Pharmacology Biochemistry & Behavior, Vol. 30, pp. 847–851. © Pergamon Press plc, 1988. Printed in the U.S.A. 0091-3057/88 $3.00 + .00

Effects of Prenatal 5-Methoxytryptamine and Parachlorophenylalanine on Serotonergic Uptake and Behavior in the Neonatal Rat

A. SHEMER, P. M. WHITAKER-AZMITIA AND E. C. AZMITIA

Department of Biology, New York University, Washington Square, New York, NY 10003

Received 11 May 1987

SHEMER, A., P. M. WHITAKER-ZAMITIA AND E. C. AZMITIA. *Effects of prenatal 5-methoxytryptamine and parachlorophenylalanine on serotonergic uptake and behavior in the neonatal rat.* PHARMACOL BIOCHEM BEHAV **30**(4) 847–851, 1988.—Parachlorophenylalanine (pCPA) or 5-methoxytryptamine (5MT) was administered to pregnant Sprague Dawley rats from day 8 (D8) of gestation till D17 and from D12 until birth respectively. Birth weights of both drug groups of neonates were approximately 20% less than the saline-injected controls. 5MT neonates showed a significant reduction of high affinity ^{3}H-5HT uptake in the brainstem at all three time points: D1, D15, D30, and a slight reduction in the forebrain reaching significance only on D30. The pCPA animals showed a significant reduction in the brainstem and forebrain on D1 and D30, but only a small nonsignificant reduction in both areas on D15. Behaviors measured on day 15 revealed that in general activity, spontaneous alternation, and passive avoidance both drug groups of neonates showed deficits: less activity, less alternation, and less avoidance.

Prenatal Serotonin 5-Methoxytryptamine Uptake Behavior Parachlorophenylalanine Development

DURING normal development, cells of the serotonin (5-hydroxytryptamine, 5HT) system have completed their replication by embyronic Day 14 [18,19] and are beginning to send axons over relatively long distances. However, projections to terminal fields and neighbouring nuclei may not be complete until up to two months after birth [20,21]. It has recently been shown that 5HT will influence the maturation of developing neurons. In invertebrate brains, 5HT applied to outgrowing neuronal processes will inhibit neurite extension and cause the filopodia to retract [15]. Evidence from a tissue culture model of neuronal outgrowth has shown that the presence of 5HT agonists can influence the maturation and the number of surviving 5HT immunoreactive cells [6].

5-Methoxytryptamine (5MT), a 5HT agonist, has been found endogenously in trace quantities in the rodent brain [8,26] and pharmacologically mimics the effects of 5HT although five times less potent [35]. No neurotoxic effects have been reported. Twenty-four hours after administration to rats (3 mg/kg) 80% of the radiolabelled 5MT was excreted in the urine [26]. 5MT produced a biphasic dose-dependent effect on the extent of outgrowth from fetal raphe mesencephalic cells after 5 days in culture [6,32]. Inhibition of transmitter maturation and cell death was observed with a low concentration of 5MT (10 nM) while with a high concentration (1000 nM) a slight increase in transmitter maturation was seen. The amount of outgrowth in these studies was quantified by measuring the high affinity uptake of ^{3}H-5HT which is generally accepted to reflect the total membrane area of the axon [6, 12, 32]. This biphasic model of outgrowth was the model that we chose as a basis for our continuing research.

We proceeded to expand the tissue culture observations to an in vivo model, by exposing the developing organism to the same 5HT agonist, 5MT, during gestation. We predicted that we would find altered levels of high affinity specific uptake in animals that had been exposed to the drug in utero. The direction of the change was hard to foresee due to the observed biphasic effects in tissue culture. We predicted reduced uptake if our dose was a low one and increased uptake if it was a high one.

Previous reports have shown that 5HT levels change with even mildly stressful manipulations such as brief isolation from the parent or handling [16]. It is plausible that environmental influences could in turn affect the internal environment of the developing organism. Thus a pharmacological manipulation during gestation could partially parallel environmentally-mediated events especially since serotonin may be functioning as a developmental signal [19].

The ontogeny of adult typical responses by the developing organism to pharmacological insult has also been investigated [24,25]. More recently, interest has focused on drug-induced responses that are broadened to include infant-typical responses that may not remain part of the adult repertoire [30]. The behaviors that we proposed to look at were

those reflecting behavioral inhibition which is known to be mediated by the 5HT system. Behavioral arousal has been shown to be sensitive to pCPA, a 5HT depletor, from postnatal D15 onwards but not earlier [24]. 5HT has been shown to be involved in spontaneous alternation [31] and passive avoidance, also a manifestation of behavioral inhibition with specific ontogenic patterns [11]. There is an increasing body of evidence that 5HT is involved in memory mechanisms and specifically in avoidance learning [1, 2, 27]. For these reasons we decided that these were relevant behaviors to investigate.

METHOD

Pregnant Sprague-Dawley rats were housed individually and maintained on a 12-hour light-dark cycle with food and water ad lib. From D12 of gestation until the day of birth the rats were given 5MT 1 mg/kg or the equivalent volume of saline once a day IP. pCPA rats were given a loading dose of 300 mg/kg on D8 of gestation and 100 mg/kg from D9 until D17. Litters were divided and cross fostered. Neonates were sacrificed on postnatal D1, D15 and D30 for biochemical analyses.

Synaptosomal Preparation

On postnatal D1, D15 and D30 brainstem and forebrain sections were removed from the neonate by blunt dissection and homogenized in 10 vol./weight (minimum volume was 1 ml ice-cold 0.32 M sucrose) with 10 strokes of a loosely fitting glass tube (Thomas No. 04715) and a teflon pestle (No. 5952). The homogenate was centrifuged (500×g, 5 min) and the supernatant carefully removed and saved. The pellet P_1 was resuspended in 2 ml of 0.32 M sucrose and centrifuged again at low speed 500×g, 5 min to obtain a second supernatant. The two supernatants were combined and centrifuged at 10,000×g for 15 min. The resulting crude synaptosomal pellet was resuspended in 10 vol. of the original tissue weight of Minimal Essential Medium (MEM) from Gibco + 1% glucose.

Uptake

Incubations were performed in triplicate in tissue culture multiwelled plates (Linbro 76-00204) with a total reaction volume of 200 microliters containing 20 microliters of the P2 suspension. The wells contained MEM as a buffer and 50 nM of ^{3}H-5HT (New England Nuclear, 26.2 Ci/mmole). Nonspecific uptake was defined as that occurring in the presence of an excess of unlabelled 5HT (5×10^{-5} M, Sigma). The incubation solution was raised to 37°C and the reaction was started by introducing 20 microliters of the P_2 suspension in triplicate. The reaction was terminated after 20 min by filtering the incubation medium through Whatman filters and washing for 15 sec with 0.1 M phosphate buffered saline, with a Titertek cell harvester (Flow laboratories). Protein levels in the P_2 fraction were determined according to the method of Lowry *et al.* [22]. Kinetic analysis was performed using concentrations of ^{3}H-5HT from 5 nM to 100 nM. Linearity of uptake over time and tissue concentration were also measured. All determinations were in triplicate and uptake expressed as CPM/mg protein/20 min.

Behavior

Activity was measured in a Lehigh Valley Electronics photoactometer drum 85 centimeters in diameter equipped with 12 photobeams. Activity scores were recorded during 5 consecutive blocks of 2 min each.

Activity was also measured in an open-field apparatus, 2×2 ft square with a 6 inch high perimeter. The "field area" was divided into 3″×3″ squares and activity was scored each time an animal moved from one square to another.

Passive avoidance was measured in a simple step-through test in which animals were allowed to cross from one side to the other of a two compartment box. Once the animal had crossed over a door was closed behind it and a shock of 0.3 mA was delivered to the feet for 10 seconds. Three days later the animals were returned to the original side of the box and latency to cross over to the side where they had been shocked was measured. Long latencies were considered a sign of memory and/or of behavioral inhibition.

Spontaneous alternation was measured in an apparatus consisting of a start box (4″ by 4″) with a door opening into an intermediate compartment (4″ by 5″) flanked on the right by a further chamber (4″ by 9″). Animals were placed in the start box, the door was raised and then from the intermediate compartment they could choose to go into the right or left chamber where they were left for 30 sec with the door closed behind them. They were then placed directly in the start box for a second trial.

TABLE 1

HIGH AFFINITY UPTAKE OF ^{3}H-5HT IN THE PRESENCE OF EXCESS UNLABELED 5HT (5×10^{-5}) INTO SYNAPTOSOMAL P_2 FRACTION FROM BRAINSTEM AND FOREBRAIN FROM NEONATAL RATS EXPOSED PRENATALLY TO pCPA OR 5MT (PERCENTAGE OF UNTREATED CONTROL VALUES)

	pCPA	5MT
Day 1	brainstem 22.6%†	49.89%†
	forebrain 61.0%†	92. 8%*
Day 15	brainstem 78.7%*	59. 5%†
	forebrain 88.4%*	71. 6%*
Day 30	brainstem 58.7%†	47. 7%†
	forebrain 68.2%†	51. 8%†

Statistical analysis was performed using the Student *t*-test for independent samples on the specific CPM/mg protein/20 min.
*$p<0.1$, †$p<0.005$.

RESULTS

High Affinity Uptake Into Synaptosomes

The ^{3}H-5HT high affinity uptake into synaptosomes was linear for 5 min at 37°C and was linearly related to tissue concentration of the P_2 suspension between 2 microliters and 30 microliters of tissue. In the 5MT drug condition the high affinity ^{3}H-5HT uptake was significantly reduced in the brainstem at all three time points measured: D1, D15, D30. In the forebrain, levels were reduced considerably less, reaching significance only on D30. For pCPA animals significant reductions in uptake were observed in both brainstem and forebrain on D1 and D30. Only nonsignificant reductions were observed on D15 in both areas (see Table 1).

Behavior

Passive avoidance. pCPA, 5MT and control animals were

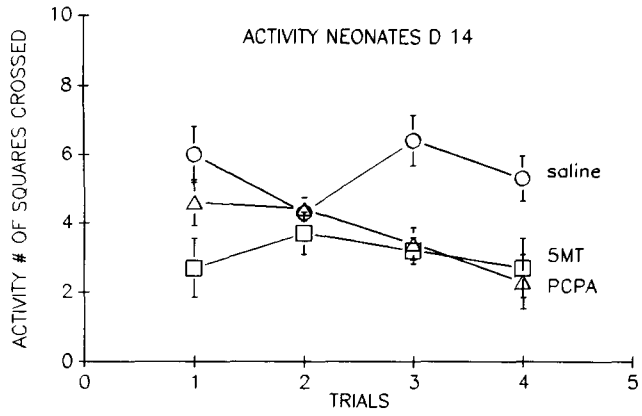

FIG. 1. Neonatal rats were tested in an open-field apparatus for 4 blocks of 2 minutes each on D14 and D15 postnatally. Neonates exposed to 5-methoxytryptamine (5MT) or parachlorophenylalanine (pCPA) were significantly less active than their controls ($p=0.005$).

trained on postnatal D20 on a step through passive avoidance paradigm. There was no difference between groups in their latency to cross: 18.0 sec compared to 22.3 sec respectively.

The drug animals scored significantly lower latencies to cross when tested 3 days later. They were less able to restrain their response to cross, or alternatively they did not remember the shock. [Saline: 227.7 sec cf. 5MT: 91.67 sec ($t=p<0.05$); cf. pCPA 122.3 sec ($t=p<0.05$).]

Activity. Activity was measured in an open-field test on days 7, 15, and 30. At 7 days there was no difference between any of the groups. At day 15 both groups of drug animals were significantly less active ($t=p<0.005$). The treated animals tended to move in spurts and then remain immobile for periods while the controls moved around more evenly over a block of 4 trials of 2 min each.

In the photoactometer drum where activity is measured in the dark, the same pattern of results was observed. We had decided to use this enclosed environment in case the open field was inhibiting the young animals, however both the pCPA animals and the 5MT animals were significantly less active than controls even in this apparatus ($t=p<0.005$) but did not differ from each other.

Spontaneous alternation. Saline animals on D15 alternated on more than 75% of the trials, whereas the 5MT animals alternated on only 16.6% of the trials. They consistently returned to the side of their first choice. There was no preference for any particular side in either of the groups. The difference between the two groups did not reach significance although the trend was similar to that observed in passive avoidance. At D30 the 5MT animals were now alternating more than 75% of the trials and were comparable to the controls. No pCPA animals were tested in this paradigm.

DISCUSSION

In our study, pCPA and 5MT both have significant effects on the biochemistry and behavior of animals exposed to these drugs during gestation. Interestingly, although the drugs have totally different modes and sites of actions, 5MT a receptor agonist, and pCPA a synthesis inhibitor, the final outcome of their presence is surprisingly similar. It appears that 5MT causes a reduction of high affinity uptake in the brain stem which is attenuated during the first month of life, but still only reaches half the normal control levels at 30 days. pCPA on the other hand causes even greater reductions in uptake on D1 but rises at D15 returning to reduced levels comparable to those of the 5MT animals by D30. Thus, both drugs cause reduced uptake at ^{3}H-5HT at birth, an indication that both drugs were inhibiting neuronal outgrowth or maturation. It is therefore clear that interfering with the endogenous levels of this neurotransmitter, either by changing its level or by stimulating its receptors, can influence outgrowth of 5HT neurons. The presence of the neurotransmitter 5HT in the brain prior to synaptogenesis indicates that its function in gestation is probably different from the one it assumes in the mature brain. There is evidence that during development serotonin may contribute to the maturation of target tissue as well as influencing the direction and distance of outgrowth. This has been shown in invertebrate studies [15], in in vivo studies [18,19] and in tissue culture [33]. As it is known that pCPA administration reduces the level of 5HT [17], it is possible that in the absence of sufficient amounts of this neurotransmitter, the 5HT system develops less extensively. This would account for the apparent inhibition of neurite outgrowth by 5HT neurons in pCPA-treated animals.

Receptors for 5HT are already present in the fetal brain [6, 32, 33] and could mediate the actions of a serotonergic agonist such as 5MT. Furthermore, in our laboratory, we have shown that the number of receptors in neonates treated prenatally with pCPA increases in response to the 5HT depletion. Conversely, the number of receptors in neonatal animals decreases in response to prenatal serotonergic stimulation with 5MT [34]. Our data therefore give further support to the claim that 5HT receptors are already functional in the immature brain. 5MT therefore, may be acting through 5HT receptors, as it did at low dose in tissue culture, to promote the growth of short proximal axonal connections and reduce the outgrowth of long axons to more distant target sites [32].

The mechanisms proposed above could account for the low uptake levels seen on day 1 in both drug conditions. On day 15 both groups show a rise in the amount of uptake compared to controls which on day 30 again drops to levels significantly lower than controls.

Locomotor activity has often been chosen as a measure that reflects the actions of maturing neurotransmitter systems in the neonate [9,25]. Studies on the ontogeny of spontaneous activity have shown a rise from birth until a peak at approximately day 15 at which time levels greatly exceed those in the adult. After day 15 there is a gradual decline until adult levels are reached at about day 30 [10]. The ontogeny of this arousal has been explained by the caudal to rostral sequence of development in which forebrain inhibitory mechanisms are last to mature [25]. Thus, at peak activity levels around day 15, arousal is unchecked while in the adult this arousal is dampened by effective inhibition. Arousal and hyperactivity have been attributed to the catecholamines [7], whereas inhibition is believed to be cholinergic and serotonergic [24,28]. Serotonergic inhibition of catecholamine arousal was shown by Mabry and Campbell [24] who used pCPA to potentiate amphetamine arousal and by Grabowska and Michaluk [14] who used apomorphine and methysergide to potentiate activity. Our drug animals exposed to chronic pCPA or 5MT in utero showed reduced activity and uptake levels. This appears to contradict the acute effects of pCPA and methysergide in the adult. However, other researchers have reported that both quipazine, an agonist, and methysergide an antagonist, produce a re-

duction in activity [23]. This effect is apparently dose-dependent which could explain the apparent discrepancy between our observations and those of others.

Reduced activity can be an indication of either accelerated or retarded development, if considered in isolation. However, in our study, as part of a group of behavioral measures, less activity is probably an indication of immaturity. In measures of passive avoidance, both drug groups showed shorter latencies than controls. This could mean that they were less capable of restraining their need for water or activity, or that they remembered less than the controls. Egger [13] has provided convincing evidence that the preweaning rat has adequate memory mechanisms, but lacks the capacity to inhibit behavior which accounts for perserverance in spontaneous alternation. Therefore, if we consider the passive avoidance data together with the data from the spontaneous alternation test in which the drug neonates tended to return to the familiar side, it appears that the animals lacked inhibition rather than had poor memory.

In conclusion, by pharmacological manipulation we have reduced apparent serotonergic outgrowth as assessed by ^{3}H-5HT uptake and have produced a neonate that has less well developed behavioral inhibition. The most parsimonious explanation for these results is that the animals were less mature at the time of assay or testing. The interpretation of immaturity would concur with the reduced uptake seen on D15 but then on D30 when uptake is still reduced the behavioral deficits are no longer evident. It is always possible that the animal is learning to compensate for any biochemical deficit, or that as the deficit declines it no longer reaches a critical level at which behavior is affected. Further research is needed to address these questions.

REFERENCES

1. Altman, H. J. Mediation of storage and retrieval with two drugs that selectively modulate serotonergic neuro transmission in memory dysfunction: an integration of animal and human research from preclinical and clinical perspectives. Ann NY Acad. Sci. 444:496–498; 1985.
2. Archer, T. Serotonin and fear retention in the rat. J. Comp. Physiol. Psychol. 96(3):491–516; 1982.
3. Azmitia, E. C.; Brennan, M. J.; Quartermain, D. Adult development of the hippocampal serotonin system of C5BL/6N mice: an analysis of high affinity uptake of ^{3}H-5HT in slices and synaptosomes. Neurochem. Int. 5(1):39–44; 1983.
4. Azmitia, E. C.; Marovitz, W. P. In vitro hippocampal uptake of tritiated serotonin (^{3}H-5-HT); a morphological, biochemical and pharmacological approach to specificity. J. Histochem. Cytochem. 28:636–644; 1980.
5. Azmitia, E. C.; Whitaker-Azmitia, P. M.; Lauder, J.; Privat, A. Primary culture of dissociated fetal mesencephalic raphe: differential stimulation of serotonergic growth by target tissue. Soc. Neurosci. Abstr. 9:10; 1983.
6. Azmitia, E. C.; Whitaker-Azmitia, P. M. Target cell stimulation of dissociated serotonergic neurons in culture. Neuroscience 20:47–63; 1987.
7. Baez, L. A.; Eskridge, N.; Schein, R. Postnatal development of dopaminergic and cholinergic catalepsy in the rat. Eur. J. Pharmacol. 36:155–162; 1976.
8. Bjorklund, A.; Falck, B.; Stenevi, U. Classification of monoamine neurones in the rat mesencephalon; distribution of a new monoamine neurone system. Brain Res. 32:269–272; 1971.
9. Campbell, B. A.; Mabry, P. D. Ontogeny of behavioral arousal: A comparative study. J. Comp. Physiol. Psychol. 81:371–379; 1972.
10. Campbell, B. A.; Mabry, P. D. The role of catecholamines in behavioral arousal during ontogenesis. Psychopharmacologia 31:253–264; 1973.
11. Campbell, B. A.; Spear, N. E. Ontogeny of memory. Psychol. Rev. 79:215–236; 1972.
12. Currie, D. N.; Dutton, G. R. ^{3}H GABA uptake as a marker for cell type in primary cultures of cerebellum and olfactory bulb. Brain Res. 199:473–481; 1980.
13. Egger, G. The relevance of memory, arousal and cue factors to development changes in spontaneous alternation by rats. Dev. Psychobiol. 65:459–468; 1972.
14. Grabowska, M.; Michaluk, J. The role of serotonin in apomorphine-induced locomotor stimulation in rats. Pharmacol. Biochem. Behav. 2:263–266; 1974.
15. Haydon, P. G.; McCobb, D. P.; Kater, S. B. Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. Science 226:561–564; 1984.
16. Hofer, M. A. Studies on how early maternal separation produces behavioral change in young rats. Psychosom. Med. 37:245–264; 1975.
17. Koe, B. K.; Weissman, A. The pharmacology of parachlorophenylalanine, a selective depletor of serotonin stores. Adv. Pharmacol. 63:387–391; 1968.
18. Lauder, J.; Bloom, F. E. Ontogeny of monoamine neurons in the locus coeruleus, raphe nucleus and substantia nigra of the rat. J. Comp. Neurol. 155(4):496–481; 1974.
19. Lauder, J.; Krebs, H. Serotonin as a differentiation signal in early neurogenesis. Dev. Neurosci. I:15–30; 1978.
20. Levitt, P.; Moore, R. Y. Developmental organisation of raphe serotonin neuron groups in the rat. Anat. Embryol. 154:241–251; 1978.
21. Lidov, H. G. W.; Molliver, M. E. An immunohistochemical study of serotonin neuron development in the rat: Ascending pathways and terminal fields. Brain Res. Bull. 8:389–430; 1982.
22. Lowry, O. H.; Rosenbrough, N. J.; Fair, A. I.; Randall, R. J. Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265–272; 1951.
23. Lucot, J. B.; Seiden, L. S. Effects of serotonergic agonists and antagonists on the locomotor activity of neonatal rats. Pharmacol. Biochem. Behav. 24:537–541; 1986.
24. Mabry, P. D.; Campbell, B. A. Ontogeny of serotonergic inhibition of behavioral arousal in the rat. J. Comp. Physiol. Psychol. 86:193–201; 1974.
25. Mabry, P. D.; Campbell, B. A. Cholinergic monoaminergic interactions in the brain. Butcher, L. L., ed. New York: Academic Press; 1978:257–270.
26. Miller, F. B.; Maickel, R. P. Fluorometric determination of indole derivatives. Life Sci. 1(9):747–752; 1970.
27. Ogren, S. O. Forebrain serotonin and avoidance learning: Behavioral and biochemical studies on the acute effect of chloramphetamine on one way active avoidance learning in the male rat. Pharmacol. Biochem. Behav. 16:881–895; 1982.
28. Randall, P. K. Environmental and pharmacological determinants of behavioral arousal in the developing rat. Doctoral dissertation, Princeton University, 1975.
29. Seiger, A.; Olson, L. Late prenatal ontogeny of central monoamine neurons in the rat: Fluorescence histochemical observations. Z. Anat. Entwgesch. 140:281–318; 1973.
30. Spear, L. P.; Enters, K.; Linville, D. Age specific behaviors as tools for examining teratogen-induced neural alterations. Neurobehav. Toxicol. Teratol. 7:691–695; 1973.
31. Swonger, A. K.; Rech, R. H. Serotonergic and cholinergic involvement in habituation of activity and spontaneous alternation of rats in a Y maze. J. Comp. Physiol. Psychol. 81:509–522; 1972.

32. Whitaker-Azmitia, P. M.; Azmitia, E. C. A pharmacological model for studying the development of serotonergic neurons in tissue culture: the role of high activity serotonergic receptors. In: Caciagli, F.; Giacobini, E.; Paoletti, R., eds. Developmental neuroscience: Physiological, pharmacological and clinical aspects. Amsterdam: Elsevier Science Publishers; 1984.
33. Whitaker-Azmitia, P. M.; Azmitia, E. C. Autoregulation of fetal serotonergic neuronal development: Role of high affinity serotonin receptors. Neurosci. Lett. 67:307–312; 1986.
34. Whitaker-Azmitia, P. M.; Lauder, J. M.; Shemer, A.; Azmitia, E. C. Postnatal changes in serotonin receptors following prenatal alterations in serotonin levels: further evidence for functional fetal serotonin receptors. Dev. Brain Res. 33:285; 1987.
35. Whitaker, P. M.; Seeman, P. High affinity ^{3}H-serotonin binding to caudate: inhibition by hallucinogens and serotonergic drugs. Psychopharmacology (Berlin) 59:1–5; 1978.