death[15] and the regression of multiple innervation[16] are retarded when synaptic transmission is blocked; and (2) terminal sprouting in partly denervated muscle is suppressed by direct electrical stimulation[9].

We consider the neurite-promoting activity described here to be a possible candidate for the role of 'sprouting factor' postulated by several authors[9,17]. Its appearance at an extremely high specific activity (10,000 units per mg protein) is a specific and local response to denervation, with a time course in chick muscles that is comparable to the period (4–6 days[8,18,19]) that elapses in partially denervated mammalian muscles before the first sprouts are detected.

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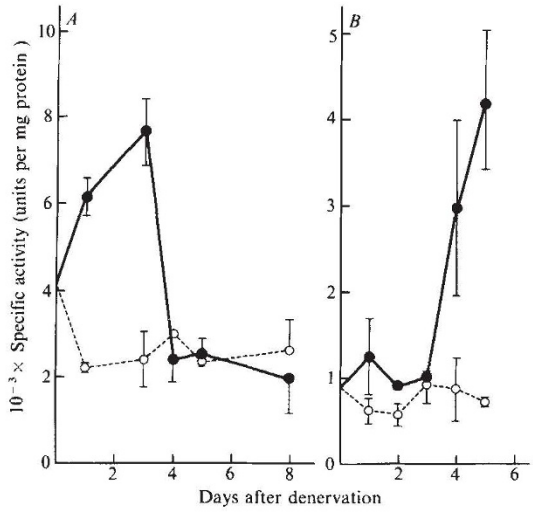

Fig. 3 Evolution with age of the specific neurite-promoting activity in muscle extracts from control and denervated chicks. 6-Day-old chicks from *A*, Warren or *B*, SV15 Vedette strains were denervated or maintained as unoperated controls (see text). ●, Chicks denervated at 6 days after hatching; ○, control unoperated chicks. Specific activities (units per mg protein) were determined from dose–response curves of the type shown in Fig. 2. Results are expressed as the mean ± range of results obtained from two different chicks, but error bars do not take account of the uncertainty in determining the 50% maximal effect from dose–response curves. For practical reasons, specific activity values for 0 days after denervation were supplied from independent experiments on 6-day-old control chicks reared under similar conditions. The total wet weights of muscles in denervated and control chicks of the same age did not in general differ significantly and in no case was a difference observed sufficient to explain the observed effects on specific neurite-promoting activity.

Table 1 Comparison of neurite-promoting activities of muscle extracts from denervated and contralateral legs

Dilution factor	Neurite outgrowth expressed as percentage of value at 2,000-fold dilution (±s.e.m.) Denervated	Contralateral
2,000	100	100
4,000	114±9	71±5
8,000	91±6	26±6
20,000	69±7	14±3
40,000	43±6	6±3
80,000	36±9	2±1

Chicks (Warren) were denervated at 6 days post-hatching by transection of the sciatic nerve. Three days later, muscle extracts were prepared from both denervated and contralateral legs. These were assayed for their activity in the neurite outgrowth assay and dose–response curves of the type shown in Fig. 2 were constructed. Here, results obtained in 12 experiments using extracts from 7 different chicks are combined. Values (% cells with neurite, corrected for F12 background) at each dilution have been normalized with respect to the value in the presence of a dilution of 1:2,000 (v/v) as 100%, and are presented as mean ± s.e.m. ($n = 6$–12). Absolute mean values (% cells with neurite, corrected) for neurite outgrowth in 2,000-fold dilutions of denervated and contralateral extracts, respectively, were 18.6±1.1% and 15.9±1.3%. All extracts were prepared in identical conditions at a constant ratio of 0.25 g wet weight of muscle for 1 ml of homogenization medium, and so no allowance was made in the table for the slight variations in total protein concentration of the resulting extracts.

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Generation of cat retinal ganglion cells in relation to central pathways

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The ganglion cells of the cat retina form classes distinguishable in terms of perikaryal size[1,2], dendritic morphology[3] and functional properties[4]. Further, the axons differ in their diameters, patterns of chiasmatic crossing and in their central connections[5–8]. Here we define, by ^{3}H-thymidine autoradiography, the order of production of cells of each class and relate the order of the 'birthdates' to the known axonal pathways. The ganglion cell classes are produced in broad waves, which overlap as cells are produced first for central then for peripheral retina. Medium-sized cells are produced before the largest cells, and small ganglion cells are produced throughout the period of cell generation. This sequence of cell production relates to the orderly arrangement of axons in the optic tract[9,10], and can also be related to the rules of chiasmatic crossing observed for each ganglion cell class[11–13].

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Tritiated thymidine (250–500 μCi, specific activity 90–110 Ci mmol^{-1}) was injected into the allantoic sac of 24 fetal cats aged 21–36 days (E21–E36) (see ref. 14 for an evaluation of this procedure). The timed pregnancies were produced by exposing an oestrous female to an experienced male for 12–16 h. The first day of exposure was counted as E0; this differs from the numbering used in earlier summaries[15–17], but is the same as that used by others[18,19]. The animals were killed by perfusion with 10% formol saline after retinal development was essentially complete, 55–260 days after birth. The eyes were removed and the retina and choroid were dissected free, flattened, dehydrated and embedded in glycol methacrylate resin (Sorvall) according to a method described elsewhere (E.H.P. and C.W., in preparation). Sections were cut radially or parallel to the retinal layers at 2-5 μm, then mounted on gelatin-coated slides, processed by standard autoradiographic techniques[20] using 14–60-day exposures, and counterstained with cresyl violet. All measurements were taken from sections cut parallel to the retinal layers.

The earliest injections labelled only medium-sized and small ganglion cells in the central retina, and slightly later injections labelled these cells over larger retinal regions. Two experiments with the earliest injections (E21) showed only a few heavily labelled ganglion cells, while many cells were labelled more lightly. The former, which can be interpreted as cells undergoing their final DNA replication at the time of injection[21], were all small or medium-sized ganglion cells according to the criteria of Stone[1] and Hughes[2]. Scattered in the region of the optic disk and visual streak, they covered a zone having a diameter equal to ~20° of visual angle, almost exclusively in the nasal retina. Injections made on days E22–23 produced more heavily labelled medium-sized and small ganglion cells in a larger central region, within ~40° of the optic disk (see Fig. 1). Heavy labelling of medium-sized and small cells extended still further towards the periphery when an injection was made on E24.

As the region covered by heavily labelled medium-sized and small cells expanded to include the whole retina, heavy labelling among large cells first occurred centrally. Whereas no large cells were heavily labelled in six animals given injections before E25, two ^{3}H-thymidine injections at E25 heavily labelled a few large cells in the central retina. Two animals given injections on E26 showed heavily labelled small and medium-sized neurones all across the retina, and heavily labelled large neurones in the central retina. These heavily labelled large neurones were widely scattered and located primarily in nasal retina. An injection at E27 labelled still more large neurones in the central retina.

After late injections, labelling among medium-sized cells, and then among large cells, receded from the central retina, became limited to the retinal periphery, and finally disappeared altogether, while labelled small ganglion cells continued to cover the whole retina. Thus, two injections on E28 labelled large and small neurones in the central retina, all cell types at intermediate eccentricities, and medium-sized and small cells in the peripheral retina. Two injections made on E29 produced heavily labelled large and small ganglion cells in all parts of the retina, while labelled medium-sized ganglion cells were restricted to the periphery (see Fig. 2). Two animals given injections on E31 had no labelled large cells <10° temporal to the area centralis, or <35° nasal to the area centralis. Medium-sized cells were labelled only near the ora serrata, while small cells were labelled all across the retina.

Injections made later than E31 (E35, 36) labelled only small cells. Some of these were small ganglion cells according to the criteria of Stone[1] and Hughes[2], indicating that these cells are still generated after production of medium-sized and large ganglion cells is complete. However, we have not determined the age at which ganglion cell production ceases.

These results show the order in which those ganglion cells that survive in the adult are produced. The results do not suggest

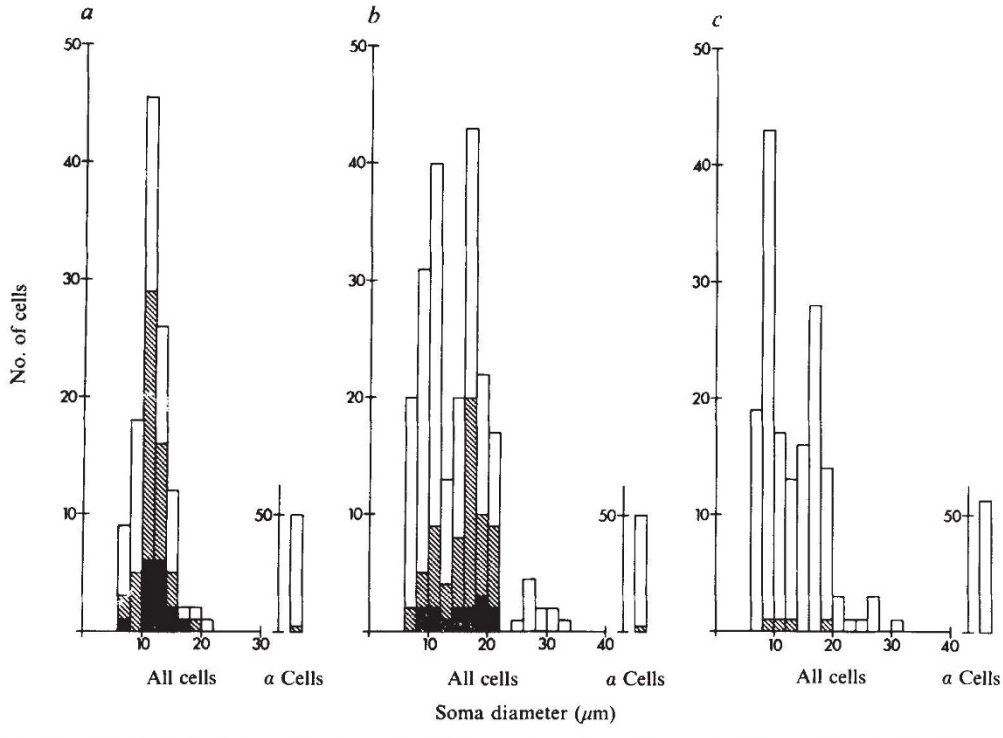

Fig. 1 Size distributions of labelled ganglion cells from an animal given 500 μCi of ^{3}H-thymidine on embryonic day 23 and killed 182 days after birth. Heavily labelled cells (shown by solid bars) are exclusively small or medium-sized, as are most lightly labelled cells (cross-hatched bars). Open bars represent unlabelled cells. Labelled cells are common in central regions of the retina (*a*, *b*) but rare in the periphery (*c*). Large (α) cells are occasionally lightly labelled, suggesting that they are formed rather later. Each histogram was prepared from three or four serial 2-μm sections from: *a*, within 500 μm of the area centralis; *b*, 1.5 mm temporal to the area centralis; and *c*, 9 mm temporal to the area centralis. Results from nasal retina were similar, although nasal retina developed earlier than temporal retina. All cells with visible Nissl substance and that had a well-defined, pale nucleolus in the plane of the section were included. Soma diameter was defined as the mean of the greatest and least diameters. Duplicate cell counts were eliminated. Heavily labelled cells contained over half the number of grains seen in the most heavily labelled nucleus in the section, and lightly labelled cells contained one-eighth to one-half the maximum number of grains but significantly above background levels. The method of ^{3}H-thymidine delivery used here produces a pulse of label with an extended tail, falling to <50% of maximum after 4 h, and <10% after 24 h[9]. Because low levels of ^{3}H-thymidine may be available long after the injection, all cells with less than one-eighth the maximum number of grains were treated as unlabelled. As the largest neurones (α cells) represent <5% of the total neurone population, separate samples of at least 50 large neurones (no duplicates) were taken from larger regions which included the area in which all neurones were counted. The fraction of the total nuclear volume included in a section, and thus the intensity of label, is inversely proportional to nuclear size[42]. Appropriate corrections have been computed[16], but as they do not significantly affect the cell distributions in these histograms, the uncorrected data are presented.

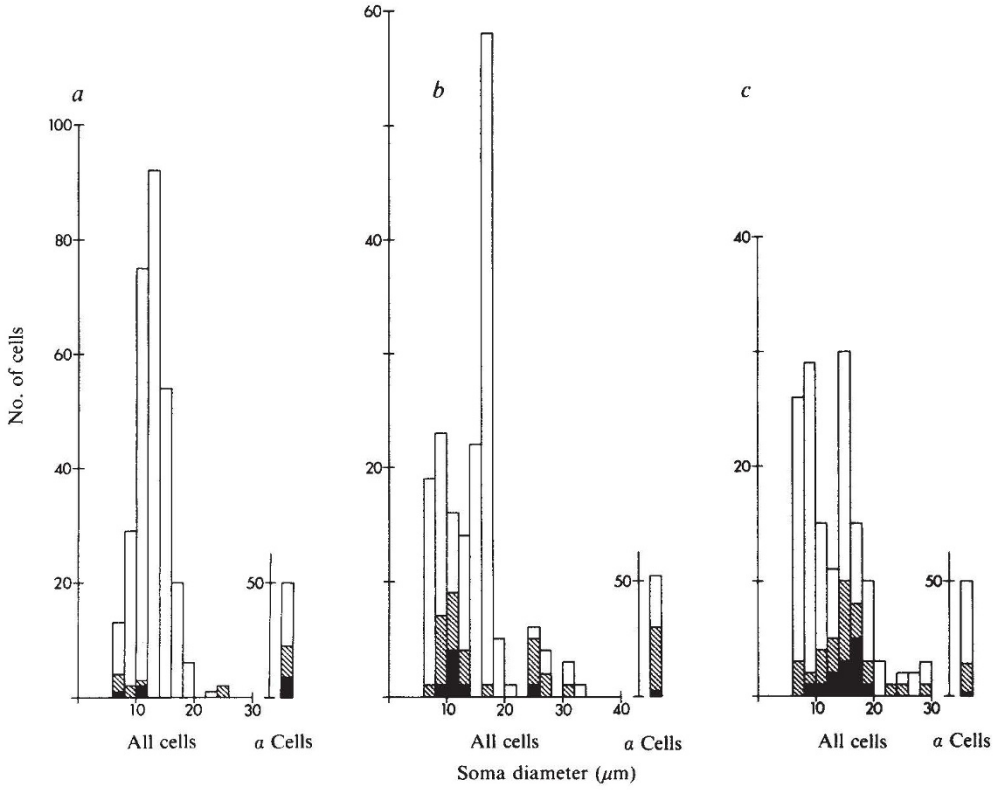

Fig. 2 Histograms prepared from three or four serial 2-μm sections: *a*, within 500 μm of the area centralis; *b*, 3 mm temporal to area centralis; *c*, 9.5 mm temporal to area centralis, from an animal that received ^{3}H-thymidine on embryonic day 29 and was killed 206 days after birth. All conventions as in Fig. 1. Ganglion cells were labelled across the entire retina. In central regions, the labelled neurones were small or large (*a*, *b*) while in peripheral retina, medium-sized neurones were also labelled (*c*). Note also that while some large ganglion cells were labelled all across the retina, the proportion of large cells that was labelled varied with retinal location.

differential cell death during development[22,23] nor do they differentiate between displaced amacrine cells and ganglion cells. However, the results do show, as do comparable results summarized by others[19], that in the cat the ganglion cells are not produced in a sequence that is in accord with the retinotopic maps formed centrally by the axon terminals (see also refs 21, 24, 25). Thus, cells whose axons have essentially the same terminal sites can be generated at widely differing times (for example, large and medium-sized cells form in register maps in the same geniculate layers), and cells born at the same time can have widely differing terminal sites. In this respect, the development of the mammalian retina differs from that of amphibians and fish, in which growth occurs mainly by a continuous addition of rings of new cells to the retinal periphery throughout post-embryonic life[26–28]. This pattern of growth allows the animal to maintain a fully functional retina while new cells are being added (S. Easter, personal communication) and also produces an order of addition of ganglion cells that represents a single retinal map. The difference in the pattern of cell addition between non-mammalian and mammalian forms may, therefore, reflect the differing functional demands on the developing retina.

Despite these differences between the cat and non-mammalian forms, fibre order within the optic tract reflects the order of ganglion cell production in all species examined[16,29–33]. The order of ganglion cell production that we have found in the cat retina is closely related to fibre order in the optic tract if one relates ganglion cell size to axonal diameter in terms established by others[5–7]. Thus, in the cat optic tract, medium-calibre axons lie deep in the tract, furthest from the pia, and represent the oldest ganglion cells. Large and small axons lie nearer the pia, and a dense group of finest axons is in a sub-pial position, representing the last addition of small ganglion cells[9,10]. Moreover, just as ganglion cells of each size class are produced in a rough central–peripheral sequence, so fibres of each size class are roughly mapped in the tract, with axons from the central retina lying generally deeper in the tract than axons from peripheral retina. The oldest retinofugal fibres can be labelled preferentially by an intraocular injection of anterograde tracers during the period of ganglion cell production[34,35]. This experiment has been done in the ferret, also a member of the order Carnivora with a visual pathway similar to that of the cat and other carnivores[36,37], and the results show that the segregation in the tract of older from newer fibres is quite sharp, and is maintained throughout most of the length of the tract[38].

The pattern of cell generation defined by our results relates significantly to the pattern of chiasmatic decussation for the axons of each cell type. It has been shown that medium-sized ganglion cells, which are among the earliest to be produced, have axons with the sharpest line of decussation, so that in the retina there is minimal spatial overlap between cells having crossed axons and those having uncrossed axons[11–13]. The axons of large ganglion cells, which are produced later, have a line of decussation that is further temporal, and these cells show more 'naso-temporal overlap'[11–13]. There is evidence that the small ganglion cells of the temporal retina that project contralaterally are the last ganglion cells to be produced in the temporal retina, as their axons run close to the surface of the tract near the pia[9,10]. The possibility that there is more than one wave of small cell production, and more than one functional class of small cells[39–41], remains to be explored in detail. It seems that, as the retina develops, the factors that produce a sharp naso-temporal segregation become weaker, and their effect moves nearer to the temporal pole, so that progressively more of the axons arising in the temporal retina take a crossed path and the line of decussation becomes less clearly defined.

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Synapse elimination in neonatal rat muscle is sensitive to pattern of muscle use

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The synaptic connections among the cells of the vertebrate nervous system undergo extensive rearrangements early in development[1,2]. During their initial growth, neurones apparently form synaptic connections with an excessive number of targets, later retracting a portion of these synapses in establishing the adult neural circuits. Because of the profound effects which experience has upon the developing nervous system[3], a question of considerable interest has been the role which the functional use of these developing synapses might play in determining the final pattern of connectivity. At the neuromuscular junction the early changes in synaptic connections are well documented, and here questions about the importance of function can be relatively easily addressed. Mammalian skeletal muscle fibres experience a perinatal period of synapse elimination so that all but one of several synapses formed on each muscle fibre are lost[4–6]. This synapse elimination is sensitive to alterations of neuromuscular use or activity. Reduction of muscle use by tenotomy[7,8] or by paralysis of the muscle with drugs blocking nerve impulse conduction[9] or neuromuscular transmission[10] delays or even prevents synapse loss, while increased use produced by stimulation of the muscle nerve[11] apparently accelerates the rate at which synapses are lost. I report here a further examination of the role of neuromuscular activity in synapse elimination. I show that chronic neuromuscular stimulation accelerates synapse elimination but that this acceleration is dependent on the temporal pattern in which the stimuli are presented: brief stimulus trains containing 100 Hz bursts of stimuli produce this acceleration whereas the same number of stimuli presented continuously at 1 Hz do not. Furthermore, the 100 Hz activity pattern which is effective in altering synapse elimination also alters two other muscle properties: the sensitivity of the muscle fibres to acetylcholine and the 'speed' of muscle contractions. These findings suggest that the ability of muscle fibres to maintain more than one nerve terminal, like other muscle properties, is sensitive to the pattern of muscle use rather than just the total amount of use.

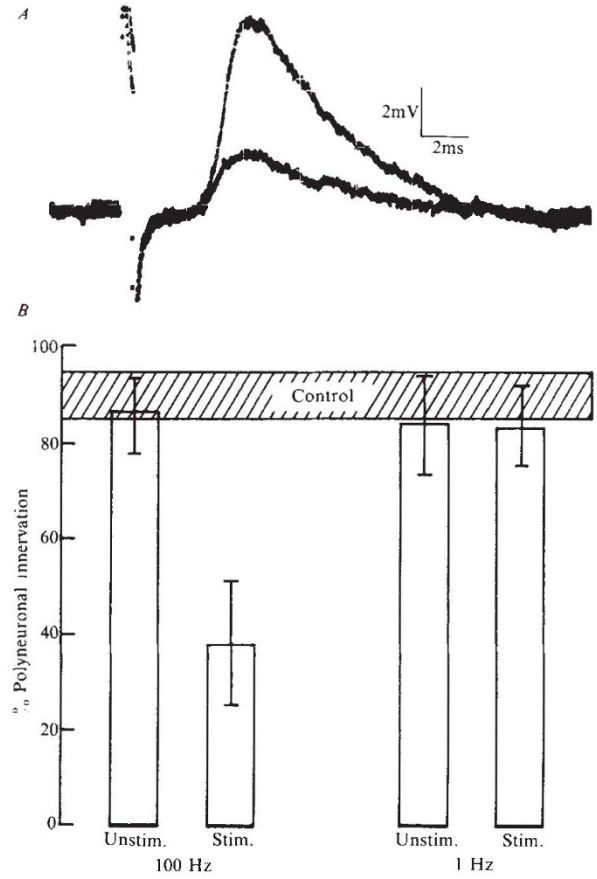

Fig. 1 *A*, Intracellular recording of endplate potentials from a muscle fibre showing the procedure used to determine whether individual muscle fibres were polyneuronally innervated. Shown are two superimposed oscilloscope sweeps, each at a different intensity of stimulation of the muscle nerve. The increase in the amplitude of the endplate potential at the higher intensity of stimulation shows that this fibre was innervated by at least two axons having slightly different thresholds to the stimulation of the muscle nerve. *B*, Histograms of percentage of muscle fibres exhibiting polyneuronal innervation in 10–11-day-old muscles following various procedures. The hatched portion of the histogram indicates the level (mean +/− s.e.) of polyneuronal innervation present in 11 muscles of normal, untreated animals reared by their mothers. The pair of bars to the left represent the polyneuronal innervation in right soleus muscles stimulated with the 100 Hz pattern from day 7 (Stim.) and in the left, contralateral muscles (Unstim.) from 8 animals. The pair of bars to the right show the polyneuronal innervation present in muscles from 3 animals whose right soleus muscles received the 1 Hz stimulation pattern from day 7 (Stim.) and whose left soleus muscles served as controls (Unstim.). At least 20 fibres were sampled by intracellular recording in each muscle. Bars indicate s.e.m.

Synapse elimination in rat soleus muscle occurs during the first 2 weeks after birth[6]. Up to days 9–10, all of the fibres are innervated by more than one motor axon. At this age, synapse elimination begins to result in the appearance of singly innervated fibres, and by days 15–16 almost all of the fibres are singly innervated. To investigate the effects of chronic stimulation on this time course of synapse elimination, Teflon-coated multistranded stainless steel wires were drawn underneath the skin of 7-day-old rat pups anaesthetized with ether. The bared tips of these wires were implanted into the muscles anterior and posterior to the soleus in the right leg. Stimulus pulses of 1.0 ms duration and 4–6 mA were passed between the wires to activate the soleus muscle; these pulses were at least three times the intensity necessary to promote maximal plantar flexion. Stimuli were presented chronically to the pups for the next 3 to 4 days. To assure the animals suffered no pain from the stimuli, their spinal cords were transected at T11. In agreement with previous findings[12], cord transection in these

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