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NEUROMUSCULAR TRANSMISSION IN NEW-BORN RATS

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SUMMARY

1. End-plate potentials (e.p.p.s) were recorded intracellularly from the isolated phrenic/diaphragm preparation of the rat during the first few weeks of life.

2. Most e.p.p.s at birth were complex and resulted from the summation of two to four units, which could be separated by their different latencies and thresholds to stimulation of the phrenic nerve.

3. The e.p.p.s became simpler during the second week of the rats' life, and by 16–18 days old the e.p.p.s consisted of single units, and resembled the e.p.p.s of adult rat muscle.

4. It is proposed that the units of the e.p.p. resulted from the stimulation of separate nerve axons and that all but one of the synapses on each muscle fibre were lost during the second week of life.

INTRODUCTION

Diamond & Miledi (1962) have shown that the muscle fibres of foetal and new-born rats differ in many ways from adult muscle fibres. Acetylcholine sensitivity is present along the length of the fibres, and the frequency of miniature e.p.p.s is lower than in the adult. There is a gradual change during the first few weeks of extra-uterine life to the adult pattern.

Because of these changes it was decided to investigate the end-plate potentials in the muscle of the new-born rat. This paper reports the results of study of neuromuscular transmission in the diaphragm of the neonatal rat, and the changes in e.p.p. activity during the first 2–3 weeks of extra-uterine life.

METHODS

Litters of Wistar Albino rats were bred, the date of birth noted, and the rats used one by one during the first 3–4 weeks of life. (This may have accelerated the development and reduced mortality in the rest of the litter, particularly when most of the

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litter had been removed.) The rats were killed by rapid decapitation with or without anaesthesia. The thorax was quickly removed, and the intact diaphragm and phrenic nerves dissected free under cooled mammalian Ringer solution.

The preparation was mounted on a paraffin wax coated Perspex slide and placed in a bath at $30 \pm 1°$ C. The muscle was covered by mammalian Ringer solution (Liley, 1956) flowing at 200–300 ml./hr which was bubbled with 5% CO_2 in O_2 immediately before addition to the bath. The phrenic nerve was stimulated by a Grass S 8 stimulator connected through two Grass stimulus isolation units (SIU 4678) to fine silver wire electrodes on the surface of the fluid in the bath. Tubocurarine was added to the mammalian Ringer solution to prevent twitching, and the calcium concentration was increased to 10 mM, by the addition of calcium chloride. Complete nerve block was a frequent complication using normal Ringer, perhaps as a result of the strong stimuli necessary to excite these small unmyelinated axons, but the incidence appeared to be reduced by increasing the calcium concentration. All recordings were, therefore, obtained using mammalian Ringer containing 10 mM-Ca^{2+}.

Recordings of resting and end-plate potentials was by means of conventional glass capillary micro-electrodes filled with 3 M-KCl, having resistances between 5 and 10 MΩ, and connected through a negative capitance cathode follower to a dual beam oscilloscope. The potentials were also displayed on a slave cathode ray tube, and photographed on 35 mm film.

RESULTS

The muscle fibres of new-born rats are small in diameter and have a lower resting potential than adults rats (Harris & Luff, 1969) and after penetration this often fell quickly to very low levels presumably due to damage. Because the fibres are small in diameter, and their margins appear indistinct, repenetration of the same fibre could not be assured. In addition, the rise and fall times of the e.p.p.s are slower than those of the larger and older rat, and the electronic decay along the fibre is less. Fibres were penetrated near their mid points and recordings selected on the basis of a fast rise time.

There were variations in results between litters of rats and between some rats in the same litter. This may be a reflexion of the differences in development of the individual rats, as judged by weight, growth of hair, activity and eye opening.

Nerve conduction velocities were measured in only a few fibres, and were subject to a wide variation. However, the mean value of conduction velocities at a temperature of 30° C estimated from the latent periods of nerve conduction was about 1 m/sec at birth, rising in 2 weeks to about 10 m/sec. These surprisingly low values are consistent with those of Lewis (1968). No estimate of synaptic delay in neonatal rat muscle is available, but subtracting typical values obtained from adult muscle from the latent periods measured does not significantly affect the estimates of conduction rates.

The photographs show typical intracellular recordings of e.p.p.s close to the middle of a muscle fibre, while gradually increasing or decreasing

the strength of the stimulus applied to the phrenic nerve. Increasing the stimulus strength results in the addition of 'units' to the e.p.p., and these summate to form a complex e.p.p. (Fig. 1).

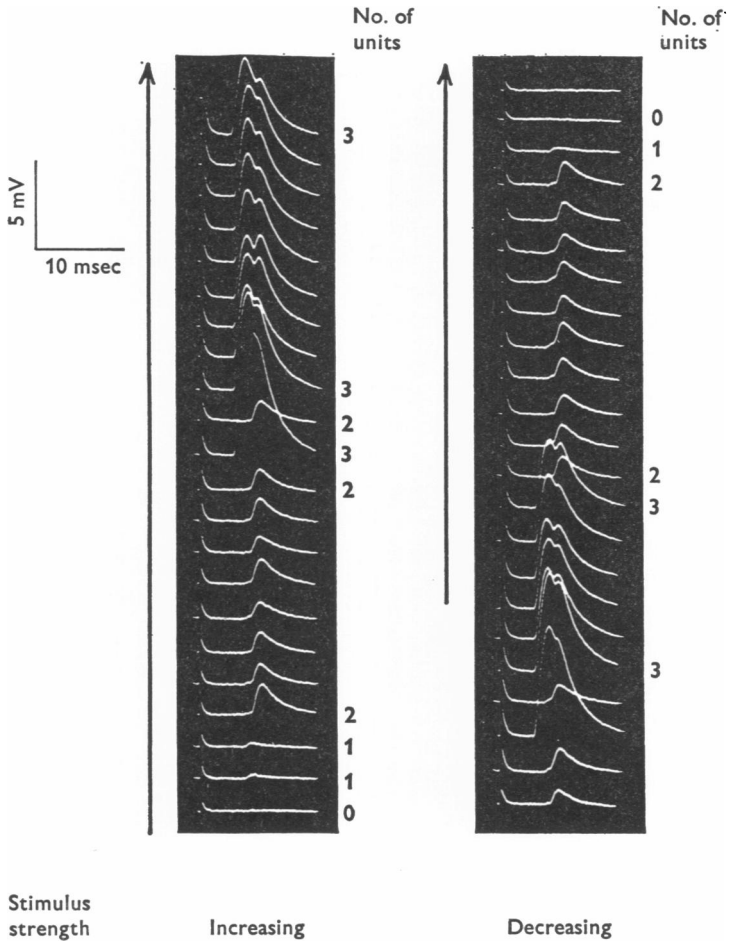

Fig. 1. Typical e.p.p.s of a curarized diaphragm muscle in a rat, 10 days old weighing 16 g. The diaphragm was in mammalian Ringer solution containing tubocurarine and 10 mM-Ca^{2+}. The e.p.p. consisted of three units which are added to the e.p.p. by progressively increasing the stimulus strength, and subtracted from the e.p.p. in reverse order, by reducing the stimulus strength. The resting potential was 60 mV on insertion and 45 mV while recording.

Sometimes these units were almost simultaneous and could not be temporally separated. Their separate existence could only be inferred from an abrupt jump in the e.p.p. amplitude while increasing the stimulus

strength. On the other hand, a few fibres had e.p.p.s which consisted of widely separated 'units' which did not summate (Fig. 2).

In most muscle fibres there was a relationship between the latency and the stimulus strength, such that the units with the longer latency appeared

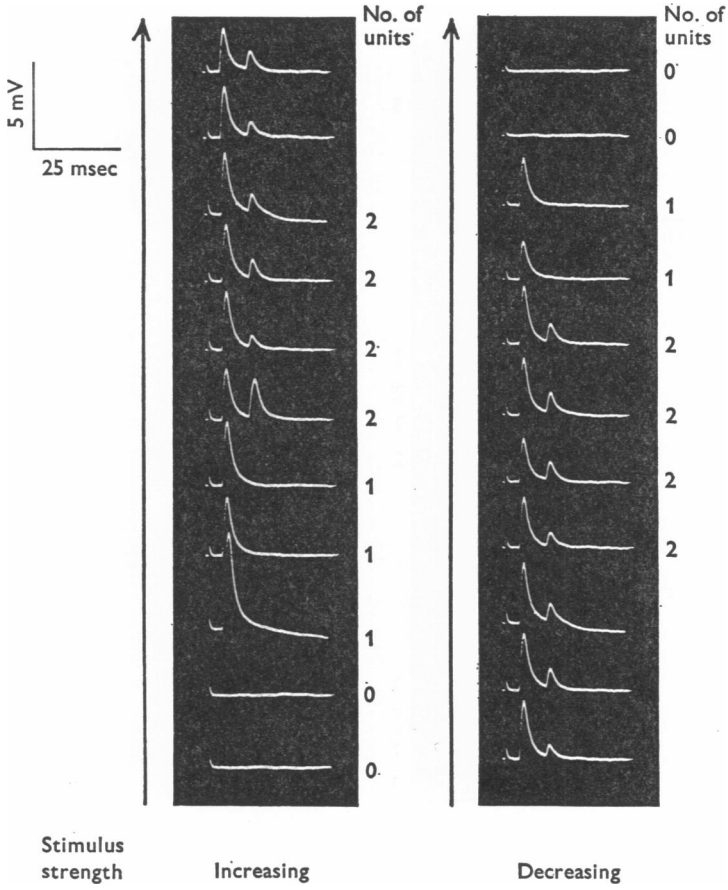

Fig. 2. E.p.p.s from the curarized diaphragm muscle of a rat 10 days old weighing 16 g. They consist of two units, widely separated by their different latent periods. The resting potential was 60 mV on insertion and 45 mV during recording.

at a higher stimulus strength (Fig. 2). However, in some fibres the units with a longer latency resulted from a lower stimulus strength (Fig. 3).

The relationship between the amplitude of the individual units of any one fibre also varied widely. In some fibres one unit was much larger than the others (e.g. Fig. 1) whereas other fibres had e.p.p. units of a similar size (Fig. 3). There was no obvious relationship between amplitude and

latent period or between amplitude and threshold to nerve stimulation of the individual units.

In normal mammalian Ringer there was no consistent rundown of e.p.p. amplitudes during a train of stimuli, as occurs in the muscle of adult rats. However, increasing the calcium concentration in the Ringer to 10 mM

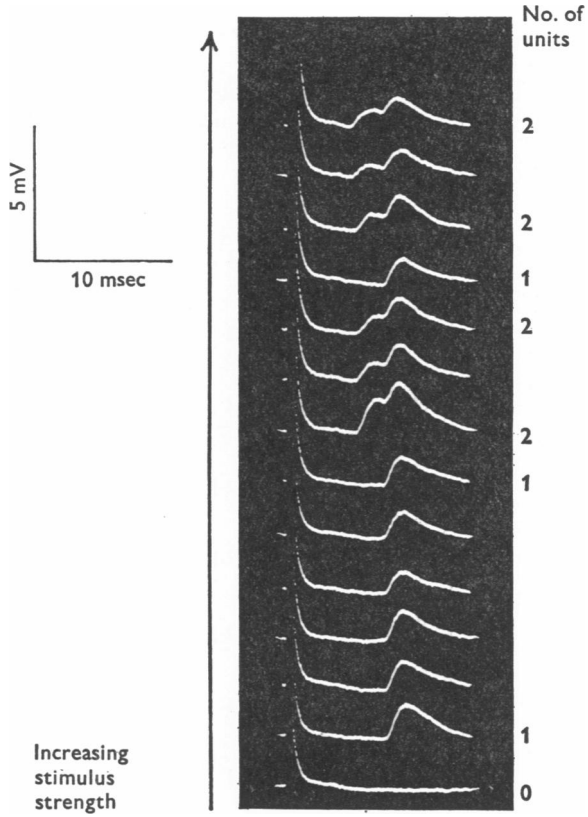

Fig. 3. E.p.p.s from the curarized diaphragm muscle of a 10-day-old, 16 g rat. The resting potential was 70 mV on insertion and 50 mV during the recording.

resulted in a rundown of e.p.p. amplitudes during a train of stimuli. This progressive decrease in amplitude of the first few e.p.p.s occurred independently in each of the units in one fibre (Fig. 2), indicating that they were separate functional units with independent transmitter release.

Recording from most muscle fibres during the first week of life revealed e.p.p.s with multiple units, usually two, three or four. Some fibres had more than four units, but the narrow spread of thresholds to nerve stimulation, and latent periods, made it difficult to separate the units. A few

muscle fibres had single unit e.p.p.s. Fig. 4 illustrates a muscle fibre action potential arising from the first three summated e.p.p.s of two 'units'.

There was a gradual change in this pattern of multiple unit e.p.p.s during the second week of the rat's life. The average number of units of one e.p.p. decreased, and the proportion of fibres having single unit e.p.p.s increased, until by 16–18 days old, multiple units were uncommon. This period coincides with eye opening in the rat, and a general increase in activity.

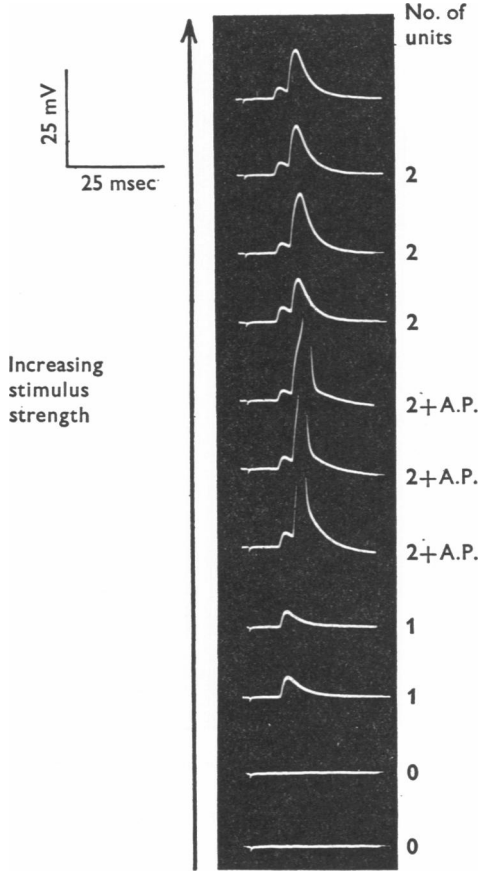

Fig. 4. E.p.p.s from the curarized diaphragm muscle of a rat 13 days old, weighing 17 g. The first three of the e.p.p.s formed by the summation of the two units are above the threshold and result in action potentials. The progressive rundown in e.p.p. amplitudes results in the subsequent summated e.p.p.s being sub-threshold. The resting potential was 70 mV on insertion and 60 mV while recording.

DISCUSSION

Because of the extensive selection of fibres necessary to achieve high resting potentials and a high e.p.p. amplitude-to-noise ratio, and the possibility of undetected selective block of some nerve fibres, it was thought unwise to reduce the recorded data to average number of nerve endings per muscle fibre, or to the proportion of muscle fibres with multiple innervation.

Although accurate quantitative assessment of the changes in neuromuscular junctions during the first weeks of life in the rat did not prove possible, there were clear qualitative differences between the muscle fibres of new-born rats, and those of older rats. In the majority of the diaphragmatic muscle fibres of new-born rats examined, the e.p.p. was complex and consisted of a number of units which could be added to, or subtracted from, the e.p.p. by increasing or reducing the stimulus strength. These observations indicated that there were functional connexions between more than one axon in the nerve trunk, and a single muscle fibre, and that these axons have differing latent periods and thresholds to nerve stimulation.

There can be several explanations for these connexions. Waxman (1968) has described 'tight' junctions between several nerve axons sharing a common myelin sheath in the sciatic nerve of developing and adult mice. Junctions between axons before they reach the muscle fibres could thus provide several pathways of excitation from different axons to the final common pathway down a single axon. However, the observed units were often of widely differing amplitude and sometimes occurred simultaneously, whereas multiple action potentials in a single axon and its nerve endings would result in e.p.p.s of a similar amplitude separated by at least the refractory period of the axon.

Kelly & Zacks (1968) have described 'close' junctions between muscle fibres in the rat foetus with gaps of 20–100 Å, which, if functional, could result in the simultaneous recording of e.p.p.s from more than one fibre by a micro-electrode inserted into one fibre. This could well explain the observations, but the authors state that the intermuscular close junctions disappear at birth.

Spacially separate and individually innervated end-plates are unlikely, since Diamond & Miledi (1962) demonstrated that m.e.p.p.s in foetal and new-born rat muscle were produced at about the middle of the fibre, although they could be recorded along the entire length of the fibre. Also, multiple end-plates on a scale necessary to explain these observations have not been described in the histological studies of the new-born rats (Kelly, 1966; Terräväinen, 1968).

The observed complex e.p.p.s became simpler during the first 2 weeks

of life, and by 16–18 days e.p.p.s in almost all fibres consisted of single potentials with an all-or-nothing character. This change could either be the result of an increase in the total number of muscle fibres by longitudinal splitting, so that there were equal numbers of muscle fibres and nerve branches, or it could result from a degeneration of some nerve branches. It could, perhaps, be brought about by a combination of these two factors.

The increase in the total number of muscle fibres in the biceps brachii muscle of the albino mouse during early post-embryonic growth (Goldspink, 1962) could be regarded as evidence for the first of these possibilities. However, this increase results from the differentiation of myoblasts, and not from the splitting of myotubes and muscle fibres (Chiakulas & Pauly, 1965). It has been shown in other species that during late embryonic development there is a decrease in the number of nerve fibres in peripheral nerves (Hughes, 1965; Prestige, 1967) and a loss of ventral horn cells (Hughes, 1961). However, there is no evidence available at present that this occurs in the phrenic nerve of the rat after birth.

For these reasons it is suggested that a pattern of multiple and perhaps repeated innervation of individual muscle fibres at birth changes to one of single fibre innervation during the second week of life, probably by the loss of superfluous nerve branches.

It has been proposed (Weiss, 1963; Hughes, 1968) that nervous pathways in the mature animal occur as the result of selection from a larger number of random connexions. The present observations of multiple neuromuscular connexions present at birth and reducing to single connexions during early post-natal development are consistent with these views, although the possible mechanisms of this selection remain obscure.

In the absence of neuromuscular block, many of the junctions demonstrated in this study would have resulted, on activation, in a muscle fibre action potential and contraction. If many of these junctions are indeed temporary, their effectiveness is surprising and significant. Perhaps the junctions must become fully functional before being rejected or selected, so that the relationship in the central nervous system between the effector unit, and the afferent fibres from the receptors stimulated by its action, can be demonstrated. Inappropriate junctions might then atrophy and degenerate, whilst appropriate ones, perhaps fed back by reflex activity, might hypertrophy and mature.

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