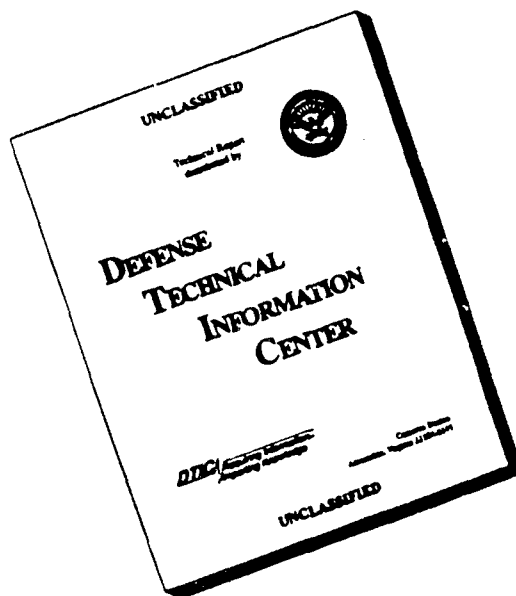


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TURNOVER OF ACETYLCHOLINE RECEPTORS: MECHANISMS OF REGULATION

ANNUAL REPORT

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AUGUST 1, 1987

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→ The synthesis, insertion and degradation of acetylcholine receptors (AChRs) of skeletal muscle cells are closely regulated both by the muscle cells and by the motor nerves that supply them. The goal of this project is to elucidate the mechanisms of regulation of the AChRs, both at the neuromuscular junction and at extrajunctional regions. We have used ¹²⁵I-α-bungarotoxin (¹²⁵I-α-BuTx) as a label to follow the metabolic turnover of AChRs both in vivo and in tissue culture. Recent experiments have used cDNA that specifically binds to the mRNA for the α-subunit of the AChR to measure mRNA (gene regulation), as well. <i>Myasthenia Gravis Transgenic Mouse (MG-TM) neuromuscular system; AChR synthesis</i> During the past year, our studies have shown: 1) Synthesis and insertion of AChRs of the normal neuromuscular junction is rapid. New receptors are inserted at the rate of 16% within 24 hrs. and 20% at 48 hrs. This rapid rate (continued)			
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is sufficient to compensate for the degradation of both the rapidly turned over ACh receptors and the stable ACh receptors at the neuromuscular junction.

2) Rapidly turned over AChRs serve as precursors for the stable AChRs at the neuromuscular junction.

3) The motor nerve plays a key role in the process of stabilization of junctional AChRs.

4) Denervation results in a rapid increase in messenger RNA for the α -subunit of the AChR (mRNA-AChR).

5) Botulinum toxin, which blocks quantal release of ACh, results in a denervation-like increase in mRNA-AChR; however, this increase is less marked than that which results from surgical denervation. We surmise that both quantal and non-quantal ACh transmission may be involved in the regulation of mRNA-AChR.

6) ACh transmission plays a role in maintenance of stability of junctional AChRs. Blockade of quantal ACh release by botulinum toxin results in a denervation-like acceleration of loss of pre-existing stable AChRs. This effect occurs later than that of surgical denervation, suggesting that both quantal and non-quantal ACh transmission may be involved in the nerve's regulation of AChR stability.

These findings are leading to an understanding of the turnover of AChRs at the neuromuscular junction. From the military point of view, they provide important information regarding the recovery of blocked or damaged neuromuscular junctions.

Foreword

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. (NIH) 86-23, Revised 1985).

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I. 1. Introduction:

As described in our original proposal, the major goal of this investigation is to learn more about the mechanisms that regulate the synthesis and turnover of junctional and extrajunctional acetylcholine receptors (AChRs). During the past year, we have made significant progress in many aspects of these studies. The results of these are detailed in II. Progress. Our findings, detailed in Section II indicate the following:

- a. Junctional AChRs are rapidly synthesized.
- b. The rapidly turned over junctional AChRs (RTOs) are precursors of the stable junctional AChRs.
- c. The motor nerve plays an important role in "stabilizing" a proportion of the RTOs, converting them to the stable form.
- d. ACh transmission plays a role in maintenance of stability of junctional AChRs.
- e. We have acquired cDNA probes for several subunits of the AChR, and are using them in studies of AChR turnover.
- f. Preliminary evidence indicates that ACh transmission plays a key role in regulating transcription of the mRNAs for the AChR subunits.
- g. We have published results indicating that several cations (lithium, calcium, sodium) down-regulate the synthesis of AChRs in a skeletal muscle cell culture model.

2. Brief Restatement of Overall Research Problem and Rationale

Both the distribution and turnover of acetylcholine receptors (AChRs) of mammalian skeletal muscles are regulated to a large extent by the motor nerves. In innervated muscles, AChRs are localized almost exclusively at neuromuscular junctions (Axelsson and Thesleff, 1959; Miledi, 1960; Albuquerque et al., 1974; Fertuck and Salpeter, 1974; Kuffler and Yoshikami, 1975). Following denervation, a great increase of extrajunctional AChRs occurs (Miledi, 1960; Miledi and Potter, 1971; Lee, 1972; Chang et al., 1975; Hertzell and Fambrough, 1972; Pestronk et al., 1976a,b), presumably due to increased synthesis of AChRs (Fambrough, 1970; Grampp et al., 1972; Brookes and Hall, 1975b; Devreotes and Fambrough, 1976), resulting from increased transcription of the appropriate mRNAs (Merlie et al., 1984; Goldman et al., 1985).

Junctional ACh Receptors:

AChRs of neuromuscular junctions differ in many respects from extrajunctional AChRs. They are densely packed, located mainly at the peaks of post-junctional folds (Mathews-Bellinger and Salpeter, 1978). They differ physiologically, physicochemically and immunologically from extrajunctional AChRs (Brookes and Hall, 1975a; Lindstrom et al., 1976; Neher and Sakmann, 1976;

Sakmann and Brenner, 1978; Schuetze and Fishbach, 1978; Weinberg and Hall, 1979; Dwyer et al., 1981; Brenner and Sakmann, 1983) (as described in the original proposal).

One of the most important characteristics of junctional AChRs, which is a major focus of this research, is their metabolic stability, with a half-life previously reported to be between 6 and 13 days (in rodents) (Berg and Hall, 1975; Chang and Huang, 1975; Stanley and Drachman, 1978; Linden and Fambrough, 1979; Bevan and Steinbach, 1983). We have recently reported that the AChRs at innervated neuromuscular junctions are actually comprised of two subpopulations with strikingly different rates of turnover (Stanley and Drachman, 1983a, 1987). The majority of junctional AChRs are stable, with a half-life of 11 to 12 days. The remainder, which we now estimate to be 20 to 25% of the total, are rapidly turned over (RTOs) with a half-life of approximately 1 day. This finding is based on our detailed analyses of degradation curves of ¹²⁵I-labeled AChRs, using an *in vivo* mouse model (Stanley and Drachman 1983a, 1987). This result, which we have repeatedly confirmed in the course of our subsequent studies described below, leads to several conclusions and predictions:

First, it predicts that the rate of synthesis and insertion of junctional AChRs should be more rapid than previously estimated, in order to replace the rapidly degraded AChRs.

Second, this may explain the rapid recovery from certain neuromuscular toxins. Recovery from irreversible AChR blocking agents (such as α -bungarotoxin (α -BuTx)) is known to occur far more quickly than would be expected on the basis of dissociation of the toxin.

Third, it suggests that the turnover of RTOs alone accounts for the majority of the overall junctional receptor turnover. Although the population of rapidly turned over AChRs is only 20 to 25% of the total AChR population, its rate of turnover is 10 times as rapid as the rate for the stable AChRs.

Fourth, and perhaps most significantly, the rapidly degraded (RTO) subpopulation of junctional AChRs appear to be precursors of the stable AChRs.

Our progress to date (see below Section II-Tasks 1,2,3) strongly supports:
a) the rapid synthesis of junctional AChRs (Ramsay and Drachman, 1988)
b) the concept that the RTOs are converted to stable AChRs

Neural control of junctional ACh receptors:

There is abundant evidence that many of the properties of junctional AChRs are regulated to a large extent by the motor nerves (Salpeter and Loring, 1986; Schuetze and Role, 1987). For example, the ionic channel properties of short open times and high ionic conductances are dependent on motor innervation (Mehe, and Sakman 1976; Sakmann and Brenner, 1978; Schuetze and Fishbach 1978; Schuetze et al., 1978; Sellin, 1981; Brenner, 1983). Clustering of AChRs occurs at the site of contact between the motor nerve endings and the muscle cell membrane (Takeuchi, 1963; Anderson and Cohen, 1977; Bevan and Steinbach, 1977; Burden, 1977; Reiness and Weinberg, 1981). Some nerve-induced modification of the membrane (possibly the basement membrane) is thought to determine the high

density accumulation of AChRs at this site (Burden et al., 1979). Stability of junctional AChRs is also dependent on the motor nerve. Both the initial appearance of stable AChRs during development (Burden, 1977; Reiness and Weinberg, 1981), and the continued maintenance of AChR stability (Chang and Huang, 1975; Bevan and Steinbach, 1977; Brett and Younkin, 1979; Levitt and Salpeter, 1981; Stanley and Drachman, 1981) require some influence of the motor nerve.

One of our major results during the past year has been the experimental demonstration that the motor nerve "stabilizes" a proportion of the rapidly turned over junctional AChRs, converting them to the stable form (see Progress, Section II, Task 2).

Little is presently known about the mechanisms by which these changes in junctional AChRs are brought about. There is some evidence that the initial localization of the nerve-muscle junction and clustering of AChRs involve nerve-muscle contact, rather than neurotransmission (Steinbach et al., 1975). On the other hand, normal channel properties of junctional AChRs may require ACh transmission (Sjellin and Thesleff, 1981). One of the hypotheses that we are testing in these studies is that ACh transmission may have a role in the initial stabilization and the maintenance of stability of AChRs at the neuromuscular junction.

We now have preliminary evidence suggesting that ACh transmission plays a role in maintenance of stability of junctional AChRs (see Section II, Task 7).

Extrajunctional ACh Receptors:

In normally innervated muscles, the density of AChRs at extrajunctional regions is very low -- typically less than 20 α -BuTx binding sites per μm^2 for the soleus muscles of rodents (Pestronk et al., 1976a,b; Fambrough, 1979). However, the density of extrajunctional AChRs is increased in skeletal muscle cells that lack innervation (immature, or denervated mature muscle) (Axelsson and Thesleff, 1959; Miledi, 1960; Miledi and Potter 1971; Diamond and Miledi, 1962; Dryden, 1970; Fambrough and Rash 1971; Lee, 1972; Letinsky, 1975; Pestronk et al., 1976a,b; Bevan and Steinbach, 1977; Drachman et al., 1984). The high density of extrajunctional AChRs in these situations is thought to be due to a high rate of receptor synthesis (Fambrough, 1970; Grampp et al., 1972; Brookes and Hall, 1975b; Devreotes and Fambrough, 1976). Recent evidence indicates that there is a high rate of transcription of the genes for the various subunits of AChR, resulting in increased amounts of the appropriate mRNAs in denervated muscle (Merlie et al., 1984; Goldman et al., 1985). cDNA probes are now available for these mRNAs (Merlie et al., 1983; LaPolla et al., 1984; Boulter et al., 1985).

We have made progress in acquiring cDNA probes for the α -, β -, and delta-subunits, and are using them as sensitive and particularly relevant probes in our studies of AChR turnover (see Section II-Task 4).

Neural Control of Extrajunctional ACh Receptors:

It is clear that the motor innervation plays a dominant role in regulating

the synthesis of extrajunctional AChRs (see above, and Edwards, 1979; Fambrough, 1979). The questions of a) how the nerve's influence is mediated, and b) how the message is translated at the level of the muscle cell have been subjects of intense interest, and are addressed in our studies (see Progress). We have previously demonstrated the critical role of ACh transmission from the motor nerve in mediating the nerve's "trophic" influence in regulating expression of extrajunctional AChRs. All 3 forms of ACh release -- impulse dependent, spontaneous quantal, and spontaneous non-quantal -- appear to contribute to this effect (Drachman et al., 1982). Heretofore, the key evidence for the role of AChR has been derived from experiments in which pharmacological agents have been used to block the various forms of ACh transmission, and the number of surface AChRs has been measured by ^{125}I - α -BuTx binding. The availability of the new cDNA probes for AChR mRNAs greatly improves our ability to determine the mechanisms of AChR regulation, because: 1) changes in mRNA occur much earlier than changes in the surface AChRs; and 2) the changes in the appropriate mRNAs appear to reflect the regulatory influences more directly.

During the past year, we have made progress in these studies suggesting that ACh transmission plays a key role in regulating transcription of these mRNAs (see Section II, Task 4). Since this method provides a rapid indication of the nerve's regulatory influence, it should allow us to obtain more direct answers to these critical questions of neural regulation of AChRs.

At the level of the muscle cell, there is now a growing body of evidence that various cations may have important regulatory effects on the metabolism of ACh receptors of skeletal muscles.

During the past year, we have published the results of studies showing that several cations (lithium, calcium, sodium) down-regulate the synthesis of AChRs in a skeletal muscle cell culture model (Pestronk and Drachman, 1987). We have now begun to use the cDNA probes to study the regulation of AChR synthesis in the muscle cell culture system. Our preliminary findings suggest that innervation-like effects can be produced in this system by: a) cholinergic agonists; b) various cations; and c) phorbol esters (see Progress, Section II-Task 4).

II. Progress (8/1/86 - 7/31/87)

Overall Progress

During the past year we have made excellent progress in our studies of regulation of AChRs in skeletal muscle. We are keeping up well with the anticipated timetable. We have completed studies supporting several of the central features of our hypothesis of neurally regulated stabilization of rapidly turning over AChRs at the neuromuscular junction. Furthermore, we are applying newer methods of molecular biology and immunochemistry, which are yielding important information regarding the fundamental goals of this project.

Task 1. To determine the time course of new AChR synthesis and insertion.

During the past year, we have completed the remaining experiments in this project, prepared a manuscript, and submitted it for publication (Ramsay et al.,

in press).

This study is based on the idea that in order to maintain a constant number of AChRs at the neuromuscular junction, the rapidly degraded AChRs (RTOs) must be replaced at a correspondingly rapid rate, much faster than previously supposed. This study was designed to evaluate the rate of synthesis and insertion of junctional AChRs after irreversible blockade of pre-existing AChRs with α -BuTx. During the initial year of this contract, we carried out several parts of this study:

We found that the synthesis and insertion of junctional AChRs was initially extremely rapid; by 24 hours, 16% of the junctional AChRs had been synthesized and inserted, while 28% were present at 48 hours. The remainder of the time curve was slower, as predicted by our previous studies of degradation of AChRs.

Control experiments showed that this rapid reappearance of α -BuTx binding sites could not be attributed to "un-binding" of α -BuTx so as to reexpose the original sites.

In order to complete this study, we have carried out two additional series of experiments during the year just ended (1986-87):

1) Degradation of junctional AChRs in the sternomastoid (SM) muscle of the mouse (Fig. 1).

Our previous studies of AChR degradation had been carried out in the diaphragm of the mouse. The synthesis experiments summarized above required a muscle (a) that could be completely blocked with α -BuTx, and (b) in which the junctional AChRs formed a discrete band suitable for dissection and measurement. For these reasons, we used the sternomastoid muscle. It was therefore necessary to ascertain whether the sternomastoid (SM) muscle showed a pattern of degradation of RTOs and stable junctional AChRs similar to that previously found in the diaphragm. We have now carried out studies of degradation of AChRs in the sternomastoid muscles, as follows:

Methods

^{125}I - α -BuTx (1.4 μg in 10 μl) was injected into the left SM muscles of 123 female Swiss mice. (Note that a slightly larger amount of labeled α -BuTx was used for AChR saturation because of possible inactivation of a fraction of the ^{125}I - α -BuTx during the iodination procedure.) At various times, from 3 hours to 19 days after labeling, groups of 6 to 17 mice were killed, and the left SM muscles removed. Care was taken to wash the muscles free of any unbound radioactivity: The muscle was flushed by injecting 0.2 ml of wash medium, and was then rinsed repeatedly until no further radioactivity was detected in the wash medium. End-plate specific radioactivity was then determined as previously described (Stanley and Drachman, 1983a, 1987).

The time point 3 hours after labeling was taken as "zero time." All subsequent counts were expressed as a fraction of the total end-plate counts present at the zero time, and the means were plotted on a log scale against time. Straight lines were fitted to the points by the method of least squares, and

half-lives of AChRs were calculated from their slopes, as previously described (Stanley and Drachman, 1983a, 1981, 1987).

Results:

The degradation curve (Fig. 1) showed an initial rapid loss of radiolabel during the first three to four days, followed by a much slower constant rate of loss thereafter. This indicated the presence of 2 classes of receptors - i.e. - RTOs and stable AChRs - in the SM muscle. The half life of the stable AChRs was 10.6 days. The calculated half-life of the RTOs was 18.05 hrs (Fig. 1 Inset). These findings, which are closely similar to those in the diaphragm, confirmed that the SM muscle has a subpopulation of junctional AChRs that are rapidly degraded as well as the population of stable AChRs.

2) Effect of α -BuTx injection on the number of junctional AChRs.

Since the method used to measure newly synthesized and inserted AChRs entails preliminary blockade by α -BuTx, we tested the possibility that the blockade procedure itself might induce a change in the number of junctional AChRs. For this purpose, the initial injection consisted of ^{125}I -labeled α -BuTx to block as well as label the AChRs in SM muscles of 13 mice. The SM muscles of these mice were relabeled after 24 hours or 48 hours by reinjection with ^{125}I - α -BuTx, and a group of controls were also labeled. After 6 hours, the SM muscles were removed, thoroughly washed for 48 hours, and the end-plate specific radioactivity determined as described above. There was no significant difference ($p > 0.1$) in the amount of bound radioactivity in the BuTx-blocked muscles, as compared with the controls (controls, 89.1 femtomoles of ^{125}I - α -BuTx/muscle ± 3.3 (SEM); 24 hours, 82.2 ± 5.3 ; 48 hours 95.3 ± 3.8) which indicates that α -BuTx blockade does not induce a change in the number of AChRs.

Interpretation:

The results of this study show that synthesis and insertion of junctional AChRs is appropriately rapid to compensate for the degradation of both the RTOs and the stable AChRs at the neuromuscular junction. New receptors are inserted at the rate of 16% within 24 hours and 28% at 48 hours. This is significantly faster than the rate needed to replace the stable AChRs alone, which would require replacement of only 5.5% of the total receptor population during the first day.

This finding of rapid appearance of a subpopulation of AChRs at neuromuscular junctions is consistent with previous reports from this laboratory (Stanley and Drachman, 1983a, 1987) and others (Bevan and Steinbach, 1983) of rapid degradation of a subpopulation of junctional AChRs in the diaphragm. Since the present experiments required the sternomastoid muscle, we have now studied the pattern of degradation of AChRs in the sternomastoid as described above, and shown that it closely parallels that of the diaphragm. We have also carried out control experiments which showed: a) that the initial injection of α -BuTx totally blocked pre-existing AChRs; b) that "un-binding" of toxin could not account for the rapid reappearance of α -BuTx binding sites; c) that blockade of the AChRs per se could not induce an increase in junctional AChRs.

The evidence thus indicates that new AChRs are rapidly synthesized and inserted into the neuromuscular junction, sufficient to replace those lost by both rapid and slow degradation. This confirms the concept of two subpopulations of AChRs at the normal innervated neuromuscular junction.

We have presented evidence elsewhere that a proportion of the RTOs serve as precursors for the stable AChRs at the neuromuscular junction (Stanley and Drachman 1983b, 1987 and tasks 2 and 3.) Further, the stabilization process requires some influence of the motor nerve. It will be important to determine: (a) the mechanism by which the motor nerve converts RTOs to stable AChRs, and (b) the biochemical or structural differences between RTOs and stable AChRs.

Task 2. To determine whether denervation prevents post-insertional stabilization of junctional AChRs.

We have postulated that the RTOs are precursors of stable receptors at the neuromuscular junction, and that the stabilization process depends on some influence of the motor nerve (Stanley and Drachman, 1983b). In this study, we have tested this hypothesis in the mouse sternomastoid muscle by: 1) labeling junctional ACh receptors with ^{125}I - α -BuTx; 2) denervating the SM muscle; 3) following the fate of the RTOs through a 6 day period when they were either degraded or converted to stable ACh receptors.

Our hypothesis predicts that denervation should prevent the conversion of RTOs to stable receptors, and would therefore result in a deficit in the number of stable AChRs in the denervated muscles as compared with the innervated muscles. Our results as described below strongly support this hypothesis (Fig. 2, Table 1).

Methods:

1. Labeling of junctional AChRs with "enhanced populations of RTOs."

As noted previously, the RTO subpopulation normally constitutes only a relatively small fraction (approximately 15%) of the total AChR population at the neuromuscular junction. In order to detect changes in stabilization of these AChRs, it is helpful to follow a population with an enhanced proportion of labeled RTOs. For this purpose, the SM muscles of 20-25 gram female Swiss mice were injected with 0.75 μg α -BuTx, to block all pre-existing AChRs. Seven days later, the "new" AChRs are labeled with ^{125}I - α -BuTx. At that time approximately 30% of the AChRs in this pool are RTOs.

2. Unilateral Denervation; measurement of stabilization of AChRs.

Three separate series of experiments involving 135 mice were used for this experiment. The SM muscles were bilaterally blocked with α -BuTx, as above, to produce enhanced populations of RTOs.

6 days later, the left SM muscles in all mice were denervated surgically. 24 hours later (in order to allow the effect of denervation to take place) the SM muscles were bilaterally labeled by injection of ^{125}I - α -BuTx.

Groups of mice were killed, 3 hours after labeling, and then at daily

intervals for 5 days. Both SM muscles were removed from each animal, and endplate-specific radioactivity was measured as previously described (Stanley and Drachman, 1983a, 1987).

Results:

The results are presented in Fig. 2 and Table 1.

The major finding of this study is that denervation resulted in a significant deficit of AChRs that occurred promptly in the muscles with a mixed population of RTOs and stable AChRs. The deficit, which is attributed to failure of stabilization of AChRs, was 6.6% by 1 day after labeling (2 days post-denervation), and increased to 10.4% by 4 days after labeling (5 days post-denervation).

Control experiments described below show that the denervation-induced AChR deficit cannot be attributed either to a) asymmetry of mouse SM muscles, or to b) accelerated loss of stable AChRs per se.

Control Experiments

a) Symmetry of SM muscles:

Methods: Since the results of the above experiments are based on comparisons of the left (denervated) and right (innervated) SM muscles in the experimental female Swiss mice, it was important to determine whether there is a consistent discrepancy or significant variability in the numbers of junctional AChRs in the paired (left and right) muscles of the mice. We therefore labeled junctional AChRs with "enhanced populations of RTOs," exactly as in the experimental group, but the muscles were not denervated. The radioactivity bound to junctional AChRs was measured as above, and the ratio of radioactivity bound to the left/right SM muscles expressed as a percentage.

Results: The results showed that there was no consistent difference between the number of junctional AChRs in the left and right SM muscles (see Fig. 2 and Table 1).

b) Effect of denervation on stable AChRs: Denervation causes acceleration of degradation of stable junctional AChRs, but this does not occur until after a latent period. Since the present experiment follows the fate of AChRs during a 6 day period after denervation, an important control is to evaluate the effect of denervation on the turnover of stable AChRs during the 6 day period.

Methods: Labeling of stable AChRs: AChRs are first labeled by injection of ^{125}I - α -BuTx. By the end of 6 days (i.e. - >6 half-lives), the number of labeled RTOs remaining is negligible (2). The labeled AChRs then consist entirely of the stable subpopulation.

At this time (i.e. - 5 days after labeling), the left SM muscle was surgically denervated. Groups of mice were killed at intervals of 3 hours to 7 days later. The radioactivity bound to their junctional AChRs was measured as above, and the ratio of radioactivity bound to the left/right SM muscles

expressed as a percentage.

Results: The results showed no deficit of stable AChRs in the denervated SM muscle, as compared to the innervated side, during the first 5 days after denervation. However, on the 6th day, the degradation rate increased, and a significant deficit of AChRs then appeared in the denervated SM muscle. These results show that the early deficit of AChRs seen in the experimental group (above) cannot be attributed to loss of stable AChRs.

Statistics:

Because of the crucial importance of this experiment, and our anticipation that the differences might be relatively small, we used: a) large numbers of animals in each group; b) several methods of parametric and non-parametric analysis, including the Wilcoxon ranked pairs test, the Mann-Whitney U test (one tailed), analysis of variance, and analysis of covariance. We thus compared groups on each individual day of the experiment, and also compared trends of the curves generated by the experimental and control experiments.

The results of statistical calculations showed that the differences between denervated and non-denervated muscles were highly significant ($p < 0.002$) for the experimental group at all time points from 1 day after labeling through 5 days. By contrast, none of the control groups except the 6 day post-denervation stable AChRs showed significant differences between left and right muscles.

Analysis of variance showed a highly significant difference between the experimental and each of the two control groups ($p < 0.01$).

Analysis of covariance showed a highly significant difference between the experimental curve and each of the control curves ($p < 0.002$).

Interpretation:

We have postulated that the RTOs are precursors of the stable junctional receptors, and that the stabilization process depends on an influence from the motor nerve. Our hypothesis predicts that denervation should prevent the stabilization of RTOs, and thus result in a deficit of stable ACh receptors at the neuromuscular junction.

The results of the present experiments strongly support this interpretation. At the time of denervation, and at the time of labeling the AChRs, there was no difference in the number of AChRs in the SM muscles of large groups of mice. However, by 24 hours later, there was a significant deficit on the denervated side, which increased over the course of the next 4 days. This deficit is attributable to failure of conversion of RTOs to stable receptors.

The deficit cannot be accounted for by rapid loss of the pre-existing stable receptors, since our control experiments showed that acceleration of degradation of stable AChRs does not begin until 6 days after denervation---much later than the appearance of the deficit. We also ruled out a trivial artifact of asymmetry of mouse SM muscles to explain the deficit on the left side.

Thus, our results strongly support the hypothesis that the RTOs are

precursors for the stable receptors, and that an intact motor nerve supply is necessary for their conversion to stable receptors. This concept has certain interesting implications regarding the normal biology and pathogenesis of disorders of the neuromuscular junction. From the military point of view this has important application to recovery from toxic inactivation of AChRs.

1. The present findings are consistent with previous evidence that motor innervation plays an important role in the stabilization process. During development, AChRs are not stabilized prior to innervation of skeletal muscle (Schuetze and Role, 1987). After denervation, the degradation rate of pre-existing stable receptors accelerates (Stanley and Drachman 1981; Bevan and Steinbach, 1983; Salpeter and Loring, 1985; Schuetze and Role, 1987).

2. The stabilizing action of the motor nerve must be rapid. In the denervated muscles, the majority of the receptor deficit is apparent by 24 hours.

3. The mechanism by which the motor nerve conveys its stabilizing influence remains to be determined. One of the goals of this project is to study how the motor nerve stabilizes junctional AChRs. Elsewhere in this annual report (see task 7) we present preliminary evidence that ACh transmission may be involved in the maintenance of stability of junctional AChRs.

4. The differences between rapidly and slowly turned-over AChRs have as yet been defined only in terms of their metabolic stability. Undoubtedly this must reflect some critical biochemical or structural differences between the two types of receptors. Possible changes that might stabilize AChRs include chemical modifications of the AChR molecules (for example by phosphorylation, acylation or methylation); attachment of AChRs to cytoskeletal elements; alterations of the surrounding microenvironment of the synaptic membrane; or localization of the stable AChRs to a region of the membrane that turns over slowly (Salpeter and Loring, 1985).

5. The present findings, together with the results presented previously (Stanley and Drachman, 1987) suggest that stabilization of AChRs at the neuromuscular junction occurs after transcription, translation, and insertion of the AChR molecules. RTOs and stable receptors would therefore have a common origin, and it would be unnecessary to postulate the synthesis of two different species of AChR molecules at mature innervated neuromuscular junctions. (This point should not be confused with recent evidence which suggests that extrajunctional AChRs in immature muscles may have a different subunit composition (Mishina et al., 1986).)

6. From a teleological point of view, the conversion of RTOs to stable AChRs has several advantages. The fact that RTOs are continuously being replaced at a rapid rate affords considerable protection against loss of AChRs from whatever cause. This rapid turnover can account for the observation that recovery from blockade of AChRs with irreversible blocking agents such as α -BuTx occurs more quickly than expected. If all the AChRs were turned over slowly with a half life of the stable AChRs, it would take approximately 6 days to recover the 25 to 30% that are required to maintain synaptic transmission. The rapid turnover of a sizeable subpopulation of RTOs results in recovery of neuromuscular transmission within 2 to 3 days, consistent with experimental observations.

7. The fact that the majority of junctional receptors are stable, and turn over at a slow rate is also advantageous to the economy of the muscle cell. If all the junctional AChRs turned over at the rapid rate of RTOs, it would greatly increase the resources expended in maintaining the complement of AChRs.

Summary:

The results of our studies to date suggest a working concept of the turnover of junctional AChRs that we have illustrated in cartoon form in Figure 3. A single molecular species of AChRs is synthesized and inserted at the neuromuscular junction, representing the pool of RTOs. The large majority of RTOs are lost through rapid degradation. A fraction of RTOs are modified to become stable AChRs. This "stabilization" process requires some action of the motor nerve. Thus, the neuromuscular junction contains both RTOs and stable AChRs, but all the receptors originate as RTOs. The completion of this project has represented a major commitment during the past year. We are now in the process of writing a definitive paper on it, and plan to incorporate these findings in our proposed mathematical model of junctional AChR kinetics.

Task 3: To determine whether depletion of rapidly turning over AChRs results in a deficit of stable AChRs.

This project was completed last year, and a publication (Stanley and Drachman, 1987) is in press:

Task 4 (Objective II): To determine the effects of cations on AChR metabolism in vitro.

During the first year of this project, we carried out a study of the effects of the cations lithium, calcium and sodium on the metabolism of extrajunctional AChRs, using a rat skeletal muscle tissue culture system. Our findings showed that each of the cations reduced the apparent "synthesis" of extrajunctional AChRs in this system, as measured by ^{125}I - α -BuTx binding. These findings have now been published (Pestronk and Drachman, 1987).

Task 4a: Use of cDNA Probes to Study Regulation of Extrajunctional AChR Synthesis: Measurement of Messenger RNAs for Receptors.

During the past few years, cDNA probes have become available for the RNA messages for most of the subunits of AChRs of several species (Merlie et al., 1983; LaPolla et al., 1984; Boulter et al., 1985; Mishina et al., 1986). These powerful tools enable one to estimate the amount of the relevant mRNA directly, by hybridization techniques (Merlie et al., 1984; Goldman et al., 1985). They have major advantages in studying certain aspects of regulation of AChR synthesis, including:

a) Changes in message levels occur promptly, well before changes in surface AChRs, thus facilitating experiments that can only be carried out on a short term basis.

b) The changes in appropriate mRNAs are thought to be closer to the level at which regulation of synthesis of AChRs takes place, as compared with the more remote and indirect effect of change in the amount of AChR expressed on the surface membrane.

Because of these important conceptual and practical advantages, we have devoted a major effort to acquiring the technology and skills for preparing and using cDNA probes for rodent AChR. We are applying these methods to two projects that are directly germane to the goals of this contract - i.e., understanding the mechanisms of regulation of AChRs:

- 1) The role of ACh transmission in the regulation of extrajunctional AChR synthesis, in vivo.
- 2) The role of cations, messengers, and neurotransmitters in the regulation of AChR synthesis, in vitro.

Methods:

RNA extraction from whole skeletal muscle - Total RNA is extracted from skeletal muscle by the following procedure: the muscle is minced, and homogenized in 30 vols of 50 mM Tris, pH 7.5, - 100 mM NaCl - 5 mM EDTA - 1% SDS, with a Brinkman polytror at setting #5 for 3 10-second intervals. Proteinase K is then added at a final concentration of 250 µg/ml, and the mixture incubated for 60-90 minutes at 37°C. It is then extracted twice with phenol:chloroform, 1:1, washed twice, with chloroform, and RNA is precipitated by the addition of 1/10 vol 3M NaAcetate and 2.2 volumes ethanol at -20°C overnight. The RNA pellet is dissolved in water, and the concentration measured spectrophotometrically at OD 260, with the reference value of OD 1.0 = 40 µg/ml.

RNA extraction from cultured cells - 60 mm dishes are washed twice with PBS, and cells are scraped off the dish with a rubber policeman. 5 dishes are pooled, and cells collected by centrifugation. They are resuspended in lysis buffer (0.14 M NaCl; 1.5 mM MgCl₂; 10 mM Tris, pH 8.6; 0.5% Triton-x-100; 10 mM vanadyl-ribonucleoside complexes) on ice for 5-10 minutes, then centrifuged. The supernatant is removed, and incubated with an equal volume of 0.2 M Tris, pH 7.5; 25 mM EDTA; 0.3 M NaCl; 2% SDS, and 250 µg/ml Proteinase K for 30 minutes at 37°C. The remaining steps are the same as for skeletal muscle.

Preparation of mRNA: mRNA is eluted from poly-oligo-d (T) columns as described (Maniatis et al., 1982), and quantitated spectrophotometrically.

RNA agarose gel electrophoresis and Northern blotting: Total RNA (5-10 µg) or mRNA (0.1-0.5 µg) is denatured in a formamide-formaldehyde solution and applied to a 1.5% agarose-6% formaldehyde gel. After electrophoresis is completed, the gel is marked, and overlaid with a piece of nitrocellulose paper. Transfer is carried out as described (Maniatis et al., 1982). The nitrocellulose is air dried, then heated at 80°C for 2 hrs, and stored.

Preparation of "Slot Blots": Slot blots can be used for measurement of specific mRNAs, provided that the cDNA probe being used is known to bind only to a single band of RNA on gel electrophoresis. We first established on Northern blots of agarose gels that the cDNA probe for the α -subunit of AChR binds only to one band of RNA from innervated and denervated skeletal muscles, and muscle cell cultures (Fig. 4).

Slot blotting has many advantages over Northern blots for our experiments: a) Slot blots require 10 to 100-fold less RNA than for Northern blots; b) binding of RNA in this system is far more efficient (quantitative), as compared to Northern blots; c) more samples can be run simultaneously (72 vs 20 for

Northerns), thus permitting reliable intra-experiment comparisons.

Total RNA (1 μ g) is denatured in a formamide - formaldehyde solution, and applied to a well of an S&S "Minifold" slot blotting apparatus into which a nitrocellulose filter is inserted. The well is rinsed with buffer three times, and then the nitrocellulose filter is removed, air dried for 1 hour at room temperature, then heated to 80°C for 2 hours and stored.

Preparation of labeled cDNA: A cDNA clone for the α -subunit of the acetylcholine receptor was obtained as an M13 insert from Dr. J. Merlie of the Washington University School of Medicine. JM 103 cultures are transfected with this DNA. Total DNA is extracted, and the RF form of M13 is isolated by cesium chloride centrifugation, to be labeled by nick translation.

Nick translation: The double stranded insert containing the cDNA for the AChR subunit was excised from the RF form of the M13 vector by digestion with EcoRI. The insert was isolated by agarose gel electrophoresis. The insert was labeled by incorporation of 32 P-dCTP, using a standard "nick translation" procedure (Maniatis et al., 1982). The probe was then boiled to denature the DNA, and added to the hybridization solution.

RNA-DNA hybridization: Nitrocellulose blots are prehybridized for 4 hours at 42°C in 50% formamide-3 x SSC-10 x Denhardt's -0.1% SDS-100 μ g/ml salmon sperm DNA. The labeled probe is added to a similar solution, except that it contains 5x SSC, and hybridization carried out for 24-72 hours at 42°C. The blots are then washed for 10 minutes at 60°C in 2x SSC-0.1% SDS. They were then washed for 45' at 60°C in 0.2x SSC - 0.1% SDS. The blots are then exposed to X-ray film for appropriate periods. Binding of DNA is quantitated on a scanning densitometer (LKB).

Experiments:

Experiment 1: The role of ACh transmission in the regulation of extrajunctional AChR synthesis in vivo.

Denervation of skeletal muscle is known to result in an increase in the amount of mRNA for the AChR. The purpose of these experiments is to determine whether this effect of denervation can be attributed to loss of ACh transmission.

In these experiments, we either a) surgically denervated the rat soleus muscle by sciatic nerve avulsion; or b) blocked quantal ACh transmission, using botulinum toxin. Groups of rats were killed at intervals of 24 hours to 12 days later. We measured the mRNA for the α -subunit of AChR, using either Northern blot, or more recently the sensitive "slot-blot" techniques.

We first determined the time course of the change following denervation. 58 soleus muscles of female Sprague-Dawley rats were denervated by sciatic nerve section. At time intervals from 1 to 12 days later, groups of animals were killed and the specific mRNA in the soleus muscles was measured as described above. Our results show that the level of mRNA for the α -subunit is just detectable in innervated muscles by Northern blot analysis and is easily seen on slot blots. By 1 day post-denervation, an approximately 10-fold increase per muscle is already apparent. The increase reaches a peak at 3 days, and then plateaus for the remainder of the 12 day period examined (Fig. 5).

We next compared the effects of blockade of quantal ACh transmission using botulinum toxin, with those of denervation. In two series of experiments, soleus muscles of 50 female Sprague-Dawley rats were injected with 1.5×10^{-9} gm of Type A botulinum toxin in 30 μ l of Ringer solution. Fifty soleus muscles were denervated by sciatic nerve section and avulsion. Groups of rats were killed, and the soleus muscles were removed at intervals of 1 1/2 to 7 1/2 days later for measurement of mRNA. Control (innervated) muscles were also removed and assayed at the same times.

The results in the two series of experiments were closely similar. The rise of mRNA following denervation was much more rapid than that following botulinum toxin treatment. By 1 1/2 days after treatment, the specific mRNA had risen more than 7-fold in the denervated muscles, and slightly less than 4-fold in the botulinum-treated soleus muscles. The AChR-mRNA level peaked by 2 1/2 days after denervation, and plateaued thereafter. By contrast, the specific AChR-mRNA levels in the botulinum-treated muscles consistently lagged behind those in the denervated muscles until the 7 1/2 day time point, when they reached nearly the same levels (Fig. 6).

Interpretation:

These results are interpreted as follows:

Denervation results in an increase of mRNA for the α -subunit of AChR, confirming results published by other laboratories (Merlie et al., 1984; Goldman et al., 1985).

Blockade of quantal ACh release by botulinum toxin produces a denervation-like increase in mRNA for the ACh receptor (α -subunit).

This increase lags behind that produced by surgical denervation.

Control experiments showed that botulinum toxin's blockade of quantal ACh release is maximal by 3 hours, producing complete paralysis to electrical stimulation. Our experiments were carried out so as to inject the muscles with BOT at least 4 hours earlier than the surgical denervation in the comparable muscles; therefore, the lag in increase of mRNA cannot be attributed to delayed onset of ACh blockade by BOT.

Thus, the effect of botulinum toxin is similar, but not equivalent, to that of denervation.

Since botulinum toxin blocks the quantal release of ACh from motor nerve endings, but does not block non-quantal ACh release, this suggests that the nerve's regulatory effect may be mediated by both quantal and non-quantal ACh release.

We plan to test the effects of total blockade of quantal and non-quantal ACh transmission, using α -BuTx.

Finally, these results demonstrate that the measurement of mRNA by the slot blot technique is an appropriately sensitive method for detecting even moderate changes in regulation of AChR metabolism.

Thus far, we have measured only the mRNA for the α -subunit of AChR. We have now obtained, and are presently preparing labeled cDNA probes for the β - and δ -subunits in addition. We plan to compare the effects of denervation and

ACh blockade, using these probes as well, since the β - and delta-subunit mRNAs may reflect the neural influence differently, and perhaps more sensitively than the α -subunit.

Experiment 2: The role of cations, messengers, and neurotransmitters in the regulation of AChR synthesis, *in vitro*.

During the past year, we have begun to carry out experiments to determine whether certain innervation-like treatments with cations and neurotransmitters can down-regulate the amount of mRNA for the α -subunit of AChR.

Previous studies in our laboratory (Pertronk and Drachman, 1987) have shown that the cations lithium, calcium and sodium, can down-regulate the expression of AChRs on the surface of myotubes in muscle culture. There is evidence that the ACh analogue carbachol and the phorbol ester TPA may also down-regulate extrajunctional AChRs, similar to the effect of innervation. Using the cDNA probes, we are now able to examine these questions more precisely, earlier, and at a more fundamental level (at the level of mRNA rather than insertion of a surface protein).

Methods: Thus far, we have carried out two preliminary sets of experiments using these agents in muscle cultures. Rat muscle cells were grown in culture dishes under standard conditions (controls), or with the addition of 1.5 mM lithium plus 0.1 μ M A23187 (ionophore); 100 μ M carbachol; or the phorbol ester 160×10^{-9} M TPA, for 24 hours. The muscle cells were then removed from the dishes and the mRNA for the α -subunit of AChR measured as described above.

Control experiments have shown that these agents do not damage the cultured muscle cells, as assessed by the criteria:

1. Morphology, using phase microscopy.
2. Measurement of total protein per culture dish (Lowry method).
3. Measurement of creatine kinase (CK) specific for skeletal muscle.
4. Protein synthesis, as measured by incorporation of 3 H-leucine.

Results: Results of these preliminary experiments (Fig. 7) show that each of these three treatments causes a reduction of the specific mRNA for the α -subunit of the AChR.

These preliminary findings add support to the concept that ACh transmission and entry of cations into muscle cells may serve the "neurotrophic" function of down-regulation of synthesis of extrajunctional AChRs.

Task 7. To determine whether ACh transmission plays a role in maintenance of stability of junctional AChRs.

We and others have previously shown that stable junctional AChRs are degraded slowly provided that the muscle remains innervated. Following denervation, after a lag period, the rate of degradation of pre-existing junctional AChRs becomes accelerated (Stanley and Drachman, 1981; Bevan and Steinbach, 1983; Salpeter and Loring, 1985). This experiment is designed to determine whether ACh transmission plays a role in maintenance of the stability of these AChRs.

Methods: Pre-existing AChRs in the flexor digitorum brevis (foot) muscles of female Swiss mice were labeled by bilateral injections of $0.5 \mu\text{g } ^{125}\text{I}-\alpha\text{-BuTx}$. Five days later, when only stable AChRs remained (since all the labeled RTOs had been degraded), they were either a) denervated surgically, or b) injected repeatedly with botulinum toxin ($1.5 \times 10^{-10} \text{ gm}$ on day 0, and $1.0 \times 10^{-10} \text{ gm}$ on days 5, 12 and 19) to block quantal ACh transmission. Groups of mice were killed at intervals of 0 to 29 days, and the radioactivity bound to junctional AChRs measured by gamma counting.

Results: Thus far, we have carried out two sets of preliminary experiments: one to determine the time course of denervation-accelerated degradation of junctional AChRs in the FDB muscles, and the second to examine the effect of botulinum treatment. These preliminary studies have used approximately 70 white Swiss mice. The initial findings (Fig. 8) suggest the following:

In the FDB muscle, denervation induces accelerated degradation of stable AChRs beginning by about day 12.

Botulinum toxin treatment (i.e. - blockade of quantal ACh transmission) induces accelerated degradation of stable AChRs in the FDB muscle.

The onset of this effect is delayed, compared to that of denervation. It appears to begin 20 days after initiation of botulinum treatment!

This suggests that ACh transmission plays an important role in the maintenance of stability of junctional AChRs. However, quantal ACh transmission appears not to account for the entire effect of the nerve on AChR stability.

We plan to repeat these experiments. We will use the FDB muscle as well as another muscle in which accelerated degradation of stable AChRs occurs earlier after denervation (e.g. - the sternomastoid or the soleus). If the present results are confirmed, we will next evaluate the effect of complete blockade of quantal plus non-quantal ACh transmission, using $\alpha\text{-BuTx}$. We anticipate that this strategy should reproduce the exact time-course of the effect of denervation on stable junctional AChRs.

Task 7a: Mobility of Junctional AChRs: Movement induced by nerve terminal outgrowth.

This study was undertaken to examine another aspect of the turnover of stable junctional AChRs: i.e. - their ability to move during nerve terminal outgrowth. It relates directly to the influence of ACh transmission on turnover of stable AChRs.

It is well known that blockade of ACh release by botulinum toxin results in sprouting of nerve terminals (Duchen and Stritch, 1968). More recently, we have shown that the post-synaptic membrane also enlarges during this process, as shown by combined silver-cholinesterase staining (Pestronk and Drachman, 1978, 1985). In this study, we have tested two hypotheses: a) that the AChR-containing area of the junction may also enlarge; and b) that pre-existing stable AChRs might become mobile, and actually move within the postjunctional membrane, during the process of nerve terminal sprouting (Yee and Pestronk, 1987).

Methods:

Botulinum toxin injections: To induce sprouting, Type A botulinum toxin,

diluted in mammalian Ringer solution (1.2×10^{-9} gm in 30 μ l) was injected directly into the surgically exposed soleus muscles of adult female Sprague-Dawley rats.

Immunocytochemical staining of AChRs: This method depends on the binding of α -BuTx to AChRs, followed by immunocytochemical staining of the α -BuTx. We either: a) labeled the AChRs *in vivo*, by direct intramuscular injection of 2 μ g of α -BuTx in 30 μ l of Ringer solution; or b) labeled the AChRs *in vitro* by incubating cryostat-cut longitudinal sections of frozen muscle with 0.2 μ g/ml α -BuTx. The remainder of the immunocytochemical staining procedure is described elsewhere (Yee and Pestronk, 1987), and depends on the use of a specific rabbit anti- α -BuTx antibody followed by a standard PAP staining procedure.

Pre-existing AChRs: To follow the movement of pre-existing stable AChRs, the receptors were first labeled with α -BuTx *in vivo* as above, and treated with botulinum toxin to induce sprouting 3 days later, when virtually all the remaining labeled receptors were stable AChRs (see above, task 2).

New AChRs: To demonstrate newly inserted AChRs, we first blocked pre-existing AChRs with α -BuTx, 6 days after treatment with botulinum toxin. One day later, 125 I- α -BuTx was injected into the same muscle, to label new AChRs that had subsequently been inserted.

Staining of Neuromuscular Junctions: Cholinesterase at neuromuscular junctions was stained as previously described (Pestronk and Drachman, 1978, 1985).

Results and Comments: The outstanding results of this study showed that:

The AChR-containing postsynaptic membrane enlarged from a normal value of 39 ± 0.8 μ m in length, to 51 ± 1 μ m long within 7 days after botulinum toxin treatment.

Pre-existing AChRs participated in the elongation, moving outward to accompany the sprouting nerve terminals (Fig. 9).

In addition, new AChRs were added throughout the entire length of the enlarged junctions.

These findings show that inhibition of ACh transmission can result in: a) enlargement of the AChR-containing postsynaptic membrane at the neuromuscular junction; and b) striking movement of pre-existing stable AChRs.

This work provides an independent new line of evidence that ACh transmission plays an important role in maintenance of the stability of junctional AChRs.

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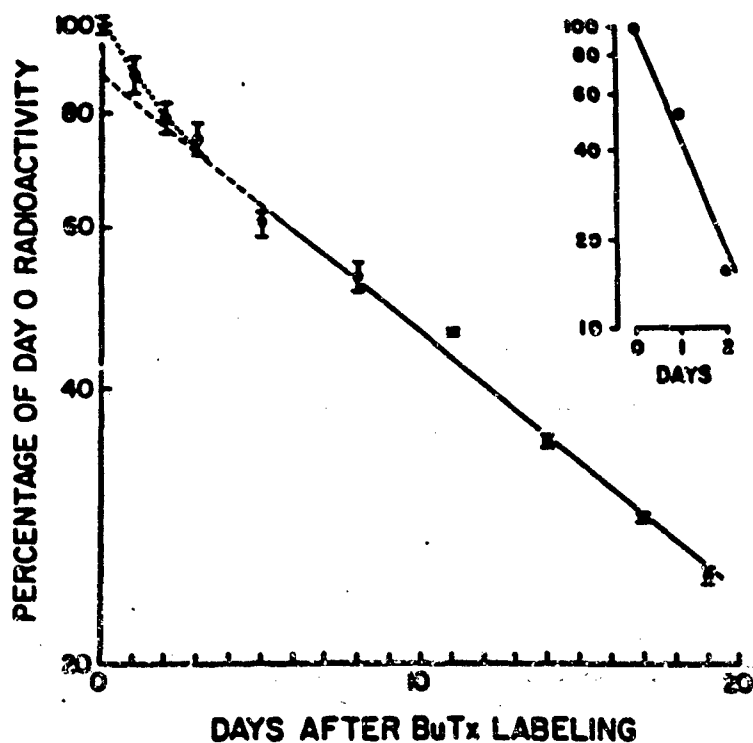


Fig. 1 Degradation of AChRs in sternomastoid muscles. The left sternomastoid muscle was labeled by direct injection of ^{125}I - α -BuTx. At intervals from 3 hours to 19 days later, groups of mice were killed, and the endplate-specific radioactivity was counted. Bound radioactivity is expressed as a percentage of the endplate counts present at zero time (3 hr after labeling). Note initial rapid loss of radioactivity (attributable to RTOs), followed by a steady slower rate of loss (of stable AChRs). Inset - loss of RTOs after contribution of stable AChRs has been subtracted. Half-life = 18.05 hrs.

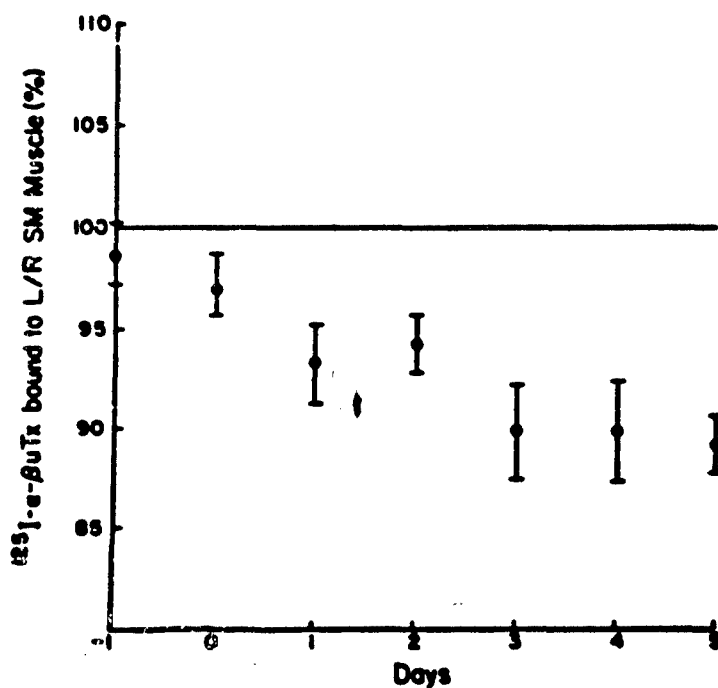


Fig. 2 Denervation prevents stabilization of junctional AChRs. Sternomastoid (SM) muscles of mice were treated to produce neuromuscular junctions with enhanced populations of RTOs. The left SM muscle was denervated on day -1, and both muscles were labeled with $^{125}\text{I}-\alpha\text{-BuTx}$ on day 0. Mice were killed at daily intervals, the radioactivity remaining bound to each SM muscle was counted, and the radioactivity bound to the denervated (left) muscle was expressed as a percentage of the radioactivity bound to the innervated (right) muscle for each mouse. Means $\pm$ SEM are shown. Note that there was a significant deficit in bound $^{125}\text{I}-\alpha\text{-BuTx}$ (i.e. - AChRs) in the denervated muscles by 24 hours after labeling ($p < 0.002$).

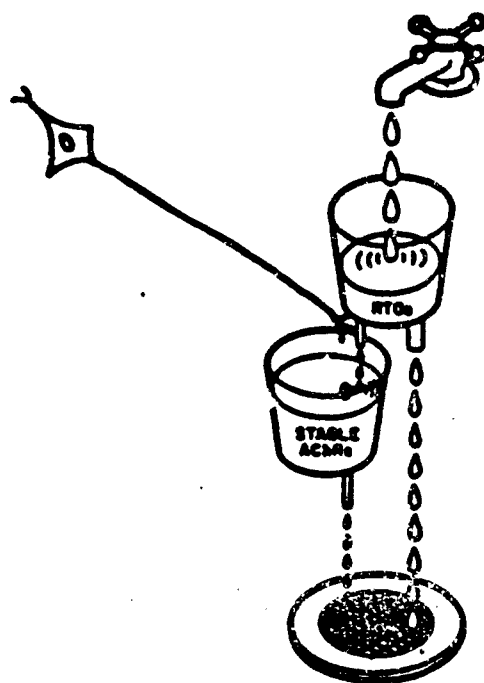


Fig. 3 Cartoon illustrating working hypothesis of turnover of AChRs at neuromuscular junctions.
AChRs are synthesized, assembled and inserted at the neuromuscular junction from a single source, contributing to the pool of RTOs. The majority of RTOs are lost through degradation. A fraction of RTOs are modified as a result of some action of the motor nerve, and join the pool of stable AChRs.



Fig. 4 Effect of denervation and botulinum toxin treatment on α -subunit mRNA. Northern blot showing hybridization of α -subunit cDNA against soleus muscle RNA. Lanes 1A and 1B have 20 μ g RNA per well, lanes 2A-3B have 10 μ g RNA. 1A: 24 hours after denervation; 1B: 24 hours after BOT injection; 2A: 48 hours after denervation; 2B: 48 hours after BOT injection; 3A: 96 hours after denervation; 3B: 96 hours after BOT injection.

Note that at all time points the increase of AChR-mRNA is greater for denervation than for botulinum treatment. Also, hybridization reveals only single bands of mRNA.

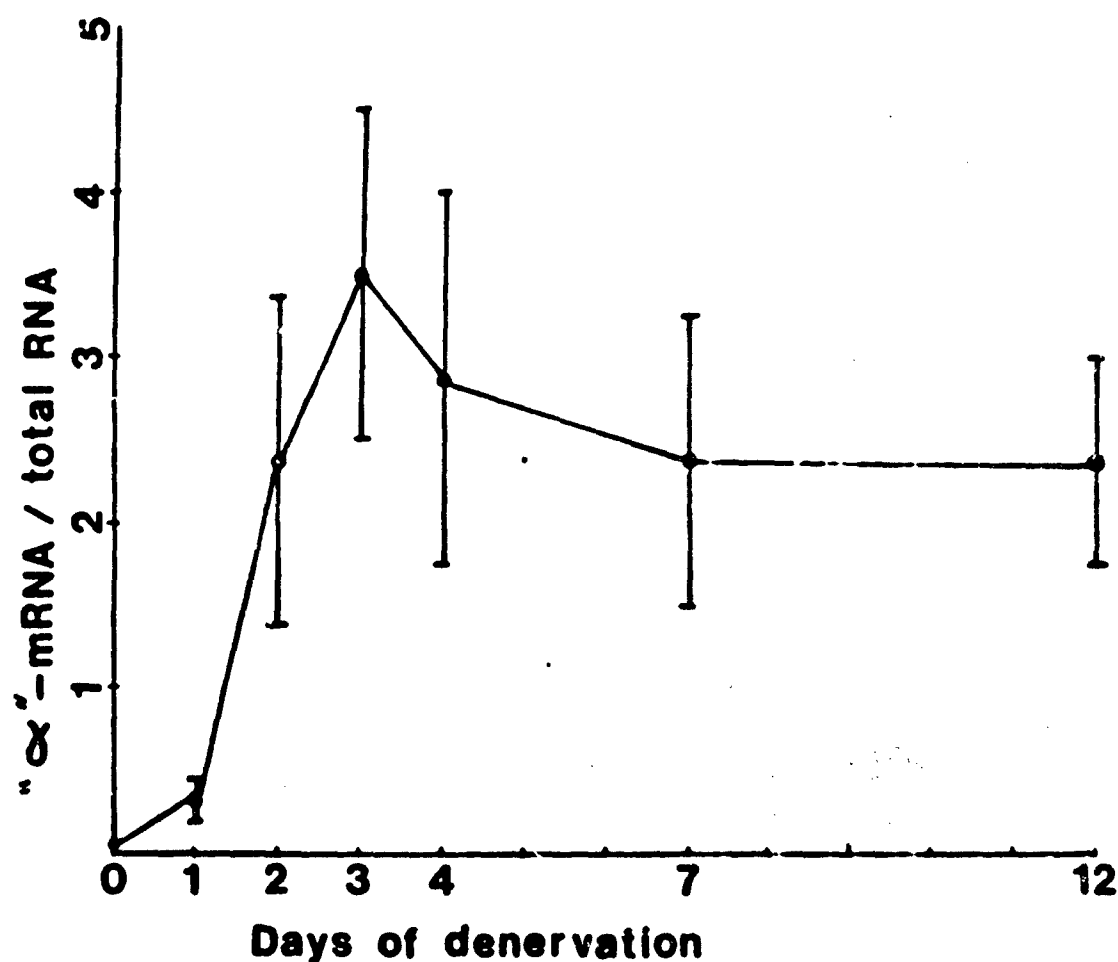


Fig. 5 Time course of the effect of denervation of α -subunit mRNA levels. Rats were denervated by avulsion of the sciatic nerve, and RNA was extracted from the soleus muscle at the indicated times. Each point represents the mean $\pm$ S.D. for 8-10 muscles. mRNA was estimated from the measured density of the autoradiograph of a Northern blot, which is proportional to the mRNA of the α -subunit. Results based on the α -subunit mRNA per microgram of total RNA, and are expressed as scanner units. Note the rapid rise, and subsequent plateau.

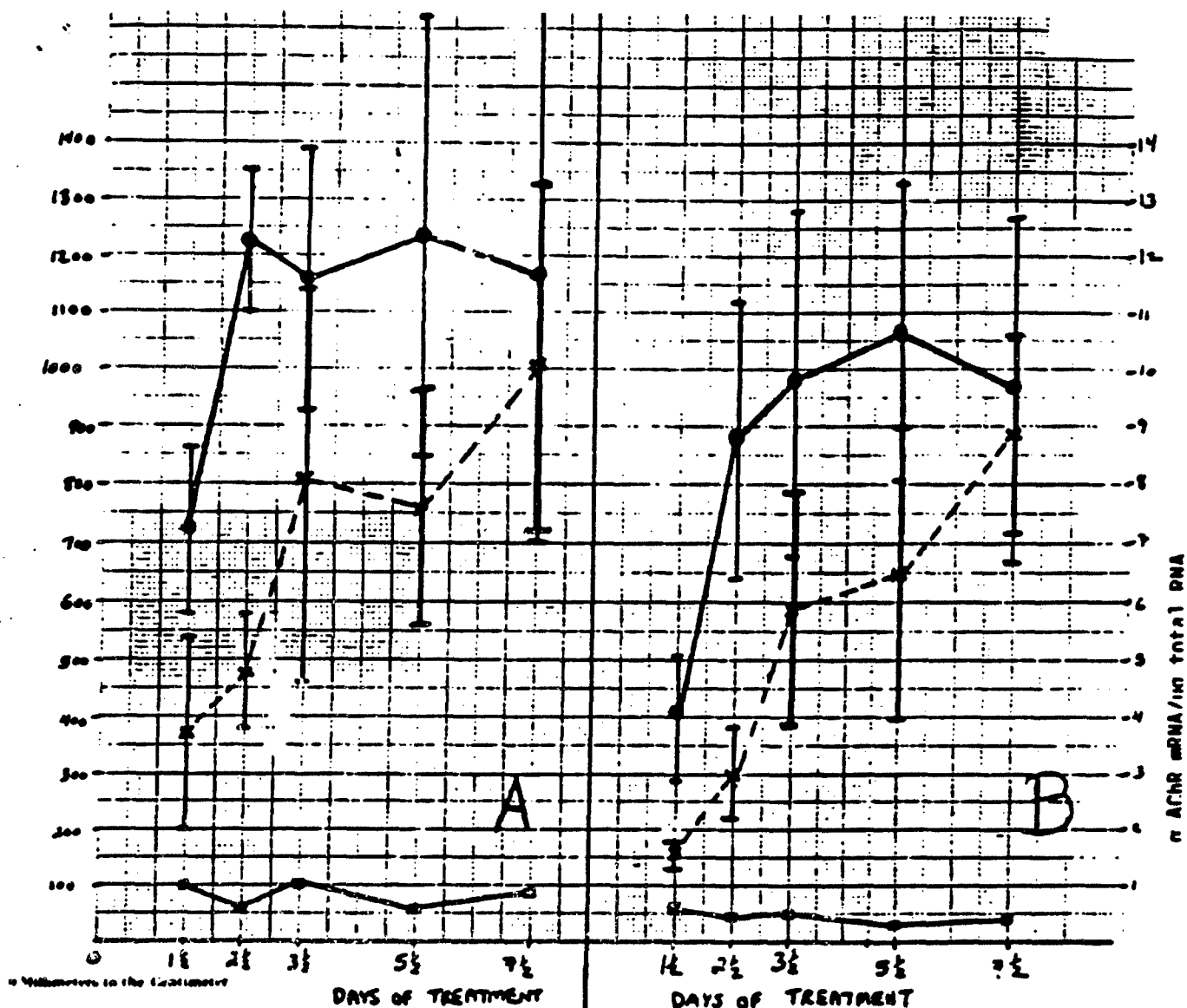


Fig. 6 Effect of denervation and botulinum toxin treatment on α -subunit mRNA levels over time.

Rats were denervated by avulsion of the sciatic nerve, or injected with botulinum toxin. Total RNA was extracted from the soleus muscle at the indicated times. Each point represents the mean $\pm$ S.D. of 5 muscles, except for control points, which are the mean of 2 muscles. mRNA levels were estimated by scanning densitometry of autoradiograms of Northern blots. Results are given in terms of α -subunit mRNA per whole muscle (A); or in α -subunit mRNA per μ g total RNA (B) (expressed in arbitrary scanner units).

● — ● = denervation
 x — — — x = botulinum treatment
 □ — — — □ = control

Note that the effect of denervation is greater than that of 3OT.

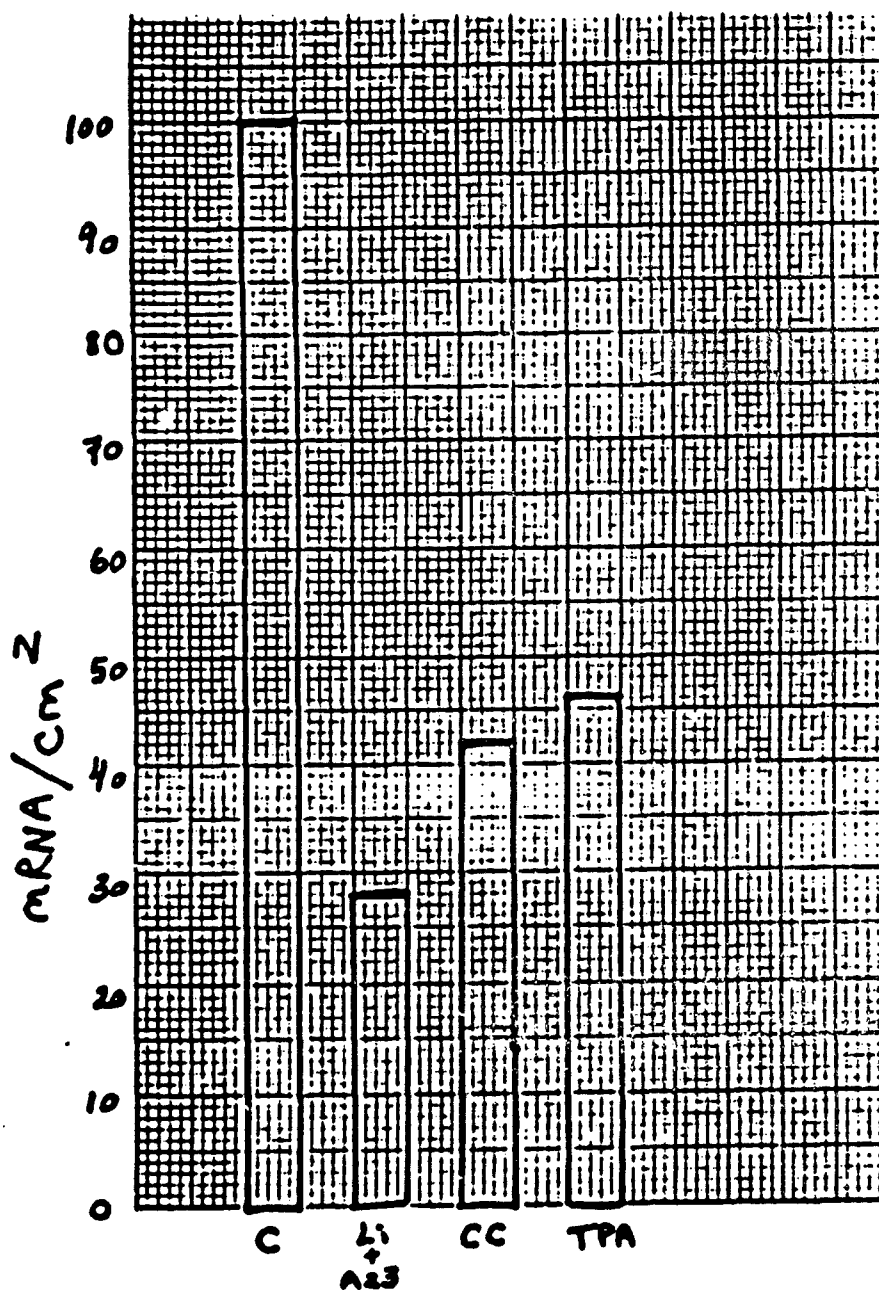


Fig. 7 Effect of various treatments on mRNA levels in cultured skeletal muscle.

Cultured skeletal muscles were incubated for 24 hours in medium alone (c) or medium plus L1 + A23187, carbachol (CC), or TPA (see task 4a). Total RNA was extracted, and mRNA levels were estimated by scanning densitometry of autoradiograms from Northern blots. Results were calculated in arbitrary scanner units of mRNA/surface area of cultured muscle, and are expressed as % of the controls (c).

Note the decrease of α -subunit mRNA produced by these treatments (see text, Task 4a).

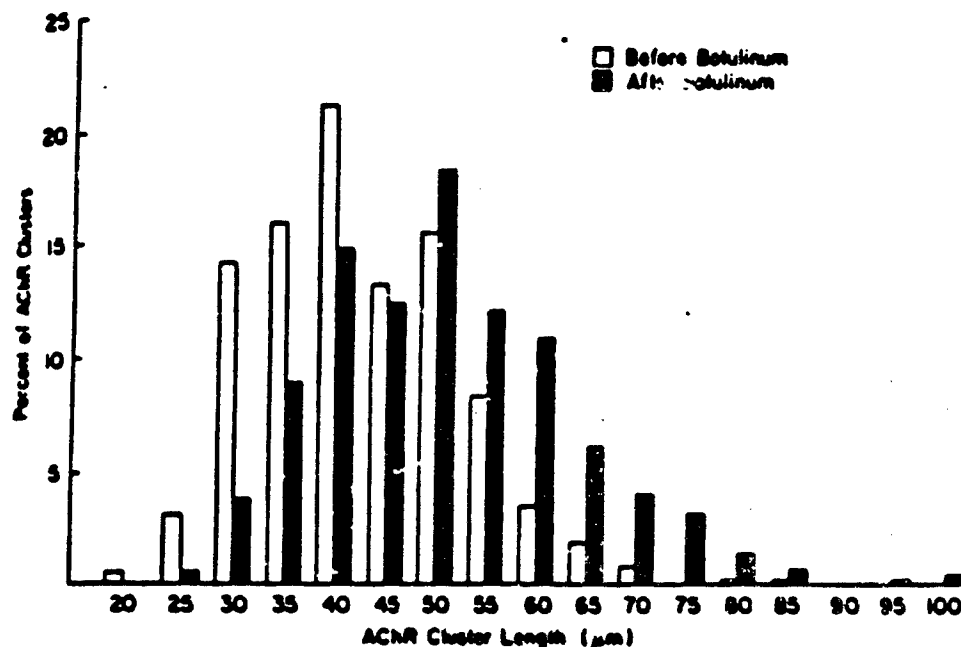


Fig 9. Lengths of clusters of preexisting AChRs in botulinum-treated and control muscles.

"Preexisting" junctional AChRs were labeled with α -BuTx *in vivo* 3 days before treatment of soleus muscle with botulinum toxin. The muscles were removed and studied another 7 days later. Control muscles were labeled with α -BuTx but not treated with botulinum. At least 40 AChR clusters per muscle were measured. The histograms were drawn using 436 AChR clusters from 8 botulinum-treated muscles and 412 AChR clusters from 8 control muscles.

Note the increased population of longer junctional AChR clusters in botulinum-treated muscles. The mean length of clusters of "preexisting" junctional AChRs was $53 \pm 2 \mu\text{m}$ for botulinum-treated muscles compared with $44 \pm 1 \mu\text{m}$ for control muscles. Thus, preexisting AChRs are redistributed during NMJ elongation (see text, Task 7a).

Table I
Denervation Prevents Stabilization of Junctional AChRs

Group	Time (days)				
	1 day	0	1	2	3
Experimental Group: Mixed Population of AChRs Left SM denervated		97.0 ± 1.7 (n=36)	93.4 ± 1.7 (n=19)	94 ± 1.4 (n=22)	89.8 ± 2.2 (n=19)
"Stable" AChR Controls Left SM denervated	98.3 ± 1.4 (n=17)	99.1 ± 2.6 (n=13)	101.1 ± 3.4 (n=13)	100.7 ± 3.1 (n=13)	98.1 ± 4.0 (n=7)
Non-denervated Controls	98.6 ± 1.6 (n=44)	101.5 ± 3.0 (n=14)	94.5 ± 2.8 (n=15)	99.4 ± 2.2 (n=15)	99.4 ± 5.8 (n=15)
					100.1 ± 3.8 (n=7)
					89.6 ± 2.5 (n=19)
					88.9 ± 1.5 (n=20)
					89.7 ± 4.4 (n=5)

The amount of ^{125}I - α -BuTx bound to the left SM muscle is shown as a percentage of that of the right SM (mean ± SEM) at the times indicated, for the experimental group and each of the controls.

Experimental group. Mixed population of RTDs and Stable AChRs. Left SM was denervated on day -1, and then labeled on day 0.

"Stable" AChR controls. Labeled 6 days before denervation. Left SM was denervated at -1 day.

Non-denervated controls. Neither SM was denervated. Labeled on day 0.

Note the deficit of AChRs due to failure of stabilization of AChRs in the experimental group. The deficit was significant ($p < 0.02$) by day 1, and increased throughout the experimental period. The control groups are explained in the text.

Some of these data are presented graphically in Fig. 2

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