change accompanying phosphorylation of Ser^{103} occurs at a distant, voltage-sensing, transmembrane region. Our finding that the greatest effect of PDD occurs at small depolarizations indicates that phosphorylation has a physiological function because K^+ conductance would be reduced at potentials close to rest.

The change in voltage sensitivity associated with phosphorylation strongly suggests that the protein is a channel subunit. This conclusion agrees with the finding that mutations in the hydrophobic domain can alter ion selectivity (*9*). Furthermore, the results of our site-directed mutagenesis support a structural model for the I_{sK} protein in which the NH_2-terminus is outside the cell, there is a single transmembrane domain, and the COOH-terminus is within the cell (Fig. 1). Thus, the alteration in the voltage dependence of I_{sK} observed in the mutation at Ser^{69} is consistent with that residue lying within the membrane, and the effects of the mutation at Ser^{103} show that this amino acid must be intracellular.

It remains uncertain which K^+ currents recorded from mammalian cells result from activation of this molecular species of the K^+ channel. Sensitivity to phorbol esters may be helpful in the identification of this channel as it occurs in mammalian cells.

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Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System

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Neurogenesis in the mammalian central nervous system is believed to end in the period just after birth; in the mouse striatum no new neurons are produced after the first few days after birth. In this study, cells isolated from the striatum of the adult mouse brain were induced to proliferate in vitro by epidermal growth factor. The proliferating cells initially expressed nestin, an intermediate filament found in neuroepithelial stem cells, and subsequently developed the morphology and antigenic properties of neurons and astrocytes. Newly generated cells with neuronal morphology were immunoreactive for γ-aminobutyric acid and substance P, two neurotransmitters of the adult striatum in vivo. Thus, cells of the adult mouse striatum have the capacity to divide and differentiate into neurons and astrocytes.

THE GENERATION OF NEURONS IN the mammalian central nervous system (CNS), with few exceptions, occurs during early development (*1*). Mitogenic growth factors, such as basic fibroblast growth factor (bFGF) and nerve growth factor (NGF), may participate in the production of CNS neurons (*2, 3*). Epidermal growth factor (EGF) is a powerful mitogen of numerous non-neuronal cells and enhances wound healing and tissue regeneration in various adult organs such as skin, liver, and intestinal epithelium (*4*). In the CNS, mitogenic and trophic actions of EGF on embryonic and early postnatal cells indicate its importance in neuronal development (*5*). The demonstration of EGF- and EGF receptor–immunoreactivity in the adult rodent and human CNS (*6*) prompted us to examine whether EGF-responsive cells could be isolated from the adult mouse CNS.

The striata of 3- to 18-month-old adult mice were enzymatically dissociated and plated in serum-free culture medium containing 20 ng of EGF per milliliter. Cells were seeded in 35-mm-diameter culture dishes (1000 viable cells per plate) in the absence of supplementary substrate or adhesion factors (*7*). After 2 days in vitro (DIV) most of the cells had died; however, 15 ± 2 cells per plate (n = 4 independent culture preparations; the striata of two adult mice were pooled in each of the four experiments) were undergoing cell division (Fig. 1A). Cell division continued for an additional 2 to 3 DIV (Fig. 1, B and C), after which the proliferating clusters of cells detached and formed (6 to 8 DIV) a sphere of proliferating cells (Fig. 1D). Cell division and proliferation were not observed in the absence of EGF, nor were they mimicked by bFGF (20 ng/ml), platelet-derived growth factor (20 ng/ml), or NGF (100 ng/ml). In addition, if cells were seeded on a substrate that had been treated with poly-L-ornithine, proliferation was not observed in the presence of EGF. These findings suggest that the presence of both EGF and a nonadhesive substrate is required to initiate cell division of these isolated adult striatal cells.

To assess the antigenic properties of cells within these 6- to 8-DIV spheres, we transferred them to poly-L-ornithine–coated cover slips, allowed them to adhere for 1 hour, and processed them for indirect immunocytochemistry (*8*). Virtually all cells in the spheres were immunoreactive for nestin (Fig. 1, E and F; n = 8 independent culture

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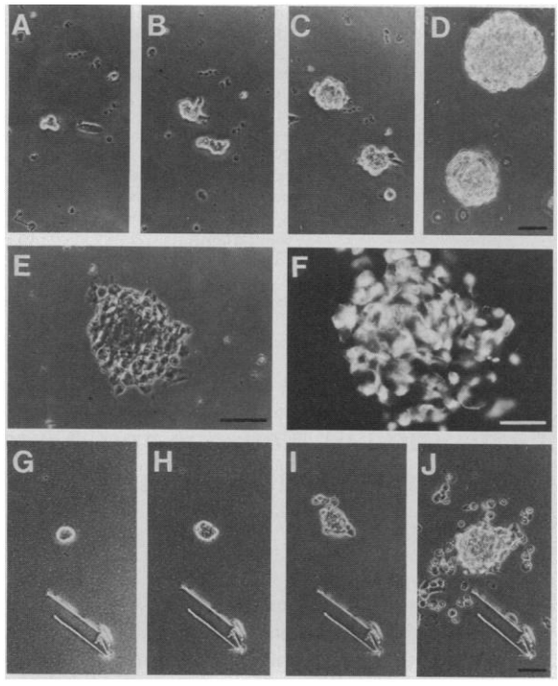

Fig. 1. EGF-induced proliferation of cells isolated from the adult mouse striatum. (**A**) After 2 DIV, cells that had undergone cell division were first observed. Cell division continued at 3 (**B**) and 4 (**C**) DIV, although dividing cells (beginning to form a cluster) migrated slowly across the substrate. (**D**) After 6 to 8 DIV, spheres of cells lifted off the substrate and floated in suspension. Line in substrate (A through C) serves to identify the field. (**E**) One hour after plating onto poly-L-ornithine, a 6-DIV sphere attached to the substrate. (**F**) The cells in (E) were immunostained with antibody to nestin; virtually all cells were immunoreactive for nestin. (**G** through **J**) Single cells, derived from dissociated 6- to 8-DIV spheres, were plated in single wells of a 96-well plate. A large, hypertrophic cell after 2 DIV (G) began to divide and form a cluster of cells during the subsequent 3 (H), 4 (I), and 6 (J) DIV. Scratches in substrate serve to identify the field. Scale bars: (A through D) bar in (D) denotes 50 μm; (E), 50 μm; (F), 25 μm; (G through J) bar in (J), 50 μm.

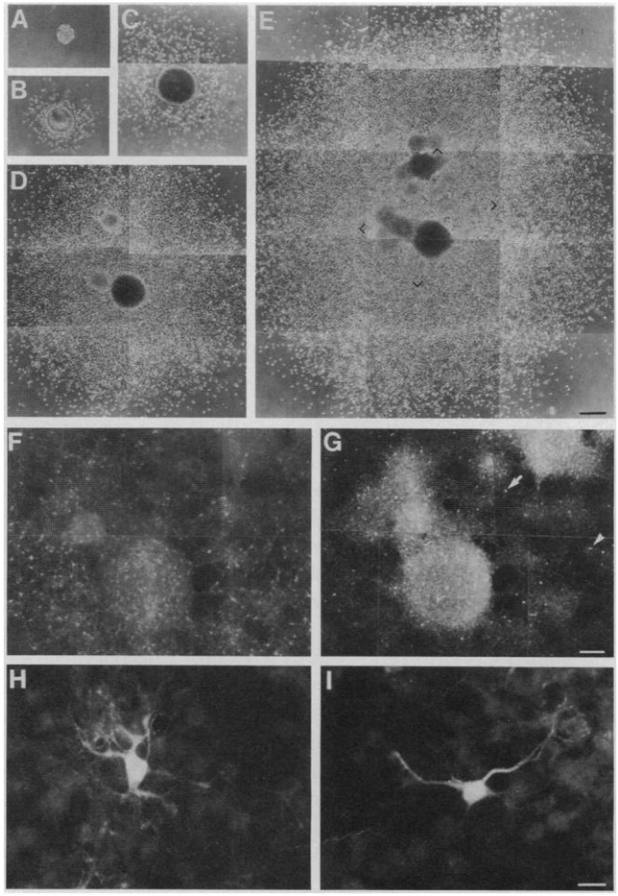

Fig. 2. NSE- and GFAP-positive cells generated from a single sphere of EGF-induced, undifferentiated adult striatal cells. (**A**) A single sphere immediately after plating. (**B** through **D**) The same sphere after 1 (B), 3 (C), and 14 (D) DIV, respectively. (**E**) After 21 DIV, the transferred sphere and associated cells were fixed and processed for dual-antigen immunocytochemistry. (**F** and **G**) Fluorescence micrographs of the portion of field delineated in (E), after dual-labeling of the same field with antibody to GFAP (F) and antiserum to NSE (G), respectively. Arrowhead and arrow indicate NSE-positive cells enlarged in (**H**) and (**I**), respectively. Scale bars: (A through E) bar in (E) denotes 250 μm; (F and G) bar in (G), 100 μm; (H and I) bar in (I), 10 μm. Single spheres (6 DIV), generated as described (7), were transferred with a pipette to poly-L-ornithine–coated glass cover slips in the base of 12-well culture dishes and cultured in serum-free medium containing EGF (which was replenished twice weekly thereafter). After 21 DIV, cover slips were processed for immunocytochemistry as described (*8*), with the following modifications. The primary antibodies (applied together) were a mouse monoclonal antibody to GFAP (1:100; Boehringer Mannheim) and rabbit antiserum to NSE (1:300; Dakopatts, Carpenteria, California), and the secondary antibodies (applied together) were fluorescein-conjugated goat antibody to mouse IgG (1:100; Jackson Immunochemicals) and rhodamine-conjugated goat antibody to rabbit IgG (1:100; Jackson Immunochemicals).

preparations), an intermediate filament found in neuroepithelial stem cells (*3, 9*). In contrast, cells in the 6- to 8-DIV spheres were not immunoreactive for neurofilament (168 kD), neuron-specific enolase (NSE), and glial fibrillary acidic protein (GFAP) (*10*). To determine whether cells within the 6- to 8-DIV spheres could continue to proliferate in secondary cultures, spheres were mechanically dissociated and replated as single cells in the wells of 96-well plates (*11*). In the presence of EGF, single cells proliferated and formed new spheres (Fig. 1, G through J); the majority of cells within these secondary spheres were also immunoreactive for nestin (*10*). When 200 to 250 of these cells were plated in a 35-mm dish, in the presence of EGF and in the absence of supplementary substrate or adhesion factors, 67 ± 4% (n = 3 independent culture preparations) of the cells formed new spheres. As above, if EGF was omitted from the serum-free culture medium, proliferation was not observed. In addition, when EGF was removed from the medium after proliferation had been initiated, no further proliferation was observed. These findings suggest that in vitro conditions may be established for the continual proliferation of undifferentiated cells originally derived from the adult mammalian CNS.

We next examined whether, given a suitable substrate, cells generated from EGF-induced spheres would develop the morphological and antigenic properties of the principal cell types of the CNS. Single 6- to 8-DIV spheres were transferred with micropipettes to poly-L-ornithine–coated glass cover slips (in 12-well plates) and cultured in the continued presence of EGF-containing, serum-free medium (Fig. 2A). During the subsequent 21 DIV, cells were observed migrating from the sphere, which continued to proliferate in the presence of EGF (Fig. 2, B through E). After 21 DIV, the proliferating sphere and cells that had migrated from the core were processed for dual-antigen, indirect immunocytochemistry; both GFAP- (Fig. 2F) and NSE- (Fig. 2G) immunoreactive cells were present. These findings were reproduced in eight independent culture preparations. Incorporation of bromodeoxyuridine (1 μM, applied between 10 and 13 DIV) into NSE-immunoreactive cells (Fig. 3, A and B) supports our conclusion that these cells were born during the culture period. The EGF-induced pattern of proliferation precluded an accurate count of the total number of cells produced. Nevertheless, in the GFAP plus NSE dual-labeling experiments, 61 ± 7% of the labeled cells were immunoreactive for GFAP, whereas 39 ± 5% were immunoreactive for NSE (as assessed by counting 827 immunoreactive

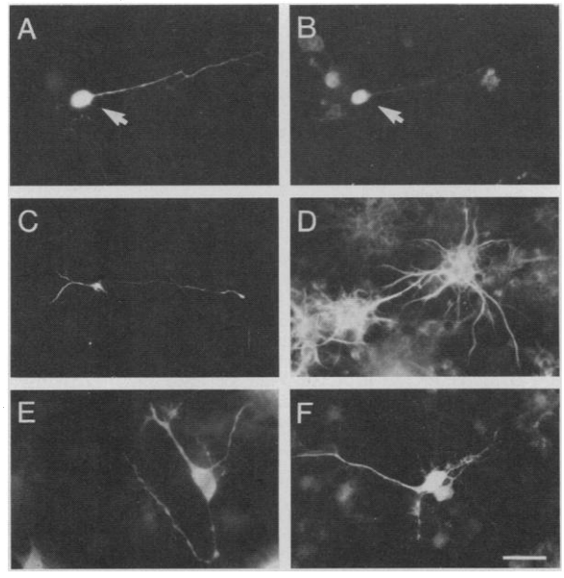

Fig. 3. Morphology and phenotype of cells generated by EGF-induced proliferation of undifferentiated adult striatal cells. Fluorescence photomicrographs of cells in cultures of single, EGF-generated spheres after 21 DIV on poly-L-ornithine. (**A**) An NSE-immunoreactive cell (arrowhead) that had incorporated bromodeoxyuridine (**B**), the distinct morphology of neurofilament (168 kD)-immunoreactive (**C**) and GFAP-immunoreactive (**D**) cells, and cells with neuronal morphology that were immunoreactive for γ-aminobutyric acid (**E**) and substance P (**F**). Bar, 20 μm. Culture and immunocytochemistry of single spheres were carried out as described (*7, 8*) with the following modifications. Primary antibodies used included mouse monoclonal antibody to bromodeoxyuridine (undiluted; Amersham), monoclonal antibody to neurofilament (168 kD) antibody (1:50; clone RMO 270), rabbit antiserum to γ-aminobutyric acid (1:3000; Incstar, Stillwater, Minnesota), or rabbit antiserum to substance P (1:100; Incstar).

cells in four independent culture preparations). The majority of cells not immunoreactive for GFAP or NSE were immunoreactive for nestin (*10*).

In EGF-generated cultures, the NSE-immunoreactive cells generally had small, rounded somas with fine processes (Figs. 2, H and I, and 3A). In contrast, GFAP-immunoreactive cells were stellate, with large somas and several thick processes (Fig. 3D). Cells with rounded soma and long, thin processes also were immunoreactive for neurofilament (168 kD) (Fig. 3C). To determine whether these cells contained CNS neurotransmitters, we tested for the presence of amino acids, biogenic amines, and neuropeptides with indirect immunocytochemistry. Cells with neuronal morphology that were immunoreactive for γ-aminobutyric acid (Fig. 3E) and substance P (Fig. 3F) were present throughout 21-DIV cultures of spheres and associated cells (n = 3 independent culture preparations); these are two of the major neurotransmitters of the adult striatum in vivo (*12*). In contrast, these cultures did not contain cells that were immunoreactive for glutamate, serotonin, tyrosine hydroxylase, methionine-enkephalin, neuropeptide Y, or somatostatin (*13*), the latter three being present in the adult striatum. The reason for the restricted expression of phenotypes is unknown, but it is possible (i) that EGF-responsive progenitor cells are limited to produce only γ-aminobutyric acid– and substance P–containing cells or (ii) that other phenotypes may appear at different times or under different culture conditions.

Our results demonstrate that EGF induces the proliferation of a small number of cells, isolated from the striatum of the adult mouse brain, that produce clusters of cells with antigenic properties of neuroepithelial stem cells. Under appropriate conditions these cells can be induced to differentiate into astrocytes and neurons with phenotypes characteristic of the adult striatum in vivo. We have recently isolated an EGF-responsive, multipotent stem cell from the embryonic striatum that exhibits a pattern of proliferation and differentiation that is indistinguishable from that described in this study (*10*). Taken together, these findings suggest that a population of embryonic stem cells may survive in the adult brain in a dormant, nonproliferative state. Alternatively, these cells may be similar to a population of subependymal cells in the dorsolateral region of the lateral ventricle that proliferates in the adult mouse, although the fate of half of the progeny is death (*14*). Neurogenesis in the mouse striatum is complete after the first few postnatal days (*15*). The inability of these stem cells to continue to proliferate in vivo may be due to inhibitory influences of the adult mammalian CNS. Inhibition of initial EGF-induced proliferation on plates treated with poly-L-ornithine suggests that the substrate may be one of these inhibitory influences.

Although neuroepithelial stem cells may be manipulated in vitro after isolation from fetal brain tissue (*16*), our findings indicate that the adult brain may serve as an alternative source. The ability to induce EGF-responsive stem cells to proliferate in suspension in vitro, and to reinitiate proliferation in a large percentage of the progeny, can provide a plentiful source of undifferentiated CNS cells from the adult for studies of their basic properties and for use in experimental models of autologous or heterologous CNS transplantation. Identification of factors that induce or inhibit the in situ proliferation and differentiation of these cells may allow for their eventual manipulation in the intact adult mammalian CNS to replace cells lost to injury or disease.

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7. Dissociation of adult mouse striata was carried out according to the method of Kay and Wong (*17*) with the following modifications. Mouse striata [female, pathogen-free CD1 (CF1 and CFW strains yielded identical results), albino, 3 to 18 months old; Charles River, St. Constant, Québec] were dissected and hand-cut with scissors into 1-mm coronal sections and transferred into artificial cerebrospinal fluid (aCSF) that contained 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.35, ~280 mosmol) and was aerated with 95% O_2–5% CO_2 at room temperature. After 15 min the tissue sections were transferred to a spinner flask (Bellco Glass) with a magnetic stirrer filled with low-Ca^{2+} aCSF that contained 124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.35, ~280 mosmol), was aerated with 95% O_2–5% CO_2 at 32° to 35°C, and contained 1.33 mg/ml of trypsin [9000 BAEF (benzoyl-L-arginine ethyl ester) units per milligram], 0.67 mg/ml of hyaluronidase (2000 unit/mg), and 0.2 mg/ml of kynurenic acid. After 90 min, tissue sections were transferred to normal aCSF for 5 min before trituration. Tissue was transferred to DMEM/F-12 (1:1, Gibco) medium containing 0.7 mg/ml ovomucoid (Sigma) and was triturated mechanically with a fire-narrowed Pasteur pipette. Cells were plated (1000 viable cells per plate) in noncoated 35-mm culture dishes (Costar) containing serum-free medium (*18*) and EGF (20 ng/ml) [purified from mouse submaxillary gland (Collaborative Research) or human recombinant EGF (Gibco/BRL)].
8. After 6 DIV, floating spheres were transferred with wide-bore pipettes onto poly-L-ornithine (15 μg/ml) (Sigma) –coated glass cover slips in 24-well culture dishes. After 1 hour, the medium was replaced with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, followed by three 10-min rinses in PBS. Attached spheres were incubated with a rabbit polyclonal antiserum directed against nestin (Rat 401). Cover slips were incubated in antibody to nestin (1:1500 in PBS/0.3% triton-X-100/10% normal goat serum) for 2 hours at 37°C. Cover slips were rinsed three times (10 min) with PBS and incubated with rhodamine-conjugated goat antibody to rabbit immunoglobulin G (IgG) (1:100; Jackson Immunochemicals) for 30 min at room temperature. After three final washes in PBS, coverslips were mounted with Fluorsave (Calbio-

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Evidence for a Computational Distinction Between Proximal and Distal Neuronal Inhibition

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Most neurons have inhibitory synapses both "proximally" near the spike-initiating zone and "distally" on dendrites. Although distal inhibition is thought to be an adaptation for selective inhibition of particular dendritic branches, another important distinction exists between proximal and distal inhibition. Proximal inhibition can attenuate excitatory input absolutely so that no amount of excitation causes firing. Distal inhibition, however, inhibits relatively; any amount of it can be overcome by sufficient excitation. These properties are used as predicted in the circuit-mediating crayfish escape behavior. Many neuronal computations require relative inhibition. This could partly account for the ubiquity of distal inhibition.

MOST CENTRAL NEURONS RECEIVE inhibitory inputs on their basal dendrites or somata, where they are well situated to shunt distally generated excitatory postsynaptic potentials (EPSPs) before they spread to the axonal spike initiating zone. Such "proximal inhibition" was discovered first, but it is now known that distal dendrites also commonly receive extensive inhibitory as well as excitatory inputs (*1*). A reason often proposed for such "distal inhibition" is that it would allow for selective inhibition of particular dendritic branches (*2*). While studying inhibitory control of the lateral giant (LG) command neurons for crayfish tail-flip escape behavior, we have come to appreciate a different and perhaps more generally important reason for distal inhibition.

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Two kinds of suppression of LG escapes are known: (i) Escape cannot be initiated while an escape response is in progress. The responsible suppressive influence is called recurrent inhibition (*3*). Because initiating a new response while another is in progress would be maladaptive, recurrent inhibition should be absolute: No matter how strong the provocation, the inhibition should prevent a response. (ii) The likelihood of escape is reduced when crayfish are feeding or are restrained so that escape would be ineffectual (*4*). The responsible process, tonic inhibition, is strategic in nature: Although ignoring a modest threat to continue feeding may be adaptive, sufficient provocation should still cause escape. Thus, it should be possible to override tonic inhibition.

Both recurrent and tonic inhibition are known to be directed to the LG neuron itself and thus might be produced by the same inhibitory neurons. However, a consideration of the way excitation and inhibition interact suggests that proximal inhibition best confers the absolute suppression required for recurrent inhibition, whereas distal inhibition can more readily be overcome by strong excitation, as required for tonic inhibition.

The reason is illustrated in Fig. 1, which shows predictions from a steady-state, two-compartment model of LG (equivalent circuit insets). Attenuation of EPSPs from distal to proximal compartment was by a factor of 10, consistent with known physiology and anatomy. The top curve in Fig. 1, A1 and A2, shows the depolarization produced by excitation in the absence of inhibition. Depolarization increases with increasing excitation until the EPSP at its distal origin approaches the excitatory synaptic reversal potential and saturates. Thus, the curve plateaus.

Proximal inhibition decreases EPSP amplitude by a nearly constant factor independent of the amount of excitation (Fig. 1, A1 and B1). Thus, if the inhibitory strength were sufficient to reduce the plateau of the curve in Fig. 1, A1, below the critical firing level of the proximal compartment (for example, the dashed line), then no amount of additional excitation would fire the cell. In other words, the inhibition would be absolute, as required for recurrent inhibition.

Distal inhibition behaves differently. Because it reduces EPSPs in the distal compartment itself, it tends to counter the excitatory saturation, allowing excitatory levels that in the uninhibited situation would produce no further potential change to do so, until the inhibition is overcome and excitation again saturates. Thus, any amount of distal inhibition can be overcome by further excitation, as reflected in Fig. 1, A2, by the fact that all curves crossed the critical firing level with strong enough excitation. Consequently, distal inhibition can be overridden, as required for tonic inhibition. We refer to the different functional properties of inhibition conferred by proximal and distal synapses as "absoluteness" and "relativity," respectively.

These arguments imply that recurrent inhibition of LG should operate proximally, whereas tonic inhibition should be distal. Is this the case? To evaluate this, we compared the conductance increases produced by recurrent and tonic inhibition in the proximal dendrites of LG (Fig. 2A). If the hypothesis is correct, the proximal conductance increase associated with recurrent inhibition should be much greater than that associated with comparable amounts of tonic inhibition.

Recurrent inhibition was produced by firing the medial giant neurons, which cause an escape response without LG neuron involvement (*5*); inhibition was measured near its maximum, about 10 ms after medial giant firing (*3*) (Fig. 2, B1). We measured recurrent inhibition in the absence of tonic inhibition by isolating the abdominal ner-