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DEVELOPMENT OF NICOTINIC RESPONSES IN THE RAT ADRENAL MEDULLA AND LONG-TERM EFFECTS OF NEONATAL NICOTINE ADMINISTRATION

R.N. ROSENTHAL[1] & T.A. SLOTKIN

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

1 The development of nicotinic responses in the rat adrenal medulla was examined at various ages from 1 to 50 days of age by testing the ability of nicotine (10 mg/kg, s.c.) to deplete catecholamines and induce tyrosine hydroxylase.

2 Catecholamines were depleted 25% 3 h after injection of nicotine at all ages tested, but the degree of tyrosine hydroxylase induction 24 h after nicotine increased with age.

3 These data indicate that functional nicotinic receptors are present in the neonatal adrenal medulla before the development of functional splanchnic innervation, but that the development of the ability to induce tyrosine hydroxylase is not coupled directly to the development of secretory mechanisms.

4 The long-term effects of a single dose of nicotine (10 mg/kg, s.c.) administered to one day old rats were also examined.

5 After the short-term catecholamine depletion caused by nicotine, there were persistent elevations of catecholamines and tyrosine hydroxylase until 23 days of age; however, dopamine β-hydroxylase remained elevated into young adulthood.

6 These data indicate that neonatal nicotine administration can produce long-term changes in adrenal catecholamine biosynthetic enzymes.

Introduction

Nicotine stimulates the adrenal medulla by at least three distinct mechanisms: direct stimulation of nicotinic receptors in the chromaffin cells (Schneider, 1969), stimulation *via* sympathoadrenal reflexes (Patrick & Kirshner, 1971a), and, to a lesser degree, hormonal stimulation from the adrenal cortex (Rubin & Warner, 1975). During the maturation of the rat adrenal medulla, catecholamines, their biosynthetic enzymes and storage vesicles, undergo a series of changes which are in part dependent upon the levels of neuronal input to the gland (Patrick & Kirshner, 1972; Slotkin, 1973a,b; 1975). The developing adrenal medulla does not respond to splanchnic stimulation in rats less than a week old, as shown by the inability of insulin to evoke reflex secretion of catecholamines (Slotkin, 1973b) and by the absence of trans-synaptic induction after reserpine administration (Bartolomé & Slotkin, 1976). This could result either from an inherent lack of ability of the adrenal to respond to nicotinic stimulation, or from failure of the splanchic nerve to deliver trans-synaptic signals to the adrenal. It is therefore essential to establish the pattern of development of nicotinic responses in the rat adrenal.

Previous studies indicate that maturing adrenergic systems respond to drugs differently from those of adults. Administration of morphine to pregnant and nursing rats produces long-term deficits in the offspring of adrenal catecholamines and catecholamine biosynthetic enzymes, a pattern unique to the immature animal (Anderson & Slotkin, 1975a). Reserpine or tetrabenazine administration to neonatal rats produces brain and adrenal catecholamine depletions that are more intense, more rapid in onset, and of longer duration than those produced in mature animals (Kulkarni & Shideman, 1966; Bartolomé & Slotkin, 1976). Furthermore, there appear to be periods in development during which exposure to drugs produces permanent alterations in adrenergic function (Bartolomé, Seidler, Anderson & Slotkin, 1976). In this study, the long-term effects of a single dose of nicotine administered to one day old rats have been examined in the developing adrenal medulla and central nervous system.

[1] Present address: Downstate Medical Center, School of Medicine, Brooklyn, New York 11203 U.S.A.

Methods

Pups from timed pregnant Sprague-Dawley rats (Zivic-Miller) were given 10 µl subcutaneous injections of nicotine 10 mg/kg or 0.9% w/v NaCl solution (saline). In studies to delineate the development of nicotinic responses, pups received single injections at various ages and were weighed and killed 3 h and 24 h later. To determine long-term effects, pups were given a single injection at one day of age, and the animals were weighed and killed at intervals of several days through young adulthood.

Adrenal glands were homogenized with a Polytron (Brinkmann Instruments) in 1.5–2.2 ml of 0.3 M sucrose containing 25 mM Tris (pH 7.4) and 10 µM iproniazid (irreversible monoamine oxidase inhibitor); 0.1 ml of homogenate was deproteinized with 1.9 ml of 3.5% perchloric acid and centrifuged at 26,000 *g* for 10 min; the supernatant was then analyzed for catecholamines by the trihydroxyindole method using an autoanalyzer (Merrills, 1963). An additional 0.5 ml of homogenate was added to 0.5 ml of water containing 2000 units/ml beef catalase, and then assayed in duplicate for dopamine β-hydroxylase by the method of Friedman & Kaufman (1965), using 10 µM [^{3}H]-tyramine as substrate and 0.5 mM para-hydroxymercuribenzoate to inactivate endogenous inhibitors (Duch, Viveros & Kirshner, 1968). The remaining homogenate was centrifuged at 26,000 *g* for 10 min to sediment catecholamine storage vesicles, and 0.1 ml portions of the supernatant used for duplicate assays of tyrosine hydroxylase according to Waymire, Bjur & Weiner (1971), using 100 µM [^{14}C]-tyrosine as substrate. In order to obtain sufficient material for analysis before 11 days of age, gland pairs from 2–4 pups were pooled for each sample.

To examine central effects of neonatal nicotine administration, brains were weighed and homogenized in 9 volumes of Tris (pH 7.2) and duplicate 0.1 ml aliquots of homogenate assayed for tyrosine hydroxylase activity as described above, except that final concentrations of 0.1% Triton X-100 and 0.7 mM $CaCl_2$ were added to the assay to optimize activity. The rest of the homogenate was diluted with an equal volume of 10 mM Tris (pH 7.2), centrifuged at 26,000 *g* for 15 min and duplicate 0.9 ml portions of the supernatant used for assay of ornithine decarboxylase activity according to Anderson & Schanberg (1972), with 9.25 µM [^{14}C]-ornithine as substrate.

Data are reported as means ± standard errors, with levels of significance calculated by paired and unpaired *t* tests. Group means of control and experimental animals were paired by age over the time periods specified; degrees of freedom were calculated as the number of paired means minus one. This mode of pairing enables comparisons to be made of developmental patterns over the entire course of maturation or over multiple time periods as opposed to comparisons only of individual age points in the unpaired *t* test.

Figure 1 Adrenal catecholamines 3 h after administration of nicotine (10 mg/kg, s.c.) to rats of various ages. Data represent means of the number of determinations in parentheses, vertical lines show s.e. means; all points are significantly different from saline-treated controls by unpaired *t* test ($P < 0.05$ or better).

Figure 2 Adrenal tyrosine hydroxylase activity 24 h after administration of nicotine (10 mg/kg, s.c.) to rats of various ages. Data represent means of the number of determinations in parentheses, vertical lines show s.e. means; all points are significantly increased over saline-treated controls by unpaired *t* test ($P < 0.05$ or better).

Tyramine-[G-^{3}H] (5 Ci/mmol), L-tyrosine-[1-^{14}C] (55 mCi/mmol), and DL-ornithine-[1-^{14}C] (40 mCi/mmol) were purchased from New England Nuclear Corporation, and nicotine, iproniazid phosphate, beef catalase, and parahydroxymercuribenzoate from Sigma Chemical Company.

Results

Development of nicotinic responses

Three hours after nicotine administration to one day old rats, adrenal catecholamines were depleted by approximately 25% (Figure 1); nicotine given to rats at 4, 7, 11, 15, 35 or 50 days of age produced a similar

Figure 3 Adrenal dopamine β-hydroxylase activity 24 h after administration of nicotine (10 mg/kg, s.c.) to rats of various ages. Data represent means of the number of determinations in parentheses, vertical lines show s.e. means; none of the differences is significant from controls by unpaired t test.

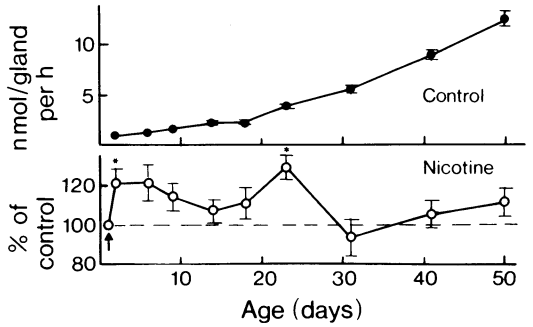

Figure 5 Adrenal tyrosine hydroxylase activity in rats administered saline or nicotine (10 mg/kg, s.c.) at one day of age (at arrow). The first point after the arrow is at 24 h post-injection. Points and vertical lines represent means ± s.e. means of 11–24 determinations at each age; * significant differences from controls by unpaired t test ($P < 0.05$ or better).

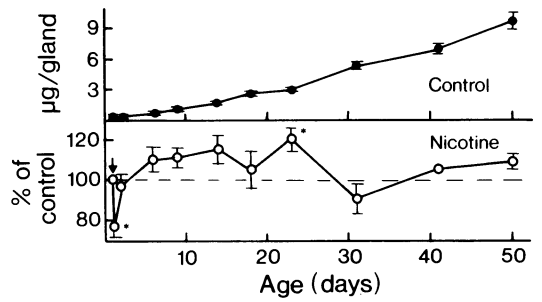

Figure 4 Adrenal catecholamines in rats administered saline or nicotine (10 mg/kg, s.c.) at one day of age (at arrow). The first point after the arrow is at 3 h post-injection. Points and vertical lines represent means ± s.e. means of 11–24 determinations at each age; * significant differences from controls by unpaired t test ($P < 0.05$ or better).

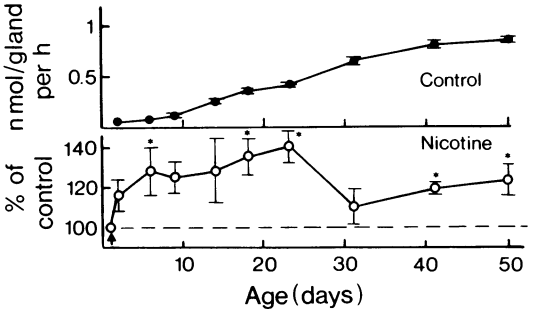

Figure 6 Adrenal dopamine β-hydroxylase activity in rats administered saline or nicotine (10 mg/kg, s.c.) at one day of age (at arrow). The first point after the arrow is at 24 h post-injection. Points and vertical lines represent means ± s.e. means of 11–24 determinations at each age; * significant differences from controls by unpaired t test ($P < 0.05$ or better).

catecholamine depletion. In contrast, tyrosine hydroxylase induction 24 h after nicotine administration was higher in 50 day old rats (80% increase) than in one day old rats (21% increase, $P < 0.001$ *vs* 50-day induction, Figure 2). There appeared to be a significant correlation between age and ability to induce tyrosine hydroxylase ($r = 0.78$), except after injection at 15 days of age, where a smaller induction was noted than after 11 or 35 days.

At no age was significant induction of dopamine β-hydroxylase obtained 24 h after nicotine administration (Figure 3), and little or no alteration in body or brain weight or in brain tyrosine hydroxylase was seen either 3 or 24 h after nicotine (data not shown).

Long-term effects of neonatal administration

Over the course of development, adrenal catecholamines in control rats increased from approximately 0.3 µg/gland to nearly 10 µg/gland in adulthood (Figure 4). After the acute (3 h) phase of adrenal catecholamine depletion caused by nicotine administration to one day old rats, levels recovered to normal by 2 days of age, and then tended toward elevation until 23 days of age ($P < 0.01$ by paired t test). From 31 to 50 days of age, adrenal catecholamines appeared to be normal (not significant by paired t test).

Adrenal tyrosine hydroxylase activity in control

Table 1 Long-term effects of nicotine (10 mg/kg, s.c.) administered at one day of age on body and brain weights, and on brain tyrosine hydroxylase activity

Age (days)	Body weight (g)				Brain weight (g)		Brain tyrosine hydroxylase (nmol/brain per h)			
	Control		Nicotine		Control	Nicotine	Control		Nicotine	
2	9.5±0.2	(54)	9.4±0.2	(54)	0.361±0.004 (18)	0.365±0.003 (18)	4.2±0.4	(6)	5.0±0.2	(6)
6	15.9±0.7	(42)	16.2±0.4	(42)	0.686±0.007 (12)	0.674±0.009 (12)	7.8±0.4	(6)	8.2±0.5	(6)
9	24.0±1.0	(36)	23.0±0.8	(36)	0.988±0.013 (12)	0.943±0.009 (11)†	12.7±0.6	(6)	12.3±0.3	(6)
14	35.9±1.1	(12)	35.1±1.6	(12)	1.33±0.03 (6)	1.29±0.02 (6)	32.2±1.1	(6)	32.5±1.2	(6)
18	48.0±1.8	(18)	45.6±2.6	(18)	1.53±0.03 (6)	1.42±0.01 (6)**	—		—	
23	67.7±4.6	(18)	62.9±3.2	(18)	1.64±0.05 (6)	1.61±0.01 (6)	61.3±1.6	(6)	72.1±2.9	(6)*
31	112±4	(12)	111±5	(11)	1.76±0.01 (6)	1.62±0.02 (5)§	46.6±0.9	(6)	41.4±0.2	(5)§
41	184±10	(12)	182±9	(11)	1.92±0.04 (6)	1.83±0.02 (5)	40.7±1.9	(6)	44.8±1.1	(5)
50	247±16	(14)	239±14	(14)	1.81±0.06 (6)	1.79±0.03 (6)	43.0±2.7	(6)	44.2±2.0	(6)

Data represent means ± s.e. means of the number of determinations in parentheses.
* $P < 0.02$ vs. control by unpaired t test; ** $P < 0.01$; † $P < 0.005$; § $P < 0.001$.

Table 2 Effects of nicotine (10 mg/kg, s.c.) administered at one day of age on brain ornithine decarboxylase activity

Age (days)	Brain ornithine decarboxylase (nmol/brain per h)	
	Control	Nicotine
2	0.64±0.04 (12)	0.74±0.04 (12)
6	0.55±0.04 (6)	0.53±0.02 (6)
9	0.56±0.03 (6)	0.58±0.03 (6)

Data represent means ± s.e. mean of the number of determinations in parentheses.

rats increased 13-fold over the course of development (Figure 5). Twenty four hours after neonatal nicotine administration, adrenal tyrosine hydroxylase activity was elevated 20%, and a tendency toward elevation persisted until 23 days of age ($P < 0.02$ by paired t test); from 31 to 50 days of age, no significant induction was noted. In contrast, induction of adrenal dopamine β-hydroxylase persisted after 23 days of age ($P < 0.001$ until 23 days, $P < 0.05$ 31–50 days by paired t test) and was significant even into young adulthood (Figure 6).

Neonatal nicotine administration produced a minor retardation (5%) in developmental gains in body and brain weights ($P < 0.05$ and $P < 0.01$ by paired t test, respectively); however, whole brain tyrosine hydroxylase activity appeared generally to be unaltered (not significant by paired t test, Table 1).

No significant differences in the developmental fall-off of whole brain ornithine decarboxylase activity were seen at 2, 6, or 9 days of age, after neonatal nicotine administration (Table 2).

Discussion

In adult animals, administration of agents which stimulate nicotinic receptors directly or which evoke central stimulation of the sympatho-adrenal axis (e.g. morphine, reserpine), causes release of adrenomedullary catecholamines (Schneider, 1969; Viveros, Arqueros, Connett & Kirshner, 1969; Anderson & Slotkin, 1975b; Slotkin & Seidler, 1975) and compensatory trans-synaptic induction of the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) (Thoenen, Mueller & Axelrod, 1969; Molinoff, Brimijoin, Weishilboum & Axelrod, 1970; Slotkin & Kirshner, 1973). In contrast, neonatal rats given reserpine show catecholamine depletion but not TH induction (Bartolomé & Slotkin, 1976), a phenomenon which could result from either absence of functional splanchnic innervation (Comline & Silver, 1966; Slotkin, 1973b) or from a lack of nicotinic receptors,

or from a lessened inductive capability. In the present study, nicotine evoked an equivalent adrenomedullary response in adult and developing rats in terms of catecholamine depletion, but the degree of TH induction increased with age. These data show that functional nicotinic receptors are present in the neonatal adrenal gland before the establishment of functional innervation, which takes place at 7–10 days (Slotkin, 1973b; Bartolomé & Slotkin, 1976). However, there appears to be a difference in sensitivity between nicotinic receptor-mediated secretion of catecholamines and induction of TH, implying that secretion and induction are not coupled directly. This hypothesis is supported by observations in adult rats that low doses of nicotine or morphine can cause TH induction without an evident decrease in catecholamines (Slotkin & Seidler, 1975; Anderson & Slotkin, 1976), and that secretion without induction can occur in neonatal adrenals (Barolomé Slotkin, 1976). Since adrenal TH activity increased 13-fold over the first 50 days of development, nicotine-induced TH induction in the developing rat may be limited by the already high levels of transcription and/or translation (Thoenen, 1974). Alternatively, in the absence of innervation, receptor-mediated induction of TH inherently may be less effective than the induction seen in older animals; this latter hypothesis is supported in part by the observation that the rate of increase in TH is slower in denervated adult adrenals than in innervated glands during chronic nicotine administration (Seidler & Slotkin, 1976). However, immature adrenals are not simply the equivalent of denervated adult adrenals, since in the latter the acute catecholamine-secretory response to nicotine also is diminished (Seidler & Slotkin, 1976) while in neonates the TH induction is reduced without affecting catecholamine secretion.

It is of interest that, despite the general trend toward greater TH induction in older animals, a smaller TH induction was seen at 15 days than at 11 days ($P < 0.01$); similar results have been reported for reserpine (Bartolomé & Slotkin, 1976), suggesting that more subtle developmental changes may be superimposed upon the general pattern.

DBH activity was not induced 24 h after nicotine at any age tested. This probably results from the longer time period required for DBH induction, as well as exocytotic loss of soluble DBH (Patrick & Kirshner, 1971a; Slotkin & Seidler, 1975).

In long-term studies, a single dose of nicotine administered to 1-day old rats caused acute (3 h) catecholamine depletion, and subsequent persistent elevations of catecholamines, TH and DBH; however, while catecholamines and TH returned to normal by 31 days of age, DBH activity remained 20–30% above normal into young adulthood, suggesting a permanent increase in the number of storage vesicles (Viveros *et al.*, 1969; Slotkin, 1973b; Bartolomé *et al.*, 1976). Three hypotheses could explain this phenomenon: first, the chronically increased DBH could be due to trans-synaptic induction *via* permanently increased sympathoadrenal outflow. However, one would then also expect a permanent induction of TH to a larger extent than for DBH; the smaller change in TH indicates that trans-synaptic induction alone cannot account for the magnitude of change in DBH. Second, since nicotine causes release of adrenocorticotrophic hormone (ACTH), as well as direct secretion of adrenocortical steroids (Kershbaum, Pappajohn, Belley, Hirabayashi & Shafiiha, 1968; Suzuki, Ikeda, Narita, Shibata, Waki & Egashira, 1973; Rubin & Warner, 1975), a primary effect upon the pituitary-adrenal axis could cause long-term corticoid-mediated changes in TH and DBH; this is unlikely because ACTH apparently increases adrenal TH and DBH only in hypophysectomized animals (Mueller, Thoenen, Axelrod, 1970; Weinshilboum & Axelrod, 1970), and because the effect is usually greater for TH than for DBH (in contrast to the present findings). Further to rule out corticoids as a primary mediator of adrenomedullary developmental alterations, the effect of nicotine on brain ornithine decarboxylase (ODC) was evaluated. Anderson & Schanberg (1975) have shown that corticoids produce a marked lag in the developmental fall-off of brain ODC. In the present study, a normal ODC pattern was seen, suggesting that adrenocortical secretion is unaffected.

The third hypothesis which could explain long-term elevations in adrenal DBH after neonatal nicotine administration is that the adrenal medulla itself is altered. In this regard, in addition to trans-synaptic or corticoid-mediated regulation of DBH, another mechanism appears to exist which is mediated directly in the adrenal medulla and which is unique to DBH. In adult rats, this mechanism can be unmasked by denervation of adrenals, followed by treatment with reserpine (Patrick & Kirshner, 1971b), chlorisondamine (Slotkin, Seidler, Lau, Bartolomé & Schanberg, 1976), or morphine (Anderson & Slotkin, 1976); while induction of DBH is seen under these conditions, no TH induction occurs. Thus, in addition to trans-synaptic effects, a long-term developmental change in DBH regulation in the adrenal medulla itself may be responsible for the persistent elevation in activity of that enzyme.

In conclusion, adrenomedullary nicotinic receptors are present in the neonatal rat before development of functional splanchnic innervation, but the ability to induce TH is lower than that in adults. A single dose of nicotine administered at one day of age can produce long-term changes in control of catecholamine biosynthetic enzymes.

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