Relationship of APP mRNA Transcripts and Levels of NGF and Low-Affinity NGF Receptors to Behavioral Measures of Age-related Cognitive Dysfunction

Mark D. Lindner,¹ Steven I. Dworetzky,² Craig Sampson,² and Rebekah Loy^{1,3}

¹University of Rochester School of Medicine and Dentistry Neurogerontology Unit, Monroe Community Hospital, Rochester, New York 14620, ²Bristol-Myers Squibb Pharmaceutical Research Institute, Department of Biophysics and Molecular Biology, Wallingford, Connecticut 06492, and ³Batavia V.A. Medical Center, Batavia, New York 14020

This study was designed to examine the relationship between cognitive function and endogenous levels of NGF, low-affinity NGF receptor (LNGFR), and amyloid precursor protein (APP) mRNAs. Using 3 month ($n = 5$), 18 month ($n = 40$), and 29 month ($n = 17$) Fischer-344 male rats, cognitive function was assessed with the Morris water maze, reverse transcription and polymerase chain reaction were used to quantify APP mRNAs, and NGF and LNGFR levels were determined with an ELISA.

Cognitive function declined progressively with age from 3 months to 18 months, and from 18 months to 29 months, but only RNA content in the tissue declined significantly from 3 months to 18 months. Between 18 month and 29 month rats there were small but statistically significant decreases only for Kunitz protease inhibitor (KPI)-inclusive mRNAs and cortical NGF levels. There was a small but statistically significant correlation between cognitive function and %KPI (the amount of KPI APP mRNAs relative to the total amount of APP mRNA), with lower %KPI related to more impaired spatial learning. No other statistically significant correlation or linear relationship could be detected between cognitive function and any of the other neurological measures or any combination of these measures (i.e., hippocampal levels of APP 695 mRNA, cortical and hippocampal levels of NGF, and cortical, hippocampal, and basal forebrain levels of LNGFR).

Although there were no strong linear relationships between cognitive function and our neurological measures, a post hoc exploratory cluster analysis suggests that there may be a brief transient increase in tissue levels of %KPI, NGF, and LNGFR during the initial period of cognitive deterioration before substantial neuronal degeneration has occurred. To test this possibility, levels of these substances in the tissue, and the number of remaining neurons in which

they are being produced will need to be measured independently.

[Key words: NGF, low-affinity NGF receptor, amyloid precursor protein mRNA, polymerase chain reaction, ELISA, Morris water maze, aging]

Two biological measures closely associated with the progression of Alzheimer's dementia are the increased density of senile plaques (Roth et al., 1966; Blessed et al., 1968), and the atrophy/degeneration of cholinergic basal forebrain neurons (Whitehouse et al., 1981; McGeer et al., 1984) that have low-affinity receptors for NGF (LNGFR) (Kordower et al., 1989; Mufson and Kordower, 1989; Mufson et al., 1989a; Loy et al., 1990). The dementia-related loss of LNGFR-positive neurons is greatest in the posterior basal forebrain, which projects to the hippocampus and cortex (Higgins and Mufson, 1989; Mufson et al., 1989b), and reduced NGF levels in those target tissues may play a causal role in cholinergic/LNGFR neuronal degeneration and dementia (Appel, 1981). Amyloid is the central constituent of senile plaques, and plaques may form as a consequence of either an overproduction and/or aberrant metabolism of the amyloid precursor protein (APP) (Robakis et al., 1991), or as a consequence of differential expression of APP mRNA transcripts (Harrison et al., 1991). Results consistent with both of these possibilities have been reported in Alzheimer's patients (Jacobsen et al., 1991; Tanaka et al., 1992).

Experimental manipulations of APP and NGF also suggest that endogenous levels of these substances may be interrelated. NGF alters the ratios of APP mRNAs during neuronal differentiation of PC12 cells such that the level of APP 770 mRNA is reduced and the level of APP 695 mRNA is increased (Smith et al., 1991). Both NGF and low concentrations of APP increase neurite length and branching in PC12 cells, and antibodies to APP 695 block the neurite-promoting effects of NGF (Milward et al., 1992). The neurotoxic carboxy-terminal fragment of amyloid binds specifically and with high affinity to NGF-treated PC12 cells (Kozlowski et al., 1992), and NGF potentiates the neurotoxicity of amyloid (Yankner et al., 1990).

Consistent with results from clinical studies of Alzheimer's disease, both degeneration of LNGFR-positive neurons in the basal forebrain (Koh et al., 1989; Fischer et al., 1992) and altered levels and transcript ratios of APP mRNA (Higgins et al., 1990; Greene et al., 1992) have been observed in aged rats with cognitive deficits (impaired performance in spatial learning tasks). Although it remains to be determined how valid aged and learn-

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Correspondence should be addressed to Mark D. Lindner, Ph.D., Cyto-Therapeutics, Two Richmond Square, Providence, RI 02906.

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ing-impaired rats are as a model of Alzheimer's disease, for the present this seems to be the best rodent model available. Therefore, the purpose of the present study was to determine whether endogenous levels of NGF and LNGFR in the basal forebrain, cortex, and hippocampus, or hippocampal levels of APP mRNA transcripts are closely related to behavioral measures of age-related cognitive deficits.

Materials and Methods

Animals. Male Fischer-344 rats from Harlan Sprague-Dawley were acclimated to the colony room for more than 30 d before behavioral testing began, at which time they were aged 18 months ($n = 40$), and 29 months ($n = 17$). A small group of young 3 month rats ($n = 5$) was included to provide a reference point. Rats were group housed in stainless steel cages with wire-mesh floors, and food and water were available *ad libitum*.

Morris water maze. The Morris water maze, a black 152-cm-diameter pool filled to a depth of 35 cm with 21°C water, containing a small 10 cm² platform submerged 1.2 cm below the surface of the water, was located in a temperature-controlled 22°C room. There were large high-contrast visual cues throughout the room. Tracking and computation of dependent measures were performed by an automated water maze system (San Diego Instruments, San Diego, CA). Swim distance, the length of the path that the animal swims to find the platform, is the most appropriate measure of performance in this task (Morris, 1984; Lindner and Gribkoff, 1991; Lindner et al., 1992), and it was used as the dependent measure. In order to remove the influence of swim speed on swim distance in trials where the platform was not located during the 2 min time limit, a maximum swim distance of 36 m (the median swim distance on all trials where the platform was not located) was assigned on all such trials and on trials where rats located the platform within the 2 min time limit but swam more than 36 m (Lindner et al., 1992).

Each rat received the same sequence of random starting positions and initial directions. Trials were a maximum of 120 sec in duration, and rats remained on the platform for 20 sec at the end of each trial. In order to prevent hypothermia, rats were placed in a 38.5°C warm-water bath during the 20 min intertrial interval and placed under an infrared heat lamp for 15 min after the second trial of the day, before being returned to their home cages (Lindner and Gribkoff, 1991). During acquisition training all rats received two trials each day for 10 d with the platform located in the same position. The platform was removed from the pool for a single 120 sec probe trial conducted the day following acquisition training. During the probe trial, the following measures were recorded: (1) the number of platform annulus crossings, (2) the proximity ratio (the proportion of the swim distance within an annulus surrounding the platform which included 25% of the surface area of the pool), and (3) the degree of thigmotaxis (Lindner and Schallert, 1988) (the proportion of the swim distance in which the rat was in contact with the wall of the pool). On the day following the probe trial, swim distance was recorded during two "cued" trials in which the platform was moved randomly to different locations while marked with a large (250 cm²) visual cue.

Tissue dissection. Within 10 d after the end of behavioral testing, rats were decapitated and within 5 min the septum and vertical limb of the diagonal band [VDB; between 1.7 mm anterior and 0.3 mm posterior to bregma (Paxinos and Watson, 1986), caudal to the genu of the corpus callosum, and medial to the lateral ventricles], both hippocampi, and a large piece of neocortex that included the frontal (from 0.3 mm posterior to bregma), temporal, parietal, and occipital lobes of the right hemisphere, were dissected on ice, quick frozen in liquid nitrogen, and stored at -70°C.

Reverse transcription and PCR. Reverse transcription (RT) and polymerase chain reaction (PCR) were used to quantify mRNA levels of APP 695, 751, and 770 in the right hippocampus, based on the methods of Gilliland et al. (1990). The oligonucleotide primers were sense (5'-TGCCACCACTACCACAACCTACCACTGAGTC-3') and antisense (5'-CCTCTCTTTGGCTTTCTGGAAATGGG-3'). The PCR technique was used to delete 25 nucleotides from the APP 695 isoform, which was subcloned into the TA cloning vector (In-Vitrogen). This internal standard utilized the same primers required for the APP isoforms and resulted in a smaller PCR product.

Hippocampal tissue was homogenized and the RNA was isolated

according to Chomczynski and Sacchi (1987). All RNA samples were quantified by UV spectrophotometry and stored at -70°C. RNA (2 µg) was used for each RT. The final concentrations of the components in the RT mixture were 20 U of RNasin (Promega), 1 mM dNTPs (Pharmacia), 100 pmol of random hexamer (In-Vitrogen), 1.5 mM MgCl₂, 100 mM Tris-Cl, pH 8.3, 50 mM KCl, and 400 U of Moloney virus reverse transcriptase (GIBCO-Bethesda Research Labs). After addition of each component the reactions were incubated at room temperature for 10 min, 42°C for 60 min, followed by inactivation at 94°C for 10 min. Each RT reaction contained enough sample for 10 separate amplifications of the APP isoforms, that is, 10 µl for each PCR amplification. Each PCR reaction mix contained 0.2 mM dNTPs (from RT reaction), 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 100 pmol of each oligonucleotide primer [exons 6 and 9 of the rat APP gene, which span the Kunitz protease inhibitor (KPI) and OX-2 domains], 2.5 U of amplitaq polymerase (Perkin-Elmer), 5 µCi of ³²P-dCTP, and the internal DNA standards ranging from 1 attogram up to 100 attograms. The PCR parameters were 94°C for 45 sec, 60°C for 90 sec, and 72°C for 120 sec at 25 cycles followed by a 7 min 72°C extension period. Amplified product (15 µl) was separated on a 6% native polyacrylamide gel. The gel was vacuum dried and analyzed on an AMBIS radioanalytical imaging system. Standard curves were prepared from the known amount of internal standard DNA added to each tube and the cpm's were detected by radioanalytical imaging.

NGF ELISA. NGF levels in the left hippocampus and the right cortex, and NGF receptor levels in the left hippocampus, the cortex, and the VDB were determined with two-site enzyme-linked immunosorbent assays (ELISA; adapted from Weskamp and Otten, 1987; Mobley et al., 1989; Lindner et al., 1993). Immunoplates (Nunc, Maxisorb F96) were coated with 100 µl of goat anti-mouse NGF polyclonal antibody (GAM; 20 µg/ml) in coating buffer (3.07 mM Na₂SO₄, 34.88 mM NaHCO₃, 16.13 mM Na₂CO₃, pH 9.6), and incubated overnight at 4°C. The GAM solution was removed, and the plates washed with washing buffer (10 mM PBS, pH 7.4, and 0.05% Tween 20), incubated at room temperature for 1 hr with 200 µl of blocking buffer [5% fetal calf serum (FCS) in PBS], and then washed three times with washing buffer.

Samples were prepared by homogenizing frozen tissues with a hand-held Polytron PT 1200 (Kinematica, AG) for 20 sec in 1:10 (w/v) sample buffer [0.5% BSA, aprotinin (20 KIU/ml; Sigma), 0.0987 mM phenylmethylsulfonyl fluoride, and 0.0982 mM benzethonium chloride in PBS] and centrifuged for 30 min at 15,000 × g at 4°C, and then Tween 20 (final concentration of 0.05%) was added to the supernatant. For each brain area, tissues for all subjects were included in a single assay, and one standard curve was used for all samples. Half of each homogenate was used in the NGF receptor assay below. NGF, purified from mouse submaxillary gland (Mobley et al., 1986), was serially diluted to produce a standard curve with concentrations of 0–100 pg/100 µl. Standards and sample solutions were each added to four replicate wells and were left on the plate overnight at 4°C. (Preliminary studies showed no binding to wells coated with normal rabbit serum, so these controls were omitted from this assay and the LNGFR assays described below.) The plates were washed with PBS.

Wells were coated with 100 µl of rabbit anti-mouse NGF monoclonal antibody (IG3, 0.012 mg/ml) in 1% FCS, 0.1% Tween 20, and PBS, and the plates were incubated overnight at 4°C. After the plates were washed with PBS, 100 µl of biotinylated goat anti-rabbit antibody (Vector; 0.3 µg/ml in PBS containing 1% FCS and 0.1% Tween 20) was added to each well, and the plate incubated overnight at 4°C. Plates were then washed three times in PBS, and incubated for 2 hr at room temperature with 100 µl of HRP-streptavidin (Zymed; 1:2000 in PBS containing 1% FCS). Plates were then washed three times with PBS, and developed with 100 µl/well of a freshly made solution containing 0.04% O-phenylenediamine and 0.012% hydrogen peroxide in a phosphate-citrate buffer, pH 5.0, by incubating the foil-covered plates for 20 min at room temperature. The plates were lightly agitated during all incubations in both NGF and LNGFR assays, and the reactions of both assays were stopped by adding 50 µl of 2.5 M H₂SO₄ to each well. Optical densities were measured with an MR 600 Microplate Reader (Dynatech Laboratories, Inc.) using a 490 nm filter and a 590 nm reference filter. Least-squares regression analysis was performed with SIGMAPLOT 5.01 (Jandel) to determine the parameters of a four-parameter logistic log function (Rodbard and McClean, 1977; Plikaytis et al., 1991) for the standard curve.

NGF receptor ELISA. The ELISA for LNGFR used the same incubation and wash times and procedures as the NGF ELISA except for

the following. Plates were initially coated with 100 μ l of anti-Rex at a concentration of 1 μ g/ml in PBS (20 mM phosphate-buffered saline, pH 8.0). Tween 20 (0.05% in PBS) was used as the washing buffer. The blocking buffer was 200 μ l of 0.25% BSA, 7.69 mM sodium azide, 0.9 mM EDTA, 1% normal horse serum (NHS), and 0.05% Tween 20 in borate-buffered saline, pH 8.5. Sample homogenates from the NGF ELISA protocol above, and Rex [the ectodomain of the rat p75 NGF receptor (Weskamp and Reichardt, 1991)] serially diluted to produce a standard curve with concentrations from 0 to 100 pg/100 μ l, were each added to four replicate wells and incubated for 3 hr. A different blocking buffer was used in the second blocking step: 200 μ l of 0.05% Tween 20, 1% NHS, and 0.01% horse anti-mouse antibody (HAM) in PBS. The plates were washed and loaded 100 μ l/well with 8.97 μ g/ml of 192-IgG in PBS. Plates were washed with 100 μ l biotinylated HAM (Vector; 0.15 μ g/ml in PBS containing 1% NHS). Tween 20 (0.1%) was added to each well and plates were incubated 1 hr at room temperature. Plates were washed six times with washing buffer and incubated for 2 hr at room temperature with 100 μ l HRP-streptavidin (Zymed; 1:2000 in PBS containing 1% NHS).

Data analysis. The data were analyzed with SAS procedures for general linear models, with options for repeated-measures where appropriate, and Cronbach's coefficient alpha was computed to determine reliability (SAS Institute Inc., 1989). ω^2 was computed as a measure of effect size (Dodd and Schultz, 1973). Planned contrasts were conducted for differences between 3 month and 18 month rats, and between 18 month and 29 month rats. A post hoc cluster analysis was conducted using the SAS procedure PROC FASTCLUS (SAS Institute Inc., 1989). Data are presented in the text and in all figures as means and standard errors of the mean.

Results

Morris water maze

All rats began swimming within a few seconds after first entering the water, and were able to keep their heads above water and swim throughout the pool without apparent difficulty. Subjects could be very reliably distinguished from one another in terms of swim speed (Cronbach's reliability coefficient, $r = 0.917$), and age accounted for a large proportion of the variance in swim speeds ($\omega^2 = 0.65$). Swim speeds were 0.39 ± 0.007 , 0.33 ± 0.004 , and 0.29 ± 0.005 m/sec for the 3, 18, and 29 month rats, respectively; this represented a statistically significant difference [$F(2,59) = 57.67$, $p = 0.0001$].

Acquisition. On the first day of acquisition training, all age groups had long swim distances of approximately 24 m, which were not significantly different from each other [$F(2,59) = 0.43$, $p = 0.65$]. Over the 10 d of acquisition training, all groups showed evidence of improvement; the main effect of days was statistically significant [$F(9,531) = 15.02$, $p = 0.0001$] (Fig. 1A). Younger rats performed better than older rats; the main effect of age was statistically significant [$F(2,59) = 8.87$, $p = 0.0004$]. In addition, 18 month rats were impaired relative to 3 month rats [$F(1,59) = 9.13$, $p = 0.004$], and 29 month rats were impaired relative to 18 month rats [$F(1,59) = 5.45$, $p = 0.02$]. The total proportion of variance accounted for by age [ω^2 values for age (0.253), plus the age $\times$ days interaction (0.029)] = 0.282.

Probe trial. There was a significant difference between the groups in terms of the number of annulus crossings (the number of times the previous platform location was crossed over) [$F(2,59) = 6.23$, $p = 0.0035$] (Fig. 1B). The 18 month rats had fewer annulus crossings than the 3 month rats [$F(1,59) = 5.21$, $p = 0.026$], and 29 month rats had fewer annulus crossings than 18 month rats [$F(1,59) = 4.99$, $p = 0.03$]. There were also significant differences between the groups in terms of the proportion of their swim path near the previous platform location (proximity ratio) [$F(2,59) = 24.11$, $p = 0.0001$] (Fig. 1B). The 18 month rats had smaller proximity ratios than 3 month rats [$F(1,59) = 22.41$, $p = 0.0001$], and 29 month rats had smaller proximity

ratios than 18 month rats [$F(1,59) = 17.09$, $p = 0.0001$]. Finally, the groups were significantly different in terms of the measure of thigmotaxia [i.e., the proportion of their swim path in which their snout or whiskers were in contact with the wall of the pool (Lindner and Schallert, 1988)] [$F(2,59) = 3.06$, $p = 0.05$] (Fig. 1B). The measure of thigmotaxia was not significantly higher in 18 month rats than in 3 month rats [$F(1,59) = 0.37$, $p = 0.54$], but 29 month rats did have significantly higher measures of thigmotaxia than 18 month rats [$F(1,59) = 5.05$, $p = 0.03$].

Overall performance in the probe trial, represented by the combination of standardized values of annulus crossings, proximity ratios, and degree of thigmotaxia, showed that there was a significant main effect for age [$F(2,59) = 12.34$, $p = 0.0001$; $\omega^2 = 0.268$]. The 18 month rats were impaired relative to 3 month rats [$F(1,59) = 8.51$, $p = 0.005$], and 29 month rats were impaired relative to 18 month rats [$F(1,59) = 11.71$, $p = 0.001$]. Acquisition swim distances were correlated with performance in the probe trial [$r(62) = -0.48$, $p = 0.0001$].

Cued platform. There were no significant differences between groups in terms of their swim distance to the cued platform [$F(2,59) = 2.95$, $p = 0.06$] (Fig. 1A). Fisher's Z transformations (Fisher, 1934) show that the effect for age was substantially larger during maze acquisition ($\omega^2 = 0.282$) and during the single probe trial ($\omega^2 = 0.268$) than in the cued platform task ($\omega^2 = 0.055$; p values < 0.05).

APP mRNA

APP mRNA/ μ g total RNA. The three amplified DNA products corresponding to the APP transcripts were easily visualized after separation by PAGE, and all three forms were clearly noted to be present in all samples. APP 695 mRNA made up 97.4% of all APP mRNA, and the groups were not different with respect to their levels of APP 695 mRNA [$F(2,59) = 1.70$, $p = 0.19$] (Fig. 2A). The levels of APP 695 mRNA were not significantly different between 3 month and 18 month rats [$F(1,59) = 1.19$, $p = 0.28$], or between 18 month and 29 month rats [$F(1,59) = 2.77$, $p = 0.11$; power = 0.90]. APP 751 mRNA represented 2% of all APP mRNA, and 29 month rats had significantly less APP 751 mRNA than 18 month rats [$F(1,59) = 4.56$, $p = 0.04$] (Fig. 2A), but there was no significant difference between 3 month and 18 month rats [$F(1,59) = 0.41$, $p = 0.53$]. APP 770 mRNA represented only 0.6% of all APP mRNA, and 29 month rats had significantly less APP 770 mRNA than 18 month rats [$F(1,59) = 5.81$, $p = 0.02$] (Fig. 2A), but there was no significant difference between 3 month and 18 month rats [$F(1,59) = 0.71$, $p = 0.41$].

APP mRNA/mg tissue. The right hippocampi weighed an average of 82.6 mg, and the groups were not different with respect to hippocampal weights [$F(2,59) = 1.40$, $p = 0.25$]. However, the amount of RNA recovered per milligram of tissue did decline with age [$F(2,59) = 9.95$, $p = 0.0002$]. Contrasts showed that the 36% decrease in total RNA content from 0.89 ± 0.07 to 0.57 ± 0.02 μ g/mg tissue for the 3 month and 18 month rats, respectively, was significant [$F(1,59) = 19.67$, $p = 0.0001$], but at 0.62 ± 0.04 μ g/mg tissue the 29 month rats did not have any less RNA than 18 month rats [$F(1,59) = 1.63$, $p = 0.21$].

The effects of age on APP mRNA levels in hippocampal tissue were the same as the effects of age on APP mRNA levels with respect to total RNA presented above. Rats (29 months) did not have significantly lower APP 695 mRNA levels than 18 month rats [$F(1,59) = 1.86$, $p = 0.18$], but 29 month rats had significantly less of both APP 751 mRNA [$F(1,59) = 3.71$, $p =$

Figure 1. Morris water maze data. *A*, Swim distance during acquisition training and during final "cued" trials (note that time of probe trial is indicated with vertical dashed line). *B*, Swim performance during probe trial (platform removed). 1, number of annulus crossings; 2, proportion of swim path near previous platform location; 3, proportion of swim path in contact with wall of pool.

0.05] and APP 770 mRNA [$F(1,59) = 4.96, p = 0.03$] than 18 month rats (Fig. 2*B*).

%KPI. In order to be consistent with previous studies and in order to determine whether the levels of KPI-inclusive forms of APP mRNA were actually reduced relative to the levels of total APP mRNA, the ratios of KPI-inclusive APP mRNA levels to total APP mRNA levels were analyzed. The ratio of KPI-containing forms of APP mRNA (751 and 770) relative to the total amount of APP mRNA (%KPI) was significantly different with respect to age [$F(2,59) = 5.02, p = 0.01$]. While 3 month rats did not differ from 18 month rats with respect to the %KPI [$F(1,59) = 0.70, p = 0.41$], 29 month rats had a significantly smaller %KPI than 18 month rats [$F(1,59) = 8.14, p = 0.007$] (Fig. 2*C*).

NGF/LNGFR levels

NGF and LNGFR levels for each age group, in pg/mg tissue, are presented for the different brain areas in Table 1. Cortical NGF levels were not different between 3 month and 18 month rats [$F(1,59) = 1.29, p = 0.26; \omega^2 = 0.0044$], but NGF levels in the cortex were significantly higher in the 18 month rats than in the 29 month rats [$F(1,59) = 5.70, p = 0.02$]. Although this effect was statistically significant, the magnitude of the effect was relatively small ($\omega^2 = 0.071$). No statistically significant

Figure 2. Extrapolated APP mRNA transcripts from right hippocampus. *A*, APP mRNA levels presented with respect to total RNA. *B*, APP mRNA levels presented in terms of milligrams of tissue. *C*, APP 751 and 770 mRNA levels presented as percentage of total APP. *D*, Standard curve (note that APP 751 and 770 mRNA levels fall near the lower limit of sensitivity).

effects of age were evident in any of the other NGF or LNGFR measures, either for the main effects of age [all $F(2,59) < 1.47, p$ values > 0.24], or for the contrasts between the 3 month and 18 month rats, and between 18 month and 29 month rats [all $F(1,59) < 1.58, p$ values > 0.21].

Reliability

The detection and quantification of linear relationships between variables with the use of correlation/regression analyses, as planned in this study, are highly dependent on the ability to discriminate reliably between samples within each set of data. The degree to which samples could be discriminated was determined with the reliability coefficient, Cronbach's coefficient alpha (Nunnally, 1978; Cohen and Cohen, 1983; SAS Institute Inc., 1989). Rats could be reliably discriminated from one another in terms of their performance during acquisition of the spatial learning Morris water maze, and tissue samples could be reliably discriminated from one another with respect to all measures of NGF and LNGFR (Table 2).

The APP 695 mRNA levels among all rats ($n = 62$), and among the 18 month rats ($n = 40$), were sufficiently reliable to warrant correlational analyses (Table 2). The APP mRNA levels for the 751 and 770 forms, measured at less than 2 attograms (Fig. 2*D*), could not be reliably discriminated from one another

Table 1. NGF and LNGFR levels (pg/mg tissue)

Area and NGF/LNGFR	3 month (n = 5)	18 month (n = 40)	29 month (n = 17)
Cortical NGF	5.45 ± 4.77	14.96 ± 3.37	2.77 ± 0.695
Cortical LNGFR	0.647 ± 0.132	0.639 ± 0.070	0.739 ± 0.129
Hippocampal NGF	1.47 ± 0.27	1.59 ± 0.32	2.16 ± 0.66
Hippocampal LNGFR	3.08 ± 0.63	2.30 ± 0.20	2.74 ± 0.25
VDB LNGFR	2.80 ± 0.47	3.02 ± 0.22	3.21 ± 0.25

(Nunnally, 1978), which meant that they were not amenable to correlation/regression analyses. Therefore, %KPI was used in this study for correlation/regression analyses, even though its reliability could not be precisely determined.

Correlation/regression analyses

%KPI and APP 695 mRNA levels were significantly correlated with each other [$r(62) = 0.43$, $p = 0.0005$]. Except for cortical NGF levels, which were not significantly correlated with any other biological measure, all measures of NGF and LNGFR were significantly correlated with each other. LNGFR levels in the VDB were correlated with LNGFR levels in the cortex [$r(62) = 0.46$, $p = 0.0002$], LNGFR levels in the hippocampus [$r(62) = 0.81$, $p = 0.0001$], and NGF levels in the hippocampus [$r(62) = 0.49$, $p = 0.0001$]. LNGFR levels in the hippocampus were correlated with LNGFR levels in the cortex [$r(62) = 0.36$, $p = 0.004$] and NGF levels in the hippocampus [$r(62) = 0.44$, $p = 0.0003$]. Finally, hippocampal NGF levels were correlated with LNGFR levels in the cortex [$r(62) = 0.86$, $p = 0.0001$].

Chronological age was correlated with acquisition swim distance [$r(62) = -0.44$, $p = 0.0003$], and there was a small but statistically significant correlation between %KPI and acquisition swim distance among all rats [$r(62) = -0.27$, $p = 0.03$], and among the 18 month rats [$r(40) = -0.32$, $p = 0.04$]. None of the other biological measures were significantly correlated with acquisition swim distance (Fig. 3). Furthermore, stepwise multiple regression analyses showed that, except for %KPI, there was no statistically significant linear relationship between any combination of neurological variables and acquisition swim distance.

In order to determine whether nonlinear relationships might be present between the neurological and behavioral measures, an exploratory cluster analysis was performed post hoc, including all neurological measures and acquisition swim distance.

The cluster analysis detected three natural groupings or clusters of subjects (Fig. 4): (1) rats that performed well in the water maze and had low levels of %KPI and all measures of NGF and LNGFR ($n = 34$), (2) rats that were mildly impaired in the spatial learning Morris water maze and had elevated levels of %KPI and all measures of NGF and LNGFR ($n = 8$), and (3) rats that were more severely impaired in the spatial learning Morris water maze but had reduced levels of %KPI, NGF, and LNGFR ($n = 20$). Except for the level of APP 695 mRNA, all biological variables included in this analysis had significant quadratic trends across these three groups or clusters of subjects: (1) %KPI [$F(1,59) = 7.78$, $p = 0.007$], (2) cortical NGF [$F(1,59) = 3.80$, $p = 0.05$], (3) cortical LNGFR [$F(1,59) = 58.62$, $p = 0.0001$], (4) hippocampal NGF [$F(1,59) = 72.28$, $p = 0.0001$], (5) hippocampal LNGFR [$F(1,59) = 10.15$, $p = 0.002$], and (6) VDB LNGFR [$F(1,59) = 21.0$, $p = 0.0001$] (Fig. 4). Trend analysis on acquisition swim distance revealed significant linear [$F(1,59) = 40.84$, $p = 0.0001$] but not quadratic trends across clusters.

Discussion

Cognitive function, as assessed with the spatial learning Morris water maze, generally declined progressively with chronological age from 3 months to 18 months, and from 18 months to 29 months. There was also a large range in performance between individuals, and even rats of the same age could be reliably discriminated from one another on the basis of their spatial learning ability. These individual differences seem ideal for determining the biological basis of senescence or physiological aging (as opposed to chronological aging) in cognitive function (Collier and Coleman, 1991; Fischer et al., 1992).

The RNA content in the tissue was significantly lower in 18 month rats relative to 3 month rats, which is consistent with age- and dementia-related decreases in overall rates of protein synthesis (Ekstrom et al., 1980; Fando et al., 1980; Davis et al., 1981; Davis and Squire, 1984; Marotta et al., 1986). However, there were no significant differences between 3 month and 18 month rats with respect to any of the neurochemical measures this study was designed to examine. In 29 month rats relative to 18 month rats, the levels of NGF in the cortex and KPI-inclusive mRNA were reduced regardless of whether KPI-inclusive mRNA was presented with respect to total RNA, in terms of its concentration in the hippocampal tissue, or as a proportion of the total amount of APP mRNA. A statistically significant but small correlation was detected between %KPI and spatial learning ability, with lower levels of %KPI being related to more impaired spatial learning. No other statistically significant correlation or linear relationship could be detected between spatial learning ability and any of the other neurological measures or any combination of these measures (i.e., hippo-

Table 2. Cronbach's coefficient alpha^a

Variable	Total (n = 62)	18 month (n = 40)	29 month (n = 17)
Maze acquisition	0.77	0.67	0.77
APP 695 mRNA	0.96	0.90	0.00
%KPI	NA	NA	NA
Cortical LNGFR	0.97	0.95	0.99
Cortical NGF	0.98	0.98	0.94
Hippocampal NGF	0.96	0.97	0.95
Hippocampal LNGFR	0.98	0.99	0.96
VDB LNGFR	0.95	0.95	0.95

^a r^2 = proportion of variance accounted for.

Figure 3. Scatter plots of acquisition swim distance with measures of NGF and LNGFR.

campal levels of APP 695 mRNA, cortical and hippocampal levels of NGF, and cortical, hippocampal, and basal forebrain levels of LNGFR).

It seems unlikely that large correlations between our neurological and behavioral measures went undetected because our data were invalid or unreliable. The validity of acquisition swim distance as a measure of cognitive function is supported by the facts that (1) age-related deficits were not detected in the cued version of the task, which is similar to the acquisition and probe trial tasks in terms of the noncognitive demands of the task (i.e., its dependence on motivation and its demands on motor and visual function), and (2) acquisition swim distances were correlated with several measures of performance in a probe trial. In addition, all data sets were highly reliable (i.e., swim distances, hippocampal levels of APP 695 mRNA, cortical and hippocampal levels of NGF, and cortical, hippocampal, and basal forebrain levels of LNGFR) with the exception of %KPI (whose reliability could not be determined). It is conceivable that measures of cognitive function might be correlated with measures from brain areas not included in the present study (e.g., APP mRNA levels in cortex and NGF/LNGFR levels in nucleus basalis).

Figure 4. Bar chart showing results of cluster analysis. Note that spatial learning performance continuously declines from cluster 1 through cluster 3, but all biological measures except the APP 695 mRNA are low in cluster 1, substantially elevated in cluster 2, and lower again for cluster 3 (statistically significant quadratic trends).

Unfortunately, the present results do not help resolve the conflicting reports already present in the literature regarding APP mRNA changes as a function of age and cognitive ability. Jacobsen et al. (1991) and Palmert et al. (1988) reported that levels of APP 695 mRNA are increased and levels of APP 751 and 770 mRNA are decreased in Alzheimer's patients compared to controls, but Robakis et al. (1991) did not detect APP 751 mRNA in rats and concluded that KPI-inclusive forms of APP mRNA may be related to plaque formation in humans, and Tanaka et al. (1992) reported that APP 770 and 751 mRNAs were increased relative to the amount of APP 695 mRNA in Alzheimer's patients. Higgins et al. (1990) reported that learning-impaired aged rats have increased levels of KPI-containing APP mRNA, but Greene et al. (1992) reported increased levels of APP 695 mRNA in learning-impaired aged rats.

Published studies of the relationship between NGF and cognitive function are, likewise, in conflict. Henriksson et al. (1992) reported that learning-impaired aged rats have lower levels of hippocampal NGF, but Hellweg et al. (1990) reported that cortical NGF levels were higher in learning-impaired aged rats. Goedert et al. (1986) reported no difference in temporal/parietal NGF mRNA levels in Alzheimer's patients, and Allen et al. (1991) reported no difference in hippocampal or cortical NGF levels in Alzheimer's patients. On the other hand, Crutcher et al. (1993) reported an increase in cortical NGF levels in Alzheimer's patients, and in our lab (R. Loy, M. D. Lindner, and R. W. Hamill, 1993 unpublished observations) we find a decrease in cortical NGF and LNGFR levels in Alzheimer's patients.

To our knowledge, this is the first study quantifying tissue levels of LNGFR. It seems surprising that tissue levels of LNGFR do not decline markedly as cognitive function deteriorates since morphological measures of the degeneration of ChAT/LNGFR-positive neurons are consistently related to the degree of cognitive dysfunction (Fischer et al., 1989, 1992; Koh et al., 1989). However, there is some evidence that LNGFR production increases, at least during the initial period of LNGFR-positive neuronal degeneration (Lindner et al., 1993), and a combination of increasing LNGFR production per neuron with decreasing numbers of LNGFR-positive neurons could explain why tissue levels of LNGFR are not linearly related to behavioral measures of cognitive dysfunction in the present study.

There is also some evidence that transient increases in tissue levels of NGF may occur during the initial period of cognitive

deterioration (Hellweg et al., 1990), and the post hoc cluster analysis conducted in the present study supports the possibility that tissue levels of %KPI, NGF, and LNGFR may all exhibit transient increases during the initial period of cognitive deterioration, perhaps before substantial neuronal degeneration has occurred. To test this possibility, future studies could independently quantify tissue levels of neurochemical measures and the number of neurons producing these substances. In addition, since transient increases in levels of NGF (Needels et al., 1985; Nieto-Sampedro and Cotman, 1985; Gasser et al., 1986; Hengerer et al., 1990; Ballarin et al., 1991), LNGFR mRNA (Brunello et al., 1990), APP and APP mRNA levels have been observed following neuronal injury (Otsuka et al., 1991; Scott et al., 1991; Wallace et al., 1991; Shigematsu and McGeer, 1992), studies could test the possibility that an early critical period of neuronal vulnerability may occur during physiological aging and cognitive decline.

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