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Changes in the Neuronal Membranes of Mice Related to Steroid Hormone Influences

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WHITING, K. P., C. J. RESTALL AND P. F. BRAIN. *Changes in the neuronal membranes of mice related to steroid hormone influences.* PHARMACOL BIOCHEM BEHAV **59**(4) 829–833, 1998.—Changes in the biochemical composition of synaptosomal plasma membranes (SPM) isolated from mouse brains have been measured. The protein, phospholipid, and cholesterol contents all increased over the first 30 days of postnatal life, with the cholesterol to phospholipid molar ratio (one of the major determinants of lipid fluidity) also increasing in direct relation to the decrease in lipid fluidity. The fatty acid composition of SPM also changes with the increase in 18:0, and the decrease in 18:2, 18:3, and 22:4, in keeping with the increase in membrane order. Steroid hormones alter lipid fluidity to a greater degree in fluid membranes, indicating that the nongenomic effects of steroids will be most prevalent in membranes during the early prenatal period and for the first days following birth. The potential effects of xenobiotics on membrane fluidity are also discussed. © 1998 Elsevier Science Inc.

Sexual differentiation Development Steroid hormones Mice Synaptosomal plasma membranes Xenobiotics

DIFFERENCES in the general morphology, brain structure, and behavior between male and female mammals are collectively termed "sexual differentiation," and this process is dependent on the successful completion of a series of steps initially under genetic control. The chromosomal sex largely regulates gonadal development, which controls the sexual phenotype through secretions of steroid hormones, namely testosterone and 17β-estradiol. In animals such as rodents, with a short gestation, sexual dimorphism occurs during the late neonatal period and continues for the first few days of postnatal life (11). The early actions of steroids on peripheral target tissues and on the central nervous system are classified as organizational effects. Subsequent exposure to these steroids activates, modulates, or inhibits the function of these existing neural circuits.

The classically accepted mechanism of action of steroid hormones is via their binding to intracellular receptors, initiating gene transcription and subsequent protein synthesis (4,36,40) However, in, for example, the mouse brain, steroid receptor systems are not fully established at birth, and the various synapses only become fully functional between days 12 and 28 postnatally (1,27,28). This suggests that the early regulation of sexual differentiation by steroid hormones (in rodents at least) is partly controlled by nongenomic mechanisms. Three potential nongenomic steroid actions have been recently proposed (6,23,30). The steroid may interact with membrane-bound receptors, initiating adenylyl cyclase activity, and second messenger cascades, which could then activate protein kinases, calcium channels, or result in the exocytosis of membrane vesicles (2,26,34,35). Steroids also bind to neurotransmitter receptors, principally the $GABA_A$ receptor, altering neuronal activity (21,22,29,31). The lipophilic nature of steroids may result in their intercalation into the lipid bilayer of target cell membranes, altering the fluidity and, therefore, the function of the membrane through changes in membrane-bound enzyme activity. Changes have been observed in the ordering of membranes on intercalation of cholesterol and a number of steroid hormones (8–10). These hormone effects are less in membranes with a greater cholesterol content (unpublished data).

Membrane fluidity is strongly influenced by the cholesterol-to-phospholipid molar ratio and by the fatty acid composition of the lipid bilayer. The greater the cholesterol content and the longer and more saturated the fatty acyl chain length, the lower is the membrane fluidity. Changes in the biochemical composition of membranes, including the plasma membrane of chick embryo heart, rat liver microsomal membranes, and synaptosomal plasma membranes (SPM) have been noted over maturation (18,20,39). In the current work

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TABLE 1

PROTEIN, PHOSPHOLIPID, AND CHOLESTEROL CONTENTS OF SYNAPTOSOMAL PLASMA MEMBRANES ISOLATED FROM MOUSE BRAINS AGED 1 TO 30 DAYS POSTNATALLY

Postnatal Day	Total protein (mg/g Brain Weight)	Phospholipid (μmol/mg Protein)	Cholesterol (μmol/mg Protein)	Molar Ratio of Cholesterol to Phospholipid	Fluorescence Anisotropy ($r \times 10^3$)
1	0.70 ± 0.15	1.25 ± 0.06	0.65 ± 0.11	0.52	185 ± 5
2	0.75 ± 0.18	1.23 ± 0.08	0.75 ± 0.08	0.61	188 ± 10
3	2.10 ± 0.12	1.19 ± 0.17	0.69 ± 0.07	0.58	190 ± 7
4	2.76 ± 0.10	0.86 ± 0.15	0.62 ± 0.04	0.72	186 ± 14
6	3.01 ± 0.19	2.37 ± 0.08	1.54 ± 0.13	0.65	194 ± 5
8	3.43 ± 0.14	1.77 ± 0.10	1.29 ± 0.11	0.73	193 ± 9
10	4.06 ± 0.25	1.21 ± 0.09	0.82 ± 0.06	0.68	195 ± 6
15	4.18 ± 0.18	1.01 ± 0.11	0.76 ± 0.06	0.75	203 ± 12
20	4.79 ± 0.10	1.48 ± 0.11	1.19 ± 0.08	0.80	214 ± 9
25	5.08 ± 0.13	1.46 ± 0.12	1.15 ± 0.11	0.79	212 ± 13
30	5.10 ± 0.16	1.61 ± 0.13	1.37 ± 0.09	0.85	218 ± 4

The cholesterol-to-phospholipid molar ratio and the fluorescence anisotropy are also shown. The data are the mean of four independent measurements and the standard deviations from the mean are shown.

both the biochemical composition and the fluidity of neuronal membranes have been measured over the first 30 days of postnatal life. Any changes in membrane composition are related to steroid hormone influences on membranes, with the potential to determining if the nongenomic actions of steroids on membranes could be involved in sexual differentiation.

METHOD

Animals

Synaptosomal plasma membranes were prepared from whole brains of Alderley Park strain mice (original stock obtained from Zeneca Plc, Dorset, UK) using the procedure of Enna and Snyder (12). The animals were housed in cages measuring 33 × 15 × 13 cm, in a facility maintained at 18–22°C with a 12 L:12 D cycle. Food and water were available ad lib. Measurements were made for both males and females for postnatal days 1 to 30. To obtain enough synaptosomal material, it was necessary to combine 10 brains for the samples at 1 to 6 days. Five mouse brains were used for the samples at days 8, 10, and 15, with three used for days 20, 25, and 30.

Biochemical Analysis

The protein content of each preparation was measured using the method of Bradford (5), and expressed per gram of brain wet weight. The membrane lipid was isolated using a Folch wash (13), and the total phospholipid measured using the method of Bartlett (3). The SPM cholesterol composition was measured using a colorimetric assay obtained from the Sigma Chemical Company, Dorset, UK. Fatty acid analysis of the membranes was performed using gas liquid chromatography, following preparation of fatty acid methyl esters.

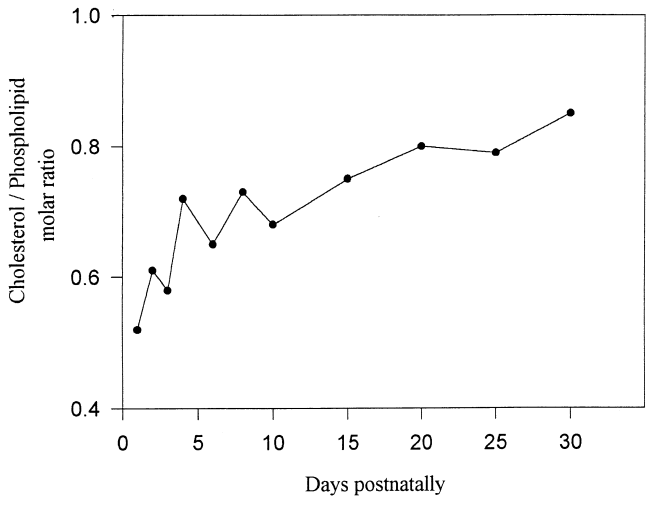

FIG. 1. Changes over development in the cholesterol to phospholipid molar ratio of synaptosomal plasma membranes isolated from mouse brain. The data presented are the average of four readings with standard deviations from the mean indicated.

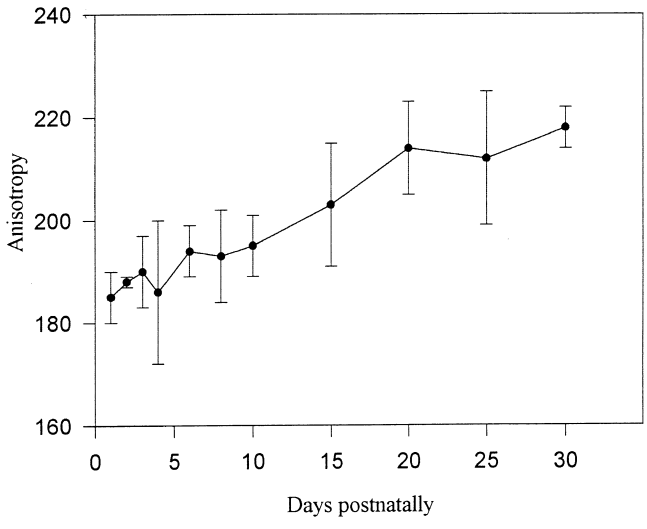

FIG. 2. Changes over development in the fluorescence anisotropy of synaptosomal plasma membranes isolated from mouse brains. The data presented are the average of four readings with standard deviations from the mean indicated.

TABLE 2

THE FATTY ACID COMPOSITION OF SYNAPTOSOMAL PLASMA MEMBRANES ISOLATED FROM THE BRAINS OF DEVELOPING MICE

Fatty acid	Postnatal Day								
	3	4	6	8	10	15	20	25	30
16:0	24	22	13	11	12	8	6	4	8
18:0	12	8	13	11	9	12	15	17	23
18:1	4	5	11	13	18	16	20	23	20
18:2	6	7	6	7	1	2	3	1	2
18:3	7	9	12	4	9	4	1	2	1
20:4	9	13	13	18	11	15	13	13	10
22:4	19	20	10	16	22	19	12	10	12
22:6	10	8	12	10	14	16	23	22	19

The data are the mean of four independent measurements and are expressed as the percent of the total comprised by each fatty acid.

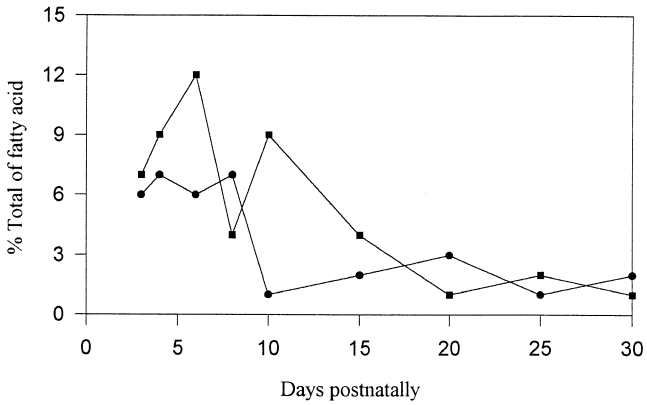

FIG. 4. Changes in the percentage composition of 18:2 (●) and 18:3 (■) in synaptosomal plasma membranes from mouse brains. The data presented are the average of four readings.

Lipid Fluidity Measurements

The lipid fluidity of each SPM preparation was measured using the technique of steady-state fluorescence polarization (32). A fluorescent probe, 1,6-diphenyl-1,3,5 hexatriene (DPH) obtained from the Aldrich Chemical Company, Dorset, UK, was dissolved in tetrahydrofuran (THF) and added to each membrane suspension to give a molar ratio of 1 DPH molecule to 500 molecules of membrane lipid. The final concentration of THF did not exceed 0.01% (v/v), and had no effect on the fluorescence anisotropy. The mixture was incubated for 30 min in the dark to allow the intercalation of the DPH into the lipid bilayer. The probe was exposed to polarized light using an excitation wavelength of 360 nm. The emission intensities were measured both parallel and perpendicular to the plane of the polarized beam (I_{VV} and I_{VH}, respectively) at a wavelength of 430 nm. All fluorescence measurements were performed using a Perkin–Elmer LS5B luminescence spectrometer fitted with a polarization accessory.

The fluorescence anisotropy (*r)* of the lipid bilayer is given by:

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$

When a sample is exposed to horizontally polarized light, the emission components I_{HV} and I_{HH} are theoretically equal. However, the polarization response of the instrument causes this ratio to deviate and a wavelength correction factor (*G)* has to be introduced. This involves exciting the sample with horizontally polarized light and measuring the vertical and horizontal components. *G* is given by:

$$G = \frac{I_{HV}}{I_{HH}}$$

The emission measured horizontally to the vertically exciting light is then multiplied by this correction factor.

Statistics

Statistical analysis was performed using the SPSS for Windows software package. Correlations between parameters were determined in a nonparametric analysis by use of the Spearman Correlation Coefficient using a two-tailed analysis.

RESULTS

There were no significant differences in the biochemical compositions or in the lipid fluidity of SPM between male and

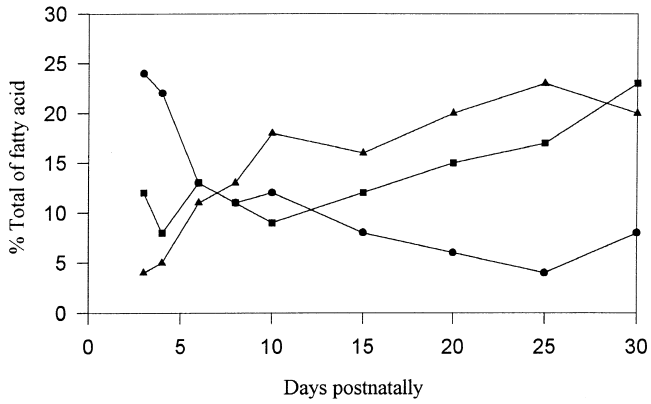

FIG. 3. Developmental changes in the percentage composition of 16:0, (●), 18:0 (■), and 18:1 (▲) in synaptosomal plasma membranes isolated from mouse brains. Values are the average of four readings.

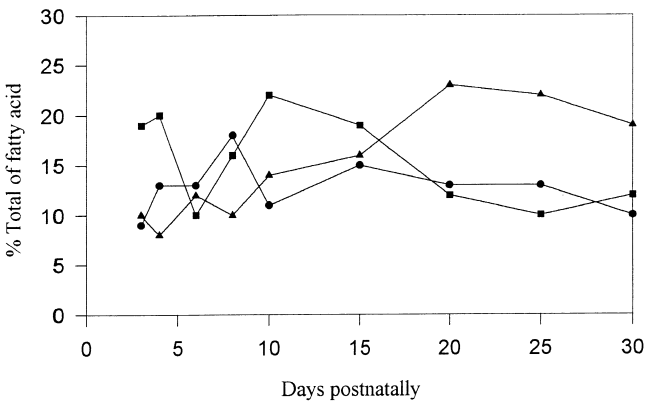

FIG. 5. Percentage compositional changes in the synaptosomal plasma membrane content of 20:4 (●), 22:4 (■), and 22:6 (▲). Values are the average of four readings.

female animals, and so the data from two independent male and female experiments were combined and shown in Table 1. The total protein content of the SPM (in mg per gram wet weight of brain) increased with development, rising sharply until day 10, followed by a steady increase in content until day 30 (correlation coefficient 1, significance <0.001). The cholesterol content, expressed as μm per mg of protein, also increased with the days postnatal (correlation coefficient 0.6364, significance 0.035). Increases were also seen in the phospholipid content, but this was less significant (correlation coefficient 0.3, significance 0.37). However, calculation of the cholesterol-to-phospholipid molar ratio, which is one of the major determinants of lipid fluidity, showed a steady increase until day 30 (correlation coefficient 0.9364, significance <0.001). The fluorescence anisotropy of the SPM increased over maturation (correlation coefficient 0.9545, significance <0.001), indicating a decrease in lipid fluidity, in keeping with the increase in the ratio of cholesterol to phospholipid (Figs. 1 and 2).

The changes in the fatty acid composition of the SPM from the four data sets were combined, and the mean percentage total of each fatty acid are shown in Table 2. The membrane composition of the saturated fatty acid, palmitic acid (16:0) decreased over development (correlation coefficient -0.9128, significance 0.001), while stearic acid (18:0) increased (correlation coefficient 0.7133, significance 0.032). The percentage composition of the unsaturated fatty acids oleic (18:1) and 22:6 increased (correlation coefficient 0.954, significance <0.001 and correlation coefficient 0.8787, significance 0.002, respectively). Although both linoleic (18:2) and linolenic (18:3) acids make up only a minor percentage of the total fatty acid content, the proportion of both these compounds decreased further with the age of the animals. In the case of linoleic acid, the correlation coefficient was -0.695, with a significance of 0.038, while for linolenic acid a correlation coefficient of -0.7849 and a significance of 0.012 was observed. The changes in the proportion of 22:4 were less significant (correlation coefficient -0.4389, significance 0.237).

In general, the greater the degree of unsaturation, and the shorter the chain length of the membrane fatty acids then the greater is the expected lipid fluidity. This is because the presence of double bonds introduces kinks in the fatty acyl chains, thereby reducing the degree of interaction with the surrounding fatty acids. The increase in 18:0 and the decrease in the content of 18:2, 18:3, and 22:4 is in keeping with the observed decrease in membrane fluidity (Figs. 3–5).

DISCUSSION

The changes in both biochemical composition and fluidity of neuronal membranes over development are in agreement with previous studies using brain cell tissues and other membranes (18,20). However, no difference was observed in the SPM between male and female animals, which, given the differences in brain morphology between the species, was initially surprising. Male rodents are exposed in utero to the androgen, testosterone, and its metabolite 17β-estradiol, which is known to increase the fluidity of membranes [(7), unpublished data], whereas females at this early stage of development produce little hormone and are protected from exposure to maternal estrogens by α-fetoprotein (19). One might, therefore, have expected male SPM to have a greater membrane fluidity than females due to estrogenic exposure, although the biochemical composition would be unchanged. However, this was not found to be the case. One possible explanation for the lack of difference between males and females may be that the fluidity of membranes is highly regulated because, during the early stages of sexual dimorphism, in particular, fluid membranes are required for neuronal differentiation (14,15). Following synaptic maturation, more ordered membranes are needed for the regulation of synaptic activities (16). Alternatively, if the steroid hormones act nongenomically via changes in the membrane environment of target tissues, induced fluidity changes would occur almost instantaneously following hormone exposure and have a short latency of effect.

Steroid hormones induce changes in lipid fluidity and alter both protein mobility and activity. These changes are more evident in fluid membranes with a low cholesterol content, (unpublished data). The presence of cholesterol seems to inhibit the intercalation of the steroid molecules into the lipid bilayer, resulting in more binding to the external membrane surface. If changes in membrane fluidity induced by steroid hormones contribute to their effects on sexual differentiation, these alterations will be more evident at the start of the critical perinatal period of development when the neuronal membranes are most fluid. Sexual differentiation is particularly vulnerable to disruption by environmental substances, which can disrupt endocrine systems, changing behavior, fertility, and sexual differentiation (24). These compounds can mimic the effects of the endogenous hormones by recognizing their binding site, or block the receptors, preventing hormone binding. Chemicals can also react directly or indirectly with the hormones, altering their pattern of synthesis or changing receptor levels. It is unlikely to be coincidental that these xenobiotics have their greatest influence during the critical period of differentiation. The changing cholesterol content of neuronal membranes may also result in there being a short time when these exogenous chemicals can act to their full potential. Decreasing membrane fluidity may reduce or prevent their effects from occurring. Antiestrogens, such as tamoxifen, alter membrane fluidity on incorporation into membranes (17,33,38) and, therefore, these compounds may act via changes in the membrane environment of target tissues.

The exact mechanisms of sex steroid hormone action on sexual differentiation are still unknown. The existence and function of receptor systems during the differentiation period, and the viability of synaptic and neurotransmitter systems are currently debated (16,25,37). These early hormonal effects might partially be due to changes in the lipid environment of target cell membranes, inducing changes in enzyme activity and, therefore, membrane function.

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