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MATERNAL METHADONE ADMINISTRATION: DEFICIT IN DEVELOPMENT OF ALPHA-NORADRENERGIC RESPONSES IN DEVELOPING RAT BRAIN AS ASSESSED BY NOREPINEPHRINE STIMULATION OF $^{33}P_i$ INCORPORATION INTO PHOSPHOLIPIDS *IN VIVO*

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Abstract—The effects of perinatal methadone exposure on the development of noradrenergic responses in the brain were examined by assessing the ability of intracisternally administered norepinephrine to stimulate $^{33}P_i$ incorporation into phospholipids *in vivo*; the effect of norepinephrine is mediated by alpha$_1$-receptors juxtaposed to noradrenergic nerve terminals. Although there was no difference in basal (unstimulated) incorporation of $^{33}P_i$, a deficit in norepinephrine-induced stimulation of incorporation was found throughout the preweanling period in offspring of dams treated daily with methadone beginning in midgestation. This effect was not seen when methadone was given during the postnatal period. Since perinatal methadone exposure also delays development of presynaptic catecholaminergic nerve terminals in the brain, these results support the view that perinatal exposure to methadone depresses overall central noradrenergic synaptic function; however, the effects on presynaptic development and on receptor-mediated responses appear to be separable in that they display differences in the critical age periods of sensitivity to perturbation by the drug.

Perinatal methadone exposure increases the risk of infant mortality, delays growth, and interferes with development of the nervous system. In animal models, offspring of methadone-treated dams display deficits in ontogenesis of a variety of biochemical markers for presynaptic nerve terminals in the central nervous system, preceded or accompanied by distortion of patterns of cellular maturation and differentiation [1–6]. Thus, the presynaptic uptake mechanisms for brain biogenic amines develop at a slower rate in perinatally-addicted animals, as do neurotransmitter biosynthetic enzymes and synaptic storage vesicles. The effects on these biochemical markers for central synaptogenesis suggest that neuronal ontogeny is delayed by methadone exposure, an effect which appears to be generalized in that a variety of different transmitter systems are affected in a similar fashion [2, 3, 6–8]. The alterations of neuronal development are largely independent of the undernutrition associated with maternal methadone administration, and differences in both cellular maturation and in synaptic biochemical markers are prominent even when control dams are pair-fed to match the food intake of methadone-treated animals [3, 7].

The development of a variety of behavioral patterns also appears to be slowed by perinatal methadone, and the periods of drug exposure in which the fetus/neonate is at greatest risk for impaired performance resemble those for maximal effects on presynaptic nerve terminal development [2, 6–9]. This correlation notwithstanding, there has been no conclusive demonstration that methadone-induced alterations of biochemical development of presynaptic nerve terminals in the central nervous system result directly in perturbations of synaptic function. For example, delayed synaptic development could be compensated by postsynaptic supersensitivity, resulting in no net change in function; alternatively, the deficits in synaptic biochemistry may simply not be of sufficient magnitude to compromise function. Therefore, before a direct connection can be drawn between methadone-induced alterations in biochemical development of synapses and its effects on behavioral variables, it is necessary to demonstrate in some fashion that function has indeed been adversely affected in central synapses whose presynaptic biochemical development is delayed by methadone. In the current study, the reactivity of central noradrenergic synapses has been examined in perinatally-addicted animals, using a methadone dosage regimen known to affect nerve terminal development and behavior [1–9], by assessing the ability of intracisternally administered norepinephrine to stimulate $^{33}P_i$ incorporation into phospholipids, an effect mediated by alpha-noradrenergic receptors juxtaposed to noradrenergic terminals [10–13].

METHODS

Timed-pregnant Sprague–Dawley rats (Zivic Miller, Allison Park, PA) were housed individually in plastic breeding cages and given food and water *ad lib*. Perinatal exposure to methadone was produced by giving the drug to pregnant and nursing

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dams as follows: 2.5 mg/kg s.c on day 10 of gestation, 3.5 mg/kg on day 11 of gestation, and 5 mg/kg daily thereafter continued throughout the experiment, a regimen which maximizes effects on development of the nervous system without causing excessive mortality [4–6, 14–17]. Controls received equivalent volumes of saline (1 ml/kg). Postnatal exposure of pups to methadone was produced by direct injection starting at 1 day of age; the dose was 2.5 mg/kg s.c. for the first injection, 3.5 mg/kg for the second, and 5 mg/kg thereafter. This regimen was chosen because it maximizes effects on development of neurochemical and behavioral variables while minimizing maternal and neonatal mortality [1–9]. Pups from litters within each treatment group were randomized at birth and redistributed to the dams; this was repeated 24 hr before each experiment. All experiments were conducted 24 hr after the preceding maternal or postnatal methadone injection.

$^{33}P_i$ incorporation into brain phospholipids. When necessary (> 7 days of age), animals were lightly anesthetized with ether, and a small incision was made to permit the intracisternal administration of drugs and $^{33}P_i$. Animals received 2 μCi/g brain of carrier-free $^{33}P_i$ premixed either with 0.1% ascorbic acid vehicle (controls), or with a maximally effective dose [10–12] of norepinephrine (40 nmoles/g brain) in a total injection volume of 17 μl/g brain; to enable dosing per unit brain weight, weights were estimated *a priori* from the relationship between body and brain weight established in previous studies with this dosage regimen of methadone [2, 4–8], and actual brain weights obtained after killing indicated no significant deviations from predicted values. Exactly 5 min after the injection of $^{33}P_i$, the rats were decapitated and the brains were removed rapidly, weighed, and homogenized (Polytron) in 10 vol. of chloroform–methanol (1:1, v:v). After centrifugation at 5000 *g* for 15 min, an aliquot of the supernatant fraction was taken, and chloroform was added to achieve a chloroform–methanol ratio of 2:1 v:v. After mixing, 0.2 vol. of 0.73% NaCl was added and the solution was mixed vigorously and centrifuged. The aqueous layer and interface were aspirated, and a 1-ml aliquot of the chloroform layer was counted. This procedure isolates the major phospholipids while removing inorganic phosphate, phosphorylated proteins and other contaminants [18]; extraction of unincorporated $^{33}P_i$ added directly to brain homogenates was essentially zero (< 50 dpm/g brain for addition of 2 μCi/g). Results were calculated as dpm incorporated per gram original wet weight of brain, and the degree of stimulation of incorporation by norepinephrine was taken as the difference between animals treated with norepinephrine + $^{33}P_i$ and those receiving $^{33}P_i$ alone (basal incorporation). By use of the intracisternal injection procedure, norepinephrine was restricted to the neuronal side of the blood–brain barrier, thus ensuring a reliable *in vivo* assessment of cellular responses to receptor activation in the neuronal compartment. The effect of intracisternal norepinephrine on phospholipid incorporation of $^{33}P_i$ into this chloroform–methanol extract is known to result from excitation of alpha$_1$-noradrenergic receptors associated with noradrenergic synapses in the central nervous system, as demonstrated by studies with noradrenergic agonists and antagonists and by regional distribution studies [10–13]; this biochemical marker for receptor activation has proven useful in elucidating development of central noradrenergic pathways [12].

Data analysis. Results are presented as means and S.E.M. with levels of significance calculated by two-way analysis of variance. In addition, the frequency distribution of values for norepinephrine stimulation of incorporation was determined and evaluated by the Fisher exact test [19]; this test detects subpopulations of affected animals against a background of animals with essentially normal values, a situation which has been noted previously [7, 20] for some of the effects of methadone on brain development. One standard deviation from control means of the appropriate age was chosen as the critical value to denote a "low" (value < control mean minus 1 S.D.) or "high" (value > control mean plus 1 S.D.) stimulation-for-age, and in each case the methadone-exposed group was compared both to controls and to the random incidence predicted from the normal distribution curve. Values for basal or norepinephrine-stimulated incorporation of $^{33}P_i$ were essentially the same in the two types of control groups (maternal saline or postnatal saline), and therefore control data are presented as a single curve; however, statistical comparisons were based solely on the control group appropriate to each mode of methadone exposure.

Materials. Carrier-free ^{33}P-orthophosphoric acid was obtained from the New England Nuclear Corp. (Boston, MA) and *l*-norepinephrine HCl was purchased from the Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

Basal (unstimulated) incorporation of $^{33}P_i$ into brain phospholipids was 10,000 dpm/g brain in 2-day-old rats and rose to more than 20,000 dpm/g by weaning (Fig. 1); there were no overall differences in basal incorporation among controls, maternal methadone, or postnatal methadone groups.

Intracisternal administration of norepinephrine produced a small stimulation (3000–4000 dpm/g) of $^{33}P_i$ incorporation in 2-day-old control rats and in rats injected with methadone postnatally (Fig. 2); however, no stimulation was seen in 2-day-old rats whose mothers received methadone. In keeping with the time course of development of central alpha$_1$-noradrenergic receptors [12, 21], norepinephrine-induced stimulation of $^{33}P_i$ incorporation into phospholipids in the controls rose during the preweaning period to a maximum of 15,000 dpm/g by the end of the third postnatal week. A consistent and uniform deficit in stimulation was seen in the maternal methadone group over the course of development through 21 days of age ($P < 0.005$ by analysis of variance). In contrast, pups receiving methadone by direct postnatal injection did not show a consistent pattern of alteration of stimulated incorporation, with values somewhat elevated during the first postnatal week but fairly normal or depressed thereafter (overall effects not statistically significant). Analysis of the frequency distribution of the magnitude of

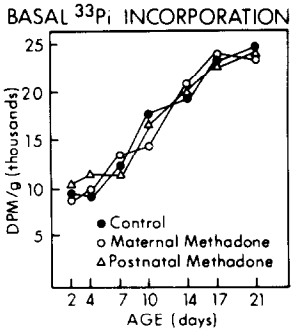

Fig. 1. Basal incorporation of $^{33}P_i$ into phospholipids of developing rat brain in control rats, rats whose mothers received methadone beginning in midgestation, and rats receiving methadone by direct injection postnatally. Data represent means of twelve to sixteen control rats at each age and six to eight rats in each of the methadone groups. Standard errors were generally < 500 dpm/g at early ages and < 2000 dpm/g in older animals. Neither methadone group is significantly different overall compared to controls.

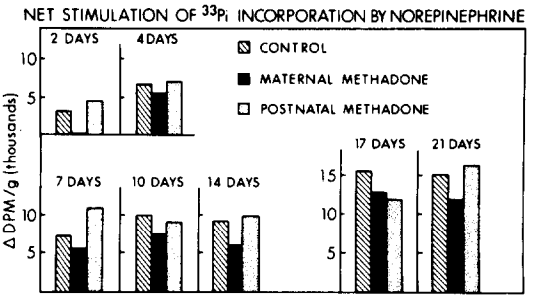

Fig. 2. Stimulation of $^{33}P_i$ incorporation into brain phospholipids caused by intracisternal administration of norepinephrine. Stimulation was calculated as incorporation with norepinephrine minus incorporation without. Data represent means of twelve to sixteen controls and six to eight methadone-exposed rats in each group. Standard errors were < 500 dpm/g at early ages and < 2000 dpm/g in older animals. Stimulation is significantly lower overall in the maternal methadone group compared to controls ($P < 0.005$ by analysis of variance) but is not in the postnatal group.

norepinephrine stimulation confirmed the shift in the characteristics of the maternal methadone-exposed population (Table 1); a marked increase in the overall incidence of abnormally low stimulation-for-age occurred at the expense of values in the high and mid-range. The incidence of high or low values in the controls was not significantly different from random occurrence, and a small, but significant increase in the incidence of high stimulation values was seen in the postnatal methadone group; the latter reflected primarily the earliest age points at which the slight increase in mean stimulation was seen in the postnatal group.

Both maternal methadone administration and direct postnatal administration of drug produced deficits in organ growth of 10–15%, essentially identical to those in earlier reports using the same dosage regimens [2, 4–6] (data not shown); thus, the deficits in norepinephrine stimulation of $^{33}P_i$ incorporation are even larger if compared on a whole organ basis rather than per unit tissue weight.

These results provide direct biochemical evidence that perinatal exposure to methadone produces perturbation of the functioning of central noradrenergic synapses. The *in vivo* stimulation by norepinephrine of $^{33}P_i$ incorporation into membrane phospholipids, an effect mediated by $alpha_1$-noradrenergic receptors, was lowered in the brains of developing rats whose mothers received methadone beginning in midgestation. This effect was not due to a general biochemical deficit in phospholipid metabolism, as only the norepinephrine-stimulated component of synthesis was affected while the basal incorporation rate was normal. Thus, the slowing by perinatal methadone of presynaptic nerve terminal development noted previously [2, 3, 6–8] is not compensated by supersensitivity of receptor-mediated responses but rather is exacerbated by a reduction in response capability.

Similar interference with receptor development and receptor-mediated responses has been reported following treatments with other types of drugs. Destruction of developing central catecholaminergic terminals during the postnatal period by neonatal

Table 1. Frequency distribution of magnitude of stimulation-for-age of $^{33}P_i$ incorporation into brain phospholipids*

Treatment	Percentage of animals within each range of stimulation-for-age		
	Low	Mid-range	High
Control	17	65	18
Maternal methadone	45†	51†	4†
Postnatal methadone	17	55	28†
Random (normal distribution)	16	68	16

* Data represent cumulated distributions of animals from 2 through 21 days of age, totalling 112 in the control group, 49 in the maternal methadone group, and 66 in the postnatal methadone group. One standard deviation above or below the control mean value for stimulation at each age was used to define "high" or "low" categories.

† Significant differences from both control distribution and random incidence at $P < 0.05$ or less by Fisher's exact test.

intracisternal treatment with 6-hydroxydopamine results in reduced numbers of receptors and deficits in phospholipid-labeling responses [12]. With more subtle interference with synaptic development, two types of effects have been noted which depend upon the age at which exposure takes place; for example, postnatal blockade of dopaminergic receptors with neuroleptics results in receptor supersensitivity just as is found in adults, whereas exposure prenatally *reduces* receptor numbers and receptor-mediated responses [22]. These findings with other agents raise the questions of: (a) whether a similar critical period exists in which methadone exerts an effect on development of receptor-mediated responses, and (b) whether this effect is a direct result of slowing of nerve terminal development or instead is independent of the presynaptic deficit. Postnatal administration of methadone directly to developing pups failed to cause a reduction (and may have caused a slight enhancement) of norepinephrine stimulation of phospholipid synthesis, despite the fact that postnatal methadone exposure is as effective as perinatal maternal methadone in slowing presynaptic terminal development [2, 3, 6–8]. Thus, the actions of maternal methadone on development of receptor-mediated responses appear to be separable from the effect on presynaptic maturation in that they display a different critical period in which development is sensitive to perturbation by the drug.

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