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Review

MORPHOLOGICAL AND NEUROCHEMICAL PLASTICITY OF RAT MESENCEPHALIC TRIGEMINAL NEURONS

Angel D. Dandov[1*], Dimitrinka Y. Atanasova[2,3], and Nikolai E. Lazarov[1,3]

[1]*Department of Anatomy and Histology, Medical University, Sofia, Bulgaria;*

[2]*Department of Anatomy, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria;*

[3]*Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

The mesencephalic trigeminal nucleus (Me5) is a unique structure in the central nervous system (CNS), made up of pseudounipolar sensory neurons. It is also a suitable paradigm for studying the plastic alterations in neurons. It is known that the Me5 neurons utilize various neurotransmitters under normal conditions, though little information is available about the morphological and chemical events taking place in the nucleus after injury. This review provides concise description of the structural adaptive changes in Me5 neurons following peripheral axotomy of the masseteric nerve. Furthermore, it validates NADPH-diaphorase activity in them, and using immunohistochemistry for glutamate (Glu), substance P (SP), calcitonin-gene related protein (CGRP), neuropeptide tyrosine (NPY) and galanin (GAL), it deals with the altered neurochemical phenotype of the injured neurons. Our results distinctly show that the Me5 neurons in the rat are extremely sensitive to peripheral injury and we demonstrate their distinct structural and neurochemical plasticity. The adaptive morphological alterations comprise of both qualitative and quantitative alterations in the axotomized Me5 population which are statistically significant when compared with the number and phenotype of the neurons on the contralateral intact side. Besides, the axotomy-induced alterations in the neurochemical character of Me5 are best signified by the down-regulation of the classical neurotransmitters under normal conditions, and the up-regulation of nitric oxide synthase and de novo *synthesis of certain neuroactive substances such as NPY, SP, GAL and VIP. It can be inferred that the described phenomena only occur in the nucleus in cases of injury and changes in the environmental cues, and serve as adaptive mechanisms and powerful trophic factors for the neuronal survival in the Me5. There is, undoubtedly, still a long way to go in order to clarify the dynamic and plastic alterations occurring in the CNS in health and disease, and also explain their role in such important functions as pain, perception, learning, cognition and memory.* ***Biomed Rev*** **2019;30:63-81**

Keywords: axotomy, mesencephalic trigeminal neurons, neurotransmitters, plasticity, rat

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*Correspondence: Dr Angel D. Dandov, Department of Anatomy and Histology, Faculty of Medicine, Medical University-Sofia, 1 Sv Georgi Sofiiski Str., BG-1431 Sofia, Bulgaria. Tel.: +359-2-9172 602, E-mail: adandov@medfac.mu-sofia.bg

INTRODUCTION

The primary sensory neurons whose bodies are located in cell clusters, called ganglia, transmit somatosensory information to neurons in the central nervous system (CNS) from different types of afferent receptors in the periphery. In contrast to the spinal ganglia and the major part of cranial sensory ganglia, one of the most characteristic features of the trigeminal sensory system is that the bodies of primary trigeminal afferent neurons are located both in the trigeminal ganglion (TG) and in the brain, in particular, the mesencephalic nucleus of trigeminal nerve (Me5) (reviewed in 1-7). Some researchers of the trigeminal sensory system claim that the TG is a cranial analogue of a peripheral spinal ganglion in the peripheral nervous system (PNS) (8). On the other hand, the Me5 is the only known brain nucleus which contains the perikarya of primary afferent neurons (9, 10). The Me5 is also unique in that its cells comprise one clearly differentiated functional class of trigeminal sensory neurons, which exclusively subserve proprioception. The TG cells receive afferent information mainly from pressure and stretch mechanoreceptors, thermoreceptors, and nociceptors, located in the area of the face, mouth, and nasal cavity (11, 12). It is also well known that TG neurons receive proprioceptive information from the muscles of mastication, more specifically, from the jaw-closing and jaw-opening muscles (13). Their central axons form synaptic contacts on several groups of second-order neurons, which, in turn, transmit impulses to the somatosensory cortex, passing through the thalamus (14, 15). Some of the second-order neurons, however, form local networks in the CNS and their central processes do not reach the cortex (16,17).

The Me5 neurons mainly innervate the muscle spindles in the masticatory and extrinsic eye muscles (18-20), as well as other receptor types in the periodontal ligaments (21-27) and the dental pulp (28, 29).

The primary trigeminal afferent neurons share a common embryonic origin, but have different fate during their development. The various populations of cells, which comprise TG and Me5 can be identified based on their morphological characteristics, neurotransmitter profile and electrophysiological peculiarities. From a morphological point of view, it is considered that the neurons of Me5 are very similar if not even identical with the cells in the cranial and spinal ganglia (9, 10). It is also accepted that the peripheral processes of Me5 neurons have the same conduction velocity of the nerve impulse as the low-threshold mechanoreceptive afferent neurons in the TG (23, 26, 30). However, it still remains unexplained to what extent these neurons differ regarding their neurochemical characteristics, as well as whether or not they share an identical neurotransmitter profile. The elucidation of this issue is of key significance, since the characteristics of neurotransmission and synthesis of neuroactive substances in different neuronal populations is often directly related to their target projections. It is assumed that the trigeminal primary afferent neurons manifest signs of innervation-specific neurochemical expression, a concept called neurochemical coding (31).

At present, it is well known that a peripheral nerve injury induces dynamic and adaptive changes in the structure and neurochemical content of neurons in the areas innervated by the injured nerve, a phenomenon commonly known as neuroplasticity, and more specifically, structural and chemical plasticity (32). It is also known that the Me5 neurons are very sensitive to peripheral nerve injury, which results in a significant cell loss (33, 34). On the other hand, their survival in altered environmental conditions largely depends on the presence of certain neurotrophic factors (7, 34-36).

This review outlines the morphological and neurochemical changes in the Me5 in response to peripheral nerve axotomy.

LOCATION AND NORMAL MORPHOLOGY OF THE MESENCEPHALIC TRIGEMINAL NUCLEUS

The Me5 in rats is a bilateral longitudinal column of about 1000-1500 neurons extending for 4-5 mm in the rostral part of pons, and along the whole rostrocaudal length of the mesencephalon. The predominant part (60-80%) of Me5 neurons are located in the rostral pons, where they are grouped in 2-9 cells in the triangle between the locus coeruleus and medial parabrachial nucleus (Fig. 1), medially bordering the superior cerebellar peduncle *(brachium conjunctivum)*. In a rostral direction, at the midbrain level, the scattered Me5 neurons are observed as a thin and bent marginal plate of perikaryal profiles, laterally fencing off the periaqueductal gray (Fig. 2). The Me5 in rats comprises of two distinct subpopulations of nerve cells. The majority are large-sized, while a subset of them are small in size spherical or ovoid pseudounipolar neurons (Fig. 1B, 2B), the latter observed along the entire length of the nucleus. Besides, small-sized and spindle-shaped multipolar neurons are observed in the caudal pontine part of the nucleus (Fig. 1B).

MORPHOLOGICAL CHANGES IN THE MESENCEPHALIC TRIGEMINAL NUCLEUS AFTER PERIPHERAL AXOTOMY

Following experimental unilateral cut of the masseteric nerve and a survival period of 7 days, the Me5 neurons on the ipsi-

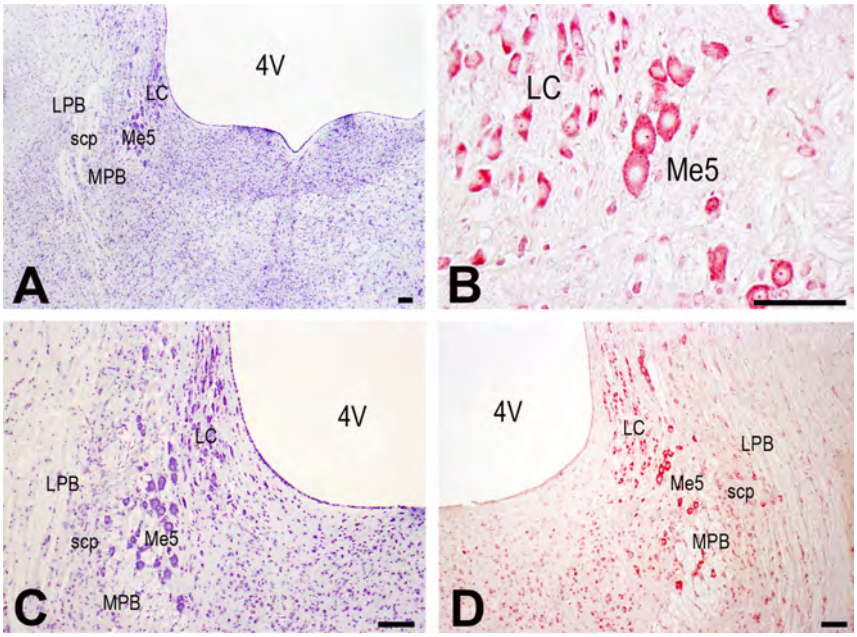

Figure 1. *Location of the neuronal population in the caudal part of the Me5 in rats.*
(A) A low magnification of the nucleus at the level of pons, demonstrating the location of Me5 neurons adjacent to the locus coeruleus (LC) and parabrachial nuclei. (B-D) Neutral red-stained Me5 sections at a high (B) and low magnification (D), and stained with cresyl violet (C), showing the location of Me5 perikarya in aggregates between the LC and the medial parabrachial nucleus (MPB). Lateral parabrachial nucleus (LPB), pedunculus cerebellaris superior (scp), ventriculus quartus (4V). Scale bars = 50 µm.

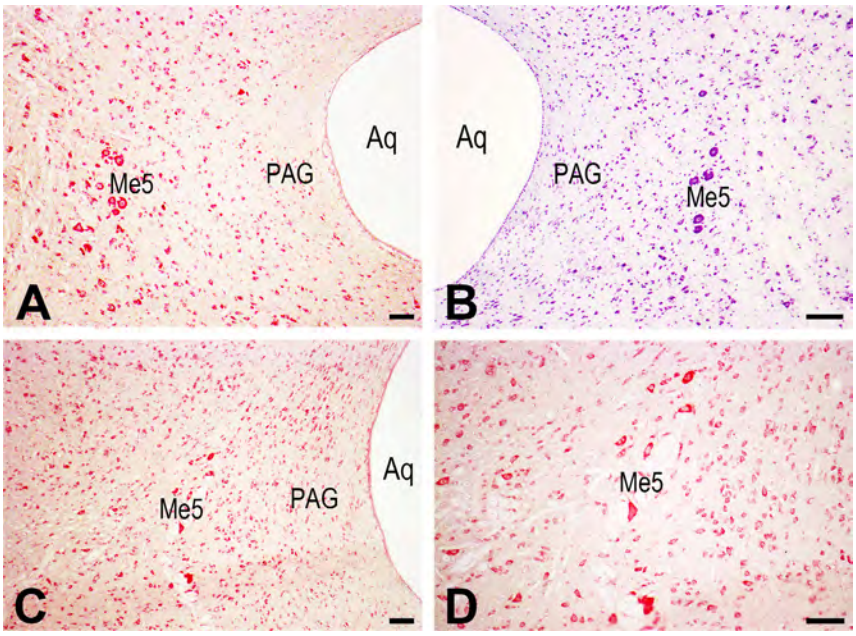

Figure 2. *Location of the neuronal population in the rostral part of the Me5 in rats.*
(A-C) A low magnification of the nucleus at the level of the mesencephalon, demonstrating the location of Me5 neurons, laterally from the periaqueductal gray (PAG). (D) A higher magnification of the mesencephalic part of the nucleus, indicated in (C), showing dispersed profiles of Me5 neurons. Aq, aqueductus cerebri. Staining with neutral red (A, B, D) and cresyl violet (B). Scale bars = 50 µm.

lateral axotomized side demonstrated insignificant decrease of their number, compared with the Me5 neurons on the contralateral intact half (Fig. 3A). A similar finding was registered along the entire length of the nucleus, both at the level of pons and mesencephalon. At that time, at a higher magnification we found the first morphological signs of chromatolysis in the perikarya of the axotomized Me5 neurons (Fig. 3B). The chromatolytic changes in the nucleus were more evident in the large-sized Me5 neurons compared to those in the smaller pseudounipolar and multipolar Me5 neurons. They involved both the cell nucleus and the cytoplasm, and were characterized with fading and in some neurons also with completely absent basophilic-stained Nissl bodies in their perikarya. The nuclei of damaged neurons were enlarged and displaced peripherally, and the heterochromatin appeared disintegrated and despiralized (Fig. 3B).

Degenerative changes were also observed in the axonal processes distal from the site of the nerve cut. They demonstrated the typical pattern of the so-called Wallerian degeneration and were manifested with a disintegration of the axonal skeleton, derangement in the axolemma integrity, and subsequently affecting the myelin sheath.

Two weeks after the axotomy, the number of injured Me5 neurons on the side of nerve cut continued to decrease compared to the contralateral control side (Fig. 3C). At that time, together with the persistent and already described morphological cell changes, basophilic granulations and degenerative profiles could be observed in the perikarya of the damaged Me5 neurons (Fig. 3D).

After a survival period of 21 days, the reduction in Me5 neuronal number at the level of pons on the side of the injury was clearly visible. In fact, the number of axotomized neurons was significantly smaller, compared to the population of intact neurons on the control side (Fig. 4A). Their cell bodies, regardless of size and shape, demonstrated all signs of neuronal degeneration (Fig. 4B). Similar findings were

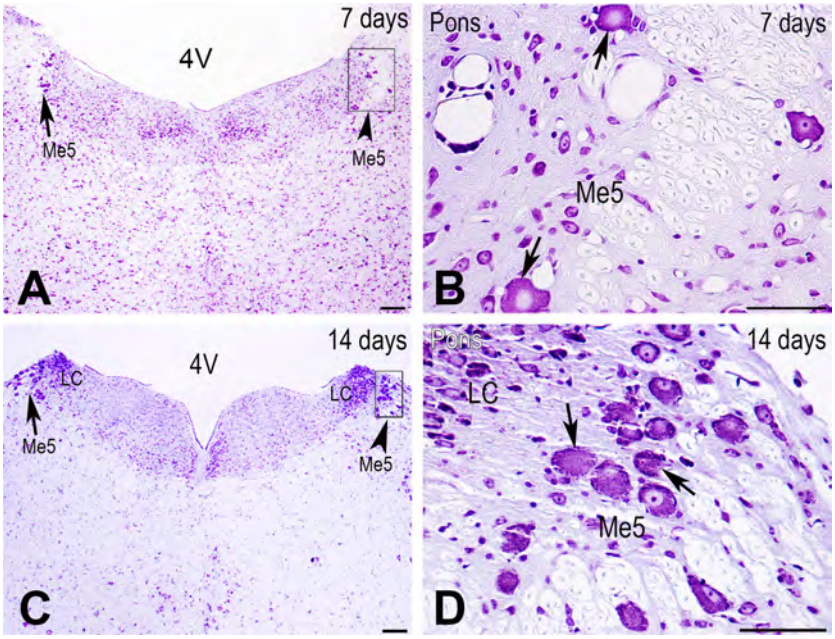

Figure 3. *(A) A microphotograph through the pontine part of the Me5 in rat, 7 days after unilateral transection of the masseteric nerve.*

The Me5 neurons at the side of axotomy (arrowhead), show insignificant decrease in number compared with intact half (arrow). (B) A higher magnification of the outlined zone in (A), demonstrating chromatolytic changes in the large axotomized Me5 neuronal profiles (arrows). Initial disintegration of the Nissl substance and heterochromatin in the nucleus is observed seven days after the intervention. (C) A microphotograph of the caudal part of the Me5 in rats, 14 days after unilateral transection of the masseteric nerve. The number of Me5 neurons on the side of axotomy (arrowhead) is reduced compared to the intact half (arrow). (D) A higher magnification of the outlined rectangle shown in (C), which demonstrates the chromatolytic changes at this stage in the axotomized Me5 neurons. Note the presence of basophilic granulations in the degenerated neuronal profiles (arrows). LC, locus coeruleus; 4V, ventriculus quartus. Staining with cresyl violet. Scale bars = 100 μm in (A, C) and 50 μm (B, D)

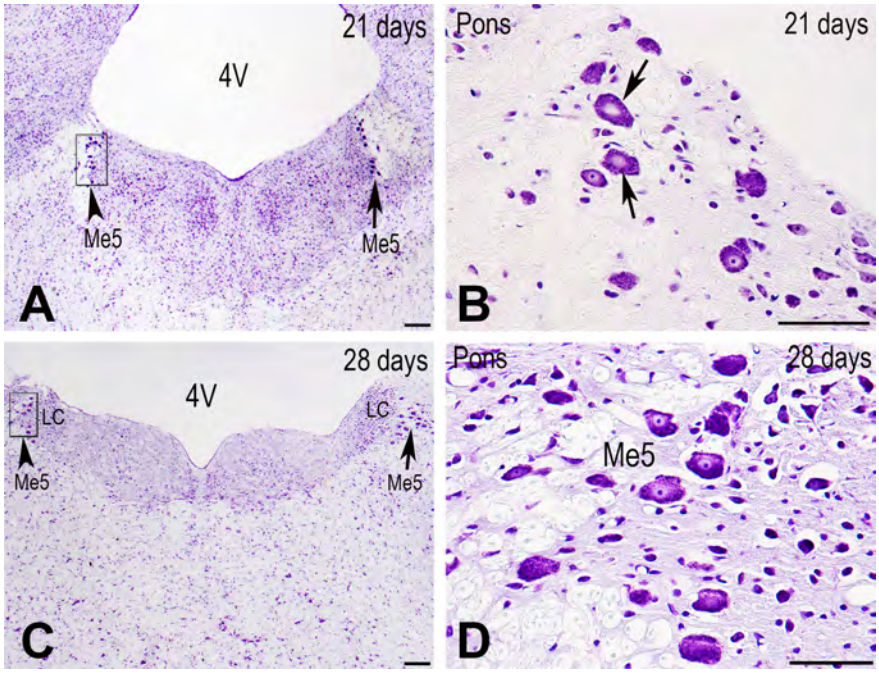

Figure 4. *(A) Microphotograph of the transition part of the pons and mesencephalon of the Me5 in rats, 21 days after unilateral axotomy of the masseteric nerve.*
The number of axotomized Me5 neurons (arrowheads) is apparently reduced, compared to the intact neurons (arrows). (B) A higher magnification of the outlined rectangle of (A), demonstrating degenerated Me5 neurons (arrows) 21 days after unilateral axotomy. (C) A microphotograph of a lower magnification of the caudal part of the Me5 in rats, 28 days after unilateral transection of the masseteric nerve. The number of Me5 neurons on the side of axotomy (arrowhead) is smaller than the intact half (arrow). (D) Higher magnification of the outlined zone in (C), demonstrating chromatolytic changes in the large axotomized Me5 neuronal profiles (Me5). LC, locus coeruleus; 4V, ventriculus quartus. Staining with cresyl violet. Scale bars = 100 µm in (A, C) and 50 µm (B, D).

observed more rostrally, at the level of the mesencephalon where the axotomized Me5 neurons were apparently reduced, compared to the intact side. Here the damaged Me5 neurons demonstrated patterns of transneuronal degeneration with clearly visible chromatolytic granules and a dispersion of the nuclear chromatin.

On the 28th day after the intervention, on the axotomized side of nucleus at the level of pons, a significant reduction in the number of Me5 neurons could still be registered. That was greater when compared to the number of degenerated neurons on the 7th postoperative day, and than the neurons in the non-treated control half (Fig. 4C). At the same time, the level of neuronal degeneration was slightly decreased (Fig. 4D), though this phenomenon was still present in the days after the intervention. The described morphological changes did not demonstrate significant differences between the neurons located in the caudal and rostral part of the nucleus.

After a period of continuous survival of 56 days following the intervention, the number of Me5 neurons on the side of axotomy was still smaller, although no significant and distinct differences could be registered when compared with the neurons on the intact side. Besides, no visible changes in the morphology of Me5 neurons were visualized on the ipsilateral and contralateral side (Fig. 5A, B). A similar finding for these chronological changes in the number and morphology of Me5 neurons was done when comparing the axotomized Me5 neurons with the contralateral neurons in the same nucleus on the control side, where sham surgery with incision and subsequent suturing of the skin with no sectioning of the peripheral nerve was performed (Fig. 5 C, D).

NEUROCHEMICAL CHANGES IN THE MESENCEPHALIC TRIGEMINAL NUCLEUS AFTER PERIPHERAL AXOTOMY

Plastic changes in the expression of classical neurotransmitters

The glutamatergic nature of Me5 neurons in normal conditions

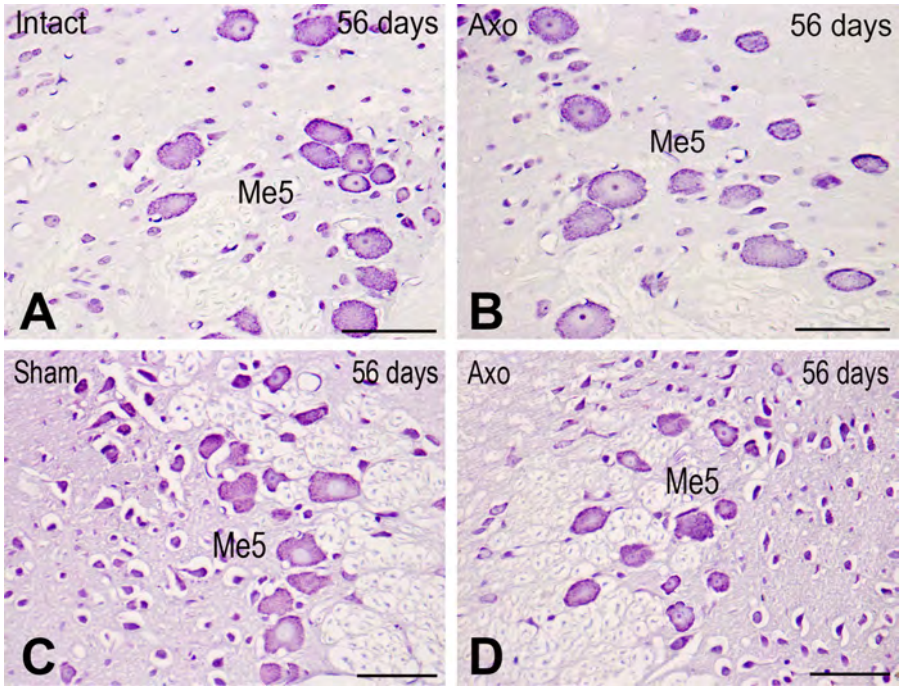

Figure 5. *A higher magnification of the intact (A) and the outlined in a rectangle axotomized side (B) of the Me5, 56 days after unilateral axotomy.*
The number and morphology of the Me5 neurons on both sides are almost identical. The histological appearance of Me5 neurons (Me5) on the shame-operated side (C), and with axotomy (D), 56 days after the intervention. The number and morphology of the Me neurons on both sides are almost the same. Nissl staining. Scale bars = 50 µm.

has been repeatedly documented. Therefore, in this study we followed the changes in the expression of established amino acid transmitters after experimental unilateral transection of the masseteric nerve, and subsequent immunohistochemical examination to demonstrate their presence in the nucleus. One week after the axotomy, on the side of injury , we found reduced Glu immunoreactivity in the injured neurons along the entire length of the nucleus, compared to its expression patterns in the intact Me5 neurons. A distinct tendency to weaker immunostaining intensity was registered on 14th, 21st, and 28th day after the intervention, while 56 days after it the number and intensity of immunostaining of the injured neurons was similar to the one of intact neurons.

Our efforts to establish axotomy-induced changes in the expression of tyrosine hydroxylase (TH), a rate-limiting enzyme in catecholamine synthesis, were not successful. Indeed, we did not find TH immunoreactivity in the axotomized Me5 neurons following any period of survival after peripheral axotomy. Only nerve fibers and their terminals showed immunopositive reaction for this enzyme in the intact Me5.

The histochemical reaction for visualization of NADPH-diaphorase showed that seven days after the intervention, the number of nitrergic Me5 neurons had increased on the side of injury compared to the intact one (Fig. 6A, B). A similar finding was observed when comparing the number of positive Me5 neurons on the axotomized side with those in the sham-operated contralateral side (Fig. 6 C, D). The tendency for increase in the number of axotomized nitrergic Me5 neurons was observed until the end of the first postoperative month. However, two months after the intervention, no visible difference in the number of NADPH-diaphorase-positive Me5 neurons between the operated and intact half was established.

Plastic changes in the expression of neuroactive peptides

Previous experiments demonstrated that under normal conditions the intact Me5 neurons did not express neuropeptide immunoreactivity in their cell bodies. The peripheral axotomy, however, changed their phenotype to *de novo* expression of some neuropeptides. In our study we tested the expression of some sensory neuropeptides such as SP and CGRP, and other peptides like VIP, NPY and GAL, which are usually expressed

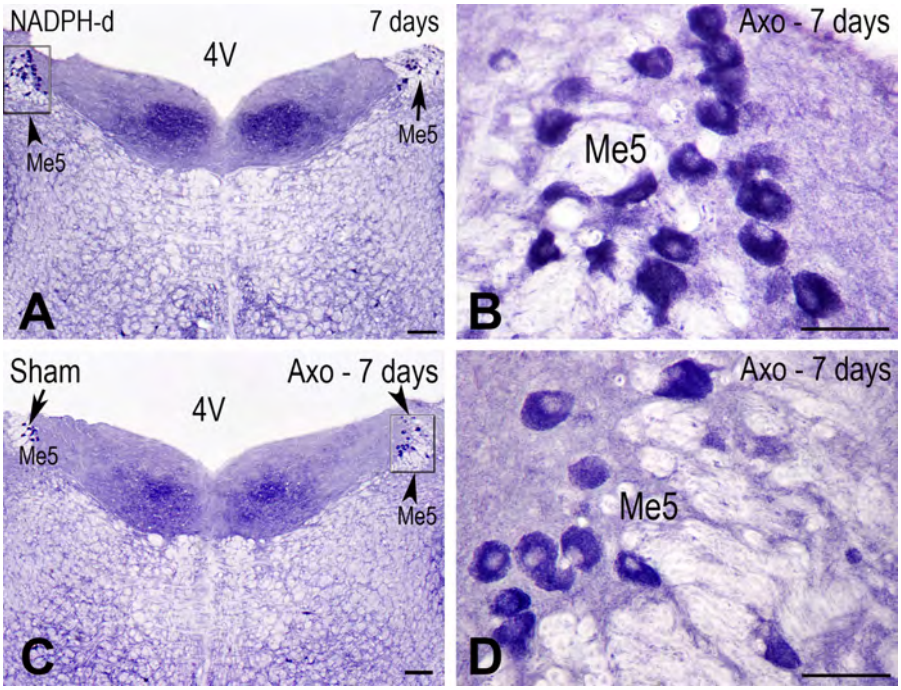

Figure 6. *Histochemical reaction for NADPH-diaphorase in the caudal part of the Me5 in rats. Seven days after the axotomy, the number of reactive Me5 neurons on the side of injury (arrowhead) is larger than the contralateral nucles (arrow) (B). A higher magnification of (A), demonstrating the difference in the number of axotomized NADPH-diaphorase reactive Me5 neurons. (C) 7 days after the intervention, the number of axotomized NADPH-diaphorase reactive Me5 neurons (arrowhead) is larger compared to the sham-operated side (arrow). (D) A higher magnification of the nucleus on the side of injury. Scale bars = 100 µm in (A, C) and 50 µm in (B, D).*

in the perikarya of sympathetic neurons.

We were not able to find the presence of SP and CGRP in the cell bodies and/or the processes of the axotomized Me5 neurons along the entire rostrocaudal extent of the Me5, in any of the studied periods of survival after unilateral peripheral axotomy.

The immunoreaction to GAL in animals, subject to unilateral axotomy, was markedly positive in the ipsilateral side, while it was negative contralaterally. Positive immunostaining was also observed in the neurons of the adjacent nuclei, the locus coeruleus and medial parabrachial nucleus. GAL-immunoreactivity was distinct in the large-sized axotomized Me5 neurons along the entire rostrocaudal length of the nucleus, and in individual small neurons in its pontine part. Immunopositivity was already noted on the 7th day after the nerve cut, persisted two to four weeks, and then faded away about the 56th day following the intervention (Fig. 7A, B).

The immunoreactive pattern for the two other studied neuropeptides,VIP and NPY, was also similar, though the intensity of the observed immunoreactivity in the axotomized Me5 neurons was relatively weaker. More specifically, 14 days after the axotomy the immunoreaction for NPY was with the same intensity as for GAL. However, we noted a slight tendency for decrease, which was apparent on the 28th day after the procedure (Fig. 7 C, D).

Plastic Changes in the Expression of Calcium-Binding Proteins

In the early stages after unilateral cut of the masseteric nerve, we found no visible changes in the immunohistochemical pattern of some neuron-specific calcium-binding proteins such as parvalbumin and calbindin D-28 in the Me5 neurons on the affected side. The immunostained neurons on the both sides were of middle and large size with a distinct pseudounipolar morphology. It was observed that Me5 neurons in the intact nucleus were immunoreactive to parvalbumin and calbindin with almost the same intensity of staining. However, it is noteworthy that the calbindin-immunoreactive neurons in the Me5 were fewer in number compared to the parvalbumin-immunopositive ones. After a survival period of 7 days, no

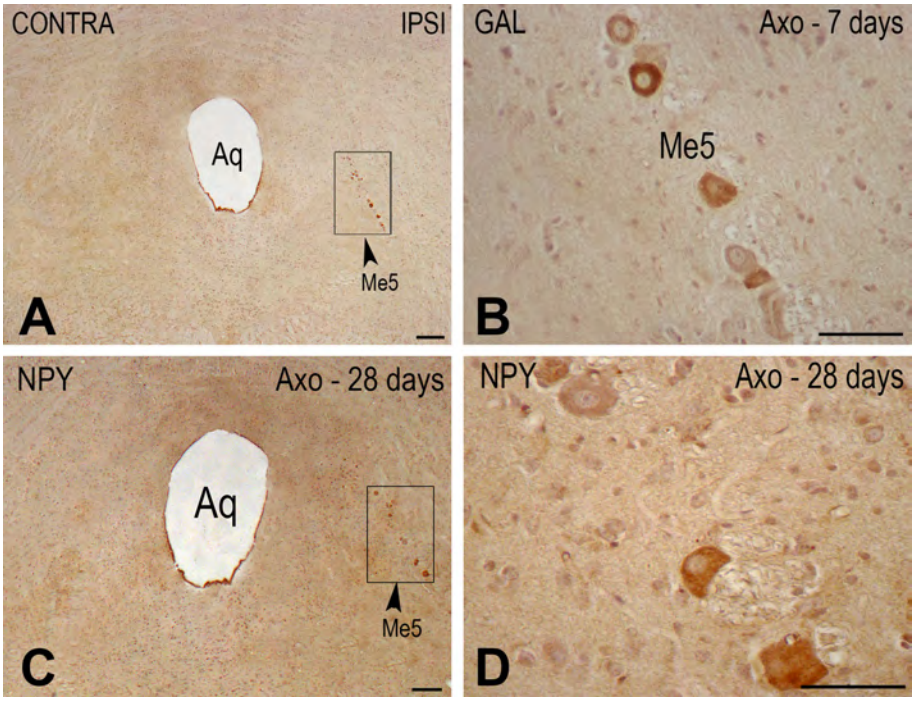

Figure 7. *(A) Immunohistochemical reaction for GAL in Me5 in rats, at the level of the mesencephalon, 7 days after experimental unilateral transection of the masseteric nerve. Note the presence of GAL-immunoreactive Me5 neurons (arrowhead) on the ipsilateral side and their absence in the intact contralateral half. (B) Higher magnifications of the rectangle in (A), which demonstrates GAL-immunoreactive axotomized Me5 neurons, 7 days after the intervention. (C, D) NPY-immunoreactive axotomized Me5 neurons (arrowhead) in the mesenecephalic part of the nucleus, 28 days after peripheral axotomy. Aq, aqueductus cerebri. Scale bars = 100 µm in (A) and 50 µm in (B, C, D).*

noticeable changes were registered in the expression profile of the two studied calcium-binding proteins in the intact and axotomized Me5 neurons. Two weeks after the axotomy, a decrease in the expression levels in the axotomized Me5 neurons along the entire length of the nucleus was observed, though this was significantly more pronounced for parvalbumin than for calbindin (Fig. 8). In fact, the number of parvalbumin-containing Me5 neurons ipsilaterally to the nerve cut was smaller than the one observed in the control animals, while the number of calbindin-containing neuronal profiles remained unchanged. The registered differences concerned the intensity, and not the number of immunopositive neurons. Moreover, they were distinct when comparing both with the contralateral intact Me5 neurons and those on the ipsilateral side of the control sham-operated animals. In addition, 56 days after the intervention, no decrease in the expression of calcium-binding proteins was established ipsilaterally, and their level remained almost the same when compared to the first days following peripheral axotomy.

Statistical analysis revealed an insignificant decrease in the number of Me5 neurons in 7-day axotomized animals, compared to the intact and sham-operated control groups. Interestingly, only at the level of pons we observed a statistically significant decrease in the number of neurons in Me5 from 16.393 ± 0.403 in the control group to 14.920 ± 0.443 in the group with 7 day survival (Fig. 9). The average number of Me5neurons in the control group at the level of pons-mesencephalon was 12.615 ± 0.851 compared to 11.273 ± 0.359 in the axotomized group; at the level of the inferior colliculi it was 6.125 ± 0.295 compared to 5.417 ± 0.229 in the axotomized group; and at the level of the superior colliculi it was 4.429 ± 0.297 compared to 3.636 ± 0.279 in the axotomized group. On the other hand, no statistically significant differences $p > 0.05$ were observed in the control and sham-operated animals.

The significant changes in the neuronal number were more pronounced 14 days following the intervention. The analysis showed that from caudally to rostrally, the number of Me5 neurons on the axotomized side changed from 13.200 ± 0.416 in the pons, to 9.063 ± 0.335 in the pons-mesencephalon transi-

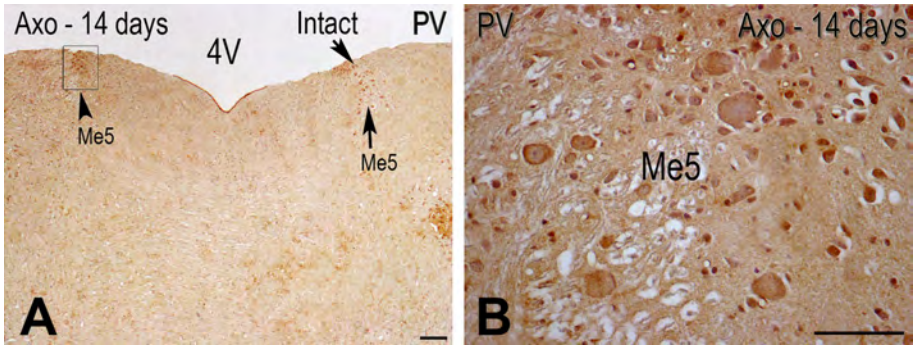

Figure 8. *(A) Immunohistochemical demonstration of PV in the pontine part of the Me5, 14 days after unilateral peripheral axotomy. Note the apparently decreased number of PV-immunoreactive Me5 neurons of axotomized side (arrowhead) compared to the intact half (arrow). (B) Higher magnifications of the axotomized PV immunoreactive Me5 neurons in (A). 4V, ventriculus quartus. Scale bars = 50 µm.*

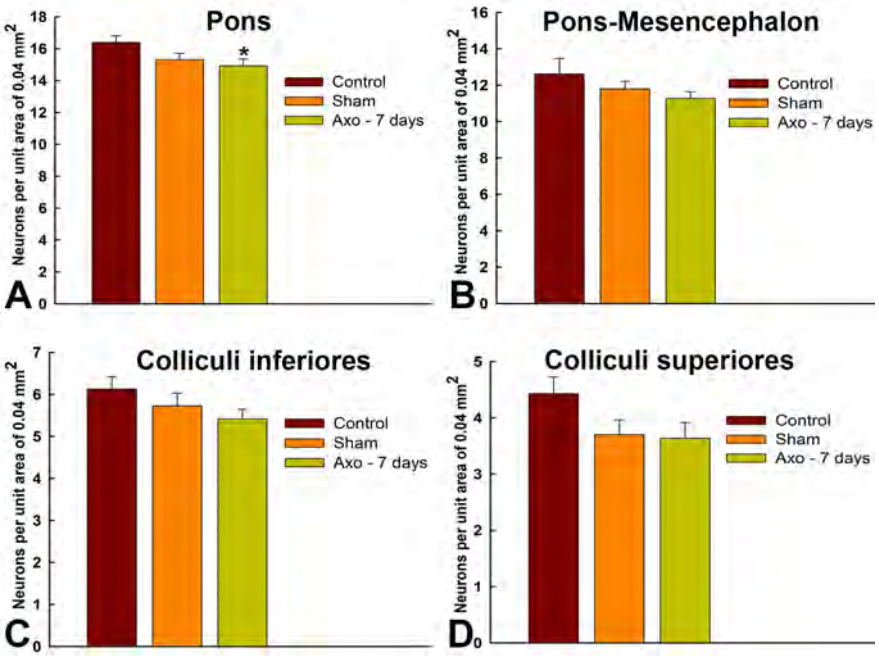

Figure 9. *Schematic presentation of the average number of neurons in the control (Control), sham-operated group (Sham), and the axotomized and survived for 7 days rats (Axo - 7 days) at the level of the pons (A), pons-mesencephalon (B), inferior colliculi (C), and superior colliculi (D). The data present the mean value and standard error of mean, and they are compared by Student's t-test (the number of studied animals in each group was n = 5). *p < 0.05 compared to the control group.*

tion area, 4.444 ± 0.377 at level of the inferior colliculi, and 3.125 ± 0.227 in the superior colliculi (Fig. 10). Besides, in all studied areas the neuronal loss on the side of axotomy was statistically significant ($p < 0.05$), compared to the control and sham-groups at the respective levels.

The effect of axotomy was most strongly noticeable on the 21st day along the whole rostocaudal extent of the nucleus. In particular, the neuronal loss was most significant, as follows: 11.923 ± 0.265 in the pons, 8.222 ± 0.173 in the pons-mesencephalon transition area, 3.824 ± 0.231 at level of the inferior colliculi, and 2.875 ± 0.125 at level of the superior colliculi (Fig. 11). The neuronal loss on the side of axotomy was statistically significant ($p < 0.05$) when compared to both the control and sham-operated groups at the respective levels. Starting with the 28th day after the intervention, a slight increase in the average number of Me5 neurons in the respec-

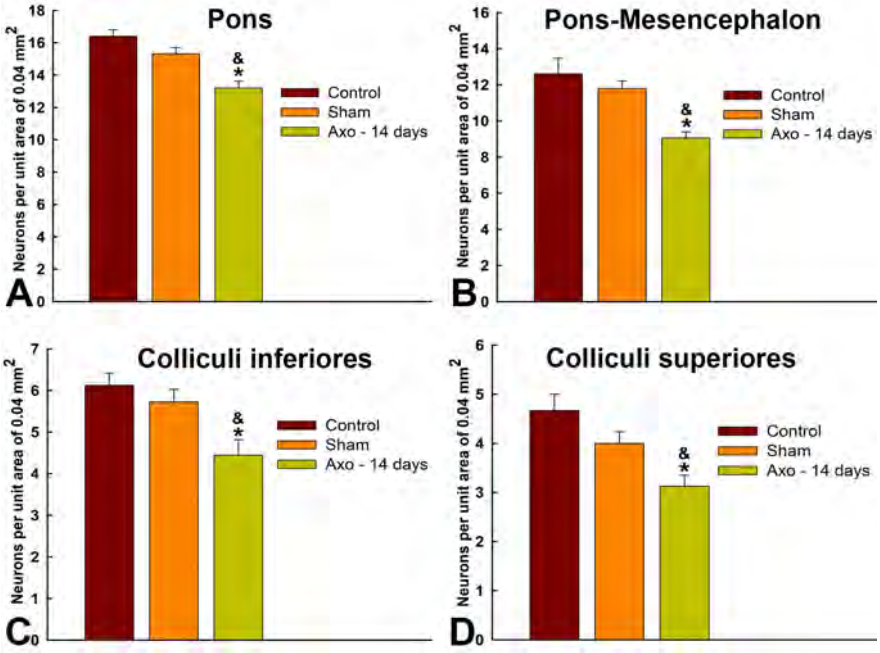

Figure 10. *Graphical visualization of the average number of neurons in the control (Control), sham-operated (Sham), and axotomized group at 14 days (Axo - 14 days) at the level of the pons (A), pons-mesencephalon (B), inferior colliculi (C), and superior colliculi (D). The data are compared with Student's t-test and demonstrate the mean value and standard error of the mean (n = 5). *p < 0.05 compared to the control group; &p < 0.05 compared to the sham-operated group.*

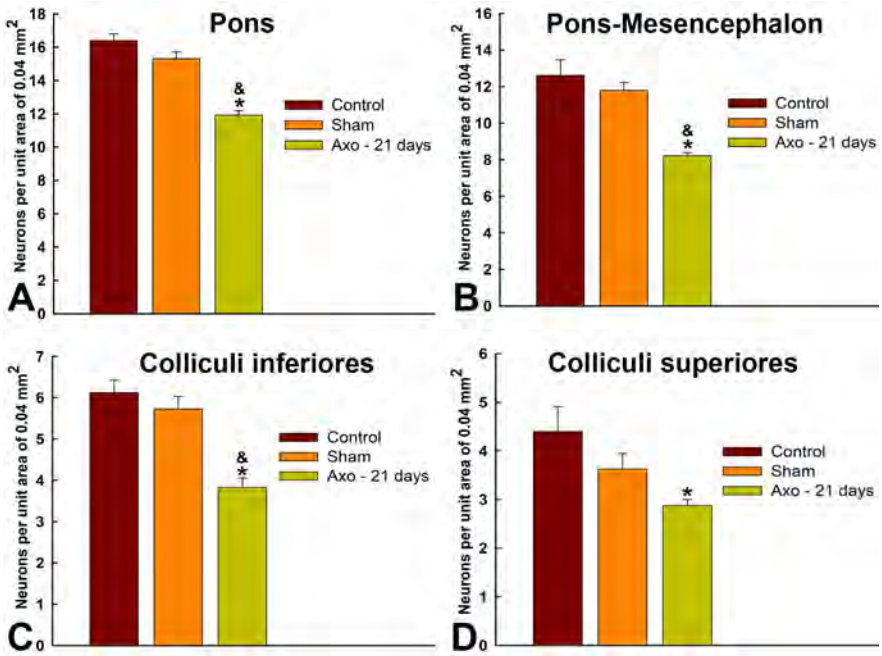

Figure 11. *Statistical presentation of the average number of neurons in the control (Control), sham-operated (Sham), and axotomized groups at 21 days (Axo - 21 days) at the level of the pons (A), pons-mesencephalon (B), inferior colliculi (C), and superior colliculi (D). The data demonstrate the mean value and standard error of mean (n = 5). *p < 0.05 compared to the control group; &p < 0.05 compared to the sham-operated group.*

tive areas was noted. This tendency was obvious on the 56th survival day. Specifically, at that time the number of the Me5 neurons on the side of axotomy was almost the same as on the 7th postoperative day (Fig. 12). Statistically significant differences were only registered between the control and axotomized group of animals at the levels of the pons and inferior colliculi. To summarize, we did not register statistically significant differences ($p = 0.062$) in the average number of neurons, per unit of area of 0.04 mm^2 in all the examined areas in the control and sham-operated groups, as well as in the axotomized animals during different postoperative periods. On the other hand, the average number of neurons per unit of area decreased with statistical significance until the 21st day after the intervention, and subsequently began to increase gradually until the 56th day.

MORPHOFUNCTIONAL ASPECTS OF MESENCEPHALIC TRIGEMINAL NEURONAL PLASTICITY

One of the most striking features of the Me5 is its plastic nature. The data from a series of recent studies undoubtedly show that changes in environment result in concomitant, delayed in time, and long-term damages in the morphological and neurochemical phenotype of the Me5 neurons. In response to those external factors, Me5 neurons react with adaptive morphochemical alterations, which direct their activity towards survival and regeneration of the injuries. Currently, it is well known that central and peripheral nerve injury changes the neuronal phenotype from its usual status of interneuronal synaptic signaling and communication to regeneration, including down- and upregulation of cell physiological events and *de novo* synthesis of some biologically active substances, which are not expressed in the adult neurons in normal environmental circumstances. These changes are most likely associated with an adequate morphological and neurochemical cell response to the nerve injury (37).

Our data demonstrate that the unilateral cut of the masseteric nerve causes development of noticeable morphological changes in the cell bodies and processes of the damaged Me5 neurons. These are manifested mainly with chromatolysis and transneuronal degeneration of the affected perikarya, and also with degenerative changes in the distal parts of the transected peripheral nerve. This degeneration is an early cellular reaction and it is visualized as an initial sign of morphological damage, far before the registration of cell loss. However, it may consequently lead to a significant neuronal death. These changes are a useful marker for distinguishing of the surviving neurons, and therefore, the counting of nucleoli in the axotomized and

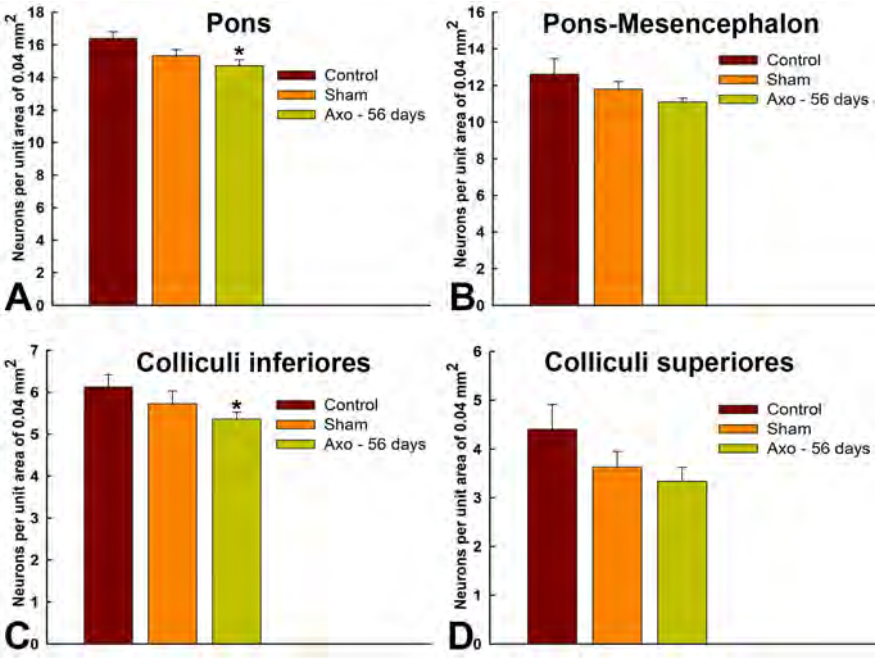

Figure 12. *Graphical presentation of the average number of neurons per area of 0.04 mm^2 in the control (Control), sham-operated (Sham), and axotomized groups of rats at 56 postoperative days (Axo - 56 days) at the level of the pons (A), pons-mesencephalon (B), inferior colliculi (C), and superior colliculi (D). The data demonstrate the mean value and standard error of the mean (n = 5). *p < 0.05 compared to the control group.*

intact neurons may be a reliable method for comparison and identification of survived and dead nerve cells in normal and experimental animals (33).

The existing evidence shows that the lesions of the Me5 or mesencephalic trigeminal tract causes degeneration of the nerve fibers, innervating the masticatory muscles and demonstrates how these lesions lead to chromatolysis of the Me5 neurons (38-41). This morphological finding is also supported by early ultrastructural studies, which demonstrate the presence of axotomy-induced degenerated and necrotic Me5 neurons (42). Consequently, more recent studies confirm that the Me5 neurons are rather sensitive to peripheral nerve injury, and they develop degenerative argyrophilia, which results in distinct cell death (33,34). Similar changes are also reported soon after the intervention in the TG cells in rats, following transection of the infraorbital nerve and they are manifested with a retrograde degeneration in their cell bodies, called transganglionic degeneration (43).

A previous quantitative study of Me5 in rats established that the peripheral axotomy of the masseteric nerve causes a certain (10.5-22.7%) reduction in the number of the damaged Me5 neurons, and that most of them die in the period between the 10th and 30th postoperative day (33). Our morphometric studies show a similar tendency and prove that this is principally true for the whole rostrocaudal extent of the nucleus. The data collected from the neuron counting of separate segments of the Me5, i.e. the pontine part, pons-mesencephalon transition area, superior and inferior colliculi in the mesencephalon doubtlessly confirm the fact that the number of axotomized Me5 neurons, when compared to the neurons on the intact side and sham-operated groups, begins to decrease 7 days after the intervention and continues to reduce slightly until the third week afterwards. Such reduction in the number of damaged Me5 neurons is statistically significant for each Me5 segment within this time frame. At the end of the first month following the peripheral axotomy, some tendency for an increase in the number of Me5 neurons on the side of injury is observed, albeit it keeps being smaller than the neurons counted in the control animals at this stage. At the end of second month following axotomy of the masseteric nerve, the number of the registered surviving neurons is similar to their number at the end of the first postoperative week. Beyond any doubt, this phenomenon is not due to recovery through regeneration of the number of axotomized Me5 neurons but to the fact that they probably cannot be visualized with the applied methods.

A key postulate in neurobiology states that nerve cells pay for their high specialization with a loss of their ability to divide postnatally. Regardless that in the present century enough convincing evidence has been gathered which questions this postulate, at least in certain brain regions such as the hippocampus and the olfactory bulb of the telencephalon in rodents and primates (summarized in 44), there is still a lack of firm confirmation for adult neurogenesis in this brain area. A logical explanation for the visualization of a larger number of neurons at the end of the second month after axotomy could be found in the fact that after the first week following the intervention, the Me5 neurons show obvious signs of neuronal degeneration. Above all, this is expressed in the disintegration of the rough endoplasmic reticulum (RER), the ultrastructural equivalent of Nissl bodies, which results in a loss of the tinctorial abilities of the cytoplasm for staining with cresyl violet. It is obvious that during the period between the second and fourth week after the peripheral axotomy, part of the injured Me5 neurons cannot be visualized by the Nissl method. Following the switch-on of the defense mechanisms in the damaged neurons in the second month after the injury, they gradually regain at least a part of their RER and their inherent abilities for active protein synthesis. Thus, they stain again with the Nissl method and are visualized with conventional histological and immunohistochemical techniques. The changes we observed confirm the findings of Raappana and Arvidsson (33) that the peak of neuronal loss is between the second and fourth postoperative week. Supporting these findings is the fact that the period of greatest neuronal loss chronologically coincides with the time when the most significant changes are registered in the levels of the neuroactive substances expressed by the axotomized Me5 neurons (45-48). These data make us conclude that the process of adaptation to the changed environmental conditions is accompanied by significant cell loss. In this sense, it is quite probable that axonal signals induced by the neuronal damage activate some genetic signal pathways in the perikarya leading to either of two possible opposing events, i.e. cellular death or regenerative response of the damaged neurons resulting in their survival. On the other hand, earlier studies provide convincing evidence that the peripheral nerve injury "obliges" the surviving Me5 neurons to modify their natural activity by switching from a state of maintenance of normal cellular functions and neurotransmission to unusual adaptive phenomena such as regeneration and survival (36, 49). There are also data claiming that the changes induced by nerve injury involve increased neurotrophic requirements (34).

NEUROCHEMICAL PLASTICITY OF THE MESENCEPHALIC TRIGEMINAL NEURONS

It is well known that in the course of their embryonic and early postnatal development the trigeminal primary sensory neurons need neurotrophins, though with time they mature and become less dependent on the presence of neurotrophic signals. For their survival in unusual external conditions, however, sensory neurons need to acquire specific patterns in order to respond adequately to the changed environment. This happens through the expression of other growth and regulatory signals such as neurotransmitters and neuropeptides. The role of these neuroactive substances as maintaining factors has continuously aroused the interest of neurobiologists, since nowadays it is known for certain that they share common signal pathways with the growth factors and proto-oncogenes regulating the neuronal proliferation, migration, survival, growth, differentiation, and gene expression (50).

Our current results show a significant chemical plasticity of the Me5 neurons, manifesting as a bidirectional change, i.e. up- or down-regulation in the levels of main transmitters in Me5, as well as through the *de novo* synthesis of certain neuropeptides following axotomy. The data from a series of studies unambiguously show that some neurotransmitters and neuropeptides are not only a means for short-term transsynaptic transmission of information, but can also act as long-term morphogenic signals and trophic factors, which maintain the neuronal growth, plasticity, and survival (50, 51).

The study of neurotransmitter plasticity of primary sensory neurons marked its peak at the end of the last century, when a number of research groups devoted considerable efforts in the elucidation of the phenomenon in different animal species. The data from a series of immunohistochemical and *in situ* hybridization studies described the changes in the levels of normally expressed neuronal calcium-binding proteins and gaseous transmitters after axotomy of a peripheral nerve (45, 46, 52-55). For the first time, we establish in our study a decreased immunoreactivity, i.e. a decreased number of immunopositive neurons and a low staining intensity for Glu in axotomized Me5 neurons. Previous studies have shown that this excitatory amino acid is the main neurotransmitter candidate in the Me5 neurons in rats (56), guinea pigs (57), and cats (7, 48). A plausible explanation of the observed low expression of Glu could be that peripheral injury causes a functional shift from neurotransmission to survival and regeneration of the axonal processes in the damaged neurons. In this sense it is logical to accept that the levels of neurotransmitters playing a chief role in the transmission of nerve signals between neurons upon normal conditions apparently lowers upon environmental changes, and the efforts of the damaged neurons are directed towards their survival by minimizing of their usual functional activities. This conclusion could also be extrapolated to other atypical transmitters, such as the gaseous molecule nitric oxide for which there are sufficient data that it is expressed by Me5 neurons under normal environmental conditions (58,59). In support of this finding, an increase in the number of NADPH-diaphorase-containing Me5 neurons has been reported 4-6 days after cutting of the infraorbital nerve (55). Similar data have also been obtained after unilateral peripheral axotomy of the masseteric nerve in rats: three days after the intervention the Me5 neurons demonstrated NADPH-diaphorase-positivity, reaching a maximal number on the seventh day after the axotomy and keeping such a high number until the eighth postoperative week. At the same time, the maximal number of axotomy-induced nitric oxide synthase (NOS)-immunoreactive neurons remain unchanged for two weeks after the intervention and within four weeks afterwards the nitrergic neurons disappeared (60). Our results confirm such a tendency of early increase in the number of NADPH-diaphorase-reactive Me5 neurons, persistence of nitrergic neurons until the end of the first month after the intervention, and their slow return to the intact condition by the end of the second postoperative month. This phenomenon could be explained with the assumption that the constantly increased level of NOS in Me5 neurons is due to slowly progressing neuronal death after the peripheral nerve injury, because it is a logical outcome from the increased sensitivity of the neurons to calcium-mediated neurotoxicity under unfavorable surrounding conditions. Alternatively, we could speculate that probably the endogenous production of nitric oxide underlies a protective mechanism of the neurons against nerve injury, and thus supports the survival and regeneration of the Me5 neurons.

Newer studies on the chemical plasticity of the trigeminal primary afferent neurons have shown that the axotomy of the inferior alveolar nerve causes significant reduction of the level of two sensory neuropeptides, SP and CGRP, in the perikarya of damaged trigeminal ganglionic cells in the ferret (61). In our studies, however, we did not succeed to find induced by axotomy expression of these two peptides in the injured Me5 neurons in rats. Besides interspecies differences, a possible explanation of this negative finding could be the assumption that the *de novo* synthesis of SP and CGRP is too weak and/

or these proteins are transported too slowly for any possible detection with immunohistochemical methods. The application of *in situ* hybridization could provide more specific data for the presence of these peptides in the damaged Me5 neurons, at least at mRNA level, while the blocking of the axoplasmic flow with colchicine would also increase the possibility for their visualization at protein level. Similar positive findings for the expression of CGRP, but not of SP, in about 20% of the peripherally axotomized Me5 neurons in rats have been reported by other authors (46). By using Fluorogold tracing of the projections of the transected nerve the authors manage to demonstrate that both types of primary proprioceptors, innervating the masticatory muscle spindles and periodontal baroreceptors, undergo CGRP up-regulation.

On the other hand, we clearly denote that the Me5 neurons, which do not synthesize neuropeptides under normal conditions, as a consequence of peripheral nerve axotomy begin to express other neuropeptides at mRNA and protein level, which functionally are accepted as inhibitors of the sensory transmission. This refers both to the involvement in this process of the neurons, predominantly located in the pontine part of Me5, which innervate the periodontal ligament and are considered to be baroreceptors, and to these dispersed throughout the whole nucleus, which take part in the innervation of the masticatory muscles. Our data show that the transection of a peripheral nerve induces immunoreactivity for NPY, GAL, and VIP in the Me5 neurons in rats one week after the axotomy, and that the expression reaches maximum intensity two weeks following the injury. These and some of our previous results (62, 63) contribute to the findings of other authors (45), who find expression for VIP in the damaged Me5 neurons at mRNA level, but not at protein level. This is also supported by the findings from subsequent experiments of Larsen *et al* (54), who demonstrate an early expression of another peptide from the family of VIP, PACAP (pituitary adenylate cyclase-activating polypeptide) in the axotomized Me5 neurons, in coexistence with NPY and GAL at that. Similar neuropeptide expression evoked by trauma has been demonstrated chronologically in axotomized spinal ganglion neurons in rats as well (64). The authors state that the spinal ganglion cells of a large size are immunoreactive, thus they are functionally accepted as proprioceptive. The established period of time of the *de novo* neuropeptide synthesis corresponds to the changes found in the neuropeptide mRNA and protein content of Me5 neurons in cats (7, 35, 36), as well as results about the neurochemical response to axotomy in Me5 in rats (45, 46). It comprises the onset of the so-called peptide up-regulation between the 1st and 3rd postoperative day and its continuous peak between the 2nd and 4th week following the intervention. Later, the significant increase in the peptide levels persists until the 28th postoperative day, followed by a discernible and slow, yet constant peptide down-regulation (52). We register a recovery of the peptide levels close to the control values 56 days after the peripheral nerve injury. Comparing these data with the results from the above-mentioned studies, it seems probable that PACAP participates in the early neuronal response to peripheral injury and its initial adaptive effect is taken up at a later stage by other newly synthesized peptides such as NPY and GAL.

These data make us conclude that the involvement of neuropeptides in orofacial proprioception is observed only in abnormal conditions and this phenomenon is true for all primary afferent neurons. It is obvious that the expression of neuropeptides in the Me5 neurons is plastic and the proprioceptive neurons in Me5 can up-regulate these peptides as a consequence from the injury of a peripheral nerve. It is yet to be clarified what is the functional significance of this phenomenon and what is its specific correlation with the cell death.

A well-known fact in synaptology is that the release of a transmitter from the axonal terminal must be compensated by its *de novo* synthesis in the perikaryon (37). Therefore, it is logical to accept that neuropeptides such as SP, which normally participate in the sensory transmission, are suppressed upon selective axotomy, while the expression of peptides, acting as trophic factors, are induced by the intervention and they participate actively in the response to injury and the process of neuronal regeneration (65). It seems probable that the suppression of sensory signal transmission and down-regulation of the excitatory neurotransmitters aims to minimize the effect of peripheral nerve injury. Moreover, we consider that the newly synthesized neuropeptides could also play a supporting role in the adaptive process as neurotrophic factors, produced in response to the injury, thus protecting the axotomized Me5 neurons. Beyond any doubt, the actual neurotrophins as growth factors are included in the regulation and maintenance of the phenotype and morphofunctional condition in mature age. This assumption is supported by their demonstrated presence in more than 60% of the Me5 neurons in adult rats (66), which suggests their probable participation in the process of survival of the neurons. It is currently known that the additional endogenous application of neurotrophins can intensify

the regeneratory response in peripherally axotomized neurons (37), though their specific role is still to be clarified.

The axotomy-induced neurochemical changes include a changed Me5 profile and alteration of the neuronal calcium-binding proteins. It is mainly related to the level of parvalbumin, and, to a lesser extent, of calbindin. After a latent period of several days, the adaptive response of the axotomized Me5 neurons includes significant decrease in the level of parvalbumin, and also a slighter one of calbindin. The absence of any alterations in the initial days could be explained with the fact that some time following the injury is needed to overcome the threshold for their immunohistochemical registration. These data correspond to the results from previous studies on the effect of peripheral axotomy on the level of calcium-binding proteins in Me5 in rats (53), and coincided with the described initial changes in the axotomized Me5 neurons in other animal species (7,36). In cats, however, the level of these calcium-binding proteins remains unchanged during the first week after the axotomy and decreases during the second postoperative week (67). Nonetheless, such changes are not found in the neurons of spinal ganglia after transection of peripheral somatic nerves or injury of visceral nerves (68, 69). It is most likely that these discrepancies are due to interspecies differences, but it could also be that the masseteric nerve is more vulnerable to injury than the inferior alveolar nerve, which passes through calcified tissue in a bone canal (33).

We believe that both a reduction in the number of Me5 neurons and decreased intensity of their immunological staining after peripheral axotomy may contribute to these neurochemical changes. However, their functional interpretation requires a different approach. Because the two studied calcium-binding proteins are located in large-sized primary sensory neurons, a subpopulation with known high calcium content, it only seems logical to suggest that these proteins play a role in the buffering of intracellular calcium, as it is assumed by Blaustein (70). Current evidence suggests that the calcium-binding proteins can function as intracellular calcium transporters or as a buffering system for cellular protection of the neuronal activity in normal conditions (71, 72). Thus, for example, Wakisaka *et al* (53) assume that calbindin is involved in calcium-buffering mechanisms in the sensory ganglia after peripheral injury. Moreover, its involvement in this process is accompanied by NPY-immunoreactivity in the trigeminal primary afferent neurons, where the calcium regulation is of particular significance (73). In this regard, Wakisaka *et al* (53, 74) find a partial coexistence of calbindin and NPY in trigeminal ganglion cells in rats, where both of these neuroactive substances participate in buffer mechanisms for calcium ions in the injured primary sensory neurons. It can be inferred that the changes in the expression of calcium-binding proteins can be attributed to an adequate cellular response, because the sensitivity of the axotomized Me5 neurons to intracellular calcium seems to differ from that of intact Me5 neurons. It can also be assumed that the reduced levels of calcium-binding proteins together with the newly synthesized NPY contribute to the survival of axotomized Me5 in the process of adaptation following peripheral axotomy.

CONCLUSIONS

In summary, it can be inferred that the neuronal population in the Me5 in rats is very sensitive to peripheral nerve injury, and shows clear structural and neurochemical plasticity. It causes morphological alterations in the neurochemical phenotype of the injured Me5 neurons, which are dynamic, with a delayed onset, and long-term lasting. Moreover, the adaptive morphological phenomena include quantitative and qualitative structural changes in the axotomized Me5 neurons, which include a statistically significant neuronal loss on the side of injury. In addition, the qualitative changes in Me5 are manifested with transneuronal degeneration and chromatolysis of the perikarya of axotomized neurons, and degeneration of their peripheral processes. The neurochemical changes induced by the axotomy are observed in the down-regulation of normally expressed in the nucleus classical transmitters such as GLU and the neuronal calcium-binding proteins like parvalbumin and calbindin, up-regulation of the gaseous transmitter nitric oxide, and *de novo* synthesis of some neuroactive peptides including NPY, GAL, and VIP in the damaged Me5 neurons. The newly synthesized peptides participate in the orofacial proprioception only in abnormal conditions. They most likely suppress the presynaptic level of transmission in axotomized neurons. Furthermore, the established morphological and neurochemical changes in the axotomized Me5 neurons are manifested at the end of first postoperative week, persist until the end of fourth week, and slowly begin to subside to return to their usual levels about the end of eighth week after the intervention. Last but not least, the morphological changes in the axotomized Me5 neurons are an important adaptive quality, aimed at their survival in abnormal conditions. The neurochemical changes are another relevant factor for the survival of the axotomized Me5 neurons and they also contribute for regeneration of their axons in the course of the adaptive process.

The morphofunctional and neurochemical changes illustrate the amazing interactive nature and the remarkable neuronal plasticity of the Me5 neurons, not suspected until now. The changed phenotype can find partial explanation in their unique ectopic brain location and their functional role in the proprioceptive sensitivity of the orofacial area. It seems quite probable that this unusual location of the cell bodies of the Me5 neurons in CNS is of significance for their adequate reaction to peripheral nerve injury.

CONFLICT OF INTEREST

None.

REFERENCES

1. Cajal S. Beitrag zum Studium der Medulla Oblongata, des Kleinhirns und des Ursprungs der Gehirnnerven. Johann Ambrosius Barh, Leipzig, 1896. DOI:10.5962/bhl.title.61878
2. Cajal S. *Histologie du Système Nerveaux de l'Homme et des Vertèbrès*, vol. 1 A. Maloine, Paris, 1909. DOI:10.5962/bhl.title.48637
3. Scharf JH. Sensible Ganglien. In: Möllendorff M, Bargmann W, editors. *Handbuch der mikroskopischen Anatomie des Menschen* Bd. 4/3, Springer-Verlag, Berlin, 1958; 290–300.
4. Brodal A. *Neurological Anatomy in Relation to Clinical Medicine*, 3rd ed., Oxford University Press, New York, 1981. ISBN-13: 978-0195026948
5. Usunoff KG, Marani E, Schoen JH. The trigeminal system in man. *Adv Anat Embryol Cell Biol* 1997; 136: 1-126. DOI:10.1007/978-3-642-60779-0
6. Marani E, Usunoff KG. The trigeminal motonucleus in man. *Arch Physiol Biochem* 1998; 106: 346-354. DOI:10.1076/apab.106.5.346.4364
7. Lazarov NE. The mesencephalic trigeminal nucleus in the cat, *Adv Anat Embryol Cell Biol* 2000; 153: 1-103. DOI:10.1007/978-3-642-57176-3
8. Darian-Smith I. The trigeminal system. In: Iggo A, editor. *Handbook of Sensory Physiology, Somatosensory System*, Springer, Berlin, 1973; 271-314. DOI:10.1007/978-3-642-65438-1_10
9. Johnston JB. The radix mesencephalica trigemini. *J Comp Neurol* 1909; 19: 593-644. DOI:10.1002/cne.920190602
10. Freeman W. The relationship of the radix mesencephalica trigemini to the extraocular muscles. *Arch Neurol Psychiatry* 1925; 14: 111-113.
11. Dubner R, Sessle B, Storey A. *The Neural Basis of Oral and Facial Function*, Plenum Press, London, 1978. DOI:10.1016/0002-9416(79)90109-X
12. Davies AM. The trigeminal system: an advantageous experimental model for studying neuronal development. *Development* 1988; 103: 175-183.
13. Shigenaga Y, Sera M, Nishimori T, Suemune S, Nishimura M, Yoshida A, Tsuru K. The central projections of masticatory afferent fibers to the trigeminal sensory nuclear complex and upper cervical spinal cord. *J Comp Neurol* 1988; 268: 489-507. DOI:10.1002/cne.902680403
14. Kruger L, Young RF. Specialised features of the trigeminal nerve and its central connections. In: Samii M, Janeta PJ, editors. *The Cranial Nerves*. Springer, Berlin, 1981; 273-301. DOI:10.1007/978-3-642-67980-3_37
15. Pfaller K, Arvidsson J. Central distribution of trigeminal and upper cervical primary afferents in the rat studied by anterograde transport of horseradish peroxidase conjugated to wheat germ agglutinin. *J Comp Neurol* 1988; 268: 91-108. DOI:10.1002/cne.902680110
16. Yoshida A, Yasuda K, Dostrovsky JO, Bae YC, Takemura M, Shigenaga Y, Sessle BJ. Two major types of premotoneurons in the feline trigeminal nucleus oralis as demonstrated by intracellular staining with horseradish peroxidase. *J Comp Neurol* 1994; 347: 495-514. DOI:10.1002/cne.903470403
17. Yoshida S, Oka H. Membrane properties of dissociated trigeminal mesencephalic neurons of the adult rat. *Neurosci Res* 1998; 30: 227-234. DOI:10.1016/s0168-0102(98)00003-0
18. Alvarado-Mallart MR, Batini C, Buisseret-Delmas C, Corvisier J. Trigeminal representations of the masticatory and extraocular proprioceptors as revealed by horseradish peroxidase retrograde transport. *Exp Brain Res* 1975; 23: 167-179. DOI:10.1007/bf00235459
19. Capra NF, Anderson KV, Atkinson RC. Localization and morphometric analysis of masticatory muscle afferent neurons in the nucleus of the mesencephalic root of the trigeminal nerve in the cat. *Acta Anat (Basel)* 1985; 122: 115-125. DOI:10.1159/000145992
20. Shigenaga Y, Yoshida A, Mitsuhiro Y, Tsuru K, Doe K. Morphological and functional properties of trigeminal nucleus oralis neurons projecting to the trigeminal motor nucleus of the cat. *Brain Res* 1988; 461: 143-149. DOI:10.1016/0006-8993(88)90732-9

21. Jerge CR. Organization and function of the trigeminal mensencephalic nucleus. *J Neurophysiol* 1963; 26: 379-392. DOI:10.1152/jn.1963.26.3.379
22. Byers MR, Holland GR. Trigeminal nerve endings in gingiva, junctional epithelium and periodontal ligament of rat molars, as demonstrated by autoradiography. *Anat Rec* 1977; 188: 509-523. DOI:10.1002/ar.1091880409
23. Byers MR. Sensory innervation of periodontal ligament of rat molars consists of unencapsulated Ruffini-like mechanoreceptors and free nerve endings. *J Comp Neurol* 1985; 231: 500-518. DOI:10.1002/cne.902310408
24. Byers MR, O'Connor TA, Martin RF, Dong WK. Mesencephalic trigeminal sensory neurons of cat: axon pathways and structure of mechanoreceptive endings in periodontal ligament. *J Comp Neurol* 1986; 250: 181-191. DOI:10.1002/cne.902500205
25. Shigenaga Y, Yoshida A, Mitsuhiro Y, Doe K, Suemune S. Morphology of single mesencephalic trigeminal neurons innervating periodontal ligament of the cat. *Brain Res* 1988; 448: 331-338. DOI:10.1016/0006-8993(88)91272-3
26. Byers MR, Dong WK. Comparison of trigeminal receptor location and structure in the periodontal ligament of different types of teeth from the rat, cat, and monkey. *J Comp Neurol* 1989; 279: 117-127. DOI:10.1002/cne.902790110
27. Linden RWA, Millar BJ, Halata Z. A comparative physiological and morphological study of periodontal ligament mechanoreceptors represented in the trigeminal ganglion and the mesencephalic nucleus of the cat. *Anat Embryol* 1994; 190: 127-135. DOI:10.1007/BF00193410
28. Amano N, Yoshino K, Andoh S, Kawagishi S. Representation of tooth pulp in the mesencephalic trigeminal nucleus and the trigeminal ganglion in the cat, as revealed by retrogradely transported horseradish peroxidase. *Neurosci Lett* 1987; 82: 127-132. DOI:10.1016/0304-3940(87)90116-9
29. Yoshino K, Andoh S, Kawagishi S, Yamauchi M, Jones TE, Amano N. Innervation of the tooth pulp by the mesencephalic trigeminal nucleus in the cat: a retrograde horseradish peroxidase study. *Brain Res* 1989; 503: 152-155. DOI:10.1016/0006-8993(89)91717-4
30. Byers MR, Matthews B. Autoradiographic demonstrations of ipsilateral and contralateral sensory nerve endings in cat dentin, pulp and periodontium. *Anat Rec* 1981; 201: 249-260. DOI:10.1002/ar.1092010205
31. Costa M, Furness JB, Gibbins IL. Chemical coding of enteric neurons. *Prog Brain Res* 1986; 68: 217-239. DOI:10.1016/S0079-6123(08)60241-1
32. Hökfelt T, Zhang X, Wiesenfeld-Hallin Z. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci* 1994; 17: 22-30. DOI:10.1016/0166-2236(94)90031-0
33. Raappana P, Arvidsson J. The reaction of mesencephalic trigeminal neurons to peripheral nerve transection in the adult rat. *Exp Brain Res* 1992; 90: 567-571. DOI:10.1007/bf00230940
34. Ichikawa H, Jin HW, Terayama S, Yamaai T, Matsuo S, Sugimoto T. The reduction of proprioceptors in the mesencephalic trigeminal tract nucleus after neonatal masseteric nerve transection; effect of brain-derived neurotrophic factor. *Brain Res* 2007; 1153: 98-102. DOI:10.1016/j.brainres.2007.03.060
35. Lazarov NE. Comparative analysis of the chemical neuroanatomy of the mammalian trigeminal ganglion and mesencephalic trigeminal nucleus. *Prog Neurobiol* 2002; 66: 19-59. DOI:10.1016/s0301-0082(01)00021-1
36. Lazarov NE. Neurobiology of orofacial proprioception. *Brain Res Rev* 2007; 56: 362-383. DOI:10.1016/j.brainresrev.2007.08.009
37. Navarro X, Vivó M, Valero-Cabré A. Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol 2007; 82: 163-201. DOI:10.1016/j.pneurobio.2007.06.005
38. Allen WF. Application of the Marchi method to the study on the radix mesencephalica trigemini in the guinea pig. *J Comp Neurol* 1919; 30: 169-216. DOI:10.1002/cne.900300202
39. Corbin KB. Probst's tract in the cat. *J Comp Neurol* 1940; 77: 455-467. DOI:10.1002/cne.900770302
40. Szentágothai J. Anatomical considerations of monosynaptic reflex arc. *J Neurophysiol* 1948; 11: 445-454. DOI:10.1152/jn.1948.11.5.445
41. Dault SH, Smith RD. A quantitative study of the nucleus of the mesencephalic tract of the trigeminal nerve of the cat. *Anat Rec* 1969; 165: 79-87. DOI:10.1002/ar.1091650109
42. Imamoto K. Electron microscopic observations in the trigeminal mesencephalic nucleus following neurotomy of the third division of the trigeminal nerve. *Arch Histol Jpn* 1972; 34: 361-374. DOI:10.1679/aohc1950.34.361
43. Aldskogius H, Arvidsson J. Nerve cell degeneration in the trigeminal ganglion of the adult rat following peripheral nerve transection. *J Neurocytol* 1978; 7: 229-250. DOI:10.1007/bf01217921

44. Lepousez G, Nissant A, Lledo PM. Adult neurogenesis and the future of the rejuvenating brain circuits. *Neuron* 2015; 86: 387-401. DOI:10.1016/j.neuron.2015.01.002
45. Umemoto S, Noguchi K, Kawai Y, Senba E. The expression of neuropeptides and their mRNAs in the trigeminal mesencephalic nucleus following masseteric nerve transection. *Brain Res Mol Brain Res* 1994; 23: 93-99. DOI:10.1016/0169-328x(94)90215-1
46. Yoshida A, Ichikawa H, Sugimoto T. CGRP in peripherally axotomized mesencephalic trigeminal neurones of the rat. *Neuroreport* 1995; 6: 837-840. DOI:10.1097/00001756-199504190-00003
47. Lazarov N, Dandov A, Stoyanova I, Chouchkov C. Axotomy-induced alterations in the neurochemical content of cat mesencephalic trigeminal nucleus neurons. *It J Anat Embryol [Suppl]* 1999; 104: 386.
48. Lazarov N, Dandov A. Ultrastructural patterns of glutamate and GABA immunolabelling in the cat mesencephalic trigeminal nucleus. *C R Acad Bulg Sci* 2000; **53**: 109-112.
49. Dandov AD, Atanasova DY, Lazarov NE. Morphological changes in the rat mesencephalic trigeminal nucleus following peripheral axotomy. *C R Acad Bulg Sci* 2016; 69(6): 801-806.
50. Lipton SA, Kater SB. Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci* 1989; 12: 265-270. DOI:10.1016/0166-2236(89)90026-x
51. Copray JCVM, Liem RSB. Survival and neurite formation of mesencephalic trigeminal neurones of the rat in vitro. *Arch Oral Biol* 1993; 38: 547-557. DOI:10.1016/0003-9969(93)90119-7
52. Arvidsson J, Rappaana P, Diez M, Hökfelt T. Expression of neuropeptides in the rat mesencephalic trigeminal nucleus after peripheral axotomy. *Neuroreport* 1994; 5: 1269-1272. DOI:10.1097/00001756-199406020-00029
53. Wakisaka S, Takikita S, Youn SH, Kurisu K. Partial coexistence of neuropeptide Y and calbindin D28k in the trigeminal ganglion following peripheral nerve axotomy of the inferior alveolar nerve in the rat. *Brain Res* 1996; 707: 228-234. DOI:10.1016/0006-8993(95)01262-1
54. Larsen JO, Hannibal J, Knudsen SM, Fahrenkrug J. Expression of pituitary adenylate cyclase-activating polypeptide (PACAP) in the mesencephalic trigeminal nucleus of the rat after transsection of the masseteric nerve. *Mol Brain Res* 1997; 46: 109-117. DOI:10.1016/s0169-328x(96)00279-3
55. De Gandarias JM, Mendez PL, Vegas L, Echevarría E, Maza JL, Casis L. Infraorbital nerve transection increases NADPH-diaphorase activity in the rat mesencephalic trigeminal nucleus. *J Hirnforsch* 1999; 39: 567-571.
56. Copray JCVM, Ter Horst GJ, Liem RSB, van Willigen JD. Neurotransmitters and neuropeptides within the mesencephalic trigeminal nucleus of the rat: an immunohistochemical analysis. *Neuroscience* 1990; 37: 399-411. DOI:10.1016/0306-4522(90)90410-6
57. Chandler SH. Evidence for excitatory amino acid transmission between mesencephalic nucleus of V afferents and jaw-closer motoneurons in the guinea pig. *Brain Res* 1989; 477: 252-264. DOI:10.1016/0006-8993(89)91413-3
58. Lazarov N, Dandov A. Distribution of NADPH-diaphorase/nitric oxide synthase in the trigeminal ganglion and mesencephalic trigeminal nucleus of the cat. A histochemical and immunohistochemical study. *Acta Anat* 1998; 163: 191-200. DOI:10.1159/000046498
59. Stoyanova II, Lazarov NE. Localization of nitric oxide synthase in rat trigeminal primary afferent neurons using NADPH-diaphorase histochemistry. *J Mol Histol* 2005; 36: 187-193. DOI:10.1007/s10735-005-1694-3
60. Varathan V, Shigenaga Y, Takemura M. Nitric oxide synthase/nicotinamide adenine dinucleotide phosphate-diaphorase in the brainstem trigeminal nuclei after transection of the masseteric nerve in rats. *J Neurosci Res* 2001; 66: 428-438. DOI:10.1002/jnr.1235
61. Elcock C, Boissonade FM, Robinson PP. Changes in neuropeptide expression in the trigeminal ganglion following inferior alveolar nerve section in the ferret. *Neuroscience* 2001; 102: 655-667. DOI:10.1016/s0306-4522(00)00508-x
62. Dandov A, Draganov M, Lazarov N. Synthesis of galanin and neuropeptide Y in the rat mesencephalic trigeminal nucleus after injury of n. massetericus. *Trakia J Sci* 2005; 3: 1-5.
63. Dandov A, Penkova Zh, Lazarov N. De novo expression of neuropeptides in the mesencephalic trigeminal nucleus of the rat after masseteric nerve injury. *Acta Morhol Antropol* 2010; 15: 27-30.
64. Noguchi K, De León M, Nahin RL, Senba E, Ruda MA. Quantification of axotomy-induced alteration of neuropeptide mRNAs in dorsal root ganglion neurons with special reference to neuropeptide Y mRNA and the effects of neonatal capsaicin treatment. *J Neurosci Res* 1993; 35: 54-66. DOI:10.1002/jnr.490350108

65. Nielsch U, Keen P. Reciprocal regulation of tachykinin- and vasoactive intestinal peptide-gene expression in rat sensory neurones following cut and crush injury. *Brain Res* 1989; 481: 25-30. DOI:10.1016/0006-8993(89)90481-2
66. Jacobs JS, Miller MW. Expression of nerve growth factor, p75, and the high affinity neurotrophin receptors in the adult rat trigeminal system: evidence for multiple trophic support systems. *J Neurocytol* 1999; 28: 571-595. DOI:10.1023/a:1007019422675
67. Lazarov NE, Dandov A, Stoyanova I, Chouchkov CN. Calcium-binding proteins in the mesencephalic trigeminal nucleus of the cat. *Arch Physiol Biochem* 1998; 106: 370-377. DOI:10.1076/apab.106.5.370.4366
68. Honda CN. Differential distribution of calbindin-D28k and parvalbumin in somatic and visceral sensory neurons. *Neuroscience* 1995; 68: 883-892. DOI:10.1016/0306-4522(95)00180-q
69. Medici T, Shortland PJ. Effects of peripheral nerve injury on parvalbumin expression in adult rat dorsal root ganglion neurons. *BMC Neurosci* 2015; 16: 93. DOI:10.1186/s12868-015-0232-9
70. Blaustein MP. Calcium transport and buffering in neurons. *Trends Neurosci* 1988; 11: 438-443. DOI:10.1016/0166-2236(88)90195-6
71. Baimbridge KG, Miller JJ, Parkes CO. Calcium-binding protein distribution in the rat brain. *Brain Res* 1982; 239: 519-25. DOI:10.1016/0006-8993(82)90526-1
72. Celio MR. Calbindin D-28k and parvalbumin in the rat nervous system. *Neuroscience* 1990; 35: 375-475. DOI:10.1016/0306-4522(90)90091-h
73. Sasaki Y, Wakisaka S, Kurisu K. Effects of peripheral axotomy of the inferior alveolar nerve on the levels of neuropeptide Y in rat trigeminal primary afferent neurons. *Brain Res* 1994; 664: 108-114. DOI:10.1016/0006-8993(94)91960-7
74. Wakisaka S, Youn SH, Miyawaki Y, Kurisu K. The effects of peripheral nerve injury of the masseteric nerve on the levels of calcium binding proteins and neuropeptide Y, and their correlation in the mesencephalic trigeminal nucleus of the rat. *Brain Res* 1996; 735: 249-256. DOI:10.1016/0006-8993(96)00585-9