

## POLYAMINES INCREASE IN SYMPATHETIC NEURONS AND NON-NEURONAL CELLS AFTER AXOTOMY AND ENHANCE NEURITE OUTGROWTH IN NERVE GROWTH FACTOR-PRIMED PC12 CELLS

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**Abstract**—Following axonal damage, sympathetic neurons are capable of regenerating and reinnervating their target tissues. Some years ago exogenous administration of polyamines was shown to enhance this regeneration. Recently, it was found that axonal injury leads to a dramatic up-regulation of the expression of arginase I in sympathetic neurons. This enzyme catalyzes the conversion of arginine to ornithine, which can subsequently be converted to the diamine putrescine and, ultimately, to the polyamines spermidine and spermine. In the present study, using an antiserum that reacts with both spermidine and spermine, we have found an increase in polyamine levels in both neurons and non-neuronal cells in the superior cervical ganglion 2 and 5 days following transection of the ganglion's postganglionic trunks. Using PC12 cells primed with nerve growth factor and then stripped off the culture dish and replated as a model system for axotomized sympathetic neurons, we found that spermidine treatment, with or without nerve growth factor, resulted in an increased percentage of cells with a neurite whose length was at least twice the diameter of the neuron's cell body. These increases could be seen within 48 h and were still evident after 8 days. Together, these data support the possibility that endogenous polyamines are involved in the normal regeneration which occurs following sympathetic axonal damage. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** spermidine, regeneration, superior cervical ganglion, PC12 cells, arginase.

Adult peripheral neurons are capable of considerable regeneration after axonal damage (Lieberman, 1971). Understanding the mechanisms underlying this plasticity and finding agents that enhance regeneration are high priorities for current neuroscience research. In the 1980s, Dornay et al. (1986) demonstrated that, following axotomy of neurons of the superior cervical sympathetic ganglion (SCG), administration of exogenous polyamines acceler-

ates the reinnervation by these neurons of their target tissues. This result raised the possibility that endogenous polyamines play a role in facilitating regeneration in this system. This possibility was further strengthened by our recent finding that axotomy of the SCG leads to a 22-fold increase in the mRNA for arginase I, an enzyme involved in polyamine biosynthesis (Boeshore et al., 2004). In addition, axotomy led to increases in the level of arginase I protein in these ganglia and in the levels of the polyamine spermidine and its precursor putrescine. No change was found in the levels of the polyamine spermine (Boeshore et al., 2004). In the present study, as part of an effort to elucidate the role of endogenous polyamines, we have used immunohistochemistry to determine the cell type(s) in which polyamines are localized within the axotomized SCG and have examined a possible functional effect of spermidine on neurite outgrowth. Cai et al. (2002) reported that raising polyamine levels by overexpression of arginase I in neonatal cerebellar neurons in culture allowed these CNS neurons to overcome the normal inhibitory effect of myelin on axon outgrowth. Notably, in their study, neurite outgrowth in the absence of myelin was unaffected by arginase overexpression. Axon outgrowth in the presence of myelin was seen also when cerebellar neurons were treated with putrescine. Since the axons of regenerating SCG neurons encounter little, if any, myelin, it is of interest to determine if spermidine can increase neurite outgrowth in cultured SCG neurons in the absence of myelin. Due to the low yield that is obtained when dissociating and culturing adult sympathetic neurons, we chose to examine the effect of polyamines on neurite outgrowth in PC12 cells, a commonly used model for sympathetic neurons (Greene, 1977; Rukenstein and Greene, 1983). PC12 cells were exposed to nerve growth factor (NGF) and then stripped off the culture dish and replated in order to mimic the changes that occur after axotomy of sympathetic neurons.

### EXPERIMENTAL PROCEDURES

#### Animals and surgical procedures

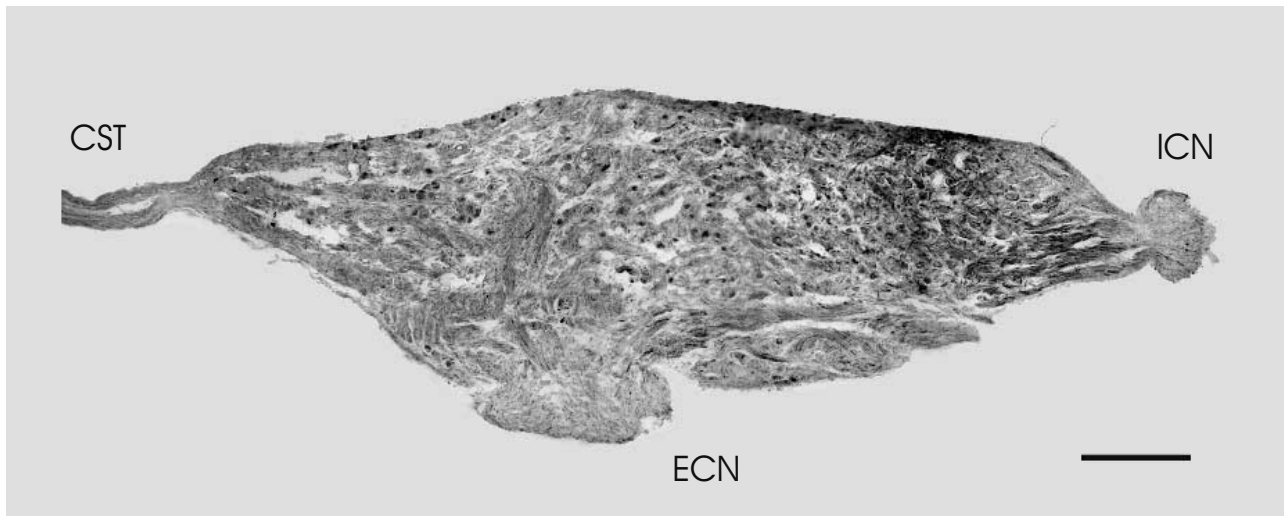
Adult male Sprague–Dawley rats (150 g; Zivic Miller Laboratories, Allison Park, PA, USA) were anesthetized by i.p. injection of a mixture of ketamine, xylazine, and acepromazine. Bilateral axotomy of the SCG was performed by transecting the internal and external carotid nerves, approximately 2 mm distal to the SCG. Sham-operated animals had the internal and external carotid nerves exposed but not transected. Four to six animals were included in each group. All protocols received prior approval of the Case Western Reserve University Institutional Animals Care and

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**Abbreviations:** BSA, bovine serum albumin; DFMO,  $\alpha$ -difluoromethylornithine; NGF, nerve growth factor; PBS, phosphate-buffered saline; PBS-A, 0.2% bovine serum albumin in phosphate-buffered saline; SCG, superior cervical ganglion.



**Fig. 1.** Distribution of polyamine like-immunoreactive cells in a longitudinal section from an SCG 2 days after axotomy. Two days after transection of the two major postganglionic nerve trunks of the SCG or after a sham-operation, the ganglion was fixed and sections were cut and reacted with an antiserum raised against spermine conjugated to BSA. Immunoreactive neurons are widely distributed throughout the ganglion. The darkly stained neuropil in the area of the ganglion near the exit of the internal carotid nerve (ICN) reflects labeled cells with the morphology of Schwann cells. CST, preganglionic cervical sympathetic trunk; ECN, external carotid nerve. Scale bar=500  $\mu\text{m}$ .

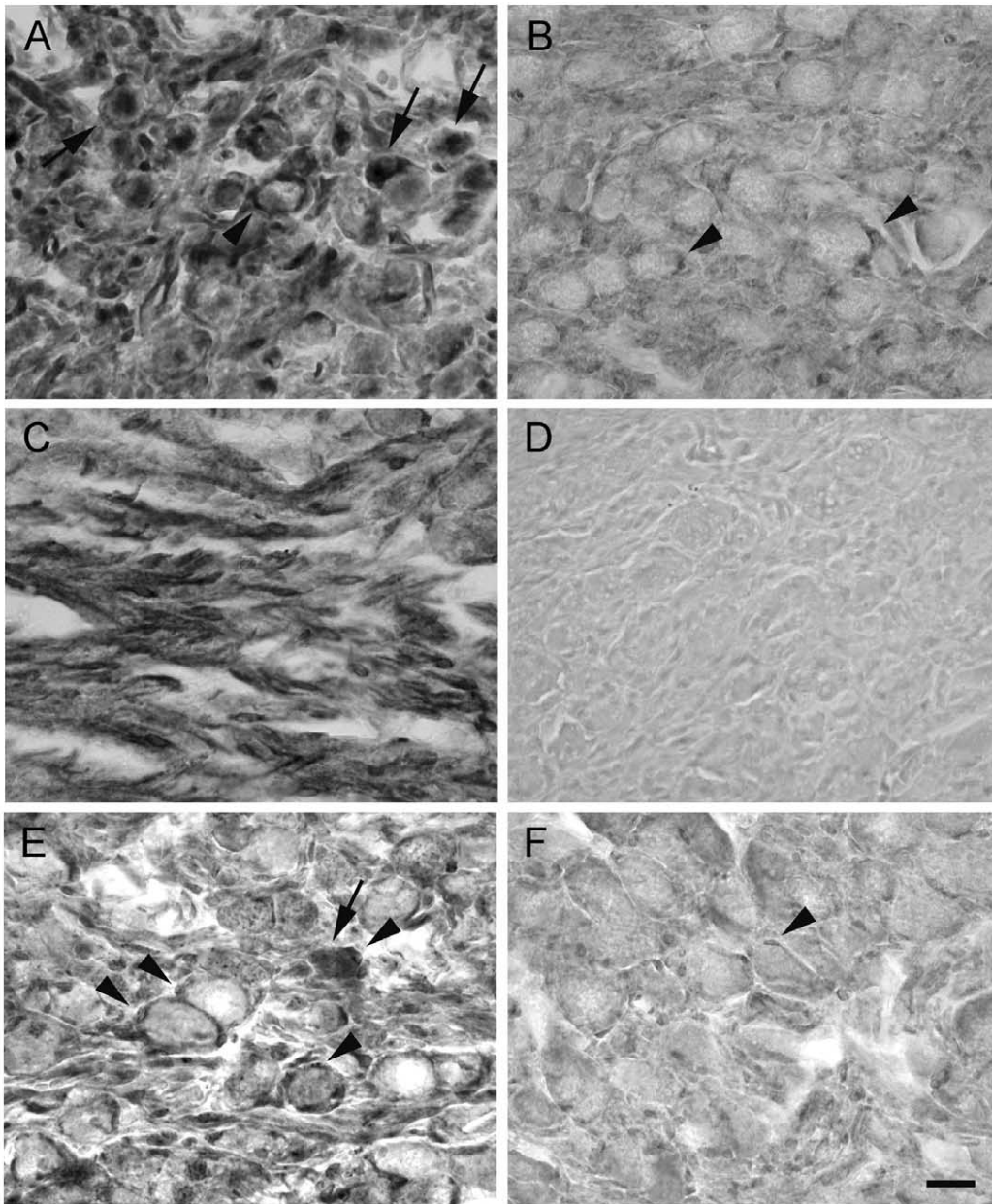
Use Committee and conformed to the regulations of the National Institute of Health. The minimum number of animals necessary to produce reliable data was used, and all efforts were made to minimize animal suffering.

### Immunohistochemistry

Six hours, 2 days and 5 days following surgery, SCG were removed and perfused with 4% paraformaldehyde. Following fixation, the SCG were desheathed, cryoprotected in sucrose, embedded in OCT, sectioned on a cryostat at 10  $\mu\text{m}$ , mounted on slides, and stored at  $-20^{\circ}\text{C}$ . For immunohistochemistry, the slides were thawed, incubated in 1% sodium borohydride for 15 min followed by two washes in phosphate-buffered saline (PBS), and pre-incubated in 10% donkey serum and 0.3% Triton X-100 in PBS for 30 min. The rabbit anti-spermidine/spermine antiserum (Laube and Veh, 1997; Laube et al., 2002) was diluted 1:50 in 10% donkey serum and 0.3% Triton X-100 in PBS and applied to the slides for 24–36 h at room temperature. The next day the slides were washed two times in PBS (20 and 40 min each) and pre-incubated in 0.2% bovine serum albumin (BSA) in PBS (PBS-A) for 1 h. The slides were then incubated for 24 h in biotinylated donkey anti-rabbit Fab<sub>2</sub> fragment with minimum cross-reactivity to rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:2000 in PBS-A. The slides were then washed two times in PBS (20 and 40 min each), pre-incubated in PBS-A for 30 min, and then incubated for 6 h in the ABC complex (Vector Elite ABC in PBS-A; Vector Laboratories, Burlingame, CA, USA), followed by three 5 min washes in PBS. The polyamine labeling was visualized by incubating the slides with diaminobenzidine and nickel chloride (Peroxidase Substrate Kit; Vector Laboratories) for 3–5 min. The diaminobenzidine–nickel chloride incubation was halted by washing the slides in distilled water, and the slides were coverslipped with Citifluor mounting media (Ted Pella, Redding, CA, USA). Immunoreactive cells were visualized with a 40 $\times$ /1.70 Plan Fluotar objective on a Leitz transmitted light microscope (Leica Microsystems Inc., Bannockburn, IL, USA), and images were captured with a Hamamatsu ORCA 100 cooled CCD camera (W. Nuhsbaum, Inc., McHenry, IL, USA) interfaced with C-imaging software (Compix, Inc, Cranberry, PA, USA).

### PC12 cell culture methods

PC12 cells (ATCC, Manassas, VA, USA) were cultured in F12K medium (ATCC) supplemented according to the manufacturer's recommendations with heat-inactivated horse serum (15%), fetal bovine serum (2.5%), and penicillin–streptomycin (100 units/ml and 100  $\mu\text{g}/\text{ml}$ , respectively). All supplements were purchased from Invitrogen (Carlsbad, CA, USA). This “high serum” medium was used throughout the study. Cells were maintained on collagen IV-coated 75  $\text{cm}^2$  culture flasks (Becton Dickinson, San Diego, CA, USA) at 37  $^{\circ}\text{C}$  in a humidified atmosphere with 5%  $\text{CO}_2$ , and the medium was changed every 3 days. For NGF priming experiments, PC12 cells were treated with NGF (NGF-7S; Sigma; 50 ng/ml of medium) for 14 days (Greene, 1977). Half of the culture medium was changed every 3 days, and the NGF concentration was kept constant. While still in the 75  $\text{cm}^2$  flasks, the NGF-primed cells were washed four times with NGF-free medium (same as above). Cells were then detached from the flask with forceful trituration using NGF-free medium, centrifuged (500 $\times$ g for 5 min) and resuspended in fresh medium. Centrifugation and resuspension in fresh medium was repeated three times. Aliquots of NGF-primed cells were frozen in medium containing 10% DMSO for future use (Rukenstein and Greene, 1983). The frozen NGF-primed cells were thawed at 37  $^{\circ}\text{C}$  in a water bath, centrifuged, and resuspended in medium. The cells were plated on 35 mm six well, Collagen IV-coated culture dishes (Becton Dickinson) with medium at a low density ( $3 \times 10^3$  cells/well) to facilitate morphometric analysis. Four experimental conditions were examined ( $n=3$  wells for each experimental condition): untreated (medium alone), spermidine (10  $\mu\text{M}$  in medium; Sigma, St. Louis, MO, USA), NGF (50 ng/ml in medium), or NGF and spermidine (50 ng/ml NGF and 10  $\mu\text{M}$  spermidine in medium). Half of the medium volume was changed every 3 days, keeping the concentration of NGF and spermidine constant. Neurite outgrowth was monitored using an Axiovert 405 M microscope (10 $\times$  Plan-Neofluor, Ph 1, NA 0.30 objective; Zeiss, Gottingen, Germany) and images were taken of six fields surrounding the central area of each of the wells with a Princeton Instruments



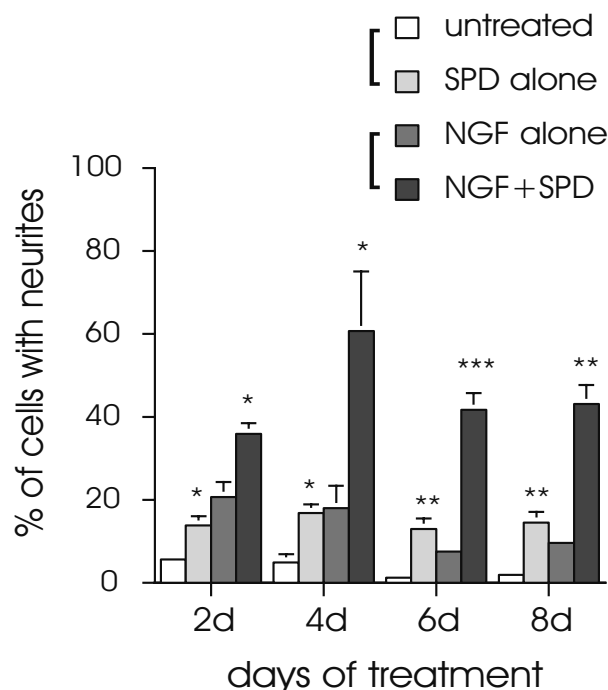
**Fig. 2.** Polyamine-like immunoreactivity in neurons and non-neuronal cells in the SCG. At high power, 2 days (A) and 5 days (E) after axotomy, cell bodies with dark immunoreactivity in the center of the ganglion consisted of both neurons (arrows) and non-neuronal cells (arrowheads), the latter probably satellite cells. In sections from ganglia 2 days (B) or 5 days (F) after a sham-operation, light immunoreactivity was found only in “satellite-like” cells, which surrounded principal neurons (arrowheads). (C) Near the origins of the postganglionic nerve trunks, darkly labeled cells with the morphology of Schwann cells were found 2 days after axotomy. (D) No immunoreactivity was seen on a section from a ganglion 2 days after axotomy when the primary antiserum was omitted from the reaction. Scale bar=25  $\mu$ m.

Micro MAX cooled CCD camera (Roper Scientific, Trenton, NJ, USA) at days 2, 4, 6, and 8. From each of the images, the total number of cells with processes greater than two times the shortest diameter of the cell body was counted and expressed as a percentage of the total number of cells within the image. For all groups, a minimum of 250 cells were examined. In addition, for each cell with processes greater than two times the diameter of the cell body, the total neurite length was measured using Metamorph (version 4.6; Universal Imaging Corporation, Downingtown, PA, USA). Differences between groups were examined using Student's *t*-test, except as noted.

## RESULTS

### Localization of polyamines in the SCG

The antiserum used in this study was raised against a BSA-spermine conjugate. This antiserum reacts equally with BSA-spermidine as with BSA-spermine in an ELISA assay, but it reacts only weakly with BSA-putrescine (Laube and Veh, 1997). In confirmation of a previous study in which polyamines were measured by HPLC (Boeshore



**Fig. 3.** Percentage of PC12 cells with neurites. NGF-primed PC12 were cultured under four conditions: with normal medium (“untreated”), with spermidine alone (“SPD”), with NGF alone, and with NGF plus spermidine. Cultures were photographed at 2, 4, 6, and 8 days, and the percentage of cells with at least one neurite that was longer than two times the diameter of the cell was determined. In this figure, and in all the histograms in Fig. 6, statistical comparisons are made between the spermidine alone groups and the untreated groups and between the spermidine plus NGF groups and the NGF alone groups. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

et al., 2004), increased polyamine-like immunoreactivity was seen in sections from axotomized ganglia. The darkly immunostained cells were widely distributed throughout the SCG 2 days after axotomy (Fig. 1).

In the brain, polyamine-like immunoreactivity has been found in both neurons and non-neuronal cells (Laube and Veh, 1997; Laube et al., 2002). Similarly, at both 2 and 5 days after axotomy, immunoreactivity in sections of the SCG was found both in principal neurons and in non-neuronal cells within the ganglion (Fig. 2A, E). The non-neuronal cells had the morphology and localization of satellite (Fig. 2A, E; arrowheads) and Schwann cells (Fig. 2C). The proportion of labeled cells that were neurons appeared to be greater at 2 days than at 5 days (Fig. 2A, E). In contrast, sections from ganglia 2 or 5 after a sham-operation showed only weak immunostaining in non-neuronal cells surrounding principal neurons, probably satellite cells (Fig. 2B, F). No staining was seen in sections from the same ganglion that were not treated with the primary antibody (Fig. 2D). Six hours after axotomy, no evidence was seen for a change in immunoreactivity compared with that seen in sections from sham-operated ganglia (data not shown).

#### Neurite outgrowth in PC12 cells

PC12 cells primed with NGF (as described in Experimental Procedures) were cultured with normal (i.e. high serum

containing) medium, medium containing 10  $\mu$ M spermidine, medium containing 50 ng/ml NGF, or medium containing both spermidine and NGF. To determine whether spermidine affected neurite outgrowth, the spermidine alone group was compared with the untreated group, and the spermidine plus NGF group was compared with the NGF alone group. The percentages of cells with neurites longer than twice the shortest diameter of the cell body were greater when cells were cultured in the presence of spermidine than when they were cultured with normal medium (Fig. 3; Fig. 4A,B, and Fig. 5A, B). The percentage of cells with neurites was also higher in the spermidine plus NGF group than in the group cultured in the presence of NGF alone (Fig. 3; Fig. 4C, D, and Fig. 5C, D). Within each treatment group, none of the groups showed a significant change over time, although the  $P$  value for the change over time for the NGF alone group was 0.056 by one way repeated measures analysis of variance. The differences between the spermidine and untreated groups and between the spermidine plus NGF and the NGF alone groups were statistically significant at all four time points examined from 2 days until 8 days (Fig. 3).

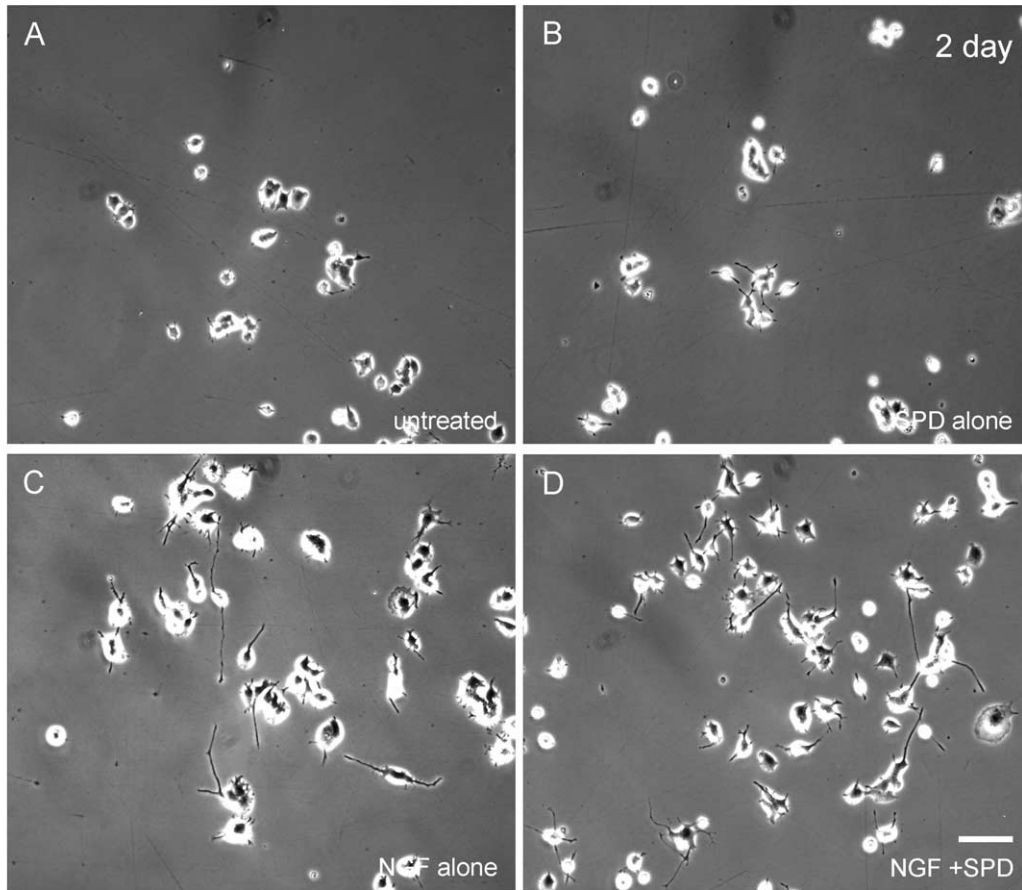
When the total lengths of the neurites per cell were measured, the values for cells treated with spermidine alone were greater than those in the untreated group at 2, 6, and 8 days in culture (Fig. 6A). In contrast, in the presence of NGF, the total length of neurites for cells treated with spermidine plus NGF was significantly less than those treated with NGF alone at 2 days and 8 days. The mean number of branches per cell was also determined and was found to be significantly higher at 2, 6 and 8 days for the cells treated with spermidine alone than for the cells treated with control medium (Fig. 6B). At most of the time points, no difference was found in branching between the NGF alone and the NGF plus spermidine groups.

The total number of cells that were quantitated within the six fields was not significantly different on day 2 among the four experimental conditions, and the number of cells in the spermidine alone or spermidine plus NGF conditions did not change during the time in culture (Fig. 6C). At day 8, however, the number of cells was significantly higher in the control medium than in the spermidine alone medium, and in the NGF alone medium than in the NGF plus spermidine medium (Fig. 8), indicating that proliferation occurred in the groups that were not exposed to spermidine.

## DISCUSSION

### Previous studies on a role for polyamines in peripheral nerve regeneration

Early interest in a possible role for polyamines in nerve regeneration came from three types of studies. The first was the finding that the activity of ornithine decarboxylase, the enzyme which catalyzes the conversion of ornithine to the diamine putrescine, increased following axonal injury in a number of neural systems in which regeneration takes place. Secondly, in some instances, pharmacological blockade of polyamine synthesis was found to inhibit re-



**Fig. 4.** Micrograph of PC12 cells after 2 days in culture. NGF-primed PC12 cells were photographed after 2 days in culture in the presence of (A) normal medium ("untreated"), (B) spermidine ("SPD"), (C) NGF, and (D) NGF plus spermidine. Scale bar=100  $\mu$ m.

generation. Finally, administration of exogenous polyamines enhanced regeneration in most of these systems.

In the goldfish retina, for example, ornithine decarboxylase activity was increased 3 days after optic nerve crush (Kohsaka et al., 1981; Schwartz et al., 1981). In addition, axonal transport of putrescine and spermidine was enhanced in regenerating retinal ganglion cells (Ingoglia et al., 1977). However, an attempt to decrease regeneration in this system by administration of an irreversible inhibitor of ornithine decarboxylase,  $\alpha$ -difluoromethylornithine (DFMO), was unsuccessful (Kohsaka et al., 1982).

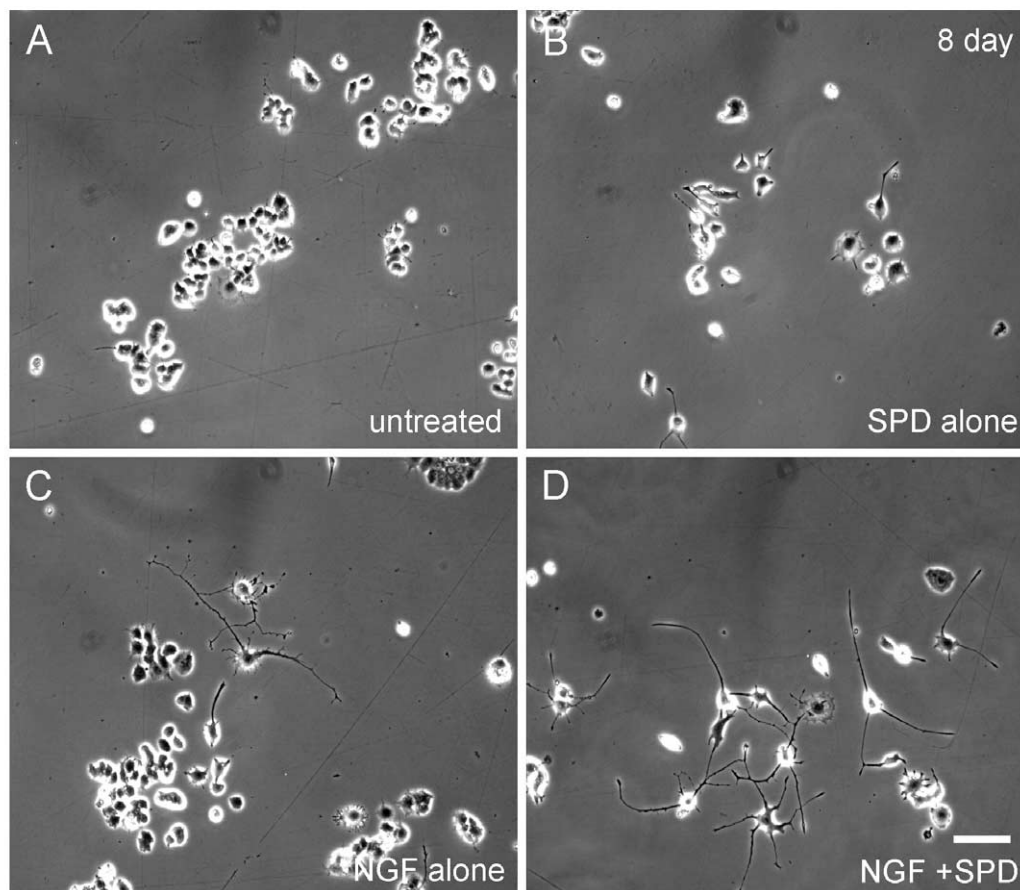
Increased ornithine decarboxylase activity also occurs in sensory, motor and sympathetic neurons after axotomy (Gilad and Gilad, 1981, 1983b; Ando et al., 1984; Tetzlaff and Kreutzberg, 1985; Kanje et al., 1986; Wells, 1987; Soiefer et al., 1988). Regeneration of the axons of neurons in the frog dorsal root ganglion was inhibited when animals were injected with DFMO (Kanje et al., 1986; Edbladh et al., 1990). An inhibitory effect of DFMO was also found on regeneration of sensory and motor neurons following the crushing of the rat sciatic nerve (Kauppila and Stenberg, 1989). In addition, in the case of the rat sciatic nerve, administration of spermine or spermidine was found to enhance the rate of regeneration of sensory and motor axons (Sebillé and Bon-

doux-Jahan, 1980; Kauppila et al., 1988; Kauppila, 1992). Finally, administration of putrescine, spermidine and spermine, together with an inhibitor of diamine oxidase (an enzyme involved in polyamine catabolism), accelerated the return of vibrissae movement after facial nerve crush (Gilad et al., 1996).

Experiments *in vivo* indicate that polyamines also play a role in sympathetic nerve regeneration. When DFMO was administered together with an inhibitor of *S*-adenosylmethionine decarboxylase (a third enzyme involved in polyamine biosynthesis), chromatolysis in neurons of the SCG, an early aspect of the cell body response to axonal injury, was blocked. In addition, there was almost a 50% decrease in the number of neurons in the ganglion 1 week after nerve transection, indicating a role for polyamines in neuronal survival after axotomy (Gilad and Gilad, 1983a). Injection of a mixture of putrescine, spermidine and spermine enhanced the reinnervation after postganglionic nerve crush of the pineal gland and iris, two targets of neurons in the SCG.

#### Changes in polyamine biosynthesis in axotomized SCG

Axotomy of the SCG leads to changes in three enzymes involved in polyamine biosynthesis, arginase I, ornithine

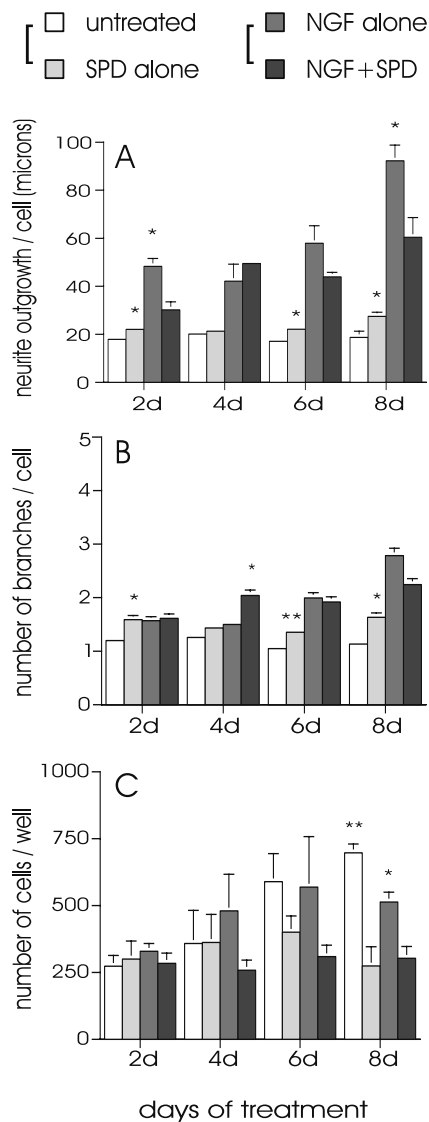


**Fig. 5.** Micrograph of PC12 cells after 8 days in culture. NGF-primed PC12 cells were photographed after 8 days in culture in the presence of (A) normal medium ("untreated"), (B) spermidine ("SPD"), (C) NGF, and (D) NGF plus spermidine. Scale bar=100  $\mu$ m.

decarboxylase, and S-adenosylmethionine decarboxylase (Gilad and Gilad, 1981, 1983b; Boeshore et al., 2004). With regard to the first of these, 2 days after the postganglionic nerve trunks are transected, arginase I mRNA levels were increased 22-fold, and this increase was accompanied by a dramatic increase in arginase I protein (Boeshore et al., 2004). Arginase I is a cytosolic enzyme that catalyzes the conversion of arginine to ornithine and urea. While ornithine has a number of roles in cellular metabolism (Albina et al., 1993; Mills, 2001; Witte and Barbul, 2003; Cederbaum et al., 2004), one of them is as a precursor for the synthesis of putrescine (Wu and Morris, 1998), a reaction catalyzed by the enzyme ornithine decarboxylase. As already noted, the activity of ornithine decarboxylase is also increased in sympathetic neurons after axotomy, with a two-fold increase detectable within 2 h, a maximum 4.5-fold increase seen at 10 h, and a two-fold elevation being maintained thereafter for 5 days (Gilad and Gilad, 1981, 1983b). In a gene microarray study, however, we found no evidence for an increase in ornithine decarboxylase mRNA after axotomy (Boeshore et al., 2004). Previous studies indicated that the increase in ornithine decarboxylase activity seen in the SCG after axotomy is accompanied by an increase in enzyme protein (Wells, 1986). These findings are consistent with studies

from other systems indicating that much of the regulation of ornithine decarboxylase occurs at the translational level (Shantz and Pegg, 1999). Finally, mRNA for S-adenosylmethionine decarboxylase decreases 2.7-fold in the SCG 2 days after axotomy. Decarboxylated S-adenosylmethionine is the aminopropyl donor in the conversion of putrescine to spermidine and in the conversion of spermidine to spermine (Pegg and McCann, 1992).

While increases in expression of arginase I and ornithine decarboxylase would tend to increase polyamine biosynthesis, whereas decreases in S-adenosylmethionine decarboxylase would tend to do the opposite, the overall effect of axotomy on polyamines in the SCG is to increase levels of putrescine and spermidine (Boeshore et al., 2004). Ornithine decarboxylase is usually considered to be the rate-limiting enzyme in polyamine synthesis, although this is not always the case (e.g. Guroff and Dickens, 1983). Under certain conditions, increases in arginase activity can lead to increases in polyamine levels (Wu and Morris, 1998; Kepka-Lenhart et al., 2000; Li et al., 2001; Cai et al., 2002). However, the fact that there are changes in the SCG in both arginase I and ornithine decarboxylase after axotomy leaves open the question as to whether one or both of these enzymes is responsible for the increases in putrescine and spermidine levels that occur.



**Fig. 6.** Effect of spermidine on neurite length, branching, and cell number. (A) For each PC12 cell that had a neurite longer than two times the cell body diameter, the total length of all the neurites of the cell was determined. For the NGF alone group ( $P < 0.001$ ) and the NGF plus spermidine group ( $P < 0.02$ ), the total length increased significantly over time when tested by a one way repeated measures analysis of variance. In each of the histograms in this figure, the asterisks refer to comparisons between the spermidine alone groups and the untreated groups and between the spermidine plus NGF groups and the NGF alone groups. \*  $P < 0.05$ . (B) For each PC12 cell that had a neurite longer than two times the diameter of the cell, the number of neurite branches was determined. At all time points except at 4 days, the number of branches per cell was higher when the cells were treated with spermidine alone than when they were treated with control medium. \*  $P < 0.03$ , \*\*  $P < 0.003$ . (C) Total number of cells examined per culture well. Six microscopic fields were photographed per culture well at  $10\times$ , and the total number of PC 12 cells (i.e. with and without neurites) was determined. In the control group ("untreated"), the number of cells increased over time ( $P < 0.02$ ) by a one way repeated measures analysis of variance. At 8 days, the number of cells in the control group was significantly greater than that in the spermidine group, and the number of cells in the NGF group was significantly greater than that in the NGF plus spermidine group. \*  $P < 0.02$ , \*\*  $P < 0.006$ .

Histochemical and autoradiographic studies localized the changes in ornithine decarboxylase primarily to the axotomized neurons in the SCG (Gilad and Gilad, 1983b; Wells, 1986), with some labeling of non-neuronal cells (Wells, 1986). The change in arginase I immunoreactivity was localized to neurons in the ganglion and to macrophages in the injured nerve trunks (Boeshore et al., 2004). In the present study, we have localized the change in polyamine levels to both neurons and non-neuronal cells in the SCG. While the localization of ornithine decarboxylase and arginase suggests that the primary site of polyamine synthesis within the SCG is in axotomized neurons, we cannot rule out non-neuronal cells as additional sites of polyamine synthesis particularly in sham-operated animals. Studies in the rat CNS, using the same antiserum used in the present study, have revealed labeling of both neurons and non-neuronal cells (primarily astrocytes; Laube and Veh, 1997; Laube et al., 2002). Interestingly, studies with radioactive polyamines indicate that these compounds can be transferred from neurons to perineuronal non-neuronal cells (Ingoglia et al., 1982; Lindquist et al., 1985) and polyamine uptake systems have been characterized in astrocytes (Dot et al., 2000; Masuko et al., 2003).

#### Effects of polyamines on neurite outgrowth in cell culture

Studies of the role of polyamines in neurite outgrowth in cell culture began with the finding of Greene and McGuire (1978) that, although NGF produced a large and rapid increase in ornithine decarboxylase activity, pharmacological inhibition of this enzyme did not inhibit NGF-induced neurite outgrowth. Interpretation of this finding is complicated by the subsequent finding that, despite this increase in ornithine decarboxylase activity, NGF produced no change in polyamine levels in PC12 cells (Guroff and Dickens, 1983; Mudumba et al., 2002), suggesting that ornithine decarboxylase is not the rate-limiting enzyme under these conditions. In any case, the fact that NGF does not produce its effects on neurite outgrowth via polyamines does not rule out the possibility that polyamines can stimulate neurite outgrowth in these cells. Nevertheless, two former studies failed to find effects of polyamines on neurite outgrowth in PC12 cells. Guroff and Dickens (1983) reported finding no effects of putrescine, spermidine or spermine on NGF induced neurite outgrowth, though details of the experiments and their results were not given. A recent study reported no significant increase in neurite outgrowth in PC12 cells with putrescine, spermidine, or spermine in the absence of NGF; however, treatment with  $N^6$ -acetylspermidine or with an inhibitor of  $N^6$ -acetylspermidine deacetylase did enhance neurite outgrowth (Mudumba et al., 2002).

We chose to examine the effects of polyamines on NGF-primed PC12 cells. In this procedure, cells grow neurites in the presence of NGF, then these neurites are stripped off by removing the cells from the culture dish, and finally the cells are replated and the regrowth of neurites is studied. Thus, this procedure mimics what a sympathetic

neuron goes through during normal development and after axotomy in the adult. In addition, we maintained our cells in a high serum containing medium throughout our experiments so that we could look at long term effects of polyamines. Under these conditions, spermidine increased the percentage of PC12 cells with neurites both by itself and when it was used together with NGF. While we did not set out to study the effects of spermidine on cell proliferation, our data suggest that spermidine inhibits proliferation of PC12 cells. Although polyamines stimulate proliferation of many cell types, it is interesting that in the house cricket, putrescine but not spermidine or spermine stimulate neurogenesis, while spermidine and spermine but not putrescine stimulated neuronal differentiation (i.e. process outgrowth; Cayre et al., 2001). In the present study, spermidine did not increase the total length of the neuritic arbor in process-bearing cells when given together with NGF. Rather, the neuritic arbor was somewhat longer at most time points examined when cells were cultured with NGF alone. The data indicate that spermidine increases the probability that an NGF-primed PC12 cell will regrow a neurite. These findings strengthen the possibility that polyamines play a role in sympathetic nerve regeneration after axonal damage and suggest that they might stimulate the initiation of axonal regrowth. Little is known about the biochemical mechanisms of action of polyamines on the nervous system, and one can only speculate as to the mechanism(s) by which spermidine stimulates neurite outgrowth. One possibility, however, is that polyamines act by altering the cytoskeleton either via an effect on gene expression (Kaminska et al., 1992) or on cytoskeletal assembly (Grant and Oriol-Audit, 1985).

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## REFERENCES

- Albina JE, Abate JA, Mastrofrancesco B (1993) Role of ornithine as a proline precursor in healing wounds. *J Surg Res* 55:97–102.
- Ando M, Miwa M, Kato K, Nagata Y (1984) Effects of denervation and axotomy on nervous system-specific protein, ornithine decarboxylase, and other enzyme activities in the superior cervical sympathetic ganglion of the rat. *J Neurochem* 42:94–100.
- Boeshore KL, Schreiber RC, Vaccariello SA, Sachs HH, Salazar R, Lee J, Ratan RR, Leahy P, Zigmond RE (2004) Novel changes in gene expression following axotomy of a sympathetic ganglion: a microarray analysis. *J Neurobiol* 59:216–235.
- Cai D, Deng K, Mellado W, Lee J, Ratan RR, Filbin MT (2002) Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. *Neuron* 35:711–719.
- Cayre M, Malaterre J, Strambi C, Charpin P, Ternaux JP, Strambi A (2001) Short- and long-chain natural polyamines play specific roles in adult cricket neuroblast proliferation and neuron differentiation in vitro. *J Neurobiol* 48:315–324.
- Cederbaum SD, Yu H, Grody WW, Kern RM, Yoo P, Iyer RK (2004) Arginases I and II: do their functions overlap? *Mol Genet Metab* 81 (Suppl):38–44.
- Dornay M, Gilad VH, Shiler I, Gilad GM (1986) Early polyamine treatment accelerates regeneration of rat sympathetic neurons. *Exp Neurol* 92:665–674.
- Dot J, Lluch M, Blanco I, Rodriguez-Alvarez J (2000) Polyamine uptake in cultured astrocytes: characterization and modulation by protein kinases. *J Neurochem* 75:1917–1926.
- Edbladh M, Edstrom A, Persson L (1990) The role of ornithine decarboxylase and polyamines in regeneration of the frog sciatic nerve. *Exp Neurol* 107:63–68.
- Gilad GM, Gilad VH (1981) Increased choline kinase activity in the rat superior cervical ganglion after axonal injury. *Brain Res* 220:420–426.
- Gilad GM, Gilad VH (1983a) Polyamine biosynthesis is required for survival of sympathetic neurons after axonal injury. *Brain Res* 273:191–194.
- Gilad GM, Gilad VH (1983b) Early rapid and transient increase in ornithine decarboxylase activity within sympathetic neurons after axonal injury. *Exp Neurol* 81:158–166.
- Gilad VH, Tetzlaff WG, Rabey JM, Gilad GM (1996) Accelerated recovery following polyamines and aminoguanidine treatment after facial nerve injury in rats. *Brain Res* 724:141–144.
- Grant NJ, Oriol-Audit C (1985) Influence of the polyamine spermine on the organization of cortical filaments in isolated cortices of *Xenopus laevis* eggs. *Eur J Cell Biol* 36:239–246.
- Greene LA (1977) A quantitative bioassay for nerve growth factor (NGF) activity employing a clonal pheochromocytoma cell line. *Brain Res* 133:350–353.
- Greene LA, McGuire JC (1978) Induction of ornithine decarboxylase by nerve growth factor dissociated from effects on survival and neurite outgrowth. *Nature* 276:191–194.
- Guroff G, Dickens G (1983) The effects of nerve growth factor on polyamine metabolism in PC12 cells. *J Neurochem* 40:1271–1277.
- Ingoglia NA, Sturman JA, Eisner RA (1977) Axonal transport of putrescine, spermidine and spermine in normal and regenerating goldfish optic nerves. *Brain Res* 130:433–445.
- Ingoglia NA, Sharma SC, Pilchman J, Baranowski K, Sturman JA (1982) Axonal transport and transcellular transfer of nucleosides and polyamines in intact and regenerating optic nerves of goldfish: speculation on the axonal regulation of periaxonal cell metabolism. *J Neurosci* 2:1412–1423.
- Kaminska B, Kaczmarek L, Grzelakowska-Sztabert B (1992) Inhibitors of polyamine biosynthesis affect the expression of genes encoding cytoskeletal proteins. *FEBS Lett* 304:198–200.
- Kanje M, Fransson I, Edstrom A, Lowkvist B (1986) Ornithine decarboxylase activity in dorsal root ganglia of regenerating frog sciatic nerve. *Brain Res* 381:24–28.
- Kaupilla T (1992) Polyamines enhance recovery after sciatic nerve trauma in the rat. *Brain Res* 575:299–303.
- Kaupilla T, Stenberg D (1989) alpha-Difluoromethylornithine (DFMO) disturbed the sensorimotor functional recovery from a sciatic lesion. *Neurosci Lett* 104:121–124.
- Kaupilla T, Stenberg D, Porkka-Heiskanen T (1988) Putative stimulants for functional recovery after neural trauma: only spermine was effective. *Exp Neurol* 99:50–58.
- Kepka-Lenhart D, Mistry SK, Wu G, Morris SM Jr (2000) Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages? *Am J Physiol Regul Integr Comp Physiol* 279:R2237–2242.
- Kohsaka S, Schwartz M, Agranoff BW (1981) Increased activity of ornithine decarboxylase in goldfish following optic nerve crush. *Brain Res* 227:391–401.
- Kohsaka S, Heacock AM, Klinger PD, Porta R, Agranoff BW (1982) Dissociation of enhanced ornithine decarboxylase activity and optic nerve regeneration in goldfish. *Brain Res* 256:149–156.
- Laube G, Veh RW (1997) Astrocytes, not neurons, show most prominent staining for spermidine/spermine-like immunoreactivity in adult rat brain. *Glia* 19:171–179.

- Laube G, Bernstein HG, Wolf G, Veh RW (2002) Differential distribution of spermidine/spermine-like immunoreactivity in neurons of the adult rat brain. *J Comp Neurol* 444:369–386.
- Li H, Meininger CJ, Hawker JR Jr, Haynes TE, Kepka-Lenhart D, Mistry SK, Morris SM Jr, Wu G (2001) Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells. *Am J Physiol Endocrinol Metab* 280:E75–82
- Lieberman AR (1971) The axon reaction: a review of the principal features of perikaryal responses to axon injury. *Int Rev Neurobiol* 14:49–124.
- Lindquist TD, Sturman JA, Gould RM, Ingoglia NA (1985) Axonal transport of polyamines in intact and regenerating axons of the rat sciatic nerve. *J Neurochem* 44:1913–1919.
- Masuko T, Kusama-Eguchi K, Sakata K, Kusama T, Chaki S, Okuyama S, Williams K, Kashiwagi K, Igarashi K (2003) Polyamine transport, accumulation, and release in brain. *J Neurochem* 84:610–617.
- Mills CD (2001) Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. *Crit Rev Immunol* 21:399–425.
- Mudumba S, Menezes A, Fries D, Blankenship J (2002) Differentiation of PC12 cells induced by *N*<sup>6</sup>-acetylspermidine and by *N*<sup>6</sup>-acetylspermidine deacetylase inhibition. *Biochem Pharmacol* 63:2011–2018.
- Pegg AE, McCann PP (1992) *S*-adenosylmethionine decarboxylase as an enzyme target for therapy. *Pharmacol Ther* 56:359–377.
- Rukenstein A, Greene LA (1983) The quantitative bioassay of nerve growth factor: use of frozen 'primed' PC12 pheochromocytoma cells. *Brain Res* 263:177–180.
- Schwartz M, Kohsaka S, Agranoff BW (1981) Ornithine decarboxylase activity in retinal explants of goldfish undergoing optic nerve regeneration. *Brain Res* 227:403–413.
- Sebille A, Bondoux-Jahan M (1980) Motor function recovery after axotomy: enhancement by cyclophosphamide and spermine in rat. *Exp Neurol* 70:507–515.
- Shantz LM, Pegg AE (1999) Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway. *Int J Biochem Cell Biol* 31:107–122.
- Soiefer AI, Moretto A, Spencer PS, Sabri MI (1988) Axotomy-induced ornithine decarboxylase activity in the mouse dorsal root ganglion is inhibited by the vinca alkaloids. *Neurochem Res* 13:1169–1173.
- Tetzlaff W, Kreutzberg GW (1985) Ornithine decarboxylase in motoneurons during regeneration. *Exp Neurol* 89:679–688.
- Wells MR (1986) Autoradiographic measurement of relative changes in ornithine decarboxylase in axotomized superior cervical ganglion neurons. *Exp Neurol* 92:445–450.
- Wells MR (1987) Changes of ornithine decarboxylase activity in dorsal root ganglion cells after axon injury: possible relationship to alterations in neuronal chromatin. *Exp Neurol* 95:313–322.
- Witte MB, Barbul A (2003) Arginine physiology and its implication for wound healing. *Wound Repair Regen* 11:419–423.
- Wu G, Morris SM Jr (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* 336:1–17.

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