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NERVE GROWTH FACTOR-INDUCED INCREASE IN [^{3}H]THYMIDINE INCORPORATION INTO PAROTID AND SUBMANDIBULAR GLANDS OF YOUNG RATS AND ITS PARTIAL BLOCKADE BY PROPRANOLOL OR PARTIAL SIALOADENECTOMY

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Abstract—Administration of nerve growth factor (NGF) twice daily for 2 days to young rats (11 days old at the time of the initial injection) resulted in an 8.1-fold increase in [^{3}H]thymidine levels of the parotid gland, and a 9.7-fold increase in levels of the submandibular gland when compared to untreated controls. Isoproterenol (ISO), a β-adrenergic receptor agonist, caused an 8.7-fold increase in [^{3}H]thymidine incorporation into DNA of the parotid gland, and a 10.7-fold increase in [^{3}H]thymidine in the submandibular gland when compared to controls. The increase in thymidine was accompanied by parotid gland enlargement as well as an increase in cell surface β1–4 galactosyltransferase, an enzyme whose expression has been associated previously with acinar cell proliferation. Administration of NGF and ISO together were not additive in their effects on the parotid and submandibular glands. The introduction of propranolol, a β-adrenergic receptor antagonist, completely negated the ISO effects on the salivary glands but was only partially effective in blocking the NGF effects on the glands. An assay of parotid levels of norepinephrine showed NGF treatment to cause an increase in gland-associated levels of neurotransmitter. Removal of the submandibular/sublingual glands prior to administration of ISO prevented the above changes in the parotid gland. NGF administered to partially sialoadenectomized rats was also less effective in inducing parotid gland hypertrophy and hyperplasia. Simultaneous administration of NGF and ISO to the partially sialoadenectomized rats had an additive influence on [^{3}H]thymidine incorporation, galactosyltransferase expression and gland hypertrophy. The results suggest that NGF influences salivary gland cell growth in part through activation of cell-surface β-adrenergic receptors.

Nerve growth factor (NFG§) and epidermal growth factor (EGF) are synthesized in large quantities in rodent and murine submandibular gland [1–4]. Both are also present in the circulation. The physiological significance of NGF or EGF when released into saliva and the circulation by hormones and stimulation with adrenergic agonist [5, 6], however, is unknown. When injected into animals, or provided to cells in tissue culture, NGF promotes the growth of neurons, whereas EGF is mitogenic in various tissues and cells [4, 7, 8].

The salivary glands of the neonatal rat are both histologically and developmentally immature [9–13]. The normal physiological responses of the gland during maturation are mediated through development of the autonomic innervation [14, 15]. Treatment with isoproterenol (ISO), a β-adrenergic agonist, between 9 and 36 days postpartum leads to precocious morphological development of the parotid and submandibular glands [16–19]. ISO has also been noted to accelerate changes in protein composition and response of adenylate cyclase by increasing intracellular cyclic AMP (cAMP) pools [20–23].

When injected into adult animals, ISO causes parotid and submandibular gland hypertrophy and hyperplasia of the acinar cells [24, 25]. The physiological change in the parotid gland of hyperplasia is accompanied by a dramatic increase in the synthesis and accumulation at the cell surface of the enzyme β1–4 galactosyltransferase (EC 2.4.1.38) [26–28]. Numerous reports have implicated cell surface galactosyltransferase in mediation of such developmental and proliferative events as cell adhesion [29, 30], recognition [31], differentiation [32, 33] and embryogenesis [34]. This enzyme has also been shown to be present in a number of different cancer cells [35, 36]. Galactosyltransferase at the surfaces of contacting cells has also been suggested to be a possible mediator of growth control. Roth and White [37] presented evidence implicating these enzymes in the contact inhibition exhibited by nontransformed tissue culture cells. Indeed, in the ISO-treated parotid gland, cell proliferation can be retarded dramatically by the introduction of specific substrates for the

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§ Abbreviations: NGF, nerve growth factor; 4β-galactosyltransferase, β1–4 galactosyltransferase; UDP-gal, uridine diphosphate-galactose; TCA, trichloroacetic acid; ISO, isoproterenol; DES, sialoadenectomy; EGF, epidermal growth factor; NE, norepinephrine; IN, propranolol; DHA, dihydroalprenolol; and QNB, quinuclidinylbenzilate.

enzyme [27, 28], the most significant of these being the milk protein α-lactalbumin.

In the present study, we investigated the role of NGF in mediating salivary gland hypertrophy and hyperplasia of young animals. The growth-promoting role of NGF was compared to that of ISO in order to determine the mechanism by which it mediates cell proliferation in the developing parotid gland. This was accomplished by using the β-adrenergic antagonist, propranolol, or by using partially desalivated animals, since both maneuvers prevent ISO-induced growth of salivary glands.

EXPERIMENTAL PROCEDURES

Materials. *dl*-Isoproterenol, trichloroacetic acid, UGP-galactose, propranolol, CMP-sialic acid, fetuin, nerve growth factor, and ovalbumin were purchased from the Sigma Chemical Co. (St. Louis, MO). UDP-[^{14}C]-galactose (300 Ci/mmol), [^{3}H]thymidine and CMP-[^{3}H]-sialic acid (60 Ci/mmole) were obtained from the Amersham Corp. (Arlington Heights, IL). [^{3}H]Dihydroalprenolol ([^{3}H]DHA, 30–70 Ci/mmol) and [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB, 30–60 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Long–Evans rats, 10 to 13-days-old, were from the University of Alabama at Birmingham breeding colony maintained by Dr. C. A. Schneyer. Animals were maintained on solid lab chow and water *ad lib.* or permitted to continue suckling. All other reagents were of ultrapure quality obtained through commercial sources.

Experimental drug regimen of animals. Animals were divided into two groups: the first group was control rats with intact submandibular/sublingual glands, while the second group had undergone submandibular/sublingual gland ablation at the initiation of the drug regimen. Animals subsequently received twice daily injections of 0.1 mL of propranolol (3 or 9 mg/kg body wt), or NGF (10 ng/kg body wt), or ISO (50 mg/kg body wt). These compounds were also given in combination as indicated in the Results.

Preparation of parotid acinar cell membranes. Parotid glands were removed from rats following anesthesia with pentobarbital (1%). Total membrane fractions were prepared at 4° by homogenization of intact parotid tissue from the various treatments in 10 mM Tris–HCl buffer, pH 8.0, using a Biohomogenizer from Biospec Inc. followed by lysis in a Dounce Apparatus [38]. Then, low-speed centrifugation at 1000 *g* was performed to remove connective tissue as well as unlysed cells. The resulting slurry was centrifuged at 100,000 *g* for 1 hr to pellet total membrane. Protein assays were performed by a modification of the Lowry method with bovine serum albumin as standard [39]. Plasma membranes were isolated by the procedure of Arvan and Castle [40], a protocol designed specifically to isolate plasma membranes from rat parotid gland. The total membrane pellet was resuspended in 0.5 mM $MgCl_2$, 1 mM $NaHCO_3$ (pH 7.4) containing 0.7 mM EDTA. The membrane slurry was centrifuged at 825 *g* for 15 min, and the pellet was resuspended in the same buffer containing 0.3 M sucrose and recentrifuged at 12,500 *g* for 15 min. The pellet was adjusted to 1.38 M sucrose (125 M), overlayed with 0.3 M sucrose, and centrifuged for 2 hr in a Beckman swinging bucket rotor SW27 at 50,000 *g*. Plasma membranes were removed from the interface, diluted to 0.35 M sucrose in 0.5 mM $MgCl_2$, 1 mM $NaHCO_3$ (pH 7.4) containing 1.7 mM EDTA, and centrifuged in an SW40 rotor for 2 hr at 150,000 *g*. Fractions enriched for Golgi membranes were obtained from the supernatant fraction of the 12,500 centrifugation [28, 40]. This supernatant fraction was diluted to 12 mL in 0.3 M sucrose and centrifuged at 100,000 *g* for 1 hr. The final membrane pellets were resuspended in 10 mM Tris buffer (pH 8.0) for subsequent assays for protein and glycosyltransferase activity.

Glycosyltransferase assays. The activities of two glycosyltransferases were measured as previously described by Humphreys-Beher *et al.* [27]. In brief, membrane fractions were obtained as described above and resuspended in 10 mM Tris–HCl, pH 8.0, to give a final protein concentration of 200 μg/mL. The assay mixture (total volume 50 μL) contained 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.3, 25 mM $MnCl_2$, 0.5% Triton X-100, 1 mM UDP-[1-^{14}C]-galactose, 0.5 mg ovalbumin (or 10 mM GlcNAc) and 0.05 mg of the membrane preparation for the assay of β1–4 galactosyltransferase [3]. Assays for sialyltransferases were performed using the same buffer conditions. One millimolar CMP-[9-^{3}H]-sialic acid and 10 μg asialo-fetuin were included as sugar donor and acceptor molecules respectively. Sialic acid was removed from glycoprotein acceptors by acid hydrolysis [41]. Incorporation of ^{14}C or ^{3}H into glycoprotein acceptors was determined by TCA precipitation of the reaction mixtures onto glass-fiber filters.

Incorporation of thymidine into DNA. In vivo DNA synthesis was followed in control and experimentally-treated rat parotid glands by monitoring the incorporation of [^{3}H]thymidine into TCA-precipitable counts. Injections of NGF, ISO, propranolol, or combinations of these compounds using the above regimen were followed by the intraperitoneal administration of 10 μCi [^{3}H]thymidine/20 g rat at 48 hr. The animals were killed 5 hr following this injection, and their glands were removed and homogenized at 4°. One hundred microliters was removed for TCA precipitation on glass-fiber filters followed by scintillation counting for ^{3}H-incorporation. Part of the sample was removed for a protein assay. Sets of untreated animals were used to determine the basal rates of DNA synthesis.

In vivo *and* in vitro *estimation of β-adrenergic receptor occupancy.* [^{3}H]DHA, a β-adrenergic receptor antagonist, was injected into animals in an attempt to estimate the level of functional β-adrenergic receptors present on acinar cells of parotid glands. One millimole of [^{3}H]DNA (diluted with unlabeled dihydroalprenolol to give a final concentration of 1000 cpm/10 fmol) was injected following treatment with the drug regimen described earlier. After 3 hr, the animals were killed and the glands collected. The tissue was homogenized in

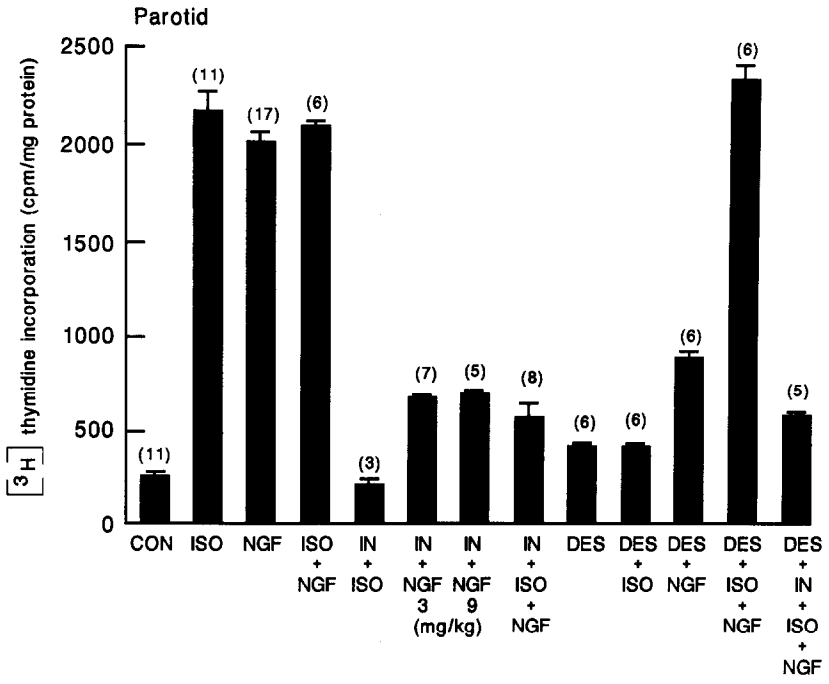

Fig. 1. ISO or NGF effects on [^{3}H]thymidine incorporation in parotid gland of intact, partially desalivated, or IN-treated rats. Values are means ± SE for parotid gland of 13-day-old rats. Abbreviations are as follows: CON (no drugs administered, glands intact); ISO (*dl*-isoproterenol HCl, 50 mg/kg body wt, i.p., two times daily); NGF (nerve growth factor, 10 ng/kg body wt, i.p., two times daily); ISO + NGF (both drugs given i.p. in doses listed above); IN + ISO (In = propranolol, given 20 min prior to ISO, in a dose of 3 mg/kg body wt); IN + NGF (propranolol, given 20 min prior to NGF, in a dose of 3 or 9 mg/kg body wt); IN + ISO + NGF (dosages as described above); DES (partial desalivation involving removal of both submandibular and sublingual glands); DES + ISO or + NGF (drugs administered in doses described above, beginning 2 days after desalivation). Numbers in parentheses = number of rats.

2 mL of 10 mM Tris–HCl, pH 7.4. One hundred microliters was precipitated onto glass-fiber filters using Tris buffer. Part of the sample was used for protein determination as described above. The quantity of radioligand was found to be saturating for β-adrenergic receptors of the parotid gland by prior injection of various concentrations of radioligand.

For *in vitro* assay of receptors, gland homogenates for both [^{3}H]QNB and [^{3}H]DHA binding were prepared by centrifugation at 20,000 *g* for 30 min (4°) [42]. The pellet containing the membrane fraction was resuspended in 100 μM dithiothreitol. Membranes were resuspended by a combination of vortex vibration followed by Dounce homogenization. Protein concentrations were determined subsequently as described above by a modification of the Lowry protein assay using bovine serum albumin as standard [39]. Binding of [^{3}H]QNB and [^{3}H]DNA was linearly dependent on membrane concentration within this dilution of both the parotid and submandibular glands [43]. Binding assays were performed in duplicate using 1.0 mL of diluted membrane and 1.0 nM [^{3}H]QNB or [^{3}H]DHA. The reaction mixture was incubated for 90 min at 37° and terminated by the addition of 3 mL of ice-cold 0.9% NaCl. Quantitation of binding was performed by precipitation of membranes from the above slurry onto glass-fiber filters, washed three times with 5 vol. of cold phosphate-buffered saline (PBS), and counted for radioactivity by liquid scintillation. Non-specific binding was determined by the inclusion of 1.0 μM atropine 10 min prior to the addition of labeled QNB. Non-specific binding for DHA was determined by the inclusion of 10 μM (−)-propranolol prior to the addition of radiolabel.

Quantitation of norepinephrine release associated with NGF treatment. For determination of norepinephrine, tissues were first homogenized (using a Brinkmann Polytron) in 2 mL cold 0.05 M perchloric acid which contained 10 mg/L sodium metabisulfite as an anti-oxidant. Following centrifugation at 3°, the catecholamines in the protein-free supernatant fraction were adsorbed with 100 mg of acid-washed alumina in 3 M Tris buffer (pH 8.6). After washing with water, the catecholamines were eluted with 1 mL of 0.5 M perchloric acid (with 10 mg/L sodium metabisulfite).

The level of norepinephrine was analyzed using a modification of a method described by Krstulovic [44] employing HPLC and electrochemical detection. The separation was achieved with a Supelco $C_{18}RP_3$ μM column and a Waters pump. The mobile phase, a phosphate–citric acid buffer (0.347 M KH_2PO_4 and 0.03 citric acid), contained 3 mM sodium acetyl sulfate as an ion-pair and methanol

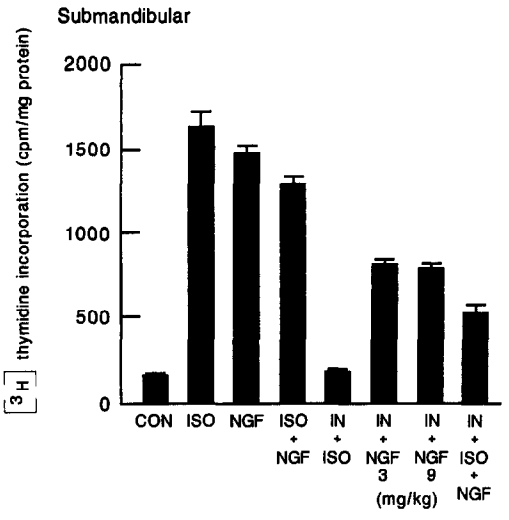

Fig. 2. ISO or NGF effects on [^{3}H]thymidine incorporation in submandibular glands of intact, partially desalivated, or IN-treated rats. Values are means ± SE for submandibular gland of 13-day-old rats. All abbreviations are the same as those in Fig. 1 except for the fact that DES groups are not included.

(14%) as a modifier. The eluting compounds were detected with a Bioanalytical Systems ampermometric detector set at a potential of + 0.5 V. Student's *t*-test was used for statistical analysis of the data.

RESULTS

The data in Fig. 1 show that NGF administered daily to young rats (11 days old at the time of initial injection) caused an 8.1-fold increase from controls in [^{3}H]thymidine incorporation into DNA of parotid gland when measurements were made 2 days later. This was similar in magnitude to the increase (8.7-fold) effected by administration of large doses (50 mg/kg body wt) of ISO for 2 days (values for NGF and ISO did not differ statistically, $P > 0.05$). Administration of NGF to young animals also caused increases in parotid gland wet weight similar to those observed previously with ISO (Table 1). However, the injection of both compounds into the same rats did not result in an additive effect on either gland enlargement or DNA synthesis ($P > 0.05$). Both NGF and ISO caused an increase in the enzyme 4β-galactosyltransferase, along with previously demonstrated ectopic expression in the plasma membrane fractions of acinar cells during transition from stasis to active proliferation (Table 1) [27, 28, 38].

When the β-adrenergic antagonist propranolol (IN) was administered 20 min prior to injection of ISO, the normally induced gland enlargement and [^{3}H]thymidine incorporation were prevented completely. In contrast to this, when propranolol was given in either a dose of 3 or 9 mg/kg, prior to injection of NGF, values for [^{3}H]thymidine incorporation remained approximately 35% of those observed with NGF alone. There was also evidence of a limited increase in gland weight (33 mg compared with 25 mg for NGF/propranolol and control animals respectively). Total galactosyltransferase activity as well as localization of enzyme activity in the plasma membrane fraction showed an increase when compared to controls.

The data in Fig. 1 and Table 1 also show the effects of submandibular gland ablation on ISO- and NGF-induced [^{3}H]thymidine incorporation of the parotid gland. [^{3}H]Thymidine incorporation into DNA of parotid gland of partially sialoadenectomized rats given NGF for 2 days was 44% of the mean values observed in intact rats given NGF for the same period of time. Similarly, gland enlargement and galactosyltransferase expression were decreased (51% of NGF-treated controls). However, as present data as well as previous data on other age groups have shown [42, 45], when the partial sialoadenectomy preceded ISO administration (2 days), the usual ISO-induced increase in [^{3}H]thymidine incorporation, gland weight gain and altered galactosyltransferase expression were abolished almost completely. The [^{3}H]thymidine values for parotid of rats with partial sialoadenectomy and those given ISO in addition to sialoadenectomy were the same. The values for sialoadenectomized animals (no ISO) and sialoadenectomized animals given ISO were nonetheless somewhat higher than those observed in controls, since partial sialoadenectomy itself causes a small increase in [^{3}H]thymidine incorporation [45]. The increase induced by the partial gland ablation was reflected in the 44% increase observed in the partially sialoadenectomized rats given NGF. Administration of both NGF and ISO to partially desalivated rats resulted in an increase in [^{3}H]thymidine values to levels in excess of those of intact rats given ISO and NGF (Fig. 1). While parotid gland enlargement did not exceed that observed with control animals, the effect of these compounds together in sialoadenectomized rats was additive (Table 1). With the administration of propranolol to either intact or sialoadenectomized rats prior to the injection of ISO and NGF, [^{3}H]thymidine values for parotid gland were the same in both groups of animals, and similar to values observed in rats administered propranolol and NGF (Fig. 1).

While the parotid gland showed the most dramatic physiological changes in response to NGF, the submandibular gland also showed alterations in DNA synthesis. [^{3}H]Thymidine incorporation into submandibular gland of young rats given NGF for 2 days showed a 9.7-fold increase over control levels, while ISO given for the same time showed a 10.7-fold increase over control levels of incorporation (Fig. 2). When these compounds were given together, values for [^{3}H]thymidine were somewhat less (8.5-fold increase over control levels). However, when propranolol was administered 20 min prior to NGF, the [^{3}H]thymidine levels showed only a 5.3-fold increase over controls, in contrast to values with IN and ISO where values were identical to those of controls.

In vivo and *in vitro* quantitation of β-adrenergic and cholinergic receptors was performed in an effort to delineate the possible mechanism of NGF mediation of parotid gland enlargement and cell

Table 1. Glycosyltransferase expression in parotid gland acinar cells after isoproterenol treatment

Treatment	Gland wt*	4β-Galactosyltransferase†			Sialyltransferase‡		
		Total	Golgi	Plasma membrane§	Total	Golgi	Plasma membrane§
Control	25 ± 2	0.07 ± 0.01	2.63 ± 0.14	0.02 ± 0.01	0.17 ± 0.09	3.69 ± 0.21	0.01 ± 0.01
DES	26 ± 3	0.07 ± 0.01	2.51 ± 0.17	0.01 ± 0.01	0.21 ± 0.11	3.40 ± 0.25	0.02 ± 0.01
ISO	65 ± 3	0.37 ± 0.07	2.71 ± 0.19	1.66 ± 0.03	0.16 ± 0.03	3.59 ± 0.17	0.01 ± 0.01
DES/ISO	29 ± 3	0.09 ± 0.02	2.45 ± 0.17	0.03 ± 0.02	0.19 ± 0.07	3.61 ± 0.17	0.01 ± 0.01
NGF	68 ± 4	0.29 ± 0.02	2.68 ± 0.13	1.81 ± 0.05	0.14 ± 0.04	3.65 ± 0.23	0.01 ± 0.01
DES/NGF	35 ± 4	0.14 ± 0.04	2.70 ± 0.13	0.30 ± 0.04	0.17 ± 0.05	3.52 ± 0.21	0.02 ± 0.01
ISO/IN	27 ± 3	0.10 ± 0.03	2.81 ± 0.21	0.02 ± 0.01	0.16 ± 0.03	3.49 ± 0.21	0.01 ± 0.01
DES/ISO/IN	26 ± 4	0.11 ± 0.03	2.81 ± 0.15	0.03 ± 0.01	0.14 ± 0.06	3.56 ± 0.23	0.01 ± 0.01
NGF/IN	33 ± 4	0.20 ± 0.03	2.67 ± 0.19	0.38 ± 0.01	0.20 ± 0.03	3.59 ± 0.23	0.02 ± 0.01
DES/NGF/IN	35 ± 5	0.16 ± 0.05	2.71 ± 0.17	0.18 ± 0.01	0.19 ± 0.03	3.61 ± 0.20	0.01 ± 0.01
ISO/NGF	69 ± 3	0.43 ± 0.04	2.68 ± 0.16	1.83 ± 0.02	0.19 ± 0.05	3.59 ± 0.15	0.01 ± 0.01
DES/ISO/NGF	59 ± 5	0.49 ± 0.05	2.59 ± 0.16	1.74 ± 0.03	0.16 ± 0.06	3.69 ± 0.11	0.01 ± 0.01
ISO/NGF/IN	30 ± 3	0.10 ± 0.03	2.74 ± 0.18	0.22 ± 0.01	0.20 ± 0.04	3.63 ± 0.13	0.02 ± 0.01
DES/ISO/NGF/IN	33 ± 4	0.10 ± 0.04	2.55 ± 0.13	0.02 ± 0.01	0.21 ± 0.03	3.71 ± 0.17	0.02 ± 0.01

Values are based on the average of 7 experimental animals ± SE; IN dosage = 9 mg/kg body wt; other dosages and descriptions are given in Table 2.

* Weight is given in mg.

† Values are given in nmol/min/mg protein utilizing 1 mg/mL ovalbumin as exogenous acceptor.

‡ Values are given in nmol/min/mg protein utilizing 1 mg/mL asialo-fetuin as exogenous acceptor.

§ Enrichment of plasma membrane fractions was determined by the assay of the parotid gland cell plasma membrane marker enzyme, glutamyltranspeptidase [40]. Plasma membranes prepared in this fashion resulted in a 10-fold enrichment in the specific activity for this enzyme over that present in the control membrane preparation (4000 units/mg protein on the average for treated animals and 375 units/mg, respectively, control).

Table 2. Properties of membranes from parotid and submandibular glands of 13-day-old rats injected with NGF, ISO, or IN/NGF

Treatment	No. of rats	Receptor density [^{3}H]DHA binding (fmol/mg membrane protein)		[^{3}H]QNB binding (fmol/mg membrane protein)	
		Parotid	Submandibular	Parotid	Submandibular
None	6	38 ± 0.3	27 ± 0.8	176 ± 0.3	136 ± 0.8
ISO	4	38 ± 0.3	28 ± 0.0	175 ± 0.9	135 ± 1.0
NGF	5	38 ± 0.4	27 ± 0.5	175 ± 0.2	136 ± 0.5
NGF + ISO	6	37 ± 0.4	27 ± 0.4	175 ± 1.0	135 ± 0.9
IN + NGF	4	38 ± 0.4	27 ± 0.3	176 ± 0.6	134 ± 0.4

Values are means ± SE. ISO (50 mg/kg body wt), NGF (10 ng/kg body wt), and IN (3 mg/kg body wt) were injected twice daily for 2 days. Abbreviations: ISO = isoproterenol; NGF = nerve growth factor; and IN = propranolol.

Table 3. Binding levels of [^{3}H]propranolol to parotid gland acinar cells

Treatment	[^{3}H]Propranolol bound (cpm/mg protein)
Control	3475 ± 25
ISO	475 ± 50
DES	3500 ± 75
NGF	1265 ± 50
DES/NGF	1300 ± 40
IN (9 mg/kg)	610 ± 30
DES/IN (9 mg/kg)	645 ± 45
NGF/IN (9 mg/kg)	620 ± 20

Values are means ± SE determined from four experimental animals. Assays were performed in duplicate. DES = removal of submandibular-sublingual glands. Dosages of ISO and NGF are given in Table 2.

proliferation. As shown in Table 2, short-term administration of NGF and ISO had no effect on the level of receptor density when measured by [^{3}H]DHA or [^{3}H]QNB binding. This is also consistent with previous results indicating that short-term removal of the submandibular gland does not alter receptor density of the parotid gland of young rats [42, 45]. Intact control and partially desalivated rats bound the same amount of labeled propranolol (3475 cpm/mg protein and 3500 cpm/mg protein respectively); this was similar to the quantity bound from *in vitro* parotid membrane assays (Table 3). The level of [^{3}H]propranolol retained was equivalent to approximately 34 fmol/mg protein for parotid gland β-adrenergic receptors (see Table 2 for corresponding *in vitro* values). One hour after ISO treatment, [^{3}H]propranolol binding decreased to 13.7% of control values. Injection of unlabeled propanolol showed no further effect on radioligand binding. Animals injected with NGF also demonstrated decreased capacity to bind [^{3}H]propranolol; however, this level was 36% that of untreated animals.

To determine if receptor occupancy could account for the difference in *in vivo* and *in vitro* receptor assays, the level of norepinephrine in the parotid gland was analyzed from NGF-treated animals. Animals treated with NGF demonstrated a norepinephrine concentration of 610 ± 92 ng/g of tissue (9 rats) as compared to 195 ± 7 (7 rats) for control levels. In older animals (35 days of age), this NGF-induced increase in parotid gland norepinephrine levels was diminished substantially and was only 15% higher than in control rats (639 ng/g tissue compared to 554 ng/g tissue for NGF-treated and control animals).

DISCUSSION

Present data show that NGF had a growth stimulatory effect on parotid and submandibular glands of young rats, since [^{3}H]thymidine incorporation was enhanced greatly when comparison was made with controls. Earlier work showed that EGF also has a small stimulatory effect on proliferative activity of these organs [42, 46] but NGF-induced growth was very much greater than that induced by EGF. In fact, NGF-induced proliferative activity as assessed by [^{3}H]thymidine incorporation was virtually equal to that induced by administration of large doses of the β-adrenergic agonist, ISO [10, 24, 25]. It is unexpected to find another substance that can induce the marked increase in ^{3}H-incorporation that is induced by ISO. Both ISO and NGF appear to activate the same group of receptors since, with the administration of ISO and NGF to the same animal, values for [^{3}H]thymidine incorporation or galactosyltransferase activity from parotid gland did not differ ($P > 0.05$) from those observed with either compound alone. Moreover, it appears that the principal receptor involved in mediating the biochemical and morphological responses induced by NGF as well as ISO are β-adrenoceptors. The conclusions based on data from parotid gland can also be made from the observations on the submandibular gland. Only the proportions differ slightly and this is probably a reflection of the fact that the number of β-adrenergic receptors present in the organs differ from one another [43]. However, while all of the receptors mediating the ISO-induced responses are

β-adrenoceptors (since propranolol inhibited isoproterenol-induced changes completely), NGF-induced growth was only partially mediated via β-adrenoceptors (since propranolol did not suppress completely NGF-induced thymidine incorporation). Thus, while values for [^{3}H]thymidine, gland wet weight, and galactosyltransferase activity for parotid gland of rats given propranolol prior to ISO were the same as those of controls, mean thymidine values for parotid gland of rats given propranolol prior to NGF were 35% of the values observed when NGF was given without the β-antagonist present, indicating that at least 65% of the NGF-induced increase must be mediated through β-adrenoceptors.

The use of partially sialoadenectomized animals provided further evidence to show that NGF-induced thymidine increases were not solely mediated by activation of β-adrenoceptors. Thus, present data as well as earlier data on animals of other ages [42, 45] and other species [8, 46] showed that ISO failed to induce DNA synthesis of parotid gland in partially desalivated rats; thymidine levels were the same as those of desalivated rats not given ISO. Introduction of NGF, on the other hand, induced an increase in thymidine levels of the parotid gland in the partially desalivated rats that was not solely β-adrenoceptor mediated [46]. Since propranolol again completely blocked ISO (but not NGF)-induced DNA synthesis, the elevated thymidine levels in parotid of partially desalivated rats given propranol prior to ISO plus NGF must be attributed to non-β-mediated effects of NGF. Determination of other factors influencing salivary gland growth response is under investigation.

The parotid and submandibular glands of the neonatal rat depend on neurological maturation to produce the physiological and biochemical changes necessary for functional development [10–12]. Nerve growth factor is a peptide hormone that is essential for the development and differentiation of sympathetic nerves and other cell types of the central nervous system [47, 48]. Therefore, the administration of NGF to neonatal animals could be expected to exert its growth promoting influence on parotid glands of control animals by causing precocious nerve development and maturation of the sympathetic nerves to the gland. Thus, NGF was found to increase norepinephrine concentrations of parotid gland when the animals were still very young (13 days of age) and, at 13 days, these levels were elevated to [NE] observed for normal 35-day-old rats. The number of β-adrenoceptors, however, was not changed by NGF. Thus, accelerated differentiation of the adrenergic nerves but not of β-adrenoceptors may therefore be implied.

In conclusion, it is clear that NGF exerts a growth promoting effect on rat salivary glands of young rats that is principally, but not exclusively, mediated through activation of β-adrenoceptors. The fact that ISO induced DNA synthesis in parotid glands only when the submandibular glands were present, or, in their absence, when NGF (or EGF [42, 45]) had been administered, suggests that peptide growth factors have an important role in the sequence of events leading to activation of β-adrenoceptors and subsequent DNA synthesis.

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