reoviruses under controlled conditions of host and environment and found that high transmission is a genetically determined property controlled by the L2 dsRNA segment. Release of high levels of virus (shedding) from the gastrointestinal tract is also a property controlled by the L2 segment, suggesting that the differences in transmission are mediated by the level of shedding. These findings indicate that a single reovirus gene can influence the capacity of the virus to be transmitted in the environment. A further understanding of the L2 gene and how other genes affect its properties should provide new insights into the behavior of viruses in the natural environment.

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Suppression of Neurite Elongation and Growth Cone Motility by Electrical Activity

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Electrical activity may regulate a number of neuronal functions in addition to its role in transmitting signals along nerve cells. The hypothesis that electrical activity affects neurite elongation in sprouting neurons was tested by stimulating individual snail neurons isolated in cell culture. The findings demonstrated that growth cone advance, and thus neurite elongation, is reversibly stopped during periods when action potentials are experimentally evoked. A decrease in filopodial number and growth cone area was also observed. Thus, action potentials can mediate the cessation of neurite outgrowth and thereby may influence structure and connectivity within the nervous system.

ELECTRICAL ACTIVITY PLAYS A PROMinent role in communication within the nervous system. It has long been suggested that electrical activity might also play a role in shaping the morphology and connectivity of the nervous system both during development and in adult neuroplastic events (*1*). One plausible mechanism for such regulation is through the control of neurite outgrowth. While, in recent years, chemical factors have been shown to influence neurite outgrowth (*2*), a similar role for electrical activity has not been unequivocally demonstrated. However, electrical activity has been shown to regulate many other cellular processes including: the synthesis of acetylcholine receptors (*3, 4*), the synthesis of neurotransmitters and enzymes used in the production of neurotransmitters and hormones (*5*), the formation and pattern of synaptic connections (*6*), and sprouting at the neuromuscular junction (*7*). Electrical fields have been implicated in influencing the direction of neurite outgrowth (*8*), and shifts in the membrane potential induced by changes in ion concentrations or the addition of ionophores and growth factors can cause changes in the state of differentiation and the amount of neurite outgrowth in culture (*9*). By stimulating specific neurons growing in cell culture, we directly tested the hypothesis that action potentials can affect neurite outgrowth. Our findings show that the generation of action potentials is a sufficient signal to halt neurite outgrowth abruptly and reversibly.

We used cultured neurons from the snail *Helisoma*, as they afford a high level of resolution in the analysis of neurite outgrowth. Single identified neurons were removed from buccal ganglia and plated in cell culture (*10*). Within 3 days of plating, these neurons extended neurites via large growth cones that typically measured 20 μm in width. A direct test of the hypothesis required that three experimental conditions be met. First, growth cone advance should be quantitatively measured during stimulation of the neuron. Second, action potentials must be evoked at will and their occurrence monitored. Third, the techniques used to stimulate the neurons and record the responses must not interfere with the motile properties of the growth cones. We have now been able to satisfy all of these conditions in a single set of experiments with a total of 143 growth cones from 21 neurons. Quantitative measurements of the advance of each growth cone were readily obtained from photographs taken at constant time intervals (*11*). Direct stimulation was accomplished by extracellularly stimulating the somata of individual neurons during the period of neurite outgrowth. We initially tested two stimulation techniques. Penetrating the cell body with an intracellular microelectrode frequently stopped neurite elongation; in contrast, extracellular patch pipettes (*12*) attached to the soma membrane by a gigohm seal did not alter elongation (see below). Consequently, we used extracellular patch pipettes in these experiments. Because the patch pipette could not reveal the magnitude of membrane potential change during stimulation, we performed control experiments to evaluate the effect of current pulses delivered through the patch pipette by simultaneously recording from the soma with an intracellular microelectrode (Fig. 1).

Neurons were stimulated under current-clamp conditions with 20-msec pulses (amplitudes of 1 to 6 nA, depending on the seal resistance). Current pulses delivered through the patch pipette produced small depolarizations (approximately 10 mV in amplitude), which evoked action potentials (Fig. 1) as measured intracellularly (*13*). Furthermore, we could monitor these action potentials with the patch pipette as a result of capacitive coupling between the pipette and the cell. Thus, individual neurons could be stimulated extracellularly and the evoked action potentials could be recorded through the same noninvasive pipette.

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To assay the effect of action potentials on neurite elongation, we compared the rate of growth cone advance and morphology during a 2- or 3-hour observation period for identified neuron B19. A single extracellular patch pipette was used for stimulation and recording of action potentials. The first hour always served as a control period during which the extracellular patch pipette was sealed to the soma membrane but no current pulses were delivered. During the entire second hour, 20-msec current pulses at 4 per second were applied through the patch pipette, and action potentials were evoked. In some experiments, a third period without current pulses was used to monitor recovery. Growth cone advance during the control period prior to cell stimulation (Fig. 1) proceeded at a constant rate [mean, 8.7 ± 0.7 μm/hour for 64 growth cones in seven neurons (*14*)].

During the period when action potentials were evoked, growth cone advance ceased. Advance often stopped within 15 minutes of the onset of stimulation. The reduction in growth rate resulting from stimulation was significant (mean, -0.08 ± 0.7 μm/hour for 64 growth cones in seven neurons; $P < 0.001$, matched-pair t test). During stimulation, 92 percent of the growth cones showed a decrease in their growth rate. Furthermore, over half of the growth cones showed either zero growth rate or a slight retraction. During the third period when the stimulus was turned off, growth cone advance resumed with growth rates returning to their prestimulation values (mean, 8.7 ± 0.8 for 17 growth cones). All growth cones recovered; the amount of time necessary for recovery, however, varied among neurons from 15 minutes to 2 hours. Taken together (Fig. 2), these data demonstrate that the generation of action potentials reversibly suppresses neurite elongation.

The changes in neurite elongation caused by stimulation were accompanied by changes in growth cone structure (Fig. 3). Stimulation caused a significant decrease in the number of growth cone filopodia (unstimulated mean, 8.6 ± 0.9, and stimulated mean, 5.2 ± 0.6, for 51 growth cones in six neurons; $P < 0.001$). The number of filopodia decreased continuously during the stimulation period. Other changes in growth cone morphology also occurred. Growth cones of unstimulated *Helisoma* neurons are characteristically broad, flat, phase-dark structures. In contrast, stimulated neurons had growth cones that were phase-bright with retracted lamellipodia that caused a decrease in the surface area of the growth cone (Fig. 3B). The changes in growth cone morphology produced by stimulation were reversed when stimulation ended. Growth cones resumed their growing morphology within 2 hours after stimulation stopped. Thus, action potentials generated in the cell body not only suppress neurite elongation but also change growth cone structure.

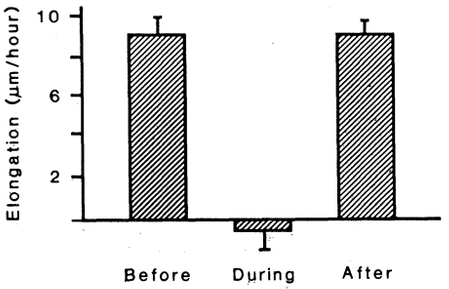

Fig. 2. Mean rates of growth cone advance before, during, and after stimulation with extracellular patch pipettes. During the stimulation period the rate of elongation was significantly less than that for the other periods (see text).

None of the above changes were observed in parallel control experiments in which a patch pipette (i) remained in contact with the soma for a period of time equal to observation periods used above but current pulses were not applied (*15*) or (ii) was situated directly above but not touching the soma and current pulses passed at two to three times the amplitude (10 nA) used above to test for possible field effects (12 growth cones). Furthermore, we verified that the patch of soma membrane through which stimulus pulses were applied had not ruptured during our experiments by observing the capacitance from pulse transients under voltage-clamp conditions at the end of each experiment. In all cases only the small amount of capacitance associated with the pipette was evident. Capacitance was greatly increased when additional suction was applied to the pipette to break the membrane patch.

The experiments described above were repeated with a second method of stimulation and recording (*16*) in which whole-cell current-clamp conditions were obtained by rupturing the membrane patch (*17*). Under these conditions, we again found that growth cone advance in neuron B19 was significantly inhibited during the generation of soma action potentials just as was shown for extracellular stimulation. Growth rates decreased from 12.5 ± 1.1 μm/hour before stimulation to 4.7 ± 1.4 μm/hour after stimulation ($P < 0.002$; 18 growth cones). The whole-cell clamp method also allowed us to use other identified neurons that could not be stimulated with an extracellular patch pipette because the membrane patch ruptured when currents sufficient to evoke action potentials were delivered. We used the whole-cell stimulation protocol with a different identified neuron, B5, and found that

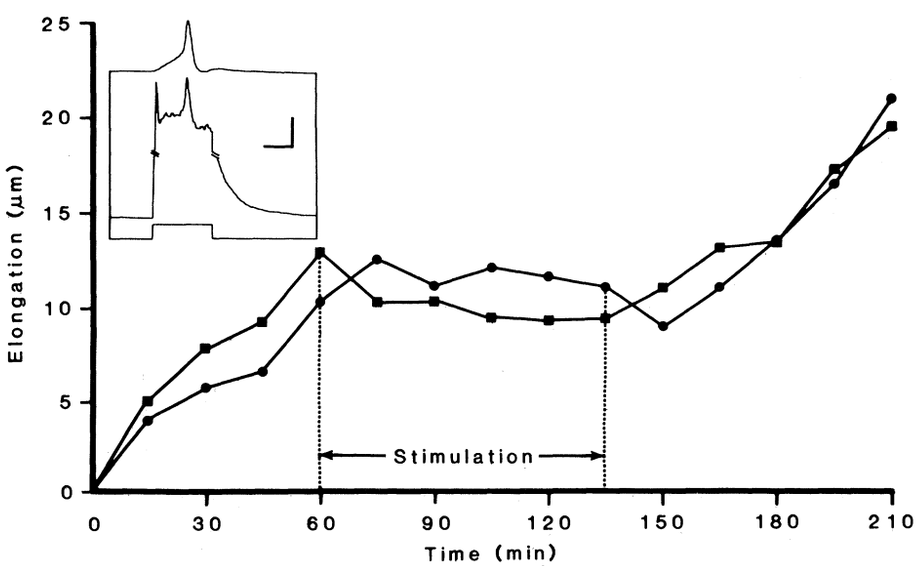

Fig. 1. The effect of action potentials on growth cone advance. Inset: control experiment to determine stimulus parameters. An extracellular patch pipette through which stimulus pulses were applied under current-clamp conditions was sealed to the soma of neuron B19, and an intracellular microelectrode (top trace) was used to monitor membrane potential. A 20-msec current pulse (bottom trace; amplitude 1.0 nA in this example) through the extracellular pipette (voltage recording on middle trace) caused a small depolarization that evoked an action potential in the soma, as recorded by both electrodes. Calibration: 45 mV, 10 msec. Generation of action potentials (with only the patch pipette present) reversibly suppressed growth cone advance. During the first hour (control period), no pulses were passed through the patch pipette, and neurite elongation proceeded at a constant rate (8.7 μm/hour). Action potentials evoked by current pulses during the second period stopped neurite elongation. Pulses were turned off during the third period and neurite elongation resumed. Data from two growth cones are shown.

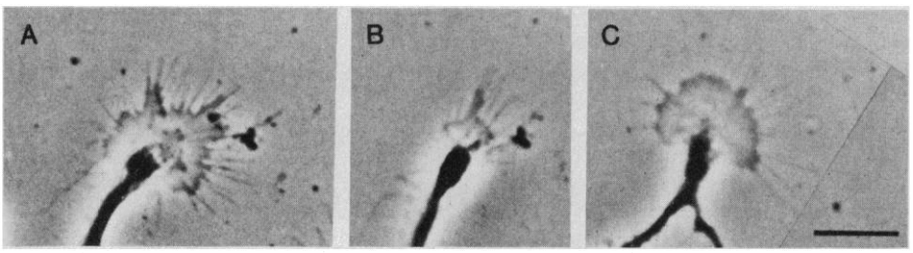

Fig. 3. Stimulus-evoked changes in growth cone morphology occur concomitantly with changes in neurite elongation. (A) Before stimulation, a growing growth cone has a characteristic broad, flat lamellipodium from which 27 filopodia project. (B) After 1 hour of stimulation of the soma, the lamellipodium has retracted and the number of filopodia has decreased to 8. (C) Several hours after stimulation has ended, the growth cone has resumed its normal growing morphology. Scale bar, 20 μm.

growth cone advance in this neuron was also inhibited during the stimulation period. Before stimulation the growth rate was 20.4 ± 1.4 μm/hour, whereas after stimulation the rate dropped to 2.3 ± 1.7 μm/hour ($P < 0.001$; 11 growth cones). The effects of action potentials on neurite elongation in neurons B19 and B5 when the whole-cell current-clamp method was used were indistinguishable from those obtained with the extracellular patch pipette. Thus, action potential generation suppresses neurite elongation in different identified neurons. A recent report now demonstrates what we interpret as the inverse of the present studies. Tetrodotoxin blockade of action potentials in tadpole retinal ganglion cells increased arborization at the terminals (*18*). Therefore, electrical activity must normally suppress outgrowth, just as we have shown.

It is important to consider our findings within the context of normal activity levels. Within the buccal ganglion, the average action potential frequency in B19 varied from 4 to 6 per second; peak rates exceeded 25 per second during single bursts of activity. In contrast, in the above experiments the frequency of spontaneous activity in neuron B19 isolated from its synaptic partners in cell culture was very low (mean, 0.2 ± 0.2 per second for 17 neurons). Although we did not extensively investigate variations in the stimulus parameters, extracellular stimulation at 2 per second also inhibited elongation. Thus, the changes in neurite outgrowth induced by the evoked activity (4 per second) occurred within physiologically relevant limits.

A number of plausible mechanisms can be suggested to account for the regulation of growth cone behavior by action potentials. Action potentials may act at remote sites, such as the soma or neurite. Alternatively, action potentials may propagate to the growth cone (*19*) and have local actions on motility. This possibility is supported by the observation that isolated growth cones themselves can morphologically respond to perturbation of their environment (*20*). The effects of action potential activity might be caused by changes in intracellular ion concentrations, perhaps in a fashion analogous to the mechanism of release of neural transmitters. This interpretation is reinforced by our recent findings that the generation of action potentials results in large calcium influxes in the growth cone, just as expected at the presynaptic terminal (*21*). In addition to the effect of action potentials it is also possible that more subtle changes in membrane potential may have effects (*22*). Diffusion, axoplasmic transport, and even progressive depolarization of the cell body, while undoubtedly important in other contexts, are all unlikely to be relevant here, given the time course of the response to action potentials and the considerable distance between the site of stimulation and the location of the growth cones (often greater than 250 μm).

An implication of our results is that electrical activity in neural circuits may prevent further neurite outgrowth from synaptic partners. Accordingly, electrical interactions between neurons during the formation of connections may inhibit outgrowth and thus influence the consolidation of functional circuitry.

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10. A small incision was made in the sheath of trypsin-treated buccal ganglia. Individual neurons were removed with a suction pipette, plated onto polylysine-coated glass cover slips attached to the bottom of 35-mm petri dishes and cultured in conditioned medium [P. G. Haydon, C. S. Cohan, D. P. McCobb, H. R. Miller, S. B. Kater, *J. Neurosci. Res.* **12**, 135 (1985); R. G. Wong, R. D. Hadley, S. B. Kater, G. C. Hauser, *J. Neurosci.* **1**, 1008 (1981)].
11. Individual neurons and their growth cones were monitored during each experiment. Neurons were viewed with phase-contrast optics and illuminated only during photography. Photographs were taken at 15-minute intervals; the negatives were projected onto a screen and the position of each growth cone was marked for each frame.
12. Our motivation in using extracellular pipettes was to disturb the cell interior as little as possible. A similar method has been shown to keep the intracellular environment unchanged [M. Lindau *et al.*, *Soc. Neurosci. Abstr.* **11**, 953 (1985)]. Patch pipettes had tip diameters of about 4 μm and input resistances of 3 megohms. Pipette solution contained 65 m*M* NaCl, 1 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, and 5 m*M* Hepes; the *p*H was adjusted to 7.4 with NaOH.
13. Calculations showed that 1/20 or less of the steady-state pipette current actually crossed the patch to enter the cell; the rest of the current passed through the seal. Cell input resistance measured with the intracellular microelectrode had values in the range of 257 megohms. A 10-mV intracellular depolarization caused by a 1-nA pulse indicated that 40 pA of current must have entered the cell through the patch. Capacitive current also may have helped to depolarize the cell.
14. All data are cited as means ± SEM. Statistical comparisons were made with the *t* test for matched-pair data, which minimizes the effect of sample variability by comparing the same growth cones before and after stimulation.
15. In control experiments with the patch pipette in contact with the neuron but no pulses applied, the rates of growth cone advance for the first hour of observation (mean, 17.0 ± 1.1 μm/hour) were not significantly different from rates for the second hour of observation (mean, 15.5 ± 1.1 μm/hour, $P > 0.1$ for 38 growth cones). In addition, in 3-hour control experiments during which no stimulus pulses were delivered through the attached extracellular pipette, no inhibition of growth cone advance was observed (12 growth cones).
16. Although the membrane patch was tested to ensure that it was intact at the beginning and end of each experiment, the unlikely possibility existed that the passage of current through the patch during extracellular stimulation transiently damaged the membrane patch (that is, caused it to be leaky).
17. Although whole-cell clamp provided an important additional control it had the disadvantage that the contents of the pipette could perfuse the cell. However, the extracellular and whole-cell stimulation methods together complemented each other as a means of confirming the validity of our results. Pipette solution for whole-cell current clamp contained 35 m*M* KCl, 5 m*M* $MgCl_2$, 5 m*M* EGTA, and 5 m*M* Hepes; the *p*H was adjusted to 7.3 with KOH.
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21. Using the dye Fura-2 and a charge-coupled device (CCD) video camera, C. S. Cohan, J. A. Connor, and S. B. Kater (in preparation) have found large, local influxes in calcium at the growth cone in response to the generation of action potentials.
22. Subthreshold pulses applied in whole-cell current-clamp conditions tended to slow growth cone advance but much less than suprathreshold pulses applied to the same growth cones (nine growth cones).

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