Sodium Channels in Normal and Regenerated Feline Ventral Spinal Roots

Henry W. Querfurth, Regina Armstrong, and Robert M. Herndon

Center for Brain Research, University of Rochester School of Medicine and Dentistry, Rochester, New York

Regenerated and remyelinated nerve fibers have shorter internodes and thus more nodes than normal mature fibers. This requires either a decrease in the number of sodium channels per node or an increase in the number of channels per fiber or both. The purpose of this investigation was to determine what happens to sodium channel number, as estimated by ³H-saxitoxin (STX) binding, in regenerated fibers and to relate this to nodal number. Five adult cats underwent cryoaxotomy of ventral root levels L5, L6, L7, and S1 on the left side. After regeneration for 16–45 weeks, binding parameters were determined. On the right (control) side, binding was consistent with that in unoperated animals ($b = 1.3$, $B_{\max} = 10.2 \pm 0.4$ fmol/mg wet, $K_d = 0.6 \pm 0.1$ nM). However, the regenerated nerves showed a 3.5-fold increase in maximal binding ($b = 1.3$, $B_{\max} = 36.1 \pm 0.5$, $K_d = 0.45 \pm 0.4$).

Computer-aided histologic analysis of the regenerated roots revealed (1) a decrease in fiber size; (2) a significant decrease in internodal length for fibers in a given size class; and (3) a 1.35-fold increase in total fibers per root. These factors account for a 2.36-fold increase in nodes per milligram (wet). The number of STX binding sites per regenerated node was calculated to be 1.95×10^6 (1.31, 3.07, 95% confidence limits), whereas it was 1.26×10^6 (0.78, 2.02) for the control roots. The difference was not significant ($p > 0.05$).

It is concluded that, in regeneration, the increase in nodal number is accompanied by an increase in sodium channels, so that the number of channels per node is normal or slightly increased. There is a marked increase in channels per fiber and an even greater increase in channels per anterior horn cell. The implications of these data for nodal reorganization in remyelination are discussed.

Recent evidence suggests that ionic channel reorganization must play an important role in recovery of function following demyelination. Several observations indicate that remyelination and restoration of conduction velocity are not essential for restoration of function following demyelination in multiple sclerosis (Namerow and Thompson, 1969; Ghatak et al., 1974; McDonald, 1974a, b; Wisniewski et al., 1976; Herndon et al.,

1977; Ritchie and Rogart, 1977b, c; Prineas and Connell, 1979; Rasminsky, 1980). The importance of paranodal function for conduction and activity-dependent excitability in the course of experimental demyelination supports the view that changes in sodium channel numbers and distribution are critical to restoration of function (Morgan-Hughes, 1968; Smith and Hall, 1980, 1982; LaFontaine et al., 1982).

The concept of channel reorganization was developed on the basis of several electrophysiological reports (Huxley and Stampfli, 1949; McDonald, 1963; McDonald and Sears, 1970; Rasminsky and Sears, 1972; Chiu and Ritchie, 1982) and binding data using the specific sodium channel marker saxitoxin (³H-STX) (Ritchie and Rogart, 1977a; Waxman and Ritchie, 1981), which collectively indicate that there are insufficient sodium channels in the internodal regions of normal axons to support regenerative currents after the loss of myelin. This inference was strengthened by demonstrating the delayed appearance of excitability in demyelinated axolemma (Bostock and Sears, 1976; Foster et al., 1980).

The factors that control internodal length, nodal structure, and sodium channel number and distribution in regenerated or remyelinated fibers are poorly understood. When axons regenerate following axotomy (Sanders and Whitteridge, 1946; Culp and McKenzie, 1981) or when they remyelinate after primary demyelination (Allt, 1969, 1976; Gledhill et al., 1973; Prineas and Connell, 1979), the normal direct fiber size (D)–internodal distance (I) relationship is lost, and the newly formed internodal lengths are uniformly reduced in size with a reciprocal increase in the number of nodes (Hiscoe, 1947; Visoso and Young, 1948). Whether the total sodium channel number per fiber remains constant despite an increased number of nodes or whether the number of channels increases with the increase in nodes has been the subject of speculation. The carefully correlated biochemical and morphologic studies necessary to determine what changes occur and how they interrelate have not been reported.

The purposes of this investigation were (1) to reassess the number of sodium channels per node in mammalian peripheral nerve using ³H-STX binding under equilibrium conditions (Ritchie et al., 1976) and to compare this with data from gating current studies (Chiu, 1980); and (2) to determine what changes occur in sodium channel number as estimated by ³H-STX binding in regenerated ventral roots and correlate this with changes in nodal number to determine whether the number of channels per node or the number of channels per fiber remains constant in regenerated fibers or whether a more complex relationship exists. An accurate determination of the number of channels per node can be calculated only in a study in which both toxin binding and nodal histology are correlated.

Received Apr. 25, 1986; revised Oct. 17, 1986; accepted Dec. 23, 1986.

This work was supported by PHS Grant GM 07356 and NS 15283. We thank Ted Begenesich, Ph.D., Henry Davis, Ph.D., and Mr. Steven Fram for their help in the statistical and programming aspects of this work. We also express gratitude for the help of Mrs. Mary Capozzi and Mrs. Nancy Dintruff in the preparation of the manuscript.

Correspondence should be addressed to Henry W. Querfurth, M.D., Ph.D., Division of Neurology, Box RG-27, University of Washington, University Hospital, Seattle, WA 98195.

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Figure 1. *A*, High-power light micrograph ($\times 1200$) of a typical sample area within a control ventral root (cat 18 L5R, 20 weeks) that would be manually digitized to collect axonal, fiber, and extracellular space perimeters and areas. The 2 distinct fiber size populations are evident. Scale, 50 μm . *B*, High-power micrograph ($\times 1200$) of the contralateral ventral root (cat 18 L5L). The large fibers are somewhat reduced in size and have thinner myelin sheaths. The evidence for branching in this photo is manifest in several locations by the presence of myelinated satellite axons surrounding a larger axon and even contained within the same basal lamina (center, upper right, and lower right). The endoneurial space is increased. Scale, 50 μm .

Materials and Methods

The cat lumbosacral ventral roots L5, L6, L7, and S1 were chosen for the following characteristics: (1) scant epi- and perineurial sheathing to minimize trauma in preparation and facilitate equilibrium binding of ^3H -STX, (2) a relatively uncomplicated histology reflecting a relatively low degree of contamination by unmyelinated fibers, (3) little branching, and (4) favorable conditions for functional regeneration after interruption (Thulin and Carlsson, 1969; Gamble, 1976; Bray et al., 1981).

Surgery. Preliminary ^3H -STX binding data were obtained from the ventral and dorsal roots of 2 normal cats and morphometric data from another cat. Subsequently, 7 cats were premedicated with 0.1 mg/kg atropine, anesthetized with 25 mg/kg sodium pentobarbital, i.v., and intubated. Respiration was maintained on room air using a respirator. Temperature was held at 37°C by a heating pad coupled to a rectal

thermistor. Using sterile technique, a laminectomy was performed, the dura was opened, and the ventral roots identified. Succinylcholine was infused in a dose of 0.56 mg/kg, and a ventral root was lifted onto a copper probe placed 5–6 mm from the root-cord junction (Fig. 1) and frozen by applying a piece of dry ice to the shaft of the probe. After freezing for 10 sec, the root was allowed to thaw and the probe removed. Ventral roots L5, L6, L7, and S1 on the left side were cryoaxotomized in this manner. The area was packed with gel foam, and the muscle, fascia, and skin layers closed. Neurological examinations were then performed periodically in the operated cats until clinical improvement ceased. In both the binding experiments and histologic methods to follow, the right (unoperated) ventral root served as control for the left (axotomized) ventral root.

STX binding. Crystalline STX was tritiated by New England Nuclear and purified by the methods of Ritchie et al. (1976); purity was serially determined during the period of the experiments by the method de-

scribed by Levinson (1975). A radio purity of 0.63 ± 0.04 with a half-life of 3.5 months and a true specific activity of 15.2×10^{15} dpm/mol were obtained for the single batch used on the operated animals.

Normal cats and operated cats that had reached maximal recovery were anesthetized and perfused with saline for binding and morphometric studies. The lumbosacral cord was extracted and kept in rabbit Locke solution at 3°C. The intradural roots L5–S1 were identified and removed and their length–weight relationships recorded before teasing sample fascicles for nodal quantitation and ^3H -STX binding. For each concentration of STX from 0.25 to 25 nM, several fascicles weighing 10–25 mg were randomly selected from a pool of mixed fascicles teased from the nerve roots. They were then incubated in glass vials containing ^3H -STX in 1.8 ml rabbit Locke. Radioactive ^{14}C -mannitol was added as an extracellular space marker. STX binding is determined both in the presence and absence of TTX; the TTX completely inhibits the saturable uptake of tritiated STX; thus the difference between the 2 values gives the saturable uptake. Accordingly, 10 μM TTX was added to another identically prepared sample. The samples were incubated in an ice bath with agitation for 5–8 hr, after which binding was terminated by blotting and the tissue weighed. The sample was then solubilized in 0.6 ml Protosol (New England Nuclear) at 55–60°C and counted in 10 ml of toluene–POPOP adjusted to pH 7 in a Searle Tricarb scintillation counter. Aliquots from the remaining solution were counted to determine the final STX concentration. Graphs were constructed by pooling data from the individual binding experiments.

Quantitative ventral root histology. A sampling procedure was developed to estimate for each ventral root from all cats (1) the total myelinated fiber count, (2) the axonal perimeters and their distribution, (3) the myelin thickness and its relation to fiber diameter, and (4) the percentage of total root cross-sectional area occupied by endoneurial space. Because of the large number of fibers, a fairly accurate, semiautomated quantitative system was used to assess these parameters.

A short segment of ventral root was obtained near the dura along each root L5, L6, L7, and S1. These were immersion-fixed in 1% paraformaldehyde, 2% glutaraldehyde, 1% acrolein, and were then postfixed in 2% osmium tetroxide, embedded in Poly 812, sectioned, and stained with a modified Stevenel's stain. A low-power micrograph was taken of the entire section and of a calibration slide at the same magnification to determine cross-sectional area. High-power micrographs (1000–1350 $\times$) were made of a standardized grid sample area based on a previous pilot study (comprising approximately 13% of total root area) and calibrated. The perimeter of the entire root and then of the individual fibers (both internal and external) in each sample area were manually traced on a digitizing tablet interfaced to an Apple II+ computer. The algorithm employed calculates areas and perimeters based on the trapezoidal rule and the Pythagorean theorem, respectively, and is similar to that reported by Cowan and Wann (1973). The resulting array of fiber and axonal perimeters for each profile encountered in the sample area was then grouped according to size into 1 μm bins. A total fiber count based on the fiber density within the sample area(s) and the total cross-sectional area were estimated for the root in question.

An additional animal (the seventh) was used to examine the entire root to control for changes in the total number of fibers (and branches) at various points along the intradural course of the root. This animal (C9) was perfused 42 weeks after surgery, and the axotomized unilateral L6, L7, and S1 ventral roots were fixed, embedded, and sectioned every 3 mm from the cord exit to the dural junction for serial estimates of total fiber count.

The spinal cord from animal C9 was removed *en bloc*, fixed, and embedded in paraffin. Ten micron sections were obtained and stained for Nissl with thionin–acetate. Anterior horn cells were counted in every tenth section from the S1 and L6 level under low-power light microscopy (100 $\times$). Only cells with clearly defined processes located ventral to the canal with a central nucleus and a uniform dense nucleolus were enumerated. An estimate of anterior horn cell loss from a proximal cryoaxotomy was then made. The resulting root-fiber per motor neuron ratio would be of interest in the final tally of anterior horn cell sodium channel production due to peripheral injury.

Internodal length measurements. Fascicles not used in binding experiments were fixed with 0.4% formaldehyde in 0.1 *N* phosphate buffer and were then stained with 0.5% osmium tetroxide in 0.1 *N* phosphate at pH 7.2 and stored in diluted glycerol. Single fibers or bundles of 2–3 fibers containing up to 10 internodes were teased from the fascicles. Three diameter measurements were made for each internode and averaged to yield a representative diameter. The internodal lengths from

a given cat were all pooled according to fiber diameter in 1 μm bins and the mean and SD plotted for each class. No distinction was made between them as to spinal level.

Statistical methods. The binding curves were fitted to the equation (Colquhoun et al., 1972)

$$B_{\text{tot}} = b [\text{STX}]_f + B_{\text{max}} [\text{STX}]_f / [\text{STX}]_f + K_d$$

The slope parameter b was obtained by a linear-regression fit to the binding data obtained in the presence of 10 μM TTX. The parameters B_{max} and K_d were obtained using a Marquart nonlinear least-squares algorithm (Bevington, 1969). The fitted binding data incorporated the pooled, single-observation results from several cats with no distinction as to level of origin. A variance estimate for each of the binding points was not feasible. An estimated SD of the fitted parameter B_{max} , as well as a calculated SD for those points above 4 nM STX, was available from which to calculate confidence limits (CL). The more conservative value was always employed. The number of binding sites per node (X) was derived from the ratio of the STX receptor number per milligram of ventral root ($C = B_{\text{max}} A_v$) to the total nodal number per milligram of ventral root [N].

The representative number of nodes [N] per milligram of operated and of control ventral root was derived using the individual nodal numbers (n_i) calculated for each of the 4 matched roots obtained from the 5 operated cats with a correction for interroot variability. For this purpose, multiple linear regression was performed on the log-transformed values for $n_{i,j,k}$ [i = levels L5, L6, L7, S1; j = cats 5, 7, 16, 17, 18; k = 1 (regenerated), 0 (control)] to arrive at the coefficients and associated SE that relate the additive effects of each of the isolated variables identified (i, j, k, ik_i) to the predicted value [N]. In addition, a factorial analysis of variance tested for significance in the contribution that the various isolated effects made to the total observed variance. These 2 sets of computations were performed on a DEC-10 computer utilizing the Minitab statistical package (Minitab Project, Pennsylvania State University). Ninety-five percent confidence limits for [N] were calculated from the SE of the coefficients. Finally, Student's t test was used to test the hypothesis that there were no differences between the number of channels per node in normal and regenerated ventral roots.

Results

Clinical examination

All 6 cats (5, 7, 10, 16, 17, 18) displayed a flaccid left leg suspended in a flexed position at the hip in the immediate postoperative period. This included the denervation of all major muscle groups in the distribution of the sciatic, tibialis, and common peroneal nerves (Reinhard and Jennings, 1961). The femoral nerve (L5) was moderately involved, and the gluteal nerve appeared to be least affected, with early return in the ability to circumduct the leg. During the next 4 months, 5 animals regained the customary elevated heel posture for locomotion; this indicated reinnervation of the gastrocnemius muscles, which receive supply from all the levels axotomized (Romanes, 1951). One animal (16) recovered completely by 16 weeks and was sacrificed at 16½ weeks, at which time the left gastrocnemius muscle weighed 32.2 gm, which was 92% of the control side. Cat 10 recovered poorly and developed complications and was excluded from the study. The remaining cats had some evidence of residual abnormality on examination at the time of sacrifice (19, 20, 45, 45 weeks postop).

Histology

An example of the cross-sectional histologic appearance from one pair of ventral roots is shown in Figure 1*A* (control) and 1*B* (regenerated), from cat 18 (20 weeks) at the L5 level. The normal ventral root histology with a homogeneous fiber size distribution (Bergman and Afifi, 1974) is exemplified by Figure 1*A*. The percentage of unmyelinated fibers in the ventral root has been estimated from electron micrographs to be 29% of the total. They are very small (0.5 μm) and originate in the dorsal root ganglion (Coggeshall et al., 1974). Their percentage in re-

Figure 2. *A*, Computed fiber-size histogram for S1 ventral root digitized from a sample light-micrograph area such as in Fig. 1 (cat 17 S1R at 19 weeks). Axonal perimeter (μm) is given along abscissa. The equivalent fiber diameter is the perimeter divided by (πg) , where $g = d/D = 0.63$. P1 and P2 correspond to the bimodal population classes, as shown with diameters of 7–8 μm and 20 μm , respectively. *B*, Histogram for contralateral axotomized root (S1L). The spectrum is shifted toward smaller perimeters with a predominant “P1” pattern (diameter, 4 μm), although a less populated class of fibers is appreciable at an intermediate size range (“P2”; 14 μm diameter).

generated ventral roots is not known but is assumed to be unchanged. A correction for their presence was made. Even a substantial change in unmyelinated fibers would have only a minor effect in results.

Figure 1*B* is a cross section from the contralateral regenerated ventral root. The fibers fall into a smaller, but more dispersed size class. Regeneration and branching are demonstrated, respectively, by noticeably thinner myelin sheaths, and occasional groupings of several small profiles enclosed within 1 sheath, a so-called band of Bungner (Guth, 1956; Allt, 1976).

The fiber-size histogram in Figure 2 is representative of the root pairs analyzed in this series. The control root axonal sizes have a bimodal distribution that is characteristic of motor nerves (Shawe, 1955; Sjöstrand et al., 1969). The regenerated fibers are predominantly grouped into a small-caliber class but also reveal a discernible second population of intermediate-sized fibers, consistent with restoration of bimodality (Sjöstrand et al., 1969; Thulin and Carlsson, 1969). The histologic parameters obtained for all 4 root pairs of 1 cat (17) are summarized in Table 1. The mean value of $g = 0.63$ (axon/fiber diameter) in the far-right

column did not differ between the control and regenerated populations, and this is taken as evidence for mature remyelination.

In Table 1, the cross-sectional area of the regenerated root proper has diminished in most instances by around 36%. The length/weight ratio also decreased but by a smaller amount. The percentage of extracellular (or endoneurial) space in all regenerated roots had increased by approximately 20–30%; however, only a slight increase in extracellular fluid per milligram wet weight was obtained using the extracellular space marker. Similar absolute endoneurial compartment size and greater fascicle density from connective tissue proliferation in the regenerated roots account for the apparent discrepancy better than differences in blotting or marker penetration.

The estimated total numbers of fibers in each ventral root from representative cat 17 are also listed in Table 1. For control roots, the numbers ranged from 3300 to 5600, comparable with values from other studies of feline ventral roots (Sjöstrand et al., 1969; Berthold, 1978). In the regenerated roots, the total fiber counts increased from 10 to 70% (average, 1.45-fold). Acknowledging an unknown percentage of anterior horn cell drop-

Table 1. Summary of the parameters obtained from the morphology gathering program for 1 operated cat (17)

Level	Nerve Area (mm ²)	Area sampled (%)	<u>Length (mm)</u>	ECS (%)	Est. total fibers	In-crease (%)	<u>P1</u>		<u>P2</u>		Int./ext. diam-eter, $g = d/0$
			Weight (mg)				MAP (μ)	%	MAP (μ)	%	
L5R	0.75	13.0	1.25	30	3316	—	12.2	55	45.9	45	0.64
L5L ^a	0.47	13.1	1.22	52	5406	60	9.1	83	34.3	17	0.63
L6R	1.09	9.3	0.75	35	4370		11.2	52	40.4	48	0.59
L6L ^a	0.60	10.0	0.69	57	6126	40	7.7	50	22.8	50	0.60
L7R	1.53	10.1	0.63	36	5672		12.5	47	43.7	53	0.63
L7L ^a	1.05	7.1	0.45	76	6499	10	10.3	76	27.3	24	0.65
S1R	0.99	8.1	0.72	31	4569		14.4	42	42.8	58	0.69
S1L ^a	1.35	7.5	0.65	80	7742	70	7.9	76	27.2	24	0.61

All spinal levels are included. From left to right, total root area is given in mm² and is usually less in the axotomized roots. The area sampled refers to the percentage of total root area that was bound by the sampling strip (see Materials and Methods). The length/weight ratio (r) of wet whole ventral root was decreased for the axotomized nerves. ECS refers to the percentage of endoneurial space. Branching after regeneration is reflected by the 10–70% increase in fibers per root. The mean computed axonal perimeters (MAP) for P1 and P2 (the small and large components of the bimodal size distribution, respectively) and their percentage contributions to the total fiber pool are also tabulated.

^a Regenerated.

Figure 3. *A*, Computed graphic relationship between axonal size and myelin thickness for control ventral root at 19 weeks (S1R; cat 17). A discontinuous jump at 30 μ m perimeter is apparent, corresponding to the transition between P1 and P2 (see Fig. 7*A*). *B*, Contralateral regenerated ventral root (S1L) maintains a fairly constant myelin thickness over all size classes. Means ± 1 SD are shown.

out, this clearly represents branching of the remaining fibers. Distal grafts have been reported to have 1.42 times the number of myelinated fibers in the central stump (Sunderland and Lavarack, 1953). A detailed serial morphologic examination in another operated animal (C9) provided additional evidence justifying the use of cross section for total fiber counts obtained from the far distal portion of the root as representative of the whole root distal to the lesion site. Including all level pairs and

cats ($N = 19$), the operated roots have 1.35 ± 0.52 times the number of myelinated fibers as do control ventral root.

Anterior horn cell counts were made from serial sections at 100 μ m intervals at 2 spinal levels from cat C9. A total of 1181 cells was identified. The largest neurons (40–60 μ m), which reside in the large lateral nuclei, were noticeably reduced in number by the axotomy on that side. These cells form the bulk of lumbosacral motor efferents (Romanes, 1951). Neurons found

Figure 4. *A*, Internodal length–fiber diameter relationship for the S1R and L7R control side lumbosacral motor roots from cat 5 at 45 weeks. Note that internodal length is in millimeters, whereas fiber diameter is in microns. The data represents the mean of all pooled single internodes within each 2 μ m bin size class ± 1 SD. The least-squares fit is drawn and the resulting relationship given along the lower-right margin. A total of 125 internodes were enumerated. *B*, Contralateral L5L, L6L, and S1L axotomized fibers from cat 5. A total of 231 internodes were entered. Note the uniform nodal spacing across size classes $> 8 \mu$ m.

Table 2. Summary of nodal calculations given as number per mg nerve (*r*) for all ventral roots (top) and all cats (left) entered in the study

Cat	Control				Operated (left hemilaminectomy)			
	L5	L6	L7	S1	L5	L6	L7	S1
6								
Right	4628	4809	3845	11,193				
Left	3574	5478	4113	8739				
17	4870	3957	4002	3510	18,111	10,953	7910	13,614
16		4326	5399	6114		11,252	10,550	11,273
5			5355	8114			6668	36,923
7	3598	3309			12,896	3987		
18	4414	4584			13,089	8952		
Mean	$\bar{n} = 5139 \pm 1973$				$\bar{n} = 12,783 \pm 8052 (2.5 \times)$			

A 2.5-fold increase in mean, uncorrected nodal number ($\bar{n}$) is calculated for the axotomized roots. The formula used for calculation of nodal number is given below. Multiple linear regression and analysis of variance was performed on the data to derive the coefficients for the expected nodal number [*N*] and to determine the relative contribution of several variables to the total observed variance, respectively. Variance effects between right and left in the control animal, between the operated cats, and between spinal level $\times$ axotomy were not significant. The effect of axotomy and subsequent regeneration was the most significant contributor to the total variance ($p < 0.0001$). The S1 level was responsible for a significant degree of between-levels variance ($p < 0.05$).

Formula for calculating nodal number:

$$n_{i,j,k} = r \int_{D=0}^{\infty} mI^{-1} dD \approx r(m_1/I_1 + m_2/I_2)$$

where *r* is mm/mg (wet weight), *D* is equivalent fiber diameter (= axon perimeter/ πg , where *g* is int/ext), *I* is internodal length [= *f*(*D*)], *m* is the estimated contribution to total fiber number in root made within a given fiber size increment.

in the medial motor nuclei were smaller in size (10–30 μ m) and relatively less affected in number by the unilateral surgery. Some of these may even belong to a class of spinothalamic neurons (Willis and Coggeshall, 1978), which may partially explain their immunity from the postchromatolytic necrosis undoubtedly experienced by the larger nuclear group. They were included in the counts nevertheless. The hemiventral horn whose axons were interrupted suffered a 35% (S1) to 36% (L6) loss in motor neuron number.

In Figure 3, the relationship between myelin thickness and axonal size is presented for control and regenerated ventral roots from the same cat. P1 has myelin that is about 1.5 μ m thick, whereas P2 myelin is 2.5–3 μ m thick. A minority of regenerated fibers, >30 μ m in size, had relatively thinner myelin sheaths. Single fibers were teased from all 4 levels (right and left) of each cat and assessed for internodal lengths and mean fiber diameter. The results are plotted for cat 5 (45 weeks) in Figures 4*A* (control) and 4*B* (regenerated). The line drawn through the control points is a linear least-squares fit; however, a curvilinear relationship with an asymptote at large fiber diameters might describe the data as well. The literature supports both interpretations (Hursh, 1939; Schnepf and Schnepf, 1971; Fried et al., 1982). The regenerated fibers had, as anticipated from the introduction, diminished but constant internodal lengths, especially for fibers greater than 8 μ m. This is taken as evidence for the completeness of the lesion and the universality of the regenerative process (Hiscoe, 1947; Vizoso and Young, 1948).

The total number of nodes of Ranvier per milligram of each individual ventral root can be calculated (Table 2) from the length/weight ratios, total number, and size distribution of myelinated fibers within each ventral root and their respective internodal lengths. The calculated mean number ($\bar{n}$) of such nodes for control and operated roots indicated at Table 2 (bottom) is, however, skewed. The analysis of variance demonstrated that the S1 level was responsible for the large variance ($p < 0.05$).

Perhaps its smaller diameter fibers conferred upon it smaller nodal spacing and hence more nodes. The expected value derived from the regression analysis for the pooled nodal number incorporated a correction factor for the S1 effect based on its net weight contribution. The final result [*N*] was that for control and normal ventral roots there are expected to be 4629 (3435, 6475:95% CL) nodes/mg and for the axotomized group a total of 10,963 (7935, 16,056:95% CL) nodes/mg. This represents a 2.36-fold increase over the untreated roots and is highly significant.

³H-STX binding

The ³H-STX binding results in the control ventral roots from all 5 operated animals are given in Figure 5. The fitted results are $B_{\max} = 10.2 \pm 0.4$ fmol/mg wet, $K_d = 0.55 \pm 0.12$ nM⁻¹, *b* (linear slope) = 1.34 fmol/mg · nM. The SD of the fit was 3.67 fmol/mg and the coefficient of determination, which reflects the “goodness” of fit, was 0.92 (Nettler and Wasserman, 1974). The fitted coefficient for $B_{\max}$ from the specific binding data was 10.8 fmol/mg. The maximal binding results are in agreement with those from 2 normal unoperated cats (11.6 ± 1.4 fmol/mg) (Fig. 6*A, B*). The binding to dorsal roots was higher at 38.4 fmol/mg (unpublished observation).

The data for the contralateral regenerated ventral roots are plotted in Figure 7*A*. The fitting parameters are now $B_{\max} = 36.1 \pm 0.46$, $K_d = 0.45 \pm 0.4$, *b* = 1.34. The fit SD and coefficient are, respectively, 7.99 fmol/mg and 0.88. The scatter in the data reflects the inhomogeneities in (1) the length/weight relationship as a result of fibrosis, (2) toxin equilibration, (3) differential response among root levels, or (4) binding to a variable population of non-neuronal elements. It reflects a clearly significant ($p < 0.01$) 3.54-fold increase in STX receptor number per milligram of mature regenerated ventral root over the contralateral control group. The maximal binding observed in the operated ventral roots was unaffected by the duration of regeneration

Figure 5. A, ^3H -STX uptake data to the control ventral roots (L5-S1) from all 5 operated animals expressed as 10^{-15} mol/mg wet weight of mixed ventral root fascicle (ordinate). The total binding (upper curve) and nonspecific binding (lower line) in the presence of 1000-fold excess TTX ($10 \mu\text{M}$) are shown as a function of the final STX concentration (abscissa). The total binding data was fitted (see text) to the form $B_{\text{tot}} = b[\text{H-STX}] + B_{\text{max}}[\text{H-STX}]/[\text{H-STX}] + K_d$, where b is the slope of the nonspecific binding data calculated by linear least-squares method. Each point represents 1 experiment. The fitted parameters are given.

between 16 and 45 weeks. The saturable binding for both the regenerated and control plus normal ventral roots is plotted together in Figure 7B for comparison. The affinity constant for ^3H -STX has not changed in the newly formed receptors.

To calculate the number of channels per node of Ranvier, it was necessary to multiply the fitting parameter B_{max} (fmol/mg)

by Avogadro's number (N_A), after the small correction for unmyelinated fiber binding. This numerator (C) divided by the expected nodal number per milligram [N] yields the final answer (X) and 95% confidence limits as follows:

$$\text{Control: } (10.2 \pm 0.5 \text{ fmol/mg} \times N_A)/4629 \text{ nodes/mg} = 1.26 \times 10^6 \text{ STX receptors/node } [0.79, 2.02]$$

$$\text{Regenerated: } (36.1 \pm 0.5 \text{ fmol/mg} \times N_A)/10,963 \text{ nodes/mg} = 1.95 \times 10^6 \text{ STX receptors/node } [1.31, 3.07]$$

The control binding is very similar to the value obtained from other normal cats (1.22×10^6) (unpublished observation). There appear to be 1.54 times as many STX receptors in the newly formed nodes.

It will be noted that the confidence intervals overlap. Two-tailed hypothesis testing concluded that the difference in STX receptors per node observed between control and regenerated ventral roots did not reach significance ($p > 0.10$).

Discussion

The various sodium channel and STX binding parameters reported for normal unmyelinated and myelinated nerve fibers using several different methods are summarized in Table 3. Sodium channel density estimates as given by ^3H -STX binding and electrophysiological methods are in excellent agreement for unmyelinated squid axons. Even in frog node, ^3H -STX receptor number is well within an order of magnitude range of channel estimates. For mammalian fibers of the same species, however, there is a trend toward higher ^3H -STX binding per node than the analyses of sodium channel currents would predict. Our estimate of 1.22×10^6 STX binding sites per node for normal cat ventral root is 1.7-fold greater than that reported for rabbit

Figure 6. ^3H -STX binding to unoperated feline L7 ventral root segments. Four L7 ventral roots from 2 cats C6, C7 were divided into equal segments of 5 mg each and treated as in Figure 5 except at concentrations below saturation. A, Specific binding data with fitted curve and parameters. B, Scatchard plot of same data fitted by linear least-squares method. A higher K_d value is noted for pure L7 fascicles compared with mixed control fascicles from a larger series of operated cats. The small difference in affinity, due possibly to differences in toxin batch, tissue source, and handling, did not affect B_{max} .

Table 3. Summary table of sodium channel estimates by the various methods

Model	³ H-STX equilibrium binding			$\bar{Q}_{\max}/N$ gating currents			Current variance Channels/node
	Sites/node	Sites/ μm^2	K_d	Channels/ node	Channels/ μm^2	$\bar{G}_{\text{Na}}$	
Rabbit vagus		110	1.8 ^a				
<i>Loligo forbesi</i>		553 ± 120 ^b 522–324 ^c			483 200–300 ^f	1.2 nS/ μm^2 ^{d,k}	
<i>pealei</i>		166 ± 20	4.3 ^d				
Frog node	132,000	2000 ^{c,e}	5.3 ^{f,g}	246,000	5212 ^s	15–20 nS/ μm^2 ^m (0.57–0.97 μS)	34,400
Cat node	1.22 × 10 ⁶	18.5 × 10 ³	1.6 ^h			0.36 μS ⁿ	
Rabbit node	720,000	10–12 × 10 ^{3e}	3.3 ^{i,j}	82,000		0.22–0.76 μS ^{n,o}	
Rat node							18,700 ± 800
Remeylinated rabbit sciatic node	(2.3 × increase in binding/unit length; 1.3 × increase in binding/unit weight)						

The various preparations (models) employed are listed at left. The values obtained for a particular method are subdivided into relevant parameters. $\bar{G}_{\text{Na}}$ is maximal nodal sodium conductance. γ is the single-channel conductance.

- ^a Ritchie et al. (1976).

^b Levinson (1975).

^c Keynes et al. (1975): from kinetics.

^d Strichartz et al. (1979).

^e Nodal area taken to be 66 μm^2 .

^f Ritchie (1978).

^g Ritchie (1979).

^h This study.

ⁱ Ritchie and Rogart (1977a).

^j Ritchie et al. (1981). Conditions: lysolecithinized, homogenized, 5 nM STX, 2 months recovery.
- ^k Rojas and Keynes (1975).

^l Armstrong (1981).

^m Nonner et al. (1975).

ⁿ Chiu et al. (1979).

^o Chiu (1980).

^p Sigworth (1980).

^q Neumcke and Stampfl (1982).

^r Conti et al. (1975).

^s Nodal area, 50 μm^2 .

^t Conti et al. (1976).

Figure 7. A, ³H-STX uptake by the contralateral regenerated ventral lumbosacral roots to those shown in Figure 5. Same 5 cats (16–45 weeks). Each point represents 1 experiment. Note difference in ordinate scale. The maximal binding from the fit is 36.1 (3.5 × greater than control); however, the affinity (K_d) is not appreciably different. Experiment performed exactly as for Figure 5 with same toxin batch and at 2–3°C. B, Fitted specific binding for axotomized (upper) and contralateral (lower) control ventral roots plotted together for comparison. The maximal binding ratio is given along at right. The extra points included with the lower curve (controls) are data taken from a series of unoperated cats.

Table 3. Extended

Current variance (cont.)		S_{Na} (F) noise			
γ	$\bar{G}_{Na}$	Channels/ node	Channels/ μm^2	γ	$\bar{G}_{Na}$
			330		
6.4 pS	0.22 μS^2	100,000	2000 ^c	6.8 pS	0.683 μS^2
14.5 pS ^a					

sciatic nerve using the same technique (Ritchie and Rogart, 1977a; and see Table 3); however, confidence limits were not given for the latter. Although our value of B_{max} is only 50% of that reported by Ritchie and Rogart (1977a) and Ritchie (1978), the larger receptor/node ratio results from a relatively smaller nodal number per milligram of feline nerve, which is only 4985 as compared with 14,200 in the rabbit sciatic nerve. Interspecies differences and differences in myelinated fiber size distributions between ventral root and sciatic nerve and, therefore, nodal spacing probably account for this observation (Yates et al., 1976). In addition, larger-diameter fibers, as those in the cat, may have a larger nodal area and more channels per average node than in rabbit. A tendency for larger-diameter fibers to have a higher peak I_{Na} and maximal conductance has been noted (Table 3 in Nonner et al., 1975; Chiu, 1980; Table 1 in Sigworth, 1980). Furthermore, it is not likely that our relatively high receptor/node estimate is attributable to an inflated B_{max} because a value of 37.1 ± 4.3 fmol/mg has been reported for cat sciatic nerve (Waxman and Ritchie, 1981). The latter B_{max} is understandably large, as sensory fibers probably account for a greater proportion of binding in mixed nerve than do motor fibers (see STX binding results).

The large number per feline node that we report, nonetheless, remains plausible after consideration of steric restraints. Dividing our receptor number by nodal areas of 36 and 107 μm^2 , corresponding to the bimodal fiber classes, a receptor density of 18,500/ μm^2 is obtained. If the diameter of a sodium channel is approximated to that of the isolated TTX binding polypeptide, 5 μm (Miller et al., 1982), we calculate a 36% nodal areal occupancy by STX receptors. A 60% occupancy was estimated by Ritchie and Rogart (1977a). The use of Berthold's (1968) values for nodal areas corresponding to P1 (4 μm^2) and P2 (20 μm^2), in accordance with a 50% axonal constriction of the node, would increase both reported densities an additional 5-fold.

This study confirms the trend of higher receptor than channel density reported in mammals and again raises the issue of whether all the observed STX binding is indeed to nodal sodium channels or if the additional difference is accountable by some other phenomenon.

The possible absence of a 1:1 STX binding site and sodium

channel stoichiometry has been reviewed by Catterall et al. (1980) and has been offered as a possible explanation. Glial binding of STX may account for a portion of the discrepancy as demonstrated by TTX sensitive depolarization and ^{22}Na influx (Villegas et al., 1976; Lazdunski et al., 1980) and residual STX binding months after proximal nerve ligation (Ritchie et al., 1981; Ritchie and Rang, 1983). Such binding may have both specific and nonspecific components and if present to a very large extent, especially in regenerated nerves, could pose significant limitations on the interpretation of our results. Shrager et al. (1985) have suggested that Schwann cells may be involved in the manufacture of sodium channels for insertion into the axolemma based on their findings of voltage-sensitive currents and saturable 3H -STX uptake in cultured cells. They calculate 25,000 STX sites (but only 920 channels) per rabbit Schwann cell. At a ratio of one such cell per node, the glial contribution to the observed binding for normal rabbit nerve would amount to only 3.5%. This alone would not account for the discrepancy between receptor and channel estimates in mammalian nerves. Unless it can be shown that feline Schwann cells bind 10-fold more STX than do rabbit Schwann cells, there are no compelling reasons to suspect a significant glial contribution to the total binding in normal cat.

In our regenerated nerves, there is a 2.36-fold increase in nodes and at least this degree of Schwann cell proliferation as well. The total binding contribution made by the latter could therefore be slightly greater. It would be substantial if regenerated Schwann cells possessed more receptors than normal glial cells. STX binding on the order of 32 fmol/mg wet weight of *in vitro* cultured Schwann cells (Shrager et al., 1985) and 40 fmol/mg wet weight of *in vivo* preparations of Schwann cells in degenerated distal stumps (Ritchie and Rang, 1983) may seem large, but these preparations lack viable axons and myelin. The contribution by glial soma and paranodal processes to the wet weight and receptor number in our roots is not known; however, we feel that conclusions can be drawn even when STX binding customarily assigned to nodal axolemma excludes a correction for an unknown, but probably less significant, Schwann cell factor.

Ritchie et al. (1981) studied changes in sodium channel number in peripheral nerve that had undergone remyelination following lysolecithin demyelination. By 50 d after injection in the rat sciatic nerve, the authors noted that at least 70% of the fibers were thinly myelinated and that a 25 mm length of homogenized nerve including the lesion site bound 1.3 times as much 3H -STX/mg or 2.3 times/mm when compared with control (Table 1). By 200 d, the corresponding figures were 1.9 and 2.7 times normal. These increases are similar to the increase reported here. Although quantitative morphology and adjustment for binding to sensory fibers was not performed, Ritchie et al. offered the interpretation that the number of channels per node remains constant in remyelinated nerve.

Our results based on axotomized nerve primarily demonstrate that, despite a substantial increase in the number of nodes in regenerated axons, the number of STX receptors associated per node does not decrease and may even increase slightly. There is a roughly 6.2-fold increase in receptors per neuron. This result is the product of a 1.54-fold increase in receptors per node, an average 1.94-fold increase in nodes per fiber, and a 1.35-fold increase in fibers (branches of surviving axons) accompanied by a 35% reduction in anterior horn cells. This is expected to put an increased metabolic load on the regenerated neuron that

would include the supply of additional membrane channels and other structural components (Griffin et al., 1981; Parhad et al., 1982). Since the amount of sodium influx per impulse is almost directly proportional to the number of sodium channels, there would be an additional increased demand on the sodium–potassium pump that must maintain the ionic gradients necessary for conduction. This in turn produces an increase in demand for oxidative metabolism and, presumably, a requirement for more mitochondria. The smaller axoplasmic volume combined with the increase in sodium flux might also result in more rapid accumulation of intracellular sodium and loss of potassium. Thus, the energy reserve for conduction (in the form of the ionic gradient) of the regenerated fibers could be substantially reduced under special conditions, such as sustained high-frequency impulse trains. Nevertheless, reorganization in response to injury may serve to reduce impedance mismatch and thus increase the safety factor for transmission at the junction of myelinated and demyelinated axonal segments (Waxman and Brill, 1978).

The current study has significant implications for the mechanism of channel reorganization in demyelination and remyelination. Our results strongly suggest that redistribution of existing channels is not the sole manner by which new nodes become excitable. They are more consistent with the synthesis of new channels and their incorporation into the axolemma proportional to the rise in nodal number. This adaptation occurs over a fairly long period and may not be applicable in the acute setting. Computer simulations of conduction suggest that only 4% of the nodal density of channels needs to be present in a demyelinated area to sustain continuous conduction. Hence, according to Waxman's (1978) estimate, redistribution of preexisting channels may be all that is required in an acute segmental demyelination. However, diffusion of nodal sodium channels over the entire cat internode without new synthesis would lead to a channel density 0.2% of that in the node or about 36 channels/ μm^2 , which appears to be insufficient to sustain continuous conduction. Limited diffusion of channels in the very acute stages of injury probably does occur since ferric ion–ferrocyanide staining is noted to spread from the nodal confines as early as 1 hr after application of antigalactocerebroside serum (Saida et al., 1980).

In acutely disrupted mammalian paranodes, the total sodium current does not change—which is inconsistent with rapid new synthesis and insertion of channels but not with limited diffusion (Chiu and Ritchie, 1982). Evidence from external longitudinal current recordings for continuous conduction in demyelinated and myelinated spinal fibers (Bostock and Sears, 1976; Rasminsky and Kearney, 1976) is consistent with either new channel synthesis and nondiscontinuous insertion, diffusion of preexisting channels, or technically limited spatial resolution.

The possibility of significant sodium channel synthesis and STX receptor binding in regenerating nerves by proliferating Schwann cells (in addition to those supplied by the perikaryon to the nodal axolemma) is a theoretical one, but the numbers are not convincing. Furthermore, the appearance of “phi nodes” in demyelinated rat ventral roots appears to be independent of Schwann cell differentiation, nodal register, or inactivation by irradiation (Smith et al., 1982; see also Feasby et al., 1981, and Oaklander et al., 1984). An understanding of the factors controlling the synthesis and redistribution of sodium channels following injury could lead to more effective treatment of demyelinating diseases.

Conclusion

In response to axotomy and regeneration in cat ventral roots, the following responses occur:

1. A 1.35-fold increase in the total number of myelinated fibers with a decrease in average fiber size.
2. A 2- to 3-fold increase in nodes per unit length for larger diameter fibers and an overall 2.36-fold increase in nodes per unit weight of lumbosacral ventral root.
3. A 3.5-fold increase in ^3H -STX binding per unit weight of aggregate ventral root.
4. A 6.2-fold increase in nodes per anterior horn cell which results from neuronal dropout and axonal branching of surviving neurons.
5. An increase in the number of binding sites per node in regenerated peripheral nerve from 1.26×10^6 to 1.95×10^6 (not statistically significant).

The implications of such channel reorganization include an increased metabolic load, both on the synthetic capacity of the neuron and on the oxidative metabolism of the fiber.

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