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Expression of BDNF and trkB as a function of age and cognitive performance

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Abstract

The expression of the neurotrophic factor BDNF increases during learning-related events and is decreased in the hippocampus of Alzheimer's Disease patients, suggesting that it plays a role in learning, memory, and/or age-related memory deficits. We examined the expression of BDNF and its high affinity receptor, trkB, in young and aged Sprague–Dawley rats. BDNF and trkB mRNA were measured by semi-quantitative in situ hybridization and BDNF protein was measured by ELISA. Significant decreases with age were detected for BDNF mRNA in the pons, BDNF protein in the midbrain, and trkB mRNA in many areas of the brain. Rats were evaluated on the Morris water maze before sacrifice so that BDNF and trkB levels could be related to cognitive status. Regression analyses revealed that decreased trkB mRNA in the pons significantly predicted impaired memory performance in aged rats. These results suggest that decreases in trkB mRNA with age are more widespread than decreases in BDNF, and that BDNF decreases are restricted to more caudal brain regions.

Keywords: Aging; Brain-derived neurotrophic factor; trkB; Neurotrophin; Memory

1. Introduction

The neurotrophin brain-derived neurotrophic factor (BDNF) and its receptor, trkB, are found throughout the adult rat brain. Especially high levels of BDNF and trkB mRNA and protein are found in the hippocampal formation [3,7,31,45,60,61]. The hippocampus and associated cortical structures have been shown to play important roles in learning and memory, and are the primary structures affected by Alzheimer's Disease [for overview see Ref. [20]]. BDNF mRNA is reduced in surviving hippocampal neurons of Alzheimer's patients compared to age-matched controls [9,36,44]. In contrast, increased levels of BDNF mRNA have been associated with improved learning in the Morris water maze [15]. BDNF mRNA also increases after induction of long-term potentiation (LTP) in the hippocampus [6,41] and BDNF causes increases in hippocampal excitability [24,32,49]. BDNF's high levels in the adult hippocampus, reduction during Alzheimer's disease, and increase during learning-associated processes suggests that BDNF may play a role in learning and memory.

Because BDNF may play a role in age-related cognitive impairments, it is important to understand how BDNF and its high affinity receptor, trkB [28,51,52], change with age and with age-associated memory impairments. BDNF mRNA and protein have already been shown not to change significantly in the hippocampus and cortex of aged rats [30,35]. However, BDNF and trkB levels have not been related to the learning and memory abilities of the rats. In addition, BDNF and trkB levels have not been studied broadly throughout the brain of aged rats. If decreased BDNF levels were shown to be related to learning and memory impairments in aged animals, perhaps regional manipulations to alter BDNF levels would improve memory.

The present study measured BDNF and trkB mRNAs, and BDNF protein, in younger adult and aged rats. All rats were tested for memory ability, and their memory scores were related to their regional BDNF and trkB levels. BDNF and trkB levels were measured in various parts of the brain by in situ hybridization, and by ELISA (BDNF only). Significant changes in BDNF protein, BDNF mRNA, and/or trkB mRNA occurred in many brain regions with

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age, and a few of these alterations, most notably trkB mRNA in the pons, significantly predicted cognitive performance.

2. Materials and methods

2.1. Subjects

All subjects were male Sprague–Dawley rats housed one to two per cage in a temperature and humidity-controlled animal colony with food and water available ad libitum. Animals were housed on a 12:12 h light–dark cycle (lights on 0700 h). Nine young animals (3–4 months old) and nine middle-aged animals (12–13 months old) were used as non-aged controls. Large groups of 22–26 month old rats were screened for behavioral ability and general health. Specifically, animals were only included if they maintained stable weights, ate and drank normal amounts of food and water, moved around their cages when experimenters entered the colony, explored actively on an open field, groomed, swam competently, visually tracked a moving pen, showed a startle response to a sudden loud hand clap, responded well to being handled, and showed no obvious signs of ill health such as alopecia or dehydration. Eighteen aged rats were selected for study, half of which were memory-unimpaired and half of which were memory-impaired.

2.2. Behavioral testing

Rats were behaviorally tested on a modification of the Morris water maze task [34]. Young and middle-aged rats were behaviorally tested at the same time as the aged rats. Testing took place daily between 1000 and 1400 h, with each animal being tested within approximately 1 h of the same time each day. Animals were placed in the behavioral testing area for approximately 1 h prior to testing so that they could acclimate. Animals received one trial per day for 7 days in which they searched for a submerged escape platform in a pool of opaque water approximately 1.5 m in diameter. If an animal did not find the platform in 5 min, it was assigned an escape latency of 300 s and was led to the platform by hand. After the 7 days of acquisition, all animals received a spatial probe trial for retention. Each animal was placed in the pool for 30 s with the goal platform removed. The amount of time that the animal spent in the quadrant which had previously contained the escape platform was measured. The proportion of time the animal spent in the goal quadrant was used as the retention score for each animal. Half of the aged animals selected for tissue analysis were considered memory-unimpaired, and performed no worse than one standard deviation below the mean for the young rats. The other half of the aged animals selected for study were considered memory-impaired, and performed more than one standard deviation below the mean for young animals. Animals were tested in two cohorts; one for in situ hybridization and one for BDNF ELISA.

Water maze acquisition performance was analyzed using a 2 (group) $\times$ 7 (trial) mixed factorial analysis of variance (ANOVA). Water maze retention performance was analyzed using Student's independent groups *t*-test. Water maze retention compared across all four subgroups was analyzed using a One-way ANOVA.

2.3. In situ hybridization

Eight non-aged and eight aged rats were used for in situ hybridization studies of BDNF and trkB mRNA in the rat brain with age. Brains were inspected when removed, and all brains were grossly normal. Rats were anesthetised with ketamine/xylazine (48 mg/kg ketamine and 9.6 mg/kg xylazine i.m.). Once anesthetised, rats were exsanguinated and perfused transcardially with heparinized 0.9% saline kept at 4°C, followed sequentially by 4% paraformaldehyde in acetate buffer (pH 6.5) and 4% paraformaldehyde in borate buffer (pH 9.5) at 4°C. Brains were removed and post-fixed in 4% paraformaldehyde in borate buffer overnight. Brains were then placed in 30% sucrose in borate buffer for 5–7 days until sectioned. Brains were frozen and sectioned coronally on a sliding microtome at 40 μm. Sections were stored at -20°C in cryoprotectant [55] made with DEP-C treated water.

Sections were hybridized with cDNA probes for BDNF mRNA and trkB mRNA directed to the kinase domain. Probes were labeled with ^{35}S-dCTP. A 1:24 series of sections were hybridized from each rat brain. All sections from the 16 rats (eight from each group) were hybridized through the same solutions during the same run for each probe. Free-floating sections were washed with 2 $\times$ SSC for approximately 4 h to rinse off any remaining cryoprotectant. They were then pre-hybridized in hybridization buffer at 48–50°C for 3 h. RNAse A amounting to 10 mg was added to one vial just before pre-hybridization as a negative control. The sections from this vial showed no specific hybridization. Ten million c.p.m. of denatured ^{35}S-labeled probe in 200 ul of hybridization buffer was added to each vial of tissue. Sections were hybridized overnight at 48–50°C with agitation. The next day, sections were washed with 2 $\times$, 1 $\times$, 0.5 $\times$ and 0.25 $\times$ SSC for 10–15 min each at 48–50°C, and with 0.1 $\times$ SSC at room temperature for 30 min. Sections were then transferred to 50 mM phosphate buffer until mounted onto 4% gelatin-coated slides.

The slides from all 16 rats were placed on the same Hyperfilm X-ray for each probe. All slides were placed on film for four different exposures (2, 5, 7, and 10 days). Optical densities (O.D.) of the film images were measured for each of the three probes in each brain region measured. All analyses were conducted using exposures for which O.D. were in the linear range and all O.D. were normal-

Table 1
Regions measured for the in situ hybridization study using Paxinos and Watson [42] (portions of areas in parentheses may have been included)

Region	Coordinates	Areas measured
Anterior to bregma		
Olfactory bulb	Approximately 6.7	Entire section
Frontal cortex	2.7 to 3.2	Fr2 (Cg1)
Striatum	0.2 to 0.7	CPu
Septum	0.2 to 0.48	MS, LSI, Ld, VDB, HDB, mfbb
Posterior to bregma		
Hypothalamus	Approximately 3.6	DM, VMH, Arc, PH
Thalamus	3.8 to 4.16	LPMR, LPLR, Po, VPM, VPL, PF, Rt
Hippocampus	3.8 to 4.16	Hil, PoDG, DG, CA1, CA2, CA3
CA1/CA2	3.8 to 4.16	CA1, CA2
CA3	3.8 to 4.16	CA3
Dentate gyrus	3.8 to 4.16	DG, PoDG, Hil
Occipital/parietal cortex	3.8 to 4.16	Oc2MM, Oc2ML, Oc2L, Par1
Retrosplenial cortex	4.8 to 5.3	RSG, RSA
Temporal cortex	4.8 to 5.3	Te1, Te3
Entorhinal cortex	4.8 to 5.3	Ent (APir)
Substantia nigra	5.3 to 5.8	SNC, SNR
Superior colliculus	7.64 to 7.8	Zo, SuG, InG, InWh, PpG (ECIC, InCo)
Dorsal raphe	7.64 to 7.8	DR, CGLV
Locus coeruleus	9.68 to 10.04	LC (Me5)
Pons	9.68 to 10.04; 8.72 to 8.8	PnC, PnV, PnO, PPTg (DMTg)
Cerebellum	10.04 to 10.30	2, 3, 4, 5, Sim

ized to unhybridized tissue on the film. Brain regions quantified included the olfactory bulb, frontal cortex, occipital/parietal cortex, temporal cortex, retrosplenial cortex, entorhinal cortex, striatum, septum, hippocampus, thalamus, hypothalamus, substantia nigra, dorsal raphe/periaqueductal gray, superior colliculus, pons, locus coeruleus, and the cerebellum. Table 1 shows the regions which were quantified, using Paxinos and Watson's atlas [42] as a reference. For all bilateral structures, one measurement was taken for each side, and the two sides were averaged. Three measurements were taken in the pons and averaged for each rat.

O.D. values were analyzed for each brain region using Student's independent groups *t*-test. In addition, regression analyses were performed to determine if BDNF or trkB mRNA levels in each brain region were significantly correlated with or predictive of memory (retention) score either for all animals or for aged animals only.

2.4. BDNF ELISA

Ten non-aged and 10 aged rats were selected for measurement of BDNF protein by ELISA as previously described [26]. Rats were anesthetised with ketamine–xylazine, as described for the in situ hybridization animals. Animals were sacrificed by decapitation and brains were removed. All brains were grossly normal. Brains were dissected into eight brain regions (olfactory bulbs, cortex, hippocampus, striatum, diencephalon, midbrain, hindbrain, and cerebellum). Tissue was frozen on dry ice and stored at −80°C until used. Samples were weighed and then homogenized in 10 vols. of homogenization buffer using a polytron. Samples were spun at 14,000 r.p.m. for 30 min at 4°C. The supernatant was removed and diluted 1:1 with a 0.2% Triton solution. The sample was further diluted 1:25 in a 0.1% Tween–BSA solution and frozen at −20°C until the ELISA was run. BDNF-like immunoreactivity was measured by a two-site ELISA. The capture antibody was a monoclonal antibody to BDNF, and the reporter antibody was a biotinylated rabbit polyclonal antibody to BDNF (Amgen, Thousand Oaks, CA). A BDNF standard curve was run in the Tween–BSA solution using concentrations of BDNF from 0.005 ng/ml to 2.5 ng/ml. Amounts of BDNF protein detected in the samples were normalized to the standard curve.

BDNF ELISA values were analyzed with a Student's independent groups *t*-test to examine any differences between groups. In addition, regression analyses were performed to determine if BDNF ELISA values in any area could significantly predict memory score in either all animals or in aged animals only.

3. Results

3.1. Behavioral results

Results of the Morris water maze showed that, overall, aged rats learned the task more slowly than non-aged rats, and with more variability. This delayed acquisition was reflected in a significant overall effect of age on water

maze acquisition (7 (trial) × 2 (group) mixed factorial ANOVA, $F(1,34) = 6.517$, $p < 0.02$, Fig. 1A). By the end of the training period, aged rats were performing as well as non-aged rats, and showed no overall impairment in retention scores ($t(34) = 0.894$, $p > 0.37$, Fig. 1B). When subdividing the rats to ensure an even mix of impaired and unimpaired aged rats, and young vs. middle-aged non-aged rats, we observed that aged memory-impaired animals performed significantly worse than any of the other three groups on the retention trial (One-way ANOVA, $F(3,52) = 16.937$, $p < 0.0001$, individual group comparisons performed using the Tukey–Kramer post hoc test with a significance level of $p < 0.05$, Fig. 1C).

3.2. In situ hybridization

In situ hybridization analysis revealed many age-related changes in trkB and BDNF mRNA. These changes were not uniform throughout the brain. Instead, there was a specific pattern of changes by brain region for each mRNA.

There were no statistically significant changes in BDNF mRNA in any area of the brain measured except the pons. Specifically, BDNF mRNA was significantly decreased in the aged group when compared to non-aged controls ($t(14) = 3.169$, $p < 0.007$, Table 2).

Significant differences between groups were found in many areas of the brain for trkB mRNA (see Table 2). TrkB mRNA decreased significantly in the retrosplenial cortex ($t(14) = 2.430$, $p < 0.03$). Other areas of cortex did not suffer the same magnitude of decrease, showing a regional specificity for the trkB mRNA decline with age. In addition, significant decreases in trkB mRNA were detected in thalamus ($t(14) = 2.404$, $p < 0.03$), and hypothalamus ($t(14) = 2.869$, $p < 0.013$) with age. Significant decreases with age were also observed in the hippocampus (overall hippocampus: $t(13) = 2.166$, $p < 0.05$). These decreases varied slightly between hippocampal regions. The dentate gyrus showed the largest decrease in trkB mRNA ($t(13) = 2.699$, $p < 0.02$), CA3 showed an intermediate decrease ($t(13) = 2.242$, $p < 0.044$), and the decrease in CA1 did not achieve significance ($t(13) = 1.869$, $p < 0.085$). No decreases were detected for BDNF mRNA in any of these regions, illustrating that the decreases measured were probe-specific.

3.3. BDNF ELISA

The BDNF ELISA revealed significant decreases in BDNF protein with age only in the midbrain ($t(18) =$

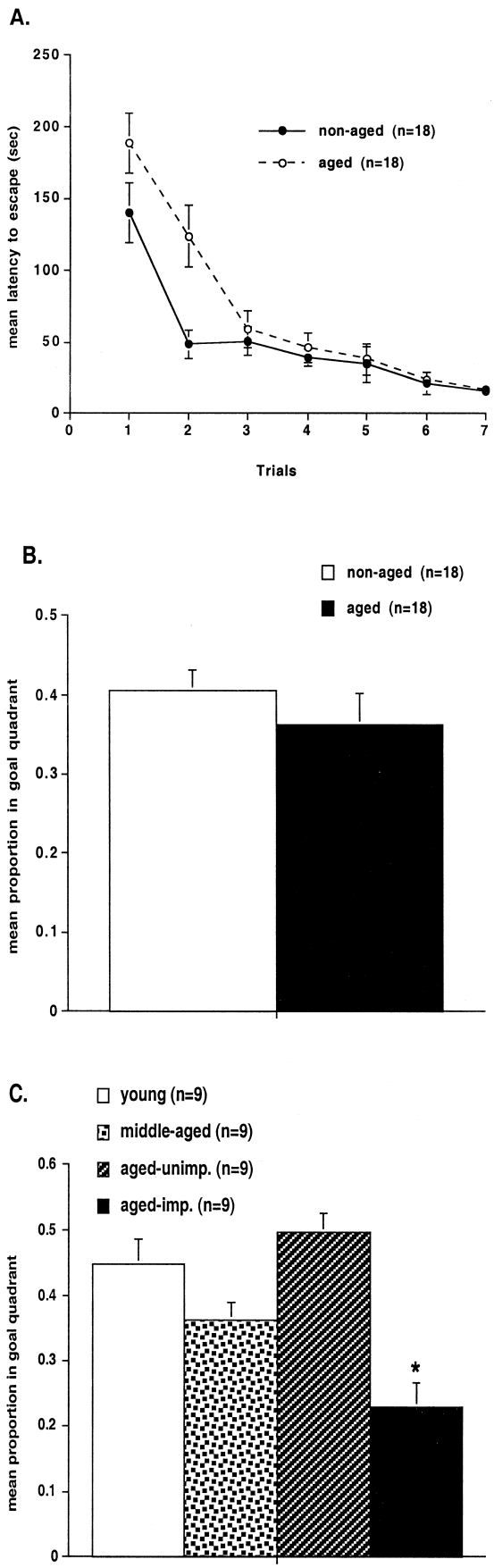

Fig. 1. Water maze results. (A) Water maze acquisition for non-aged vs. aged rats. (B) Water maze retention on the spatial probe trial for non-aged vs. aged rats. (C) Water maze retention on the spatial probe trial for the four subdivided groups of animals, * significantly different than young controls, $p < 0.05$; the middle-aged animals were significantly different than the aged-unimpaired rats, $p < 0.05$.

Table 2
Percent change for aged rats compared to younger controls in all areas of the brain measured for BDNF and trkB mRNAs

Brain Region	BDNF (%)	$p =$	TrkB (%)	$p =$
Olfactory bulb	+43	0.09	−18	0.35
Frontal cortex	+5	0.81	−11	0.46
Striatum	−14	0.15	−5	0.77
Septum	−9	0.60	−6	0.66
Hypothalamus	+14	0.60	**−35**	**0.01***
Thalamus	+20	0.39	**−30**	**0.03***
Hippocampus	+9	0.63	**−28**	**0.05***
CA1/CA2	+9	0.62	−25	0.08
CA3	+3	0.89	**−29**	**0.04***
Dentate gyrus	+10	0.56	**−31**	**0.02***
Occipital/parietal cortex	+6	0.73	−18	0.23
Retrosplenial cortex	+27	0.10	**−33**	**0.03***
Temporal cortex	−22	0.12	−20	0.43
Entorhinal cortex	−20	0.18	−26	0.14
Substantia nigra	−18	0.24	−10	0.67
Superior Colliculus	+9	0.39	−22	0.08
Dorsal raphe	−11	0.32	−5	0.65
Locus coeruleus	+22	0.10	+2	0.93
Pons	**−24**	**0.01***	−11	0.59
Cerebellum	−17	0.14	−21	0.28

Statistically significant changes are bolded and indicated by an $*(p < 0.05)$.

3.312, $p < 0.004$, Fig. 2). BDNF ELISA data from the other brain regions are included to demonstrate the relative specificity of the BDNF decrease in the midbrain.

3.4. Regression analyses

Regression analyses examining the ability of mRNA or BDNF protein levels to predict memory ability (using the retention scores) were conducted for both the in situ hybridization analyses and the BDNF ELISA values. Because we detected small decreases with age for many measures, the regressions using animals from all age groups were confounded with age as a factor, and should, therefore, be interpreted with caution. Significant prediction of memory ability using all rats was detected for trkB mRNA in the striatum ($R^2 = 0.257$, $p < 0.05$, data not shown), locus coeruleus ($R^2 = 0.270$, $p < 0.05$, data not shown), and pons ($R^2 = 0.621$, $p < 0.01$, data not shown). No

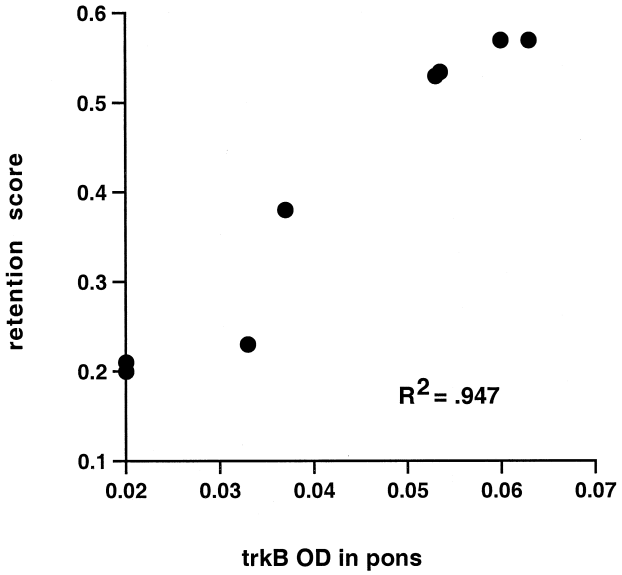

Fig. 3. Scatterplot showing the significant regression of retention score as a function of trkB mRNA in the pons for aged animals only.

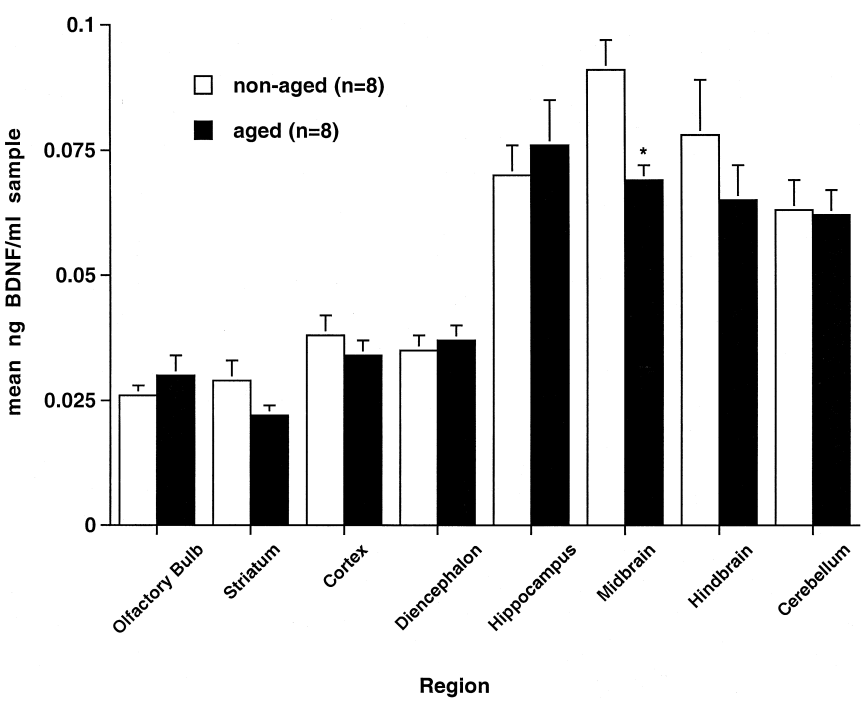

Fig. 2. Results of BDNF ELISAs for non-aged vs. aged rats in all eight areas of the brain measured, * significantly different than non-aged controls, $p < 0.05$.

BDNF mRNA O.D. values were able to significantly predict memory scores in the rats. For the BDNF ELISA, the hindbrain values were able to significantly predict memory scores ($R^2 = 0.265$, $p < 0.05$, data not shown). In all cases, decreases in trkB or BDNF levels were correlated with decreased memory ability.

Regression analyses were conducted on only the aged brains to allow for a true evaluation of what factors could predict age-related memory impairments. Because all animals were the same age, age-related changes could not be confounding factors. For the trkB probe, only the pons values significantly predicted memory ability ($R^2 = 0.947$, $p < 0.001$, Fig. 3), such that more trkB mRNA in the pons was related to a higher memory score. No regional BDNF mRNA or protein levels were able to significantly predict memory score in aged rats.

4. Discussion

In the present study, few decreases in BDNF mRNA or protein were observed, and those that were found were limited to caudal areas of brain. In contrast, significant decreases in trkB mRNA were observed in many areas of the brain. In addition, decreases in trkB mRNA in the pons were significantly predictive of cognitive performance in aged rats.

4.1. BDNF and trkB in the hippocampus

The finding here that neither BDNF mRNA nor protein significantly decrease in the hippocampus or cortex with age is consistent with previous results [25,30,35]. In contrast, trkB mRNA decreased significantly in the hippocampus of aged rats. This finding does not disagree with a previous report [30] finding no decrease in trkB mRNA with age, because we used a probe directed against the signal-transducing kinase domain of trkB, while they used a probe directed against the extracellular domain. Their probe hybridized both to the full-length signal tranducing transcript and to the non-catalytic truncated form. Preliminary data in our lab also show no significant decrease of trkB mRNA with age in the hippocampus using the extracellular probe [Croll et al., unpublished data], suggesting a selective loss of the message encoding the full-length trkB. Neither BDNF nor trkB in the hippocampus were predictive of memory performance. Because animals were evaluated on the hippocampally-mediated spatial water maze, BDNF or trkB may not be involved in the hippocampal component of age-related memory impairments. It is possible that trkB mRNA decreases do not lead to TrkB protein decreases, that TrkB levels are not rate-limiting in the hippocampus, or that high levels of TrkB activation are not necessary for successful performance of this task. These findings may also explain why administration of exogenous BDNF does not improve water maze performance in aged rats [16,43].

Many other structures play roles in either learning and memory or in performance factors which may contribute to age-related memory impairments. For this reason, we analyzed BDNF and trkB levels throughout the brain.

4.2. BDNF measurements

In situ hybridization revealed no significant decreases in BDNF mRNA except for in the pons. The decreased BDNF mRNA in the pons was related only to age, and did not predict memory ability.

Unlike our findings for BDNF mRNA, BDNF protein was decreased significantly only in the midbrain with age. BDNF immunostaining is most intense in the terminal areas of cells positive for BDNF mRNA, rather than in the cell bodies themselves, suggesting anterograde transport of BDNF [2,7,60]. Therefore, the decreased BDNF protein in the midbrain could be located in terminal fields of cells projecting to the midbrain from pons, in which we detected decreased BDNF mRNA. Although it is unclear exactly which systems are responsible for these decreases in BDNF mRNA, it is possible that the ascending systems thought to be responsible for attention and arousal could be involved. Although BDNF protein levels were not significantly decreased in the hindbrain of aged rats, they significantly predicted overall retention performance. That is, BDNF levels decreased in the hindbrain with poor performance in the water maze probe trial, but not with age. Attention/arousal deficits and distractibility have previously been proposed to represent major contributing factors in age-related learning impairments [39,59]. Based on our data, it is possible that infusions of BDNF directly into the midbrain or hindbrain of aged or memory-impaired rats could be of benefit, although the exact mechanism of the improvement is uncertain because of the many functions associated with these structures (e.g., attention, mood, motor skills, etc.).

4.3. trkB measurements

In contrast to BDNF, trkB mRNA was decreased with age in many areas of the brain. The finding that BDNF does not decrease in most regions suggests that the decreases we report for trkB are not non-specific RNA decreases with age. Most decreases in trkB mRNA with age were found in the forebrain. Both the hippocampus and cortex showed significant decreases in trkB mRNA levels. The cortex and hippocampus have long been implicated not only in learning and memory, but in pathological age-related changes. The retrosplenial cortex was the only region of cortex in which trkB mRNA decreases with age achieved statistical significance. The retrosplenial cortex may be especially susceptible to oxidative stress [10],

which has been proposed as a mechanism of brain aging [for reviews see Refs. [33,56]]. Because the regression analyses showed no significant relationship between retention score and trkB mRNA in cortex, the decrease is age-related rather than dementia-related.

Significant age-related decreases in trkB mRNA were also detected in the striatum, thalamus, hypothalamus, and pons. All of these regions have been associated with functions which show age-related declines [5,21,46]. The role of trkB activation in each of these functions is not well-understood. It is possible, however, that the loss of trkB mRNA with age could contribute to the age-related disruptions of these functions.

4.4. BDNF or trkB and age-related cognitive performance

We described relationships between memory performance and trkB mRNA or BDNF protein in the locus coeruleus, striatum, and pons. These areas have all been shown to influence learning and memory or the performance of learning tasks [for reviews and examples see Refs. [8,12–14,19,27,47,48,53,58]]. Because mRNA levels often decreased with age, as well as with memory impairment, age was a possible confound in these regression analyses. Analyses performed only on the aged animals could not have age as a confound, and therefore, selectively revealed relationships between mRNA levels and age-related memory impairments. There were no significant relationships between BDNF mRNA or protein levels and age-related memory impairments in any area of the brain, suggesting that age-related memory deficits in these rats were not related to alterations in BDNF. In contrast, the trkB probe in the pons significantly predicted age-related memory impairments, such that trkB decreases were associated with memory deficits. The pons may have a role in learning and memory through its mediation of attention [12,53].

BDNF has been shown to alter the phenotype of many neurons, including cholinergic, serotonergic, catecholaminergic, and peptidergic cells [1,11,22,29,37,38,50,54]. Age-related, and/or dementia-related, changes have been described for all of these neurotransmitters and neuromodulators and/or their receptors [for examples and reviews, see Refs. [4,17,18,23,40,57]]. It is, therefore, possible that decreased amounts of either BDNF or trkB contribute to the disruption of neuroactive chemicals. Therefore, any relationship between BDNF or trkB and age-related declines could either be direct, or could be the indirect result of interactions with multiple systems.

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