Postnatal development of substance P-immunoreaction in the trigeminal caudalis of neonatally capsaicin-treated mice*

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Summary. The trigeminal subnucleus caudalis (Vc) is a critical relay site for processing nociceptive afferent input from the orofacial area in addition to its modulation by neuroplastic change. Although an administration of capsaicin in neonates induces a selective destruction of substance P (SP)-immunoreactive nerve fibers, little information is available regarding its detailed effects on the Vc, particularly during postnatal development. The present study examined postnatal changes in the distribution of SP in the Vc and trigeminal ganglion (TG) by immunohistochemical techniques in naïve (NV) and neonatally capsaicin-treated (CP) mice, combined with a quantitative analysis. The neonatal mice received a single subcutaneous injection of capsaicin (50 mg/kg) at 48 hours after birth. The neural density of the SP-immunoreaction decreased to approximately a quarter of that in 1-week-old NV mice but increased to three-quarters of that in the NV in the superficial area after postnatal week 2. A double staining with SP and myelin basic protein confirmed the absence of any SP-immunoreaction in the myelinated nerve fibers

in both NV and CP mice. The SP-immunoreaction never overlapped with non-peptidergic IB4-labeled neurons in the Vc and TG of either group. Neither the size distribution of SP-positive neurons nor their relative ratio in the TG differed between NV and CP mice at the ages of postnatal weeks 1 and 8. These findings indicate two putative origins for the emergent SP-immunoreaction in the superficial layer of the Vc of the CP mice: the surviving trigeminal neurons with SP against capsaicin treatment and/or intrinsic neurons/interneurons in the Vc without SP under normal conditions.

Introduction

A systemic administration of capsaicin to neonatal animals causes attenuation of the nociceptive reflex against noxious chemical and thermal stimuli in their postnatal stage (Fitzgerald, 1983; Buck and Burks, 1986; Waite and de Prementier, 1997; He *et al.*, 2000), as evidenced by the elimination of unmyelinated afferent fibers (cf. Fitzgerald, 1983; Jancsó *et al.*, 1997). This treatment reduces substance P (SP)—a slow neurotransmitter that serves as a modulator of nociceptive transmission (Yoshimura 1996)—in the spinal dorsal horn (Helke *et al.*, 1981; Hammond and Ruda, 1991). Previous studies have shown that capsaicin administration causes the destruction of primary peptidergic neurons by interfering with the action of nerve growth factor (NGF) (Otten *et al.*, 1983) and/or the mitochondrial damage in surviving neurons (Szöke *et al.*, 2002), resulting in a reduction of SP content in adults (cf. Helke *et al.*, 1981). Although an investigation using an HRP-tracing method suggested that capsaicin is the main target for small-diameter unmyelinated neurons in neonates

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(Waite and de Prementier, 1997), the capsaicin-treated animals displayed a re-appearance of SP-immunoreaction in the dorsal horn during the postnatal period despite the continuous suppression of nociceptive responses (Hammond and Ruda, 1991). Thus, the neurotoxic effects of the systemic administration of capsaicin to neonatal animals remain unclear in detail.

The trigeminal subnucleus caudalis (Vc) plays an important role as a critical relay nucleus for the processing of nociceptive signals from the orofacial area as well as for nociceptive afferent input modulation mediated by descending inhibition to the Vc or by intrinsic neurons (for review, Sessle, 2000). Recently, we succeeded in demonstrating characteristic postnatal changes in the spatio-temporal distribution of the SP-immunoreaction in the Vc of normal mice (Aita *et al.*, 2005) and, further, a postnatal development and generation of an SP-mediated excitation propagation mechanism by optical imaging (Seo *et al.*, 2005). These findings readily suggest that neonatal capsaicin treatment alters the spatial distribution and postnatal development of SP in the Vc. However, little information is available regarding the relationship between the SP immuno-expression pattern and nociceptive responses as well as the modulation mechanism of trigeminal nociceptive response in capsaicin treated animals.

The present study was therefore undertaken to examine the postnatal changes in SP distribution in the Vc of capsaicin-treated mice by immunohistochemistry and quantitative analysis using a computer-assisted image analyzer. A double staining with SP and either *Griffonia simplicifolia* IB4 lectin or myelin basic protein (MBP) was also applied to clarify the relationship between SP and the non-peptidergic neuron (Sugimoto *et al.*, 1997; Petruska *et al.*, 2000)/myelinated fibers (Hamano *et al.*, 1996) in the Vc and trigeminal ganglion of naïve (NV) and neonatally capsaicin-treated (CP) mice.

Materials and Methods

All experiments were approved and performed based on the guidelines of the Niigata University Intramural Animal Use and Care Committee (approval number 161).

1. Animal and tissue preparations

A total of 32 mice was divided into two groups of NV and CP mice ($n=16$ each group). Since our previous electrophysiological studies have revealed nociceptive transmission features in the trigeminal area of mice with a depletion of C-fibers (Seo *et al.*, 2003, 2005), the same

capsaicin treatment was employed in this study. Capsaicin (8-methyl-N-vanillyl-6-nonenamide; Wako Pure Chemical Industries, Ltd., Osaka) was dissolved in an emulsion of 10% ethanol and 10% Tween 80 in isotonic saline. Under anesthesia with inhalation of halotane vapor (Takeda Chemical Industries, Ltd., Osaka), the neonatal mice received a single subcutaneous injection of capsaicin (50 mg/kg) within 48 h after birth. Untreated mice served as the NV group.

At a determined time (postnatal 1, 2, 4 and 8 weeks; $n=4$ for each age per each group), the animals were intraperitoneally anesthetized with chloral hydrate (400 mg/kg), and perfused with 4% paraformaldehyde containing 0.2% picric acid in a 0.1 M phosphate buffer (pH 7.4). The removed medullary brain stem and trigeminal ganglion were horizontally cut to a respective 40 and 20 μm thickness using a freezing microtome and treated as free-floating sections.

2. SP-immunohistochemistry for quantitative analysis

Protocols for immunostaining and statistical analysis have been provided in our previous report (Aita *et al.*, 2005). Briefly, after an inhibition of endogenous peroxidase with 0.3% H_2O_2 in absolute methanol, frozen sections were treated with Triton X-phosphate buffered saline (TX-PBS, pH 7.4) containing 2.5% normal goat serum (Vector Lab., Burlingame, CA, USA). The sections were primarily incubated with a polyclonal antiserum against SP (1:10,000, Chemicon International, Temecula, CA, USA). They were reacted with two consecutive incubations with a biotinylated goat anti-rabbit IgG (1:1,000, Vector Lab.) and an avidin-peroxidase complex solution (ABC kit; Vector Lab.) at room temperature (Hsu *et al.*, 1981). Final visualization of the immunoreaction sites used 0.04% 3-3'-diaminobenzidine (DAB) and 0.003% H_2O_2 in a 0.05 M Tris buffer (pH 7.6) with nickel ammonium sulfate (0.1%) intensification. The immunostained sections were thaw-mounted onto poly-L-lysine-coated glass slides, air-dried, and counter-stained with 0.03% methylene blue.

The Vc consists of four layers—an outer pure fiber layer, a thin layer containing many cell bodies and a fiber network, a translucent layer, and a deep layer containing many fibers and cells—that respectively correspond to the tract (Tr), marginal layer (Mar), substantia gelatinosa (SG), and magnocellular layer (Mag). Since the rostral pole of the SG in the Vc appeared as a semilunar-shaped translucent layer, it is difficult to compare and analyze the morphological features at different depths among different animals. Therefore, in this study, we compared the densities of the SP-immunoreactivity in areas approximately 1000 μm caudal to the most rostral part of the Vc, where

Fig. 1. Representative microphotographs of SP-immunoreactivity in the Vc of naïve (a–h) and capsaicin treated (i–p) mice at 1 week (a, e, i, m), 2 weeks (b, f, j, n), 4 weeks (c, g, k, o) and 8 weeks of age (d, h, l, p). Note the SP-immunoreaction in the superficial layer. Tr: trigeminal spinal tract, Mar: marginal layer, SG: substantia gelatinosa, Mag: magnocellular layer. Scale bars: 200 μm (a–d, i–l), 100 μm (e–h, m–p).

each layer showed relatively flat laminar structures. Since these areas were located caudally to the obex, the measured areas corresponded to the Vc or the Vc/cervical spinal cord transition zone (Meng *et al.*, 1997; Sugimoto *et al.*, 1997). Because the size of the Vc remained unchanged after postnatal week 2, the average widths of the Mar and SG in the horizontal sections were approximately 40 and 80 μm , respectively. However, the combined width of the

Mar and SG was approximately 40 μm in the 1 week-old mice. Therefore, we set 40 μm as the minimum width and standard for analyzing the immunopositive areas when clarifying the postnatal changes in the distributions of the SP-immunoreaction in the Vc.

For quantitative analysis ($n=4$ per group for each developmental stage), we measured the density of the SP-immunoreaction in the Vc using an image analyzer (KS300;

Fig. 2. Neural densities (%) of the SP-immunoreaction (mean + S.D) from the border between the trigeminal spinal tract and the marginal layer (Mar) in the Vc for 1-week, 2-week, 4-week and 8-week-old mice. Open and hatched columns indicate the densities of SP-immunoreaction in the naïve and capsaicin treated mice, respectively. The square bars under each graph indicate the depth in the Vc and the corresponding layer. SG: substantia gelatinosa, Mag: magnocellular layer, Ret: the medullary dorsal reticular formation.

Carl Zeiss, Jena, Germany) according to our previous report (Aita *et al.*, 2005). The averaged percentage ratios of the SP-immunoreactive areas to the defined surface areas were shown as the mean $\pm$ S.D. Statistical analysis of comparisons of SP-immunoreaction at the same depth in the Vc among the different age groups was performed by one-way ANOVA. The null hypothesis was rejected whenever the statistical risk probability rated below 5% ($p < 0.05$).

3. Double Immunohistochemistry for SP and MBP (myelin basic protein)

Free floating sections were treated with TX-PBS containing a mixture of 2.5% normal donkey (Jackson Lab., Baltimore Pike, PA, USA) and goat (Vector Lab.) sera for 1 h at room temperature. They were reacted with a mixture of antisera to SP raised in a guinea pig (1:100, American

Fig. 3. Merged images showing the trigeminal subnucleus caudalis (Vc; **a–d**) and trigeminal ganglion (TG; **e–h**). Double fluorescent immunohistochemistry. **a** and **b**: Double labeling with SP (red) and IB4 (green) in the Vc. 8-week-old naïve (**a**) and capsaicin treated (**b**) mice. Note the different distribution of SP-immunoreaction and IB4-labeling. **c** and **d**: Double labeling with SP (green) and myelin basic protein (MBP; blue). There is no co-localization of SP and MBP in the Vc. Eight-week-old naïve (**c**) and capsaicin treated (**d**) mice. **e–h**: Double labeling with SP (red) and IB4 (green) in the TG. Neither naïve nor capsaicin treated mice (**e, f**: 1 week old; **g, h**: 8 week old) show any overlapping of SP and IB4 in the trigeminal neuron. Tr: trigeminal spinal tract, Mar: marginal layer, SG: substantia gelatinosa, Mag: magnocellular layer. **a–d** and **e–h** at the same magnification, respectively. Scale bars indicate 50 μm .

Research Products, Belmont, MA, USA) and MBP raised in a rabbit (Histofine, Nichirei, Tokyo) at 4°C for 2 days. These reacted sections were incubated with fluorescein thiocyanate (FITC)-conjugated AffiniPure F(ab')₂ fragment donkey anti-guinea pig (1:200, Jackson Lab.) and bitotylated anti-rabbit IgG (Vector Lab.) for 1 h. Finally, they were reacted with 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated avidin (10 $\mu\text{g}/\text{ml}$, 1:100, Vector Lab.). These immunoreacted sections were thaw-mounted onto poly-L-lysine-coated glass slides, cover-slipped with Vectashield (Vector Lab.), and examined with a fluorescent microscope (Axioplan 2; Carl Zeiss).

4. Double labeling with IB4 and SP

The frozen sections were reacted with normal donkey serum (Vector) for 1 h. After incubation with a rabbit polyclonal antiserum against SP (Chemicon International) followed with CyTM 3-conjugated AffiniPure F(ab')₂ fragment donkey anti-rabbit IgG (1:500, Jackson Lab.), the immunostained

sections were labeled with FITC conjugated *Griffonia simplicifolia* IB4 (10 $\mu\text{g}/\text{ml}$, 1:100, Sigma, St. Louis, MO, USA) for 1 h at room temperature. They were observed with a fluorescent microscope (Axioplan 2; Carl Zeiss).

5. Quantitative analysis of SP-positive or IB4-labeled neurons in trigeminal ganglion

The cross-sectional areas of SP-positive, IB4-labeled, and all neurons in the trigeminal ganglion showing the nucleolus profiles were calculated from 4 sections per animal (3 animals per group at postnatal 1 and 8 weeks) using an NIH image (<http://rsb.info.nih.gov/ij/download.html>), and their size distribution (frequency (%)) was measured. The relative ratio of SP-labeled neurons to total trigeminal neurons per section was also calculated at postnatal 1 and 8 weeks. Statistical analysis was performed between NV and CV mice by a *t*-test. A *p* value of less than 0.05 was considered statistically significant.

Results

Immunostaining with antisera against SP demonstrated age-specific changes in the distribution of SP-immunoreactive nerve fibers in the Vc of both NV and CP mice during the observation period (Fig. 1). In 1-week-old mice, the immature cyto-architecture made it impossible to distinguish between the Mar and SG, but these two areas could be clearly identified after postnatal 2 weeks.

NV mice at 1 week of age exhibited distributions of neural elements with an SP-immunoreaction that were dense in the Mar and the superficial part of the SG ($79.5 \pm 10.5\%$), but slight or absent in the superficial Mag (Fig. 1a.). In the area more than $500 \mu\text{m}$ in depth of Mag, the SP-immunoreaction began to increase by deepening, showing a U-shaped distribution pattern (Fig. 2). After postnatal 2 weeks, the SP-immunoreaction did not show remarkable changes in the superficial area. However, it

gradually decreased in density in the deep part of Mag with aging (Fig. 1b, 2) and almost vanished from the Mag at postnatal weeks 4 and 8 (Fig. 1c, d, 2).

CP mice showed completely different postnatal changes in the distribution of the SP-immunoreaction throughout this experimental period (Fig. 1). Compared with NV mice, the CP mice received a smaller innervation of SP-positive neural elements. In 1-week-old CP mice, the density of the SP-immunoreaction in the Mar and superficial part of SG was $20.7 \pm 8.8\%$, which comprised approximately a quarter of that in 1-week-old NV mice ($79.5 \pm 10.5\%$) (Fig. 1i, 2). There was a significant difference in the density of the SP-immunoreaction at 1 week of age between the CP and NV mice (*t*-test, $p < 0.05$). In contrast, the SP-immunoreaction in the superficial part increased in density in 2-, 4- and 8-week-old CP mice compared with that in 1-week-old CP mice ($51.8 \pm 14.0\%$ in 2-week old, $40.6 \pm 18.1\%$ in 4-week old and $44.5 \pm 17.0\%$ in 8-week old, respectively) (Fig. 1i–p, 2). In the deep part of the Mag of CP mice, the

Fig. 4. Size distribution of trigeminal ganglion neurons in naïve (NV) and capsaicin treated (CP) mice. Size frequency histograms of trigeminal neurons show the same pattern of cell size frequency among these groups. The mean sectional areas in both types of mice increase with aging, but the NV mice exhibit a larger cross-sectional area than CP mice at both periods.

SP-immunoreaction was undetectable as observed in NV mice throughout this experimental period (Fig. 2).

A double labeling with SP and either IB4 or MBP in the Vc demonstrated a quite different localization of SP-positive and IB4-labeled neural elements (Fig. 3a–d). IB4-labeling was found in superficial layers of the Vc—exclusively in the inner layer of the SG corresponding to lamina III in the spinal dorsal horn—whereas the SP-immunoreaction was recognizable in the more superficial layer, Mar (Fig. 3a, b). IB4-labeled neural elements never overlapped with SP-immunoreactive neurons either in the NV or CP mice at the age of postnatal week 8 (Fig. 3a, b). In a double immunostaining with SP and MBP, myelinated nerves were present in the trigeminal spinal tract and superficial part of Mar in the Vc while SP-positive neural elements appeared in the Mar of both the NV and CP mice (Fig. 3c, d). Neither NV nor CP mice showed a double reactivity for MBP or SP-immunoreaction in the Mar and SG. The CP mice displayed a neuronal reticular construction intensely-stained with MBP-antiserum in the Mag layer as compared with that in NV mice (Fig. 3c, d). The SP-immunoreaction failed to co-localize with IB4-labeling in both NV and CP mice in the trigeminal ganglion (Fig. 3e–h).

The mean value of the cross-sectional area of total trigeminal neurons showed $309.8 \pm 119.9 \mu\text{m}^2$ (a total of 1276 neurons) and $413.0 \pm 254.8 \mu\text{m}^2$ (a total of 1400 neurons) at postnatal 1 and 8 weeks, respectively, in NV mice. On the other hand, CP mice exhibited a lower value of the mean cross-sectional area: $272.8 \pm 111.9 \mu\text{m}^2$ (a total of 1412 neurons) and $354.2 \pm 171.9 \mu\text{m}^2$ (a total of 904 neurons) at postnatal 1 and 8 weeks, respectively. The medians of the cross-sectional area were $292.7 \mu\text{m}^2$ and $250.9 \mu\text{m}^2$ at postnatal 1 week, and $346.1 \mu\text{m}^2$ and $315.0 \mu\text{m}^2$ at postnatal 8 weeks (NV versus CP mice). A size distribution of the trigeminal ganglion is shown in Figure 4.

The mean cross-sectional area of SP-immunoreactive neurons in the trigeminal ganglion of the NV and CP mice was $216.2 \pm 72.4 \mu\text{m}^2$ (a total of 216 neurons) and $217.3 \pm 63.4 \mu\text{m}^2$ (a total of 121 neurons) at postnatal 1 week. However, its value at postnatal 8 weeks showed $245.8 \pm 77.2 \mu\text{m}^2$ (a total of 99 neurons) and $246.2 \pm 83.2 \mu\text{m}^2$ (a total of 72 neurons), respectively. There was no significant difference in size between the NV and CP mice at both postnatal weeks (t -test, $p > 0.05$). These data indicate that SP-positive cells in the trigeminal ganglion with capsaicin treatment could be categorized as a

Fig. 5. Size distribution of SP-positive neurons in trigeminal ganglion. Size frequency histograms of SP-positive trigeminal neurons show the same pattern of cell size among the groups.

small diameter cell. SP- positive trigeminal neurons showed a similar size distribution pattern between NV and CP mice at the age of both 1 and 8 weeks, but trigeminal ganglion cells at postnatal week 8 tended to show a larger size distribution than at postnatal week 1 (Fig. 5). Furthermore, no difference in the mean of the cross-sectional area of IB4-labeled trigeminal ganglion cells existed between NV ($265.0 \pm 96.7 \mu\text{m}^2$ in a total of 165 neurons at postnatal 1 week and $347.2 \pm 110.0 \mu\text{m}^2$ in a total of 302 neurons at postnatal 8 weeks) and CP ($282.2 \pm 115.0 \mu\text{m}^2$ in a total of 140 neurons at postnatal 1 week and $343.6 \pm 95.7 \mu\text{m}^2$ in a total of 117 neurons at postnatal 8 weeks) mice at each postnatal stage.

The relative ratio of the SP-positive to total trigeminal neurons in NV mice was $17.0 \pm 5.2\%$ at postnatal 1 week but decreased to $7.14 \pm 2.5\%$ at postnatal 8 weeks with a significant difference. However, the relative ratio in CP mice was unchanged between postnatal 1 and 8 weeks ($8.57 \pm 3.1\%$ in postnatal 1 week and $8.07 \pm 3.8\%$ in postnatal 8 weeks). There was a significant difference between NV and CP mice at postnatal 1 week but none at postnatal 8 weeks.

Discussion

By use of immunohistochemistry and quantitative analysis, the present study was able to demonstrate postnatal changes in the distribution of SP-immunoreaction in the Vc of CP mice. Current data on NV mice was comparable with our previous findings of spatial and temporal changes in the distributions of SP-immunoreactivity in the Vc after birth (Aita *et al.*, 2005).

The neonatal injection of capsaicin is known to induce a selective degeneration of unmyelinated primary afferent fibers to a large extent (Jancsó *et al.*, 1977), including laminae I and II of the Vc as well as the spinal dorsal horn (Fitzgerald, 1983), despite various capsaicin treatments. This treatment induces an apoptosis of trigeminal ganglion cells (Sugimoto *et al.*, 1998) and mitochondrial damage in certain neurons (Szöke *et al.*, 2002), resulting in a loss of small B type ganglion cells (Fitzgerald 1983, Waite and de Prementier, 1997; Szöke *et al.*, 2002) and substantia gelatinosa neurons in the spinal dorsal horn (Yang *et al.*, 2003). Neonatal capsaicin animals attenuated c-fos expression in the trigeminal nucleus by single or repetitive injections, suggesting that the unmyelinated afferent pathway remains to be destroyed in adult animals (He *et al.*, 2000). These findings may explain the reduced nociceptive response in the adult animals of neonatally capsaicin treated animals (Buck and Burks, 1986; Khasar and Livine, 1996).

A most notable finding in this study is the emergent SP-

immunoreaction in the superficial layer of Vc in the CP mice. A similar phenomenon has been reported in the spinal cord (Hammond and Ruda, 1991); it was observed that the reduced SP density recovered after 6 weeks of the age in neonatal capsaicin treatment rats. However, this report did not mention postnatal changes in the spatial distribution of SP there, due to limitations of the observation period. As far as we know, this is the first report to show characteristic postnatal changes in the SP-immunoreaction in the Vc of the CP mice.

There are four possible explanations for the emergent SP-immunoreaction in the superficial layer of the CP mice. First, the SP-immunoreaction recovered in the superficial layer may have originated from the non-peptidergic neurons in the Vc or trigeminal ganglion. Second, the emergent SP-immunoreaction could be produced by large or medium-sized (myelinated) neurons in the Vc. Third, damaged trigeminal neurons could survive to produce SP. Finally, SP-negative intrinsic Vc neurons/interneurons under normal conditions are capable of producing SP via phenotypical changes by neonatal capsaicin treatment.

Nociceptive neurons in the dorsal root ganglion can be divided into three populations: 1) small-diameter IB4 positive cells which give rise to unmyelinated fibers, 2) small-diameter peptidergic cells which give rise to unmyelinated fibers (IB4-negative), and 3) medium-diameter peptides-containing cells which have A δ axons (Priestley *et al.*, 2001). Neonatal capsaicin, in the same way as in this study, may affect unmyelinated nerve terminals (Waite and de Prementier, 1997). Current observation showed that SP-positive neuronal elements never co-localized with IB4-labeled fibers in the Vc and trigeminal ganglion of CP mice, indicating that the non-peptidergic neurons did not produce SP to be conveyed to the superficial layers of the Vc.

MBP immunoreactivity appeared dense in the Mag of the CP mice compared with NV in this study, suggesting that A-fiber projection increased there. This phenomenon is consistent with that of capsaicin treatment, but two injections with the same dose of capsaicin as in our study induced a deficit of C-fibers termination which in turn induced A-fiber terminations more dorsally, extending to lamina II (Torsney *et al.*, 2000). By use of a double immunostaining, no overlapped labeling of MBP and SP in the Vc was recognizable in either NV or CP mice, indicating that the SP-immunoreaction in the CP mice never occurred in myelinated fibers in the Vc. Taken together with the present quantitative analysis, the second hypothesis of ours cannot account for the occurrence of the emergent SP-immunoreaction in the superficial layer of the Vc in the CP mice.

Many researchers agree with the idea of a selective

degeneration of primary afferent fibers by capsaicin treatment but not their complete destruction (Buck and Burks, 1986; Holzer, 1991). Jancsó *et al.* (1977) reported a 70% reduction of primary afferent fibers, suggesting that the remaining afferent fibers can survive to maintain SP-immunoreaction. Szöke *et al.* (2002), who reported a loss of 39.4% of small sensory neurons with two-day-injections of capsaicin, found mitochondrial damage in the trigeminal ganglion of neonatal capsaicin-treated rats under a transmission electron microscope. The present numerical analysis shows that the CP mice had a similar size distribution and relative ratio of SP-positive neurons to those in the NV in the trigeminal ganglion, suggesting the survival of the damaged neurons against capsaicin treatment. Szöke *et al.* (2002) speculated that the storage of sensory neuropeptides such as SP might be impaired by unrecoverable mitochondrial damage, resulting in the disturbance of SP-release. Thus, it is reasonable to propose that capsaicin-affected neurons in the trigeminal ganglion maintain and/or re-produce SP.

The primary afferent unmyelinated fiber terminals of the Vc and spinal dorsal horn have been reported to contain SP, but the intrinsic neuron in the SG lacked (Ribeiro-da-Silva *et al.*, 1989, 1991). Previous reports have shown that peripheral inflammation induces SP-immunoreaction by a phenotypical switch, resulting in an increase in SP expression in large myelinated fibers (Neumann *et al.*, 1996) as well as silent mechanoreceptive A β and C fibers (Yu *et al.*, 2000; Xu and Zhao, 2001). These findings suggest the possibility that capsaicin treatment enables neurons without SP under normal conditions to produce certain peptides—including SP—by a phenotypical switching. This idea may explain the emergent SP in the superficial layer by phenotype-changed intrinsic neurons/interneuron of the Vc. However, we failed to find any SP-positive neurons in the SG layer of the Vc of CP as well as NV mice throughout this observation period. Therefore, it seems more likely that the emergent SP in the superficial layer by 8 weeks is due to the up-regulation of SP in the capsaicin-affected neurons of the trigeminal ganglion rather than to phenotype-changed intrinsic neurons/interneuron of the Vc. However, the involvement of phenotypical changed intrinsic neurons/interneuron of the Vc in the re-appearance of SP-immunoreaction in the superficial layer can not be excluded because Szöke *et al.* (2002) found drastic changes in the SP-immunoreaction after 20 weeks in capsaicin treated animals.

A critical discrepancy exists between the behavioral non-recovery and both morphology and neurological peptide recovery. Hammond and Ruda (1991) reported a recovery of SP-density in neonatal capsaicin animals after 6 weeks of age. In contrast, our optical imaging

study revealed that excitation propagation in the Vc -- which is mediated by SP in neonatal capsaicin animals -- does not recover even after postnatal week 7 (Seo *et al.*, 2005), as supported by physiological studies. A possible explanation is that the emergent SP in the superficial Vc is not a normal nociceptive transmitter which is released from afferent central terminals in the Vc. Therefore, the previous physiological findings of a lack in nociceptive response after capsaicin injection can be explained by the hypothesis that the emergent SP in the Vc is derived from neurons not relating to nociceptive transmission. The neurons with the emergent SP-expression might be involved in some other unknown function, such as neurotrophic effects, beside nociceptive transmission. This will constitute a subject for a future study due to the current lack of sufficient data.

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