

Biphasic Regulation of Development of the High-Affinity Saxitoxin Receptor by Innervation in Rat Skeletal Muscle

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ABSTRACT Specific binding of ^3H -saxitoxin (STX) was used to quantitate the density of voltage-sensitive sodium channels in developing rat skeletal muscle. In adult triceps surae, a single class of sites with a $K_D = 2.9$ nM and a density of 21 fmol/mg wet wt was detected. The density of these high-affinity sites increased from 2.0 fmol/mg wet wt to the adult value in linear fashion during days 2–25 after birth. Denervation of the triceps surae at day 11 or 17 reduced final saxitoxin receptor site density to 10.4 or 9.2 fmol/mg wet wt, respectively, without changing K_D . Denervation of the triceps surae at day 5 did not alter the subsequent development of saxitoxin receptor sites during days 5–9 and accelerated the increase of saxitoxin receptor sites during days 9–13. After day 13, saxitoxin receptor development abruptly ceased and the density of saxitoxin receptor sites declined to 11 fmol/mg wet wt. These results show that the regulation of high-affinity saxitoxin receptor site density by innervation is biphasic. During the first phase, which is independent of continuing innervation, the saxitoxin receptor density increases to 47–57% of the adult level. After day 11, the second phase of development, which is dependent on continuing innervation, gives rise to the adult density of saxitoxin receptors.

INTRODUCTION

Many properties of skeletal muscle cells are regulated by the motor neurons that innervate them (for reviews, see Guth, 1968; Purves, 1976; Fambrough, 1979). These include regulation of the three principal muscle cell surface proteins involved in impulse transmission: the acetylcholine receptor (AChR), acetylcholinesterase, and the voltage-sensitive sodium channel. In mammalian skeletal muscle, each of these membrane-associated components has a functionally active embryonic counterpart that may be distinguished from the adult form on the basis of its biochemical and pharmacological properties. The developmental appearance of the adult form of AChR and acetylcholinesterase is linked to innervation by the motor neuron. Conversely, denervation leads to the reappearance of the embryonic form (Fambrough, 1979; Vigny et al., 1976).

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In rat skeletal muscle, the embryonic and adult forms of the voltage-sensitive sodium channel can be distinguished by their affinity for the potent neurotoxins tetrodotoxin (TTX) and saxitoxin (STX). These small, water-soluble toxins have a high affinity for a common site on the sodium channel of adult muscle at which they bind and block ionic flux at nanomolar concentrations (Ritchie and Rogart, 1977*a*; Narahashi, 1974). On the other hand, the TTX-insensitive* sodium channels of embryonic muscle require a 200-fold higher concentration of these toxins to block ion flux and action potentials (Kidokoro et al., 1975; Catterall, 1976; Sastre and Podleski, 1976; Stallcup and Cohn, 1976). When adult muscle is denervated, TTX-insensitive channels appear and may constitute up to 25% of the total sodium conductance present after denervation (Redfern and Thesleff, 1971; Pappone, 1980). Because of the similarity in affinity for TTX of the TTX-insensitive sodium channels in uninnervated muscle cells *in vitro* and denervated muscle *in vivo*, it seems likely that these channels are similar or identical.

The effect of denervation on the TTX-sensitive* channel of adult muscle has been studied using both electrophysiological and neurotoxin binding methods. The original electrophysiological experiments measuring the reduction in the maximum rate of rise of the action potential caused by TTX and STX suggested that TTX-sensitive sodium channels were lost on denervation (Redfern and Thesleff, 1971). In fact, more recent voltage-clamp data show that at least 75% of the original TTX-sensitive sodium channels remain as the new TTX-insensitive channels appear after denervation (Pappone, 1980). In agreement with this, 60–80% of the original high-affinity STX receptor sites in adult muscle remain after denervation (Ritchie and Rogart, 1977*b*; Barchi and Weigele, 1979; Hansen Bay and Strichartz, 1980). Thus, it appears that denervation of adult muscle causes, at most, a partial loss of TTX-sensitive sodium channels. To define more clearly the role of innervation in regulation of high-affinity STX receptors, we have studied the time course of development of these receptors in newborn rat muscle and examined effects of denervation at various times during development. A preliminary report of these results has appeared (Sherman and Catterall, 1982).

MATERIALS AND METHODS

Materials

Sprague-Dawley rats were obtained from Tyler Labs, Bellevue, WA. Tetrodotoxin was purchased from Calbiochem-Behring Corp., San Diego, CA. Saxitoxin was obtained from the National Institutes of Health. ^3H -STX was prepared by the ^3H - H_2O exchange technique of Ritchie et al. (1976). Purification and characterization were performed as described by Catterall et al. (1979). The preparation of ^3H -STX used had a specific activity of 12.2 Ci/mmol and a radiochemical purity of 75%.

* The terms "tetrodotoxin insensitive" and "tetrodotoxin resistant" have been used to denote the voltage-sensitive sodium channels found in cultured, fetal, and denervated skeletal muscle. These terms are not meant to imply that these Na^+ channels are not inhibited by tetrodotoxin. Rather, the K_I (0.5–2 μM) for tetrodotoxin is about 200-fold greater than the K_I of tetrodotoxin for Na^+ channels of nerve and innervated skeletal muscle. These latter channels are denoted as "tetrodotoxin sensitive."

Measurement of ^3H -STX Binding to Muscle Homogenates

Triceps surae and diaphragm muscles were dissected free of connective tissue and large blood vessels and nerves were removed. The muscles were rinsed with sodium-free buffer, carefully drained and weighed in tared vessels. They were then minced and homogenized in 10 ml/g of sodium-free buffer with a Tek-Mar (Cincinnati, OH) Tissuemizer at the half-maximal setting for 15 s. The buffer consisted of 130 mM choline chloride, 5.4 mM KCl, 5.5 mM glucose, 0.8 mM MgSO_4 , 1.8 mM CaCl_2 , and 50 mM Hepes adjusted to pH 7.4 at 0°C with Tris base. After homogenization, the samples were passed through 100- μm woven nylon mesh to remove large pieces of connective tissue. No binding activity was present in the material trapped by the mesh. The samples were stored at -70°C for up to 3 mo without loss of binding activity.

The assay for binding of ^3H -STX was performed using a procedure similar to that described by Barchi and Weigele (1979). The binding reaction was initiated by the addition of ^3H -STX in 100 μl of homogenization buffer to 100 μl of muscle homogenate. The samples were mixed and incubated for 30 min at 0°C . The reaction was stopped by the addition of 3 ml of wash medium consisting of 163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 at 0°C with Tris base), 1.8 mM CaCl_2 , and 0.8 mM MgSO_4 . The sample was immediately passed over glass fiber filters (Whatman GF/C) to collect membranes and particulate matter. The filters were washed twice with 3 ml of wash medium and radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined by including TTX at a final concentration of 2 μM in the assay mixture. Specific binding increased linearly with homogenate concentration over the range used in these experiments.

Denervation of the Triceps Surae

Unless stated otherwise, the triceps surae was denervated under ether anesthesia by section of the sciatic nerve deep to the gluteus maximus. Re-innervation was prevented by removing a 2–4-mm segment of the nerve. In some experiments the cut proximal end of the nerve was ligated; however, this procedure did not affect the results.

RESULTS

^3H -STX Binding to Adult Muscle

Specific binding of ^3H -STX to sodium channels in homogenates of adult rat skeletal muscle was measured using a rapid filtration assay as described in Materials and Methods. The use of muscle homogenates offered several advantages over whole muscle. Equilibrium was reached rapidly with maximum binding occurring within 10 min and remaining stable for at least 1 h. The scatter in the data was small with the standard deviation generally $<10\%$. The muscle homogenates could be stored for up to 3 mo at -70°C without loss of binding activity. The assay was performed at 0°C because of the increased affinity of STX for its receptor at this temperature (Barchi and Weigele, 1979). Similarly, the assay medium contained no Na^+ since this cation will compete with STX for its binding site and increase the apparent K_D (Barchi and Weigele, 1979).

Total binding of ^3H -STX to muscle homogenates is the sum of specific binding to the STX receptor and nonspecific binding to other components of the homogenate. Specific binding is saturable, is of high affinity, and is completely blocked by excess (2 μM) TTX in the assay solution (Ritchie and

Rogart, 1977a). When specific binding is plotted against the concentration of $^3\text{H-STX}$, the resulting curve is a rectangular hyperbola that can be described by the equation:

$$B = B_{\max} [^3\text{H-STX}] / (K_D + [^3\text{H-STX}])$$

where B is the amount bound at a given $^3\text{H-STX}$ concentration, B_{\max} is the total number of sites present, and K_D is the dissociation constant of the ligand-receptor complex. Nonspecific binding is unaffected by TTX and increases linearly with $^3\text{H-STX}$ concentration (Ritchie and Rogart, 1977a). Fig. 1A shows the total and nonspecific binding of $^3\text{H-STX}$ to the triceps surae muscle of adult rat. Each point represents the mean of determinations on at least four aliquots of the homogenate. In Fig. 1B, data derived from Fig. 1A are redrawn

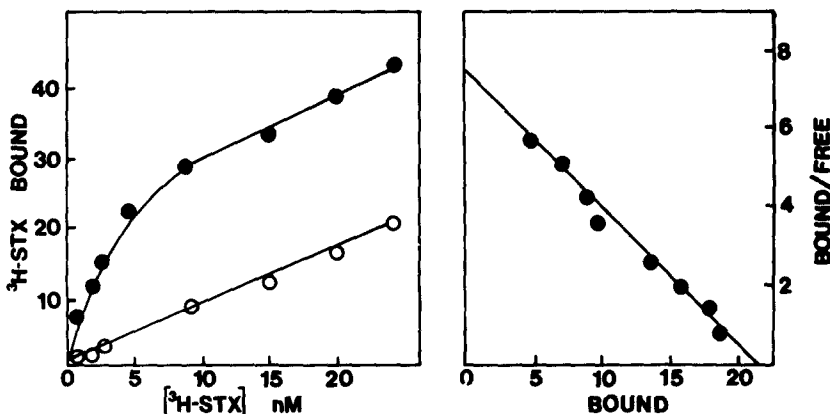


FIGURE 1. Binding of $^3\text{H-STX}$ to adult triceps surae. (A) Muscle homogenates were incubated with various concentrations of $^3\text{H-STX}$ (●) or $^3\text{H-STX}$ plus 2 μM TTX (○) for 30 min at 0°C. Bound $^3\text{H-STX}$ was measured by the rapid filtration assay described in Materials and Methods. (B) Specific binding was calculated at each concentration of $^3\text{H-STX}$ by subtracting the nonspecific binding measured in the presence of 2 μM TTX from the total binding and is presented as a Scatchard plot. The units shown on the abscissa are $\text{fmol} \cdot \text{mg wet wt}^{-1}$. The units shown on the ordinate are $\text{fmol} \cdot \text{mg wet wt}^{-1} \cdot \text{nM}^{-1}$. Least squares analysis gave values of: $K_D = 2.9$ nM and $B_{\max} = 21$ fmol/mg wet wt.

as a Scatchard plot. The data are best fit by a straight line that indicates that a single class of saturable sites is present with a $K_D = 2.9$ nM and $B_{\max} = 21.7$ fmol/mg wet wt. Similar experiments using adult rat diaphragm as the muscle source gave values of $K_D = 3.3$ nM and $B_{\max} = 24.0$ fmol/mg wet wt. These values are in good agreement with values previously reported for homogenates of rat leg muscle ($K_D = 1.4$ nM, $B_{\max} = 18$ fmol/mg wet wt; Barchi and Weigele, 1979) and for whole rat diaphragm muscle ($K_D = 3.8$ nM, $B_{\max} = 24$ fmol/mg wet wt; Ritchie and Rogart, 1977b), which indicates that homogenization does not adversely affect the STX receptor. Binding of STX to small pieces of muscle also gives similar results with K_D from 4.3 to 5.1 nM and B_{\max} from 23.8 to 52.6 fmol/mg wet wt for three different rat muscles (Hansen Bay and Strichartz, 1980).

In the following experiments, B_{\max} was estimated by assaying binding at 15 nM $^3\text{H-STX}$ in the absence and presence of 2 μM TTX. At this concentration, 83% of the receptors will have bound $^3\text{H-STX}$. Higher concentrations, although they saturate a greater percentage of the sites, give a less favorable ratio of specific to nonspecific binding.

Time Course of the Development of $^3\text{H-STX}$ Binding Sites

Although neuromuscular transmission first occurs during embryogenesis, many characteristics of the muscle fiber and neuromuscular junction continue to develop for several weeks after birth. Fig. 2 illustrates the time course of the

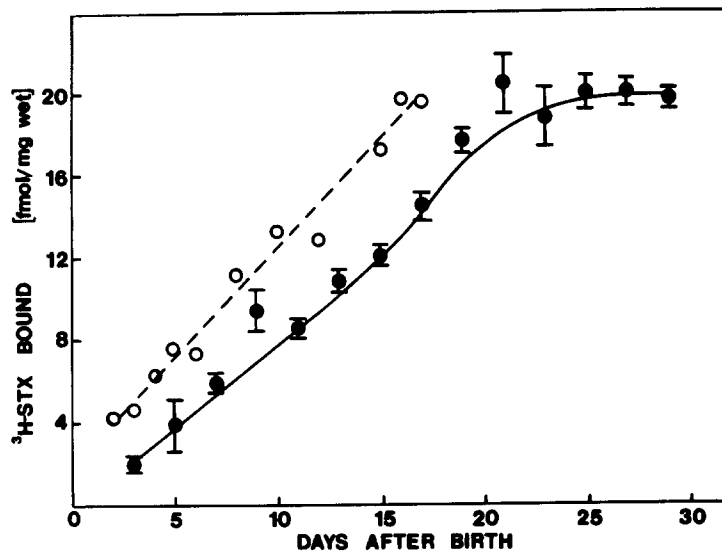


FIGURE 2. Development of $^3\text{H-STX}$ binding sites. The specific binding of $^3\text{H-STX}$ at 15 nM to homogenates of triceps surae (●) and diaphragm (○) from rat pups of various ages was measured as described in Materials and Methods. The error bars represent \pm SEM of data from at least three separate groups of animals. Error bars are not shown for the diaphragm since some of the points represent single determinations.

appearance of high-affinity $^3\text{H-STX}$ binding sites for both diaphragm and triceps surae. These data represent the mean of separate determinations from several litters of equal size. The error bars on the triceps surae data indicate \pm SEM and represent at least three independent assays at each age. Only the specifically bound component is shown. For triceps surae, binding was $\sim 10\%$ of the adult value at day 3, the earliest age assayed, and increased in a roughly linear fashion until the adult value of 21 fmol/mg wet wt was reached at day 21. No overshoot above the adult value was detected. For diaphragm, a similar linear development was observed occurring ~ 3 d earlier than in triceps surae (Fig. 2). Although a detailed study of K_D as a function of age was not performed, binding was measured at 25 nM $^3\text{H-STX}$ as well as 15 nM in

some experiments and the results indicated that 15 nM gave at least 83% saturation of the STX receptor sites throughout the developmental time course. The changes observed therefore represent changes in B_{\max} for $^3\text{H-STX}$ binding.

Effect of Denervation on the Development of TTX-sensitive Sodium Channel

Denervation of adult muscle leads to the appearance of TTX-insensitive sodium channels and extrajunctional AChR's as well as affecting many other properties of skeletal muscle (Purves, 1976). To determine the effects of

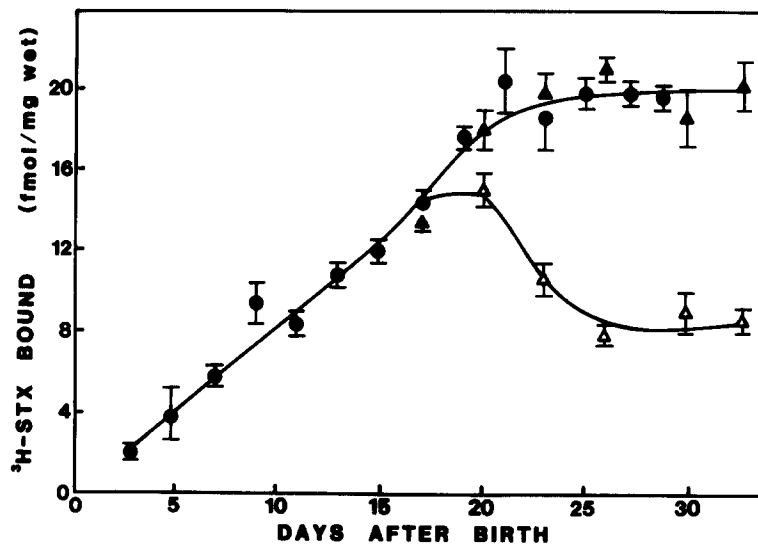


FIGURE 3. Effect of denervation at day 17 on the development of $^3\text{H-STX}$ binding sites. The triceps surae of 17-d-old animals were denervated unilaterally by section of the sciatic nerve. The muscles were removed at various times after denervation and specific binding of $^3\text{H-STX}$ was measured as described in Materials and Methods (Δ). The contralateral triceps surae served as controls and were assayed under identical conditions (\blacktriangle). The error bars represent \pm SEM of data from at least four separate experiments. To compare the effects of denervation with the time course of normal binding site development, the data from Fig. 2 have been redrawn (\bullet).

denervation on the development of the adult sodium channel, we denervated the triceps surae by sectioning the sciatic nerve deep to the gluteus maximus. The contralateral triceps surae served as the control. Fig. 3 shows the results of experiments in which the denervation was performed at day 17 after birth. Each data point represents the mean \pm SEM of separate determinations from at least four muscles. After a short lag period, the site density in denervated muscles begins a gradual decline that continues for the next 6 d until a plateau is reached at 9.2 fmol/mg wet wt or 47% of the adult value. The values for the contralateral control muscles (\blacktriangle) are in close agreement with the data for normal development (\bullet) as illustrated in Fig. 3.

Fig. 4 presents the results of similar experiments in which the denervation was performed at day 11. Each data point represents the mean \pm SEM of determinations on at least 3 groups of muscles from a total of 16 animals. The STX receptor site density of the denervated muscles (Δ) exhibited a gradual increase from 8.4 fmol/mg wet wt to 10.4 fmol/mg wet wt over the ensuing 10 d. The contralateral control muscles (\blacktriangle) remained within the range of normal development. Thus, denervation at day 11 blocks essentially all further development leading to a plateau of the STX receptor density at 53% of the adult value. This level is approximately the same as the STX receptor site

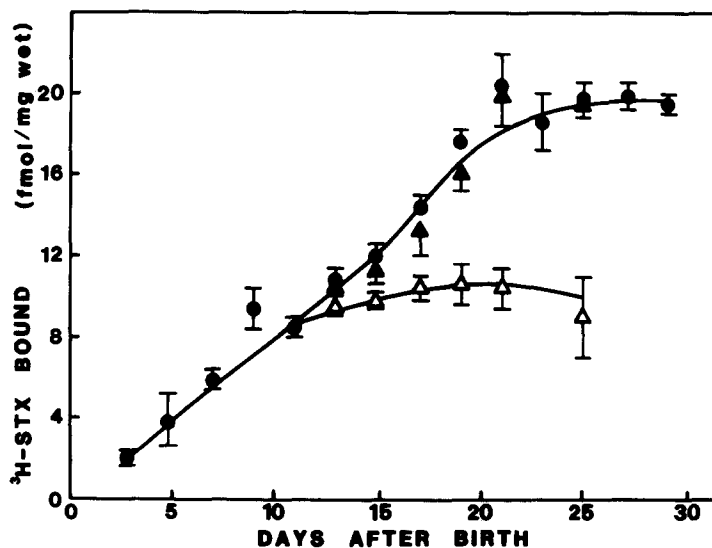


FIGURE 4. Effect of denervation at day 11 on the development of ^3H -STX binding sites. The triceps surae of day-11 animals were denervated unilaterally by section of the sciatic nerve. The muscles were removed at various times after denervation and specific binding of ^3H -STX was measured as described in Materials and Methods (Δ). The contralateral triceps surae served as controls and were assayed under identical conditions (\blacktriangle). The error bars represent \pm SEM of at least 3 separate experiments, each using muscles from 16 animals. To compare the effects of denervation with the time course of normal binding site development, the data from Fig. 2 have been redrawn (\bullet).

density on the final plateau reached after denervation at day 17. These results show that continuing innervation is required for both the development and maintenance of a STX receptor density greater than ~ 11 fmol/mg wet wt.

The changes in STX binding caused by denervation (Figs. 4 and 5) could be due to either a reduced number of STX receptors or a markedly increased K_D for STX binding. To distinguish these two possibilities, the K_D and B_{max} values for STX binding to homogenates of muscles denervated at day 11 and of contralateral control muscles from the same animals were determined at day 23 by Scatchard analysis (Fig. 5). The K_D values were found to be 2.9 nM in each case, whereas B_{max} decreased from 25 fmol/mg to 16.5 fmol/mg (Fig.

5). Therefore, the decrease in STX binding of denervated muscles relative to controls is due to a decrease in B_{\max} with no change in K_D , which indicates that only the density of STX receptors is affected by denervation.

At day 11, nearly one-half the adult density of STX receptors is present (Fig. 2). To test the effects of denervation on the early development of TTX-sensitive sodium channels, we denervated animals at day 5 and followed the time course of ^3H -STX binding for the next 3 wk (Fig. 6). The same experimental procedure was used as before. Each data point represents the mean \pm SEM of separate determinations from at least 3 groups of muscles from a total of 21 animals. In contrast with our results with denervation at

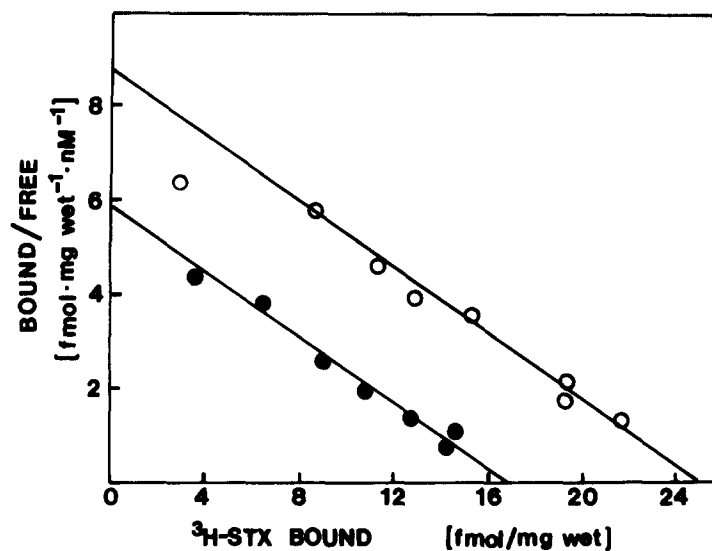


FIGURE 5. Effect of denervation at day 11 on the affinity of ^3H -STX for its receptor. The triceps surae of 10 animals were denervated unilaterally by section of the sciatic nerve 11 d after birth. The contralateral triceps surae served as controls. The muscles were removed 11 d after denervation and homogenized. Specific binding of ^3H -STX was measured at concentrations ranging from 1 to 30 nM as described in Materials and Methods and is presented here as a Scatchard plot. Denervated muscle (●) gave values of: $K_D = 2.9$ nM and $B_{\max} = 16.8$ fmol/mg wet wt. Control muscles (○) gave values of: $K_D = 2.9$ nM and $B_{\max} = 25.0$ fmol/mg wet wt.

days 11 and 17, denervation at day 5 had a biphasic effect. From day 5 through day 9, STX receptor number increased at a similar rate in both denervated and control muscles (Fig. 6). From day 9 through day 13, STX receptor number actually increased more rapidly in denervated muscles than in controls. This increased rate of STX receptor development on days 9 through 13 led to a statistically significant increase in STX receptor levels on days 11 through 15 in denervated muscles as compared with control muscles ($P < 0.01$, Student's t test). Throughout the entire developmental time course, the STX receptor site densities for contralateral control muscles from denervated animals (Fig. 6) were within the range of values observed for normal

animals. Thus, our results show that continuing innervation is not required for the first phase of development of high-affinity STX receptors from day 5 to 13 after birth and may actually slow the appearance of STX receptor sites on days 9 through 13.

However, in muscles denervated at day 5, the development of STX receptor sites stops abruptly on day 13 (Fig. 6). By day 17, the STX receptor site density of denervated muscles has dropped below that of controls and continues to fall to a plateau of 11.1 fmol/mg wet wt or 57% of the final adult value. Thus, as observed in muscles denervated at days 11 or 17, continuing

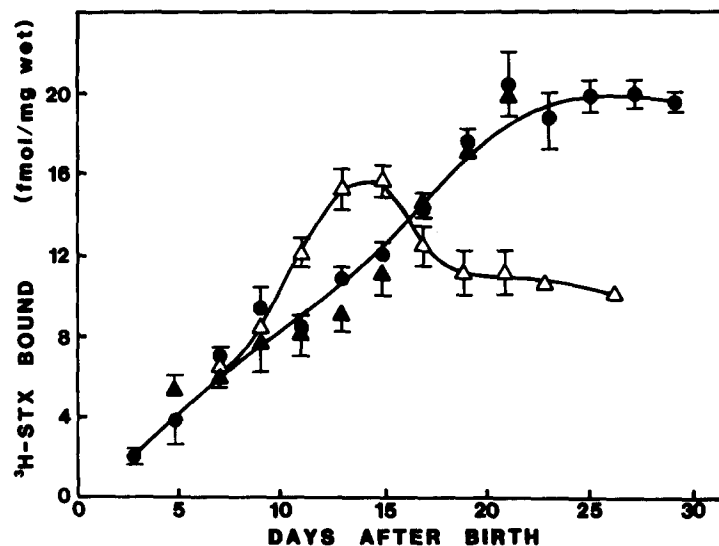


FIGURE 6. Effect of denervation at day 5 on development of ^3H -STX binding sites. The triceps surae of day-5 animals were denervated unilaterally by section of the sciatic nerve. The denervated muscles were removed at various times after denervation and specific binding of ^3H -STX at 15 nM was measured as described in Materials and Methods (Δ). The contralateral triceps surae served as controls and were assayed under identical conditions (\blacktriangle). The error bars represent \pm SEM of data from at least 3 separate experiments, each using muscles from 21 animals. The experimental data points at days 23 and 26 are from single determinations. To compare the effects of denervation with the time course of normal binding site development, the data from Fig. 2 have been redrawn (\bullet).

innervation is required for maintenance of a STX receptor site density above ~ 11 fmol/mg wet wt. Denervation at day 5, 11, or 17 results in STX receptor site development to approximately the same final value of 9.4 to 11 fmol/mg wet wt or 47–57% of the adult value.

These results define a biphasic regulation of appearance of TTX-sensitive sodium channels by innervation. Continuing innervation is not required for development of high-affinity STX receptor sites from days 5–11 after birth and actually slows the increase in site density on days 9–11. The initial innervation-independent phase of development results in the appearance of

47–57% of the adult density of STX receptor sites. This critical density of STX receptor sites is reached after denervation at 5, 11, or 17 d. After day 11, continuing innervation is required for the development and maintenance of a STX receptor site density >11 fmol/mg wet wt. This second innervation-dependent phase of development results in the achievement of the adult level of STX receptor site density by day 25 after birth. The requirement for continuing innervation for maintenance of this site density is observed in denervation experiments at days 11 and 17 (Figs. 3 and 4), as well as in adults (Ritchie and Rogart, 1977*b*; Barchi and Weigele, 1979; Hansen Bay and Strichartz, 1980).

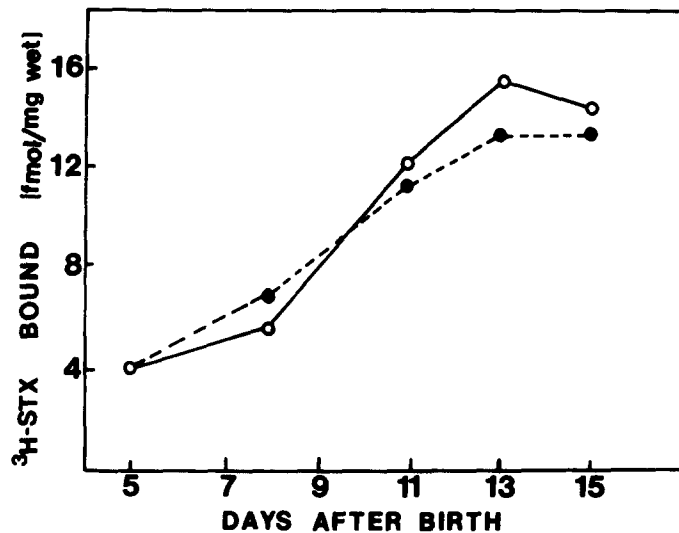


FIGURE 7. Effect of nerve stump length on the development of ^3H -STX binding sites after denervation 5 d after birth. The triceps surae of 16 animals were denervated bilaterally 5 d after birth. On one side, the sciatic nerve was sectioned deep to the gluteas maximus, leaving a relatively long nerve stump (○). On the other side, the triceps surae was denervated by sectioning the tibial nerve as close as possible to the muscle surface (●). Specific binding of ^3H -STX at 15 nM was measured at subsequent times as described in Materials and Methods.

One possible explanation for the difference in the effect of denervation at days 5 and 11 on development is that essential trophic factors continue to be released from the stumps after denervation at 5 d but not at 11 d. The length of the remaining stump after denervation has been reported to affect the rate of appearance of the extrajunctional AChR's (Emmelin and Malm, 1965; Uchitel and Robbins, 1978) as well as the disappearance of the endplate specific form of acetylcholinesterase (Fernandez et al., 1979; Ranish et al., 1980). To test this hypothesis, a number of animals were denervated bilaterally. On one side the triceps surae was denervated as before, by sectioning the sciatic nerve deep to the gluteas maximus, leaving a relatively long nerve stump (5 mm). On the other side, the triceps surae was denervated by

sectioning the tibial nerve as close as possible to the muscle surface. No difference was observed in the rate of development of binding sites (Fig. 7) in muscles having short or long nerve stumps. These results show that the long nerve stump is not required for the development of high-affinity STX receptor sites in denervated muscles. This result does not rule out an effect of trophic factors, however, since amounts adequate to support the observed development may have been present in the intramuscular branches of the nerve. Alternatively, factors acting as developmental regulators may have been released from the nerve before denervation and have had a continuing inductive effect for the following 10 d.

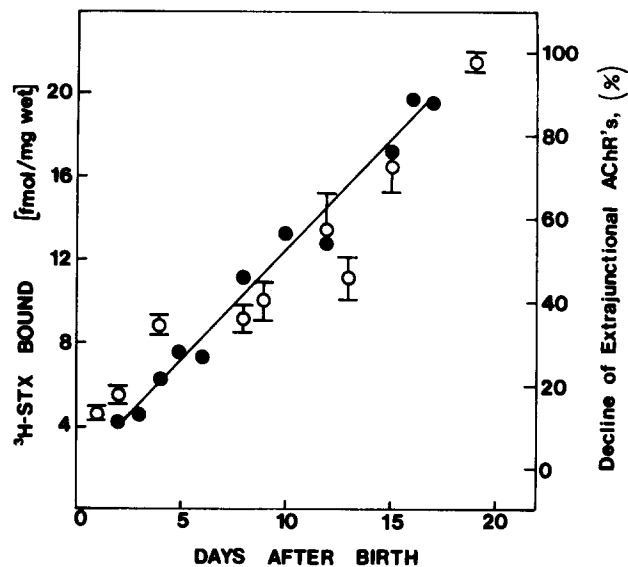


FIGURE 8. Comparison of the development of ^3H -STX binding sites and loss of extrajunctional AChR's. The specific binding of ^3H -STX to developing diaphragm (●) is the same data shown in Fig. 2. The percentage decrease of extrajunctional AChR's in rat diaphragm (○) as a function of age is derived from the data of Steinbach et al. (1979), who used the binding of [$^{125}\text{I}_2$]α-bungarotoxin to measure AChR density. Error bars represent \pm SEM.

DISCUSSION

The motor neuron has a profound influence on many properties of skeletal muscle and, in particular, regulates the amount and type of AChR's, acetylcholinesterase, and voltage-sensitive sodium channels produced by the muscle fiber (for a review see Purves, 1976). It is of interest to compare the time course for developmental changes in these three proteins which are essential for neuromuscular transmission. During development of rat diaphragm, AChR's are initially located all along the muscle fibers. As development proceeds, extrajunctional AChR's are progressively lost until finally AChR's are located only in the region of the neuromuscular junction (Bevan and Steinbach, 1977; Fambrough, 1979). Fig. 8 compares the developmental increase in ^3H -STX

binding to the decrease in extrajunctional AChR's. The solid circles are the same data shown in Fig. 2 for diaphragm. The open circles represent the data of Steinbach et al. (1979), who used several methods to estimate the percentage of AChR's that were extrajunctional in developing rat diaphragm. Their data have been averaged and are represented by a single point \pm SEM for each age. As shown, these two developmental processes have remarkably similar time courses. Both have occurred to a small extent by birth and continue in a roughly linear fashion for the next 3 wk when adult levels are achieved. Although this close temporal correlation does not necessarily imply a common regulatory mechanism for TTX-sensitive sodium channels and AChR, it seems likely that the concordant development of the adult forms of these two proteins reflects an important functional maturation in neuromuscular transmission. The endplate specific form of acetylcholinesterase appears in rat skeletal muscles over a generally similar time course (Vigny et al., 1976).

One can envision two major mechanisms by which the motor neuron could influence postsynaptic muscle properties. The first is that activity *per se* is the regulatory signal and the neuron exerts its influence by causing synaptically mediated depolarizations and contractions. The second is that trophic factors, which act as chemical or local hormonal signals, are released from the nerve endings. Current evidence indicates that both these mechanisms are important for regulation of AChR (Fambrough, 1979; Lomo and Rosenthal, 1972; Berg and Hall, 1975; Christian et al., 1978; Podleski et al., 1978; Jessell et al., 1979) and acetylcholinesterase (Oh and Mackelonis, 1978; Rubin et al., 1980).

The mechanisms by which the voltage-sensitive sodium channel is regulated have not been as well characterized. Experiments performed *in vivo* and in organ culture have been successful in modulating the TTX-insensitive channel. For example, the TTX-insensitive action potentials of denervated diaphragm maintained in organ culture disappear when the muscle is innervated by fetal spinal cord explants (Ziskind and Harris, 1979). The importance of activity *per se* as a regulator has been demonstrated by experiments performed *in vivo*, in which chronic pharmacological blockade of the neuromuscular junction led to the appearance of TTX-insensitive action potentials (Berg and Hall, 1975). Furthermore, subthreshold electrical stimulation has been reported to block this effect of chronic blockade (Greuner and Baumbach, 1976). Trophic factors have also been implicated in the control of the TTX-insensitive sodium channel by the observation that extracts from the ventral portion of the spinal cord delay the appearance of TTX-insensitive action potentials in denervated muscle maintained in organ culture (Hasegawa and Kuromi, 1976). Thus, both trophic influences and electrical activity may be important in the regulation of the TTX-insensitive sodium channel.

The effect of denervation of adult muscle on the high-affinity STX receptor has been studied in three laboratories. Hansen Bay and Strichartz (1980) found that denervation of the extensor digitorum longus of rat caused a 20% decrease in ^3H -STX binding per milligram wet weight after 7 d but this decrease was not considered statistically significant. Ritchie and Rogart (1977b) found a 32% decrease in binding to intact rat diaphragm after 7 d but

attributed this to post-denervation swelling of the muscle fibers and concluded that there was no change in the sarcolemmal density of the TTX-sensitive sodium channels. Barchi and Weigele (1979) found that after 10–14 d denervation produced a 43% decrease in ^3H -STX binding to sarcolemma isolated from rat gastrocnemius and tibialis, which suggests that a true change in sarcolemmal sodium channel density does occur. In no case was a significant change in the K_D for ^3H -STX reported.

Since denervation of adult muscle causes at most only a partial loss of the TTX-sensitive channel, it is important to determine the effect of denervation on the initial appearance of STX receptors during development. The results presented in this report indicate that denervation of developing muscle does have important regulatory effect on the density of TTX-sensitive channels. Since denervation hypertrophy does not occur in the triceps surae (Pellegrino and Franzini, 1963), the changes in ^3H -STX binding site number that we have observed represent actual changes in the sarcolemmal sodium channel density.

Our data show that the development of the TTX-sensitive sodium channel occurs in two phases. The first phase leads to 47–57% of the adult level and is independent of continuing innervation. The second phase, which gives rise to the remaining portion of binding sites, begins 11 d after birth and is dependent upon continuing innervation. After denervation at 5, 11, or 17 d, the STX receptor site density finally approaches a common critical value of ~ 9.5 –11 fmol/mg wet wt or 47–57% of the adult level. It is probable that the loss of up to 40% of STX receptor sites on denervation of adult muscle (Ritchie and Rogart, 1977; Barchi and Weigele, 1979; Hansen Bay and Strichartz, 1980) also represents a reversal of the second, innervation-dependent phase of development toward this critical level of STX receptor site density. Since the complete time course of the effect of denervation was not determined in these studies of adults, the values reported may not reflect the final extent of receptor site decrease.

Although continuing innervation is not required for the first phase of STX receptor development, it does modulate the rate of increase in STX receptor density. Our results show that denervation significantly increases the rate of accumulation of STX receptor sites on days 9 through 13. These data suggest that during this time neuronal influences normally slow the rate of increase in STX receptor site density that is an intrinsic part of muscle development. Alternatively, the increase in STX receptor site density during this time may result from a greater effect of denervation to slow the increase in muscle mass than to slow the increase in STX receptor number causing STX receptor site density measured as femtomoles per milligram wet weight to increase relative to controls. In either case, the overlap of the more rapid increase of STX receptor site density in denervated muscles on days 9 through 13 with the block of further receptor development observed after denervation at day 11 may explain the overshoot of the final plateau value of STX receptor density that occurs after denervation at day 5 (Fig. 6).

Although the first phase of STX receptor development is independent of

continuing innervation, we cannot rule out a requirement for prior innervation or continuing release of trophic factors from the remaining nerve stump as essential for this phase of development. It is clear, however, that this phase does not require neuronally driven electrical or contractile activity, since such activity is completely blocked by denervation. Further experiments are necessary to determine whether it is activity, trophic influence, or both that is required during the second, innervation-dependent phase of development.

In adult muscle, high-affinity binding sites for STX represent functional sodium channels present at the cell surface (Ritchie and Rogart, 1977a; Barchi and Weigele, 1979). Since voltage-clamp studies of developing rat muscle have not been reported, we cannot be certain that the high-affinity STX receptor sites we have measured are associated with physiologically active sodium channels. Moreover, since the binding measurements were carried out on tissue homogenates, a fraction of the STX receptor sites measured might be intracellular. Recent studies (S. Sherman and W. Catterall, unpublished data) of embryonic rat muscle cells developing in cell culture in the absence of innervation indicate that STX receptors appear in a biphasic time course similar to denervated muscle *in vivo* and are associated with a physiologically active sodium channel. In this *in vitro* preparation, there is no difference in the K_D or total binding capacity of STX when intact cells are compared to cell homogenates, which indicates that the STX binding represents exclusively sites on the surface membrane and that homogenization does not cause a change in site density due to proteolysis or exposure of concealed sites. These results provide further support for an innervation-independent development of functional sodium channels having high affinity for STX and TTX in rat muscle.

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