Degradation Rate of Acetylcholine Receptors Inserted into Denervated Vertebrate Neuromuscular Junctions

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Abstract. Many studies exist on the effect of denervation on the degradation of acetylcholine receptors (AChRs) at the vertebrate neuromuscular junction (nmj). These studies have described the behavior of either the total population of junctional receptors at different times after denervation, or of the receptors present at the time of denervation (referred to as original receptors). No experimental studies yet exist on the degradation rate of the receptors newly inserted into denervated junctions. In the previous studies, the original receptors of mouse sternomastoid muscles were found to retain the slow degradation ($t_{1/2}$) of ~8–10 d of innervated junctional receptors for up to 10 d after denervation before accelerating to a $t_{1/2}$ of ~3 d. The total junctional receptors, on the other hand, showed a progressive increase in degradation rate from a $t_{1/2}$ of 8–10 d to a $t_{1/2}$ of 1 d.

To reconcile these earlier observations, the present study examines the degradation of new receptors inserted into the nmj after denervation. To avoid possible contamination of the data with postdenervation extrajunctional receptors, we used transmission electron microscope autoradiography to study only receptors located at the postjunctional folds of the nmj. We established that the new receptors inserted into denervated junctions have a $t_{1/2}$ of ~1 d, considerably faster than that of the original receptors and equivalent to that of postdenervation extrajunctional receptors. Both original and new receptors are interspersed at the top of the junctional folds. Thus, until all the original receptors are degraded, the postjunctional membrane contains two populations of AChRs that maintain a total steady-state site density but degrade at different rates. The progressive increase in turnover rate of total AChRs therefore reflects the combined rates of the original and new receptors, as earlier postulated by Levitt and Salpeter (1981).

SEVERAL properties of the nicotinic acetylcholine receptor (AChR)[1] in vertebrate muscles change during development and are affected by denervation (see reviews by Fambrough, 1979; Salpeter and Loring, 1985). The present study deals with one such property, the degradation rate of the junctional AChR.

Embryonic receptors have a degradation $t_{1/2}$ of ~1 d. After innervation the receptors cluster at the neuromuscular junction (nmj) and become stabilized to a $t_{1/2}$ of ~8–10 d (Steinbach et al., 1979; Burden, 1977). Denervation reverses this stabilization (Loring and Salpeter, 1980). Using a gamma counting technique, Levitt et al. (1980) observed that there was a time-dependent increase in the degradation rate of junctional receptors labeled at different times after denervation. This progressive increase was inconsistent with the observations regarding the postdenervation degradation of receptors that are present at the endplate at the time of denervation (referred to as "original" receptors). The degradation rate of the original receptors remains unchanged for several days after denervation before accelerating. The exact delay time depends on the muscle studied (Levitt and Salpeter, 1981; Stanley and Drachmann, 1981; Brett et al., 1982; Bevan and Steinbach, 1983) and is ~9–10 d in the mouse sternomastoid muscle (Levitt and Salpeter, 1981; Salpeter et al., 1986). After this "lag time" the degradation rate increases to a $t_{1/2}$ of ~2.5 to 3.0 d. Since this two-phase degradation rate of original receptors could not account for the overall progressive increase in turnover of junctional AChRs seen after denervation, Levitt and Salpeter (1981) proposed that as the original receptors degrade they are replaced by another population of receptors (referred to as "new" AChR) which have a much faster turnover rate. They calculated that for a best fit to the data of Levitt et al. (1980), these new receptors should turn over with a $t_{1/2}$ of 1 d. The observed progressive increase in degradation rate after denervation would then reflect the combined rates of the two populations.

The possibility that ACh receptors with different degradation rates may be present simultaneously at the same postjunctional membrane, has important implications for understanding the mechanism for controlling turnover of integral

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

*Table I. Cold Saturation Control**

Experimental condition	No. of animals	BGT binding sites per μm^2 pjm‡
1.5 h ^{125}I-α-BGT	2	19,390 ± 156
1.5 h BGT plus 1.5 h ^{125}I-α-BGT	2	260 ± 60

* Two animals each were labeled either with ^{125}I-α-BGT for 1.5 h (*top line*) or with nonradioactive α-BGT (1.5 h) followed by ^{125}I-α-BGT for 1.5 h (*bottom line*). Table shows that muscles labeled with ^{125}I-α-BGT after nonradioactive toxin had only 2% as much label as did muscles saturated with ^{125}I-α-BGT only.

‡ *pjm*, thickened postjunctional membrane at top 1/3 of junctional folds.

membrane proteins. The model of Levitt and Salpeter (1981) therefore needed direct experimental verification, especially since it was deduced using techniques that were subsequently found to have potential problems. These were contamination by postdenervation extrajunctional receptors when using the gamma counting technique, and unequal labeling of denervated and innervated junctional AChRs after intraperitoneal injection of label (see Discussion).

In the present study these problems were avoided. The new receptors were saturated by topical application of ^{125}I-α-bungarotoxin and the degradation rate was measured directly using transmission electron microscope (TEM) autoradiography. By TEM autoradiography only receptors located on the postjunctional membrane of the denervated muscle were included in the tabulation, thus eliminating possible distortion by extrajunctional label. The results established that new receptors inserted into denervated nmjs do have a fast turnover rate with a $t_{1/2}$ of ~1 d, equal to that generally reported for postdenervation extrajunctional receptors (see reviews by Fambrough, 1979; Salpeter and Loring, 1985). The interspersion on the same postjunctional membrane of the new receptors with the original receptors having different turnover rates raises questions regarding the neural control of turnover of surface ACh receptors.

Materials and Methods

Denervation and Receptor Labeling

Sternomastoid muscles from adult female white mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used in this study. This muscle has a very well-defined endplate band and can easily be dissected out for gamma-counting or TEM autoradiography. All the surgery was performed under nembutal anesthesia (60 mg/kg of body weight).

To block the original receptors, the right sternomastoid muscles were first exposed surgically and bathed to saturation with nonradioactive α-bungarotoxin (α-BGT; 3 μM) (to be called cold saturation) as previously described (Loring and Salpeter, 1980). Full inactivation was ensured when suction electrode stimulation of the nerve at 100 Hz no longer caused any tetanic muscle contraction. Application of α-BGT was then continued for a total of 1.5 h (Fertuck et al., 1975). To further determine the extent of receptor saturation with the nonradioactive α-BGT, we compared the radioactive α-BGT binding site density of two groups of muscles: the first was bathed in 3 μM nonradioactive α-BGT for 1.5 h and then immediately in 3 μM ^{125}I-α-BGT for an additional 1.5 h, the second was bathed only in ^{125}I-α-BGT for 1.5 h.

In all muscles the nerve was cut at the time of cold saturation. Regeneration was prevented by ligating the nerve and then cutting the nerve distal to the ligature. The wound was sutured, and, 6 or 14 d later, the muscles were saturated with ^{125}I-α-BGT (as described above for cold saturation) to label the newly inserted receptors. (Receptors were labeled by saturating the muscle with ^{125}I-α-BGT instead of by injecting the label intraperitoneally, since we have found that intraperitoneal injection causes unequal labeling of denervated and innervated junctional AChRs [see Discussion].)

Determination of Degradation Rates

To eliminate contamination of the data with postdenervation extrajunctional AChRs, all analyses were done using TEM autoradiography to study only receptors located at the postjunctional folds. Deeply anesthetized animals were killed by intracardial perfusion with 4% paraformaldehyde in phosphate buffer (0.067 M, pH 7.4) at various times after radioactive labeling of new receptors. The denervated sternomastoid muscles were removed and stained for acetylcholinesterase (Karnovksy and Roots, 1964) to identify the endplate band.

Endplate band tissue was then dissected out and postfixed in OsO_4, stained with uranyl acetate, and processed for TEM autoradiography using the flat substrate procedure of Salpeter and Bachmann (1964; see Fertuck and Salpeter, 1976; Salpeter, 1981). The α-BGT binding site density was determined specifically at the postjunctional membrane of the nmjs (as described by Fertuck and Salpeter, 1976; or Matthews-Bellinger and Salpeter, 1978) and the degradation rate assessed by the rate of decrease of this site density. Loss of radioactivity after labeling with ^{125}I-α-BGT has been shown to reflect degradation of receptors (see review by Fambrough, 1979) with unbinding of α-BGT causing a negligible effect (Bevan and Steinbach, 1983; Salpeter et al., 1986).

Results

Control experiments for cold saturation showed that ~98% of the junctional receptors were inactivated with the nonradioactive α-BGT (Table I) as was also seen in previous studies using this procedure (Loring and Salpeter, 1980). Furthermore, fine structure studies established that the ligation of the nerve successfully prevented reinnervation during the time course of the degradation curves. Of a total of 250 randomly chosen endplates from 22 animals at both 6 and 14 d after denervation not one showed any preterminal nerve fibers. By Poisson statistics, one can calculate that the probability of even a 2% innervation is less than 0.01.

TEM autoradiographs (Fig. 1) showed that the labeled new receptors are distributed throughout the top of the folds. This relatively uniform distribution is maintained throughout the degradation period. A similar distribution during degradation has been seen for the original receptors (data not shown). These results indicate that no preferential localization for the new receptors relative to the original receptors can be discerned at the level of resolution of TEM autoradiography, which is ~1,500 Å under the conditions of this study (Salpeter et al., 1969).

After denervation the junctional folds retain their characteristic dense membrane at the top, which has been shown to be the receptor-rich region (Fertuck and Salpeter, 1974, 1976; Sealock et al., 1984). Occasionally the junctional folds get stretched out or folded onto themselves (see Fig. 8 in Salpeter, 1987) and could affect the size and shape of a denervated junction viewed by esterase staining or fluorescence α-BGT staining of the intact fiber. However, since the folds always remained recognizable in the electron microscope, these distortions do not affect TEM autoradiographic results.

Fig. 2 shows the degradation curve of new receptors. We chose to determine the degradation rate for new receptors at 6 and 14 d after denervation, because at 6 d the original receptors in the sternomastoid endplate are degrading with a $t_{1/2}$ of ~8 d and at 14 d the $t_{1/2}$ has decreased to ~3 d (Levitt

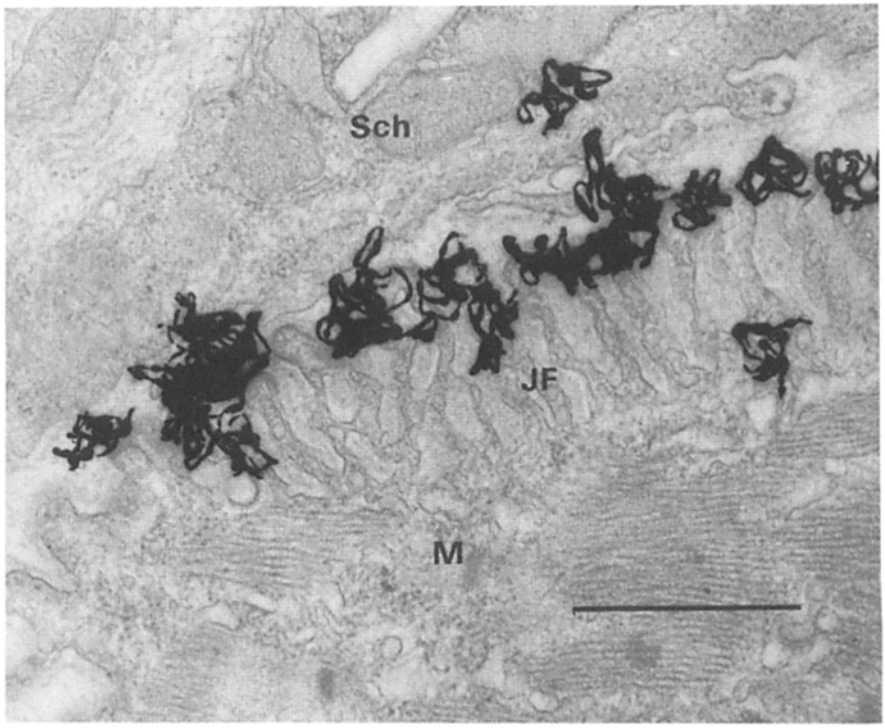

Figure 1. EM autoradiograph showing the relatively uniform distribution of newly inserted AChRs at the top of the junctional folds in a denervated endplate. Original receptors were saturated with nonradioactive α-BGT at the time that the nerve was cut. 6 d later, new receptors were labeled with ^{125}I-α-BGT. Since a similar distribution is seen for the localization of original receptors at all times during degradation (data not shown), this distribution profile indicates that a preferential localization for the new receptors is not discernible within the resolution of the TEM autoradiographic technique. *Sch*, Schwann cells; *JF*, junctional folds; and *M*, muscle. Bar, 1 μm.

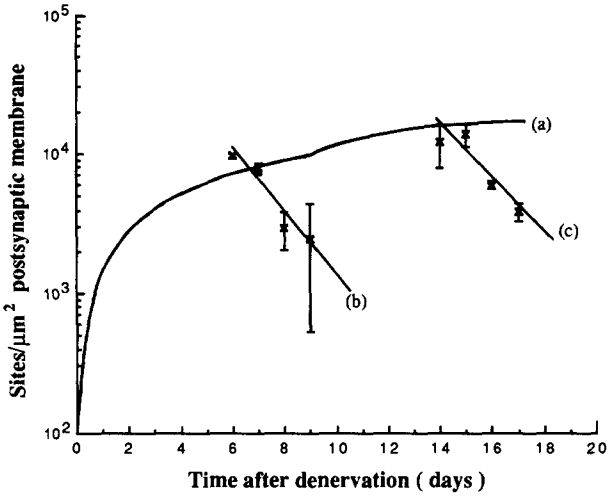

Figure 2. Insertion and degradation of new AChRs at denervated nmj. Original receptors were saturated with nonradioactive α-BGT. New receptors were labeled with ^{125}I-α-BGT 6 or 14 d after nerve cut. Residual label is plotted as binding site density (α-BGT binding sites/μm² of thickened postsynaptic membrane) measured by TEM autoradiography at different times after labeling. Three curves are shown. *a* gives calculated number of new receptors expected to be accumulated at the nmj at different times after denervation. (Calculation assumes that the site density at the nmj is 18,000 sites/μm² at the time of denervation and that the original receptors degrade with a $t_{1/2}$ of 8 d during the first 9 d of denervation and with a $t_{1/2}$ of 2.5 d thereafter.) *b* gives the degradation of new receptors labeled at 6 d after denervation. *c* gives the degradation of new receptors labeled at 14 d after denervation. Each point is the averaged site density from two to five animals, and curves are fitted by linear regression. Error bars are standard error of the mean when the sample size is greater than two. In cases where the sample size is equal to two, the error bar represents the range of the two values. Degradation $t_{1/2}$ of new receptors was calculated to be ~1 d (see text).

and Salpeter, 1981). We therefore asked whether the new receptors also showed a different behavior at these two time periods.

Interestingly, there often is little or no degradation of receptors during the first day (i.e., between 2 h and 1 d) after labeling (see also Loring and Salpeter, 1980; Salpeter and Harris, 1983). The reason for this phenomenon is unclear, but may represent a period of shock after the operating procedure involved in saturating the receptors with α-BGT. In assaying the half-life of the receptors we therefore report two values both obtained by linear regression: the first based on all the time points and the second only on those beginning 1 d after labeling. By both tallies, newly inserted receptors labeled either 6 or 14 d after denervation degrade with a $t_{1/2}$ of ~1 d, similar to the $t_{1/2}$ of embryonic and denervation-induced extrajunctional receptors (see Salpeter, 1987 for review). With all time points included, the $t_{1/2}$ values are 1.07 ± 0.25 d and 1.57 ± 0.31 d for 6 and 14 d, respectively. If only times after day 1 are included, as we believe to be more valid, they are 0.94 ± 0.27 and 1.1 ± 0.16 d, respectively.

Discussion

It has been established by several studies that metabolically stable AChRs, present at the nmj before denervation (original receptors), retain their stability for some time after denervation but then their degradation accelerates to a $t_{1/2}$ of ~3 d (see reviews Salpeter and Loring, 1985; Salpeter, 1987).

The present study establishes (*a*) that the new receptors inserted into denervated nmjs are located at the top of the junctional folds (as are innervated receptors) and thus interspersed with the original receptors; and (*b*) that these new receptors degrade with a $t_{1/2}$ of 1 d, equal to embryonic receptors and to extrajunctional receptors that develop after denervation.

The degradation rate of new receptors was earlier postulated by Levitt and Salpeter (1981) based on data obtained by a gamma counting technique. However, after the study of Levitt and Salpeter (1981) was published, we found two sets of potential complications which could have affected their calculations. The first complication could arise from the use of the gamma counting technique to determine the specific junctional label. In this technique, the muscle is divided into three pieces, one containing the endplate band. The radioactivity bound to pieces without endplates is subtracted from the radioactivity of the piece containing endplates on a per weight basis. This subtraction assumes a uniform distribution of extrajunctional label. Thus any extrajunctional receptors in a gradient around the neuromuscular junctions within the endplate band will be included in the specific junctional label. In innervated muscles this is not a problem since the extrajunctional receptors which are distributed in a very steep perijunctional gradient (Fertuck and Salpeter, 1976; Salpeter et al., 1988) have a turnover rate equal to that of the junctional receptors (Salpeter, M. M., manuscript in preparation). In denervated muscles however, the level of extrajunctional receptors increases and, especially early after denervation, distribute over a long distance in a shallow gradient (Salpeter et al., 1988) with higher density in the tissue containing the endplate band than in that without the endplate band (Levitt-Gilmour and Salpeter, 1986). The endplate-specific counts by gamma counting will therefore include counts from the elevated extrajunctional receptors in the endplate band–containing tissue. This could distort the value obtained for the degradation rate of junctional receptors if the true degradation rate of the junctional receptors is different from that of the extrajunctional receptors.

The second complication could arise from the receptor labeling procedure. In the study by Levitt and Salpeter (1981), receptors were labeled by injecting ^{125}I-α-BGT intraperitoneally. In preliminary experiments, while preparing for the present study, we found by TEM autoradiography that this procedure results in a two- to threefold higher label at innervated than at denervated junctions. However, when receptors were labeled to saturation with topical application of ^{125}I-α-BGT, as used in the present study, the junctional site density ratio of denervated to innervated muscle was ~1. Thus the AChR site density does not decrease but stays relatively constant after denervation (as also reported earlier by Frank et al., 1975; Porter and Barnard, 1975; Bader, 1981; Loring and Salpeter, 1980). Yet by intraperitoneal injection, which represents a short, nonsaturating pulse label, there is a preferential labeling of the innervated junctional AChRs. This preferential labeling was not seen in the study by Levitt and Salpeter using the gamma counting procedure, presumably because the increased extrajunctional label in the denervated muscle, discussed above, masked this effect.

The reason for the preferential label of innervated junctional receptors is not known. To our knowledge no reports exist that denervated receptors have a lower affinity for α-BGT. In fact Almon et al., (1974) found the opposite to be true. Diffusion barriers, due to scar tissue or damaged blood supply, may have developed in the denervated muscle, decreasing the access of α-BGT to receptors during the short pulse labeling of an intraperitoneal injection. Whatever the reason, this phenomenon would accentuate the extent of contamination of the endplate-specific label by extrajunctional receptors in denervated muscles labeled by α-BGT injection and assessed by the gamma counting procedure.

It was therefore important to establish the turnover rate of new receptors in denervated junctions directly. For that, the specific junctional label had to be assessed by TEM autoradiography to exclude extrajunctional contamination. Fortuitously, as the TEM autoradiographic results in this study show, the degradation rate of the new junctional receptors does indeed have a $t_{1/2}$ of ~1 d and thus is the same as that of the postdenervation extrajunctional receptors.

Preliminary results from studies in which new receptors were labeled 12 d after cold saturation and the degradation curve extended to >16 d, indicate that there is a small (~20%) component of slowly degrading receptors in the labeled pool. These slowly degrading receptors could be due in part to unbinding or destruction of the nonradioactive blocking toxin after cold saturation, which would cause some original receptors to be labeled together with the new ones. In addition, a delay in the full degeneration of the nerve after being cut, could cause a delay in the appearance of the rapidly degrading new receptors. This would mean that some receptors inserted after cold saturation would still have a slow degradation rate. Finally, there may be some slowly degrading receptors even in the absence of nerve. We are currently investigating the possible source(s) and extent of such slowly degrading receptors. Whatever the source however, the presence of these slowly degrading receptors would cause the $t_{1/2}$ measured for the new receptors in this study to be a slight overestimate and the new receptors would be degrading even faster than given here.

One can estimate the percentage by which a measured $t_{1/2}$ value ($t_{1/2}$ obs) is an overestimate since

$$\frac{1}{t_{1/2}\ \text{obs}} = \frac{f_i}{t_{1/2}\ \text{slow}} + \frac{(1 - f_i)}{t_{1/2}\ \text{fast}},$$

where f_i is the small fraction of slowly degrading receptors present during the period that the degradation rate is being measured.

If $t_{1/2}$ slow $\gg$ $t_{1/2}$ fast, then $t_{1/2}$ fast $\sim(1 - f_i)t_{1/2}$ obs.

Thus, the percentage by which $t_{1/2}$ obs is an overestimate is approximately equivalent to the percentage of the total pool that is degrading slowly.

The mechanism whereby the nerve regulates degradation is not known. From the present study we can say that the new receptors behaved as do extrajunctional receptors both at 6 and 14 d after cutting the nerve, when the original receptors have very different degradation half-lives. Thus the postdenervation degradation rates of junctional receptors seem to be related to whether the receptors had ever been stabilized by innervation or not. Since TEM autoradiography shows that the new and original receptors are interspersed in the postjunctional membrane, the control of their degradation is likely to be exerted in a microdomain, which could include the individual receptors, its surrounding membrane, and associated cytoskeleton or basal lamina.

In summary, this study established that new AChRs, inserted into a nmj after denervation, have a turnover half-life of ~1 d and are therefore in this respect equal to that of embryonic or postdenervation extrajunctional receptors. Thus, until all the original receptors are degraded, two metabolically distinct receptor populations (original and new) coexist at denervated nmj's. These two receptor populations are interspersed within the postjunctional membrane and degrade at different rates. The results confirm the "dual population" hypothesis proposed by Levitt and Salpeter (1981). Any model to explain neural control of degradation must account for this coexistence of receptors differing in degradation rate.

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