PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **179**, 143–146 (1985)

Developmental Status of Sympathetic Innervation in Relation to Calcium Accumulation by Submandibular Gland following Reserpine, Surgical Sympathectomy or Cyclocytidine (42075)

CHARLOTTE A. SCHNEYER AND JIA-HUEY YU[1]

Department of Physiology and Biophysics, University of Alabama, Birmingham, Alabama 35294

Abstract. Sympathectomy (Sx) of the submandibular gland was induced at various postnatal ages by ip administration of a single dose of reserpine or by unilateral excision of a superior cervical ganglion. If animals were 12 days old or less at the time of drug administration, [Ca] of the submandibular gland was not measurably increased 24 hr later; if rats were 14 days of age or older, [Ca] of the gland 24 hr after reserpine injection was nearly double that of untreated controls. Two days after surgical Sx, [Ca] of the denervated submandibular gland was unchanged from that of the innervated member of a pair if animals were less than 14 days of age at the time of denervation; [Ca] was twice that of glands of control rats if animals were older than 14 days of age when the denervation was performed. The anti-tumor agent, cyclocytidine (CC), given daily for 3 days in an ip dose of 500 mg/kg, also caused a two- to threefold increase in [Ca] of the submandibular gland when rats were more than 12 days of age at the time of the initial injection of the drug, but in rats younger than this age, CC caused no change in the [Ca] of the submandibular gland. Present data show that there are age-related differences in the ability of the submandibular gland to accumulate calcium following sympathetic denervation or treatment with a norepinephrine-releasing drug. These differences may be attributed either to incomplete development of calcium transport mechanisms, or incomplete development of the sympathetic innervation before 14 days of age.

The calcium concentration ([Ca]) of salivary glands of the adult rat is very high and greatly exceeds that of other soft tissues (1). The significance of these high levels is not known, but it has been shown recently that the glandular concentration of calcium can be increased to even greater levels by chemical (2) or surgical sympathectomy (3, 4). Because sympathectomy is accompanied by depletion of the neurotransmitter, norepinephrine (NE) (5), we have suggested that the increase in glandular [Ca] is related to depletion of NE (4). The developing salivary glands afford a good system for testing this hypothesis, because the sympathetic pathway to the glands is not fully developed at birth, and full maturation, including attainment of adult levels of NE, does not occur until 2 (submandibular gland) (6) to 4 (parotid gland) (7) weeks of age. If depletion of NE is the critical factor in causing an increase in [Ca], then sympathetic denervation should result in an increase in glandular concentration of calcium at all postnatal ages where physiologically responsive sympathetic nerves innervate the glands. Therefore, as early as 5 days of age, an increase in [Ca] of submandibular gland should follow sympathectomy (8). On the other hand, unless NE levels are at adult levels (or some adequately high level), sympathectomy may not be followed by an increase in glandular concentration of calcium.

In the present work, chemical and surgical denervations were performed at various postnatal ages to explore the relationship described. In addition, the anti-tumor agent, cyclocytidine (CC), which has a side action that results in sustained release of NE from sympathetic nerve endings (9) and ultimate depletion of NE, was used to further explore the phenomenon. The consequence of CC administration is an increase in the [Ca] of the submandibular gland (11). Only the submandibular gland was used for the study since [Ca] of the parotid gland shows no increase other than a very transient change

[1] Present address: Veterans Administration Medical Center, Oral Pathology Research Lab, Washington, D.C. 20422.

0037-9727/85 $1.50

that occurs within hours after chemical sympathectomy (4).

Materials and Methods. Long-Evans rats (3–120 days of age) of both sexes were maintained on mother's milk or on solid lab chow and water *ad lib* until 6–18 hr prior to acute experimentation. Chemical or surgical sympathectomy (Sx) of the submandibular gland was effected at 1 (for reserpine) or 2 (for surgical Sx) days prior to sacrifice of the rats. For surgical sympathectomy, a single superior cervical ganglion was removed from each animal. This was done at 8, 14, 15, or 16 days of age; for chemical Sx, a single dose of reserpine, 0.5 mg/kg body wt, was dissolved in 20% ascorbic acid and administered ip at 3, 8, 11, or 14 days of age. The anti-tumor agent, cyclocytidine, was given in a dose of 500 mg/kg body wt once daily for 3 days, with the initial injection made either at 9, 15, or 26 days of age. At the appropriate times, glands were excised rapidly, and weighed on a torsion balance, dried in a desiccator and then reweighed to obtain dry weight; water content was calculated. The glands were then ashed for 48 hr at 550°C, and the ash dissolved in 0.5 ml of 1 *N* HCl; calcium was then determined by titration using a Precision Systems calcette instrument. The Student *t* test was used for statistical analysis of the data.

Surgical or chemical sympathectomy was performed at various postnatal ages, and the [Ca] of the glands subsequently determined. The preferred method to induce Sx was surgical, as it produces a localized effect in contrast to the diffuse effects caused by chemical denervation. However, at ages when surgical Sx was not feasible, chemical Sx was used.

Results. Table I shows the effects of chemical denervation at early postnatal ages, whereas Table II shows the effects of surgical Sx at slightly more advanced postnatal ages. Control rats showed an age-dependent increase in [Ca], and present data are confirmatory of previous data (12). The [Ca] of the submandibular gland of reserpine-treated rats was not different from that of the submandibular gland of control rats at 4, 9, or 12 days of age. However, when reserpine was administered at 14 days of age, the [Ca] of the submandibular gland was nearly twice as high as that of glands of untreated rats.

An increase in the [Ca] of the submandibular gland of adult rats was not observed until 2 days after removal of the superior cervical ganglion (4). Therefore, the [Ca] of the submandibular gland of the neonates was examined 2 days after removal of the superior cervical ganglion. When surgical Sx was performed on rats at 8 days of age, the [Ca] of the denervated glands showed no statistically significant increase from that of contralateral control glands ($P > 0.05$). When Sx was effected at later ages, however, the [Ca] of the

TABLE I. EFFECTS OF RESERPINE ON CALCIUM CONCENTRATION OF SUBMANDIBULAR GLAND AT VARIOUS POSTNATAL AGES

No. days on RES	Age at sacrifice (days)	% Water	[Ca] of submandibular gland: meq/kg wet wt	[Ca] of submandibular gland: meq/kg dry wt	No. of rats
0	4	84.5 ± 0.6	6.1 ± 0.1	42.4 ± 3.1	4
1	4	85.8 ± 1.6	6.6 ± 0.2	46.9 ± 1.6	4
0	9	76.8 ± 0.6	7.7 ± 0.4	36.6 ± 2.2	5
1	9	79.1 ± 0.5	8.6 ± 0.8	37.1 ± 1.7	5
0	12	79.4 ± 0.6	7.9 ± 0.7	39.2 ± 2.2	6
1	12	77.3 ± 0.6	8.6 ± 0.3	49.1 ± 1.1	6
0	15	79.0 ± 0.3	9.6 ± 0.3	41.1 ± 2.4	6
1	15	77.6 ± 0.3	17.0 ± 0.4*	76.4 ± 2.1*	6
0	120	76.2 ± 0.4	9.6 ± 0.8		8
1	120	77.3 ± 0.5	35.5 ± 2.1*		8

Note. Values are means ± SE. Glands were removed 1 day after injection of a single dose of reserpine (RES) (0.5 mg/kg body wt); separate groups of rats were used for controls (saline injected).

* Indicates value for reserpine-treated gland differs significantly from that of controls ($P < 0.001$).

TABLE II. EFFECTS OF SURGICAL SYMPATHECTOMY (Sx) OF 2 DAYS DURATION ON [Ca] OF SUBMANDIBULAR GLAND AT VARIOUS POSTNATAL AGES

Age at Sx (days)	Age at sacrifice (days)	Condition	% Water	[Ca] of submandibular gland		No. of rats
				meq/kg wet wt	meq/kg dry wt	
8	10	Con	82.5 ± 1.6	6.8 ± 1.6	30.3 ± 2.2	4
		Sx	82.3 ± 1.5	7.9 ± 0.4	44.6 ± 2.0	
14	16	Con	80.0 ± 1.0	7.5 ± 0.7	36.3 ± 3.5	4
		Sx	79.3 ± 0.3	20.3 ± 1.5*	101.5 ± 5.7*	
15	17	Con	78.5 ± 0.5	8.5 ± 1.1	38.5 ± 6.7	6
		Sx	79.5 ± 0.3	21.7 ± 2.3*	89.5 ± 6.6*	
16	18	Con	76.0 ± 1.1	9.5 ± 1.1	45.3 ± 6.1	4
		Sx	75.0 ± 0.7	22.8 ± 1.1*	91.4 ± 3.5*	
118	120	Con	76	11.4 ± 0.8		10
		Sx	77	41.9 ± 0.8*		

Note. Values are means ± SE; each group represents sympathectomized (Sx) and contralateral normally innervated gland (Con).

* Indicates value for Sx gland differs significantly from that of Con ($P < 0.001$).

denervated gland was more than twice that of controls (Table II).

Table III shows that administration of CC did not cause any change in glandular concentration of calcium from that of controls, if rats were 12 days of age. When the initial injection of CC was made at 15 days of age, the [Ca] of the glands of the CC-treated rats was more than three times higher than that of the submandibular glands of untreated rats. CC, given at 26 days of age, also induced a marked increase in the [Ca] of the submandibular gland.

With reserpine, surgical Sx, or administration of CC, the [Ca] showed increases similar in magnitude whether expressed per unit of wet or dry tissue. Water content of the glands showed a small age-associated decrease that was most pronounced at the early postnatal ages (Tables I, II, III). Water content of control and experimental glands did not differ significantly from each other ($P > 0.05$).

Discussion. Although, in the adult rat, chemical or surgical sympathectomy, or injection of CC, is followed by an increase in concentration of calcium (11) and depletion of NE, of the submandibular gland (2, 4–11), present data show that none of these manipulations resulted in an increase in [Ca] of the submandibular gland of the early

TABLE III. EFFECTS OF CYCLOCYTIDINE (CC) ON [Ca] OF RAT SUBMANDIBULAR GLAND AT VARIOUS POSTNATAL AGES

Age at sacrifice (days)	Treatment	% Water	[Ca] of submandibular gland		No. of rats
			meq/kg wet wt	meq/kg dry wt	
12	O	82.1 ± 0.4	7.9 ± 0.5	45.6 ± 2.0	6
12	CC	80.1 ± 0.8	7.9 ± 0.5	39.5 ± 1.9	6
18	O	79.3 ± 0.3	9.5 ± 0.7	36.3 ± 3.5	8
18	CC	78.1 ± 0.4	32.1 ± 1.9*	149.5 ± 9.9*	8
29	O	78.0 ± 0.4	8.7 ± 0.8	39.1 ± 3.1	4
29	CC	76.3 ± 1.4	19.6 ± 2.1*	85.5 ± 7.5*	4

Note. Values are means ± SE. CC administered in dose of 500 mg/kg daily for 3 days.

* Indicates value for CC-treated glands differs significantly from that of controls ($P < 0.001$).

neonatal rat (4–11 days of age). On the other hand, if rats were more than 12 days of age at the time of the initial injection of reserpine, CC, or removal of a superior cervical ganglion, the [Ca] of the submandibular gland was found to be increased. If the increase in [Ca] is related to the experimentally induced depletion of NE, it is not surprising that administration of reserpine to rats less than 5 days of age caused no increase in the [Ca] of the submandibular gland. It has been demonstrated that catecholamine-containing neurones are not seen in the parenchyma of the submandibular gland before 5 days of age, and electrical stimulation of the superior cervical ganglion does not elicit secretion of peroxidase until this age (8). However, between 5 and 11 days of age, the number of catecholamine-containing neurones (8), the levels of catecholamines (13), and secretion of peroxidase increase greatly (8). In view of the fact that a functional, albeit incompletely developed, sympathetic innervation is present between 5 and 11 days of age, it is surprising that the maneuvers that deplete the levels of NE and cause an increase in the [Ca] of the submandibular gland (4–11) in the adult rat fail to do so in these neonatal rats. It is possible that the accumulation of glandular calcium follows the described manipulations only if the sympathetic innervation is already completely developed (8). It is also possible that calcium transport mechanisms do not become functional in the developing submandibular cells before 14 days of age at which time they become susceptible to the lack of NE. However, even in rats 14–29 days of age, the ability of the submandibular gland to accumulate calcium is generally less than that of the adult, suggesting that calcium transport mechanisms, though functional, may not be fully developed before 29 days of age. The data show that there are age-related differences in the ability of the submandibular gland to accumulate calcium following maneuvers that lead, in the adult, to reduction in levels of the sympathetic neurotransmitter. To establish that in the neonate also, an increase in the [Ca] is related to depletion of NE, direct measurements of NE must be done.

This work was supported by Grants DE 06185 and DE 02110 from the National Institute of Dental Research, NIH, Bethesda, Md. 20205.

1. Dreisbach RH. Secretion of calcium by rat submandibular gland. Amer J Physiol **196**:645–648, 1959.
2. Wood DL, Martinez JR. The chronically reserpinized rat as a possible model for cystic fibrosis. VI. Synergistic effects of isoproterenol on Ca^{++} and protein in the submaxillary gland. Pediatr Res **11**:827–832, 1977.
3. Bazerque PM, Perec CJ, Terradas SE. Calcium in salivary glands of the rat after autonomic denervation. J Dent Res **51**:918–922, 1972.
4. Schneyer CA, Yu JH, Jirakulsomchok D. Calcium concentration of salivary glands and saliva after chemical or surgical sympathectomy. J Auton Nerv Sys **8**:171–177, 1983.
5. Benmiloud M, von Euler US. Effects of bretylium, reserpine, guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland. Acta Physiol Scand **59**:34–43, 1963.
6. Cutler LS, Christian CP, Bottaro B. Development of stimulus-secretion coupling in salivary glands. In: Hand A, Oliver C, eds. Methods and Perspectives in Cell Biology. New York, Academic Press, Vol 23: pp531–545, 1981.
7. Ludford JM, Talamo BR. Beta adrenergic and muscarinic receptors in developing rat parotid glands. J Biol Chem **255**:4619–4627, 1980.
8. Bottaro B, Cutler LS. An electrophysical study of the postnatal development of the autonomic innervation of the rat submandibular gland. Arch Oral Biol **29**:237–242, 1984.
9. Schneyer CA, Galbraith WM, Mellett LB. Unexpected beta-adrenergic effects of the anti-tumor agent, cyclocytidine, on rat salivary glands. Proc Soc Exp Biol Med **148**:1206–1211, 1975.
10. Schneyer CA, Galbraith WM. Inhibition of cyclocytidine-induced enlargement of parotid and heart by propranolol or sympathectomy. Proc Soc Exp Biol Med **150**:394–400, 1975.
11. Schneyer CA, Yu JH. Calcium concentration of saliva and salivary glands of rat after cyclocytidine. Proc Soc Exp Biol Med **175**:16–20, 1984.
12. Schneyer CA, Yu JH. Postnatal changes in calcium and amylase of rat salivary glands, including calcium changes with senescence. Arch Oral Biol **30**:137–140, 1985.
13. Ikeno T, Hashimoto S, Nemoto K, Kuzuya H. Monoamine oxidase activities on biogenic amines in the salivary glands of young growing rats. Arch Oral Biol **25**:71–73, 1980.
14. Giachetti A, Shore PA. Permeability changes induced in the adrenergic neurone by reserpine. Biochem Pharmacol **19**:1621–1626, 1970.
15. Dreisbach RH. Calcium binding by rat submaxillary gland in vitro. Biochem J **82**:71–75, 1962.

Received October 15, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted January 28, 1985.