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Astrogliosis in different zones of the spinal cord gray matter after sciatic nerve axotomy in the newborn rat: A morphometric and immunohistochemical study

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Summary. Astrocytic response following unilateral sciatic nerve axotomy was examined in the spinal gray matter of newborn rats. Using an antiserum to glial fibrillary acidic protein (GFAP), immunoreactive astrocytes were studied in the ventral, dorsal and transitional region between the dorsal and ventral gray matters (TDVG) at intervals of one day, one week, two weeks and one month postaxotomy. The axotomized side showed an obvious increase in the number of immunoreactive astrocytes at one week, two weeks and one month after surgery. The numerical density per area of the glial cells (N(a)) was determined in all groups on both the intact and axotomized sides, and it increased in all groups at the axotomized sides. The percentage of glial cell increase (Pgi) was also determined. At the ventral horn Pgi increased at day one and continued to increase in all groups, while the increase in TDVG and the dorsal horn occurred at later time points.

The total motoneuron count in the ventral horn at the axotomized and intact sides was done at all time points, and the percentage of motoneuron reduction (Pmr) was calculated, the highest Pmr being noticed at one month (41%). A nonlinear regression for Pmr and Pgi showed that the rate of Pgi was approximately double that of Pmr.

The rate of glial cell increase at each time point (one day, one week, two weeks and one month groups) was calculated, and the highest rate of glial cell increase in the ventral horn occurred one week after axotomy, while the highest rate in the dorsal horn and TDVG occurred at the second week. The conclusion of the study is that there may be an initial post-axotomic proliferative phase of the glial cells, which was followed by a differentiation phase. Also a gradient of an increase in the rate glial cell proliferation was noticed from the ventral horn toward the dorsal horn, maybe due to stimulation by a paracrine factor.

Key words: Astrocyte, Gliosis, Sciatic nerve, Axotomy

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Introduction

Axotomy of the sciatic nerve is reported to cause apoptosis in the motoneuron of the spinal cord (Rossiter et al., 1996), the dead cell must be removed and replaced by glial material (Dusart et al., 1991) Glial cell activation was reported to start as early as 24 hours postaxotomy (Tetzlaff et al., 1988). Time course study indicated that there was a progressive increase in the glial reaction in neuronal supply of the axotomized nerves (Anders and Johnson, 1990). This reaction was also reported in the spinal cord after sciatic nerve axotomy in both the ventral and dorsal horns (Gilmore et al., 1990).

Gilmore et al. (1990) used immunolabeling of GFAP and reported the presence of glial reaction in the spinal cord of axotomized sciatic nerve. The time course of glial reaction in the axotomized segments was documented as early as two days post-operation. However, Anders and Johnson (1990) used olfactory nerve axotomy and an earlier response (24 hr) was reported, and a similar finding was reported in facial motoneurons (Tetzlaff et al., 1986). Other investigators have used different models to evaluate glial cell response such as excitotoxic lesion in the CNS (Dusart et al., 1991), electrically lesioned rat brain (Ahmed et al., 1996), brain lesion (Janeczko, 1989), brain following chronic hypoxia (Zimmer et al., 1991) and corticospinal lesion (Kost-Mikucki and Oblinger, 1991). Garrison et al. (1991) used the sciatic nerve constriction as a model to evaluate the response of the spinal cord and reported that GFAP increased following spinal cord injury in the lumbar region.

There are few investigations on the gliosis of the ventral and dorsal horns, and there is no information about the reaction of the glial cells in TDVG of the spinal cord following sciatic nerve axotomy. Therefore, it may be relevant to investigate the possibility of glial cell activation in TDVG following sciatic nerve transection. The purpose of this study is to evaluate the post-axotomic gliosis in the ventral, TDVG and dorsal zones. Morphometric methods were used here to evaluate the rate of gliosis at each time point, as well as

the general rate of gliosis using nonlinear regression analysis.

Materials and methods

Twenty newborn Sprague-Dawley rats at five days of age were divided into four groups (five animals per group) and kept in the university animal facility in standard conditions with free access to food and water. Adequate measures were taken to minimize pain and discomfort to animals according to the "Giudelines for animal experimentation" of the ethical committee of the School of Medical Sciences at Tarbiat Modarres University. The animals were anesthetized by hypothermia, their right sciatic nerves were axotomized at the mid-thigh region and they were sacrificed on day one, one week, two weeks and one month postaxotomy. They were perfused with heparinized saline and fixed in buffered formal saline. The spinal segments (L4-L6) were identified (Gelderd and Chopin, 1977), removed, immersed in the same fixative and transversely cut. The tissue was processed in paraffin and 5 μm transverse sections were obtained and either stained with Hematoxylin and Eosin, or used for immunolabeling. The ventral, TDVG and dorsal zones were identified according to Brichta and Grant (1985).

The section used for immunohistochemstry was treated with 10% hydrogen peroxide for 30 min at 37 °C, followed by washing with phosphate buffered saline (PBS: 0.01 M, pH 7.4), then the antigen was unmasked with trypsin and incubated in rabbit serum (incubation time: 10 min at 37 °C). The indirect immunoperoxidase method was used. Rabbit polyclonal anti-GFAP antibody was used as the primary antibody (incubation time: 4 hrs at 37 °C) (Dako). The sections were washed with PBS then labeled with secondary goat anti-rabbit antibody conjugated with peroxidase (Dako) for the same incubation time used for the primary antibody. Then they were washed with PBS, and incubated in the substrate solution (5% diaminobenzidine in tris-buffer solution, 0.05 M pH 7.6, with hydrogen peroxide) for 10 min. at 37 °C. After that, they were washed with PBS and the staining was intensified with 0.5% copper sulfate in normal saline. The sections were counterstained in hematoxylin and mounted in synthetic resin.

Glial cell morphometry was carried out on Hematoxylin and Eosin-stained sections using nuclear morphology as the basis for differentiating glial cells from other cells (De Girolami et al., 1994). The number of sections in each animal, and the number of fields in each section, to obtain a valid sampling, was calculated according to the DeHoff method (Ahrene and Dunnill, 1982). The slides and the fields were selected using a random table. The numerical density of glial cells per area was calculated using an unbiased method (Gundersen et al., 1988) as follows:

$$\text{The numerical density of glial cells per area} = \frac{Q(\text{prof.})}{A(\text{sect})}$$

Where Q is the number of profiles (prof.) in a given area (A) of tissue section (sect).

The percentages of glial cell increase were estimated as follows:

$$\text{The percentage of glial cell increasee} = \frac{Na(AS) - Na(IS)}{Na(IS)} \times 100$$

Where Na(AS) and Na(IS) are the numerical densities (Na) of glial cells at the axotomized side (AS) and intact side (IS) respectively.

Then the rate of glial cell increase was estimated.

The motoneuron counting was carried out on other groups (five newborns per group) of animals which were sacrificed at the same scheduled time and L4-L6 segments were sampled, fixed in Bouin's fixative and processed for paraffin embedding. Serial sections were cut and stained with Cresyl violet stain. The total motoneuron count was carried out according to the method of Li et al. (1998), where the motoneuron counting was done on both the axotomized and intact sides in the ventral horn at all time points. Briefly, the count of the surviving motoneurons was carried out in every 5th section and the cell counts were multiplied by 5 to calculate the total motoneuron count on a given side (axotomized or intact). The spinal motoneurons were identified as large profiles ($\geq$10 μm) with a distinct deeply stained nucleolus.

The percentage of motoneuron reduction (Pmr) was calculated as follows:

$$Pmr = \frac{TMC\text{-}AS - TMC\text{-}IS}{TMC\text{-}IS} \times 100$$

Where TMC is the total motoneuron count at the axotomized side (AS) or the intact side (IS).

The linearity test for regression for the data of Pmr was done according to Woolf (1968). Pmr and Pgi were plotted against the time points and nonlinear regressions were carried out to evaluate the rate of neuronal replacement by glial cells.

The normality of the data was tested using the K-S test, and the analysis of the variance and Tukey's test were used for statistical analysis.

Results

Immunohistochemical results showed that there were many cells labeled with anti-GFAP antibody at the axotomized side while there was less labeling at the intact one. The increase in the expression of GFAP was progressive during the time course, where there was an increase in the number of GFAP-positive cells at one week, two weeks and one month postaxotomy and the highest was noticed after one month at the axotomized side (Figs. 1, 2). TDVG and the dorsal horn of the spinal cord showed labeling with anti-GFAP antibody and the highest labeling was noticed in the one month group after nerve transection (Figs. 3, 4). Figure 5 shows a few astrocytes labeled with anti-GFAP antibody at the intact side. The normal control showed no immunoreactive

Table 1. The mean and the standard deviation of the mean of the number of glial cells per mm^2 (numerical density of glial cells per area) in the ipsi-lateral (axotomized) and contra-lateral (intact side) of the operated animals in different groups (i.e. time points).

	VENTRAL HORN		TDVG		DORSAL HORN		NORMAL
	IS	AS*	IS	ASS*	IS	AS*	
G1	231.7±7.6	242.2±7.7	228±7.6	230±7.3	235.9±8	228.4±7.6	233±9.4
G2	264.8±8.4	349.9±10.9	244.6±6.9	282.9±8.4	40.3±7.6	263.8±8.1	229±6.3
G3	323.2±9.2	504.5±14.2	308.6±9.2	425.5±12.2	294.7±8.1	378.9±11	230±7.9
G4	392.1±14.2	783±17.8	371.6±12.2	552.9±13.7	389.4±13.7	556.2±14.2	231±3.8

TDVG: The transitional region between dorsal and ventral gray matters; IS: Intact side; AS: Axotomized side; *: The comparison between the axotomized and intact sides in all groups were significant except in G1 (one day). G1, G2, G3 and G4 are one day, one week, two weeks and one month respectively.

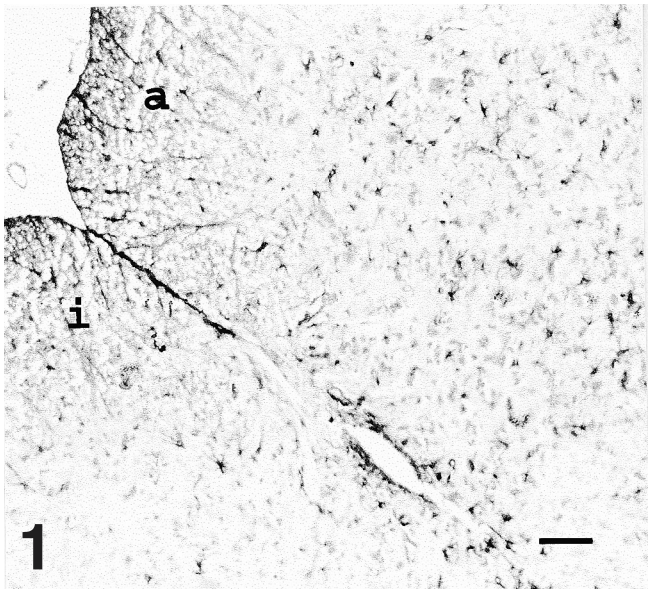

Fig. 1. A photomicrograph of the spinal cord of a newborn rat after one month of surgery shows the GFAP-positive cells in the ipsi-lateral side (axotomized). The astrocytes can be seen at the ventral horn, the transitional region between the dorsal and ventral gray matters and the ventral region of the contra-lateral side of the spinal cord. a: axotomized side, i: intact side. Scale bar: 81 μm.

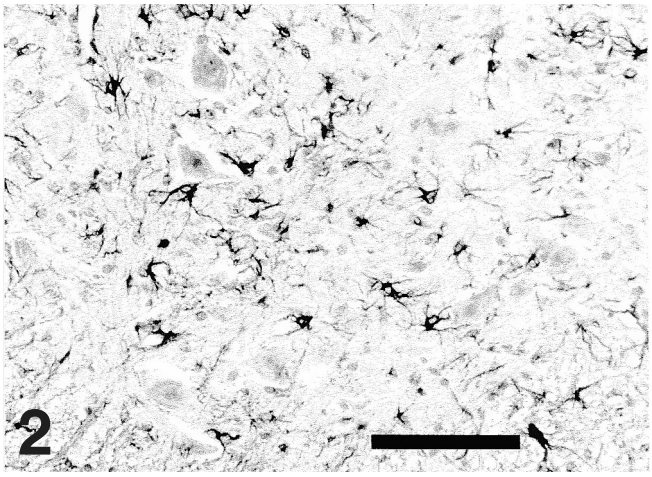

Fig. 2. A photomicrograph of the ipsi-lateral side (axotomized) of a newborn rat after one month of surgery at the ventral horn of the spinal cord stained with anti-GFAP Ab. Many cells are labeled. Scale bar: 50 μm.

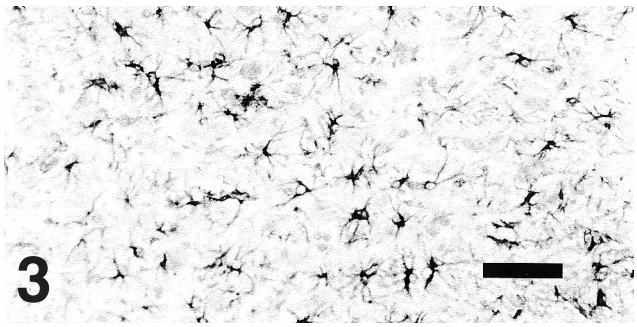

Fig. 3. A photomicrograph of the ipsi-lateral side (axotomized) of a newborn rat after one month of surgery, at the transitional region between the dorsal and ventral gray matters of the spinal cord stained with anti-GFAP Ab. Many cells are labeled. Scale bar: 25 μm.

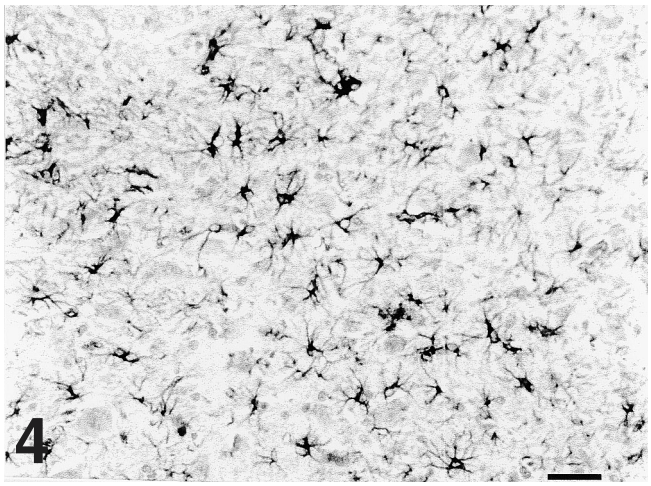

Fig. 4. A photomicrograph of the ipsi-lateral side (axotomized) of a newborn rat after one month of surgery at the dorsal horn of the spinal cord labeled with anti-GFAP Ab. Many cells are labeled. Scale bar: 25 μm.

glial cells (Fig. 6).

Morphometric results indicated that the number of glial cells in mm^2 (numerical density per area of glial cells: N(a)) in the ventral, TDVG and dorsal regions after one day of axotomy were not significantly different from that of the intact side. N(a) results at the intact side were significantly lower than those of the axotomized sides in all regions after one week, two weeks and one month of axotomy (see Table 1). The comparisons between the ventral horn and TDVG as well as the dorsal horn were significant in all groups except on day one post-axotomy.

Figure seven shows the percentage of increase in the number of glial cell at the axotomized side in relation to that of the intact side at each time point. There was an increase in the glial cells at the axotomized side (Fig. 8). The rate of glial cell increase at each time in the ventral horn was noticed at the first week (27.6) while those of TDVG and the dorsal horn occurred at the second week

Table 2. The total motoneuron count in the axotomized and intact sides (L4-L6 segments) in the ventral horn at all time points.

GROUP	TMC-IS*	TMC-AS
G1	2720±129	2182±174
G2	2630±167	1988±209
G3	2590±158	1771±172
G4	2403±128	1418±184

TMC-IS: Total motoneuron count at the intact side of the ventral horn. TMC-AS: Total motoneuron count at the axotomized side of the ventral horn. * : The comparison between the axotomized and intact sides in all groups were significant. G1, G2, G3 and G4 are one day, one week, two weeks and one month respectively.

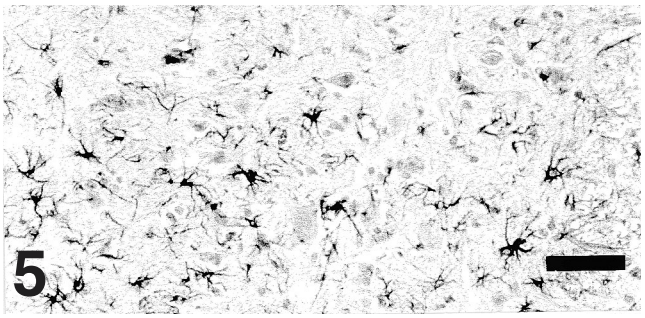

Fig. 5. A photomicrograph of the contra-lateral side (intact) of a newborn rat after one month of surgery at the ventral horn of the spinal cord stained with anti-GFAP Ab. There are Fewer cells labeled with anti-GFAP Ab than ipsi-lateral side. Scale bar: 25 μm.

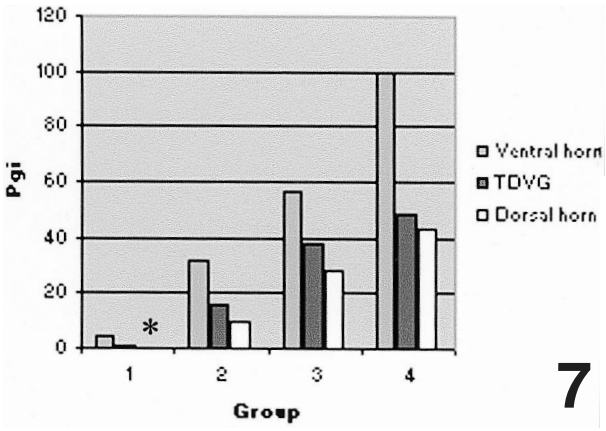

Fig. 7. A graph presents the percentage of increase in the glial cell number in the axotomized side in relation to the intact side at the ventral horn, the transitional region between dorsal and ventral gray matters(TDVG) and the dorsal root; in group 1 (one day), group 2 (one week), group 3 (two weeks) and group 4 (one month). The value in the column marked with an asterisk is meaningless because it is negative and must be ignored.

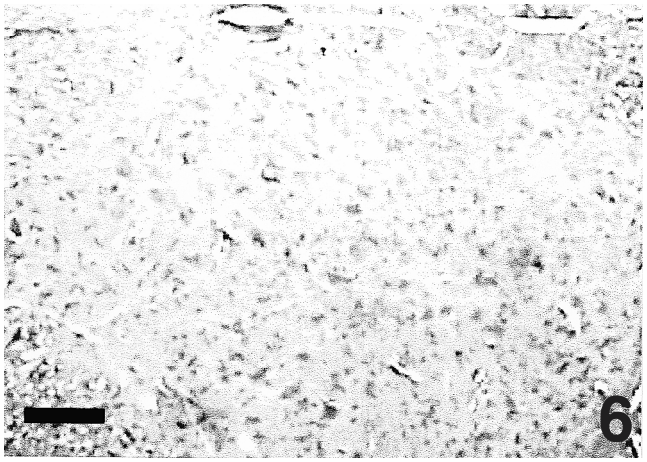

Fig. 6. A photomicrograph of the left side of a newborn rat from the normal control group stained with anti-GFAP Ab. Scale bar: 135 μm.

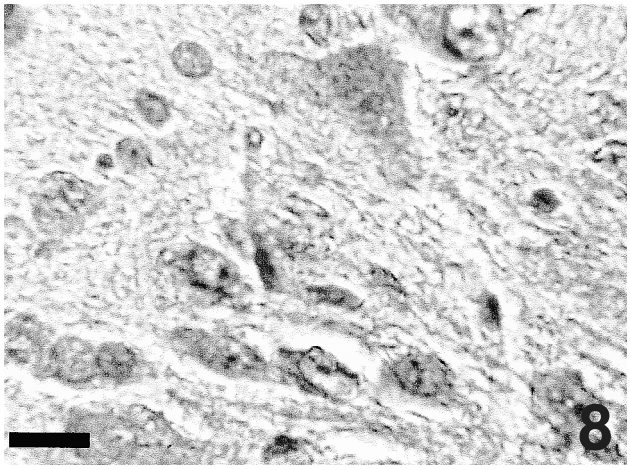

Fig. 8. A photomicrograph of the ipsi-lateral side (axotomized) of a newborn rat after one month of surgery stained with Hematoxylin-Eosin stain. Many glial cells can be seen in the ventral horn. Scale bar: 12.5 μm.

(22.3 and 19.25 respectively). Pgi in the dorsal horn at day one was negative and its value was ignored because the cells were stimulated for proliferation and the negative value represents a reduction in the number.

Figure nine shows the motoneorons were fewer at the axotomized side than that of the intact side (Fig. 10). The results of the total motoneuron count in the ventral horn (Table 2), and the percentage of motoneuron reduction are presented in Figure 11. There was a statistically significant difference between the axotomized and intact sides at all time points. The highest percentage of motoneuron reduction (Pmr) was noticed at one month (41%). The statistical comparison among the groups showed a significant decrease in the total motoneuron counts at all time points. A similar trend of increase was noticed in the percentage of GFAP immunoreactive cell to the total number of glial cells.

The data of Pgi was tested for linearity of regression which showed that the relationship between time and PSN was nonlinear ($p<0.05$). Nonlinear regression was carried out and an exponential model was fitted for Pmr against the time points which was ($y=20.4e^{a}$, $a=0.023x$) and for Pgi was ($y=21.02e^{a}$, $a=0.054x$). The constant number associated with x variable is a time constant (day is the unit of the constant) which indicates that the rate of increase in Pgi is approximately double that of Pmr.

Discussion

The immunoperoxidase results confirmed that there was astroglial activation following sciatic nerve axotomy, which is consistent with other investigators' findings (Gilmore et al., 1990).

The morphometric results showed that there was an increase in the numerical density of glial cells in the axotomized groups which indicated that there was a proliferative phase following sciatic nerve axotomy. This was also supported by the autoradiographic study on brain injury, which showed an increase in the labeling of titriated thymidine of brain trauma (Janeczko, 1989). The proliferation of glial cells following brain injury was found in the center of the lesion, which was labeled within 2 hrs. Also, Janeczko (1992), in another study, documented that the proliferation phase started 24 hrs after injury, then reached its peak on day 4 and declined after 8 days; 50% of the cells on day 4 were GFAP positive, which is consistent with this study, and suggests that there are two phases in glial cell response to axotomy; proliferation and differentiation. In the spinal cord, the proliferation of astrocyte precursor was reported in vitro and in vivo (Guenard et al., 1996; Tao

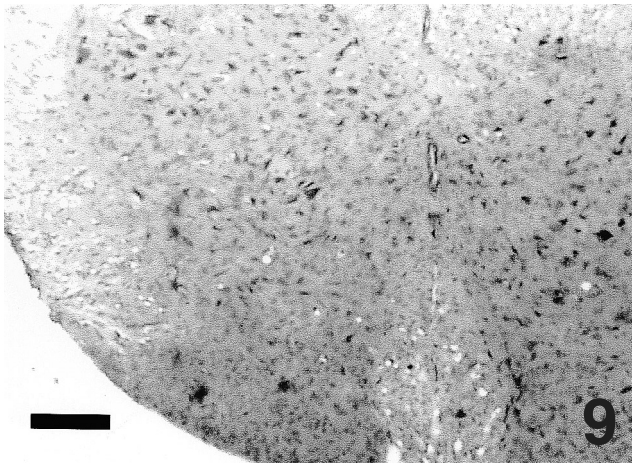

Fig. 9. A photomicrograph of the contra-lateral side (intact) of a newborn rat after one month of surgery stained with Cresyl violet stain. Many motoneurons can be seen in the ventral horn. Scale bar: 160 μm.

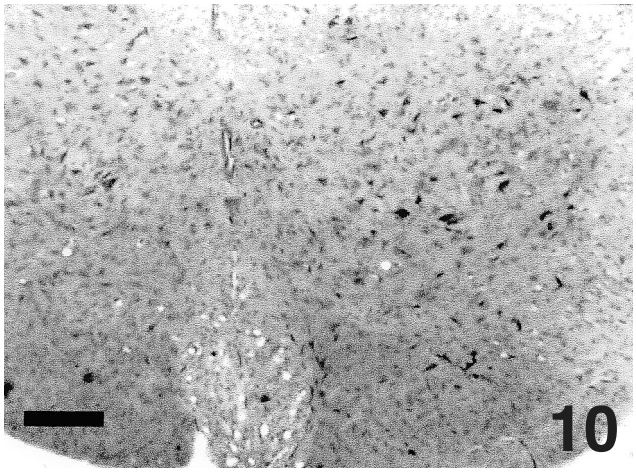

Fig. 10. photomicrograph of the ipsi-lateral side (axotomized) of a newborn rat after one month of surgery stained with Cresyl violet stain. Few motoneurons can be seen in the ventral horn. Scale bar: 160 μm.

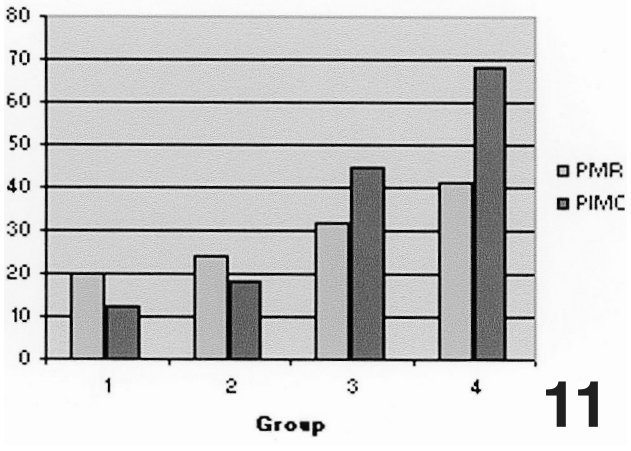

Fig. 11. A graph presents the percentage of motoneuron reduction (PMR) at all time points in axotomized and intact sides, and the percentage of GFAP immunoreactive cell to the total number of glial cells (PIMC); in group 1 (one day), group 2 (one week), group 3 (two weeks) and group 4 (one month).

and Aldskogius, 1999).

In this study, there was an increase in the percentage of glial cell at the ventral horn of the axotomized side. The highest increase was in the one month group where there was a two-fold increase in N(a) of glial cells as compared with the same group at the intact side. The highest rate of increase was noticed in the period between one day and one week (27.64%). This shows that at the first week there was a sustained increase in the numerical density of glial cells, which then declined. This confirmed the autoradiographic study of Janeczko (1989) who reported that the highest labeling was during the first week. Moreover, the morphometric results in this study show that the glial cell count continued to increase but at a lower rate. This is consistent with the percentage of immunoreactive glial cell, which was relatively low at 1 day and 1 week where glial cells underwent proliferation, and increased in the 2-week and one month groups where glial cells underwent differentiation. Ju et al. (1994) reported similar results in the axotomized facial nerve where the glial cell reaction was noticed as early as day 1 postaxotomy and reached the maximal levels within 7 days. This is different from the findings of this investigation which may be due to the age of the axotomized rats (14 days) or the site of axotomy (facial nerve).

The immunolabeling with anti-GFAP antibody was higher in the two-week and one month groups which agreed with other investigators' finding who used either immunohistochemistry (Gilmore et al., 1990) or GFAP gene expression (Svensson et al., 1993). In this investigation, the labeling was noticed at day one, which is consistent with other investigators' findings who reported the labeling of the astrocytes with GFAP at day one postaxotomy in the ventral horn of the spinal cord (Goldberg et al., 1987; Anders and Johnson, 1990). Graeber and Kreutzberg (1986) explained that the labeling of the astrocytes with GFAP happened as a result of the transformation of these cells into GFAP-producing type. Lee et al. (2000) indicated that there was a glial stem cell in the central nervous system. The glial progenitor cell was reported in the spinal cord by other investigators (Fok-Seang and Miller, 1992; Horner et al., 2000) and these findings support the hypothesis that the reactive astrocytes were either originated from mature cells or from precursor cells of astrocyte which were activated after axotomy then underwent two phases: the growth phase, where the number of cells increases; and the differentiation phase, where the cells start to express GFAP (Mikucki and Oblinger, 1991). The post-axotomic glial cell proliferation was confirmed by in vitro and in vivo techniques (Svensson et al., 1993; Guenard et al., 1996).

The results of the percentage of glial cell increase in TDVG and dorsal gray matter were not as high as that of the ventral region, which may be due to growth factor deprivation in the latter. On the other hand, there was no significant difference in Pgi between the dorsal region and TDVG, which may indicate the existence of similar spatial events in both regions. The temporal events at the axotomized side can be explained from the results of glial cell-rate increase. The total motoneuron count showed that there was an increase in Pmr in post-axotomized side where the highest reduction was at one month (41%). This agreed with the results of other investigators (Woolf, 1968; Schmalbruch, 1984). The result of the exponential model showed that the rate of neuronal loss was (b=0.023) while the rate of glial cell increase was (b=0.054) i.e. 0.023 and 0.054 fraction of a day in Pmr and Pgi respectively, which indicated that the rate of glial cell increase was approximately twice that of the rate of percentage of motoneuron reduction. This may be due to the difference between the size of the lost neurons and that of the replacing glial cells. Suzuki reported that a long-term amputation of the arm resulted in a reduction in the number of ventral horn neurons which is consistent with the finding of this investigation (Suzuki et al., 1993). Moreover, the glial cells were increased in the intact side in all regions including TDVG which may be due to reduction in interneurons (Suzuki et al., 1993), as a result of retrograde transneuronal degeneration which followed degeneration of the anterior horn cells (Suzuki et al., 1995). The result of glial cell activation in the newborn noticed in this study had similarity to that of the adult where no GFAP immunoreaction was noticed after 24 hrs following visual cortex lesion. Massive neuronal death occurred after 3 days while GFAP immunoreactivity distinctly increased at 3 days (Agarwala and Kalil, 1998). In spinal cord lesion, labeled reactive astrocytes were most evident 5 weeks after injury (McBride et al., 1988). The peripheral chronic phrenicotomy injuries in adult rats were reported to induce astrocyte reactivity peaking after 4 weeks. Comparison of animals that had been deafferented in the early neonatal period with those of adults indicated that the GFAP immunoreactive response persisted in the neonates (Gould et al., 1997).

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