Distribution of hemokinin-1 in the rat trigeminal ganglion and trigeminal sensory nuclear complex

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ABSTRACT

Objective: A new mammalian tachykinin peptide encoded in a TAC4 gene was identified and designated as hemokinin-1 (HK-1). A representative of the tachykinin peptide family is substance P (SP), and the function of SP has been well characterized as a pain transmitter or modulator, while it is possible that HK-1 is involved in pruriceptive processing, but, as yet, the distribution of HK-1 peptide in the trigeminal sensory system is still unknown. Thus, the aim of the present study was to elucidate the distribution of HK-1, while comparing the expression of SP, in the trigeminal ganglion and trigeminal sensory nuclear complex.

Design: The trigeminal ganglion and the brain stem of male SD rats were used in the immunohistochemical study. Since the amino acid sequence in the carboxyl-terminal regions of HK-1 and SP is common, polyclonal antibodies of HK-1 and SP derived from 6 amino acids consisting of amino-terminal regions of these peptides were produced in guinea pig and rabbit, respectively. The immunohistochemical staining of HK-1 and SP was conducted using frozen sections of the trigeminal ganglion and brain stem in rats.

Results: Immunohistochemical studies revealed the expression of HK-1 in small- and medium-sized trigeminal ganglion neurons, in the paratrigeminal nucleus, and in lamina I of the trigeminal nucleus caudalis, while there was no immunoreactivity of HK-1 in the trigeminal nucleus principalis, trigeminal nucleus oralis, and trigeminal nucleus interpolaris. *Conclusion:*

These findings indicate that HK-1 is a target molecule for treatment of itch in the orofaicial regions.

Keywords: hemokinin-1; immunohistochemistry; trigeminal sensory nuclear complex; trigeminal ganglion; pruritus; substance P

1. Introduction

A new mammalian tachykinin peptide encoded in a preprotachykinin gene, TAC4, was identified in mouse bone marrow cells and designated as hemokinin-1 (HK-1) (Zhang et al., 2000). HK-1 exhibits structural homology with known members of the tachykinin family, of which peptides share a carboxyl-terminal F-X-G-L-M-amide motif and more varied amino-terminals (Page, 2004; Page, 2005; Page, 2006).

A representative of the tachykinin peptide family is substance P (SP), and its function has been well characterized. SP and its proper receptor, neurokinin 1 (NK1) receptor, are mainly expressed in the superficial layer of the spinal dorsal horn, in which nociceptive primary afferents terminate (Hökfelt et al., 1975; Brown et al., 1995; Greco et al., 2007; Naono-Nakayama et al., 2011). SP is found in and released from primary afferents, and is believed to function as a pain transmitter or modulator (Henry, 1976; De Koninck and Henry, 1991). Meanwhile, the function of HK-1 remains poorly understood, but recent investigations have suggested

its involvement in processing of pruritus (Naono-Nakayama et al., 2014; Funahashi et al., 2014) or neuropathic pain (Matsumura et al., 2008; Sakai et al., 2012).

Additionally, there is currently no information available about the distribution of HK-1 peptide in either the spinal cord or the trigeminal sensory system, although the expression of mRNA derived from TAC4 gene encoding HK-1 has been reported in several regions, including the brain, spinal cord, dorsal root ganglia, brain stem, and trigeminal ganglion (Kurtz et al., 2002; Duffy et al., 2003; Matsumura et al., 2008). These findings suggest the expression of HK-1 in the trigeminal sensory system, as well as in the spinal cord. In the trigeminal sensory system, peripheral axons of trigeminal ganglion neurons are distributed in the orofacial regions, and central axons terminate in the trigeminal sensory nuclear complex that is structurally and functionally divided into the trigeminal sensory nucleus principalis (Vp) and three subnuclei of the spinal trigeminal nucleus: trigeminal nucleus oralis (Vo), trigeminal nucleus interpolaris (Vi), and trigeminal nucleus caudalis (Vc) (Olszewski, 1950).

Scratching is a widely-used behavior marker for evaluating pruritus, and administration of a pruritic stimulus to the cheek, an orofacial region, as well as the nape of the neck, elicits scratching behavior and neuronal activity in trigeminal nucleus caudalis neurons (Shimada and LaMotte, 2008; Akiyama et al., 2010; Klein et al., 2011; LaMotte et al., 2011; Akiyama et al., 2016). These findings suggest that transmitters or modulators used in the spinal cord

may also be involved in pruritus signaling in the trigeminal sensory system. Recently, pharmacological studies have demonstrated the involvement of HK-1 in pruriceptive processing in the spinal cord (Naono-Nakayama et al., 2014; Funahashi et al., 2014). Thus, it is possible that HK-1 may similarly contribute to pruriceptive processing in the trigeminal sensory system. Undoubtedly, the clarification of HK-1 distribution in this system provides a clue to elucidate the underlying mechanisms of pruriceptive processing in the trigeminal sensory system.

Therefore, the aim of the present study was to elucidate the expression of HK-1 in the trigeminal ganglion and trigeminal sensory nuclear complex. SP and HK-1 are peptides belonging to the same tachykinin family, and share of a common sequence of amino acids in the C-terminal, but not the N-terminal regions. Thus, to avoid the cross-reactivity, two different antibodies derived from the N-terminal regions of SP and HK-1 were produced from two different species, rabbit and guinea pig, respectively. The immunoreactivity of these two peptides was investigated in the trigeminal ganglion and trigeminal sensory nuclear complex, and the their coexpression was examined in trigeminal ganglion neurons.

2. Materials and methods

2.1 Experimental design

The experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used (Zimmermann, 1983). Male Sprague-Dawley rats, weighing 200-250 g, were acclimated for a period of at least one week at the Experimental Animal Center of the University of Miyazaki, and maintained under a 12/12 h light/dark cycle with food and water available.

2.2 Antibodies against HK-1 and SP

The amino acid sequences of HK-1 (1-11) and SP (1-11) are Arg-Ser-Arg-Thr-Arg-Gln-Phe-Tyr-Gly-Leu-Met-amide and Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-amide, respectively. The amino acid sequence of HK-1 proposed by Zhang et al. (2000) was adopted in this study, since it remains to be determined whether arginine exists at the N-terminus of HK-1 (Page, 2004). Since the C-terminal regions of HK-1 and SP share an amino acid sequence common to the tachykinin peptide family, Phe-X-Gly-Leu-Met, antibodies against HK-1 or SP were produced using peptides derived from the N-terminal regions of each of these two peptides for reducing the possibility of cross-reactivity of antibodies. The 1st to 6th consecutive amino acid sequences of the N-terminal regions of HK-1 (1-6): Arg-Ser-Arg-Thr-Arg-Gln, and SP (1-6): Arg-Pro-Lys-Pro-Gln-Gln, were

synthesized, and, HK-1 (1-6) and SP (1-6) peptides, were injected into guinea pig and rabbit, respectively, for producing polyclonal antibodies against these peptides. IgG isolated from their sera was purified, and the final concentration of IgG was adjusted to 2 mg/ml (TAKARA BIO INC, Kusatsu, Shiga, Japan). Antibodies derived from each terminal fragment of HK-1 and SP were designated as HK-1 (1-6) antibody and SP (1-6) antibody, respectively.

2.3 Immunohistochemistry of HK-1 and SP

Animals were anesthetized with an overdose of sodium pentobarbital, and perfused intracardially with saline (200 ml), followed by 4% cold paraformaldehyde in 0.1 M phosphate buffer (PB) (500 ml) for 30 min. The brain stem and trigeminal ganglion were removed, postfixed for 1 h in the same fixative, and cryoprotected in 10% sucrose in PB for 1 h and then in 30% sucrose in PB overnight. Frozen serial sections of the brain stem and trigeminal ganglion, 50 μm in thickness, were prepared, collected in phosphate-buffered saline (PBS; pH 7.4), and processed as free-floating sections for immunohistochemical staining against HK-1 and SP. All sections were incubated in hydrogen peroxide and Triton X-100, and then in normal goat serum. Part of sections were immersed in a solution of polyclonal guinea pig anti-HK-1 (1-6) antibody (1:15,000 or 1:5,000) overnight at 4 °C, and then reacted with biotinylated goat anti-guinea pig

IgG and avidin-conjugated horseradish peroxidase (VECSTAIN ABC kits, Burlingame, CA, USA). The remaining sections were incubated in polyclonal rabbit anti-SP (1-6) antibody (1:15,000 or 1:5,000) or monoclonal rabbit anti-NeuN antibody (1:10,000, Abcam, Cambridge, MA, USA) overnight at 4ºC, and then reacted with biotinylated goat anti-rabbit IgG and avidin-conjugated horseradish peroxidase (SAB kit; Nichirei Biosciences Inc., Tokyo, Japan). All sections with an avidin-biotin complex were visualized using diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, Mo, USA) and hydrogen peroxide. The sections placed on Frontier-coated glass slides (Matsunami Glass, Kishiwada, Japan) were mounted using NEW M·X mounting agent (Matsunami Glass, Kishiwada, Japan) and coverslips, after dehydration by ethyl alcohol and penetration by xylene. Preabsorption of HK-1 (1-6) and SP (1-6) antibodies with HK-1 (1-11) and SP (1-11) peptides (Genenet Co., Fukuoka, Japan), respectively, or omission of the antibody from the protocol, eliminated positive staining.

Trigeminal ganglion cells were stained with various degree of density. Then, cells labeled heavily and contained a clearly defined nucleus were regarded as cells positive to HK-1, SP and NeuN, and their sizes were measured for generating cell-size histograms of HK-1-positive cells, SP-positive cells and NeuN-positive cells in the trigeminal ganglion. Trigeminal sensory nuclear complex was divided into Vp, Vo, Vi and Vc. The dorsal horn of Vc was further divided into lamina I, lamina II, lamina III,

lamina IV and lamina V, and especially lamina II was subdivided into lamina IIo consisting of the outer one-third of this lamina and lamina IIi consisting of the inner two-thirds of this lamina.

In addition, some sections were incubated in hydrogen peroxide and Triton-X, and reacted with polyclonal guinea pig anti-HK-1 (1-6) antibody (1:10,000 or 1:5,000) and then goat anti-guinea pig IgG conjugated with Alexa Fluor 488 (1:10,000, Abcam, Cambridge, MA, USA), polyclonal rabbit anti-SP (1-6) antibody (1:10,000 or 1:5000) and then donkey anti-rabbit IgG conjugated with Alexa Fluor 594 (1:10,000, Abcam, Cambridge, MA, USA), or monoclonal rabbit anti-NeuN antibody (1:10,000, Abcam, Cambridge, MA, USA) and then donkey anti-rabbit IgG conjugated with Alexa Fluor 594 (1:10,000, Abcam, Cambridge, MA, USA). Double-fluorescent staining was performed between HK-1 (1-6) and SP (1-6) antibodies, or HK-1 (1-6) and NeuN antibodies. Images were visualized using a Keyence Biorevo BZ-9000 microscope and a Keyence Fluorescence Microscope BZ-X710, captured via the BZ-II Analyzer application and the BZ-H3C Analyzer application (Osaka, Japan), respectively, and cell size was measured from the resulting digital image.

3. Results

3.1 Characterization of HK-1 immunoreactive cells in the trigeminal ganglion

Cells showing immunoreactivity to an antibody derived from HK-1 (1-6) or SP (1-6) were evenly distributed in the trigeminal ganglion (Figs. 1A and 1D).

The specificity of the HK-1 (1-6) antiserum or SP (1-6) antiserum was confirmed by absorption experiments, in which primary guinea pig antibody against HK-1 (1-6) and primary rabbit antibody against SP (1-6) were preabsorbed with an excess of HK-1 (1-6) or HK-1 (1-11) peptide and SP (1-6) or SP (1-11) peptide, respectively. The immunoreactivity of the antibody derived from HK-1 (1-6) in the trigeminal ganglion was completely absent after preabsorption with HK-1 (1-6) or HK-1 (1-11) peptide (Fig. 1B), whereas the addition of SP (1-11) peptide to HK-1 (1-6) antibody produced little change in HK-1 immunoreactivity (Fig. 1C). Similarly, there was little change in the immunoreactivity of the SP (1-6) antibody in the trigeminal ganglion after preabsorption with HK-1 (1-11) peptide (Fig. 1E), whereas SP (1-6) immunoreactivity was completely diminished by preabsorption with an excess of SP (1-6) or SP (1-11) peptide (Fig. 1F). Similarly, the absorption experiments were conducted by using the same antibodies, HK-1 (1-6) antiserum and SP (1-6) antiserum, in the sections derived from the medulla oblongata. The immunoreactivity of HK-1 (1-6) was diminished by preabsorption of HK-1 (1-6) peptide, not but SP (1-6) peptide, while SP (1-6) immunoreactivity was disappeared by preabsorption of SP (1-6) peptide, not but HK-1 (1-6) peptide (data not shown).

Trigeminal ganglion cells displaying immunoreactivity for antibodies derived from HK-1 (1-6) (Fig. 2A), SP (1-6) (Fig. 2B) and NeuN (Fig. 2C) were evenly distributed within the areas of the ophthalmic nerve, maxillary nerve and mandibular nerve forming the trigeminal ganglion nerve. There was little difference in cell sizes between HK-1-positive (Fig. 2D, HK-1-positive) and SP-positive (Fig. 2D, SP-positive) cells. Indeed, cell distribution histograms for HK-1-positive or SP-positive cells showed similar patterns to each other, and the mean sizes of HK-1-positive and SP-positive cells were 571 μm^2 ± 19.0 (mean ± SEM) and 518 μm^2 ± 14.9 (mean ± SEM), respectively. In addition, HK-1-positive cells and SP-positive cells were small or medium size based on cell-size distribution of NeuN-positive cells (Fig. 2D, NeuN-positive).

To clarify the relationship between cells expressing HK-1 or SP and cells expressing HK-1 or NeuN in the trigeminal ganglion, colocalization of HK-1 and SP, and HK-1 and NeuN was observed in each trigeminal ganglion cell. Some cells investigated showed both HK-1 and SP immunoreactivity, while other SP-positive cells, especially small-sized cells, did not show HK-1 immunoreactivity (Fig. 3A). Additionally, all cells expressing HK-1 also showed NeuN immunoreactivity, a neuronal marker, whereas other NeuN-positive cells did not show HK-1 immunoreactivity (Fig. 3B). Indeed, 5.6% of cells (8/142) expressing NeuN was positive to HK-1 immunoreactivity.

3.2 Distribution of HK-1 or SP immunoreactivity in the trigeminal sensory nuclear complex

The degree of HK-1 immunoreactivity was largely dependent on the nucleus consisting of the trigeminal sensory nuclear complex. The immunoreactivity of HK-1 was entirely absent in the trigeminal nucleus principalis (Fig. 4A, Vp), trigeminal nucleus oralis (Fig. 4B, Vo), or trigeminal nucleus interpolaris (Fig. 4C, Vi). On the other hand, HK-1 immunoreactivity was prominent in the paratrigeminal nucleus (Fig. 4D, Vpara) and trigeminal nucleus caudalis (Fig. 4E, Vc). In the paratrigeminal nucleus, HK-1 immunoreactivity was evenly distributed. In contrast, the dense immunoreactivity of HK-1 was distributed in lamina I of the trigeminal nucleus caudalis (Fig. 4F), but sparse within lamina II and absent in laminae III, IV, and V of the Vc (Figs. 4E and 4F).

SP immunoreactivity in the trigeminal sensory system was similar to that of HK-1. There was no immunoreactivity of SP in the Vp (Fig. 5A), Vo (Fig.

5B), and Vi (Fig. 5C). SP immunoreactivity was prominent throughout the Vpara (Fig. 5D), as well as in the Vc, especially laminae I and II (Fig. 5E). To be more precise, the dense immunoreactivity of SP was distributed in lamina I and lamina IIo of the trigeminal nucleus caudalis (Fig. 5F).

4. Discussion

In this study, the expression of HK-1 was demonstrated for the first time using immunohistochemical analysis of the trigeminal ganglion and the trigeminal sensory nuclear complex of rats.

The present results showed that the HK-1 immunoreactivity in the trigeminal ganglion was markedly reduced by preabsorption of HK-1 peptide, whereas there was little change in HK-1 immunoreactivity following pretreatment with SP peptide. This indicates that the polyclonal antibody derived from HK-1 (1-6) peptide used in this study recognizes the HK-1 peptide, but not the SP peptide. HK-1 and SP belong to the same tachykinin peptide family, in which the C-terminal regions share a common sequence of amino acids, F-X-G-L-M, suggesting that polyclonal antibodies produced by HK-1 (1-11) and SP (1-11) peptides may include a small amount of antibodies that recognize these common amino acids in the C-terminal region, and consequently results in the reduction of the antibody specificity. Based upon the results of the preabsorption study, it is likely that the use of antibodies produced by peptides free from this common amino acid sequence is required for selectively detecting each peptide.

HK-1 and SP were mainly expressed in small- and medium-sized cells of the trigeminal ganglion. This result of SP was consistent with the findings of many previous studies (Hökfelt et al., 1975; Bae et al., 2015). Similarly, the expression of HK-1 immunoreactivity in trigeminal ganglion cells was

comparable with that of the TAC4 gene encoding HK-1 in the trigeminal ganglion (Duffy et al., 2003) and dorsal root ganglion (Matsumura et al., 2008), indicating that HK-1 peptide is produced in trigeminal ganglion cells. Most of trigeminal primary afferent neurons expressing SP are unmyelinated afferents (Bae et al., 2015), and this study revealed that the majority of HK-1-positive cells also expressed SP. Thus, it is likely that most of cells expressing HK-1 are also unmyelinated afferents. In addition, HK-1-positive cells expressed NeuN, a neuronal marker protein, indicating that the HK-1 peptide is produced in neurons in the trigeminal ganglion neurons, but not in microglial cells (Matsumura et al., 2008; Sakai et al., 2012). Taken together, these findings suggest that SP and HK-1 peptides are produced in the same trigeminal ganglion neurons, and are simultaneously released from central terminals of unmyelinated primary afferents into the trigeminal nuclear complex as transmitters or modulators. It is well known that SP is a pain neurotransmitter or neuromodulator, whereas HK-1 is involved in pruriceptive processing (Naono-Nakayama et al., 2014; Funahashi et al., 2014), suggesting that HK-1 released from the central terminals of trigeminal ganglion neurons may have the crucial role in pruriceptive processing. On the other hand, the localization of HK-1 in the trigeminal ganglion indicates that the peripheral terminals of trigeminal ganglion neurons expressing HK-1 distribute in the peripheral tissues. Indeed, the expression of TAC4 mRNA encoding HK-1 was detected in peripheral tissues such as skin, muscle, lung and liver (Kurtz et al., 2002; Duffy et al., 2003). Additionally, inflammatory

histopathological alterations in the joint homogenates after adjuvant-induced arthritis were smaller in TAC4$^{-/-}$ mice (Borbély et al., 2013), and inflammatory parameters in the lung after acute pneumonitis were diminished in Tac4$^{-/-}$ mice (Hajna et al., 2015). Taken together with data of HK-1 expression in trigeminal ganglion neurons, it is likely that HK-1 derived from ganglion neurons may play, at least in part, a crucial inflammatory role in the orofacial regions.

Since HK-1-positive primary afferent neurons expressed SP, it is likely that HK-1 and SP are simultaneously released from central terminal of trigeminal primary afferents by pruritic or noxious stimulation. Consequently, HK-1 and SP may bind to the same receptor (Morteau et al., 2001), suggesting that the HK-1-preferred receptor and NK1 receptor may express on the same neurons. Thus, it is reasonable that trigeminal nucleus caudalis neurons responsive to pruritic stimulation are also excited by stimulation using algogenic substances (Akiyama et al., 2010). In contrast, antagonists of the HK-1-preferred receptor and NK1 receptor inhibited the induction of pruriceptive processing and nociceptive processing, respectively (Naono-Nakayama et al., 2014; Funahashi et al., 2014). This finding indicates that the HK-1-preferred receptor and NK1 receptor play crucial roles in pruritic processing and nociceptive processing, respectively, despite the fact that both SP and HK-1 can bind to the NK1 receptor (Morteau et al., 2001). Unfortunately, the underlying mechanisms involved in the HK-1-preferred receptor and NK1 receptor in pruriceptive processing and

nociceptive processing, respectively, remains to be elucidated. Primary afferents derived from trigeminal ganglion neurons terminate in the trigeminal sensory nuclear complex. HK-1 and SP immunoreactivities were specifically localized in the paratrigeminal nucleus and the trigeminal nucleus caudalis, but not the trigeminal nucleus principalis, trigeminal nucleus oralis, or trigeminal nucleus interpolaris. This finding is consistent with the findings in a previous study on the distribution of gastrin-releasing peptide, an itch-related peptide, in the trigeminal sensory nuclear complex (Takanami et al., 2014). Thus, it is likely that the paratrigeminal nucleus and trigeminal nucleus caudalis expressing HK-1 play crucial roles in pruriceptive processing in the trigeminal sensory system.

The distribution of HK-1 and SP immunoreactivities in the trigeminal nucleus caudalis was dependent on each lamina; indeed, HK-1 was densely labeled in lamina I, while SP was localized through lamina I and lamina IIo (Greco et al., 2007). This finding suggests that pruriceptive information derived from HK-1 and nociceptive information derived from SP may be differentially processed in the trigeminal nucleus caudalis.

On the other hand, physiological analysis revealed the localization of cells responsive to pruritic stimulation in the spinal cord and the trigeminal nucleus caudalis. Several spinothalamic tract neurons responsive to histamine, a pruritogen, were localized in lamina I (Davidson et al., 2007; Davidson et al., 2012), and the majority of neurons responsive to some pruritogens were distributed in lamina I of the spinal cord (Akiyama et al.,

2014). Furthermore, injection of several pruritogens, including histamine and serotonin, into the cheek produced a unit response in lamina I neurons of the trigeminal nucleus caudalis (Klein et al., 2011), and pruriceptive trigeminoparabrachial tract neurons were located in lamina I of the trigeminal nucleus caudalis (Jansen and Giesler, 2015). These physiological data concerning the localization of pruritic responsive cells in the trigeminal nucleus caudalis are similar to the distribution of HK-1 in the trigeminal nucleus caudalis shown in this study, suggesting that pruriceptive information in the trigeminal sensory system may be processed in lamina I of the trigeminal nucleus caudalis. Additionally, the expression of c-Fos protein, a marker protein of neuronal activation, induced by intradermal injection of pruritogens was mainly distributed in lamina I of the spinal dorsal horn (Akiyama et al., 2009a; Akiyama et al., 2009b; Funahashi et al., 2014) and trigeminal nucleus caudalis (Akiyama et al., 2016). These results further support the above-mentioned proposal. Taken together, these findings show that the distribution of HK-1 in the trigeminal nucleus caudalis is very similar to the location of cells responsive to pruritus and c-Fos expressing cells in the trigeminal nucleus caudalis after pruritic stimulation, suggesting that HK-1 has a crucial role in pruriceptive processing in lamina I of the trigeminal nucleus caudalis.

5. Conclusions

The present studies showed the expression of HK-1 in the trigeminal ganglion neurons and in the paratrigeminal nucleus and lamina I of the trigeminal nucleus caudalis, indicating that HK-1 may be a transmitter or modulator released from primary afferents innervating orofacial regions and may have crucial roles in pruriceptive processing in the paratrigeminal nucleus and the trigeminal nucleus caudalis. Thus, it seems likely that HK-1 can serve as a target molecule for treatment of itch in the orofacial regions.

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Conflict of interest

All authors declare that there are no financial, personal, or other potential conflicts of interest to report.

Ethical approval

The experimental protocol used in the present study was approved by the Institutional Animal Care and Use Committee of the University of Miyazaki (approval number 2015-515).

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Figure legends

Figure 1. Specificity of the HK-1 (1-6) antibody and SP (1-6) antibody in the rat trigeminal ganglion. A, D; Immunohistochemistry of HK-1 (A) and SP

(D) was observed in the rat trigeminal ganglion. B, E; A complete absence of HK-1 immunoreactivity was recognized after preabsorption with an excess of HK-1 (1-11) peptide (B), while there was little change in HK-1 immunoreactivity after preabsorption with SP (1-11) peptide (C). Conversely, SP immunoreactivity was almost entirely unchanged after preabsorption with

HK-1 (1-11) peptide (E), while SP immunoreactivity was completely reduced by preabsorption with SP (1-11) peptide (F). Scale bar = 50 μm.

Figure 2. Histogram of size distributions of HK-1-positive, SP-positive or NeuN-positive cells in the rat trigeminal ganglion. Sizes of HK-1-positive cells and SP-positive cells were mainly small and medium, based on that of NeuN-positive cells. Scale bar = 50 μm.

Figure 3. Colocalization of HK-1 with SP or NeuN in trigeminal ganglion cells. (A) Most HK-1-positive cells (Green) exhibited the expression of SP (Red) in the trigeminal ganglion. (B) Some cells expressing HK-1 (Green) exhibited the expression of NeuN (Red). Scale bar = 100 μm.

Figure 4. Distribution of HK-1 immunoreactivity in the trigeminal sensory nuclear complex. A-C: HK-1 immunoreactivity was absent in the trigeminal nucleus principalis (Vp), the trigeminal nucleus oralis (Vo), and the trigeminal nucleus interpolaris (Vi). D-F: Arrows indicate HK-1 immunoreactivity in the paratrigeminal nucleus (D, Vpara) and lamina I of the trigeminal nucleus caudalis (E-F). In F, the outer dotted line indicates a border between spinal tract of trigeminal nerve and lamina I, and the inner dotted line indicates a border between lamina II and lamina III. Scale bar = 500 μm in A, B, C, E; 50 μm in D, F.

Figure 5. Distribution of SP immunoreactivity in the trigeminal sensory nuclear complex. A-C: SP immunoreactivity was absent in the trigeminal nucleus principalis (Vp), the trigeminal nucleus oralis (Vo), and the trigeminal nucleus interpolaris (Vi). D-F: Arrows indicate SP immunoreactivity in the paratrigeminal nucleus (D, Vpara) and lamina I and the outer layer of lamina II of the trigeminal nucleus caudalis (E-F). In F, the outer dotted line indicates a border between spinal tract of trigeminal nerve and lamina I, and the inner dotted line indicates a border between lamina II and lamina III. Scale bar = 500 μm in A, B, C, E; 50 μm in D, F.

Figure 1

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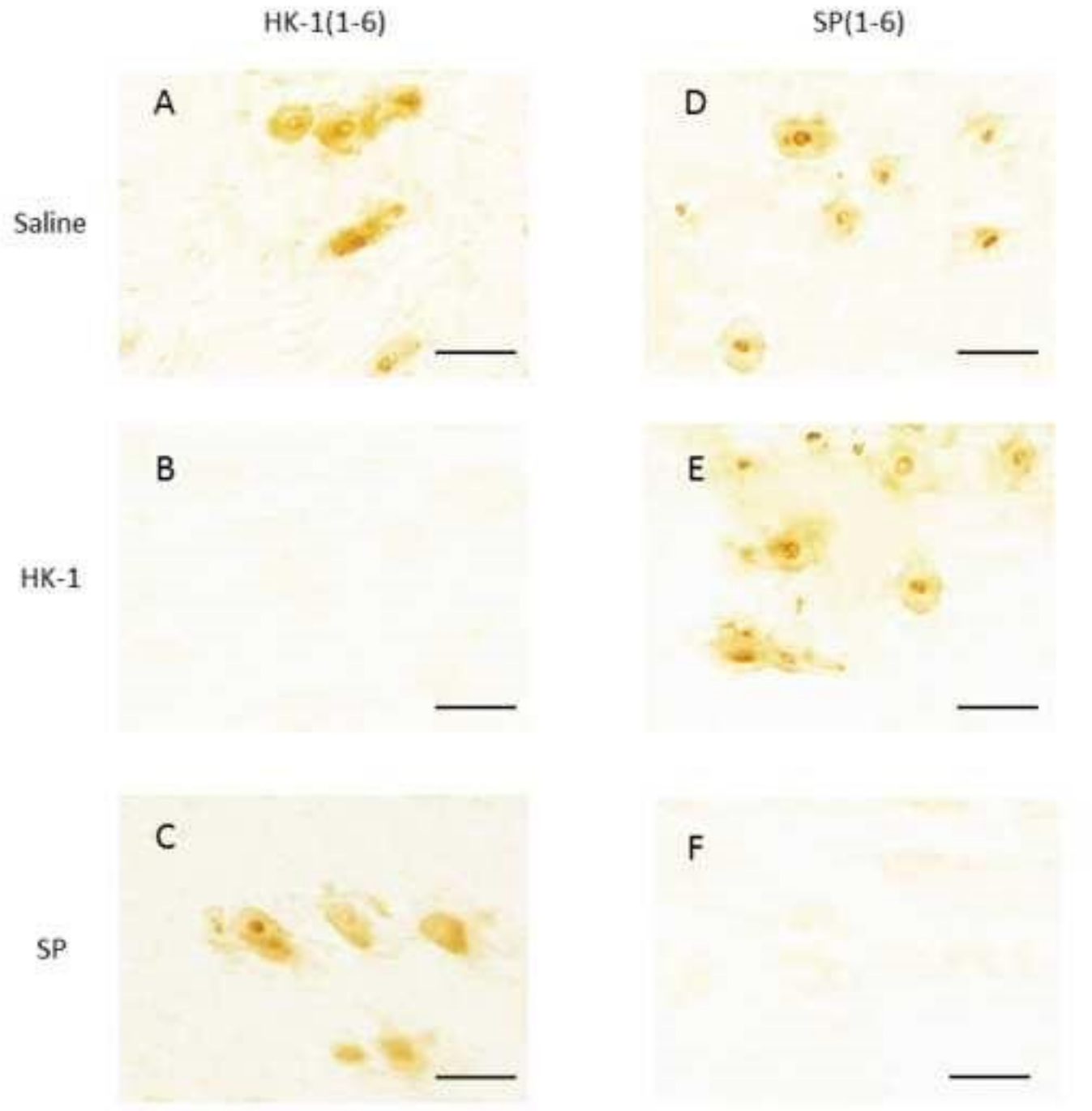

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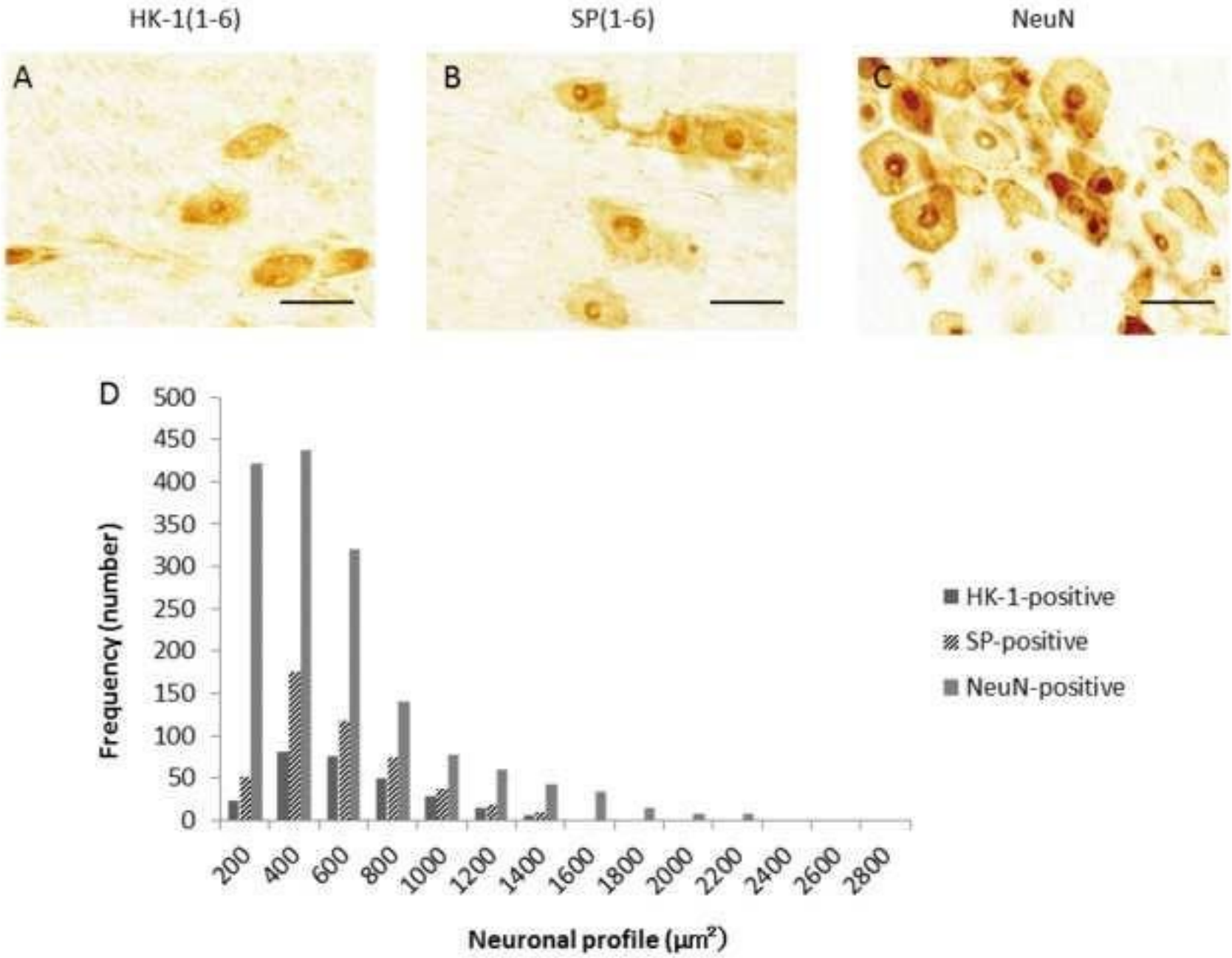

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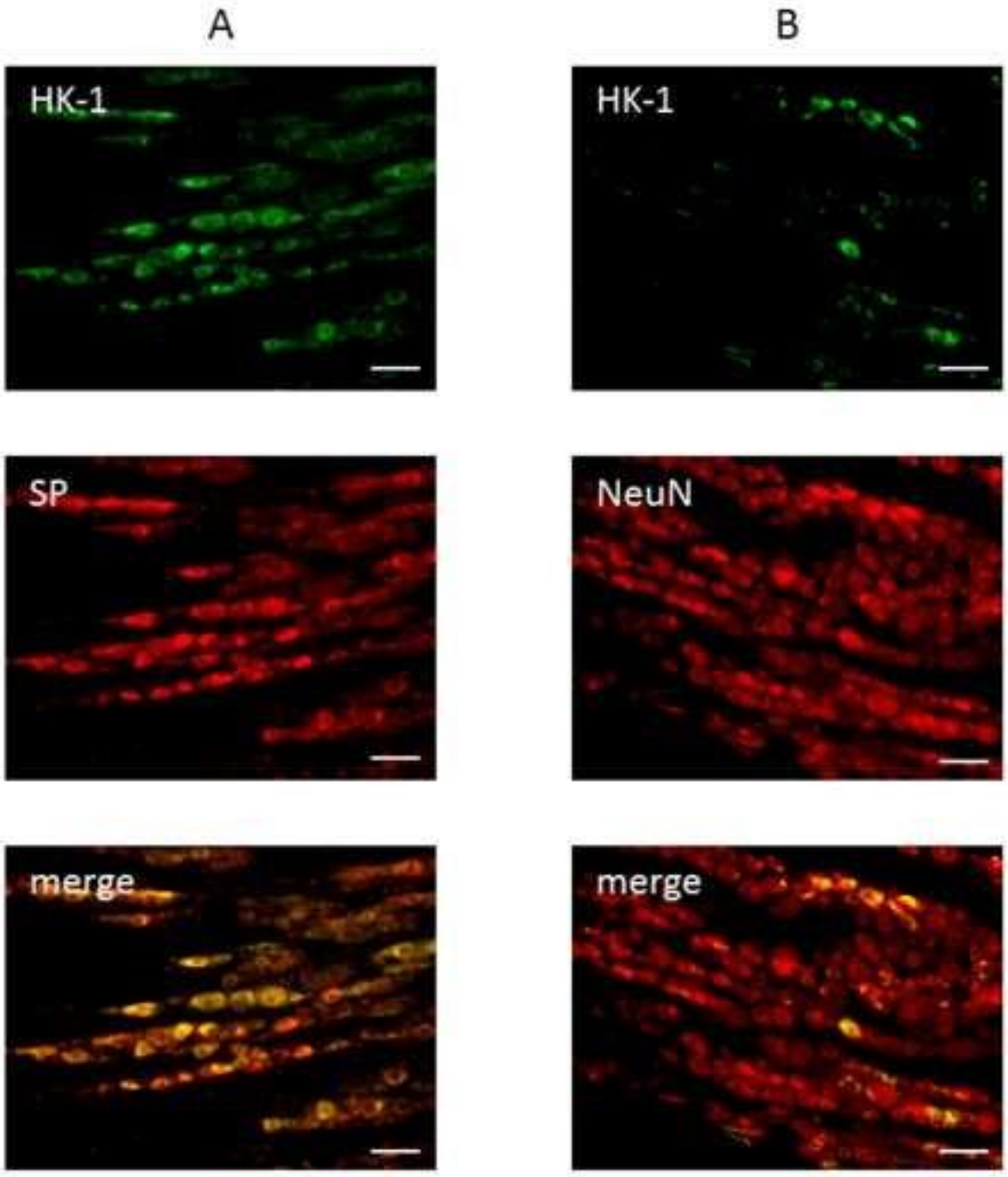

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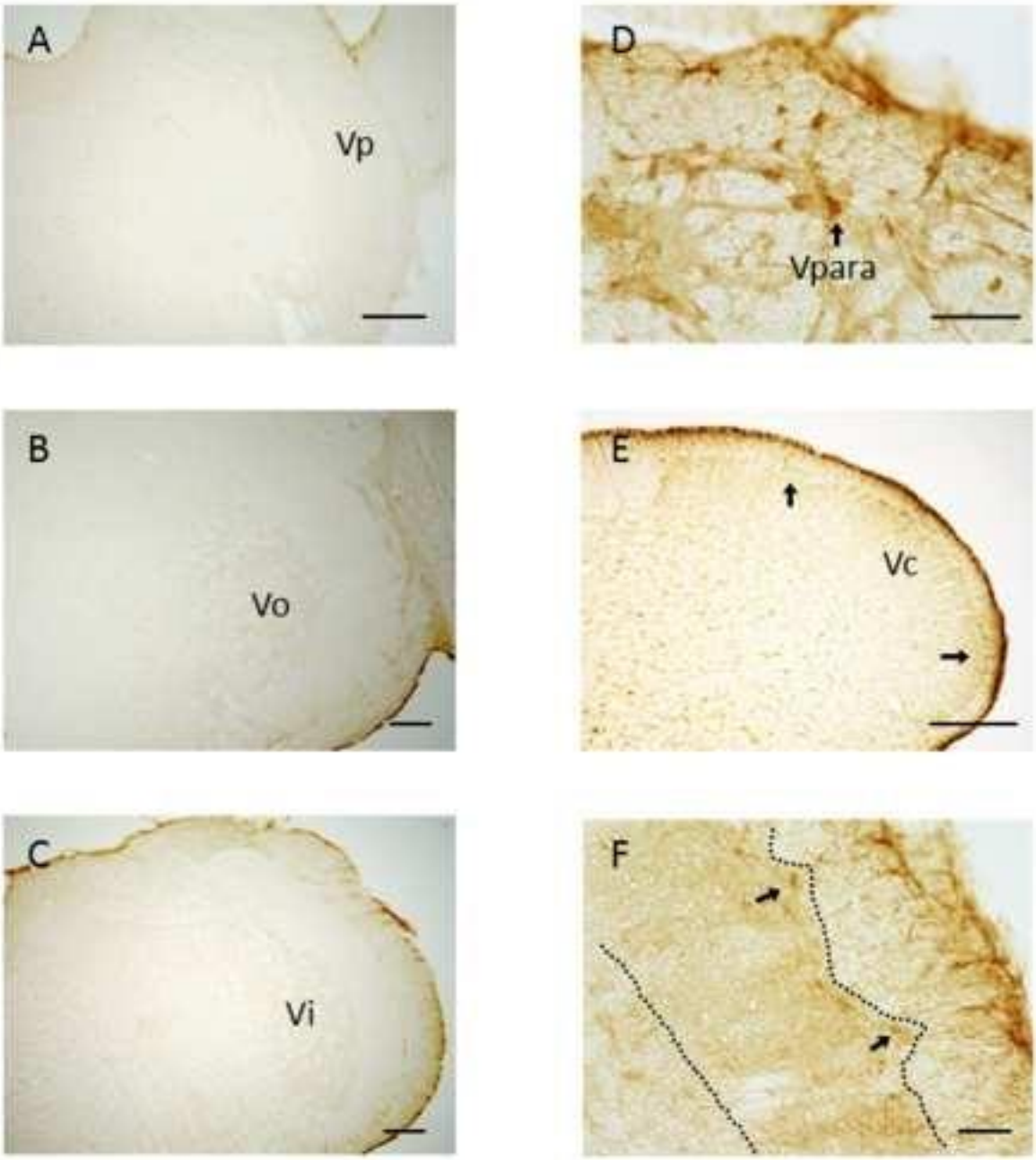

Figure 5

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