Biochemical Pharmacology, Vol. 25, pp. 2387–2390. Pergamon Press, 1976. Printed in Great Britain.

EFFECTS OF EARLY POSTNATAL GUANETHIDINE ADMINISTRATION ON ADRENAL MEDULLA AND BRAIN OF DEVELOPING RATS

JORGE BARTOLOMÉ, MARÍA BARTOLOMÉ, FREDERIC J. SEIDLER, THOMAS R. ANDERSON* and THEODORE A. SLOTKIN

Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N.C. 27710, U.S.A.

(*Received* 28 *October* 1975; *accepted* 26 *March* 1976)

Abstract—Starting at 2 days of postnatal age, rats were injected with guanethidine (50 mg/kg, s.c.) once daily for 5 days and the adrenals were analyzed for catecholamines (CA), tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH); the brain was analyzed for TH and ornithine decarboxylase (ODC). Guanethidine treatment produced a 40–80 per cent increase in the adrenal CA, TH and DBH values, with return to normal by 3–4 weeks of age. Pretreatment of neonates with chlorisondamine (10 mg/kg, s.c.) prevented the stimulatory effects, indicating that guanethidine might act by direct nicotinic stimulation in neonates. In contrast, administration of guanethidine to adult rats had little or no effect on adrenal CA, TH or DBH, indicating that the stimulatory effect is unique to the developing animal. In the brain, administration of guanethidine resulted in an initial deficit and subsequent enhancement of ODC activity, suggesting a delay in cellular proliferation. TH activity was stimulated initially but was subnormal at later stages; the latter phenomenon may be related to incomplete function of the neonatal blood–brain barrier permitting a consequent central neurotoxic effect of guanethidine.

Administration of the antihypertensive drug, guanethidine, to developing animals produces markedly different effects from those seen in adults. Morphological, biochemical and functional studies conducted in rats and mice have indicated that guanethidine given chronically during the first 10–20 days of development produces an irreversible and almost complete peripheral sympathectomy [1, 2]; in adults, guanethidine administration must be maintained for much longer periods to produce adrenergic nerve damage [3]. In the mature rat, guanethidine is unable to cross the blood–brain barrier and, therefore, exerts little effect on the central nervous system (CNS) [4]. However, in newborn rodents the blood–brain barrier appears to be incompletely developed, thus allowing access of the drug to the brain compartment. Consequently, administration of the drug to mice during development causes long-lasting decreases in catecholamines in the CNS [5].

One adrenergic tissue whose developmental mechanisms have been well studied is the adrenal medulla [6–11]. During maturation of the rat adrenal medulla, catecholamine levels, tyrosine hydroxylase and dopamine β-hydroxylase undergo a series of changes which are in part dependent on the levels of neuronal input to the gland [9–12]. At birth, innervation of the rat adrenal medulla appears to be nonfunctional [11, 13]. As a result, drugs like morphine and reserpine, which in adult rats elicit trans-synaptic induction of tyrosine hydroxylase and dopamine β-hydroxylase, fail to do so when given to neonates [13, 14]. Accordingly, it seemed likely that guanethidine, although having little effect on the adult adrenal [15], when administered early in development could produce effects on catecholamines and catecholamine biosynthetic enzymes not observed in the adult.

METHODS

Treatment of rats. Timed pregnant Sprague–Dawley rats (Zivic–Miller) were housed in individual breeding cages and allowed food and water *ad lib.*; litter size ranged from eight to ten pups. Starting at 2 days of postnatal age, pups were injected with guanethidine (50 mg/kg, s.c.) once daily for 5 days while controls received vehicle (0.9% NaCl, 0.1% ascorbic acid, pH 7.4, prepared daily). Rats were killed by decapitation at various time intervals from 3 to 49 days of age. In some experiments, pups were pretreated (30 min) with chlorisondamine (10 mg/kg, s.c.) before receiving guanethidine, and sacrificed 24 hr after the second injection.

Studies on adrenals. Adrenal glands were excised and homogenized (glass-to-glass) in 2.5 ml of ice-cold 0.3 M sucrose containing 0.025 M Tris (pH 7.4) and 10^{-5} M iproniazid (irreversible monoamine oxidase inhibitor). At early stages of development, adrenals from several pups were pooled to obtain sufficient material. One-tenth ml of the homogenates was withdrawn and deproteinized with 1.9 ml of 3.5% perchloric acid, centrifuged at 26,000 *g* for 10 min, and supernatants were analyzed for catecholamines (CA) by the trihydroxyindole method using an autoanalyzer [16]. One-half ml of the remaining homogenates was added to an equal volume of water containing 2000 units/ml of beef catalase, and used for duplicate assay of dopamine β-hydroxylase activity by the method of Friedman and Kaufman [17], with 10^{-5} M [^{3}H]tyramine as substrate and *p*-hydroxymercuribenzoate (optimal concentration, 0.5 mM) to inactivate endogenous inhibitors. The remainder of the homo-

* Present address: School of Dentistry, University of North Carolina, Chapel Hill, N.C., U.S.A.

genates was centrifuged at 26,000 *g* for 10 min to sediment the CA storage vesicles, and the supernatants were used for duplicate determinations of tyrosine hydroxylase activity by the method of Waymire *et al.* [18], with 100 μM [^{14}C]tyrosine as substrate.

Studies on whole brain. Brains were homogenized in 9 vol of 0.01 M Tris (pH 7.2) and duplicate 0.1-ml aliquots of the homogenates were used for assay of tyrosine hydroxylase activity as described above, except that 0.1% Triton and 0.7 mM $CaCl_2$ (final concentrations) were added to optimize activity. The rest of the homogenate was diluted with an equal volume of 0.01 M Tris (pH 7.2), centrifuged for 10 min at 26,000 *g* and the supernatant used for assay of ornithine decarboxylase activity according to Anderson and Schanberg [19], with 12 μM [^{14}C]ornithine as substrate.

Statistics. Results are expressed as means ± standard errors and levels of significance are calculated by Student's *t*-test.

Materials. Tyramine[G-^{3}H] (10 Ci/m-mole), *dl*-epinephrine-7-[^{3}H] (10 Ci/m-mole), L-tyrosine-1[^{14}C] (50 mCi/m-mole) and *dl*-ornithine-1[^{14}C] (40 mCi/m-mole) were purchased from New England Nuclear Corp. Guanethidine sulfate and chlorisondamine chloride were obtained from Ciba Pharmaceutical Co. Epinephrine bitartrate was obtained from Winthrop Laboratories and iproniazid phosphate, tyramine HCl, *p*-hydroxymercuribenzoate and beef liver catalase from Sigma Chemical Corp.

RESULTS

Effects of guanethidine on adrenal medulla. During the course of development, catecholamines (CA) increased from 0.43 μg/gland at 3 days of age to 9.72 μg/gland at 49 days (Table 1). Over the same period, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) activities increased approximately 10-fold.

Administration of guanethidine to developing rats caused a significant increase in the levels of CA, TH and DBH (Fig. 1). At 3 and 5 days of age, TH was elevated approximately 30 per cent, while CA and DBH were unchanged. Between 7 and 11 days of age, all three parameters showed maximal increases of 40–80 per cent, with return to normal by 2 to 3 weeks after the last injection. The guanethidine-treated animals displayed little or no alterations in weight gain, nor were there any discernable differences in appearance or activity.

To determine whether the augmented CA and enzyme levels observed after guanethidine administration reflected a direct stimulation of the adrenal via nicotinic receptors, chlorisondamine (a long-lasting ganglionic blocker) was administered 30 min prior to guanethidine. As shown in Table 2, chlorisondamine completely blocked the TH increase. Chlorisondamine itself had no effect on adrenal TH ($P > 0.1$).

In adult rats, administration of guanethidine over the same time course had no effect on adrenal CA, TH or DBH (data not shown).

Effects of guanethidine on brain. Over the course of development, brain weights in control rats increased from 0.41 g at 3 days of age to 1.7 g at 49 days (Table 3). During the same period of time, TH levels increased approximately 10-fold.

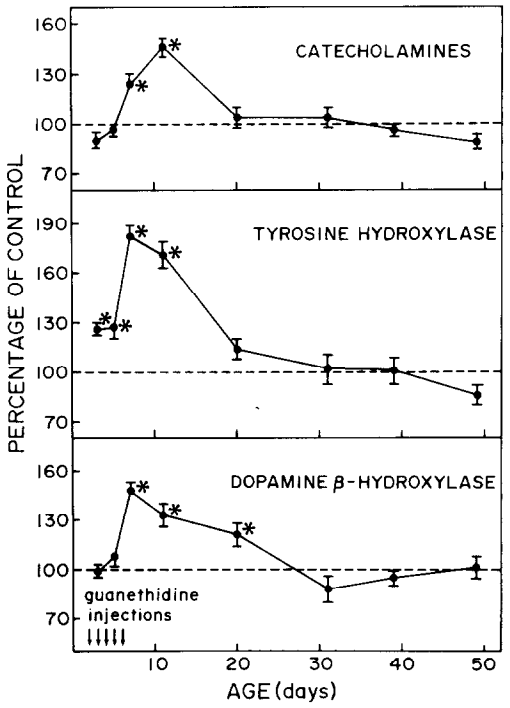

Fig. 1. Adrenal catecholamines, tyrosine hydroxylase and dopamine β-hydroxylase activities per gland in developing rats treated with guanethidine (50 mg/kg, s.c.) as percentage of control. Points and bars represent means ± S.E.M. of five to six determinations; asterisks denote significant differences from controls ($P < 0.05$ or better). Control values are shown in Table 1.

Table 1. Development in control rats of adrenal catecholamines, tyrosine hydroxylase and dopamine β-hydroxylase activities*

Age (days)	Catecholamines (μg/gland)	Tyrosine hydroxylase (nmoles $^{14}CO_2$ evolved/gland/hr)	Dopamine β-hydroxylase (nmoles [^{3}H]octopamine formed/gland/hr)	No. of determinations
3	0.428 ± 0.027	0.93 ± 0.04	0.096 ± 0.004	4
5	0.697 ± 0.024	1.27 ± 0.05	0.083 ± 0.005	6
7	0.611 ± 0.021	1.37 ± 0.11	0.145 ± 0.006	6
11	0.999 ± 0.083	1.32 ± 0.11	0.180 ± 0.012	6
20	2.53 ± 0.12	2.09 ± 0.13	0.335 ± 0.010	10
31	5.86 ± 0.31	5.47 ± 0.32	0.555 ± 0.043	6
39	6.00 ± 0.34	6.02 ± 0.18	0.601 ± 0.040	5
49	9.72 ± 0.51	9.64 ± 0.36	0.897 ± 0.073	11

* Starting at 2 days of age, control pups were injected with vehicle (0.9% NaCl, 0.1% ascorbic acid, pH 7.4, prepared fresh) once daily for 5 days. Rats killed at 3 and 5 days of age were sacrificed 24 hr after the previous injection. Data represent means ± S.E.M.

Table 2. Effects of chlorisondamine pretreatment on guanethidine-induced increases in adrenal tyrosine hydroxylase of 2-day-old pups*

First injection	Second injection (30 min later)	Tyrosine hydroxylase† (24 hr after second injection)	No. of determinations
Saline	Saline	100 ± 7	18
Chlorisondamine	Saline	86 ± 5	6
Saline	Guanethidine	127 ± 8‡	6
Chlorisondamine	Guanethidine	94 ± 5	6

* Doses of chlorisondamine and guanethidine were 10 mg/kg, s.c., and 50 mg/kg, s.c. respectively. Data represent means ± S.E.M. Control value was 1.27 ± 0.09 nmole $^{14}CO_2$ evolved/gland/hr.

† Measured as percentage of control.

‡ $P < 0.02$ vs saline–saline.

Administration of guanethidine produced slight (5–15 per cent) reductions in brain weight on a gram basis at 3 and 7 days of age (Fig. 2). TH activity was elevated at 5 days of age, but showed a subsequent deficit at 11 and 20 days, followed by a trend to subnormal values (10 per cent) throughout the rest of the experiment. Guanethidine had no effect on brain TH in adult rats (data not shown).

It has been shown that in proliferating tissues (such as developing organs) both polyamine concentrations and the activity of ornithine decarboxylase (ODC), an enzyme involved in polyamine synthesis, are elevated [20–22]. Recent studies have demonstrated that, in developing rat brain, the periods of high ODC activity correspond to maximal cell proliferation; administration of thyroxine or cortisol produces alterations in the developmental pattern of ODC activity which parallel behavioral abnormalities [19, 23], suggesting the utility of the pattern of ODC development as an early index of disturbed brain maturation. Consequently, the effects of guanethidine on ODC in developing brain were assessed to determine if the drug might pass the immature blood–brain barrier to exert effects on the developing central nervous system. ODC activity in control rats decreased from 1.2 nmoles $^{14}CO_2$ evolved/brain/hr at 3 days of age to 0.031 units at 20 days (Table 3). At 3 days of age ODC activity in guanethidine-treated pups was lower than in controls (Fig. 2); however, from 5 to 11 days of age the activity was higher than control and returned to normal by 20 days.

DISCUSSION

Administration of guanethidine to rats during the first days after birth resulted in an increase in adrenal catecholamines and tyrosine hydroxylase and dopamine β-hydroxylase activities, indicating an acceleration in the maturational gains of the key enzymes and product of this branch of the sympathetic system. The pattern is similar to that observed in adult rats after chronic administration of drugs which cause direct or reflex stimulation of the adrenal [24–26]. However, in the rat, innervation of the adrenal medulla by the splanchnic nerve is not functional until 8 to 10 days of age [11, 14], as evidenced by lack of secretion in response to insulin and lack of tyrosine hydroxylase induction in response to reserpine. Thus, the fact that guanethidine-induced increases appear prior to the establishment of connections with the central nervous system rules out the possibility that the effect is mediated via trans-synaptic signals or autonomic reflexes. Despite the non-functional nature of the innervation of the adrenal medulla during the first few days of postnatal life, functional nicotinic receptors are present [14]. Blockade of nicotinic receptors by chlorisondamine prior to guanethidine administration completely prevented tyrosine hydroxylase induction, supporting the hypothesis that guanethidine might act by direct nicotinic stimulation in neonates.

In contrast to the results obtained in neonates, guanethidine given to adult rats did not alter adrenal catecholamines, tyrosine hydroxylase or dopamine β-hydroxylase, confirming a previous report on lack of tyrosine hydroxylase induction [15], and indicating that the direct stimulatory effect is unique to the developing animal. These data suggest that either the specificity of adrenomedullary nicotinic receptors changes with development, or that in later stages the presence of functional innervation prevents the direct stimulation by guanethidine.

In the brain, guanethidine administration resulted in an alteration in the developmental pattern of

Table 3. Development in control rats of brain weight, ornithine decarboxylase and tyrosine hydroxylase activities*

Age (days)	Brain weight (g)	Ornithine decarboxylase (nmoles $^{14}CO_2$ evolved/brain/hr)	Tyrosine hydroxylase (nmoles $^{14}CO_2$ evolved/brain/hr)	No. of determinations
3	0.411 ± 0.006	1.20 ± 0.09	3.31 ± 0.11	5
5	0.561 ± 0.007	1.82 ± 0.11	6.90 ± 0.39	6
7	0.764 ± 0.009	--	10.4 ± 0.5	6
11	1.04 ± 0.01	0.243 ± 0.048	18.7 ± 0.6	6
20	1.37 ± 0.04	0.031 ± 0.003	27.5 ± 0.9	16
31	1.58 ± 0.03	—	43.9 ± 1.4	12
39	1.68 ± 0.04	--	49.6 ± 2.5	5
49	1.70 ± 0.04	—	37.1 ± 1.7	11

* Starting at 2 days of age, control pups were injected with vehicle (0.9% NaCl. 0.1% ascorbic acid, pH 7.4, prepared fresh) once daily for 5 days. Rats killed at 3 and 5 days of age were sacrificed 24 hr after the previous injection.

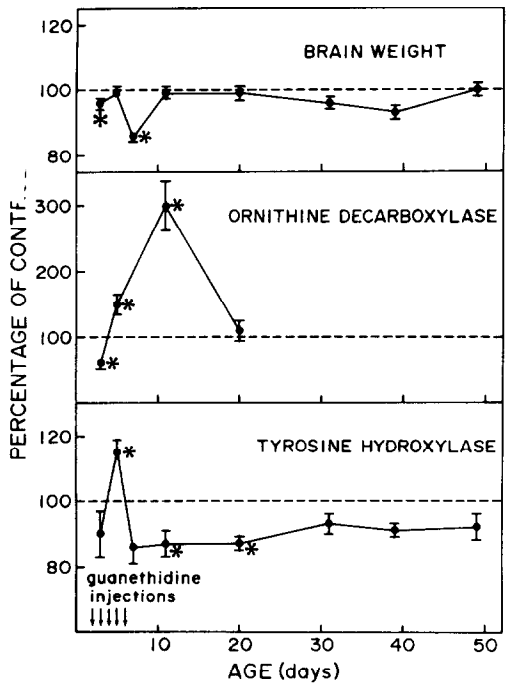

Fig. 2. Brain weights and ornithine decarboxylase and tyrosine hydroxylase activities per brain in developing rats treated with guanethidine (50 mg/kg, s.c.) as percentage of control. Points and bars represent means ± S.E.M. of five to six determinations; asterisks denote significant differences from controls ($P < 0.05$ or better). Control values are shown in Table 3.

ornithine decarboxylase activity consistent with a possible delay in cellular proliferation; Anderson and Schanberg [23] have demonstrated a similar relationship for actions of cortisol on development of brain polyamine metabolism. This effect may play a role in the initially low brain weights after guanethidine.

The effects of guanethidine on development of brain tyrosine hydroxylase activity were biphasic. First, a transient increase was observed, suggesting stimulation by the drug. Afterwards, tyrosine hydroxylase activity was subnormal; this may indicate impairment of catecholamine neurons in the central nervous system, an hypothesis supported by studies showing that administration of guanethidine to newborn mice causes a long-lasting reduction in brain catecholamines [5]. In comparison to the actions in neonates, the lack of effect of guanethidine in adult brain is consistent with the earlier observation [5] that the neonatal blood–brain barrier is not completely developed and thus is unable to protect the brain against the neurotoxic effects of the drug.

Acknowledgements—Supported by USPHS DA-00465 and by a grant-in-aid from the American Heart Association. Dr. Slotkin is the recipient of Research Scientist Development Award K01-DA-00006 from the National Institute on Drug Abuse.

REFERENCES

1. P. U. Angeletti and R. Levi-Montalcini, *Proc. natn. Acad. Sci. U.S.A.*, **69**, 86 (1972).
2. E. M. Johnson, Jr., E. M. Cantor and J. R. Douglas, Jr., *J. Pharmac. exp. Ther.* **193**, 503 (1975).
3. G. Burnstock, B. Evans, B. J. Gannon, J. H. Heath and V. James, *Br. J. Pharmac. Chemother.* **43**, 295 (1971).
4. M. Nickerson and B. Collier, in *The Pharmacological Basis of Therapeutics* (Eds. L. S. Goodman and A. Gilman), p. 553. Macmillan, New York (1975).
5. A. Liuzzi, F. H. Foppen and P. U. Angeletti, *Biochem. Pharmac.* **23**, 1041 (1974).
6. S. Daikoku, O. Takashi, A. Takahashi and M. Sako, *Tokushima J. exp. Med.* **16**, 153 (1969).
7. L. G. Elfvin, *J. Ultrastruct. Res.* **17**, 45 (1967).
8. R. S. Comline and M. Silver, *J. Physiol., Lond.* **183**, 305 (1966).
9. R. L. Patrick and N. Kirshner, *Devl Biol.* **29**, 204 (1972).
10. T. A. Slotkin, *Biochem. Pharmac.* **22**, 2023 (1973).
11. T. A. Slotkin, *Biochem. Pharmac.* **22**, 2033 (1973).
12. T. A. Slotkin, *Biochem. Pharmac.* **24**, 89 (1975).
13. T. R. Anderson and T. A. Slotkin, *Biochem. Pharmac.* **24**, 1469 (1975).
14. J. Bartolomé and T. A. Slotkin, *Biochem. Pharmac.*, **25**, 1513 (1976).
15. B. Bhagat, *Biochem. Pharmac.* **23**, (suppl. 1), 131 (1974).
16. R. J. Merrills, *Analyt. Biochem.* **6**, 272 (1963).
17. S. Friedman and S. Kaufman, *J. biol. Chem.* **240**, 4763 (1965).
18. J. C. Waymire, R. Bjur and N. Weiner, *Analyt. Biochem.* **43**, 588 (1971).
19. T. R. Anderson and S. M. Schanberg, *J. Neurochem.* **19**, 1471 (1972).
20. D. H. Russell, S. H. Snyder and V. J. Medina, *Life Sci.* **8**, 1247 (1969).
21. C. M. Caldarera, M. S. Moruzzi, C. Rossoni and B. Barbiroli, *J. Neurochem.* **16**, 309 (1969).
22. H. Tabor and C. W. Tabor, *Pharmac. Rev.* **16**, 245 (1964).
23. T. R. Anderson and S. M. Schanberg, *Biochem. Pharmac.* **24**, 495 (1975).
24. T. A. Slotkin and F. J. Seidler, *Life Sci.* **16**, 1613 (1975).
25. R. L. Patrick and N. Kirshner, *Molec. Pharmac.* **7**, 389 (1971).
26. T. R. Anderson and T. A. Slotkin, *Biochem. Pharmac.* **24**, 671 (1975).