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Perinatal exposure to methadone and the development of hepatic microsomal testosterone hydroxylation and methadone N-demethylation in the rat

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Methadone, a synthetic analgesic, is routinely used for the detoxification and maintenance of narcotic addicts, many of whom are women of childbearing age. Babies of methadone-addicted women frequently undergo withdrawal shortly after birth [1, 2]. Additionally, a variety of behavioral, neurological, and developmental abnormalities have been observed in methadone-exposed newborns, infants, and children [1–3]. Maternally ingested methadone crosses the placenta [1, 4] and can be measured in fetal tissues [4], as well as in breast milk [2] and neonatal tissues [5].

Many adverse effects of methadone exposure on perinatal outcome, growth, and development in humans have been substantiated in the rat [6–9]. The duration and timing of exposure to methadone are critical in determining both the magnitude and duration of effects on the offspring [7, 9].

In the rat, hepatic mixed-function oxygenase (MFO) activities toward certain substrates are higher in adult males than in adult females or immature animals of either sex [10, 11]. Although this sex difference is not manifested until puberty, the adult male pattern of hepatic MFO activity is programmed, or imprinted, during the perinatal period by exposure to androgen [11, 12].

Perinatal exposure to methadone has been shown to alter the subsequent development of hepatic MFO activity in both young [13] and adult [14] rats. Since methadone decreases the serum concentration of testosterone in adult male rats [15], male rat fetuses [16], and neonates [14], changes in adult MFO activity may be mediated through an effect of methadone on the imprinting process.

The present investigation utilized the rat model to test the hypothesis that perinatal exposure to methadone alters the normal development of the hepatic MFO system. The ability of male and female offspring of methadone-treated dams to metabolize drug and steroid substrates *in vitro* was studied during a critical period in development of hepatic MFO activity (10–26 days of age) and again during adulthood (90 days of age).

Materials and methods

Chemicals. HEPES (*N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid), glucose-6-phosphate (disodium salt), NADP (sodium salt), glucose-6-phosphate dehydrogenase, and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO). Methadone for injection (dolophine hydrochloride) was purchased, and methadone hydrochloride in powder form was a gift, from Eli Lilly & Co. (Indianapolis, IN). High performance liquid chromatographic solvents were purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ). 11β-Hydroxy-testosterone and 7α-hydroxy-testosterone were purchased from Steraloids, Inc. (Wilton, NH). Testosterone, 6β-hydroxy-testosterone, and 16α-hydroxy-testosterone were gifts from Dr. Judith Weisz, Division of Reproductive Biology, M. S. Hershey Medical Center. All other reagents were of analytical grade.

Animals. Male and virgin female Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were housed under constant light (7:00 a.m. to 7:00 p.m.), temperature (21 ± 1°), and humidity (50 ± 10%). Food (Diet CR RMH 3000, Agway, Inc., Syracuse, NY) and water were available *ad lib*. Females were randomly divided into two groups for daily intraperitoneal injections of either 5.0 mg/kg methadone hydrochloride or 0.9% saline (controls). In some experiments, the daily dose of methadone was increased to 7.5 mg/kg on day 10 of gestation. One week after beginning drug administration, females were placed with untreated males. Presence of sperm in vaginal smears was considered indicative of pregnancy, and that day was designated as day 0 of gestation.

Within 18 hr after birth, before the daily injection of methadone or saline, litters were weighed, culled to eight pups (equal sex distribution when possible) and cross-fostered to achieve any or all of the following treatment groups: (1) control (C)—pups from saline-injected mothers cross-fostered to saline-injected mothers, (2) prenatally exposed (5.0 pre or 7.5 pre)—pups from methadone-injected mothers cross-fostered to saline-injected mothers, (3) postnatally exposed (post)—pups from saline-injected mothers cross-fostered to 5.0 mg/kg methadone-injected mothers, and (4) prenatally/postnatally exposed (pre/post)—pups from 5.0 mg/kg methadone-injected mothers cross-fostered to 5.0 mg/kg methadone-injected mothers. In experiments with postnatally exposed animals, methadone mothers were injected throughout lactation. No dams received 7.5 mg/kg methadone after parturition. At no time during gestation or lactation was the treatment of the mothers changed. All pups were weaned at 21 days of age.

Tissue preparation. Immature rats were killed by decapitation. Livers were pooled from entire litters (eight pups) or separated by sex (two to four per group). Entire litters were pooled only when the parameters to be studied had been shown previously to be sex independent. Non-littermate adult rats were killed by cervical dislocation, and their livers were perfused with cold 0.1 M potassium phosphate buffer, pH 7.4, before excision.

Liver microsomes were prepared by differential centrifugation as previously described [17]. Microsomal pellets were stored in glycerol (20%):0.01 M HEPES, pH 7.35, until day of assay. Storage of microsomes at −70° for up to 8 weeks did not alter enzymatic activity.*

Enzyme activity determinations. Microsomal protein concentration was measured using the biuret method [18] with bovine serum albumin as the standard. Cytochrome P-450 content was measured in microsomal suspensions with an Amino DW-2 spectrophotometer in the split beam mode [17].

Microsomal methadone *N*-demethylase activity was determined by incubating microsomal protein with various concentrations of methadone in the presence of an NADPH-regenerating system [17]. Formaldehyde was measured according to the colorimetric method of Nash [19]. Apparent Michaelis constants, K_m and V_{max}, were obtained from a least squares fit of the data [20].

Microsomal hydroxylation of testosterone was determined in incubations that contained: testosterone, 120 μM; glucose-6-phosphate, 7.4 mM; NADP, 0.35 mM; glucose-6-phosphate dehydrogenase, 0.54 units/ml; $MgCl_2$, 4.0 mM; HEPES buffer, pH 7.55, 0.1 M; and microsomal protein, 0.5 mg/ml. Testosterone was added in methanol. The incubation mixture was preincubated at 37° for 1 min before the NADPH-regenerating system was added to initiate the reaction. The reaction proceeded at 37° for 8 min

* B. H. Dvorchik, unpublished observations.

and was terminated with perchloric acid and placing the flasks on ice. Blanks contained substrate and microsomal protein only. Linearity with respect to time and protein concentration was assured in preliminary experiments.

Samples were prepared for chromatography as follows. Internal standard, 11β-hydroxy-testosterone, was added to all samples, blanks, and microsomes spiked with calibration standards (7α-, 6β-, and 16α-hydroxy-testosterone). The steroids were extracted into methylene chloride which was washed with water and then evaporated to dryness under nitrogen. The residue was dissolved in methanol for injection on to the HPLC.

The HPLC system consisted of an M6000-A pump with a 440 u.v. detector set at 254 nm and a model U6K injector (Waters Associates, Milford, MA). Samples were chromatographed on a Waters radial compression module with a 10 μm radial pak (C_{18}) cartridge (8 mm × 10 cm). The mobile phase was methanol–water–acetonitrile (60:39:1) at a flow rate of 2.5 ml/min. Amounts of the metabolites in samples were extrapolated from calibration curves that were constructed by plotting the peak height ratios of individual reference standards to internal standard vs the amount of standard. The lower limits of detection were 50 ng of each metabolite. The inter- and intra-assay coefficients of variance for all metabolites were less than 6.5%.

Statistical methods. Statistically significant differences due to sex, age, or treatment, or the interaction of the factors, were detected by one-, two-, or three-way analysis of variance (ANOVA) and Newman–Keuls *t* statistic [21].

Results and discussion

Exposure of female rats to methadone decreased weight gain both before mating (194 ± 2 g vs 201 ± 2 g, $P < 0.05$) and during gestation (day 20, 306 ± 5 g vs 327 ± 4 g, $P < 0.05$). In addition, methadone-exposed pups weighed less than controls at birth (6.1 ± 0.1 g vs 6.4 ± 0.1 g, $P < 0.05$). Postnatally-exposed offspring weighed less than controls throughout lactation, but all body weight deficits were gone within 2 weeks after weaning (data not shown). These results are consistent with results of other investigators using the same protocol [6, 22]. Since food consumption of methadone-treated dams does not differ from controls during gestation or lactation [23], changes in body weight are directly attributable to methadone.

The developmental patterns of hepatic microsomal methadone N-demethylation were similar ($P > 0.05$) in controls and methadone-treated groups (5.0 pre, pre/post, Fig. 1; 7.5 pre, post, data not shown). The apparent K_m for methadone N-demethylation in immature rats was unaffected by perinatal exposure to methadone as well. However, in contrast to the V_{max}, the apparent K_m did not change as a function of age (range, 0.086 to 0.131 mM). Similar developmental patterns have been reported for the N-demethylation of ethylmorphine [24] and meperidine [25].

In adult rats, sex differences were observed for both the V_{max} (Fig. 2A) and K_m (Fig. 2B) for methadone N-demethylation. Sex differences were greater in methadone-treated groups but were not statistically significant ($P > 0.05$). Adult rats of the 7.5 pretreatment group were not available for study; therefore, the presence of a dose–response relationship for these data could not be determined. However, perinatal methadone exposure clearly did not interfere with the normal imprinting of the V_{max} for methadone N-demethylation in adult males.

The regio- and stereospecific hydroxylation of testosterone has been widely used to help characterize multiple forms of cytochrome P-450 [26–28]. Accordingly, we observed distinct age- and sex-related developmental patterns for the formation of 7α-, 6β-, and 16α-hydroxy-testosterone by hepatic microsomes (Table 1). Treatment of dams with methadone during pregnancy, lactation or throughout both periods did not alter the profile of metabolite formation in the offspring ($P > 0.05$, three-way ANOVA); therefore, all values were pooled by age and sex. Whereas in immature males the predominant sites of hydroxylation were 7α- and 6β-, in adult males the 16α-position was preferentially hydroxylated. In contrast, the primary site of hydroxylation in females of all ages studied was the 7α-position. Testosterone 6β-hydroxylation was also active in immature females, but this activity, as well as 16α-hydroxylation, decreased to levels below the limits of sensitivity of the assay in adult females. The only activity

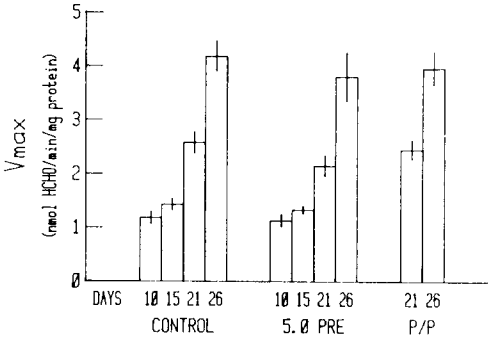

Fig. 1. V_{max} for methadone N-demethylation in livers of immature rats exposed to methadone perinatally. Values are mean ± S.E. for each age studied (days postnatally). N = 4 litters at day 10, and 8–13 at other ages. Males and females did not differ ($P > 0.05$); therefore, values were pooled. Increases in V_{max} were significant at days 21 and 26; also day 21 < 26 in all groups ($P < 0.05$, two-way ANOVA).

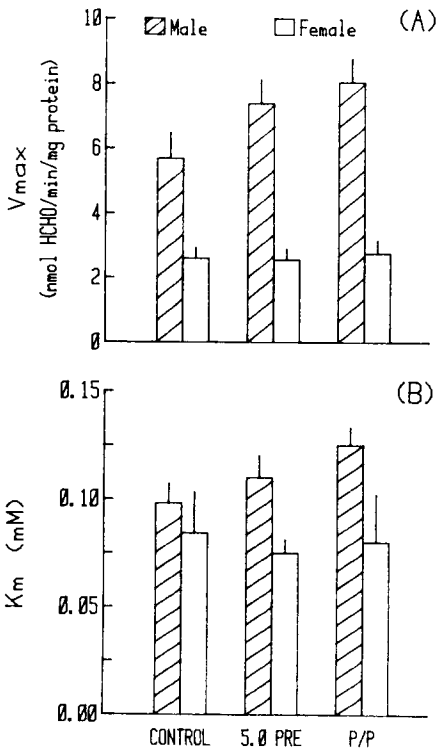

Fig. 2. Kinetic constants for methadone N-demethylation in livers of 90-day-old rats exposed to methadone perinatally. Values are mean ± S.E. N = 5 animals per group. Male values were statistically greater than female values (treatments pooled) for both V_{max} (A) and K_m (B) ($P < 0.05$, two-way ANOVA).

Table 1. Developmental pattern of testosterone hydroxylation in rat liver microsomes*,†

Age (days)	N‡	7α-Hydroxy-testosterone		6β-Hydroxy-testosterone		16α-Hydroxy-testosterone	
		Male	Female	Male	Female	Male	Female
15	13	224.4 ± 13.0	226.1 ± 15.5	192.1 ± 10.9	187.0 ± 12.4	§	§
21	34	346.5 ± 17.0	367.5 ± 22.1	176.7 ± 8.5	137.0 ± 10.1	25.7 ± 1.1	22.7 ± 1.5
26	16	316.8 ± 19.7	322.7 ± 15.2	306.2 ± 14.6	202.8 ± 13.1	40.4 ± 3.0	34.2 ± 2.6
90	11	11.0 ± 1.5	83.0 ± 5.6	43.3 ± 3.8	§	77.9 ± 5.2	§

* Perinatally exposed groups did not differ from controls or each other ($P > 0.05$, three-way ANOVA); therefore, values are mean ± S.E. of all animals studied expressed as ng product formed/min/mg microsomal protein.

† Significant differences ($P < 0.05$, three-way ANOVA) as follows: 7α-hydroxy-testosterone: (15 < 21 = 26) > 90; female > male at day 90. 6β-hydroxy-testosterone: (15 > 21 < 26) >90; male > female at all ages. 16α-hydroxy-testosterone: 21 < 26, both sexes; 26 < 90, males; male > female at all ages.

‡ Number of litters on days 15–26; number of animals of each sex on day 90.

§ Below the limits of sensitivity of the assay.

that increased between days 26 and 90 was 16α-hydroxylation, an activity known to be imprinted by neonatal testosterone [29, 30]. Normal imprinting of 16α-hydroxylation was observed in all treatment groups ($P > 0.05$, three-way ANOVA).

In the present investigation, in contrast to previous reports [13, 14], chronic exposure of developing rats to methadone did not significantly alter their ability to metabolize selected substrates *in vitro* either during the development of the hepatic MFO system (days 10–26 of age) or during adulthood. These discrepancies are most likely due to differences in experimental design, including selection of individual animals such that treatment effects cannot be separated from litter effects [13, 14, 31] and route of drug administration [14].

Although the rat model is frequently used to study the effects of methadone exposure on perinatal outcome, growth, and development [6–9], the results of the present investigation cannot be directly extrapolated to the human situation. However, these data suggest that abnormalities observed in methadone-exposed offspring may not be related to changes in the development of hepatic drug and steroid metabolism.

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Increase in serotonin$_2$ receptor density in rat cerebral cortex slices by stimulation of beta-adrenergic receptors

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Central noradrenergic and serotonergic pathways appear to modulate and balance each other. The interaction of norepinephrine and serotonin plays a role in the regulation of mood [1–3], blood pressure [4], pituitary hormone release [5], sleep patterns [6], and many other physiological and behavioral processes. Recent studies have found that the administration of β-adrenergic agonists to rats enhances behavioral responses to serotonergic stimulation [7, 8]. These serotonin-related behaviors, e.g. head twitching, resting tremor, forepaw treading, and hind-limb abduction, have been suggested to be mediated by central serotonin$_2$ (5-HT$_2$) receptors [9]. In the present study, we provide evidence for an interaction between β-adrenergic and serotonergic$_2$ receptor binding sites which may account for the serotonergic hyperactivity induced by β agonists. We report here that stimulation of β-adrenergic receptors by (-)isoproterenol in rat cerebral cortical brain slices increased the density of serotonin$_2$ receptor binding sites. Modulation of serotonin receptors by norepinephrine could represent an important component of central serotonergic and noradrenergic nervous system interaction.

Cerebral cortical slices (0.26 × 0.26 × 1–2 mm) were prepared from twelve to fifteen male Sprague–Dawley rats (150–250 g) as described previously [10] and immediately transferred to 250 ml of oxygen-saturated physiologic buffer [11] containing 0.1% (w/v) bovine serum albumin (Sigma Chemical Co.) at 37° and preincubated for 10–50 min, during which the buffer was continuously gassed with 95% O_2 and 5% CO_2. Preincubated slices were centrifuged at 200 *g* for 30 sec, the supernatant fraction was aspirated, and the slices were resuspended in 40 ml of fresh, gassed buffer at 37°. Slices were equally aliquoted into control and test groups, and each group was diluted to a total volume of 150 ml with 37° gassed buffer containing 0.5 mM sodium metabisulfite antioxidant (Sigma Chemical Co.) with or without 100 μM (−)isoproterenol (+)bitartrate (Sigma Chemical Co.). The incubation buffer was maintained at 37° in a shaking incubator and gassed (95% O_2/5% CO_2) throughout each experiment. Following the incubation, the slices were diluted 2-fold with ice-cold isotonic buffered saline and immediately homogenized using a Brinkmann Polytron. Membranes for binding assays were then prepared according to Bylund and Snyder [12], and protein concentrations were determined by the method of Lowry *et al.* [13].

Beta-adrenergic and 5-HT$_2$ receptor binding to cerebral cortical membranes (430–600 μg protein) were determined using [^{3}H]dihydroalprenolol ([^{3}H]DHA, Amersham, 50.0 Ci/mmole) and [^{3}H]spiperone (Amersham, 17.0 Ci/mmole), respectively, as described elsewhere [14, 15]. Nonspecific [^{3}H]DHA binding was determined in the presence of 5.0 μM *l*-alprenolol-*d*-tartrate (Sigma Chemical Co.), and nonspecific [^{3}H]spiperone binding was determined in the presence of 25 μM ketanserin tartrate (Janssen Pharmaceuticals).

Incubation of cerebral cortical slices with 100 μM (−)

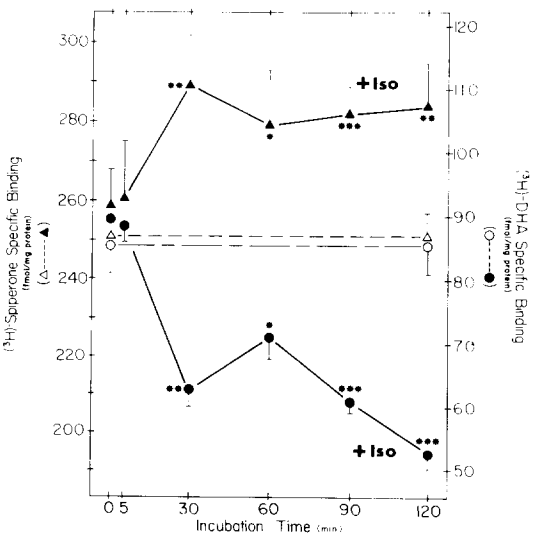

Fig. 1. Time course of isoproterenol-induced changes of β- and 5-HT$_2$-receptor binding sites in rat cerebral cortical membranes. Slices were incubated for various times at 37° with or without 100 μM (−)isoproterenol, after which membranes were prepared and assayed with a single concentration of ^{3}H-labeled ligand. Beta-adrenergic receptor binding was assayed with 1.45 nM [^{3}H]dihydroalprenolol ([^{3}H]DHA). Serotonin$_2$ receptor binding was assayed with 1.91 nM [^{3}H]spiperone. Assays were terminated by filtration through Whatman GF/B filters, and protein concentrations of 430–470 μg/ml and 430–470 μg/2 ml were used for β- and 5-HT$_2$-receptor assays respectively. Specific radioligand binding to control membranes did not vary significantly with incubation time, and the control values ± S.E.M. averaged over the entire incubation period were 85.4 ± 4.1 fmoles/mg protein for [^{3}H]DHA and 251 ± 5 fmoles/mg protein for [^{3}H]spiperone. Each point is the mean ± S.E.M. of four separate determinations for [^{3}H]DHA binding and six separate determinations for [^{3}H]spiperone binding, each determined in triplicate. The data shown are typical of results obtained in six experiments of similar design. Student's *t*-test was used to evaluate differences for significance. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$, when compared to controls.