serotonin as well as other pruritogens and algogens, including histamine, chloroquine (which elicits itch in humans and acts at the Mas-related G-protein-coupled receptor MrgprA3) (Liu et al., 2009: Han et al., 2013), capsaicin, and allyl isothiocyanate (AITC; Moser and Giesler, 2014). These response properties were similar to those of neurons in trigeminal subnucleus caudalis and upper cervical spinal, not identified by ascending projection, that responded to serotonin and other chemicals in rats (Klein et al., 2011) and mice (Akiyama et al., 2010). The characterization of neurons with cheek receptive fields is particularly relevant to the behavioral "cheek" model in rats and mice (Shimada and LaMotte, 2008; Akiyama et al., 2010; Spradley et al., 2012) . In this model, pruritogens injected intradermally in the cheek elicit hindlimb scratch bouts, whereas algogens elicit ipsilateral forelimb wipes, directed to the injection site. This model thus distinguishes between itch and pain and provides a basis to investigate the differential processing underlying these two discrete behaviors.

In addition to the spinothalamic tract, other ascending pathways have been implicated in pain transmission, but little is known regarding their role in signaling itch. One pathway of particular interest is the spinoparabrachial pathway, which originates largely from neurons in lamina I, with some in deeper dorsal horn laminae (Menetrey and De Pommery, 1991; Kitamura et al., 1993; Hwang et al., 2003; Todd et al., 2005: Al-Khater et al., 2008, Al-Khater and Todd, 2009; Polgár et al., 2010). The vast majority (>90%) of antidromically identified spinoparabrachial projection neurons in lamina I responded to noxious mechanical and thermal stimuli (Bester et al., 2000). A very recent study of antidromically identified trigeminoparabrachial projection neurons in rats revealed that many responded to intradermal application of serotonin, histamine, chloroquine, and capsaicin, implicating them in signaling itch and chemogenic pain sensations (Jansen and Giesler, 2015).

For the present study, we employed a double immunofluoroscent-labeling strategy to identify trigeminothalamic and trigeminoparabrachial projections neurons by retrograde tracing and to assess whether they responded to pruritic (histamine, chloroquine) or algesic (capsaicin) stimulation as assessed by Fos immunofluorescence. We tested the hypothesis that some ascending projection neurons would be double labeled for Fos, implicating them in the rostral transmission of itch and/or chemogenic pain. The results supported this hypothesis and were in good accordance with the recent electrophysiology data from Giesler's group showing that substantial subpopulations of trigeminothalamic and trigeminoparabrachial neurons responded to pruritogens and algogens. A preliminary report of these data has appeared elsewhere (Carstens et al., 2014).

MATERIALS AND METHODS

Fluorogold microinjections

The procedures used in this study were approved by the UC Davis Animal Care and Use Committee. Adult male C57BL/6 mice (21–38 g) were anesthetized with sodium pentobarbital (65 mg/kg ip) and prepared for the injection of the retrograde tracer Fluorogold (FG; Fluorochrome, Denver, CO). Briefly, the mouse's head was fixed in a stereotaxic frame, and the skull was exposed by midsagittal incision. With a drill, burr holes were made over target sites (i.e., right thalamus or right lateral parabrachial nucleus [LPb]). The microsyringe needle containing FG was introduced through the burr hole and stereotaxically positioned, and FG was injected into either thalamus (AP 1.8, ML 1.0, DV 3.6 and AP 1.8, ML 1.6, DV 3.6) or LPb (AP 5, ML 1.3, DV 3.8 and AP 5.2, ML 1.3, DV 3.8) in a volume of 80 nl each. The incision was closed with Vetbond (3M, St. Paul, MN).

Fos immunohistochemistry

One week after FG injections, mice were anesthetized with sodium pentobarbital (65 mg/kg ip) and received an intradermal microinjection of histamine (50 μg/10 μl; Sigma Aldrich, St. Louis MO), chloroquine (100 μg/10 μl; Sigma), capsaicin (30 μg/10 μl; Sigma), or vehicle (saline, 7% Tween 80) into the left cheek. After 2 hours, the mice were perfused transcardially with phosphate-buffered saline, followed by 4% paraformaldehyde. The caudal medulla and upper cervical spinal cord were postfixed, transferred to 30% sucrose, and cut in 30-μm sections. Sections were collected serially in 24-well containers. Every fourth section was processed for Fos immunofluorescence. In brief, every fourth section (120-μm intervals) was blocked in donkey serum (5%) and then incubated in primary c-fos antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA; H-125, antibody ID AB_2106765) at 4°C overnight, followed by incubation with Alexa Fluor 488 (1:500; Invitrogen Life Technologies, Grand Island, NY; antibody ID AB_221544) for 2 hours at room temperature.

Images were captured with a fluorescence microscope (Nikon Eclipse Ti; Technical Instruments, San Francisco CA) outfitted with a CCD camera (Andor Clara; Technical Instruments) connected to a computer running NIS-Elements software (Technical Instruments; Resource ID SciRes_000190). Cells retrogradely labeled with FG were imaged with a UV filter (excitation wavelength 350 nm, emission wavelength 420 nm). Fos-immunoreactive neurons were imaged with an FITC filter (excitation wavelength 490 nm, emission wavelengths 515–545 nm). Image intensity and contrast were adjusted to maximize the fluorescent signal on a dark background. Overlay of the images revealed double-labeled neurons having a teal hue (see Fig. 6).

The numbers of Fos-immunofluorescent and FG-positive neurons were blindly counted by two independent observers. The average fluorescence intensity for cells that were accepted was 1,989.02 analog–digital units (ADUs; range 1,396.1–3,004), whereas the average fluorescence intensity for cells that were rejected was 1,299.5 ADUs (range 1,049–1,618.6). The length of the caudal-rostral distribution of Fos-positive neurons was calculated by the formula: length of caudal-rostral distribution (mm) = (number of sections that included Fos-immunoreactive neurons × 4 – 1) × 0.03. The mean numbers of Fos-immunoreactive neurons elicited by each chemical were compared by one-way analysis of variance (ANOVA), followed by post hoc Bonferroni tests, with $P < 0.05$ set as significant. To calculate the percentages of double-labeled neurons, we averaged counts of Fos- and FG-labeled neurons for the three sections from each animal with the relatively highest counts. The percentage of projection cells in the superficial dorsal horn exhibiting Fos-immunofluorescence was calculated by dividing the mean number of double-labeled neurons by the mean number of FG-positive neurons. The percentage of Fos-positive cells in the superficial dorsal horn with ascending projections was calculated by dividing the mean number of double-labeled neurons by Fos-positive neurons.

RESULTS

Fos immunoreactivity

Intradermal microinjections of histamine, chloroquine, or capsaicin each resulted in large numbers of Fos-immunoreactive neurons in the superficial dorsal horn, extending from subnucleus caudalis caudally to the upper cervical (C1–C2) spinal cord. Figure 1 shows photomicrographs of Fos immunoreactivity following id histamine (Fig. 1C), chloroquine (Fig. 1D), and capsaicin (Fig. 1E) and absence of Fos immunoreactivity following vehicle injections of saline (Fig. 1A) or Tween 80 (Fig. 1B). Intradermal injections of histamine, chloroquine, or capsaicin each resulted in equivalent numbers of Fos-immunopositive neurons (Fig. 1F, three rightmost bars), whereas vehicles were much less effective (Fig. 1F, two leftmost bars). Fos immunoreactivity was observed primarily in the medial half of the superficial medullary and upper cervical spinal dorsal horn, distributed over a rostrocaudal range of approximately 1–2 mm (Table 1). Additional examples of the distribution of Fos immunoreactivity in the superficial upper cervical dorsal horn are shown in Figure 5.

Retrograde FG labeling

Nine mice received intracranial microinjections of FG that included the ventrobasal thalamic nuclei VPM and VPL. The rostrocaudal distributions of injected FG for these animals are indicated by the shaded regions shown on the brainstem sections in Figure 2. An example of an individual FG thalamic injection is shown in Figure 3. The dashed line indicates the necrotic core of the injection site.

Nine other mice received FG microinjections that included the parabrachial nuclei and surrounding areas such as the Koelliker-Fuse nucleus. Figure 4 shows rostrocaudal distributions of the extent of the FG injections in these animals.

Spinothalamic projection neurons were located primarily contralateral to the thalamic injection site, whereas spinoparabrachial projection neurons were observed bilaterally. Most neurons retrogradely labeled from thalamus or parabrachial nuclei were located in the superficial dorsal horn of the upper cervical (C1–C2) segments. Figure 5A–C shows individual examples of drawings of C1 sections from animals receiving FG injections in the thalamus. Blue dots indicate neurons retrogradely labeled with FG, the large majority of which were contralateral to the thalamic FG injection. Figure 5D–F shows a bilateral distribution of neurons retrogradely labeled by FG injections in the parabrachial nuclei.

Double labeling

Figure 6 shows an example of a double-labeled neuron. The left panel (FITC) is a photomicrograph of Fos-immunoreactive neurons; the middle panel shows a neuron retrogradely labeled with FG; the merged image (right panel) showing the double-labeled neuron with a

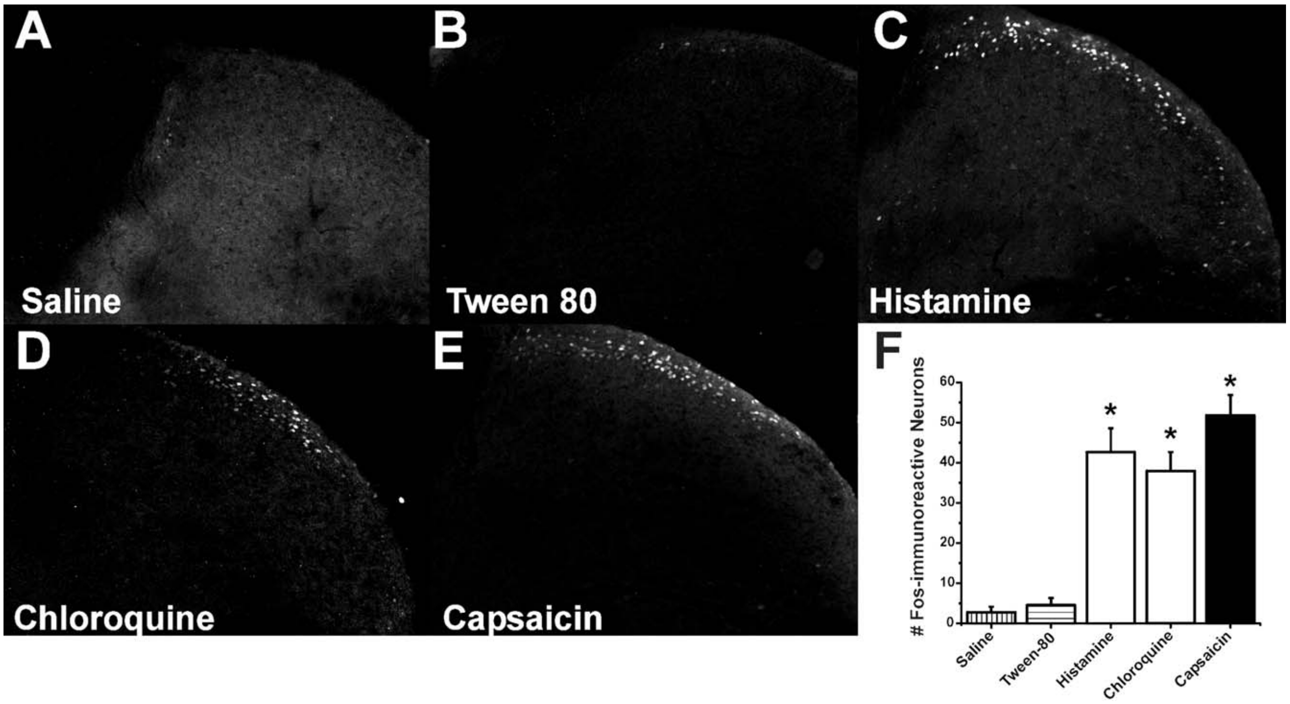

Figure 1. Fos immunoreactivity following histamine, chloroquine, and capsaicin. **A:** Saline control. Photomicrograph of C2 spinal cord section shows absence of Fos-immunofluorescence following id injection of isotonic saline in the ipsilateral cheek. **B:** 7% Tween 80 cheek injection. Note absence of Fos immunofluorscence. **C:** Histamine (50 μg) injected id into the cheek resulted in a large number of Fos-immunoreactive neurons in the superficial dorsal horn. **D:** Chloroquine (100 μg) also resulted in considerable Fos immunoreactivity. **E:** Capsaicin (30 μg). **F:** Average counts of c-fos-immunoreactive cells per section after intradermal cheek microinjection of each indicated chemical. Significantly different compared with saline or Tween 80 vehicle groups ($^*P < 0.05$, ANOVA with post hoc Bonferroni test).

teal hue. Figure 5 shows individual examples of the distributions of retrogradely labeled (blue), Fos-positive (green), and double-labeled (red dots) neurons. Figure 5A–C shows drawings of upper cervical sections from animals receiving FG injection in the thalamus and intradermal injection of histamine (Fig. 5A), chloroquine (Fig. 5B), or capsaicin (Fig. 5C). Figure 5D–F shows sections from animals receiving FG injections in parabrachial nuclei and intradermal injection of histamine (Fig. 5D), chloroquine (Fig. 5E), or capsaicin (Fig. 5G).

The percentages of double-labeled neurons are summarized in Figure 7. Figure 7A,B shows, respectively, the percentages of Fos-positive neurons with projections to the thalamus (Fig. 7A) and the percentages of FG-positive trigeminothalamic projection neurons exhibiting Fos immunoreactivity. The numbers in parentheses indicate the numbers of double-labeled cells divided by the total number of Fos-immunoreactive neurons in each treatment group. Similarly, Figure 7C,D shows percentages of Fos-positive neurons with projections to the parabrachial nuclei (Fig. 7C) and percentages of FG-positive trigemino-parabrachial projection neurons that were Fos immunopositive. Among the Fos-immunopositive neurons, very low percentages projected to either thalamus or parabrachial nuclei (Fig. 7A,C). Much higher percentages of retrogradely labeled neurons exhibited Fos immunolabeling. Thus, 43%, 8%, and 22% of neurons retrogradely labeled from thalamic injections exhibited Fos immunoreactivity

TABLE 1.

Counts of c-fos-Immunoreactive Cells Following Intradermal Cheek Injections of Histamine, Chloroquine, or Capsaicin and Their Distributions in Medial and Superficial Dorsal Horn and Along the Rostrocaudal Extent of the Caudal Brainstem and Upper Cervical Spinal Cord

	Mean No. Fos-positive (±SEM, all sections)	Medial dorsal horn (%)	Superficial dorsal horn (%)	Rostrocaudal distribution (mm)
Histamine	266 ± 27 (187–353)	78 ± 6 (59–97)	84 ± 4 (73–96)	2.03 ± 0.22 (1.29–2.61)
Chloroquine	197 ± 36 (79–323)	56 ± 9 (24–87)	93 ± 3 (84–99)	1.19 ± 0.16 (0.69–1.77)
Capsaicin	304 ± 45 (156–487)	74 ± 4 (62–89)	90 ± 4 (80–100)	1.59 ± 0.33 (0.81–2.61)

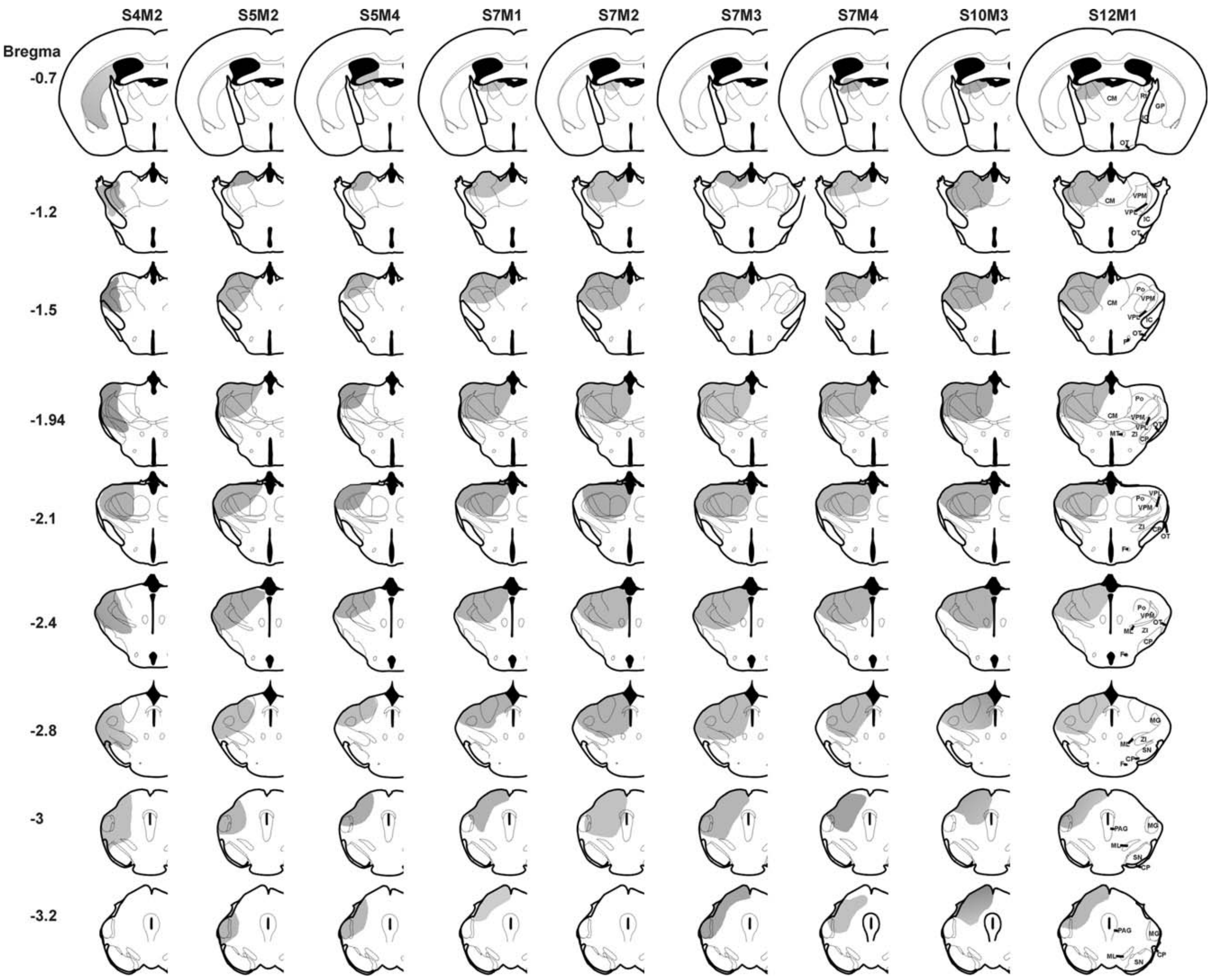

Figure 2. Thalamic FG injection sites. Columns show sections aligned from rostral to caudal for separate experiments. Gray shading indicates extent of FG fluorescence. CM, centrum medianum, Cnf, cuneiform n.; CP, cerebral peduncle; F, fornix; GP, globus pallidus; IC, internal capsule; ICP, inf. cerebellar peduncle; KF, Koelliker-Fuse n.; LC, locus coeruleus; LPB, lat. parabrachial n.; MCP, middle cerebral peduncle; MG, medial geniculate n.; ML, medial lemniscus; MPB, medial parabrachial n.; MT, mamillothalamic tract; OT, optic tract; PAG, periaqueductal gray; Po, posterior thalamus, Ptg, pedunculopontine tegmentum; Pyr, pyramid; Rt, reticular n.; Scp, superior cerebellar peduncle; SN, substantia nigra; Vc, trigeminal subnuc. caudalis; VPL, ventroposterolateral n., VPM, ventroposteromedial n.; ZI, zona incerta; 7n, seventh nerve.

following intradermal cheek injections of histamine, chloroquine, and capsaicin, respectively (Fig. 7B). For trigeminoparabrachial projection neurons, 36%, 29%, and 33% exhibited Fos immunoreactivity following intradermal injections of histamine, chloroquine, and capsaicin, respectively (Fig. 7D).

DISCUSSION

Methodological considerations

The present study employed a double-label strategy involving retrograde labeling and chemically evoked Fos immunoreactivity to assess the fraction of pruritogen- and capsaicin-sensitive neurons projecting to thalamus or parabrachial nuclei. Some methodological issues require discussion in order to assess the accuracy of the present findings.

An important issue regards the selectivity of the FG injections made here to investigate trigeminothalamic and trigeminoparabrachial projection neurons. Some of our injections spread beyond the intended ventrobasal thalamic and parabrachial targets and may have resulted in retrograde labeling of neurons projection to nontargeted sites. Our intention was to make fairly large FG injections to label as many trigeminothalamic and trigeminoparabrachial neurons as possible. Several thalamic injections spread caudally to involve the superior colliculus (Fig. 2). Spinotectal projections arise from neurons in lamina I at cervical segments, similar to spinothalamic

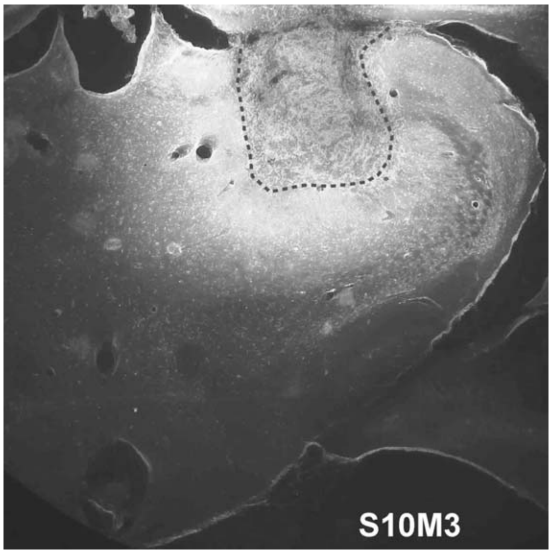

Figure 3. Example of thalamic injection site. Photomicrograph (×40) of section at level of ventrobasal thalamus showing FG (bright area) and necrotic core of injection site (dashed line).

projection neurons (Verburgh et al., 1990). Verburgh et al. suggest that some spinotectal projections may have had collateral projections to thalamus, with limited support from a double-label study (Liu, 1986) reporting spinothalamic neurons with collaterals to deep layers of the superior colliculus and adjacent periaqueductal gray (PAG). Some of the present thalamic injections encroached on the dorsal mesencephalic reticular formation and rostral lateral PAG (Fig. 2). There are several reports of collaterals of spinothalamic neurons to the PAG (Harmann et al., 1988; Zhang et al., 1990; Al-Khatar and Todd, 2009), with a recent estimate that one-third to one-half of spinothalamic neurons have collaterals to PAG (Al-Khatar and Todd, 2009). Moreover, upward of 45% of spinothalamic neurons have collateral projections to the midbrain reticular formation (Kevetter and Willis, 1983; Bice and Beal, 1997). It is thus possible that, with some of the larger thalamic injections in the present study, trigmenotectal, trigemino-PAG and trigeminoreticular neurons were retrogradely labeled and misidentified as trigeminothalamic neurons. However, the number of such misidentified neurons in our study will be substantially reduced when considering that many of them likely had collateral projections to the thalamus and can thus be considered trigeminothalamic.

Some of the present injections intended to target the parabrachial nuclei encroached on the PAG (Fig. 4). It has been reported that 85–100% of spinothalamic neurons have collaterals to the parabrachial nucleus in rats (Hylden et al., 1989; Al Khatar and Todd, 2009), with many of the projection neurons located in lamina I, as observed here for mice. Furthermore, as noted above, upward of 50% of spinothalamic and spinoparabrachial projection neurons have collaterals to PAG (Al-Khatar and Todd, 2009), suggesting that many spinal neurons project to all three structures (thalamus, parabrachial nuclei, and PAG). Thus, in the present study, the thalamic and parabrachial injections likely labeled neurons projecting to both structures. Some trigeminotectal, trigemino-PAG and trigeminoreticular neurons might have been retrogradely labeled by the larger FG injections, but many of them are likely to have had collateral projections to parabrachial nuclei and/or thalamus, thus reducing this source of error.

Another issue is that FG may be taken up by fibers passing through the injection site to retrogradely label nontargeted projection neurons (Dado et al., 1990). Given that essentially 100% of trigeminothalamic tract neurons also have collaterals to the parabrachial nuclei (see previous paragraph), we believe that this issue is moot for trigeminothalamic fibers passing through the parabrachial injection site. It is more of a problem for spinohypothalamic and spinotelencephalic projection fibers (Burstein et al., 1990a; Cliffer et al., 1991) passing through the thalamic FG injection sites. Many spinohypothalamic tract neurons were located in lamina I as well as the intermediate or ventral horn at upper cervical levels (Burstein et al., 1990a). Most of the present double-labeled cells were in lamina I of the upper cervical spinal cord. It is thus possible that some of the latter were actually trigeminohypothalamic neurons that were misidentified as trigeminothalamic.

Fos immunoreactivity is a generally accepted method to identify functionally activated neurons (Harris, 1998; Coggeshall, 2005) but is not without limitations. For example, it is unclear whether all neurons in the superficial dorsal horn are capable of expressing Fos following the chemonociceptive stimuli delivered here, potentially underestimating the extent of functional activation. Coggeshall (2005) comments that “Fos expression . . . is at present the best global marker for efficiently locating populations of neurons in the awake animal that respond to nociceptive input.” Moreover, it is possible that the presence of FG might affect Fos expression (Franklin and Druhan, 2000). In this regard, the mean numbers of Fos-immunopositive cells per section following intradermal injections of histamine, chloroquine, and capsaicin in the present study (Fig. 1F; ~40–50/section) are comparable to counts of Fos-immunopositive spinal neurons evoked by intradermal formalin in mice that did not receive intracranial FG injections (Merrill et al., 2006), suggesting that the presence of FG did not unduly reduce Fos expression. Conversely, immunohistochemical processing for Fos

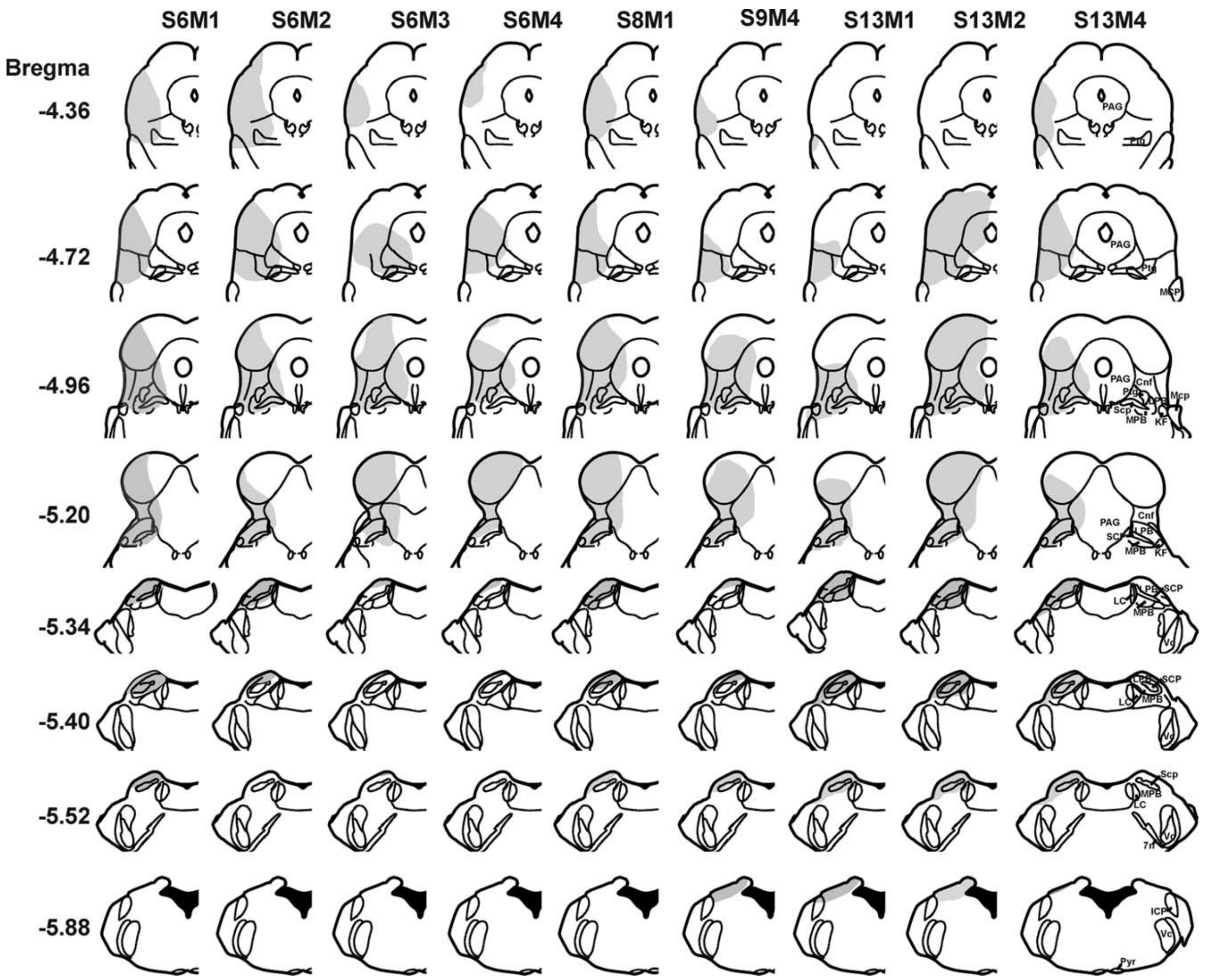

Figure 4. Parabrachial injections sites (format and abbreviations as for Fig. 2).

might have affected FG detection. However, we do not believe that washout of FG staining by the Fos staining procedure would have drastically reduced the number of FG-labeled neurons, because the percentages of double-labeled cells in our study are comparable to data obtained with a completely different electrophysiological approach (Moser and Giesler, 2014; Jansen and Giesler, 2015), as discussed further below.

A related issue is that our use of pentobarbital anesthesia during the delivery of intradermal chemical stimuli might have reduced Fos expression. Indeed, pentobarbital was recently reported to reduce spinal Fos immunoreactivity elicited by formalin in rats (Takasusuki et al., 2013). However, we previously observed that pentobarbital at doses sufficient to block motor reflexes did not inhibit responses of dorsal horn neurons to noxious stimuli (Carstens and Campbell, 1992). Moreover, there was no difference in the number of spinal neurons expressing Fos immunoreactivity elicited by formalin in propofol-anesthetized compared with unanesthetized mice (Merrrill et al., 2006). We previously assessed pruritogen-evoked Fos expression in the lumbar spinal cord of animals that were prevented from reaching experimental dry skin on the hindlimb by wearing an inverted Elizabethan collar, although we did not compare results between awake and anesthetized animals (Nojma et al., 2004). In conclusion, it is possible that our use of the general anesthetic agent pentobarbital suppressed Fos expression to a limited extent.

Ascending projections of pruritogen- and capsaicin-responsive neurons

In the present study, Fos-immunoreactive neurons were located over a 1–2-mm rostrocaudal area extending from the caudal medulla to upper cervical (C1–C2) spinal cord. Fos-positive neurons were distributed mainly in the medial dorsal horn, with a predominant distribution in superficial

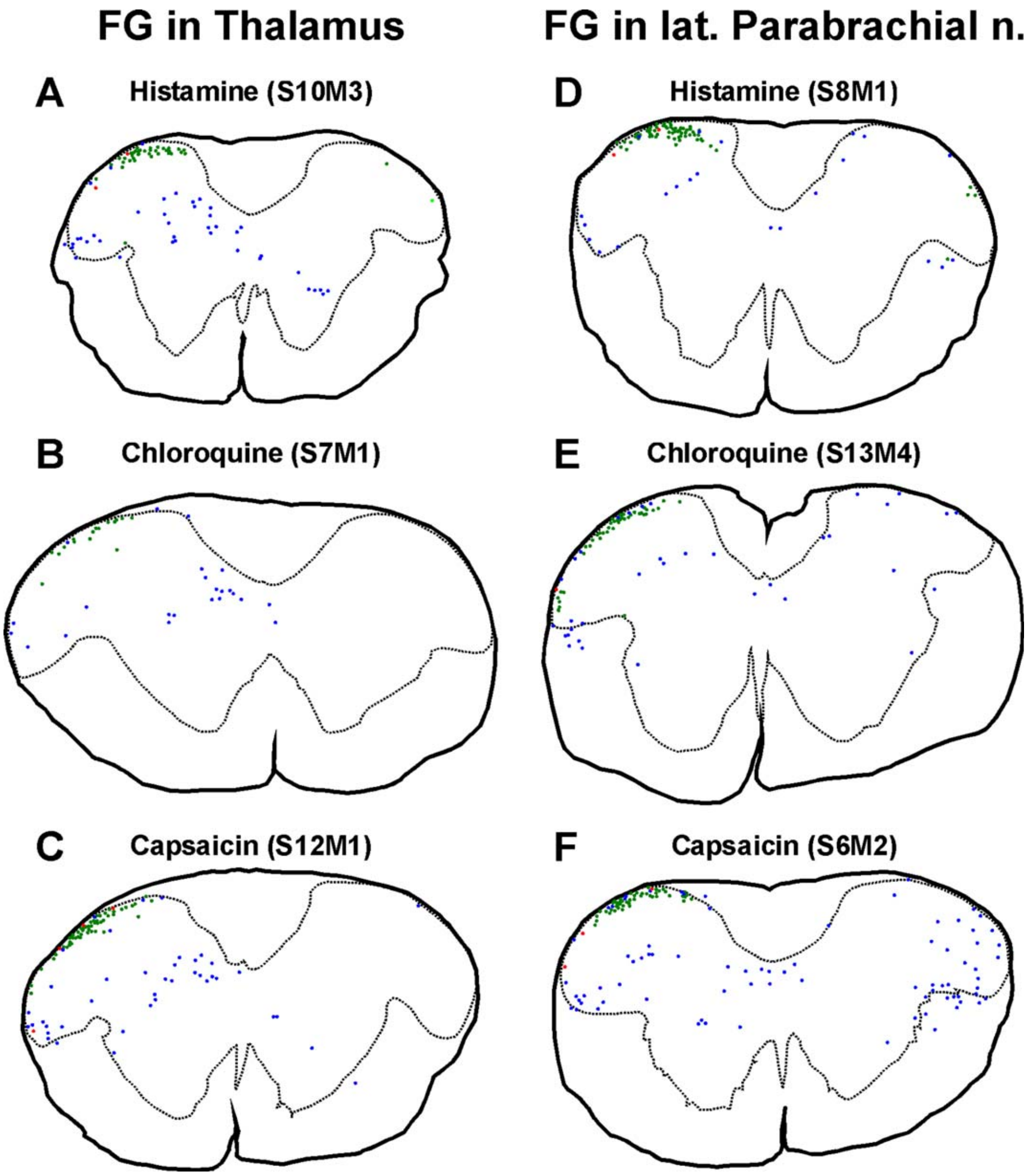

Figure 5. Examples of neurons in lower brainstem and upper cervical spinal cord retrogradely labeled with FG, imunopositive for Fos, or both (double labeled). **A:** Example showing FG retrogradely labeled neurons (blue), Fos-immunopositive neurons (green) and double-labeled neurons (red) on tracing of upper cervical spinal cord section. Mouse received FG injection in right thalamus (S10M3 in Fig. 2) and intradermal injection of histamine in left cheek. **B:** Thalamic injection of FG (S7M1, Fig. 2) and intradermal injection of chloroquine (format as in A). **C:** Thalamic injection of FG (S12M1, Fig. 2) and intradermal injection of capsaicin (format as in A). **D:** LPB injection of FG (S8M1, Fig. 3) and intradermal injection of histamine (format as in A). **E:** LPB injection of FG (S13M4, Fig. 3) and intradermal injection of chloroquine (format as in A). **F:** LPB injection of FG (S6M2) and intradermal injection of capsaicin (format as in A).

laminae. This nicely matches the distribution of sites in mouse caudal medulla and upper cervical dorsal horn at which microelectrode recordings were made of single neurons identified by their response to intradermal cheek microinjection of histamine, SLIGRL (agonist of PAR2 and MrgprC11), or AITC (Akiyama et al., 2010). This distribution is appropriately placed between the dorsomedial region of trigeminal subnucleus caudalis (Vc) and upper cervical spinal cord exhibiting Fos immunoreactivity following noxious intraoral stimuli (Carstens et al., 1995) and more lateral and ventrolateral distributions of Fos-positive neurons following noxious intranasal (Anton et al., 1991) or ocular stimulation (Bereiter et al., 2002), respectively, as assessed in rats. The distribution of Fos immunoreactivity was qualitatively, and generally also quantitatively, similar following intradermal cheek injections of histamine, chloroquine, or capsaicin. The similar distributions of Fos-positive neurons elicited by different chemicals, as

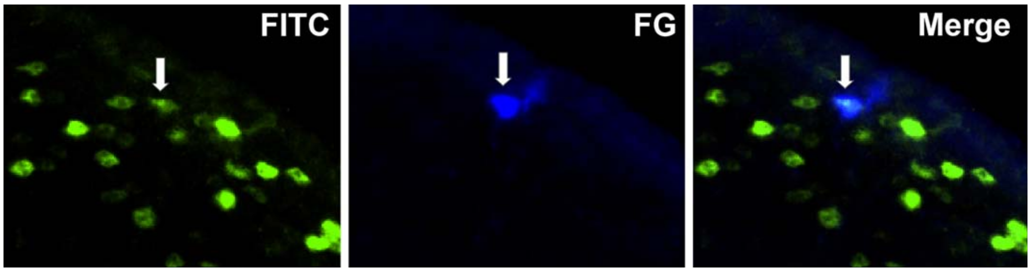

Figure 6. Example of double-labeled neuron (arrow). **Left:** Fos-immunoreactive neurons (FITC). **Middle:** One FG-labeled neuron. **Right:** Merged images, with the double-labeled neuron exhibiting a teal hue.

observed here, is consistent with our previous electrophysiological data showing that a substantial fraction (one-third or one-half) of chloroquine-responsive spinal dorsal horn neurons also responded to capsaicin or histamine, respectively (Akiyama et al., 2014). Similarly, approximately one-half of histamine-responsive Vc neurons responded to capsaicin (Akiyama et al., 2010), and two-thirds of histamine-responsive spinal dorsal horn neurons responded to

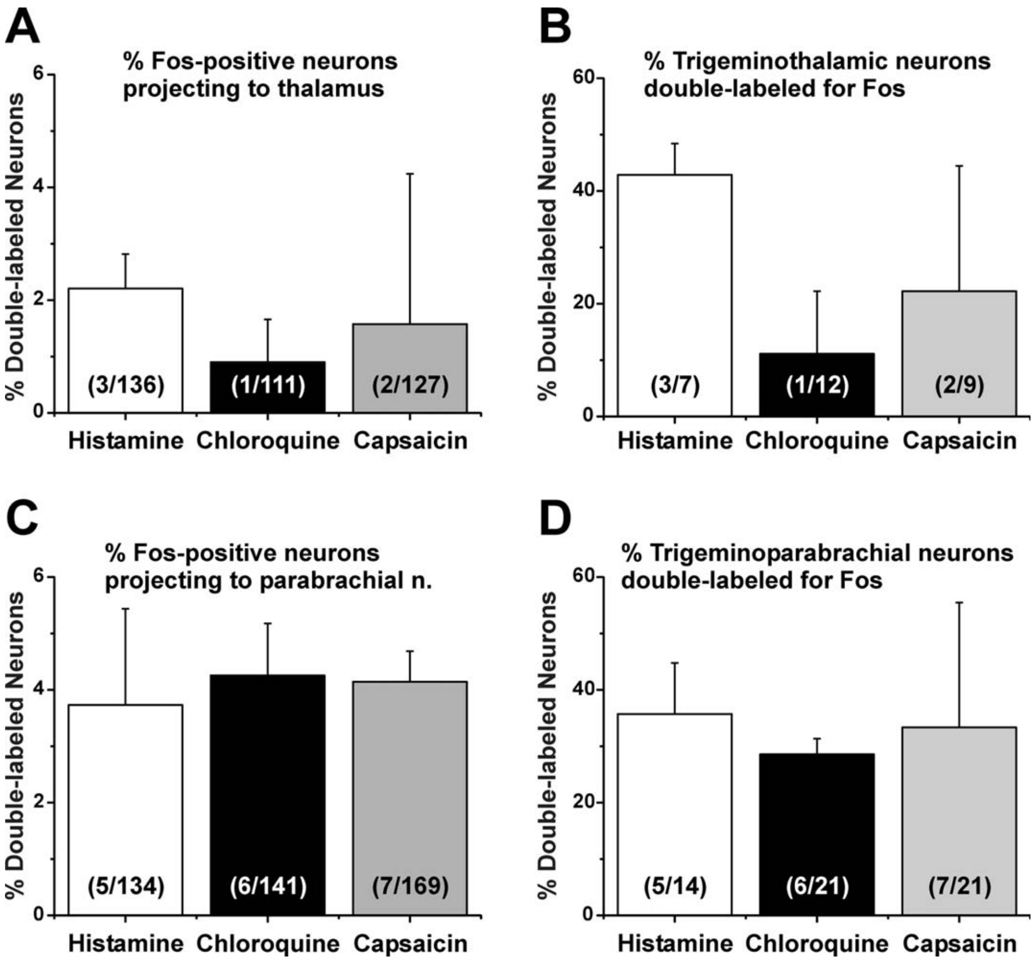

Figure 7. Percentages of double-labeled neurons. **A:** Percentages of neurons exhibiting Fos immunoreactivity evoked by intradermal histamine (□), chloroquine (■) or capsaicin (▣) that were retrogradely double labeled with FG injected in thalamus. Numbers in parentheses indicate the number of double-labeled neurons divided by the total number of Fos-immunoreactive neurons in each treatment group. **B:** Percentage of neurons retrogradely labeled from thalamus that were double labeled for Fos immunoreactivity evoked by histamine (□), chloroquine (■) or capsaicin (▣). **C, D:** As in A,B for neurons retrogradely labeled with FG injected in LPB. Numbers in parentheses indicate the number of double-labeled neurons divided by the total number of FG-labeled neurons in each treatment group.

capsaicin (Akiyama et al., 2009); chloroquine was not tested in these latter studies.

We found retrogradely labeled neurons to be located predominantly in the superficial dorsal horn at upper cervical (C1–C2) and caudal medullary levels following FG injections in the lateral parabrachial nucleus or ventral posteromedial thalamus. This is consistent with previous studies in mice (Davidson et al., 2010) and rats (Giesler et al., 1976: Burstein et al., 1990b; Menetrey and De Pommery, 1991; Kitamura et al., 1993; Hwang et al., 2003; Todd et al., 2005: Al-Khater et al., 2008, Al-Khater and Todd, 2009; Polgár et al., 2010).

The observation that only a minority of Fos-positive neurons were retrogradely labeled from the thalamus or parabrachial nuclei indicates that the vast majority of neurons activated by chemonociceptive and pruritic stimuli are not projection neurons and presumably function as interneurons presynaptic to projection neurons and/or participate in local segmental circuits. This is consistent with previous studies. In an older study, very few if any spinal interneurons retrogradely labeled transsynaptically by injection of pseudorabies virus into hindlimb biceps femoris or tail muscles were double labeled by cholera toxin subunit B injected into the ventrobasal thalamus (Jasmin et al., 1997). It was suggested that few if any spinal premotor interneurons in segmental tail flick or paw withdrawal reflex pathways give rise to ascending projections. This is generally consistent with the present study showing that few (<5%) Fos-positive neurons projected rostrally. In a recent study, knockout mice lacking the testicular orphan nuclear receptor 4 (TR4) exhibited a dramatic loss of spinal excitatory interneurons expressing the gastrin-releasing peptide receptor (GRPR), with a nearly complete preservation of ascending projection neurons (Wang et al., 2013). Despite the preserved ascending spinal projections, these mice displayed a dramatic reduction in scratching behavior elicited by different pruritogens, as well as formalin-evoked nocifensive behavior, underscoring the importance of spinal interneurons in these integrated behavioral responses. Presumably, the nonprojecting, GRPR-expressing interneurons that are activated by pruritogens and algogens function together with ascending projection neurons in circuits giving rise to the “scratch reflex” or other nocifensive behaviors.

The present results further imply that most pruriceptive neurons encountered in neurophysiological single-unit recording studies are unlikely to be projection neurons. Methods such as antidromic stimulation are required to demonstrate that a spinal or trigeminal neuron gives rise to an ascending projection. Giesler and colleagues have used antidromic stimulation to identify ascending trigeminothalamic and trigeminoparabrachial projection neurons in rats and to characterize their responses to various chemical and physical stimuli. Among identified trigeminothalamic tract neuron, 27% exhibited an excitatory response to intradermal injection of serotonin, 9% responded to chloroquine, 28% responded to histamine, 22% responded to BAM8–22 (MrgprC11 agonist), and 27% responded to capsaicin (Moser and Giesler, 2014). We observed here that 43% of ascending trigeminothalamic projection neurons exhibited Fos expression elicited by intradermal histamine, 22% by capsaicin, and 8% by chloroquine. The latter two percentages fairly closely match the electrophysiological data of Moser and Giesler (2014). The higher percentage of histamine-sensitive trigeminothalamic neurons in mice vs. rats may be due to a species difference, with histamine being algogenic rather than pruritic in rats (Jinks and Carstens, 2002).

In a subsequent study of antidromically identified trigeminoparabrachial projection neurons in the rat, 62% responded to serotonin, 30% to histamine, 35% to AITC, and 50% to capsaicin (Jansen and Giesler, 2015). Curiously, 32% responded to chloroquine, but with a decrease, rather than increase, in firing rate. We observed here that 36% of trigeminoparabrachial projection neurons exhibited Fos immunoreactivity following intradermal injection of histamine, 29% following chloroquine, and 33% following capsaicin. These findings are in reasonably good accordance with the electrophysiological data from rats (Jansen and Giesler, 2015), and support the idea that the double-labeled neurons observed here are involved in transmitting chemoceptive, including itch, information to higher centers.

In comparison, 20% of antidromically identified monkey spinothalamic tract neurons responded to histamine, and 13% responded to cowhage, with 2% responding to both (Davidson et al., 2012). These findings suggest that histaminergic and nonhistaminergic itch may be transmitted by largely separate subpopulations of spinothalamic tract neurons. It is also noteworthy that all histamine- and cowhage-responsive monkey spinothalamic tract neurons additionally responded to capsaicin and/or mustard oil (Davidson et al., 2012), as did most pruritogen-sensitive spinal and trigeminal neurons in mice (for review see Akiyama and Carstens, 2013). The separation of histaminergic and nonhistaminergic itch-signaling pathways may be less pronounced in rodents, given that higher percentages of neurons respond to both histamine and nonhistaminergic itch mediators such as chloroquine, serotonin, and SLIGRL (Akiyama et al., 2009a, 2010, 2014). The broad tuning of mouse trigeminal and dorsal horn neurons to pruritogens and algogens may at least partially account for the similar distributions of Fos immunoreactivity evoked by histamine, chloroquine, and capsaicin observed in the present study.

In rats, most (~80%) spinal neurons with ascending projections to the brainstem express the neurokinin-1 (NK-1) receptor (Todd et al., 2000, 2002) and respond to noxious thermal stimulation (Todd et al., 2005). We recently reported that 94% of upper cervical spinal neurons retrogradely labeled from the ventral posteromedial thalamus (same injection sites as shown in Fig. 2), and 89% of those retrogradely labeled from the parabrachial nuclei (same injections as in Fig. 3), exhibited immunofluorscence for the NK-1 receptor (Akiyama et al., 2015), supporting the earlier studies with rats. In comparison, only 24% of trigeminothalamic and 20% of trigeminoparabrachial projection neurons were double labeled for GRPR (Akiyama et al., 2015). The importance of NK-1 receptor-expressing superficial dorsal horn neurons in itch is further supported by our previous study showing that neurotoxic ablation of such neurons significantly attenuated scratching behavior elicited by intradermal injection of serotonin in rats (Carstens et al., 2010). In contrast, a much smaller percentage of ascending projection neurons appeared to be contacted by primary afferents and/or interneurons that release GRP as a neuropeptide transmitter. The critical role of GRPR-expressing spinal neurons in itch is supported by reports that scratching behavior elicited by intradermal injection of a variety of different pruritogens is reduced or abolished in knockout mice lacking GRPR (Sun and Chen, 2007) or in mice with neurotoxic ablation of GRPR-expressing spinal neurons (Sun et al., 2009; Mishra and Hoon, 2013). Itch-related scratching behavior was also attenuated in mice lacking brain natriuretic peptide (BNP) in primary sensory neurons as well as in mice with neurotoxic destruction of neurons expressing the BNP receptor Npra (Mishra and Hoon, 2013). Mishra and Hoon suggested that BNP, rather than GRP, is released from primary afferent pruriceptors to excite GRPR-expressing spinal neurons because intrathecal delivery of GRP still elicited scratching behavior after deletion of Npra-expressing neurons and based on recent genomic data (Goswami et al., 2014). The exact chemical anatomy of the spinal itch-signaling circuitry remains a topic of debate (Liu et al., 2014; Goswami et al., 2014). Our data suggest that GRPR-expressing neurons are upstream of NK-1 receptor-expressing projection neurons that transmit itch signals to higher centers.

CONFLICT OF INTEREST STATEMENT

None of the authors declares a conflict of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: TA, ECa. Acquisition of data: TA, ECu, TN, MIC. Analysis and interpretation of data: TA, ECa. Drafting of the manuscript: ECa. Critical revision of the manuscript for important intellectual content: ECa, TA. Statistical analysis: TA, ECa, ECu, TN. Obtained funding: TA, ECa. Administrative, technical, and material support: MIC. Study supervision: TA, ECa, MIC.

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