Metabolic Stabilization of Acetylcholine Receptors in Vertebrate Neuromuscular Junction by Muscle Activity

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Abstract. The effects of muscle activity on the growth of synaptic acetylcholine receptor (AChR) accumulations and on the metabolic AChR stability were investigated in rat skeletal muscle. Ectopic end plates induced surgically in adult soleus muscle were denervated early during development when junctional AChR number and stability were still low and, subsequently, muscles were either left inactive or they were kept active by chronic exogenous stimulation. AChR numbers per ectopic AChR cluster and AChR stabilities were estimated from the radioactivity and its decay with time, respectively, of end plate sites whose AChRs had been labeled with ^{125}I-α-bungarotoxin

(α-butx). The results show that the metabolic stability of the AChRs in ectopic clusters is reversibly increased by muscle activity even when innervation is eliminated very early in development. 1 d of stimulation is sufficient to stabilize the AChRs in ectopic AChR clusters. Muscle stimulation also produced an increase in the number of AChRs at early denervated end plates. Activity-induced cluster growth occurs mainly by an increase in area rather than in AChR density, and for at least 10 d after denervation is comparable to that in normally developing ectopic end plates. The possible involvement of AChR stabilization in end plate growth is discussed.

THE distribution and the functional properties of nicotinic acetylcholine receptors in vertebrate skeletal muscle depend strongly on the state of innervation of the muscle fibers. During the formation of a neuromuscular junction, acetylcholine receptors (AChRs)[1] are accumulated in the end plate membrane and their functional properties change under neural control (for review, see Salpeter and Loring, 1985; Schuetze and Role, 1987). Among these changes is an increase in the metabolic stability of the AChRs in the end plate membrane. At early stages of development, $t_{1/2}$ of junctional AChRs is about ∼1 d and comparable to that in noninnervated embryonic muscle membrane; in adult end plates, $t_{1/2}$ is increased to ∼10 d (Burden, 1977; Michler and Sakmann, 1980; Reiness and Weinberg, 1981). It declines again upon denervation (Loring and Salpeter, 1980; Bevan and Steinbach, 1983; Brett et al., 1982; Levitt et al., 1980; Stanley and Drachman, 1981) and is restored upon reinnervation (Salpeter et al., 1986), suggesting that the metabolic degradation rate of junctional AChRs depends on the presence of the nerve. In principle, the nerve may regulate metabolic AChR stability via "trophic" effects or by the electrical activity it induces in the muscle. Recently, it was found that pharmacological blockade of impulse conduction in the motor nerve (Cangiano et al., 1987) or of neuromuscular transmission (Avila et al., 1989) is sufficient to lower AChR stability. Conversely, the decline in metabolic AChR stability after denervation can be prevented if the denervated

muscle is kept active by chronic muscle stimulation (Brenner and Rudin, 1989). These observations indicate that AChR stabilization is dependent on muscle activity. The aim of the present paper was to investigate whether muscle activity has a similar effect enhancing the metabolic AChR stability at developing end plates as it has on mature end plates. The results show that muscle activity induces metabolic stabilization of junctional AChRs at developing ectopic end plates that had been denervated before the AChRs had been stabilized. If muscle stimulation is begun immediately after denervation, activity also promotes the growth of ectopic AChR clusters as previously observed by Lømo et al. (1984). Activity-induced cluster growth is comparable to that during normal ectopic end plate development.

Materials and Methods

The effect of denervation and of muscle stimulation was examined on the development of ectopic AChR clusters visualized by autoradiography after labeling the AChRs with ^{125}I-α-bungarotoxin (^{125}I-α-butx). Experiments were carried out on ectopic end plates in soleus muscles of male Sprague Dawley rats weighing ∼100 g at the time of the first operation.

Surgery and Stimulation

For surgery, the animals were anesthetized with nembutal (1 ml/kg) or ether. The formation of ectopic end plates was induced as described by Frank et al. (1975). Briefly, the superficial branch of the fibular nerve was transplanted onto the proximal end plate-free zone of the soleus muscle. 3 wk later, ectopic end plate formation was initiated by sectioning of the tibial nerve. Ectopic end plates then began to form within 2.5-3 d. For denervation of ectopic and original end plates, the sciatic nerve was cut. Chronic

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1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; α-butx, ^{125}I-α-bungarotoxin; psns, postsoleus nerve section.

stimulation of the soleus muscles was performed as described by Lømo et al. (1985). Stimuli of 12 mA strength, 0.5 ms duration and of alternating polarity were applied in 100 Hz trains of 1-s duration once every 100 s.

Autoradiography

For autoradiography, the animals were killed with ether. The muscles were excised, incubated in a solution containing 0.5–1 μg/ml ^{125}I-α-butx (Amersham Corp., Arlington Heights, IL; sp act, >200 Ci/mmol), extensively rinsed, fixed in 2.5% glutaraldehyde in PBS (pH 7.4), and stained for acetylcholine esterase (Karnovsky, 1964) as described by Brenner and Rudin (1989). Muscle segments containing the ectopic end plates were dispersed ultrasonically and transferred onto gelatine-coated slides (Reiness and Weinberg, 1981). After thorough rinsing in running tap water, the slides were then coated with Ilford L4 emulsion diluted 1:4 in 2% glycerol. Coating was done by dipping and withdrawing the slides from the emulsion at a constant velocity in an attempt to have all slides of one experiment coated with similar emulsion thickness. Exposure was at 4°C for 1–2 d. Autoradiograms were developed in Kodak D19 developer, fixed in sodium thiosulphate, dried and embedded in Eukitt (ABS, Basel, Switzerland). Autoradiographs were viewed on a Zeiss Standard microscope with dark field illumination at 250–1,000×.

Determination of AChR Density, AChR Cluster Area, and AChR Number per Cluster

To investigate the effects of stimulation on AChR density, AChR cluster area and AChR number, at least two sets of muscles were processed simultaneously in each experiment: one containing control ectopic end plates at 3 d postsoleus nerve section (psns) and others containing either older, normally developing ectopic end plates or end plates that had been denervated at 3 d psns and were then stimulated or left unstimulated for various times. At each end plate site, the grain density was counted at a magnification of 1,000× in dark field illumination, using the ocular grid of the microscope, and background densities were subtracted. Per cluster, one to three grain counts were averaged, and only clusters viewed completely en face were used. The densities of autoradiographic grains were 1.2–2 grains/μm^2. Grain densities from test muscles were normalized to the average density at the end plates at 3 d psns that had been processed in the same experiment. Thus, data from different experiments could be pooled even when the muscles had been labeled with different batches to toxin or when different exposure times had been used. Normalized grain densities for each denervation/stimulation protocol were then averaged and the standard error was calculated.

For determination of cluster area, the AChR clusters were photographed. Only clusters viewed en face were accepted. Their areas were measured with an electronic planimeter on photographic prints and summed to give end plate area. When clusters were further apart than 2 μm and a clear gap in high grain density was seen, they were considered as separate. One end plate was defined as the sum of all high density grain clusters located within 45 μm of one another, and only end plates with peripheral clusters at least 45-μm distant from the respective ends of a fiber segment were accepted for measurements of end plate area. With this procedure, true total end plate area was underestimated since ectopic end plate sites may be composed of AChR clusters further apart than 45 μm (Lømo et al., 1988) and clusters located on the side or underneath muscle fibers were rejected; however, it was sufficient for comparative estimates of end plate size. Observed changes in area and density by different denervations/stimulations were tested for statistical significance by means of the two-tailed t-test.

The number of AChRs per cluster normalized to that at 3 d psns was estimated by multiplying the averaged normalized values of AChR density (d) and cluster area (a), and the propagation of the standard errors was calculated according to

$$SE_{AChR} = [a^2 \cdot (SE_d)^2 + d^2 \cdot (SE_a)^2]^{1/2}.$$

Determination of AChR Half-lives

To investigate the effect of muscle activity on AChR half-lives, four to six test muscles treated according to the same denervation/stimulation protocol and 2–5 denervated but unstimulated control muscles were labeled, per experiment, with the same batch of toxin. After rinsing, some of these muscles were maintained for up to 96 h in organ culture as described previously (Brenner and Rudin, 1989) except that for culturing, muscles were dissected to a thin layer of superficial fibers containing the ectopic end plates. To exclude the possibility of changes in AChR half-lives because of culture conditions, test and control muscles were co-cultured in the same dish. At various

times after labeling, muscle were fixed. All muscles of any one experiment were processed simultaneously for autoradiography. For each experiment, the grain densities determined after various times of culturing were normalized to the average density at 0 h cultivation time. Thus, the data from different experiments employing the same denervation/stimulation protocol could be pooled. For each cultivation time, the pooled grain densities were averaged and their standard error was calculated. Metabolic stability of the labeled AChRs was determined from the rate of loss of the specific end plate grain density by fitting all data points (N) by the Levenberg-Marquardt method, assuming first order kinetics for AChR turnover. The rate constants of the AChR loss, $k \pm$ their SD, after different denervation/stimulation protocols were tested for significant differences, using the two-tailed t-test. AChR stability was expressed in terms of $t_{1/2} = \ln2/k$. The estimated half-lives were then corrected for unbinding of α-butx from the AChRs according to

$$1/t_{1/2(degrad)} = 1/t_{1/2(measured)} - 1/t_{1/2(unbind)}$$

(Salpeter et al., 1986) with $t_{1/2(unbind)} = 36$ days (Bevan and Steinbach, 1983). Preliminary experiments showed that the emulsion did not saturate up to a grain density of 2/μm^2: the range over which grain density changed linearly with increasing radioactive dose was determined by labeling normal soleus muscles with ^{125}I-α-butx and dispersing the muscle fibers on to several slides that were then processed for autoradiography as described. Radioactive dose could be changed by developing the slides after different exposure times. The results showed that under our experimental conditions, linearity was maintained up to a density of ~2 grains/μm^2.

To test further our method, we compared, using the same grain densities between 1 and 2/μm^2, the known AChR half-lives in chronically denervated versus control end plates. AChR half-lives in control and 20 d denervated muscles averaged 13.4 d and 1.9 d, respectively, in good agreement with corresponding values in the literature (e.g., Fumagalli et al., 1990). Thus, the grain counts in our paper reflect changes in radioactivity in a linear fashion and our method is sufficient to resolve changes in AChR half-lives. The reason for the more extended linearity observed in the present experiments as compared to those of Podleski and Salpeter (1988) may be that the emulsion on our slides was thicker than their monolayer.

Results

Changes in Area and Density of Junctional AChR Clusters and in Metabolic Stability of AChRs during Normal Ectopic End Plate Development

Experiments were carried out on ectopic end plates which had been surgically induced in soleus muscles of adult rats. In contrast to the single AChR accumulations at normal end plates, the AChR accumulations of ectopic end plates are composed of several AChR clusters (Lømo et al., 1988). However, the functional properties of ectopic end plate AChRs develop along a pattern similar to the one observed in normal end plate formation. Specifically, the metabolic stability of their AChRs increases as the synapse matures (Reiness and Weinberg, 1981). In different series of experiments, we determined the areas of individual AChR clusters in the ectopic innervation region, their relative densities in AChRs and the metabolic half-lives of the AChRs as a function of time after sectioning the soleus nerve or of muscle activity. Examples of autoradiograms are shown in Fig. 1; changes in AChR cluster area, AChR density, and AChR number per cluster in Fig. 2; and AChR degradations in Fig. 3.

Fig. 1 A shows an autoradiograph of an α-butx–labeled AChR cluster in the ectopic innervation region at 3 d psns. Such clusters were considered to represent ectopic end plate sites since they were seen neither in muscles whose original innervation had been left intact nor in the distal end plate-free segments of ectopically innervated fibers. The area of individual ectopic AChR clusters at 3 d psns averaged 108

± 10 μm^2 (SE, $N = 70$ clusters). Area and grain density were taken arbitrarily as 1.0 at this stage and were multiplied by one another to give a relative estimate of AChR number per cluster of 1.0 ± 0.1. All figures for cluster area, receptor density, and receptor number given below are normalized to their values at this stage. The AChR $t_{1/2}$ at 3 d psns was found to be 1.5 d and was significantly higher than $t_{1/2}$ of extrajunctional AChRs ($t_{1/2} = 1.1$ d, $P < 0.05$).

At 14 d psns, the mean area of the ectopic AChR clusters had increased to 3.0 ± 0.4 ($N = 80$, $P < 0.001$), whereas relative AChR density was increased only little to 1.3 ± 0.05 ($N = 26$, $P < 0.001$), resulting in a 4.0 ± 0.5-fold increase in relative AChR number per cluster. At the same time, the $t_{1/2}$ of the clustered AChRs was increased to 17.1 d that was significantly higher than $t_{1/2}$ at 3 d psns ($P < 0.02$).

At 35 d psns, cluster area was increased to 4.3 ± 0.7 ($N = 39$, $P < 0.001$), relative grain density was 1.9 ± 0.1 ($N = 12$, $P < 0.001$), to give an estimate of relative AChR number per cluster of 8.2 ± 1.3. The AChR half-life was not determined at this stage.

Ectopic AChR clusters are positionally stable and are resistant to muscle activity for several days even when the nerve is removed at an early stage of ectopic end plate development (Lømo and Slater, 1980; Lømo et al., 1984). In an attempt to assess the effect of muscle activity alone on junctional AChR accumulations, we have examined the effects of denervation and of muscle stimulation on ectopic AChR cluster area, AChR density and AChR stability.

Early Denervation Interrupts Developmental Changes in Ectopic AChR Accumulations

When the foreign nerve of a developing ectopic end plate was cut at different times after soleus nerve section, further development of the end plate membrane was arrested. This was shown by sectioning of the foreign nerve at 3 and 14 d psns. In muscles whose foreign nerve was cut at 3 d psns and that were subsequently left inactive for another 10 to 11 d, cluster area declined to 0.6 ± 0.1 ($N = 125$; $P < 0.002$) and relative grain density remained at 0.9 ± 0.1 ($N = 23$, $P > 0.2$), resulting in a decreased AChR number per cluster of ~0.6 ± 0.1 (all comparisons were made to ectopic end plates at 3 d psns). The half-life of the AChRs remained low at $t_{1/2} = 1.8$ d, but was significantly higher ($P < 0.02$) than that of extrajunctional AChRs in adjacent nonjunctional membrane ($t_{1/2} = 1.1$ d).

Cutting the foreign nerve at 14 d psns and then leaving the muscle inactive for 19 d produced clusters with areas of 3.2 ± 0.3 ($N = 62$), relative grain densities of 1.3 ± 0.1 ($N = 14$), and relative AChR numbers of 4.3 ± 0.5. These values are equal to those at the time of denervation ($P > 0.5$). AChR half-lives at these intermediate and late denervated ectopic end plates were not determined. They were assumed to be lowered as they are at chronically denervated original end plates (Loring and Salpeter, 1980).

Muscle Activity Reversibly Increases Metabolic AChR Stability and Produces Growth of Ectopic AChR Clusters at Denervated Ectopic End Plates

To examine the effect of muscle activity alone on junctional AChRs, a possible trophic neural influence was eliminated by cutting the ectopic innervation at 3 d psns when AChR

number and stability were still low. The muscles were then chronically stimulated for 10–11 d, and cluster size, relative grain densities and AChR numbers were compared to these parameters in muscles that had been left inactive for the same time. Stimuli were applied in 100 Hz trains of 1-s duration once every 100 s. In contrast to nonstimulated muscle, the area of the AChR clusters in stimulated muscle was increased to 3.6 ± 0.5 ($N = 37$, $P < 0.001$), whereas the density remained unchanged at 1.1 ± 0.1 ($N = 19$, $P > 0.5$), resulting in a 4.1 ± 0.7-fold increase in AChR number. Metabolic AChR stability was increased, resulting in a half-life of $t_{1/2} = 11.5$ d. This was higher than that in end plates at 3 d psns ($P < 0.02$) but not significantly different ($P > 0.05$) from that at normally developing end plates at 14 d psns ($t_{1/2} = 17.1$ d). When after fibular nerve section, muscles were left inactive for 18 d and were then stimulated for 11 d, AChR cluster areas did not increase as they did when stimulation was begun immediately after denervation, but were decreased to 0.5 ± 0.1 (SE, $N = 15$, $P < 0.002$).

Based on the delay of restabilization of junctional AChRs after reinnervation of denervated end plates, Salpeter et al. (1986) had estimated that stabilization of AChRs occurs "within hours or very few days" after reinnervation. To examine more directly how soon junctional AChRs are metabolically stabilized by muscle activity, we denervated ectopically innervated muscles at 3 d psns, i.e., when AChR stability was still low, stimulated them for 22–26 h, and, after labeling the AChRs with ^{125}I-α-butx, estimated the AChR half-life from the decay of the radioactivity at the ectopic clusters within 48–62 h of culturing. At AChR clusters of 16 stimulated muscles, residual radioactivity at the end of the culturing period ($N = 42$) had, on the average, declined to ~85% of the original ($N = 48$), corresponding to an AChR half-life $t_{1/2}$ of 12.9 d; this was significantly higher than the AChR half-life in extrajunctional fiber segments of the same muscles ($t_{1/2} = 0.8$ d; $N = 80$; $P < 0.01$) and was also higher than the $t_{1/2}$ of the AChRs in ectopic clusters from nonstimulated contralateral muscle ($t_{1/2} = 1.5$ d, $N = 22$). Two each of the stimulated and the nonstimulated muscles containing 10 and 14 end plates, respectively, had been co-cultured in the same dish in this series of experiments; their end plate AChR half-lives were 11.9 and 1.5 d, respectively. This and the difference in junctional and extrajunctional AChR degradation rates in stimulated muscle demonstrates that the effect of activity on metabolic stabilization was specific on the junctional AChRs and not because of a decrease in metabolism of the muscle fibers in the organ culture conditions. The data from the 1-d–stimulated muscles are summarized in Fig. 3 C. In conclusion, a single day of muscle activity is sufficient to metabolically stabilize AChRs at developing end plates.

The increases in metabolic half-life $t_{1/2}$ and in cluster area were reversed when stimulation was discontinued. In ectopic end plates denervated at 3-d psns, stimulated for 11 d and then left inactive for 18–22 d, the area of the AChR clusters declined to 1.0 ± 0.1 ($N = 60$) which is similar to its original value ($P > 0.5$), whereas AChR density was again not affected (1.0 ± 0.1, $N = 9$, $P > 0.5$). These changes result in an AChR number of 1.0 ± 0.2 that is similar to that at 3-d ectopic end plates. AChR half-life had fallen back to $t_{1/2} = 2.3$ d as compared to 1.5 d at young ectopic end plates at day 3 psns.
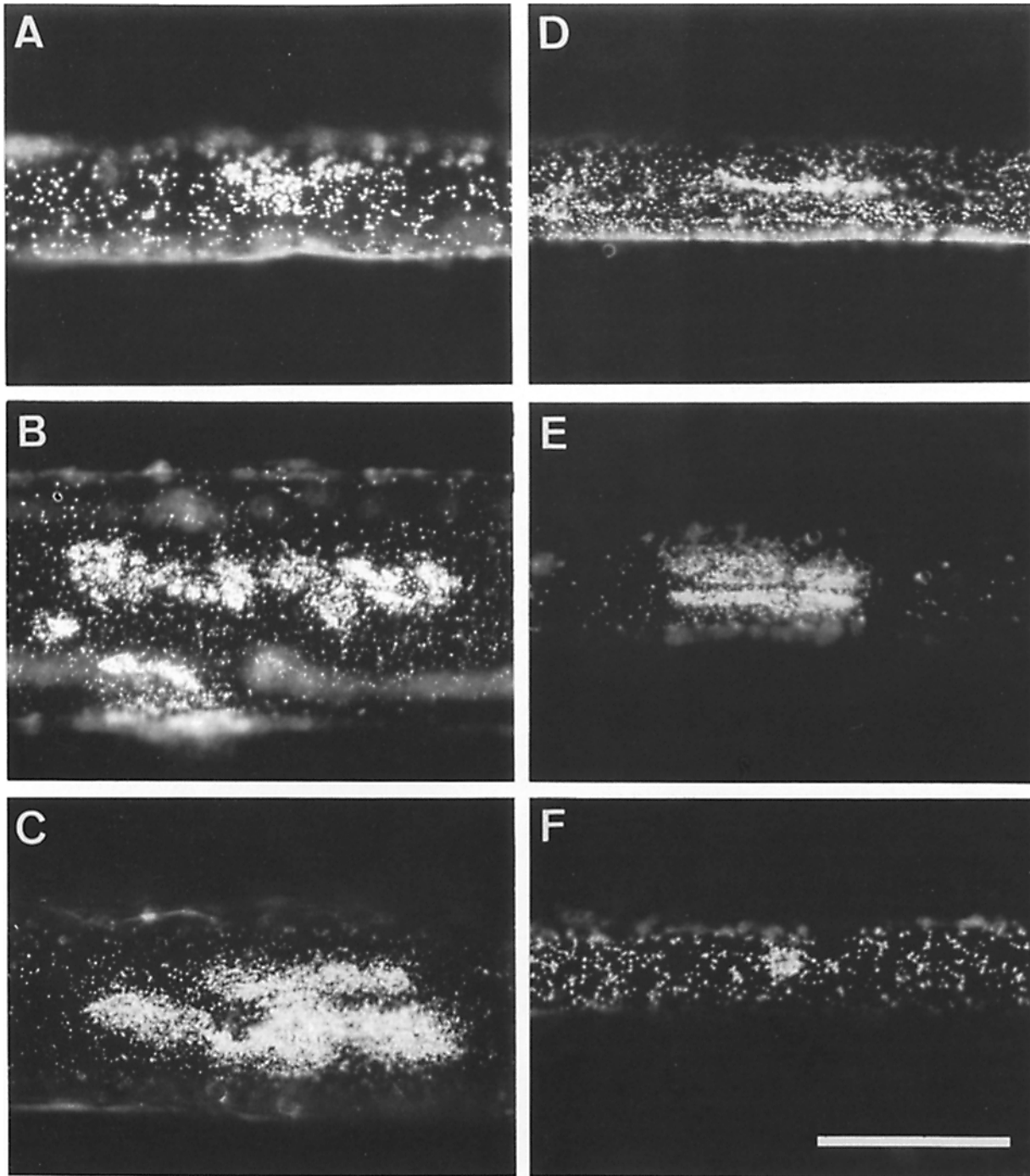
Figure 1. Autoradiograms of α-butx–labeled AChR clusters at ectopic end plates. (*A*) At day 3 psns. (*B*) At day 14 psns. (*C*) At day 35 psns. (*D*) Denervated at day 3 psns and inactive until day 14 psns. (*E*) Denervated at day 3 psns and then stimulated for 10–11 days. (*F*) As *E*, but then inactive until day 33 psns. Bar, 50 μm.

Discussion

The main result of the present work is that muscle stimulation alone is sufficient to reversibly increase the metabolic stability of the AChRs at ectopic end plates denervated early in development; a single day of stimulation is sufficient to increase their half-life to the value observed at mature end plates. At ectopic end plates that had been denervated at 3 d psns, i.e., when metabolic AChR stability was still low, $t_{1/2}$ was increased from 1.5 to 11.5 d by stimulation, and it declined again to 2.3 d after the stimulation was discontinued. Conversely, the AChRs retained their low half-life when the muscles were left unstimulated after denervation. The effects of stimulation on AChR half-lives at denervated ectopic AChR clusters after different stimulation protocols are summarized in Fig. 4. Combined with the previous observations that mere pharmacological muscle paralysis causes the half-life of junctional AChRs to decline (Cangiano et al., 1987)
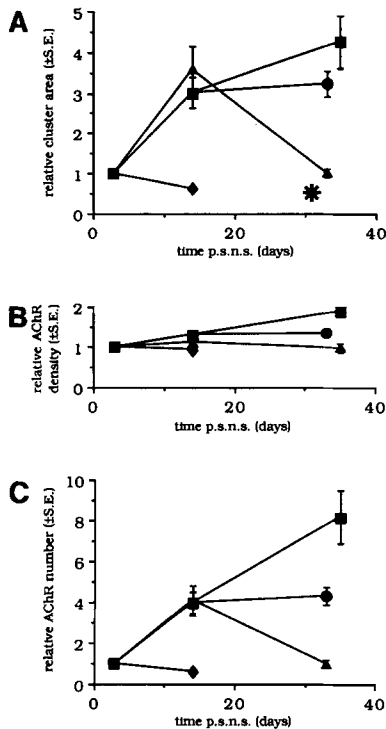
Figure 2. Changes in area, receptor density, and total receptor number of junctional AChR clusters at developing ectopic end plates during normal development and after early denervation with and without subsequent muscle stimulation. (*A*) Changes in area of individual ectopic AChR clusters. Area taken as 1.0 at 3 d psns. Each data point represents mean ± SE of 15–125 measurements; (*B*) changes in relative AChR density taken as 1.0 at 3 d psns. Each data point represents mean ± SE of 9–26 measurements. (*C*) Changes in AChR number per cluster, calculated from *A* and *B*. Relative receptor number is taken as 1.00 at day 3 psns. Error bars represent ± SE. (*Squares*) Normal ectopic development until day 35; (*circles*) normal development until day 14, denervation, inactive until day 33; (*triangles*) denervated at day 3, active until day 14, inactive until day 35 psns; (*diamonds*) denervated at day 3, inactive until day 14 psns; (*asterisk*) denervated at day 3, inactive until day 21, active until day 31.

Figure 3. Degradation of junctional AChR-^{125}I-α-butx complex as determined by quantitative autoradiography at ectopic end plates. (*A*) Degradation at day 3 psns ($t_{1/2}$ = 1.5 d) and at day 14 psns ($t_{1/2}$ = 17.1 d) of normal development, and degradation of extrasynaptic AChRs in ectopically innervated fibers after 10 d of ectopic denervation ($t_{1/2}$ = 1.1 d). (*B*) Degradation at denervated ectopic AChR clusters as a function of muscle stimulation. Ectopic innervation was cut at day 3 psns, and muscles were then left inactive until day 14 ($t_{1/2}$ = 1.7 d); or they were stimulated until day 14 ($t_{1/2}$ = 11.5 d); or, after stimulation until day 14, they were left inactive until day 31–35 ($t_{1/2}$ = 2.3 d). (*C*) Degradation at ectopic AChR clusters denervated at day 3 psns and left inactive until day 4 ($t_{1/2}$ = 1.5 d); or stimulated until day 4 ($t_{1/2}$ = 12.9 d), and degradation of extrasynaptic AChRs of the same stimulated muscles ($t_{1/2}$ = 0.8 d). AChR half-lives were determined from least-squares fits, assuming first order kinetics for AChR degradation, using the Levenberg-Marquart method and corrected for α-butx unbinding as described in Materials and Methods. Each data point represents mean of 5–40 measurements. SEMs were smaller than the symbols used in the figure.

and that muscle stimulation maintains or restores high AChR stability at chronically denervated end plates (Brenner and Rudin, 1989; Fumagalli et al., 1990), these findings strongly suggest that the nerve metabolically stabilizes the junctional AChRs in the developing end plate membrane (Michler and Sakmann, 1980; Reiness and Weinberg, 1981) by inducing electrical activity in the muscle.

Activity was not sufficient to stabilize all muscle AChRs, however. Its effect was restricted to junctional AChRs since extrajunctional AChRs in muscles stimulated for 1 d only retained their low half-life whereas the junctional AChRs were metabolically stabilized. Thus, AChR stabilization depends on additional components specific for the end plate membrane. Further indication for an interaction of AChRs with end plate components is that AChR half-life at early denervated ectopic end plates in inactive muscles was consistently higher than that in the adjacent extrajunctional membrane: $t_{1/2}$ was 1.5 d at 3 d psns, and was 1.8 d after 10 d of ectopic denervation compared to 1.1 d for extrajunctional AChRs. Shyng and Salpeter (1989) have recently reported that $t_{1/2}$ of
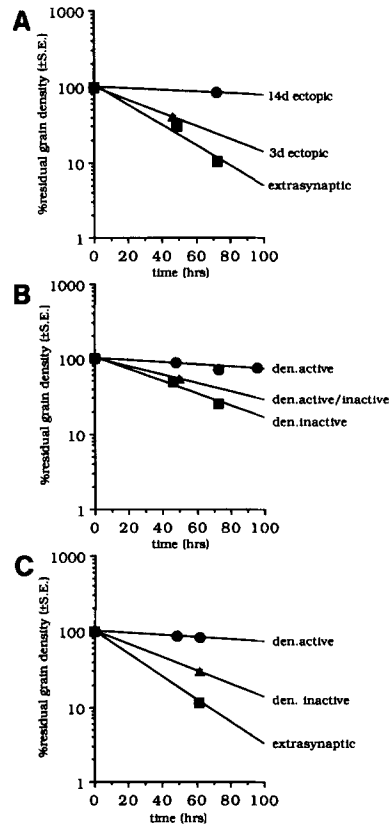
newly inserted AChRs into denervated mature end plate membrane of mouse sternomastoid muscle was similar to that of extrajunctional AChRs. The reason for this discrepancy is not clear. It may be related to the different species or muscle used in the present experiments.

Our experiments confirm earlier observations by Lømo et al. (1984) that denervated ectopic AChR clusters grow if the muscle is exogenously stimulated. The growth due to stimulation was similar to the one at normally developing ectopic end plates and, like that in normal development, originated primarily from an increase in area rather than AChR density (Weinberg et al., 1981; Michler and Sakmann, 1980). Thus, on the level of individual AChR clusters and over brief time
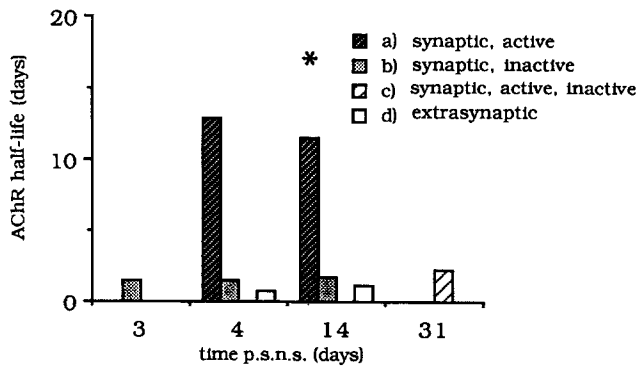
Figure 4. Effects of different stimulation protocols on metabolic half-lives of AChRs. Summary: all ectopic end plates denervated at day 3 psns and muscles subsequently stimulated or left inactive until the days psns indicated. Asterisk, $t_{1/2}$ at normally developing ectopic end plates for comparison.

spans of 1-2 wk, activity-dependent cluster growth is qualitatively and quantitatively similar to the growth of individual clusters at normally developing end plate membrane. Ectopic end plate sites are composed of several clusters, however, and although we could not be certain that with the method employed to dissociate muscle fibers, all clusters of an ectopic end plate site were seen, total number of end plate AChRs estimated by adding the areas of all clusters of a normally developing ectopic end plate site was about twice that observed after early denervation and subsequent stimulation. The reason for this may be that early denervation prevents the induction of further clusters by the nerve.

Although the activity-induced growth of denervated AChR clusters was similar to the growth at normally developing end plates up to 14 d psns, the normally developing clusters were more stable at longer times after denervation. Thus, unlike the latter, the clusters denervated early (3 d psns) in development and then growing under the influence of stimulation were not maintained when stimulation was discontinued. Furthermore, after 21 d of inactivity, even muscle stimulation failed to produce AChR cluster growth. Therefore, the long-term maintenance of ectopic clusters depends on the continued presence of the nerve, the stability of the neural "imprint" maintaining the clusters increasing with the duration of neuromuscular contact. Similarly, in neonatal mouse muscle, junctional AChRs become increasingly resistant to dispersal by denervation as the end plate matures (Slater, 1982). The postulated neural imprint may be related to agrin (Nitkin et al., 1987), a protein that is highly concentrated in the synaptic portion of the basal lamina (Fallon et al., 1985), is present in motoneurones (Magill-Solc and McMahan, 1988), and, when added to cultured muscle, induces clustering of the AChRs (Wallace, 1986; Nitkin et al., 1987).

Possible mechanisms whereby activity affects the growth of AChR clusters remain speculative at this time. It has been proposed that the number of junctional AChRs may be determined by the number of hypothetical AChR anchoring sites in the end plate membrane and that the metabolic stability of the AChRs is increased by their binding to these sites (Salpeter and Loring, 1985). In terms of this hypothesis, activity-dependent cluster growth is due to an increase in the number of anchoring sites. However, because of the long-term insta-

bility of denervated AChR clusters in stimulated muscle, additional factors released by the nerve, such as agrin (Nitkin et al., 1987), must be present to organize the anchoring sites into clusters at the site of the synapse. The role of AChR stabilization in the activity-dependent cluster growth may then be to reduce the number of new AChRs required for this growth at a stage of development, when (possibly due to the onset of muscle activity) the synthesis of AChRs in the junctional and perijunctional muscle regions declines. Indeed, the synthesis of α-subunit mRNA by the junctional muscle nuclei (Fontaine and Changeux, 1989) and the intracellular AChRs colocalizing with the neuromuscular junction (Pestronk, 1985) decrease as the synapse matures.

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