Pharmacology Biochemistry & Behavior, Vol. 7, pp. 253–258.

Ontogeny of Biogenic Amine Systems and Modification of Indole Levels Upon Adult Sexual Behavior in the Rat

BRIAN A. GLADUE, RAYMOND R. HUMPHRYS[1], JOSEPH F. DEBOLD AND LYNWOOD G. CLEMENS

Department of Zoology, Michigan State University, East Lansing, MI 48824 USA

(Received 3 June 1977)

GLADUE, B. A., R. R. HUMPHRYS, J. F. DEBOLD AND L. G. CLEMENS. *Ontogeny of biogenic amine systems and modification of indole levels upon adult sexual behavior in the rat.* PHARMAC. BIOCHEM. BEHAV. 7(3) 253–258, 1977. – Male, female and androgenized female rats treated on Days 9, 10 and 11 after birth with either 5-HTP or saline were tested for female sexual behavior in adulthood. It was hypothesized that such pharmacological manipulations of the developing serotonergic system in which Day 12 sex differences have been reported might have an influence on the expression of lordosis behavior. No effect of 5-HTP was found in either normal or androgenized females, even though fluormetric analysis indicated a marked increase in endogenous levels of 5-HT at Day 12. However, males treated with 5-HTP has significantly higher 5-HT levels than 5-HT treated control or androgenized females. In addition, fluorometric analysis of norepinephrine, dopamine and serotonin from hypothalamus, mesencephalon and cortex was performed on male and female rats on Days 8, 10, 12 and 14 postnatally to examine the development of various transmitter systems during the early postnatal period.

Sexual differentiation 5-HTP Serotonin Norepinephrine Dopamine Lordosis behavior

GONADAL steroids exert major influences upon the development of sexual behavior in the rat [5, 13, 15, 17]. The presence of aromatizable androgens during neonatal development in the rat has been shown to masculinize and defeminize males and females [2, 16, 18].

During neonatal development and shortly thereafter, differences appear between male and female rats in brain levels of serotonin (5-Hydroxytryptamine, 5-HT) [7]. From Day 10 to Day 12 of neonatal life (Day 1 being the day of birth) female rat pups exhibit significantly higher whole brain 5-HT levels than do males [7,10]. This sexual dimorphism appears to be hormonally rather than genetically determined. Males castrated at birth show brain serotonin levels comparable to those of intact females, and females androgenized at birth by an injection of testosterone propionate (TP) have serotonin levels similar to intact males [7,10].

Further, studies in this area have shown an interaction between gonadal steroids and enzyme activities relevant to neurotransmitter function. Intact male rats have significantly higher monoamine oxidase (MAO) levels than females at Day 12 [6]. MAO is the primary degradative enzyme which participates in the regulation of 5-HT turnover in the central nervous system (CNS) [1].

Female rats appear to have greater 5-Hydroxytryptophan (5-HTP) decarboxylase activity than do males neonatally and thus synthesize more 5-HT than do males [8]. It has also been demonstrated that MAO activity in the medial pre-optic area is increased in response to testosterone replacement in adult castrate males [11].

Since the potential for adult sexual behavior in the rat is influenced by hormonal events occuring perinatally and since gonadal hormones also influence 5-HT processes it is possible that concentrations of 5HT during development might be related to the process of sexual differentiation. It has been suggested that Day 12 5-HT differences between intact male and female rats might be related to the development of sexually dimorphic patterns of copulatory behavior [7]. The present study was undertaken to ascertain the adult behavioral consequences of manipulating brain levels of 5-HT in the neonatal rat. Intact male, female and androgenized female rats were treated with the immediate precursor to serotonin biosynthesis, 5-Hydroxytrytophan (5-HTP) prior to the reported period of 5-HT sex differences (Day 12). Animals treated with 5-HTP at Days 9, 10, and 11 were tested for the display of female sexual behavior (lordosis) as adults. In other groups fluorometric assays were employed to verify the elevation of 5-HT levels by 5-HTP treatment. If the high levels of 5-HT in Day 12 females are related to adult female sexual behavior we would expect 5-HTP treated animals to show higher levels of female sexual behavior than controls.

A second objective was to expand upon earlier work describing the ontogeny of brain amines in the developing rat by examining 5-HT, norepinephrine (NE) and dopamine (DA) levels in discrete regions of the nervous system during the period of reported serotonin sex differences. These baseline data regarding the ontogeny of serotonin, nor-

[1] Neurosciences Program

epinephrine and dopamine neurotramsmitter systems were obtained at 8, 10, 12, and 14 days of age.

METHOD

Experiment 1

Animals and treatments. Female pups born to Long-Evans rats (Charles River, Mass.) impregnated in our laboratory were treated on the day of birth (Day 1) with either 100 μg testosterone propionate (TP) in 0.05 cc sesame oil (androgenized females) or 0.05 cc oil alone (control females). All animals were housed with their mothers under standard laboratory conditions with food and water ad lib (light cycle: 14 hours light: 10 hours dark, lights off at 1100 hr). Subsequently, animals in all 3 groups received subcutaneous injections of either 100 mg/kg 5-Hydroxytrytophan (5-HTP, Sigma Chemical Co.) in 0.1 cc saline or the saline vehicle alone on Days 9, 10 and 11. Animals were then weaned at 21 days of age and housed by sex and treatment groups, 4–6 animals per cage (cage dimensions: 22 × 35 × 50 cm). All animals were gonadectomized in adulthood (approximately 70–90 days of age) under ether anaesthesia and after a two-week recuperative period were treated with gonadal hormones in preparation for behavioral testing.

Additionally, a separate group of intact male, female and androgenized female rats given either 100 mg/kg 5-HTP or saline on Days 9, 10 and 11. Animals were decapitated on Day 12 between 1000 and 1200 hr, their brains removed, dissected and assayed for endogenous 5-HT as described below.

Behavioral Procedure and Hormone Treatments

Females. Females were divided into the following groups: TP-SAL (Androgenized females given saline Days 9, 10 and 11), n = 7, TP-5HTP (Androgenized females given 5-HTP on Days 9, 10 and 11), n = 8, INT-SAL (normal females given saline on Days 9, 10 and 11), n = 8, and INT-5HTP (normal females given 5-HTP on Days 9, 10 and 11), n = 8. At 90–100 days of age, each animal received daily IM injections of 0.4 μg estradiol benzoate (EB) in 0.1 cc sesame oil for 3 days followed on the fourth day by a single injection of 500 μg progesterone 4 hr prior to the onset of behavioral testing.

Testing consisted of the female being placed in a testing arena with a sexually vigorous stud male. Animals were tested for the display of the lordosis response in response to the mounting by the male. Lordosis is characterized by a deep ventral arching of the back with dorsal elevation of the head and lateral tail deflection. The behavioral response occurs only during sexual receptivity. In addition, the display of proceptive soliciting behaviors (hopping and darting) as well as refusals (turning over and backward kicking) toward the male were observed and recorded. Lordosis behavior was quantified for each animal using the lordosis quotient: LQ = frequency of lordosis in response to 10 mounts/10 mounts × 100. Scores for proceptive and refusal behavior were recorded as frequencies of occurrence during a test.

After three tests at one week intervals at the 0.4 μg EB dose, the same paradigm was repeated at two additional doses of EB: 2.0 and 10.0 μg.

Males. The procedure for testing males for female sexual behavior was somewhat different in that only one dose of estrogen was used (10.0 μg EB). Otherwise the testing procedure and hormone administration was the same for males as for females, with N = 10 for each group. Thus males were given 10.0 μg EB daily for three days followed on the fourth day by a single 500 μg injection of progesterone followed 4 hr later by the behavioral test. This testing procedure was repeated at 1 week intervals for 4 weeks.

Experiment 2

Biochemical assay procedure. Fluorometric assay for serotonin (5-HT), norepinephrine (NE) and dopamine (DA) was performed in 2 groups of animals. The first group sacrificed at Day 12 consisted of intact male, female and androgenized females treated at Days 9, 10, and 11 with either 5-HTP or Saline as described above (N = 6 samples for each group). A fluorometric assay for 5-HT was undertaken for these animals to varify serotonin increase as a result of 5-HTP pretreatment. The second assay group entailed an analysis of endogenous 5-HT, NE and DA levels in intact males and females over a six-day postnatal period (Days 8–14) around that time when sex differences in 5-HT have been reported [7,10]. Hence, these fluorometric data represent the steady state levels in normal developing male and female rats.

Tissue preparation for assay. Male and female pups born in the laboratory were sacrificed at 8, 10, 12 and 14 days of age by decapitation between 1100 and 1300 hr. Three sections of brain were dissected for analysis: hypothalamus, posteriorly defined by the mammillary bodies, anteriorly by the optic chiasm, laterally by the hypothalamic sulcus and dorsally to a depth of 2–2.5 mm; mesencephalon, posteriorly defined by the pons, anteriorly up to but not including the mammillary bodies, lateral fibers tracts and dorsal colliculi were removed. The third section of brain was a slice of parietal cortex. Dissected samples were then pooled by age, brain section and sex, weighed and frozen (−20° C) for later analysis. Approximately 3 to 4 brain sections constituted a sample.

Homogenization and extraction. Brain tissue was homogenized in 10 volumes of acidified n-butanol (Regis Chemical Co.) at 0–4° C for 3 min, then centrifuged for 5 min at 2000 rpm. Amines contained in the supernatant phase were transferred to centrifuge tubes containing acidified heptane (Regis Chemical Co.) for solvent extraction. Following centrifugation and removal of the organic layer, aliquots of the acid layer were transferred for the determination of 5-HT, NE and DA.

Fluorometric assay. Serotonin was measured fluorometrically utilizing the O-pthalaldehyde (OPT) procedure of Miller, *et al.* [12]. Norepinephrine and dopamine were also analyzed fluorometrically using the methods of Miller *et al.* [12] for norepinephrine and Chang [4] for dopamine. OPT and normal standards were obtained from the Regis Chemical Co. Following the production of fluorophores, samples were read in an Aminco-Bowman Spectrophotofluorometer. Serotonin was read at excitation wavelength of 360 mu and emission wavelength of 470 mu. NE was read at excitation 385 mu and emission 485 mu, DA was read at excitation 320 mu and emission wavelength of 370 mu.

Statistical analyses. Behavioral data were analyzed using Student's *t*-test for independent means. Fluorometric data regarding endogenous neurotransmitters were converted to μg transmitter per gm tissue (wet weight) and analyzed

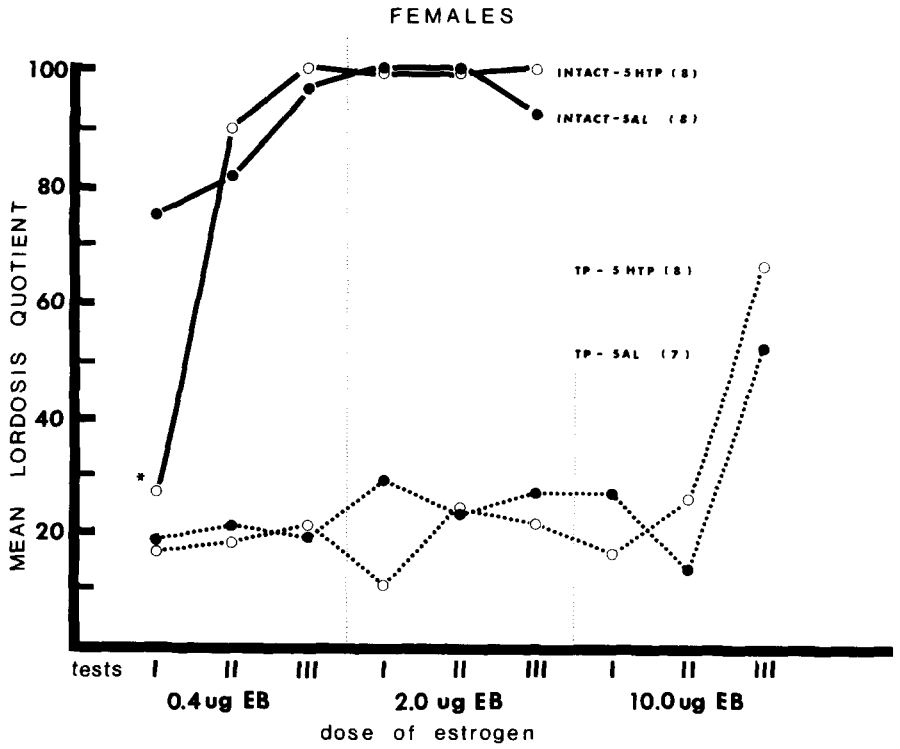

FIG. 1. Lordosis behavior in female rats in response to estrogen and progesterone in adulthood. Females treated neonatally with either oil (INTACT) or 100 μg testosterone propionate (TP). Animals were given either 5-HTP or SAL on Days 9, 10 and 11 after birth. Number of animals in each group indicated in parentheses. Numerals I, II, and III indicate week of tests at that dose of EB. *$p<0.001$.

using a multi-way ANOVA. F-tests were used for a posteriori individual comparisons [19].

RESULTS

Behavioral Testing

Females. Treatment with 5-HTP on Days 9, 10, and 11 had no overall effect on the display of lordotic behavior in either normal (INT) or androgenized (TP) females. However, there was a strong effect of a single injection of TP in decreasing the potential for females to show the lordosis posture when compared to INT females ($p<0.01$). These relationships were demonstrated over all dose ranges (Fig. 1).

Initially, control females pretreated neonatally with 5-HTP showed fewer lordosis responses at the first 0.4 μg EB test than did control females treated with saline ($p<0.001$). This difference was not evident at subsequent tests or at other doses. Control females showed such high levels of lordosis behavior at the 2.0 μg dose of EB that further testing of these females at the 10 μg EB dose seemed unwarranted.

TP-treated females failed to demonstrate any hopping and darting behavior (proceptive behavior), while control females did at all doses of EB.

Males. Control males pretreated with 5-HTP did not differ significantly from males pretreated with saline (Mean LQ ± SEM over all tests: 30.4 ± 5.1 and 29.3 ± 4.9, respectively). However, control males displayed less lordosis than control females at the 2.0 μg dose of EB. No proceptive behavior was seen during any tests of males.

Biochemical Assays

Mean serotonin levels for the saline-treated male and TP-females at Day 12 were 0.746 μg/gm and 0.740 μg/gm respectively. Mean levels of 5-HT for male and TP-females treated with 5-HTP were 3.31 and 1/94 μg/gm respectively. Thus, in male rats treated with 5-HTP, serotonin levels were elevated approximately 4–5 fold while TP-treated females experienced a 2–3 fold increase in endogenous 5-HT as a result of precursor treatment. Analysis of these data revealed that 5-HTP treated animals were significantly higher than the saline controls (t-test, $p<0.01$), and that serotonin in 5-HTP treated males was significantly higher than in 5-HTP TP-treated females (t-test, $p<0.01$).

Analysis of variance of levels of 5-HTP from animals sacrificed on either Day 8, 10, 12 or 14 of life revealed that there were significant differences by day, $F(3,141) = 14.36$, $p<0.001$, between sections, $F(1,141) = 5.82$, $p<0.01$ (Fig. 2). A posterior analyses of the means revealed that in females, 5-HT on Day 12 was significantly higher than males, $F(1,14) = 11.25$, $p<0.005$), in the hypothalamus. Furthermore, serotonin in the hypothalamus on Day 14 was found to be significantly higher than on Days 8, 10 and 12 (Day 8 vs. Day 14, $F(1,153)$ $p<0.001$; Day 10 vs. Day 14, $F(1,153) = 11.47$, $p<0.001$; Day 12 vs. Day 14,

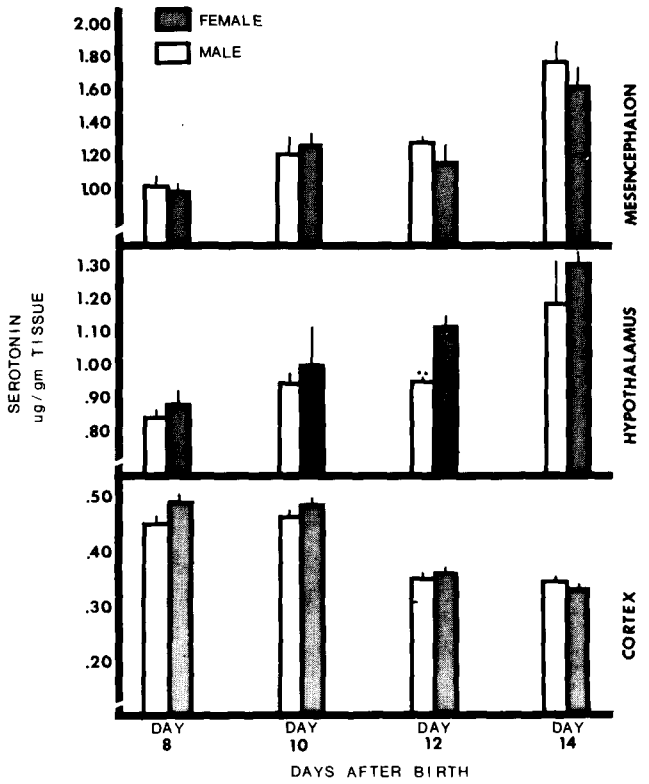

FIG. 2. Endogenous levels of serotonin (μg/gm wet weight) in various brain regions as a function of sex and postnatal age. Bars indicate mean serotonin level. Vertical lines indicate SEM. There are 8 assay samples per group. $^{**}p<0.01$.

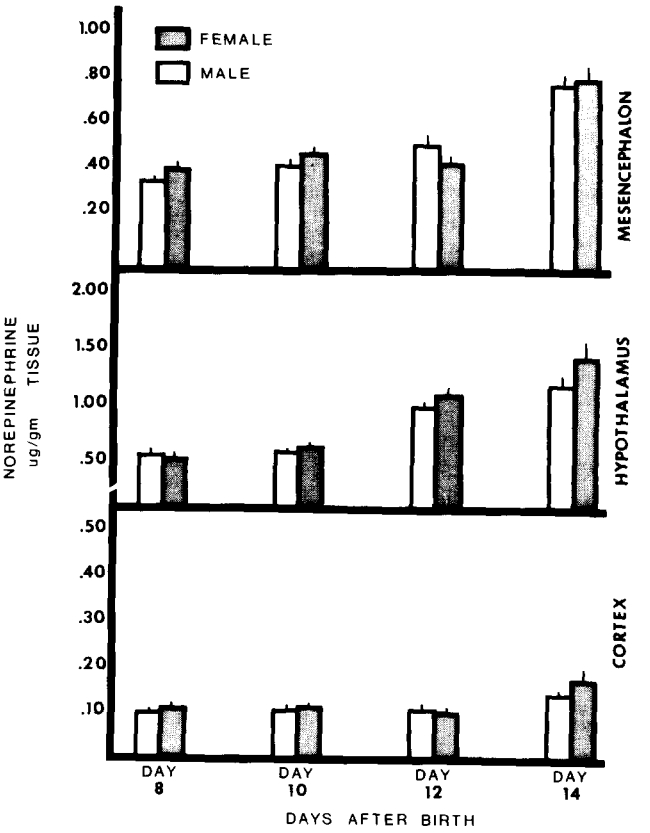

FIG. 3. Endogenous levels of norepinephrine (μg/gm wet weight) in various brain regions measured as a function of sex and postnatal age. Vertical bars represent each group. Vertical lines indicate SEM. There are 8 assay samples per group.

F(1,153) = 21.6, $p<0.001$). Mesencephalic 5-HT on Day 8 was found to be significantly lower than on Day 10, F(1,153) = 6.45, $p<0.05$. Day 10 did not differ from Day 12 in 5-HT levels. Day 14 was significantly higher than Days 8, 10 and 12, F(1,153) = 74.29, 38, 93 and 36.93 respectively with $p<0.001$ for all 3 comparisons.

Analysis of variance of steady state levels of NE revealed that there was a significant effect of section, F(2,135) = 91.26, $p<0.001$); day, F(3,135) = 11.38, $p<0.001$ but not of sex, F(1,135) = 0.062, $p<0.1$ (Fig. 3). A posteriori comparisons on the basis of NE irrespective of sex revealed that in the hypothalamus levels of NE increased as a function of postnatal life (Day 14 vs. Day 8, 10 and 12, F(1,147) = 15.04, 10.35 and 9.13 respectively, $p<0.001$ for all 3 cases). Day 10 NE was also significantly lower than Day 12, F(1,147) = 18.21, $p<0.001$. In the mesencephalon Day 14 was also significantly higher than all other days, however Days 8, 10 and 12 were not significantly different (Day 14 vs. Day 8, 10 and 12, F(1,147) = 15.04, $p<0.001$, F(1.147) = 10.35, 9.13 respectively, $p<0.001$ in all cases).

Significant effects of section, day and sex were found following analysis of variance of steady state levels for DA; F(2,132) = 22.18, $p<0.001$, F(3,132) = 37,46, $p<0.001$ and F(1,132) = 4.92, $p<0.001$ respectively. A posteriori analysis of means revealed no individual differences due to sex thus only trends were observed with females having consistently higher levels of DA (Fig. 4). In the hypothalamus, Day 14 DA was found to be significantly higher than levels on Days 8, 10 and 12; F(1,144) = 42.64, $p<0.001$; F(1,144) = 20.63 and F(1,144) = 31.38, $p<0.001$ for all cases. Mesencephalic DA on Day 8 was found to be significantly lower than on Days 10 and 12; F(1,144) = 11.76 and 4.15, $p<0.001$ and 0.05 respectively. Day 10 was found to be not significant from the levels observed on Day 12. Day 14 was found to be significantly different from Days 8, 10 and 12; F(1,144) = 52.36, 14.49, 27.12, respectively, $p<0.001$ for all cases.

DISCUSSION

The present experiment extends previous works [7,10] on endogenous levels of serotonin in intact male and female rats. A overall sex difference in serotonin levels was found at Day 12 in the hypothalamus but not in either the mesencephalon or cortex. Earlier work reported a whole brain sex difference in 5-HT at Day 12; much of this appears to result from hypothalamic 5-HT sex difference. We also found that 5-HT levels increase with time in both hypothalamus and mesencephalon but not in cortex. This is apparently a reflection of the development of the serotonergic system terminations in these areas [3,14].

Manipulation of endogenous levels of 5-HT in the neonate were without any major effect on differentiation of adult female sexual behavior. Despite fluorometric verification that 5-HTP pretreatment markedly increased endogenous levels of serotonin in Day 12 intact males, females and androgenized females, the mimicking of that

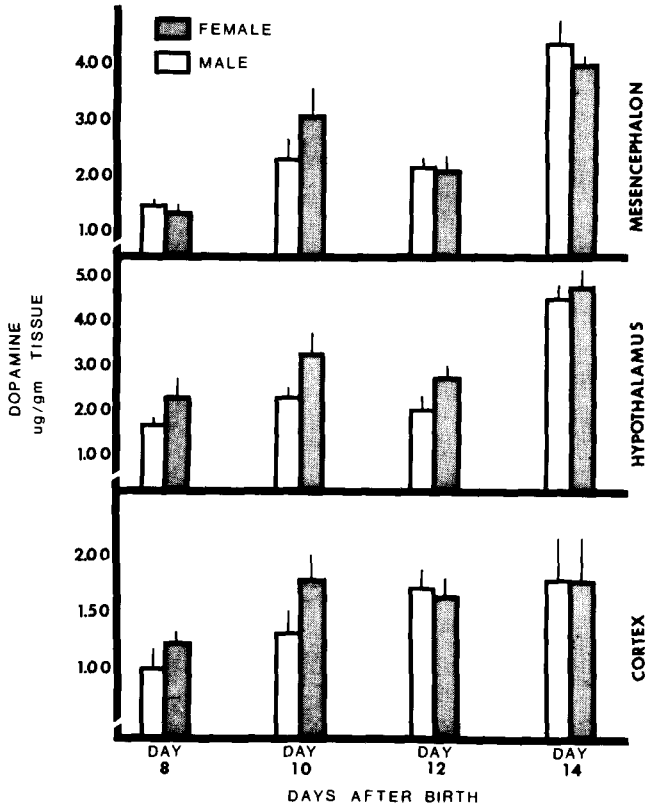

FIG. 4. Endogenous levels of dopamine in various brain regions (μg/gm wet weight) as a function of sex and postnatal age. Vertical bars indicate group means, vertical lines indicate SEM. There are 8 assay samples per group.

sex difference failed to exert any measurable influence on lordosis behavior.

Neonatal application of 6-OH-DA, a potent depletor of catecholamines to rats at 2 and 3 days of age had marked affects on NE levels in adulthood but no significant effects on measures of activity and active avoidance [9], suggesting that perturbations of neurotransmitter systems pharmacologically during development may influence the chemistry of the adult system but not necessarily adult behavior. The failure to see marked behavioral changes following changes in neurotransmitter activity may reflect considerable plasticity in the developing nervous system.

While the difference in Day 12 5-HT levels appears to be inconsequential for adult female sexual behavior, it should be noted that early hormonal exposure does influence the serotonergic system. Males treated with 5-HTP showed on Days 9, 10 and 11 higher levels of 5-HT at Day 12 than either control or androgenized females treated with 5-HTP. This sex difference may reflect a hormonally-mediated influence on the ability to process 5-HTP into the putative neurotransmitter.

In addition, results from this study demonstrate a wide range of neurotransmitter changes in the early postnatal period of development in the rat. Steady-state levels of NE in the hypothalamus doubled from Day 8 to Day 14 in both sexes, while changes in the mesencephalon over the same period were slight. However, dopamine concentrations in both hypothalamus and the mesencephalon increased drastically from Day 8 to Day 14 in both sexes. The physiological or behavioral implications of these changes are not yet clear.

The importance of brain monoamines in the development of sexually dimorphic behavior may not be reflected in alterations of a single neurotransmitter but in the balance between proposed excitatory and inhibitory transmitters [9]. Transmitters may be undergoing alterations in discrete neural loci as a result of variations of endogenous ovarian and testicular steroids. Changes in transmitter dynamics of one system may be reflected in observable alterations on other transmission system. Furthermore, such changes in neurotransmitter activity may be reflected in some sexually demorphic behavior other than that considered in this presentation.

ACKNOWLEDGEMENTS

Hormones were gifts of Schering Corporation, Bloomfield, New Jersey, USA. This work was supported by USPHS Grant No. HD-06760.

REFERENCES

1. Axelrod, J. Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. *Pharmacol. Rev.* 18: 95–113, 1966.
2. Beach, F. A. Hormonal factors controlling the differentiation development and display of copulatory behavior in the ramstergig and related species. In: *Biopsychology of Development*, edited by E. Tobach, L. Aronson and E. Shaw, New York, Academic Press, 1971, pp. 249–296.
3. Bourgoin, S., F. Artaud, J. Adrien, F. Hery, J. Glowinski and M. Hamon. 5-Hydroxytryptamine catabolism in the rat brain during ontogenesis. *J. Neurochem.* 22: 951–956, 1977.
4. Chang, C. C. A sensitive method for spectrophotofluorometric assay of catecholamines. *Int. J. Neuropharmacol.* 3: 643–649, 1964.
5. Clemens, L. G. The Neurohormonal control of masculine sexual behavior. In: *Reproductive Behavior*, edited by W. Montagna and W. Sadler, New York, Plenum Press, 1974.
6. Gaziri, L. C. J. and W. Ladosky. Monoamine oxidase variation during sexual differentiation. *Neuroendocrinology* 12: 249–256, 1973.
7. Giulian, A., L. A. Pohorecky and B. S. McEwen. Effects of gonadal steroids upon brain 5-Hydroxytryptamine levels in the neonatal rat. *Endocrinology* 3: 1329–1335, 1973.
8. Hardin, C. M. Sex differences in serotonin synthesis from 5-Hydroxytryptophan in neonatal rat brain. *Brain Res.* 59: 437–439, 1973.
9. Isaacson, R. L., W. J. Street, T. L. Petit and A. J. Dunn. Neonatal treatment with 6-OH-DA affects brain NE content but not behavior. *Physiol. Psych.* 5: 49–52, 1977.
10. Ladosky, W. and L. C. J. Gaziri. Brain serotonin and sexual differentiation of the nervous system. *Neuroendocrinology* 6: 168–174, 1970.
11. Liune, V. N., R. I. Khylchevskaya and B. S. McEwen. Effect of gonadal steroids on activities of monoamine oxidase and choline acetylase in rat brain. *Brain Res.* 293: 306, 1975.
12. Miller, F. P., R. H. Cox, W. R. Snodgrass and R. P. Maickel. Comparative effects of p-chlorophenylalanine, p-chloroamphetamone and p-chloro-N-methylamphetamone on rat brain norepinephrine, serotonin and 5-Hydroxyindole-3-acetic acid. *Biochem. Pharmac.* 19: 435, 1970.

13. Nadler, R. D. Differentiation of the capacity for male sexual behavior. *Hormones Behav.* **1**: 53–64, 1969.
14. Saavedra, J. M. 5-Hydroxy-L-Tryptophan decarboxylase activity: microassay and distribution in discrete rat brain nuclei. *J. Neurochem.* **26**: 585–589, 1976.
15. Ward, I. L. Differential effect of pre- and postnatal androgen on the sexual behavior of intact and spayed female rats. *Hormones Behav.* **1**: 25–36, 1968.
16. Ward, I. L. Sexual behavior differentiation: Prenatal hormonal and environmental control, In: *Sex Differences in Behavior*, edited by R. C. Friedman, R. M. Richart and R. L. Vander Wiele, New York, John Wiley and Co., 1974, pp. 3–17.
17. Whalen, R. E. Differentiation of the neural mechanisms which control gonadotropin secretion and sexual behavior. In: *Reproduction and Sexual Behavior*, edited by E. M. Diamond, Indiana University Press, Bloomington, 1968, pp. 303–340.
18. Whalen, R. E. Sexual Differentiation: Models, Methods and Mechanism, In: *Sex Differences and Behavior*, edited by R. M. Richart, New York, John Wiley and Co., 1974, pp. 467–481.
19. Winer, B. J. *Statistical principles in experimental design.* New York, McGraw-Hill, 1962.