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MOTONUCLEAR CHANGES AFTER CRANIAL NERVE INJURY AND REGENERATION

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INTRODUCTION

Surgical repair of peripheral nerve lesions has become ever more common since the introduction of both microsurgical techniques and autotransplants. The demonstration that the nerve growth factor (NGF) is essential for the neuronal development and maintenance of certain differentiated neurons of the peripheral and central nervous systems (21), has prompted the search, and brought to the discovery of other substances (i.e. true neurotrophic factors like brain-derived neurotrophic factor or BDNF, ciliary neurotrophic factor or CTNF, glial cell line-derived neurotrophic factor or GDNF, and natural molecules like levo-acetylcarnitine) which are proposed for improving the prognosis of neurodegenerative diseases and of peripheral nerve injuries (4, 6, 15, 29, 30). Obviously, the possibility to reach such a goal is dependent on the progress of knowledge of the events underlying the degenerative and regenerative phenomena that follow nerve injury. This is the opinion of many authors, including ourselves, who have studied the changes occurring in central motonuclei after nerve regeneration in mammals and the effects that the growth factors have on such changes (4, 6-13, 15, 24, 25, 29, 30).

The purpose of this paper is to analyze in detail the most recent experience on neuron degeneration and regeneration, accumulated over the years at our Center that specifically regard the motoneuron pools of the oculomotor (ON) and facial (FN) nerves. We also report our experience regarding the effects of acetyl-L-carnitine (ALC) treatment on parent motoneurons after axotomy of the facial and vagus nerves.

METHODS

The experiments were performed on rats and guinea pigs . The retrograde axonal transport of horseradish peroxidase (HRP) was used as the main tool for studying the organization of the brainstem motonuclei after nerve lesion. Using this technique, it is possible to determine the location, number and soma size of the parent neurons. It has been shown that there is a direct relationship between the number of regenerating neurons reinnervating a specific target and the degree of functional recovery (25). Furthermore, the degree of neuronal hypertrophy after nerve section and repair is inversely related with recovery of function (25). The details of surgical technique and histological methods were reported in early papers (6-8, 12).

1) The oculomotor (55 guinea pigs) and facial (8 adult rats) motonuclei were studied after section and immediate reconstruction of the respective cranial nerves. For the animals that underwent an oculomotor nerve lesion, HRP was injected in the ipsilateral medial rectus muscle (MRM) six months after the original operation. For the animals that underwent a facial nerve lesion, the HRP injection of the ipsilateral stylohyoid muscle (SHM) was carried out three months or 21 months after the original operation.

2) The vagus (10 adult rats) and facial (13 newborn rats) motonuclei were studied after section of the respective nerves and treatment with ALC (75 mg of ALC/Kg/day in adult rats; 6 mg of ALC/day in newborn rats). The sectioned vagus nerve was injected with HRP at the level of the proximal nerve stump after a ninety day treatment with ALC. The axotomy induced cell death in the facial nucleus was analyzed in Nissl stained brain stem sections 7 days after nerve transection during which ALC was administered.

The same types of procedures described above were carried out on 23 intact control animals (for what regards the nerve section and reconstruction studies) and on 7 animals that received saline solution (for what regards the studies on ALC effects in post-axotomy nerve degeneration). The number, diameter and somatotopic location of HRP labelled or Nissl stained motoneurons was analyzed in the two groups of experiments and in the controls. The preparations were studied under light microscopy. The results are reported as means plus or minus standard error of the means. In the experiments testing the neurotrophic activity of ALC, the difference between means was examined for significance using Student's t test and a probability of $p < 0.05$ was chosen as the level of significance.

RESULTS

1. The results of the effects of nerve section and reconstruction on the motoneuron number of the respective brainstem motonuclei are summarized in Table I.

Table I. - *Number of motoneurons in control and operated animals.*

Repaired nerve		Oculomotor	Facial
Species		adult guinea pig	adult rat
Injection site of HRP		MRM	SHM
Total labelled motoneurons (±SD)	Control	482.0 (±23.3)	214.3 (±19.8)
	Operated	363.5 (±144.3)	481.4 (±109.5)
Contralateral motoneurons	Control	0%	0%
	Operated	14.8%	8%*

MRM = medial rectus muscle; SHM = stylohyoid muscle; * This value considers only the 50% of rats with contralateral representation.

In all of the animals that underwent section and reconstruction of the oculomotor nerve, the central map of the MRM was found to be bilateral because of the presence of labelled motoneurons within the contralateral ON nucleus. These contralateral neurons represented 14.8 % of the total number of neurons in the

MRM subnucleus. The mean soma diameter was 20.82 μm in control animals and 26.12 μm in the experimental guinea pigs.

Conversely, in only 50% of the animals that underwent facial nerve section and reconstruction, the SHM showed a bilateral central representation due to the presence of labelled motoneurons within the homologous nucleus contralateral to the side of the nerve lesion. These contralateral neurons represented 8% of the total number of neurons. A significant number of labelled motoneurons was found as well outside the borders of the ipsilateral SHM subnucleus in all the animals studied, including those with a bilateral representation of the same subnucleus. The mean motoneuron diameter in the control and experimental animals was 23.7 μm and 23.9 μm, respectively.

2. The effects of ALC treatment after axotomy on the motoneuron number of the respective brainstem motonuclei are summarized in Table II. In the dorsal motor vagal nucleus of animals treated with ALC after the section without reconstruction of the nerve, the number of HRP labelled motoneurons was significantly greater than that of the untreated control animals (2004 and 1290 motoneurons, respectively; $p < 0.01$) (Fig. 1). The cell diameter was also different in the two groups, the HRP-labelled motoneurons in the treated animals being smaller (diameters of 8 to 36 μm) than that of control animals (diameters ranging from 8 to 44 μm). The percentages of motoneurons with diameters between 8 and 20 μm were 91% for the treated animals and 85% for control animals. Conversely, the percentages of

Table II. - *Number of motoneurons after section of the vagus and facial nerves in control and in acetyl-L carnitine (ALC) treated animals.*

Nerve studied		Vagus	Facial
Species		adult rat	newborn rat
Staining method		HRP injection in proximal nerve stump	Nissl method
Number of motoneurons (±SD)	Control	DMVN = 1290 (±266) § AN = 339 (±118)*	193.5 (±312.8)°
	ALC-treated	DMVN = 2004 (±208) §§ AN=475 (±195)**	1244(±1057.2)°°
	Intact	DMVN=2465 (±557)§§§ AN=594 (±94)***	4631.3(±943.5)
		§ - §§ = $p < 0.01$ § - §§§ = $p < 0.02$ §§ - §§§ = No significant * - ** = No significant ** - *** = No significant * - *** = $p < 0.05$	° - °° = $p < 0.05$

DMVN = Dorsal Motor Vagal Nucleas; AN = Ambiguous Nucleus

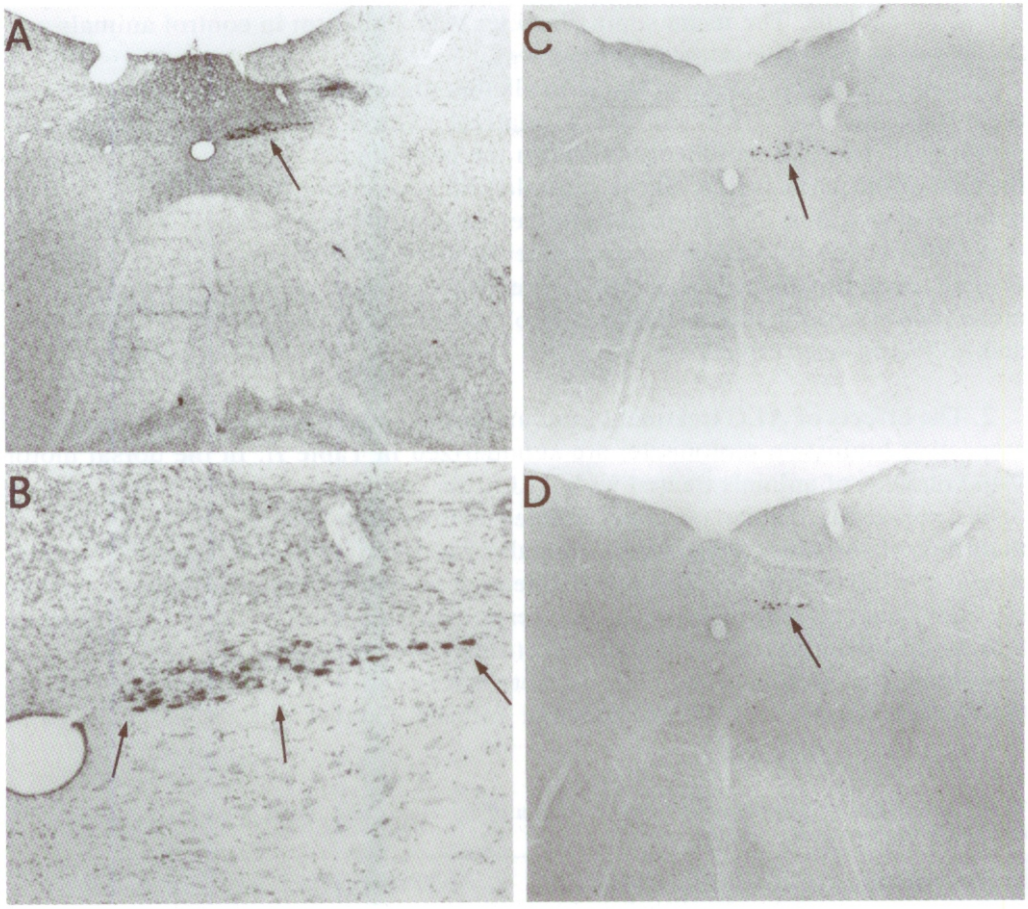

Fig. 1 - *Transverse sections of the brain stem showing horseradish peroxidase-labeled motoneurons forming the dorsal motor vagal nucleus (arrows).*

A. Intact rat (50 X). B. The same of A at higher magnification (125 X). C. Acetyl-L-carnitine (ALC)-treated rat with prolonged section of the vagus nerve (50 X). D. Control untreated rat with prolonged section of the vagus nerve (50X). No counterstained sections. Prolonged section of the vagus nerve causes significant decrease in number of motoneurons in the dorsal motor vagal nucleus in untreated rats but not in ALC-treated rats.

motoneurons between 20 and 44 μm were 9% and 15% for the same groups of animals, respectively. The mean motoneuronal area of the dorsal motor vagal nucleus (mean area of single motoneurons X mean number of motoneurons) was 24% larger in ALC treated animals than in the control group.

In the ambiguous nucleus, the number of HRP-labelled motoneurons in the untreated animals was significantly lesser than in the intact control animals, whereas the decrease in motoneuron number in the treated rats with respect to the intact controls was no significant. On the other hand, in the ambiguous nucleus the percentages of motoneurons with diameters between 8 and 20 μm were 87% for

the treated animals and 81% for control animals. Conversely, the percentages of motoneurons with diameters between 20 and 40 μm were 13% and 19% for the treated and control animals, respectively. The decrease in the mean motoneuronal area was similar for the two groups with a difference of only -5% between treated and control animals.

The effects of ALC on the motonucleus of the facial nerve transected animals was quite remarkable. The mean number of motoneurons was 1244 in the treated and 193.4 in the untreated control animals ($p < 0.05$) (Fig. 2); the mean motoneuron diameter was 21.28 μm in the treated and 18.17 μm in the untreated rats ($p < 0.05$).

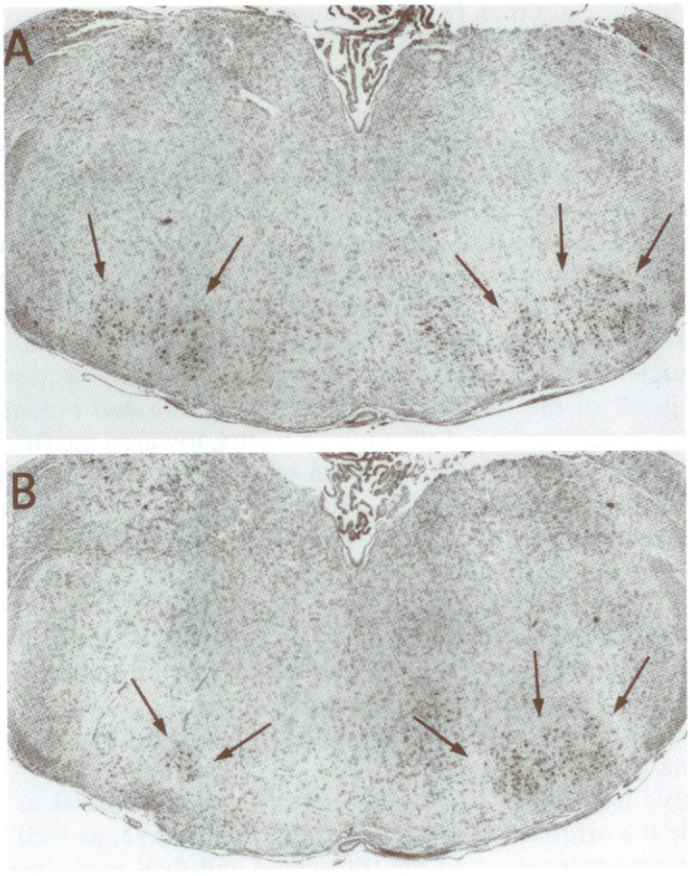

Fig. 2. - *Transverse sections of the brain stem showing the facial nucleus (arrows) of both sides.*

The facial nucleus seen on the left side corresponds to the facial nerve sectioned 7 days before in neonate rats; on the right side is the facial nucleus with intact facial nerve. A. ALC-treated rat (31X). B. Control untreated rat (31X). Nissl method. Section of the facial nerve in neonate rats causes significant decrease in number of motoneurons in the facial nucleus but in the ALC-treated rats such decrease is significantly lesser than in the untreated animals.

DISCUSSION

Our studies show the events taking place after axotomy and regeneration of a cranial nerve and the differences in motoneuron response after ALC treatment in some cranial nerve motonuclei. After section and reconstruction of the ON and FN in guinea pigs and adult rats, not only do axotomized motoneurons of the original ipsilateral motonucleus regenerate, but so do neurons located in the contralateral motonucleus. In our opinion, the bilateral motoneuron reinnervation of muscles, originally innervated only by unilateral-ipsilateral motoneurons, is a sign of neural plasticity. Plasticity of the central nervous system in motonuclear reorganization after cranial nerve regeneration is also indicated by the fact that in adult rats the mean motoneuron number in the stylohyoid subnucleus after FN section and repair is 2.20 fold greater than in control animals and "ectopic " motoneurons, i.e. neurons not normally involved in SHM innervation, are brought into evidence. Such a phenomenon was seen also in previous personal experiments on the abducens and sciatic nerves (8, 11, 12). Analogous phenomena of bilateral innervation were reported after section and regeneration of the ON in goldfish (28) and after injury of the brachial plexus in newborn rats (31). In our study, bilateral innervation was seen after section and repair of different nerves. These findings appear to indicate a commonly occurring repair mechanism. On the other hand, the difference we found between some aspects of the reorganization occurring in the ON of guinea pigs and in FN of adult rats might indicate different qualitative and quantitative plasticity capacities in different animal species and/or in different motoneuron nuclei. It seems interesting to remark that many "ectopic" stylohyoid motoneurons in all of the rats submitted to FN section and reconstruction were located dorsally to the borders of the facial nucleus, towards the floor of the fourth ventricle (8). A somewhat similar location close to the subependymal zone was also found after sciatic nerve section and reconstruction (12). Finally, a similar phenomenon was observed also in the injured musculocutaneous nerve of a primate model where the regrowth included axons also coming from the anterior horn cell areas of C3, C4 and C7, as well as C5 and C6 (19). These findings might indicate that the increase in the total motoneuron population after nerve regeneration is at least partially due to the multiplication and migration of new neurons from the subependymal layer (8). Though a direct experimental proof is still lacking, this view seems supported by recent observations putting the "no new neuron" dogma in discussion (2, 3, 14, 16-18, 20, 22, 26, 27).

An alternative hypothesis is that the motoneurons ipsilateral to the lesion but located outside the original innervational field of the nerve, as well as the contralateral motoneurons reinnervating the transected and then reconstructed nerve, do not necessarily have to be "new" neurons; they may be "quiescent" neurons that are "awakened" by regenerative signals. These signals start from the transection site, spread first in and around the ipsilateral motonucleus and then towards the homologous contralateral one. These regenerative signals, retrogradely transported or directly secreted by the injured motoneurons, most probably consist of diffusible molecules. This hypothesis is supported by the influence that the distance

between the nuclei of both sides appears to have on both the occurrence and magnitude of the phenomenon. In fact, contralateral motoneurons accounted for 14% of the total number of motoneurons of the MRM subnuclei which is located very close to the midline, while they accounted only for 8% in the case of the SHM subnuclei that are located at a greater distance from each other.

The putative regenerative signals, although generally diffusible, seem to have some form of specificity, as most of the contralateral neurons lay in the homologous area of the contralateral motonucleus. This specificity may be due to the specific transport pathways taken by these substances, and/or to the variability of the retrograde response of extrinsic neurons to axotomy (1). Recent studies have proven that certain diffusible proteins (i.e. netrin-l, netrin-2, and semaphorin family proteins) have the property of guiding growing axons of certain neurons to their targets (23, 31). Most of these substances are normally produced during the embryological development of the central nervous system, at the level of axonal target zones, in order to attract and/or repel specific types of neurons (23). In the light of these assertions, nerve regeneration could at least to a certain extent recapitulate normal development (18).

Our result confirm a significant neuroprotective effect of ALC on the motoneurons that was quite apparent in both adult rats with VN lesions and in newborn rats with FN lesions. Furthermore, our findings on the vagus nerve nucleus after VN section and treatment with ALC, seem to demonstrate a certain degree of ALC cell specificity, especially for parasympathetic cholinergic neurons. In fact, the effect of ALC was more evident at the level of the dorsal motor vagal nucleus, that is a source of general visceral parasympathetic axons, than on the ambiguous nucleus, that is a source of both special visceral axons for striated musculature and general visceral parasympathetic fibres (5). Furthermore, it has been reported that the small-sized (8 to 20 μm) neurons located in the ventral division of the ambiguous nucleus should project parasympathetic fibres to the heart and larynx, while the medium-sized cells should project to the striatal laryngeal and esophageal musculature, being located in the dorsal division of the ambiguous nucleus (5). By comparing our morphometrical data, it is apparent that ALC treated rats presented about a 2% reduction of the small-sized cells compared to 35% in the operated control rats, while the decrease in medium-sized cells was similar in the two groups.

To sum up, our findings indicate that the neuronal organization of various cranial nerves' motonuclei can massively respond to nerve section and reconstruction. This response is extremely complex but anatomically positive. Its functional relevance remains to be proved. The same types of considerations that we have made for cranial nerves, in particular for the oculomotor, facial and vagus nerves, have also been made for peripheral spinal nerves (9, 12).

SUMMARY

Little is known about the mechanisms at play in nerve regeneration after nerve injury. Personal studies are reported regarding motonuclear changes after regen-

eration of injured cranial nerves, in particular of the facial and oculomotor nerves, as well as the influence that the natural molecule acetyl-L-carnitine (ALC) has on post-axotomy cranial nerve motoneuron degeneration after facial and vagus nerve lesions.

Adult and newborn animal models were used. Massive motoneuron response after nerve section and reconstruction was observed in the motonuclei of all nerves studied. ALC showed to have significant neuroprotective effects on the degeneration of axotomized motoneurons. Complex quantitative, morphological and somatotopic nuclear changes occurred that sustain new hypotheses regarding the capacities of motoneurons to regenerate and the possibilities of new neuron proliferation. The particularities of such observations are described and discussed.

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