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Effects of Neonatal 6-Hydroxydopa on Behavior in Female Rats[1]

JACK H. McLEAN,[2] RACHAEL S. GLASSER

Department of Psychology, University of New Orleans, New Orleans, LA 70122

RICHARD M. KOSTRZEWA

East Tennessee State University, College of Medicine, Department of Pharmacology

AND

JAMES G. MAY

University of New Orleans

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Neonatal 6-hydroxydopa Female sexual behavior Activity Equilibrium Habituation Norepinephrine

THE behavioral significance of catecholamine-containing neurons in the central nervous system has been studied extensively in recent years by injections of various compounds which deplete central stores of norepinephrine (NE), dopamine (DA) and serotonin (5-HT). Valuable innovations in this method involve the discovery of drugs which offer a high degree of specificity regarding the transmitter substance depleted and the brain areas affected. For example, 6-hydroxydopa (6-OHDOPA) has been shown to alter NE levels in the brain with a high degree of selectivity [6,9]. It is typically administered either intracranially into the ventricles of adult rats [8, 12, 13, 15] or systemically in neonatal animals [9,10]. Both methods result in long-term depletion of NE in the hippocampus and cortex without long-term alteration of brain DA or 5-HT, and with no evidence of long-term effects on peripheral neurotransmitters. Differences occur between the two modes of injection in that the intraventricular-adult technique leads to NE depletion in the cerebellum and brainstem while the systemic-neonatal technique causes NE elevation in these areas [8, 12, 13, 15].

The behavioral effects of these two techniques are almost diametrically opposed. Studies with intraventricular-adult injections have reported increased pain-elicited aggression [15], transient hypophagia [8, 12, 15], adipsia [12], hypoactivity [8, 12, 13], long-term increases in emergence latencies [13] and hyperemotionality [12,13]. The systemic-neonatal method, on the other hand, has been reported to increase open field activity, reduce pain-elicited aggression and decrease passive avoidance, without affecting food or water intake [9,10]. Thus, the former technique results in animals that are less active and more emotional, while the latter produces animals that are more active and less emotional.

The purpose of the present research was to extend the range of findings of neonatal injections of 6-OHDOPA to female rats. The animals were tested in adulthood on a number of behavioral tasks, including female sexual behavior, activity, emotionality, habituation to startle, and equilibrium.

METHOD

Animals

Sprague-Dawley albino female rats were used in all studies. Rats were raised in litters culled to seven to nine animals. Littermate males were used in other research proj-

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[2]Reprint requests should be sent to Jack H. McLean, Department of Psychology, University of New Orleans, New Orleans, LA 70122.

ects. The rats were weaned on day twenty-five and housed in individual mesh cages. Subjects were maintained ad lib on Purina lab chow and water in a reversed light-dark cycle ("on" at 2200 hours, "off" at 800 hours). Males used in the sex tests were obtained at approximately 100 days of age from Research Animals Inc., Braddock, PA.

Groups of rats were treated with 6-OHDOPA (60 μg/g, IP) on postnatal days 1 (i.e. day of birth) and 3. Control animals received the diluent saline (0.9%)-ascorbic acid (0.1%). Each rat performed in each behavioral task (except for the habituation task), although the number of subjects differs from task to task due to mortality during the course of the experiment. In the habituation task an equal number of experimental and control subjects was tested.

Age of Vaginal Opening

Beginning on day 25, 37 experimental and 28 control rats were examined for vaginal opening. Subjects were given a score each day based on the degree to which the vagina had opened. A score of one indicated the vagina was not open to the outside, whereas a score of three indicated complete vaginal opening. The number of days until each rat received a rating of three was recorded. In this and all subsequent tasks, the experimenter was blind as to the group in which each animal belonged.

Estrous Cycle

Vaginal smears were taken on 35 experimental and 27 control animals beginning on day 47. The procedure was performed for 21 days between 1000 and 1200 hours. Slides were stained using Toluidine Blue O and categorized according to stage of the estrous cycle (proestrus, estrus, metestrus, or diestrus).

Sexual Receptivity

At the age of 70–80 days, 30 experimental and 26 control animals were ovariectomized under ether anesthesia. Subjects were subsequently tested between 90–120 days of age with sexually experienced males. Rats were brought into heat with a 3.3 μg IM injection of estradiol benzoate followed in 42 hours by 0.5 mg progesterone. Tests were performed approximately six hours following progesterone. The testing chamber consisted of a semicircular black arena with dimensions of 35 cm (radius) by 55.5 cm (across the front). The male was placed in the arena first and given a 10-min adaptation period before introducing the female. Scoring consisted of observer ratings for lordosis, holding and darting [1]. Three sex tests were given at approximately one week intervals. Animals were observed for 15 mounts at each session. A lordosis score was calculated by adding each lordosis rating for all 15 mounts. Holding and darting scores were obtained in the same way. In addition, a total composite receptivity score was obtained by adding across the lordosis, holding, and darting ratings for all 15 mounts.

Activity and Emotionality Measures

Open field activity was measured during four three-minute trials: Trial 1, day 70; Trial 2, day 130; Trial 3, day 140; and Trial 4, day 159. The apparatus consisted of a round 110 cm diameter open-field marked off into three concentric circles. Each concentric circle was in turn divided into equal area segments. Dependent measures included the total number of segments crossed, the number of outer segments crossed, the number of inner segments crossed, the number of rearing responses, and the number of fecal boluses emitted. Body weight was also recorded for each rat at the end of each test.

Equilibrium Task

At 145 days, 28 experimentals and 25 controls were tested for equilibrium on a stationary dowel rod 2 centimeters in diameter and 60 cm long, suspended 134 cm above the floor. The rod was marked off into 11 segments each 4 cm in length, with the remainder designated as a starting area. Below the rod was a net, with its lowest point 69 cm from the rod. The rat was given an adaption period of two minutes in her home cage which had been placed on a platform at one end of the rod. Following this, she was placed at the opposite end of the rod in the starting area and held for 10 seconds. At the end of this interval, she was released. If the rat fell, she was placed back on the rod at the site where she fell. Rats were given a total of 60 seconds on the bar. Recordings were made of the latency to cross (if less than 60 seconds), the number of falls, and the number of segments crossed.

Habituation to Acoustic Startle

At 160 days of age, 21 experimental and 21 control animals were tested individually in a sound-attenuated chamber (63.7×20.4×25.5 cm) for habituation to a loud tone. Each subject was placed in a vertical cylindrical tube positioned just below a 20.4 cm speaker, and directly above an Electronic Activity Monitor (Stoelting Model No. 31400). A three channel timer was used to present 50-msec tone bursts and to provide time marks on one channel of a strip chart recorder. The elicited activity was recorded on an adjacent channel of the recorder. The tone bursts had a frequency of 1000 Hz and were delivered at 108 db SPL. Stimuli were generated from an oscillator and presented through an audio amplifier (Grass, Model No. AM3DR).

Each subject was allowed 20 min adaptation to the apparatus prior to habituation training. No tones were presented during this interval, but prehabituation activity was recorded. Habituation training consisted of the presentation of 100 tone bursts at 15 sec intervals. Immediately after the last of these, the frequency and presentation rate of the tones were changed so that 20 2000 Hz tones (sensitizing stimuli) were presented at one-second intervals during which activity was not recorded. After this sensitizing stimulation, 30 more 1000 Hz tones were presented at 15-sec intervals, and activity was again recorded. Activity marks occurring within one sec after a tone burst were considered startle responses. The frequency of startle responses within each successive 10-trial block was counted for each subject.

Catecholamine Analysis

Animals were weighed and decapitated at approximately six months of age. Regions of the central nervous system taken for study included the amygdala, anterior cortex, hippocampus, cerebellum, hypothalamus, striatum, posterior cortex, pons-medulla, and spinal cord. Cardiac atria and ventricles were also removed for assay. Tissues were frozen on dry ice and stored at −50°C until assayed. A trihydroxyindole fluorometric method was used to assay catecholamine levels [11].

TABLE 1
SUMMARY OF OPEN FIELD TASK

Measure	Group Saline (N=25)	6-OHDOPA (N=25)
Total segments entered	24.76 (±1.26)	33.70 (±1.67)
Outer segments entered	22.28 (±1.53)	29.30 (±1.31)
Inner segments entered	2.23 (±0.20)	3.33 (±0.31)
Number of fecal boluses	2.98 (±0.31)	1.36 (±0.24)
Rearings	6.98 (±0.47)	8.72 (±0.47)

Drugs

DL-6-hydroxydopa as well as norepinephrine (free base) used in the biochemical analyses were obtained from Regis Chemical Company, Chicago, Illinois. Estradiol benzoate and progesterone used in the sex test were obtained from Nutritional Biochemical Company, Cleveland, Ohio. Other reagents and chemicals were A.C.S. grade or spectrograde.

Statistical Analyses

F-tests are reported where appropriate [17]. When heterogeneity variance was indicated (F^{max}), the Mann-Whitney U test was calculated [4]. When unequal N existed, subjects were randomly eliminated to give an equal-N analysis.

RESULTS

Mortality Data

During the six months of the experiment 8 of the 37 experimental animals, and 3 of the 28 control animals died. While these data result in expected cell frequencies too small to submit to a statistical test, visual inspection shows the observed frequencies to conform very closely to expected frequencies.

Age of Vaginal Opening

No significant difference was found between groups ($t=.938, df=63, p<0.352$) in the number of days until vaginal opening. The mean number of days for vaginal opening was 35.90 and 35.31 for the treated and control groups respectively.

Estrous Cycle

Analyses of variance were performed on data from each stage of the cycle comparing the number of days each group spent in that phase of the cycle. There were no differences between the experimental and control group at any stage of the cycle.

Sexual Receptivity

No significant differences were found between groups for darting, $F(1,50)=0.148$, $p<0.702$; holding, $F(1,50)=0.322$, $p<0.573$; lordosis, $F(1,50)=1.615$, $p<0.205$; total receptitivy, $F(1,50)=1.216$, $p<0.275$.

Activity and Emotionality Measures

As one may see from Table 1 the 6-OHDOPA animals were more active and less emotional than the saline animals. They crossed more segments overall, $F(1,48)=8.107$, $p<0.006$, more outer segments, $F(1,48)=5.116$, $p<0.23$, and more inner segments, $F(1,48)=6.334$, $p<0.015$. Further they reared more often than controls, $F(1,48)=4.216$, $p<0.046$, and defecated less, $F(1,48)=9.676$, $p<0.003$.

Body Weight

The control group weighed significantly more than the experimental group throughout the experiment, $F(1,48)=4.723$, $p<0.035$. Weight gains, however, were parallel. At age of sacrifice, the means (and SEM's) for the control and experimental groups, respectively, were 341.6 g (6.83) and 335.9 g (5.75).

Equilibrium Task

The 6-OHDOPA animals fell from the rod significantly more often than the saline animals (Mann-Whitney U test, $z=3.36$, $p<0.01$). The mean number of falls for the experimental group was 12.88, and for the control group, 7.04. While there were no significant differences between the groups on number of segments crossed ($t=1.38$, $df=51$, $p<0.174$) or latency to cross the rod ($t=1.673$, $df=51$, $p<0.101$), there was a trend for the experimental group to have more difficulty than the control on these measures (4.04 segments crossed for the experimental vs. 6.29 for the control; and 52.77 latency for the experimental vs. 48.73 for the control).

Habituation to Acoustic Startle

There was no difference between the groups in prehabituation activity, $F(1,40)=3.52$, $p>0.05$. As one may see from Fig. 1, however, the 6-OHDOPA animals were more reactive to the tones over all blocks of trials than controls. When the first ten trial blocks were submitted to analysis of variance there occurred a significant main effect for groups, $F(1,40)=14.22$, $p<0.001$ and trials, $F(9,360)=5.684$, $p<0.000$. The interaction, however, did not reach statistical significance, $F(9,360)=1.364$, $p<0.203$, indicating that the decrement in startle response frequency to the tone bursts was no greater in one group than the other. Similar analyses were carried out on trial blocks 10 and 11, and for trial blocks 11 through 13. Following the sensitizing stimulation which occurred between trial blocks 10 and 11, the treated group remained significantly more active than the control, $F(1,40)=10.70$, $p<0.002$, and both groups exhibited dishabituation as evidenced by a significant trials effect from trial block 10 to 11, $F(1,40)=6.44$, $p<0.015$. The increased activity was approximately the same for both groups, as reflected by a nonsignificant groups-by-trials interaction. $F(1,40)=0.015$, $p<0.0701$. The hyperactivity of the experimental group persisted in trial blocks 11 through 13, $F(1,40)=11.70$, $p<0.002$, although neither the trials effect, $F(2,80)=2.98$, $p<0.057$, nor the interaction, $F(2,80)=2.41$, $p<0.096$, reached statistical significance.

Catecholamine Analysis

Neonatal treatment with 6-OHDOPA produced differential effects in various regions of the brain (Figs. 2 and 3). The brainstem regions generally exhibited moderate to marked elevations of NE. In the midbrain and pons-medulla, NE was increased by 97% and 128% respectively. In the hypothalamus and cerebellum also, there was an NE increase, but

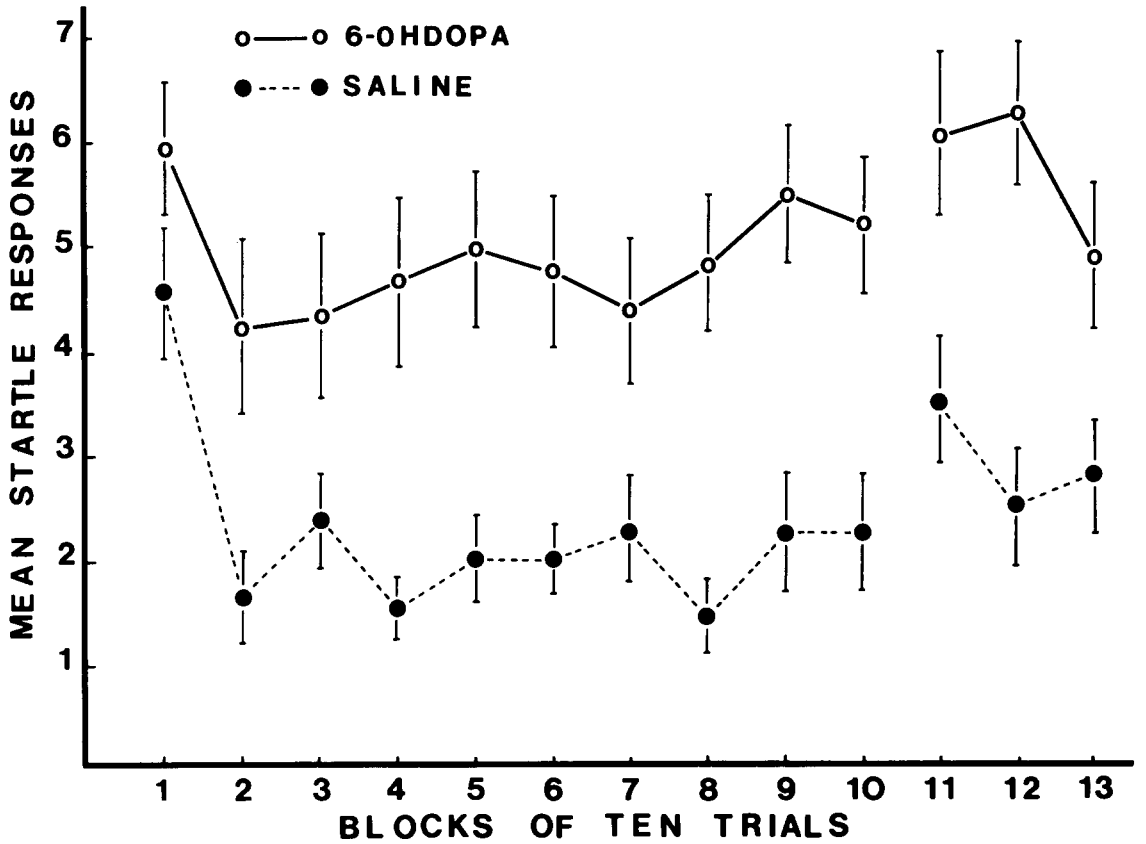

FIG. 1. Mean (±SEM) number startle responses to ten tone burst per block for rats treated with saline or 6-OHDOPA (60 μg/g, IP) on Days 1 and 3 of life (N=21 per group).

more moderate (31% and 33%, respectively). Therefore, neonatal 6-OHDOPA affected the brainstem and cerebellum by significantly elevating NE.

In contrast to the elevated NE in the brainstem, there was significant NE depletion in telencephalic structures and the spinal cord. In the amygdala NE was reduced by 58%. More severe depletion was produced in the hippocampus where NE concentration was so slight that it could not be detected by the assay. The cortex also exhibited marked NE depletion in both anterior and posterior regions (68% and 89%, respectively). Alterations in the spinal cord were similar to those of the hippocampus in that NE was reduced to such a level that it was not measurable.

In spite of these effects on NE content, striatal DA content in treated animals was not different from the controls. Striatal DA levels were 5.19 and 4.63 μg/g for the experimental and control groups, respectively. Further, the NE alterations produced by 6-OHDOPA were apparently limited to the CNS as there was no significant peripheral depletion of NE in the upper and lower ventricles of the heart (Fig. 2). Thus, the long term chemical effects of neonatal 6-OHDOPA appeared to be limited to central noradrenergic neurons.

DISCUSSION

The present study confirms and extends the effects of neonatal administration of systemic 6-OHDOPA. The biochemical effects are in agreement with previous findings [9,10] and may be characterized by NE depletion in forebrain structures and spinal cord, and NE elevation in brainstem regions and cerebellum. These results are also similar to the biochemical alterations produced by neonatal 6-hydroxydopamine (6-OHDA) [14] with one major difference. That is, neonatal 6-OHDA apparently produces more severe peripheral NE reduction than 6-OHDOPA, as treatment used in the present study resulted in no significant long-term peripheral NE changes. Hence, the behavioral effects reported here should not be ascribed to changes in the autonomic nervous system. Further, central catecholamine changes were apparently limited to NE as there was no effect on striatal DA, and previous research has shown 5-HT also to be unaltered in adults by such neonatal treatment [14]. Thus the behavioral effects reported here are likely due to alterations in central NE.

The failure of neonatal 6-OHDOPA to affect any of the components of female sexual behavior is in line with similar negative findings in the male [9]. It had originally been thought that sexual behavior might be affected since the 6-OHDOPA was injected within the critical period of development before the hypothalamus is organized according to a male or female pattern [2]. The profile of NE depletion in the present study, however, suggests that the terminals of the dorsal noradrenergic bundle are destroyed, not the ventral bundle which innervates the hypothalamus and median eminence area. Hence, neural areas responsible for sexual behavior and/or gonadotrophin release are relatively unaffected by neonatal 6-OHDOPA injections.

There is a previous report of neonatal 6-OHDA producing markedly delayed vaginal opening and prolonged estrous cycles [16]. The authors point out that their animals were

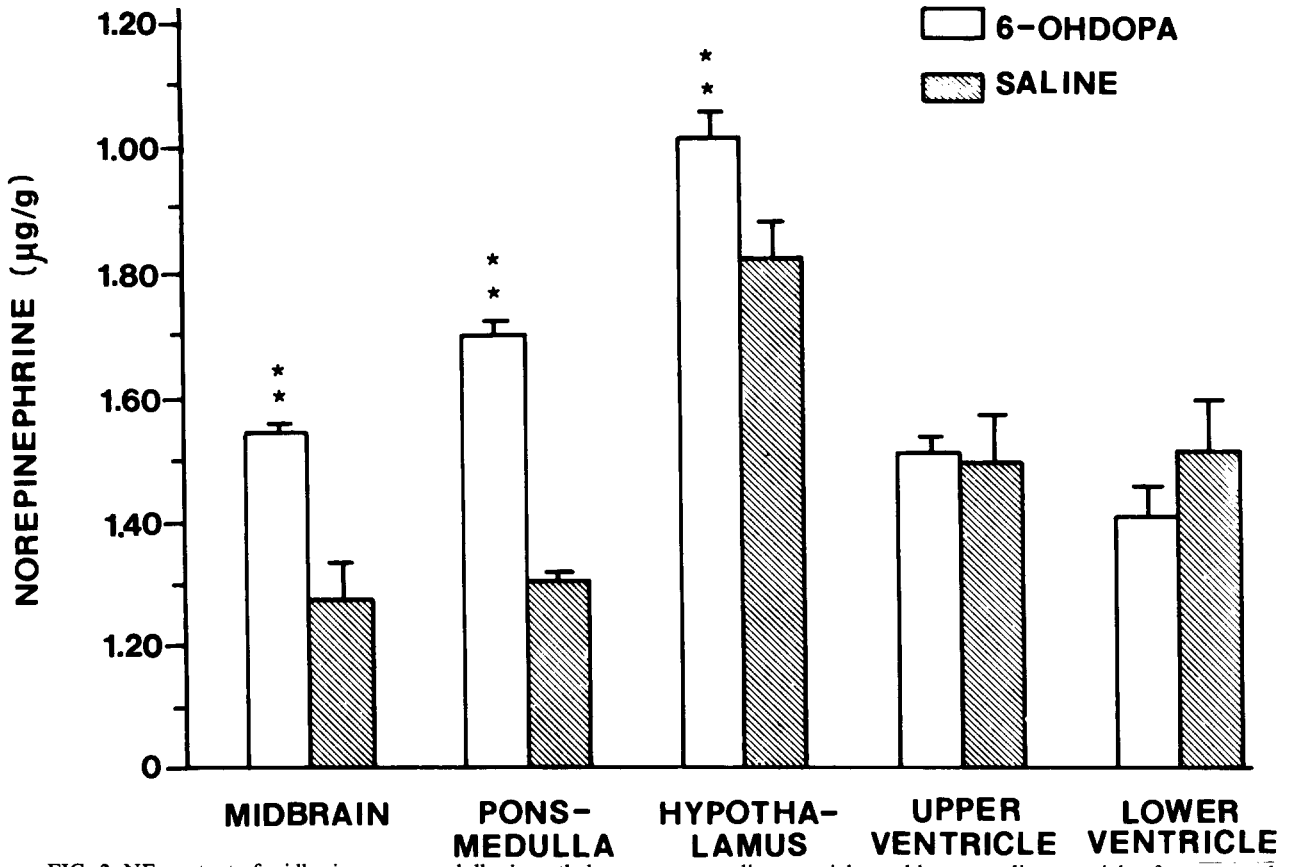

FIG. 2. NE content of midbrain, pons, medulla, hypothalmus, upper cardiac ventricle, and lower cardiac ventricle of rats treated with 6-OHDOPA (60 µg/g, IP) or saline on Days 1 and 3 of life and sacrificed at 6 months. Each column represents the mean ± SEM of 5–15 animals. †NE level below sensitivity of assay. $^{**}p<0.01$.

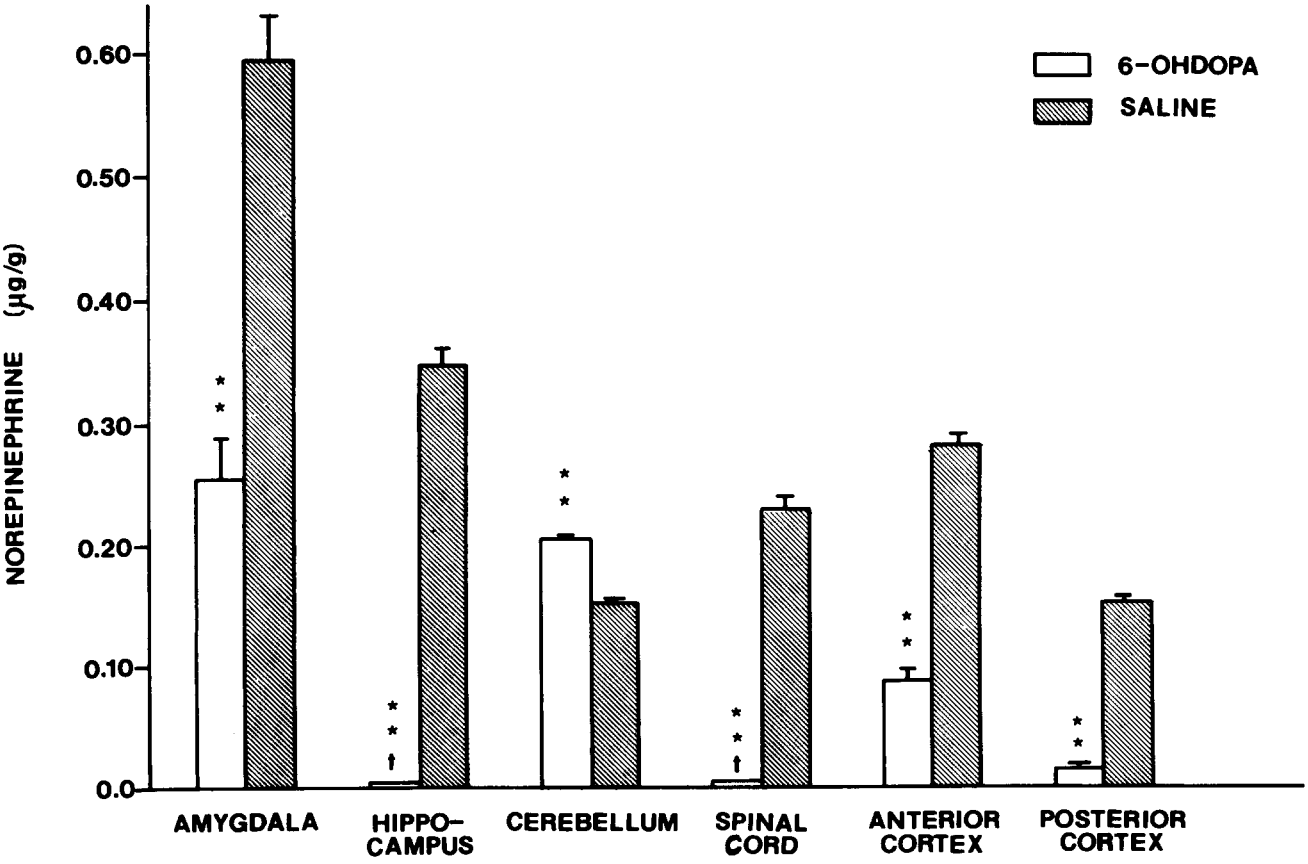

FIG. 3. NE content of amygdala, hippocampus, cerebellum, spinal cord, anterior cortex and posterior cortex. Legend as in Fig. 2.

very severely affected by the treatment, with a 30–40% weight loss. The vaginal smears in pair-fed animals, however, indicated no such prolongation of the estrous cycle. Thus, they suggested the endocrine effects were due to retardation of body growth. The experimental animals in the present study exhibited no changes in day of vaginal opening or length of estrous cycle, and as one might expect, weight loss was much less severe than in the Van Delft *et al.* study, with the experimental animals on the average 6 g lighter than the controls.

The activity and emotionality findings are in line with previous reports of neonatal 6-OHDOPA administration [9,10] in that the treated animals were less emotional and more active than the controls in the open field. The experimental animals were not only more active overall, they were also more likely to leave the perimeter of the open field and venture into the inner portion. Further, they were more likely to rear than the saline animals. Also, and in agreement with the activity data, the 6-OHDOPA animals demonstrated less emotionality as they were less likely to defecate than the controls.

In addition to alterations in activity and emotionality, the treated animals exhibited changes in equilibrium in that they were more likely to fall from the rod than were controls. Equilibrium disturbances have been reported previously following neonatal 6-OHDA [5], but in young, prepuberal animals. In the present study, however, equilibrium problems were present at approximately six months of age. The disruption of equilibrium is possibly due to the significant cerebellar NE elevation exhibited following neonatal 6-OHDOPA. Previous research has shown the elevated NE is likely due to an increase in the number of nerve terminals in the cerebellum that accumulate NE [7]. Perhaps this sprouting of cerebellar NE terminals results in equilibrium disturbances.

In the habituation task, while both groups exhibited a response decrement over trials, there was no evidence that one group habituated more rapidly than the other. The 6-OHDOPA animals, however, were more reactive to the tones over all trials than the control animals, just as they were more active in the open field. It has been proposed that the brainstem subserves habituation of responses to novel stimuli [3]. Since neonatal 6-OHDOPA treatment affects the nerve terminals of neurons whose perikaryia are in the brainstem, and since brainstem NE is elevated following neonatal 6-OHDOPA, it had been thought that rate of habituation to the tones would be different for the two groups. This hypothesis, however, was not supported. Similarly there was no difference between the groups in reaction to the sensitizing stimuli, as the increments in response frequencies exhibited from trial ten to eleven were approximately the same. Hence, the 6-OHDOPA subjects exhibited more reactivity than the controls to startle stimuli, but the two groups did not differ in habituation or sensitization.

In summary, the effects of neonatal 6-OHDOPA in the present study confirm previous biochemical findings. Similarly the general activity, emotionality, and reproductive behavior were also in line with previous work. There were no differences between the treated and control groups on habituation or sensitization to acoustic startle. The treated animals, however, were more reactive to the startle stimuli. Finally, the experimental animals exhibited impaired equilibrium when compared to the controls.

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