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An *in vitro* Model for the Effects of Estrogen on Neurons Employing Estrogen Receptor-transfected PC12 Cells

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Estrogen alters neurite outgrowth, neuritic spine development, and synaptogenesis in estrogen-responsive areas of the rat brain. However, examination of the specific effects of estrogen on neurons *in vivo* has been difficult. An *in vitro* model for the effects of estrogen on neurons was developed, using the PC12 rat pheochromocytoma cell line. Wild-type cells (PC12-WT) were stably transfected either with an expression vector coding for the full-length cDNA for the human estrogen receptor (hER), or with a control vector. Resultant clones were isolated, screened for incorporation of vector and expression of ER mRNA and protein, and analyzed for morphologic responses to estrogen.

PC12-WT, NEO9 (ER-negative), and SER8 (ER-positive) cells exposed to 100 ng/ml NGF exhibited dose-responsive neurite outgrowth within 2 d by light microscopy (LM). Coadministration of 10^{-10} to 10^{-9} M estradiol (E_2) had minimal effects on neurite outgrowth, neuritic spine development, or interneuritic connections in NEO9 or PC12-WT cells, but in SER8 cells E_2 led to additive and dose-dependent increases in neurite outgrowth, spine development, and interneuritic connectivity. Coincubation of SER8 cells with E_2 and the antiestrogen ICI 164,384 negated estrogenic effects on spine development and interneuritic connectivity. At the electron microscopic (EM) level, intercellular abutments of NEO9 or PC12-WT cells contained few and rudimentary gap junctions, with no increase by E_2. However, SER8 cells exhibited augmented basal frequencies of gap junctions that increased with E_2 incubation. Microinjection of Lucifer yellow into PC12-WT and NEO9 cells demonstrated low frequencies of dye coupling and no change with E_2, but SER8 cells demonstrated increased dye-coupling frequency with E_2 coincubation.

The results suggest that SER8 cells recapitulate estrogen effects on neurons *in vivo*. Estrogen appears to induce an inherent neural morphologic program in estrogen receptor (ER)-containing cells. These three cell lines provide a unique *in vitro* system for studying mechanisms of estrogen–neuron interactions.

[Key words: estrogen, neurite outgrowth, spines, PC12 cells, gap junctions, sex dimorphisms]

Adult male and female rat brains differ in several biochemical and behavioral parameters (MacLusky and Naftolin, 1981; Arnold and Gorski, 1984; Gorski, 1984; Breedlove, 1992). This is evidenced by sex differences in response to estrogen. The ability of estrogen to activate changes in brain function is clearly sex specific (McEwen, 1983; McEwen et al., 1984), including alterations in neurotransmitter receptors (Fischette et al., 1983; Luine and McEwen, 1983), estrogen receptors (Lauber et al., 1991), progestin receptors (Rainbow et al., 1982), neuropeptide transcription (Romano et al., 1990), and gonadotropin-releasing hormone secretion, vis-à-vis the LH surge (Goodman, 1978; Corbier, 1985).

The above dimorphisms have neuroanatomic correlates. In the male rat, the cortical mantle of the left hemisphere is thinner than on the right, and than that noted in the female (Diamond et al., 1981; McShane et al., 1988). On a synaptic level, sex differences have been noted in the pattern of neuronal connectivity in the preoptic area (Raisman and Field, 1973; Fischette et al., 1983), arcuate nucleus (Matsumoto and Arai, 1980), and amygdala (Nishizuka and Arai, 1981). Most notable is the sex difference in the "sexually dimorphic nucleus" (SDN), a region of the medial preoptic area that is several times larger in males due to increased neuron number (Gorski et al., 1980; Jacobson et al., 1981; Döhler et al., 1986) and also exhibits greater neuritic growth and extent (Hammer and Jacobson, 1984). These sex-dependent alterations in neurite outgrowth are thought to be secondary to the effects of neonatal estrogen. The rat hypothalamus and cortex undergo perinatal neuronal organization under the influence of estrogen (formed *in situ* by aromatization of neonatal testicularly derived androgens), a process called "defeminization" (McEwen et al., 1977; Gorski et al., 1980). For instance, neonatal castration of male rat pups results in a symmetrical cortical mantle (Diamond, 1984). Further, castration of, or administration of an aromatase inhibitor to, neonatal male rats results in decreased size of the adult SDN (both neuron number and synaptic density) (Diamond, 1984; Hammer and Jacobson, 1984), while perinatal androgen or estrogen exposure to neonatal females increases adult SDN size (Jacobson et al., 1981). Conversely, castration or aromatase inhibition of neonatal males facilitates lordotic behavior and LH surges in response to adult estrogen exposure (McEwen et al., 1979), while androgen exposure of neonatal females suppresses these param-

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eters in adulthood (Clemens et al., 1978; Parsons et al., 1984). On a molecular level, sex dimorphisms in mRNA levels for the neural-specific proteins growth-associated protein 43 kDa (GAP-43) and synaptosomal-associated protein 25 kDa (SNAP-25) have been noted in the adult rat cortex, which are also determined by the neonatal sex hormonal milieu (Lustig et al., 1993). Although controversial, the presence of plasma α-fetoprotein, which binds estrogens avidly in the perinatal period, presumably prevents ovarian-derived plasma estrogen from crossing the blood–brain barrier in females, thus preventing defeminization (Toran-Allerand, 1984). Therefore, it is the ability of plasma androgen to be aromatized to estrogen in the rat brain *in situ* that dictates whether defeminization will occur.

Estrogen exerts effects on neuritic outgrowth, spine formation, and synaptogenesis in the *neonatal* rat brain. The addition of E_2 to the medium of fetal mouse hypothalamic explants enhances the growth and arborization of neurites (Toran-Allerand et al., 1980, 1983), while addition of antiestrogen antibodies negates the arborization effect (Toran-Allerand, 1980). In fetal neuron cultures, estrogen induces transcription of mRNA for the axon-specific protein tau, and stabilization of microtubules, leading to axonal outgrowth (Ferreira and Caceres, 1991; Diaz et al., 1992), and dendritic outgrowth and arborization (Lorenzo et al., 1992). Ultrastructural studies of the ventromedial hypothalamus (VMH) indicate that the number of axosomatic and axospinous synapses are similar between the sexes at birth, but by adulthood there is a clear male > female sex dimorphism of both types of synapses. Castration of neonatal males reduces the number of adult synapses in these areas to the female level, while testosterone treatment of neonatal females increases the adult synaptic number to that of intact males (Matsumoto and Arai, 1986; Matsumoto, 1991).

Estrogen also alters neural organization in ER-positive areas of the *adult* rat brain. Several reports have documented increased numbers of synaptic terminals and contacts in the ventromedial hypothalamus (Matsumoto and Arai, 1979; Carrer and Aoki, 1982; Clough and Rodriguez-Sierra, 1983; Pozzo Miller and Aoki, 1991) and midbrain central gray (MCG) (Chung et al., 1988) in adult ovariectomized (OVX) rats given EB. Estrogen administration to OVX or peripubertal rats increases the number and density of dendritic spines in the VMH and hippocampus (HPC) (Frankfurt et al., 1990; Gould et al., 1990; Segarra and McEwen, 1991). Similarly, the number of dendritic spines in the VMH and HPC varies with the estrous cycle (Frankfurt et al., 1990; Woolley and McEwen, 1992), suggesting that the neural circuitry of the VMH is inherently plastic, and is constantly remodeled by the fluctuations of peripheral plasma E_2 (Pérez et al., 1993).

Although these *in vivo* studies document the effects of estrogen on neural structure and plasticity, the data are only indirectly correlated to hormonal changes in physiology, cognition, and behavior. These approaches do not answer molecular or mechanistic questions of how neurons are affected by estrogen. To provide a tool for such investigations, we have created an *in vitro* system for studying the effects of estrogen on neuronal development. The PC12 cell line served as the basis for such a model. These cells, derived from a rat pheochromocytoma (Greene and Tischler, 1976), have been used to study neuritogenesis and neural gene expression. In the presence of NGF, these otherwise chromaffin cells become postmitotic, and initiate neurite outgrowth (Greene and Shooter, 1980) similar to that seen *in vivo* and in brain tissue explants in culture. Furthermore, these cells are easily transfected by mammalian expression vectors with subsequent mRNA expression and alteration of their phenotype (Yankner et al., 1990). Wild-type PC12 cells (PC12-WT) express a small amount of ER mRNA in the basal state as determined by reverse transcriptase–PCR (data not shown), but their morphology is altered in response to estrogen only minimally. Federoff et al. (1988) have shown that PC12-WT cells do not alter GAP-43 mRNA transcription in response to estrogen, again suggesting that estrogen responsivity in PC12 cells is routinely minimal. Thus, these cells are a suitable parent line for studying the effects of estrogen on neurons *in vitro*. By stably transfecting PC12-WT cells with an expression vector coding for the human ER (hER), we hoped to confer estrogen responsivity and morphologic modifications in accordance with the effects noted above. The construction, molecular characterization, and morphologic analysis of this model are now described.

Materials and Methods

All reagents were purchased from Sigma (St. Louis, MO), all cell culture products were purchased from GIBCO/Bethesda Research Labs (Grand Island, NY), and all electron microscopy reagents were purchased from EM Science (Gibbstown, NJ) unless otherwise specified.

Expression vectors. For hER transfection, these studies utilized the expression vector pCMV-ERα-neo, which was described previously and shown to confer estrogen responsivity on ER-negative MDA-231 breast cancer cells by coding for the full-length 1.8 human estrogen receptor (hER) (Jiang and Jordan, 1992). This 7.35 kilobase (kb) vector consists of a strong constitutive cytomegalovirus immediate-early gene promoter and enhancer, which is known to express in PC12 cells (Donis et al., 1993), spliced to a 4.0 kb polycistronic DNA coding for both the hER and the enzyme neomycin aminoglycoside phosphotransferase (*neo*) for selection. As a negative control vector, these studies utilized the pCMV-neo vector containing the same CMV promoter and neomycin aminoglycoside phosphotransferase, but without the hER coding region (Jiang and Jordan, 1992). These vectors were a gift from Dr. V. Craig Jordan. Vectors were prepared for transfection by cesium chloride ultracentrifugation.

Cells and stable transfection. PC12-WT cells normally require a substratum of collagen, laminin, or polylysine to adhere and extend neurites, but a clone (provided by Dr. Howard Federoff) has been expanded that will grow on tissue culture plastic without treatment. These PC12-WT cells were grown as previously described (Federoff et al., 1988) in Dulbecco's Minimal Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and 5% horse serum (JRH Bioscience, Lenexa, KS). Cells were grown to 50% confluence and stably transfected by the calcium phosphate method (Chen and Okayama, 1987), using 25 μg of each vector for transfection. Transfectants were grown for 2 d, and then selected by addition of G418, a neomycin analog (Bethesda Research Labs), 400 mg/ml, to the media for 3 weeks. Resultant transfected clones were isolated using cloning cylinders (Bellco, Vineland, NJ), and expanded in phenol red (PR)-free DMEM with charcoal-stripped FBS and horse serum (to remove all estrogens), under constant G418 pressure to select for strong expressors of the transfected neomycin resistance gene. Approximately 12 clones from each transfection were expanded and analyzed for estrogen responsivity and ^{3}H-E_2 binding. Clones selected for further study were labeled NEO9 (an ER-negative line), and SER8 (an ER-positive line).

ER mRNA expression. For Northern analysis of ER mRNA expression, all three cell lines (PC12-WT, NEO9, SER8) were grown in phenol red (PR)-free DMEM with 10% charcoal-stripped FBS and 5% charcoal-stripped horse serum (NEO9 and SER8 also with G418, 400 mg/ml) for 6 d. In addition, MCF-7 breast cancer cells (as an ER-positive control) were grown in Minimal Essential Media with 5% calf serum. PolyA$^+$ RNA was prepared using the method of Badley et al. (1988). Cells were washed in cold PBS-aurin-tricarboxylic acid (Boehringer, Indianapolis, IN) and harvested by cell scraping in lysis buffer, and polyA$^+$ RNA was isolated by incubation with oligo-dT-cellulose (Boehringer) and centrifugation. RNAs from each line were electrophoresed on a 1% agarose–formaldehyde gel at 25 V overnight, and transferred to a Hybond-N (Amersham, Arlington Heights, IL) membrane by vac-

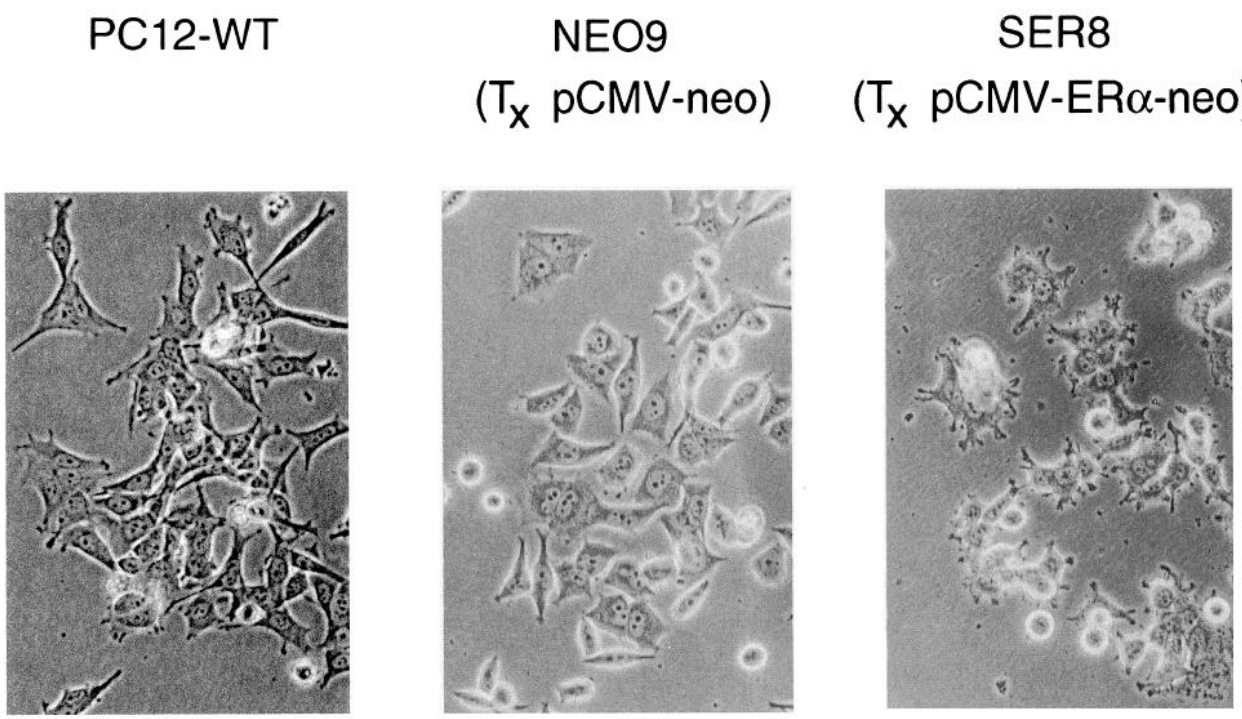


Figure 1. Morphology by inverted phase-contrast LM (400×) of PC12-WT, NEO9, and SER8 cells, when grown in DMEM with 10% FBS, 5% horse serum, without exposure to NGF. The SER8 cells demonstrate increased lamellopodia on their surfaces in the basal state.

uum transfer for 3 hr. The membrane was UV cross-linked (Stratagene, La Jolla, CA) at 1200 J, and hybridized with the ^{32}P-hER cDNA (full-length) in hybridization buffer (5× SSC, 24 mM Na_3PO_4, 5× Denhardt's solution, 50 μg/ml denatured salmon sperm DNA, 50% formamide, 10% dextran sulfate) overnight. The blot was washed with 2× SSC, 0.2% SDS three times at 25°C, then 0.1× SSC, 0.2% SDS at 65°C for 10 min, and exposed to autoradiographic film.

Cytosolic ^3H-E_2 binding assay. Assay for cytosolic ER by ligand-binding assay was performed as described previously (Lustig et al., 1989). All three lines (plus MCF-7 as an ER-positive control) were grown in PR-free DMEM with charcoal-stripped serum (+G418 for NEO9 and SER8) for 6 d. Cells were trypsinized and harvested, resuspended in 4 ml of buffer containing 0.32 M sucrose, 10 mM KPO_4, 10 mM sodium molybdate, and 10 mM monothioglycerol, homogenized using a Teflon homogenizer (Wheaton), and centrifuged at 105,000 × *g* for 30 min. Aliquots of resultant cytosol (100 μl) were incubated overnight at 4°C with 0.1–5 nM ^{3}H-E_2 (Amersham) (dissolved in 10 mM Tris-HCl, 1.5 mM Na_2EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM bacitracin) in the presence or absence of 200-fold excess diethylstilbestrol. Incubates were eluted on short LH-20 columns with TEGDB buffer, and the eluate counted in a β-scintillation counter and standardized for cytosolic protein concentration using the Bradford method (Bradford, 1976).

Quantitative light microscopy. For each experiment, 100 cells per dish were randomly counted by one investigator (R.H.L.) according to a protocol devised to minimize counting bias. Cells were counted at 430× magnification. Cells which remained round, did not flatten on plastic, and did not express any lamellopodia or neurites were not counted. Cells whose somata abutted other somata were considered crowded, and therefore extended neurites suboptimally, also were not included in the analysis. All other cells were included in the analysis. A potential maximum of six cells per high-power field were counted, and then the field was shifted in a discernable pattern, to prevent recounting the same cell. Cells were assessed for the following parameters: (1) frequency of neuritic outgrowth (number of cells with neurites per 100 cells counted); (2) frequency of short neurites (number of cells with neurites whose lengths were less than two cell body widths/100 cells counted) and density (number per cell) of short neurites; (3) frequency of long neurites (number of cells with neurites whose lengths were greater than two cell body widths/100 cells counted) and density (number per cell) of long neurites; (4) frequency of neuritic spines (varicosities along the length of neurites, perpendicular to the neurite, and very short in length; number of cells with neuritic spines per cells with neurites) and density of spines (number of spines per cell with spines); and (5) frequency of interneuritic interaction [cells whose neurites appear by light microscopy (LM) to be communicating, where a single neurite appears to travel uninterrupted between two separate cell bodies; number of interneuritic interactions per 100 cells counted].

Statistical evaluations were performed by (1) χ^2 analysis of the effect of estrogen on the frequency of each parameter at each NGF concentration, with subsequent Fisher's Exact Test; and also by (2) analysis of variance of the effect of estrogen on the density of each parameter, with subsequent post-hoc Bonferroni analysis.

Quantitative electron microscopy. Cultures were fixed in 4% glutaraldehyde-PBS, postfixed in 1% OsO_4, dehydrated in graded ethanols, and embedded in Epon. Sections were prepared on a Reichert-Jung Ultracut S. Sections were stained with uranyl acetate and lead citrate, and visualized using a JEOL CX-100 electron microscope at 10,000× magnification. Quantification of ultrastructural characteristics were performed in a blinded fashion on serial electron microscope (EM) negatives.

Dye coupling. Cells growing on polylysine-coated glass coverslips were visualized by differential-interference contrast (DIC) optics (Zeiss Axiovert 35M). Cells whose processes abutted on another cell were microinjected with 5% Lucifer yellow in 50 mM potassium glutamate buffer, using an Eppendorf 5170 micromanipulation and 5242 microinjection apparatus. Injection volume did not exceed 10% of the cell's basal volume. The transfer of dye from the injected cell into the secondary abutting cell was determined by epifluorescence.

Results

Selection of transfected clones and alterations in basal morphology

Figure 1 shows the morphology of PC12-WT, NEO9, and SER8 cells when grown in standard phenol red (PR)-containing medium. PC12-WT cells are pleomorphic, reflecting their inherent heterogeneity, with lamellopodia and early and immature neuritic formation, while NEO9 cells appear more homogeneous, with fewer membrane specializations. In contrast, SER8 cells have a different phenotype, with marked lamellopodial formation along the cell membrane, even in the basal state.

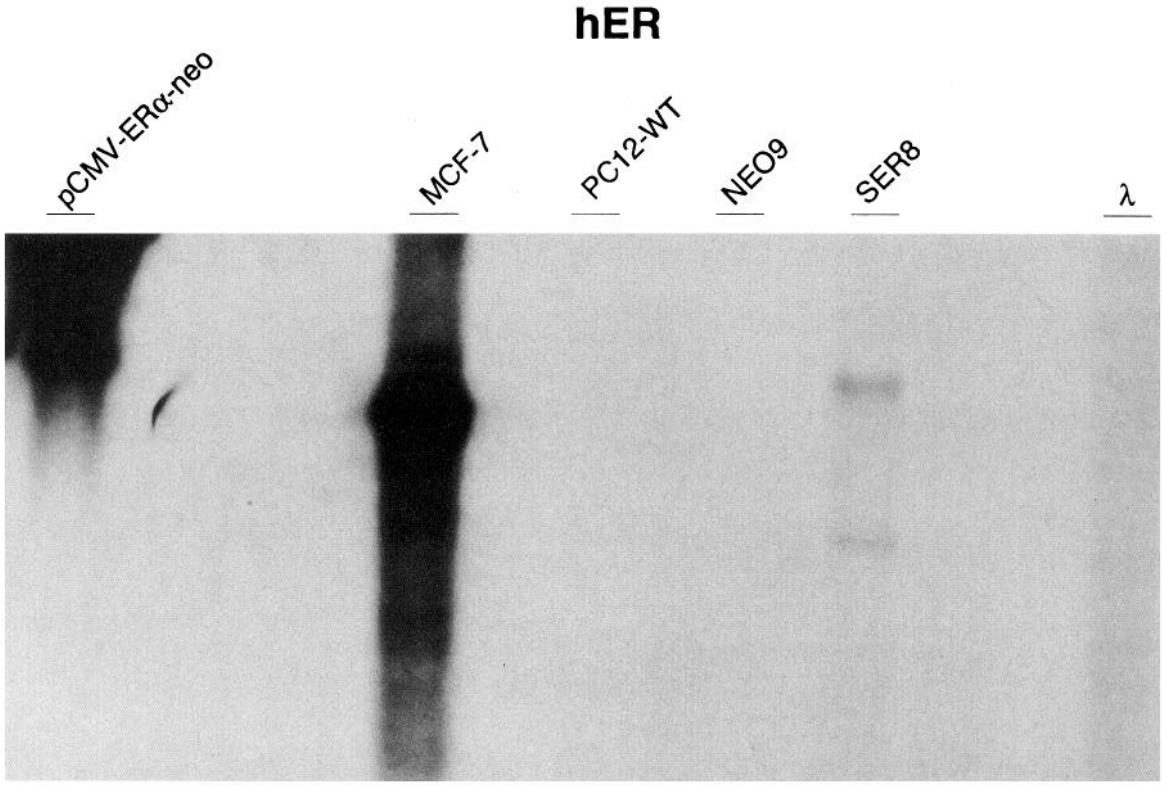


Figure 2. Northern blot of polyA$^+$ RNA extracted from MCF-7 breast cancer cells (ER-positive control), and PC12-WT, NEO9, and SER8 cells. Blot was probed with a 1.8 kb full-length ^{32}P-labeled cDNA coding for the hER. MCF-7 cells demonstrate an intense ER mRNA band at 6.2 kb. PC12-WT and NEO9 cells do not demonstrate any bands that hybridize with the hER cDNA probe, but SER8 cells demonstrate two light bands: one at 7.4 kb, which corresponds to the size of the transfected pCMV-ERα-neo vector, and a second band at 3.2 kb.

Analysis of ER mRNA expression

Northern blots were prepared from polyA$^+$ RNA from the different cell lines, and hybridized with ^{32}P-labeled cDNA for hER. Results are shown in Figure 2. ER-positive MCF-7 breast cancer cells demonstrate an intense band at 6.2 kb, previously shown to correspond to the hER (Jiang and Jordan, 1992), while PC12-WT and NEO9 cells do not demonstrate a band. SER8 cells do not have as much ER mRNA as do MCF-7 cells, but there are two bands at 7.4 and 3.2 kb, suggesting that SER8 cells express hER mRNA.

Cytosolic ER assay by ^{3}H-E_2 binding

To document functional ER protein, cytosol was prepared from each cell line and incubated with ^{3}H-E_2 to determine specific binding. Resultant femtomoles bound is expressed as a Scatchard plot in Figure 3. MCF-7 cells (positive control) demonstrated ^{3}H-E_2 specific binding with a K_d of 2.2 nM, and a B_{max} of 533 fmol/mg protein. PC12-WT and NEO9 cells demonstrated less and no specific binding, respectively, and a Scatchard plot could not be drawn. However, SER8 cells demonstrated specific binding with a K_d of 6.6 nM, and a B_{max} of 152 fmol/mg protein. Thus, the SER8 cells expressed an ER species with binding characteristics similar to MCF-7 cells, but with a diminished total ER concentration.

Light microscopic alterations of PC12 cell phenotype after NGF and estrogen incubation

To determine if estrogen has any effect on the morphologic phenotype of PC12 cells, the three lines were plated at a density of 35,000–40,000 cells per 35 mm dish. Cells were allowed to attach overnight, and then incubated with graded doses of NGF (0, 1, and 100 ng/ml), along with graded doses of E_2 (0, 10^{-10}, 10^{-9} M) in 0.1% ethanol for 2 d. Cells were fixed in 4% glutaraldehyde-PBS, examined under high-power light microscopy (LM), and quantified for total neurites, short neurites, long neurites, neuritic spines, and interneuritic interactions (see Materials and Methods). Representative cells are shown in Figure 4*a–c*. In Figure 4*a*, addition of NGF to PC12-WT cells caused them to extend neurites, and the coadministration of E_2 led to some lamellopodia formation, with occasional neuritic spines and interneuritic interaction. In Figure 4*b*, NEO9 cells demonstrated equivalent neuritic outgrowth with NGF, but coadministration of E_2 had no effect on spine formation or interneuritic interaction. In Figure 4*c*, in the absence of NGF, SER8 cells showed baseline lamellopodia formation, and the addition of E_2 led to the formation of some neurites. Administration of NGF led to neuritic outgrowth with some spine formation and interneuritic interaction in the absence of E_2, and addition of E_2 augmented these parameters, suggesting that E_2 was able to synergize with NGF. These findings are quantitated in Figure 5. E_2 demonstrated an additive effect with low-dose NGF on the frequency of both short and long neurite outgrowth in SER8 cells ($P < 0.05$), although the effect was not as noticeable at the higher dose of NGF, but there was no effect of estrogen in either PC12-WT or NEO9 cells. Furthermore, E_2 induced an increased frequency of neuritic spine formation and interneuritic interaction in SER8 cells, which again was most notable at low levels of NGF ($P < 0.05$), but again minimal and no effects were noted on PC12-WT and NEO9 cells, suggesting that these frequency

parameters are induced by estrogen. The density of each of the parameters analyzed did not demonstrate any estrogen effect.

Blockade of estrogen effect on SER8 cell morphology by coadministration of an antiestrogen

To determine the nature of the E_2 effect on SER8 cells, a corollary LM experiment was conducted in which the three cell lines were harvested, plated, and exposed to 100 ng/ml NGF with the following steroid treatments for 2 d: (1) 0 M E_2; (2) 10^{-9} M E_2; (3) 10^{-9} M E_2 + 2 × 10^{-8} M ICI 164,384 (ICI, Cheshire, UK), a pure antiestrogen that binds to the ER (Gottardis et al., 1989), 20-fold excess over E_2 dose; and (4) 10^{-9} M E_2 + 2 × 10^{-7} M ICI, a 200-fold excess. Cells were again fixed, and underwent quantitative light microscopy, as above. The quantitative results of this study are shown as histograms in Figure 6. Since this dose of NGF was maximal, these was no augmentation of neuritic outgrowth with E_2 in any cell line. ICI coadministration had little effect on PC12-WT or NEO9 cells. However, the aforementioned E_2-induced increases in spine formation and interneuritic interaction in SER8 cells were negated in a dose-dependent fashion by the coadministration of ICI.

Estrogenic induction of gap junctions

Electron microscopy (EM). To characterize the estrogen-induced interneuritic connections, the ultrastructure of cells from all three lines exposed to NGF and E_2 was examined. In particular, interneuritic abutments were visualized. In general, there were two types of abutments that differed in their ultrastructural appearance. Both types of abutments were present in all cell lines, and in the presence and absence of E_2. The first type of abutment was nonspecialized in appearance, with the cell membranes of the two neurites simply coming into apposition. The other type of abutment was highly specialized in appearance, with many thickened and darkly stained cell membranes on either side of the abutment. The appearances of the membranes on both sides of the abutment were similar, and showed no detectable asymmetries. Very high magnification and image tilting at the electron microscope indicated a layered appearance to the membrane. These observations led to the inference that these specialized abutments were gap junctions. Examples of these membrane specializations are depicted in Figure 7. It is also noted that there were no true synapses observed in any of the cell lines or estrogen conditions.

Consistent with the LM work, quantification of the number of abutments and the proportion that resembled gap junctions varied between cell lines and estrogen exposure (Table 1). PC12-WT and NEO9 cells only occasionally exhibited putative gap junctions, with no increase in frequency or length with E_2 administration. SER8 cells exhibited more frequent putative gap junctions at the abutments of cells in the absence of E_2, and markedly increased both their frequency and length with E_2 ($P < 0.05$).

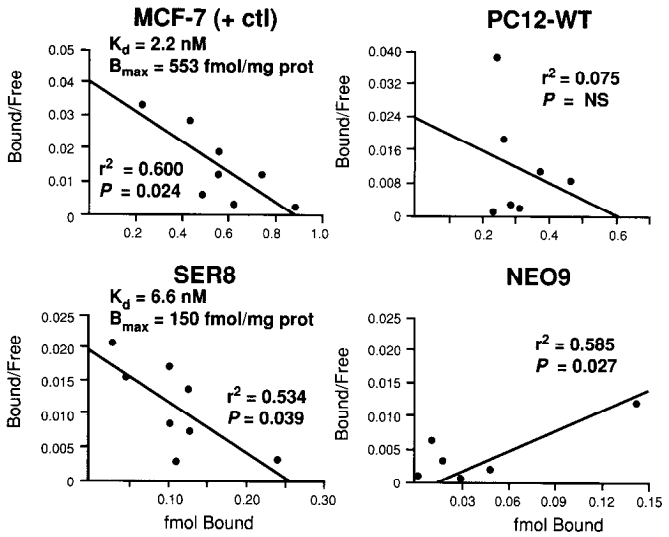


Figure 3. Scatchard plots calculated from ^{3}H-E_2 specific binding for MCF-7 (ER-positive control) cells, along with PC12-WT, SER8, and NEO9 cells. MCF-7 and SER8 cells both demonstrate a linear correlation ($P < 0.05$), suggesting only one binding site, with K_d calculations of 2.2–6.6 nM, which approximate that reported for hER in the literature.

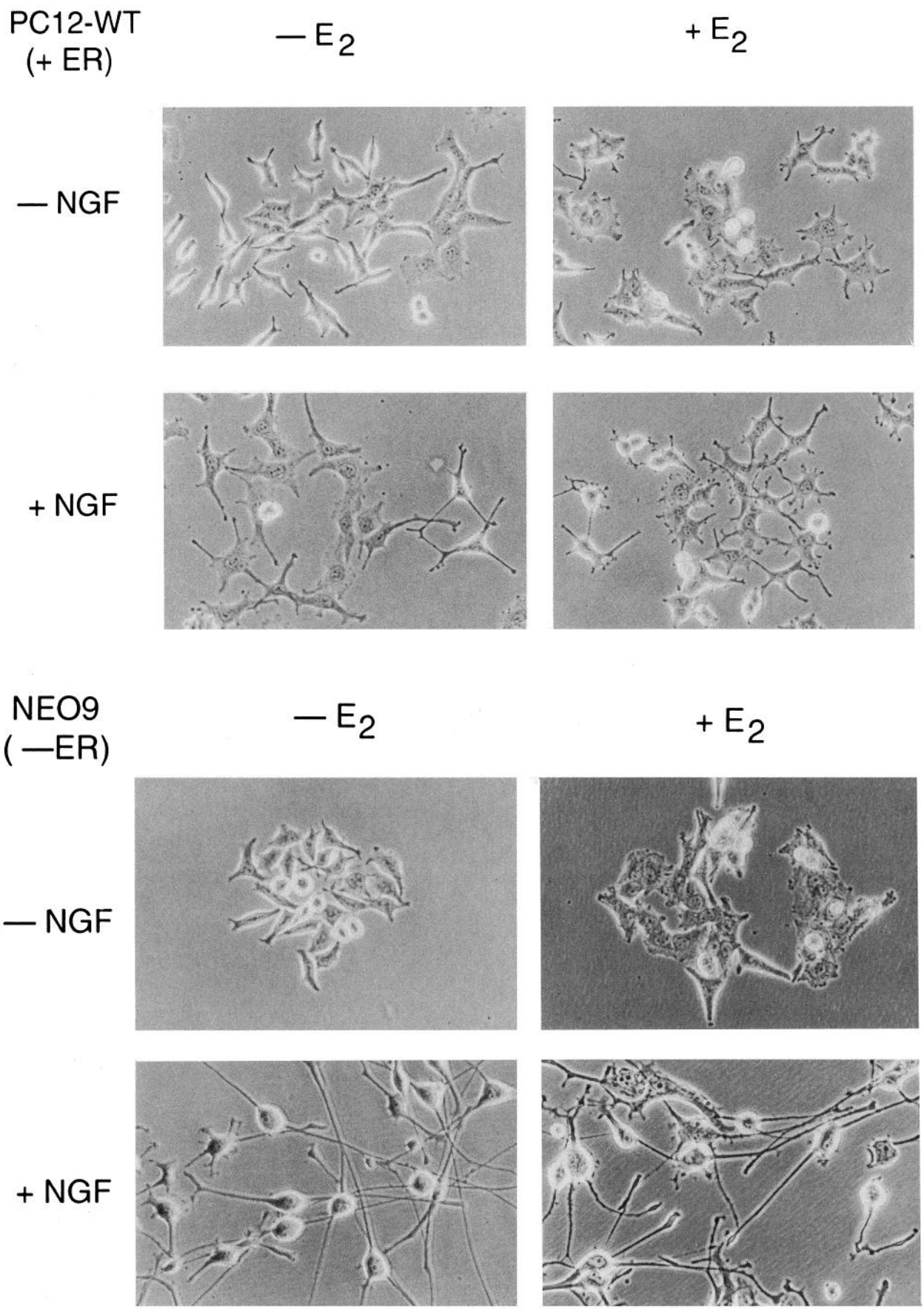
Dye coupling. To determine whether the specialized abutments observed under EM were indeed functional gap junctions, the transfer of dye between cells was assessed. Cells of each line were plated on polylysine-coated glass coverslips, exposed to 100 ng/ml NGF with or without 10^{-9} M E_2 for 2 d, and visualized by DIC microscopy. Abutments of neurites were determined visually. The cell body of one of the two neurites was microinjected with 5% Lucifer yellow. The neurites were defined as dye coupled if the second abutting but noninjected cell glowed under the epifluorescence beam 1 min after the first cell was injected. Examples of dye coupling are shown in Figure 8. PC12-WT and NEO9 cells demonstrated only rare dye coupling, either basally or with estrogen treatment. Conversely, SER8 cells showed relatively frequent dye coupling in the basal state, and a marked increase with E_2 treatment. Quantitative analysis of dye coupling is shown in Table 1. PC12-WT and NEO9 cells dye coupled with minimal frequency, with essentially no change with estrogen treatment. However, SER8 cells showed higher basal dye-coupling frequency, which more than doubled after E_2 administration ($P < 0.05$). The frequency of SER8 cell dye coupling was consistent with the EM data shown above.

Table 1. Effects of estrogen on gap junction frequency, gap junction length, and dye coupling

	PC12-WT		NEO9		SER8	
Parameter	0 M E_2	10^{-9} M E_2	0 M E_2	10^{-9} M E_2	0 M E_2	10^{-9} M E_2
Frequency of abutments with gap junctions (%)	8.2	21.0	15.7	12.7	21.0	46.9*
Length of abutments with gap junctions (%)	6.8	8.3	2.8	2.6	13.6	51.8*
Frequency of dye coupling	2/20	3/20	1/20	1/20	4/20	9/20*

Cells were grown for 2 d in DMEM with 100 ng/ml NGF, in the absence or presence of 10^{-9} M E_2. Gap junction length and frequency were determined by electron microscopy, and dye coupling was determined by DIC microscopy and fluorescence.

* Fisher's exact test $P < 0.05$.


PC12-WT
(+ ER)
— E_2
+ E_2
— NGF
+ NGF
NEO9
(—ER)
— E_2
+ E_2
— NGF
+ NGF

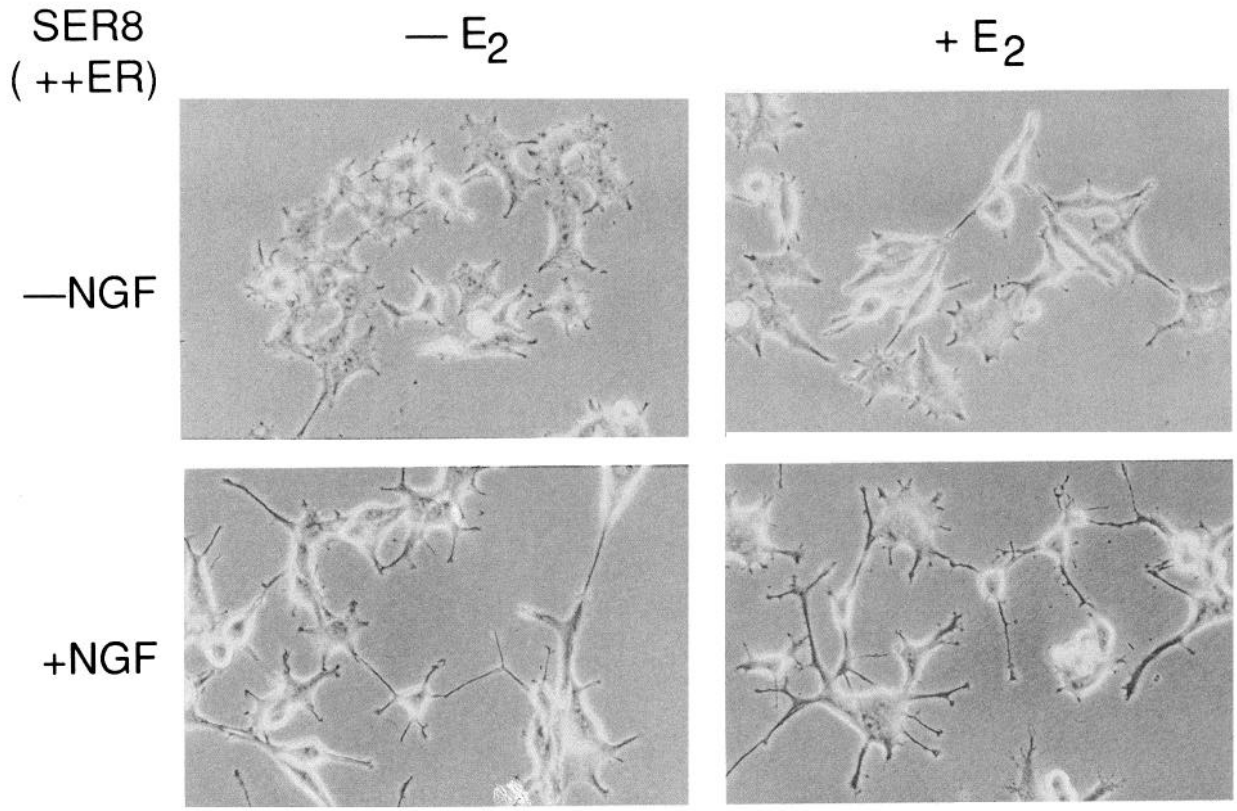


Figure 4. Continued.

Discussion

The results of these experiments show that estrogen has very specific effects on PC12 cells. In hER-expressing SER8 cells, estrogen has the ability to increase neurite outgrowth in the absence of NGF, and to synergize with NGF to augment the frequency of short and long neurite outgrowth. Several investigators (Toran-Allerand, 1980; Toran-Allerand et al., 1983; Diaz et al., 1992; Lorenzo et al., 1992) have clearly demonstrated the ability of estrogen to induce or augment neurite outgrowth in ER-positive neurons. As a corollary to this phenomenon, estrogenic regulation of mRNA levels for the axonal growth protein GAP-43 in VMH has also been noted (Lustig et al., 1991; Shughrue and Dorsa, 1993). The neuritic growth responses of SER8 cells to estrogen, even in the absence of NGF, are consistent with the hypothesis that estrogen is able to induce neurite outgrowth in ER-positive neural cells *in vitro*. Of note was the ability of estrogen to increase the frequency, but not the density, of these parameters. This suggests that estrogen increases the propensity for cells to elongate their processes, which alters their potential for interaction but does increase their inherent ability to form these processes. A similar effect of estrogen on neurite outgrowth was recently reported in an ER-transfected neuroblastoma cell line (Ma et al., 1993), suggesting that various cells of neural crest origin may retain the capacity to respond to estrogen by increasing the propensity for augmenting neurite outgrowth.

In addition, estrogen induces neuritic spine formation and interneuritic interaction in PC12 cells. Thus, the effects of estrogen on SER8 cells appear to be similar to the effects of estrogen on ER-positive neurons and areas of the rat brain *in vivo* (Gould et al., 1990; Ferreira and Caceres, 1991; Segarra and McEwen, 1991; Diaz et al., 1992; Lorenzo et al., 1992; Woolley and McEwen, 1992). The ability of an antiestrogen to negate the E_2 effect in a dose-responsive fashion implies that the E_2 effects on SER8 cells are mediated through standard estrogen–ER ligand–receptor interactions, rather than through any secondary phenomenon (e.g., direct membrane effects, cell metabolism, etc.).

Jones et al. (1986) showed that estrogen induces ribosomal RNA (rRNA) levels in VMH and amygdala (both ER-positive areas of the adult rat brain) within 6 hr. Steward and Falk (1991) have proposed that newly synthesized RNA is transported to the dendritic spine as polyribosomes, where they form clusters

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Figure 4. Effects of NGF and estradiol (E_2) on the morphology by LM (400×) of three lines of PC12 cells. In the NGF-deprived state (*upper two panels* of *a–c*), cells only occasionally establish neurites. NGF 100 ng/ml administration (*lower two panels* of *a–c*) induces neurite outgrowth in all lines. PC12-WT cells (*a*) and NEO9 cells (*b*) both exhibit neurite outgrowth in response to NGF, but E_2 coadministration has minimal or no morphologic effect with respect to neurites, spines, or interneuritic connections. In SER8 cells (*c*), even without NGF, E_2 induces neurite outgrowth, and when given in conjunction with NGF, E_2 is able to augment neurite outgrowth and also increase neuritic spine formation and interneuritic connections.

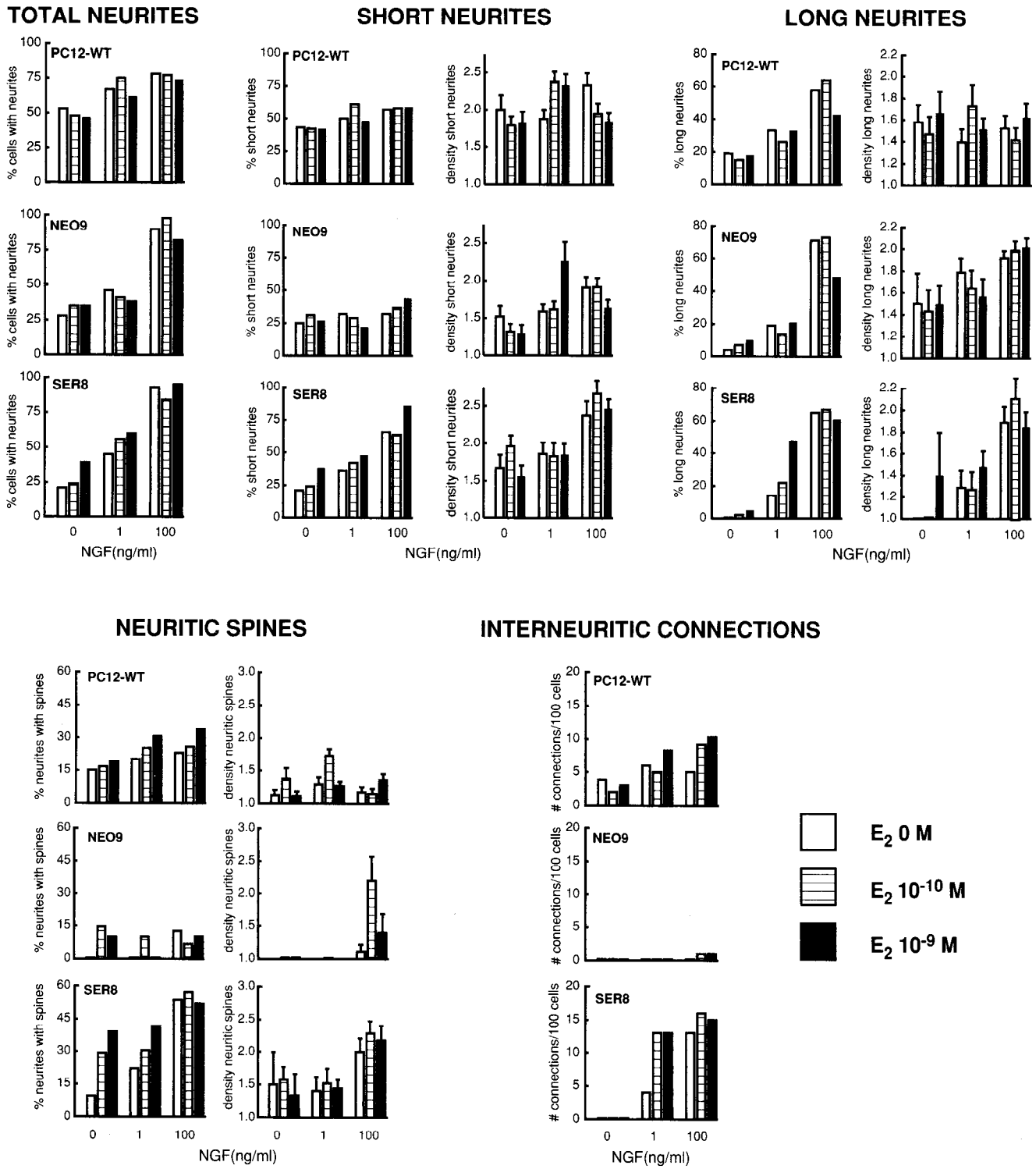

Figure 5. Quantitative effects of graded doses of NGF and E_2 on the morphological response of PC12 cells. NGF induces neurite outgrowth in all lines. PC12-WT and NEO9 cells show minimal and no response to graded doses of E_2. In SER8 cells, E_2 is additive in a dose-responsive fashion with NGF in inducing neurite outgrowth, and E_2 also increases neuritic spine formation and interneuritic connections.

and provide the biochemical machinery for local protein synthesis in the spine (Steward, 1983; Steward and Reeves, 1988; Rao and Steward, 1991; Steward and Falk, 1991). Axonal growth cones make contact with the spine, and this complex forms into an axospinous synapse (Steward et al., 1988). Spines and polyribosomes both increase during the period of synaptogenesis in hippocampus (Steward et al., 1988). The relative lack of spines in both the PC12-WT and NEO9 cells thus corresponds with previous reports of a relative lack of ribosomes within the PC12 neurite (Jacobs and Stevens, 1986). However, the abundance and the estrogenic regulation of neuritic spines in hER-positive SER8 cells suggest the ability of estrogen to enact a neural mor-

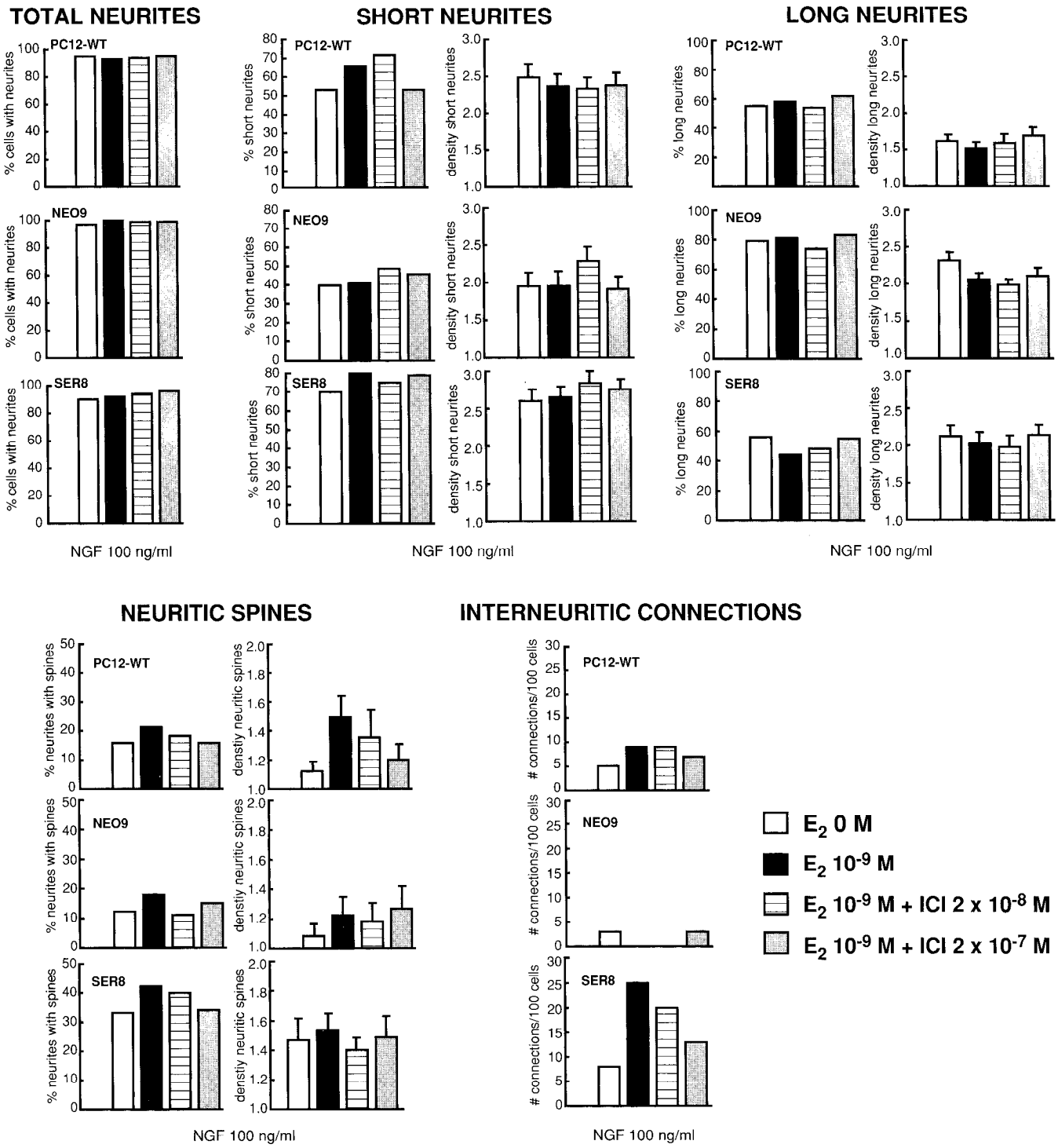

Figure 6. Quantitative effects of the antiestrogen ICI 164,384 (*ICI*) on the morphological response of PC12 cells to estradiol. ICI had little effect on the neuritic responses of PC12-WT and NEO9 cells to E_2, but increasing doses of ICI in SER8 cells decreased the ability of E_2 to induce neuritic spines and interneuritic interactions.

phologic program necessary to promote intercellular communication, which may be inherent in many types of neural cells. The evaluation of the estrogenic regulation of ribosomes, synaptic vesicles, and synapse-associated proteins in these cell lines will be the subject of future studies.

In addition, E_2 alters the communication between SER8 cells by inducing the formation of gap junctions, allowing for electrolyte, small particle transfer, and electrical coupling between cells. Estrogen has previously been noted to induce gap junctions in the uterus (MacKenzie and Garfield, 1985; Petrocelli and Lye, 1993); this phenomenon is thought to be important in parturition, when electrical coupling of myometrial cells allows for synchronous contraction of the uterus (MacKenzie and Garfield, 1985; Lye et al., 1993). Thus, there is precedence for the estrogenic induction of gap junctions in excitable cells. Documentation of the ability of estrogen to induce neural gap junc-

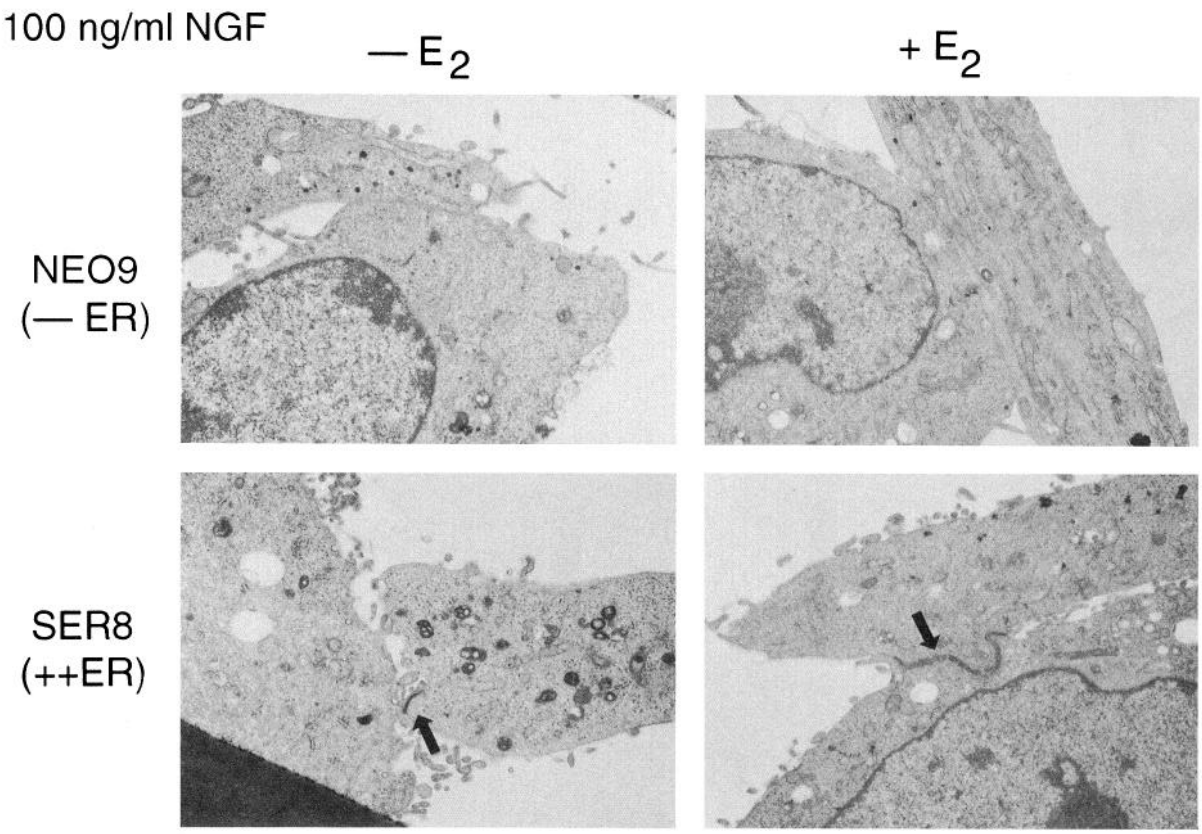

Figure 7. EM evidence for gap junctions in PC12 cells (10,000×). NEO9 cells treated with 100 ng/ml NGF only rarely showed membrane specializations consistent with gap junctions in the absence or presence of E_2. The predominant form of abutments in these cell lines was that of direct membrane apposition. Conversely, SER8 cells treated with NGF demonstrated these specializations (depicted by *arrows*) in the absence of E_2, and increased their number and length markedly in response to E_2 coadministration.

tions *in vivo* is less clear. Of note are studies by Hatton et al. (1990, 1992) indicating bidirectional changes in dye coupling in response to estrogen versus lactation in the supraoptic oxytocinergic system. Similarly, Theodosis and Poulain (1989) found alterations in neural–glial interactions in the paraventricular nucleus during lactation. These data suggest that estrogen-induced alterations in gap junctions in ER-positive areas may modulate the hypothalamic response to parturition.

The formation of gap junctions has been shown to be important in early intercellular communication between neurons during development, particularly at a time when synaptogenesis is beginning (Naus et al., 1988; Dermietzel et al., 1989; Peinado et al., 1993). The relative frequency of dye coupling between neurons in the rat neocortex *in vivo* during days P1–P12, with subsequent diminution (Peinado et al., 1993), suggests that gap junctions are a transient and short-lived phenomenon. Their egress at the same time that cortical synaptogenesis is occurring suggests a temporal relation between electrical and chemical transmission, and suggests an ontogenic role for gap junction expression in the sex- and hormone-dependent dimorphisms in neural architecture, synaptogenesis, and reproductive physiology. However, such an association is extremely difficult to test *in vivo* because of the heterogeneity of the brain and the inability to follow a gap junction serially *in vivo*. However, this hypothesis may be testable *in vitro* by monitoring the potential metamorphosis of gap junctions into synapses in SER8, NEO9, and PC12-WT cells by EM and dye-coupling methods.

Molecular studies of connexin expression in the neonatal rat brain have implicated estrogen as a potential regulatory signal of gap junction function. Matsumoto et al. (1991) have shown that connexin-32 mRNA can be found by *in situ* hybridization in areas of the neonatal rat brain that are ER-positive, for example, the hippocampus and anterior hypothalamus. Similarly, Naus et al. (1988, 1990) found high levels of connexin-32 mRNA in developing rat neocortex and hypothalamus (both ER-positive areas in the neonate) that decrease after day 19, when ER levels fall (Shughrue et al., 1990; Toran-Allerand et al., 1992), suggesting that gap junctions may be an early form of communication between hormone-responsive neurons in the neonatal rat brain, which may later be modified or cleared when synaptogenesis becomes the predominant form of intercellular communication.

Various connexins may have specific cellular localizations. Matsumoto et al. (1991), using *in situ* hybridization with a ^{35}S-labeled connexin-32 (liver-derived) cDNA probe, demonstrated silver grains over neurons in the hippocampus, anterior hypothalamus, and other limbic structures. However, Yamamoto et al. (1992), using a site-specific antibody to connexin-43 (heart-derived), demonstrated by ICC and EM that while there was a clear differential distribution of this connexin during neural development (including staining in the hypothalamus), there was a clear predilection of the antibody for glial and ependymal cells, with no staining in either oligodendrocytes or neurons. It is not clear if the discrepancy is protein or method specific. Naus and colleagues have demonstrated regional differences in the expression of the two connexins; connexin-43 mRNA is more

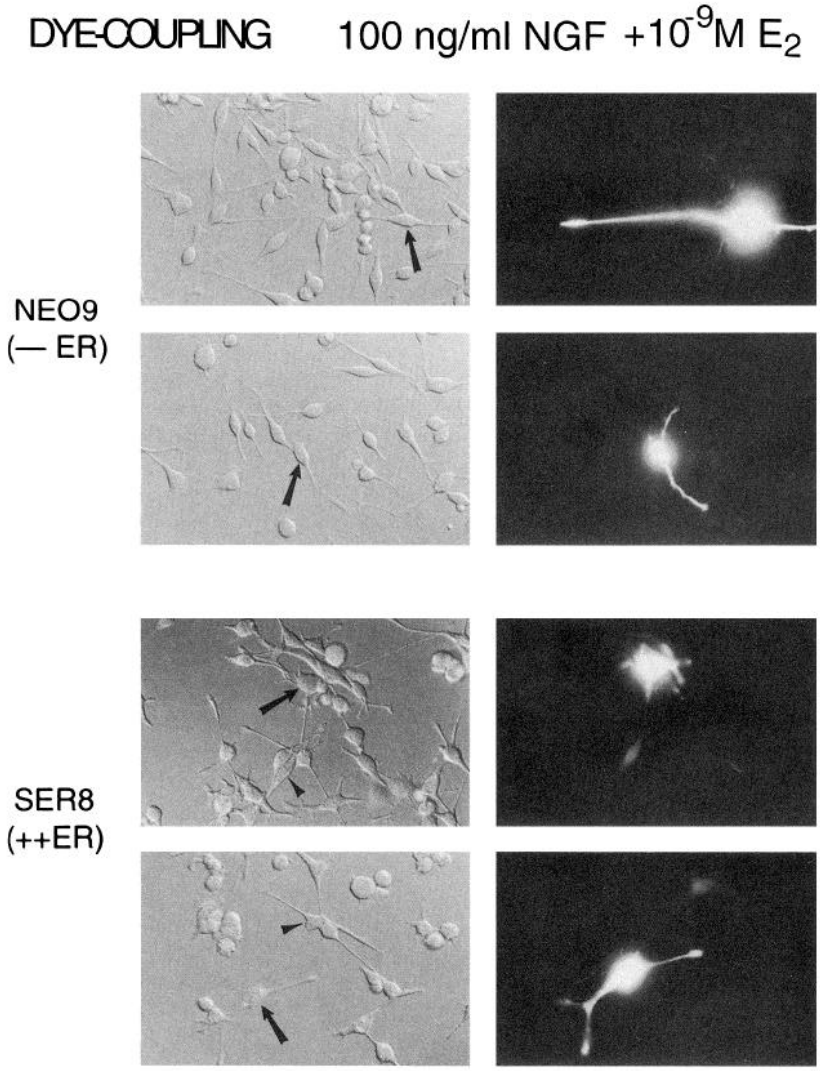

Figure 8. Representative examples of the absence or presence of dye coupling after NGF, 100 ng/ml, and E_2, 10^{-9} M, for 2 d in NEO9 and SER8 cells (400×). The *left panels* depict cell morphology with DIC microscopy, and the *right panels* depict injected and dye-coupled cells glowing under epifluorescence. *Arrows* depict injected cells, while *arrowheads* depict dye-coupled cells. NEO9 cells dye-coupled only rarely and E_2 was ineffective in increasing dye coupling, as demonstrated by the lack of secondary epifluorescence of cells in this figure. SER8 cells dye coupled frequently in the absence of E_2, along with increasing dye-coupling frequency with concomitant E_2 treatment.

ubiquitous, and connexin-32 mRNA is more localized, particularly in the hypothalamus (Naus et al., 1990). Lastly, Dermietzel et al. (1989) localized connexin-26 to non-neuronal tissue in the embryonic brain; however, a recent report by Matesic et al. (1993) documents the expression of connexin-26 in the cell line GT1-7, suggesting neuronal localization. Experiments delineating the type and hormonal regulation of connexin mRNA expression in SER8 cells are currently underway.

In conclusion, these three cell lines form the basis of an *in vitro* model for estrogen effects on neurons. We anticipate exploiting them to examine the effects of estrogen in a homogeneous neural cell population. Biochemical and gene regulation studies can be performed in culture, as well as developmental studies regarding changes after prolonged NGF and estrogen exposure as well as withdrawal. Ultrastructural and electrophysiologic studies can also be accomplished. Finally, mechanistic studies evaluating the role of specific proteins or mRNAs in sex- and hormone-dependent neural development can be addressed.

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