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Activity-Dependent Regulation of Dendritic Spine Density on Cortical Pyramidal Neurons in Organotypic Slice Cultures

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SUMMARY

In order to examine the effects of activity on spine production and/or maintenance in the cerebral cortex, we have compared the number of dendritic spines on pyramidal neurons in slices of P0 mouse somatosensory cortex maintained in organotypic slice cultures under conditions that altered basal levels of spontaneous electrical activity. Cultures chronically exposed to 100 μM picrotoxin (PTX) for 14 days exhibited significantly elevated levels of electrical activity when compared to neurons in control cultures. Pyramidal neurons raised in the presence of PTX showed significantly higher densities of dendritic spines on primary apical, secondary apical, and secondary basal dendrites when compared to control cultures.

The PTX-induced increase in spine density was dose dependent and appeared to saturate at 100 μM. Cultures exhibiting little or no spontaneous activity, as a result of growth in a combination of PTX and tetrodotoxin (TTX), showed significantly fewer dendritic spines compared to cultures maintained in PTX alone. These results demonstrate that the density of spines on layers V and VI pyramidal neurons can be modulated by growth conditions that alter the levels of spontaneous electrical activity. © 1994 John Wiley & Sons, Inc.

Keywords: cerebral cortex, sensorimotor, development, picrotoxin, tetrodotoxin.

INTRODUCTION

A fundamental issue in modern neurobiology is defining the role electrophysiological activity plays in neural development. The dendritic trees of neurons are the primary recipients of electrophysiological input, and as such, are in a position to be affected by alterations in activity. A number of studies have utilized the sensory enrichment/deprivation paradigm to demonstrate that activity can regulate dendritic morphology and spine density (Coleman and Riesen, 1968; Fifkova, 1968; Valverde, 1971; Ryugo et al., 1975a,b,c; Juraska, 1982; van Huizen et al., 1985, 1987; McMullen and Glaser, 1988; Bryan and Riesen, 1989; Müller et al., 1993). In the visual cortex, various studies employing uniocular deprivation (Fifkova, 1968; Rothblat and Schwartz, 1979), binocular deprivation (Globus and Scheibel, 1967; Valverde, 1967, 1971; Coleman and Riesen, 1968; Freire, 1978), and enucleation (Ryugo et al., 1975b; Heumann and Rabinowicz, 1982) have shown that decreased visual input to the cortex during critical periods of development can result in a corresponding decrease in dendritic spine density on pyramidal neurons. Similar studies in the somatosensory (Ryugo et al., 1975a; Harris and Woolsey, 1979; Bryan and Riesen, 1989), auditory (McMullen and Glaser,

1988), and motor (Bryan and Riesen, 1989) cortices also demonstrate that decreased afferent activity can affect the levels of dendritic spine production and maintenance.

To more directly examine the effects of activity on dendritic spine density during early development, we have compared the number of dendritic spines on pyramidal neurons in slices of somatosensory cortex maintained in organotypic slice cultures under conditions in which levels of spontaneous activity were varied. In a previous study we reported that the acquisition of several cortical neuronal characteristics, including dendritic arborization, cellular morphology, and electrophysiological membrane properties, develop normally in the absence of extrinsic influences (Annis et al., 1993). Results of the present study show that growth conditions that alter the levels of spontaneous electrical activity in culture affect the density of spines on cortical pyramidal neurons.

METHODS

Preparation of Organotypic Slice Cultures

The slice culture technique used in this study was adapted from that of Gähwiler (Gähwiler, 1981, 1988). Coronal slices of sensorimotor cortex (two to three slices from each brain) were prepared as described previously (Annis et al., 1993) from newborn (day of birth = P0) mice of the ICR strain. Briefly, freshly cut 400-μm thick Vibratome slices were cut into a dish of cold EOL1 medium (Annis et al., 1990) and then mounted onto coverslips coated with poly-D-lysine and dialyzed rat tail collagen (Bornstein, 1958). Coverslips with attached slices were placed into 16-mm culture tubes with 500 μl of either EOL1-defined medium or EOL1 supplemented with one or a combination of various receptor/channel blockers including 1 μM picrotoxin (PTX); 10 μM PTX; 100 μM PTX; 500 μM PTX; 1 mM PTX; 100 μM PTX and 0.5 μM tetrodotoxin (TTX); or 30 μM 2-amino-5-phosphonopetanoic acid (APV). The tubes were sealed with 18-mm gas-permeable caps (Biomedical Polymers) and placed into a 5.0% CO_2 incubator at 36.0°C and left stationary for 24 h. After the initial 24-h attachment period, cultures were rotated on a roller drum, in the incubator at 10 rev/h. The medium was subsequently changed every other day, and fresh medium was prepared once a week. Cultures received the same medium with or without pharmacological agents, throughout the 14-day culture period.

Extracellular Recordings

On day 14 *in vitro*, selected cultures were removed to a recording chamber and bathed in the growth medium.

Ten continuous extracellular recording trials, for a duration of 30 s each, were made in layers V and VI of three representative cultures from each experimental group. Recordings were made at room temperature using large tip (80–100 μm) tungsten microelectrodes with the cultures bathed in the growth medium. The majority of records were obtained at depths of 25–100 μm from the surface (upper one-third to one-half of the culture). Spontaneous activity from three cultures in each group was monitored using a Dagan 2400 extracellular pre-amplifier (X1000) and digitized using a Phillips PM3352 8-bit digital oscilliscope. Digitized data was downloaded to a Macintosh computer with custom acquisition software and raster plots were constructed as previously described (Spitzer and Semple, 1993).

Intracellular Injection of Lucifer Yellow

After 14 days *in vitro*, slice cultures were fixed for 1 h in 4% buffered paraformaldehyde and stored in 0.1 M phosphate-buffered saline (PBS). Before intracellular injection of Lucifer yellow (LY), slice cultures were stained with 0.000125% ethidium bromide (EB) as a fluorescent counterstain for identifying cortical laminae. Cultured slices were viewed on a fixed-stage fluorescent microscope with fluorescein filter cubes for simultaneous visualization of LY and EB. Sharp intracellular electrodes were pulled on a Sutter Instrument Brown & Flaming micropipette puller and filled with a combination of 5% Lucifer yellow lithium salt (Molecular Probes) and 1% Lucifer yellow cadaverin-x (Molecular Probes). Intracellular impalements were made under visual control in layers V and VI of the cortex; injections were made in cells in the upper one-half to one-third of the culture. Injections were made with a 2- to 7-nAmp pulse of negative current for 0.25 s, followed by a 1-s interpulse period. This injection protocol was continued for up to 5 min or until the tips of distal dendrites could be observed. Slice cultures with filled neurons were then reimmersed in 4% paraformaldehyde for 1–3 h and stored in PBS for processing.

Processing of Lucifer Yellow-Filled Neurons

Slice cultures ($n = 90$) with LY-filled neurons were first washed 1 × 10 min in PBS. In order to minimize background staining, cultures were incubated for 20 min in a 0.5% hydrogen peroxide solution followed by washing in PBS with 0.1% Triton-X-100 (3 × 15 min) and incubation overnight at 4°C in a 1:500 dilution of Extravidin (Sigma) in PBS/Triton-X-100. Cultures were subsequently washed in PBS (3 × 10 min) and pre-incubated in 50 ml of PBS containing 25 mg diaminobenzidene, 1.75 ml of 1% cobalt chloride, and 1.0 ml of 1% nickel ammonium sulphate. Following this pre-incubation, 25

ml of fresh pre-incubation solution with 5 μl of 3% hydrogen peroxide were added and the DAB reaction was allowed to proceed for 15 min. Slices were washed for 10 min in PBS, dehydrated, mounted, and coversliped in DPX.

Analysis

125 LY-filled neurons were analyzed from 90 slice cultures (obtained from 40 animals) either on a Bio-Rad laser confocal microscope (MRC-600) or a Nikon light microscope with either a 60× or 100× oil immersion lens. Image processing of confocal images was conducted on a Gateway 2000 workstation using Optimas and Photoshop software.

To determine neuronal cross-sectional areas, sections of cultured slices or filled pyramidal neurons were examined on a BioQuant 3 image analysis system at a magnification of 800×. In Nissl-stained material, every neuron (identified by the presence of a pale nucleus and a nucleolus) in layers V and VI, within a given field was measured; the areas of 25 neuronal somata per culture were measured from three cultures in each group.

Spine density was determined for LY-filled pyramidal neurons in the deep layers (layers V and VI) of the cortex. Estimates of spine density were made by counting the number of spines per 25 μm on proximal apical dendrites (within 50 μm of the soma), secondary apical dendrites (daughter branches from the apical trunk), or secondary basal dendrites (daughter branches from primary basal dendrites). Counts were made blind with respect to the growth conditions, and average spine density counts were made for each neuron based on two to five samples/branch order/cell (except primary apical dendrite which had one count/cell). All data are expressed as means ± S.E.M., and differences between data groups were evaluated with either the student's t test or ANOVA, where appropriate. Significance was determined at the 95% confidence level, using two-tailed tests.

RESULTS

Spontaneous Activity in Culture

Extracellular electrophysiological recordings were performed to assess overall levels of electrical activity in cultures grown for 14 days under three growth conditions. Control cultures, maintained for 14 days without any pharmacological agents, displayed a low, basal level of spontaneous activity, characterized by 10- to 15-mV single spikes followed by interspike periods of 1–15 s [Fig. 1(A, B)]. In contrast, cultures treated with 100 μM PTX, which blocks GABA$_A$ receptors, showed very high levels of spontaneous activity characterized

by epileptiform burst discharges with 20- to 30-mV spikes and interspike intervals of 0–2 s [Fig. 1(C, D)]. In cultures treated with 100 μM PTX and 0.5 μM TTX, which blocks sodium-dependent action potentials, no spontaneous activity was observed [Fig. 1(E, F)].

Cellular Morphology

Overall health of slice cultures grown under the three conditions was assessed by Nissl-stained cryostat sections of cultures from each group. Low-magnification photomicrographs reveal similar histotypic organization between the control [Fig. 2(A)], 100 μM PTX [Fig. 2(B)], and 100 μM PTX/0.5 μM TTX- [Fig. 2(C)] treated cultures. Higher-magnification analysis demonstrates that layer V and VI Nissl-stained neurons in the 100 μM PTX [Fig. 2(E)] and the combination 100 μM PTX and 0.5 μM TTX- [Fig. 2(F)] treated cultures appeared similar in size and staining quality to those found in control cultures [Fig. 2(D)]. Measurements of cross-sectional somal area indicated no statistically significant difference between the three experimental groups. Average areas ranged from 88–92 sq. μm.

Individual pyramidal neurons in layers V and VI were iontophoretically injected with Lucifer yellow-cadaverin-x to assess possible differences in morphological development as a result of receptor blockade. Qualitatively, pyramidal neurons from control [Fig. 3(A)], 100 μM PTX [Fig. 3(B)], or 100 μM PTX and 0.5 μM TTX- [Fig. 3(C)] treated cultures appeared similar in terms of dendritic length and degree of branching. Quantitative studies of numbers of first- through third-order dendrites of pyramidal neurons and soma size revealed no statistically significant differences between any of the three growth conditions (Table 1). Low-power confocal microscopy in the transmitted light mode (as used in Fig. 3) does not provide for the resolution of fine structures, such as spines. Higher-magnification images under oil immersion, as seen in Figure 4, were necessary to visualize spines.

Examination under higher magnification revealed that pyramidal neurons in the 100 μM PTX-treated cultures [Fig. 4(B)] possessed more spines on their apical and basal dendrites than did similar neurons from control cultures [Fig. 4(A)]. Pyramidal neurons in PTX-treated cultures also appeared to possess more sessile (Jones and Powell, 1969) or stubby spines than control [Fig.
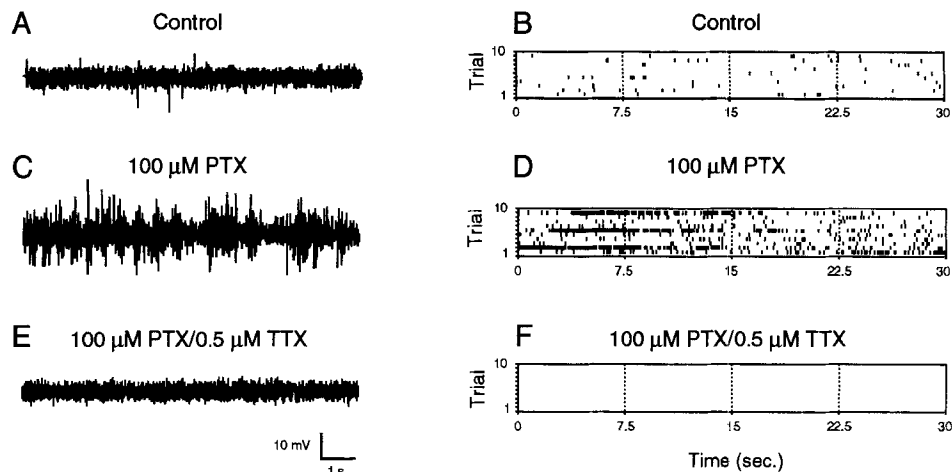
Figure 1   Altered levels of spontaneous activity in response to pharmacological manipulation. Extracellular recordings from cortical slice cultures maintained 14 days *in vitro,* under control (A, B), 100 μM PTX- (C, D), and 100 μM PTX/0.5 μM TTX- (E, F) treated conditions. Representative raw data traces can be seen in (A), (C), and (E), while raster plots of 10 trials of 30-s duration each presented in (B), (D), and (F).

4(A, B)]. In cultures in which spontaneous activity was blocked by tetrodotoxin treatment [Fig. 4(C)], numbers of dendritic spines were at or below that observed on control neurons.

Quantitative Analysis of Spine Development

Density of dendritic spines, defined as the number of spines/25 μm of dendritic length, was determined for primary apical, secondary apical, and secondary basal dendrites of layer V and VI pyramidal neurons. All counts were made blind with respect to the growth conditions. In the first set of experiments, a total of 45 slice cultures were maintained for 14 days in either 30 μM APV (n = 15), 100 μM PTX (n = 16), or normal EOL1 medium (n = 14) as a control. Average spine densities were nearly twofold greater on dendrites from 100 μM PTX-treated cultures than from controls [Fig. 5(A)]. A similar twofold difference was seen on secondary apical [Fig. 5(B)] and secondary basilar dendrites [Fig. 5(C)], as well. In contrast, no difference from control was detected in cultures treated with 30 μM APV [Fig. 5(A, B, C)].

In order to assess whether the PTX-induced increase in spine density was dose dependent, a total of 51 cultures were maintained in various concentrations of PTX and their spine densities examined. After 14 days *in vitro,* neurons maintained in 1.0 μM PTX showed no statistically significant dif-

ference in spine number when compared to control [Fig. 6(A, B, C)]. Pyramidal neurons from cultures grown in 10 μM PTX demonstrated a greater number of spines, as compared to neurons from control cultures, on primary apical [Fig. 6(A)], secondary apical [Fig. 6(B)], and secondary basal [Fig. 6(C)] dendrites. The largest increase in spine density occurred at the 100-μM dosage of PTX, in all three dendritic classes [Fig. 6(A, B, C)]. The number of spines per 25 μm of dendrite was significantly lower on primary [Fig. 6(A)] and secondary [Fig. 6(B)] apical dendrites in cultures treated with 500 μM PTX, when compared to the 100 μM dosage, while no statistically significant change was seen in the secondary basal dendrites [Fig. 6(C)]. Cultures treated with 1-mM dosages of PTX showed signs of necrosis and cellular degeneration and therefore cells were not analyzed for changes in spine density.

In order to determine whether changes in spine density were attributable to PTX-induced changes in electrophysiological activity, and not due to nonspecific drug effects, experiments were conducted in which cultures were raised in the presence of 100 μM PTX with 0.5 μM TTX added to block all sodium-dependent electrical activity. Dendritic spine density was significantly lower in all three dendritic classes, in neurons from PTX-/TTX-treated cultures when compared to neurons from cultures raised in 100 μM PTX alone [Fig. 7(A, B, C)]. When compared to control levels of spine
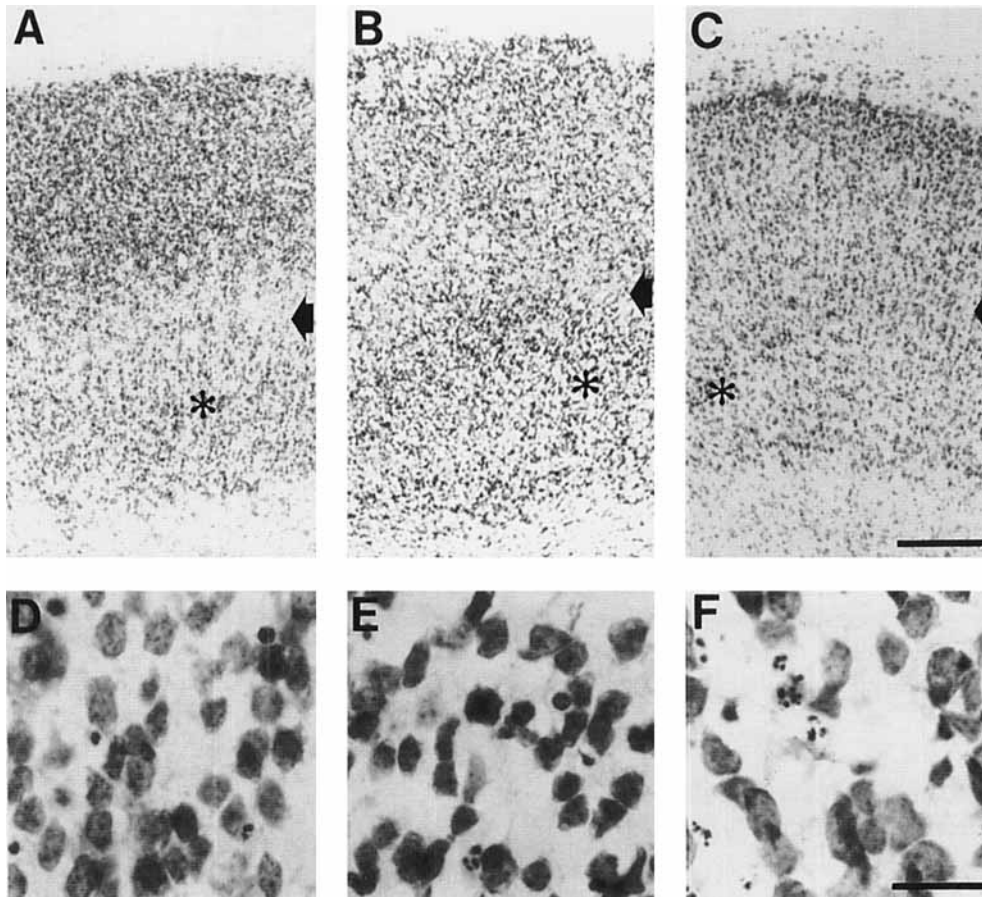
Figure 2 Histological organization of slice cultures after pharmacological manipulation. Photomicrographs of 25-μm thick Nissl-stained cryostat sections through 14-day-old cultures raised under control (A, D), 100 μM PTX- (B, E), and 100 μM PTX-/0.5 μM TTX- (C, F) treated conditions. Low-power micrographs (A, B, and C) demonstrate organotypic organization of slice cultures raised under the three conditions (arrowheads indicate border of layers V and VI; scale bar = 200 μm in C). Asterisks indicate regions where higher-magnification micrographs (D, E, and F) were obtained. These photomicrographs reveal the similar size of neurons located in layer VI (scale bar = 20 μm in F).

density, PTX/TTX neurons from treated cultures demonstrated a statistically significant reduction in spine density on secondary basal [Fig. 7(C)] and secondary apical [Fig. 7(B)] dendrites.

DISCUSSION

The first 2 weeks of postnatal life are a time of rapid development in the rodent neocortex. Complex cortical circuits are established and consolidated, while at the cellular level, neurons undergo extensive dendritic arborization, elongation and spine production (Ramon y Cajal, 1911; Wise et al., 1979; Miller, 1981, 1988; Miller and Peters,

1981; Petit et al., 1988; Horner, 1993). In order to assess whether levels of altered electrophysiological activity can influence the acquisition of spines, experiments were conducted which quantitatively examined dendritic spine density of layer V and VI pyramidal neurons grown under conditions of altered spontaneous electrical activity. The results of this study indicate that elevated levels of spontaneous activity associated with the blockade of GABA_A receptors lead to a twofold increase in the number of dendritic spines. Decreased levels of activity, created by the blockade of all sodium-dependent electrical activity, resulted in a 30%–40% reduction in spine density on secondary apical and basal dendrites, when compared to controls. The
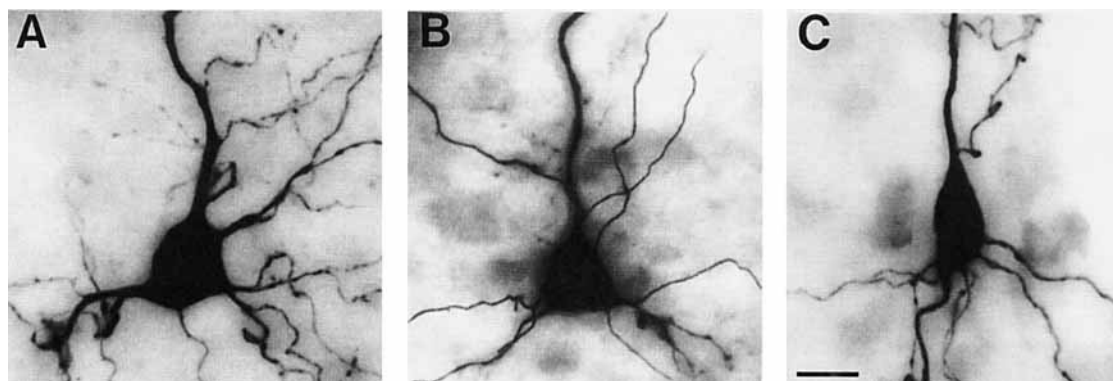
Figure 3 Effects of altered activity on cell morphology. Confocal images of layer V and VI Lucifer yellow-filled pyramidal neurons in control (A), 100 μM PTX- (B) and 100 μM PTX-/ 0.5 μM TTX-treated cultures after 14 day *in vitro*. Scale bar = 10 μm.

results of this study, along with other studies employing sensory stimulation/deprivation paradigms, suggest that levels of electrophysiological activity within the cerebral cortex contribute to the regulation of dendritic spine density on pyramidal neurons.

Effects of Chronic Pharmacological Manipulation on Cortical Pyramidal Neurons

For over 3 decades, investigators have examined the question of whether electrical activity plays a role in the structural development of the cerebral cortex (Coleman and Riesen, 1968; Fifkova, 1968; Valverde, 1971; Ryugo et al., 1975a; McMullen and Glaser, 1988; Bryan and Riesen, 1989). Experiments conducted in the visual (Coleman and Riesen, 1968; Fifkova, 1968; Valverde, 1971; Ryugo et al., 1975b), somatosensory (Ryugo et al., 1975a; Harris and Woolsey, 1979; Bryan and Riesen, 1989), motor (Bryan and Riesen, 1989), and auditory (McMullen and Glaser, 1988) cortices *in*

vivo, have shown that the blockade or manipulation of sensory stimuli can affect the normal development of dendrites and the acquisition of dendritic spines. In the present study, cortical activity levels were directly manipulated through the selective blockade of various receptors and/or channel types, in organotypic slice cultures. The noncompetitive $GABA_A$ receptor antagonist picrotoxin was used to increase levels of electrophysiological activity by blocking inhibitory GABA-ergic transmission (Connors et al., 1982; van Huizen et al., 1987; Corner and Ramakers, 1992; Müller et al., 1993). Epileptiform activity was induced in the cortical slices chronically exposed to 100-μM concentrations of PTX. This epileptiform activity is presumably attributable to disinhibition of the network of excitatory, intrinsic connections established in these slice cultures *in vitro* (Wolburg and Bolz, 1991; Distler and Robertson, 1993) thus confirming that PTX had the expected electrophysiological effect. These repetitive bursts of action potential discharges were rarely if ever seen in cultures maintained in untreated growth medium;

Table 1 Numbers of First-, Second- and Third-Order Dentritic Branches and Somal Areas of Layer V and VI Pyramidal Neurons Raised under Control, 100 μM PTX- and 100 μM PTX-/0.5 μM Treated Conditions

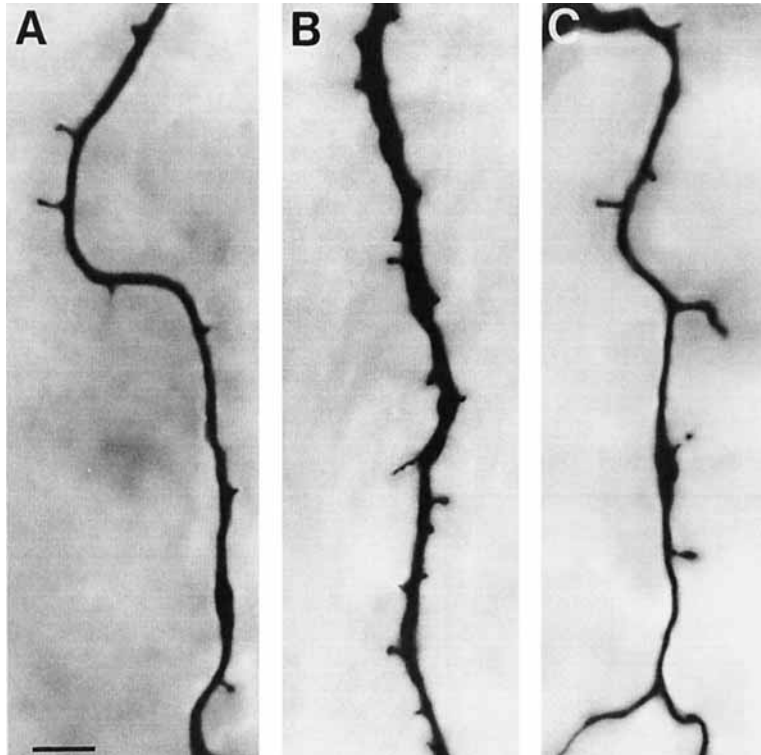| Condition	First-Order Dendrite	Second-Order Dendrite	Third-Order Dendrite	Cell Area (sq. μm)
Control	3.5 ± 0.17	9.3 ± 0.50	9.9 ± 0.94	134.4 ± 7.5
	$n = 10$	$n = 10$	$n = 10$	$n = 10$
100 μM PTX	3.5 ± 0.22	10.4 ± 0.54	11.1 ± 0.96	145.9 ± 11.1
	$n = 10$	$n = 10$	$n = 10$	$n = 10$
100 μM PTX	3.6 ± 0.22	10.2 ± 0.99	10.1 ± 1.0	152.6 ± 10.4
0.5 μM TTX	$n = 10$	$n = 10$	$n = 10$	$n = 10$

Figure 4 Confocal images of pyramidal cell secondary basal dendrites after 14 days under control (A), 100 μM PTX- (B) and 100 μM PTX-/0.5 μM TTX conditions. Note increased density of spines in (B) with higher incidence of sessile or "stubby" spines. Scale bar = 2 μm.

control cultures showed a low level of spontaneous activity, similar to that seen in other organotypic slice culture systems (Wolfson et al., 1989). In order to determine whether the PTX effects observed in this study were attributable to elevated levels of electrophysiological activity and to determine the effect of low spontaneous activity on spine density, a series of cultures were grown in the presence of both PTX and TTX. This growth condition resulted in complete cessation of spontaneous electrical activity throughout the culture and a concommitant decrease in spine density. All electrophysiological analyses were conducted at the PTX dose resulting in the maximal effect on spine density. Further experiments will be necessary to determine the specific relationship between submaximal PTX doses and levels of electrical activity.

Nissl-stained sections of cultures indicated that altered activity levels did not adversely affect the gross organization of the slices after 14 days *in vitro*, a finding further supported by measurements of dendritic branch order and somal area of Lucifer yellow-filled pyramidal neurons within layers V

and VI. Further, the data from both groups were comparable to values measured from cultured and acute slice neurons in a previous study (Annis et al., 1993). Average somal areas of LY-filled pyramidal neurons indicated that filled neurons were larger than the cellular population as a whole, as seen in Nissl stain. This difference may be due to the increased likelihood of impaling a larger neuron over a smaller one and by the larger somal size of pyramidal neurons compared to other morphological classes of neurons. These findings, in aggregate, support the notion that the pharmacological manipulations conducted in this study did not adversely affect the gross morphological development of the neuronal populations.

In contrast to our findings that PTX treatment did not affect somal size or dendritic branching, chronic exposure of the cultures to 100 μM PTX in the normal nutritive medium resulted in a twofold increase in the number of dendritic spines, after 14 days *in vitro*. A dose–response analysis of PTX sensitivity demonstrated that the PTX-induced increase in spine density was saturable with the maximal response occurring at 100 μM. A recent study
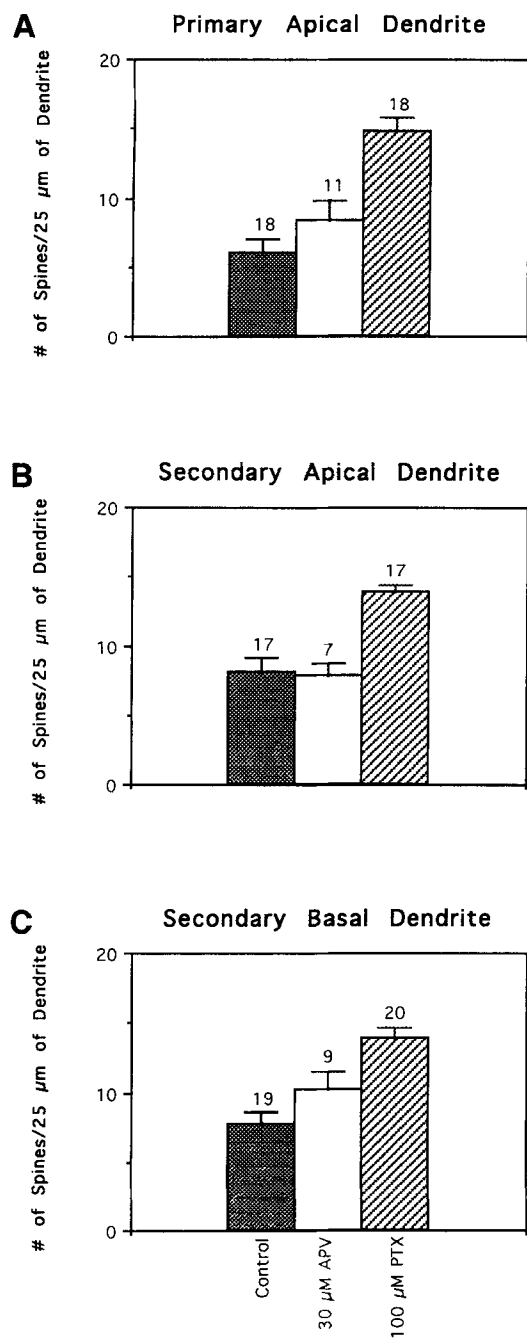
Figure 5 Changes in spine density in response to pharmacological manipulation. The average number of spines/25 μm of primary apical (A), secondary apical (B), and secondary basal (C) dendrites raised under control, 100 μM PTX- or 30 μM APV-treated conditions for 14 days (mean ± S.E.M., n indicated for each condition). Significant increases in the number of dendritic spines/25 μm of dendrite were observed on all dendritic classes examined in 100 μM PTX-treated cultures ($p <$ 0.0001, Student's t test), whereas no significant difference from control could be detected in 30-μM APV-treated cultures.
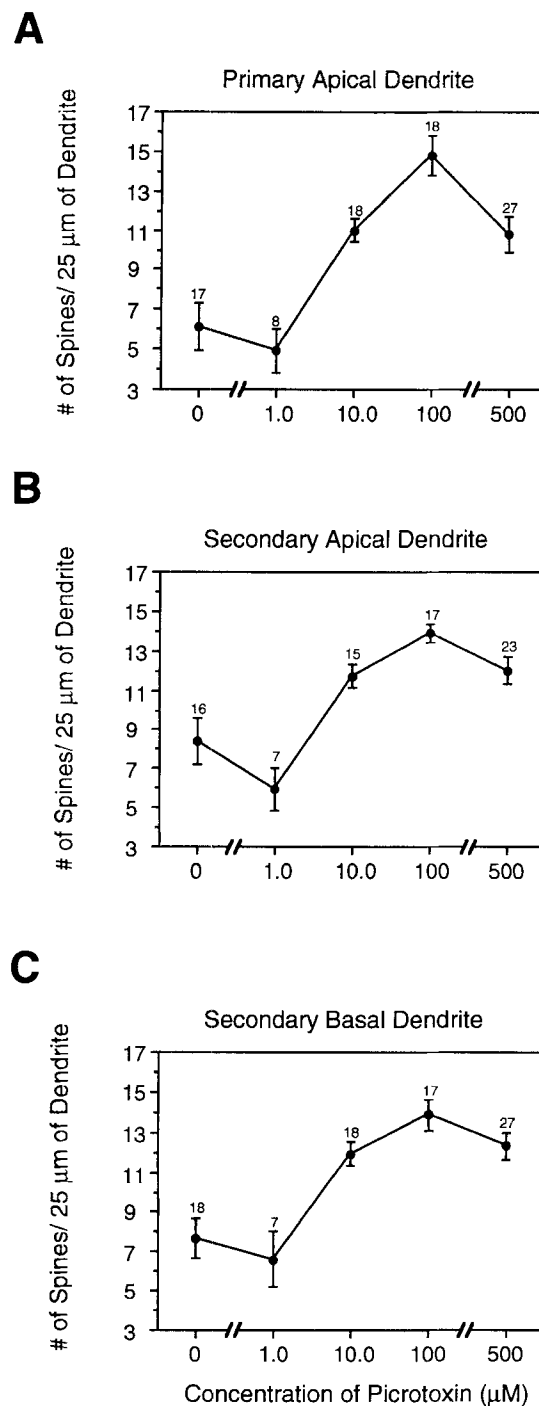
Figure 6 Dose dependence of PTX treatment. PTX dose dependence was examined for primary apical (A), secondary apical (B), and secondary basal dendrites (C) under control, 1.0 μM, 10 μM, 100 μM, 500 μM, and 1 mM PTX concentrations (mean ± S.E.M.; n indicated for each condition). A saturable effect was observed in all three dendritic classes with the maximal effect occurring at the 100 μM dosage ($p < 0.0001$, Student's t test, 95% confidence ANOVA). 1-mM dosages of PTX resulted in necrosis and neurons were not analyzed.
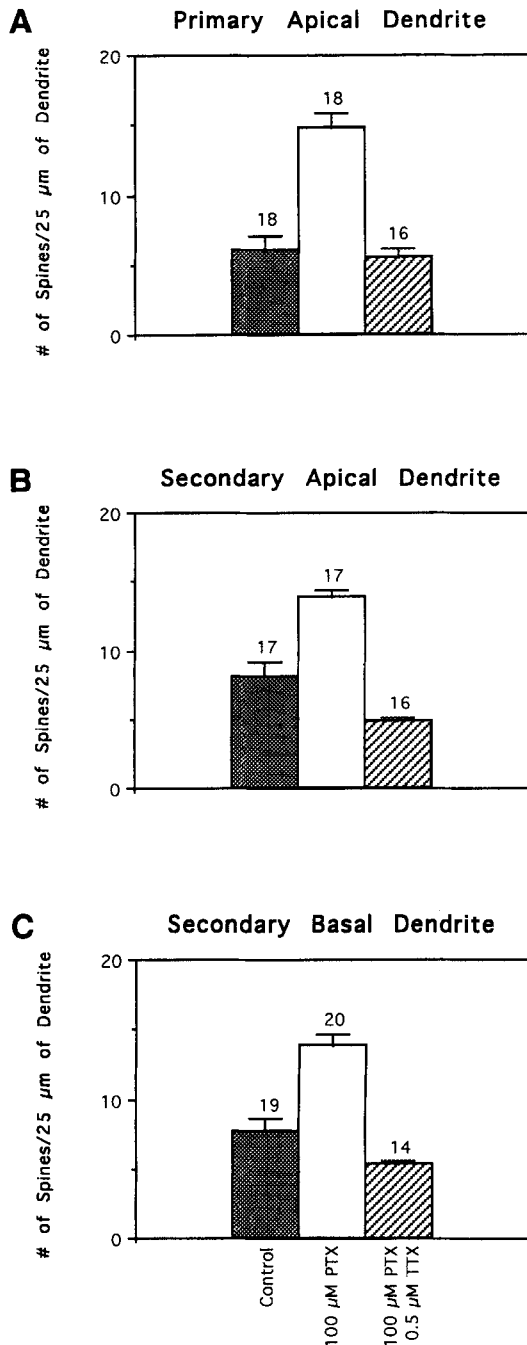
Figure 7 Effects of low spontantaneous activity on spine density. Cultures treated with a combination of 100 μM PTX and 0.5 μM TTX showed significantly fewer spines on their primary apical (A), secondary apical (B), and secondary basal (C) dendrites than did cultures treated with 100 μM PTX alone ($p < 0.0001$, Student's t test) (mean ± S.E.M.; n indicated for each condition). PTX-/TTX-treated secondary apical ($p < 0.01$, Student's t test) (B) and secondary basal ($p < 0.05$, Student's t test) (C) dendrite spine densities were significantly lower than control.

by Müller et al. (1993) has shown that, in hippocampal slice cultures, epileptiform activity created by 500 μM PTX treatment causes a significant decrease in apical dendrite spine density on hippocampal pyramidal neurons. This study used rat tissue, 6–9 days old at the time of culturing that was maintained for 12–20 days in a combination of serum-supplemented and semisynthetic medium; PTX was added only during the final 3 days of the experiment. This experiment raises the question of whether regulation of dendritic spine density in regions of the brain other than the cortex occur by a different mechanism or whether the interplay of differing local circuitries affect spine density differently.

Mechanisms Underlying Activity-Dependent Regulation

Changes in the density of dendritic spines could be the result of several synaptic and/or nonsynaptic events associated with increased electrophysiological activity. It is known that epileptiform activity can result in increased expression of neurotrophic factors (Gall, 1992). Elevated levels of these factors could promote dendritic growth and synaptic connectivity, including spine formation. It is also possible that the establishment of an excitatory synapse on the dendritic shaft of a pyramidal neuron or the activation of a postsynaptic density by excitatory neurotransmitters may be sufficient to initiate spine formation. Conversely, if excitatory activity is diminished, there may no longer exist the synaptic driving force/cue necessary to create or maintain the normal number of dendritic spines. To determine whether this is indeed the case, it would be important to use electron microscopy to establish whether all of the spines in PTX-treated cultures are associated with presynaptic structures.

This process of spine formation could be initiated by one or more cellular mechanisms. One potential mechanism is the activation of calcium-dependent enzymes as a result of increased calcium influx at the sites of excitatory synapses (Horner, 1993). Our study showing that blockade of the NMDA receptor with APV alone has no significant effect on spine density would suggest that calcium influx through the NMDA receptor is not involved in spine formation in the untreated growth condition. To determine whether calcium influx through the NMDA channel is involved in the PTX-induced increases in spine density, experi-

ments could be conducted where both the GABA and NMDA channels are blocked via PTX and APV. It would also be of interest to raise cultures in the presence of low levels of magnesium to remove the magnesium block from the NMDA receptor and thus increase activity via a second, non-GABA-ergic, mechanism.

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