References and Notes

1. R. Doll and R. J. Peto, *J. Natl. Cancer Inst.* **66**, 1193 (1981).
2. W. R. Bruce, A. T. Varghese, R. Furrer, P. C. Land, in *Origins of Human Cancer*, H. N. Hiatt, J. D. Watson, J. A. Winstein, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1977), pp. 1641–1644.
3. I. Gupta *et al.*, *Biochemistry* **22**, 241 (1983).
4. W. R. Bruce *et al.*, *Naturwissenschaften* **69**, 557 (1982).
5. N. Hirai, D. G. I. Kingston, R. L. van Tassell, T. D. Wilkins, *J. Am. Chem. Soc.* **104**, 6149 (1982).
6. T. D. Sorensen, *ibid.* **87**, 5075 (1965).
7. D. Bethell and V. Gold, *Carbonium Ions* (Academic Press, New York, 1967), pp. 126–128.
8. E. C. Miller and J. A. Miller, in *Chemical Carcinogens*, C. E. Searle, Ed. (American Chemical Society, Washington, D.C., 1976), pp. 736–762.
9. J. McCann and B. M. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 950 (1976).
10. P. Magnus and G. Roy, *J. Chem. Soc. Chem. Commun.* (1979), p. 822.
11. W. P. Weber, *Silicon Reagents for Organic Synthesis* (Springer-Verlag, Berlin, 1983), p. 58.
12. J. L. Speier, *J. Am. Chem. Soc.* **70**, 4142 (1948).
13. ———, B. F. Daubert, R. R. McGregor, *ibid.*, p. 1117.
14. I. Gupta *et al.*, *J. Org. Chem.*, in preparation. An appropriate analog of compound **8**, that is, $CH_2(OR)CH(OR)CH_2OCH_2Si(CH_3)_3$, could not be made directly from $CH_2(OR)CH(OR)CH_2OH$ and halomethyltrimethylsilane because of Brook rearrangement; silicon-protected $CH_2(OR)CH(OR)CH_2OCH_2SnBu_3$ was used instead [W. C. Still, *J. Am. Chem. Soc.* **100**, 1481 (1978); D. Seyferth and S. B. Andrews, *J. Organomet. Chem.* **30**, 151 (1971)].
15. W. E. Steinmetz *et al.*, *J. Phys. Chem.* **83**, 1540 (1979).
16. K. L. D'Amico, C. Manos, R. L. Christensen, *J. Am. Chem. Soc.* **102**, 1777 (1980).
17. The structures of the intermediates and the final products were confirmed spectroscopically (I. Gupta *et al.*, *J. Org. Chem.*, in preparation).
18. F. Paltauf, in *Ether Lipids*, H. K. Mangold and F. Paltauf, Eds. (Academic Press, New York, 1983), pp. 49–84.

* To whom requests for reprints should be sent.

30 January 1984; accepted 16 April 1984

Voltage-Dependent Sodium Channels in an Invertebrate Striated Muscle

Abstract. *Striated skeletal muscles from the planktonic arrowworm* Sagitta elegans *(phylum Chaetognatha) were voltage-clamped. The muscles displayed classical voltage-dependent sodium channels that (i) showed peak transient currents when the membrane was depolarized 90 millivolts from rest, (ii) opened rapidly with peak currents flowing within 0.4 milliseconds at 4°C, (iii) showed voltage-dependent inactivation with 50 percent inactivation at +25 millivolts from rest, and (iv) were blocked by 500 nanomolar tetrodotoxin.*

Many cells, notably nerves and muscles, are termed excitable because they generate and propagate action potentials. Most invertebrate axons (*1*) as well as vertebrate axons (*2*) and skeletal muscles (*3*) rely on sodium ions as the predominant inward charge carrier during depolarization. In contrast, invertebrate muscles investigated so far seem to use calcium ions to generate action potentials (*4, 5*). Voltage-dependent calcium channels seem to be more evolutionarily primitive than sodium channels in that they are present in unicellular organisms (*6*) and are the first channels to appear during the embryonic development of excitable cells (*7*). At some point during evolution there must have been selective pressures favoring the expression of sodium channels not only in neurons but also in muscle membranes as well. Because of the large size of their skeletal muscles, most invertebrate preparations examined to date are protostomates (annelids, arthropods, or mollusks), which are not considered to have evolved along the same path as that giving rise to the chordates (*8*). Therefore, using a new patch-clamp technique that allows voltage clamping of small cells, we have examined the ionic basis of the action potential in the muscles of a primitive deuterostome, the arrowworm *Sagitta elegans* (phylum Chaetognatha) (*9*). We now report that in this species, sodium constitutes the predominant inward charge carrier during depolarization.

Groups of five to ten intact *Sagitta* (*10*) were placed in seawater or artificial seawater (ASW) and shocked with bipolar extracellular electrodes, which always elicited vigorous twitches. Removing calcium from the ASW did not prevent the animals from twitching in response to electrical stimulation during the 20-minute test period. In contrast, removing the sodium resulted in paralysis within 60 seconds. Animals remained flaccid and unresponsive to electrical stimulation until sodium was returned, at which point they regained the ability to swim. Addition of the specific sodium channel blocker tetrodotoxin (500 n*M* TTX) (*11*) to the medium resulted in rapid paralysis. These data suggest that sodium, but possibly not calcium, is required in the normal chain of neuromuscular events that ultimately lead to contraction.

We next examined the electrophysiological properties of the longitudinal muscles (*12*). The shape and kinetics of an action potential often reflects the ionic mechanisms underlying its generation. Figure 1 shows examples of action potentials obtained from a frog (*Rana temporaria*), an insect (*Manduca sexta* Lepidoptera), and *Sagitta*. The insect action potential (Fig. 1A) is mediated by calcium (*5*), as is suggested by the slower time course and broader spike than the sodium-generated action potential of the frog (Fig. 1B). The action potential recorded from *Sagitta* (Fig. 1C) both rises and falls rapidly and lacks the characteristic calcium plateau on the falling phase. The shape of the *Sagitta* action potential suggests that calcium is not the predominant inward charge carrier.

To better examine the ionic mechanisms underlying the action potential in *Sagitta*, the muscle membrane was voltage-clamped (*13–15*). Figure 2A shows typical current responses seen when the membrane was depolarized by 10 to 120 mV in steps of 10 mV. There was a fast inward current which inactivated and a slower outward current, both of which were voltage-dependent. The fast kinetics and apparent inactivation of the inward current resemble those of TTX-sensitive sodium channels observed in other cells (*16*). Inactivation was examined in more detail by measuring the fractional amplitude of the inward cur-

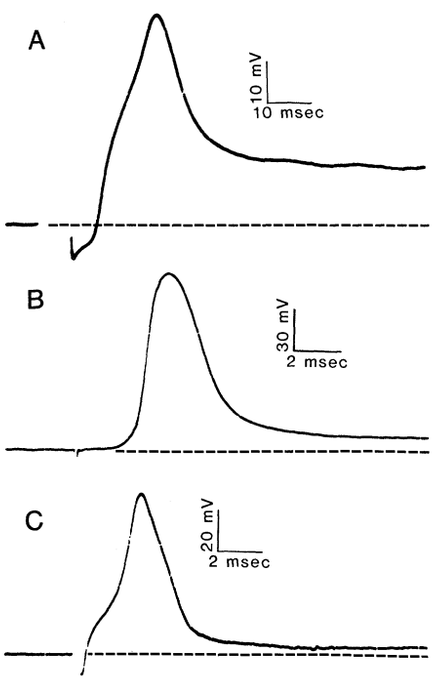


Fig. 1. Comparison of (A) insect (*Manduca sexta*), (B) frog (*Rana temporaria*), and (C) chaetognath (*Sagitta elegans*) action potentials. The insect action potential is generated by an influx of calcium (*5*), whereas that of the frog is due to the entry of sodium (*3*). The shape and kinetics of the *Sagitta* action potential are similar to that of frog. (A) The falling phase of the action potential took approximately 500 msec to return to baseline. The dashed line represents a resting potential for this fiber of −50 mV. (B) The dashed line represents a resting potential for this fiber of −90 mV. (C) The dashed line represents a resting potential for this fiber of −72 mV.

rent following 40-msec depolarizing pulses of various amplitudes (Fig. 2B). The inward current showed a rapid voltage-dependent inactivation characteristic of sodium channels. Further evidence identifying this channel as a sodium channel is given in Fig. 2C, where the inward current has been greatly diminished by exposing the muscle to 500-n*M* TTX. Attempts to analyze membrane responses in salines with cation substitution, such as $tris^+$ for Na^+ and Ba^{2+} for Ca^{2+}, were unsuccessful because we were unable to obtain adequate seals between the membrane and patch electrode.

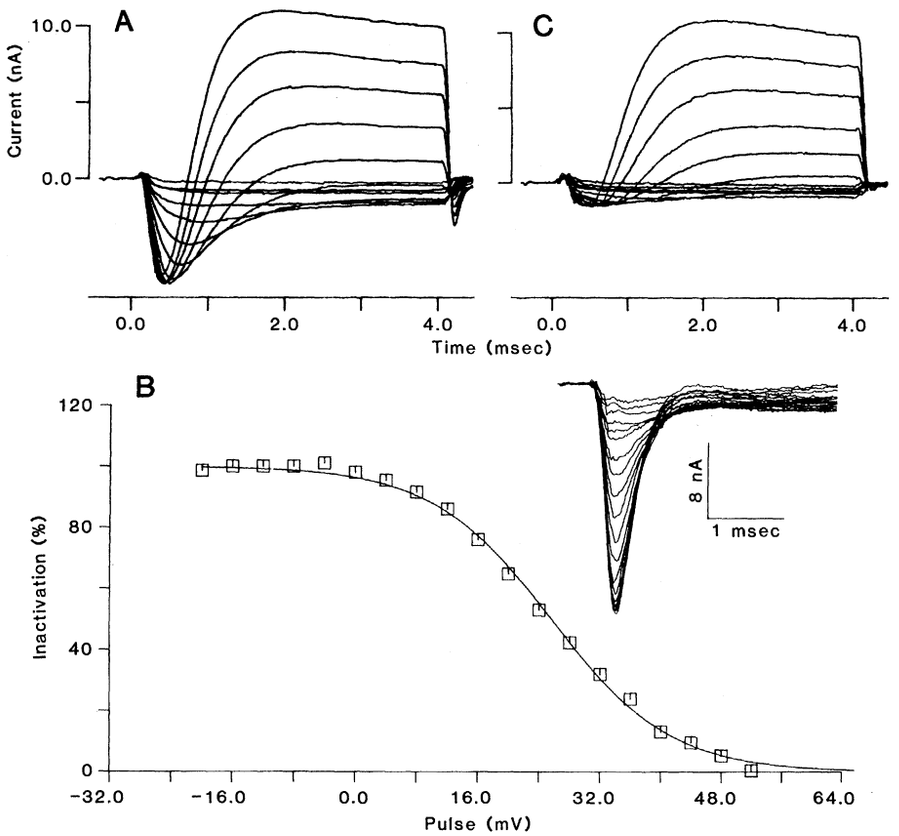
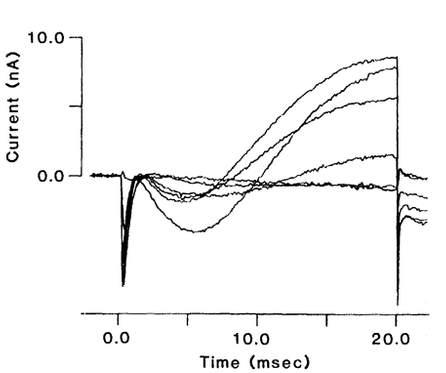


Fig. 2. Voltage-clamp analysis of *Sagitta* muscle. Parts A and C were obtained with the same electrode and membrane patch. All records begin at the holding potential. (A) The current flowing through a patch of membrane depolarized from 10 to 120 mV in steps of 10 mV. Each trace represents an average of five repetitions. (B) An analysis of inactivation of the inward current produced by 40-msec conditioning pulses from 0 to 80 mV in steps of 4 mV. Each point represents the average of ten repetitions. The line is the least-squares fit to the data points according to the equation $[1 + \exp\text{-}(V_{1/2} - V)/a)]^{-1}$ with $V_{1/2} = +25$ mV and $a = 7.9$ mV. The inset shows the actual current records during a 4-msec test pulse. At 4°C, there was a voltage-dependent inward conductance that failed to inactivate; in parts A and C it is shown as a pedestal. Because this pedestal disappeared at higher temperatures, this record was taken at 12°C to more accurately examine the inactivation of the presumptive sodium channels. The resting potential of *Sagitta* muscle was -66.7 ± 1.1 mV ($n = 14$). (C) The effects of 500 n*M* TTX on current responses to depolarization of *Sagitta* muscle. The patch and stimulus are identical to those of part A. TTX abolished most of the inward current.

Fig. 3. The current responses of *Sagitta* muscle membrane to long steps of depolarization at elevated temperature. A membrane patch with prominent inward currents was held at 15°C and 20 msec depolarizing steps were applied from 30 to 130 mV in steps of 20 mV. Each trace represents the average of six repetitions. The records have been corrected for leakage and capacitive artifacts. The fast inward current was sensitive to TTX, whereas the second inward current was reduced by cobalt. The delayed outward current was diminished by 4-aminopyridine. These data suggest that the currents flow through sodium, calcium, and potassium channels, respectively.



The outward current, which repolarizes the muscle, is probably carried by K^+. Its kinetics and voltage dependence suggest that it flows through channels of the delayed rectifier type (*16*). Exposing the preparation to 5 m*M* 4-aminopyridine rapidly decreased the flow of outward current (not shown) as has been observed in other preparations (*17*).

The outward current did not remain constant during depolarization but declined slightly (Fig. 2, A and C). This feature was seen in many of the patches examined and suggests potassium channel inactivation, external potassium accumulation, or the development of a second, slower inward current. In *Sagitta* muscle, as has been seen in frog (*15*), the absolute and relative densities of sodium and potassium currents vary from one membrane patch to another. In addition, in some patches a second, slower inward current was observed with depolarizing steps of longer duration (20 msec) at higher temperatures (15°C) (Fig. 3). The kinetics, direction, and voltage dependence of this inward current suggest a Ca^{2+} conductance. Furthermore, the current was insensitive to 500-n*M* TXX but greatly reduced in the presence of 10 m*M* Co^{2+} (data not shown). When this channel was blocked with cobalt ions, the decline in the potassium current was greatly reduced.

These data suggest that *Sagitta* muscle, like that of chordates, uses TTX-sensitive sodium channels as the predominant mechanism for depolarization and potassium channels for repolarization. In both *Sagitta* and vertebrate muscle (*18*) there is also a delayed inward Ca^{2+} flux, whose role is less well defined. The use of sodium to generate the action potential in chaetognaths contrasts with other invertebrate muscles examined, in which calcium is the predominant carrier of inward current (*4, 5*). Another possible exception is the observation that some long-term denervated crayfish muscle fibers develop TTX-sensitive action potentials (*19*).

In many cells calcium entry not only depolarizes the membrane, but also stimulates a cellular response such as secretion or contraction. Vertebrate cardiac and smooth muscles, as well as many invertebrate muscles, have a reduced sarcoplasmic reticulum. This feature obligates most of these cells to use extracellular calcium in the process of activating the contractile proteins for the generation of tension (*20*). All of these preparations therefore rely to some extent on voltage-dependent calcium channels.

The longitudinal muscles of the chaetognaths have a well-developed sarcoplasmic reticulum (*21*) and are therefore presumably not obligated to use extracellular calcium for contraction, as they have an internal source of the ion. They could use sodium rather than calcium currents to depolarize the muscle and trigger force generation mainly by release of calcium from the sarcoplasmic reticulum. One possible selective pressure that would favor the use of sodium rather than calcium currents would be the faster kinetics afforded by the use of the former, which would in turn allow faster muscle responses. This feature is of obvious value to predatory animals such as chaetognaths (*8, 9*). At this point one can only speculate about the evolution of sodium channels in muscle. It is possible that sodium action potentials are a trait of all deuterostome muscles that have a well developed sarcoplasmic reticulum. Alternatively, sodium channels could have evolved independently in the muscles of both sagitta and the chordates. With the development of the loose patch clamp, it will now be possible to examine the small muscles of other invertebrate phyla and perhaps provide some insight into this question.

LAWRENCE M. SCHWARTZ*
WALTER STÜHMER†
Department of Physiology and Biophysics, School of Medicine, University of Washington, Seattle 98195

References and Notes

1. A. L. Hodgkin and A. F. Huxley, *J. Physiol. (London)* **116**, 449 (1952).
2. A. F. Huxley and R. Stampfli, *ibid.* **112**, 496 (1951).
3. W. L. Nastuk and A. L. Hodgkin, *J. Cell. Comp. Physiol.* **35**, 39 (1950).
4. S. Hagiwara and L. Byerly, *Annu. Rev. Neurosci.* **4**, 69 (1981); S. Hagiwara and K. Naka, *J. Gen. Physiol.* **48**, 141 (1964); D. A. Weisblat, L. Byerly, R. L. Russell, *J. Comp. Physiol.* **111**, 93 (1976); Y. Kidokoro, S. Hagiwara, M. P. Henkart, *ibid.* **90**, 321 (1974).
5. F. M. Ashcroft and P. R. Stanfield, *J. Physiol. (London)* **323**, 93 (1982).
6. Y. Naitoh, R. Eckert, K. Friedman, *J. Exp. Biol.* **56**, 667 (1972).
7. P. I. Baccaglini and N. C. Spitzer, *J. Physiol. (London)* **271**, 93 (1977); C. S. Goodman and N. C. Spitzer, *Nature (London)* **280**, 208 (1979).
8. R. D. Barnes, *Invertebrate Zoology* (Saunders, Philadelphia, ed. 3, 1974).
9. L. H. Hyman, *The Invertebrates* (McGraw-Hill, New York, 1959), vol. 5, pp. 1–71.
10. *Sagitta elegans* were obtained from plankton tows at a depth of 200 m from Puget Sound and kept at 4°C. *Sagitta* are approximately 1.5 cm long and torpedo shaped, with a diameter of about 1.0 mm. Experiments were performed in artificial seawater (ASW): 460 m*M* NaCl, 6.7 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4$, 4.4 m*M* Na_2HPO_4, 10 m*M* Hepes buffer, *p*H 7.4. (Sodium-free ASW had the same composition except that tris-chloride replaced NaCl). Unless stated otherwise, all experiments were performed at 4° to 6°C.
11. J. W. Moore, M. P. Blaustein, N. C. Anderson, T. Narahashi, *J. Gen. Physiol.* **50**, 1401 (1967).
12. For electrophysiological examination, animals were opened mid-dorsally and the viscera were removed to expose the longitudinal muscles running the length of the body. These muscles have 2.4-μm repeating sarcomeres and a diameter of approximately 15 μm. These preparations were then mounted in a plexiglass chamber and flooded with ASW. Resting and action potentials were recorded with intracellular glass microelectrodes filled with 3*M* KCl and having resistances of 7 to 15 megohms. Action potentials from *Sagitta* were elicited with extracellular bipolar platinum electrodes. Action potentials were generated in pharate adult *Manduca* by stimulating the ventral nerve cord with bipolar platinum electrodes while recording from the lateral intersegmental muscle of the fifth abdominal segment. The composition of the saline was 35 m*M* KCl, 9.2 m*M* NaCl, 4.2 m*M* $CaCl_2$, 16 m*M* $MgCl_2$, 172 m*M* glucose, 2.5 m*M* phosphate buffer, *p*H 6.7. The action potential in the frog sartorius muscle was produced by stimulating the muscle directly with bipolar platinum electrodes. The saline consisted of: 115 m*M* NaCl, 2.5 m*M* KCl, 1.8 m*M* $CaCl_2$, 3 m*M* phosphate buffer, *p*H 7.25.
13. Voltage-clamp measurements were performed with the extracellular loose-patch clamp as described elsewhere (*15*). Briefly, glass capillaries with fire-polished tips approximately 10 μm in diameter were filled with saline and pressed against a fiber to electrically isolate a patch of the sarcolemma. Voltage steps were applied to the electrode, and the resulting current flow was measured and corrected for leakage and capacitive artifacts. A holding potential of −20 mV was applied to the patch. In some experiments, muscles were initially treated for 5 minutes at 0°C with 1 mg of type III collagenase (Sigma) (E.C. 3.4.24.3) per milliliter of ASW to disrupt the connective tissue over the muscle to achieve tighter seals between the electrode and membrane. The results obtained were identical to those of muscles without collagenase treatment.
14. W. Stühmer and W. Almers, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 946 (1982); W. Stühmer, W. Roberts, W. Almers, in *Single Channel Recording*, B. Sakmann and E. Neher, Eds. (Plenum, New York, 1983), pp. 123–132.
15. W. Almers, P. R. Stanfield, W. Stühmer, *J. Physiol. (London)* **336**, 261 (1983).
16. B. Hille, in *Handbook of Physiology*, section 1, vol. 1, E. R. Kandel, Ed. (Williams & Wilkins, Baltimore, 1977), pp. 99–136.
17. S. H. Thompson, *J. Physiol. (London)* **265**, 465 (1977).
18. J. A. Sanchez and E. Stefani, *ibid.* **283**, 197 (1978); W. Almers and P. T. Palade, *ibid.* **312**, 159 (1981).
19. J. Lehoueller, J. Cundras, J. Bruner, *Neurosci. Lett.* **37**, 227 (1983).
20. S. Suzuki and H. Sugi, in *The Role of Calcium in Biological Systems*, L. J. Anghiteri and A. M. Tuffet-Anghiteri, Eds. (CRC Press, Boca Raton, Fla., 1982), vol. 1, pp. 201–217.
21. M. Camatini and L. Lanzauvecchia, *Atti. Accad. Naz. Lincei Mem. Cl. Sci. Fis. Mat. Nat. Sez. 3a*, **41**, 392 (1966).
22. We thank W. Almers and B. Hille for both a critical reading of the manuscript and helpful comments throughout the course of this study. We also thank M. R. Landry, C. Green, and the Water Column Group for providing animals. L.M.S. held a fellowship from the American Heart Association of Washington. Supported by PHS grant AM17803 to W. Almers.

* Address after 15 August: Department of Biology, Wilson Hall, University of North Carolina, Chapel Hill 27514.
† Present address: Max-Planck-Institüt für Biophysikalische Chemie, D-3400 Göttingen-Nikolausberg, Am Fassberg, Federal Republic of Germany.

9 January 1984; accepted 13 March 1984

Increased Numbers of Thoracic Dorsal Root Axons in Rats Given Antibodies to Nerve Growth Factor

Abstract. *Sensory axons were counted in untreated 1-month-old rats and in littermates that were injected with antibodies to nerve growth factor. There were 45 percent more unmyelinated and 17 percent more myelinated axons in dorsal roots of the fifth thoracic spinal segment in treated rats. This suggests that the number of sensory axons can be changed by postnatal inactivation of nerve growth factor.*

Nerve growth factor (NGF) affects the growth and development of sensory and sympathetic neurons (*1, 2*). On the sensory side, this compound is necessary for the survival of dorsal root ganglion cells and the outgrowth of sensory processes in vitro (*3*). Furthermore, it has been suggested that NGF is transported from peripheral endings to appropriate neuronal cell bodies and that this serves as a signal that innervation is being maintained (*4*). In vivo, treatment with antibodies to NGF before birth reduces the number of primary sensory cells (*5*), but there is no demonstrable postnatal effect on these cells (*5, 6*). It was recently discovered, however, that postnatal manipulation of NGF levels changes the number of axons in a peripheral nerve (*7*) and in the dorsal root after spinal injury (*8, 9*). This suggests that sensory axons might be influenced by postnatal inactivation of NGF even if the number of sensory cells does not change.

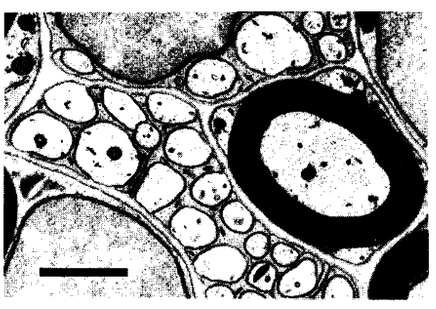


Fig. 1. Electron micrograph of a cross section of a dorsal root, showing a myelinated axon (right) surrounded by smaller, unmyelinated axons. Because of their distinctive morphology, myelinated and unmyelinated axons can readily be counted with the electron microscope. Scale bar, 1 μm.

Pregnant Sprague-Dawley rats were obtained from Texas Inbred Mouse Company. When the pups were born they were given daily injections (3 μl/g subcutaneously) of antibodies to NGF (anti-NGF; undiluted rabbit antiserum) at a site near the dorsal fat pad for 4 weeks. The anti-NGF was prepared against the purified mouse β-NGF subunit as described by Beck and Perez-Polo (*10*). In bioassays of embryonic chick sensory ganglia or of human neuroblastoma cells, a 1:500 dilution of the