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THE REORGANIZATION OF SYNAPTIC CONNEXIONS IN THE RAT SUBMANDIBULAR GANGLION DURING POST-NATAL DEVELOPMENT

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SUMMARY

1. The innervation of neurones in the submandibular ganglion of neonatal and adult rats has been studied with intracellular recording, and light and electron microscopy.

2. Intracellular recordings from neurones in isolated ganglia from adult animals showed that about 75% of the ganglion cells are innervated by a single preganglionic fibre.

3. However, multiple steps in the post-synaptic potential (about five on average) were elicited in ganglion cells from neonatal animals by graded stimulation of the preganglionic nerve. The same result was obtained when the preganglionic fibres were stimulated at their emergence from the brainstem, indicating that neonatal neurones are innervated by several different preganglionic nerve cells.

4. The number of preganglionic fibres innervating individual ganglion cells gradually decreased during the first few weeks of life, and by about 5 weeks each ganglion cell was generally contacted by a single preganglionic axon.

5. Synapses were made on short protuberances in the immediate vicinity of the neuronal cell bodies in both neonatal and adult ganglia as shown by staining presynaptic boutons with the zinc-iodide osmium method, injection of horseradish peroxidase into ganglion cells, and electron microscopical examination.

6. Electron microscopical counts of synaptic profiles per ganglion cell perimeter showed that the number of synaptic contacts made on ganglion cells actually increased during the first few post-natal weeks, when the number of axons innervating each neurone was decreasing.

7. These results show that in the rat submandibular ganglion there is a reorganization of neuronal connexions during the first few weeks of life which results in a transition from multiple to generally single innervation of ganglion cells.

INTRODUCTION

The ways in which nerve cells establish and maintain synaptic connexions with one another are largely unknown. The rules that govern these processes must account not only for the specificity of neuronal connexions, but also for the numerical balance of presynaptic endings and post-synaptic sites. Some of the principles underlying this balance have been suggested by studies of the developing neuromuscular junction (Redfern, 1970; Bagust, Lewis & Westerman, 1973; Bennett & Pettigrew, 1974, 1975, 1976; Brown, Jansen & Van Essen, 1976). A general feature of the developing neuromuscular junction appears to be transient multiple innervation of individual end-plates, with subsequent elimination of a portion of the synaptic contacts initially formed. In most mammalian muscles this process results in each adult muscle fibre being contacted by a single motor nerve terminal. Since synapse elimination can occur without a reduction in the number of motor units in mammalian muscle (Brown *et al.* 1976), the transition from multiple to single innervation probably involves the rearrangement of innervation, rather than the death of presynaptic cells. The aim of the present work was to examine whether a similar rearrangement of synaptic connexions occurs between developing neurones. The submandibular ganglion of the rat was chosen because preliminary experiments showed it to be remarkably simple in organization, most neurones being innervated in maturity by a single preganglionic axon.

The results of intracellular recording during the first few days of post-natal life show that immature ganglion cells are innervated by multiple preganglionic axons. As at the neuromuscular junction, there is a gradual decline in the number of presynaptic axons contacting each post-synaptic cell, leading to the establisment of the adult pattern of generally single innervation within about a month of birth. The similarity of the process of synapse elimination in developing mammalian muscle and in the immature submandibular ganglion suggests that this may be a general feature of neural ontogeny.

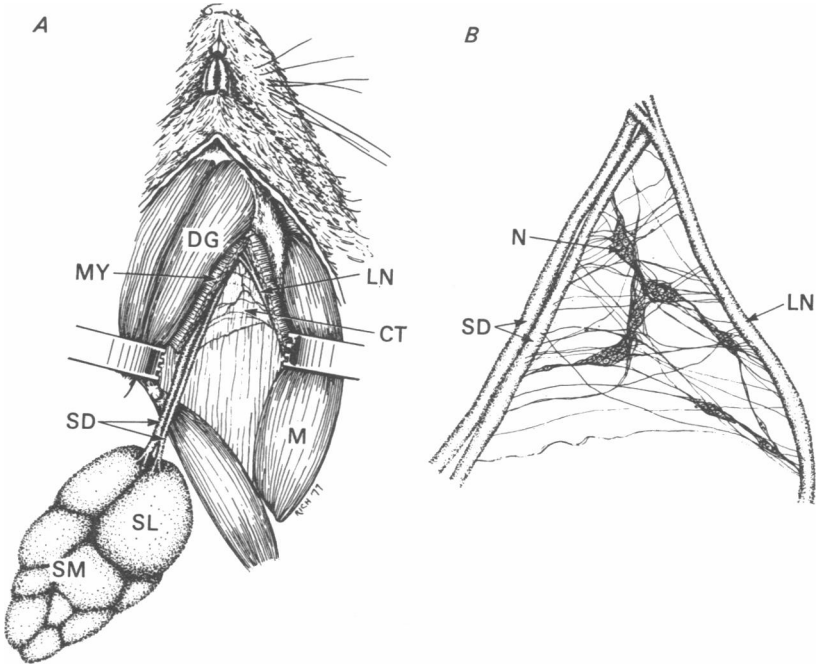
METHODS

Dissection

Twenty-six female albino rats (Wistar strain) 1–35 days of age and seventeen young adult animals (8–14 weeks of age, 160–220 g) were anaesthetized with chloral hydrate (0·35 gm/kg, I.P.) and perfused through the heart with mammalian Ringer fluid (Liley, 1956). A ventral mid line incision was made in the neck, and the submandibular and major sublingual glands and their ducts were freed by blunt dissection to the disappearance of the ducts beneath the mylohyoid muscle (Text-fig. 1*A*). This muscle was cut to expose the ducts rostrally to the point at which the lingual nerve crosses them from the lateral side. The ducts, lingual nerve and con-

nective tissue between them, were removed from the animal and placed in oxygenated Ringer fluid.

The triangular connective tissue sheet between the ducts and the lingual nerve contains a number of neurones which innervate the salivary glands, and is subsequently referred to as the submandibular ganglion (Text-fig. 1 *B*) (see also below).



Text-fig. 1. The submandibular ganglion of the rat (left ganglion, ventral aspect). *A*, the ganglion cells, which innervate the submandibular (SM) and sublingual (SL) salivary glands, lie in a thin connective tissue sheet (CT) beneath the mylohyoid muscle (MY) (which has been cut between the digastric (D) and masseter (M) muscles). The connective tissue sheet is bordered by the lingual nerve (LN) laterally, and the salivary ducts (SD) medially. *B*, when the connective tissue between the lingual nerve (LN) and the salivary ducts (SD) is removed and examined at higher magnification, clusters of neurones (N), can be seen within the sheet. The preganglionic axons enter the sheet in many small branches from the lingual nerve. These branches run either directly to the salivary ducts, or through the clusters of ganglion cells. The post-ganglionic axons follow the salivary ducts to the salivary glands.

In four 3-week-old rats the preganglionic fibres were dissected proximally to their emergence from the brainstem in the nervus intermedius. The submandibular ganglion was denervated in five additional adult rats by cutting the chorda tympani near the medial side of sphenoid spine 4–10 days before removing the ganglion.

Recording methods

Ganglia were pinned out in a small chamber (vol. = 0·5 ml.) perfused with oxygenated Ringer fluid at a rate of approximately 2 ml./min, and viewed with an

inverted differential interference contrast microscope (Biovert, Reichert) through a cover-slip 0·1 mm thick which formed the bottom of the recording chamber. The lingual nerve and salivary ducts were taken into close fitting glass suction electrodes for stimulation with single pulses (0·5–1·0 msec, 10–100 V).

Impalements of ganglion cells were made with glass micro-electrodes bent at a 75° angle within a few millimetres of their tips to allow easy movement within the working distance of the microscope condenser (7 mm). Electrodes were filled with 0·5 M potassium citrate, and had resistances of 80–120 MΩ. In all experiments the Ca^{2++} concentration of the bathing fluid was increased from 2 to 8 m-mole/l. to improve the stability of intracellular recordings. Impalements were facilitated by passing brief pulses of hyperpolarizing current through a bridge circuit in series with the recording electrode. Only neurones giving action potentials of 60 mV or more in response to intracellular injection of depolarizing current were included in the results.

The number of discrete steps in the excitatory post-synaptic potential (e.p.s.p.) in response to graded stimulation of the preganglionic nerve was taken as a measure of the number of preganglionic fibres innervating each cell. Counting the number of steps was often easier if action potentials superimposed on the synaptic response were eliminated by passing depolarizing current through the recording electrode to initiate a spike; the synaptic response was then timed so that it occurred during the refractory period of the directly elicited action potential (see Text-fig. 4, for example). Although generally reliable, estimates of the number of fibres innervating a neurone obtained by this method were subject to several uncertainties. For example, if a fibre making a large synaptic contribution was activated at a low intensity of stimulation, a small synaptic potential from another axon with a higher threshold could be obscured because of shunting. That this sometimes occurred was evident from antidromic stimulation of fibres making *en passant* synaptic contacts. A further difficulty with this method was that the latency of e.p.s.p.s often decreased as the strength of stimulus increased. Presumably this occured because the action potential was initiated closer to the point of recording. As a consequence, changes in latency could not be used as a criterion of additional recruitment, and fibres with the lowest threshold often appeared to be the most slowly conducting (see, for example, Text-figs. 4*B* and 5*C*). These deficiencies would lead to an underestimate of the number of axons innervating a neurone, but would not affect the observation that the complexity of the synaptic response changed markedly with age.

Anatomical methods

(a) *Zinc-iodide osmium staining.* Preganglionic boutons were stained with a mixture of zinc-iodide and osmium tetroxide (Maillet, 1962). Ganglia from nine neonatal (1–35 days old), and eleven young adult rats were incubated overnight in the staining solution, dehydrated in graded ethanol solutions, cleared in xylene, and mounted in synthetic mounting medium (Fisher Preservaslide) on glass slides.

(b) *Intracellular injection of horseradish peroxidase.* Twenty-eight neurones in adult ganglia and twenty-four in neonatal ganglia were pressure-injected with horseradish peroxidase (type VI, Sigma, St Louis) and stained with benzidine dihydrochloride. The procedure used was similar to that of Muller & McMahan (1976) with the following modifications. To ensure a stable and agranular reaction product (the brown reaction product; Straus, 1964), ganglia fixed in glutaraldehyde were immersed in a 2 % benzidine dihydrochloride solution in 0·01 M-Tris maleate buffer (pH 7·4) at room temperature. One drop of 3 % H_2O_2 was added after 10 min. When the injected cells were blue-brown, the ganglia were washed in distilled water, and dehydrated at room temperature by passage through graded ethanol solutions.

This procedure usually removed the granular blue reaction product which formed initially. The tissue was cleared in xylene and mounted whole in Preservaslide under a cover-slip. The cells and their processes were traced with the aid of a camera lucida.

(*c*) *Measurements of cell area.* To determine the size of ganglion cells at different ages, semi-thin sections (1 μm) of ganglia from 2-day-old, 2- to 3-week-old and adult rats were stained with toluidine blue. The sections were taken from the blocks prepared for ultrastructural examination (see below). The perimeters of 100 nucleated neuronal profiles from each age group were traced by camera lucida using an oil-immersion objective ($\times$ 1200), and the area of each profile calculated by a computer-assisted planimeter (Cowan & Wann, 1973).

(*d*) *Electron microscopy.* Thirty-two additional ganglia from twenty-two neonatal and ten young adult rats were pinned in dishes with Sylgard resin bottoms, and immersed in half strength Karnovsky fixative (Karnovsky, 1965) for 30 min. The ganglia were transferred to full strength fixative for an hour, and were then post-fixed in 2 % osmium tetroxide in cacodylate buffer at pH 7·2 for an additional 3 hr. Following dehydration in graded ethanol solutions, the ganglia were imbedded in Araldite (Ciba). Thin sections were mounted on 200-mesh grids, and stained with lead citrate and uranyl acetate.

The number of synaptic profiles per cell perimeter was taken as an index of the relative number of profiles contacting cells at different ages. Synapse counts were made only on cell perimeters which contained a nuclear profile and were entirely within a grid square. Neurones whose perimeters bordered closely upon other neurones were avoided, since in these cases it was sometimes difficult to assign synapses to one cell or the other. To be counted as a synapse, a profile had to show pre- and post-synaptic membrane thickenings, and presynaptic agranular vesicles focused on a presynaptic membrane specialization (see Pl. 3).

To compare the size of presynaptic elements from neonatal and adult ganglia, fifty randomly chosen synaptic profiles from ganglia in 2-day-old rats, and fifty profiles from adults were photographed and printed at a total magnification of 48,900. The cross-sectional area of the presynaptic terminals and the extent of the pre- and post-synaptic membrane thickenings were measured by a computer assisted planimeter.

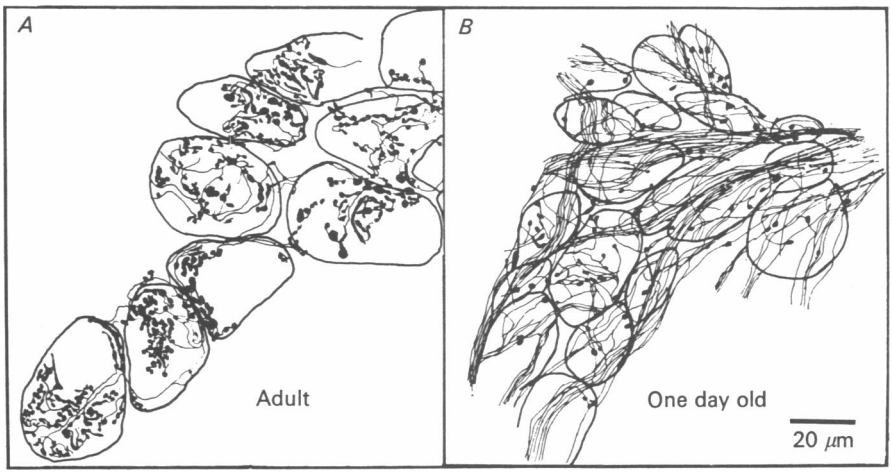
RESULTS

The preparation

The anatomy of the parasympathetic innervation of the mammalian submandibular and sublingual salivary glands has been briefly described by a number of workers (Langley, 1890; Szentágothai, 1957; Snell, 1958; McMahan & Kuffler, 1971). In the rat, about half of the ganglion cells which innervate the glands are located in the thin connective tissue sheet defined here as the submandibular ganglion (Text-fig. 1*B*), while the remainder are grouped along the salivary ducts, or occasionally within the submandibular gland itself (Snell, 1958). The preganglionic axons orginate in the superior salivatory nucleus and run in the nervus intermedius, facial nerve, chorda tympani, and lingual nerve from which they enter the connective tissue sheet by way of numerous small branches. Within the sheet the preganglionic nerves branch extensively. Many of the branches run through clusters of ganglion cells, while others course directly to the

ducts. The ganglion usually consists of three to ten clusters, each containing from five to about 250 neurones (Text-fig. 1*B* and Pl. 1). Occasional neurones occur in isolation.

Individual nerve cell bodies and adjacent Schwann cell nuclei were easily seen with differential interference contrast optics. The clusters of ganglion cells are often one cell thick (Pl. 1) and their arrangement is in many ways similar to certain parasympathetic ganglia of amphibians (McMahan & Kuffler, 1971; McMahan & Purves, 1976). Ganglion cells were



Text-fig. 2. Camera lucida drawings of groups of neurones from the submandibular ganglion stained with zinc-iodide osmium. *A*, the cell bodies of neurones in adult ganglia show numerous boutons in apparent contact with the neuronal surface. The boutons are variable in shape, size and location on the cell bodies. Photomicrographs of single focal planes of three neurones stained with zinc-iodide osmium are shown in Pl. 2*A*, *B*, *C*. *B*, neonatal ganglia stained with zinc-iodide osmium show relatively few boutons, but an abundance of fine fibres (see Pl. 2*D*, *E*).

generally the same in size and appearance; this argued against the presence of interneurones ('small intensely fluorescent' cells) found in some parasympathetic and sympathetic ganglia in both mammals and lower vertebrates (Siegrist, Dolivo, Dunant, Foroglou-Kerameus, De Ribaupierre & Rouiller, 1968; Mathews & Raisman, 1969; Williams & Palay, 1969; McMahan & Purves, 1976). The absence of interneurones containing dense core vesicles was confirmed by electron microscopy (see below).

To determine the distribution of preganglionic boutons on the neurones, ganglia were stained with zinc-iodide osmium. In adult ganglia, dense pleomorphic boutons were observed on the surface of many neurones (Text-fig. 2*A*; Pl. 2*A–C*). The fibres interconnecting the boutons were often quite

thick, making it difficult to estimate the number of individual endings on some cells. On cells where counts could be made, the number of boutons ranged from 25 to 80 with mean of 44 per neurone (n = 110). This number is relatively large: in the mudpuppy cardiac ganglion there are an average of 22 boutons per cell (McMahan & Purves, 1976), and an average of only 12 per cell in the parasympathetic ganglion of the frog atrial septum (McMahan & Kuffler, 1971). There was no indication that boutons contacted nerve cells other than in the immediate vicinity of the cell body.

Electron microscopical examination of the ganglion showed that the population of neurones was homogeneous by ultrastructural criteria, although Schwann cells and connective tissue cells were also present. Synapses on ganglion cells were usually on small fingers of cytoplasm within a few microns of the cell body surface (Pl. 3). Profiles of presynaptic boutons in adult ganglia contained densely packed, agranular vesicles about 50 nm in diameter, as well as a few dense core vesicles up to 150 nm in diameter, and numerous mitochondria (Pl. 3*A*, *B*). These profiles resemble cholinergic preganglionic terminals in other autonomic ganglia (see for example, Taxi, 1965). Gap junctions, which occur between parasympathetic ganglion cells in some lower vertebrates (McMahan & Purves, 1976) were not seen in the submandibular ganglion. However, symmetrical desmosome-like junctions were observed between some neurones; these resembled junctions seen in other autonomic ganglia (Gabella, 1972).

The anatomy of the submandibular ganglion as demonstrated by zinc-iodide osmium staining and electron microscopy suggested that the nerve cells do not have an extensive dendritic aborization. To confirm this, neurones were injected with horseradish peroxidase. Cells visualized in this way had few if any large dendrites, but numerous smaller processes extended from the perikaryon and the initial portion of the axon (Text-fig. 3, Pl. 4). It is likely that most of the presynaptic profiles seen in electron microscopical sections contacted these short processes. Horseradish peroxidase injections failed to demonstrate axonal branching beyond the initial segment, even though postganglionic axons could often be followed for long distances (Text-fig. 3; see also Pl. 5 and below). The short processes extending from the initial portion of the axons were probably not postganglionic collaterals (see the next section).

Intracellular recordings from adult neurones

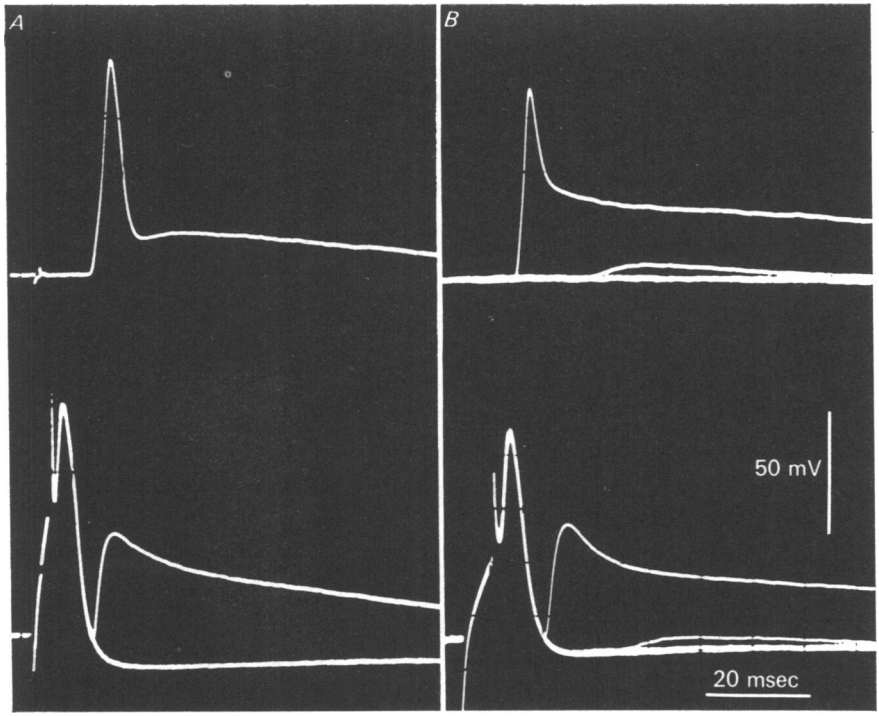
In general, the electrophysiological properties of submandibular neurones are similar to those of other mammalian autonomic cells which have been studied with intracellular recording (see, for example, Blackman, Crowcroft, Devine, Holman & Yonemura, 1969). Action potentials elicited

by depolarizing current injection through the recording micro-electrode, or by ortho- or antidromic stimulation, had amplitudes of 60–114 mV; resting potentials ranged from −40 to −73 mV, and input resistances were between 55 and 200 MΩ.

Text-fig. 3. Camera lucida drawing of ganglion cells injected with horseradish peroxidase. *A*, an adult neurone showing numerous small processes extending from the cell body and the initial portion of the axon. The axon could be followed out of this field for distance of several hundred microns, and remained unbranched. *B*, ganglion cell from a 1-week-old rat. The cellular geometry of immature ganglion cells was similar to that of adult neurones; the axon of this neurone and other immature cells also remained unbranched as far as they could be followed. Pl. 4*A* and *B* show single focal planes of these same neurones.

In the majority of adult neurones, a single suprathreshold excitatory post-synaptic potential (e.p.s.p.) was elicited by graded stimulation of the lingual nerve (Text-fig. 4*A*). Changes in the strength, duration, or polarity of the stimulus did not alter the amplitude or shape of the post-synaptic potential. In twenty-seven of 121 adult neurones impaled (22 %), a second e.p.s.p. was observed, and in four neurones (3 %) a third could be seen. These additional e.p.s.p.s were subthreshold in all but two cases (Text-fig. 4*B*). Thus, although adult neurones were sometimes innervated by more than one preganglionic fibre, suprathreshold innervation from a single axon was the rule.

Stimulation of the nerve fibres which run with the salivary ducts also elicited e.p.s.p.s., as well as antidromic action potentials, in many ganglion cells. Antidromic action potentials were distinguished from regenerative responses arising from synaptic activation by collision with directly initiated action potentials. To see if ganglion cells received innervation from sources other than the preganglionic fibres (for example, from post-



Text-fig. 4. Intracellular recordings from adult ganglion cells. *A*, recording from a singly innervated neurone. Upper trace: stimulation of the lingual nerve (at artifact) elicited a suprathreshold synaptic response. Resting potential was −50 mV. Lower trace: Same neurone as in *A*. In order to observe the e.p.s.p. without the regenerative response, depolarizing current (5 msec pulse) was injected through the recording electrode to initiate an action potential; the synaptic response was timed so that it occurred during the refractory period of the directly elicited action potential (two traces superimposed). A unitary e.p.s.p. such as this was observed in 75% of adult neurones. *B* recording from a multiply innervated neurone. Upper trace: gradually increasing the stimulus to the lingual nerve elicited two steps with separate thresholds in the synaptic response. The smaller synaptic potential was well below threshold (three traces superimposed). Resting potential was −44 mV. Lower trace: one large and one very small e.p.s.p. were seen when the synaptic response was timed so that it occurred in the refractory period of a directly elicited action potential; this was typical of multiply innervated adult ganglion cells (three traces superimposed).

ganglionic axon collaterals or afferents arising in the salivary glands), ganglia from five animals were denervated by cutting the chorda tympani, and examined 4–10 days later when the preganglionic fibres had degenerated. In neurones impaled in these ganglia there was no response to stimulation of the lingual nerve, and stimulation of the nerve fibres that run with the salivary ducts elicited only antidromic action potentials. Thus ganglion cells appear to be innervated solely by preganglionic fibres arising from the lingual nerve, some of which make *en passant* contacts and run on to synapse with other neurones along the salivary ducts or in the salivary glands. Antidromic stimulation confirmed the results of orthodromic stimulation: in the majority of neurones in which e.p.s.p.s. could be elicited by stimulating the salivary ducts, a single suprathreshold e.p.s.p. was observed.

Intracellular recordings from neonatal neurones

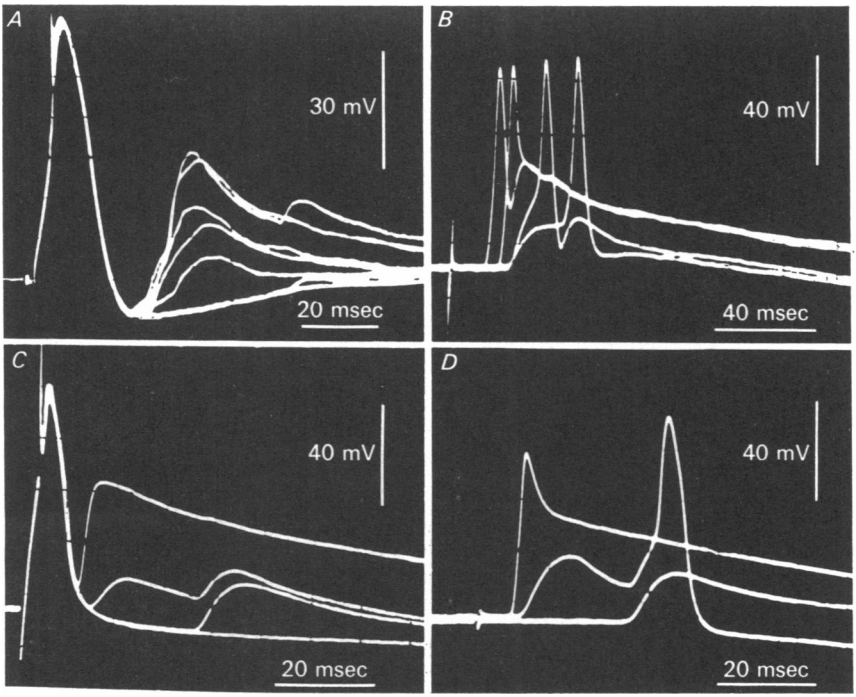
Although neonatal neurones were more difficult to impale, action potential amplitudes, resting potentials, and input resistances were similar to those of adult neurones. However, the results of graded preganglionic stimulation of neonatal ganglion cells were quite different than those obtained in adults. Complex e.p.s.p.s were invariably elicited in neonatal neurones by gradually increasing the strength of lingual nerve stimulation (Text-fig. 5). The amplitude, shape, and latency of the synaptic response changed in discrete steps as the stimulus strength was increased and often more than one of the steps in the synaptic response gave rise to an action potential (Text-fig. 5*B*, *D*).

The estimated number of steps in the e.p.s.p.s. was greatest during the first week of life (range 3–7, mean = $4{\cdot}7 \pm 1{\cdot}1$ (S.E. of mean), $n = 60$), and gradually decreased thereafter (Text-fig. 6). By the fifth post-natal week the number of steps was similar to that in neurones from adult ganglia (range 1–3, mean = $1{\cdot}3 \pm 0{\cdot}5$, $n = 50$).

Multiple steps in the synaptic response of neonatal neurones are presumably due to several preganglionic axons innervating each cell. However, other explanations are possible. For instance, complex e.p.s.p.s. might occur if neonatal neurones were electrically coupled. Electrotonic junctions have been demonstrated between immature muscle fibres (Kelly & Zacks, 1969; Dennis & Ort, 1976; Blackshaw & Warner, 1976), and between adult parasympathetic neurones in the mudpuppy (McMahan & Purves, 1976; Roper, 1976). To examine the possibility of electrical coupling in the submandibular ganglion, adjacent neurones were impaled using two independent electrodes, and current was injected into one cell while recording from the other. Weak electrical coupling was observed between some pairs of neurones in both neonates and adults. There was,

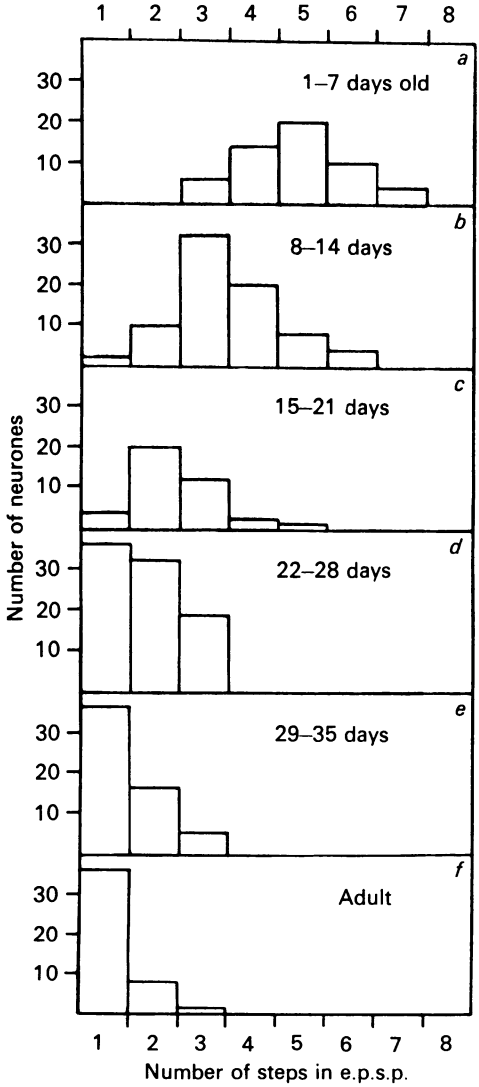
however, at least a fiftyfold attenuation of the potential in coupled neurones. This degree of coupling is far too small to account for the complex e.p.s.p.s observed in young animals. The apparent absence of gap junctions between ganglion cells in electron microscopical sections (see above) is consistent with the failure to demonstrate strong electrical coupling.

Multiple steps in the post-synaptic response might also be due to branches of the same preganglionic axon arising above the point of lingual nerve



Text-fig. 5. Intracellular recordings from neonatal ganglion cells. *A*, neurone from a 7-day-old rat. Gradually increasing the preganglionic stimulus stength elicited multiple steps with different thresholds in the post-synaptic response. In this tracing, the synaptic response was timed so that it occurred during the refractory period of a directly elicited action potential which eliminated the superimposed regenerative responses. At least six separate steps can be observed (seven traces superimposed). Resting potential was -41 mV. *B*, neurone from a 14-day-old rat. In this cell, graded stimulation to the lingual nerve elicited 4 post-synaptic responses with different thresholds. Two were just at threshold and two were suprathreshold (four traces superimposed). Resting potential was -46 mV. *C*, neurone from a 20-day-old rat showing a decrease in the complexity of the synaptic response. There are three discrete steps with separate thresholds and latencies (four traces superimposed). Resting potential was -48 mV. *D*, intracellular recording from same neurone as *C*. Two of the steps in the synaptic response gave rise to action potentials.

stimulation. To explore this possibility, the preganglionic fibres were stimulated at their emergence from the brainstem in the nervus intermedius. In four ganglia from 3-week-old rats, stimulation of the nervus intermedius produced complex synaptic responses composed of discrete steps with separate thresholds. This result was indistinguishable from the response to lingual nerve stimulation. Thus neonatal neurones are innervated by



Text-fig. 6. Histogram of the number of innervating fibres estimated from number of steps in the synaptic response recorded in neurones from birth to 35 days of age (*a–e*). The number of steps in fifty adult neurones is shown in *f*.

several different fibres emerging from the brainstem. In two experiments, suction electrodes were applied to both the nervus intermedius and to the lingual nerve just proximal to the ganglion. Every e.p.s.p. step elicited by stimulation of the nervus intermedius was also observed when the lingual nerve was stimulated. Therefore once a preganglionic fibre emerges from the brainstem it probably sends only one preterminal branch to a neurone. Axonal branches arising within the brainstem and contacting the same ganglion cell cannot be ruled out, but this seems unlikely.

Finally, multiple steps in the e.p.s.p.s of neonatal neurones could occur if postganglionic axon collaterals made synapses with other ganglion cells as occurs, for example, in the cardiac ganglion of the mudpuppy (McMahan & Purves, 1976; Roper, 1976). It was difficult to denervate neonatal ganglia and to rule out axon collaterals by subsequent antidromic stimulation as in adult ganglia (see above). However, horseradish peroxidase injections of ganglion cells from 1-week-old animals demonstrated that the post-ganglionic axons resembled those in adult ganglia: aside from several short processes arising from the initial segment, they remained unbranched as far as they could be followed (Pl. 5).

In summary, electrical coupling of ganglion cells, multiple branches from individual preganglionic fibres, and recurrent axon collaterals are probably not responsible for the multiple steps in the post-synaptic responses recorded in neurones from young animals. Rather, each nerve cell appears to be innervated by several preganglionic neurones at birth, this number gradually decreasing during the first 5 weeks of life.

Anatomical aspects of synaptic reorganization

Ganglia from neonatal rats were similar in appearance to adult ganglia. Intracellular injection of horseradish peroxidase showed the cellular geometry to be similar as well. Although the twenty-four peroxidase injected ganglion cells from 1-week-old rats were generally smaller than the twenty-eight adult neurones injected, most had many short processes ($< 5\,\mu$m), as adult cells (Text-fig. 3*B* and Pl. 4*B* and 5). A few neonatal neurones had occasional longer, thicker processes (for example cell 3 in Pl. 5) which were not observed in adult ganglia. It is possible that the retraction of these somewhat larger dendrites could result in a decrease in the number of axons innervating a ganglion cell. Most injected neurones, however, did not have large processes during the early post-natal period when all the cells are multiply innervated.

Although the cellular geometry of neonatal and adult ganglion cells was generally the same, the arrangement of preganglionic boutons stained with zinc-iodide osmium on neonatal neurones differed considerably from the

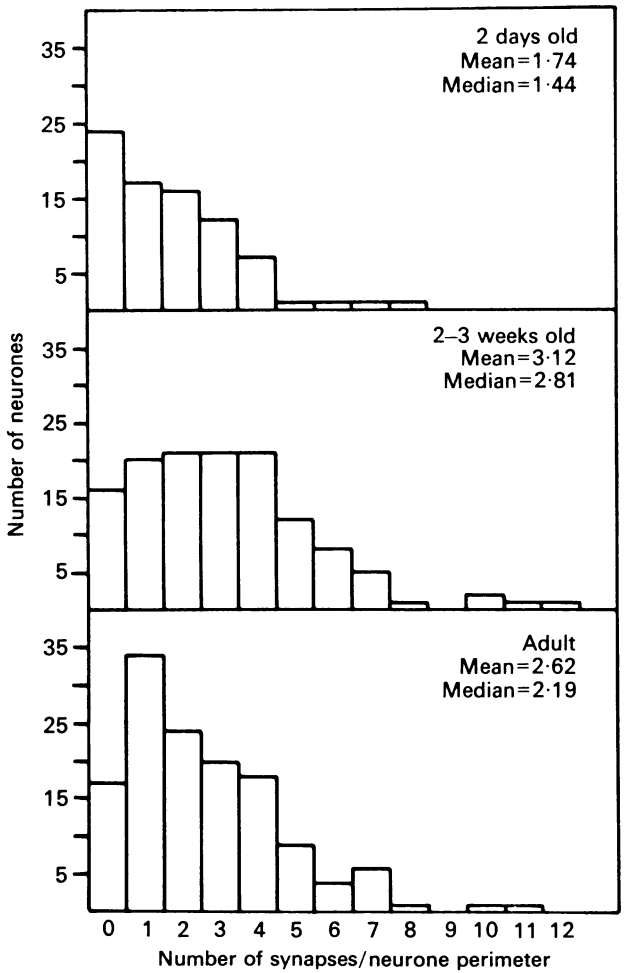
arrangement in adults (Text-fig. 2 and Pl. 2). In neonatal ganglia surprisingly few boutons were observed, and unlike adult ganglia, numerous fine fibres were stained. The difference in the staining pattern of neonatal and adult ganglia suggested that each neonatal neurone received a smaller number of synaptic contacts, although many more axons appeared to be available. The paucity of boutons in neonatal ganglia could, however, be due to the method itself. That this technique is to some degree capricious is shown by the presence of some ganglion cells without any impregnated boutons in many zinc-iodide osmium preparations.

To quantitate the relative number of synaptic contacts received by neonatal and mature ganglion cells, synapses were counted in electron microscopical sections of neonatal and adult ganglia. The number of synaptic profiles per cell perimeter was lower in 2-day-old rats than in 2- to 3-week-old or adult rats, but there was no significant difference in the number of synapses contacting two 3-week-old neurones and the number contacting adult ganglion cells (Text-fig. 7). The actual difference in the number of synapses per cell in 2-day-old and more mature rats may be larger than suggested by the results shown in Text-fig. 6 because the cross-sectional area of ganglion cells increased roughly threefold between birth and maturity (Text-fig. 8). Thus, assuming presynaptic profiles of constant size, a larger fraction of the total number of synapses per cell would be counted on each perimeter in ganglia from young animals. Measurements from electron micrographs showed that the cross-sectional area of presynaptic profiles and the length of the presynaptic specilization in fact were relatively constant between birth and maturity. The mean cross-sectional area of fifty presynaptic profiles from 2-day-old ganglia was $0{\cdot}99 \pm 0{\cdot}10\ \mu m^2$, compared with $1{\cdot}01 \pm 0{\cdot}13$ in adult ganglia ($n = 50$). The average length of the presynaptic specialization was $0{\cdot}55 \pm 0{\cdot}04\ \mu m$ in the 2-day-old ganglia, and $0{\cdot}50 \pm 0{\cdot}03$ in adults. Thus during post-natal development the number of synapses made on individual ganglion cells increases while the number of preganglionic axons contacting each neurone decreases.

Ultrastructural study also indicated that individual synaptic profiles from 2-day-old rats differed from those in older animals in that the presynaptic profiles were less densely packed with vesicles and mitochondria (Pl. 3*C* and *D*). This appearance is generally similar to immature profiles described in other autonomic preparations (Brenner & Johnson, 1976; Landmesser & Pilar, 1972). Degenerating nerve terminals (usually characterized by increased affinity for osmium, aggregation of vesicles, autophagic vacuoles, and lysosomes – Hámori, Láng & Simon, 1968) were occasionally seen in both neonatal and adult ganglia, but were not more prevalent in developing ganglia. The absence of degeneration may mean that elimination of synapses occurs through the gradual resorption of

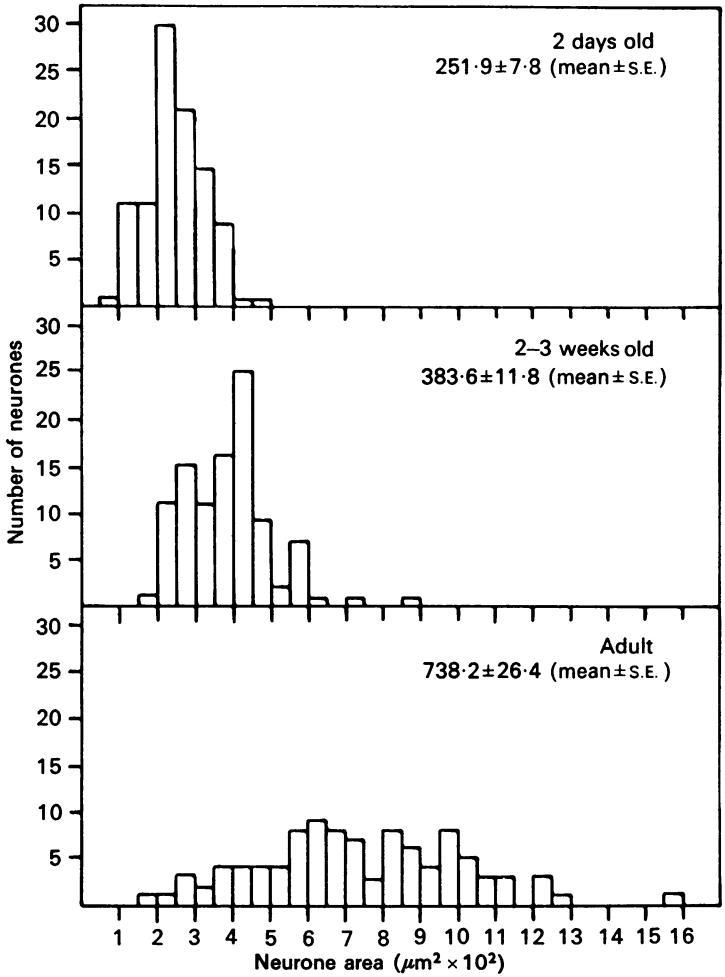
terminals, as may occur at the developing neuromuscular junction (Korneliussen & Jansen, 1976; see, however, Rosenthal & Taraskevich, 1977). Preganglionic cell death, however, cannot be ruled out.

As in adult ganglia, the synaptic profiles in 2-day-old rats contacted small fingers of cytoplasm in the immediate vicinity of the cell bodies. Multiple

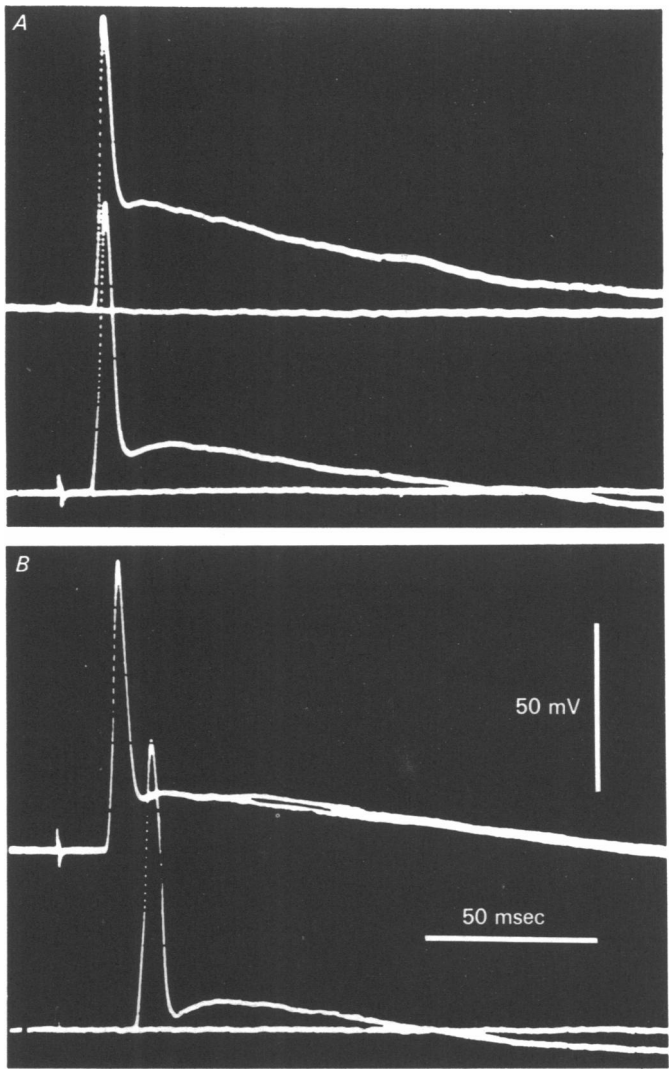


Text-fig. 7. Distribution of the number of synapses per neurone perimeter from 2-day-old, 2- to 3-week-old and adult rats. The median (and 95 % confidence interval; Noether, 1973) for 2-day-old rats was 1·44 (0·93–1·94), for 2- to 3-week-old rats 2·81 (2·33–3·39), and for adult rats 2·19 (1·71–2·69). The difference in the number of synapses per neurone perimeter between 2-day-old rats and either the 2- or 3-week-old or adult rats was highly significant ($P < 0·001$; Wilcoxon two-tailed test). The difference between the 2- to 3-week-old rats and adults was not significant ($P < 0·05$).

synaptic contacts innervating the same post-synaptic specilization were not observed. This suggests that presynaptic axons probably do not share the same post-synaptic sites during the period of multiple innervation, as occurs at the developing neuromuscular junction (Bennett & Pettigrew, 1974; Brown *et al.* 1976).



Text-fig. 8. Histogram of the cross-sectional areas of one hundred nucleated ganglion cell profiles from 2-day-old, 2- to 3-week-old and adult rats. The mean cell cross-sectional area increased about threefold from birth to maturity. The greater variance of the areas of adult neurones suggests that some cells increase in size much more than others.



Text-fig. 9. Simultaneous recordings from a pair of adult ganglion cells. *A*, upper and lower traces are intracellular recordings from two nearby ganglion cells innervated by the same preganglionic fibre. At one stimulus strength neither cell fires; however, a slight increase in the stimulus to the lingual nerve elicits suprathreshold post-synaptic responses in both cells. Cells innervated by the same preganglionic fibre always failed together when the stimulus was adjusted to threshold levels, and had nearly identical latencies. *B*, a pair of adjacent neurones innervated by separate preganglionic fibres. At one stimulus strength the cell monitored in the upper trace fires, while the other cell (lower trace) does not. Increasing the stimulus strength elicited a post-synaptic potential in the cell monitored in the lower trace; its latency was also different from the post-synaptic potential elicited in the other cell. Two traces are superimposed in both *A* and *B*.

The arrangement of innervation within clusters of ganglion cells

As the number of fibres innervating each ganglion cell decreases during development, one of the fibres contacting a neurone becomes its dominant source of innervation. By simultaneously impaling pairs of ganglion cells in a cluster, it was possible to determine whether or not a single preganglionic fibre 'captured' not only individual neurones, but entire clusters of cells. Two cells were considered innervated by the same preganglionic axon if the post-synaptic potentials elicited in the ganglion cells appeared at exactly the same stimulus strength, had the same latency ($\pm$ 0·5 msec), and always failed together when the stimulus was adjusted to threshold levels (Text-fig. 9*A*, *B*).

In these experiments one electrode remained in a 'reference' cell, while the other electrode successively impaled nearby neurones. With each new impalement, the pair was tested to see if the same preganglionic fibre innervated both cells. In 4 of 25 clusters studied in adult ganglia, all the cells recorded from in the cluster were innervated by a single preganglionic fibre. Most of the clusters, however, were innervated by two to eight different fibres. Although preganglionic fibres usually innervated multiple neurones within a cluster, neighbouring ganglion cells were not necessarily captured by the same preganglionic fibre; rather, adjacent cells were often innervated by different axons.

DISCUSSION

The results of this study show that during the first post-natal month there is a gradual reorganization of the innervation in the rat submandibular ganglion. At birth each ganglion cell is innervated by several (three to seven) preganglionic fibres, while by 5 weeks of age most of the neurones are innervated by a single axon. Synaptic reorganization during development is probably a widespread phenomenon. Muscle fibre endplates in amphibia (Letinsky, 1974; Bennett & Pettigrew, 1975), birds (Bennett & Pettigrew, 1974) and mammals (Redfern, 1970; Bagust *et al.* 1973; Bennett & Pettigrew, 1974; Brown *et al.* 1976) are transiently innervated by multiple motor axons during development. There is also some evidence for synapse elimination during the development of connexions within the central nervous system (Conradi & Skoglund, 1970; Ronnevi & Conradi, 1974; Delhaye-Bouchaud, Crepel & Mariani, 1975; Crepel, Mariani & Delhaye-Bouchaud, 1976).

In the submandibular ganglion, as at the neuromuscular junction, synapse elimination is not haphazard: the majority of synaptic contacts from all but one axon innervating a neurone are usually removed. The

transition from multiple to predominantly single innervation of ganglion cells could occur because preganglionic neurones are dying, or because the peripheral connexions of a stable number of preganglionic neurones are reorganized. Preganglionic cell death has been suggested as a cause of the loss of synapses during embryonic development in the chick ciliary ganglion (Landmesser & Pilar, 1974, 1976). However, presynaptic cell death seems a less likely mechanism in post-natal life since in most vertebrates the major phase of neural loss is embryonic (Glücksmann, 1951; Cowan, 1973). Moreover, Brown *et al.* (1976) have shown that the elimination of polyneuronal innervation from muscle fibres in early post-natal life occurs without a change in the number of motor axons innervating rat soleus muscle.

Although a similar mechanism may underlie synapse elimination in the developing submandibular ganglion and at the neuromuscular junction, an important difference between the innervation of muscle fibres and submandibular ganglion cells is the location of the presynaptic terminals. On each neonatal muscle fibre innervation is confined to the endplate where several axons share the same post-synaptic site (Bennett & Pettigrew, 1974; Brown *et al.* 1976; see also Rotshenker & McMahan 1976). In contrast the post-synaptic sites on ganglion cells are spatially separate, and each of these is apparently occupied by only one preganglionic axon terminal. Yet in maturity each submandibular ganglion cell, as each mammalian skeletal muscle fibre, is generally innervated by a single dominant axon. Thus the total number of spatially separate post-synaptic sites on a ganglion cell appears to be functionally equivalent to a single endplate during the process of synapse elimination.

The mechanism of synapse elimination is not understood. The experiments of Brown *et al.* (1976) suggest that during the development of the neuromuscular junction there is an intrinsic tendency for presynaptic fibres to retract since synapse elimination occurs even when the number of innervating axons is drastically reduced by partial denervation. In the submandibular ganglion, the number of fibres innervating each ganglion cell decreases while the number of synapses per cell is increasing. This argues against an intrinsic tendency for each preganglionic neurone to reduce the number of synaptic contacts it makes during development: assuming a constant number of preganglionic axons and ganglion cells, the total number of synapses made by each preganglionic neurone must increase during the period of synapse elimination.

Some competitive interaction among the multiple axons initially innervating each post-synaptic cell seems likely. Elimination of synapses in the developing submandibular ganglion might be explained if preganglionic fibres compete for a limited amount of trophic substance associated with

the post-synaptic cell: growth and increased uptake of a factor by one axon might result in the local regression of terminals of other axons as they receive a smaller and smaller share of the trophic agent. A trophic dependence of adult ganglionic synapses on some property of the post-synaptic cells might also explain collateral sprouting after partial denervation (Murray & Thompson, 1957), and the reversible loss of synapses following post-ganglionic axotomy (Matthews & Nelson, 1975; Purves, 1975; Njå & Purves, 1978). Additional mechanisms, however, probably would be required to explain why each ganglion cell tends ultimately to be innervated by a single preganglionic axon.

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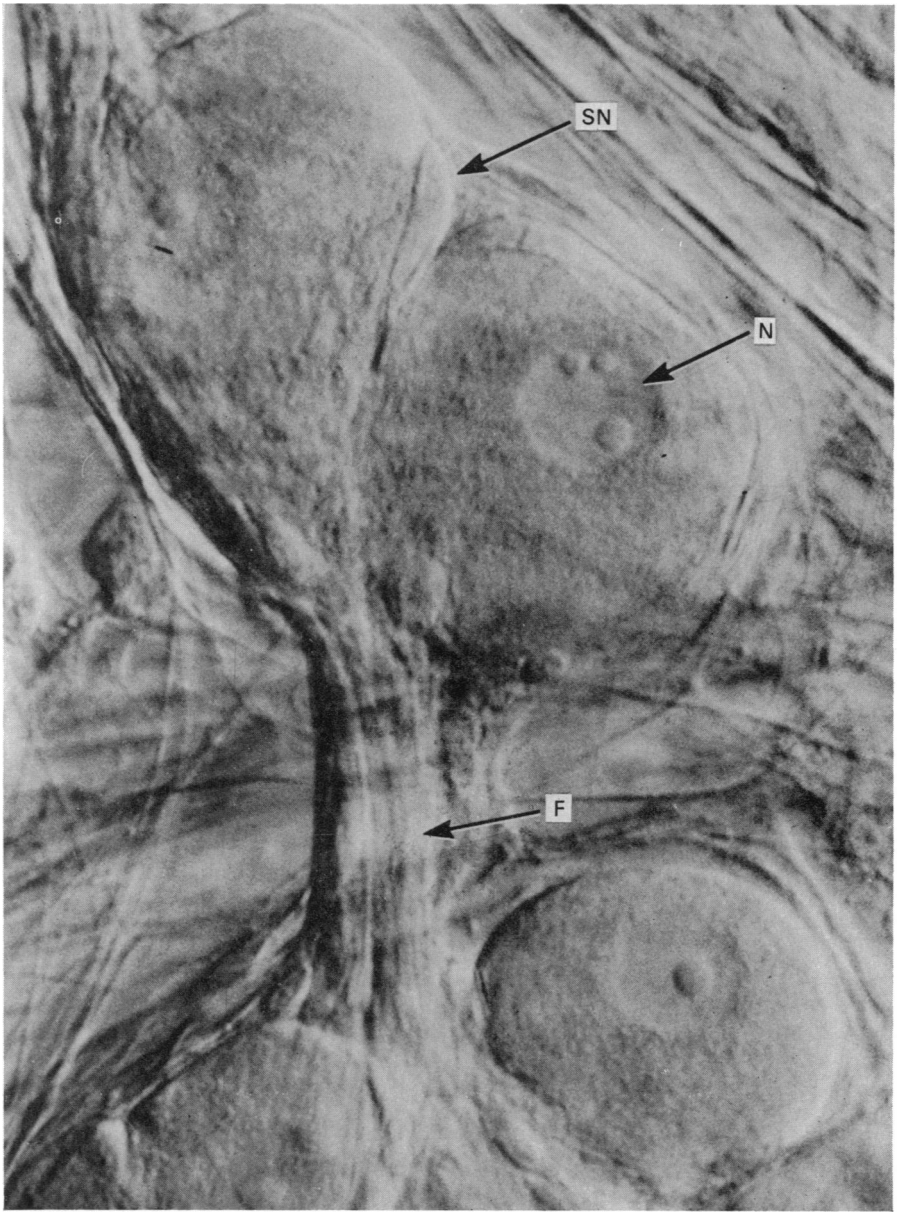
EXPLANATION OF PLATES

PLATE 1

Neurones in the living submandibular ganglion viewed with differential interference contrast optics (× 880). Cellular details of the ganglion are readily visible. The nuclei and nucleoli (N) of the neurones are apparent, as are numerous nerve fibres (F). Schwann cells surround the neurones; their nuclei (SN) often cap the neurone cell bodies. The three neurones in this photomicrograph are part of a cluster of about fifteen cells.



J. W. LICHTMAN

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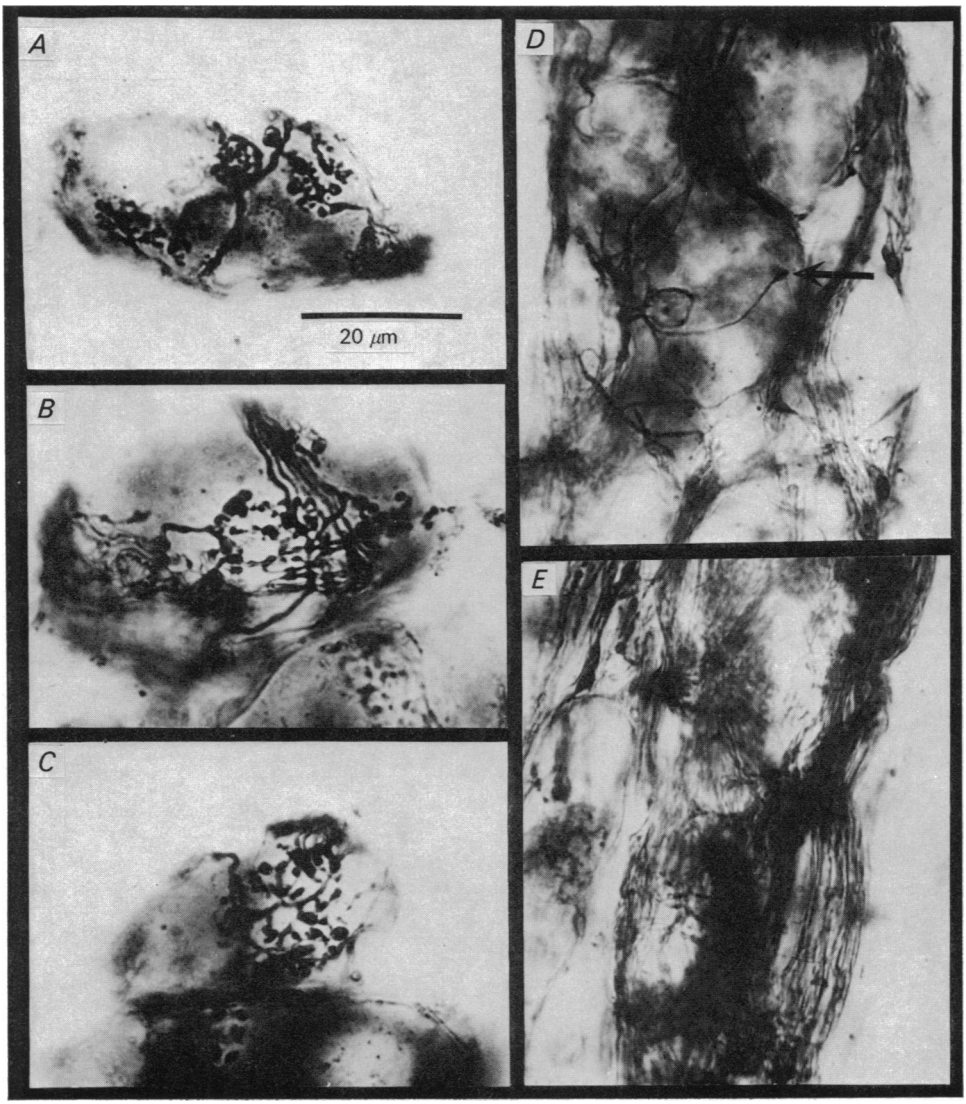
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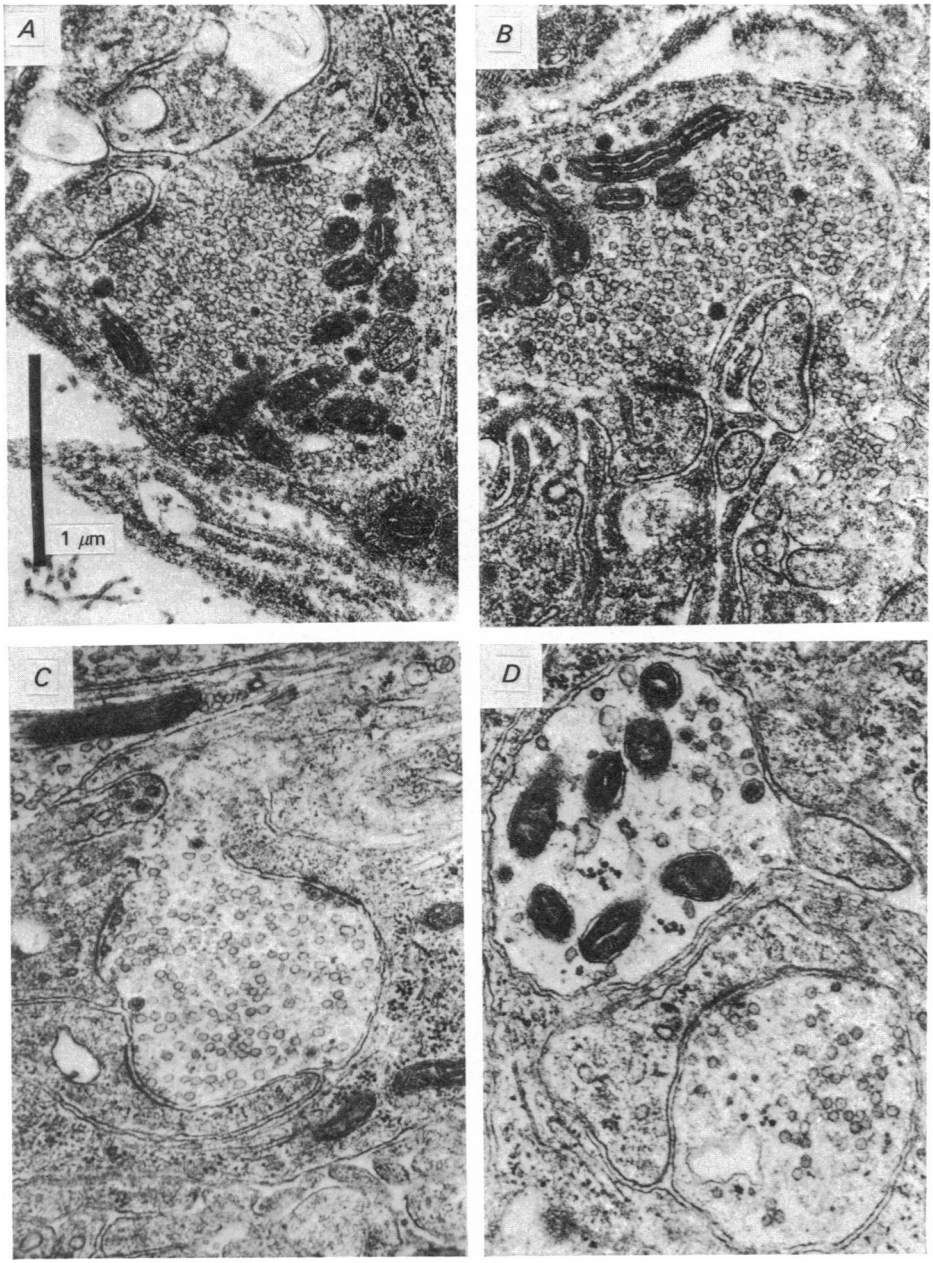
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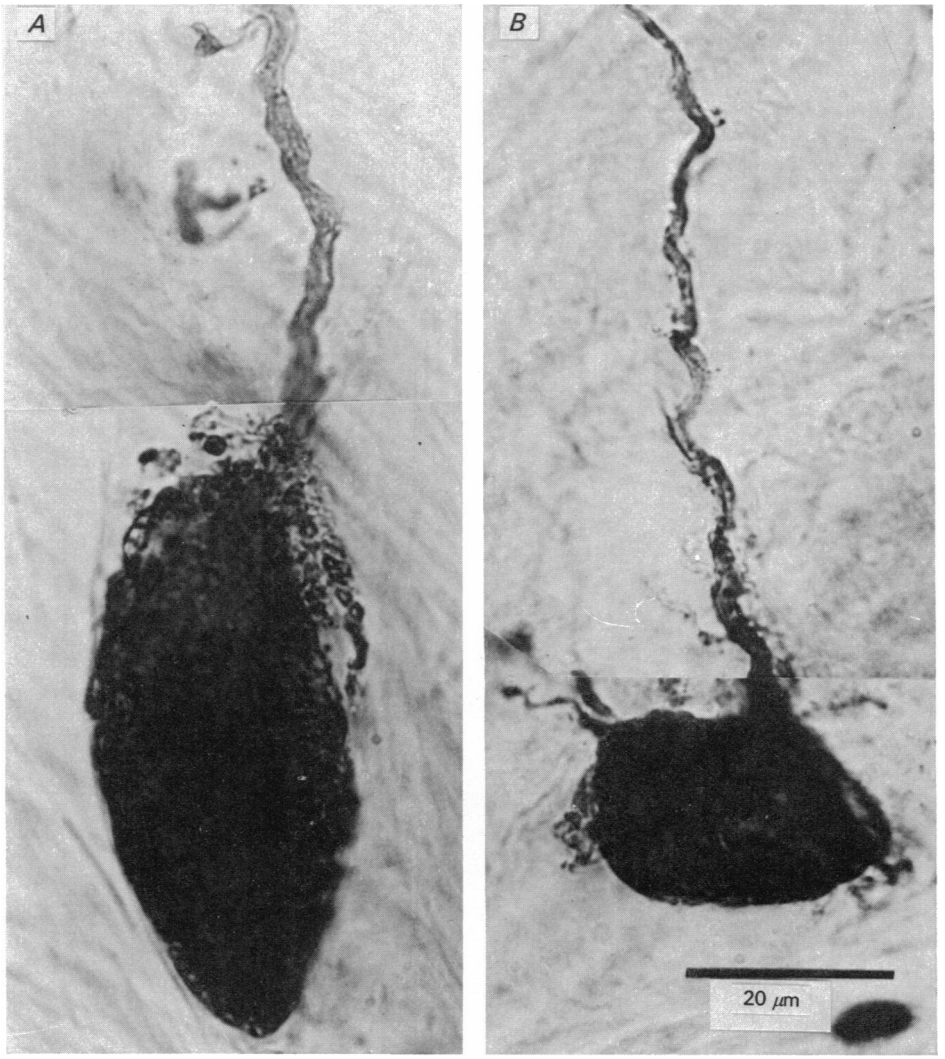
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20 μm

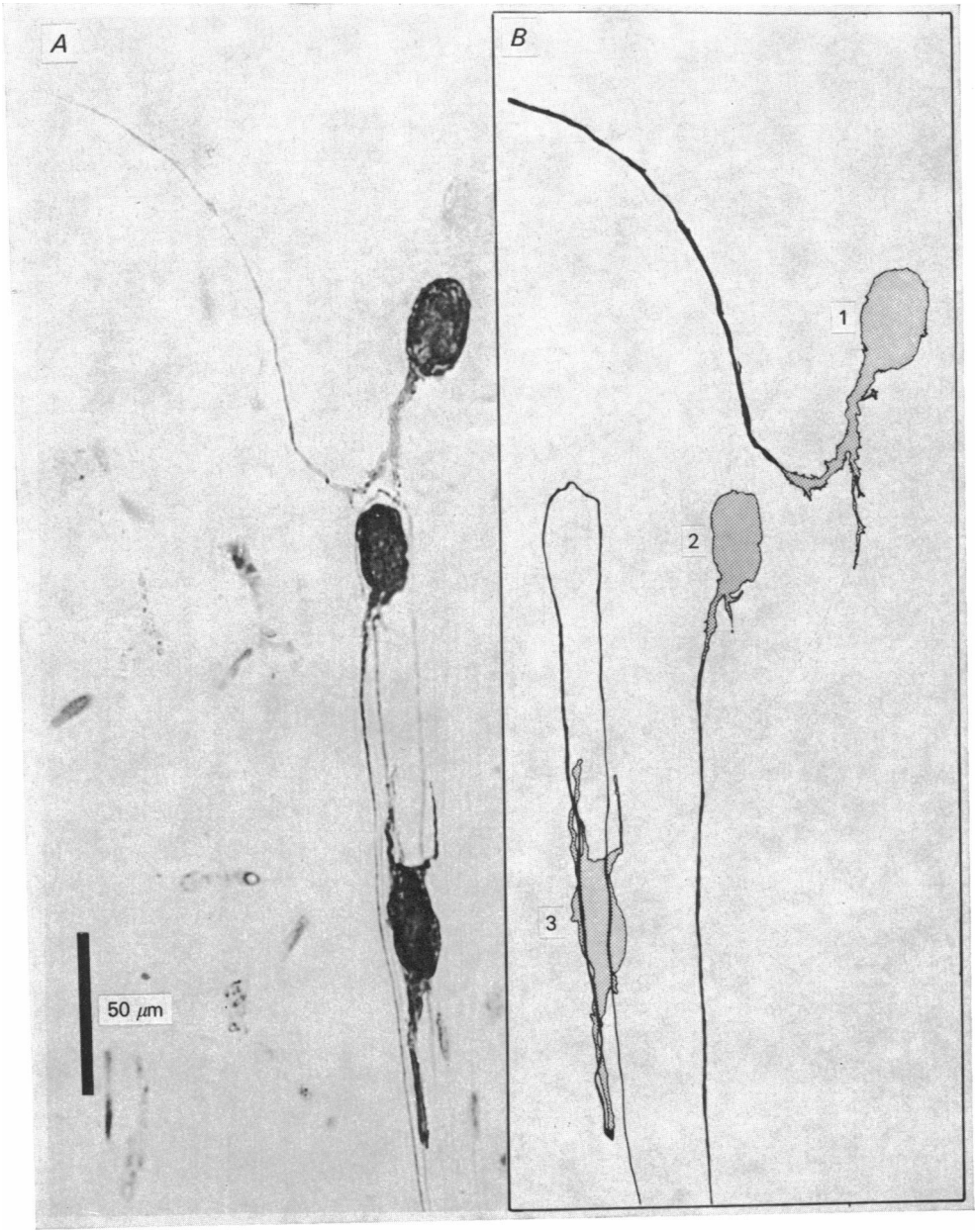
A
B
1
2
3
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PLATE 2

Photomicrographs of submandibular ganglion cells from adult (*A–C*) and 1-day-old rats (*D* and *E*) stained with zinc-iodide osmium. *A*, *B* and *C* show the pericellular network of preganglionic terminals on three adult cells. Counting the number of boutons per ganglion cell was often difficult because of the thickness of the terminal parts of the preganglionic fibres. Neonatal ganglia appeared quite different than adult ganglia treated with this method. Before the third post-natal week, fewer boutons (arrow in *D*) and many more fibres were evident than in older animals. A dense reticulum of fine fibres obscured much of the cellular detail on many neonatal cells (*E*).

PLATE 3

Electron micrographs of synaptic profiles from adult (*A*, *B*) and 2-day-old rats (*C*, *D*). Synaptic profiles on both neonatal and adult neurones were located on small processes within a few microns of the cell perimeter. Neonatal profiles generally contained fewer mitochondria and were less densely packed with vesicles (for example, compare *A* and *B* to *C* and *D*).

PLATE 4

Neurones injected with horseradish peroxidase from adult (*A*) and a 1-week-old rat (*B*). Many small protuberances originate from the cell body and the initial portion of the axon of both neonatal and adult neurones (see Text-fig. 3 for camera lucida drawings of these cells). Axons show no branches other than the short protuberances arising from the initial segment.

PLATE 5

Three neurones injected with horseradish peroxidase in a cluster of about fifteen cells from a 1-week-old rat (*A*), together with a camera lucida drawing of the cells separated from each other so that the axons can be followed more easily (*B*). The neurones have no axon collaterals within the cluster, and remain unbranched for as far as they could be followed (several hundred microns). Unlike adult cells, several neonatal neurones had one or more somewhat larger processes (cell 3, for example). Even though all three injected cells run to the salivary glands, their axons initially extend in different directions.