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PRENATAL PROGRAMMING OF HEPATIC MONOAMINE OXIDASE BY 5,5-DIPHENYLHYDANTOIN

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Abstract—During ontogeny, rat liver monoamine oxidase gradually increased in activity in both sexes until the pubertal period when female activity rose 1.5- to 2.0-fold higher than male activity. Oral administration of 5,5-diphenylhydantoin (10 mg/kg body wt) to pregnant rats on days 7, 9, 12, 14 and 16 of gestation had no effect on the monoamine oxidase activity of immature male or female offspring. The enzyme activity in adult male offspring, from females prenatally treated with diphenylhydantoin, was elevated to a level similar to that of adult females. Subcutaneous injection of diphenylhydantoin (10 mg/kg body wt) for 5 days prior to death failed to induce changes in monoamine oxidase activity in either pre- or postpubertal males or females. Thus, prenatal administration of diphenylhydantoin can program changes in adult male monoamine oxidase activity. The serum levels of testosterone in the male offspring of prenatally treated females, on days 5 and 63 postpartum, were the same as those of their respective controls, demonstrating that the changes caused by diphenylhydantoin are not due to diminished levels of testosterone.

Advances in developmental biochemistry in recent years have demonstrated links between endocrine and behavioral functions [1–3]. The regulation of sex-linked hormone secretion and protein synthesis corresponding to various stages of organism growth has been identified as occurring in two prime periods: (1) the perinatal period, during which the irreversible 'organizational' actions take place and (2) during and after sexual maturation when 'activational' effects are observed [4]. Biogenic amines play a key role in the development and control of endocrine function, as neurotransmitters or as direct effectors of hormone synthesis and release [5, 6]. Since neonatal hormone exposure is critical to the subsequent sexual differentiation of many hepatic enzyme levels, it was of interest to investigate the development of an amine-metabolizing system and the 'organizational' influences to which it is subject.

One of the important steps controlling the levels of biologically active monoamines is oxidative deamination carried out by monoamine oxidase (MAO; amine:oxygen oxidoreductase [deaminating] [flavin-containing]; EC 1.4.3.4). This enzyme functions in the metabolism of monoamine neurotransmitters. MAO is recognized as existing in two forms, A and B, which have differing substrate specificities and differing susceptibilities to various types of inhibitors [7]. A second suggested role is in the inactivation of toxic amines, both endogenous and exogenous, which may cause hypertensive reactions [8, 9]. Monoamines have also been implicated in the direct inhibition or stimulation of hormone release [5, 6].

Although MAO is widely distributed through mammalian tissue, our knowledge of the development and control of MAO levels is still sparse. In several species MAO activity levels show a difference between adult males and females; however, the time course and control of this differentation have not been characterized adequately [10].

A variety of enzymes are affected by sexual differentiation during the postpubertal activational period, stimulated by the release of androgens or estrogens at puberty. Histidase (EC 4.3.1.3) [11], glutathione *S*-transferase (EC 2.5.1.13) [12], UDP glucuronyltransferase [13], ethylmorphine-*N*-demethylase [14] and several steriod-metabolizing enzymes [15, 16] also demonstrate pubertal differentiation. In many cases this differentiation can be prevented by hormonal or surgical intervention during the perinatal period. Thus, the maturation of the hypothalamic-hypophysial-gonadal axis can be altered during the critical perinatal period by either exogenous or endogenous agents. The expression of these alterations as hormone secretion or differential enzyme synthesis, for example, is not apparent until after sexual maturation. This process has been termed 'programming' or 'imprinting' [17]. Both Gustafsson *et al.* [16] and McEwen [4] have suggested mechanisms, whereby a fetal female metabolism is 'masculinized' by the release of androgen during the perinatal organizational period, which alter hypothalmic and pituitary function.

In this study, we have examined the levels of hepatic MAO at various stages in development to assess the usefulness of MAO as a marker for sexual differentiation. We also examined the effects of a prenatally administered xenobiotic, 5,5-diphenylhydantoin (DPH), on the development of enzyme activity. We used DPH because of its reported effects on the central nervous system, the probable source of a sexual programming mechanism, and its accumulation in the fetal and maternal brain [18–21]. We wished to see if the normal programming of enzyme activity could be altered prenatally in a manner similar to the alterations observed in histidase using diethylstilbestrol (DES) [22].

DPH has been widely used as an anticonvulsant and is one of a class of drugs, the hydantoins, which have been shown to be teratogens in both animals and

humans [23–26]. A number of actions have been proposed for DPH including alteration of sodium [27] and calcium [28] fluxes, decreased neurotransmitter synthesis and release [29, 30], and effects on corticosteroid metabolism [31], any of which might affect the imprinting process.

MATERIALS AND METHODS

Birth-dated Sprague–Dawley CD-stock random bred albino rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used. The animals were maintained on synthetic diets (NIH Feed-31) and water *ad lib.* Animals were housed in a controlled environment (temperature 21°; lights on 6:00 a.m. to 6:00 p.m.), weaned at 21–23 days post partum and housed four per cage. Stock 5,5-diphenylhydantoin (10 mg/ml) was prepared in 0.9% saline and administered by oral gavage on days 7, 9, 12, 14 and 16 of gestation. Control animals received saline on the same schedule.

Animals were decapitated, bled and the livers rapidly removed and placed on ice. The mitochondrial fraction was prepared from a 10% (w/v) liver homogenate in 0.1 M KH_2PO_4 (pH 7.4) containing 0.32 M sucrose and 3 mM EDTA (buffer A) in a motor-driven glass Potter–Elvehjem homogenizer equipped with a teflon pestle. The homogenate was spun at 900 *g* for 10 min, and the supernatant fraction was decanted and spun at 12,000 *g* for 10 min. The crude mitochondrial pellet was resuspended in buffer A and was stored frozen at −76° to be used later for assay (less than 5 percent loss on storage). The cytosol fraction, used for histidase and glutathione *S*-transferase enzyme assays, was prepared from a 20% (w/v) liver homogenate in 10 mM Tris (pH 7.2) containing 14 mM $MgCl_2$ and 0.6 M KCl, by centrifugation at 105,000 *g* for 60 min. The supernatant fraction was frozen for later use at −76° (no loss of histidase or glutathione *S*-transferase activity on storage). Trunk blood was collected after decapitation and allowed to clot for 30 min. at room temperature before centrifuging at 400 *g* for 15 min. The supernatant fraction was decanted and frozen at −76° until assay.

The MAO assay (based on the method of Wu and Dyck [32]) measures the amount of [1-^{14}C]tyramine which is converted to the product, [1-^{14}C]*p*-hydroxyphenylacetaldehyde, by a mitochondrial sample in the presence or absence of the MAO inhibitor clorgyline (*N*-[2,4-dichlorophenoxy-*n*-propyl]-*n*-methylpropargyline) [7]. Clorgyline inhibits MAO type A at 10^{-7} M and MAO A plus B at 10^{-4} M. Thus, incubation in 10^{-7} M clorgyline will give MAO B activity which, subtracted from the MAO A plus B figure (obtained in the absence of inhibitor), gives MAO A activity. Samples were incubated in 0.02 M KH_2PO_4 (pH 7.5) containing 3.75×10^{-4} M or 3.75×10^{-7} M clorgyline, or no addition, for the assay blank, MAO type B and MAO A plus B respectively. After addition of the sample (to give between 1.5 and 3.5 mg protein/ml of incubation mixture) and incubation for 10 min at 37°, the reaction was initiated by the addition of 1.0 mM tyramine containing [1-^{14}C]tyramine. After a further 10 min at 37°, the reaction was terminated by the addition of an aliquot of 0.1 M diethylhexylphosphoric acid in chloroform. The chloroform:water emulsion was mixed and then separated by centrifugation at 2000 *g* for 15 min, and an aliquot of the aqueous phase was added to 0.2 ml of 1 N perchloric acid in a scintillation vial. After mixing, 10 ml of scintillation fluid (6.0 g PPO and 0.075 g POPOP/l of toluene)* was added and the ^{14}C-labeled product was counted on a Beckman LS 9000 scintillation counter. The reaction is linear over the time used and with respect to the range of protein concentrations used. Units of enzyme activity are given as nmoles of product formed/mg of protein/min.

Histidase was assayed by the method of Lamartiniere and Feigelson [33] on a Gilford model 250 recording spectrophotometer maintained at 37° and using L-histidine as a substrate. Glutathione transferase was assayed using 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as substrates prepared in dimethylsulfoxide [34].

Serum gonadotrophin levels were determined by radioimmunoassay by A. F. Parlow, Harbour General Hospital, Torrance, CA, using NIAMDD standards. Follicle-stimulating hormone (FSH) is expressed as ng/ml of serum in terms of NIAMDD-Rat FSH-RP-1, thyroid-stimulating hormone (TSH) in terms of NIAMDD-Rat TSH-RP-1, and testosterone in ng/ml of serum.

Protein concentration were assayed by the method of Lowry *et al.* [35]. 5,5-Diphenylhydantoin, histidine, tyramine, chlorodinitrobenzene and dichloronitrobenzene were purchased from the Sigma Chemical Co., St. Louis, MO. Clorgyline (M and B. 9302) was a gift of May & Baker Ltd., Dagenham, England. [1-^{14}C]tyramine (58.76 mCi/m-mole) was obtained from New England Nuclear, Boston, MA. All other chemicals used were of the highest purity available.

Analysis of variance procedures were used to assess the significance of sex effects, treatment effects and sex/treatment interactions. Pairwise comparisons were made by Fisher's LSD test.

RESULTS

Ontogeny of hepatic MAO. Animals were chosen randomly from the offspring of a series of date-bred litters on days 2, 5, 14, 21, 28, 35 and 63 postpartum, and hepatic MAO was assayed. Figure 1A shows the development of total (A plus B) MAO activity up to day 63 in both male and female animals. MAO activity toward the substrate tyramine rises slowly in the liver of both males and females until day 28 at which point the activity in females rises sharply and continues to increase in the postpubertal period, leveling off by day 105 (not shown). Panels B and C of Fig. 1 illustrate the separate development of MAO types A and B. Although both forms undergo sex-related changes in activity levels, the overall increase in male MAO activity is in the A form of the enzyme during the period studied, while in the female 66 percent of the total increase in activity is due to the A form and 34 percent is due to increased B activity. Thus, the two forms of MAO appear to undergo similar but independent development during growth. The differentiation between male and female MAO activities which appears in the fifth week is seen in the changes in both body and liver weights.

* PPO = 2,5-diphenyloxazole; and POPOP = 1,4-Bis (5-phenyloxazol-2-yl)benzene.

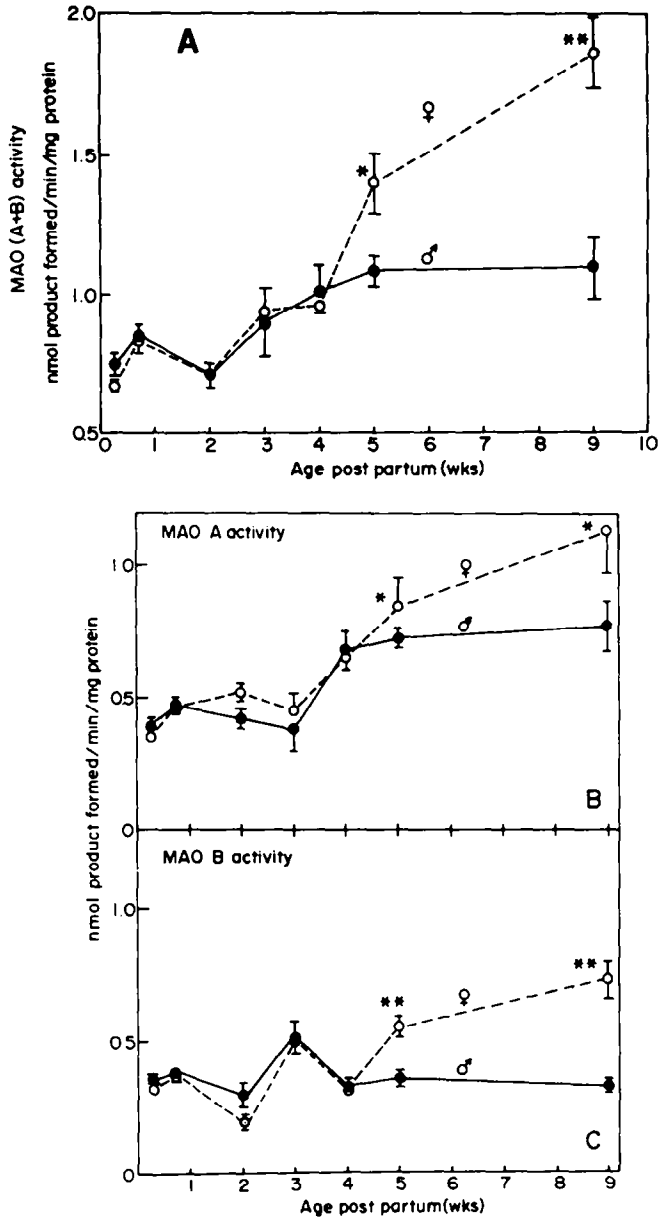

Fig. 1. Ontogeny of male and female rat liver MAO activity. Changes in male(●) and female (○) MAO activity toward tyramine between days 2 and 63 postpartum. Panel A: MAO (A + B) activity; panel B: MAO A activity; and Panel C: MAO B activity. Results are expressed as means ± S.E.M.; n = 8. The single asterisk (*) indicates $P < 0.05$, and the double asterisk (**) $P < 0.01$.

Prenatal DPH effects. DPH was administered by oral gavage in 0.9% saline at a concentration of 10 mg/kg body wt to random-bred pregnant females on days 7, 9, 12, 14 and 16 of gestation. After birth, littermates (referred to as 'DPH male' or 'DPH female' hereafter) were killed on days 5, 21 and 63. Hepatic MAO activities of the offspring are given in Fig. 2. There are no significant differences in MAO activity between males and females on days 5 and 21 in either control or DPH animals. By day 63, however, sexual differentiation has taken place, and control females show significantly higher activity than control males. DPH females show no changes compared to female controls, but in the DPH males MAO activity is increased 56 percent over the normal male. At 100 days postpartum, the DPH male still shows a 50 percent greater level of MAO activity compared to the normal male. Thus, prenatal DPH administration has changed the level of hepatic MAO in the adult male to the adult female level. The proportions of the A and B forms in the 5- and 21-day-old animals follow the developmental pattern illustrated in Fig. 1. The majority of the activity increase in the 63-day-old DPH male is the A form (76 percent) (Fig. 3). Neither body nor liver weights show any significant differences between control and treated animals at any of these times. The same treatment was repeated using a 3 mg/kg body wt dose, but no significant changes were observed between control and

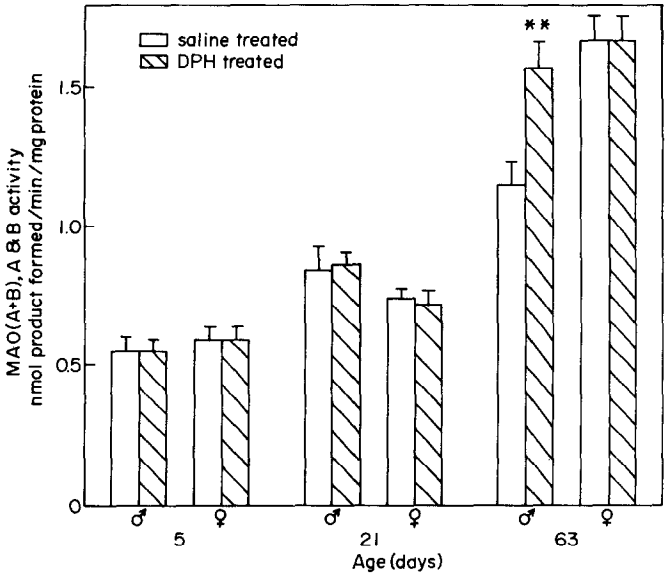

Fig. 2. Effects of prenatally administered DPH on male and female hepatic MAO activity between days 5 and 63 postpartum. MAO activity (toward tyramine) of the male and female offspring of females treated with saline (open bars) or DPH (10 mg/kg body wt) on days 7, 9, 12, 14 and 16 of gestation (cross-hatched). MAO (A + B) activity was from animals killed on days 5, 21 and 63. Results are expressed as means ± S.E.M.; n = 8. The double asterisk (**) indicates $P < 0.01$ vs saline-treated male offspring.

treated animals at day 63. Prenatal intervention with DPH, therefore, prevents the sexual differentiation of hepatic MAO.

Hepatic cytosol samples from the same group of control and DPH animals were assayed for histidase and glutathione *S*-transferase activities on day 63. In contrast to MAO, no significant differences between control and DPH-treated animals were observed for either enzyme.

Direct effects of DPH on MAO. To assess the direct effects of DPH on hepatic MAO activity, male and female rats were injected with DPH either pre- or postpubertally and MAO was assayed after treatment. Prepubertal rats (14 days old) were given subcutaneous injections of DPH (10 mg/kg body wt) in 0.9% saline daily for 5 days. Twenty-four hr after the last treatment the animals were killed and MAO activity was measured. No differences were seen between the saline and

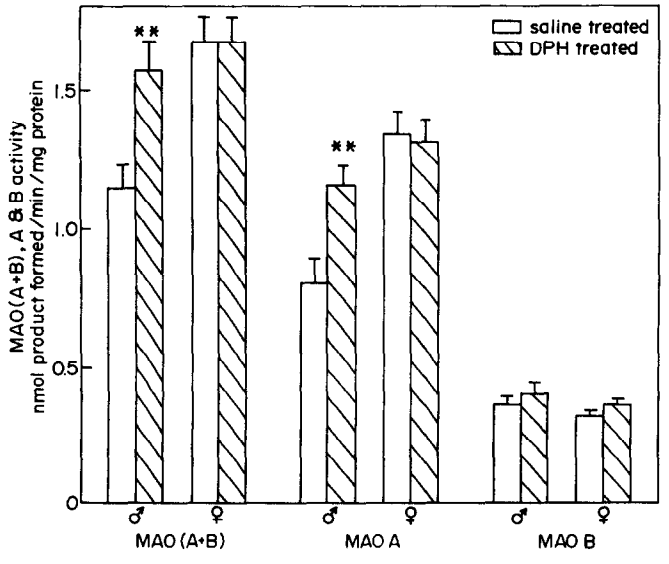

Fig. 3. Effects of prenatally administered DPH on male and female MAO (A + B), A and B activities on day 63 postpartum. MAO (A + B), A and B activities on day 63 postpartum (toward tyramine) of the male and female offspring of females treated with saline (open bars) or DPH (10 mg/kg body wt) (cross-hatched bars) on days 7, 9, 12, 14 and 16 of gestation. Results are expressed as means ± S.E.M.; n = 8. The double asterisk (**) indicates $P < 0.01$ vs saline-treated offspring.

Table 1. Serum hormone levels in the 63-day-old male rat*

	FSH (ng/ml)	TSH (ng/ml)	Testosterone (ng/ml)
Normal male	488 ± 46	578 + 77	5.33 ± 1.17
DPH male	325 ± 32†	947 ± 207	5.62 ± 1.45
Normal female	174 ± 27‡		

* Serum levels of FSH, TSH and testosterone at 63 days in normal males and females, and the male offspring of females treated with DPH (10 mg/kg body wt) during gestation. Units are ng/ml of serum. Results are expressed as the means ± S.E.M.; n = 5.

† $P < 0.05$ vs normal male.

‡ $P < 0.01$ vs normal male.

Table 2. Serum hormone level in 5-day-old male rats*

	FSH (ng/ml)	TSH (ng/ml)	Testosterone (ng/ml)
Normal male	446	446	0.6
DPH male	429	495	0.7

* Serum levels of FSH, TSH and testosterone at 5 days after birth in normal males and the male offspring of females treated with DPH (10 mg/kg body wt) during gestation. Units are ng/ml of serum. Samples assayed were pooled serum from eight animals in each category.

DPH-treated animals. In postpubertal animals treated in a similar manner, no changes could be seen between saline and DPH-treated animals. DPH is unable, therefore, to induce changes in MAO activity.

Serum hormone levels. Table 1 shows the serum levels of FSH, TSH and testosterone in normal males, females and DPH males at 63 days. Serum FSH levels are decreased toward the normal female level, TSH is elevated, and the testosterone level remains unaltered in the DPH male.

Table 2 shows the serum levels of FSH, TSH and testosterone in normal and DPH males at 5 days postpartum. There are no differences in the levels of any of these hormones between normal and DPH males at this age.

DISCUSSION

The ontogeny of rat liver MAO determined from our experiments shows great similarities with the developmental pattern of other enzymes which show differentiation after sexual maturation. There is a gradual rise in the MAO activity level in both male and female animals until the pubertal period when the female activity rises sharply to level off at 1.5 to 2.0-fold higher than the male. In both sexes the MAO type A:B ratio is maintained at 50:50 from birth until the fifth week postpartum when the ratio changes to approximately 60:40 for both sexes. While neonatal testicular androgens have been recognized as one of the tools by which development is irreversibly programmed for expression of male metabolism after pubertal activation [4, 15, 16], there is evidence that prenatal intervention can also be effective [32]. Thus, the critical organizational period for programming may occur during gestation for some enzymes. Most experiments altering enzymic programming have used deprivation or administration of hormones (including synthetics such as DES) which have defined target molecules and in some cases target cells or tissues. We have shown here that DPH, which has no reported affinity for hormone receptors, can alter the sexual differentiation of MAO when administered prenatally. DPH does perturb thyroid metabolism [36], and while it does have some affinity for thyroxine-binding globulin, the binding constant is very low compared to T_4 [37]. DPH also seems to have little effect on nuclear T_4 and T_3 binding in human lymphocytes when administered at therapeutic dose levels [38]. Given the inter-tissue similarity of hormone receptors, DPH is unlikely to affect nuclear binding elsewhere.

Prenatal administration of DPH (at two to three times the human therapeutic dose level) to pregnant rats resulted in a change of the adult male hepatic MAO level from 61 to 95 percent of the adult control female, but histidase and glutathione *S*-transferase were unaffected. Ethylmorphine-*N*-demethylase, which is induced by DPH and can be programmed by testosterone [14, 39] is also unaffected.* These experiments, therefore, not only reinforce the extension of a critical organizational period into mid-gestation, but also point to the existence of programming mechanisms capable of selective alteration by exogenous agents. Thus, both the fetus and the neonate are sensitive to agents which cause subtle and selective effects not detectable until after sexual maturity.

Several reports have shown that neonatal deprivation of androgen causes enzyme activities to develop toward the normal female level rather than the normal male level [14–16]. The effect caused by DPH is not due to diminished serum levels of testosterone, because assays show similar levels in the normal and DPH male at both 5 and 63 days after birth. The decreased FSH level in the DPH male is another indicator that an androgen deprivation mechanism is not responsible for the elevations of MAO activities since FSH levels have been shown to increase rather than decrease after neonatal castration [40]. We believe that DPH may interfere with the perinatal androgenic programming that we have observed for MAO.† This may be either in the form of alterations in pituitary or tissue hormone responsiveness or by alterations in a central regulatory locus such as the hypothalamus.

One of the more specific implications for the alteration of sexual differentiation of MAO activity involves the role of genetic factors in the cause or susceptibility to psychiatric disorders. There is substantial evidence that suggests a genetic etiology for schizophrenia [41], for certain affective disorders [42], and for certain personality disorders (e.g. sociopathy; [43]). Many of the biochemical theories which address the problems of psychiatric disorders assign a central role to alterations in the neuronal catecholamine systems [44, 45]. The ability of exogenous agents, encountered during gestation, to change irreversibly the level of MAO which is involved in the maintenance of steady state levels of neurotransmitters, suggests the need for caution and further investigation of data assigning a genetic role in the etiology of mental illness.

* Dr. E. Lui, personal communication.

† N. P. Illsley and C. A. Lamartiniere, manuscript in preparation.

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