move north or south after crossing the ocean-freshwater boundary [for example, some anadromous salmon leaving the Fraser River, British Columbia, eventually move 5° to 15° north in the ocean (*22*)]. Third, selection for migration will also be affected by other factors (*1*), including the presence of competitors in a habitat, which influence food abundance, and predators and disease, which affect survivorship. These factors are not considered in our analysis because of the lack of such data for each latitude. However, it would be of interest to investigate the significance of these factors in explaining the variation in Fig. 2. The remaining paradox, that in some waters both anadromous and catadromous species co-exist (*1*), may require survivorship data or more detailed foraging data.

In spite of these potentially complicating factors, the food availability hypothesis appears to be consistent with the global geographic pattern of diadromy in fishes. We therefore conclude that food availability is an important factor in explaining not only where diadromous fishes occur, but also why fish migrate across the ocean-freshwater boundary, as well as their direction of movement.

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Nicotinic Antagonists Enhance Process Outgrowth by Rat Retinal Ganglion Cells in Culture

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Functional nicotinic cholinergic receptors are found on mammalian retinal ganglion cell neurons in culture. The neurotransmitter acetylcholine (ACh) can be detected in the medium of many of these retinal cultures, after release presumably from the choline acetyltransferase–positive amacrine cells. The postsynaptic effect of endogenous or applied ACh on the ganglion cells can be blocked with specific nicotinic antagonists. Here it is shown that within 24 hours of producing such a pharmacologic blockade, the retinal ganglion cells begin to sprout or regenerate neuronal processes. Thus, the growth-enhancing effect of nicotinic antagonists may be due to the removal of inhibition to growth by tonic levels of ACh present in the culture medium. Since there is a spontaneous leak of ACh in the intact retina, the effects of nicotinic cholinergic drugs on process outgrowth in culture may reflect a normal control mechanism for growth or regeneration of retinal ganglion cell processes that is exerted by ACh in vivo.

NICOTINIC CHOLINERGIC DRUGS have long been studied for their role in synaptic transmission in the nervous system. Besides their function in intercellular communication, trophic effects of acetylcholine (ACh) and other presumptive neurotransmitters have been postulated in the mammalian central nervous system. For example, the activation of ACh, norepinephrine, and *N*-methyl-D-aspartate (NMDA) receptors appears to facilitate the synaptic plasticity that affects ocular dominance columns after monocular visual deprivation (*1*). Factors that inhibit neuronal growth have often been associated with glial cells (*2*). Recently, however, in invertebrate and submammalian vertebrate nervous systems, growth-inhibiting properties have been attributed to classical neurotransmitters (*3*). Hence, neuronal interactions, possibly mediated by neurotransmitters, may be important determinants of the cytoarchitecture of the central nervous system (*4*).

In some cell culture systems neurotransmitters leak into the culture medium (*5*), and spontaneous release of ACh occurs in retinal cultures such as those described here (*6*). This release of ACh resembles at least to some degree a naturally occurring event in the intact retina (*7*). Thus, in tissue culture it may be possible to investigate the chemical nature of neuronal interactions associated with cell growth in a precisely controlled environment.

In the present study we used cultures of postnatal rat retinal cells because they provide a system in which neurite outgrowth can be monitored from an identified central neuron, the retinal ganglion cell (*8, 9*). We found that the addition of nicotinic antagonists to the culture medium for the first 24 hours after plating resulted in a striking increase in process outgrowth by the ganglion cells. During the same period, there was no effect of nicotinic antagonists on the survival of the ganglion cells compared to controls, suggesting that the effect was in some way specific for the growth of neurites and not merely influencing the overall health and welfare of the cells.

Retinal cultures were produced as described previously from 7- to 12-day-old rat pups (*8*) and grown in a specific batch of rat serum; under these conditions the culture fluid was found to have endogenous levels

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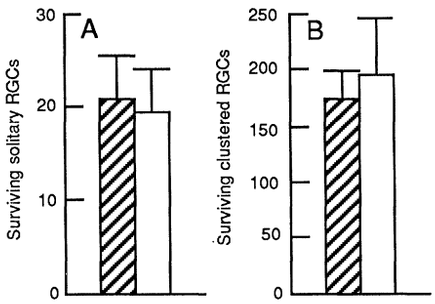

Fig. 1. Lack of effect of *d*-tubocurarine (10 μ*M*, white bars) on the survival of solitary (**A**) or clustered (**B**) retinal ganglion cells in culture for 24 hours compared to control (hatched bars). The ordinate represents the mean number of ganglion cells surviving in a dish; the experiment was performed in quadruplicate. The error bars are standard deviation.

Table 1. Effect of antagonists to acetylcholine and GABA on outgrowth of processes by solitary rat retinal ganglion cells in culture. Each experiment consisted of a single dissociation of cells cultured in multiple dishes. A ganglion cell was counted as having a neuronal process only if it was longer than the diameter of the cell body. To allow for slight variations among experiments, we analyzed the data summarized in the percentage column with an extension of Fisher's exact test for several 2 × 2 tables, as described previously (*8*). Each table contained the number of ganglion cells with and without processes in two different treatment media for a given retinal dissociation. Each drug was added at a concentration of 10 μ*M*.

Treatment	Number of cells with processes (%)	Total process length per cell (μm)	Number of experiments	Cells counted
Control	37.4	91.7	8	463
d-Tubocurarine	65.4*	133.2	7	342
Mecamylamine	70.0*	130.5	4	178
Atropine	39.2	101.0	5	306
Picrotoxinin	39.8	74.8	2	119

*Significantly greater than the value for the control ($P > 0.001$; Fisher's exact test for a 2 × 2 matrix with Bonferroni's correction for multiple comparisons).

of ACh probably emanating from the choline acetyltransferase–positive cells in these cultures (*6*). On the basis of studies in the intact retina, the ACh-synthesizing neurons probably represent cholinergic amacrine cells that normally synapse on ganglion cells (*10*). The amount of ACh spontaneously released from the presumptive cholinergic amacrine cells was sufficient to produce a nondesensitizing depolarization of nearby ganglion cells. In dense platings, such as those used here (>2×10^5 cells per 1.25×10^{-4} m^2 culture well), nearly every ganglion cell was affected in this manner, although the magnitude of the responses varied widely from cell to cell (*6*).

We found that culturing the cells for the first 24 hours in the presence of nicotinic blockers such as *d*-tubocurarine (10 μ*M*; Fig. 1) or mecamylamine (10 μ*M*) had no significant effect on the survival of the retinal ganglion cells compared to controls. Two populations of retinal ganglion cells were examined in these studies: (i) solitary ganglion cells that had no physical connections to other cells in the culture dish and constituted 10 to 15% of the total ganglion cells, and (ii) clustered ganglion cells that were connected to other cells either by direct contact of the soma or via processes and made up 85 to 90% of the total ganglion cell population (*8, 11*). The survival of neither of these populations of ganglion cells was affected after 24 hours (Fig. 1, A and B). Survival was assessed by cell counts on phase-bright ganglion cells identified with fluorescent labels and verified with electrical recordings and the ability to cleave fluorescein from fluorescein diacetate (*8*).

Nicotinic antagonists had a striking effect, however, on the outgrowth of processes by solitary retinal ganglion cells (*12*). As shown in Table 1, the proportion of ganglion cells [scored as described previously (*8, 13*)] growing at least one process longer than the diameter of the cell body nearly doubled, and the total length of processes per cell increased by 45% compared to controls. In contrast, the muscarinic antagonist atropine (10 μ*M*) did not substantially enhance process outgrowth; this concentration of atropine also did not alter the cholinergic current measured during whole-cell recording of retinal ganglion cells with patch electrodes (*14*). Moreover, picrotoxinin (10 μ*M*), which blocks the large currents induced by γ-aminobutyric acid (GABA) in these cells (*15*), did not affect process outgrowth. These results suggest a degree of specificity to the effect of nicotinic antagonists on the plasticity of process outgrowth by retinal ganglion cells.

Approximately two-thirds of the retinal ganglion cells incubated in *d*-tubocurarine (10 μ*M*) grew at least one process longer than the diameter of the cell body and often much longer. In contrast, only about a third of the retinal ganglion cells in cultures lacking *d*-tubocurarine produced a process as long as the diameter of the cell soma, and even then these processes were shorter than those growing in *d*-tubocurarine (*16*). As illustrated in Figs. 2 and 3, quantitative analysis of 4762 processes on 716 solitary retinal ganglion cells revealed that the nicotinic antagonist compared to controls resulted in a significant increase in the mean length per process (*17*). The number of processes per ganglion cell, however, was not significantly different in *d*-tubocurarine and control (by paired *t* test, *t* statistic = 0.6395, $P > 0.5$); the mean number of processes per cell (±SEM) was 6.05 (±1.00) for *d*-tubocurarine and 6.50 (±1.49) for control. The fact that *d*-tubocurarine was associated with longer process lengths indicates that elongation (or prevention from shrinkage and pruning) but not initiation of process growth was affected by the nicotinic antagonist. These observations may help in elucidating the cellular mechanism of drug action in future studies.

Alternative interpretations besides nicotinic blockade for the effect of *d*-tubocurarine on process outgrowth in cultures containing endogenous ACh are possible. For example, *d*-tubocurarine could have nonspecific effects; this drug is known to block several other types of ionic channels in addition to the nicotinic receptor-channel complex, although at higher concentrations than we have used here (*18*). This possibility is unlikely, however, because (i) another nicotinic antagonist, mecamylamine, yielded concordant results, and (ii) process outgrowth was not affected by nicotinic antagonists added to cultures lacking detectable levels of endogenous ACh. The concentration of endogenous ACh in the retinal cultures appeared to depend on culture conditions, for example, on the batch of rat serum used in feeding the cells (although ACh was measured after removal of the serum, so it did not directly contribute to ACh levels) (*6*). Retinal cultures lacking endogenous ACh had a greater proportion of ganglion cells that grew processes than dishes with endogenous ACh (*19*). These findings are consistent with the notion that endogenous levels of ACh, when present and not blocked by nicotinic antagonists, are inhibitory to the growth of retinal ganglion cell processes (*20*).

There are many possible mechanisms for the effect of ACh on the outgrowth of processes by retinal ganglion cells. Since there are nicotinic receptors on retinal ganglion cells, the simplest explanation for our results would involve a direct action of ACh on these neurons, although a more indirect influence cannot be ruled out. The mechanism of action of ACh may relate to its

electrical effects on retinal ganglion cells, which we have studied using the patch-clamp technique (*14*). For example, the depolarization engendered by ACh could be responsible for the lack of process growth; a precedent for this mode of action has been encountered in an invertebrate neuron in which depolarizing current pulses sufficient to trigger action potentials were found to inhibit neurite elongation (*21*). Nevertheless in the mammalian central neurons studied here, K^+, glutamate, or kainate did not decrease process outgrowth despite their depolarizing the cells (*22*). Thus, it is unlikely that ACh was inhibiting process outgrowth by simply depolarizing the cells. Other possible actions of ACh that might influence process outgrowth include specific second messenger effects mediated by ionic fluxes; for example, effects of ACh-triggered Ca^{2+} entry on genes involved in the growth response (*23*) are currently under investigation.

Whatever the mechanism by which ACh appears to inhibit process outgrowth, these results show that an endogenous neurotransmitter may influence the plasticity of neurites. Accordingly, chemical interactions between neurons may inhibit neurite elongation and thus influence possible functional connections and circuitry within the mammalian central nervous system. For example, the decrease in process elongation engendered by ACh could be a mechanism for halting, stabilizing, or consolidating dendritic growth at an appropriate location so that synapse formation could occur. Along these lines, recent morphological evidence has shown that mammalian retinal ganglion cell processes undergo conjoint growth and remodeling prior to the formation of their final connections on amacrine cells (*24*). Our findings indicate a possible mechanism for this restriction of process location: inhibition of further extension (or concomitant shrinkage) of neurites by release of the putative neurotransmitter from the future presynaptic element. Most importantly, nicotinic cholinergic antagonists can reverse the apparent inhibitory effects of ACh on process growth. Thus, our results may have implications for modifying the formation and regeneration of pathways within the mammalian central nervous system both under normal conditions and in states that might afflict cholinergic neurons and their nicotinic targets, such as retinal ganglion cells affected by Alzheimer's disease (*25*).

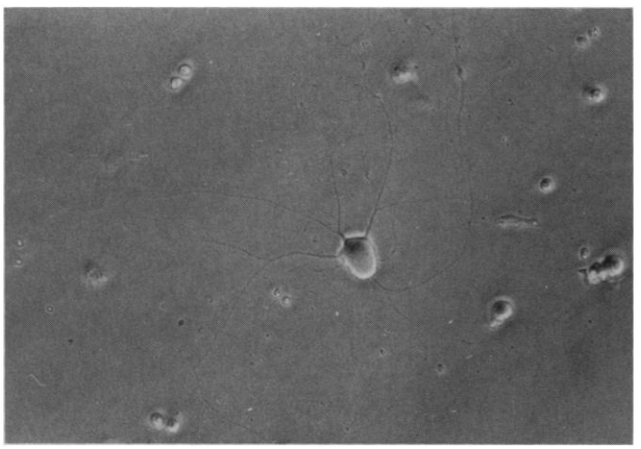

Fig. 2. Phase-contrast micrograph of a rat retinal ganglion cell with particularly exuberant outgrowth that had been cultured for 24 hours prior to quantifying its processes. As previously described (*8*), the illustrated cell was identified under epifluorescence microscopy as a ganglion cell by the presence of blue fluorescent dye that had been retrogradely transported before dissociation. This cell (16 μm greatest diameter) was chosen because of its easily photographed processes; most ganglion cells did not elaborate such extensive processes under any circumstances.

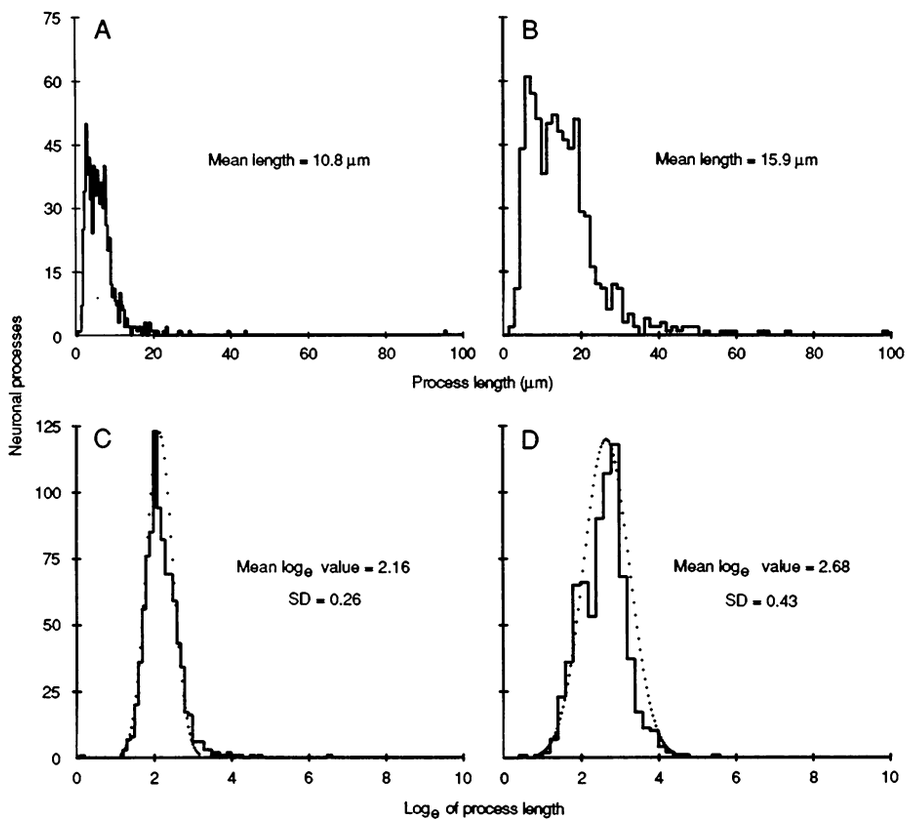

Fig. 3. Analysis of process outgrowth by solitary retinal ganglion cells in culture for 24 hours in control dishes (**A** and **C**) and in dishes incubated in media containing 10 μ*M* *d*-tubocurarine (**B** and **D**). The distribution of process length versus frequency (A and B) was not normal but the logarithm of the lengths (C and D) was (*13, 17*). The mean of the logarithm of the lengths was significantly greater in the cultures incubated in *d*-tubocurarine ($P < 0.0001$, two-tailed t test). In the eight control dishes in this experiment 158 solitary ganglion cells were analyzed containing 936 total processes; in the eight dishes treated with *d*-tubocurarine 150 solitary ganglion cells with 724 total processes were analyzed. Five other experiments yielded similar results.

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9. Retinal ganglion cells were identified by the presence of a fluorescent dye, granular blue, which 2 days before enucleation had been injected into the superior colliculi and transported retrogradely to the ganglion cells [for a color photograph see (*8*)]. The presence of dye permitted the unequivocal identification of the ganglion cells after dissociation of the retina with the enzyme papain. In previous studies the retinal ganglion cells were also labeled after dissociation by means of immunocytochemical techniques with monoclonal antibodies against Thy-1 (*8*). The same population of retinal ganglion cells was identified by Thy-1 antibodies and by retrograde transport of fluorescent dye (*8*), ensuring the reliability of either technique. For convenience, the retrograde transport method was used routinely. By postnatal day 5 all of the central (axonal) connections of pigmented rat retinal ganglion cells have been formed. Thus, axonal processes that grow in culture from ganglion cells obtained from animals at this stage or later must represent regeneration rather than initial outgrowth. Electron micrographs of the ganglion cells in culture have confirmed that at least some of the neurites are axonal (S. A. Lipton and R. D. Madison, unpublished observations), indicating that they have regenerated. In contrast, many of the dendritic connections within the retina are still forming at the age of the animals used in this study. Hence, dendritic processes that grow in culture may represent the initial developmental outgrowth of these structures.
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11. About half of the clustered retinal ganglion cells had spontaneous electrical activity presumably due to synaptic input from other cells in the dish [S. A. Lipton, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9774 (1986)]. Together the solitary and clustered populations of ganglion cells accounted for approximately 1% of the total cells in a typical culture dish; this proportion resembles the composition of the intact postnatal retina.
12. Outgrowth from clustered retinal ganglion cells could not be adequately assessed because processes were more difficult to visualize within the clusters (*8*).
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16. As shown in (*6, 14*), there is a wide range in the electrical responsiveness among retinal ganglion cells to nicotinic agonists, probably reflecting the variation in cholinoreceptive sites among the cells. Thus, the fact that about a third of the ganglion cells bear processes in the absence of nicotinic inhibitors and another third do not exhibit outgrowth even in the presence of these antagonists (Table 1) does not necessarily indicate that these cells are totally refractory to the effects of ACh. Rather, different ganglion cells are possibly more or less sensitive to the presence of ACh depending on the number of receptor sites, producing a fair degree of heterogeneity in response to ACh both in electrophysiological properties and in growth characteristics.
17. As described previously (*13*), the lengths of processes did not form a normal distribution, but the logarithm of the lengths did. Therefore, the logarithmic distribution was analyzed by normal statistics. This showed that the lengths of the processes were significantly longer when the incubation medium included *d*-tubocurarine.
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19. Of the ganglion cells in cultures containing endogenous ACh, 37.4% grew processes (Table 1); in contrast, in three experiments in cultures without detectable endogenous ACh, 58.0% grew processes. This difference is highly significant ($P < 0.001$) by Fisher's exact test, as performed in (*8*). Also, for cultures without endogenous ACh, *d*-tubocurarine (10 μM) had no significant effect on process outgrowth compared with controls; 63% of the ganglion cells grew processes in medium containing *d*-tubocurarine while, as stated above, 58% grew processes in the control dishes.
20. If nicotinic agents are indeed inhibitory to the growth of processes by retinal ganglion cells, then it should be possible to recreate this effect in cultures that lack substantial levels of endogenous ACh by adding exogenous nicotinic agonists. After submission of our manuscript, a preliminary report of this experiment appeared. Monitored with AVEC-DIC microscopy, direct application of nicotine (100 μM) onto growth cones of presumptive ganglion cells from the chick retina resulted in the inhibition of activity or even retraction of filopodia and neurites [K. L. Lankford, M. I. Fonseca, W. L. Klein, *Soc. Neurosci. Abstr.* **13**, 258 (1987)].
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Dynamic Changes in Inhibin Messenger RNAs in Rat Ovarian Follicles During the Reproductive Cycle

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The alterations in morphology and function of the ovarian follicle as it matures, ovulates, and becomes a corpus luteum are dramatic. A variety of steroid and polypeptide hormones influence these processes, and the ovary in turn produces specific hormonal signals for endocrine regulation. One such signal is inhibin, a heterodimeric protein that suppresses the secretion of follicle-stimulating hormone from pituitary gonadotrophs. Rat inhibin complementary DNA probes have been used to examine the levels and distribution of inhibin α- and β_A-subunit messenger RNAs in the ovaries of cycling animals. Striking, dynamic changes have been found in inhibin messenger RNA accumulation during the developmental maturation of the ovarian follicle.

THE PROGRESSIVE GROWTH, OVULATION, and luteinization of ovarian follicles are highly integrated processes coordinated by regulatory signals including steroid and peptide hormones from the brain, anterior pituitary, adrenals, and ovaries. The two primary regulators of ovarian function are the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (*1*). FSH and LH are usually secreted in tandem under the influence of the hypothalamic peptide gonadotropin-releasing hormone (GnRH) (*2*). However, normal physiological situations exist where the secretion of FSH and LH are dissociable (*3*).

FSH secretion can be specifically suppressed by the steroid-free portion of ovarian follicular fluid in many species (*4, 5*). Follicular fluid can inhibit both the primary and secondary FSH surges, as well as the rise in serum FSH concentrations that follows unilateral or bilateral ovariectomy (*5, 6*). Purification of this activity, termed inhibin, from follicular fluid resulted in the identification of a heterodimeric glycoprotein,

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