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The Neurosteroid 3α-Hydroxy-5α-Pregnan-20-One Induces Cytoarchitectural Regression in Cultured Fetal Hippocampal Neurons

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The neurosteroid 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP) acts as a potent allosteric modulator and a direct activator of the GABA–chloride channel complex. This neurosteroid has also been found to protect against seizures that arise from blockade of the GABA–chloride channel complex. Because 3α,5α-THP protects against excitotoxin-induced seizure activity and because seizure activity has been found to be associated with aberrant hippocampal nerve cell growth, the rapid effect of the neurosteroid 3α,5α-THP upon nerve cell growth was investigated using videomicroscopy of hippocampal neurons in culture. Within 40 min of exposure 3α,5α-THP induced a significant decrease in the area and length of neurites. A concomitant decrement in the number and length of filopodia decorating neuritic extensions also occurred within the 40 min of 3α,5α-THP exposure. Both rapid and slow retrograde movement of intracellular organelles was observed in 3α,5α-THP–treated neurons. 3α,5α-THP–induced regression of neuritic extensions occurred only in nerve cells that had not yet established contact with other nerve or glial cells in culture. Established structural connections between neurons or glia did not erode during 3α,5α-THP exposure. Neither the inactive stereoisomer 3β-hydroxy-5β-pregnan-20-one nor progesterone had a significant effect upon any of the morphological parameters assessed. In approximately 25% of the cells in which 3α,5α-THP had induced regression, subsequent exposure to 17 β-estradiol induced profuse filopodial growth within 60 sec of exposure. In cultures similar in age to those used in the morphological studies, 3α,5α-THP induced a significant increase in $^{36}Cl^-$ uptake within 10 sec. The magnitude of $^{36}Cl^-$ uptake was comparable to that induced by exposure to 100 μM GABA. In older, more mature cultures in which the nerve cells had established structural connections, 3α,5α-THP protected cells from picrotoxin-induced nerve cell death. These results demonstrate that 3α,5α-THP can induce regression of neuronal morphology within a relatively rapid time frame. 3α,5α-THP induction of $^{36}Cl^-$ uptake within 10 sec suggests that activation of neurosteroid-regulated chloride channels is an initial step in the biochemical mechanism underlying the retraction induced by this progesterone metabolite steroid. In select instances, 17 β-estradiol induced an extremely rapid reversal of the filopodial regression produced by 3α,5α-THP. Collectively, these findings indicate that steroid factors acting singly and in combination can induce significant changes in nerve cell morphology within a time frame that is consistent with 3α,5α-THP and 17 β-estradiol regulation of excitability.

[Key words: neurosteroids, progesterone metabolite steroids, nerve cell growth, hippocampal neurons, epilepsy, estrogen]

Recent discoveries in the field of neurosteroids have led to a resurgence of research efforts to better understand the influence of steroids acting at membrane receptors on CNS function (Harrison and Simmonds, 1984; Barker et al., 1986; Majewska et al., 1986; Gee et al., 1987, 1988; Harrison et al., 1987; Lambert et al., 1987; Morrow et al., 1987, 1990; Gee, 1988; Turner et al., 1989; Purdy et al., 1990; reviewed in Paul and Purdy, 1992). These recent discoveries are based on the now classical work of Selye (1941, 1942), who observed anesthetic effects of the steroids progesterone and corticosterone.

Results from several laboratories have shown that glial cells in the brain are capable of de novo synthesis of select steroids (Weidenfeld et al., 1980; Holzbauer et al., 1985; Hu et al., 1987; Jung-Testas et al., 1989a,b; Krieger and Scott, 1989; Baulieu and Robel, 1990; Karavolas and Hodges, 1990, 1991). These findings lead Baulieu, Robel, and colleagues to propose the concept of neurosteroids (Le Goascogne et al., 1987; Jung-Testas et al., 1989). Specifically, these investigators observed that glial cells were capable of cholesterol, progesterone, and pregn-5-ene-3β,20α-diol synthesis. They further found the conversion of progesterone into the metabolites pregnane-3,20-dione and 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP). Most recently, it has been found that accumulation of 3α,5α-THP can reach levels as high as 30 ng/ml in pregnant rats (Paul and Purdy, 1992).

Biochemical and electrophysiological studies performed during the same time frame indicated that metabolites of progesterone could directly induce chloride flux at micromolar concentrations and at lower concentrations could act as an allosteric modulator of GABA-mediated chloride ion conductance (Harrison and Simmonds, 1984; Majewski et al., 1986; Gee et al., 1987, 1988; Harrison et al., 1987; Lambert et al., 1987; Morrow et al., 1987; reviewed in Gee, 1988). Collectively, results of these studies have shown that the naturally occurring metabolite of

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progesterone, 3α,5α-THP, and the naturally occurring metabolite of deoxycorticosterone, 3α,21-dihydroxy-5α-pregnan-20-one, can directly interact with receptors to regulate chloride channel activity in a GABA-dependent manner at low concentrations and in a GABA-independent manner at higher concentrations.

Because 3α,5α-THP had been shown to enhance chloride conductance through the GABA–chloride channel complex and because this complex has been implicated in the control of epilepsy, the ability of 3α,5α-THP to protect against seizure activity was investigated. Results of these studies showed that 3α,5α-THP protected against chemically induced seizures at concentrations that were more potent than either benzodiazepines or barbiturates (Gee et al., 1988; Paul and Purdy, 1992).

A correlative relationship between seizure activity and change in nerve cell morphology has been reported. Several laboratories have found aberrant growth of hippocampal circuitry following kindling-induced seizures (Tauck and Nadler, 1985; Cronin and Dudek, 1988; Geinisman et al., 1988; Sutula et al., 1988; Represa et al., 1989; Ben Ari and Represa, 1990). Mossy fiber sprouting was found both in postmortem brain tissue derived from children who suffered with childhood epilepsy (Represa et al., 1989) as well as in surgically derived tissue samples from human adults (reviewed in Ben Ari and Represa, 1990). Collectively, these results strongly suggest that seizure activity results in aberrant nerve cell growth.

Because seizure activity is associated with aberrant nerve cell growth and since 3α,5α-THP protects against seizure activity, the hypothesis that 3α,5α-THP would inhibit nerve cell growth was formulated. Experiments to test this hypothesis utilized videomicroscopy to monitor cytoarchitectural changes of individual nerve cells following exposure to 3α,5α-THP. Results of this study demonstrated that 3α,5α-THP induced neurite and filopodial regression within a time frame that is consistent with its seizure-suppressive effects, that 3α,5α-THP induced chloride uptake into cultured hippocampal neurons, that 3α,5α-THP protected against picrotoxin-induced nerve cell death, and that, in select instances, 17 β-estradiol reversed the regressive effect of 3α,5α-THP.

Materials and Methods

Culture preparation. Hippocampal cultures were prepared as described by Bartlett and Banker (1984). Briefly, hippocampi were dissected from the brains of embryonic day 18 (E18) rat fetuses, treated with 0.25% trypsin in serum-containing media for 15 min at room temperature, and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. Between 20,000 and 40,000 cells were seeded onto polylysine-coated (10 μg/ml) coverslips (22 mm in diameter). Nerve cells were grown in phenol red–free Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with 10 mM $NaHCO_3$, 5 U/ml penicillin and 5 μg/ml streptomycin (GIBCO), 10% F12 nutrient medium (GIBCO), and 10% fetal calf serum (GIBCO). Medium was phenol red free since it has been shown that phenol red can act as an estrogen agonist in culture (Berthois et al., 1986). Nerve cells were used following 1–4 d in culture.

Preparation of steroids. Steroids (Steraloids, Inc.) were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in serum-containing culture media to desired concentration. Final concentration of DMSO added to the cultures was 0.01%, a concentration that had no effect upon nerve cell growth.

Observations of living nerve cells. Nerve cells attached to polylysine-coated coverslips were removed from the culture dish and rapidly mounted into a perfusion chamber. Media exchange took place every 10 min. Steroid-containing medium was added at the end of the 20 min equilibration period. Volume of the chamber was 200 μl and complete media exchange was accomplished within 30 sec; pH was maintained at 7.4.

Videomicroscopic recording of individual living nerve cells was accomplished using a Dage-MTI camera equipped with a Newvicon tube linked to an Olympus BH-2 microscope and a Panasonic time-lapse video recorder (model AG-6050). Recordings were made using phase-contrast optics with a 100× objective and a 1.25 or 1.5 multiplier with 100 W tungsten source passed through a green filter. Recordings occurred between 24 and 96 hr following seeding of the culture.

Morphological and statistical analysis of cytoarchitectural data. Morphological analysis was achieved using a BioQuant Image Analysis system, which permitted quantitation of length, area, and total number of filopodial extensions. Statistically significant differences were determined by a mixed-design analysis using a repeated-measures ANOVA for within groups, followed by an ANOVA for between groups, followed by a Newman–Keuls post hoc analysis. Potential bias in selection of nerve cells to receive experimental treatment was evaluated by a comparative analysis between filopodial length of control and 3α,5α-THP–treated neurites. Filopodial length was chosen because this morphological parameter is uniformly distributed independent of cell type, whereas neurite or branch length is highly dependent upon cell type. Statistical analysis of filopodial length (7.4 ± 0.6 μm, $n = 144$) at time 0 of control neurites and filopodial length (7.5 ± 0.7 μm, $n = 144$) at time 0 of 3α,5α-THP–treated neurites revealed no significant difference ($p < 0.98$) between the groups. This result indicates that bias was not introduced into the selection of neurons for experimental treatment. Moreover, analysis for statistically significant differences based on a change from original length or number obviates any contribution of intrinsic morphological limits determined by cell type. Prior to pooling the data obtained with 250 nM and 1 μM 3α,5α-THP, a Student's t test was performed. Results of this analysis indicated no significant difference between the magnitude of response of neurites treated with 250 nM and 1 μM THP in either neurite area ($p < 0.18$), neurite length ($p < 0.65$), or number of filopodia ($p < 0.31$). Thus, the data obtained from the two concentrations were pooled.

$^{36}Cl^-$ uptake. Cultures were prepared as described above with the exception that cells were seeded onto polylysine-coated (10 μg/ml) plastic coverslips (22 × 22 mm, Arthur H. Thomas). $^{36}Cl^-$ uptake combined the strategies described in Thampy and Barnes (1984) and Suzdak et al. (1986). Briefly, neurons were cultured for 3 d in media described above. Coverslips were removed from tissue culture medium and incubated at 37°C for 15 min in fresh DMEM-free medium saturated with a 5% CO_2/95% air mixture. Coverslips were then rinsed for 3–4 sec at room temperature in Tris-HEPES–buffered saline (pH 7.4) containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM *d*-glucose. Immediately after the rinse the coverslips were drained on tissue paper and then transferred to Tris-HEPES–buffered saline containing $^{36}Cl^-$, 0.96 μCi/ml, 100 μM GABA (Sigma), or 1 μM 3α,5α-THP. After 10 sec incubation at room temperature with $^{36}Cl^-$ and experimental substances, uptake was terminated by rapid transfer of the coverslip to ice-cold Tris-HEPES buffer containing 100 μM picrotoxin (Sigma) and rinsed 3 × 2 sec in picrotoxin-containing buffer. Coverslips were transferred to scintillation vials containing 1 ml of 0.5 NaOH and incubated at room temperature for 4 hr. A 200 μl aliquot was withdrawn for protein determination using the Pierce Protein Assay Reagent (Rockford, IL), and scintillation fluid added to the remaining 800 μl of sample for scintillation counting. Data are expressed as nmol $^{36}Cl^-$/μg protein and were analyzed for significant differences using a Student's t test.

Cytotoxicity. Hippocampal nerve cell cultures were treated for 15 hr with either 1 mM picrotoxin (Sigma), 1 μM 3α,5α-THP, or a combination of 1 mM picrotoxin and 1 μM 3α,5α-THP. Picrotoxin was dissolved in serum-containing media while 3α,5α-THP was first dissolved in DMSO and diluted in serum-containing media to desired concentration. The final concentration of DMSO in the culture was 0.01%, a concentration that had no effect on nerve cell growth. Control cultures were treated with serum-containing media with 0.01% DMSO. Cell death determination was achieved by counting trypan blue–positive nerve cells using phase-contrast microscopy at 40× magnification. A one-way ANOVA followed by a Newman–Keuls post hoc analysis was used to determine significant differences between the groups.

Results

General features of 3α,5α-THP responsivity

The effect of 3α,5α-THP (1 μM) upon neurite growth was evident in most neurites tested, in that 70% of the neurites observed showed responsivity. The percentage of 3α,5α-THP–responsive

Table 1. Steroid specificity of effects upon parameters of nerve cell growth

Condition	Neurite area (μm)	Neurite length (μm)	Number of filopodia
Control	−3.38 ± 3.17 (18)	−1.76 ± 0.83 (20)	−0.95 ± 0.50 (20)
3β,5β-THP	−5.53 ± 12.58 (30)	−1.95 ± 0.75 (30)	−2.47 ± 0.66 (30)
Progesterone	−9.10 ± 8.03 (22)	−1.64 ± 1.25 (22)	−2.82 ± 1.03 (22)

All data are expressed as change from baseline value at end of 40 min observation period for each experimental condition. Values represent mean ± SEM (n = number of neurites analyzed).

neurons remained consistent throughout and across days in culture, up to 4 d. Morphological characteristics in culture were not a predictive measure of which cell type would be responsive. Thus, even though a cell would have many of the hallmark morphological characteristics of a hippocampal pyramidal cell, these cytoarchitectural qualities did not serve as a consistent marker of 3α,5α-THP responsivity. Neurites that were in an active growth phase as well as neurites that appeared to be in a quiescent growth state exhibited 3α,5α-THP responsivity. Moreover, the responsivity of one neuritic extension on a nerve cell usually but not always was associated with retraction in the other neuritic extensions of the same neuron. Lastly, both morphologically determined axons and dendrites showed 3α,5α-THP responsivity.

3α,5α-THP influences upon morphological characteristics of neuritic extensions

Neurite area of 3α,5α-THP–treated neurons showed a significant decrease in total area within 20 min of exposure to the steroid [$F = 5.25$; df 2, 76; $p < 0.007$] (Figs. 1, 2). Moreover, the decrement in neurite area at 30 and 40 min was significantly greater than that which was apparent at 20 min. Reduction of neurite area appears to reach maximum between 30 and 40 min since the loss in area at 40 min was not significantly greater than that which occurred at 30 min. Neurite area in control neurons did not change significantly during the 40 min observation period.

A significant decrement in neurite length also occurred in response to 40 min exposure to 3α,5α-THP [$F = 5.67$; df 2, 80; $p < 0.004$] (Figs. 1, 3). As with neurite area, the loss in neurite length at 40 min was significantly greater than that which occurred following 20 min exposure to 3α,5α-THP (Fig. 3). Retraction of the neurite shown in Figure 1 indicates that large-scale retraction is associated with cytoplasmic regression toward the cell body while the cytoskeletal fibers remain intact. This response suggests the potential for reversal of neurite retraction. Control neurites exhibited no significant change in neurite length during the 40 min observation period.

In response to 3α,5α-THP, the number of filopodia/neurite decreased significantly [$F = 6.09$; df 2, 80, $p < 0.003$] within 40 min (Fig. 4*A*). Similar to the decrease in neurite area and length, the loss in number of filopodia at 40 min was significantly greater than that which occurred following 20 min exposure to 3α,5α-THP (Fig. 4*A*). Filopodial length also decreased significantly following 40 min exposure to 3α,5α-THP (*, $p < 0.02$, Fig. 4*B*). In control neurites, the number of filopodia decorating neurites tended to also decrease, but not significantly (1 filopodium/40 min; Fig. 4*A*), while the length of the remaining filopodia increased during the 40 min observation period (0.7 μm ± 0.3; Fig. 4*B*).

In a number of cells studied, one neurite had established a physical contact with a neurite from an adjacent cell. In these instances, treatment with 3α,5α-THP did not induce an erosion of the established structural contact. This observation was confirmed in a separate series of experiments described below that examined the ability of 3α,5α-THP to protect against picrotoxin-induced cell death. In these experiments, cells were treated with 1 μM 3α,5α-THP for 24 hr without evidence of erosion of structural connections between neurons and or glial cells. Interestingly, a structural contact at one neurite did not preclude the responsivity of other unconnected neurites to the regressive effect of 3α,5α-THP.

Mobility of intracellular phase-dense bodies in dendrites and axons

As seen in Figure 1, many phase-dense bodies (PDBs) are apparent throughout the extent of the neurite. PDBs were frequently apparent in both control and 3α,5α-THP–treated neurites under study. However, movement of PDBs was most apparent in 3α,5α-THP–treated neurites. PDBs, ranging in size between 2.1 and 4.8 μm², could be observed traversing the entire length of the neurite by rapid saltatory and bidirectional movements at a rate between 1.8 and 0.60 μm/sec. PDBs also exhibited merger with larger PDBs while other PDBs would merge and then reemerge from such a union. During the evacuation of the cytoplasmic compartment of the neurite, the PDBs accumulated into large masses that underwent slower retrograde movement toward the cell body (1.42 μm/min; Fig. 1). PDB movement was also apparent in 3α,5α-THP–treated neurites that had contacted other cells. In these instances, PDBs continued to be rapidly motile but did not undergo retrograde movement away from the site of structural contact.

Steroid specificity

The stereoisomer of 3α,5α-THP, 3β-hydroxy-5β-pregnan-20-one (3β,5β-THP), was tested for effects upon hippocampal nerve cell growth. This stereoisomer 3β,5β-THP had no significant effect upon any of the morphological parameters assessed (n = 30 neurites). Similarly, neurites (n = 22) treated with progesterone for 40 min showed no significant change in morphology (see Table 1).

3α,5α-THP effects on $^{36}Cl^-$ uptake

Addition of 3α,5α-THP (1 μM, the same concentration used in the morphological studies) induced a significant ($p < 0.01$) increase in $^{36}Cl^-$ uptake above basal following a 10 sec exposure (Fig. 5). GABA (100 μM) also induced a significant increase in $^{36}Cl^-$ uptake above basal but was less in magnitude than the uptake induced by 3α,5α-THP. In an attempt to determine whether $^{36}Cl^-$ uptake persisted over the 40 min 3α,5α-THP exposure as in the morphological studies, $^{36}Cl^-$ uptake was measured following a 40 min exposure to 3α,5α-THP. Results of this study revealed that 1 μM 3α,5α-THP-treated cultures were not significantly different (3.9 ± 0.44 nmol/μg protein, n = 12;

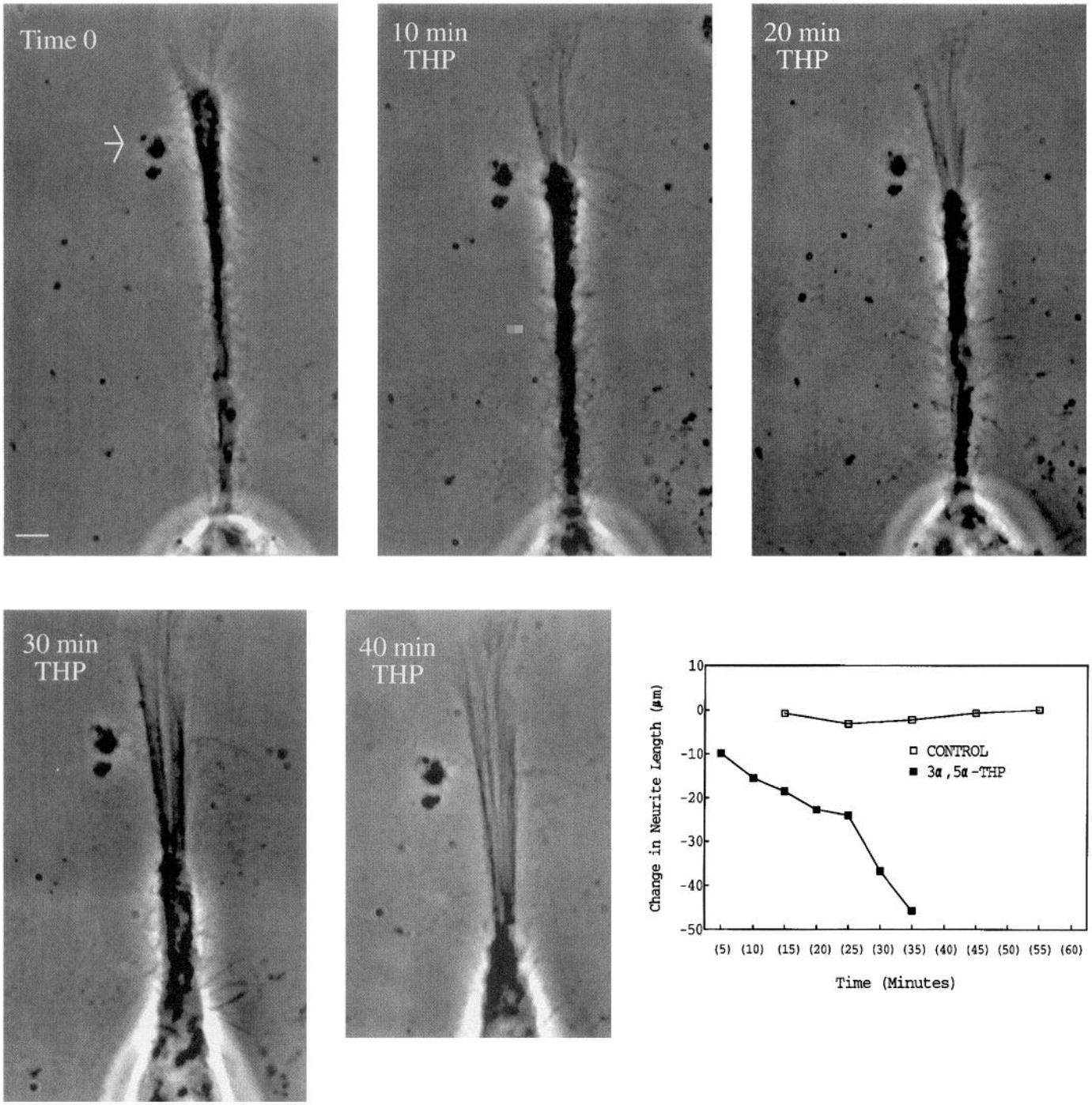

Figure 1. Photomicrograph of a hippocampal nerve cell neurite prior to (*Time 0*) and during *10, 20, 30,* and *40 min* of exposure to 1 μM 3α,5α-THP. The relative time of recording (in min) of the same neurite is denoted in the *upper left corner* of each panel. A cluster of debris marked by the *white arrow* serves as an invariant reference point. Criterion for photo composition was inclusion of the tip of the cytoskeleton of the neurite to the cell body. Thus, *Time 0, 10,* and *20 min* are composites of three photographs, while *30 min* is a composite of two photographs and *40 min* is a single photograph. To maintain the same magnification for all images, *30* and *40 min* images are shorter in length and consistent with regression of the cytoplasmic domain of the neurite back to the cell body. *Lower right panel* indicates the rate of neurite regression (1.4 μm/min). Scale bar, 5 μm.

$p < 0.5$) from basal $^{36}Cl^-$ uptake (4.32 ± 0.53 nmol/μg protein, $n = 8$). These results indicate that $^{36}Cl^-$ uptake is not constant during the 40 min exposure to 3α,5α-THP and that mechanisms for chloride extrusion from the cell operated effectively during the 40 min of exposure to 3α,5α-THP.

3α,5α-THP effects on picrotoxin-induced cytotoxicity

Exposure to 1 mM picrotoxin for 15 hr induced a significant decrease in nerve cell survival in 6-d-old hippocampal neurons in culture (Fig. 6). Although both nerve and glial cells developed

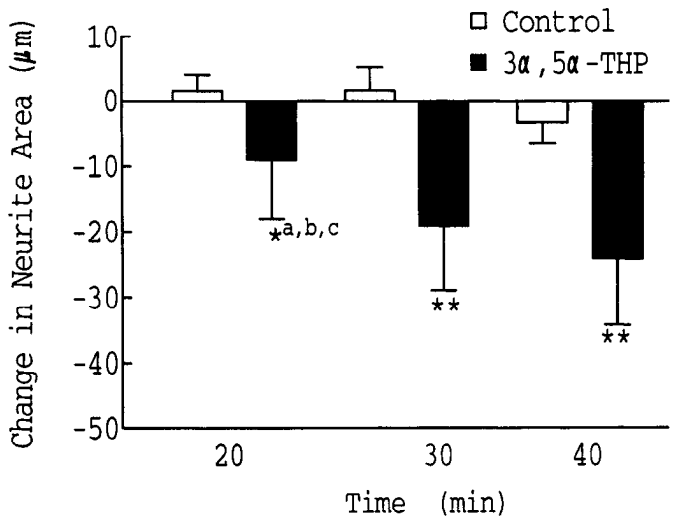

Figure 2. Influence of 3α,5α-THP upon neurite area. Neurite area was defined as the surface area of the projection including filopodial extensions but not including the intervening space between filopodia. Data are presented as a change from the area of the neurite assessed following a 10 min baseline observation period. Control neurites underwent media exchanges identical to that of 3α,5α-THP–treated neurites and were observed for the same length of time. $n = 18$ for control neurites; $n = 22$ for 3α,5α-THP (250 nM or 1 μM)–treated neurites. *, $p < 0.05$; **, $p < 0.001$; *a*, significantly different from control; *b*, significantly different from 30 min 3α,5α-THP exposures; *c*, significantly different from 40 min 3α,5α-THP exposure.

widespread indicators of cell death, only nerve cell death was quantified since determination of the boundaries between individual glial cells in 6-d-old control cultures was often not possible. By 15 hr of picrotoxin exposure a significant number of neurons stained with trypan blue [$F = 13.43$; df 3, 10; $p < 0.0008$]. Nerve cell survival in 3α,5α-THP (1 μM)–treated cultures was comparable to that which occurred in control cultures. Cultures treated simultaneously with 1 μM picrotoxin and 1 μM 3α,5α-THP were protected against picrotoxin-induced nerve cell death (Fig. 6).

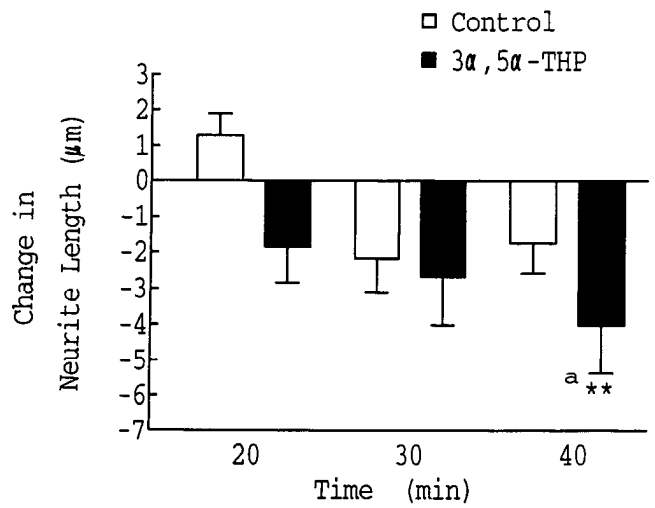

Figure 3. Influence of 3α,5α-THP upon neurite length. Data are presented as a change in neurite length (μm) assessed following 10 min baseline observation period. Statistically significant differences occurred between control ($n = 20$) and 3α,5α-THP (250 nM or 1 μM)–treated neurites ($n = 20$) at 40 min (**, $p < 0.001$) and between 20 min and 40 min exposure to 3α,5α-THP (a**, $p < 0.001$).

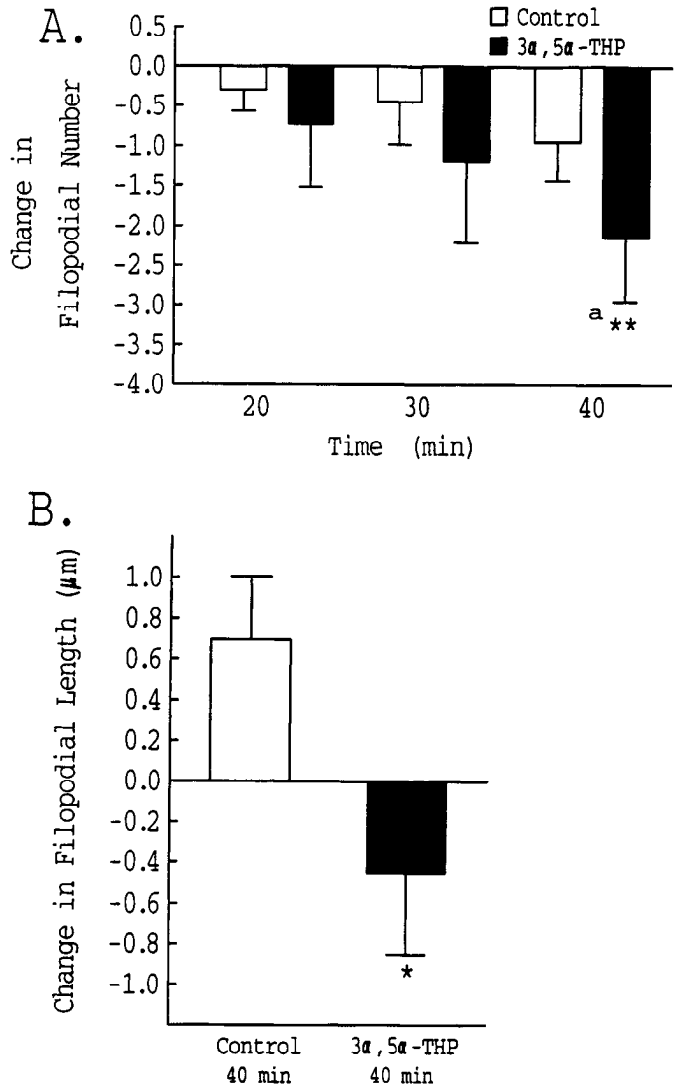

Figure 4. Influence of 3α,5α-THP upon filopodial number (*A*) and filopodial length (*B*). Data are presented as a change in filopodial number and length assessed following the 10 min baseline observation period. *A*, Statistically significant differences occurred in the number of filopodia between control ($n = 20$) and 3α,5α-THP (250 nM or 1 μM)–treated neurites ($n = 20$) at 40 min (**, $p < 0.001$) and between 20 min and 40 min exposure to 3α,5α-THP (a**, $p < 0.02$). *B*, Filopodial length decreased significantly compared to matched timed control filopodia ($n = 144$) following 40 min 3α,5α-THP exposure ($n = 144$; *, $p < 0.02$).

In both the 3α,5α-THP–treated and the picrotoxin/+3α,5α-THP–treated cultures nerve cells developed visible varicosities in their neuritic extensions (Fig. 7). Varicosity development occurred in neurons with and without structural contacts with other cells and also occurred in glial cells.

Reversal of 3α,5α-THP–induced filopodial regression by 17 β-estradiol

Every neurite ($n = 22$) that was treated with 3α,5α-THP for 40 min was subsequently treated with 1 μM 17 β-estradiol. Of those 22 neurites, five neurites showed an extremely rapid induction of filopodial growth within seconds of 17 β-estradiol exposure (Fig. 8). A comparison of the change in filopodial number that occurred during the 10 min baseline observation period, the change in filopodial number that occurred during the 40 min 3α,5α-THP exposure, and the change in filopodial number that

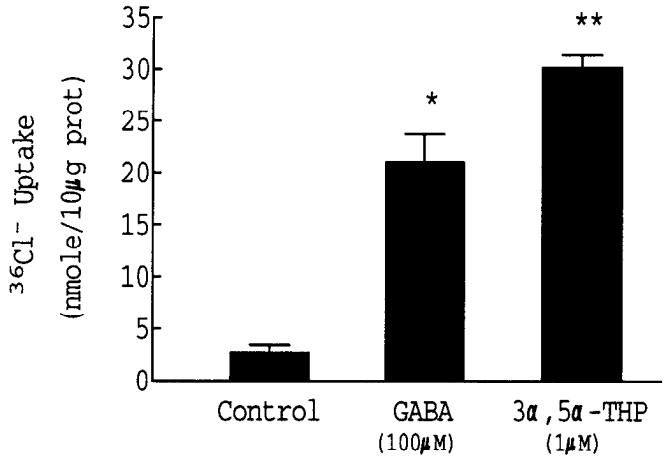

Figure 5. Effect of 3α,5α-THP on uptake of $^{36}Cl^-$ by hippocampal nerve cell cultures. Three-day-old hippocampal nerve cell cultures were exposed to 100 μM GABA or 1 μM 3α,5α-THP in the presence of $^{36}Cl^-$ (9.6 μCi/ml) for 10 sec. $^{36}Cl^-$ uptake was measured as described in Materials and Methods. Data are from one experiment and are representative of three separate experiments. Values represent mean ± SEM, $n = 3$ cultures/per condition (*, $p < 0.05$; **, $p < 0.01$; significantly different from basal uptake).

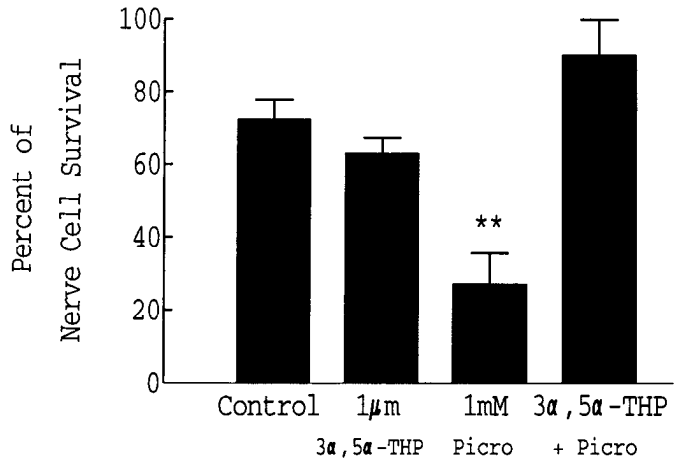

Figure 6. 3α,5α-THP protection against picrotoxin-induced nerve cell death. The number of trypan blue dye–excluding cells/100 morphologically identified neurons was determined (mean + SEM, $n = 5$ cultures per condition) after 15 hr of exposure to 1 mM picrotoxin (*Picro*), 1 μM 3α,5α-THP or 1 mM picrotoxin + 1 μM 3α,5α-THP. Control cells received no treatment. Data are presented as percentage of surviving nerve cells. All cultures were 6 d of age and contained both neurons and glial cells (**, $p < 0.01$, significantly different from all other conditions).

occurred during a 90 sec exposure to 17 β-estradiol is shown in Figure 9.

A similar spontaneous response was never observed in control neurons that were observed for an equal amount of time, under the same constant illumination as those treated with steroids and that received the same number and temporal sequence of media exchanges ($n = 20$). Similarly, progesterone (1 μM) pretreatment for 40 min followed by exposure to 1 μM 17 β-estradiol also did not induce a spontaneous outgrowth of filopodia ($n = 18$). Lastly, the reverse condition in which neurites were pretreated with 17 β-estradiol followed by 3α,5α-THP exposure also did not result in spontaneous filopodial outgrowth ($n = 5$).

Discussion

The undulating process of cytoarchitectural emergence and retraction constitutes a normal growth pattern during nerve cell development (Aletta and Greene, 1988; Smith, 1988). Under normal conditions, the retraction/emergence process can occur within minutes for filopodia and hours or days for changes in neurite length and area to become apparent. In this study, control neurites exhibited very little variability in growth during the 60 min of observation. In marked contrast, in 3α,5α-THP–treated neurons, a decrement was observed in each morphological parameter within 20 min that reached significance by 40 min. Because of the rapidity of the 3α,5α-THP–induced regression, because no significant change was observed in control neurites, and because the level of significance at 40 min ranged from $p < 0.02$ to $p < 0.0001$, it is highly unlikely that the regression of neuronal morphology is due to chance alone or to factors associated with the observation paradigm.

The concentration of 3α,5α-THP required to induce morphological retraction (250 nM to 1 μM) clearly exceeds plasma levels. Indeed, these concentrations are greater than the highest levels obtained during pregnancy (~100 nM). One can invoke the usual pharmacological explanation to such a situation by relying on the fact that local synaptic concentrations of any substance can greatly exceed the levels found in volumes of measure such as milligrams of protein or milliliters of plasma. Whether this situation is in fact the case remains to be determined. The requirement for supraphysiological levels of 3α,5α-THP is probably related to the presence of steroid-binding proteins such as albumin present in the serum, where it is well documented that only about 2% of sex steroids exist in a free, pharmacologically active form, with the remainder being bound to sex hormone binding globulin and albumin. The presence of these steroid-binding proteins in the serum in all likelihood significantly reduced the total amount of free steroid. Moreover, the minimally effective concentration of 250 nM 3α,5α-THP is only 1.5-fold higher than the levels found in plasma during pregnancy (~100 nM). The possibility that the regressive effects of 3α,5α-THP are mediated merely by perturbations in membrane fluidity is unlikely, since neither 1 μM 3β,5β-THP nor 17 β-estradiol induced retraction and 30% of the cells treated with 3α,5α-THP showed no filopodial or neuritic regression. Moreover, no steroid studied in a previously published report (Brinton, 1993) induced regression at the same concentration.

In addition to the retraction of neuronal morphology, 3α,5α-THP–treated neurites displayed retrograde movement of PDBs that populated the cytoplasmic domain of neuronal extensions. Prolonged treatment with 3α,5α-THP induced the development of varicosities in both nerve and glial cell extensions. The composition of such PDBs and varicosities was explored by Koenig et al. (1985). These investigators found that PDBs and varicosities contained α-spectrin, actin, and calmodulin in addition to cytomembranes. They also found that the predominant structure of the varicosities was a large aggregation of anastomosing tubular, smooth endoplasmic reticulum embedded in an amorphous matrix. The rapid formation of PDBs and the associated retrograde transport of cytomembranes, structural proteins, growth-associated enzymes and smooth endoplasmic reticulum following 3α,5α-THP exposure are consistent with regression of neuritic structure.

Thus, the data presented here document four main findings. (1) 3α,5α-THP can, in responsive cells, induce significant retraction of neuronal morphology within minutes following exposure. Moreover, the morphological regression induced by 3α,5α-THP is mirrored in the retrograde movement of neuritic organelles. In addition, the response of retraction induced by

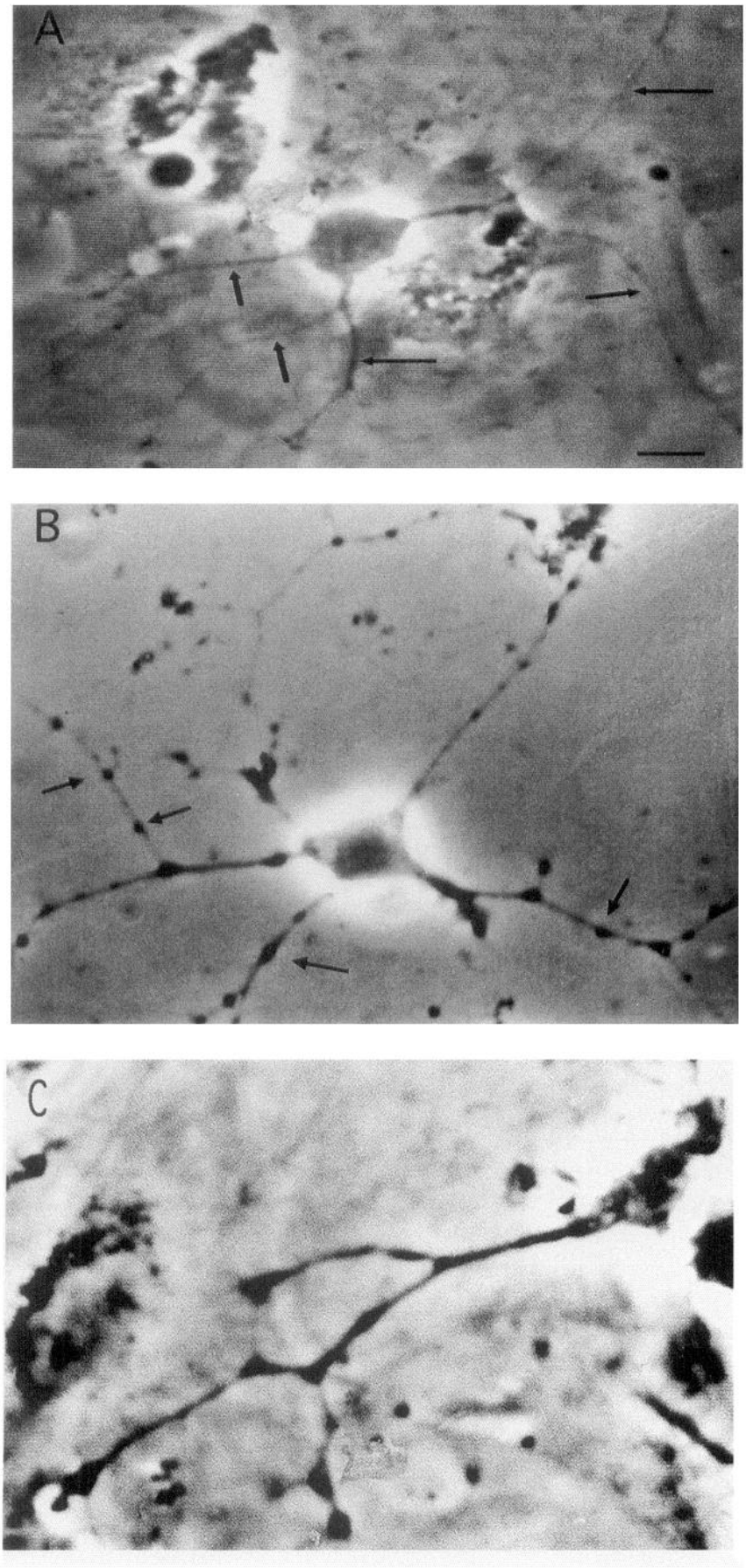

Figure 7. Development of varicosities and maintenance of structural contact in 6-d-old hippocampal neurons treated for 15 hr with 1 μM 3α,5α-THP. *A,* Photomicrograph of a 6-d-old control hippocampal neuron. *Arrows* point to neuritic extensions that are free of varicosities. *B,* Hippocampal neuron treated with 1 μM 3α,5α-THP for 15 hr. *Arrows* point to a few of the many varicosities that developed over the 15 hr of exposure to 3α,5α-THP. *C,* Hippocampal nerve cell treated with 1 μM 3α,5α-THP for 15 hr that developed varicosities but that remained attached to underlying glial cells. Note that cells treated with 3α,5α-THP (*B* and *C*) contain pronounced varicosities in cellular extensions, whereas the control cell does not show evidence of varicosity development. Scale bar, 25 μm.

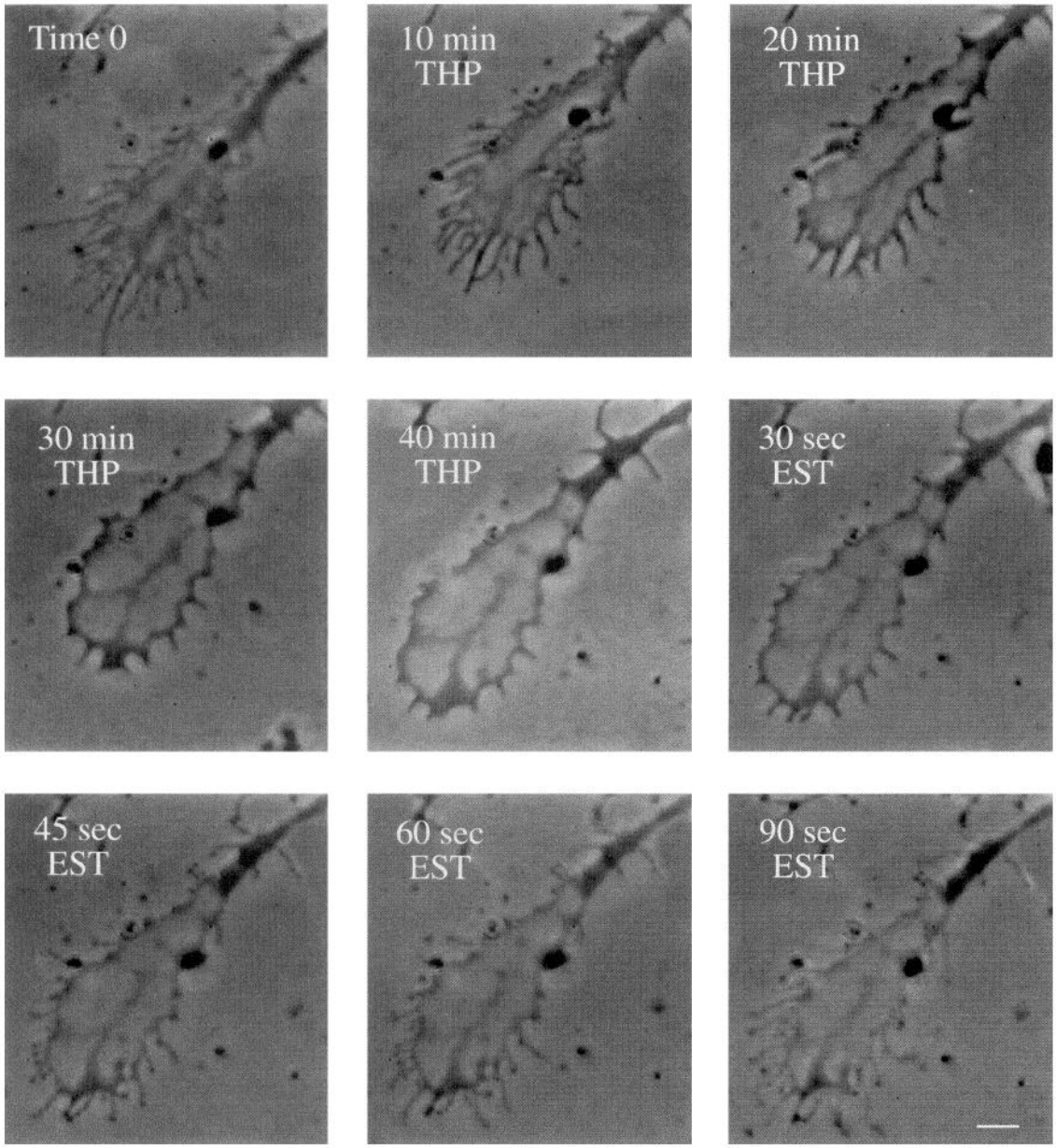

Figure 8. Photomicrograph of a hippocampal nerve cell growth cone prior to time 0) and during 10, 20, 30, and 40 min of exposure to 1 μM 3α,5α-THP (*THP*) followed by 30, 45, 60, 90 sec of exposure to 1 μM 17 β-estradiol (*EST*). The relative time of recording (in min or sec) of the same growth cone is denoted in the *upper left corner* of each panel. Note the regression of filopodia during THP exposure and the reemergence of filopodial during EST exposure. Scale bar, 5 μm.

3α,5α-THP appears to be regulated by the formation of structural contact with other cells such that structural contact renders the neurite unresponsive to the regressive effects of 3α,5α-THP but not to the development of varicosities. (2) Chloride influx could be partly involved in the process of cytoarchitectural retraction since 3α,5α-THP induced a significant influx of chloride within 10 sec of exposure. (3) 3α,5α-THP can protect neurons against picrotoxin-induced cytotoxicity. (4) The 3α,5α-THP–induced regression of filopodia can, in select instances, be reversed by subsequent exposure to 17 β-estradiol.

Potential intracellular mechanism regulating 3α,5α-THP–induced neurite and filopodial regression

The 40 min time frame for morphological retraction to reach significance could involve a genomic mechanism of action, similar to the mechanism of action for many steroids. However, we have demonstrated in an earlier study that 3α,5α-THP does not bind to progesterone nuclear receptors (Gee et al., 1988). In addition, we and others have also demonstrated that the 3α,5α-THP binding site is a unique regulatory site on the GABA-chloride channel complex (Gee et al., 1987, 1988; Morrow et al., 1987, 1990). Morrow and her colleagues (1987, 1990) have extensively characterized 3α,5α-THP–induced chloride uptake in cortical synaptosomes and have shown significant potentiating effects within 5 sec of exposure to the steroid.

Several laboratories investigated the underlying mechanism of 3α,5α-THP–induced chloride uptake using single-channel analysis. Lambert et al. (1987) found prolongation of the burst duration of GABA–chloride channels following exposure to 1 μM 3α,5α-THP. Harrison and Simmonds (1984) demonstrated

that progesterone steroid metabolite regulation of chloride channels is specific to the GABA–chloride channel complex since no effect upon glycine-mediated transmission was observed. These investigators also found that a 2 min exposure to alphaxalone (3α-hydroxy-5α-pregnane 11,20-dione, a steroid very similar to 3α,5α-THP) enhanced GABA-mediated transmission for 3 hr. This finding demonstrates that long-lasting effects upon chloride channel activity can be achieved with short exposures to high concentrations of progesterone steroid metabolites. Such a phenomenon may be operating in the case of 3α,5α-THP–induced $^{36}Cl^-$ uptake. However, because 40 min of exposure to 3α,5α-THP did not result in an increase in $^{36}Cl^-$ uptake above basal levels, it is not clear whether the initial influx of chloride induced by 3α,5α-THP within 10 sec of exposure is related to the neurite regression or is an initial signal that activates a secondary mechanism that ultimately mediates neurite regression. The fact that chloride influx was not different than basal at 40 min does indicate, however, that the cytoarchitectural retraction induced by 3α,5α-THP is not a result of intracellular changes in osmolarity or an inability to maintain chloride ion balance.

It is becoming increasingly clear that different cell types and different stages of development are associated with different subunit composition of the chloride channel complex (Schofield et al., 1987; Levitan et al., 1988; Montpied et al., 1989; Puia et al., 1990; Lan et al., 1991; MacLennan et al., 1991; McKernan et al., 1991; Paul and Purdy, 1992). Supporting the developmental changes in $GABA_A$ receptor subunit composition, Barker and colleagues (Maric et al., 1992) have shown that cortical cells exhibit unique electrophysiological responses to GABA and neurosteroids that change within the course of a few days (E13–E19) during embryogenesis. Thus, it appears reasonable to suggest that developmental and cell-specific factors operate to mediate the responsivity to neurosteroid exposure. Indeed, such developmental regulated responsivity was evident in cultured neurons utilized in the present study. Neurites that had not made structural contact exhibited regression potential, whereas those that had made contact did not.

Comparison between the effects of 3α,5α-THP and 17 β-estradiol

As the data presented in this study indicate, 17 β-estradiol can reverse the regression induced by 3α,5α-THP. In a previous report, 17 β-estradiol was found to induce filopodial growth within 5–10 min of exposure at concentrations identical to the ones used in this study (Brinton, 1993). 17 β-Estradiol–induced outgrowth was specific in that neither 17 α-estradiol, testosterone, progesterone, or corticosterone promoted outgrowth. 3α,5α-THP pretreatment markedly reduced the time course for 17 β-estradiol activation of filopodial growth exposure from minutes to seconds. The cellular selectivity for 17 β-estradiol reversal of 3α,5α-THP–induced filopodial regression is consistent with previous findings regarding 17 β-estradiol effects upon filopodial growth (reviewed in Brinton, 1993), regulation of dendritic spine growth (Gould et al., 1990), and the electrophysiological effects (Teyler et al., 1980; Foy et al., 1982; Nabekura et al., 1986; Minami et al., 1990; Wong and Moss, 1991).

The highly selective effect of 17 β-estradiol on filopodial outgrowth is not unique. Based on the studies of neurotrophic factors and neurotransmitters in both the PNS and CNS, it appears that ensembles of neurons exhibit unique and highly specific patterns of neurotrophic factor responsivity. This characteristic holds true for neurotransmitter systems that act as

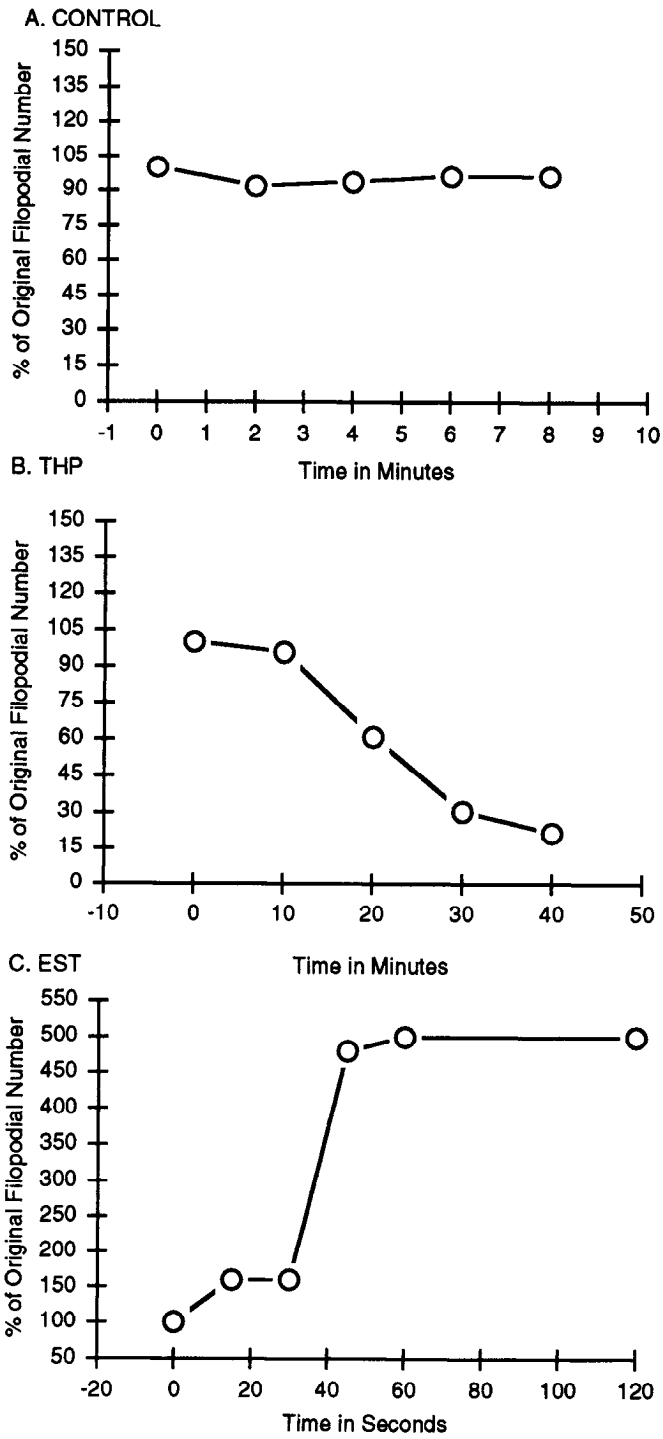

Figure 9. Comparative analysis of change in filopodial number following exposure to 1 μM 3α,5α-THP (*THP*) and 1 μm 17 β-estradiol (*EST*). Data are quantitative analysis of growth cone shown in Figure 8. A similar temporal pattern of THP-induced regression followed by reemergence of filopodia within seconds was observed in other estrogen responsive cells. *A* depicts change in filopodial number that occurred during baseline observation. Note that the percentage of original number of filopodia remains constant around 100%, indicating that very little change occurred during the baseline period. *B* depicts change in filopodial number that occurred during exposure to 1 μM 3α,5α-THP (*THP*). *C* depicts change in filopodial number that occurred during exposure to 1 μm 17 β-estradiol (*EST*). Note that in control and THP panels (*A, B*) time is recorded in minutes, while in EST panel (*C*) time is recorded in seconds.

neurotrophic agents (Berg, 1984; Brinton and Gruener, 1987; Mattson, 1988; Lipton and Kater, 1989; Brinton, 1990) and for growth factors such as NGF, significantly and selectively influence neurite outgrowth and survival (reviewed in Berg, 1984; Levi-Montalcini, 1987; Barde, 1989).

The exact mechanism for the rapid effect of 17 β-estradiol hippocampal nerve cell outgrowth has yet to be identified; however, regulation of channel function appears a likely candidate. Indeed, Nabekura et al. (1986) found that 17 β-estradiol increased potassium conductance in medial amygdala neurons. Numerous other studies have documented that 17 β-estradiol has potent and rapid excitatory effects upon nerve cells in the hippocampus (Teyler et al., 1980; Foy et al., 1982; Wong and Moss, 1991) as well as in other brain regions (Nabekura et al., 1986; Minami et al., 1990).

Functional and basic science implications of 3α,5α-THP–induced morphological regression

The conversion of progesterone to progesterone metabolite steroids by hypothalamic tissue (Karavolas and Hodges, 1990, 1991), the synthesis of 3α,5α-THP and other progesterone metabolites by cultured fetal glial cells (Jung-Testas et al., 1989), the localization of the steroid-converting enzyme 3α-hydroxysteroid oxidoreductase to glia in brain (Krieger and Scott, 1989), and the documentation that 3α-hydroxysteroid oxidoreductase is present in fetal brain, that fetal brain is one of the first tissues to produce 3α,5α-THP (Ward et al., 1992), and the formation of the 3α,5α-THP by the fetal-placental unit, all suggest that steroid metabolites can play an important role in neural development. Indeed, plasma levels can reach 30 ng/ml (~100 nM) during the third trimester of pregnancy and remain elevated for months (Paul and Purdy, 1992). Moreover, it was demonstrated by Holzbauer et al. (1985) that, in addition to synthesis in the brain, both the ovary and the adrenal gland synthesize 3α,5α-THP. These investigators also showed that levels of 3α,5α-THP increased dramatically following chronic osmotic challenge. Thus, there are an ample number of sites and physiologically relevant circumstances for 3α,5α-THP synthesis both during development and throughout the life-span of the animal to postulate reasonably that 3α,5α-THP may indeed play an important role in neural development and in sculpting neuronal architecture throughout the life of the animal.

In addition to potentially being involved in normal physiological processes, supraphysiologic concentrations of 3α,5α-THP are conceivably relevant to the potential therapeutic application of this progesterone metabolite. In behavioral studies of both antianxiety (Wieland et al., 1991) and antiepileptic properties of 3α,5α-THP, injections of 20–100 mg/kg are not uncommon (Belelli et al., 1989). Clearly, administration of 3α,5α-THP to women during the follicular phase of the menstrual cycle or to pregnant women will raise already high existing levels of 3α,5α-THP. Results of this study suggest that high levels of 3α,5α-THP could potentially effect fetal nerve cell growth.

From a clinical perspective, one must question whether the therapeutic doses of 3α,5α-THP necessary to protect against seizure activity will influence normally occurring growth such as that which may occur with learning and memory (Brinton and Gruener, 1987; Brinton, 1990; reviewed in Brinton, 1993). While addressing that question, it is important to note that 3α,5α-THP did not erode structural connections even when those connections were newly formed. In addition, the combination of 3α,5α-THP and picrotoxin produced a nerve cell survival rate that was comparable to that of control cultures and even showed a modest enhancement of survival rate. While the complexity of neuronal morphology under these conditions remains to be determined, structural contacts between neurons and glial cells were still clearly apparent following 15 hr of exposure to 1 μM 3α,5α-THP. These data would suggest that existing neural circuits are not susceptible to 3α,5α-THP–induced retraction. Thus, it is not unreasonable to speculate that a therapeutic regimen of 3α,5α-THP, which would tightly titrate inhibition of excessive nerve cell excitation but which would preserve appropriate nerve cell excitability, could result in normal nerve cell growth under desirable circumstances such as during learning and memory.

From a basic science perspective, the ability of a progesterone steroid metabolite, 3α,5α-THP, to induce significant morphological changes within minutes of exposure has fundamental implications for conceptualization of both the time requirements for steroid-induced cytoarchitectural modifications as well as the role of steroid metabolites in sculpting neuronal circuitry. Temporally, the present data suggest that steroids and steroid metabolites can induce modifications of neuronal architecture in substantially less time then that required for morphological changes resulting from nuclear receptor activation of genomically mediated events. Collectively, the present data suggest that steroid metabolites can have a profound influence on neuronal morphology. Such cytoarchitectural modifications could have significant implications for the development and function of neural circuitry.

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