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The Development of Brain Biogenic Amines, Cyclic Nucleotides and Hyperactivity in 6-OHDA-Treated Rat Pups

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CONCANNON, J. T., J. M. BRAUGHLER AND M. D. SCHECHTER. *The development of brain biogenic amines, cyclic nucleotides and hyperactivity in 6-OHDA-treated rat pups.* PHARMACOL BIOCHEM BEHAV **18**(4) 477–482, 1983.— Developmental changes in the behavior and brain biochemistry of rat pups were investigated in rats administered intracisternal injections of 6-hydroxydopamine (6-OHDA) or its vehicle at 5 days of age. Although pups of both groups were equivalent in their activity at 15 days of age, 6-OHDA-induced hyperactivity emerged at 20 and 30 days of age in a between-group design in which rats were only tested at one age. Body weight measurements revealed that 6-OHDA-treated rats were underweight at 15, 25 and 30 days of age. Furthermore, at 20 days of age, total activity was inversely related to body weights in the 6-OHDA-treated pups. Whole-brain levels of dopamine (DA) were decreased at every age by the 6-OHDA treatment, whereas norepinephrine (NE) levels were virtually unaffected by 6-OHDA at these same ages. Total activity was inversely correlated with whole-brain DA levels at 20 and 30 days of age when 6-OHDA-treated pups were hyperactive. Measures of cerebellar and "rest-of-brain" adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP) were not uniformly altered by either the 6-OHDA treatment or by maturation. Results are discussed both in terms of brain biochemistry modulation of hyperactivity and the contribution of decreased body weights induced by 6-OHDA to the production of hyperactivity.

Hyperactivity 6-Hydroxydopamine Dopamine Norepinephrine Cyclic nucleotides Cyclic AMP Cyclic GMP Activity Developing rats Hyperkinesis Animal models

MANY investigators have attempted to model the human hyperkinetic syndrome [22] by administering the neurotoxin 6-hydroxydopamine (6-OHDA) to neonatal rat pups and observing the time-course of their behavioral activity [4–7, 19, 28, 29, 31, 32]. These attempts have met with varying degrees of success [24] in terms of (a) producing cardinal feature(s) of the syndrome; (b) bearing some temporal relationship to pathogenesis in humans; and (c) decreasing the hyperactivity by psychostimulant [26, 27, 29] medication. However, one outstanding feature seldom, if ever, realized is the ability to produce a change in brain biochemistry with 6-OHDA that correlates with the degree of hyperactivity produced [16].

One major impediment to uncovering reliable correlations between whole-brain DA depletion and activity is methodological. That is, the majority of studies conducted have utilized "within-subject" designs in which a single subject is repeatedly tested over several days [4–8, 10, 16, 18, 19, 28, 31, 32]. Furthermore, 6-OHDA-treated animals are usually sacrificed several days after the last activity observation day, and inferences are made concerning the post-sacrifice DA levels and activity data collected days to weeks earlier (cf. references above). Hence, it is not surprising that few, if any, researchers have successfully correlated brain DA levels with activity in these within-subject designs [16].

One aim of the present investigation, then, was to examine developmental hyperactivity induced by neonatal 6-OHDA treatment using a "between-group" design, with animals being sacrificed for biochemical determinations immediately after a single activity test on selected days of age. This procedure facilitates interpretation of the correlation between brain biochemistry and behavior, since they are collected from each animal on the same day of age. In addition, it allows for a clearer understanding of the relationship between body weight and behavior which may be important since 6-OHDA can reduce body weight [24] in addition to producing behavioral hyperactivity [28]. Hence, one may determine whether hyperactivity is related to DA depletion *per se* or due to its indirect effect on body weight [13] using this design. Another objective of the present study was to analyze levels of the cyclic nucleotides, adenosine 3′,5′-monophosphate (cyclic AMP) [23] and guanosine 3′,5′-monophosphate (cyclic GMP), in 6-OHDA- and vehicle-treated rats as a possible "marker" for the role of "second messengers" [11, 15, 20] in developmental hyperactivity.

METHOD

Animals

Sprague-Dawley-derived (Charles River) rats, born and raised in the Department colony, served as subjects. The parents were paired in single plastic breeding cages and the male was removed as soon as it was physically apparent that the female was pregnant. Within 2 days after birth, litters

were culled to 8 rat pups with an equal number of males and females, where possible. A total of 27 liters of animals were used in this experiment. On occasion a litter with less than 8 pups was fortified by addition of animals culled from other litters born on the same day. Throughout all phases of breeding and behavioral observation, animals were housed under controlled temperature and a 12-hour light/12-hour dark cycle. To keep the presence of mother constant across all ages, pups were not weaned. Food and water were provided ad lib.

Procedure

At 5 days of age, rat pups were toe-clipped for identification and were randomly assigned to one of three treatment groups: (a) systemic distilled water (W) and intracisternal (IC) distilled water; (b) systemic desmethylimipramine (DMI) and intracisternal ascorbic acid (0.4 mg/ml ascorbate in distilled water); or (c) systemic DMI and intracisternal 6-hydroxydopamine (6-OHDA) [28]. All pups were lightly anesthetized with ether prior to the IC injections. In addition, a light cover of petroleum jelly was applied to the head and neck region of each pup to prevent leakage of cerebrospinal fluid after the IC injection which was 30 sec in duration. Activity was determined in separate groups of animals at 15, 20, 25 and 30 days of age, between the hours of 1300 and 1600, using the time-sampling technique described in detail by Shaywitz *et al.* [28].

Starting at 15 days of age, and at 5 day intervals thereafter, separate groups of rat pups were removed from the mother and individually placed into 33×27×17 cm clear plastic cages for behavioral observation. Each cage was scanned every min for 1 hr, thus generating 60 measures for each animal on a particular observation day. At each observation time, behavior was recorded using one of the following mutually exclusive and exhaustive categories [28]—sleeping, inactive, ambulating, climbing, rearing, eating, drinking, sniffing, grooming, or scratching. The last 8 categories were combined for the "Total Activity" score [28]. Similar results were found for "Very Active" behaviors (ambulating, climbing, rearing, eating and drinking combined), i.e., when "in place" activity was deleted from analysis. This activity was determined by one of two observers, completely naive concerning treatment condition, who showed at least 90% agreement on the activity categorization.

Drugs and Dosage Rationale

6-OHDA HBr (Aldrich Chemical Co, Milwaukee, WI), 150 μg, calculated as free base, was dissolved immediately before administration in 25 μl of distilled water with 0.4 mg l-ascorbic acid/ml added to retard oxidation. The two vehicle-treated littermate control groups received an injection of an equivalent volume of either distilled water or ascorbic acid. Hence, all treatments were represented within each litter [4, 5, 21] using heterogeneous litter conditions [21]. The 6-OHDA or ascorbate injections were preceded 60 min earlier by an intraperitoneal (IP) injection of 30 mg/kg DMI HCl (as base) dissolved in distilled water and administered in a constant volume of 1.0 ml/kg body weight. The IC distilled water injection was preceded 60 min earlier by an IP injection of an equivalent volume of distilled water. This DMI/6-OHDA treatment regimen has been reported to consistently produce selective depletion of central dopamine levels with little or no effect on central norepinephrine levels [4, 5, 28].

Biochemical Determinations

Separate groups of pups were decapitated at 15, 20, 25, and 30 days of age immediately after their activity testing. The brains were rapidly removed and dissected midsagitally after the cerebellum was removed. These three brain parts were then immediately submerged into liquid nitrogen and were subsequently kept at −70°C for biogenic amine and cyclic nucleotide analysis. One-half of the "rest of brain" (minus cerebellum) was used for dopamine (DA) and norepinephrine (NE) determinations. The other half of the "rest of brain" and the whole cerebellum were utilized for cyclic AMP and cyclic GMP assays.

Levels of DA and NE were analyzed by high-pressure liquid chromotagraphy (HPLC), according to the method of Felice *et al.* [9], and as previously employed in this laboratory [4–6]. Data were expressed in terms of ng/g tissue of DA and NE. Cyclic nucleotide content in frozen brain tissue was determined by radioimmunoassay (RIA) essentially as described by Harper and Brooker [12]. Briefly, 10–50 mg of frozen brain tissue was homogenized in 1 ml of 5% trichloroacetic acid at 4°C with a polytron tissue homogenizer. The homogenate was centrifuged and the resulting supernatant fraction was extracted 3 times with 4 volumes of water-saturated ether. Cyclic AMP and cyclic GMP were then determined by RIA as previously described [1,2]. In some cases, samples were acetylated prior to RIA [12]. Authenticity of the cyclic nucleotide extracted was verified by recovery of "cold" cyclic nucleotide added to the homogenate and the destruction of assayable cyclic nucleotide by the addition of excess phosphodiesterase. The trichloroacetic acid pellet was then dissolved in 1N NaOH and the protein was determined by the method of Lowry [14]. Results were based on duplicate or triplicate determinations from single tissue specimens from all of the animals in all treatment groups. Cyclic nucleotide levels were expressed as pmoles of cyclic AMP or cyclic GMP/mg protein and are reported separately for the cerebellum and the "rest of brain".

Statistical Methods

Measurements of each category of activity were calculated as percentage of occurrence during the 60-min observation period (i.e., number of times active divided by 60 times 100). For brevity of reporting, and for direct comparison to the reports of Shaywitz [26–28], only the category of "total activity" was analyzed using a 2 (treatment: 6-OHDA vs. combined controls) × 4 (age) between-group unweighted means analysis of variance (ANOVA). (Preliminary observations failed to reveal any sex differences or differences between the 2 control groups. Hence, these factors or levels within factors were not considered in the ANOVAs). Subsequent to this analysis, all between-group comparisons were made utilizing Duncan's Multiple Range Test derived from the appropriate error term. Similar ANOVAs were performed for (1) DA levels, (2) NE levels, (3) cyclic AMP in "rest of brain", (4) cyclic AMP in cerebellum, (5) cyclic GMP in "rest of brain", (6) cyclic GMP in cerebellum, (7) cyclic GMP/cyclic AMP ratio in "rest of brain", and (8) cyclic GMP/cyclic AMP ratio in cerebellum. Throughout the experiment $p<0.05$ was considered to be statistically signifi-

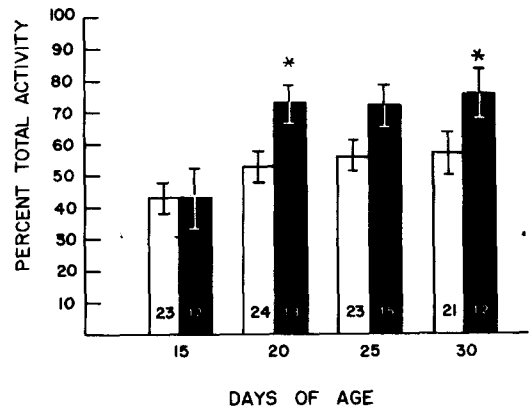

FIG. 1. Mean (±S.E.M.) percent total activity for rat pups during development. Ordinate: activity represented as a percent of total observations during a 60-min period. Abscissa: postnatal age in days. Open columns, vehicle-treated; solid columns, 6-OHDA-treated. Numbers inside columns refer to sample sizes. *Differs from vehicle-treated animals, $p<0.05$ using Duncan's Multiple Range Test.

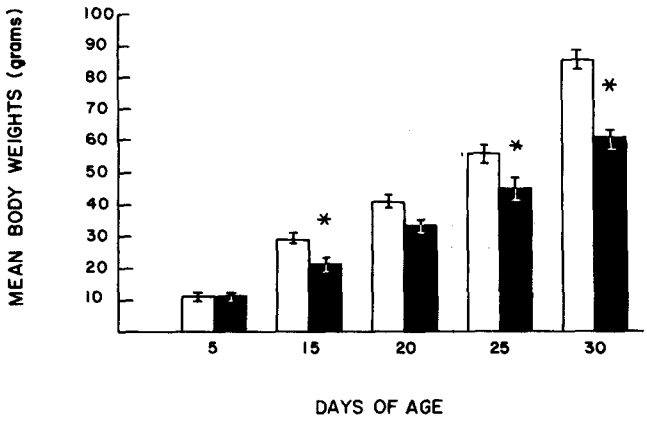

FIG. 2. Mean (±S.E.M.) body weights (in grams) for rat pups during development. Symbols have same meaning as in Fig. 1.

cant. Sample sizes ranged from 12 to 23 subjects per age per treatment (i.e., 6-OHDA vs. vehicle).

RESULTS

Activity Levels

Depicted in Fig. 1 is the percentage of "Total Activity" [28] as a function of Age and Type of Brain Injections (6-OHDA vs. vehicle). These results indicate that the control animals exhibited a slight increase in activity from 15 to 20 days of age, followed by a plateau in activity at moderate levels. The 6-OHDA-treated animals are similar in their activity at 15 days of age, but tend to be hyperactive thereafter. However, the hyperactivity observed in the 6-OHDA-treated rat pups was statistically significant from controls only at 20 and 30 days of age. As with our previous reports using a within-subject paradigm, 6-OHDA-induced

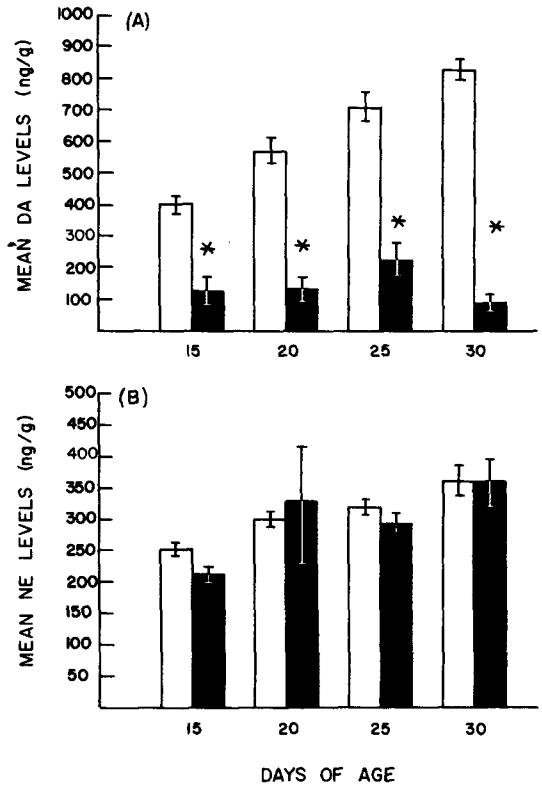

FIG. 3. Mean (±S.E.M.) "whole-brain" levels (ng/g) of (A) dopamine and (B) norepinephrine during development. Symbols have same meaning as in Fig. 1.

hyperactivity did not abate as the animals approached adulthood, i.e., at the 30-day-old observation time [4,6].

Body Weights

Figure 2 presents mean body weights as a function of Age and Brain Treatment. The two groups of animals initially had almost identical body weights, and the rate of growth is similar through day 25, although the 6-OHDA-treated animals weighed significantly less beginning at 15 days of age. At 30 days of age, the weight differences are most pronounced. It is unlikely that weight differences alone, however, accounted for the differences in activity, since 6-OHDA-treated animals were significantly hyperactive at 20 days of age, although they did not consistently weigh less than the vehicle-treated animals at this time. Furthermore, although total activity and body weight were inversely correlated at 20 days of age ($r=-0.63$, $p<0.05$) in the 6-OHDA-treated animals, this relationship failed to reach statistical significance at 30 days of age ($r=-0.57$, $p<0.10$), another day on which hyperactivity was detected.

Biochemistry

Dopamine and norepinephrine levels. Figure 3 presents the dopamine (top panel) and norepinephrine (lower panel) levels as a function of Age and Brain Treatment. (The actual DA and NE values are presented in Table 1). For normal animals, DA levels increased about 200% from 15 days of age

TABLE 1

MEAN (±SEM) LEVELS OF DA AND NE (ng/g TISSUE) AND OF c-AMP AND c-GMP (pmol/mg PROTEIN) AND THE c-GMP/c-AMP RATIOS IN "WHOLE BRAIN" AND CEREBELLUM OF PUPS

		Age (Days) 15	20	25	30
V:	DA/"whole-brain"	404.26 ±33.62	569.80 ± 41.75	705.58 ±49.07	831.03 ±34.15
6:	DA/"whole-brain"	*127.27 ±39.73	*133.91 ±45.00	*220.83 ±52.08	*92.40 ±18.45
V:	NE/"whole-brain"	251.03 ±11.82	293.79 ±15.57	322.19 ±15.40	365.21 ±18.92
6:	NE/"whole-brain"	211.35 ±15.72	330.58 ± 86.72	288.58 ±22.04	367.51 ±25.23
V:	c-AMP/"whole brain"	107.76 ± 9.04	100.43 ± 14.79	132.18 ±19.82	123.50 ±16.25
6:	c-AMP/"whole-brain"	115.54 ±17.61	90.78 ± 9.72	98.06 ±15.54	121.14 ±14.10
V:	c-AMP/cerebellum	84.02 ± 8.68	230.31 ±128.83	216.98 ±30.94	193.92 ±24.81
6:	c-AMP/cerebellum	92.52 ±13.52	115.70 ± 13.16	138.04 ±14.32	234.68 ±28.92
V:	c-GMP/"whole-brain"	2.06 ± 0.19	2.07 ± 0.27	1.75 ± 0.14	2.05 ± 0.31
6:	c-GMP/"whole-brain"	*3.02 ± 0.91	1.82 ± 0.16	1.95 ± 0.20	1.75 ± 0.21
V:	c-GMP/cerebellum	3.77 ± 0.68	5.20 ± 1.19	6.93 ± 0.81	5.11 ± 0.75
6:	c-GMP/cerebellum	4.74 ± 1.48	6.34 ± 1.99	5.39 ± 1.10	6.67 ± 1.46
V:	$\frac{\text{c-GMP}}{\text{c-AMP}}$/"whole-brain"	0.020± 0.002	0.023± 0.002	0.051± 0.032	0.021± 0.003
6:	$\frac{\text{c-GMP}}{\text{c-AMP}}$/"whole-brain"	0.024± 0.004	0.021± 0.001	0.024± 0.003	0.016± 0.002
V:	$\frac{\text{c-GMP}}{\text{c-AMP}}$/cerebellum	0.050± 0.009	0.038± 0.004	0.041± 0.005	0.027± 0.004
6:	$\frac{\text{c-GMP}}{\text{c-AMP}}$/cerebellum	0.047± 0.007	0.054± 0.012	0.038± 0.006	0.029± 0.006

V=vehicle; 6=6-OHDA.
*$p<0.05$ vs vehicle control, Duncan's Multiple Range Test.

to 30 days of age. The 6-OHDA-treated animals are significantly below control values at all ages, although the difference obviously increases with age. The NE levels, on the other hand, indicate a 70% increase from day 15 to day 30 in both groups of animals. Lastly, NE levels were not statistically different between the two groups of rat pups at any age.

An analysis of the relationship between DA levels and total activity for 6-OHDA-treated animals was conducted at 20 and 30 days of age, the two days on which hyperactivity was detected. The inverse correlations at these two ages (r's=−0.89 and −0.73, respectively) were statistically significant, while correlations at days 15 (r=−0.33) and 25 (r=−0.16) were not statistically significant. The relationship between total activity and other biochemical indices was not examined at these ages due to lack of between-group differences in any index other than whole-brain DA levels (see below).

Cyclic nucleotide levels and ratios. Presented in Table 1 are the levels of cyclic AMP and cyclic GMP in the "rest-of-brain" and the cerebellum, as well as the ratio of cyclic GMP to cyclic AMP [30] in these brain regions. The results of these analyses were rather inconsistent in terms of generating striking age- or treatment-dependent effects. However, two reliable effects were detected: (1) the cyclic GMP/cyclic AMP ratio declined reliably as a function of age in the cerebellum, and (2) cyclic GMP levels in the "rest-of-brain" were elevated in the 6-OHDA-treated animals at 15 days of age.

DISCUSSION

The results of the present investigation indicate that neonatal 6-OHDA treatment produced hyperactivity using a between-subject design and that the degree of activity was inversely correlated with whole-brain levels of dopamine for two age groups. To the best of our knowledge this is the first time that this inverse correlation has been convincingly demonstrated on a between-group basis. In addition, it was shown that hyperactivity was present at the last observation time (i.e., 30 days of age) as is the case with some within-subject paradigms. Hence, hyperactive behavior did not return to the control level as animals approached adulthood [4,6]. The reason for failure of the 6-OHDA-treated rats to return to control levels of activity [28] is presently unknown, although similar findings have recently been reported [4, 6, 8, 16, 18]. Furthermore, there were no apparent sex differences in hyperactivity [4], which is probably the result of the level of DA depletion. That is, only males were hyperactive when DA was depleted 38% [4], while both sexes were hyperactive when DA was depleted 77–89% (on days 20 and 30, present study).

Since parts of this study were correlational, it is difficult to prove that DA depletion, and it alone, caused hyperactivity. This is particularly true in the present study since 6-OHDA treated animals were not hyperactive at 15 and 25 days of age, even though DA levels were lowered considerably at these two ages. Differences in activity between the 6-OHDA and normal groups failed to reach statistical significance at 25 days of age, although it may have occurred with larger sample sizes or with a greater degree of DA depletion. Failure to find hyperactivity at 15 days of age is more problematic but is a representative finding [4–6, 8, 10, 16, 19, 26] regardless of the amount of DA depletion found at sacrifice, which was long after 15 days in the studies quoted above.

Hence, DA depletion probably interacts with some presently undefined developmental event(s) to produce hyperactivity.

In this study, the basal levels of the cyclic nucleotides were virtually unaffected by maturation [23] or by the 6-OHDA treatment. In fact, the 6-OHDA treatment altered only the levels of cyclic GMP/"whole-brain" in the 15-day-old animals. At this time, cyclic GMP was elevated while activity was unchanged by neonatal 6-OHDA administration. Perhaps differences which tended to emerge would be magnified had we specifically measured dopamine-stimulated nucleotides. Indeed, perusal of the literature indicates that basal levels of nucleotides, particularly cyclic AMP, are usually not elevated by 6-OHDA administration in adult rats [3, 17, 33], whereas they are greatly elevated when the tissue preparation is stimulated with selective ligands [3, 17, 33]. Additionally, levels of nucleotides may have been altered in brain regions that were not assayed in the present study. Both of these possibilities are under investigation.

The final point to be discussed concerns the correlation between body weight and activity in the 6-OHDA-treated rats, a potential confound in animal models of hyperactivity [13]. These inverse correlations at fairly high levels of dopamine depletion suggest that it may be imprudent to lower dopamine levels to less than 25% of controls, as is often the case in adult rat studies, in 6-OHDA-treated pups. Under these conditions, one runs the risk of not only decreasing body weight but discovering the inverse correlations between activity and body weight as presently found (e.g., on day 20). Without additional information, e.g., additional age groups, one is uncertain whether hyperactivity is the direct result of DA depletion or whether it is an indirect effect of under- or malnutrition (cf. [13]). Indeed, previous research by Shaywitz *et al.* [25] has shown that hyperkinetic children have homovanillic acid (HVA: the primary dopamine metabolite) levels that are 60% of controls, suggesting that hyperactivity in children may result from only a 40% reduction in brain dopamine. Furthermore, we have previously detected hyperactivity using 6-OHDA in male rat pups when dopamine was depleted by only 40% [4], and this depletion was not accompanied by a decrease in body weight. These combined findings suggest yet another corollary of a valid animal model of hyperkinesis; namely, to reduce DA to levels typical for those existing in the human situation while not producing excessive decrements in body weight by the brain manipulation.

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