Pharmacology Biochemistry & Behavior, Vol. 8, pp. 251–257. Printed in the U.S.A.

Methylazoxymethanol Acetate: Effect of Postnatal Injection on Brain Amines and Behavior

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(Received 14 July 1977)

LAI, H., R. M. QUOCK, W. MAKOUS, A. HORITA AND L. S. JEN. *Methylazoxymethanol acetate: effect of postnatal injection on brain amines and behavior.* PHARMAC. BIOCHEM. BEHAV. 8(3) 251–257, 1978. – The antimitotic drug, methylazoxymethanol acetate (MAMA), was injected into newborn rats during the first four days of life. At 48 days of age, these rats weighed one-third less than controls, as did the cerebella of their brains, but the rest of their brains weighed only 7% less than those of controls. The cerebellar structure of the drug-injected rats was highly disorganized. Purkinje cells were scattered haphazardly in the granular layer instead of forming a monolayer. More foldings and short folia were found in the cerebella of drugged animals. In spite of these large morphological differences, the total amounts of norepinephrine and serotonin in the cerebella of the drugged rats were not different from those of the control rats. Behavioral effects of postnatal injection of MAMA include retarded development of the righting reflex, i.e., the drugged pups took longer time to right themselves when placed on their backs during the first nine days after birth; and secondly, MAMA reduced locomotor activity measured 45 days after birth.

Methylazoxymethanol acetate Cerebellum Norepinephrine Serotonin Righting reflex Locomotor activity

THE ANTIMITOTIC drug, methylazoxymethanol acetate (MAMA), when injected into pregnant females, causes microencephaly in their offspring [10, 12, 34, 35]. The main detrimental effects caused by administration of the drug to newborn animals is on the development of the cerebellum [9, 11, 12, 14, 15, 16, 18, 31, 33, 36] and of the retina [14, 33, 34]. This paper deals with the effect of MAMA on the cerebellum.

The greatest effects that early postnatal injection of MAMA has on the cerebellum is in the development of the granular layer, for MAMA inhibits the multiplication of the granule cells and their migration from the external granular to the internal granular layer [9, 14, 16, 18, 33]. In mice, these effects cause deformation of the cerebellar folia and a complete depletion of granule cells, whereas in rabbits and rats they cause a marked reduction of the number of granule cells, with a general loss of cerebellar size and mass. The Purkinje cells are scattered in the internal granular layer instead of forming a monolayer, and they also develop abnormal dendritic pattern [36]. Unit recordings from Purkinje cell in cerebella of rats cultured in MAMA show that the reduction in granular cells causes an increase of reciprocal innervations among Purkinje cells [8]. Motor deficits associated with the effects of MAMA on the cerebella vary with species [14]: mice and hamsters develop ataxia and gait disturbance; rabbits show ataxia and paralysis of the hind limbs; but rats show no gross motor deficit in spite of the reduced number of cells and the reduction in size of the cerebellum.

This paper deals with the effects of the disruption of the cerebellum on motor activity in the rats, specifically on locomotor activity and on the development of the righting reflex.

A second question addressed by this paper is what happens to the amount of transmitter in the cerebellum when its cellular organization is disrupted during development, as occurs when MAMA is injected just after birth. A combination of histological, pharmacological and biochemical studies suggest that norepinephrine and serotonin are putative neurotransmitters that mediate inhibitory effects on cells of the cerebellum [6, 17, 19, 25, 27]. Noradrenergic fibers originating from the dorsolateral part of the locus coeruleus innervate Purkinje cells [27], and serotonergic fibers originating in the raphe nucleus innervate granule cells [6]. The differentiation of cells in these nuclei occurs much earlier in development than the cells they innervate in the cerebellum [22,28], and the noradrenergic fibers arrive at the cerebellum several days before birth in the rat when the cerebellum is still immature [32]. It has been suggested that such innervation plays a possible neurotropic role in

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0091-3057/78/0803-0251$00.90

the neurogenesis of their receptive cells [22,23]. It is interesting to see whether the amounts of these transmitters are affected by the disruption in development of the cerebellum by MAMA.

METHOD

Five litters of Long-Evans rats from the vivarium of the Department of Psychology at the University of Washington were used. Only litters of nine to eleven pups were used. On each of the first four days after birth 10 mg/kg of MAMA was injected subcutaneously into half of the animals in each litter. Equal volumes of physiological saline were injected into their litter mates, which served as controls. The rats were weaned on the 21st day after birth and then housed in groups in the vivarium.

Behavior

Each day after birth, immediately before the daily injection, the righting reflexes of the pups were tested by placing them on their backs and measuring the time taken for them to right themselves. Any given trial was terminated after 20 sec if the rat had not righted itself within that period of time. This test was repeated daily until the rat righted itself within one second.

On the 45th to the 47th day after birth the locomotor activity of the rats was measured (Stoelting Activity Monitor No. 31400M). The mean number of counts per minute was obtained for each animal during the three daily sessions of 10 min each.

Biochemical Assays

On the 48th day after birth, the rats were sacrificed by cervical dislocation, and their brain removed. The brain was separated from the spinal cord at the level of the medulla. (Blood clots were removed from the brain.) Finally, the cerebellum was separated from the rest of the brain. The whole process of dissection usually took less than five minutes.

The cerebellum and the rest of each brain were then separately assayed to determine the content of norepinephrine, dopamine and serotonin by means of the method originally invented by McCaman *et al.* [26] and modified by Iwamoto (personal communication) for mammalian brain tissue. Brain tissue was homogenized in two times its volume of ice-cooled 0.3 N perchloric acid. The homogenate was then centrifuged for 10 min at 8000 g in an ultracentrifuge. 0.8 ml of the supernatant was added to a tube containing 2 ml phosphate buffer (0.25 M Na_2HPO_4 – 7 mM EDTA) and 1.2 ml chloroform containing 0.1 M di-ethylhexylphosphoric acid. The tube was then shaken for 2 min and centrifuged at 500 g for 5 min. The upper aqueous layer was aspirated carefully, and 0.8 ml of the bottom layer was transferred to a tube containing 0.6 ml or 0.2 N HCl and 2 ml n-heptane. The tube was then shaken for 2 min and centrifuged at 500 g for 5 min. The upper layer was aspirated and 0.2 ml of the lower layer was transferred to each of two tubes. 0.8 ml of acetate buffer (0.2 N sodium acetate–7 mM EDTA) and 50 μl of iodine reagent (0.4 g iodine and 1.6 g potassium iodide in 160 ml double distilled water) were added to one of the tubes. The tube was vortexed, and three min later 50 μl sulfite reagent (450 mg sodium sulfite in 2 ml double distilled water and 8 ml 2.75 N sodium hydroxide solution) was added. The tube was vortexed, and 3 min later 50 μl of 5 N acetic acid was added. The tube with its contents was then heated in a water bath for 5 min at 100° C. Norepinephrine was read in a spectrofluorometer at λ_{ex} of 392 nm and λ_{em} of 485 nm. The sample was returned to the tube and heated in the water bath for another 10 min. Dopamine was then read at λ_{ex} of 322 nm and λ_{em} of 375 nm.

To the second tube 0.1 ml of O-phthalaldehyde reagent (5 mg O-phthalaldehyde in 10 ml methanol) and 0.8 ml concentrated HCl were added. The tube was vortexed and heated for 30 min in a water bath at 85° C. Serotonin was read at λ_{ex} of 356 nm and λ_{em} of 480 nm in a spectroflurometer. Standards of norepinephrine, dopamine and serotonin were run with the samples. Standard curves were plotted for each amine and used to calculate the amount of amine in the brain samples.

Histology

Rats deeply anesthetized with ether were perfused with light-fix solution (4% paraformaldehyde in phosphate buffer). Their brains were removed from the skulls and fixed in 4% light-fix solution for 3–5 days and then 26 μ sections were cut. These sections were stained with cresyl violet.

RESULTS

Body Weight and Growth

Rats treated with MAMA showed retarded growth of body weight. On the 48th day after birth, when the chemical assay was performed, the mean weight of the MAMA-treated rats was 70.5 g, as opposed to 109.9 g for their controls. The formation of fur and pigmentation was also retarded in the MAMA-treated rats. Eight percent of the MAMA-treated rats died before the 48th day after birth, while none of the controls did.

Righting Reflex

The time taken for the rats to right themselves at different times after birth is shown in Fig. 1. Evidently the MAMA-treated rats took more time to right themselves on any given day than did the controls, and thus it took longer for the righting reflex to be completely developed in the MAMA-treated rats. All of the control rats righted themselves within 1 sec on the 7th day after birth, but it took the MAMA-treated rats three more days to reach this criterion.

Observation of the rats revealed that the MAMA-treated rats struggled vigorously when placed on their backs. In fact, the MAMA-treated rats appeared to perform more body movements when placed on their backs than did the control animals. The righting reflex involves extending a forelimb while the other forelimb is flexed, and then the body is twisted to the side of the flexed limb until the body rolls over. Some control rats demonstrated this sequence of movement very soon after birth, but it was not observed in the MAMA-treated rats at as early an age. Instead, the MAMA-treated rats made uncoordinated movements that involved all four legs and swaying from side to side.

Locomotor Activity

Figure 2 shows that the MAMA-treated animals were consistently less active than the control rats during the 3 days of testing. The behavior observed within the activity

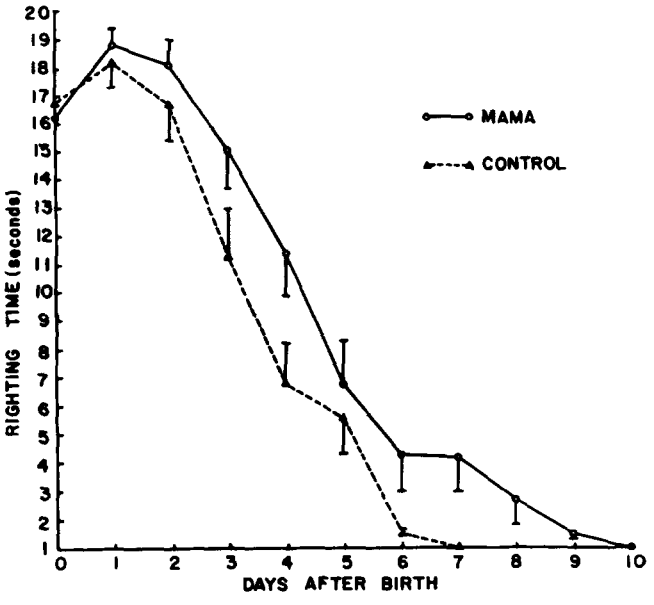

FIG. 1. Righting time (second) of MAMA-treated (○) and Control (△) rats on the first ten days of life. All control animals righted themselves within one second on the seventh day, while it took ten days for the MAMA-treated animals to reach this criterion. Bars indicate SEM.

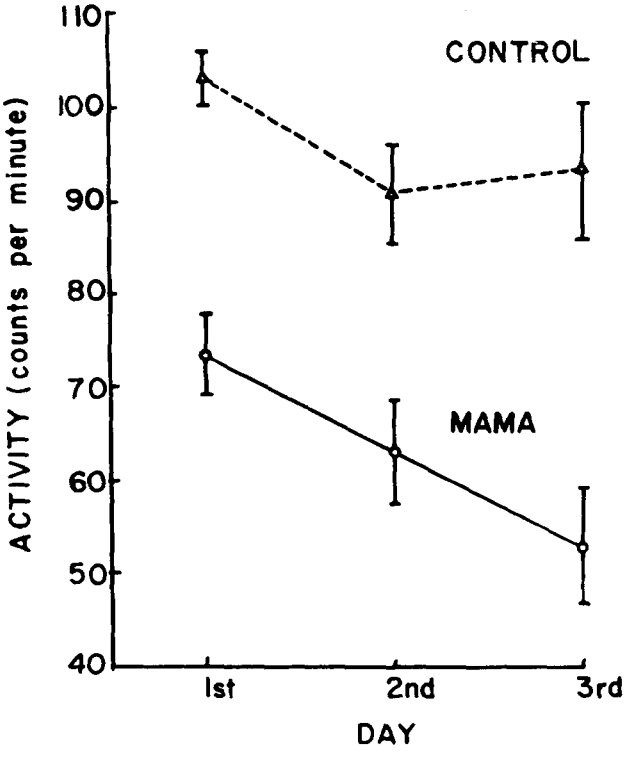

FIG. 2. Locomotor activity (counts per minute) of MAMA-treated (○) and control (△) rats at 45–47 days of age. Each rat spent ten minutes in the activity meter on each day. Bars indicate SEM.

meter consisted mainly of exploratory behavior, such as sniffing, rearing and walking.

Histology

The gross morphology of the cerebella of the two groups of animals is so different that it is hard to find corresponding regions to compare. The average weight of the cerebellum of the MAMA-treated animals (0.151 g) is significantly smaller than that of the control animals (0.219 g) ($p<0.01$, Student's t-test). The rest of the brain of the MAMA-treated animals (1.295 g) also weighs less than that of the controls (1.389 g) ($p<0.05$). Although the cerebella of the MAMA-treated animals are smaller than those of the control rats, they contain more foldings and short folia (Fig. 3a, b). The Purkinje cells in the cerebella of the MAMA-treated rats (Fig. 4a) are scattered haphazardly through the granular layer instead of forming a monolayer between the granular and molecular layer as they do in normal rats (Fig. 4b).

Biochemical Assays

Figure 5 compares the amounts of catecholamines and serotonin in the cerebellum (Fig. 5a) and the rest of the brain (Fig. 5b) in the MAMA-treated and control rats. No dopamine was observed in the cerebellum. Even though the average amount of serotonin in the cerebella of the MAMA-treated rats is 46% higher than that of the control rats, the difference is not significant at the 0.05 level. Neither the amount of norepinephrine in the cerebellum nor the amounts of catecholamines and serotonin in the rest of the brain differ between the two groups of animals.

DISCUSSION

The morphological changes of the cerebellum observed here are similar to those previously reported in rats and other animals given postnatal injection of MAMA [15, 31, 33]; in newborn rats subjected to X-irradiation [4, 5, 24]; and in mice affected by cerebellar mutant genes [13, 20, 21]. The morphology of the cerebellum of reeler mice is strikingly similar to that of the MAMA-treated rats, showing a reduced number of granule cells and haphazardly scattered Purkinje cells at all depths of the cerebellum. The sum of these findings suggested that organization of Purkinje cells in the cerebellum requires an interaction with the developing microneurons that must occur in the first few days of life. Disruption of these microneurons at this time appears to cause a permanent disruption of organization, which is associated with impeded migration of the Purkinje cells to their proper location in the cerebellum.

In one aspect, our results are opposite to those reported by a previous investigator [36] which reported fewer rather than more foldings and short folia in the cerebellum of rats treated with MAMA. We cannot explain this difference except to point to strain differences and to the fact that our series of injections were given one day earlier, starting on the day of birth, than those of the previous investigation.

The observation of the righting movements made by the two groups of rats suggested that lack of coordination accounts for the difference between groups rather than muscular weakness or general debility. The fact that the cerebellum is developing at the time the righting reflex develops [1, 2, 3] suggests that the effects of MAMA on

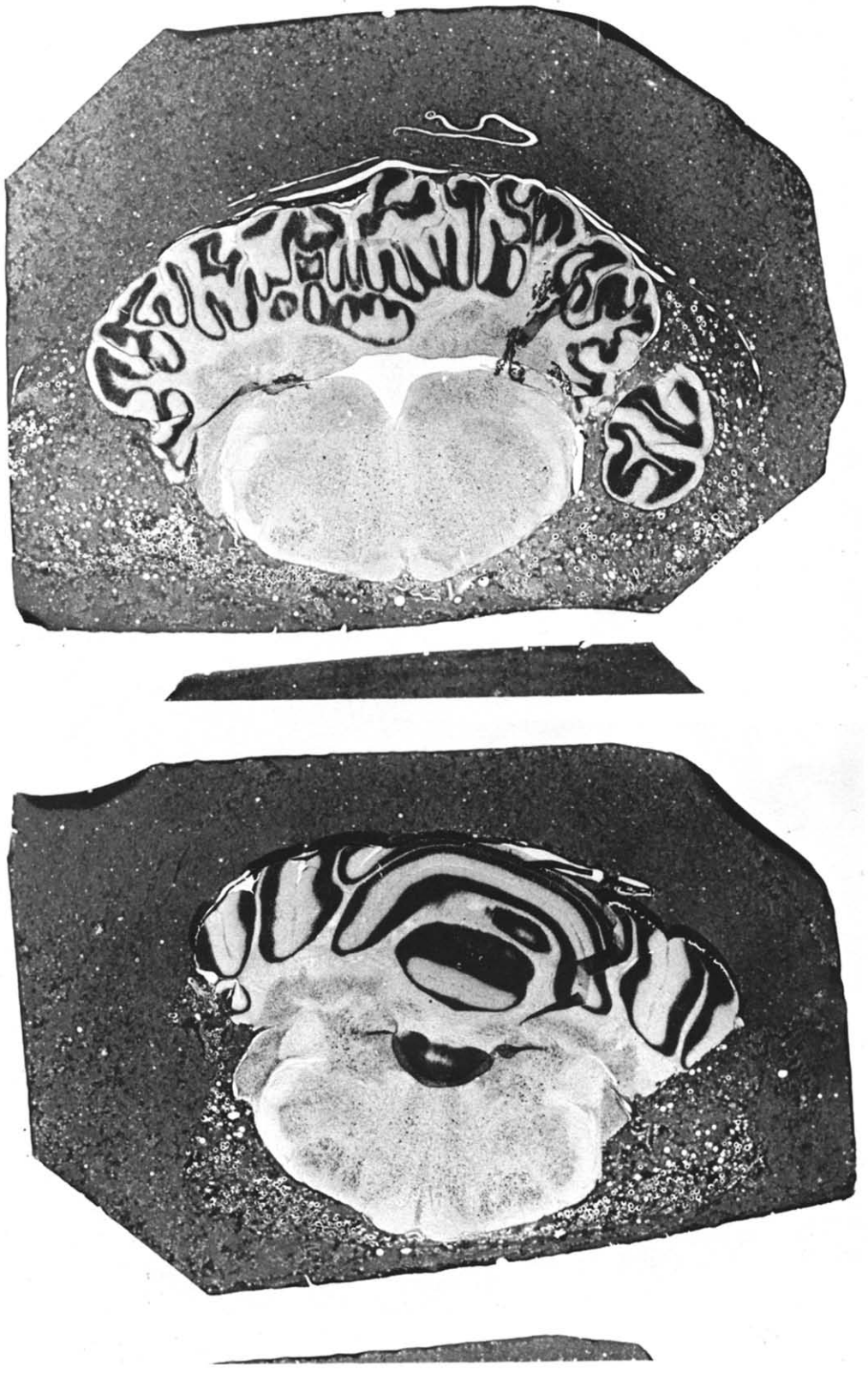

FIG. 3. (a) Cross section through the cerebellum of a MAMA-treated rat, showing irregular foldings and short folia. (b) Cross section through the cerebellum of a control rat.

FIG. 4. (a) Cresyl violet stain of cerebellar folia of a MAMA-treated rat; Purkinje cells (P) are scattered in the granular layer. Mo– Molecular layer, Gr– granular layer. (b) Cresyl violet stain of cerebellar folia of a control rat; Purkinje cells (P) form a monolayer between the granular (Gr) and molecular layer (Mo).

the cerebellum may be the cause of the retarded development of righting.

The hypoactivity of the rats treated with MAMA brings out another similarity between rats treated with MAMA and those exposed postnatally to X-irradiation, for the latter are also hypoactive and suffer from cerebellar malformation [4,7]. The lack of any easily detectable motor deficit, such as ataxia, in the microcerebellar rats, in spite of the radical change of cerebellar morphology is perhaps noteworthy. Presumably more sensitive tests of motor behavior would uncover such deficits.

As summarized above, MAMA transforms the gross morphology of the cerebellum, it disrupts the organization of the Purkinje cells, it reduces the weight of the cerebellum by 31%, and it reduces the number of granule cells. Nevertheless, there is no difference in the amounts of

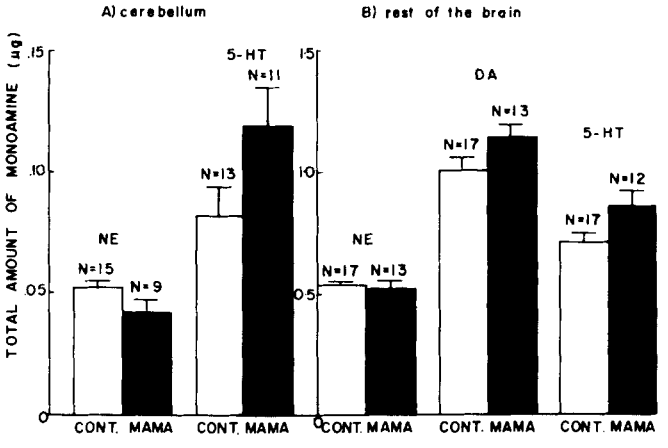

FIG. 5. (a) Total amounts of norepinephrine and serotonin (µg) in the cerebella of the MAMA-treated and control animals at 48 days of age. A *t*-test shows no significant difference between the two groups of animals. Bars indicate SEM. (b) Total amount of norepinephrine, dopamine and serotonin (µg) in the rest of the brain of the MAMA-treated and control rats on 48 days of age. A *t*-test shows no significant difference between the two groups of animals. Bars indicate SEM.

norepinephrine and serotonin in the cerebellum between the MAMA-treated and the control rats. There are other studies showing that the cerebellar content of norepinephrine is highly resistant to disruptive influence, especially during infancy. It has been shown that norepinephrine terminals that have degenerated owing to the administration of 6-hydroxydopa on the third day after birth completely regenerate in the cerebellum at 70 days of age [37]. The cerebellar content of norepinephrine is also not affected in the reeler mice, which have deformed cerebella similar to that caused by treatment with MAMA [21]. Noradrenergic neurons of the cerebellar cortex increase their terminal arborization in response to lesions of the superior cerebellar peduncle [29,30]. This type of growth plasticity has also been demonstrated in serotonergic neurons. For example, it has been shown that serotonergic terminals are capable of forming collateral sprouts to transplanted tissue [28]. These studies and the results of our study generally support the notion that monoaminergic neurons in the brain are plastic and that they can establish connections even after disruption to their site of innervation.

ACKNOWLEDGEMENT

This research was supported in part by a grant from the National Science Foundation (GB-43452).

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