Acetylcholine Synthesis and Release following Continuous Intracerebral Administration of NGF in Adult and Aged Fischer-344 Rats

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NGF promotes the survival and enhances the neurotransmitter phenotype of basal forebrain and striatal cholinergic neurons in brain of rat. The objective of the present study was to determine whether stimulations of the cholinergic neuronal markers ChAT and high-affinity choline uptake are reflected in enhanced synthesis and release of ACh. Enhancement of ACh release in brain of adult and aged rats could result in increased cholinergic neurotransmission, and altered animal behavior. NGF (1.2 µg/d) was administered intracerebroventricularly for 2 weeks by osmotic minipump to male Fischer-344 rats aged 4 and 24 months. Cholinergic neuronal functional parameters were measured in frontal cortex, hippocampus, and striatum. In young adult rats, increased ChAT and choline uptake activities were accompanied by enhanced ACh synthesis, basal and depolarization-induced release of both endogenous and newly synthesized transmitter, with the largest effect generally being observed in striatum. In aged animals, the responses to NGF were less uniform. Whereas the pattern for changes in ChAT activity was similar to that seen in younger animals, choline uptake activity was increased only in frontal cortex and striatum. Coincidentally, ACh synthesis was also increased only in these two brain regions. ACh content of synaptosomes was not affected by age or NGF treatment, and the ACh levels in microwaved samples of striatum and basal forebrain were not affected by NGF treatment. However, profound deficits in both basal and evoked release of newly synthesized ACh were observed in the aged rats. NGF treatment had no significant effect on the basal release of newly synthesized ACh in aged rats. Treatment enhanced depolarization-induced release of both newly synthesized and endogenous transmitter in hippocampus and striatum, but not in frontal cortex. Although this release was increased up to 50% compared to aged-matched controls, the levels achieved were still only 40% of those observed in the normal adult controls.

[Key words: NGF, ACh, aging, Fischer-344, cholinergic, ChAT, high-affinity choline transport, chronic administration]

NGF promotes differentiation, survival, and expression of neurotransmitter phenotype of sympathetic and sensory neurons in the PNS (Levi-Montalcini, 1982) and forebrain cholinergic neurons in the CNS (Whittemore and Seiger, 1987; Hefti et al., 1989; Lapchak et al., 1993). It has been hypothesized that some degenerative neurologic disorders, including Alzheimer's disease, could be due to a deficiency in endogenously produced trophic factors or to decreased responsiveness of neuronal populations to these agents (Appel, 1981; Hefti and Weiner, 1986). This has led to speculation that neurotrophic factors may eventually be used to treat some neurodegenerative disorders either by direct local administration, by pharmacological modification of the synthesis *in vivo*, or by release from genetically engineered transplanted cells (Lapchak et al., 1993).

In both *in vitro* and *in vivo* models, NGF enhances cholinergic neuronal markers in basal forebrain and striatal neurons. NGF increases ChAT (acetyl-CoA:choline-*O*-acetyltransferase; EC2.3.1.6) specific activity in basal forebrain (Gnahn et al., 1983) and striatum (Mobley et al., 1985) in brain of neonatal rats. Studies that have used cultured neurons as a model have also observed increases in ChAT activity (Honneggar and Lenoir, 1982; Hefti et al., 1985). Importantly, intracerebral administration of NGF prevents retrograde degeneration of septal cholinergic neurons and promotes repair following partial or complete fimbrial lesion when given immediately following or within a few weeks after injury (Hefti, 1986; Williams et al., 1986; Hagg et al., 1988), and improves performance of aged rats on spatial memory tasks (Fischer et al., 1987). We have demonstrated recently that cholinergic neurons in unlesioned brain of both adult and aged rats respond to chronic administration of exogenous NGF with increases in high-affinity choline transport, the presumed rate-limiting step for ACh synthesis (Jope, 1979), as well as with increased ChAT activity (Williams and Rylett, 1990).

An important question that requires attention is whether the increased cholinergic neuronal markers, most frequently ChAT, that have been measured in response to NGF administration *in vivo* and *in vitro* are reflected in enhanced ACh synthesis and release, and increased cholinergic transmission in brain. In cholinergic neurons in culture, both increased ACh content and release have been measured following incubation of basal forebrain neurons with NGF (Takei et al., 1988, 1989), but little is known about the effect of chronic *in vivo* administration of NGF on these measures, in particular in the unlesioned adult and aging brain. Kewitz et al. (1990) reported transient elevations in ACh concentration in brains of neonatal rats injected with single high doses of NGF, but important differences may exist

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between NGF production and responsiveness in newborn brain compared to the adult or aged. Recently, Lapchak and Hefti (1991) demonstrated that intraventricular administration of human recombinant NGF partially protected against degeneration of lesioned septohippocampal cholinergic neurons in adult rats with partial maintenance of ^3H -ACh synthesis and release. The objective of the present study was to determine whether ACh synthesis and release from basal forebrain and striatal cholinergic neurons, measured *ex vivo*, were altered following chronic intracerebroventricular administration of exogenous NGF for 2 weeks to brain of young adult and aged Fischer-344 male rats.

Materials and Methods

Materials

Male Fischer-344 rats, 4 and 24 months of age, were obtained from National Institutes on Aging colony at Harlan/Sprague-Dawley (Indianapolis, IN). Alzet osmotic minipumps, model 2002, were supplied by Alza Corp. (Palo Alto, CA). Renin-free 2.5S mouse NGF was obtained from Bioproducts for Science (Indianapolis, IN). Choline iodide, hemicholinium-3, globulin-free crystalline bovine serum albumin, Tris base, eserine sulfate, rat serum albumin, luminol, choline oxidase, peroxidase, acetylcholinesterase, choline kinase, ATP, sodium tetraphenylboron, glycylglycine buffer, acetylcholine chloride, dithiothreitol, and cysteine were purchased from Sigma Chemical Co. (St. Louis, MO). ^3H -choline chloride (80 Ci/mmol) was from Amersham Radiochemical Corp. (Oakville, Ontario, Canada). ^{14}C -acetylcoenzyme A (58 mCi/mmol) and HEPES were obtained from ICN. γ - ^{32}P -ATP (10 Ci/mmol) was purchased from Du Pont-New England Nuclear (Mississauga, Ontario, Canada). Acetylcoenzyme A (lithium salt) was purchased from Boehringer-Mannheim. 3-Heptanone was from Aldrich Chemical Co. Intracerebral infusion cannulas were purchased from Plastics One (Roanoke, VA).

Animal care

Animals were housed with a daily photoperiod of 12 hr of light between 0600 and 1800 hr (22°C), and free access to water and food. Anesthesia for surgery was induced by intramuscular injection of a mixture of ketamine (4 mg/ml), xylazine (Rompun; 1.3 mg/ml), and acepromazine (0.25 mg/ml). Animals were 4 and 24 months of age at the time of cannula implantation and start of vehicle or NGF infusion.

NGF preparation and administration

The concentration of pure NGF in a stock solution of PBS was determined by molar extinction (1.64 OD₂₈₀/mg/ml), and aliquots of 1 mg/ml were stored at -70°C until use. Working solutions were prepared at the time of surgery by dilution in Dulbecco's phosphate-buffered saline containing 0.1% rat serum albumin; this diluent also served as vehicle in the vehicle-infused animals with albumin at 1 mg/ml added as a nonspecific protein. Drug infusion cannulas were constructed and implanted at 1.2 mm lateral to bregma into the right lateral ventricle using a procedure modified from Williams et al. (1987). Alzet osmotic pumps were used to deliver sterile NGF or vehicle solutions for 2 weeks; the NGF dose of 1.2 $\mu\text{g}/\text{d}$ was found previously to stimulate basal forebrain ChAT activity maximally in axotomized young adult Sprague-Dawley female rats (Williams et al., 1989).

Assay of cholinergic functional parameters

Cholinergic neurochemical function was investigated through measurement of (1) synthesis of ^3H -ACh, (2) release of newly synthesized ^3H -ACh, (3) release of endogenous ACh, (4) high-affinity choline uptake activity, (5) ChAT activity, and (6) content of ACh in brain areas and nerve terminal preparations.

Synthesis of acetylcholine and ^3H -choline uptake. Capacity for synthesis of ^3H -ACh was measured in synaptosomes incubated with ^3H -choline. Synaptosomes from hippocampus, frontal cortex, and striatum of right hemisphere were prepared according to the procedure of Gray and Whittaker (1962); an aliquot of the total homogenate was removed for assay of ChAT activity, frozen on dry ice, and stored at -80°C (see methods below). Nerve terminals were resuspended in cold HEPES-

buffered Krebs-Ringer solution (in mM: NaCl, 124; KCl, 5; CaCl_2 , 1.5; MgCl_2 , 1.3; HEPES-NaOH, pH 7.4, 20; glucose, 10) to yield the crude synaptosome suspension; of this suspension, an aliquot was removed for measurement of synaptosomal ACh content (see methods below). Aliquots of synaptosomal suspensions were equilibrated to 37°C , and then incubated in the presence of 0.5 μM ^3H -choline for 5 min to allow the formation of ^3H -ACh; hemicholinium-3 (10 μM) was added to some aliquots to allow determination of hemicholinium-insensitive choline accumulation as correction for background. At the end of the incubation, synaptosomes were collected by centrifugation, and then washed to remove excess tritium. Portions of the washed radiolabeled synaptosome samples were removed for determination of release of newly synthesized ^3H -ACh (see methods below).

Following the final wash, synaptosomes were lysed by the addition of 5% trichloroacetic acid (TCA) for the extraction and measurement of tritiated choline metabolite content and ^3H -ACh synthesis. Following centrifugation, an aliquot was removed from each sample for determination of ^3H -choline uptake into nerve terminals. The remaining supernatants were shaken with water-saturated diethyl ether to remove TCA, and then extracted with sodium tetraphenylboron in 3-heptanone to isolate choline esters in the organic phase. An aliquot of the organic phase was back-extracted with 0.4 N HCl, and the resulting aqueous phase, now containing tritiated choline and ACh, was reduced to dryness *in vacuo*.

^3H -choline and ^3H -ACh were separated and quantified following enzymatic conversion of ^3H -choline to ^3H -phosphorylcholine, using a modification of the method of Goldberg and McCaman (1975). To the dried samples was added 100 μl of a reaction solution composed of choline kinase, 0.2 U/ml; ATP, 15.5 mM; MgCl_2 , 18.5 mM, in 93 mM glycylglycine buffer, pH 8.3. Samples were incubated for 30 min at 37°C , and then extracted with sodium tetraphenylboron in 3-heptanone (10 mg/ml). Aliquots of the organic phase containing ^3H -ACh and the aqueous phase containing ^3H -choline as phosphorylcholine were added to scintillation cocktails for quantitation of tritium. Data was normalized to sample protein content measured by the method of Lowry et al. (1951), and expressed as picomoles of ^3H -ACh or ^3H -choline per milligram of protein.

Release of newly synthesized acetylcholine. The release of newly synthesized ACh was measured from synaptosomes following labeling of ACh pools with ^3H -choline. Basal ^3H -ACh release was monitored over two 10 min intervals, followed by K^+ -depolarization-evoked release of ^3H -ACh measured over one 5 min interval; KCl was added to synaptosome samples at a final concentration of 40 mM. Supernatants were saved following each incubation for measurement of released ^3H -ACh, as were pellets for extraction for measurement of ^3H -ACh content and sample protein estimation.

^3H -ACh in supernatants was separated from ^3H -choline and measured using a separate modification of the choline kinase method of Goldberg and McCaman (1975). Of the supernatants, 300 μl portions were added to 75 μl of a reaction solution composed of choline kinase, 2.5 mU; ATP, 50 mM; MgCl_2 , 6.25 mM, in 250 mM glycylglycine buffer, pH 8.5. Samples were incubated at 37°C for 30 min, and then 300 μl of sodium tetraphenylboron in 3-heptanone (30 mg/ml) was added to extract the ^3H -ACh. Aliquots of the organic phase containing ^3H -ACh and aqueous phase containing ^3H -choline as phosphorylcholine were prepared for scintillation counting. ^3H -ACh and ^3H -choline content remaining in the synaptosomal pellets from these samples was quantified using the method described above for synthesis of acetylcholine.

Release of endogenous acetylcholine. Potassium-evoked release of endogenous ACh was measured from slices of hippocampus, frontal cortex, and striatum (300 μm , right hemisphere) by the chemiluminescent method of Israel and Lesbats (1982). Between one and three tissue slices were placed into a cuvette containing 900 μl of Krebs-Ringer bicarbonate buffer (in mM: NaCl, 132; KCl, 5; MgCl_2 , 1.3; CaCl_2 , 1.5; NaH_2PO_4 , 1.2; NaHCO_3 , 22; glucose, 10; pH 7.4; gassed with 95% O_2 , 5% CO_2) at 37°C with the additions of 10 μl of luminol (5.4 mg/ml stock concentration), 30 μl of peroxidase (400 U/ml stock), 30 μl of choline oxidase (50 U/ml stock), and 30 μl of acetylcholinesterase (125 U/ml stock). Cuvettes were placed in a darkened chamber with electronic stirrer apposed to a photomultiplier tube coupled to an amplifier and chart recorder. After a stable baseline was achieved, KCl was added (final concentration, 40 mM), and the release of ACh was measured as light emitted. Each response was calibrated individually by the addition of at least four ACh standards (25–150 pmol) to the cuvette. ACh release from each slice(s) was determined from the standard curve constructed

Table 1. Cholinergic neuronal markers in young adult and aged male Fischer-344 rats

Age group	Brain area	ChAT	High-affinity choline uptake	ACh synthesis	Basal ³ H-ACh release	Evoked ³ H-ACh release	Endogenous ACh release
4 month	Cortex	40.0 ± 2.8	6.1 ± 0.5	4.1 ± 0.2	73.4 ± 6.6	258.1 ± 19.2	124.0 ± 7.4
	Hippocampus	47.6 ± 2.5	12.3 ± 0.7	7.6 ± 0.2	115.4 ± 6.6	441.9 ± 20.8	100.9 ± 10.2
	Striatum	164.4 ± 4.5	32.6 ± 2.4	21.1 ± 1.2	431.8 ± 29.3	849.6 ± 61.6	235.9 ± 11.4
24 month	Cortex	39.7 ± 1.8	5.3 ± 0.5	3.6 ± 0.4	26.8 ± 2.8**	77.0 ± 10.5**	112.4 ± 11.2
	Hippocampus	49.3 ± 2.7	9.8 ± 1.0	6.7 ± 0.7	65.6 ± 18.2**	131.7 ± 15.1**	84.5 ± 5.5
	Striatum	132.5 ± 8.6*	23.7 ± 2.1**	17.5 ± 1.7	179.8 ± 44.8**	219.2 ± 13.3**	172.9 ± 15.4*

Groups were composed of eight control young adult rats and four control aged rats, with the following exceptions: ChAT assay, young adult $n = 12$; endogenous ACh release, young adult control $n = 7$, aged controls $n = 8$. Units of measurement: ChAT, nmol/mg protein/hr; high-affinity choline uptake, pmol/mg protein/5 min; ACh synthesis, pmol/mg protein/5 min; basal and evoked ³H-ACh release, fmol/mg protein/min; endogenous ACh release, peak value in pmol ACh/mg protein. Statistical significance compared to 4 month control group determined by unpaired Student's t test: *, $p \leq 0.05$; **, $p \leq 0.01$.

from the ACh standards by linear regression, and normalized to the slice protein content.

Measurement of acetylcholine content. Neuronal ACh content was measured in synaptosomes and brain samples from vehicle-treated control and NGF-treated rats. Synaptosomes removed immediately after preparation were extracted with 5% TCA and extracted as described above for synthesis of ACh until and including the step for drying samples under vacuum. Brain samples obtained from rats killed by head-focused microwave (Gerling Moore, Palo Alto, CA) were weighed and homogenized in 1 M formic acid/acetone (15:85, v/v), and then centrifuged; aliquots of supernatants were reduced to dryness *in vacuo*.

ACh was hydrolyzed by acetylcholinesterase and converted enzymatically to ³²P-phosphorylcholine with ³²P-ATP, following the conversion of choline to phosphorylcholine with ATP, according to the radioenzymatic method of Goldberg and McCaman (1975). To the dried samples was added 32 μ l of a reaction mixture composed of ATP, 0.8 mM; dithiothreitol, 5 mM; MgCl₂, 11.5 mM; choline kinase, 2.5 mU; in glycylglycine buffer, 50 mM, pH 8.0. Samples were incubated at 30°C for 25 min to phosphorylate choline. A second reaction mixture (10 μ l) composed of acetylcholinesterase, 2.5 U, and ³²P-ATP, 0.5 μ Ci, in buffer was added to hydrolyze ACh and allow production of ³²P-phosphorylcholine. Incubation was continued for 20 min, and then stopped by the addition of 100 μ l of cold 50 mM NaOH. ³²P-phosphorylcholine was separated from unreacted ³²P-ATP by pouring samples over Amberlite CG400 (formate form) columns and eluting with 50 mM NaOH. Eluents were counted for ³²P, normalized to synaptosomal protein content or tissue weight, and expressed as picomoles of ACh per milligram of protein or tissue.

Choline acetyltransferase assay. ChAT activity was measured using a modification of the radioenzymatic method of Fonnum (1969), as described by Williams and Rylett (1990).

Data analysis

Assays were performed in duplicate or triplicate, with means calculated to give a single n value. Data are expressed as mean $\pm$ SEM values. Statistical analysis was by one-way ANOVA combined with Duncan's multiple-range test for post hoc analysis, or by unpaired two-tailed Student's t test. The criterion set for statistical significance was $P \leq 0.05$.

Results

Values for measurements of cholinergic neuronal parameters in the vehicle-treated control young adult (4 month) and aged (24 month) male Fischer-344 rats are shown for comparison in Table 1. There are clear age-related decreases in several of the parameters that appear to be brain region specific. The activities of ChAT and high-affinity choline uptake and release of endogenous ACh were significantly decreased in striatum, but not in frontal cortex or hippocampus. Dramatic, 60–70%, age-related decreases were observed in all three brain regions for both basal and evoked release of newly synthesized ACh. ACh synthesis

in aged rats did not differ from that observed in the younger animals.

Effect of NGF on cholinergic neuronal phenotypic markers

ChAT and high-affinity choline uptake appear to be expressed selectively in cholinergic neurons, and therefore can serve as phenotypic markers for these neurons. Similar to measurements made previously (Williams and Rylett, 1990), we observed that chronic intracerebral administration of NGF for 2 weeks significantly elevated ChAT-specific activity in frontal cortex, hippocampus, and striatum of both young adult and aged rats, with the largest effect appearing in the striatum (Fig. 1). High-affinity, hemicholinium-sensitive choline uptake was also significantly elevated in all three brain areas of young adult rats, as illustrated in Figure 2. In aged rats, however, synaptosomal choline uptake was significantly increased compared to age-matched controls only in striatum (increased $39 \pm 4\%$, $P < 0.01$). It was shown in the previous study (Williams and Rylett, 1990) that vehicle-treatment had no effect upon ChAT or high-affinity choline transport in any of the brain regions studied in either age group.

Synthesis of ACh

Choline taken up into cholinergic nerve terminals by the sodium-coupled choline carrier is converted predominantly to ACh (Yamamura and Snyder, 1973). As the accumulation of choline by cholinergic nerve terminals appeared to be enhanced by NGF treatment, we predicted that this would be reflected as an increase in the production of the neurotransmitter. It was also of interest to determine whether age-related changes existed in the responsiveness of cholinergic neurons to NGF for this functional parameter.

Intracerebral administration of NGF for 2 weeks produced significant increases in ³H-ACh synthesis to about 40% of control in all three brain regions of 4-month-old rats, as shown in Figure 3. Synaptosomal ³H-ACh synthesis was also increased in brain of aged rats. When normalized to the young adult control group, ³H-ACh synthesis in the aged NGF-treated group was $115 \pm 8\%$, $100 \pm 3\%$, and $114 \pm 7\%$ in frontal cortex, hippocampus, and striatum, respectively. Compared to the 24 month age-matched controls, this represented increases in neurotransmitter synthesis of $38 \pm 8\%$ ($P < 0.05$) and $33 \pm 10\%$ in striatum and frontal cortex of aged rats, respectively. In parallel with the relative absence of effect on choline transport in hippocampus of aged rats, ³H-ACh synthesis was largely un-

Figure 1. Effect of NGF treatment on ChAT activity in brain of young adult and aged Fischer-344 rats. Vehicle or NGF was administered continuously into right lateral ventricle of rats by osmotic minipump for 2 weeks before measurement of ChAT activity. Groups included 4-month-old control (□, $n = 12$), 24-month-old control (▨, $n = 4$), 4-month-old NGF-treated (■, $n = 12$), and 24-month-old NGF-treated (▤, $n = 4$) animals. Data are the mean $\pm$ SEM values in percentage change from values measured in 4-month-old vehicle-treated control group. Statistical significance of differences between groups was determined on data values before conversion to percentage of control group: **, $p \leq 0.01$ compared with 4 month controls; ##, $p \leq 0.01$ compared with 24 month controls.

Figure 2. Effect of NGF treatment on high-affinity choline uptake in brain of young adult and aged Fischer-344 rats. Vehicle or NGF was administered continuously into right lateral ventricle of rats by osmotic minipump for 2 weeks before preparation of synaptosomes and measurement of hemicholinium-sensitive choline uptake activity. Groups included 4-month-old control (□, $n = 8$), 24-month-old control (▨, $n = 4$), 4-month-old NGF-treated (■, $n = 8$), and 24-month-old NGF-treated (▤, $n = 4$) animals. Data are mean $\pm$ SEM values in percentage change from values measured for 4-month-old vehicle-treated control group. Statistical significance of differences between groups was determined on data values before conversion to percentage of control group: *, $p \leq 0.05$ compared with 4 month controls; **, $p \leq 0.01$ compared with 4 month controls; ##, $p \leq 0.01$ compared with 24 month controls.

changed by NGF treatment in this brain region when compared to age-matched controls.

The relative conversion of ^3H -choline accumulated into nerve terminals to ^3H -ACh was compared in young and aged rats. Whereas between 68% and 77% of accumulated ^3H -choline was converted to ^3H -ACh in the synaptosomes of all three brain regions from 4-month-old control rats, this same measure ranged from 55% to 76% in control aged rats. There did not appear to be differences between the three brain regions. Treatment of animals for 2 weeks with NGF did not alter the relative efficiency of synthesis of ACh, with 70–78% and 60–75% of ^3H -choline becoming acetylated in 4- and 24-month-old rats, respectively. Again, there did not appear to be consistent interregion differences in either age group.

Effect of NGF on the release of endogenous and newly synthesized ACh

Release of endogenous ACh from brain slices of control and NGF-treated rats was measured using modifications of the chemiluminescent method of Israel and Lesbats (1982). A representative trace for release of ACh from striatal slices is shown in Figure 4. Chronic administration of NGF resulted in enhanced ACh release from slices of hippocampus and striatum of 4-month-old rats. ACh release from slices of all three brain areas of aged animals was also increased to values similar to young adult animals ($107 \pm 8\%$, $113 \pm 7\%$, and $107 \pm 8\%$ of 4-month-old vehicle-treated controls) (Fig. 5). Relative to 24 month age-matched vehicle-treated controls, significant increases in endogenous ACh release were measured in hippocampus ($135 \pm 9\%$, $P < 0.01$) and striatum ($146 \pm 11\%$, $P < 0.01$).

Basal and potassium-evoked release of newly synthesized ^3H -ACh from synaptosomes was also measured. As illustrated in Figure 6, NGF treatment of young adult rats resulted in significantly increased basal ^3H -ACh release from synaptosomes pre-

pared from hippocampus and striatum. In contrast, basal release of synaptosomal ^3H -ACh release was not substantially altered by NGF administration in the aged rats; basal release remained significantly depressed compared to the young adult group.

Similar results were obtained for the effect of NGF on potassium-evoked ^3H -ACh release (Fig. 7). NGF treatment of young

Figure 3. Effect of NGF treatment on ACh synthesis in brain of young adult and aged Fischer-344 rats. Vehicle or NGF was administered continuously into right lateral ventricle of rats by osmotic minipump for 2 weeks before preparation of synaptosomes and measurement of ^3H -ACh synthesis from ^3H -choline taken up into the nerve terminals. Groups included 4-month-old control (□, $n = 8$), 24-month-old control (▨, $n = 4$), 4-month-old NGF-treated (■, $n = 8$), and 24-month-old NGF-treated (▤, $n = 4$) animals. Data are mean $\pm$ SEM values in percentage change from values measured for 4-month-old vehicle-treated control group. Statistical significance of differences between groups was determined on data values before conversion to percentage of control group: **, $p \leq 0.01$ compared with 4 month controls; #, $p \leq 0.05$ compared with 24 month controls.

Figure 4. Profile of release of endogenous ACh from striatal slice of young adult Fischer-344 rats. Slice(s) were placed in darkened chambers in cuvettes containing reaction mixture (see Materials and Methods), and then ACh release was stimulated by the injection of KCl. Light emission, representing sequential degradation of released ACh to choline and hydrogen peroxide, was recorded following detection and amplification of the signal. ACh release from each slice(s) was quantified by addition of standards of known amounts of ACh, injected after the response due to endogenous release had returned toward baseline. The amplitude of the response for release from slice(s) was measured, and ACh release in picomoles was determined from a standard curve constructed from the response to standards.

adult animals resulted in significant increases in synaptosomal newly synthesized ^3H -ACh release to about 140% of control in all three brain regions. In aged rats, NGF treatment also stimulated evoked ^3H -ACh release, but the levels achieved were still only 40% of those measured in the young adult controls. Relative

Figure 5. Effect of NGF treatment on release of endogenous ACh from brain slices of young adult and aged Fischer-344 rats. Vehicle or NGF was administered continuously into right lateral ventricle of rats by osmotic minipump for 2 weeks before preparation of brain slices and luminometric measurement of release of endogenous ACh in response to potassium-induced depolarization. Groups included 4-month-old control ($\square$, cortex $n = 5$, hippocampus and striatum $n = 6$), 24-month-old control ($\square$, $n = 7$), 4-month-old NGF-treated ($\blacksquare$, $n = 9$), and 24-month-old NGF-treated ($\boxtimes$, cortex and hippocampus $n = 8$, striatum $n = 9$) animals. Data are mean $\pm$ SEM values in percentage change from values measured for 4-month-old vehicle-treated control group. Statistical significance of differences between groups was determined on data values before conversion to percentage of control group: **, $p \leq 0.01$ compared with 4 month controls; ##, $p \leq 0.01$ compared with 24 month controls.

Figure 6. Effect of NGF treatment on basal release of newly synthesized ^3H -ACh from synaptosomes of brain of young adult and aged Fischer-344 rats. Vehicle or NGF was administered continuously into right lateral ventricle of rats by osmotic minipump for 2 weeks before preparation of synaptosomes, formation of ^3H -ACh from ^3H -choline, and measurement of basal ^3H -ACh release from the nerve terminals. Groups included 4-month-old control ($\square$, $n = 8$), 24-month-old control ($\square$, $n = 4$), 4-month-old NGF-treated ($\blacksquare$, $n = 8$), and 24-month-old NGF-treated ($\boxtimes$, $n = 4$) animals. Data are mean $\pm$ SEM values in percentage change from values measured for 4-month-old vehicle-treated control group. Statistical significance of differences between groups was determined on data values before conversion to percentage of control group: *, $p \leq 0.05$ compared with 4 month controls; **, $p \leq 0.01$ compared with 4 month controls.

to the 24 month age-matched controls, evoked ^3H -ACh release was significantly increased in hippocampus (136%, $P < 0.01$) and striatum (170%, $P < 0.001$).

The relative proportions of newly synthesized ^3H -ACh re-

Figure 7. Effect of NGF treatment on potassium-evoked release of newly synthesized ^3H -ACh from synaptosomes of brain of young adult and aged Fischer-344 rats. Vehicle or NGF was administered continuously into right lateral ventricle of rats by osmotic minipump for 2 weeks before preparation of synaptosomes, formation of ^3H -ACh from ^3H -choline, and measurement of depolarization-induced release of ^3H -ACh from the nerve terminals. Groups included 4-month-old control ($\square$, $n = 8$), 24-month-old control ($\square$, $n = 4$), 4-month-old NGF-treated ($\blacksquare$, $n = 8$), and 24-month-old NGF-treated ($\boxtimes$, $n = 4$) animals. Data are mean $\pm$ SEM values in percentage change from values measured for 4-month-old vehicle-treated control group. Statistical significance of differences between groups was determined on data values before conversion to percentage of control group: *, $p \leq 0.05$ compared with 4 month controls; **, $p \leq 0.01$ compared with 4 month controls; #, $p \leq 0.05$ compared with 24 month controls; ##, $p \leq 0.01$ compared with 24 month controls.

Table 2. Fractional release of newly synthesized ^3H -ACh in control and NGF-treated rats

Age group	Treatment	Cortex		Hippocampus		Striatum	
		Basal	Evoked	Basal	Evoked	Basal	Evoked
4 month	Control	1.99 ± 0.16	6.96 ± 0.29	1.71 ± 0.07	6.55 ± 0.17	$2.71 \pm 0.11^*$	$5.32 \pm 0.15^\#$
	NGF	1.82 ± 0.08	7.12 ± 0.11	1.60 ± 0.07	6.61 ± 0.12	$2.69 \pm 0.09^\#$	$5.77 \pm 0.24^\#$
24 month	Control	0.87 ± 0.16	2.04 ± 0.27	0.96 ± 0.26	1.92 ± 0.20	1.12 ± 0.32	1.34 ± 0.18
	NGF	0.68 ± 0.07	1.84 ± 0.16	0.83 ± 0.12	2.20 ± 0.26	1.11 ± 0.32	1.63 ± 0.27

Data represent mean $\pm$ SEM of the release of ^3H -ACh during basal and potassium-evoked collection periods expressed as a percentage of the total synaptosomal ^3H -ACh content. Total content was determined as the amount of released ^3H -ACh added to the synaptosomal ^3H -ACh content at the end of the incubation: 4 month olds, $n = 8$; 24 month olds, $n = 4$. Statistical significance was assessed by one-way ANOVA combined with the post hoc Duncan's multiple-range test, with * representing $p \leq 0.05$ compared to cortex and hippocampus and # representing $p \leq 0.01$ compared to cortex and hippocampus.

leased during both resting and depolarized test periods was evaluated. Several interesting features emerged, as shown in Table 2. First, NGF treatment did not alter proportional basal or potassium-evoked ^3H -ACh release within any of the three brain regions in either age group when compared with vehicle-treated controls. Second, in aged animals there were significant decreases in the fraction of newly synthesized ^3H -ACh that was released during both basal and potassium-evoked test periods in all three brain regions compared with young adult controls. Third, in 4-month-old control animals the dynamics of release of transmitter appeared to be different in striatal synaptosomes than in either frontal cortex or hippocampus, with a significantly larger fraction of ^3H -ACh being released in striatum under resting conditions (2.71 ± 0.11 compared to either 1.99 ± 0.16 , $P < 0.05$, or 1.71 ± 0.07 , $P < 0.01$) and a significantly smaller proportion being released under depolarized conditions (5.32 ± 0.15 compared to 6.96 ± 0.29 or 6.55 ± 0.17 , $P < 0.01$). The same relationship was observed in 24-month-old animals, although statistically significant differences were not obtained. ^3H -ACh released by potassium depolarization was elevated about 4-fold over basal release in cortex and hippocampus of young adult rats (3.5- and 3.9-fold in cortex and 3.8- and 4.1-fold in hippocampus of control and NGF-treated rats, respectively). This is compared to a 2-fold depolarization-induced increase in ^3H -ACh release in striatum (2.0- and 2.1-fold increase over basal release in control and NGF-treated rats, respectively). Smaller depolarization-induced increases relative to basal release were observed in 24-month-old rats with approximately 2–3-fold increases in cortex and hippocampus (2.3- and 2.7-fold in cortex and 2.0- and 2.7-fold in hippocampus in control and NGF-treated rats, respectively) and about 1.5-fold increases in striatum (1.2- and 1.5-fold in control and NGF-treated rats, respectively).

Regression analysis was performed to determine whether there was a relationship between ACh synthesis and depolarization-evoked ^3H -ACh release, with the magnitude of the NGF-induced increase in ACh release correlating with the increase in synthesis. This appeared to be the case in the NGF-treated young adult group, with correlation coefficients in frontal cortex, hippocampus, and striatum of 0.793, 0.704, and 0.966, respectively; graphically, the regression lines were significantly different from zero ($P < 0.02$, $P = 0.05$, and $P < 0.0001$, respectively). This relationship, however, did not exist in the NGF-treated aged group. Correlation coefficients relating ACh synthesis and evoked release of ^3H -ACh were found to be -0.941 , -0.098 , and 0.948 (frontal cortex, hippocampus, and striatum, respectively), with none of the regression lines significantly different from zero.

Content of ACh

As the velocity of synaptosomal high-affinity choline uptake and ACh synthesis may vary as a function of ACh levels in synaptosomes (Jenden et al., 1976), we measured ACh content in synaptosomes in samples taken at the beginning of each experiment. In addition, we hoped that this measure would provide some information about the effect of NGF on steady-state ACh levels in brain. As shown in Table 3, in both 4- and 24-month-old groups synaptosomal ACh levels in all three brain regions of NGF-treated rats were essentially the same as control animals. Additionally, there did not appear to be age-related changes in ACh content in any of the brain regions studied.

In a separate experiment, we measured ACh levels in brains of a group of 4-month-old rats treated for 2 weeks with NGF or vehicle, and then killed by head-focused microwave. ACh content in basal forebrain of control animals was 91.0 ± 7.1 ($n = 4$) compared to 97.8 ± 3.6 ($n = 3$) pmol/mg tissue in the NGF-treated group, and 107.4 ± 7.4 ($n = 3$) in striatum of control animals compared to 116.6 ± 9.6 ($n = 3$) pmol/mg tissue in NGF-treated rats. Thus, it is clear that chronic NGF administration had little effect on brain ACh levels in the regions examined.

Discussion

Several new observations were made in the present study concerning the effects of chronic intracerebral administration of NGF on cholinergic neuron function in basal forebrain and striatum of adult and aged Fischer-344 rats: (1) ACh synthesis was increased by NGF in both 4- and 24-month-old rats in the three brain regions examined, with the exception of hippocampus in the aged group; (2) NGF treatment did not appear to alter the relative efficiency of synthesis of ACh within the nerve terminal, as indicated by comparable conversion of accumulated ^3H -choline to ^3H -ACh relative to controls; (3) endogenous

Table 3. Synaptosomal ACh content in control and NGF-treated rats

Age group	Treatment	Frontal cortex	Hippocampus	Striatum
4 month	Control	367 ± 20	577 ± 26	1073 ± 96
	NGF	442 ± 56	588 ± 65	1303 ± 101
24 month	Control	404 ± 31	656 ± 40	961 ± 68
	NGF	382 ± 12	532 ± 32	1068 ± 64

Data are expressed as mean $\pm$ SEM in pmol ACh/mg protein: 4 month olds, $n = 8$; 24 month olds, $n = 4$.

ACh release was enhanced by NGF treatment in slices from hippocampus and striatum, but not frontal cortex in both adult and aged rats; (4) basal and depolarization-induced release of newly synthesized ^3H -ACh was increased by NGF treatment of young adult rats in all three brain regions, but in the aged rats, only potassium-evoked release of transmitter from hippocampus and striatum was affected by NGF treatment; and (5) ACh content in either brain tissue or freshly prepared synaptosomal fractions was not affected by NGF administration.

An important feature emerging from the present study is the apparent differential responsiveness of CNS cholinergic neurons in aged compared to the young adult rats to chronic intracerebral administration of NGF. In the young adult brain, all cholinergic functional parameters measured were increased by NGF infusion with the greatest effect generally in the striatum. However, a less uniform response of striatal and basal forebrain cholinergic neurons was apparent in the aged animal. Again, as observed in the young adult, increases in cholinergic markers were generally greatest in striatum in the aged rat brain, with effects of NGF on projection areas of basal forebrain cholinergic neurons being less consistent. For example, high-affinity choline transport and ACh synthesis were not altered in hippocampus of the aged rat brain after 2 weeks NGF administration, but ChAT, depolarization-induced release of ^3H -ACh, and endogenous ACh were enhanced. Conversely, high-affinity choline uptake and ChAT and ACh synthesis were increased in frontal cortex of aged rat brain, but there was no change compared to age-matched controls in depolarization-induced release of either endogenous or newly synthesized ^3H -ACh release. These data, in conjunction with the regional differences in age-related decreases in cholinergic measures (Table 1), indicate that the regulation of cholinergic neurotransmission and sensitivity to exogenous NGF may be regionally specific; that is, the effects of age and NGF on interneurons of the striatum are different from those on the projection neurons in the medial septum, and different from those of the nucleus basalis magnocellularis (Williams, 1991).

It is predicted that the increases in ACh synthesis and release measured in the present study after 2 weeks NGF administration may not be maximal, particularly in the young adult group. This is based, in part, upon our previous observations that continuation of growth factor treatment until 4 weeks resulted in further increases in choline uptake and ChAT activity (Williams and Rylett, 1990), and is supported by recent studies reported by Lapchak and Hefti (1991) that extension of NGF treatment in partial fimbrial-transected young rats from 3 to 9 weeks resulted in enhanced survival of denervated septohippocampal cholinergic neurons, as demonstrated by greater ^3H -ACh synthesis by remaining neurons. It is also interesting to note that in the study of Lapchak and Hefti (1991) no change in ^3H -ACh synthesis or release was observed from hippocampus of the contralateral unlesioned hemisphere of NGF-treated young rats when compared to cytochrome c-treated controls. This contrasts with the significant enhancement in a variety of measures of cholinergic neuron function in unlesioned, presumably normal, frontal cortex, hippocampus, and striatum of both young adult and aged rat brain observed in the present study. Although the reason for this difference is unclear, one possible explanation could be the use of different strains of rats (Williams and Oostveen, 1992).

ACh content of brain tissue and freshly prepared synaptosomes from NGF-treated rats did not differ from that of control rats, suggesting that enhanced transmitter synthesis was bal-

anced by enhanced release. This is in contrast to the findings of Kewitz et al. (1990), in which ACh concentration in brain of neonatal rats was significantly and transiently (about 24 hr) elevated within 48 hr following a single high-dose intracerebral injection of NGF. While the difference in these results could relate to different doses of NGF administered in the two studies, it could also represent an age-related effect of NGF that is present in the newborn, but not adult or aged, nervous system. Further studies are required to clarify this point.

Modulation of release of neurotransmitter is one of the most important parameters in terms of changing net communication of information to target cells; hence, a primary objective in the present study was to monitor alterations in ACh release by NGF. NGF administration in the young adult group increased release of both endogenous stores and newly synthesized pools of ACh, with a significant correlation between enhanced ACh synthesis and enhanced depolarization-evoked release of ^3H -ACh in all three brain regions. This latter relationship appears not to exist in the aged group, indicating that NGF-induced effects on release of newly synthesized ACh were not coupled to transmitter synthesis; the largest increases in ^3H -ACh release were not observed mainly in animals with the highest increases in ACh synthesis. Similarly, release of both endogenous ACh (Fig. 5) and newly synthesized ^3H -ACh (Fig. 7) was significantly increased from hippocampus of NGF-treated aged rats in the absence of significant changes in transmitter synthesis (Fig. 3). This latter effect, however, did not result in a significant change in the fractional release of ^3H -ACh (2.20% in NGF-treated rats compared to 1.92% in the control group), suggesting that mobilization of transmitter was not altered by the growth factor. The effects of NGF on ACh release in the aged brain appear complex. The magnitude of the age-related decrease of ACh was not as great for the measurement of endogenous transmitter, and NGF treatment restored this parameter to the level observed in the young adult control group. In contrast, both basal and depolarization-induced ^3H -ACh release were markedly reduced in the aged group, and NGF administration, although leading to significant enhancement of these measures in some brain areas, did not restore transmitter release to levels found in the younger control animals.

The mechanisms underlying the large decreases in ACh release in CNS cholinergic neurons of aged animals have not been elucidated. In general, measurement of ACh release from either brain slices or synaptosomes reveals substantially decreased transmitter release in aged animals (for review, see Decker, 1987). It is interesting that this decreased transmitter release occurs in the absence of age-related changes in neuronal ACh content (Sherman et al., 1981; see review by Giacobini, 1988), leading to the suggestion that the mechanisms controlling neuronal ACh levels remain functional during the aging process (Kindel and Karczmar, 1981, 1982); in aged mouse brain, the turnover rate for ACh is decreased by about 30% (Kindel and Karczmar, 1982). It is probable that alterations in the mechanisms controlling the mobilization and release of ACh (cf. Collier, 1977; Prado et al., 1992) rather than deficits in ACh synthesis or content cause the observed age-related impairments of motor and cognitive performance of aged rats in various behavioral paradigms (Olton, 1990; Williams et al., 1993). Meyer et al. (1986) reported evidence indicating that the age-related decrease in ACh release may be in part the result of decreased capacity of calcium to trigger transmitter release in aged nerve terminals. However, other studies have reported that both resting and

potassium-stimulated levels of neuronal cytosolic free calcium were unaffected by aging in Fischer-344 rats (Farrar et al., 1989).

In summary, chronic NGF administration to both young and aged rats resulted in stimulation of ACh release. Although NGF treatment in the aged rat did not restore the depolarization-evoked release of newly synthesized ^3H -ACh to the young adult control levels, there were significant increases in release of newly synthesized ACh from hippocampus and striatum of aged animals when compared to the 24 month age-matched controls. This enhanced release of transmitter by NGF may underlie the alterations in behavior (both positive and negative) observed in NGF-treated aged rats (Fischer, 1987; Williams et al., 1993).

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