Brain Research, 498 (1989) 339–343
Elsevier

BRES 23725

Short Communications

Estradiol induces rapid remodelling of plasma membranes in developing rat cerebrocortical neurons in culture

L.M. Garcia-Segura[1,2], G. Olmos[1], R.J. Robbins[2,3], P. Hernandez[1], J.H. Meyer[2,3] and F. Naftolin[2]

[1]*Instituto Cajal, C.S.I.C., Madrid (Spain), and Neuroendocrine Program, Departments of* [2]*Obstetrics and Gynecology and* [3]*Medicine, Yale University School of Medicine, New Haven, CT 06510 (U.S.A.)*

(Accepted 6 June 1989)

Key words: Estradiol; Cerebral cortex; Freeze-fracture; Plasma membrane; Neuronal membrane; Development; Cell culture

Exo-endocytotic images and intramembrane particles were quantitatively assessed in freeze-fracture replicas from the plasma membrane of dispersed fetal rat cortical neurons (day 16 gestation) grown for 24 days in culture. The addition of 10^{-10} M 17β-estradiol to the culture medium resulted in a significant increase in the numerical density of exo-endocytotic images within 1 min. A further increase of the number of exo-endocytotic images associated to a significant decrease in the number of intramembrane particles was observed in cells exposed for 10 min to 17β-estradiol. Similar results were observed when the cells were exposed to 17β-estradiol for 17 days. No effects on exo-endocytotic images and intramembrane particles were observed when 17α-estradiol was added, instead of 17β-estradiol, to the cultures. These results indicate that physiological levels of 17β-estradiol can have rapid effects upon the ultrastructure of the neuronal membrane of developing cerebrocortical neurons.

Considerable experimental evidence now exists to indicate that some of the biological actions of sex steroids occur at the level of cell membranes[5]. Evidence of neuronal membrane effects of sex steroids have been obtained in electrophysiological and biochemical studies[4,13,21,22,26,31]. Furthermore, we have demonstrated rapid neuronal plasma membrane ultrastructural modifications elicited by sex steroids in freeze-fracture studies of the rat hypothalamic arcuate nucleus[10]. Within 1 min, physiological doses of 17β-estradiol increased the density of exo-endocytotic images in arcuate neuronal membranes from hypothalamic slices perfused with a medium containing the hormone. We also found that the number of exo-endocytotic images in developing arcuate neuronal membranes was increased in males and testosterone-treated females when compared to normal females[8]. These effects were not observed in Purkinje cells of the cerebellar cortex[23], suggesting that membrane effects of sex steroids are restricted to sensitive neurons. However, it is unknown whether the neuronal membranes from other extrahypothalamic areas are sensitive to estradiol. The cerebral cortex is an interesting area for the study of membrane effects of gonadal steroids since there is expression of classical nuclear receptors for sex steroids in cortical cells during development and the estradiol receptor mRNA has been localized in cerebral cortex of adult female rats[12,16,17,25,29,30]. In adults there are sex differences in the structure of cerebral cortex and in the size of the corpus callosum[1,3,11,14,31,32] present. Finally, morphological and electrophysiological studies have demonstrated effects of sex steroids on cortical neurons in the rat[19,20,24,26]. In this study we tested the hypothesis that estradiol can directly alter the structure of plasma membranes in developing rat cerebrocortical neurons.

Cortical cells from Sprague–Dawley fetal rats (day 16 gestation) were obtained using methods outlined previously[28]. Cells were placed in 2 ml of a solution containing minimal essential medium

Correspondence: L.M. Garcia-Segura, Instituto Cajal, C.S.I.C., Dr. Arce 37, 28002 Madrid, Spain.

(MEM; Earle's Salts), dextrose, penicillin, streptomycin, and 10% fetal calf serum (FCS). The cells were maintained in this medium until day 6, at which time the medium was changed to one in which the FCS was replaced by 10% heat-inactivated horse serum and 2 μM cytosine β-D-arabinofuranoside

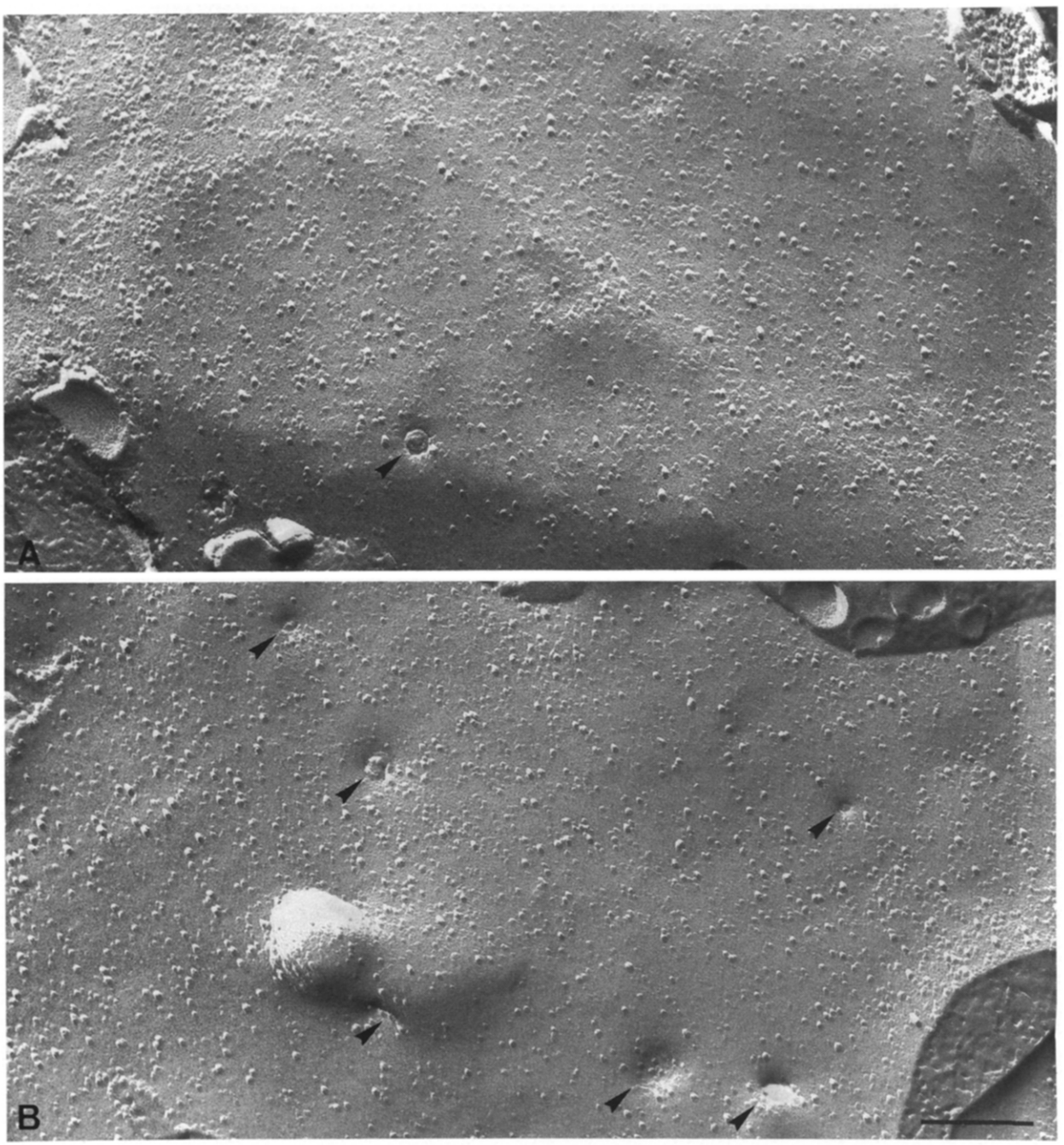

Fig. 1. Freeze-fracture replicas from the perikarya of rat cerebrocortical neurons in culture showing examples of plasma membranes similar to those evaluated quantitatively. A: from a control culture not exposed to any supplemental estrogen. B: from a culture exposed for 1 min to 10^{-10} M 17β-estradiol. The membrane from the neuron exposed to estradiol shows an increased number of exo-endocytotic images (arrowheads). Both figures are from the P-face of the membrane and are at the same magnification. Bar = 0.25 μm.

(used to minimize glial proliferation). This medium was maintained until day 12, when it was changed to one containing 10% heat-inactivated horse serum, but not cytosine β-D-arabinofuranoside. This medium was used throughout the remainder of the experiment, with changes occurring at weekly intervals to ensure culture viability.

Estradiol-treated cells were maintained in medium containing supplemental 10^{-10} M 17α- or 17β-estradiol. In long-term estradiol-treated cells, the supplemental estradiol was provided 17 days prior to cell harvesting and fixation (see below). For short-term treatments, 10^{-10} M estradiol was added 1 min or 10 min prior to cell harvesting. Control cells were not exposed to any supplemental estrogen. Cells were harvested on day 24. Harvesting was done by first aspirating the medium, and then washing the cells with iced phosphate-buffered saline. The buffer was then replaced with a solution of 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Cells were maintained in this solution for 2 h, after which the fixative was removed and replaced with 0.1 M phosphate buffer. The cells were then scraped from the bottom of the culture dish with a rubber policeman and transferred to a microfuge tube. The cells were centrifuged for 2 min, after which the supernatant was discarded. The sediments were soaked overnight in 0.1 M phosphate buffer, pH 7.4, containing 20% glycerol, then frozen in Freon 22 cooled with liquid nitrogen, fractured at −110 °C and replicated in a Balzers 400D apparatus (Balzers, Liechtenstein). The replicas were examined in a Jeol 100 B electron microscope.

TABLE I

Numerical density of exo-endocytotic images (number/1000 μm²) and intramembrane particles (number/μm²) in the plasma membrane of the perikarya of cortical cells exposed to 17α-estradiol (17αE_2) or 17β-estradiol (17βE_2) in culture

Data are means ± S.E.M. *n* is the number of cells evaluated. At least 100 μm^2 of membrane area were evaluated for each experimental condition and each membrane face.

Treatment	*Exo-endocytotic images**	*Intramembrane particles*	
		P-face	*E-face*
None (controls) ($n = 30$)	174 ± 5	858 ± 26	381 ± 34
17αE_2 1 min ($n = 10$)	182 ± 12	848 ± 43	392 ± 38
17αE_2 10 min ($n = 10$)	169 ± 16	863 ± 32	340 ± 23
17αE_2 17 days ($n = 10$)	177 ± 9	828 ± 31	378 ± 41
17βE_2 1 min ($n = 20$)	260 ± 22**	815 ± 32	330 ± 14
17βE_2 10 min ($n = 20$)	633 ± 26**	631 ± 32**	146 ± 22**
17βE_2 17 days ($n = 30$)	763 ± 21**	736 ± 48**	149 ± 18**

* Data from P and E faces were pooled for statistical analysis.
** Values significantly different ($P < 0.001$) from that of control cells.

Neuronal membranes in freeze-fracture replicas were identified by their contiguity with cross-fractured cytoplasmic profiles. To avoid the possible bias introduced by the observer, no attempt of selection was made when the replicas were photographed. Therefore, for a given replica, all the encountered replicated inner (P) and outer (E) membrane fracture faces[2] of perikarya were photographed. Photographic prints were coded and evaluated without knowledge of the experimental group from which the picture was taken. When photographic prints were analyzed, no neuronal perikarya was rejected on criteria other than unsatisfactory shadowing. The number of exo-endocytotic images and the number of intramembrane particles (IMPs) were evaluated separately in each leaflet of the plasma membrane. Exo-endocytotic images and IMPs were recorded within a test square grid superimposed on photographic prints (final magnification ×90,000 for exo-endocytotic images and ×165,000 for IMP counts, calibrated with a reference grid, 2160 lines·mm^{-1}). To avoid counting errors due to the variable radius of curvature of the plasma membrane, the calculation of the correct size of the test square grid was carried out as described previously[9]. Statistical comparisons were carried out using the Mann-Whitney *U*-test[18].

In control cells, not exposed to any supplemental estrogen, neuronal perikarya showed exo-endocytotic images in the P and E faces of the plasma membrane. Exo-endocytotic images appear as pits in the P-face and as complementary domes in the E-face. The numerical density of exo-endocytotic images was significantly ($P < 0.001$) increased in cells exposed for 1 min to 17β-estradiol when compared to control cells (Fig. 1, Table I). The number of exo-endocytotic images was further in-

creased in cells incubated for 10 min or longer with 17β-estradiol when compared to cells incubated for only 1 min (Table I). The numerical density of IMPs was slightly reduced in cells exposed for 1 min to 17β-estradiol when compared to controls (Fig. 1). This difference was not significant. However, the difference was more pronounced after 10 min of estradiol exposure; IMP content was significantly reduced in cells exposed to 17β-estradiol for 10 min and in long-term treated cells (Table I). The 17β-estradiol action on neuronal membranes appears specific, since no effects on the number of exo-endocytotic images and IMPs were observed when 17α-estradiol was used (Table I).

These findings fit with previous experiments showing an increase in the number of exo-endocytotic images in neuronal membranes from arcuate nucleus slices exposed for 1 min to 17β-estradiol[10]. Rapid ultrastructural effects of estradiol on plasma membranes have also been detected in the uterine endometrium, where the density of microvilli was increased 30 s after the injection of 17β-estradiol to ovariectomized rats[27]. The rapidity of these hormonal effects is a far shorter time than is considered necessary for cell nuclear sex hormone receptor-mediated induction of protein synthesis and raised the question of the possible non-genomic or post-transcriptional actions of sex steroids on neuronal plasma membranes[5].

That the increased number of exo-endocytotic images in neuronal membranes could be related with an increased endocytotic activity is suggested by previous studies showing a rapid induction by estradiol of endocytosis in arcuate nucleus[10]. We have also observed an increased uptake of horseradish peroxidase by cortical cultures in the presence of 17β-estradiol but not in presence of 17α-estradiol (unpublished observations). The decrease in the number of IMPs in the cells exposed for 10 min or longer to 17β-estradiol could be associated with increased exo-endocytotic activity and may therefore represent the preferential internalization of some membrane proteins induced by the hormone. A similar mechanism could be involved in the generation of sex differences in the number of IMPs in hypothalamic neurons[6,8] and may therefore represent a general effect of 17β-estradiol in sensitive neurons. The reduction in the number of IMPs in long-term estradiol-treated cortical membranes could also be associated with an effect of this hormone on protein synthesis, since the neonatal estrogenization in the rat results in a decreased incorporation of [^{3}H]lysine into cortical proteins[15].

Although the precise physiological significance of the membrane changes reported here is unknown, it is interesting to note that similar membrane modifications in arcuate nucleus are associated with a remodeling of the synaptic contacts in adult rats[7,23] and to the sexual differentiation of synaptic connectivity in developing animals[6,8]. Since membrane recognition is essential for the formation and maintenance of the precise patterns of synaptic connections, the effects of sex steroids on neuronal membrane may be involved in the sexual differentiation of synaptic connectivity in cortical neurons and in the synaptic effects of sex steroids[19]. Our results emphasize the role of the plasma membrane on the mechanisms of action of sex steroids in the central nervous system and suggest that membrane effects of sex steroids may have important influences on the development of cerebral cortex.

Supported by CSIC/CAICYT (ID751) and NIH (HD13587, NS06208) grants and by a fellowship to G.O. (FISS). We thank E. Valero and J. Sancho for technical assistance.

1 Berrebi, A.S., Fitch, R.H., Ralphe, D.L., Denenberg, J.O., Friedrich, V.L. and Denenberg, V.H., Corpus callosum: region-specific effects of sex, early experience and age, *Brain Research,* 438 (1988) 216–224.

2 Branton, D., Bullivant, S., Gilula, N.B., Karnovsky, M.J., Moor, H., Muhlethaler, K., Northcote, D.H., Packer, L., Satir, B., Satir, P., Speth, V., Staehelin, L.A., Steere, R.L. and Weinstein, R.S., Freeze-etching nomenclature, *Science,* 190 (1975) 54–56.

3 Diamond, M.C., Sex differences in the rat forebrain, *Brain Res. Rev.,* 12 (1987) 235–240.

4 Drouva, S.V., Laplante, E., Gautron, J.P. and Kordon, C., Effects of 17β-estradiol on LH-RH release from rat mediodorsal hypothalamic slices, *Neuroendocrinology,* 38 (1984) 152–157.

5 Duval, D., Durant, S. and Homo-Delarche, F., Non-genomic effects of steroids. Interactions of steroid molecules with membrane structures and functions, *Biochim. Biophys. Acta,* 737 (1983) 409–442.

6 Garcia-Segura, L.M., Baetens, D. and Naftolin, F., Sex differences and maturational changes in arcuate nucleus neuronal plasma membrane organization, *Dev. Brain Res.,*

19 (1985) 146–149.
7 Garcia-Segura, L.M., Baetens, D. and Naftolin, F., Synaptic remodelling in arcuate nucleus after injection of estradiol valerate in adult female rats, *Brain Research*, 366 (1986) 131–136.
8 Garcia-Segura, L.M., Perez, J., Tranque, P.A., Olmos, G. and Naftolin, F., Sexual differentiation of the neuronal plasma membrane: neonatal levels of sex steroids modulate the number of exo-endocytotic images in the developing rat arcuate neurons, *Neurosci. Lett.*, 91 (1988) 19–23.
9 Garcia-Segura, L.M. and Perrelet, A., Postsynaptic membrane domains in the molecular layer of the cerebellum: a correlation between presynaptic inputs and postsynaptic plasma membrane organization, *Brain Research*, 321 (1984) 255–266.
10 Garcia-Segura, L.M., Olmos, G., Tranque, P. and Naftolin, F., Rapid effects of gonadal steroids upon hypothalamic neuronal membrane ultrastructure, *J. Steroid Biochem.*, 27 (1987) 615–623.
11 Juraska, J.M. and Kopcik, J.R., Sex and environmental influences on the size and ultrastructure of the rat corpus callosum, *Brain Research*, 450 (1988) 1–8.
12 Kato, J. and Onouchi, T., Progesterone receptors in the cerebral cortex of neonatal female rats, *Dev. Neurosci.*, 4 (1981) 427–432.
13 Ke, F.C. and Ramirez, V.D., Membrane mechanism mediates progesterone stimulation effect on LHRH release from superfused rat hypothalami in vitro, *Neuroendocrinology*, 45 (1987) 514–517.
14 Lacoste-Utamsing, C. and Holloway, R.L., Sexual dimorphism in the human corpus callosum, *Science*, 216 (1982) 1431–1432.
15 Litteria, M. and Thorner, M.W., Inhibitory action of neonatal estrogenization of incorporation of [^{3}H]lysine into cortical neuroproteins, *Brain Research*, 103 (1976) 584–587.
16 MacLusky, N.J., Chaptal, C. and McEwen, B.S., The development of estrogen receptor systems in the rat brain: postnatal development, *Brain Research*, 178 (1979) 143–160.
17 MacLusky, N.J., Naftolin, F. and Goldman-Rakic, P.S., Estrogen formation and binding in the cerebral cortex of the developing rhesus monkey, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 513–516.
18 Mann, H.B. and Whitney, D.R., On a test whether one or two random variables is stochastically larger than the other, *Ann. Math. Statist.*, 18 (1947) 50–60.
19 Medosch, C.M. and Diamond, M.C., Rat occipital cortical synapse after ovariectomy, *Exp. Neurol.*, 75 (1982) 120–132.
20 Menzies, K.D., Drysdale, D.B. and Waite, P.M.E., Effects of prenatal progesterone on the development of pyramidal cells in rat cerebral cortex, *Exp. Neurol.*, 77 (1982) 654–667.
21 Moss, R.L. and Dudley, C.A., Molecular aspects of the interaction between estrogens and the membrane excitability of hypothalamic cells, *Prog. Brain Res.*, 61 (1984) 3–21.
22 Nabekura, J., Oomura, Y., Minami, T., Mizuno, Y. and Fukuda, A., Mechanism of the rapid effect of 17-β-estradiol on medial amygdala neurons, *Science*, 233 (1986) 226–228.
23 Olmos, G., Aguilera, P., Tranque, P., Naftolin, F. and Garcia-Segura, L.M., Estrogen-induced synaptic remodelling in adult rat brain is accompanied by the reorganization of neuronal membranes, *Brain Research*, 425 (1987) 57–64.
24 Pappas, C.T.E., Diamond, M.C. and Johnson, R.E., Morphological changes in the cerebral cortex of rats with altered levels of ovarian hormones, *Behav. Neural Biol.*, 26 (1979) 298–310.
25 Pelletier, G., Liao, N., Follea, N. and Govindan, M.V., Mapping of estrogen receptor-producing cells in the rat brain by in situ hybridization, *Neurosci. Lett.*, 94 (1988) 23–28.
26 Phillis, J.W. and O'Regan, M.H., Effects of estradiol on cerebral cortical neurons and their response to adenosine, *Brain Res. Bull.*, 20 (1988) 151–155.
27 Rambo, C.O. and Szego, C.M., Estrogen action at endometrial membranes: alterations in luminal surface detectable within seconds, *J. Cell Biol.*, 97 (1983) 679–685.
28 Robbins, R.J., Sutton, R.E. and Reichlin, S., Effects of neurotransmitters and cyclic AMP on somatostatin release from cultured cerebral cortical cells, *Brain Research*, 234 (1982) 377–386.
29 Sandhu, S., Cook, P. and Diamond, M.C., Rat cerebral cortical estrogen receptors: male–female, right–left, *Exp. Neurol.*, 92 (1986) 186–196.
30 Sheridan, P.J., Estrogen binding in the neonatal neocortex, *Brain Research*, 178 (1979) 201–206.
31 Teyler, T.J., Vardaris, R.M., Lewis, D. and Rawitch, A.B., Gonadal steroids: effects on excitability of hippocampal pyramidal cells, *Science*, 209 (1980) 1017–1019.
32 Van Eden, C.G., Uylings, H.B.M. and Van Pelt, J., Sex-difference and left-right asymmetries in the prefrontal cortex during postnatal development in the rat, *Dev. Brain Res.*, 12 (1984) 146–153.