On the Number and Morphometrical Parameters of the Nucleus Ambiguous Neurons After the Injury and Regeneration of the Recurrent Laryngeal Nerve in the Rat

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Introduction and objectives: In laryngeal nerves injuries it is essential to know the morpho-functional reorganization of the neurons which supply the larynx in order to be successful with the clinical techniques of functional reinnervation and/or orthotopic transplant. Due to the lack of this type of studies in the literature, we investigated the organization of laryngeal motoneurons in the nucleus ambiguous (NA) after the axotomy and regeneration of the recurrent laryngeal nerve (RLN) in adult rats.
Material and methods: We used biotinylated dextran amines (BDA, 3 kDa), this fact is an innovation in the field, because this is a novel methodological approach to this model. We studied a control group of 14 animals and 4 experimental groups of between 10 and 16 animals each one. In the experimental groups we studied the regeneration of the axotomized nerve in 4 different intervals of time after the injury: 21-28 days, 42-60 days, 90-120 days, and 150-180 days. In the control group we traced the RLN without injury while in the experimental groups we traced the axotomized RLN after each regeneration interval.
Results: The number of traced neurons in the control group was 143 (38); in the experimental groups the number was always lower than in the control (21-28 days: 14 [23] neurons; 42-60 days: 46 [49]; 90-120 days: 55 [57]; 150-180 days: 61 [60]). The morphologic parameters studied within the neuronal bodies in the experimental groups were no statistically different when compared with those in the control group.
Conclusions: Results show that the tracing of the RLN after its axotomy and regeneration, in the adult rat, involves a decrease in the number of traced neurons within the NA but no changes in their size or shape during the analysed periods.

Key words: Larynx. Axotomy. Degeneration. BDA.

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Estudio del número de neuronas del núcleo ambiguo y sus parámetros morfométricos tras lesión y regeneración del nervio laríngeo recurrente de la rata

Introducción y objetivo: Para conseguir la reinervación funcional y/o el trasplante ortotópico tras lesiones de los nervios laríngeos es esencial conocer la reorganización morfofuncional de las neuronas que inervan la laringe. Debido a la escasez de este tipo de estudios en la literatura consultada, en nuestro trabajo estudiamos la organización de las motoneuronas laríngeas del núcleo ambiguo (NA) tras sección y posterior regeneración del nervio laríngeo recurrente (NLR) de la rata adulta.
Material y métodos: El trazador utilizado fue la dextranamina biotinada (BDA) de peso molecular 3 kDa, lo que representa una innovación en la literatura científica consultada, nunca antes utilizada en este modelo. Los sujetos se distribuyeron en un grupo control de 14 animales y cuatro grupos experimentales de entre 10 y 16 animales cada uno. En los grupos experimentales se estudió la regeneración en cuatro intervalos de tiempo tras la axotomía: entre 21 y 28 días, entre 42 y 60 días, entre 90 y 120 días y entre 150 y 180 días. En el grupo control se trazó el NLR intacto y en los grupos experimentales el NLR axotomizado después de cada intervalo de regeneración.
Resultados: El número de neuronas marcadas en el grupo control fue de 143 ± 38; en los grupos experimentales el número de neuronas trazadas fue siempre menor que en el grupo control (21-28 días: 14 ± 23 neuronas; 42-60 días: 46 ± 49; 90-120 días: 55 ± 57; 150-180 días: 61 ± 60). No se encontraron diferencias estadísticamente significativas entre el grupo control y los grupos experimentales en los parámetros morfométricos analizados para los somas neuronales.
Conclusiones: Los resultados muestran que la axotomía del NLR de la rata comporta una disminución en el número de neuronas trazadas del NA, pero no afecta al tamaño ni su forma en los períodos estudiados.

Palabras clave: Laringe. Axotomía. Degeneración. BDA.

INTRODUCTION

At present the limiting factor for achieving a fully functional recovery of the larynx, after a transplant, is neither revascularization nor a possible rejection of the transplanted organ, but obtaining a functional reinnervation. In the process of reinnervation, regenerating axons take a wrong direction, thus innervating wrong muscle groups, with the result of the phenomenon known as synkinesis, which translates into an inadequate glottic mobility, which alters the laryngeal function.[1-3] Therefore, to achieve a functional reinnervation in transplants and/or larynx paralysis, a thorough knowledge of the morphological events that occur centrally and peripherally after a lesion of laryngeal innervation is required. While several studies have been published about the changes that take place in the periphery, only 2 works of the literature researched, carried out on guinea pig and rat, referred to the morphological and functional changes that occur in the ambiguous nucleus (AN) after an injury and subsequent regeneration of the recurrent laryngeal nerve (RLN). In the guinea pig, the loss of somatotopy in the AN and a steep decline in the number of neurons in the initial recovery times, which becomes moderate as the reinnervation time increases, has been described.[4] Moreover, in rats it has also been described that after injury and regeneration of the RLN the somatotopic representation of the various laryngeal muscles in the AN was disorganized, and a decrease in the number of neurons is also mentioned.[2] The loss of somatotopy described in both works has been interpreted as the basis of failure in selective reinnervation. However, neither of the works referred describe the changes in shape and/or size of the axotomized neurons.

Therefore, due to the shortage of works studying the changes that occur in the AN after an injury and subsequent regeneration of laryngeal nerves, we considered the development of this work. The aim of this study, therefore, is to describe, in rats, the changes in the number, shape, and size of neurons tracked in the AN after axotomy of the RLN, at different times of reinnervation, using biotinylated dextran amines (BDA), as neuronal tracers. BDA have never before been used in the modelling of injury and regeneration of RLN although they have been described as neural markers of high precision and high speed transport, which reveal the dendritic and axonal structure of the marked neurons.[5]

MATERIAL AND METHODS

We used 72 Sprague-Dawley male rats, of a weight between 300 and 450 grams (3-4 months old), which were distributed into 2 sets: a control series of 14 animals in which the left RLN without previous injury was traced, and an experimental series of 58 animals which were divided into 4 groups of 10 to 16 animals each, classified according to the survival time elapsed since the axotomy of the left RLN. The 4 time intervals were in the range of 21-28, 42-60, 90-120, and 150-180 days.

In the control series the RLN was injured caudally to the thyroid gland, along the fifth tracheal ring, to facilitate the capture of the tracer.[5,6] Next, the proximal end of the nerve was covered with the 3 kDa BDA (Molecular Probes, Oregon, USA) in the form of freeze-dried sample, hydrated on the tip of a thin entomology needle. In the experimental series, a complete section of the left RLN in the plane of the fifth or sixth tracheal ring was carried out, leaving the 2 ends in contact. After the different periods of postoperative survival of between 21 and 180 days had elapsed, the axotomized nerve was traced with 3 kDa BDA, distally to the injured area, to ensure that in the event of a regeneration of the sectioned fibres, those would be the ones which were traced by the BDA. The BDA was left to act for 10 minutes to allow its capture by the nerve, and then the area was cleaned with saline solution, the muscular planes were closed and the skin was sutured.[6,7] Biotinylated dextran amines (BDA) have been described as neural markers of high precision and high speed transport, which reveal the dendritic and axonal structure of the marked neurons.[5] Under certain conditions they are transported both retrogradely and anterogradely, thus enabling us, in the same animal, to visualize the central and peripheral projections of the traced fibres.[7,8] Furthermore, they are very soluble molecules in aqueous solutions, enabling an easy handling and, in the lysine form, they become joined to tissues fixed with aldehydes so that their signal is not lost with time.[5]

After 7 days from the tracing of the nerve, intracardiac perfusion with 4% paraformaldehyde in PB was conducted on the animals from both the control and the experimental groups. The brain stem was removed, cryoprotected with sucrose at 15% and 30%, and cut in the cryostat in cross-sections of 50 µm. The presence of BDA was detected by incubation with avidinabiotin-peroxidase (Vector, California, USA) and later revealed with diaminobenzidine intensified with nickel (Vector, California, USA). In some cases a contrast was performed using Nissl staining.

In both series, we examined the number of neurons marked, counted in each section in the optical microscope at ×20 increases, exploring the entire depth of the section with the micrometric. The morphometric parameters of the somas (area, perimeter, length, width, form factors, and equivalent diameter) were evaluated by the Visilog 5.4[9] software and the differences between groups in the control and experimental series were analysed by the Student *t* test.

RESULTS

In all cases studied, control and experimental, the traced neurons were to ipsilateral to the traced nerve and no differences were observed between the left and right sides.

With regard to the control series, all traced neurons were located in the AN (Figure 1A), and the majority of the neurons showed multipolar morphology (Figure 1B). The number of neurons observed was between 121 and 214, with an average of 143 (38) (Table 1 and Figure 2). The average size of neurons was 43.2 µm in greater diameter and 25.5 µm in lesser diameter, while the average area of these neurons was of 574 µm^2. The average perimeter was 118 µm; the form factor was 1.98 and the equivalent diameter was 26.8 µm

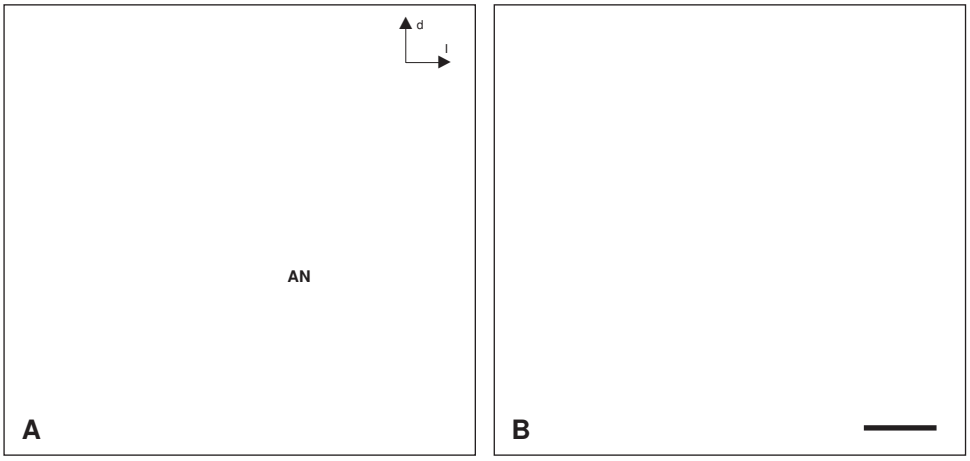

Figure 1. The trace of the recurrent laryngeal nerve ipsilaterally marks neurons in the ambiguous nucleus (AN). A: section of the oblong marrow in the level of the lower olivary nucleus. Scale: 500 µm; d: dorsal; l: lateral. B: detail of neurons in the ambiguous nucleus boxed in A. Scale: 50 µm.

Table 1. Results Obtained in the Number of Neurons After the Tracing With BDA of the Recurrent Laryngeal Nerve in Injured Groups[a]

Time After Injury	*Cases With Neuronal Marking*	*Average Number of Neurons*
Control	14/14	143 (38)
21-28 days	5/10	14 (23)
42-60 days	13/16	46 (49)
90-120 days	11/16	55 (57)
150-180 days	10/16	61 (60)

[a]For every group is indicated the number of cases in wich bearing has been obtained in the neurons of the ambiguous nucleus, as well as the average of planned neurons.

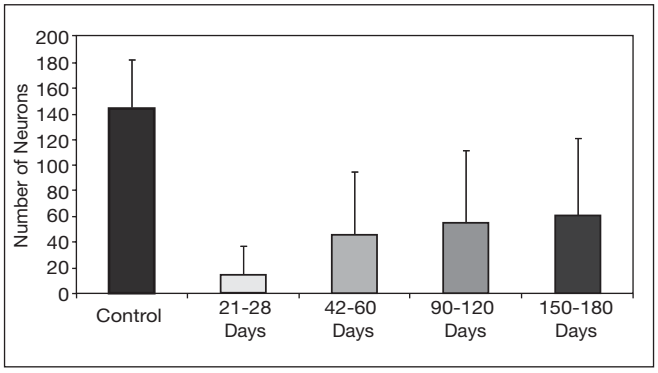

Figure 2. Average number of neurons marked with BDA after the tracing of the recurrent laryngeal nerve in the control group and in different experimental groups where the recurrent laryngeal nerve which had been severed was traced with BDA at various times after the injury. The standard deviation of each average is indicated.

(see Table 2 and Figure 3). In some sections, fibres were identified on the formation route of the apparent origin of the nerve, as well as aberrant neurons (Figure 4). No marked neurons or fibres were found in other areas than the brain stem or spinal cord.

The number of cases in which a marking of the neurons in the ambiguous nucleus was obtained, as well as the average number of neurons traced, are indicated for each group.

As for the experimental series, the following information was obtained in each group:

– Group of 21-28 days of survival after the injury. Traced neurons have only been obtained in 5 of the 10 animals in the group, with an average of 14 (23) neurons, of weak marking (Table 1 and Figure 5)

– Group of 42-60 days of survival after the injury. Traced neurons appear drawn in the AN in 13 of the 16 animals of this series, averaging 46 (50) traced neurons in the AN (Table 1 and Figure 6)

– Group 90-120 days of survival after the injury. In 11 of the 16 animals neurons were traced in the AN, the average number of neurons was 55 (58) (Table 1 and Figure 7)

Table 2. Morphometry of the Body of Neurons of the Recurrent Laryngeal Nerve Marked After Tracing With BDA[a]

Group	*No.*	*Area, µm²*	*Perimeter, µm*	*Major Diameter, µm*	*Minor Diameter, µm*	*Shape Factor*	*Equivalent Diameter, µm*
Control	243	574 (171)	118 (25)	43 (8)	26 (6)	1.9 (0.5)	27 (4)
21-28 days	145	485 (269)	99 (36)	38 (14)	21 (8)	1.8 (0.7)	24 (8)
42-60 days	341	534 (217)	101 (30)	37 (10)	23 (7)	1.6 (0.4)	26 (6)
90-120 days	203	214 (241)	60 (34)	22 (13)	14 (7)	1.8 (0.7)	14 (8)

[a]Group control and different experimental groups in wich the recurrent laryngeal nerve who had been cut was planned with BDA to different times after the injury. Is indicated the standard diversion of every average.

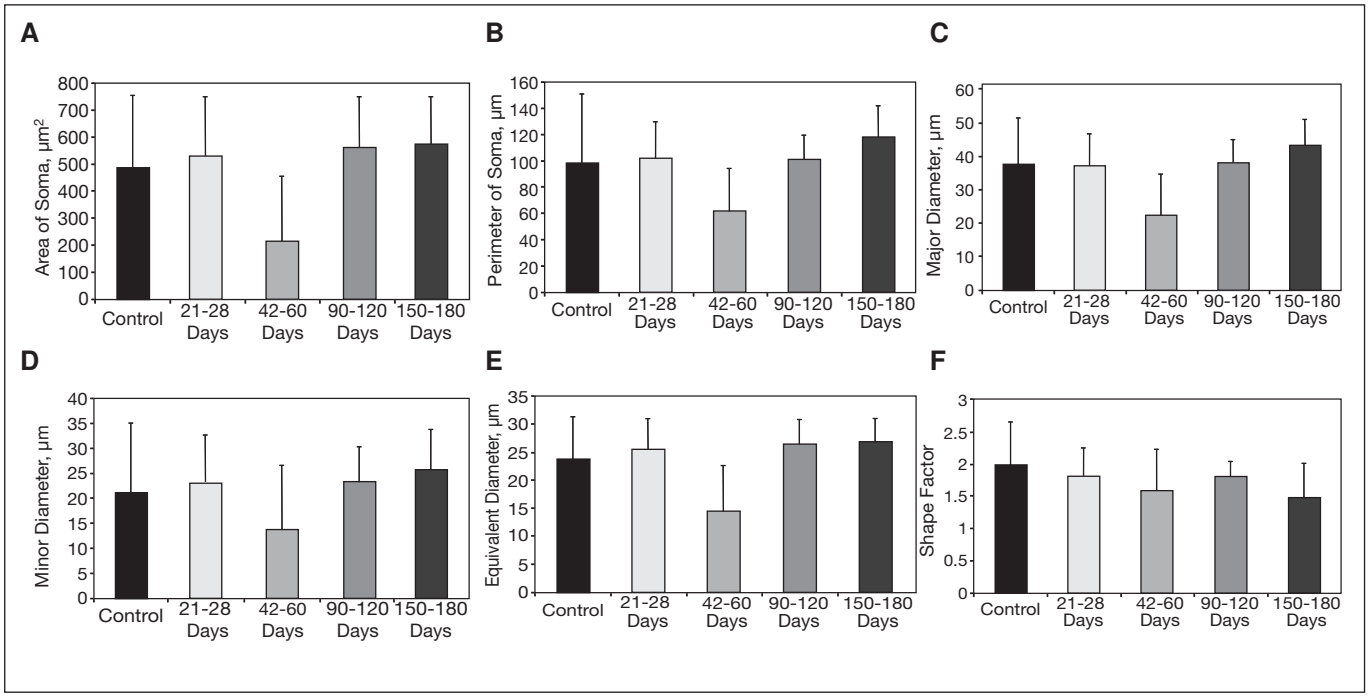

Figure 3. Composition of graphs showing the results in terms of the morphometric parameters analyzed in the control group and in the different experimental groups. The parameter analysed is showed in the X-axis and the different groups studied in the Y-axis. In all cases the standard deviation of each average is indicated. The differences were not significant in any case (Student *t*, *P*>.05).

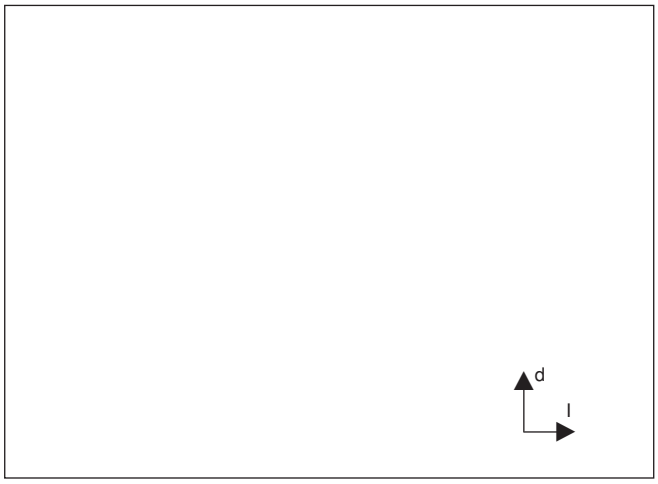

Figure 4. Section of the oblong marrow at the level of the olivary nucleus nucleus, in which aberrant neurons can be observed, marked as a result of the tracing of the recurrent laryngeal nerve. Note the spindle morphology and the position they occupy, displaced from the area that corresponds to the ambiguous nucleus. Scale: 500 µm; d: dorsal; l: lateral.

– Group of 150-180 days of survival after the injury. We identified neurons in the AN in 10 of the 16 animals, with an average of 61 (60) neurons traced (Table 1 and Figure 8)

We have not carried out a statistical comparison between the number of neurons traced in the control group and the experimental groups. This is due to the fact that in complex experimental groups, such as in the one in this study, in combination with the surgical and neural tracing techniques employed, variables are introduced which result in a final sample which is too heterogeneous to carry out any significant statistical analyses. However, we have made a biological interpretation of the data, collected in the discussion of the study.

This is not the case with the morphometric parameters studied, both in the control group and in the experimental groups (average area of the soma; form factor; perimeter; major, minor, and equivalent average diameters), in which the numerical results obtained are susceptible of statistical analysis. The data collected in the study is presented in detail in Table 2 and in Figure 3. The interpretation of this data is collected in the discussion chapter.

DISCUSSION

The results we obtained show that in the tracings of the RLN in the control group only neurons in the ipsilateral AN were identified, with none in other areas of the brain stem or spinal cord, as has been described in the rat[10,11] and in other species studied.[12-15] We counted between 121 and 214 neurons traced by AN (mean [standard deviation] of 143 [38]), consistent with quantification data from other authors.[10] The morphology of the majority of neurons identified was multipolar, as has been described in other works.[12,14,16] Neurons displaced from the AN have also been identified, labelled as "aberrant" by Ramon y Cajal.[17]

As in the control group, in the groups of the experimental series only neurons in the ipsilateral AN were identified. However, after axotomy of the RLN, the average number of neurons traced in the AN is less than the average number

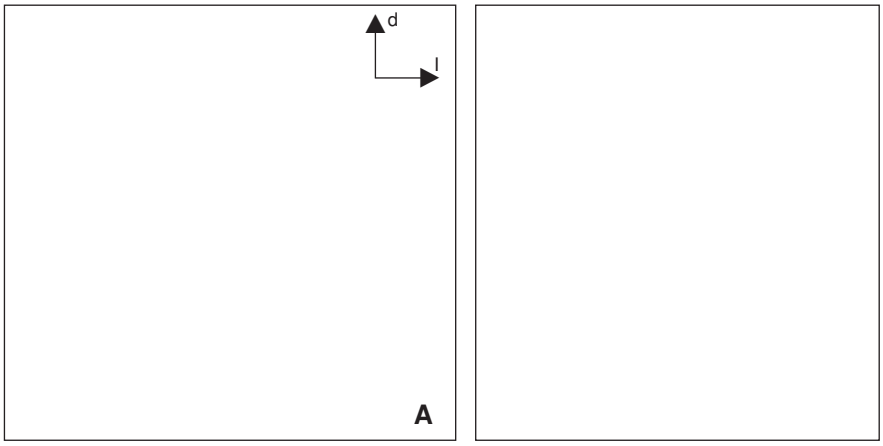

Figure 5. Trace of the recurrent laryngeal nerve between 21 and 28 days after the injury. A: overview of a section of the oblong marrow in the level of the inferior olivary nucleus, with marked neurons in the ipsilateral ambiguous nucleus. Scale: 200 µm; d: dorsal; l: lateral. B: detail of neurons marked. Scale: 50 µm.

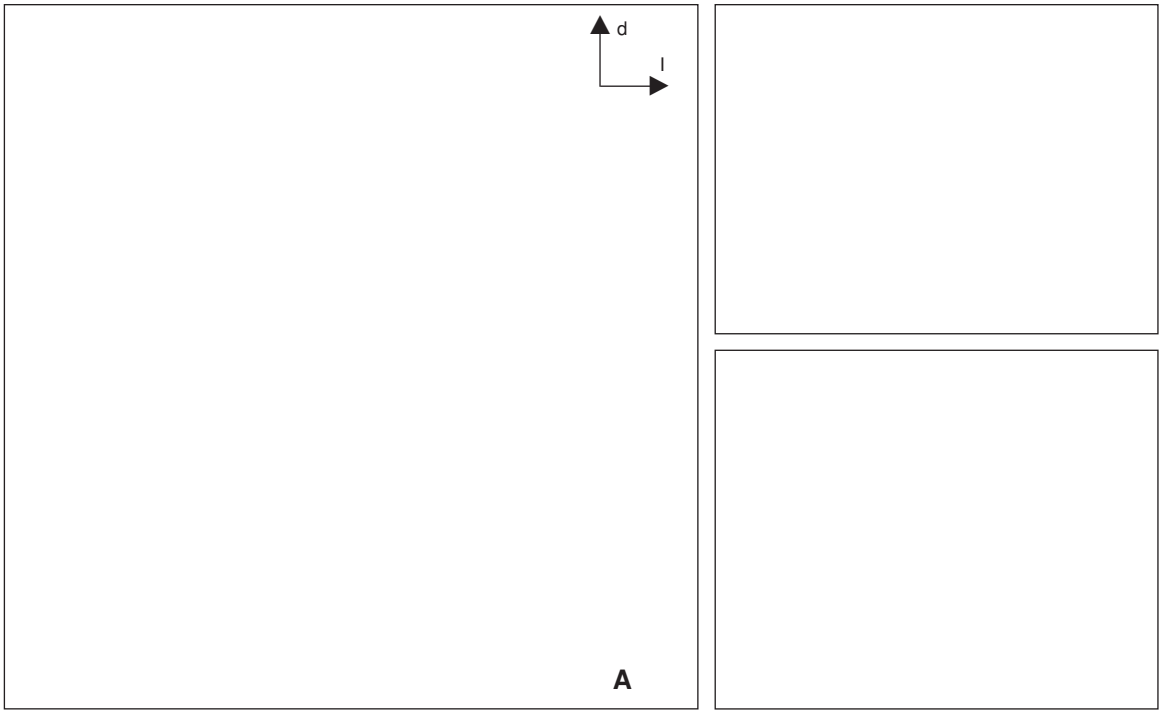

Figure 6. Trace of the recurrent laryngeal nerve, between 42 and 60 days after the injury. A: general appearance of a section of the oblong marrow, with marked neurons in the ipsilateral ambiguous nucleus. Scale: 200 µm; d: dorsal; l: lateral. B, C: detail of neurons in the ambiguous nucleus marked in A. Scale: 50 µm.

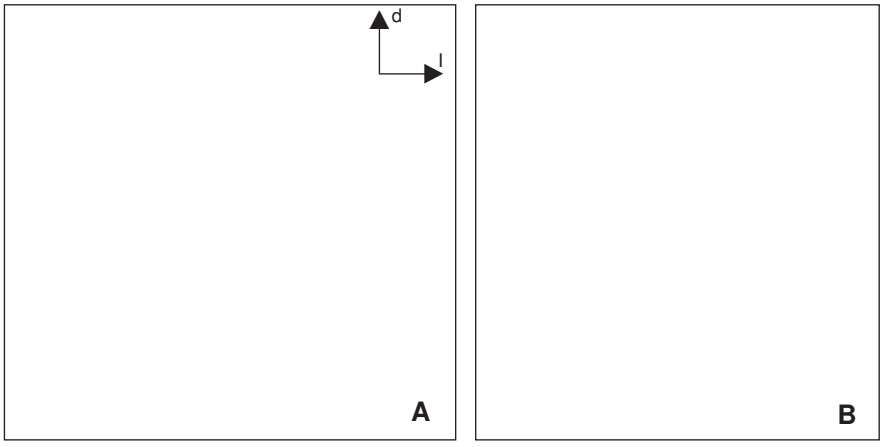

Figure 7. Trace of the recurrent laryngeal nerve, between 90 and 120 days after the injury. A: general appearance of a section of the oblong marrow in the level of the inferior olivary nucleus, with marked neurons in the ipsilateral ambiguous nucleus. Scale: 200 µm; d: dorsal; l: lateral. B: detail of neurons in the ambiguous nucleus marked in A. Scale: 50 µm.

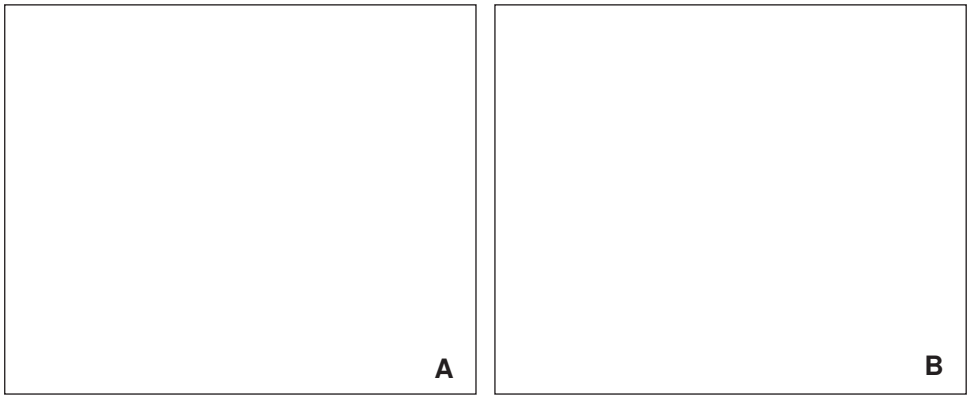

Figure 8. Trace of the recurrent laryngeal nerve, between 150 and 180 days after severed. A: general appearance of a section of the oblong marrow in the level of the inferior olivary nucleus, with marked neurons in the ipsilateral ambiguous nucleus. Scale: 200 µm. B: detail of neurons in the ambiguous nucleus marked in A. Scale: 50 µm.

of neurons in the control group, regardless of the regeneration time elapsed since the axotomy. In the group of 21-28 days the neurons marked with respect to the control group represent only 10%. However, from the group of 42-60 days a gradual increase in the number of neurons traced can be observed, and this trend continues up to the group with longer regeneration time, 150-180 days, in which a percentage of 42% neurons were traced with respect to the control group. This decrease in the number of neurons traced in injured animals coincides with previously described data in rats, in which a loss of 50% of neurons with respect to the control group was observed in the AN, 105 days after injuring the RLN.[2] Moreover, it has been observed in this study, that increasing the time allowed for regeneration enables a marking of more neurons and this fact has also been described in guinea pigs, in which, after injuring RLN the number of neurons traced in the AN, increased gradually from 20 neurons in day 14 after the injury to 90 neurons in day 180.[4]

We must emphasize that in our study we have identified instances where the tracing of the RLN distally to the area of injury did not mark any neuronal bodies in the AN; some cases occurred in all experimental groups. This could be because nerve fibres in regeneration were unable to pass distally through the fibrous area of the injury and continue through the nervous sheath, thus preventing the uptake of the tracer. It may also be because the fibres did regenerate but toward aberrant locations due to having lost the continuity between the terminal ends of the injured nerve, therefore they would not have continued on the functional path and again would have made uptake of the tracer impossible. Finally, it is possible that, for reasons unknown, no regeneration phenomena took place.

The figures we have obtained in connection with the morphometric measurements of the neurons traced, both in the control group as in the experimental groups, are susceptible of a statistical analysis, as was mentioned in the materials and methods paragraph. Along these lines, the results show that there are no significant differences between morphometric parameters analyzed in neurons in the control group and experimental groups. Biologically, this means that the size and shape of neurons in the experimental groups is maintained with respect to the neurons of the controls. However, it has been described that a set of phenomena called chromatolysis, takes place in the cell body of the axotomized neuron. This process involves a change in the shape and size of the soma.[18] The fact that in the animals from the experimental groups, the shape and size of neurons in the AN remain unchanged after the section of the RLN could be due to 2 factors: either that there have been no morphological changes in the axotomized neurons or that if such changes have occurred, they have happened before day 21 after the injury, which is when we started analyzing these neural parameters, and this would have prevented us from detecting and quantifying any changes. The latter scenario would be supported by what has been described in rats after the section of the sciatic nerve, because the size of neurons increased in 2 phases with 2 high peaks, one taking place 3 to 4 days and another 8 to 11 days after the lesion.[19] In both cases the figures are lower than earlier times of our analysis, 21 days.

In summary, the results presented show that the tracing with BDA of the previously injured RLN, with different regeneration time intervals, always identifies a number of neurons that does not reach, in the best case, 50% compared with the control group. The figure increases with the time allowed for regeneration. In addition, the size and shape of the neural bodies in the experimental groups are no different from those of the control neurons.

As a main conclusion, our study shows that after axotomy of the RLN, regeneration phenomena take place in the axotomized nerve fibres, although it seems obvious that, at least with 6 months of regeneration, not all get sectioned fibres manage to regenerate, since none of the experiments succeed in tracing the same number of neurons as in the control group. These regeneration phenomena cited are dependent on time.

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