Polysialylated Neural Cell Adhesion Molecule Supports Regeneration of Neurons in the Nucleus Ambiguus After Unilateral Recurrent Laryngeal Nerve Avulsion in Adult Rats

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Summary: Objectives. A correlation appears to exist between the expression of the polysialic acid neural cell adhesion molecule (PSA-NCAM) and repair in central nervous system (CNS) diseases. However, the expression of PSA-NCAM in the CNS after peripheral nerve injury remains unclear. This study aimed to evaluate the expression of PSA-NCAM in the ipsilateral nucleus ambiguus (NA) after unilateral recurrent laryngeal nerve (RLN) avulsion.

Materials and Methods. The left RLN of adult Sprague Dawley rats were avulsed. The expression of PSA-NCAM, PSA-NCAM/NeuN, and PSA-NCAM/Tuj1 in the brain stem was investigated using immunohistochemistry. The results were subjected to one-way analysis of variance followed by the Tukey post hoc test for statistical analyses.

Results. PSA-NCAM-positive and PSA-NCAM/NeuN and PSA-NCAM/Tuj1 double-labeled positive cells were observed 7 days post injury in the ipsilateral NA. PSA-NCAM/NeuN and PSA-NCAM/Tuj1 double-labeled cells were observed at 21 and 7 days post injury, respectively. PSA-NCAM/NeuN double-labeled cells were also found in the contralateral NA.

Conclusions. After unilateral avulsion of the RLN, the expression of PSA-NCAM in the ipsilateral NA was correlated with the proliferation and the differentiation of neural cells. PSA-NCAM expression may be used as a predictor of the initiation of repair in neural cells.

Key Words: Recurrent laryngeal nerve–Avulsion–Nucleus ambiguus–Repair–Polysialic acid neural cell adhesion molecule.

INTRODUCTION

In 1982, Finne[1] identified polysialylated glycopeptides (polysialic acids [PSAs]) as a characteristic structure in the developing brain. Since then, further studies have reported the high content of polysialylated structures in the neural cell adhesion molecule in developing brains, now known as polysialic acid neural cell adhesion molecule (PSA-NCAM).[2,3] Because of the strongly hydrophilic nature of PSA, PSA-NCAM has a larger volume than the neural cell adhesion molecule, which results in the reduction of adhesive activity between cells expressing PSA-NCAM at the cell surface.[3] In addition, the features of PSA, such as long, negatively charged and linear homopolymers, enable PSA-NCAM to regulate the differentiation of precursors, which is associated with signal transmission.[4] Consequently, the effect of PSA-NCAM on the signaling pathway influences neurite outgrowth, fasciculation, axonal guidance, branching, and synaptogenesis.[5]

PSA-NCAM is primarily expressed in the embryonic nervous system. Because of the abundance of expression, PSA-NCAM is also known as the "embryonic" isoform of the neural cell molecule involved in histogenesis. The correlation is reflected in the role of phosphorylation in the structure of neural tissue.[6] However, in the adult nervous system, PSA-NCAM is expressed at only very low levels, and in particular areas of the nervous system that retain the capacity for neural plasticity, remodeling of neural connections, or neural regeneration. These areas include the hippocampus, the subventricular zone, the thalamus, the prefrontal cortex, the amygdala,[5] the hypothalamus, habenular nuclei, the mesencephalic central gray, the lateral geniculate nucleus, and dorsal spinal laminae.[7] Current research investigating these areas demonstrates the relevance between the distribution of PSA-NCAM and alterations in function. In the adult hypothalamus, the persistence of PSA-NCAM is relevant to neuroendocrine function.[8] In the adult hippocampus and cortex, which are associated with memory, PSA-NCAM expression has been reported to be associated with synaptic potentiation, adaptation to fear or stress, memory access, and consolidation.[9,10] In the spinal cord, the removal of PSA may lead to a reduction in the sensitivity to pain.[11] The distribution of PSA-NCAM in the adult nervous system under physiological conditions is significantly correlated with physiological and structural plasticity.

When the nervous system is damaged by disease or injury, PSA-NCAM may play an important role in the process of regeneration and remodeling, which, in large part, is based on its phosphorylation properties. The function of PSA-NCAM in the brain of adult patients with Alzheimer and Parkinson diseases may be altered, which is reflected by decreased expression and is negatively correlated with phosphorylation status.[12] In transgenic PSA-NCAM-deficient mice, the reduction in neural plasticity has a causal role in the development of depressive behavior.[13] After transient middle cerebral artery occlusion in the rat brain, some involved regions exhibited

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immunohistochemically positive cells for PSA-NCAM, and part of these regions demonstrated a capacity for plasticity after injury.[14] After spinal cord injury, coordinating increases in the level of PSA-NCAM in the primary region could promote functional recovery by enhancing neural cell remyelination.[15] Changes in PSA-NCAM expression are apparent not only in the central nervous system (CNS) but also in the peripheral nervous system, and these alterations exhibit primary and secondary patterns. In an experimental model of femoral nerve injury, a significantly greater expression of PSA-NCAM in the primary region was able to improve repair by regenerating the axon, facilitating its reach to the appropriate receptor organ.[16] After transection of the infraorbital branch of the trigeminal nerve, cortical neurons become immunoreactive to PSA-NCAM.[17] Another research has found that levels of PSA-NCAM increase in the superior colliculus after optic nerve transection.[18] Extensive understanding of the physiological distribution of PSA-NCAM and the impact on the CNS after peripheral nerve injury is, therefore, imperative.

The aim of the present study was to confirm the distribution of PSA-NCAM in the nucleus ambiguus (NA) of the adult rat and whether alteration of PSA-NCAM occurs in response to recurrent laryngeal nerve (RLN) injury. More specifically, we designed the study to confirm the expression of PSA-NCAM in the bilateral NA and the attempts of neurons to remodel after RLN avulsion using immunohistochemical double-labeling methods.

MATERIALS AND METHODS

Animals and surgical procedures

Adult male Sprague Dawley rats (Beijing, China, weight 200–250 g, 8–9 weeks of age) were used in the experimental procedures and were housed in accordance with guidelines established in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 8023, revised 1978). Fifty-four rats were deeply anesthetized using pentobarbital sodium (60 mg/kg [intraperitoneal injection]) during surgery. A middle skin incision was made in the anterior of the neck and the trachea was exposed. The left RLN was microdissected from the left tracheoesophageal groove. All experimental rats were then randomly divided into two groups: the injury group (n = 45) and the control group (n = 9). In the injury group, the separated left RLN was avulsed at the seventh tracheal ring level and was removed from the distal RLN by gentle traction using microsurgery forceps. In the control (sham) group, the left RLN was performed *without* injury; the anterior neck incision and soft tissue were then sutured.

Immunohistochemistry

After RLN avulsion, the following operations were immediately performed in six rats, whereas the others required reanesthetization at 6 and 12 hours, and 1, 3, 7, 14, and 28 days. The animals were perfused transcardially with saline (warmed to 37°C), followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, cooled to 4°C). The brain stems were extracted in cold (4°C) 4% paraformaldehyde in 0.1 M PBS for 2 hours, rinsed with PBS, and cryoprotected in 30% sucrose in PBS. The brain stems were embedded in OCT compound (Sakura, Tokyo, Japan), cryosectioned (14 μm thick), and used for immunohistochemistry.

For immunohistochemistry, the sections were washed three times in PBS for 15 minutes each and immersed in the mixture of 0.1 M PBS containing 10% normal goat serum and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 60 minutes to eliminate the influence of nonspecific immunostaining. The sections were then incubated in 0.1 M PBS containing 0.1% Triton X-100 and mouse monoclonal anti PSA-NCAM antibody (1:500; Chemicon, Temecula, CA) at 4°C overnight.

To examine the relationship between PSA-NCAM-immunoreactive cells and neurons, double immunohistochemistry was performed using anti-PSA-NCAM antibody and antibodies against other neural markers. The sections were incubated overnight with anti-PSA-NCAM antibody, as mentioned previously, and with one of the primary antibodies: rabbit monoclonal to neural nuclei (1:500; NeuN, Abcam, Cambridge, United Kingdom) or rabbit polyclonal to class III β-tubulin (1:200, TuJ1; Abcam).

All sections were then washed with PBS three times and incubated with specific secondary antibodies as follows: Alexa Fluor 488 goat anti-rabbit (1:500; Molecular Probes, Eugene, OR), Alexa Fluor 568 goat anti-mouse (1:500, Molecular Probes). The sections were incubated with the secondary antibodies at room temperature for 2 hours and then washed with PBS. A scanning confocal microscope (LEXT OLS laser A; Olympus, Tokyo, Japan) was used for observation.

Statistical analysis

One-way analysis of variance was used to compare all data, followed by Tukey post hoc test (GraphPad Software, San Diego, CA); $P < 0.05$ as considered statistically significant. All data were expressed as mean ± standard deviation.

RESULTS

PSA-NCAM- and NeuN-positive cell distribution in the ipsilateral NA after injury

Regardless of whether the ipsilateral NA or the contralateral NA was observed, there were no clear changes in the number of NeuN-positive cells, and no PSA-NCAM-positive cells were apparent (Figure 1A, B). NeuN immunoreactivity (a marker for mature neurons) was observed in a gradually decreasing trend compared with the control group in the ipsilateral NA after left RLN avulsion (Figure 1A, D, G, J, M). PSA-NCAM-positive cells were found at least 7 days post injury. Furthermore, these cells were not positive for NeuN (Figure 1E, F). Double-labeled cells were present until 21 days post injury (Figure 1I, L). Although double-labeled cells were found at both 21 and 28 days post injury, there was no statistical significance between the two ($P > 0.05$).

PSA-NCAM-positive cells appeared to be immunoreactive, expanding to the axon. However, the NeuN-immunoreactive region was restricted to the cytomembrane. Generally, the colocalized areas did not include axons (Figure 1I, L; white arrow).

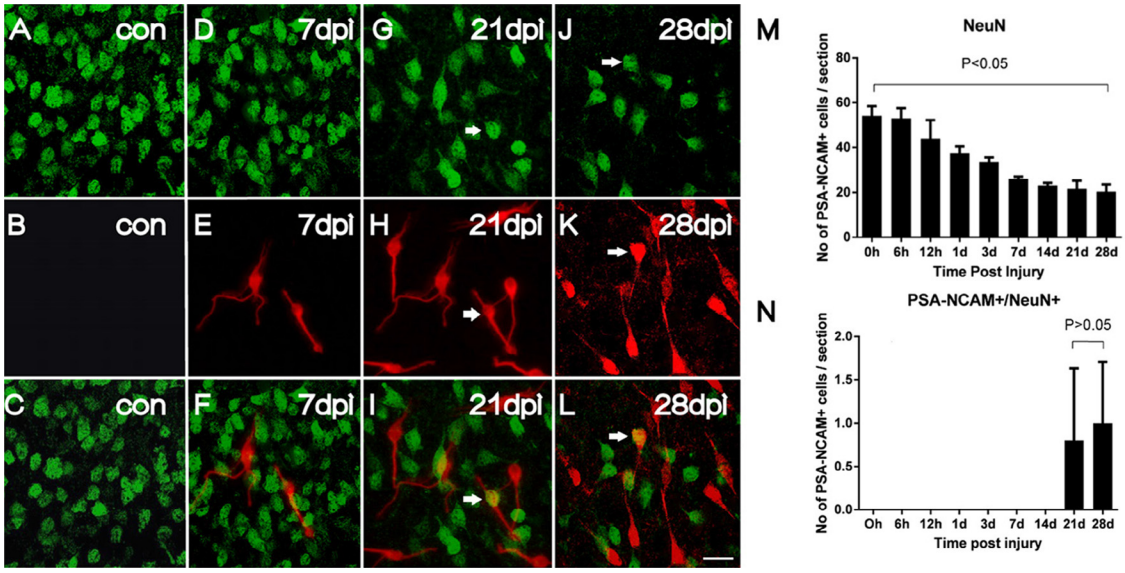

FIGURE 1. Expression of PSA-NCAM and NeuN in the ipsilateral nucleus ambiguus after left RLN avulsion. No PSA-NCAM-positive cells were apparent in the control group (**B**). At 7 days post left RLN avulsion, PSA-NCAM-positive cells (*red*) could be observed (**E**). Meanwhile, the number of NeuN-positive cells (*green*) (**D**) was reduced compared with the control group (**A**). (**F**) Merged image: there were no double-labeled NeuN and PSA-NCAM cells. (**C**) Merged image in the control group: no double-labeled cells were apparent. At 21 days post left RLN avulsion, PSA-NCAM and NeuN double-labeled cells could be observed (**I**, *white arrow*). (**G**, **H**, *white arrow*) NeuN- or PSA-NCAM-labeled cells in the same position. At 28 days post left RLN avulsion, PSA-NCAM and NeuN double-labeled cells were also apparent (**L**, *white arrow*). (**J**, **K**, *white arrow*) NeuN- or PSA-NCAM-labeled cells in the same position. (**M**) The gradually decreasing trend of NeuN-positive cells ($P < 0.01$). (**N**) Change in the number of double-labeled cells ($P > 0.05$). Values presented as mean ± standard deviation. Scale bars = 20 μm. dpi, days post injury; con, control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

PSA-NCAM- and tuj1-positive cell distribution in the ipsilateral NA after injury

Tuj1 is a marker of neurons in the initial period of development (ie, immature neurons). However, in the ipsilateral and the contralateral NAs, expression of Tuj1 in the control group was absent. At the beginning of the experiment, Tuj1-labeled cells could be found in the ipsilateral NA at 6 hours post injury. As time progressed, Tuj1-immunoreactive labeled cells gradually increased until the end of the experiment, 28 days post injury (Figure 2A, D, G). PSA-NCAM-positive cells were not observed before 7 days post injury. At 7 days post injury, not only were PSA-NCAM-labeled cells present, but also PSA-NCAM- and NeuN double-labeled cells could be observed (Figure 2B, E, C, F). The double-labeled cells also exhibited an upward trend in number ($P < 0.05$).

The PSA-NCAM-positive cells appeared to be immunoreactive while expanding to the axon. The Tuj1-immunoreactive regions were also expanded to parts of the axon. Therefore, the colocalized areas included not only the cytomembrane but also parts of the axon (Figure 2C, F; white arrow).

PSA-NCAM- and NeuN-positive cell distribution in the contralateral NA after injury

At 28 days post injury, PSA-NCAM-positive cells were found in the contralateral NA (Figure 3D). Meanwhile, PSA-NCAM-positive cells were immunoreactive for NeuN but not colocalized with Tuj1 (Figure 3F). In the double-labeled cells, the colocalized areas were the same as the ipsilateral NA, which did not include axons.

DISCUSSION

There have been no studies describing the localization of PSA-NCAM in the NA. In the adult rat, the absence of PSA-NCAM distribution suggests that properties of regeneration and remodeling are blocked. After unilateral RLN avulsion, the re-expression of PSA-NCAM in the NA can be considered a response of the CNS to injury in the peripheral nerves. The present study describes the expression of PSA-NCAM in the bilateral NA of adult rats under normal physiological conditions and after unilateral RLN avulsion, and provides evidence of neurons regenerating in the NA after RLN avulsion.

Expression of PSA-NCAM in the ipsilateral NA

We observed the localization of PSA-NCAM immunostaining in the NA of adult rats under physiological conditions, with no immunohistochemically positive cells. These results were consistent with studies investigating the localization of PSA-NCAM in the brain stem.[19] In other words, the loss of expression of PSA-NCAM in the bilateral NA suggests that the physiological mechanism of neuron remodeling in these regions had not begun until after birth. In the present study, we established an animal model of left RLN avulsion. At the beginning of the experiment, we initially found a decrease in NeuN-positive cells (we considered NeuN to be a marker of mature neurons[20]). Over

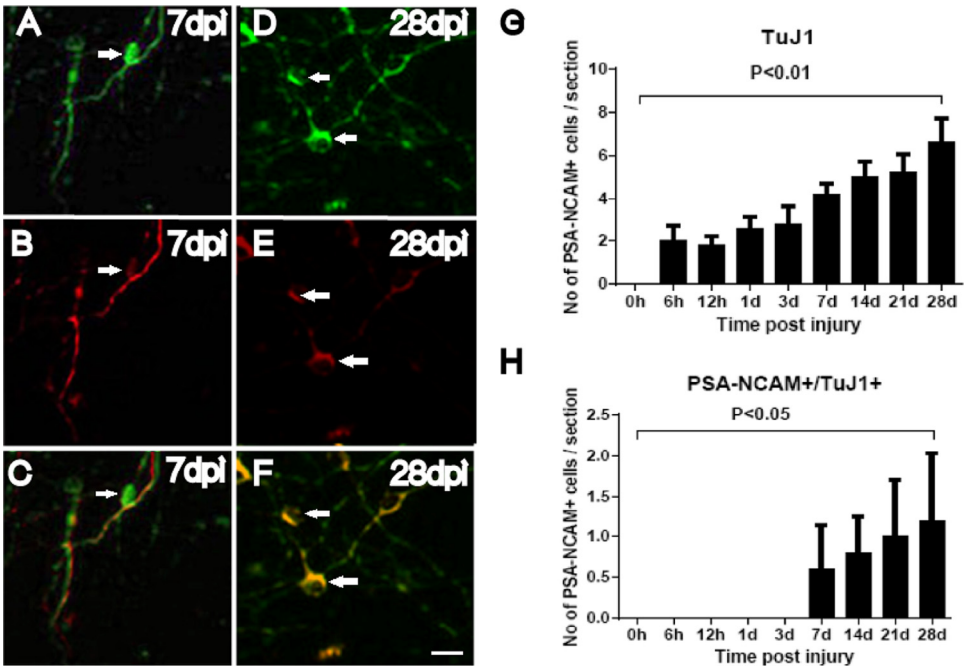

FIGURE 2. Expression of PSA-NCAM and Tuj1 in ipsilateral NA after left RLN avulsion. From 6 hours post left RLN avulsion, Tuj1-positive cells (*green*) could be observed and gradually increased in number to the end of the experiment (**A**, **D**, **G**). However, at 7 days post injury, PSA-NCAM and Tuj1 double-labeled cells were apparent (**C**, *white arrow*). (**A**, **B**, *white arrow*) Tuj1-labeled (*green*) or PSA-NCAM-labeled cells (*red*) in the same position. From 7 to 28 days post injury, double-labeled immunoreactive cells (**F**, *white arrow*) gradually increased in number (**H**) ($P < 0.05$). (**D**, **E**, *white arrow*) Tuj1-labeled or PSA-NCAM-labeled cells in the same position at 28 days post injury. Values presented as mean ± standard deviation. Scale bars = 10 μm. dpi, days post injury. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

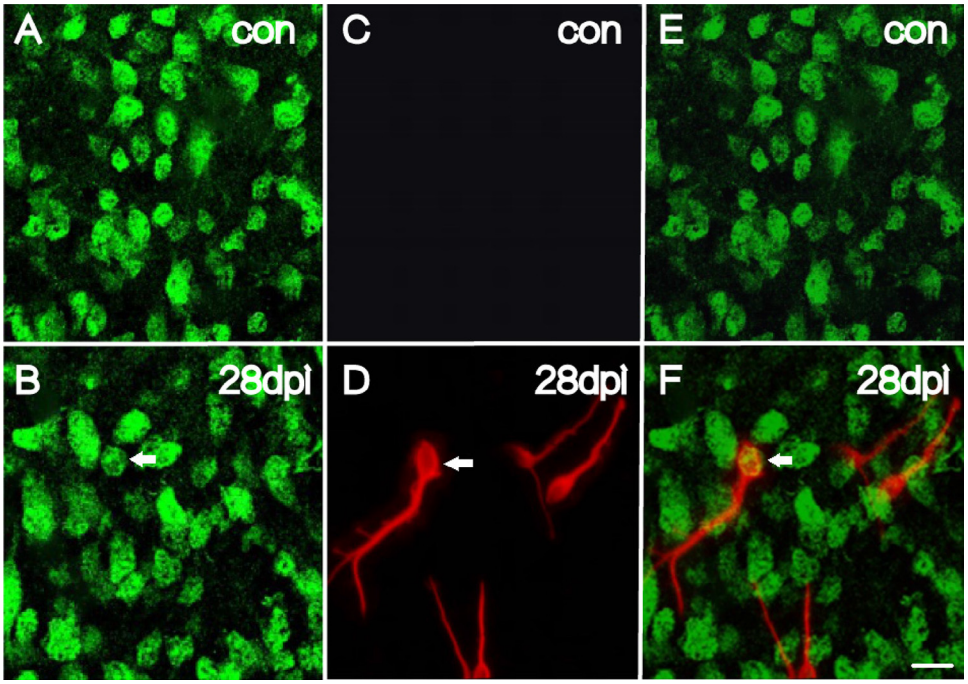

FIGURE 3. Expression of PSA-NCAM and NeuN in the contralateral NA after left RLN avulsion (**A**) and (**B**) images of NeuN-positive cells (*green*) in the contralateral NA. (**C**) No PSA-NCAM immunoreactive-labeled cells (*red*) were observed in the contralateral NA of either the control or the experimental group before 28 days post injury. Unitil 28 days post injury, PSA-NCAM labeled cells chould be observed (**D**; *white arrow*). Meanwhile PSA-NCAM and NeuN double-labeled cells could also be observed (**F**; *white arrow*). (**B**; *white arrow*) NeuN labeled cells in the same position. (**E**, **F**) Merged images. Scale bars = 20 μm. dpi, days post injury; con, control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the duration of the entire experiment, the number of NeuN-positive cells exhibited a downward trend in the ipsilateral NA and no significant change in the contralateral NA. The change in the number of mature neurons in the bilateral NA suggested that the ipsilateral NA could exhibit damage response secondary to unilateral RLN avulsion. Although PSA-NCAM-positive cells were apparent in the ipsilateral NA at 7 days, they were not present as early as 6 hours after RLN avulsion. Clearly, the re-expression of PSA-NCAM was delayed compared with the reduction in the number of neurons. After peripheral nerve injury, the delayed re-expression of PSA-NCAM in the CNS may be a feature of the secondary effects of damage.[17,18] The delayed re-expression is different from the immediate appearance of PSA-NCAM after primary craniocerebral injury, which has been reported to occur in <1 day.[21]

The delay was not only in the time point of re-expression of PSA-NCAM. Seven days after RLN avulsion, NeuN-expressing cells in the ipsilateral NA were not positive for PSA-NCAM. Except for this time point, the colocalization results were consistent with previous reports. For example, it has been reported that PSA-NCAM is a marker of immature neurons and that NeuN is a marker of mature neurons in the dentate gyrus.[22] The turning point occurred 21 days after RLN avulsion. PSA-NCAM appeared to be localized in NeuN-immunolabeled neurons, which is similar to what is observed in the superior colliculus after optic nerve transection[18] and in the caudate nucleus of patients with Alzheimer disease.[12] However, there has been no clear description of differences in time course and labeling in previous studies. First, the difference suggests that PSA-NCAM was not localized in all NeuN-immunolabeled neurons in the ipsilateral NA after unilateral RLN avulsion. Moreover, there are two possible explanations for the sequence of colocalized staining as time progressed: NeuN-positive neurons underwent structural changes to colocalize with PSA-NCAM, or PSA-NCAM-positive cells underwent structural changes to colocalize with NeuN.

Significance of PSA-NCAM re-expression in neuronal regeneration in the ipsilateral NA

Immature Tuj1-labeled cells were immunohistochemically positive only in the ipsilateral NA 6 hours after unilateral RLN avulsion, which suggests that these immature neurons were activated and began to participate in the repair process in the ipsilateral NA as early as the initial period of secondary damage. Before the emergence of PSA-NCAM, no double-labeled neurons were observed. After the emergence of PSA-NCAM, however, Tuj1 double-labeled neurons could be observed. Double-labeled neurons have also been found after low-level lead exposure in the hippocampus of adult rats.[23]

As described, neither all NeuN-positive cells nor all Tuj1-positive cells could be colocalized with PSA-NCAM. Furthermore, the mature neurons in the NA may have the potential to juvenilize after RLN injury.[24] The assumption was that neurons expressing PSA-NCAM were present at a developmental stage between extremely mature and extremely immature. These "transitional" neurons may be useful in the repair process after secondary damage to the ipsilateral NA.

Significance of PSA-NCAM expression in the contralateral NA

Alterations in the ipsilateral NA after unilateral RLN injury have been previously based on anatomical phenomenon. At the initial stages of the experiment, there were no abnormal findings in the contralateral NA. However, 28 days after the unilateral RLN was avulsed, previous understanding should be revisited, in that double immunohistochemical-positive cells with PSA-NCAM and NeuN could be visualized in the contralateral NA. It was, or at least was meant to be, affected by contralateral NA by unilateral RLN avulsion. Whether this represents a "cause and effect" is difficult to interpret, and is a different phenomenon from expression in the bilateral superior colliculus after left optic nerve transection,[18] because the ipsilateral RLN receives the efferent fibers from the ipsilateral NA. Therefore, expression in the contralateral NA may be related to the cortex. Furthermore, in experimental models of traumatic brain injury, changes in PSA-NCAM could be observed not only in the primary injury regions but also in the contralateral cortex.[21] In CNS, the regulation of injured peripheral nerves is not just in the nucleus.

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