BDNF transcripts, proBDNF and proNGF, in the cortex and hippocampus throughout the life span of the rat

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Abstract Neurotrophins are established molecular mediators of neuronal plasticity in the adult brain. We analyzed the impact of aging on brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) protein isoforms, their receptors, and on the expression patterns of multiple 5' exon-specific BDNF transcripts in the rat cortex and hippocampus throughout the life span of the rat (6, 12, 18, and 24 months of age). ProNGF was increased during aging in both structures. Mature NGF gradually decreased in the cortex, and, in 24-month-old animals, it was 30 % lower than that in adult 6-month-old rats. The BDNF expression did not change during aging, while proBDNF accumulated in the hippocampus of aged rats. Hippocampal total BDNF mRNA was lower in

12-month-old animals, mostly as a result of a decrease of BDNF transcripts 1 and 2. In contrast to the region-specific regulation of specific exon-containing BDNF mRNAs in adult animals, the same BDNF RNA isoforms (containing exons III, IV, or VI) were present in both brain structures of aged animals. Deficits in neurotrophin signaling were supported by the observed decrease in Trk receptor expression which was accompanied by lower levels of the two main downstream effector kinases, pAkt and protein kinase C. The proteolytic processing of p75NTR observed in 12-month-old rats points to an additional regulatory mechanism in early aging. The changes described herein could contribute to reduced brain plasticity underlying the age-dependent decline in cognitive function.

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Introduction

Aging is accompanied by cognitive decline in a major segment of the human population and is the primary risk factor for Alzheimer's disease (AD) and other prevalent neurodegenerative disorders (Bishop et al. 2010). Despite the fact that age-related cognitive deficits are well-documented, the underlying structural and functional changes in the brain remain largely unknown. Learning and memory impairments are generally

attributed to a decrease in neuronal plasticity of the cortex and hippocampus, two regions whose vulnerability to aging is established as well (Burke and Barnes 2006). Attention has particularly focused on the neurotrophin family, considered as genuine molecular mediators of synaptic plasticity in the adult brain (Poo 2001).

Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are the most abundant and widely distributed neurotrophins in the brain. NGF is important for the survival of forebrain cholinergic neurons (Korsching et al. 1985) which are involved in cognitive function. These neurons degenerate with age and are markedly diminished in the brains of patients with AD (reviewed in Schliebs and Arendt 2011). BDNF, on the other hand, is a key molecule of structural and functional integrity of the hippocampal formation (Thoenen 2000). Activity-dependent BDNF transcription and trafficking have been repeatedly linked to numerous physiological events and pathophysiological insults such as fear conditioning (Rattiner et al. 2004), seizures (Lauterborn et al. 1996; Chiaruttini et al. 2008), ischemia (Kokaia et al. 1998), as well as with consolidation of hippocampus-dependent memory (Tyler et al. 2002; Bramham and Messaoudi 2005). However, recent studies suggest that during prolonged periods of secretion, as observed in long-term potentiation, the endogenous activity-dependent pool of BDNF becomes rapidly depleted (Pang et al. 2004; Santi et al. 2006). Under these conditions, *de novo* synthesis from pre-existing BDNF mRNA and recycling of secreted BDNF seems to be crucial for providing threshold levels of BDNF to maintain long-lasting synaptic changes underlying memory. The mechanisms and the manner in which neurotrophin expression is regulated are therefore of considerable interest.

As all neurotrophins, both NGF and BDNF are translated from their respective single protein-coding exons and synthesized as larger, ~30–34-kDa precursors (proneurotrophins). The genes encoding for NGF and BDNF have a complex structure, and their expression appears to be highly regulated. Multiple transcripts are produced as a result of alternative promoter usage, RNA splicing, and/or the use of different polyadenylation sites (Aid et al. 2007). The biological significance of four alternate NGF mRNAs has not been fully explored. In contrast, tissue-specific regulation and differential responses to various stimuli have been extensively demonstrated

for BDNF transcripts (Timmusk et al. 1993; Aid et al. 2007; Chiaruttini et al. 2008). Recent findings that BDNF transcripts have different translational competences and that they localize in different subcellular compartments (Pattabiraman et al. 2005; Baj et al. 2011) imply that local availability of this molecule is also important.

Another intriguing aspect of neurotrophin physiology is that each neurotrophin modulates a diverse range of important functions in the nervous system. Beyond the classical employment of two functionally different classes of cell surface receptors, the tyrosine kinase receptor (Trk) and the p75NTR neurotrophin receptor (p75NTR) with their different signaling cascades, the key aspects of neurotrophin functions appear to be regulated by proneurotrophin cleavage (Greenberg et al. 2009). Neurotrophin precursors can either be cleaved intracellularly by furin and other proconvertases (Seidah et al. 1996), or they can be secreted in their uncleaved forms (Lee et al. 2001). This determines whether the factor is secreted as a cleaved “mature” neurotrophin which preferentially binds a Trk receptor and thereby supports neuronal survival via both the PI3K/Akt and MAPK pathways, or whether it is secreted as a proneurotrophin which binds to the p75NTR/sortilin complex and primarily elicits apoptosis (Teng et al. 2005). Proneurotrophin interactions with p75NTR have been implicated in axonal retraction and synapse elimination as well (Singh et al. 2008).

The premise that aging-associated cognitive deficits are mediated, at least in part, by reduced expression of neurotrophins and/or their receptor has been extensively investigated so far, albeit with mixed results since comparisons are complicated by differences in the utilized species, strains, age, and experimental procedures (reviewed in Pang and Lu 2004; Tapia-Arancibia et al. 2008). Thus far, only a few studies have comprehensively evaluated the full complement of neurotrophin-related proteins (including their receptors) in the mammalian brain, especially as it ages. In the present study, we assessed the effects of aging on both NGF and BDNF by examining expression of their mRNA and pro- and mature proteins (mNGF and mBDNF) in the cortex and hippocampus of the rat throughout its life span (at 6, 12, 18, and 24 months of age). We further analyzed the age-related regulation of multiple 5' exon-specific BDNF transcripts (the I–IXA exon-containing BDNF mRNAs), which

differentially contribute to the formation of pro- and mature BDNF proteins. Age-related changes in the expression of neurotrophin cognate receptors (Trk, p75NTR) and two downstream kinases (Akt, protein kinase C (PKC)) were examined as well.

Methods

Animals and treatments

A total of 28 Wistar rats was used. All animal handling was in accordance with the Local Animal Care Committee Guidelines and in accordance with the NIH Guide for the care and use of laboratory animals (NIH publication no. 85-23). Experimental protocols were approved by the Ethical Committee of the Institute for Biological Research, University of Belgrade. The animals were housed under standard conditions (23 ± 2 °C, relative humidity 60–70 %, 12-h light/dark cycle, free access to food and water), with $n = 4$ and $n = 2$ adult and old animals per cage, respectively; their health status was routinely checked. At the age of 6 months (adult), 12 months (middle-aged), 18 months (late-middle-aged), and 24 months (aged rats), the animals were killed and their brains quickly excised. The entire cortex and hippocampus were dissected free on ice under magnifying glass, snap-frozen in liquid nitrogen, and stored at -80 °C for subsequent mRNA and protein analyses ($n = 5$ per group). For histological studies, the whole brains of the 6- and 24-month-old rats were used ($n = 4$ per group).

RNA isolation, cDNA synthesis, semiquantitative RT-PCR

Total RNA was prepared using the TriZol reagent (Invitrogen) according to the manufacturer's instructions. Two micrograms of RNA were treated with 2 U of RNase-free DNase I (Fermentas) and reverse transcribed using a High-Capacity cDNA Archive Kit with random hexamer primers (Applied Biosystems). The reactions were performed at 25 °C for 10 min and then at 37 °C for 2 h, in a final volume of 20 μ l.

PCR reactions were performed in a total volume of 25 μ l with 150 ng cDNA, 1.5 mM MgCl₂, 0.2 mM dNTPs, and 1 U of Taq polymerase (Fermentas) in a GeneAmp PCR System 9700 (Applied Biosystems). The primer sequences were as follows: for BDNF forward primer 5'-CACTCCGACCCCGCCCGCCG-3'

and reverse primer 5'-TCCACTATCTTCCCCTTTTA-3'; for NGF forward primer 5'-CCAAGGACG CAGCTTTCTAT-3' and reverse primer 5'-CTCCG GTGAGTCCTGTTGAA-3'; for GAPDH forward primer 5'-GGAGTCAACGGATTTGGTCGTAT-3' and reverse primer 5'-AGCCTTCTCCATGGTGG TGAAGAC-3'; for β -actin forward primer 5'-TGGACATCCGCAAAGACCTGTAC-3' and reverse 5'-TCAGGAGGAGCAATGATCTTGA-3'. For rat exon I-IXA-specific BDNF transcripts, primer sequences, and conditions were used as described by Aid and colleagues (2007). All RT-PCR reactions were performed in triplicate and in at least two independent RT reactions. The PCR products were separated in 2 % agarose gels and visualized by staining with ethidium bromide. A Multi-Analyst/PC Software Image Analysis System (Bio-Rad Gel Doc 1000) was used for densitometry analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a reference gene for NGF and all exon-specific BDNF transcripts, while β -actin was used for total BDNF. The mRNA levels are expressed as relative values.

Western blot analysis

For Western blot analysis, tissues were homogenized and sonicated in 10 volumes of RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 % NP-40, 0.5 % Triton X-100, 0.1 % SDS). Protein concentrations were determined using the Micro BCA Protein Assay Kit (Pierce Biotechnology) with bovine serum albumin as a standard. Equal amounts of proteins (20 μ g per lane) were separated by electrophoresis in a 10 % SDS-polyacrylamide gel, followed by blotting onto nitrocellulose or PVDF membranes (GE Healthcare). To block nonspecific binding, the membranes were incubated for 1 h at room temperature in 5 % nonfat dry milk in TBST (150 mM NaCl, 50 mM Tris, pH 7.4, 0.05 % Tween 20). The following antibodies were incubated overnight at 4 °C in 2 % blocking solution: rabbit anti-BDNF (N-20), rabbit anti-NGF (M-20), rabbit anti-panTrk (C-14), mouse anti-p-Trk A/B (E-6), rabbit anti-p-Akt1/2/3 (Thr-308), and rabbit anti-p75NTR (N-20), all obtained from Santa Cruz, and anti-proBDNF obtained from Millipore. Rabbit anti-PKC antibody was a generous gift from Dr. Andy Czernik from Rockefeller University, USA. Following the incubation with antibodies and several rinses in TBST, the membranes were incubated for 1 h with the

appropriate Alexa-488 (1:2,000; Invitrogen) or Horse Radish Peroxidase (HRP)-conjugated secondary antibodies (1:2–5,000; Santa Cruz). HRP-immunoreactive bands were visualized by enhanced chemiluminescence (GE Healthcare) and film exposure (Kodak Biomax); the fluorescent signals were scanned under blue light ($\lambda = 450$ nm) using the STORM system for nonradioactive detection (GE Healthcare). Each blot was subsequently re-probed with goat anti-GAPDH (Santa Cruz) or rabbit anti- β -actin antibody (Sigma). Signals were quantified by densitometry using Image Quant software (v. 5.2, GE Healthcare), and the relative values were calculated and normalized to the corresponding GAPDH or β -actin signals. The expression of the target proteins in each experimental group are presented as ratios/fold changes relative to the appropriate control values obtained from 6-month-old animals.

Tissue preparation and Fluoro-Jade B histochemistry

The brain was quickly removed from the skull and stored in 4 % paraformaldehyde, 0.1 M phosphate buffered saline (PBS) for 24 h at 4 °C. The brains were cryoprotected by immersing in 30 % sucrose in 0.1-M PBS and frozen in isopentane. Coronal sections (30 μ m) were stored in an antifreeze solution at –20 °C until further use. Degenerating cells were detected by staining the brain sections with Fluoro-Jade B (Millipore). Brain sections from 6- and 24-month-old animals were mounted onto slides and incubated in each of the following solutions for the indicated times: 1 % NaOH in 80 % ethanol, 5 min; 70 % ethanol, 2 min; distilled water (dH₂O), 2 min; 0.06 % potassium permanganate, 10 min; dH₂O, 2 min; 0.0001 % Fluoro-Jade in 0.1 % acetic acid, 30 min; and dH₂O, two times for 1 min. Stained sections were dried at room temperature, protected from light and examined under an Axio Observer Microscope Z1 (Zeiss). As a positive control, sections from rats treated with two intraperitoneally administered doses of (+)MK-801 (0.5 mg/kg) at 8-h intervals and euthanized 24 h after administration of the first dose, were used.

Statistical analysis

Statistica software (v. 6.0, StatSoft) was used. Relative values obtained by RT-PCR and Western blot analysis were tested for normal distribution by the Kolmogorov–Smirnov test and then analyzed by one-way ANOVA. Post hoc comparisons were carried out with the Fisher

LSD test. Statistical significance was set at $p < 0.05$. The data are presented as the means $\pm$ SEM.

Results

Rat cortical and hippocampal proBDNF is differentially affected by aging

As a first step towards exploring the age-related changes in neurotrophin signaling, we monitored the expression of pro- and mature BDNF proteins in the cortex and the hippocampus, the two brain areas prone to age-related changes (Fig. 1a). Western blot analysis revealed that in the cortex, aging induced a gradual decline in proBDNF (32 kDa). A significant decrease was observed only in 24-month-old animals (Fig. 1a, $*p < 0.05$). In contrast to the findings obtained in the cortex, proBDNF expression in the hippocampus gradually increased during aging (by about 25 % in 24-month-old animals; Fig. 1a, $*p < 0.05$). Statistical analysis confirmed a significant effect of aging in the cortex and in the hippocampus (one-way ANOVA: cortex: $F(3, 20) = 5.357$, $p = 0.007$; hippocampus: $F(3, 24) = 3.302$, $p = 0.037$). According to statistical analysis, the expression of mature BDNF (13.5 kDa) was unaffected by aging in both brain structures.

Contribution of specific transcripts to age-related changes in BDNF mRNA expression

To determine whether changes in proBDNF are accompanied by changes in *Bdnf* gene transcription, we measured BDNF mRNA expression by RT-PCR (Fig. 1b, c and Online Resource 1). Considering that BDNF expression is highly regulated and that its gene can generate multiple transcripts, we also examined the age-related regulation of the specific exon I–IXA-containing mRNAs and compared them to the total BDNF mRNA.

A distinctive pattern of expression of BDNF transcripts was observed in the cortex and hippocampus of adult 6-month-old rats (Fig. 1b). The exon IV-containing transcript was detected only in the cortex. More diversity and the presence of transcripts 1, 2, 3, 4, and 6 as the most abundant species were observed in the adult rat hippocampus. The expression pattern was different in aged, 24-month-old animals. Interestingly, the same RNA isoforms (containing

Fig. 1 BDNF mRNA transcripts and proBDNF are differentially affected by aging in the rat cortex and hippocampus. **a** Western blot analysis of pro-/mature BDNF. Aging differentially affects proBDNF levels in the cortex and hippocampus. Protein levels are expressed as fold changes (mean $\pm$ SEM) relative to the control value (6-month-old animals). Each graph is accompanied by a representative immunoblot. Asterisk indicates $p < 0.05$ vs. 6-month-old rats. **b** RT-PCR expression analysis of exon I–IXa-containing BDNF mRNAs in 6- and 24-month-

old animals. **c** Semiquantitative RT-PCR analysis of total and exon-specific BDNF transcript expression during aging. The increase in total BDNF mRNA in the cortex is mostly due to a twofold increase in levels of transcripts 4 and 6. In the hippocampus, age severely lowered the BDNF mRNA level already in 12-month-old animals. mRNA levels are expressed relative to the level of GAPDH mRNA $\pm$ SEM. Currency sign indicates $p < 0.05$ vs 6-month-old rats

exon III, IV, or VI), were present in both brain structures, pointing to a change in region-specific regulation of the *Bdnf* gene during aging. Nevertheless, the relative

abundance of specific transcripts was different, with transcript 4 being the most abundant in the cortex and transcript 3 in the hippocampus of aged rats.

An interesting pattern of expression for total BDNF mRNA during aging was also observed in the cortex. Compared to control 6-month-old rats, significant decreases in total mRNA were detected in both 12- and 18-month-old animals (by 70 and 60 %, respectively; Fig. 1c, $*p < 0.05$). In the group of old animals, the expression of BDNF mRNA increased significantly, by about 50 %. This increase was mostly due to the twofold increases in exon IV-bearing and exon VI-bearing BDNF transcripts. The expression of exon III mRNA also increased, but to a lesser extent (50 %). In contrast to the cortex, a prominent decrease in total BDNF mRNA (for 60–70 %) was observed at all of the examined time points (Fig. 1c, $*p < 0.05$) in the hippocampus. In 12-month-old animals, we did not detect exon I mRNA (Online Resource 1), while the expression of exons II and IV decreased considerably (by 90 and 60 %, respectively). The expression of transcript 2 remained unchanged during aging. On the other hand, the level of transcript 4 increased in 24-month-old animals. Nevertheless, in comparison to control 6-month-old animals, the expression of this transcript was 30 % lower in aged animals. Exon III decreased during aging as well, but the decrease was more gradual, and a maximal decline of 45 % was registered in the 24-month-old group (Fig. 1c, $*p < 0.05$).

Aging-induced changes in NGF mRNA and protein expression are more prominent in the cortex than in the hippocampus

Comparable patterns of age-related changes were observed for NGF. Aging induced pronounced accumulation of proNGF in both brain structures (Fig. 2a, b, $*p < 0.05$; one-way ANOVA: cortex: $F(3, 24) = 30.929$, $p = 0.001$; hippocampus: $F(3, 28) = 24.260$, $p = 0.001$). In the cortex, a near twofold increase in the level of NGF precursor was detected in 12-month-old animals. ProNGF remained elevated during aging, in 18- and 24-month-old animals. The increase in proNGF expression in the hippocampus was more gradual, rising by 65 % in aged animals (Fig. 2b, $*p < 0.05$). Mature NGF was affected by aging only in the cortex. Its expression gradually decreased and in 24-month-old animals NGF was 30 % lower than in adult 6-month-old rats (Fig. 2b, $*p < 0.05$). Upregulation of *Ngf* transcription accompanied the shift in the ratio of pro- to mature neurotrophin peptides (Fig. 2c). The expression of NGF mRNA increased by 30 % in the

cortex of 18-month-old animals and by 45 % in 24-month-old rats (Fig. 2c, $*p < 0.05$). One-way ANOVA confirmed a significant effect of aging only in the cortex ($F(3, 20) = 4.768$, $p = 0.011$).

Phosphorylated Trk receptor increases in the hippocampus during aging

Since neither BDNF nor NGF was significantly affected by aging, we next examined whether the age-related deficits in neurotrophin signaling could be correlated with changes in the expression of neurotrophin cognate receptors. The biological function of mature neurotrophins is mediated by Trk receptors. Western analysis revealed a decrease in total Trk expression (140 kDa) during aging in both of the examined structures (Fig. 3). The decrease was less pronounced in the cortex. The slight but significant decrease in total Trk (by 20 %) observed in 12-month-old animals was sustained during aging (Fig. 3a, $*p < 0.05$). We also analyzed age-related changes in the activated receptor (phosphorylated at Tyr496 (pTrk)). Aging induced a pattern of pTrk expression in the cortex similar to that of the unphosphorylated receptor. A sustained aging-related activation of Trk was observed in the hippocampus. Although the expression of total protein gradually decreased, with a maximal decline of 50 % measured in 24-month-old animals (Fig. 3b, $*p < 0.05$), the level of phosphorylated receptor did not significantly change during aging. Consequently, the ratio of activated to total receptor increased during aging, increasing twofold in aged animals. One-way ANOVA revealed a significant effect of aging on Trk expression in both structures (cortex: $F(3, 20) = 37.878$, $p = 0.001$; hippocampus: $F(3, 20) = 33.333$, $p = 0.001$).

Neurotrophin receptor p75NTR is proteolytically processed during aging

Neurotrophin proforms are high-affinity ligands for p75NTR. Accumulation of both proNGF and proBDNF during aging led us to investigate age-related changes in this receptor (Fig. 4). Recent studies have shown that p75NTR is subject to proteolytic processing which involves two sequential cleavages. To address this issue, we employed a Western blot assay and determined the expression pattern of full-length p75NTR and the presence of its cleavage

Fig. 2 ProNGF increases during aging both in the cortex and in the hippocampus of rats. **a** The level of NGF protein is reduced in the cortex of aged animals, while **b** age did not significantly change the NGF level in the hippocampus. Protein levels are expressed as fold change (mean $\pm$ SEM) relative to the control value (6-month-old animals). Each graph is accompanied by a representative immunoblot. *Asterisk* indicates $p < 0.05$ vs 6-month-old rats. **c** Decrease in the level of cortical NGF is accompanied by increased transcription of the respective genes. mRNA levels were assessed by RT-PCR and are expressed relative to the level of GAPDH mRNA $\pm$ SEM. *Asterisk* indicates $p < 0.05$ vs 6-month-old rats

a CORTEX

b HIPPOCAMPUS

c

products. Aging was accompanied by a transient and robust decrease in full-length p75NTR in both brain

structures. In the cortex of 12-month-old animals, the expression of this receptor was 35 % lower than in

Fig. 3 Aging differentially affects the protein levels of total and phosphorylated Trk receptors in the hippocampus. Western blot analysis was used to determine the expression of Trk and pTrk (Tyr 496) receptors in the cortex (a) and hippocampus (b). Each

graph is accompanied by a representative immunoblot. The data are expressed as the fold change (mean $\pm$ SEM) relative to the control value (6-month-old animals). Asterisk indicates $p < 0.05$ vs. 6-month-old rats

control, 6-month-old rats. In the hippocampus, it decreased by 60 % (Fig. 4a, b, $*p < 0.05$). The decrease in full-length p75NTR was accompanied by increases in two specific cleavage products, p75ECD (55 kDa) and p75ECDf (28 kDa). In the cortex, p75ECD was the dominant species where a 25 % increase was observed (Fig. 4c, $*p < 0.05$). In the hippocampus, additional proteolysis was more active, yielding a 45 % increase in the level of p75ECDf (Fig. 4d, $*p < 0.05$). Aging further led to an increase of the full-length p75NTR and to a decrease of two cleavage fragments in both brain structures. In the cortex, the increase in full-length p75NTR almost reached the level established in control animals, whereas in the hippocampus of 24-month-old animals, the expression of total p75NTR remained 25 % lower (Fig. 5b, $*p < 0.05$). One-way ANOVA revealed a significant effect of aging on p75NTR expression in both structures (cortex: $F(3, 20) = 5.718$, $p = 0.005$; hippocampus: $F(3, 20) = 32.301$, $p = 0.001$).

Region-specific changes in phosphorylated Akt during aging

We next examined whether Trk activation triggers a particular signal transduction pathway (Fig. 5). The Akt protein (56/60 kDa) which is involved in cell survival is one of the main downstream effector kinases in

the Trk signaling pathway. The expression pattern of activated Akt which is phosphorylated at Thr308 (pAkt) was assessed by Western blot analysis. The pTrk/Trk ratio was unaffected in the cortex, implying that aging had no effect on the pAkt content (Fig. 5a). However, in the hippocampus, pAkt gradually decreased during aging. In 24-month-old animals, it declined by 30 % (Fig. 5b, $*p < 0.05$). One-way ANOVA verified a significant effect of aging on the level of active Akt ($F(3, 16) = 26.666$, $p = 0.0002$). As the decline in pAkt points to lower neuronal survival, we examined whether neuronal cell death was detectable at the histological level. To this end, brain sections were stained with Fluoro-Jade B. Fluoro-Jade B positive cells were not detected in the cortex or in the hippocampus, either in 6-month-old animals or in brain slices from aged, 24-month-old rats (Online Resource 2).

Age-related changes in PKC expression in the cortex and hippocampus

In view of the contradictory findings obtained in the hippocampus, age-related Trk activation and decrease in pAkt, we evaluated the expression of total PKC protein (80 kDa) by Western blot analysis. PKC is a critical mediator in another important downstream Trk signaling pathway involved in synaptic plasticity-related

Fig. 4 p75NTR is subject to proteolytic processing both in the cortex and hippocampus of 12-month-old rats. Western blot analysis was used to determine the protein level of full-length p75NTR (**a**, **b**) as well as the levels of two specific cleavage products: p75ECD in the cortex (**c**) and p75ECDf in the

hippocampus (**d**) of rats during aging. Graphs that show changes in protein levels are accompanied by representative immunoblots. The data are expressed as the fold change (mean $\pm$ SEM) relative to the control value (6-month-old animals). Asterisk indicates $p < 0.05$ vs 6-month-old rats

effects of neurotrophins. An aging-induced gradual decrease in the expression of PKC protein was established in both structures, with the decrease in the cortex being less prominent than in the hippocampus. The expression of PKC was 20 % lower in aged, 24-month-old animals in comparison to control animals (Fig. 5c, $*p < 0.05$). More pronounced age-related effects were observed in the hippocampus where the level of PKC was decreased by 40 % in aged animals (Fig. 5d, $*p < 0.05$).

Discussion

One of the plausible explanations for the degenerative changes and cognitive impairments observed during normal aging and in AD is loss of plasticity-related support by neurotrophins. However, although the

levels of NGF and BDNF are clearly decreased in some neurodegenerative diseases (Phillips et al. 1991; Hock et al. 2000), there is no consistent evidence to support a widespread reduction in basal neurotrophin expression in the brain as a result of aging (Pang and Lu 2004; Tapia-Arancibia et al. 2008). In contrast to earlier studies which focused on neurotrophin retrograde transport or the Trk receptor response, our work provides additional insight into the regulatory mechanisms that control the availability and thus the functions of these two abundant neurotrophins in the brain.

Age had no effect on the content of mature BDNF in either of the examined brain structures. On the other hand, mature NGF was decreased in the cortex of aged animals. Our results are in general agreement with earlier studies that were performed on the hippocampi

Fig. 5 Two effector kinases in the Trk receptors signaling pathway decrease in the hippocampus during aging. Western blot analysis was used to determine the expression of pAkt (Thr308) kinase and PKC in the cortex (**a** and **c**, respectively) and in the hippocampus (**b** and **d**, respectively). Each graph is

accompanied by representative immunoblots. The data are expressed as the fold change (mean $\pm$ SEM) relative to the control value (6-month-old animals). Asterisk indicates $p < 0.05$ vs. 6-month-old rats

of mainly Sprague–Dawley rats and which mostly employed ELISA (Lapchak et al. 1993; Croll et al. 1998; Sugaya et al. 1998; Silhol et al. 2007, 2008; Del Arco et al. 2011). Our key finding is that the age-related accumulation of proBDNF in the hippocampus is not transcriptionally driven. The age-related changes in total BDNF mRNA followed the changes in proBDNF expression in the cortex in a reverse manner. For NGF, transcriptional upregulation accompanied a decrease in the expression of mature protein.

A comprehensive analysis of *Bdnf* expression by semiquantitative RT-PCR revealed diverse, region-specific regulation of 5' exon-containing BDNF mRNAs during aging. While data on the expression of exons I, II, IV, and VI under different stimuli are available (Timmusk et al. 1993; Liu et al. 2006; Nair et al. 2007), tissue-specific expression profiles of all rat

BDNF transcripts were described only in up to 2-month-old rats (Aid et al. 2007). Furthermore, studies examining the impact of aging on *Bdnf* expression focused on the hippocampus and total BDNF mRNA. Our study extends previous observations by examining the changes of I–IXA BDNF transcripts at multiple aging points and by including the cortex. We investigated whether aging reduces the basal expression of specific BDNF transcripts and hence the capacity for their recruitment for memory-related plasticity.

Semiquantitative RT-PCR analysis revealed the unique contributions of specific BDNF transcripts to total BDNF mRNA during aging. Increased BDNF expression in the cortex of aged animals was mostly due to the increase in the expression of exon IV- and VI-containing BDNF transcripts. In the hippocampus,

the most significant decrease in total mRNA was observed in 12-month-old animals as a result of a prominent decrease in expression of transcripts 1, 2, and 4. The substantial changes detected in 12-month-old animals were more or less sustained during aging. This is noteworthy given the recent *in situ* hybridization study describing the expression patterns of transcripts 1, 2, 4, and 6 within the hippocampal subfields of aged animals (Chapman et al. 2012). According to our results, it would appear that the decrease of transcripts 1 and 2 is not exclusive to aged animals. The presence of a similar subset of transcripts in both the cortex and hippocampus of aged animals implies that changes in the constitutive expression of transcripts 3, 4, and 6 are more relevant for degenerative changes and cognitive impairments during aging in intact animals. Upregulation of exon IV- and VI-containing BDNF mRNAs in the cortex is intriguing given that these transcripts are particularly sensitive to neuronal insults that involve excitotoxic damage such as hypoglycemic coma, ischemia, and epileptic seizures (Timmusk et al. 1993; Kokaia et al. 1994). On the other hand, the overall decrease of these transcripts in the hippocampus during aging is in accordance with the age-related decrease in hippocampal synaptic plasticity. Together with the substantial downregulation of exon I/II-specific transcripts that exhibit slow but sustained transcription under constant neural activity (Hara et al. 2009), the specific decreases of transcripts 3 and 4 may be critical for the decline in plasticity in both proximal and distal dendrites of hippocampal neurons during aging (Baj et al. 2011). Moreover, an important result is that the abundance of specific mRNA subsets substantially changes during aging. Transcripts 3 and 6 appear in the cortex of aged animals. The loss of transcripts 1 and 2 early in aging is essential for the hippocampus. On the other hand, the very low expression levels of BDNF mRNAs that contain exons V, VII, and VIII were maintained during aging and in aged animals in both of the examined brain structures.

While the aging-related accumulation of proBDNF in the hippocampus may serve as a depot of mature protein, recent studies have shown that this is not the case in aged animals. The study by Silhol and colleagues demonstrated that training in a spatial learning task increased the expression of proBDNF in both young and aged rats, but that only young rats displayed a corresponding increase in mBDNF (Silhol et al. 2007). Increased proBDNF was further

associated with memory impairments in aged rats (Silhol et al. 2008). Taken together, these data suggest that the cleavage of proneurotrophins, i.e., their conversion to mature protein, is compromised in aged rats. Indeed, it was recently established that following stimulation proneurotrophins are the main releasable forms (Yang et al. 2009) and that the activity of the complex tissue plasminogen activator (tPA)/plasminogen cascade which is involved in their extracellular maturation decreases with age (Cacquevel et al. 2007). We can therefore assume that impaired cleavage of precursor protein could also account for the decrease of mNGF in the cortex of aged animals, even though proNGF was abundantly present.

In contrast to proBDNF whose cleavage is important for memory-related synaptic plasticity and which changed only in aged animals, proNGF seems to be the predominant form of NGF throughout adulthood, as was previously reported (Fahnestock et al. 2001). The presence of both precursor in aged animals and of proNGF during aging is an intriguing result, especially considering earlier studies which demonstrated that proneurotrophins elicit neuronal death by binding with high affinity to p75NTR (Lee et al. 2001; Teng et al. 2005). However, there is some controversy regarding the prosurvival role of proNGF in the brain. High proNGF concentrations can stimulate PC12 cell survival when the p75NTR/TrkA ratio is low (Fahnestock et al. 2004). It was established that the receptors balance, i.e., the relative level of Trk and p75NTR, is a crucial determinant of cell fate during development and that it is required for further modulation of neurite outgrowth/synaptic plasticity in the adult brain (Masoudi et al. 2009). ProNGF has indeed been demonstrated to induce neuronal apoptosis *in vivo* after injury or seizures when p75NTR expression is induced, shifting the balance of signaling towards apoptosis (Volosin et al. 2008).

Therefore, the regulated intramembrane proteolysis (RIP) of p75NTR in both the cortex and hippocampus of 12-month-old animals described herein is of considerable importance. RIP may also represent an additional regulatory mechanism for the suppression of p75NTR-mediated signaling during early aging in spite of the abundant presence of proNGF. In line with this, the observed decrease in Trk expression is also important for an additional shift of the Trk/p75NTR balance in aged animals.

The Trk phosphorylation status in the hippocampus increased during aging. The increase was gradual, with the most prominent ratio of pTrk/Trk detected in the oldest age group. This result complements the data obtained in the aged rat dentate gyrus (Kelly et al. 2000). Although the increased Trk phosphorylation may imply a compensatory mechanism following a substantial decrease in total protein level, we did not observe a concurrent increase in downstream signaling molecules such as pAkt or PKC in this structure, which is normally associated with neuronal survival and plasticity. That the phosphorylated (activated) Trk receptor does not suppress the ability of proNGF to elicit caspase cleavage and apoptosis via p75NTR was demonstrated in vitro (Song et al. 2010). Yet, once the downstream Akt pathway is activated, neurons attain increased resistance to proNGF-induced cell death, indicating that Trk and p75NTR signaling pathways interact downstream of the receptors but upstream of Akt. In agreement with this, we observed a continual age-related decrease in both pAkt and PKC expression. As in other studies (reviewed in Burke and Barnes 2006), we also did not detect dying neurons by Fluoro Jade-B staining. However, our results correlate well with our earlier findings that show an age-related decrease in plasticity-related proteins such as GAP-43 and synaptophysin in the cortex and hippocampus of aged rats (Mladenović et al. 2010).

Trk receptor activation, on the other hand, has been associated with p75NTR ectodomain shedding. Ligand-induced phosphorylation/activation of TACE/ADAM-10/17, and subsequent production of p75-ICD fragments in a γ -secretase-dependent manner observed in vitro (Urrea et al. 2007) could also be of importance during aging. Our preliminary data revealed an increase by 30 % of ADAM-10 mRNA expression in the cortex of 12-month-old animals that returned to the control level in aged animals (data not shown). On the other hand, the 55-kDa p75ECD fragment detected in our study was reported as a complete p75NTR ectodomain cleaved by γ -secretase in response to NGF (Weskamp et al. 2004). The presence of this fragment in middle-aged animals implies that γ -secretase-dependent ectodomain shedding occurs as well, and that shedding efficiency is lost during aging.

In conclusion, to our knowledge, this is the first study that extensively analyzes the expression of two of the most abundant neurotrophins and their receptors in the cortex and in the hippocampus of rats at different stages

of life, including aged animals. We report that aging selectively interferes with the production and availability of these plasticity-related molecular substrates, suggesting a more complex regulation of their expression than was previously assumed. The data described herein further complement a growing body of evidence which supports the hypothesis that neurotrophin signaling is altered in the aged mammalian brain.

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