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ONTOGENESIS OF CHOLINE ACETYLTRANSFERASE, TYROSINE HYDROXYLASE, MONOAMINE OXIDASE AND CATECHOL-*O*-METHYL TRANSFERASE IN THE SUPERIOR CERVICAL GANGLION OF SWINE

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Abstract—Swine have *ca* 50 per cent of the adult choline acetyltransferase (CAT) activity in the superior cervical ganglia (SCG) at birth. This activity rapidly increases to adult levels by 7–14 days of age. Tyrosine hydroxylase (TH) activity is *ca* 10 per cent of adult levels at birth. This enzyme increases at the same rate as SCG growth from 0–14 days of age and then changes 4- to 5-fold from 14–39 days of age. Monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) activities increase at the same rate as ganglion growth from birth through 150 days of age. The TH and MAO, but not CAT or COMT, activities were reduced *ca* 30 per cent in neonatal piglets treated for 5 days with 6-hydroxydopamine (50 mg/kg day, s.c.). Catecholamine levels in heart and spleen were reduced > 90 per cent by 6-hydroxydopamine, but TH, MAO or COMT was not diminished. The physically mature neonatal swine may be less susceptible to chemical sympathetecomy by 6-hydroxydopamine than immature neonatal mice or rats.

THE ACTIVITY of tyrosine hydroxylase (TH, EC 1.14.3a), the enzyme catalyzing the rate-limiting step in norepinephrine biosynthesis, increases in rat adrenal medulla and postganglionic adrenergic neurons when cholinergic presynaptic activity is augmented.[1,2] This increase apparently reflects enzyme induction and not altered regulation[1] Black *et al.*[3] demonstrated that changes in choline acetyltransferase (CAT, EC 2.3.1.6.) activity correlate with the formation of cholinergic synapses in the mouse superior cervical ganglia (SCG). The predominant increases in TH activity in the adrenergic cell bodies of the SCG of neonatal mice occurred in conjunction with the major increases in the number of cholinergic synapses. Thoenen[2] and Thoenen *et al.*,[4] on the other hand, reported that in neonatal rats the formation of cholinergic preganglionic synapses in the SCG was not prerequisite to the ontogenesis of TH or dopamine β-hydroxylase. Hendry *et al.*[5] concluded that the activity of TH, but not of monoamine oxidase (MAO, EC 1.4.3.4) or aromatic amino acid decarboxylase, was closely associated with the level of cholinergic synaptic output of the SCG of adult mice or rats and fell when synaptic activity was curtailed by denervation.

The ontogenic relationships between CAT, TH, MAO and catechol-*O*-methyl transferase (COMT, EC 2.1.1.6) were evaluated in the following study using the SCG of the developing pig. The swine provides a model for comparing the development of the autonomic nervous system in a physically mature neonate with that in the immature progeny of mice and rats.

MATERIALS AND METHODS

Animals and tissue. The piglets used in these studies represented crosses between Hampshire, Yorkshire and Duroc breeds and were farrowed in our temperature-controlled facilities. The animals were transported in temperature-conditioned vehicles a short distance (*ca* 200 yards) from the animal buildings to the laboratory and were kept in a warm (33–35° for newborn), quiet environment until they were sacrificed. The pigs were lightly anesthetized with halothane and the ganglia rapidly removed, weighed, frozen in liquid nitrogen and stored at −90° until use.

Fetal piglets were surgically delivered from sows premedicated with promazine and lightly anesthetized with halothane. The number of animals, the number of litters represented and the average body weights of each age group are shown in Table 1.

TABLE 1. AGE, NUMBER, LITTER DISTRIBUTION, BODY WEIGHT AND SEX DISTRIBUTION OF ANIMALS USED IN STUDY

Age (days)	No. pigs	No. litters	Average body wt (kg ± S.E.)	Sex M	Sex F
109-day fetuses	14	1	1·08 ± 0·05	8	6
0-8 hr	8	4	1·13 ± 0·11	4	4
3	5	3	1·54 ± 0·20	2	3
7	7	3	2·27 ± 0·17	1	6
14	10	4	3·57 ± 0·18	6*	4
39	11	3	8·79 ± 0·82	6*	5
70	6	3	39·0 ± 0·6	3*	3
150	4	2	78·0 ± 5·4	1*	3

* Castrated at 10 days of age.

Biochemical measurements. Homogenates (1 ganglia/2 ml) were prepared in cold 0·15 M KCl–5 mM Tris-HCl (pH 6·7)[7] with a Brinkman Polytron homogenizer. An aliquot of the homogenate to be used for the enzyme assays was dialyzed overnight at 5° against 5 mM Tris–HCl buffer (pH 7·0) containing 1 mM mercaptoethanol. This dialysis was to remove potential inhibitors, particularly catecholamines, of TH.[7] Both ganglia from one animal were homogenized together and used for all the assays, except those involving fetal or newborn piglets where four ganglia from two animals were pooled.

TH activity was assayed as previously described for swine adrenal glands.[6,7] The enzyme activity is expressed in terms of nmoles L-dihydroxyphenylalanine formed/tissue unit/hr.

The CAT activity was assayed by a modification of the method of Glover and Green.[8] The incubation mixture contained: 10 mM choline chloride, 200 mM KCl, 20 mM phosphate buffer (pH 7·0), 0·2 mM EDTA, 2 mM physostigmine salicylate and 0·56 mM ^{14}C-acetyl CoA (sp. act., 0·5 mCi/m-mole). A 0·05-ml aliquot of this mixture was placed in a 10 × 75 mm glass tube and warmed (37°) for 5 min before 0·05 ml of the whole ganglion homogenate was added. The reaction was incubated with shaking for 10 min at 37° and stopped with 0·05 ml of 1 N formic acid. Five-tenths ml of a solution representing the organic layer, after mixing equal parts of 100 mM K_2HgI_4 and octanone, was added to the reaction tube, mixed and centrifuged. A 0·05-ml aliquot of the upper layer was placed in 10 ml of a counting medium consisting of nine parts of 0·4% Omnifluor (New England Nuclear Corp.) in toluene and

one part methanol. The radioactivity was measured in a liquid scintillation spectrometer at an efficiency of 82 per cent. The enzyme activity is expressed as nmoles ^{14}C-acetylcholine formed/tissue unit/hr.

The MAO activity was assayed radiometrically[9] in whole ganglion homogenates. The reaction mixture consisted of: 0·1 ml of 0·5 M potassium phosphate buffer (pH 8·0), 0·2 ml tissue homogenate, 0·1 ml of 0·01 M tyramine–tyramine H^3 (G) containing 1·0 μCi/ml, and water to make 0·5 ml. This mixture was incubated for 30 min at 37°, and the reaction was stopped with 0·2 ml of 2 N HCl. Radioactive reaction products were counted in Bray's medium[10] at an efficiency of 30 per cent. The enzyme activity is expressed in terms of nmoles of radioactive product formed/tissue unit/hr. Assays using 0·01 M benzylamine (methylene-^{14}C) or 5-hydroxytryptamine (2-^{14}C) as substrates (0·1 μCi/assay) were carried out in the same manner as the tyramine procedure.

The COMT activity was assayed radiometrically[9] in whole ganglion homogenates. The reaction mixture consisted of: 0·1 ml of 0·5 M potassium phosphate buffer (pH 8·0), 0·025 ml of 0·04 M $MgCl_2$, 0·2 ml tissue homogenate, 0·05 ml of 0·01 M *S*-adenosyl methionine-*S*-adenosyl methionine-^{14}C containing 4 μCi/ml, and 0·1 ml of 0·02 M 3,4-dihydroxybenzoic acid. This mixture was incubated for 20 min at 37° and the reaction was stopped with 0·1 ml of 1 N HCl. Radioactive reaction products were extracted with 10 ml toluene. Five-ml aliquots were counted in a medium containing 0·4% Omnifluor (New England Nuclear Corp.) in toluene at an efficiency of 88 per cent. The enzyme activity is expressed as nmoles of toluene-soluble radioactive products formed/tissue unit/hr. Tissue protein levels were measured by the biuret reaction in whole homogenates.[11]

The conditions used for the enzyme assays were optimal for substrate concentration, tissue concentration and incubation time in tissues from 35-day-old swine.

The special chemicals used in these studies were obtained from Sigma Chemical Co. or from Calbiochem, while radioactive materials were purchased from New England Nuclear Corp., Dohm Products, Ltd. or International Chemical and Nuclear Corp.

RESULTS

Ontogenesis in ganglia. The relationships between the piglet age and SCG tissue weight, protein content, MAO, COMT, TH and CAT activities are shown in Fig. 1 A and B. These data were obtained from 109-day fetuses and from piglets ranging from newborn (0) through 70 days of age. Data were expressed as a percentage of values (included in figure legend) determined in ganglia from 150-day-old animals. Various segments of the age–SCG parameter relationships were analyzed by the method of least squares[12] and are summarized in Table 2. Fetal data were not included in these analyses, as they closely resembled the values obtained with newborn piglets. The slopes of lines depicting increases in MAO or COMT activities with age were similar to the slopes of the lines representing increases in tissue mass or protein content. Hence, the activities of MAO and COMT appear to increase at the same rate as the tissue growth. The slopes of the lines representing 0–70 days of age are similar to those representing 0–7 days; and, therefore, the ganglion growth rate from birth through 70 days is relatively constant.

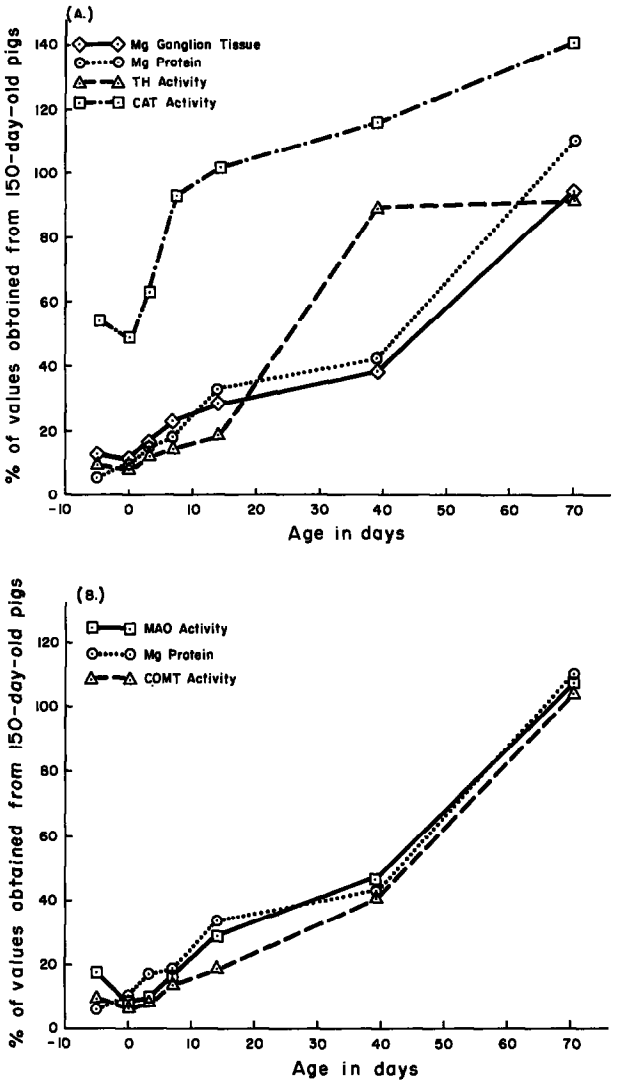

FIG. 1. Relationships between ganglion weight, protein content, CAT, MAO, COMT activities and piglet age. The data are presented as per cent of the following average, adult, 150-day-old values obtained from four pigs: ganglion weight = 123 ± 8 mg; protein = 16·3 ± 1·4 mg/ganglion; enzyme activities in nmoles product formed/ganglion/hr as follows: CAT = 772 ± 13. TN = 185 ± 29, MAO = 857 ± 41, COMT = 114 ± 7. Each point represents four to ten observations.

The CAT activity increased rapidly from 0–7 days of age and then the rate of change decreased to that of general ganglion growth from 14–70 days of age (Fig. 1A, Table 2). The calculated interception of each line with the ordinate (*a*) (Table 2) shows that the activity of CAT at birth was high (47 per cent of 150-day activity) compared to that of the other parameters (< 15 per cent of 150-day levels).

The TH activity increased at the same rate as ganglion growth from 0–14 days (Table 2, Fig. 1A), but increased very rapidly from 14–39 days. The rate of increase in activity decreased at 39 days and remained constant through 150 days of age. The

TABLE 2. SUMMARY OF STATISTICAL ANALYSIS OF AGE VS PER CENT RELATIONSHIPS AT INTERVALS OF 0–7 DAYS AND 0–70 DAYS OF AGE

Parameter (y)	Age (x) interval (days)	No. pigs	Components of $y = a + bx$ Intercept (a + 95% CI)	Slope (b + 95% CI)†	$100r^2$*
Ganglion (mg)	0–7	16	13·2 ± 2·8		
Protein (mg)	0–7	16	11·0 ± 2·6		
MAO activity	0–7	16	8·2 ± 4·6		
COMT activity	0–7	16	5·9 ± 3·1	1·2 ± 0·6	56
TH activity	0–14	26	10·2 ± 2·1	0·7 ± 0·2	58
CAT activity	0–7	16	47·4 ± 10·5	6·3 ± 2·1	74
Ganglion (mg)	0–70	43	12·6 ± 3·9	1·1 ± 0·1	88
Protein (mg)	0–70	43	8·9 ± 7·0	1·3 ± 0·2	79
MAO activity	0–70	38	6·7 ± 5·9	1·4 ± 0·2	86
COMT activity	0–70	38	2·2 ± 4·7	1·4 ± 0·1	92
CAT activity	14–70	22	91·0 ± 18·8	0·7 ± 0·4	34

* $100\,r^2$ = Coefficient of determination.
† CI = confidence interval.

high TH activity observed in 39-day-old pigs compared to that of 14-day-old animals was observed in two independent experiments and, therefore, does not seem to be artifactual.

Effects of 6-hydroxydopamine. The effects of 6-hydroxydopamine (6-OHD) on CAT, TH, MAO and COMT activities in SCG, hearts and spleen were examined in neonatal swine in order to help identify extraneuronal and intraneuronal distributions of the enzymes. Catecholamine levels were measured in hearts and spleen but not in ganglia. The drug (50 mg/kg, s.c.) was administered daily from birth through 5 days of age, and the animals were sacrificed on the morning of day 6. Four piglets from each of two litters were treated with 6-OHD, and the remaining littermates received 0·9% NaCl solution injections under the same conditions. The results of the study are summarized in Table 3. Ganglia from 6-OHD-treated piglets were smaller than those from saline-treated controls, although this may reflect differences in body size. Tissue protein concentrations were the same in the SCG of the two groups. There was more protein in hearts and spleen of 6-OHD-treated piglets, although the differences are small and the biological significance is not apparent. The CAT activity was significantly higher in SCG from 6-OHD-treated neonates, while TH activity was less than in saline controls. The TH activity was unchanged in cardiac and splenic tissue of 6-OHD-treated pigs, even though total catecholamine levels were reduced > 90 per cent by the drug treatment. The COMT activity in SCG, heart or spleen was unchanged by 6-OHD treatment. The MAO activity measured in SCG with tyramine or 5-hydroxytryptamine as substrates was significantly reduced by 6-OHD treatment, while MAO activity measured with benzylamine did not differ from that of saline controls. The MAO activity (tyramine) was higher in hearts from 6-OHD-treated animals.

DISCUSSION

The CAT activity in 109-day fetuses and newborn piglets was *ca.* 50 per cent of that observed in young swine 150 days of age. This differs from mice and rats where the CAT activity at birth was approximately 3 per cent of the maximum attained

TABLE 3. EFFECTS OF 6-HYDROXYDOPAMINE (6-OHD) TREATMENT ON CERTAIN ADRENERGICALLY-RELATED BIOCHEMICAL PARAMETERS IN GANGLIA, HEART AND SPLEEN OF 6-DAY-OLD PIGS

Parameter	Saline-treated			6-OHD-treated*		
	Ganglion	heart	Spleen	Ganglion	heart	Spleen
Number of pigs	9	9	9	8	8	8
Body wt at birth (kg ± S.E.)	1·36 ± 0·11	1·36 ± 0·11	1·36 ± 0·11	1·21 ± 0·06	1·21 ± 0·06	1·21 ± 0·06
Body wt at 6 days (kg ± S.E.)	1·93 ± 0·21	1·93 ± 0·21	1·93 ± 0·21	1·53 ± 0·17	1·53 ± 0·17	1·53 ± 0·17
Tissue wt (g ± S.E.)	0·031 ± 0·002	13·7 ± 1·4		0·022 ± 0·002‡	11·2 ± 1·2	
Tissue protein (mg/g ± S.E.)	103 ± 4	158 ± 2	173 ± 4	106 ± 2	165 ± 2‡	189 ± 5‡
Catecholamines (ng/g ± S.E.)		441 ± 60	1317 ± 235		6 ± 5‡	120 ± 23‡
CAT activity × 10^{-3} ± S.E.†	23·2 ± 1·4			28·5 ± 1·2‡		
TH activity ± S.E.†	1865 ± 200	52·8 ± 5·9	54·1 ± 7·3	1288 ± 177‡	39·8 ± 7·7	59·9 ± 5·9
COMT activity ± S.E.†	348 ± 6	339 ± 7	490 ± 7	356 ± 11	360 ± 8‡	462 ± 17
MAO (Tyr) activity ± S.E.†	6373 ± 254	250 ± 21	1723 ± 103	4308 ± 142‡	311 ± 31‡	1764 ± 28
MAO (Benz) activity ± S.E.†	2024 ± 163			1910 ± 147		
MAO (5HT) activity ± S.E.†	4845 ± 260			3011 ± 284‡		

* 6-OHD, 50 mg/kg/day s.c., administered from birth through 5 days of age. Animals were sacrificed at 6 days of age.

† Enzyme activity (as described in Methods)/g tissue/hr. S.E. = standard error of the mean.[12] Tyr = tyramine, Benz = benzylamine, 5HT = 5-hydroxytryptamine as substrates.

‡ Significantly different (P 0·05–0·01) from saline control using non-paired Student's *t*-test.[12]

at 3–4 weeks of age.[3,4] Our data indicate that one-half of the total cholinergic synapses are formed in the piglet SCG before birth, if CAT activity in the pig is a reliable index of the density of cholinergic synapses as it is in rodents.[3,4] Nevertheless, CAT activity increases five times faster than ganglion growth during the first 7 postnatal days, but the rate of change slows from 14–150 days of age. This pattern differs from that in mice and rats where CAT activity increases steadily at a rate faster than ganglion growth from birth through maturity (*ca.* 30 days of age).[3,4]

There were also striking differences in the ontogenesis of TH in the SCG of the piglet compared to neonates of mice and rats. The TH activity in the newborn pig was *ca.* 10 per cent of the 150-day level, while in the mouse or rat it represented 20–30 per cent of the adult activity. The enzyme activity in the pig increased at the same rate as general protein synthesis in the ganglion from 0–14 days of age and then increased sharply between days 14 and 39. The CAT activity was nearly maximal at this time, which suggests that the number of cholinergic synapses in the SCG of the pig reaches adult levels before TH activity in the adrenergic cell body is augmentated. The TH activity in the mouse SCG increased in two stages: a 2-fold increase from birth through 3 days of age, followed by a 4-day plateau period and a subsequent 3-fold increase from 7–12 days of age.[3] The latter rise was directly attributed to the development of cholinergic synapses, while the first increase appeared to be independent of synaptic formation and may reflect changes in a separate enzyme pool with different turnover rates and/or increased TH activity in perikarya, which respond later to synaptic influences.[3] Although TH activity in the SCG of the rat neonate increased more rapidly than general protein synthesis, the ontogenesis of the enzyme was not closely associated with the formation of cholinergic synapses.[4]

The developmental patterns for MAO and COMT in the SCG of the piglet were not specialized and resembled ganglion growth, with specific activity remaining constant with age. This is different from the pattern observed in mice[13] and chicks[14] where MAO specific activity (activity/mg protein or dry wt) increased during maturation.

The administration of 6-hydroxydopamine to mature rodents results in a selective destruction of the distal endings of the adrenergic nerves without changing the morphological characteristics of the perikarya. However, in newborn mice and rats, the daily subcutaneous administration of 6-OHD (50 mg/kg) for 7 days resulted in a selective and permanent destruction of sympathetic neuronal cell bodies which was detectable within 3 days of treatment. Glial cells and connective tissue remained after 6-OHD treatment.[15] Angeletti *et al.*[13] observed that one or two doses of 6-OHD (100 mg/kg, s.c.) administered to newborn rats or mice reduced SCG MAO activity 50 per cent by 4 days of age and *ca* 100 per cent by 8 days of age. This rapid disappearance of enzyme activity correlated well with the development of drug-induced neuronal lesions in the ganglion. Finch *et al.*[16] reported that TH activity in SCG of 10-week-old rats which had been treated daily from birth through 14 days of age with 6-OHD (150 mg/kg, s.c.) was < 25 per cent of that determined in control animals. Neonatal swine did not respond as vigorously as mice to the 6-OHD treatment regimen used by Angeletti and Levi-Montalcini (Table 3).[15] The TH and MAO activity in the SCG was reduced *ca* 30 per cent but not completely abolished after 5 days of 6-OHD treatment. The MAO activity measured with 5-hydroxytryptamine as the substrate was reduced by 6-OHD but the activity measured by benzylamine was not. This suggests

that the "A" isoenzyme contained within the adrenergic neuronal cell body is affected. Jarrott[17] observed that in the vas deferens of the rat, 5-hydroxytryptamine and tyramine were more efficacious substrates for the intraneuronal form of enzyme, while benzylamine was preferred by the extraneuronal species. The pig is physically more mature at birth than the mouse or rat and thus may be less sensitive to the ganglionic effects of 6-OHD.

Catecholamines in hearts and spleens of the piglets were reduced > 90 per cent by 6-OHD treatment (Table 3). These organs are innervated by sympathetic postganglionic endings but do not contain adrenergic parikarya. In rodents, 6-OHD presumably depletes catecholamines in peripheral sympathetic tissue by causing selective degeneration of adrenergic, postganglionic endings.[18] This may not have occurred in swine under our treatment conditions, since TH activity was not changed, although catecholamines were depleted. Perhaps the 6-OHD treatment interfered with catecholamine uptake, storage or synthesis without destroying the adrenergic endings. Furthermore, MAO was not depleted in heart or spleen by 6-OHD. Since a small portion of MAO is intraneuronal, some reduction in activity may occur if the neuron endings containing the enzyme are destroyed.

These studies show that the ontogenic patterns of cholinergic synaptic formation, TH and MAO differ in the SCG of rats, mice and swine. Neonatal pigs may be less susceptible to the destructive actions of 6-OHD on adrenergic parikarya or postganglionic endings than neonatal mice or rats. More intensive or prolonged treatment should be used to determine if 6-OHD will induce complete chemical sympathectomy in swine.

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