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INJECTABLE AND ORAL CONTRACEPTIVE STEROIDS IN RELATION TO SOME NEUROTRANSMITTERS IN THE RAT BRAIN

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Abstract—The influence of both injectable and oral contraceptive steroids on brain acetylcholine and serotonin was investigated. The effects of such steroids on some brain amino acids such as tryptophan, γ-aminobutyric acid (GABA), and glutamate (Glu) levels were determined. The steroids used in the present study were the long-acting injectable medroxyprogesterone acetate (MPA) and the orally active steroids ethinyloestradiol (EE) and norethisterone acetate (NEA). Acetylcholine content of the rat brain was not altered significantly following treatment with any of the steroids used. In contrast, all types of treatment caused a significant rise in brain serotonin levels. Tryptophan concentration was similarly increased following MPA injection, administration of NEA alone, or its combination with EE. No changes were noted, however, in brain tryptophan content following the administration of EE alone. MPA injection was found to be devoid of any effect on the balance between Glu, the excitatory amino acid, and GABA, the inhibitory one. On the other hand, all the orally active steroid regimens used significantly increased the GABA content of the rat brain. Only NEA pretreatment resulted in a significant decrease in brain Glu content. Changes in these central chemical transmitters and their possible relations to both antifertility and to the untoward effects of such contraceptive measures are discussed.

Considerable evidence suggests that the contraceptive steroids are responsible not only for the modulation of gonadotropin secretion [1–3], but also for behavior and mood [4, 5]. They may influence the secretion of the hypophyseal hormones that regulate gonadal functions through an action on the intersynaptic chemical mediators of the hypothalamus and adjacent parts. Such mediators are represented by monoamines and acetylcholine [6]. Both cholinergic systems and serotonergic fibers have been postulated to regulate the ovulatory function of the rat [7–9]. Furthermore, Fuxe *et al.* [10] reported that contraceptive drugs stimulate dopamine turnover in the hypothalamus. Altered brain monoamine metabolism as well as modified glutamate (Glu)/γ-aminobutyric acid (GABA) ratios have been claimed to be related to the behavioral side-effects of oral contraceptives [11–13]. It was thought to be of interest to study the effects of two different contraceptive preparations on the levels of acetylcholine and serotonin, as well as its amino acid precursor, tryptophan. The levels of GABA and Glu were also determined. The contraceptive steroids used were the depot medroxyprogesterone acetate (MPA) injection and the components of the oral contraceptive pill "Anovlar", i.e. ethinyloestradiol (EE) and norethisterone acetate (NEA). This study was conducted in an attempt to elucidate the possible differences in the mechanisms of action of these contraceptive measures and to correlate the incidence of mental depression of some "pill" users [14] with disturbances in the normal pattern of the Glu/GABA systems in the brain.

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MATERIALS AND METHODS

Chemicals

In this study the following drugs were used: 1-medroxyprogesterone acetate (17-α-acetoxy-6α-methyl pregn-4-ene-3,20 dione) "Depot-Provera" (The Upjohn Co., Kalamazoo, MI); and 2-ethinyloestradiol (19-norpregna-1,3,5 (10)-Trien-20-yne-3,17-diol), and 3-norethisterone acetate (17-B-acetoxy-19-norpregn-4-en-20-yn-3-one) (Schering, Kenilworth, NJ).

Treatment of animals

Virgin female albino rats weighing 120–170 g were obtained from local suppliers and were allowed food and fresh water *ad lib*. According to hormonal treatment, the rats were divided into two groups as follows.

Group A: medroxyprogesterone acetate treatment. Female rats were divided into two equal groups. The rats in one group received a single i.m. injection of 12.5 mg MPA/rat [15]. The other rats received an equivalent volume of saline and served as controls. Each animal was injected when it was in the estrous stage of its cycle. Prolonged diestrous phase was confirmed by daily vaginal smear examination throughout the treatment period. The animals were killed 30 days post-treatment to ensure optimal blood levels and maximal contraceptive effect [16, 17].

Group B: Anovlar components treatment. Female

rats were divided into four equal groups. Oral treatment was started when the animals were in the estrous stage of their cycle. The drugs were suspended in 0.5% carboxymethylcellulose and were given orally for 12 consecutive days. Group I received a daily dose of 0.05 mg EE per rat; Group II received a daily dose of 1 mg NEA per rat; Group III received a daily dose of 0.05 mg EE plus 1 mg NEA per rat; and Group IV served as control and received an equivalent volume of 0.5% carboxymethylcellulose daily for 12 consecutive days. The rats were killed 24 hr after the last dose. The dosage and duration of treatment with the oral steroids ensured a maximal contraceptive effect [18, 19]. Throughout the treatment period, the estrous phase was confirmed for rats in Group I. Smears from rats in Groups II and III were atypical. At autopsy they were composed of varying numbers of squamous and nucleated cells together with moderate numbers of leucocytes in 50 per cent of the smears.

Estimation of acetylcholine content of the whole rat brain

Brains were quickly removed at 2–4° and weighed, and acetylcholine was extracted according to the method of Richter and Crossland [20], using acetate buffer, pH 4, and eserinized acidified Ringer solution. The acetylcholine was assayed biologically using the rabbit fundal strip as described by Khayyal *et al.* [21].

Estimation of serotonin content of the whole rat brain

Brain serotonin was extracted according to the method described by Amin *et al.* [22]. It was assayed biologically on the rat fundal strip according to the method described by Vane [23].

Estimation of brain tryptophan level

Brains were quickly removed at 2–4°, weighed and homogenized in 7 vol. of ethanol (75%), and centrifuged. The clear supernatant fluid was evaporated to dryness in a water bath. After cooling, the residue was dissolved in distilled water and centrifuged. An amount of the clear supernatant fluid equivalent to 250 mg of the original wet brain tissue was used for estimation of tryptophan spectrofluorometrically according to the method described by Hess and Udenfriend [24]. The fluorescence was measured in an Aminco–Bowman spectrophotofluorometer with an activation wavelength of 365 nm and an emission wavelength of 440 nm. The amount of tryptophan present was then calculated using a predetermined standard curve.

Estimation of GABA and glutamate in the whole rat brain

The method used was a modification of the chromatographic procedures described by Maynert *et al.* [25] and Pepeu *et al.* [26]. Protein-free supernatant fluid was applied to Whatman No. 1 filter paper. The chromatogram was run by the ascending technique for 40 hr using a mixture of *n*-butanol–acetic acid–water (4:1:4) as a solvent. The optical density was measured at a wavelength of 570 mU in a Shimadzu double beam spectrophotometer u.v.-200 S. The amount of the amino acids present in each spot was then calculated using a predetermined standard curve.

Statistical analysis

The results were analyzed by Student's *t*-test. Comparison of the means with and without treatment was by the unpaired *t*-test.

RESULTS AND DISCUSSION

Central neurotransmitters, including acetylcholine and serotonin, have been found to influence the secretion of the hypothalamic-releasing factors that control the release of pituitary gonadotropins [7]. Acetylcholine stimulates the release of FSH-releasing factor and LH-releasing factor *in vitro* and *in vivo* [27, 28]. It had been claimed earlier that the metabolism of the central neurotransmitters was altered by changes in the normal balance of gonadal hormones that were reported to occur during the estrous cycle, pregnancy and castration [29, 30]. In our experiments the acetylcholine content of the rat brain was not altered significantly following treatment with any of the steroid regimens adopted (Tables 1 and 2). Similarly, other authors hold the view that Lyndiol (lynestrenol, mestranol) treatment has no effect on either the acetylcholine level or on choline acetyltransferase, its synthesizing enzyme, in various brain areas [6].

A very interesting observation was the marked

Table 1. Rat brain levels of acetylcholine, serotonin, tryptophan, γ-aminobutyric acid (GABA) and glutamate (Glu) following a single i.m. injection of medroxyprogesterone acetate (12.5 mg/rat)*

	Acetylcholine	Serotonin	Tryptophan	GABA	Glu	Glu:GABA ratio
	(μmoles/100 g wet brain tissue)					
Control, diestrous	1.69 ± 0.11 (6)	0.23 ± 0.01 (10)	1.93 ± 0.11 (9)	182.5 ± 8.7 (8)	788.4 ± 23.8 (8)	4.32 ± 0.21 (9)
Treated	1.85 ± 0.12 (6)	0.47 ± 0.02 (10)	2.46 ± 0.10 (9)	163.1 ± 4.8 (8)	753.1 ± 20.4 (9)	4.64 ± 0.19 (8)
% Change from control	+10	+104	+28	−10.9	−4.4	+7.4
P	> 0.05	< 0.001	< 0.01	> 0.05	> 0.05	> 0.05

* Results are means ± S.E.M. Rats were killed 30 days after injection. Values in parentheses indicate the number of rats. P values indicate the level of significance of the difference between control and treated animals.

Table 2. Rat brain level of acetylcholine following treatment with ethinyloestradiol (EE), norethisterone acetate (NEA) and their combination*

Drug	Dose ($mg \cdot rat^{-1} \cdot day^{-1}$)	Acetylcholine	
		μmoles/100 g wet brain	% Increase from control
Control, estrous (6)		1.88 ± 0.12	
EE (6)	0.05	2.03 ± 0.11†	18
NEA (6)	1.00	2.01 ± 0.07†	7
EE + NEA (6)	0.05 + 1.00	2.00 ± 0.07†	6

* Results are means ± S.E.M. Treatment was continued for 12 consecutive days. Values in parentheses indicate the numbers of animals used.
† Difference from control value was statistically insignificant, $P > 0.05$.

influence of all steroids tested on the levels of brain serotonin and its amino acid precursor tryptophan. All types of treatments caused a significant increase in serotonin up to 104 per cent following MPA treatment (Table 1). Changes following the orally administered steroids were of a lesser magnitude, the increase being 50 per cent of the control value following EE plus NEA administration (Table 3). These results are compatible with those reported by Baker *et al.* [31]. A combination of EE and NEA increased brain serotonin level in mice. No changes were reported however, following the administration of NEA alone. The ineffectiveness of NEA in changing brain serotonin in their study may have been due to the comparatively low doses used. The rise in brain serotonin could be secondary to changes in tryptophan, the substrate, in the tissue. Data presented in Tables 1 and 3 show that MPA injection and orally administered NEA alone, or in combination with EE, caused marked increases in tryptophan levels in the rat brain, amounting to 28, 34 and 37 per cent of the corresponding control values respectively. On the other hand, the estrogenic component of Anovlar (EE), although it affected serotonin concentration similarly, did not modify tryptophan levels significantly. This indicates that the rise in serotonin concentration that was observed was not dependent solely on an increased concentration of tryptophan in the brain but, in fact, could be attributed also to inhibition of the uptake mechanism [32] and/or decreased enzymatic inactivation of serotonin. Estradiol, alone and in combination with norgestrel, was reported to inhibit MAO enzyme activity [33, 34]. Moreover, sex steroids have been found to increase both serotonin turnover [35] and utilization in the rat brain [6]. Increased brain serotonin levels may augment the effect of serotonergic fibers that have been presumed to exercise an inhibitory effect on the release of LH-releasing factor [9] and consequently may be related to the contraceptive effect of both injectable and orally active steroids.

It is particularly difficult to assess whether altered brain serotonin values obtained in this study are related to the mental depression reported in some women taking the "pill" [14]. The etiology of such depression has been claimed to be due to disturbed central monoamine metabolism [12] as well as to altered brain GABA and Glu levels [13]. GABA

Table 3. Brain levels of serotonin and tryptophan after treatment with ethinyloestradiol (EE), norethisterone acetate (NEA), and their combination*

Drug and dose	Serotonin		Tryptophan	
	μmoles/100 g wet brain	% Increase	μmoles/100 g wet brain	% Increase
Control, estrous	0.24 ± 0.01 (10)		2.77 ± 0.21 (5)	
EE ($0.05\ mg \cdot rat^{-1} \cdot day^{-1}$)	0.46 ± 0.03† (10)	91	3.01 ± 0.21‡ (5)	12
NEA ($1.00\ mg \cdot rat^{-1} \cdot day^{-1}$)	0.42 ± 0.05§ (10)	75	3.72 ± 0.16§ (5)	34
EE ($0.05\ mg \cdot rat^{-1} \cdot day^{-1}$) + NEA ($1.00\ mg \cdot rat^{-1} \cdot day^{-1}$)	0.36 ± 0.01† (10)	50	3.80 ± 0.25¶ (5)	37

* Results are means ± S.E.M. Treatment was continued for 12 consecutive days. Values in parentheses indicate the numbers of animals used.
† Difference from control value was statistically significant, $P < 0.001$.
‡ Difference from control value was statistically insignificant, $P > 0.05$.
§ Difference from control value was statistically significant, $P < 0.01$.
¶ Difference from control was statistically significant, $P < 0.05$.

Table 4. Brain levels of γ-aminobutyric acid (GABA) and glutamate (Glu) following treatment with ethinyloestradiol (EE), norethisterone acetate (NEA), and their combination*

Drug and dose	GABA		Glu		Glu:GABA ratio	
	μmoles/100 g wet brain tissue	% Increase	μmoles/100 g wet brain tissue	% Decrease	Mean	% Decrease
Control, estrous	156.3 ± 5.8 (8)		739.5 ± 19.7 (9)		4.73 ± 0.20 (8)	
EE (0.05 mg·rat^{-1}·day^{-1})	190.3 ± 7.7† (7)	17.9	721.1 ± 19.0‡ (8)	2.4	3.78 ± 0.22§ (7)	20.0
NEA (1.00 mg·rat^{-1}·day^{-1})	188.3 ± 8.7§ (9)	20.5	662.7 ± 20.4§ (8)	10.4	3.51 ± 0.15§ (8)	25.7
EE (0.05 mg·rat^{-1}·day^{-1}) + NEA (1.00 mg·rat^{-1}·day^{-1})	185.4 ± 6.0† (9)	18.5	697.3 ± 6.9‡ (8)	5.7	3.76 ± 0.09† (8)	20.5

* Results are means ± S.E.M. Treatment was continued for 12 consecutive days. Values in parentheses indicate the numbers of animals used.
† Difference from control value was statistically significant, $P < 0.01$.
‡ Difference from control value was statistically insignificant, $P > 0.05$.
§ Difference from control value was statistically significant, $P < 0.05$.

and Glu levels greatly affect the excitability of the central nervous system [26, 36, 37]. The concentration of GABA has been found to be influenced by alteration of the hormonal balance in animals by thyroid administration [38], adrenalectomy [39], and ovariectomy [40]. In the present work, MPA injection caused a statistically insignificant decrease in both GABA and Glu levels (Table 1). On the other hand, treatment with EE or NEA, alone or in combination, resulted in a slight increase in the brain GABA level amounting to 17, 20 and 18 per cent of the control value respectively (Table 4). Regarding Glu levels, only NEA administration caused a significant decrease, amounting to 10.4 per cent of control values. When expressing the results in terms of Glu/GABA ratio, all orally active steroids employed in this study produced an identical, significant decrease in this ratio, amounting to about 20 per cent from control values, indicating that the steady-state concentration of the two amino acids was shifted in favor of GABA. Similar results have been found when using mestranol and lynestrenol [13]. The authors reported that the changes following such steroid treatment coincided with altered brain glutamic acid decarboxylase (GAD) and GABA-aminotransferase enzymes activities. Thus, although MPA did not affect the balance between the inhibitory and the excitatory amino acids in the rat brain, oral contraceptive steroids produced an imbalance in such equilibrium. This might explain both the incidence of mental depression in some women taking the "pill" and the failure of regimens containing MPA to produce such depression [4, 41].

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