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Original Contribution

SYNTHESIS OF GALANIN AND NEUROPEPTIDE Y IN THE RAT MESENCEPHALIC TRIGEMINAL NUCLEUS AFTER INJURY OF N. MASSETERICUS

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ABSTRACT

In normal circumstances, galanin (GAL) and neuropeptide Y (NPY), a peptide usually occurring in sympathetic neurons, are not found in the neurons of the mesencephalic trigeminal nucleus (MTN), a unique locale of (pseudo)unipolar sensory neurons in the central nervous system. Nonetheless, both peptides have been shown to be up-regulated in primary afferent neurons after a peripheral nerve injury. Here we demonstrate by immunohistochemistry GAL and NPY immunoreactivity in MTN neurons after an *n. massetericus* axotomy in adult rats. Following a survival period of 7 days, the MTN neurons on the ipsilateral (axotomized) side displayed pronounced immunoreactivity, particularly the perikarya belonging to the large-size subtype, while on the contralateral (untreated) side they remained negatively stained. The present results suggest that in time neuron injury launches a medley of intracellular events, leading in their turn to a *de novo* synthesis of neuroactive substances, such as GAL and NPY, which are not specific for the MTN neurons under normal conditions. The newly synthesized peptides possibly play a trophic role in the revival process or may actively take part in re-launching the sensory modalities of the MTN in the orofacial region.

Key words: Neuropeptides, Nerve transection, Trigeminal primary sensory neurons, Neurochemical plasticity, Rat

STUDY BACKGROUND

For over a decade now it has been known that the neuropeptide expression tends to be plastic Often the levels of neuropeptides are significantly increased (up-regulated) or decreased (down-regulated) due to injuries or lesions to peripheral branches of primary sensory neurons, a phenomenon named neurochemical plasticity (1). Expression of neuropeptides is dramatically changed in sensory neurons under pathological conditions, such as nerve injury and inflammation, suggesting a role in processes regulating survival and regeneration of injured neurons and in pain processing (2). There are a number of literary sources reporting on the up-regulation of galanin (GAL), neuropeptide Y (NPY) and calcitonin-gene related peptide (CGRP) following peripheral axotomy of primary sensory neurons (3, 4, 5, 6).

Primary afferents in the trigeminal system have their cell bodies both in the trigeminal ganglion and the mesencephalic trigeminal nucleus (MTN) (6, 7). The MTN is a unique structure in that it is the only nucleus in the CNS made up mostly of (pseudo)unipolar neurons of a nerve crest origin, much like those in the peripheral sensory ganglia. The MTN perikarya are located in the midbrain and the rostral portion of the pons and send out their peripheral processes to muscle spindles of the jaw-closing muscles and extrinsic ocular muscles, as well as to mechanoreceptors in the periodontal ligament (7).

GAL is a neuropeptide, which is not a member of any known family of neuropeptides, despite repeated efforts to discover related peptides. Its actions are mediated via G-protein-coupled receptors and ion channels, usually producing inhibition of secretion of a transmitter or hormone in the nervous and endocrine systems. In many respects, these inhibitory actions of GAL remind us of those of gamma-aminobutyric

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acid (GABA) and of NPY (8, 9). Data about the expression of GAL in the neurons of dorsal root ganglia and the trigeminal ganglion (TrG) are reported (10, 11, 12), although it has been repeatedly shown that its level is significantly up-regulated after a peripheral axotomy (3, 13).

NPY is the most abundant neuropeptide in the brain. It is a member of a family of proteins that include pancreatic polypeptide, peptide YY and seminalplasmin. Typically, NPY is found to be expressed in the sympathetic neurons of the autonomous nervous system, although extensive data shows its expression in the TrG after axotomy (6 and ref. therein).

In normal circumstances the MTN utilizes a variety of neuroactive substances, albeit some are expressed only under pathological or extreme conditions such as injury and pain. For instance, to date there exist no data on neuropeptide synthesis or expression in the MTN perikarya in health, notwithstanding the presence of fibre networks of varying density containing different neuropeptide substances amidst the neuronal bodies (7). Nonetheless, when subjected to injury of their peripheral processes, the MTN neurons start producing neuroactive substances and neuromodulators, which normally are not a constituent of their milieu.

Therefore, we set it as a goal of this study to establish immunohistochemically the *de novo* synthesis of two putative neuromodulators, GAL and NPY, in MTN neurons following a peripheral axotomy of the *n. massetericus*, since the latter contains peripheral projections from the nucleus.

MATERIAL AND METHODS

Three adult rats of both sexes weighing 200-300 g were used for the present study. The animals were deeply anaesthetised and two of them were subjected to a unilateral transection of the left *n. massetericus*, while the third underwent only a skin incision as sham surgery and served for a control. The untreated contralateral side also served as an additional control. Following the intervention the animals were left to recover and survive for a period of one week, and in the interim were liberally fed on chow. Thereafter, the animals were re- anaesthetised and perfused first with saline and then 4% paraformaldehyde in 0.1M phosphate buffer. The brains were quickly removed and the brainstem was cut at the level of the MTN. After post-fixation in the same fixative overnight at 4°C, the tissue blocks were immersed in a cryoprotective solution containing 20% sucrose in 0.1 M TBS until they sank. The samples were cut on a freezing microtome at 20 μm and the sections were collected in a free-floating state in TBS.

For the GAL and NPY immunostaining the sections were processed in accordance with the avidin-biotin-peroxidase complex (ABC) method (14). Briefly, the sections were initially treated with 1.2% H_2O_2 in absolute methanol to block endogenous peroxidase and then preincubated in 3% normal goat serum in 0.01 M PBS containing 0.3% TritonX-100 for 30 minutes. Afterwards, they were incubated in the primary polyclonal antibodies against GAL (Serotec Ltd., UK) and NPY (INCSTAR Co., Stillwater, Minnesota, USA) respectively at a dilution 1:1000 in the preincubation medium, first at room temperature on a rotator for 24 h and then in a fridge at 4°C for another 24 h. The sections were then treated with biotinylated goat antirabbit IgG diluted 1:50 and the ABC complex for 2 h each at room temperature. Finally, the peroxidase activity was visualized using 3,3'-diaminobenzidine (DAB) as chromogen. The sections were mounted on gelatin coated glass slides, cleared in xylene and cover-slipped with Entelan. The subsequent observation of the resultant reaction and photographing were performed with AxioCam on a Zeiss AxioPlan 2 light microscope.

RESULTS

In the MTN of the animals subjected to unilateral transection of *n. massetericus*, a pronounced immunoreactivity (IR) was observed on the ipsilateral side of the axotomy, while no IR was seen contralaterally (**Figures 1 and 3**). The IR was confined to the MTN neurons as well as to its surrounding nuclei – the locus ceruleus and the medial parabrachial nucleus. More specifically, in the case of GAL, the IR was notably expressed in the population of MTN neurons throughout the whole length of the nucleus, where they were visualized as darkly-stained round-to-ovoid shaped perikarya against a negative background (Figure 2). The GAL-IR was evenly dispersed throughout the neuronal perikarya. In the case of NPY the IR was clearly visible in the MTN neuronal population along the entire extent of the nucleus, although its intensity was somewhat weaker than that of the GAL-IR (**Figure 4**).

Figure 1 *- GAL-IR in the pontine MTN (arrowhead) on the axotomized ipsilateral (IPSI) side. The contralateral (CONTRA) MTN remains immunonegative. Magnification x 50.*

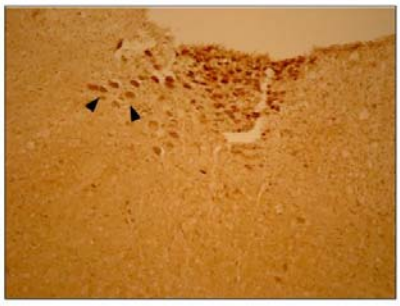

Figure 2 *- A larger view, showing the GAL-immunoreactive oval-shaped MTN neurons (arrowheads) on the axotomized side. Magnification x100.*

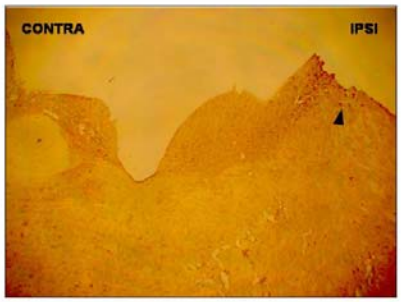

Figure 3 *– NPY-IR in the pontine MTN (arrowhead) on the axotomised ipsilateral (IPSI) side at the pontine level. The contralateral (CONTRA) MTN is immunonegative. Magnification x 50.*

No GAL- or NPY-IR was observed in the MTN of the control animal, neither ipsilaterally nor contralaterally to the axotomized nerve.

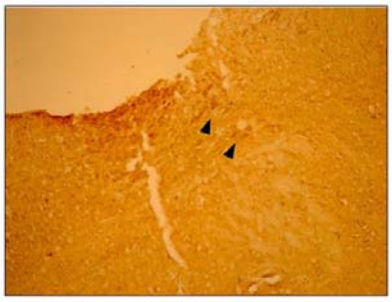

Figure 4 *- A larger view, showing the NPY-immunoreactive MTN neurons (arrowheads) on the axotomized side. Magnification x100.*

DISCUSSION

In this study we demonstrate the *de novo* synthesis and expression of two putative peptide neurotransmitters, GAL and NPY, in the MTN of the rat after peripheral transection of the *n. massetericus*. It is worth noting that only a direct surgical insult to the *n. massetericus*, or the masseter muscle itself (6) but not a skin incision of the area produces such an up-regulation, showing that a direct nerve insult on the peripheral axons is required to induce and initialise such a synthesis.

Our results are in agreement with the already published data by other authors confirming the induction of synthesis and up-regulation of neuropeptides in the MTN neurons of the rat (3, 4) and cat (7, 10) following injury of their peripheral axons. These investigations undoubtedly show that there is a constantly present up-regulation of neuropeptides in axotomized MTN neurons, in particular GAL and NPY, which apparently arises about a week after the intervention and reaches its peak fourteen days thereafter. Moreover, the GAL and NPY levels are observed to be reversibly down-regulated approximately four weeks following the injury, obviously when the healing process is completed, and likewise in an untreated animal, these two neuropeptides become absent from the neurochemical content of the MTN.

It has been repeatedly shown that under normal conditions the proprioceptive MTN neurons do not synthesize neuropeptides but start expressing mRNA and IR after peripheral axotomy (15). Such messages and neuropeptides are primarily evoked in the MTN one week post-intervention, although the maximal level of expression is reached 14 days post operatively (7). Therefore this study

demonstrates that a peptide involvement in the proprioceptive function mainly develops in abnormal conditions and that there exists an exhibited injury-induced up-regulation of GAL and NPY synthesis.

The fact that MTN neurons react to peripheral axotomy by a *de novo* synthesis of neuropeptides such as GAL and NPY leads to the notion that these substances may play a key role in the trophic responses of neurons to the altered environmental cues. In this respect this study is in conformity with other sources reporting on the significant changes in the neurochemical content of primary sensory neurons following peripheral axotomy (3, 10). We support the view of these authors that apparently nerve damage causes the surviving neurons to shift their functional activities from normal maintenance and neurotransmission away to sustaining survival and regeneration. One characteristic of these phenomena is a resultant induction in the synthesis of GAL and NPY in the MTN perikarya, which, it can be inferred, have a definite role for the neuronal adaptive mechanisms and metabolic events under abnormal conditions. As already mentioned, the levels of the newly synthesized neuroactive substances hardly remain static and vary in accordance with the environmental changes and notably time, thus implying that the neurochemical plasticity of MTN neurons is a time-dependent event. The present results suggest that in time neuron injury launches a medley of intracellular events, leading in their turn to a *de novo* synthesis of GAL and NPY, which otherwise are not specific for the MTN neurons. The newly synthesized peptides possibly play a trophic role in the revival process or more possibly, they actively take part in re-launching the sensory modalities of the MTN in the orofacial region.

Finally, we have to be aware that, along with the structural changes that the MTN neurons undergo after peripheral injury, apparent alterations are found in their neurochemical content. Hence it can be concluded that neuroplasticity as an adaptive property is another major attribute of axotomized neurons under abnormal conditions. It is our view that the newly synthesized neuropeptides, GAL and NPY, possibly play a supportive role as neurotrophic factors in the course of the adaptive processes that initiate and develop in response to injury. Thus they may protect the peripherally axotomized MTN neurons in their pathway back to re-establishing the usual functional modalities. It remains a matter of further investigation in the rat to find out when in time the peak of GAL and NPY expression after the nerve injury is reached in the MTN and when a reverse process of down-regulation and return to normal neurochemical content is completed.

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