J. Physiol. (1981), **320**, *pp.* 435–447
With 1 plate and 4 text-figures
Printed in Great Britain

AGE CHANGES IN NEUROMUSCULAR JUNCTION MORPHOLOGY AND ACETYLCHOLINE RECEPTOR DISTRIBUTION ON RAT SKELETAL MUSCLE FIBRES

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(*Received 17 February 1981*)

SUMMARY

1. Neuromuscular junctions in the sternomastoid muscles of female Lewis rats were examined in animals up to 917 d old.

2. The average number of myelinated branches of terminal axons entering a junction increased with age of the animal, up to 400 d. This change could be described by a simple kinetic model which assumed that there was no influence of age on the ability of motoneurones to produce or maintain terminal branches, but that axons could produce or maintain only a limited number of branches.

3. There was no change in the over-all junctional length with age, but there was a significant increase in the number of discrete regions of high ACh receptor density in junctions from older animals.

4. There was a gradual decrease in the number of ACh receptors per junction with age after about 500 d, and muscles from some rats older than 500 d had detectable numbers of extrajunctional ACh receptors.

5. The changes in the neuromuscular junction with increased age occurred gradually over adult life.

INTRODUCTION

Several investigators have reported that neuromuscular junctions of aged mammals show morphological differences from junctions of young adult mammals. More myelinated branches of the same terminal axon enter junctions from old cats (Tuffery, 1971) and mice (Lefkowicz, 1979). Also, the cholinesterase-staining pattern is frequently broken or abnormal at junctions from old rats (Gutmann & Hanzlikova, 1965). However, no information is available about the rate at which these morphological alterations appear.

We studied neuromuscular junctions from rats at a number of ages from 100 to 917 d after birth. We examined junctional innervation using a silver stain, and the location of cholinesterase activity with a histochemical stain. The location and shape of regions of muscle membrane containing a high density of acetylcholine (ACh) receptors was studied using a fluorescent derivative of α-bungarotoxin, and the number of ACh receptors was estimated from the binding of iodinated α-bungarotoxin.

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The primary goal of this work was to determine the time course over which morphological changes occur in the junction. We posed one basic question: do these changes occur at constant rates throughout adult life, or are there age-dependent alterations in the rates at which changes occur? If the changes occur at a constant rate then junctional aging, at least in terms of these morphological parameters, is likely to be the result of the gradual accumulation of changes rather than the result of the action of some 'aging process' present only after a certain age. Our data indicate that age-associated morphological changes in the junction are the result of the slow accumulation of alterations throughout the life of the animal.

METHODS

Female Lewis rats (Simonson Laboratories, Gilroy, CA) were used in all experiments. Animals were killed with ether. Both sternomastoid muscles were removed and pinned in a plastic dish lined with Sylgard 184 (Dow-Corning, Midland, MI). The muscles were bathed in modified Eagle's medium buffered to pH 7·2 with 15 mM-*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid and containing 1% heat-inactivated fetal calf serum ('medium'). Muscles were incubated with α-bungarotoxin solutions (see below), washed thoroughly, then fixed in 4% paraformaldehyde (w/v) in 100 mM-sodium phosphate buffer (pH 7·0) for 60–120 min at room temperature.

The age of our animals was measured as their chronological age. Only two animals died, rather than being killed for examination, both between 700 and 800 d after birth. Female rats maintained under conventional conditions (comparable to ours) have mean survival times ranging from 600 to 900 d (Altman & Katz, 1979). Only fibres from the white region of the muscle were studied. These are largely 'white-fast' fibres (84%; Delhunty & Dlutkowski, 1979).

The methods used have been presented in detail in Steinbach (1981*a*, *b*).

The number of ACh receptors was calculated from the curare-protectable binding of iodinated α-bungarotoxin. Purified α-bungarotoxin was a gift from J. Patrick, The Salk Institute. It was radioactively labelled with ^{125}I and the di-iodo derivative (I-αBT) was isolated by the methods of Vogel, Sytkowski & Nirenberg (1972). The phrase 'ACh receptor' is used, although the number of curare-protectable I-αBT-binding sites was actually determined.

The rate of degradation of ACh receptors was estimated from the rate of degradation of bound I-αBT, by the methods described by Merlie, Heinemann & Lindstrom (1979).

The distribution of regions of high ACh receptor density was determined by fluorescent microscopy of muscle incubated with mono-tetra methylrhodamine-labelled α-bungarotoxin (R-αBT). Purified α-bungarotoxin was conjugated (Anderson & Cohen, 1974) with tetramethyl rhodamine isothiocyanate (B.B.L. Laboratories, Cockeysville, MD) and the monoconjugated form (R-αBT) was isolated by column chromatography (Ravdin & Axelrod, 1977).

Nerve axons and neuromuscular junctions were visualized using a silver stain. The method of Namba, Nakamura & Grob (1967) was modified to permit staining of small bundles (1 mm or less in diameter) or muscle fibres *en block*.

All statistical analysis was performed using standard methods on a small laboratory computer. The ability of kinetic models to describe changes in junctional innervation was determined by non-linear regression. The predicted frequencies were calculated for all three classes simultaneously and the 'best-fitting' parameter values were determined by minimizing the total sum of the sums of the squared deviations between observed and predicted values.

RESULTS

Muscle weight

Data on rat weight, muscle wet weight and mean fibre width are shown in Table 1. In general, all three parameters increased rapidly until 100 d after birth (Steinbach, 1981*b*), and more slowly thereafter. The oldest rats showed a variable reduction in these parameters.

Nerve terminals

Nerve terminals and motor axons were stained with a silver technique. Junctions were classified by the number of myelinated branches which entered them (Tuffery, 1971). In this classification, a T1 junction has a single myelinated branch entering

TABLE 1. Measured values for individual animals in which two or more parameters were measured. The column headed NMJ/TA gives the ratio of junctions in a terminal spray to the number of axons supplying that spray. The final column gives the calculated mean extrajunctional receptor density, obtained from the curare-protectable binding and the mean fibre width (Steinbach, 1981*a*); the standard deviation was calculated from the standard deviations of the measured parameters used in the calculations. Zeroes indicate that the binding of iodinated αBT was not significantly different in the presence or absence of curare ($P > 0.05$). The estimates of junctional receptor numbers enclosed in parentheses were not corrected for binding of iodinated αBT in the presence of curare, because the muscles came from animals which had been injected with I-αBT for degradation studies (see text).

				Junction					
	Weight		Fibre	AChE	AChR/				Extra-junc-tional
Rat age (d)	Rat (g)	Muscle (mg)	width* (μm) (50)	length* (μm) (50–150)	junction* ($\times 10^{-6}$) (5)	Pieces/ junction† (25–45)	T1 % (50–150)	NMJ TA	AChR/ μm^{2}* (5)
101	165	250	—	57±12	27±6	2·3	78	—	0
101	177	270	—	56±12	31±7	2·6	72	—	0
119	187	—	49±10	50±10	—	—	76	—	—
227	—	—	50±9	65±14	—	—	73	1·02	0
231	217	255	—	63±11	47±15	3·4	55	1·00	0
231	255	280	—	59±15	40±15	1·4	60	1·02	0
231	218	270	—	67±16	36±4	2·0	60	—	—
240	213	295	58±12	51±14	—	—	52	—	—
463	296	353	—	—	40±11	—	62	—	0
648	—	—	51±11	70±13	—	—	37	1·04	—
673	398	370	72±14	56±13	28±7	4·0	48	1·00	1·3±0·8
673	329	360	61±11	59±13	30±8	4·8	50	1·01	1·8±1·7
697	—	—	—	54±14	(25±12)	—	—	—	—
697	375	—	49±12	63±16	(30±16)	—	—	—	—
806	—	—	81±20	64±15	26±12	—	41	—	3·2±3·5
823	335	—	52±11	61±17	(19±15)	—	57	1·00	—
844	435	—	42±12	64±14	(6±3)	—	56	1·00	—
917	271	274	—	54±20	10±2	8·0	49	1·04	0
917	213	180	—	56±11	34±4	4·0	51	1·06	0
917	377	304	44±11	62±21	41±5	7·0	48	1·00	1·0±1·0

* Mean±S.D.; numbers in parentheses at heads of columns give the number of observations used to compute the values.

† Median.

it, a T2 junction has two myelinated branches of the same terminal axon, and so on (see Pl. 1). A 'duplex' junction is one in which two myelinated branches of the same terminal axon form two closely spaced junctions on the same fibre. Duplex and T4 junctions were both very rare at all ages (< 2%), and so have been grouped with the T3 junctions in Fig. 1. The data in Fig. 1 show that there is an age-related decrease in the frequency of T1 junctions.

Few muscle fibres were innervated by more than one terminal axon. A total of six doubly innervated junctions was seen (< 1 % of the total classified) and the frequency ranged from 0 to 1 % in a given muscle. There was no tendency for muscles from old

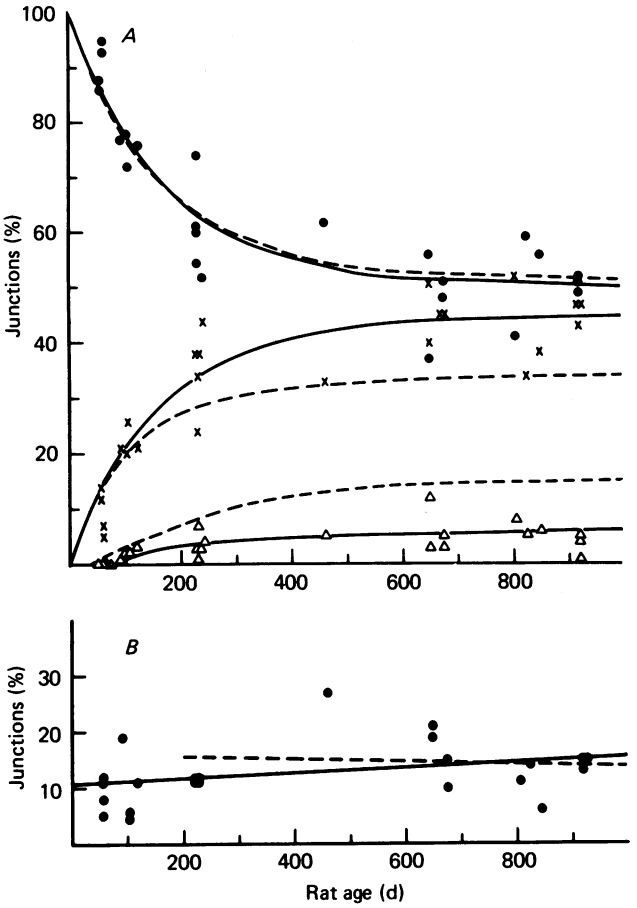

Fig. 1. Junctional innervation. Muscles from rats of different ages were silver-stained and 50–300 junctions from each muscle were classified according to the number of myelinated branches entering them (see text). *A* shows the percentage of junctions supplied by one myelinated branch (T1, ●), two myelinated branches (T2, ×) or three or more branches (△). The data were analysed in terms of two simple kinetic models described in the text. The dashed lines show the best fit with scheme (1), the continuous lines the best fit with scheme (2). The same samples were also examined for the presence of unmyelinated nerve branches to the junction (growth contributions; see text). *B* shows the percentage of the junctions in a muscle which showed growth configurations. The lines show linear regressions of the data on age, from the time of birth (continuous line) or from 200 d old (dashed line). In either case the regression coefficient was not significantly different from 0 ($P > 0{\cdot}2$ by the t test).

rats to have more doubly innervated fibres. As a further test for the presence of multiply innervated fibres, fifty single fibres were teased from muscles stained for cholinesterase activity from each of the 917 d old rats. None of these fibres showed more than one junctional region, so multiple innervation must be infrequent even in muscles from aged rats.

Some collateral branching was noted in terminal axons from rats of all ages. When the terminal innervation ratio (Coers & Woolf, 1959) was computed for muscles from rats of a number of ages there was at most a small increase in the ratio in muscles from older rats (Table 1).

The percentage of junctions showing 'growth configurations' was constant throughout life (Fig. 1). Tuffery (1971) defined a growth configuration as an unmyelinated nerve branch which leaves a terminal axon at a node of Ranvier and enters the neuromuscular junction supplied by that axon (see Pl. 1*B*). In general, a larger proportion of T1 junctions showed growth configurations than that of T2 junctions, and T3 junctions with sprouts were rare. Very few terminal sprouts (nerve branches leaving a junction) were seen. The major classification system (see above) depends on the number of myelinated branches, and so increases in complexity reflect both the formation of a sprout and its subsequent myelination.

The data in Fig. 1*A* were analysed to determine the kinetic constants appropriate to describe the changes in terminal innervation, and to determine whether the constants changed with age. Barker & Ip (1966) and Tuffery (1971) have reported that some axon branches degenerate, so the simplest kinetic model to describe the data is

$$\mathrm{I} \underset{2l}{\overset{k}{\rightleftharpoons}} \mathrm{II} \underset{3l}{\overset{k}{\rightleftharpoons}} \mathrm{III}. \qquad (1)$$

In scheme 1, I represents a T1 junction, II a T2 junction and, for simplicity, any junction more complicated than T2 is grouped in III and treated as a T3 junction. k represents the rate constant for the appearance of an additional myelinated branch, while l represents the rate constant for the loss of a myelinated branch. This model assumes several things: first, that myelinated branches both form and regress; secondly, that the terminal axons are a homogeneous population – each terminal axon has the same ability to form or maintain myelinated branches; thirdly, that denervation is rare – that T1 junctions rarely lose a myelinated branch.

In scheme (1) it is assumed that the formation or loss of a myelinated branch is independent of the number of already existing branches and also independent of age. This simplest model does not describe the data well. The dashed curves in Fig. 1 show that there are actually more T2 and fewer T3 junctions than predicted. Such a disagreement at all ages suggests that either branches form less readily on T2 junctions than T1, or that branches are lost more readily from T3 junctions than T2. A possible explanation for this is that a junction has the ability to maintain or to generate only a limited number of myelinated terminal branches, while the rates for formation and regression do not change with age.

The continuous lines in Fig. 1 show that a modified model is able to describe the data quite well. The continuous lines in Fig. 1 were generated by scheme (2), in which the rate of formation of new branches to T2 junctions and the rate of loss of branches from T3 junctions were reciprocally altered.

$$\mathrm{I} \underset{2l}{\overset{k}{\rightleftharpoons}} \mathrm{II} \underset{3lQ}{\overset{k/Q}{\rightleftharpoons}} \mathrm{III}. \qquad (2)$$

The continuous lines in Fig. 1 were generated using scheme (2) and the following parameter values: $k = 0{\cdot}00296\ \mathrm{d}^{-1}$, $l = 0{\cdot}00164\ \mathrm{d}^{-1}$ and $Q = 2{\cdot}19$. According to this fit, an additional myelinated branch would form at an existing T2 junction only one half as readily as at a T1 junction, while the half-life of a branch at a T3 junction would be about one third that for a branch at a T2 junction.

Junctional size

The size of the neuromuscular junction was measured as the over-all length of the region which stained for cholinesterase (ChE) activity (Pl. 1). The mean lengths did not change with age after growth ceased between 100 and 200 d post-natally (Fig. 2). However, inspection of the data in Fig 2 suggests that individual rats differ from each other in terms of their mean junctional lengths. This was tested by a one-way analysis of variance in data grouped according to age. In age groups 227–240 d ($n = 5$) and

600–700 d ($n = 5$) the analysis showed that the probability was less than 0·001 that the differences in mean lengths arose only from variation in sampling from a single homogeneous population of lengths. For the sample from 800–900 d ($n = 3$) $P < 0{\cdot}100$ and for the sample at 917 d ($n = 3$) $P < 0{\cdot}025$.

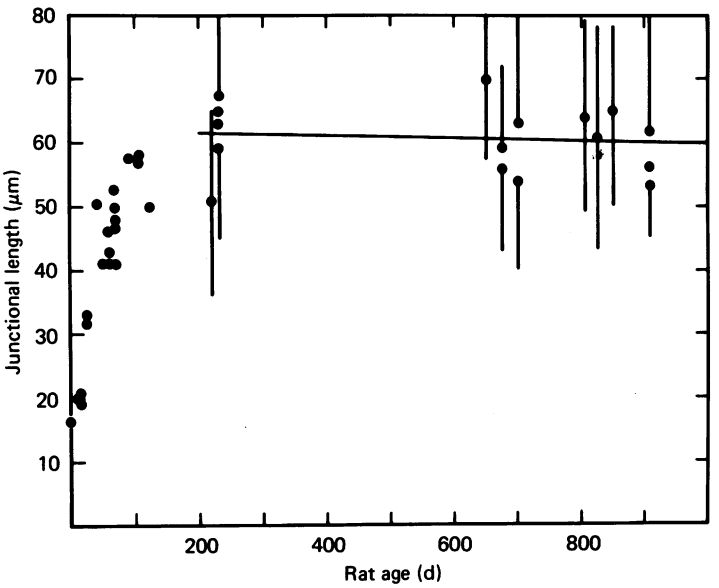

Fig. 2. Length of cholinesterase-staining regions. The mean length (±1 S.D.) of the lengths of 50–200 cholinesterase-staining regions is shown for individual animals. (Data for animals less then 200 d old, shown without error bars, are from Steinbach, 1981*b*). The line shows the linear regression line of the mean lengths on age; the slope of the line is −0·002 μm/d, which is not significantly different from 0 ($P > 0{\cdot}5$, by the t test). In seven animals the mean lengths for each of the two sternomastoid muscles were determined. In six the means were not significantly different ($P > 0{\cdot}2$ by the t test), in the seventh the difference had $0{\cdot}1 > P > 0{\cdot}05$. The mean lengths, therefore, do not differ between the two muscles of the same animal. In eight muscles from animals aged 119–844 d longer junctions were found on wider muscle fibres. Linear regression lines of junctional length on fibre width showed slopes ranging from 0·28 to 0·85, with no apparent dependence on age of the animal. In all cases the slopes were significantly different from 0 ($P < 0{\cdot}01$, t test).

Distribution of regions of high ACh receptor density

We used a fluorescent derivative of a α-bungarotoxin, R-αBT, to visualize post-junctional regions which contained a high density of ACh receptors. The staining pattern of junctions from older rats was qualitatively normal: brightly-stained regions were clearly demarcated with crisp, regular outlines (Pl. 1). The impression was formed that the junctions from older rats had a larger number of brightly staining regions than did junctions from young rats (Pl. 1). This impression was quantified by counting the numbers of separate regions of high receptor density in junctions that could be seen clearly and were essentially *en face*. Cumulative frequency histograms of these data are shown in Fig. 3. The median number of pieces per junction increases with the age of the animal, but there does not seem to be any age dependence of the rate at which pieces accumulate.

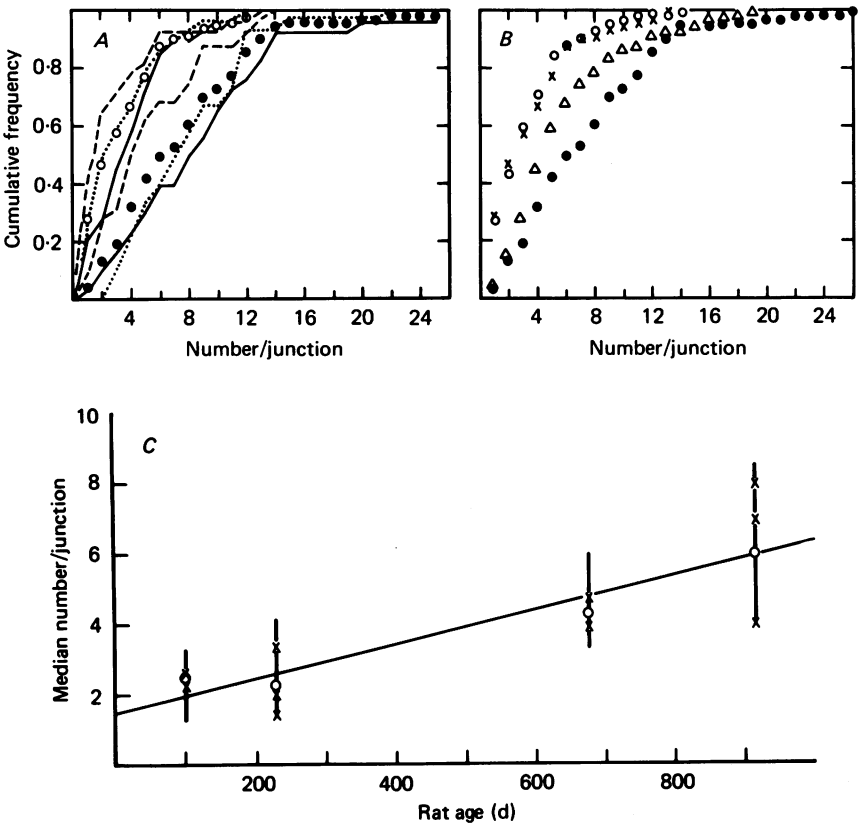

Fig. 3. Numbers of discrete regions of high receptor density in junctions. Fibres were teased from muscles stained with fluorescently labelled αBT, and the number of discrete regions which fluoresced brightly in junctions were counted in twenty-five to forty-five junctions in each muscle. *A* shows cumulative frequency histograms for the data from three individual 231 d old rats (left-hand group of lines, the open circles show the cumulative histogram for the total sample of 231 d old animals), and from three 917 d old rats (right-hand lines and filled circles). The plots are constructed so that the ordinate shows the frequency of junctions which have X or fewer discrete regions, where X is the abscissal value. *B* shows total sample cumulative frequency histograms (cf. the circles in *A*) for junctions from animals of four ages. To obtain these curves, all of the data obtained from different rats of a given age were pooled and the total sample histogram calculated. The ages of the rats (the number of rats and the total number of junctions) were as follows; ○, 101 d (2,89); ×, 231 d (3,90); △, 673 d (2,90); ●, 917 d (3,92). It is clear that there is a progressive increase in the median number of regions per junction with age. To assess the significance of this shift, the Kolmogorov-Smirnov test was applied to the data. The change in the distribution was significant between 231 d and 648 d or 917 d ($P < 0{\cdot}01$) and marginally significant between 648 d and 917 d ($P < 0{\cdot}05$). *C* shows a plot of median values (obtained by interpolation on cumulative frequency plots) for individual samples (×) and for the total samples at given ages (○, the bars show 99% confidence intervals around the total sample medians). The straight line shows the linear regression of the individual medians on age, the slope of the line is 0·006 pieces/d and the slope is significantly different from 0 ($P < 0{\cdot}01$, by the t test).

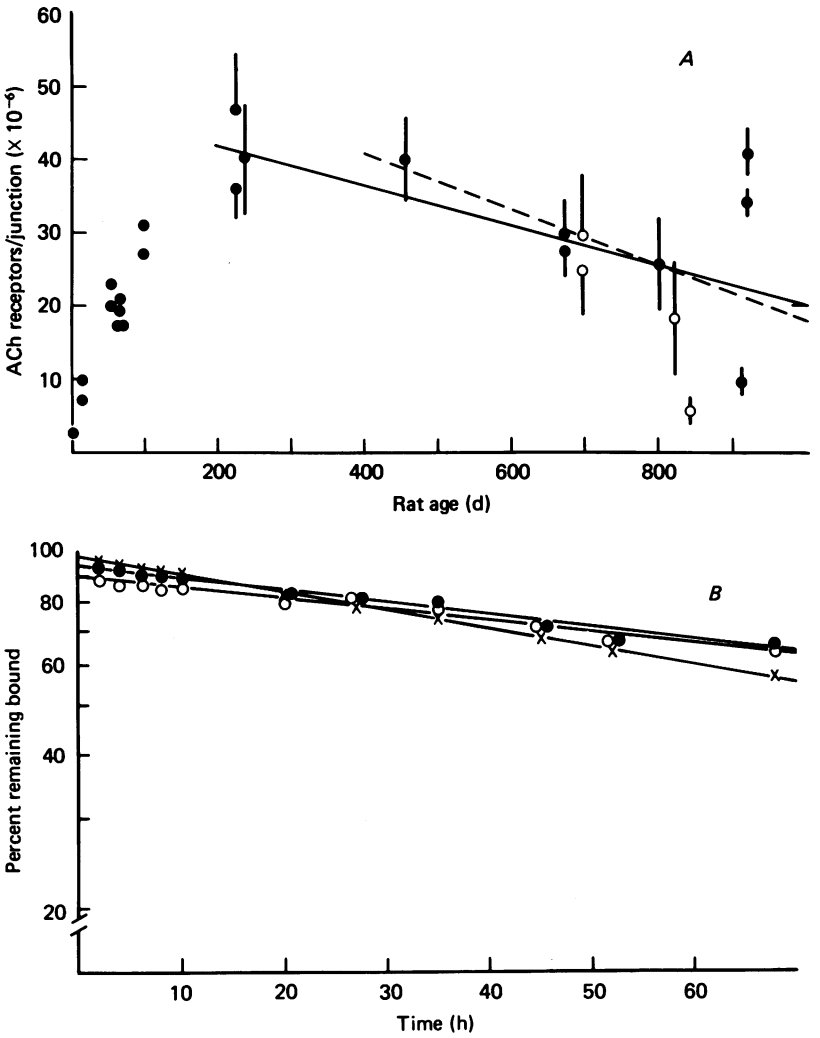

Fig. 4. *A*, numbers of junctional ACh receptors. The numbers of junctional ACh receptors were calculated from the curare-protectable binding of iodinated αBT to junctional regions. This shows the arithmetic mean (±1 S.E. of mean) number of junctional receptors. (The open points were obtained from animals used for studies of receptor degradation, and so no data were available for curare protectability.) Data from animals less than 200 d old (shown without error bars) are from Steinbach (1981*b*). The straight line shows the linear regression of mean junctional ACh receptor number on age for rats older than 200 d. It has a slope of $-2{\cdot}8 \times 10^4$ receptors/junction per day, significantly different from 0 ($P < 0{\cdot}025$, *t* test). The over-all mean number of junctional ACh receptors from rats 200–500 d old was 41×10^6 ($\pm 3 \times 10^6$). The dashed line shows the linear regression obtained when the results obtained from rats aged 200–500 d were arbitrarily assigned an 'age' of 450 d. This line has a slope of $-3{\cdot}9 \times 10^4$ receptors/junction per day ($P < 0{\cdot}025$). *B*, degradation of ACh receptors on rat diaphragm muscle. This shows a semi logarithmic plot of the amount of iodinated αBT remaining bound to rat diaphragm muscles as a function of time (for experimental details see Merlie *et al.* 1979). Data acquired in the same experiment using muscles from three animals are shown: ×, 70 d old animal; ○, 823 d old animal; ●, 844 d old animal. The lines are linear least squares regression lines fit to

ACh receptor numbers

The average number of ACh receptors per junction was determined in muscles from rats of different ages. Several old rats had significantly reduced numbers of junctional ACh receptors, but some of the oldest rats studied had normal numbers of junctional ACh receptors (Fig. 4*A*). The reason for this variability is not known. There is a statistically significant trend towards a reduced number of ACh receptors with increasing age after adulthood. It is not clear whether the reduction in the number of junctional receptors results from a decrease in the density of receptors in the sub-junctional region. Older rats had a larger number of 'pieces' in their junction, while the average junctional lengths did not increase (see above). Therefore, a reduction in the total receptor number could occur simply because the sub-junctional area decreased. For instance, in the three rats which were 917 d old, the junctions of the rat with the smallest mean number of junctional ACh receptors were composed of smaller individual pieces.

Extrajunctional ACh receptors

Sternomastoid muscles from four out of six old rats examined had a significant amount of curare-protectable extrajunctional αBT binding, although the calculated extrajunctional ACh receptor density is quite low (Table 1). The extrajunctional binding is probably not due to the presence of a few denervated fibres, for the following reasons. First, single fibres were teased from these four muscles incubated with iodinated αBT and autoradiographed. There were no fibres out of a total of 163 which showed the high extrajunctional grain density found on denervated fibres (Hartzell & Fambrough, 1972). Secondly, the calculated density was only about 1 % of the density on denervated fibres (Hartzell & Fambrough, 1972; Fambrough, 1974), so if all of the extrajunctional binding were due to the presence of denervated fibres only a small number could be present. The binding was determined using five small fibre bundles containing 10 to forty fibres per bundle, so that one or two denervated fibres would have been present in the entire sample, and hence only one bundle should have high binding and the rest low. This was not the case; the bundles showed rather uniform numbers of counts bound per unit extrajunctional length. For these reasons, the extrajunctional binding is unlikely to result from the presence of a few denervated fibres in the muscle, and it also is unlikely that there is a large percentage of denervated fibres at any given time in these muscles.

Degradation of ACh receptors

The degradation rate for ACh receptors was estimated from the rate of loss of radioactivity from the diaphragm muscles of rats which had been injected with 1-αBT (see Merlie *et al.* 1979). In two 673 d old rats and two rats aged 823 d and 844 d (Fig.

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the semi logarithmically transformed data, the apparent receptor half-lives obtained from the lines are 87 h (×), 146 h (○) and 125 h (●). In a similar experiment, radioactivity was lost from diaphragm muscles of two rats 697 d old with half-times of 261 h and 298 h while it was lost from a diaphragm of a 100 d old rat with a half time of 236 h. The mean half-life for the degradation of receptors on young adult diaphragm muscle in similar experiments is 138 h (range 94–236 h) (Steinbach, Merlie, Heinemann & Bloch, 1979).

4*B*), there was no indication of a systematic alteration in the rate of degradation for ACh receptors.

DISCUSSION

Our observations show that neuromuscular junctions in aged rats differ in several respects from neuromuscular junctions in young adult rats. The changes, however, appeared to develop gradually and in a continuous fashion over the adult life of the animal. For general reviews of aging in the neuromuscular system see Gutmann & Hanzlikova (1972, 1976).

The interpretation of our observations would be affected if there is a significant loss of muscle fibres or nerve axons in old animals. The sternomastoid muscle of the mouse apparently does not lose muscle fibres, at least not by 750 d post-natal (Rowe, 1969). In rat, only hind-limb muscles and nerve have been studied extensively. There is evidence that loss of some muscle fibres occurs in hind-limb muscles from old rats (Berg, 1956; Gutmann & Hanzlikova, 1966; Fujisawa, 1974; Caccia, Harris & Johnson, 1979; Tauchi, Yoshika & Kobayashi, 1971). There are also reports of ventral root degeneration (Berg, Wolf & Simms, 1962; Gilmore, 1972), although the number of myelinated axons apparently does not decrease in the sciatic nerve of aged rats (Caccia *et al.* 1979) or mice (Stanmore, Bradbury & Weddell, 1978). In general, studies which have examined both hind-limb and forelimb muscles have found that forelimb muscles show fewer abnormalities in old animals (Berg, 1956; Tauchi *et al.* 1971; Coers, Telerman-Toppet & Gerard, 1973; Fujisawa, 1974). It is not known whether the age-associated changes in hind-limb muscles are related to age *per se*, or, as Berg *et al.* (1962) suggest, to a disease process. We saw no signs of extensive collateral reinnervation in sternomastoid muscles from old rats and no muscle fibres with a high extrajunctional ACh receptor density, which suggests that extensive denervation does not occur. For these reasons, we will analyse our data on the assumption that no major change occurs in the numbers of muscle fibres in the sternomastoid muscle, nor in the number of motoneurones supplying the muscle.

We found that the mean junctional length did not change with age after 200 d. Other authors have reported a small increase in length (Gutmann & Hanzlikova, 1965) a small decrease in length (Gutmann & Hanzlikova, 1965; Pestronk, Drachman & Griffin, 1980) or no change in length (Tuffery, 1971) in junctions from various hind-limb muscles.

Our analysis of silver-stained preparations has confirmed the observations made by Tuffery (1971) in cat muscle, and by Lefkowicz (1979) in mouse muscle. In addition, the present data have been obtained from animals at a number of ages, allowing us to conclude that the changes observed occur gradually over the adult life of the animal. We have analysed the data in terms of a simple kinetic model, which is both plausible to us and can describe the data well. Our analysis suggests that a terminal axon has a lower probability of growing an additional myelinated branch, and a higher probability of losing one, as the number of terminal branches increases but the rates for formation and regression do not depend on age. There is no significant dependence on age of the frequency of junctions with 'growth configurations', as Tuffery (1971) also reported on comparing young adult and old cat neuromuscular junctions. Drahota & Gutmann (1961) have reported that the ability

of old animals to reinnervate denervated muscle is decreased, while Pestronk *et al.* (1980) report a decrease in the ability of hind-limb motor axons to sprout in aged rats, assayed either from the sprouting of terminal axons in the soleus muscle following intramuscular injection of botulinum toxin or from the rate of growth of axons past the site of a sciatic nerve crush. It is possible that there is a decline in the ability of motoneurones to grow or maintain terminal axons, but that this decline is sufficiently slow that it is only revealed as a diminished response to major stimulus. Alternatively, sprouting could be hampered by non-neural factors, for example by an increase in connective tissue which could slow growth mechanically. In any case, our data on junctional innervation can be described well by a model which assumes that terminal axon branches form and regress, and which excludes any effect of age on the ability of neurones to grow or maintain myelinated terminal axon branches.

When junctions were stained with R-αBT, junctions in muscles from older animals had more discrete regions which fluoresced intensely. However, there was no indication that the rate of increase in the number of pieces per junction itself depended on age. Fujisawa (1976) reported that some terminal branches in junctions from aged rat hind-limb muscles showed 'degenerative changes', while other branches in the same junction appeared normal, although Gutmann, Hanzlikova & Vyskocil (1971) found no degeneration of terminal axon branches in junctions in the levator ani muscle of aged male rats. It is possible that such a localized deterioration could result in the appearance of more discrete regions of high post-junctional receptor density.

The average number of ACh receptors did decline with age after about 500 d, although there was quite a bit of variability in this number between rats of similar ages. There was no apparent change in the rate of degradation of ACh receptors. The decline in numbers could reflect an age-associated change in some process at the junction. Still, at least some of the loss in receptors probably results from the 'fragmentation' of the receptor-dense region. The fragmentation, in turn, does not seem to change in rate with age, so that even the decline in numbers of receptors per junction may reflect a process which operates at a constant rate throughout the life of the animal. Pestronk *et al* (1980) reported that there was no decrease in the number of receptors per junction in soleus muscles from aged rats.

Muscles from some of the older rats showed a low but measurable amount of extrajunctional ACh receptors. Pestronk *et al.* (1980) also reported an increase in the amount of extrajunctional binding of iodinated αBT in soleus muscles from old rats. It is known that inactivity of innervated skeletal muscle induces an increase in the density of extrajunctional ACh receptors in rat muscle (Pestronk, Drachman & Griffin, 1976; Lavoie, Collier & Tenenhouse, 1977; Mills, Bray & Hubbard, 1978), while rats become less active with age (Elias, 1979). Therefore the increase in extrajunctional ACh receptor density could result from the over-all activity of the animal.

There have been only a few physiological studies of junctions in older animals. Kelly (1978) found that the frequency of miniature end-plate potentials (m.e.p.p.s) and the quantal content for evoked release increased rapidly in rats between birth and 100 d, then showed a further small increase until 200 d, followed by a small, but significant, decline at 375 d. Vyskocil & Gutmann (1972) and Gutmann *et al.* (1971) compared m.e.p.p. frequencies between young (60–100 d old) and old (about 900 d old) rats and

found that m.e.p.p. frequencies were significantly reduced at junctions from old rats. These results suggest that physiological changes in the junction occur gradually after adulthood. There was no morphological change which occurred with the same time course as these physiological changes (e.g. commencing at 200 d).

Over-all, our data show that there are many changes in the neuromuscular junction with the age of the animal. The changes which we have observed appear to accumulate gradually during the adult life of the animal. Our analysis of the data has not given an indication that the chronological age of the animal alters the *rates* at which the changes occur.

This research was supported by grants from the M.D.A., the N.I.H. and the N.S.F. to J.H.S. J.H.S. is a Sloan Foundation Fellow.

REFERENCES

ALTMAN, P. L. & KATZ, D. D. (1979). *Inbred and genetically defined strains of laboratory animals. 1. Mouse and rat.* Fedn. Am. Soc. exp. Biol. Bethesda: N.I.H.

ANDERSON, M. J. & COHEN, M. W. (1974). Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. *J. Physiol.* **237**, 385–400.

BARKER, D. & IP, M. C. (1966). Sprouting and degeneration of mammalian motor axons in normal and deafferented skeletal muscle. *Proc. R. Soc.* B **163**, 538.

BERG, B. N. (1956). Muscular dystrophy in aging rats. *J. Gerontol.* **11**, 134–139.

BERG, B. N., WOLF, A. & SIMMS, H. S. (1962). Degenerative lesions of spinal roots and peripheral nerves in aging rats. *Gerontologia* **6**, 72–80.

CACCIA, M. R., HARRIS, J. B. & JOHNSON, M. A. (1979). Morphology and physiology of skeletal muscle in aging rodents. *Muscle and Nerve* **2**, 202–212.

COERS, C., TELERMAN-TOPPET, N. & GERARD, J-M. (1973). Terminal innervation ratio in neuromuscular disease. I. Methods and controls. *Archs Neurol., Paris* **29**, 210–214.

COERS, C. & WOOLF, A. L. (1959). *The Innervation of Muscle: a Biopsy Study.* Oxford: Blackwell.

DELHUNTY, A. F. & DLUTKOWSKI, M. (1979). Fiber types in red and white segments of rat sternomastoid muscle. *Am. J. Anat.* **156**, 57–62.

DRAHOTA, Z. & GUTMANN, E. (1961). The influence of age on the course of reinnervation of muscle. *Gerontologia* **5** 88–107.

ELIAS, M. F. (1979). Aging studies of behavior with Fischer 344, Sprague-Dawley and Long-Evans rats. In *Development of the Rodent As a Model System of Aging*, ed. GIBSON, D. C., ADELMAN, R. C. & FINCH, C. N.I.H. Publication 79–161. Bethesda: N.I.H.

FAMBROUGH, D. M. (1974). Acetylcholine receptors: revised estimates of extrajunctional receptor density in denervated rat diaphragm. *J. gen. Physiol.* **64**, 468–472.

FUJISAWA, K. (1974). Some observations on the skeletal musculature of aged rats. Part I. Histological aspects. *J. Neurol. Sci.* **22**, 252–266.

FUJISAWA, K. (1976). Some observations on the skeletal musculature of aged rats. III. Abnormalities of terminal axons found in motor endplates. *Exp. Gerontol.* **11**, 43–47.

GILMORE, S. A. (1972). Degeneration in ventral and dorsal roots in aging laboratory rats – a light microscopic study. *Anat. Rec.* **174**, 251–258.

GUTMANN, E. & HANZLIKOVA, V. (1965). Age changes of motor endplates in muscle fibres of the rat. *Gerontologia* **11**, 12–24.

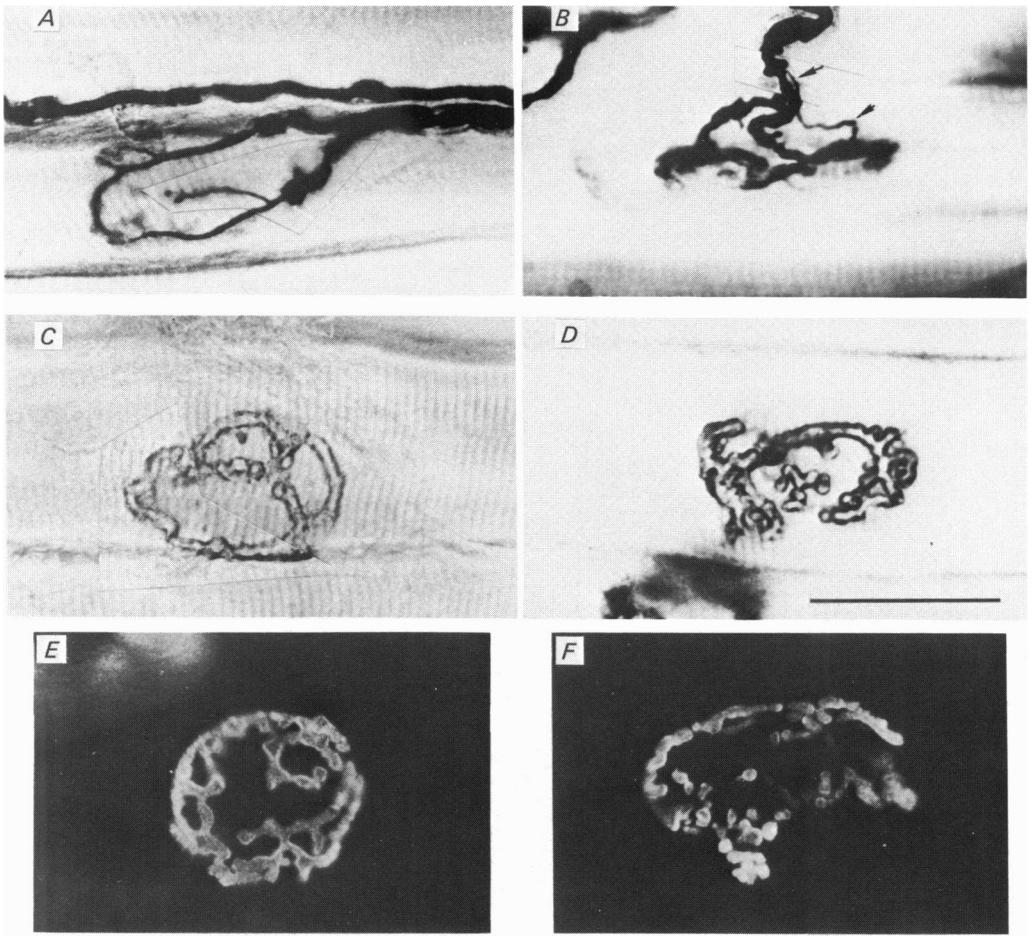
GUTMANN, E. & HANZLIKOVA, V. (1966). Motor unit in old age. *Nature, Lond.* **209**, 921–922.

GUTMANN, E. & HANZLIKOVA, V. (1972). *Age Changes in the Neuromuscular System.* Bristol: Scientechnica Ltd.

GUTMANN, E. & HANZLIKOVA, V. (1976). Fast and slow motor units in ageing. *Gerontology* **22**, 280–300.

GUTMANN, E., HANZLIKOVA, V. & VYSKOCIL, F. (1971). Age changes in cross striated muscle of the rat. *J. Physiol.* **216**, 331–343.

HARTZELL, H. C. & FAMBROUGH, D. M. (1972). Acetylcholine receptors: distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. *J. gen. Physiol.* **60**, 248–262.


A
B
C
D
E
F

KELLY, S. S. (1978). The effect of age on neuromuscular transmission. *J. Physiol.* **274**, 51–62.

LAVOIE, P. A., COLLIER, B. & TENENHOUSE, A. (1977). Role of skeletal muscle activity in the control of muscle acetylcholine sensitivity. *Expl Neurol.* **54**, 148–171.

LEWKOWICZ, S. J. (1979). Motor innervation of the gastrocnemius muscle of wobbler and dystrophic mice. *J. Neurol. Sci.* **43**, 405–419.

MERLIE, J., HEINEMANN, S. & LINDSTROM, J. (1979). Acetylcholine receptor degradation in adult rat diaphragms in organ culture and the effect of anti-acetylcholine receptor antibodies. *J. cell Biol.* **254**, 6320–6327.

MILLS, R. G., BRAY, J. J. & HUBBARD, J. I. (1978). Effects in activity on membrane potentials in rat muscle. *Brain Res.* **150**, 607–610.

NAMBA, T., NAKAMURA, T. & GROB, D. (1967). Staining for nerve fiber and cholinesterase activities in fresh frozen sections. *Am. J. clin. Path.* **47**, 74–78.

PESTRONK, A., DRACHMAN, D. B. & GRIFFIN, J. W. (1976). Effects of muscle disuse on acetylcholine receptors. *Nature, Lond.* **260**, 352–353.

PESTRONK, A., DRACHMAN, D. B. & GRIFFIN, J. W. (1980). Effects of aging on nerve sprouting and regeneration. *Expl. Neurol.* **69**, 65–72.

RAVDIN, P. & AXELROD, D. (1977). Fluorescent tetramethyl rhodamine derivatives of α-bungarotoxin: preparation, separation, and characterization. Analyt. Biochem. **80**, 585–592.

ROWE, R. W. D. (1969). The effect of senility on skeletal muscles in the mouse. *Exp. Gerontol.* **4**, 119–126.

STANMORE, A., BRADBURY, S. & WEDDELL, A. G. M. (1978). A quantitative study of peripheral nerve fibres in the mouse following the administration of drugs. 1. Age changes in untreated CBA mice from 3 to 21 months of age. *J. Anat.* **127**, 101–115.

STEINBACH, J. H., MERLIE, J., HEINEMANN, S. & BLOCH, R. (1979). Degradation of junctional and extrajunctional acetylcholine receptors by developing rat skeletal muscle. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3547–3551.

STEINBACH, J. H. (1981*a*). Neuromuscular junctions and α-bungarotoxin-binding sites in denervated and contralateral cat skeletal muscles. *J. Physiol.* **313**, 513–528.

STEINBACH, J. H. (1981*b*). Developmental changes in acetylcholine receptor aggregates at rat skeletal neuromuscular junctions. *Dev. Biol.* (In the Press.)

TAUCHI, H., YOSHIKA, T. & KOBAYASHI, H. (1971). Age changes of skeletal muscles of rats. *Gerontologia* **17**, 219–227.

TUFFERY, A. R. (1971). Growth and degeneration of motor endplates in normal cat hindlimb muscles. *J. Anat.* **110**, 221.

VOGEL, Z., SYTKOWSKI, A. J. & NIRENBERG, M. W. (1972). Acetylcholine receptors of muscle grown *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **69**, 3180–3184.

VYSKOCIL, F. & GUTMANN, E. (1972). Spontaneous transmitter release from nerve endings and contractile properties in the soleus and diaphragm muscle of senile rats. *Experientia* **28**, 280–281.

EXPLANATION OF PLATE

Appearance of neuromuscular junctions at different ages. This shows neuromuscular junctions stained by various procedures. The left column shows junctions from young adult animals, the right column junctions from older animals. *A* shows a silver-stained junction (T2) from a 231 d animal, *B* a T2 junction with a growth configuration (arrows) from a 917 d animal. *C* and *D* show junctions stained for cholinesterase activity from a 101 d (*C*) and a 823 d (*D*) animal. *E* and *F* show junctions stained with fluorescent αBT from a 231 d and a 917 d old animal, respectively. The bar in *D* indicates 50 μm for all parts of this plate. The silver stains were analysed to provide data on junctional innervation (Fig. 1), the lengths of cholinesterase-stained regions were measured to estimate junctional size (Fig. 2), while the pattern of the fluorescent αBT stain was analysed in terms of the number of discrete regions of bright fluorescence in each junction (Fig. 3).