GLUTAMATE REGULATES NEURITE OUTGROWTH OF DESCENDING BRAIN NEURONS IN CULTURE FROM LARVAL LAMPREY

A Thesis presented to the Faculty of the Graduate School

University of Missouri-Columbia

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

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by

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December 2005

The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled.

GLUTAMATE REGULATES NEURITE OUTGROWTH OF DESCENDING BRAIN NEURONS IN CULTURE FROM LARVAL LAMPREY

Presented by Sarah Kathleen Ryan

A candidate for the degree of Masters of Neuroscience

And hereby certify that in their opinion it is worthy of acceptance.

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ACKNOWLEDGEMENTS

First I would like to thank my advisor, Dr. Andrew McClellan, for his continuous support of my work and ability to clear confusion when it arose. I would also like to thank Dr. Anand Chandrasekhar and Dr. Grace Sun, my committee members, for their time and advice on my project.

Many people have contributed to this thesis in one way or another. I am thankful for the support from my lab and members of the department when days just weren't turning out right.

Special thanks to all for the people who came before me, their work or the cell culture set up and preliminary experiments will not go forgotten. I would like to thank John Armstrong, a previous graduate student, for his work developing the cell culture techniques, and Carl Groat, a previous graduate student, for refining the cell culture techniques to a reliable standard. I would like to thank Soo-Kyung Hong, a previous graduate student, and her undergraduate assistants, Lindsay Shotts, and Depika Nehra, for their work on experiments that are summarized in Figures 5-8 and a portion of Figure 4.

I would like to thank the people who worked together with me on various projects. Thanks especially to Lindsay Shotts for coming back to the lab as a full time technician for the first year of my graduate research. Lindsay and I worked side-by-side collecting data that resulted in Figures 9-13. Thanks to Anjali Patel and Mychi Lee, who were undergraduates working in the laboratory, for helping with technical assistance and data analysis. Thanks to Georgia Marsh for assistance during the initial stages of my graduate work.

A thanks additionally goes out to Adam Magg and Jeff LaCroix for the computer assistance and program development.

In addition, I would like to thank Nila Emerich, for all that she is.

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LIST OF ABBREVIATIONS

5HT – serotonin
AC – adenylate cyclase
AM – acetoxymethyl
AMPA – alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARRN – anterior rhombencephalic reticular nuclei
BAPTA – 1,2-bis (2-aminophenoxy) ethane N,N, N'N'-tetra-acetic acid
BL – body length
cAMP – cyclic AMP
CNS – central nervous system
CTX – ω-conotoxin MVIIC
DAG – diacylglycerol
dbcAMP – dibutyryl cyclic AMP
DiI – 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate
DMF – dimethyl sulfoxide
DMSO – dimethylsulphoxide
EAA – excitatory amino acids
ER – endoplasmic reticulum
GABA – γ-aminobutyric acid
GLU – L-glutamate
H89 – N-[2-(p-Bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride
HRP – horseradish peroxidase
IBMX – 3-isobutyl-1-methylxanthine
IP3 – inositol-1,4,5-trisphosphate
KA – kainate
KYN – kynurenic acid
MRN – mesencephalic reticular nucleus
MRRN – middle rhombencephalic reticular nuclei
NMDA - N-methyl D-aspartic acid
PIP2 – phosphatidylinositol 4,5-bisphosphate
PKA – protein kinase A
PKC – protein kinase C
PLCβ – phospholipase Cβ
PRRN – posterior rhombencephalic reticular nuclei
ROI – region of interest
RS – reticulospinal
sAHP – slow afterhyperpolarization
TTX – tetrodotoxin

ABSTRACT

In spinal cord-transected larval lamprey, descending brain neurons, most of which are reticulospinal (RS) neurons, regenerate their axons across a transection site and contribute significantly to behavioral recovery. In the present study, DiI-labeled descending brain neurons in larval lamprey were dissociated and plated in cell culture media. Neurites emerged from isolated descending brain neurons within ~2-4 days and grew at an average growth rate of ~0.07 μm/min. Focal application of 5 mM or 25 mM L-glutamate to growth cones of neurites caused these processes to stop growing and often to retract, and 10 mM kynurenic acid abolished glutamate-induced neurite retraction. Glutamate-induced neurite retraction appeared to be due, in part, to calcium influx via voltage-gated calcium channels, since application of high potassium media onto growth cones inhibited neurite outgrowth, an effect that was blocked by 2 mM cobalt or 100 μM cadmium. Furthermore, application of glutamate to growth cones in the presence of 2 mM ω-conotoxin MVIIC still inhibited neurite outgrowth, suggesting that calcium influx via chemically-gated channels may also contribute. In particular, application of NMDA to growth cones elicited neurite retraction. Finally, application of glutamate or high potassium media to the cell bodies of descending brain neurons in culture inhibited outgrowth of multiple neurites from the same neuron.

Focal application of glutamate, while in the presence of tetrodotoxin (TTX), inhibited neurite outgrowth. Interestingly TTX alone also inhibited neurite growth, suggesting the neurons in culture may be spontaneously active.

Bath application of 0.5 mM L-glutamate reliably elicited neurite retraction similar to that produced by focal application 25 mM glutamate to individual growth cones. Glutamate-induced neurite retraction appears to be due, in part, to an increase in intracellular calcium levels, which may involve second messengers such as cAMP. Specifically, in the present study bath application of 10 mM dibutyryl cyclic AMP (dbcAMP), a cAMP analogue, or 62.5 μM Forskolin, an activator of cAMP, induced neurite retraction. In addition, 100 μM 3-isobutyl-1-methylxanthine (IBMX), which inhibits breakdown of cAMP, inhibited neurite outgrowth, suggesting that cAMP is normally produced in descending brain neurons in culture. Finally, in the presence of 10 μM H89, which blocks the actions of protein kinase A (PKA), glutamate no longer induced neurite retraction, suggesting that cAMP and PKA are part of the signaling pathway for glutamate-induced neurite retraction.

In summary, results from the present study suggest that glutamate inhibits neurite outgrowth of descending brain neurons in culture by acting on glutamate receptors, mediating calcium influx via both voltage-gated and chemically-gated channels, increasing intracellular calcium levels, and activating cAMP. Similar intracellular signaling mechanisms may be important for regulating axonal regeneration following spinal cord injury in the lamprey.

Keywords: growth cone, reticulospinal neurons, regeneration, spinal cord injury

INTRODUCTION

Descending brain neurons, including reticulospinal (RS) neurons as well as several other specific types of brain neurons, project to the spinal cord and have inputs to spinal motor networks. Following severe spinal cord injury, such as a complete transection, axons of descending brain neurons are disrupted, and animals are paralyzed below the lesion. In adult higher vertebrates, such as birds and mammals, paralysis following spinal cord injury is usually permanent because descending axons do not regenerate beyond the injury site (reviewed in Schwab and Bartholdi, 1996). In contrast, in several lower vertebrates, including lamprey (Rovainen, 1976; Selzer, 1978), fish (Bernstein, 1964; Bernstein and Gelderd, 1970; Coggeshall et al., 1982; Sharma et al., 1993; Yamada et al., 1995), and certain amphibians (Butler and Ward, 1967; Davis et al., 1989), descending brain neurons regenerate their axons across a spinal cord lesion site and make functional synapses so as to restore behavioral functions (reviewed in McClellan, 1998).

The lamprey begins to recover locomotor function within about two weeks after a complete, rostral spinal cord transection, and by eight weeks the locomotor movements and muscle activity are virtually identical to those in normal animals (Davis et al., 1993). With increasing recovery times, progressively greater numbers of axotomized RS neurons, which make up ~80% of descending brain neurons, extend their axons across the lesion site and for progressively greater distances below the lesion (Davis and McClellan, 1994a,b). Double labeling experiments indicate that unidentified RS neurons, which are

thought to activate spinal locomotor networks (Shaw et al., 2001), regenerate their axons (Zhang and McClellan, 1999), and regeneration of these injured axons, rather than the addition of new projections, is the main mechanism for recovery of function (Zhang et al., 2002).

The cellular and molecular mechanisms that regulate axonal regeneration following spinal cord injury are not well understood. In particular, in whole animals it is difficult to control the variables that might participate in this regulation. Consequently, the outgrowth of neurites from isolated neurons in culture is often used as a model to study the factors that regulate axonal elongation, axonal guidance, and synapse formation during regeneration as well as development.

Diffusible factors, such as neurotransmitters and neuronal growth factors, can regulate not only the rate but also the direction of neurite outgrowth in culture (reviewed in Mattson, 1988; Lipton and Kater, 1989; Goodman, 1996; Zheng et al., 1996; Gallo et al., 1997; Gallo and Letourneau, 2000). In the mollusk *Helisoma*, serotonin (5HT) or dopamine applied to the culture media elicits retraction of filopodia and inhibition of neurite elongation of certain buccal ganglion neurons (e.g. B19) in culture but has little effect on other neurons (Haydon et al., 1984,1987; Cohan et al., 1987; Mattson and Kater, 1987; McCobb et al., 1988b). For other buccal ganglion neurons (e.g. B5), L-glutamate and somatostatin enhance neurite outgrowth (Bulloch, 1987; Jones and Bulloch, 1988).

In mammals, application of agonists for excitatory amino acid (EAA) receptors, such as L-glutamate, kainate (KA), N-methyl-D-aspartic acid (NMDA), or quisqualate (AMPA receptor agonist), to dendrites of hippocampal pyramidal neurons in culture results in retraction of these processes (Mattson et al., 1988a). In addition, higher concentrations of these agents were found to decrease the growth rates of axonal neurites. Glutamate-induced retraction of dendritic neurites is negated by bath applied GABA (Mattson and Kater, 1989) or fibroblast growth factor (Mattson et al., 1989). For mouse spinal cord neurons in culture, glutamate, acting through kainate/AMPA receptors, also induces neurite retraction (Owen and Bird, 1997). Glutamate, kainate, or AMPA applied to embryonic retinal neurons in culture causes a reduction in the numbers and outgrowth of neurites (Catsicas et al., 2001). Finally, retinal neurons in culture retract their neurites in response to dopamine (Lankford et al., 1988).

Intracellular calcium is known to affect many functions in biological cells, and not surprisingly it can regulate growth cone motility and neurite outgrowth (Kater and Mills, 1991). Electrical stimulation of Helisoma neurons arrests neurite outgrowth (Cohan and Kater, 1986). It has been suggested that electrical activity arrests neurite outgrowth by increasing intracellular calcium (Cohan et al., 1987). For example, this arrest results from a rise in intracellular calcium concentration within the growth cones (Haydon et al., 1984; Cohan and Kater, 1986, Cohan et al., 1987). The effects of 5HT can be mimicked by direct electrical stimulation of neurons in culture (Cohan and Kater, 1986; Fields et al., 1990) or by chronic application of high extracellular potassium (Rehder and

Kater, 1992), and can be negated by bath-applied acetylcholine (McCobb et al., 1988a), which has an inhibitory effect on Helisoma buccal ganglion neurons (e.g. B19). These experiments support Mattson and Kater's "set-point hypothesis" (1987), which state that there is an optimal range for calcium concentrations that produce neurite outgrowth. However Garyantes and Regehr (1992) have shown with calcium imaging from their work on rat superior cervical ganglion neurons that "electrical activity is not a universal signal for neurons to stop growing and that a rise in internal calcium does not always arrest the migration of growth cones".

Second messengers, such as cyclic AMP (cAMP), inositol-1,4,5-trisphosphate (IP3), and diacylglycerol (DAG), are important for regulating many biochemical processes in cells and are known to affect intracellular calcium levels, which can modulate neurite outgrowth. Although there are variations in these second messenger pathways, only the best-studied pathways will be discussed here. One of the most well studied cAMP pathways involves the synthesis of cAMP in response to activation of adenylate cyclase (AC) that is induced by ligands binding to membrane-bound receptors and activation of G proteins. The second messenger cAMP produces its effects by activating protein kinase A (PKA), and the cAMP:PKA complex can open nearby calcium channels and induce calcium influx as well as release calcium from intracellular stores, such as the endoplasmic reticulum (ER) (Levitan and Kaczmarek, 1997; Kao et al., 2002). Calcium can also act as a second or third messenger, and can activate protein kinases, which can open membrane calcium channels and/or

bind to ryanodine receptors on the ER to release calcium. In addition, calcium can independently bind to the ryanodine receptors directly to release calcium from intracellular stores.

Another well-studied pathway is that of IP3 and DAG, which are derived from PIP2 in response to ligands binding to membrane-bound receptors, activation of G proteins, and subsequential cleavage by phospolipase Cβ (PLCβ). The second messenger IP3 binds to receptors on the ER and releases calcium ions into the cytosol (Berridge et al., 2003) that are involved in intracellular initiation and propagation of calcium signaling (Venance et al., 1997). The second messenger DAG acts on protein kinase C (PKC).

To shape the duration and level of intracellular calcium signals a variety of cells, including neurons, largely rely on intracellular calcium stores, such as the ER and mitochondria (for review, see Berridge, 1998; Meldolesi and Grohovaz, 2001). Clearing intracellular calcium ions, to restore resting levels, is accomplished by various methods, specifically by binding to cytoplasmic proteins, uptake into the ER, uptake into mitochondria, and extrusion across the plasma membrane by the Na^{+}-Ca^{2+} exchanger and a plasma membrane Ca^{2+}-ATPase (Carafoli and Longoni, 1987; Thayer and Miller, 1990; Miller et al., 1990; Friel and Tsien, 1994; White and Reynolds, 1995; Herrington et al., 1996; Babcock et al., 1997; Babcock and Hille, 1998; Duchen, 1999; Pozzan and Rizzuto, 2000).

Various second messengers have been found to affect intracellular calcium levels and influence neurite outgrowth. Increases in cAMP can induce

(Johnson et al., 1988; Sánchez et al., 2004; Chierzi et al., 2005; Peng et al., 2003) or suppress neurite growth (Mattson et al., 1988c) in a variety of cells. Blocking PKA activity and formation of the cAMP:PKA complex inhibits neurite elongation in human neuroblastoma cells (cell line SH-SY5Y) (Sánchez et al., 2004). Increases in IP_3 or intracellular calcium concentration or direct electrical stimulation of mouse neuroblasroma cells in culture results in neurite retraction (Bolsover et al., 1992). In addition, neurite outgrowth of chick dorsal root ganglia is inhibited due to inhibition of IP_3 signaling or depletion of internal Ca^{2+} stores (Takei et al., 1998).

In spinal cord-transected lamprey, it will be important to identify the diffusible factors (e.g. neuronal growth factors and neurotransmitters) and non-diffusible factors (e.g. substrates) that contribute to restoration of brain-spinal cord connections, and to assess whether "these factors are operational in reduced nerve cell preparations", such as neuronal cell culture (McClellan, 1994). Over the last several years, we have developed a neuronal cell culture system to study the roles that these factors might play in regulating neurite outgrowth and synaptogenesis of dissociated descending brain neurons from larval lamprey. Results from the present study demonstrate that after a few days in culture, these neurons developed neurites with growth cones that elongated over the course of several days to several weeks. Glutamate, when focally applied to growth cones, inhibited neurite outgrowth or caused neurite retraction, and these effects appear to be due to calcium influx via both chemically-gated channels (e.g. NMDA channels) and voltage-gated calcium channels. In addition,

activation of cAMP signaling pathways is necessary for glutamate-induced inhibition of neurite outgrowth. This new cell culture system of descending brain neurons from larval lamprey will provide a controlled environment in which to test the cellular and molecular mechanisms for neurite outgrowth and synaptogenesis that might also be important for functional axonal regeneration following spinal cord injury. Preliminary accounts of certain parts of this study have appeared in abstract form (Hong et al., 2002; Ryan et al., 2004).

METHODS

Animal Care

Larval sea lamprey (*Petromyzon marinus*; 143 animals, 80-110 mm in length) were used in the present study and maintained at 23-25°C in 2.5 or 10 gallon aquaria. The procedures in this study have been approved by the Animal Use and Care Committee at the University of Missouri (protocol reference number 1506-1).

Pre-labeling of Descending Brain Neurons

Animals were anesthetized in tricaine methanesulphonate (MS-222; ~200 mg/l; Sigma Chemical, St. Louis, MO), a 5-10 mm dorsal incision was made at 20% body length (BL, normalized distance from the head), and the spinal cord was exposed and completely transected. A small piece of Gelfoam (Upjohn; Kalamazoo, MI), soaked in an 8% solution of the lipid soluble anatomical tracer 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) and dimethyl sulfoxide (DMF; Sigma Chemical), was placed between the cut ends of the spinal cord. The incision was closed and sealed with cyanoacrylate (Super Glue Gel; Loctite Company, Rocky Hill, CT). Subsequently, animals were returned to aquaria and maintained for ~2-4 weeks to allow for retrograde labeling of descending brain neurons.

Dissection, Dissociation, and Maintenance of Cell Culture

Following retrograde labeling, animals were anesthetized, and the brains and rostral spinal cords were removed in sterile lamprey Ringer's solution (110 mM NaCl, 2.1 mM KCl, 2.6 mM $CaCl_2$, 1.8 mM $MgCl_2$, 4 mM dextrose, 10 mM

HEPES; pH 7.4), similar to a dissection method previously described (Davis and McClellan, 1994a). The choroid plexus and meninges were removed, the cerebellar commissure was transected, and the obex was extended caudally. The brains were viewed with an inverted microscope (Leitz Fluovert; Leica Microsystems, Wetzlar, Germany) equipped with a 50W mercury lamp and filters for fluorescence (DiI Filter: 510-560 nm excitation; 573-647 nm emission, Chroma Technologies, Brattleboro, VT) to confirm the presence of DiI labeling of descending brain neurons (Fig. 3B), particularly RS neurons in the following reticular nuclei (Fig. 3A): mesencephalic reticular nucleus (MRN); anterior (ARRN), middle (MRRN), and posterior (PRRN) rhombencephalic reticular nuclei (Davis and McClellan, 1994a,b).

The brains were transected at the rostral mesencephalon and caudal rhombencephalon, and two #11 scalpel blades were used in a criss-cross cutting technique (Freshney, 1992) to mince the brain tissue into blocks of approximately 200-500 μm. The tissue blocks were digested in sterile collagenase solution (1.25 mg/ml; Sigma Chemical) for 30 min and then in sterile protease solution (0.5 mg/ml; Sigma Chemical) for 16 min. After enzymatic treatment, the tissue blocks were transferred for 15 min. to sterile custom L15 media containing 10% fetal bovine serum (Gibco, Life Technologies; Grand Island, NY), 2 mM glutamine, and penicillin-streptomycin-neomycin (2.5 units/ml-2.5 μg/ml-5.0 μg/ml, respectively; Sigma Chemical). The custom media was made from L15 media powder containing no salts (Gibco, Life Technologies), and the following components were added: 110 mM NaCl; 2.1 mM KCl; 2.6 mM $CaCl_2$; 1.0 mM

$MgCl_2$; 0.8 mM $MgSO_4$, 5.5 mM sodium pyruvate; 4.0 mM dextrose; 2.5 mM D-glucose; 10 mM HEPES; pH 7.4. Sterile Pasteur pipettes with fire-polished tips of 500 μm and then 300 μm inside diameters were used to triturate the tissue. The cell suspension from a single brain was placed either in a single well of six-well culture plates or in 35 mm Petri dishes, both of which had a poly-D-lysine substrate (Biocoat; Bectin-Dickinson Labware, Bedford, MA) and contained ~2 ml of sterile L15 media with serum. Within a few hours, most of the neurons had attached to the substrate. One half of the media was changed at four days after plating, and thereafter every three days.

Cell bodies with growing neurites were examined with an inverted microscope equipped with fluorescence to identify DiI-labeled descending brain neurons (Fig. 3C). The x,y coordinates of DiI-labeled neurons were displayed on a custom electronic device and were recorded so that it was possible to return to the same neurons at a later time. Only DiI-labeled neurons with processes were selected for the present study (n = 306 neurons from 143 brains). The lids of the culture dishes were removed during pressure ejection of agents onto growth cones (see below). Since this would eventually result in contamination, for these types of experiments, a maximum of three neurons that were widely separated from each other were examined within a single culture dish. In most cases, only one neurite per given neuron was measured and analyzed.

Experimental Manipulations

For most experiments, a neurite and its parent cell body were identified in the field of view of an inverted microscope at 200X or 400X. With an S-VHS

video camera (KR2222; Panasonic, Yokohama, Japan) and video image capture device (Dazzle Digital Video Creator; SCM Microsystems, Fremont, CA), time-lapse images (800 x 600) were captured every ~2-10 min. into a computer. At a later time, neurite growth rates were calculated from these sequential time-lapse images (see below). For the various experimental manipulations, neurite retraction was defined as average outgrowth rates that were negative and significantly less than zero (i.e. negative), while inhibition of growth was defined as growth rates that were significantly less than pre-control values but not significantly less than zero. In addition, the separate image files could be merged to form time-lapse movie files (Video Mach; www.Gromada.com). For obtaining fluorescence images of DiI-labeled neurons, a cooled CCD camera was used (Retiga 1300C; Q Imaging, Burnaby BC Canada).

For each type of experimental manipulation, before agents were pressure ejected onto growth cones, pre-control rates of neurite outgrowth for individual DiI-labeled neurons were determined, usually for ~1-4 hours, with time-lapse video microscopy (Fig. 5A; see below). During ejection of agents onto growth cones, neurite outgrowth rates were measured, and subsequently, post-control measurements of neurite outgrowth rates were determined. In contrast to bath application of agents, pressure ejection allows focal application of agents to restricted parts of neurons in culture.

Glutamate-induced inhibition of neurite outgrowth. For one type of experiment (n = 15 neurons), micropipettes were filled with Ringer's solution, and Fast green was added to visualize the ejection bolus. The rates of neurite

outgrowth were determined during pressure ejection of the above solution onto growth cones to examine the purely mechanical effects of the application procedure. Briefly, the tips of micropipettes were broken off to ~2-5 μm under a dissecting microscope, the micropipettes were mounted on a manipulator (Newport Corp., Fountain Valley, CA), and the tips were lowered into the media until they were in close proximity (25.6 ± 10.7 μm; range: 12.2 – 75.6 μm) to the growth cone of a neurite. Subsequently, the micropipette solution was pressure ejected (4-6 ms pulses at 1-2 Hz, 20 psi; each ejection bolus ~25 μm dia. or ~8 pl) onto growth cones for ~1-2 hours, and time-lapse images were captured of the associate neurite. Finally, neurite outgrowth was further monitored for a post-control period of ~1-10 hours.

For a second set of experiments, micropipettes were filled with 5 mM or 25 mM L-glutamate (GLU, Sigma Chemical) in L15 media without serum, and Fast green was added to visualize the ejection bolus. It should be noted that focal application of glutamate to RS neurons in intact lamprey brains elicits depolarization (Matthews and Wickelgren, 1979; Dryer, 1988). In the present study, GLU was pressure ejected onto growth cones of individual neurites for ~0.5-3 hours (Fig. 5B; n = 12 or 29 neurons, respectively), as described above for control experiments. Unlike bath application of glutamate, repetitive pressure ejection of this neurotransmitter may have allowed neurons to repolarize, which could potentially remove inactivation from voltage-gated channels and allow them to reopen. Following application of agents to growth cones, neurites were further monitored for a post-control period of ~1-12 hours.

The above glutamate concentrations are higher than those normally used for continuous bath application in lamprey experiments (i.e. 0.5-1.0 mM) (Grillner et al., 1981). In the present study, the average GLU concentrations near growth cones certainly were much lower than 5 mM or 25 mM (Curtis, 1964; see Discussion). For example, pressure ejection of 5 mM GLU has been used for pharmacological microstimulation in brain locomotor areas to elicit spinal locomotor activity in *in vitro* brain/spinal cord preparations (Paggett et al., 2004). Also, for a number of reasons, it is likely that pressure ejection of glutamate onto a growth cone was largely affecting the particular neuron of interest and not other neurons in the culture dish (see Results and Discussion).

A third type of experiment (n = 11 neurons) was performed to test whether glutamate exerts its effects on neurite outgrowth via glutamate receptors or other non-specific mechanisms. Specifically, 10 mM kynurenic acid (Sigma Chemical), a broad spectrum ionotropic excitatory amino acid (EAA) receptor blocker (Fig. 1), was added to the culture media, and the rates of neurite outgrowth were monitored for ~1-2 hours. Subsequently, with kynurenic acid still in the culture dish, 25 mM L-glutamate in media without serum was pressure ejected onto growth cones, and neurite outgrowth rates were measured.

Mechanisms for glutamate-induced inhibition of neurite outgrowth. In the present study, focal application of L-glutamate to growth cones caused inhibition of neurite outgrowth and sometimes retraction. In theory, these effects might be due to calcium influx via chemically-gated channels, such as NMDA channels (MacDermott et al., 1986), and/or influx through voltage-gated calcium channels

as a result of depolarization (El Manira and Bussieres, 1997). To determine if calcium influx via voltage-gated channels might contribute to glutamate-induced inhibition of neurite outgrowth, high potassium media (31 mM, Sigma Chemical) without serum was pressure ejected onto growth cones, similar to the methods described above (n = 8). If it is assumed that the concentration of potassium in the vicinity of growth cones was about one fifth of the above value (~6.2 mM; see above discussion regarding glutamate concentrations), according to the Nernst potential, this would result in a depolarization of ~30 mV, which probably was sufficient to elicit action potentials (Rouse et al., 1998) and to open voltage-gated calcium channels (El Manira and Bussieres, 1997). In addition, to test if possible effects of high potassium on neurite outgrowth were due to calcium influx, 2 mM $CoCl_2$ (n = 6, Sigma Chemical) or 100 μM $CdCl_2$ (n = 5, Sigma Chemical), which block voltage-gated calcium channels in lamprey (Fig. 1) (Christenson et al. 1993) and other animals, was added to the Petri dish as well as the high potassium pressure ejection solution. As a control, the effects of $CoCl_2$ (n = 6) or $CdCl_2$ (n = 8) alone on neurite outgrowth were measured.

To determine if calcium influx via chemically-gated channels might contribute to glutamate-induced inhibition of neurite outgrowth, 25 mM L-glutamate was focally applied to growth cones under conditions in which 2 mM ω-conotoxin MVIIC (Fig. 1) (CTX, Sigma Chemical), a blocker of both N and P/Q voltage-gated calcium channels (McDonough et al., 1996; Bussieres and El Manira, 1999), was present in both the Petri dish and the pressure ejection micropipette (n = 11). As a control, high potassium media was focally applied to

growth cones with CTX present in both the Petri dish and pressure ejection micropipette to determine if this calcium channel blocker abolished most of the calcium influx resulting from depolarization (n = 5). Finally, 1 mM N-methyl D-aspartate (NMDA, Sigma Chemical) was pressure ejected onto growth cones in the absence (n = 6) or presence (n = 6) of CTX to determine if calcium influx via NMDA channels was sufficient to inhibit neurite outgrowth.

Local versus global effects of glutamate on neurite retraction. For all the procedures described above, agents were pressure ejected onto growth cones to test whether local calcium influx could inhibit neurite outgrowth. To test whether more global changes in calcium influx could affect neurite outgrowth, 25 mM glutamate (n = 7) or high potassium media (n = 7) was pressure ejected onto the cell bodies of descending brain neurons in culture. For some experiments, only one neurite per neuron was selected and measured in detail (n = 7), while for other experiments the effects of the above manipulations on multiple neurites of the same neuron were measured (n = 6).

Role of action potentials in glutamate-induced inhibition of neurite outgrowth. To determine if action potentials and the associated calcium influx via voltage-gated channels might contribute to glutamate-induced inhibition of neurite outgrowth, 3 μM tetrodotoxin (TTX, n=8, Sigma Chemical), a blocker with very high affinity for voltage-gated Na+ channels, was added to the culture dish for 60 min., and the effects on neurite outgrowth were determined. Subsequently, 25 mM L-glutamate was focally applied to growth cones, still in the presence of TTX, to determine neurite outgrowth rates.

Glutamate-induced changes in calcium levels. To confirm that glutamate-induced neurite retraction involves an increase in intracellular calcium levels, DiI-labeled descending brain neurons were loaded with Calcium Green-1, a calcium indicator dye (n = 18). A stock solution of 2 mM Calcium Green-1 AM (Molecular Probes) was made in 20% Pluronic in DMSO (F-127, Molecular Probes). A volume of 10 μl of this solution was added to the 2 ml of media in the culture dishes (20 μM final Calcium Green concentration), and the neurons incubated in this solution for 2.5 hrs. in the dark, after which the dish was rinsed with fresh media several times. Manipulations started 30 min after the washes. First, in neurons loaded with Calcium Green, neurite outgrowth was measured as described below, and neurons with positive growth rates were selected. Second, an image intensifier (KS-1381, VideoScope, Dulles, VA) connected to an S-VHS video camera was attached to an inverted microscope, which was equipped with filters for Calcium Green fluorescence (41001 filter: 535-550 nm excitation; 610-675 nm emission, Chroma Technologies). A 50W mercury lamp was used, and the excitation light was attenuated by 1/16 by neutral density filters to prevent phototoxicity damage to the labeled neurons. Prior to application of glutamate to neurons in culture, 60 control images of Calcium Green fluorescence of neuronal cell bodies, captured at 1 Hz, were acquired and averaged to determine the baseline fluorescence. Subsequently, 25 mM L-glutamate was pressure ejected onto the cell bodies of neurons in culture for 30 min, and during this time Calcium Green experimental fluorescence images from cell bodies were acquired at 1 Hz. In addition, in some experiments, 1 mM glutamate was bath applied during

calcium imaging. We expected that activation of RS neurons would very likely cause an increase influx of calcium, binding to Calcium Green, and an increase in fluorescence.

To display changes in fluorescence during activity of descending brain neurons, graphical waveforms were created of changes in fluorescence versus time. In order to process the image data, a video converter (VideoMach, Version 2.7.2) was used to convert the JPEG files (24 bit true color RGB) into TIFF files (8-bit grayscale files). These JPEG images were obtained without stimulation (control images), as well as during activation of neurons (experimental images). An average control image was calculated using an image processing program (Scion Image for Windows, Beta 4.0.2) by averaging the control images. This "average control image" (C_{Avg}) was used to determine the mean pixel values before stimulation,

$$C_{Avg} = \frac{\sum_{i=1}^{n} C_i}{n}$$

where "C" is the series of control images, "i" is the image number in the set, and "n" is the total number of control images.

In the average control images, regions of interest (ROI) or contours were mark around single neurons to focus on changes in fluorescence in these specific areas of the images. To more easily determine the outlines of the ROI, the average control images were inverted. Initially, the mean pixel values from each ROI in the experimental images were determined (F_n, where n = frame number). The mean pixel values for the average control images ROI (F_o) were

subtracted from each ROI of the experimental images to yield a fluorescent change versus time ($dF = \Delta F = F_n - F_o$). This difference was divided by the mean pixel values of the control image (F_o) and multiplied by 100 to obtain percentile values versus time ($\Delta F/F$).

$$dF/F = \Delta F/F = ((F_n – F_o)/ F_o)\ 100\%$$

To eliminate the changes in fluorescence resulting from changes in background, the above procedure also was preformed on four areas around the ROI. Then the pixel values from the surrounding areas were averaged and subtracted from the pixel value of the ROI contour surrounding the descending brain neuron.

Noise from the intensifier as a result of substantial image amplification was then reduced to sharpen the difference images. A 3, 9, or 29 point “sliding average” was applied to the $\Delta F/F$ waveforms vs. time (in seconds) in order to “smooth” the waveforms and reduce the noise from the intensifier,

$$R_n = (\Delta F/F_{n-m} \ldots. + \Delta F/F_n \ldots. + \Delta F/F_{n+m})/(2m+1)$$

where R is the resulting image after the “sliding average” is performed, m is the range around the new calculated fluorescence average value at time point n, which is the center point of the sliding average. Usually the 29 point “sliding

average" was used because the 3 and 9 point averages did not substantially reduce the noise.

Control experiment for the effects of DMSO. Some agents that were used in sections below are not readily soluble in aqueous solutions and needed to be dissolved in other solvents, such as dimethylsulphoxide (DMSO, Fisher Scientific, Fair Lawn, NJ). As a preliminary control, different concentrations of DMSO were add to culture dishes to determine the maximum concentration that had no significant effects on neurite outgrowth.

Influence of second messengers on glutamate-induced neurite retraction. To determine if cyclic AMP (cAMP), an intracellular second messenger, alone could inhibit neurite outgrowth, agents that operate via cAMP signaling pathways were tested (Fig. 2). In one type of experiment, 10 mM dibutyryl (dbcAMP, Sigma Chemical), a membrane permeable cAMP analogue (n = 17), or 62.5 μM Forskolin (Tocris, Ellisville, MO), an adenylate cyclase activator that stimulates the synthesis of cAMP (n = 21), were bath applied to the culture dishes. In a different experiment with different neurons than used above, 100 μM 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical), an inhibitor of cAMP phosphodiesterase that suppresses breakdown of naturally occurring cAMP (n = 23), was bath applied to increase the intracellular levels of cAMP in neurons.

In order to determine if cAMP contributes to glutamate-induced inhibition of neurite outgrowth, glutamate was bath applied to inhibit neurite outgrowth in the presence of a cAMP blocker. For this purpose, in order to record growth rates of neurites from several neurons in the same culture dish during

manipulations of second messengers, L-glutamate was bath applied to the culture dish instead of pressure ejection onto individual growth cones. An initial control experiment was performed to determine the lowest bath concentration of glutamate that induced significant neurite retraction of all neurites, similar to the effects of focal application of 25 mM glutamate to single neurites (see Results). The agent H89 (10 μM; Sigma Chemical), a specific inhibitor of cAMP-dependent protein kinase A (PKA) (n = 19), was bath applied for 60 min, and subsequently with H89 still in the bath, the glutamate concentration found to be effective in the control experiment was bath applied. Thus, H89 should not block the synthesis of cAMP but should prevent cAMP from activating PKA.

Measurements of Neurite Outgrowth and Statistics

For a given neurite and experimental condition, the captured time-lapse images were displayed sequentially on a computer screen with custom image software. The x,y coordinate values that defined the cell body diameter and the distance between the micropipette tips and growth cones were marked and used to calculate these two parameters. In addition, eleven points were marked along each neurite from the origin of a neurite to the tip of the growth cone. The x,y coordinates for points along neurites were then imported into a spreadsheet and used to calculate the lengths of neurites as well as the incremental and average rates of neurite outgrowth or retraction (i.e. velocity). To determine if neurite outgrowth was significantly inhibited during application of agents to neurons in culture, the mean experimental rates of neurite outgrowth were compared to pre-control and post-control growth rates using repeated measures one-way ANOVA

and the Tukey-Kramer multiple comparisons post-test. To determine if application of agents resulted in significant neurite retraction, mean experimental rates for neurite outgrowth that were negative were compared to zero using either a one sample t-test or Wilcoxon rank sum test. Statistically significant differences were assumed for $p \leq 0.05$.

RESULTS

The data presented here were collected by several individuals who need to be recognized. Figures 1, 2, 3 and 20 are general summary figures. Data for Figures 5-8 were collected by Soo-Kyung Hong, who is a former graduate student, with help from Lindsay Shotts and Depika Nehra, who were undergraduate students at the time. Data for Figures 9-13 were collected by Sarah Ryan and Lindsay Shotts, a full-time technician, working side-by-side. Data for Figures 14-19 were collected by Sarah Ryan. Figure 4 represents data collected for all experiments described in this thesis.

DiI Labeling of Descending Brain Neurons Before and After Dissociation

Approximately 2-4 weeks after DiI was applied to the spinal cord at 20% BL and before dissociation, in whole mount brain tissue, DiI-labeled descending brain neurons and some axons were clearly visible in most cell groups (Fig. 3A,B). In particular, reticulospinal (RS) neurons were labeled in all reticular nuclei, including large identified Müller and Mauthner cells. Following dissociation of the tissue, DiI-labeled cells that represented descending brain neurons could be identified in culture (Fig. 3C). In general, most of the DiI-labeled neurons in culture that were examined in the present study were small-to-medium size cells with average diameters of 25.4 $\pm$ 6.6 μm (range 10.8 – 54.6 μm; n = 296 neurons) (Fig. 4A). Thus, very few of the DiI-labeled cells were relatively large descending brain neurons (e.g. $>$70 μm dia.), such as most of the Müller and Mauthner cells (Davis and McClellan, 1994b).

Within a few hours after plating the dissociated tissue from a single brain, about 400-800 DiI-labeled neurons as well as about 4400-6000 non-labeled cells that had neuron-like processes were present in and attached to the bottom of the culture dish. In larval lamprey, approximately 1250 descending brain neurons project to 20% BL in the spinal cord (Davis and McClellan, 1994a), the site of DiI application in the present study.

Time Course of Neurite Outgrowth

Soon after plating, many of the DiI-labeled neurons displayed processes, most of which retracted within about 24-48 hours. New neurites with growth cones began to emerge from DiI-labeled neurons after ~2-4 days in culture and often continued to grow for up to ~2-4 weeks, at which time the cultures usually began to deteriorate. For experiments in the present study, the cultures were 5.6 $\pm$ 4.2 days old (range: 2 - 28 days). The growth cones could be moderately complex, with lamellipodia and many fine fillopodia, or could be relatively simple with a club-like ending, similar to that reported *in vivo* during axonal regeneration in the lamprey spinal cord (Lurie et al., 1994). Descending brain neurons in culture had an average of 1.6 $\pm$ 0.8 main neurite processes (range: 1 – 6) (Fig. 4B). During pre-control periods prior to application of agents to isolated neurons, the neurites of DiI-labeled descending brain neurons in culture that were examined in the present study had average lengths of 96.6 $\pm$ 115.2 μm (range: 16.2-1117.8 μm) (Fig. 4C) and grew at variable average growth rates of 0.07 $\pm$ 0.11 μm/min (range: -0.12 to +0.57 μm/min) (Fig. 5A). Thus, on average, the majority of neurites exhibited net elongation, but they often displayed a

combination of extension and retraction during “exploratory” movements along the substrate. The above growth rates were obtained from cultured neurons that encompassed a broad range of growth cone sizes, shapes, and dynamics.

Control Experiment for the Mechanical Effects of Pressure Ejection

Since pressure ejection of glutamate onto growth cones inhibited neurite outgrowth (see below), control experiments were performed to test whether the mechanical effects of pressure ejection alone could affect neuronal outgrowth. During pre-control periods that lasted an average of 119 $\pm$ 47 min (range: 50–186 min; n = 15 neurons), the rates of neurite outgrowth were measured. Subsequently, micropipettes were filled with Ringer’s solution, and the tips were positioned an average of ~25 μm from growth cones, similar to the distances used when glutamate was pressure ejected onto growth cones (see below). Focal pressure ejection of Ringer’s solution onto growth cones for 103 $\pm$ 29 min (range: 56–150 min) did not significantly affect the rates of neurite outgrowth compared to pre-control measurements (0.002 $\pm$ 0.028 μm/min vs. 0.011 $\pm$ 0.028 μm/min; n = 15, $p > 0.05$, ANOVA; Fig. 6A, 7A, Hong et al., 2002). These results suggest that the mechanical actions from pressure ejection alone do not significantly affect neurite outgrowth and cannot account for glutamate-induced inhibition of neurite outgrowth.

Effects of Focal Application of Glutamate to Growth Cones on Neurite Outgrowth

During pre-control periods that lasted an average of 148 $\pm$ 140 min (range: 66–600 min; n = 12), rates of neurite outgrowth were measured. Subsequently, 5

mM L-glutamate in media without serum was pressure ejected onto the growth cones of neurites for an average of 184 ± 99 min (range: 92–400 min). This manipulation elicited neurite retraction of some neurons, and the average rates for neurite outgrowth became negative (-0.021 ± 0.076 μm/min) but were not significantly less then pre-control values (0.022 ± 0.064 μm/min; n = 12, p = 0.22, ANOVA; Fig. 6B, 7B, Hong et al., 2002). In contrast, focal pressure ejection of 25 mM L-glutamate in media without serum onto growth cones for an average of 74 ± 37 min (range: 20–194 min) resulted in significant inhibition of experimental neurite outgrowth (-0.482 ± 0.880 μm/min; max. of –2.7 μm/min) compared to pre-control values (0.047 ± 0.125 μm/min; n = 29, $p \leq 0.002$, ANOVA; Fig. 5B, 6C, 7C, Hong et al., 2002). Furthermore, the experimental growth rates were significantly less than zero ($p \leq 0.01$, Wilcoxon rank sum), indicative of neurite retraction (see Methods). Usually, within ~10 min of the onset of pressure ejection of 25 mM glutamate, neurite retraction was clearly visible.

To determine if application of glutamate to the growth cone of one neurite produced effects on multiple neurites of the same neuron, six neurons were selected with two neurites (Table 1). During application of glutamate to the growth cone of one neurite, the outgrowth rate of this neurite was inhibited significantly ($p \leq 0.05$, Sign test), while the second neurite did not display significant inhibition of neurite outgrowth ($p > 0.05$, Sign test). Thus, glutamate affected the neurite of interest, and diffusion to other neurites of the same neuron had little or no effect.

If L-glutamate elicited neurite retraction by acting on receptors for EAA, these effects should be abolished in the presence of appropriate receptor blockers. When 10 mM kynurenic acid, a non-specific ionotropic EAA receptor blocker (Fig. 1), was present in the culture dishes for an average of 112 ± 31 min (range: 46–152 min), neurite outgrowth was not significantly affected compared to pre-control conditions in the absence of this blocker ($n = 11$, $p > 0.05$, ANOVA; Fig. 6D, 7D, Hong et al., 2002). With kynurenic acid still present in the culture dishes, pressure ejection of 25 mM L-glutamate onto growth cones for 95 ± 46 min (range: 60–216 min; $n = 11$), resulted in neurite growth rates (0.077 ± 0.060 μm/min) that were not significantly different than those in the presence of kynurenic acid alone (0.031 ± 0.050 μm/min; $p > 0.05$, ANOVA; Fig. 6D, 7D, Hong et al., 2002). Following the termination of pressure ejection of glutamate and still in the presence of kynurenic acid, many neurites were still elongating during the post-control period (Fig. 6D, 7D, Hong et al., 2002).

Mechanisms of Glutamate-Induced Inhibition of Neurite Outgrowth

Intracellular calcium levels in growth cones are an important regulating factor for neurite outgrowth of many types of neurons (see Introduction and Discussion for references). In the present study, glutamate-induced inhibition of neurite outgrowth and neurite retraction might be due, in part, to calcium influx via chemically-gated channels and/or voltage-gated calcium channels (see Discussion). To test whether calcium influx via voltage-gated channels alone might contribute to glutamate-induced inhibition of neurite outgrowth, high potassium media (31 mM; see Methods) without serum was pressure ejected

onto growth cones to locally depolarize these processes (n = 8). Figure 9 shows that focal application of high potassium media to the growth cone of a DiI-labeled descending brain neuron in culture elicited neurite retraction (Fig. 9A-D). Compilation of data from all experiments of this type indicated that high potassium resulted in a significant inhibition of neurite outgrowth compared to pre-control growth rates (Fig. 10A; $p \leq 0.05$, ANOVA), but experimental growth rates were not quite significantly less than zero ($p = 0.065$, t-test). In the presence of 2 mM $CoCl_2$ (n = 6) or 100 μM $CdCl_2$ (n = 5), calcium channel blockers (Fig. 1), high potassium-induced inhibition of neurite outgrowth was abolished (Fig. 10B,C). In separate control experiments, 2 mM cobalt (-0.007 $\pm$ 0.319 μm/min; n = 6) or 100 μM cadmium (0.159 $\pm$ 0.158 μm/min; n = 8) alone in the bath did not result in significant changes in neurite growth rates compared to pre-control conditions (0.207 $\pm$ 0.146 μm/min and 0.259 $\pm$ 0.141 μm/min respectively; $p > 0.05$; ANOVA). Together, these results suggest that calcium influx via voltage-gated channels can suppress neurite outgrowth.

To test whether calcium influx via chemically-gated channels alone might contribute to glutamate-induced inhibition of neurite outgrowth, 25 mM L-glutamate was pressure ejected onto growth cones in the presence of 2 mM ω-conotoxin MVIIC (CTX), a specific blocker of N and P/Q voltage-gated calcium channels that, unlike divalent cations, does not block calcium influx via chemically-gated channels (Fig. 1; see Discussion) (McDonough et al., 1996; Bussieres and El Manira, 1999). Under these conditions with CTX present, glutamate still resulted in significant inhibition of neurite outgrowth (Fig. 11A; n =

11; $p \leq 0.01$, ANOVA). In contrast, CTX did block high potassium-induced inhibition of neurite outgrowth (Fig. 11B; n = 5; $p > 0.05$, ANOVA), suggesting that CTX blocked most of the calcium influx via voltage-gated channels that resulted from depolarization. Thus, calcium influx via L-type calcium channels appears to be minimal. In separate control experiments, 2 mM CTX alone (0.208 $\pm$ 0.194 μm/min) in the bath did not significantly change neurite growth rates compared to pre-control conditions (0.187 $\pm$ 0.129 μm/min; $p > 0.05$; n = 16; ANOVA). In summary, application of glutamate to growth cones in the presence of CTX probably resulted in calcium influx largely via chemically-gated channels, such as NMDA-activated channels that are known to pass calcium in lamprey neurons (Wallén and Grillner, 1987). For example, pressure ejection of 1 mM NMDA onto growth cones resulted in significant neurite retraction (Fig. 12A; n = 6; $p \leq 0.05$, t-test). Interestingly, focal application of NMDA in the presence of CTX did not appear to cause significant inhibition of neurite outgrowth (Fig. 12B; n = 6; $p > 0.05$, ANOVA; see Discussion).

Effects of Calcium Influx in Neuronal Somata on Neurite Outgrowth

For all of the above procedures, agents were pressure ejected focally onto growth cones to elicit local influx of calcium. To examine whether more global changes in intracellular calcium might regulate outgrowth of neurites, 25 mM glutamate (n = 7) or high potassium media (n = 7) were pressure ejected onto the cell bodies of DiI-labeled descending brain neurons in culture. One neurite per neuron was selected and measured in detail during the above manipulations. Under these conditions, either glutamate or high potassium applied to neuronal

somata significantly inhibited neurite outgrowth (Fig. 13A,B; $p \leq 0.01$, t-test). In addition, neurite growth rates during application of GLU were significantly less than zero, indicative of neurite retraction († - $p \leq 0.05$; t-test), while for high potassium application, growth rates were not quite significantly different than zero ($p = 0.054$, t-test).

In general, application of agents to a growth cone of a neuron inhibited outgrowth of the associated neurite but had little effect on other neurites from the same neuron (Table 1). To determine if application of glutamate to cell bodies produced global effects on multiple neurites, six neurons were selected with two neurites (Table 2). During application of glutamate to the cell bodies of these neurons, both neurites for each neuron exhibited negative outgrowth rates and significant retraction ($p \leq 0.05$, Sign test).

Role of Action Potentials on Glutamate-Induced Inhibition of Neurite Outgrowth

To test if glutamate-induced retraction of neurites might be dependent on action potentials and the associated influx of Ca^{++} through voltage-gated calcium channels, 25 mM L-glutamate was pressure ejected onto growth cones in the presence of 3 μM TTX. Under these conditions, glutamate inhibited neurite outgrowth compared to pre-control values (Fig. 14, $n = 8$, $p \leq 0.01$, ANOVA). However, unlike the effects of glutamate alone, application of glutamate in the presence of TTX did not result in growth rates that were significantly less than zero ($p > 0.05$, t-test). Interestingly, TTX alone in the culture dish appeared to

inhibit neurite outgrowth (Fig. 14, n = 8, $p \leq 0.01$, Sign test), suggesting the neurons in culture may be spontaneously active.

Glutamate-Induced Changes in Intracellular Calcium Concentrations

To examine the changes in intracellular calcium levels during glutamate-induced neurite retraction, neurons in culture were loaded with Calcium Green-1 AM and subsequently, 25 mM L-glutamate was pressure ejected onto the cell bodies (Fig. 15A,B, n = 18) (see Methods). When visualized with an image intensifier, only about 33% of DiI-labeled descending brain neurons appeared to be clearly labeled with Calcium Green relative to the background, and these were the neurons that were analyzed for fluorescence changes. In some neurons, application of glutamate to cell bodies resulted in an average increase in $\Delta F/F$ of ~2.5% (Fig. 15A), suggesting a small but detectable increase in intracellular calcium. In the remaining experiments, $\Delta F/F$ was approximately zero throughout the duration of glutamate application or decreased by an average of ~2.5% (Fig. 15B). The absence of an increase in $\Delta F/F$ during application in some neurons may have been due to insufficient Calcium Green to detect changes in intracellular calcium levels (see Discussion). All changes in $\Delta F/F$ occurred within the first 15 min, and for simplicity, graphical representations show the $\Delta F/F$ of the control images followed by the first 15 min in which L-glutamate application was applied to neurons.

Control experiment for the effects of DMSO

Some agents used in sections below are not readily soluble in aqueous solutions and needed to be dissolved in other solvents, such as

dimethylsulphoxide (DMSO). As a preliminary control, different concentrations of DMSO were added to culture dishes to determine the maximum bath concentration that did not significantly effect neurite outgrowth. At a concentration of 1% in media (n = 7), DMSO appeared to produce some inhibition on neurite outgrowth, but the effects were not significant compared to pre-control conditions in media alone (Fig. 16A, p = 0.08; unpaired t-test with Welch correction). In contrast, a 0.5% concentration of DMSO (n = 7) did not produce significant changes in neurite outgrowth (Fig. 16B, p = 0.39; ANOVA), and this concentration was used to dissolve certain agents for testing.

Activation of Second Messengers on Glutamate-induced Neurite Retraction

Glutamate-induced retraction of neurites of lamprey descending brain neurons may be due, in part, to an increase in cAMP signaling levels. To determine if cAMP signaling alone can induce neurite retraction, agents that operate via cAMP signaling pathways were tested. Application of 10 mM dbcAMP (n = 17), 62.5 μM Forskolin (n = 21), or 100 μM IBMX (n = 23) inhibited neurite outgrowth compared to pre-control values (Fig. 17A-C, *** - $p \leq 0.002$; ANOVA), suggesting that enhancing cAMP signaling alone can induce inhibition of neurite outgrowth. In addition, neurite growth rates during application of dbcAMP and Forskolin were significantly less than zero, indicative of neurite retraction (Fig. 17A-C, †† - $p \leq 0.01$; t-test), while growth rates in the presence of IBMX were not significantly less than zero ($p = 0.25$, t-test). These results may indicate that dbcAMP and Forskolin artificially increased cAMP signaling levels

above those that naturally accumulate when cAMP degradation is blocked by IBMX.

Inhibition of Second Messengers on Glutamate-induced Neurite Retraction

In order to determine growth rates of several neurons in a single culture dish, control experiments were conducted in which glutamate was bath applied to determine the lowest bath concentration that induced significant neurite retraction of all neurites, similar to the effects of focal application of 25 mM glutamate to single neurites. Bath application of 0.25 mM L-glutamate for an average of 60 min did not induce significant inhibition of neurite outgrowth (Fig. 18A; 0.013 $\pm$ 0.010 μm/min, n = 6, $p > 0.05$, t-test) compared to pre-control values (0.008 $\pm$ 0.007 μm/min). In contrast, bath application of 0.5 mM L-glutamate for an average of 60 min resulted in significant inhibition of neurite outgrowth (Fig. 18B, -0.017 $\pm$ 0.029 μm/min, n =23, *** - $p = \leq 0.002$, t-test) compared to pre-control values (0.020 $\pm$ 0.015 μm/min). Furthermore, the experimental growth rates were significantly less than zero (Fig. 18B, †† - $p \leq 0.01$; t-test), indicative of neurite retraction, similar to the effects of focal application of 25 mM glutamate to single growth cones.

To determine if an increase in cAMP signaling levels might contribute to glutamate-induced inhibition of neurite outgrowth, 0.5 mM L-glutamate was bath applied to culture dishes after 10 μM H89 had been bath applied for 60 min (Fig. 19, n = 19). Under these conditions, glutamate no longer resulted in neurite retraction or inhibition of neurite outgrowth, effects that were observed reliably during application of glutamate alone. In a control experiment, 10 μM H89 alone

(0.015 $\pm$ 0.015 μm/min) did not significantly change neurite growth rates compared to pre-control conditions (0.015 $\pm$ 0.001 μm/min; $p > 0.05$; $n = 19$; ANOVA). Taken together, the above results suggest that cAMP is necessary (Fig. 19) and sufficient (Fig. 17A-C) for glutamate-induce neurite retraction.

Figure 1. The effects of L-glutamate on neurite outgrowth as well as the extracellular channels/receptors blockers that were used in the present study. R1 = NMDA ionotrophic receptor, R2 = Kainate/AMPA ionotropic receptor, R3 = metabotropic receptor, G = G protein, VGCC = voltage-gated calcium channel, KYN = kynurenic acid, CTX = ω-conotoxin MVIIC, Co = cobalt, Cd = cadmium.

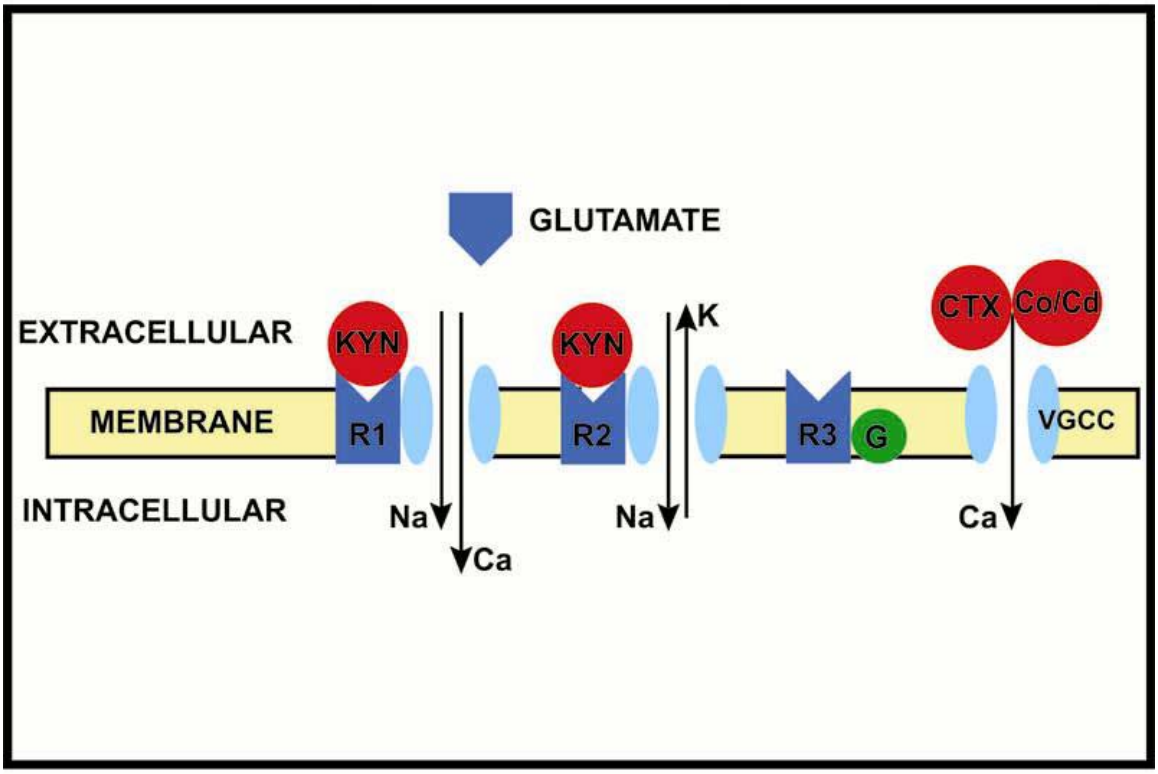

GLUTAMATE
EXTRACELLULAR
KYN
KYN
K
CTX
Co/Cd
MEMBRANE
R1
R2
R3
G
VGCC
INTRACELLULAR
Na
Ca
Na
Ca

FIG. 1

Figure 2. Pathways by which cAMP may regulate neurite outgrowth as well as agents that activate or inhibit this pathway that were used in the present study. R3 = metabotropic receptor, G = G protein, AC = adenylate cyclase, PKA = protein kinase A, ER = endoplasmic reticulum, dbcAMP = dibutyryl cyclic AMP, IBMX = 3-isobutyl-1-methylxanthine, H89 = N - [2 - (p-Bromocinnamyl-amino) ethyl] – 5 - isoquinolinesulfonamide dihydrochloride. Arrows correspond to stimulatory effects while bars correspond to inhibitory effects.

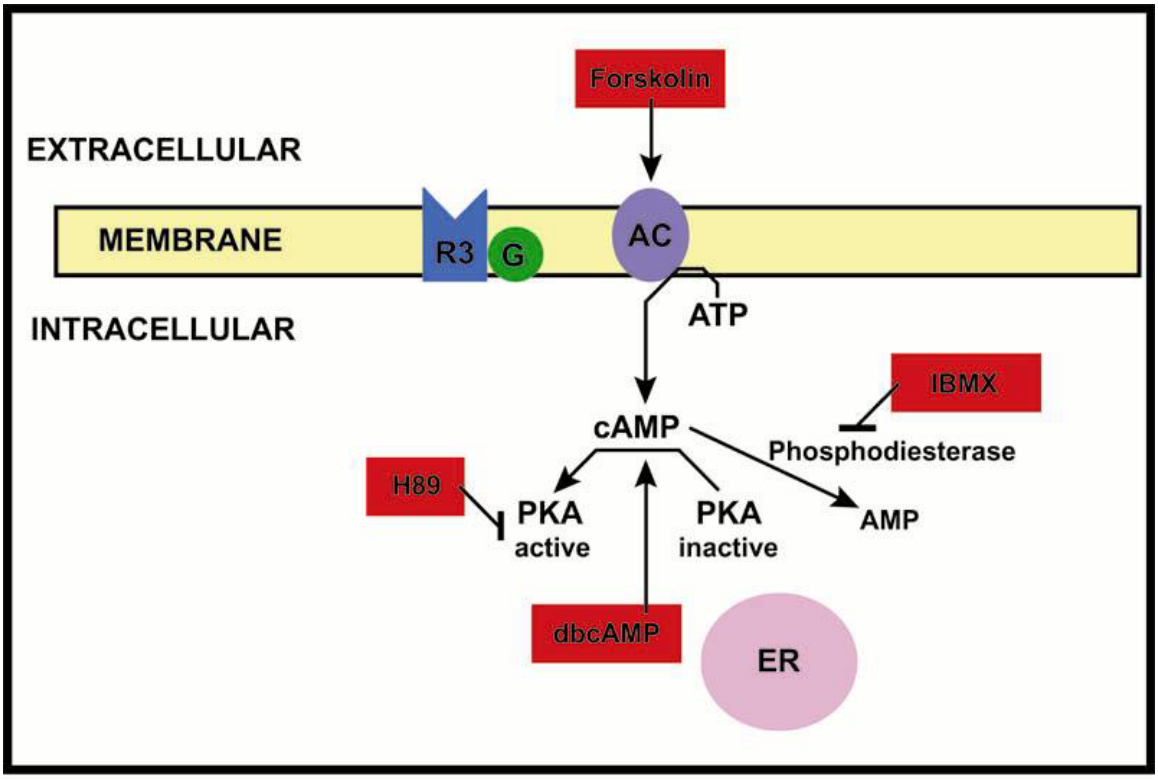

Forskolin
EXTRACELLULAR
MEMBRANE
R3
G
AC
ATP
INTRACELLULAR
IBMX
cAMP
Phosphodiesterase
H89
PKA
active
PKA
inactive
AMP
dbcAMP
ER

FIG. 2

Figure 3. (A) Diagram of dorsal view of lamprey brain. (upper) Descending brain neurons (small dots - unidentified neurons; large dots - Müller and Mauthner cells) retrogradely labeled by application of HRP to the spinal cord at 20% BL. (lower) Outlines of cell groups containing descending brain neurons. Reticulospinal (RS) neurons are in the mesencephalic reticular nucleus (MRN), as well as the anterior (ARRN), middle (MRRN), and posterior (PRRN) rhombencephalic reticular nuclei. Other cell groups: Diencephalic (Di) as well as anterolateral (ALV), dorsolateral (DLV), and posterolateral (PLV) vagal groups. (B) In a live whole-mount of the brain prior to dissociation, DiI-labeled reticulospinal (RS) neurons were clearly visible in the (B1) right ARRN and (B2) left PRRN. (C) Descending brain neuron in culture (soma in upper right quadrant) under (C1) brightfield and the same neuron (C2) shown labeled with DiI. Scale bar = 100 μm.

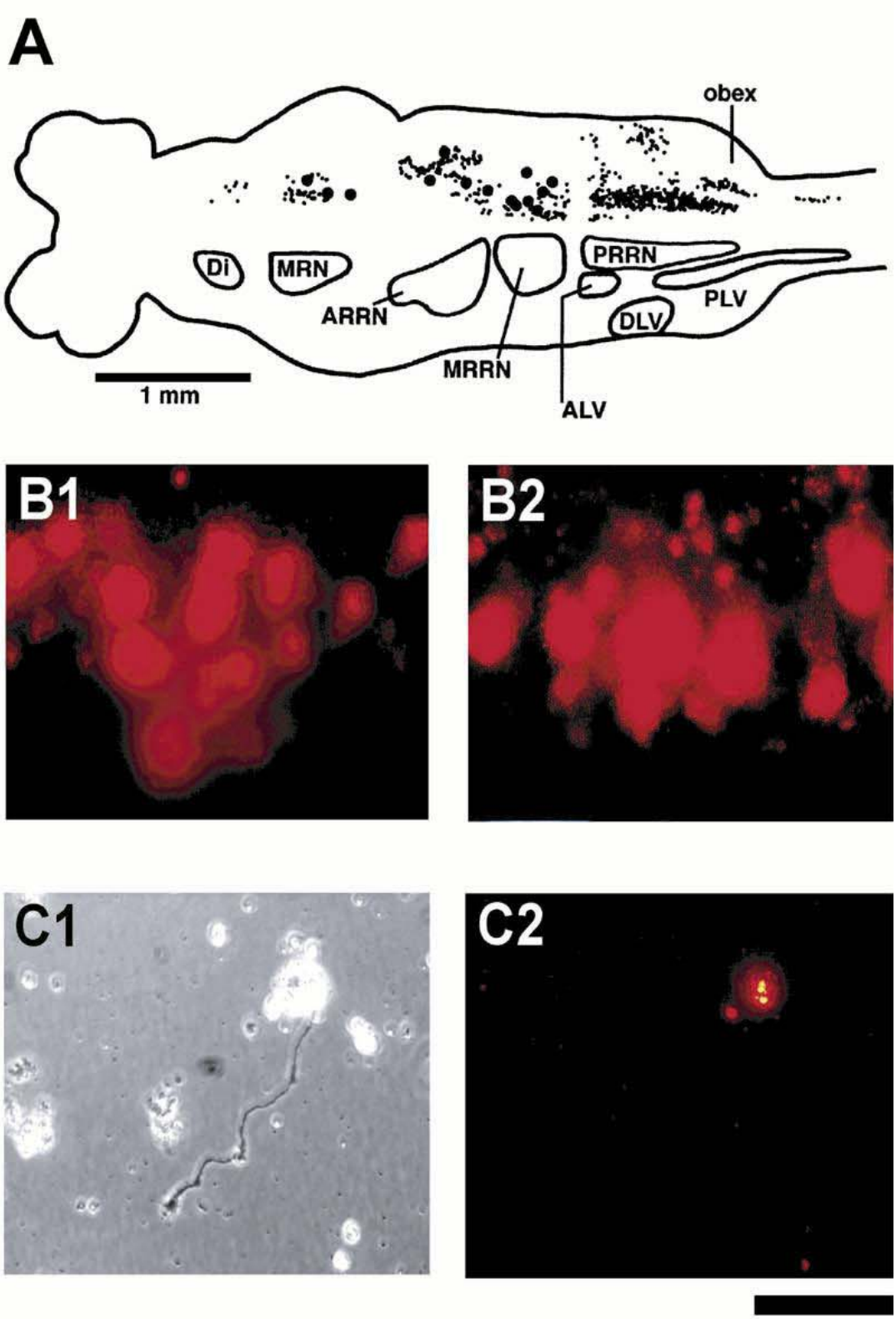


FIG. 3

Figure 4. Histograms of the distributions of (A) soma diameters, (B) numbers of main neurite processes, and (C) neurite lengths of DiI-labeled descending brain neurons in culture that were examined in the present study. Note that the neurite lengths are only for those processes that were specifically analyzed and do not include other neurites on the same neurons.

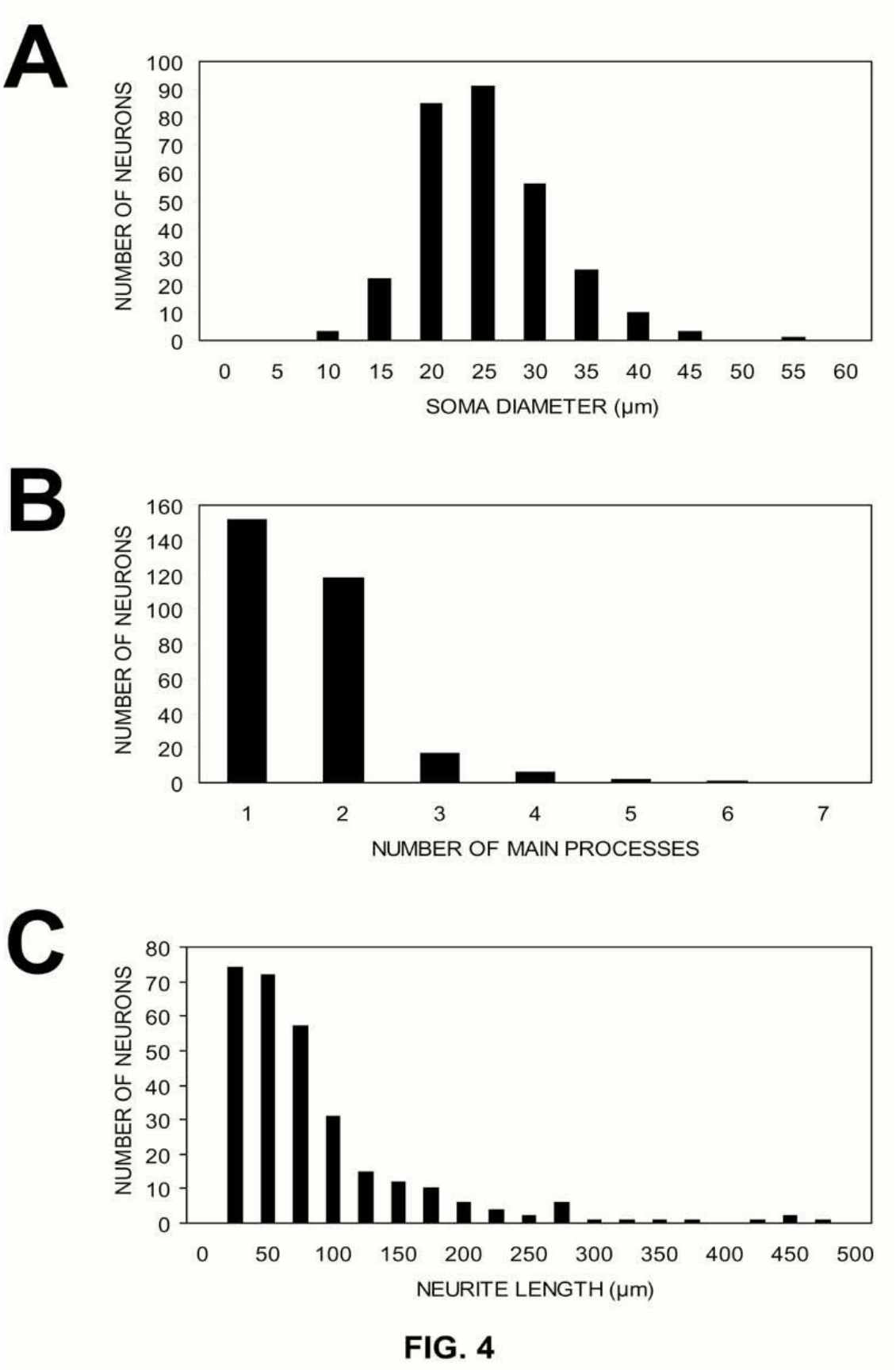

A
NUMBER OF NEURONS
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55 60
SOMA DIAMETER (µm)
B
NUMBER OF NEURONS
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7
NUMBER OF MAIN PROCESSES
C
NUMBER OF NEURONS
80
70
60
50
40
30
20
10
0
0 50 100 150 200 250 300 350 400 450 500
NEURITE LENGTH (µm)

FIG. 4

Figure 5. (A) Four sequential images (A1-A4), each separated by ~60 min, from a time-lapse recording showing a DiI-labeled descending brain neuron in culture (arrowheads = cell body) and a neurite that ends in a growth cone (black arrows). The images clearly show that the growth cone extended from its initial position into the left, upper quadrant of the field of view (compare arrow and dotted line in A4). (B) Four sequential images (B1-B4), each separated by ~16 min, from a time-lapse recording showing a DiI-labeled descending brain neuron (black arrowheads = cell body) in culture and neurites that end in growth cones (black arrows). Pressure ejection of 25 mM glutamate in media without serum from a micropipette (white arrows) onto one of the growth cones resulted in very clear neurite retraction. Scale bar = (A) 50 μm, (B) 50 μm.

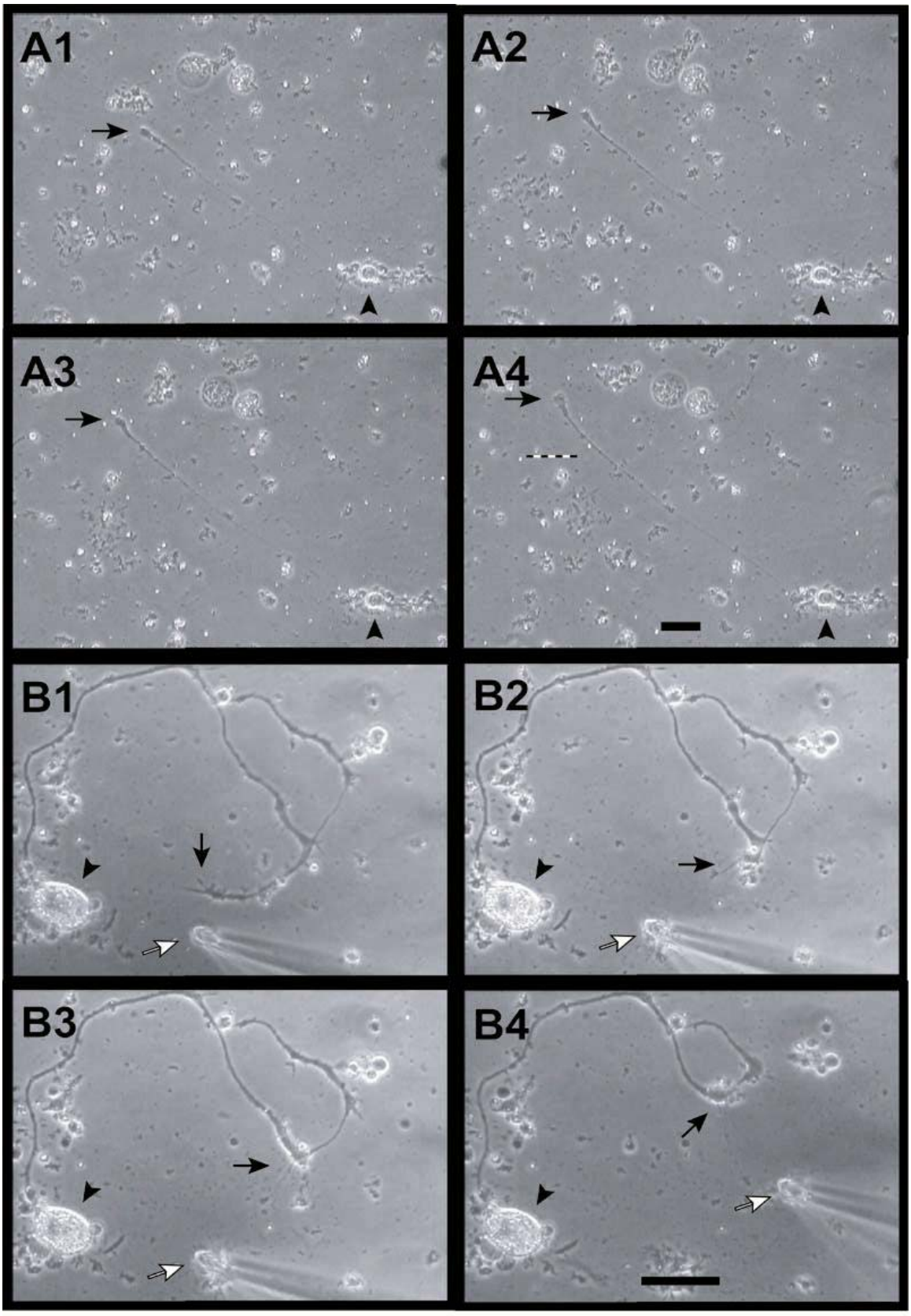


FIG. 5

Figure 6. Graphs showing examples of neurite lengths vs. time before, during, and after pressure ejection of (A) Ringer's solution, (B) 5 mM L-glutamate, (C) 25 L-glutamate, and (D) 25 mM L-glutamate in the presence of 10 mM kynurenic acid. (A-C) Each trace represents the length of a different neurite and consists of three line segments: 1 = Pre-Control; 2 (thick lines) = experimental during which agents were pressure ejected onto growth cones during solid bars; and 3 = Post-Control (see Fig. 7). (D) Three traces represent the lengths of three different neurites and consist of four line segments: 1 = Pre-Control neurite growth with normal media in the dish; 2 = neurite growth in the presence of 10 mM kynurenic acid (KYN) alone; 3 = growth during application of 25 mM glutamate to growth cones in the presence of KYN (thick lines during solid bars); and 4 = Post-Control growth in the presence of KYN alone.

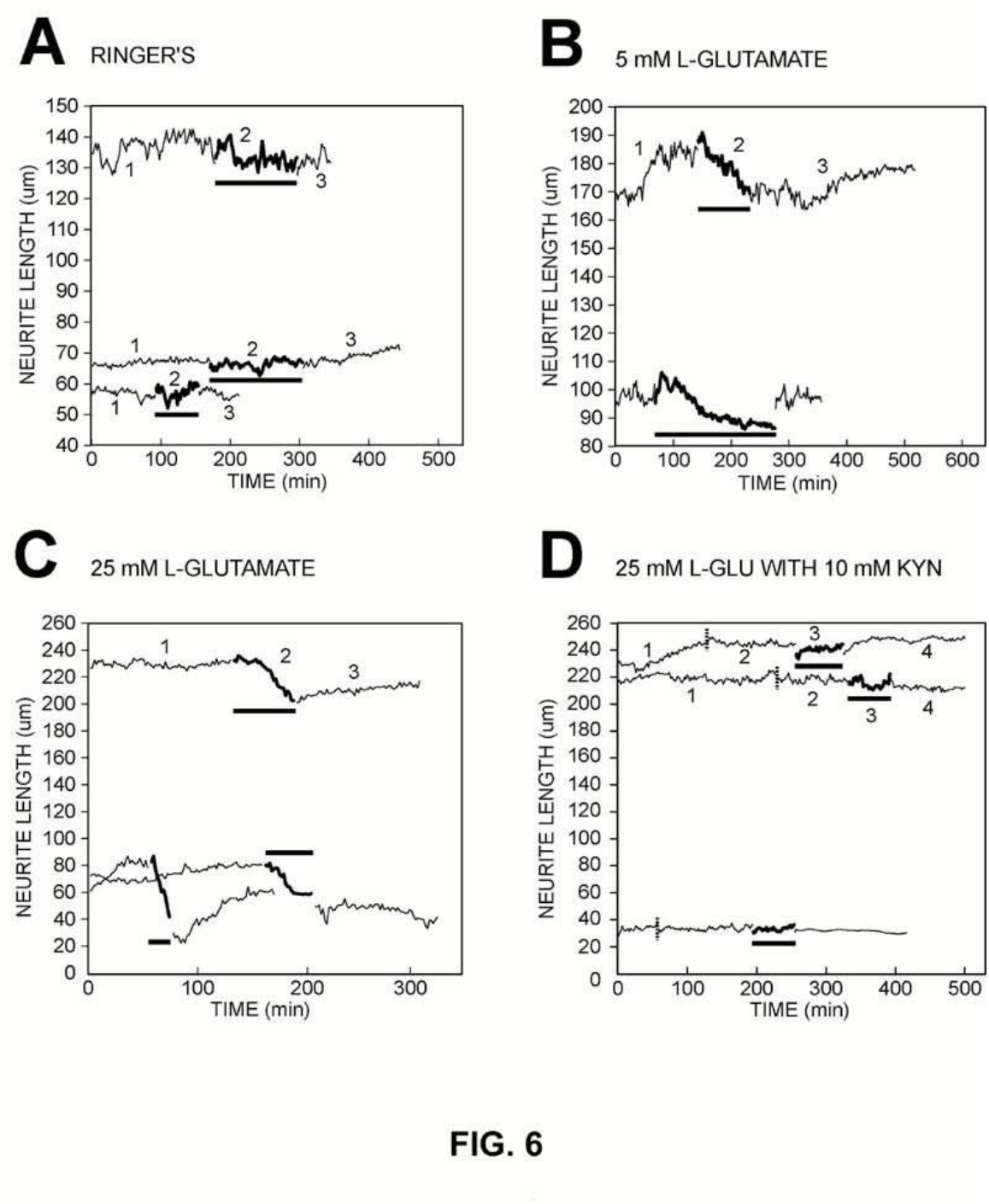

A
RINGER'S
B
5 mM L-GLUTAMATE
C
25 mM L-GLUTAMATE
D
25 mM L-GLU WITH 10 mM KYN
NEURITE LENGTH (um)
TIME (min)

FIG. 6

Figure 7. Average neurite growth rates (bars = means, vertical lines = SD) during different experimental conditions. (A-C) Mean growth rates before (left bars, Pre-Control), during (middle bars) and after (right bars, Post-Control) pressure ejection of the following agents onto growth cones of neurites: (A) Ringer's solution (n = 15); (B) 5 mM L-glutamate (n = 12); and (C) 25 mM L-glutamate (n = 29). (D) (left bar) Pre-control growth rate in normal media and (second bar from left) growth rate in the presence of 10 mM kynurenic acid (KYN). (second bar from right) Growth rate during application of 25 mM glutamate to growth cones in the presence of 10 mM kynurenic acid (n = 11). (right bar) Post-control neurite growth rate following the above manipulations and still in the presence of KYN. In this and all similar bar graph figures, the plus/minus numbers next to each bar indicate the numbers of neurites that displayed positive (+)/negative (-) growth rates. During pressure ejection of 25 mM glutamate, there was significant inhibition of neurite growth rates compared to Pre-Control values (*** - $p \leq 0.002$, one-way ANOVA). In addition, these particular growth rates were significantly less than zero (t-test or Wilcoxon rank sum test, †† - $p \leq 0.01$), indicative of neurite retraction.

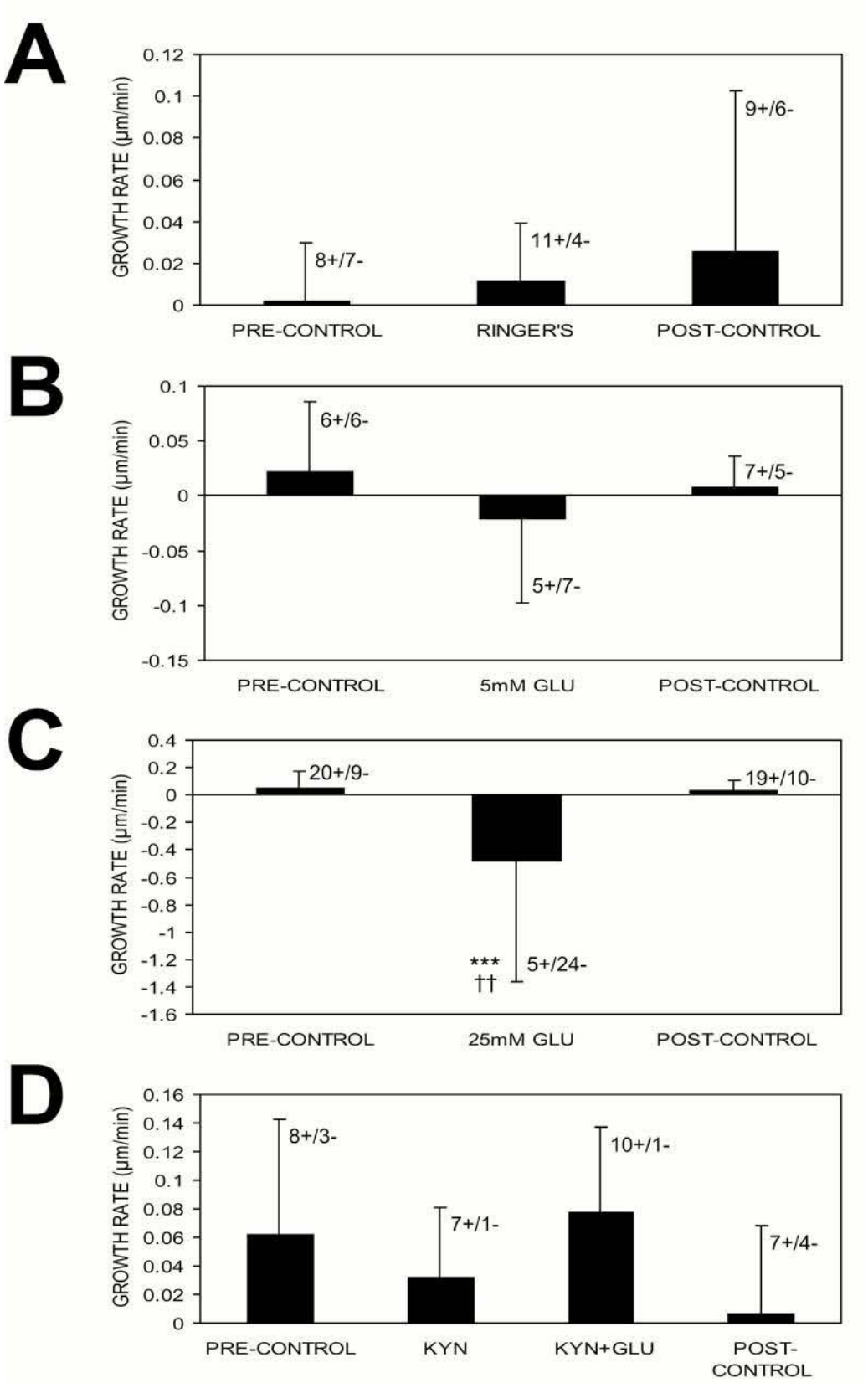

A
GROWTH RATE (µm/min)
0.12
0.1
0.08
0.06
0.04
0.02
0
8+/7-
11+/4-
9+/6-
PRE-CONTROL
RINGER'S
POST-CONTROL
B
GROWTH RATE (µm/min)
0.1
0.05
0
-0.05
-0.1
-0.15
6+/6-
7+/5-
5+/7-
PRE-CONTROL
5mM GLU
POST-CONTROL
C
GROWTH RATE (µm/min)
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
20+/9-
19+/10-
*** ††
5+/24-
PRE-CONTROL
25mM GLU
POST-CONTROL
D
GROWTH RATE (µm/min)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
8+/3-
7+/1-
10+/1-
7+/4-
PRE-CONTROL
KYN
KYN+GLU
POST-CONTROL

FIG. 7

Figure 8. Successive time-lapse images (A→D), separated by ~6 min, of a DiI-labeled descending brain neuron (arrowheads = cell body) during pressure ejection from a micropipette (white arrows) of 25 mM L-glutamate onto a growth cone (black arrows). Pressure ejection of glutamate onto a single growth cone inhibited outgrowth of that neurite, but did not noticeably affect the lengths of other neurites originating from the same neuron. (compare black arrows in A → D; see Table 1). Scale bar = 50 μm.

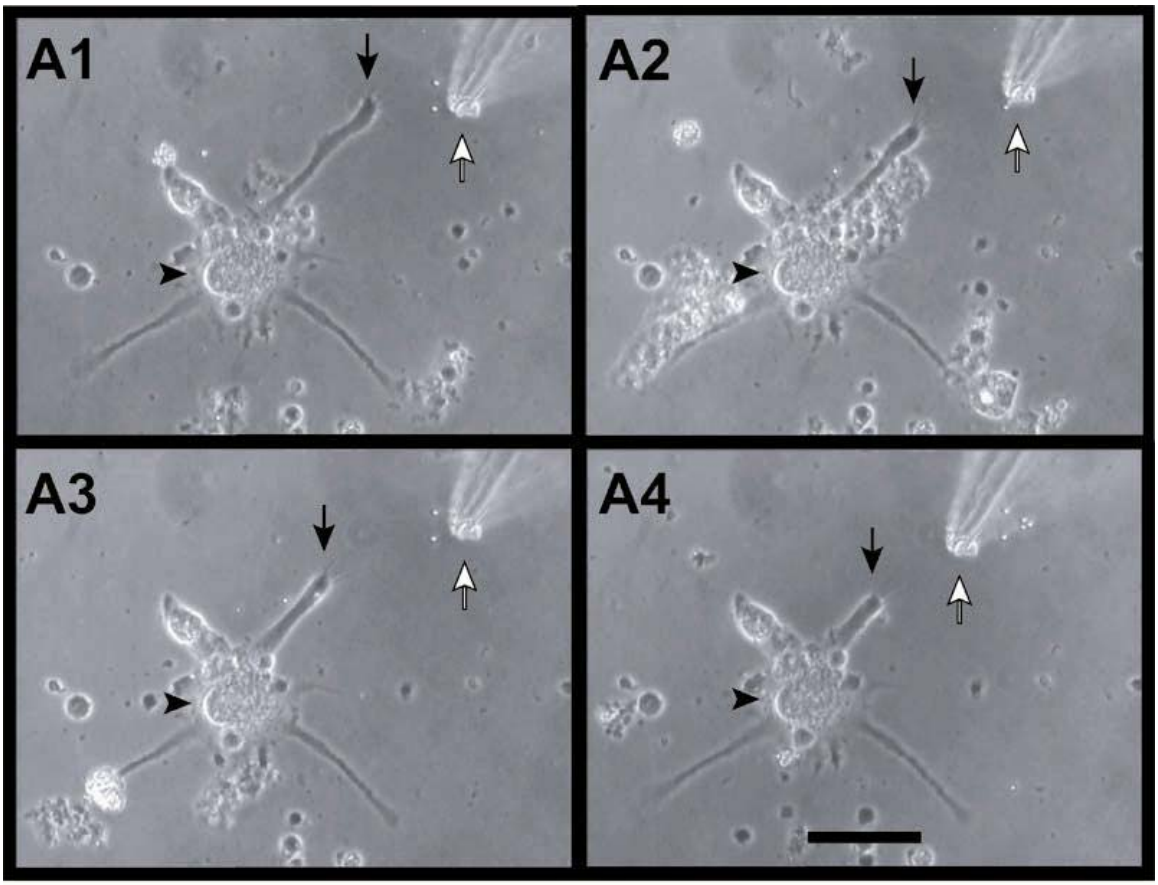


FIG. 8

Table 1

Effects of focal application of glutamate to single growth cones on growth rates of multiple neurites from the same neuron

NEURON	SOMA DIAMETER	PRE-CONTROL GROWTH RATES[a]	LENGTH BEFORE[b]	EXPERIMENTAL GROWTH RATES[c]
A	28.82096	0.3046 ± 0.3046 0.0119 ± 0.3392	82.56286 97.02139	-2.2732 ± 2.2756 0.1611 ± 0.3881
B	29.58659	0.0012 ± 1.1405 0.0090 ± 0.4695	81.62180 50.98103	-0.3911 ± 1.2420 0.0460 ± 0.5679
C	29.45519	0.0164 ± 0.5995 0.0090 ± 0.1468	73.29633 48.21057	-0.0638 ± 0.3500 0.0031 ± 0.9320
D	20.80696	0.0084 ± 0.2263 0.0143 ± 0.2010	22.49567 20.08931	-0.0304 ± 0.3292 0.0596 ± 0.1591
E	22.28105	0.0625 ± 0.1693 0.0252 ± 0.2049	48.71337 61.81232	-0.0039 ± 0.1343 0.0143 ± 0.1589
F	31.41429	0.0203 ± 0.4014 0.0025 ± 0.3232	129.1177 65.66854	-0.1608 ± 0.2566 0.1048 ± 0.3694

a = average pre-control growth rates for neurons with two major neurites

b = initial lengths of both neurites for each neuron

c = average experimental growth rates for both neurites of each neuron during application of 25 mM L-glutamate to the growth cone of the first neurite in the list for each neuron

Statictics: Application of L-glutamate to one growth cone of a neuron in culture significantly inhibited outgrowth of that neurite ($p \leq 0.05$, Sign test), but did not significantly inhibit outgrowth of the second neurite originating from the same neuron ($p > 0.05$, Sign test).

Figure 9. Successive time-lapse images (A → D), separated by ~40 min, of a DiI-labeled descending brain neuron (arrowheads = cell body) during pressure ejection from a micropipette (white arrows) of high potassium media (31 mM) onto a growth cone (black arrows). Pressure ejection of high potassium media onto growth cones inhibited neurite outgrowth (compare black arrows in A → D). Scale bar = 100 μm.

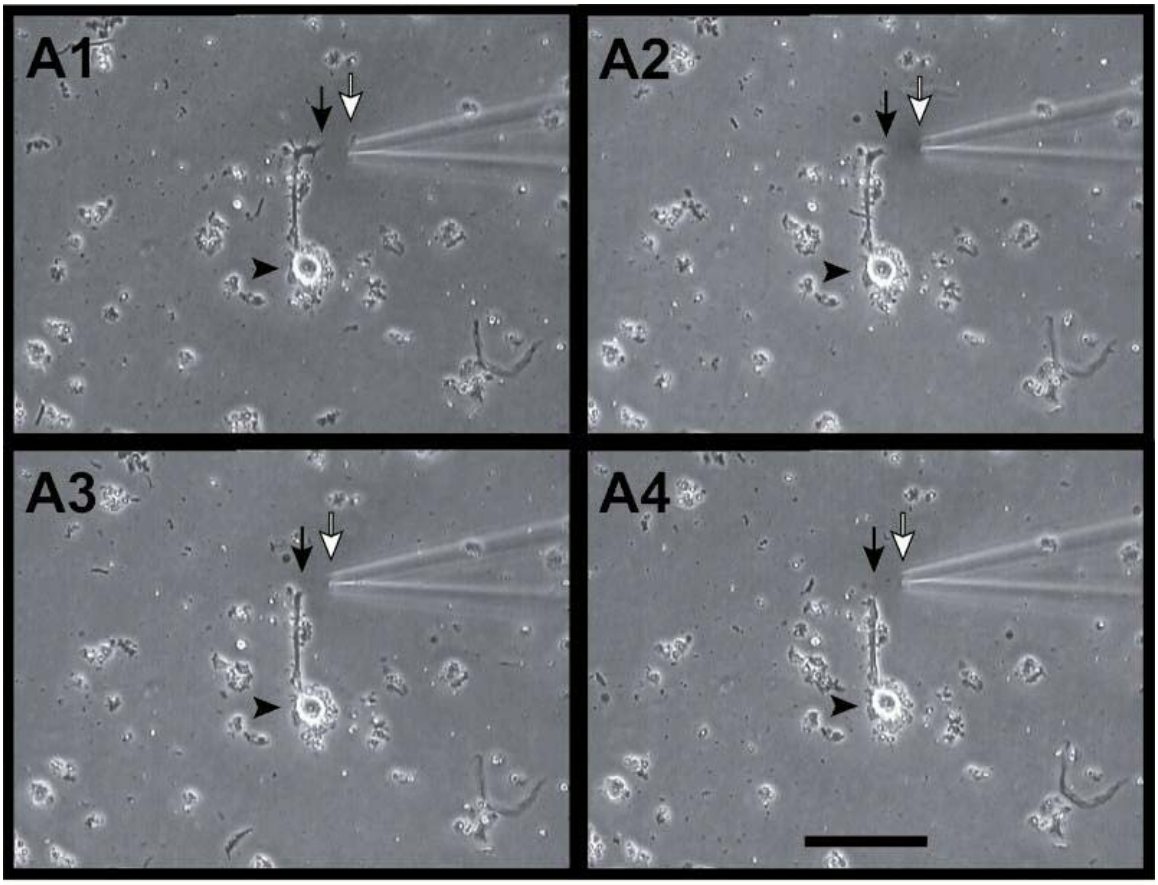


FIG. 9

Figure 10. Mean neurite growth rates (bars = means; vertical lines = SDs) before (Pre-Control), during (Exp), and after (Post-Control) pressure ejection onto growth cones of (A) high potassium (31 mM; K+) media alone (n = 8), (B) high potassium in the presence of bath-applied 2 mM cobalt (n = 6), and (C) high potassium in the presence of bath-applied 100 μM cadmium (n = 5) (see Methods). Post-control neurite growth rates for high potassium and 2 mM cobalt (B, right bar), and high potassium and 100 μM cadmium (C, right bar) were still in the presence of cobalt and cadmium, respectively. (A) Pressure ejection of high K+ media alone (middle bar) resulted in significant inhibition of neurite growth rates compared to pre-control values (* - $p \leq 0.05$), but experimental growth rates were not quite significantly different than zero ($p = 0.065$, t-test). (B,C) Neurite growth rates during pressure ejection of high K+ media with (B) cobalt or (C) cadmium (middle bars) were not significantly different than pre-control values ($p > 0.05$; ANOVA). It should be noted that cobalt (-0.007 ± 0.319 μm/min; n = 6) or cadmium (0.159 ± 0.158 μm/min; n = 8) alone in the bath did not result in significant changes in neurite growth rates compared to pre-control conditions (0.207 ± 0.146 μm/min or 0.259 ± 0.141 μm/min, respectively; $p > 0.05$; ANOVA).

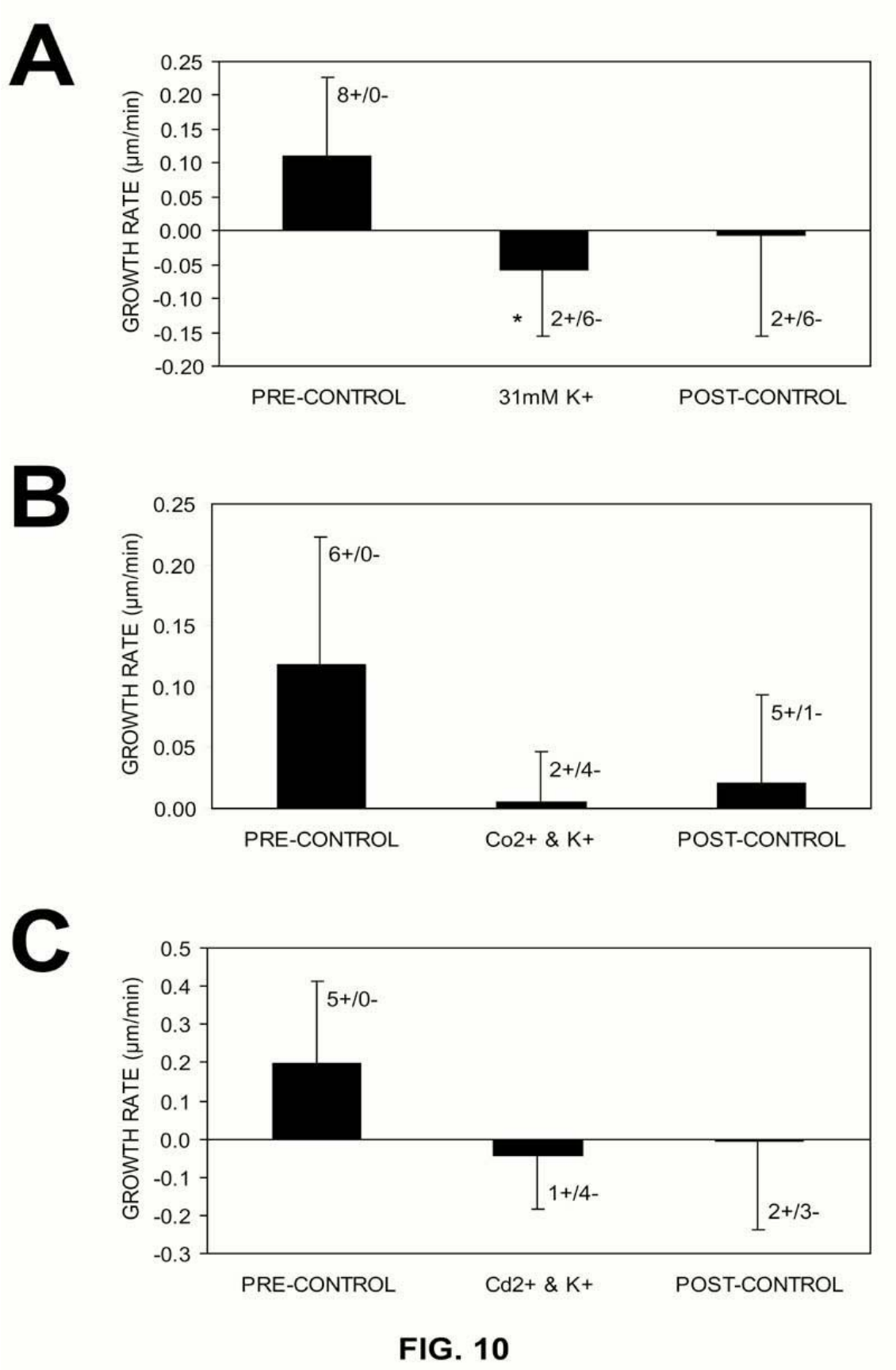

A
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
GROWTH RATE (µm/min)
8+/0-
*
2+/6-
2+/6-
PRE-CONTROL
31mM K+
POST-CONTROL
B
0.25
0.20
0.15
0.10
0.05
0.00
GROWTH RATE (µm/min)
6+/0-
2+/4-
5+/1-
PRE-CONTROL
Co2+ & K+
POST-CONTROL
C
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
GROWTH RATE (µm/min)
5+/0-
1+/4-
2+/3-
PRE-CONTROL
Cd2+ & K+
POST-CONTROL

FIG. 10

Figure 11. Mean neurite growth rates (bars = means; vertical lines = SDs) before (left bars, Pre-Control), during (middle bars), and after (right bars, Post-Control) pressure ejection of the following agents onto growth cones of neurites: (A) 25 mM L-glutamate (GLU) in the presence of bath-applied 2 mM ω-conotoxin MVIIC (CTX) (n = 11); (B) high potassium media (31 mM; K+) in the presence of bath-applied 2 mM ω-conotoxin MVIIC (CTX) (n = 5) (see Methods). Post-control neurite growth rates were still in the presence of CTX. (A) Pressure ejection of GLU in the presence of CTX still resulted in significant inhibition of neurite outgrowth compared to pre-control values (** - $p \leq 0.01$; ANOVA). (B) Neurite growth rates during pressure ejection of high potassium media in the presence of CTX were not significantly different than pre-control values ($p > 0.05$; ANOVA). It should be noted that CTX alone (0.208 ± 0.194 μm/min) in the bath did not significantly change neurite growth rates compared to pre-control conditions (0.187 ± 0.129 μm/min; $p > 0.05$; ANOVA).

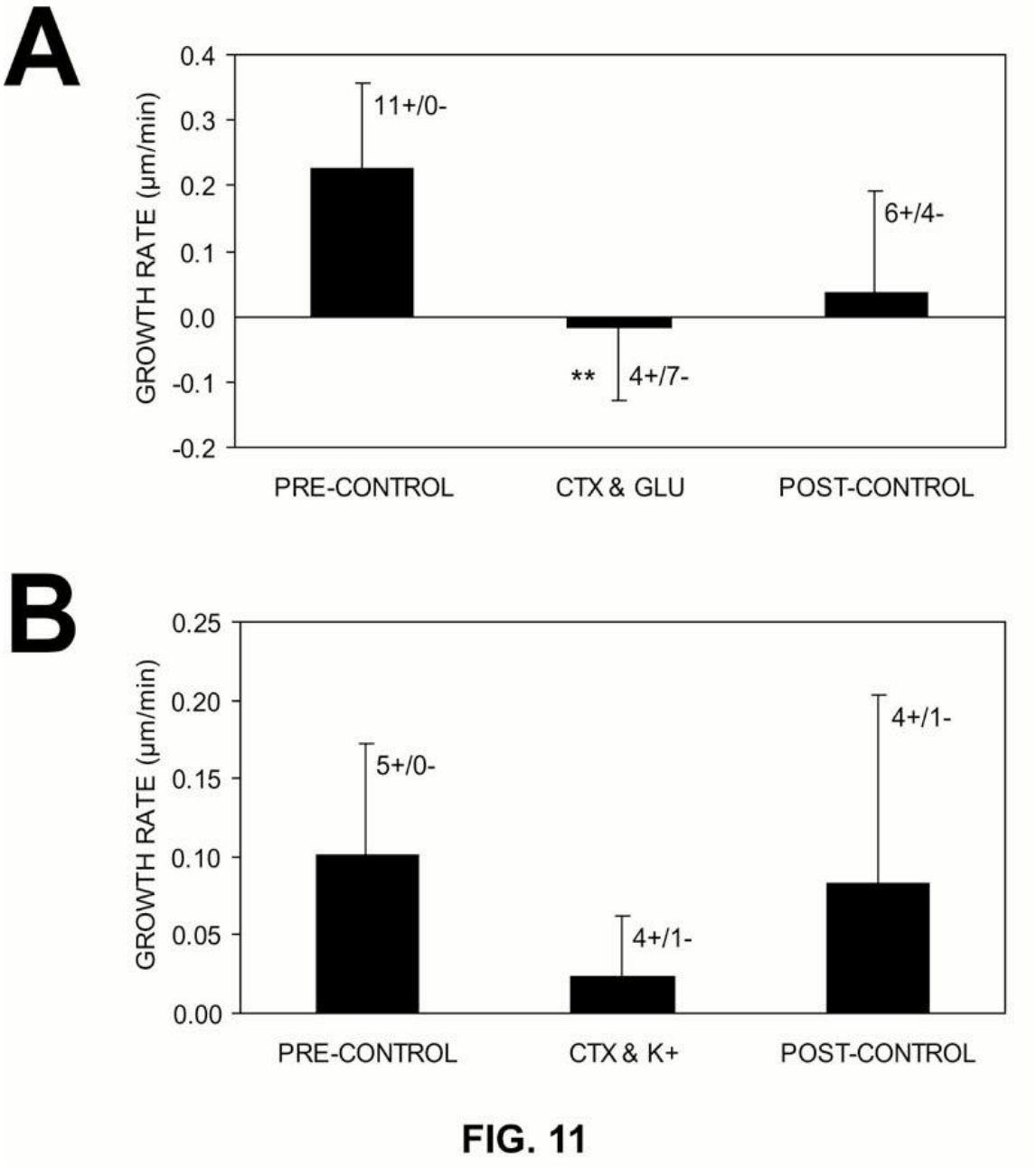

A
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
GROWTH RATE (µm/min)
11+/0-
6+/4-
**
4+/7-
PRE-CONTROL
CTX & GLU
POST-CONTROL
B
0.25
0.20
0.15
0.10
0.05
0.00
GROWTH RATE (µm/min)
5+/0-
4+/1-
4+/1-
PRE-CONTROL
CTX & K+
POST-CONTROL

FIG. 11

Figure 12. Mean neurite growth rates (bars = means; vertical lines = SDs) before (left bar, Pre-Control), during (middle bar), and after (right bar, Post-Control) pressure ejection of the following agents onto growth cones of neurites: (A) 1 mM NMDA alone (n = 6); (B) 1 mM NMDA in the presence of bath-applied 2 mM ω-conotoxin MVIIC (CTX) (n = 6) (see Methods). (A) Pressure ejection of NMDA resulted in significant inhibition of neurite growth rates compared to pre-control values (** - $p \leq 0.01$; ANOVA). In addition, neurite growth rates during application of NMDA were significantly less than zero, indicative of neurite retraction († - $p \leq 0.05$; t-test). (B) Neurite growth rates during pressure ejection of NMDA in the presence of CTX were not significantly different than pre-control values ($p > 0.05$; ANOVA).

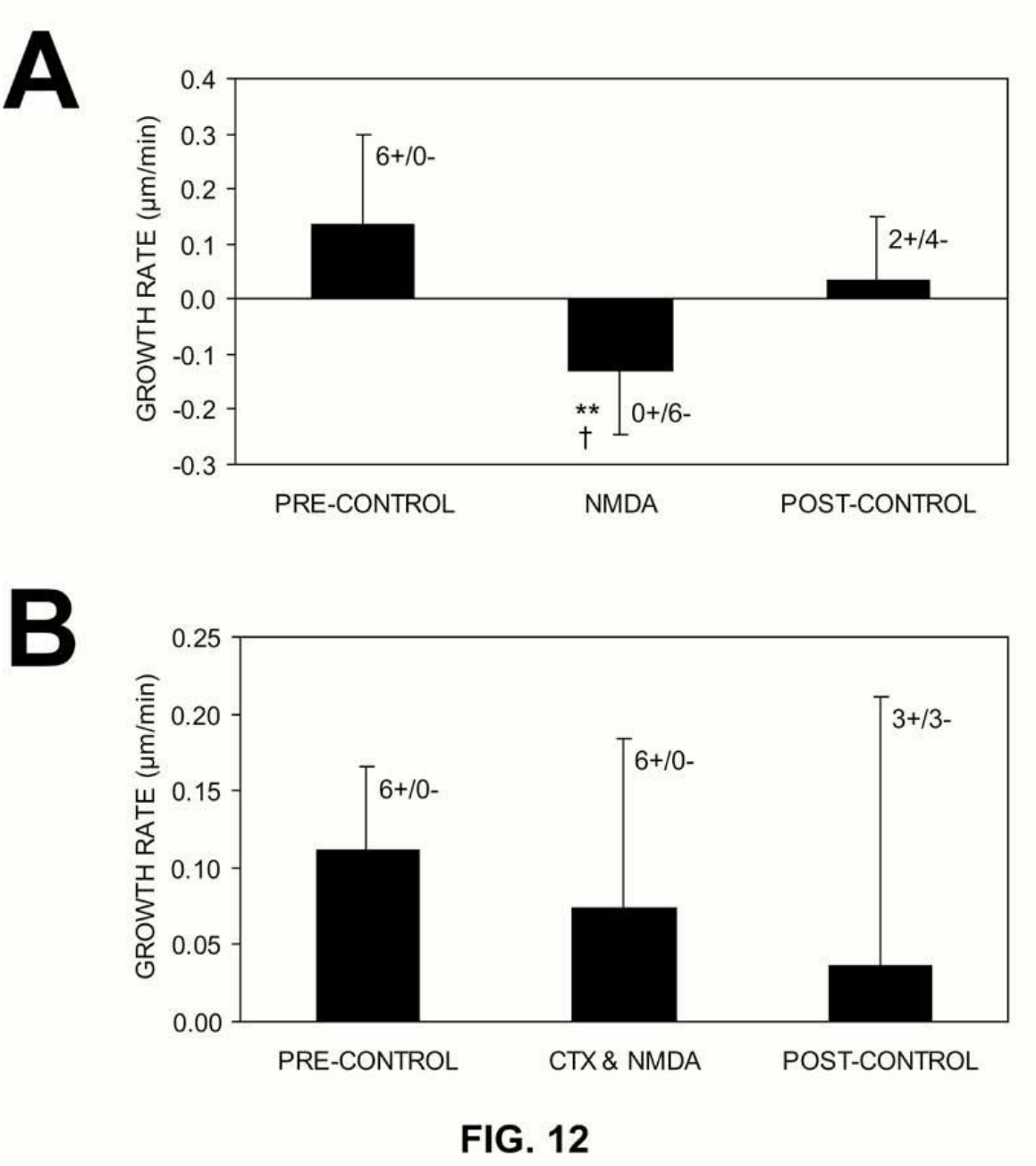

A
GROWTH RATE (µm/min)
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
6+/0-
2+/4-
**
†
0+/6-
PRE-CONTROL
NMDA
POST-CONTROL
B
GROWTH RATE (µm/min)
0.25
0.20
0.15
0.10
0.05
0.00
6+/0-
6+/0-
3+/3-
PRE-CONTROL
CTX & NMDA
POST-CONTROL

FIG. 12

Figure 13. Mean neurite growth rates (bars = means; vertical lines = SDs) before (left bars, Pre-Control), during (middle bars, GLU or K+) and after (right bars, Post-Control) pressure ejection of the following agents onto the cell bodies of DiI-labeled neurons in culture: (A) 25 mM L-glutamate (GLU) in media (n = 5); (B) 31 mM potassium (K+) in media (n = 7). Pressure ejection of L-glutamate or high potassium media onto neuronal cell bodies inhibited outgrowth of the neurite of interest compared to pre-control values (** - $p \leq 0.01$; t-test). In addition, neurite growth rates during application of GLU were significantly less than zero, indicative of neurite retraction († - $p \leq 0.05$; t-test), while for high potassium application, growth rates were not quite significantly different than zero ($p = 0.054$, t-test). For application of either glutamate or high potassium, the growth rates of other neurites whose lengths were not measured also were inhibited (see Table 2).

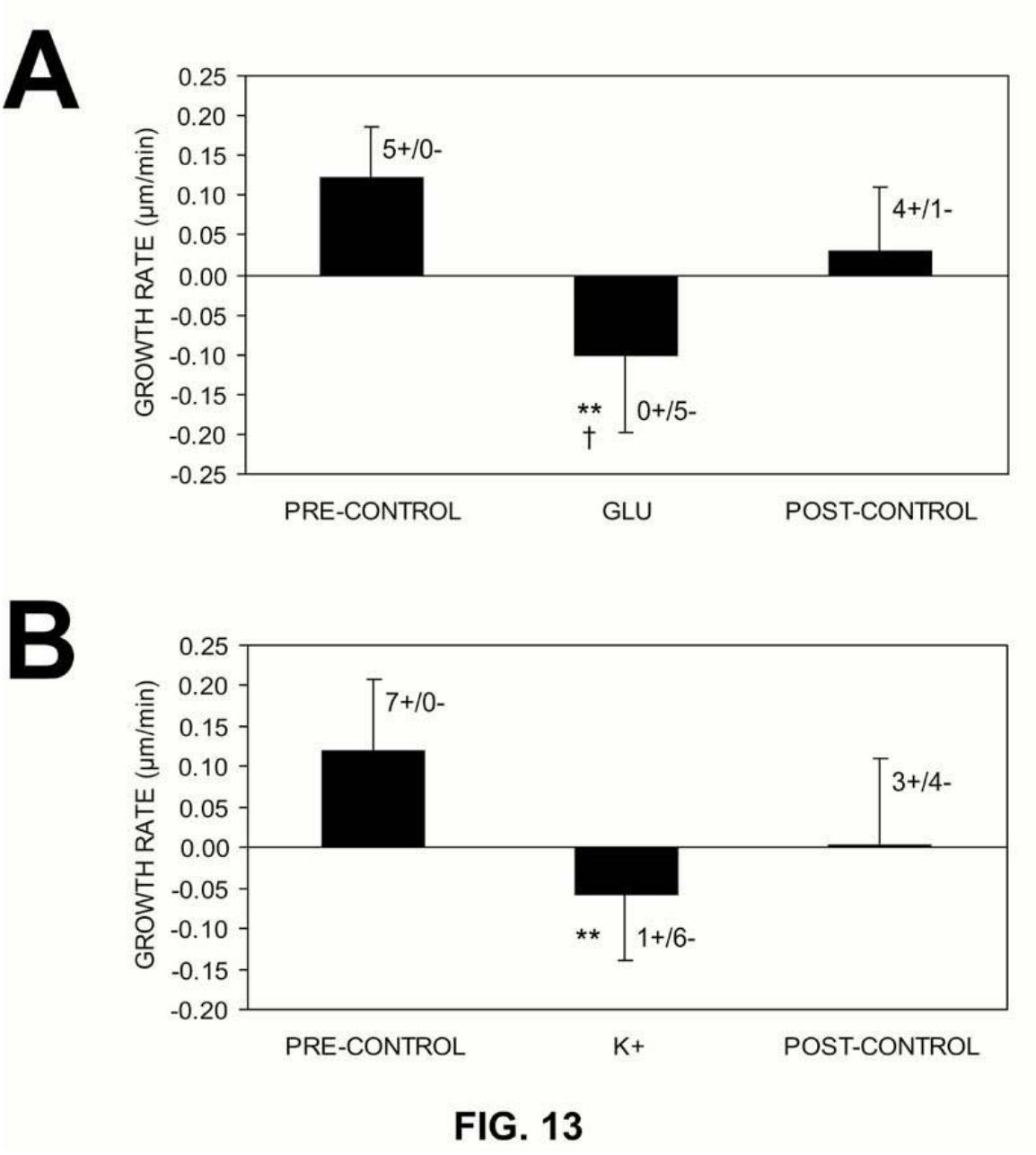

A
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
GROWTH RATE (µm/min)
5+/0-
4+/1-
** †
0+/5-
PRE-CONTROL
GLU
POST-CONTROL
B
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
GROWTH RATE (µm/min)
7+/0-
3+/4-
**
1+/6-
PRE-CONTROL
K+
POST-CONTROL

FIG. 13

Table 2

Effects of application of glutamate to neuronal cell bodies on growth rates of multiple neurites from the same neuron

NEURON	SOMA DIAMETER	PRE-CONTROL GROWTH RATES[a]	LENGTH BEFORE[b]	EXPERIMENTAL GROWTH RATES[c]
A	35.16306	0.0617 ± 0.2983 0.0493 ± 0.1971	85.97248 71.31626	-0.0668 ± 0.3697 -0.0849 ± 0.2213
B	27.61746	0.1739 ± 0.5959 0.0233 ± 0.0641	46.77947 91.15091	-0.0604 ± 0.4228 -0.1112 ± 0.1092
C	18.15357	0.1050 ± 0.3435 0.0149 ± 0.1824	83.51624 52.59206	-0.0529 ± 0.2284 -0.0315 ± 0.2281
D	27.21359	0.0217 ± 0.1717 0.0479 ± 0.1550	60.38480 53.44588	-0.0806 ± 0.0678 -0.0755 ± 0.1204
E	24.16720	0.0142 ± 0.1215 0.0092 ± 0.1220	33.30718 19.87966	-0.0298 ± 0.2272 -0.0615 ± 0.1180
F	21.49187	0.0695 ± 0.4956 0.0442 ± 0.3391	111.2172 75.14671	-0.0546 ± 0.3845 -0.0411 ± 0.1922

a = average pre-control growth rates for neurons with two major neurites
b = initial lengths of both neurites for each neuron before application of glutamate
c = average experimental growth rates for both neurites of each neuron during application of 25 mM L-glutamate to neuronal cell bodies for each neuron
Statistics: Application of L-glutamate to neuronal somata significantly inhibited outgrowth of both neurites of each of the neurons ($p \leq 0.05$, Sign test)

Figure 14. Mean neurite growth rates (bars = means; vertical lines = SDs) before (left bar, Pre-Control; second bar from left, TTX alone), during (second bar from right, TTX + GLU) and after (right bar, Post-Control) application of 25 mM L-glutamate to growth cones. Bath application of TTX alone in the culture dish (second bar from left) appeared to inhibit neurite outgrowth (n = 8, ** - $p \leq 0.01$, Sign test), but growth rates were not quite significantly different than zero ($p = 0.088$, t-test). Pressure ejection of L-glutamate in the presence of bath applied 3 μM TTX (second bar from right) inhibited neurite outgrowth compared to pre-control values (left bar) (n = 8, ** - $p \leq 0.01$, ANOVA).

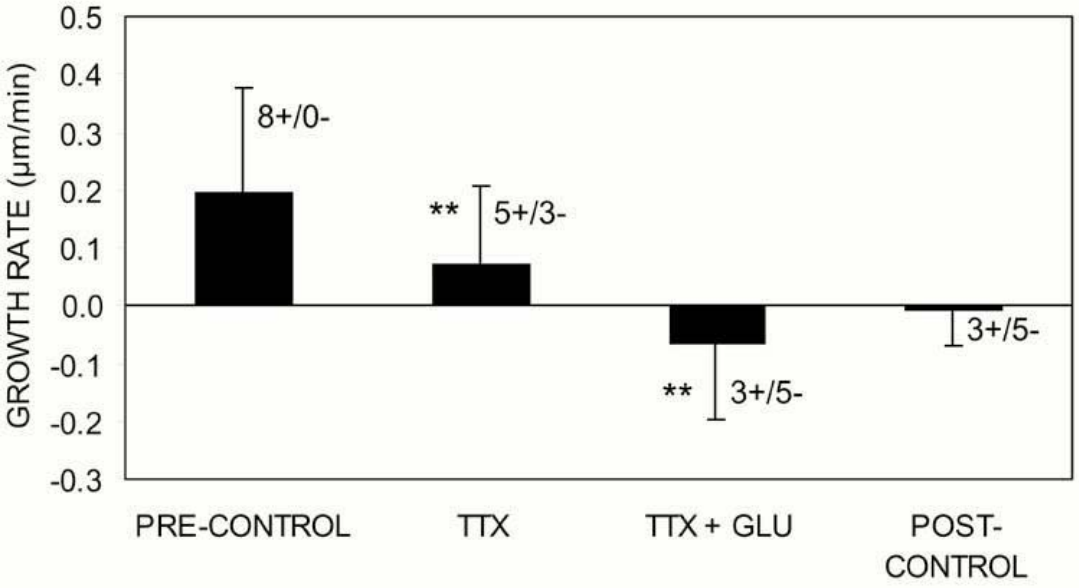
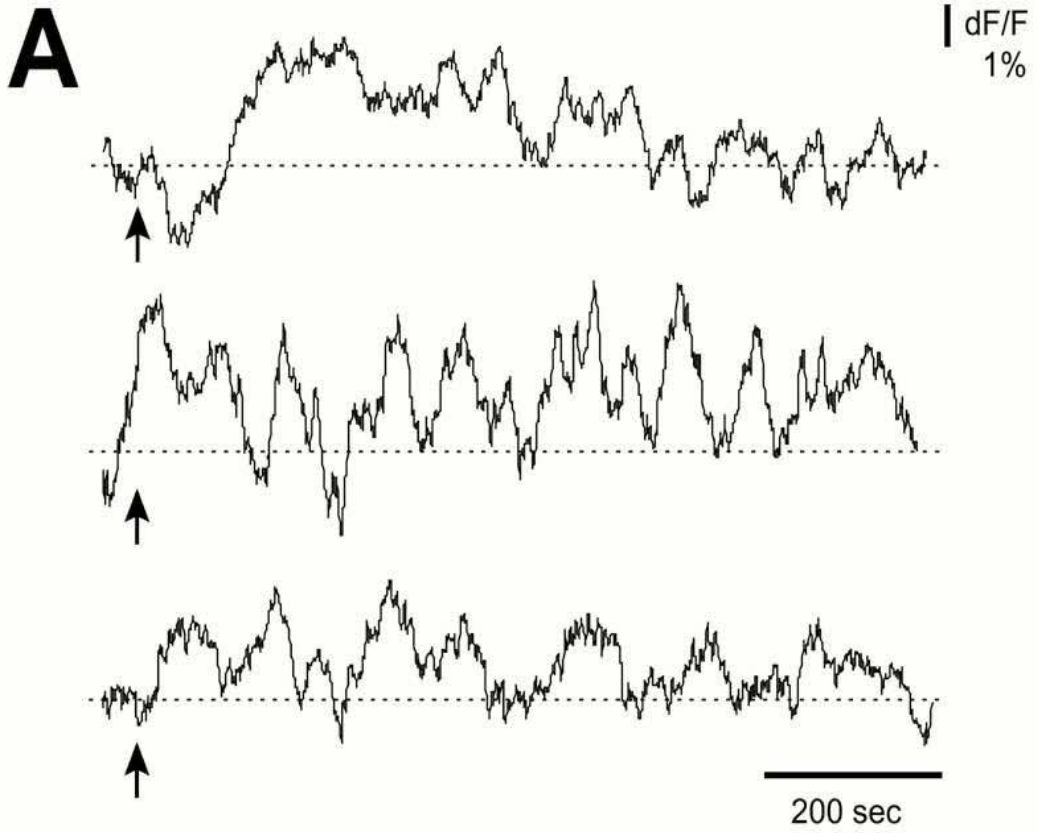

0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
GROWTH RATE (µm/min)
8+/0-
**
5+/3-
**
3+/5-
3+/5-
PRE-CONTROL
TTX
TTX + GLU
POST-CONTROL

FIG. 14

Figure 15. Graphs of Calcium Green-1 fluorescence traces versus time from RS neurons before and after pressure ejection of 25 mM L-glutamate onto the soma (arrows indicate time of L-glutamate application). (A) Fluorescence traces from four different neurons that displayed an increase in $\Delta F/F$ in response to L-glutamate. (B) Fluorescence traces from four different neurons whose $\Delta F/F$ values did not change substantially in response to glutamate.



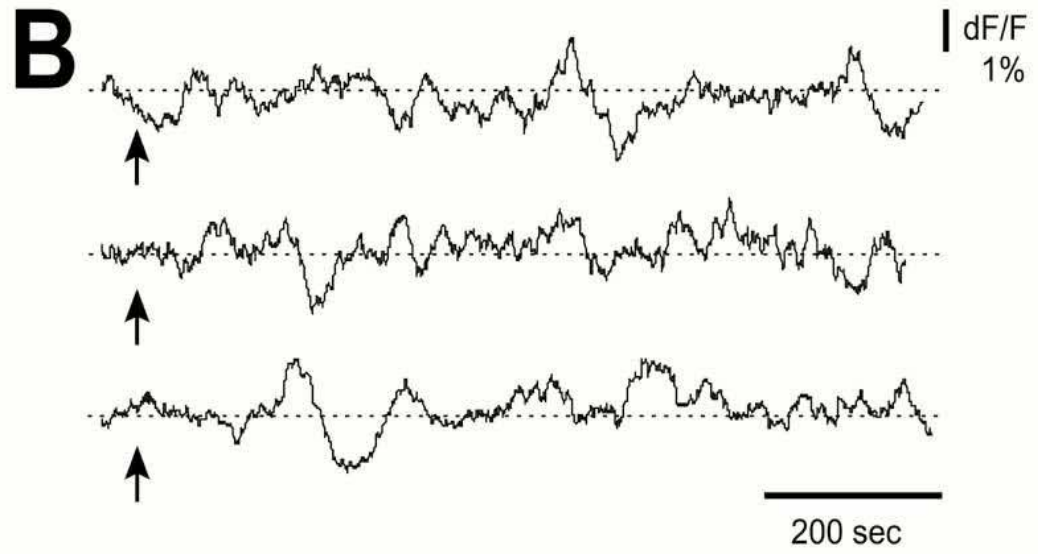


FIG. 15

Figure 16. Mean neurite growth rates (bars = means; vertical lines = SDs) before (left bars, Pre-Control) and during (right bars, 1% DMSO, or 0.5% DMSO) exposure to (A) 1% dimethylsulphoxide (DMSO, n = 7), or (B) 0.5% DMSO in media (n = 7). (A) A concentration of 1% DMSO in media appeared to produce some inhibition on neurite outgrowth, but the effects were not quite significant compared to pre-control conditions in media alone ($p = 0.08$; unpaired t-test with Welch correction). (B) A concentration of 0.5% DMSO did not produce significant changes in neurite outgrowth compared to pre-control conditions in media alone ($p = 0.39$; ANOVA).

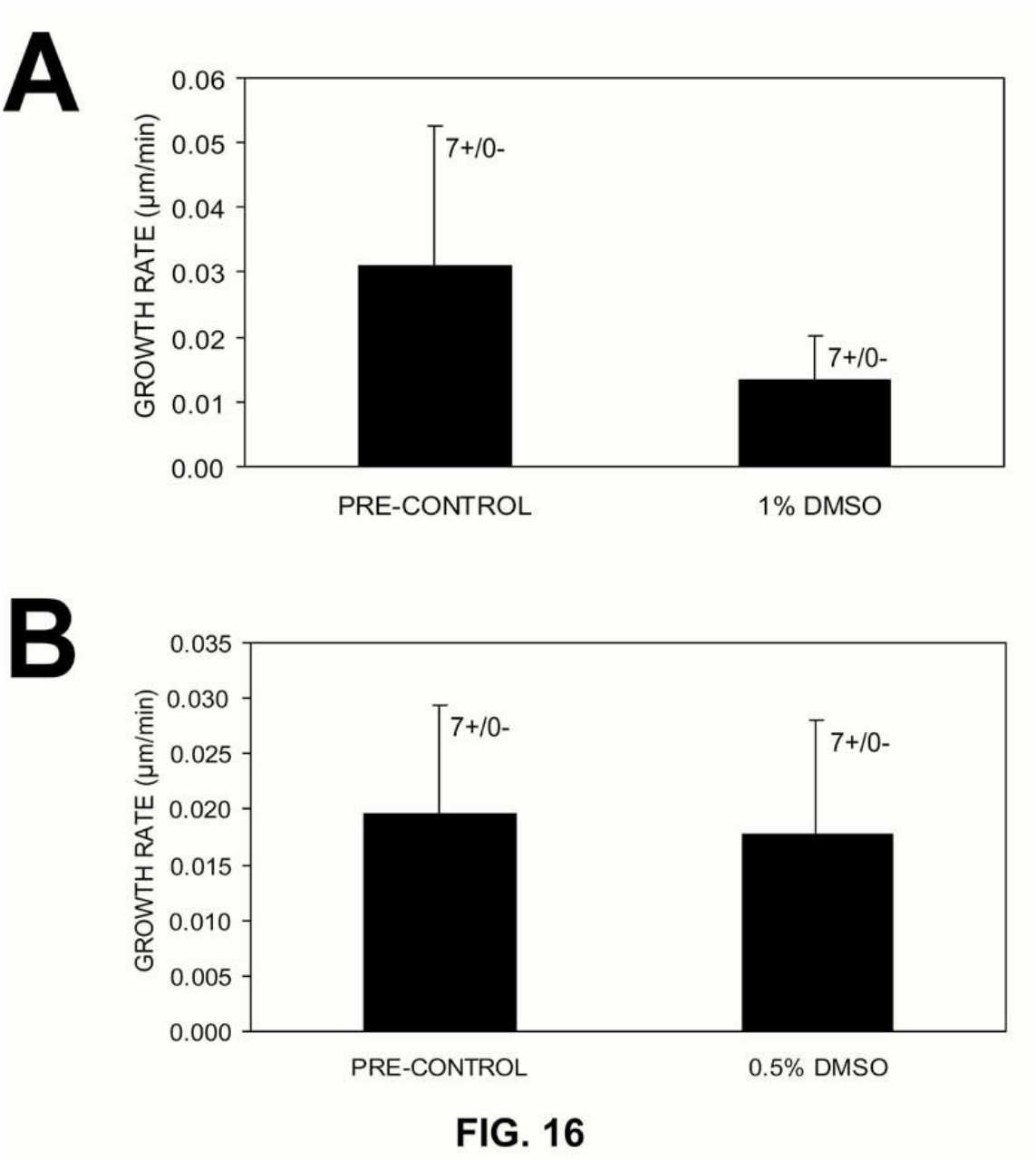

A
GROWTH RATE (µm/min)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
7+/0-
7+/0-
PRE-CONTROL
1% DMSO
B
GROWTH RATE (µm/min)
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
7+/0-
7+/0-
PRE-CONTROL
0.5% DMSO

FIG. 16

Figure 17. Mean neurite growth rates (bars = means; vertical lines = SDs) before (left bars, Pre-Control), during (middle bars) and after (right bars, Post-Control) application of the following agents onto DiI-labeled neurons in culture: (A) 10 mM dbcAMP (n = 17); (B) 62.5 μM Forskolin (n = 21); or (C) 100 μM IBMX (n = 23). Application of dbcAMP, Forskolin, or IBMX inhibited neurite outgrowth compared to pre-control values (*** - $p \leq 0.002$; ANOVA). In addition, neurite growth rates during application of dbcAMP or Forskolin were significantly less than zero, indicative of neurite retraction (††† - $p \leq 0.002$; t-test), while for IBMX application, growth rates were not significantly less than zero ($p = 0.2478$, t-test).

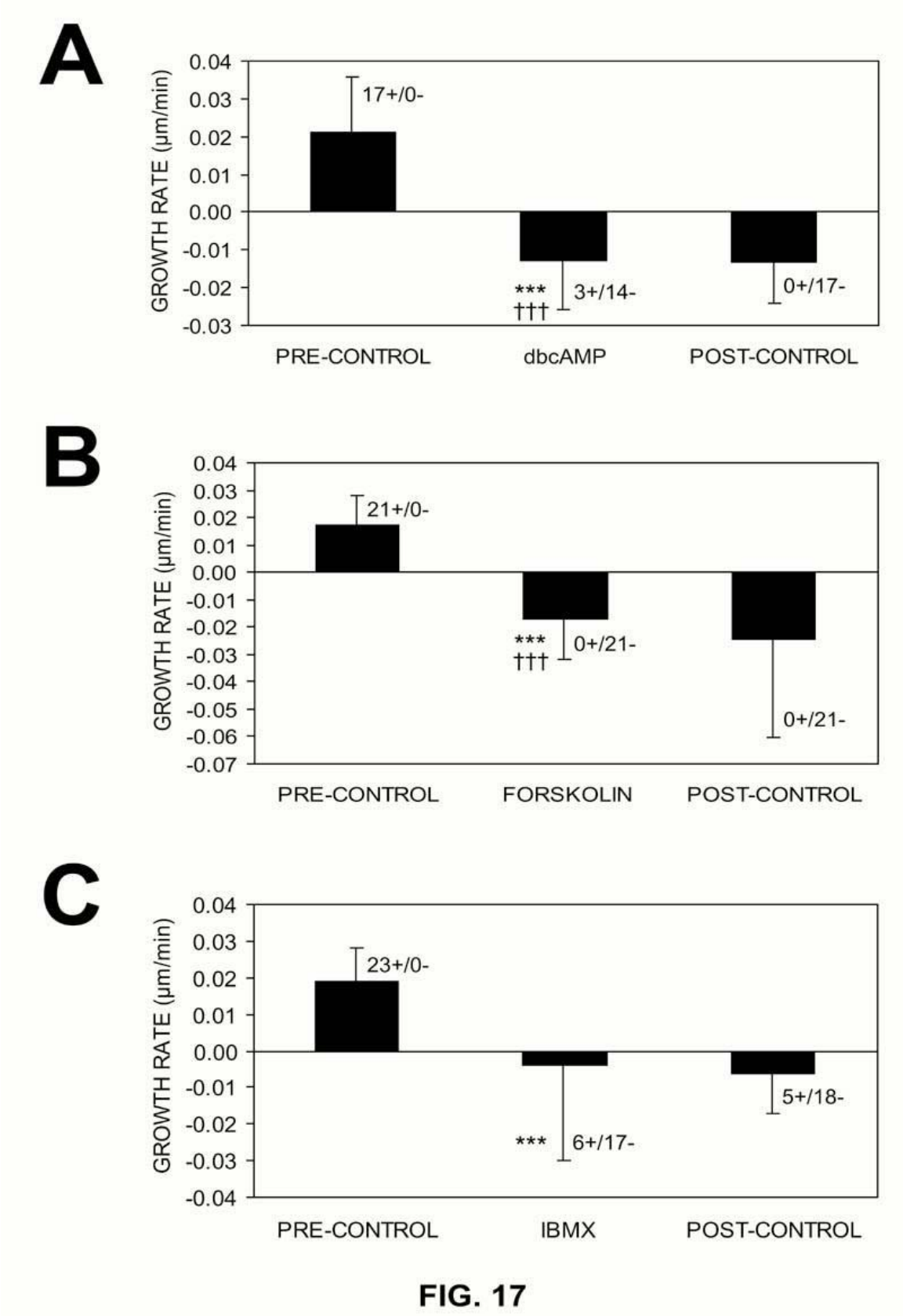

A
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
GROWTH RATE (µm/min)
17+/0-
***
†††
3+/14-
0+/17-
PRE-CONTROL
dbcAMP
POST-CONTROL
B
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
-0.07
GROWTH RATE (µm/min)
21+/0-
***
†††
0+/21-
0+/21-
PRE-CONTROL
FORSKOLIN
POST-CONTROL
C
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
-0.04
GROWTH RATE (µm/min)
23+/0-
***
6+/17-
5+/18-
PRE-CONTROL
IBMX
POST-CONTROL

FIG. 17

Figure 18. Mean neurite growth rates (bars = means; vertical lines = SDs) before (left bars, Pre-Control) and during (right bars) bath application of the following concentrations of L-glutamate (GLU) onto DiI-labeled neurons in culture: (A) 0.25 mM GLU (n = 6); or (B) 0.5 mM GLU (n = 23). (A) Bath application of 0.25 mM glutamate did not induce significant inhibition of neurite outgrowth ($p > 0.05$, t-test) compared to pre-control values. (B) Bath application of 0.5 mM glutamate resulted in significant inhibition of neurite outgrowth compared to pre-control values (*** - $p = \leq 0.002$, t-test). Furthermore, these experimental growth rates were significantly less than zero (†† - $p \leq 0.01$; t-test), indicative of neurite retraction.

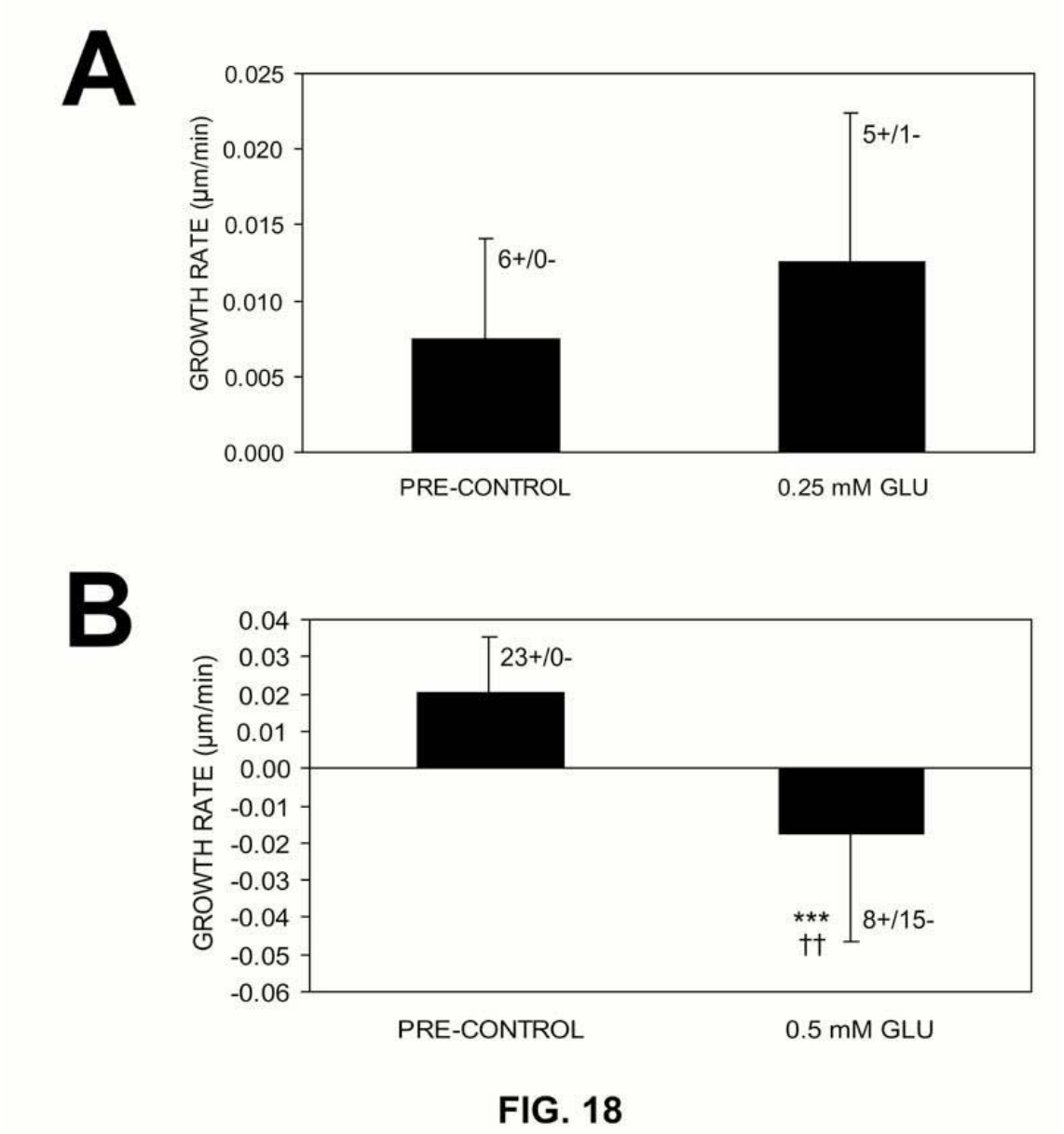

A
0.025
0.020
0.015
0.010
0.005
0.000
GROWTH RATE (µm/min)
6+/0-
5+/1-
PRE-CONTROL
0.25 mM GLU
B
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
GROWTH RATE (µm/min)
23+/0-
***
††
8+/15-
PRE-CONTROL
0.5 mM GLU

FIG. 18

Figure 19. Mean neurite growth rates (bars = means; vertical lines = SDs) before (left bar, Pre-Control), during (middle bar, H89 + GLU) and after (right bar, Post-Control) bath application of 0.5 mM L-glutamate onto DiI-labeled neurons in culture in the presence of 10 μM H89 (n = 19; see Methods). Under these conditions, glutamate no longer resulted in retraction or inhibition of neurite outgrowth ($p > 0.05$; ANOVA). In a control experiment, 10 μM H89 alone (0.015 ± 0.015 μm/min) did not significantly change neurite growth rates compared to pre-control conditions (0.015 ± 0.001 μm/min; $p > 0.05$; n = 19, ANOVA).

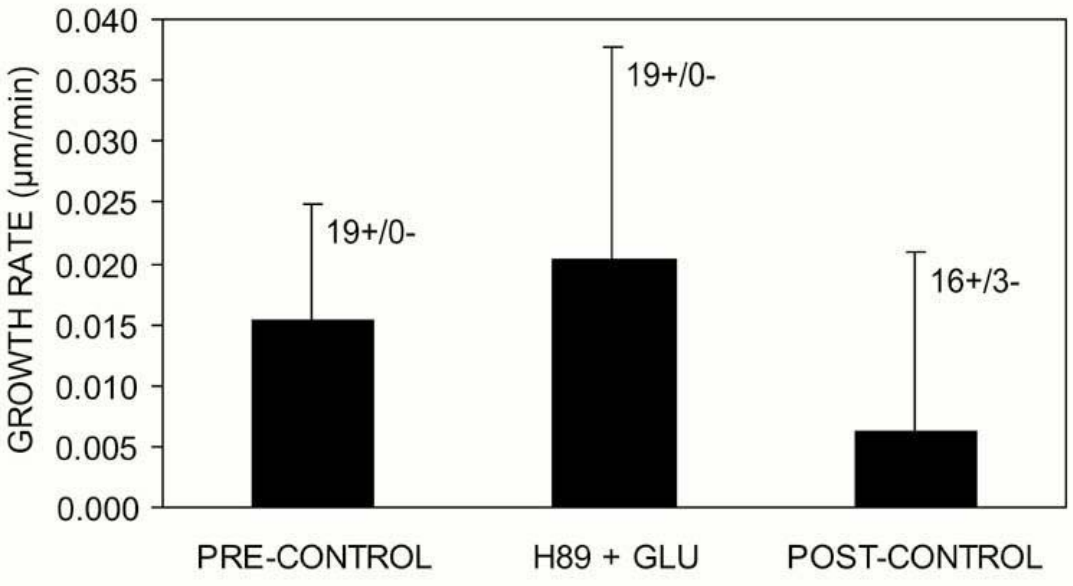

GROWTH RATE (µm/min)
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
19+/0-
19+/0-
16+/3-
PRE-CONTROL
H89 + GLU
POST-CONTROL

FIG. 19

Figure 20. Working model of the mechanisms by which L-glutamate may activate cAMP pathways that regulate neurite outgrowth. Glutamate results in a calcium influx via both chemically-gated and voltage-gated channels and intracellular calcium is proposed to activate calcium-dependent AC and cAMP signaling pathways. Proteins activated by cAMP:PKA then could regulate the rate of neurite elongation by a number of mechanisms (see Discussion). R1 = NMDA ionotrophic receptor, R2 = Kainate/AMPA ionotropic receptor, R3 = metabotropic receptor, G = G protein, AC = adenylate cyclase, VGCC = voltage-gated calcium channel, PKA = protein kinase A, ER = endoplasmic reticulum.

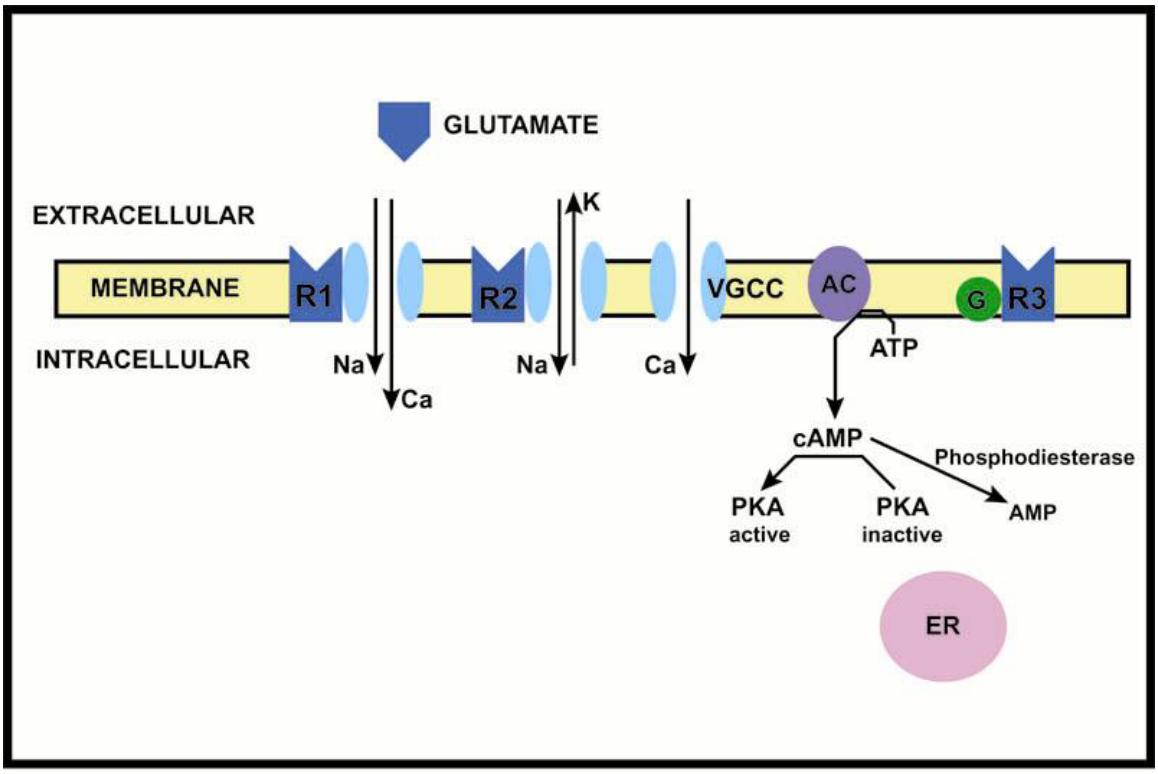

GLUTAMATE
EXTRACELLULAR
MEMBRANE
INTRACELLULAR
R1
R2
R3
K
Na
Ca
Na
Ca
VGCC
AC
G
ATP
cAMP
Phosphodiesterase
PKA
active
PKA
inactive
AMP
ER

FIG. 20

DISCUSSION

Effects of Glutamate on Neurite Outgrowth of Lamprey Brain Neurons

Dissociated descending brain neurons, of which ~80% are RS neurons, were pre-labeled with DiI and grew at an average growth rate in culture of ~0.07 μm/min. Similar neurite growth rates in this general range have been reported in both invertebrates (~0.15 μm/min; Cohan and Kater, 1986) and mammals (~0.03 – 0.08 μm/min; Mattson et al. 1988a; Wilson and Keith, 1998; Wilson et al., 2000). Furthermore, following spinal cord transection and during the first 3-6 weeks of axonal regeneration, axons of descending brain neurons grow at average rates of ~0.2–0.3 μm/min (Davis and McClellan, 1994a).

Focal pressure ejection of Ringer's solution onto growth cones did not significantly affect neurite outgrowth rates, suggesting that the purely physical actions of pressure ejection do not alter neurite outgrowth (Fig. 6A, 7A). Focal pressure ejection of 5 mM L-glutamate onto growth cones elicited neurite retraction in some of the neurons tested (Fig. 6B, 7B), while focal pressure ejection of 25 mM L-glutamate solution produced statistically significant neurite retraction (Fig. 6C, 7C). In the presence of 10 mM kynurenic acid, a broad spectrum ionotropic EAA receptor blocker, 25 mM glutamate applied to growth cones no longer elicited neurite retraction (Fig. 6D, 7D). Thus, glutamate elicits neurite retraction of descending brain neurons, including RS neurons, by acting on ionotropic EAA receptors and not by non-specific mechanisms. There were no indications that metabotropic receptors contribute significantly to glutamate-induced inhibition of neurite outgrowth.

Glutamate applied to growth cones appears to inhibit neurite outgrowth by eliciting calcium influx via both chemically-gated channels and voltage-gated calcium channels. First, focal depolarization of growth cones by application of high potassium media inhibited neurite outgrowth (Fig. 10A), and cobalt or cadmium, general calcium channel blockers, abolished this effect (Fig. 10B,C). Second, application of glutamate to growth cones in the presence of CTX, a specific voltage-gated calcium channel blocker that , unlike divalent cations, does not block chemically-gated channels, still resulted in inhibition of neurite outgrowth (Fig. 11A), probably due to calcium influx via chemically-gated channels, such as NMDA channels which are known to pass calcium in lamprey neurons (Wallén and Grillner, 1987). For example, application of NMDA to growth cones also inhibited neurite outgrowth (Fig. 12A). Interestingly, CTX blocked the above effects of NMDA but did not abolish glutamate-induced inhibition of neurite outgrowth (Fig. 12B). Since the conductance of NMDA channels is voltage dependent (Mayer and Westbrook, 1987), glutamate acting on all three ionotropic EAA receptors may result in greater depolarization and more calcium influx via NMDA channels than application of NMDA alone. Alternatively, it is possible that kainate and AMPA channels, which would be activated by glutamate but not NMDA, allow some influx of calcium (Catsicas et al., 2001).

Although in the present study the glutamate concentrations in the micropipettes were higher than those normally used for continuous bath application in lamprey experiments (i.e. 0.5-1.0 mM; Grillner et al., 1981), the

average concentrations near growth cones probably were much lower and likely were in the physiological range. First, if it is assumed that the micropipette tips in the present study are continuous point sources, which is an over estimate for repetitive pressure ejection pulses (~5 ms pulses every 0.5-1.0 s), it can be shown that the glutamate concentration will decrease very rapidly with distance from the micropipette tips (Curtis, 1964). For example, pressure ejection of glutamate onto a single growth cone inhibited the growth rates of the associated neurite but had little or no effect on other neurites of the parent neuron (Fig. 8, Table 1). Second, bath-applied glutamate can continuously bind to receptors on the entire surface of neurons in culture, and it is possible that these receptors may become partially desensitized from continuous application by glutamate. In contrast, repetitive pressure ejection of glutamate onto growth cones will largely affect receptors on only parts of a neuron and in an intermittent fashion. Third, for hippocampal neurons in culture, the glutamate concentrations that were used for pressure ejection and that caused neurite retraction were ~50 times those for continuous bath application to the culture dish (Mattson et al., 1988a). Likewise, for Xenopus cultured tectal neurons, pressure ejection of 20 mM glutamate caused significant calcium influx and sprout suppression, while as little as 20 μM was effective when continuously applied to culture dishes (Miskevich et al., 2002).

Although glutamate or high potassium was pressure ejected onto neurites for relatively long periods of time, they probably did not have global effects on other neurons in the culture dishes. For example, for application of glutamate,

each ejection bolus was ~8 pl (~25 μm dia.), and if pressure ejection pulses were applied at 2 Hz for 2 hours in ~2 ml of media, the final glutamate concentrations in the culture dish would be ~300 nM (for ejection of 5 mM glutamate) or ~1.5 μM (for 25 mM glutamate). In addition, application of glutamate or high potassium to one growth cone of a descending brain neuron in culture usually did not affect the lengths of other neurites from the same neuron (Fig. 8; Table 1). In contrast, application of glutamate or high potassium to the cell bodies of neurons in culture inhibited outgrowth of multiple neurites from the same neuron (Fig. 13A,B; Table 2).

At present, it is not clear why some manipulations elicit neurite retraction while other manipulations inhibit neurite outgrowth (see Methods). It is possible, that different mechanisms mediate these two general effects or that the effects represent different degrees of the same mechanism. In addition, it is not clear why the same manipulation can sometimes have similar qualitative but somewhat different quantitative effects on different neurons. Some of this variability may be due to heterogeneity of descending brain neurons.

Effects of Action Potentials on Glutamate-Induced Neurite Retraction

Application of 25 mM glutamate in the presence of TTX inhibited neurite outgrowth (Fig. 14), but TTX alone also inhibited neurite outgrowth, suggesting that there is some spontaneous activity and calcium influx in the cultured neurons. One interpretation of these results is that TTX alone decreases spontaneous activity and lowers calcium influx below the optimal range for neurite outgrowth, and that application of glutamate in the presence of TTX

increases neural activity and raises calcium levels above the optimal range. If true, these results may support the “calcium set point” hypothesis, which states that an optimal level of calcium is required for optimal neurite outgrowth (Mattson and Kater, 1987). To test this in the present study, varying concentrations of glutamate, between 0 - 25 mM, could be applied to growth cones in the presence of TTX to determine if intermediate glutamate levels induce neurite outgrowth.

Glutamate-Induced Changes in Calcium Concentrations

In the present study, application of 25 mM glutamate to Calcium Green-loaded descending brain neurons resulted in $\Delta F/F$ values of ~2.5% in some neurons (Fig. 15A). This increase in fluorescence presumably corresponds to increases in intracellular calcium concentrations, suggesting that glutamate-induced neurite retraction is due, in part, to an increase in intracellular calcium concentration. However, some neurons were poorly labeled with Calcium Green and some neurons that displayed adequate labeling did not appear to display an increase in fluorescence in response to glutamate. Calcium Green-1 AM, which is lipid soluble, enters neurons, but once inside the cell, the acetoxymethyl (AM) ester must be cleaved so that the indicator is no longer lipid soluble and will remain in the cytosol (Molecular Probes, Product Information sheet). In the present study, it is possible that lamprey neurons contain low levels of the nonspecific esterases to cleave the AM ester entirely, such that some of the indicator diffused out of the neurons during washout.

Influence of Second Messengers on Glutamate-induced Neurite Retraction

The glutamate-induced neurite retraction of lamprey descending brain neurons may be due, in part, to an increase in cAMP signaling levels in addition to influx of calcium through channels. In several types of neurons, cAMP increases calcium influx (Schubert et al., 1978; Connor and Hockberger, 1985). In the present study, increased cAMP signaling levels induced by bath application of dbcAMP, a membrane permeable cAMP analogue or Forskolin, an adenylate cyclase activator that stimulates the synthesis of cAMP, resulted in neurite retraction (Fig. 17A,B), similar to that produced in Helisoma buccal ganglion neurons (Mattson et al., 1988c). Second, an increase in the intracellular levels of cAMP signaling levels, induced by bath application of IBMX, an inhibitor of cAMP phosphodiesterase that suppresses breakdown of naturally occurring cAMP, caused inhibition of neurite outgrowth of lamprey descending brain neurons (Fig. 17C), similar to that produced Helisoma neurons (Mattson et al., 1988c), suggesting that enhancing the natural accumulation of cAMP can inhibit neurite outgrowth. These results may indicate that dbcAMP and Forskolin artificially increased cAMP signaling levels above those that naturally accumulate when cAMP degradation is blocked by IBMX.

Third, to determine if an increase in cAMP signaling levels alone might contribute to glutamate–induced inhibition of neurite outgrowth, L-glutamate was bath applied while H89, a specific inhibitor of cAMP-dependent PKA, was present in the bath. It should be noted that statistically significant neurite retraction was produced by bath application of 0.5 mM glutamate, which is comparable to those normally used for continuous bath application in lamprey neurophysiology

experiments (i.e. 0.5-1.0 mM; Grillner et al., 1981). The agent H89 does not block the synthesis of cAMP but should prevent the cAMP:PKA complex from exerting its effects (Levitan and Kaczmarek, 1997; Kao et al., 2002). Bath application of 0.5 mM glutamate in the presence of H89 no longer resulted in retraction or inhibition of neurite outgrowth (Fig. 19). Thus, glutamate appears to mediate neurite retraction of descending brain neurons, in part, by activating cAMP and enabling the cAMP:PKA complex.

Comparison to Other Studies

In a number of cell culture systems, neurotransmitters, including glutamate, can regulate not only the rate but also the direction of neurite outgrowth. For example, following bath application of 50 μM glutamate to hippocampal pyramidal neurons in culture, dendritic outgrowth rates and lengths were reduced, while axonal elongation rates and lengths were suppressed at somewhat higher glutamate concentrations (Mattson et al., 1988a). Bath applied glutamate (10-100 μM) to cultured spinal cord neurons dissected from mouse embryos inhibited neurite outgrowth and growth cone activity (Owen and Bird, 1997). In cultures of spinal motoneurons from embryonic rats, bath applied glutamate (usually 100 μM), acting via AMPA/kainate receptors, reduced the numbers and outgrowth of dendritic neurites (Metzger et al., 1998). In the mollusck *Helisoma*, serotonin (5HT) and dopamine cause neurite retraction of buccal ganglion neuron B19 in culture (Haydon et al., 1984,1987; McCobb et al., 1988b), and these effects are negated by bath-applied acetylcholine (McCobb et al., 1988a), which has an inhibitory effect on B19. For neuron B5 in Helisoma, L-

glutamate and somatostatin enhance neurite outgrowth (Bulloch, 1987; Jones and Bulloch, 1988).

In both invertebrates and vertebrates, intracellular calcium concentration can be an important regulator of growth cone morphology, neurite outgrowth, and directional navigation (reviewed in Kater et al., 1988; Kater and Mills, 1991; Zheng et al., 1996; Gomez and Spitzer, 2000; Zheng, 2000). For example, in the snail *Helisoma*, application of 5HT to B19 results in depolarization, calcium influx, increase in intracellular calcium, and neurite retraction (Haydon et al., 1984; Cohan et al., 1987; Mattson and Kater, 1987; McCobb et al., 1988b; Murrain et al., 1990). In addition, decreases in calcium influx and presumably intracellular calcium also can inhibit growth cone motility and neurite outgrowth (Mattson and Kater, 1987). Thus, at least for some neurons there appears to be an optimal range of internal calcium for growth cone motility and neurite outgrowth, a concept known as the "set point hypothesis" (reviewed in Kater et al., 1988; Lipton and Kater, 1989). However, the optimal range of calcium for neurite outgrowth can vary widely between neurons (Torreano and Cohan, 1997). Finally, in response to an increase in intracellular calcium, fillipodia of some *Helisoma* neurons initially display elongation but later retract (Rehder and Kater, 1992; Davenport and Kater, 1992).

In mammals, retraction of dendrites of hippocampal pyramidal neurons in culture in response to EAA receptor agonists is negated by cobalt, a calcium channel blocker, while calcium ionophores or elevated extracellular potassium mimic the effects (Mattson et al., 1988a; Mattson and Kater, 1989). For

embryonic retinal neurons in culture, activation of AMPA/kainate receptors increases intracellular calcium and reduces the numbers and outgrowth of neurites (Catsicas et al., 2001). In the case of embryonic rat spinal motoneurons in culture, application of glutamate induces calcium influx and neurite retraction via AMPA/kainate activated channels, and these effects are reduced in the presence of joro spider toxin-3, which blocks calcium channels associated with specific AMPA receptors (Metzger et al., 1998). However, in dorsal root ganglion neurons and sympathetic neurons, growth cone behavior does not appear to be well correlated with changes in intracellular calcium (Ivins et al., 1991; Garyantes and Regehr, 1992).

Several studies have measured changes in fluorescence of calcium indicator dyes in neurons. In the lamprey, electrical stimulation of spinal or descending brain neurons at 20 Hz for 4 s elicited a 5-20% increase in fluorescence of Calcium Green-1 dextran amine (McClellan et al., 1994). Brief application of glutamate to cultured cortical neurons from adult mouse produced 20% $\Delta F/F$ responses using Fura-2 AM or Fura-4 AM (Stosiek et al., 2003). Fluorescence levels of Fura-2 AM in rat olfactory receptor neurons in culture increase ~50% in response to 10 μM Forskolin but increase ~250% in response to 10 μM Forskolin + 100μM IBMX (Otsugura et al., 2005). Bath application of 1-10 μM glutamate to rat suprachiasmatic nucleus neurons in culture initiates a rapid increase in fluorescence of Fura-2 AM to 110%, followed by an average sustained elevation of fluorescence of a 75% (Quintero and McMahon, 1999).

Second messengers that affect the cytoskeleton mediate at least some of the effects of neurotransmitters on neurite outgrowth. For hippocampal neurons in culture, glutamate and activators of protein kinase C both cause retraction of dendritic and axonal neurites, while activators of adenylate cyclase enhance outgrowth of these processes (Mattson et al., 1988b). Continuous application of glutamate to dendritic neurites of hippocampal neurons causes a sustained increase in intracellular calcium, a decrease in microtubules, and neurite retraction (Wilson and Keith, 1998; Wilson et al., 2000). For certain *Helisoma* neurons, both 5HT and agents that elevate cAMP suppress neurite elongation, and both require influx of calcium to mediate their actions (Mattson et al., 1988c). Finally, in *Helisoma* neurons, an influx in calcium results in actin bundle loss (Welnhofer et al., 1999; also see Lankford and Letourneau, 1991).

Relationship of the Present Results to Axonal Regeneration in Larval Lamprey

In larval lamprey following spinal cord transection, axotomized RS neurons undergo a number of changes in biophysical properties, including an increase in spike threshold, increased spike frequency adaptation, and a decrease in the slow afterhyperpolarization (sAHP) (McClellan, 2003), the latter of which is mostly mediated by calcium-activated potassium channels in the lamprey (Wallén et al. 1989). Also, blocking calcium channels in uninjured RS neurons mimics some of the firing properties of axotomized neurons. These results suggest that axotomized RS neurons may adopt high spike thresholds

and a reduction in calcium influx to maintain intracellular calcium concentrations at optimal levels for axonal regeneration (McClellan, 2003).

For mammalian neurons in culture, contact of neurites with mature CNS myelin, which normally inhibits axonal regeneration, induces an influx of calcium and growth cone collapse (Bandtlow et al., 1993). Furthermore, blocking calcium influx with ω-conotoxin can prevent growth cone collapse when neurites contact CNS myelin (Moorman and Hume, 1993). Thus, at least for some neurons, intracellular calcium concentrations in growth cones appear to be one of the factors that regulate axonal regeneration.

Model for glutamate-induced neurite retraction in the larval lamprey

The present study demonstrates that glutamate–induced retraction is dependent on calcium influx, and that cAMP is necessary and sufficient for this effect. At present, it is not known how glutamate activates the intracellular signaling pathway that influences neurite outgrowth. In the present experiments, glutamate first binds to ionotropic receptors that result in calcium influx via voltage-gated and chemically-gated channels. Glutamate-induced neurite retraction is blocked by kynurenic acid, which blocks ionotropic but not metabotropic glutamate receptors (Catsicas et al., 2001). We hypothesize that calcium influx and an increase in intracellular calcium may activate calcium-dependent adenylate cyclases (reviewed in Defer et al., 2000; Hanoune and Defer, 2001; Cooper, 2003) that stimulate the synthesis of cAMP (Zigmond et al., 1999) (Fig. 20). The resultant cAMP:PKA complex would then activate certain proteins in the neurons that may influence neurite outgrowth. For example,

neurites elongate at the growth cone by assembly of tubulin that is produced in the soma, assembled into microtubules, and transported to the growth cone by active transport and/or diffusion. Proteins activated by the cAMP:PKA complex can inhibit assembly and/or enhance disassembly of microtubules (Zigmond et al., 1999). Alternatively, activated proteins may result in a large, secondary increase in intracellular calcium, either through ion channels (Levitan, 1985) or release from intracellular stores by binding to ryanodine receptors on the ER. This large increase in intracellular calcium may directly affect the formation of microtubules and the rate of neurite outgrowth. Thus, one key issue is whether the main increase in intracellular calcium precedes or follows activation of cAMP. This could be tested by loading neurons with a calcium buffer (e.g. BAPTA) to maintain relatively low levels of intracellular calcium, and then cAMP pathways could be activated to determine if neurite outgrowth is still inhibited.

SUMMARY

Results from the present study suggest that glutamate inhibits neurite outgrowth of RS neurons in culture by acting on glutamate receptors, mediating calcium influx via both voltage-gated and chemically-gated channels, increasing intracellular calcium levels, and activation of cAMP. For larval lamprey, it will be important to determine the cellular and molecular mechanisms by which glutamate affects neurite outgrowth of RS neurons, including the intracellular signaling pathways. Also, the effects of various neurotransmitters, growth factors, second messenger pathways, and substrates on neurite outgrowth and synapse formation of RS neurons in culture will be studied. Determination of the

cellular and molecular mechanisms that control neurite outgrowth of RS neurons in culture may lead to a better understanding of functional axonal regeneration in spinal cord-transected lamprey and may also provide insights regarding some of the conditions necessary for axonal regeneration in higher vertebrates, including humans.

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