

Degradation Rates of Acetylcholine Receptors Can Be Modified in the Postjunctional Plasma Membrane of the Vertebrate Neuromuscular Junction

Miriam M. Salpeter, David L. Cooper, and Tami Levitt-Gilmour

Section of Neurobiology and Behavior, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

Abstract. Denervation of vertebrate muscle causes an acceleration of acetylcholine receptor turnover at the neuromuscular junction. This acceleration reflects the composite behavior of two populations of receptors: "original receptors" present at the junction at the time of denervation, and "new receptors" inserted into the denervated junction to replace the original receptors as they are degraded (Levitt, T. A., and M. M. Salpeter, 1981, *Nature (Lond.)*, 291:239–241). The present study examined the degradation rate of original receptors to determine whether reinnervation could reverse the effect of denervation.

Sternomastoid muscles in adult mice were denervated by either cutting or crushing the nerve, and the nerves either allowed to regenerate or ligated to prevent regeneration. The original receptors were labeled

with ^{125}I - α -bungarotoxin at the time of denervation, and their degradation rate followed by gamma counting. We found that when the nerve was not allowed to regenerate, the degradation decreased from a $t_{1/2}$ of ~ 8 – 10 d to one of ~ 3 d (as reported earlier for denervated original receptors) and remained at that half-life throughout the experiment (~ 36 d). If the axons were allowed to regenerate (which occurred asynchronously between day 14 and day 30 after nerve cut and between day 7 and 13 after nerve crush), the accelerated degradation rate of the original receptors reverted to a $t_{1/2}$ of ~ 8 d. Our data lead us to conclude that the effect of denervation on the degradation rate of original receptors can be reversed by reinnervating. The nerve can thus slow the degradation rate of receptors previously inserted into the postsynaptic membrane.

DURING vertebrate embryonic development, innervation of skeletal muscle results in the formation of neuromuscular junctions (nmjs)¹ with a unique morphology and molecular organization. Best studied are the changed distribution and properties of the postsynaptic nicotinic acetylcholine receptor (AChR). One of the properties under neural control is the metabolic degradation rate of the AChR. Embryonic receptors are relatively unstable metabolically and degrade with a $t_{1/2}$ of ~ 1 d. After innervation of the nmj, the junctional receptors become metabolically stable and degrade with a $t_{1/2}$ of ~ 8 – 10 d (see references 6 and 19 for reviews). The maintenance of the metabolic stability of the junctional AChRs is very dependent on the physical presence of the nerve (1, 3, 10, 11, 14, 21).

After denervation, AChRs at the mammalian nmj show a progressive increase in degradation rate (10) due to the composite behavior and changing ratio of two populations of AChRs—those present at the junctions at the time of denervation ("original receptors") and those inserted at the nmj after denervation ("new receptors") (11). The original receptors have a complex behavior: initially, their degradation rate is similar to that of innervated controls ($t_{1/2}$ ~ 8 – 10 d). How-

ever that degradation rate accelerates with time, acquiring a $t_{1/2}$ of ~ 2.5 – 3 d (1, 3, 11, 21) by ~ 10 d after denervation.

It is not yet known how the nerve influences the degradation rate of junctional AChRs. In the present study, we asked whether reinnervation can reverse the effect of denervation on the original receptors. We found that it could. Regeneration of the cut nerve caused the degradation rate of the accelerated original receptors to become slow again. The nerve can thus slow the degradation rate of receptors that are already inserted into the postsynaptic membrane. The implication for understanding neural control of receptor turnover is discussed.

Materials and Methods

Denervation and Receptor Labeling

We used sternomastoid muscles from adult female white mice aged 4–6 mo (from Charles River Breeding Laboratories, Inc., Wilmington, MA). In this muscle, endplates lie in a compact band and are thus easy to dissect for gamma counting or to find in sections for electron microscopic studies. For that reason, this muscle has been used in previous studies from our laboratory, which provide the background for the studies reported here.

Animals were anesthetized with sodium nembutal (5 mg/ml, injected intraperitoneally 1 cc per 100 g body weight), the right sternomastoid muscle was exposed and denervated, leaving the contralateral side as an innervated

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; BGT, α -bungarotoxin; nmj, neuromuscular junction.

control. Three methods of denervation were used to give different rates of regeneration: (a) the nerve was cut near the muscle and the proximal end trimmed (14–30 d to regenerate); (b) the nerve was crushed by squeezing several times (5 s each) with fine forceps (7–13 d to regenerate); (c) the nerve was ligated with a human hair proximal to the cut to prevent regeneration. The nerve with the ligation was deviated and tucked under an adjacent muscle. Denervation was initially monitored, especially after nerve crush, by determining that stimulating the nerve with a suction electrode (7) did not cause muscle contraction.

After denervation, the wound was sutured, and within 20 min of denervation, each animal was injected intraperitoneally with ^{125}I - α -bungarotoxin (^{125}I -BGT) (9) (4.1 μg in Krebs ringers per 100 g body weight), to label the original receptors. (Iodinated toxin was prepared bimonthly in the laboratory [13].) This dose of BGT is not saturating and labels only $\sim 20\%$ of the receptors. The duration of the ^{125}I -BGT is expected to be brief and mostly cleared from the blood by <20 h (23, 24, and unpublished data from our laboratory).

Determination of Degradation Rates

At different times after the injection (up to 34 d), four to five animals per time point were anesthetized and killed by intracardial perfusion with 4% paraformaldehyde in phosphate buffer. The right and left sternomastoid muscles were removed and weighed. The endplate region was located by staining for acetylcholinesterase (8) and separated by dissection from the non-endplate regions of the muscle. The radioactivity remaining in the muscle was then assessed by a Beckman 4000 Gamma Counter (Beckman Instruments, Inc., Palo Alto, CA). Specific endplate counts were determined by subtracting the radioactivity bound to the muscle segments without endplates from the endplate region on a per weight basis as previously described (10). To eliminate noise resulting from animal-to-animal variation in the amount of BGT injected, and in the specific activity determination of different batches of ^{125}I -BGT, the data were expressed as a ratio of denervated to innervated (D/I) specific endplate counts determined separately for each animal.

The degradation rate of the labeled original junctional AChRs can be assessed by the rate of loss of the specific endplate radioactivity bound to the endplate band (5, 15). We usually express the degradation of the AChR in terms of half-life which is related to the degradation rate (k) as $k = \ln 2/t_{1/2}$. The measured rate of loss (k meas.) is a composite of two processes: the true degradation rate of the AChR (k degr.), and the unbinding rates of ^{125}I -BGT from the receptors (k unbi.). These are related by the sums of their rates

$$k \text{ meas.} = k \text{ degr.} + k \text{ unbi.} \\ \text{or } 1/(t_{1/2} \text{ meas.}) = 1/(t_{1/2} \text{ degr.}) + 1/(t_{1/2} \text{ unbi.}).$$

It can be seen that the faster the degradation (i.e., the larger the k degr.), the less influence the unbinding rate has on the accuracy of the measured value.

We will present our experimental results as the measured k or $t_{1/2}$ values. To estimate the true degradation rates, a reasonable value for k or $t_{1/2}$ (unbi.) is 0.0008 h^{-1} (or $t_{1/2}$ of 36 d) obtained by Bevan and Steinbach for nmjs under similar experimental conditions (1). (The lower $t_{1/2}$ unbi. value of 13 d obtained in cultured muscle by Reiness et al. [17] was found to be incompatible with results from nmjs in vivo by Levitt and Salpeter [11].)

Fine Structure

After gamma counting, the muscle segment containing the endplate band was cut into small pieces, fixed in 1% OsO_4 in phosphate buffer, block stained with 2% aqueous uranyl acetate at room temperature (1 h), dehydrated, and embedded in Epon. Sections from at least six different regions of each muscle were sectioned, until 5–15 endplates were found for each muscle and ~ 20 –40 endplates per time point. The endplates were photographed at a magnification of 10,000 with a Philips 201 or 300, and printed at a final magnification of 25,000. Junctional profiles were then scored as being fully innervated, fully denervated, or partly innervated (i.e., if thin nerve processes were seen in the vicinity of the junctional folds).

Results

Measure of Denervation and Reinnervation

All muscles tested showed no contracture by indirect stimulation immediately after denervation. The morphological

analysis (see Materials and Methods) showed that after nerve crush, axon terminals were fully degenerated by 3 d and fully regenerated between days 7 and 13. The cut nerves regenerated more slowly and over a longer period. On day 16, $\sim 40\%$ of the junctions were fully regenerated and another 25% showed some nerve twigs. Regeneration was not complete until \sim day 30, however. The ligated and cut nerves showed no regeneration even after 34 d.

In addition we examined the muscle weights, assuming that a denervated muscle would weigh less than its innervated contralateral control. Although variable, the muscle weights were consistent with the above observations. The muscles denervated by nerve crush lost weight but recovered quickly. In these muscles the weight ratio (denervated/innervated) was initially 0.99 ± 0.04 . It dropped to 0.7 ± 0.07 by day 9 and returned to 0.9 ± 0.2 by day 18. The muscles denervated by nerve cut also began with a ratio of 0.99 ± 0.04 on day 1, dropped to a low of 0.52 ± 0.03 on day 15, and recovered incompletely to a ratio of 0.73 ± 0.02 by day 30. The muscles denervated by nerve cut, ligation, and deviation also began with a weight ratio of over 0.9 on day 1, and dropped to 0.54 ± 0.04 on day 15, as did the muscle denervated by nerve cut. The weight ratio of the ligated muscles continued to drop, however, and was down to 0.32 ± 0.03 on day 30.

Our data show that we were comparing three populations of denervated endplates: one with rapid regeneration (nerve crush), a second with slower regeneration (nerve cut), and a third with no regeneration during the time course of the experiment (nerve ligated, cut, and deviated).

Effect of Reinnervation on Receptor Degradation

Fig. 1 plots the ratio of endplate counts remaining in the three groups of denervated endplates relative to that at the innervated muscle. Note that up to ~ 7 d after denervation no significant difference can be seen between the three denervated groups and between them and unity. The ratio begins to decrease thereafter. In the muscles in which the nerve was not allowed to regenerate (cut and ligate), the ratio continues to decrease until 30 d. After that time, the endplate-specific counts approached background values and no later times could be assessed. In the muscles denervated by nerve cut, regeneration occurred between days 16 and 30 (see above), and the ratio ceased to drop by about day 20. Finally, in the group denervated by nerve crush, regeneration occurred between day 7 and day 13, and the ratio dropped very little, ceasing to drop at approximately day 13.

To assess the relative half-lives at the different stages of the regeneration process, the data from Fig. 1 were replotted in Fig. 2. Again, to avoid scatter due to animal-to-animal variation in injection dose, the data were retained as a ratio of denervated to innervated (D/I) specific endplate counts, determined separately for each animal. The innervated side was set to fall on a curve with a measured $t_{1/2}$ of 8 d (i.e., $2^{-t/8\text{d}}$) as has been determined to be the case for this protocol in several studies in our laboratory (e.g., 11) and for the present data. The residual label on the denervated muscle was then calculated from the D/I ratio, set to 100% on day 1, and plotted on a semilogarithmic scale.

Mathematically, Fig. 2 plots the expression

$$\text{Residual label on denervated muscle} = \\ (D/I) \times (2^{-t/8\text{d}}) \times (100\%).$$

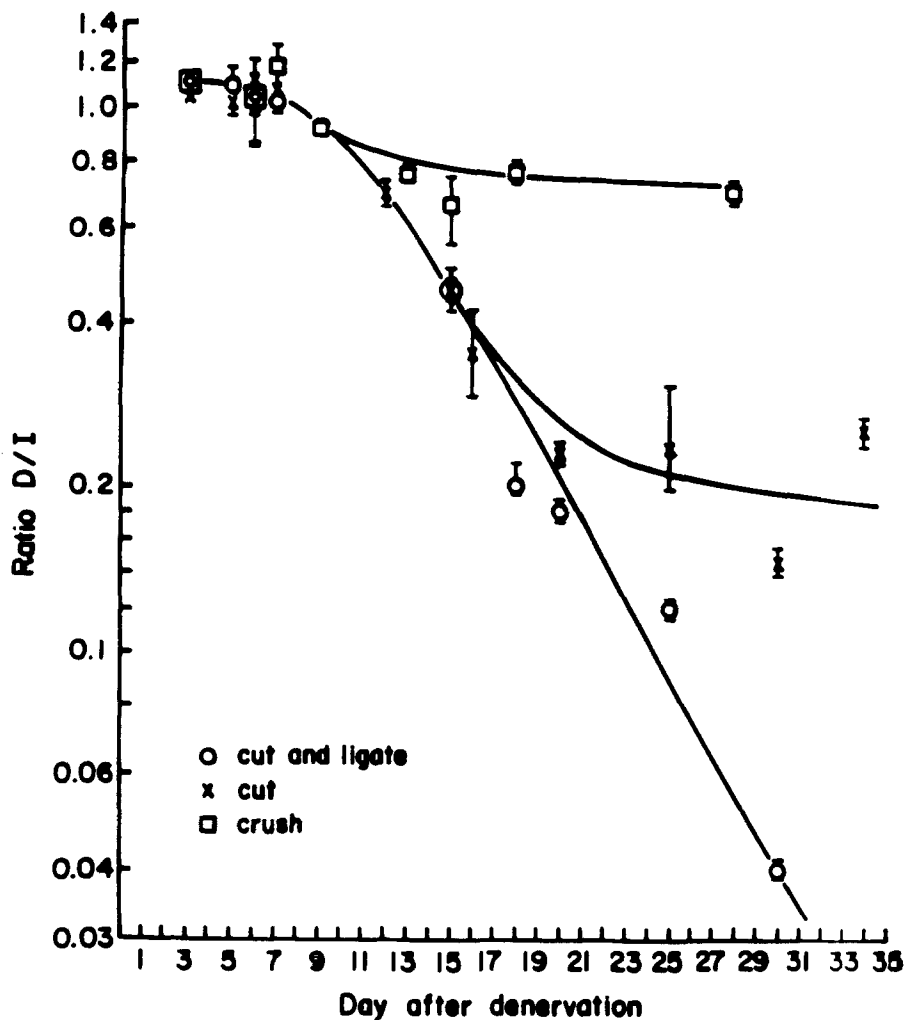


Figure 1. Receptors were labeled by injection with ^{125}I -BGT at the time of denervation, and the specific endplate band label remaining at different times after denervation measured by gamma counting (see Materials and Methods). The residual label at the denervated muscle was then expressed as a ratio to that at the innervated muscle. (Regeneration of these muscles was then assessed by the fine structure at the endplate as given in the text.) Muscles denervated by nerve crush (\square) regenerated between postdenervation days 7 and 13; muscles denervated by nerve cut (\times) regenerated between days 14 and 30; muscles denervated by nerve ligation and cut (\circ) did not regenerate during the course of the experiment. (No 34-d value could be obtained for this group since it fell below background.) Each time point represented three to five experiments. Error bars represent the standard error of the means. The ratio is constant (curve is horizontal) when the denervated and innervated junctions lose label at the same rate, and decreases if the denervated junction loses label at a greater rate than the innervated side does. Data are replotted to determine degradation half-life in Fig. 2.

Note in Fig. 2 that initially (up to about day 7), the three groups have a measured degradation $t_{1/2} \sim 7.4 \pm 0.6$ d. By about day 8–10, their degradation rates have accelerated to a $t_{1/2} \sim 3.1 \pm 0.11$ d, similar to that previously described for this muscle (11). Soon thereafter the three curves diverge, however. In the muscles denervated by ligating, cutting, and deviating the nerve (nonregenerating), the receptors retained a $t_{1/2}$ of 3.1 ± 0.11 d throughout the experimental period. In the muscles denervated by nerve crush, the degradation rate was equal to that at innervated muscles approximately by day 13. In muscles denervated by cutting the nerve, the degradation rates of the receptors first increased, and then decreased again (reaching innervated values) with a time course consistent with that seen for the neural regeneration. In these muscles, the receptor degradation began to diverge from the non-reinnervating muscle by day 14, but showed a $t_{1/2}$ of $\sim 7.4 \pm 1.2$ d only after day 23.

Discussion

The main result of the present study is that the degradation rate of receptors already in the postjunctional membrane at adult denervated junctions becomes slow in response to reinnervation.

The original receptors (i.e., receptors present at the junc-

tion at the time of denervation) were used in this study since it was previously reported that the degradation rate of these receptors increases after denervation (1, 3, 11, 21). The original receptors are also easier to study by gamma counting than are new receptors inserted into a denervated junction, since at the time that the original receptors are labeled, no post-denervation extrajunctional receptors are yet present. The gamma counting results are therefore not confounded by gradients of extrajunctional receptors which can give erroneous values for endplate-specific counts (see e.g., 12).

In the present study we confirmed earlier reports that the degradation rate of original junctional receptors accelerates after denervation. Furthermore we found that the degradation rate of these prelabeled junctional receptors decreased again at a time consistent with the reinnervation of the muscle. We obtained a change from a measured $t_{1/2} \sim 8$ d at the time of denervation to one of ~ 3 d by 10 d after denervation with a return to a measured $t_{1/2}$ of ~ 8 d at the time of reinnervation. When the measured values are corrected for ^{125}I -BGT unbinding (see Materials and Methods) using the values obtained by Bevan and Steinbach (1) for k unbi. of 0.0008 h^{-1} or $t_{1/2}$ unbi. of 36 d, the measured $t_{1/2}$ of 7.4 and 8 d for innervated and reinnervated muscle would correct to a degradation $t_{1/2}$ of 9.3 and 10.3 d; and the measured $t_{1/2}$ of 3.1 d obtained for the postdenervation accelerated receptors

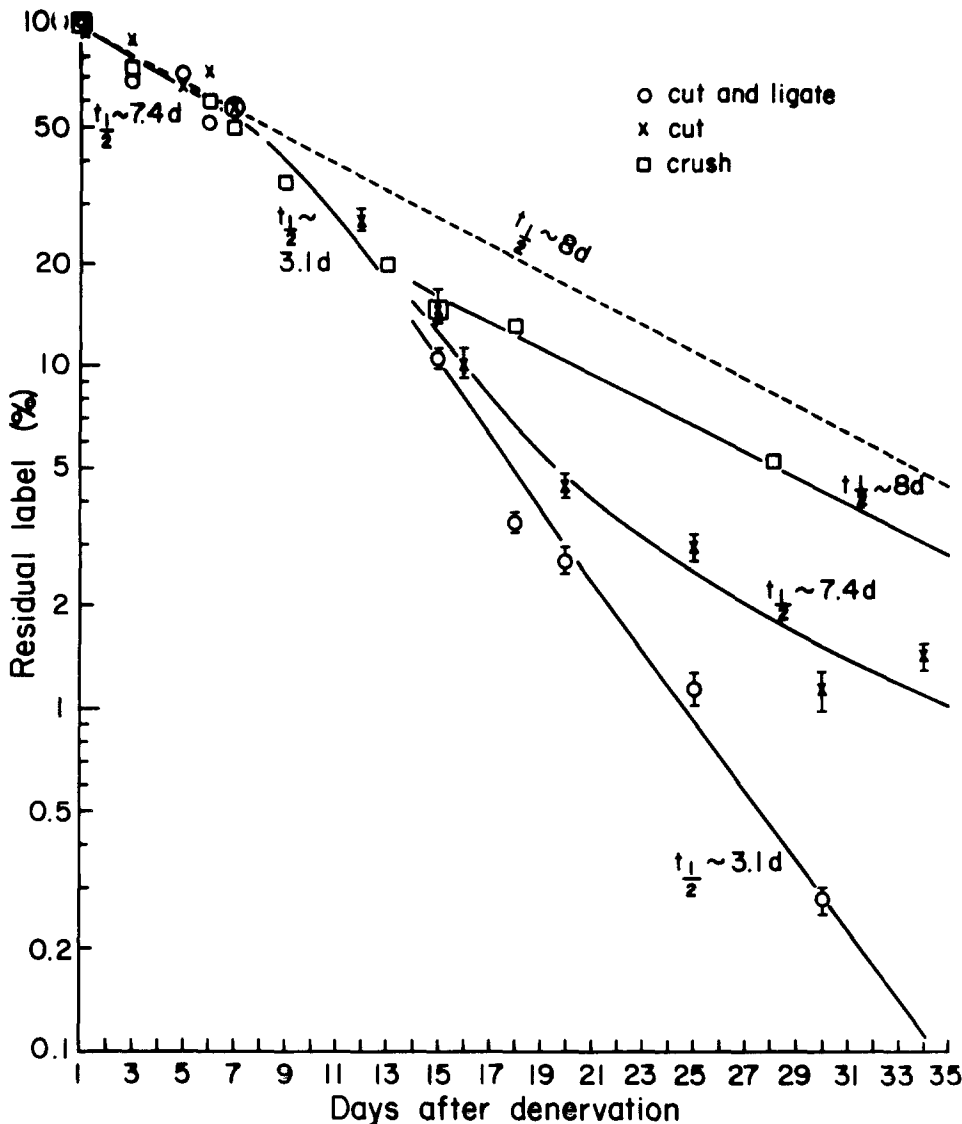


Figure 2. Data as in Fig. 1, however expressed as the residual label remaining at the denervated muscles at different times after denervation (obtained as described in Materials and Methods). Half-life ($t_{1/2}$) values were obtained by linear regression for different regions of the curves and indicated on the curves. The dashed line gives a $t_{1/2}$ of 8 d previously established for innervated junctional receptors under the same experimental conditions. (As indicated also in the caption to Fig. 1, muscle denervated by nerve crush [□] regenerated between days 7 and 13; muscle denervated by nerve cut [X] regenerated between days 14 and 30; muscles denervated by nerve ligation and cut [○] did not regenerate during the course of the experiment. [No 34-day value could be obtained for this group since it fell below background.] Error bars were omitted from values for the first 7 d, since these caused too much overlap between the different conditions, making the curve difficult to read. In all other instances, error bars were omitted if they fell within the size of the symbol.

would correct to a degradation $t_{1/2}$ of 3.4 d. The corrected values accentuate the difference between the half-life of original receptors after denervation and that before denervation or after reinnervation.

The results in the present study can be compared and may help clarify data reported previously. The curve in Fig. 1 for the D/I ratio from muscles denervated by nerve cut resembles (and extends) those in Fig. 1 from Levitt and Salpeter (11) and Fig. 10 from Bevan and Steinbach (1). As already pointed out by Levitt and Salpeter (11), with such data one cannot distinguish between a model in which there is a delay before the degradation rate accelerates, and one (which they thought may be more likely) in which the degradation rate begins to accelerate immediately and reaches a peak value at some time after innervation. In model one, the data for the sternomastoid muscle would be compatible with a sharp change in degradation rate at ~8–10 d after denervation. In model two, the peak acceleration rate would be reached somewhat later, at ~12–14 d.

Bevan and Steinbach assessed the wash out from organ-cultured diaphragm muscle and showed that in that system the degradation rate did begin to accelerate immediately af-

ter denervation and peaked at ~10 d. Although Bevan and Steinbach only tested diaphragm muscle in organ culture, there is no reason to believe that other muscles do not behave qualitatively as the diaphragm does, and model two may apply to all vertebrate junctions. It is of interest that their in vivo studies (with a protocol similar to ours) show that the original receptors of the diaphragm (Fig. 8, their paper) accelerate more quickly than do those of other muscles (Fig. 10, their paper). The behavior of the original receptors of those latter muscles is similar to that seen in the sternomastoid reported here.

A puzzling result in the study by Bevan and Steinbach was the fact that after the degradation rate of the diaphragm muscle in vivo peaked at ~10 d, it then declined (Fig. 7, *b* and *c*, their study). Since in our experiment, such a behavior was found after reinnervation, but not when reinnervation was prevented, it is conceivable that the decline seen by Bevan and Steinbach may be due to reinnervation of the diaphragm muscle before being removed. It could also reflect muscle fibers dying in organ culture.

The results presented here demonstrate that the degradation rate of original receptors is very sensitive to the pres-

ence of the nerve. An exact determination could not be made whether actual nerve-muscle contact is necessary or whether nerve factors from regenerating growth cones are sufficient to affect turnover rates. Furthermore, it is not clear how much delay exists between the time of reinnervation and the stabilization of the receptor degradation rate. However, the slowing of the receptor degradation rate appears to occur within hours (or very few days) after reinnervation is seen. This indicates that in this mouse muscle, the regenerating nerve can exert its influence in a period similar to that seen during embryonic development of other mammalian muscle (16, 23) (which is much faster than that reported for chick muscle [4]).

Since these receptors were labeled at the time of denervation, the present results show that the degradation rate of receptors can be both increased and decreased after they are inserted into the plasma membrane. The mechanism controlling degradation of this receptor must thus involve modifications of the receptor in the membrane or of its immediate microenvironment and does not involve synthesis of new receptors. A similar control mechanism may also be involved in neural control of channel open time. Brenner and Sakmann (2) and Schuetze and Vicini (20) noted that the switch in receptor channel open time from ~3–4 ms, seen in embryonic and extrajunctional AChR, to the ~1 ms seen in mature junctional receptors, occurs in a time that is short relative to the AChR turnover time. They therefore conclude that this switch must reflect a modification of receptors already inserted into the membrane. The mechanism whereby receptor open time is controlled must however be different from that involved in control of receptor degradation rate, although both can be exerted on receptors in the membrane. Once the channel open time of junctional receptors has become short, under the influence of the nerve, this property is maintained after denervation during a time longer than the turnover time of the receptors (2). The postinnervation degradation rate, on the other hand, is very dependent on the continued presence of the nerve, as seen by the accelerated degradation of the original receptors after denervation (Figs. 1 and 2), and the conclusion that new receptors, inserted into a denervated junction, may have a half-life closer to that of embryonic receptors (11).

It has been reported that ~20% of the receptors at the endplate band of rodent innervated diaphragm muscle degrade rapidly ($t_{1/2}$ of 1 d) (1, 22). This can be due to the presence of junctional receptors which are gradually being converted to the slowly degrading species (22), suggesting a conversion from fast to slowly degrading receptors in the membrane during normal receptor turnover. Alternately it could reflect the presence of extrajunctional receptors within the endplate band that is being counted (1) and does not represent a receptor population yet to be stabilized.

Salpeter and Harris (18) calculate from electron microscopic autoradiographic data that at the junctional folds of the normally innervated mouse sternomastoid muscle, not more than ~5% of receptors could be degrading rapidly, and thus if a conversion from rapid to slow degradation occurs in vivo, it must occur in much less than 2 d after insertion. The number of newly inserted "rapidly degrading" receptors normally present at the innervated nmj before stabilization remains to be established. In the present study, we demon-

strate that the nerve can change the degradation rate of the original junctional receptors while they are in the membrane at denervated and reinnervated junctions. Whether a similar mechanism exists for newly inserted receptors awaits verification.

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