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GSK-3β and oxidative stress in aged brain. Role of poly(ADP--ribose) polymerase-1

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Abstract

Glycogen synthase kinase-3 (GSK-3) plays important roles in the regulation of glycogen and protein synthesis. In Alzheimer's disease it is responsible for hyperphosphorylation of tau. However, the role of GSK-3β in brain aging and in neurodegenerative diseases is not fully elucidated. Our aim was to determine the protein level of GSK-3β and its active, tyrosine 216-phosphorylated form in adult and aged brain parts. Moreover, lipid and protein oxidation and nuclear NF-κB translocation were measured and correlated with the activity of PARP-1, the nuclear target for free radical signalling. The GSK-3β/PARP-1 relationship was investigated. Adult (4 months) and old (24 months) rats were used. PARP-1 inhibitor 3-aminobenzamide (3-AB) was injected subcutaneously for 5 days in a dose of 10 and 30 mg/kg b.w. On the 8th day object recognition test and open field test were performed. Biochemical, radiochemical, immunochemical and spectrophotometric methods were applied. Our data indicated similar protein level and activity of GSK-3β in aged and adult brain cortex, hippocampus, striatum and cerebellum. A significantly higher level of p65 NF-κB subunit was found in the nuclei of aged hippocampus. Moreover, our previous study presented higher PARP-1 activity in aged hippocampus and brain cortex versus adult. These results indicated an enhancement of oxidative stress and altered susceptibility of macromolecules to oxidative stress in aged brain. Subsequently it was found that 3-AB significantly changed the level of active form of GSK-3β(Tyr216) in the hippocampus and brain cortex and at high dose decreased the locomotor activity of aged rats. These results indicated that PARP-1 may play an important role in the regulation of GSK-3β. Under massive oxidative stress PARP inhibitor(s) may protect the brain against both excessive poly(ADP-ribosy)lation and GSK-3β activation.

Key words: *PARP, GSK-3, aging, brain, oxidative stress*

Introduction

Oxidative stress has been implicated in cognitive impairment in brain aging and in neurodegenerative diseases [4,14,21]. Free radical-induced DNA strand breaks stimulate poly(ADP-ribose) polymerase-1 (PARP-1) and lead to the depletion of βNAD+ and ATP and thus to cell death [21]. Moreover, PARP-1 interacts with transcription factors that are key regulators of cell death, survival and inflammation, such as p53 or NF-κB [20], and this interaction seems to undergo post-translational regulation [25]. This sug-

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gests that PARP-1 may change the balance of pro- and anti-survival signalling pathways, depending on the energetic status of a cell and the degree of damage. PARP-1 inhibition through Akt activation could be responsible for protection of mitochondria in oxidative stress. Phosphoinositide-3-kinase (PI3-K) inhibitors diminished the protective effect of PARP inhibition [15]. The dependence of PARP-1 activity upon signal transduction mechanisms such as the PLC/IP_3 pathway [1,10] suggests that additional regulating mechanisms may exist that influence cell survival in the CNS through modulation of poly(ADP--ribosyl)ation.

Among numerous cell fate-related proteins that interact with PARP-1, one of the new and most intriguing targets is the serine-threonine protein kinase, glycogen synthase kinase 3 (GSK3). Kovacs et al. [15] have shown that inhibition of PARP with various new PARP inhibitors facilitated recovery after ischaemia/reperfusion in a heart failure model. The inhibitors of PARP promoted activation of Akt which, in turn, phosphorylated and inactivated GSK-3β. Similar results showing the dependence of Akt and GSK-3β activity upon PARP-1 inhibitors were obtained in CNS neurons by Wang et al. [24] using the "classical" inhibitor of poly(ADP-ribosyl)ation, 3-aminobenzamide.

GSK3 is a central player in several different signal transduction pathways in mammalian cells. GSK3 has been implicated in the control of numerous cellular responses including gene transcription, mRNA translation, intracellular signalling and glycogen synthesis. During the last few years it has become evident that GSK3 is a key enzyme in insulin and Wnt signalling pathways. This enzyme is implicated in the pathomechanism of Alzheimer's disease, diabetes, cancer and inflammatory processes (Fig. 1). GSK3 phosphorylates over 50 proteins and is involved in regulation of many cellular functions: cell cycle, cell polarity, migration and apoptosis. The number of cell functions regulated by GSK3 suggests that the activity of GSK3 must be tightly regulated. However, the mechanism is not fully understood. This kinase is expressed in mammalian cells in two highly homologous isoforms, GSK3α and GSK3β, although a splice variant of the β-form has also recently been described. A variety of agents inhibit GSK3 by triggering its phosphorylation at the N-terminal serine residue (Ser9 in GSK3β and Ser21 in GSK3α), and this can be catalysed by several different protein kinases that are switched on in response to signals that activate PI-3K or the classical mitogen-activated protein kinase (MAPK) cascade, or elevate cAMP and PKA [5]. In contrast to the inhibitory action of Akt at Ser9, tyrosine phosphorylation at residue 216 (Tyr279 of GSK3α) enhances kinase activity. Dephosphorylation or mutation of Tyr216 to phenylalanine decreases enzymatic activity [12]. However, the mechanism by which Tyr216 becomes phosphorylated in cells is not yet elucidated [5].

Dysregulation of GSK-3 activity has been involved in human pathologies such as neurodegenerative diseases. Inhibition of the neuroprotective pathway mediated by PI-3K/Akt leads to activation of GSK3β.

The results of Hooper et al. [11] suggest a new role for GSK-3β in neurodegenerative diseases. Inhibitory Ser9 phosphorylation is increased when long--term potentiation (LTP) is induced in CA1 or dentate gyrus. The overexpression of GSK-3β impairs LTP in a lithium-sensitive manner. These results suggest a possible direct role of GSK-3beta in memory impairment caused by AD, a disease characterized by significant GSK-3 deregulation.

The role of GSK3β function in the brain is so far not fully understood. The aim of our study was to investigate the expression and activity of GSK3β in different parts of adult and aged brain. Moreover, the level of lipid and protein oxidation and NF-κB translocation into the nucleus were measured and correlated with the activity of PARP-1, the nuclear target for free radical signalling. Then the relationship between GSK3β and PARP-1 was investigated using PARP inhibitor.

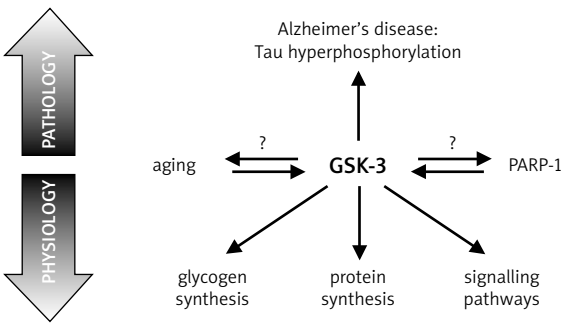

Fig. 1. The roles of GSK-3 in physiological and pathological aging

Materials and Methods

Animals

Male Wistar rats were used for the experiments. The animals were delivered by the Animal House of the Medical Research Centre, Warsaw, Poland and were of SPF (*specific pathogen-free*) health category. The Local Ethics Committee that followed the European Communities Council Directive accepted the use of these animals for the described experiments. 4-month-old animals weighing 200-250 g were used as adults, and 24-27-month-old animals weighing >300 g were used as old. In each of the experiments, a group of six rats was treated (subcutaneously) with 3-aminobenzamide (10 mg/kg and 30 mg/kg) for five days. All rats were subjected to behavioural tests and decapitated.

Object recognition task

The test was done as described previously [3,7] modified according to [13]. The apparatus was a wooden box (65 × 45 × 45cm) placed in a sound-isolated room with constant illumination. A day before the testing, rats were subjected to a habituation session, whereby they were allowed to explore the apparatus for 5 min. The next day, they were subjected to a 5-min acquisition trial during which they were individually placed in the open field in the presence of one object A (cup). 3-minute retention trials were carried out 2 h later. During this trial, object A' (a duplicate of object A in order to avoid olfactory traits) and another object B (bowl) were placed in the open field, and the times (tA and tB) the animal took to explore the two objects were recorded. Recognition index (RI) was defined as (tB/(tA' + tB)) × 100. When RI <50, it was concluded that the rat did not recognize familiar object A', and if RI >50 the rat remembered the object.

Open field test

This test was done according to Kosiorek et al. [13] to check the effect of compounds we have examined on locomotor activity of the rat. Locomotor (crossings of squares) and exploratory (rearings) activity was measured in an open field, which was a square 100 cm white floor divided by eight lines into 25 equal squares and surrounded by a 47 cm high wall. The animals were placed in the centre of the open field box and crossings of squares and rearings were counted for 5 min.

Determination of the oxidation of macromolecules

Carbonyl groups (CG) concentration, a marker for protein oxidation, was determined after derivatization with dinitrophenylhydrazine (DNPH), and lipid peroxidation was evaluated as thiobarbituric acid reactive substances (TBARS), as described previously [6]. Lipid and protein oxidation in adult and aged brain was subsequently investigated after subjection to oxidative stress evoked by incubation of homogenates with 25 μM $FeCl_2$ and 10 μM ascorbate for 15 min. at 37°C.

Immunochemical measurements (Western-blot)

The brains were dissected quickly after decapitation and the hippocampus, cortex and cerebellum were isolated on an ice-cold Petri dish. The structures were homogenized in 10 mM Tris-HCl and mixed with 5× Laemmli sample buffer and denatured for 5 min at 95°C. After a standard SDS-PAGE on 10% (7.5% for PARP-1) polyacrylamide gel, proteins were transferred onto nitrocellulose membrane, and the membrane was washed for 5 min in TBS-Tween (TBS-T; 0.05%). After incubations with antibodies and autoradiography, densitometric analysis and marker size-based verification was performed with TotalLab software.

Measurement of GSK3β and GSK3β (Tyr216) protein

The membrane was blocked for 1 h at room temperature (RT) in 5% non-fat milk in TBS-T (for GSK3β) or in 0.5%BSA/TBS-T (for GSK3β(Tyr216)). Then the membranes were incubated overnight in 1/2500 solution of monoclonal anti-GSK3β Ig (BD Transduction Laboratories, clone 7) in 5% non-fat milk in TBS-T at 4°C or in 1/1000 solution of monoclonal anti-GSK3β(Tyr216) Ig (BD Transduction Laboratories, clone 13a) in 0.1%BSA/TBS-T. The membrane was washed 3 times in TBS-T and incubated for 1 h at RT in 1/4000 horseradish peroxidase (HRP)-linked anti-mouse IgG (Amersham) in 2% non-fat dry milk. Then the membrane was washed three times with TBS-T.

Measurement of the level of PARP-1 protein

The membrane was blocked for 1 h at RT in 1% BSA in PBS-T and then incubated overnight in 1/500 solution of monoclonal anti-PARP-1 IgG (Sigma, clone C-2-10) in 0.5% BSA at 4°C. The membrane was washed 3 times in PBS-T and incubated for 1 h at RT in 1/2500 HRP-linked anti-mouse IgG (Amersham) in 5% non-fat dry milk. Then the membrane was washed three times with PBS-T and once in PBS.

Measurement of p65 NF-κB protein

Membrane with proteins was blocked for 15 h in 5% non-fat milk in PBS-T (atypically with 0.1% Tween-20) at 4°C and then incubated overnight at 4°C in 1/100 solution of polyclonal anti-p65 antibody (Sigma) in 5% non-fat milk and washed 3 times for 5 min. in PBS-T. The membranes were then incubated for 1 h at RT in 1/8000 anti-rabbit HRP-linked IgG (Sigma) in 5% milk, washed 3 times in PBS-T and once in PBS.

Measurements of β-actin level

After stripping, β-actin was detected on membranes as loading control. The membranes were washed for 5 min in TBS-T, blocked with 60 min in 5% non-fat milk/TBS-T, incubated for 2 h in 0.1% BSA/TBS-T containing mouse monoclonal anti-β-actin antibody (1:400; Mab (C4); MP Biomedicals, Inc.), washed 3 × 5 min with TBS-T, 60 min in 5% non-fat milk/TBS-T containing secondary antibody (1:4000; Anti-mouse IgG NA931V, Amersham Biosciences), 3 × 5 min in TBS-T, 5 min in TBS).

Statistics

Student t-test and one-way ANOVA with Newman-Keuls post-hoc test were used; $p<0.05$ was considered significant. All experiments were carried out on 3-6 animals. The presented data are means ± SEM.

Results

Lipid and protein oxidation was measured as the level of aldehydes and ketones reacting with thiobarbituric acid (TBARS) and as carbonyl groups, respectively. It was found that concentration of TBARS and carbonyl groups was similar in all investigated parts of the adult and aged brain and it was not significantly changed during aging (Fig. 2A and B, respectively). However, after incubation of homogenates of different brain parts with $FeCl_2$ significantly lower TBARS level was observed in all investigated parts of the aged brain. The level of carbonyl groups was lower only in the hippocampus. Moreover, our experiments demonstrated that the level of NF-κB p65 subunit considerably increased in nuclear fraction of aged hippocampus (Fig. 3).

Our previous results demonstrate that PARP-1 activity is significantly elevated by 52% in aged rat hippocampus compared to the adult tissue [21]. The activity of this enzyme in the cerebral cortex was also significantly enhanced by 64% while in the cerebellum and striatum it was non-significantly elevated by 24% and 16%, respectively [21].

We recently confirmed these results using immunochemical poly(ADP-ribose) measurements (data not shown). In the following set of experiments, GSK3β immunoreactivity was measured in different parts of the adult and old brain. Our data indicated that GSK3β protein level was similar in the hippocampus, cerebellum, striatum and brain cortex and it did not change during aging (Fig. 5A). We observed (Fig. 5B) that the level of active, phosphorylated GSK3β form (Tyr216) did not differ significantly in any investigated brain parts, with the exception that aging decreased the active form of GSK3β (Tyr216) in the striatum.

Administration of PARP inhibitor 3-AB (10 and 30 mg/kg) caused a significant decline of GSK-3β immunoreactivity in the brain cortex (Fig. 5A). Moreover, a decline of the level of GSK-3β (Tyr216) was observed in the brain cortex and hippocampus (Fig. 5B).

Object memory acquisition and consolidation did not change in aged rats treated with 3-AB compared to control, non-treated animals (Fig. 6). However, locomotor activity measured by open field test was considerably decreased after administration of higher dose (30 mg/kg b.w.) 3-AB.

Discussion

Accumulation of damage exerted to DNA and other macromolecules by reactive oxygen species (ROS) is considered as one of the most probable mechanisms of brain aging. The hippocampus and neocortex are among its most vulnerable parts and undergo selective damage during neurodegenerative diseases.

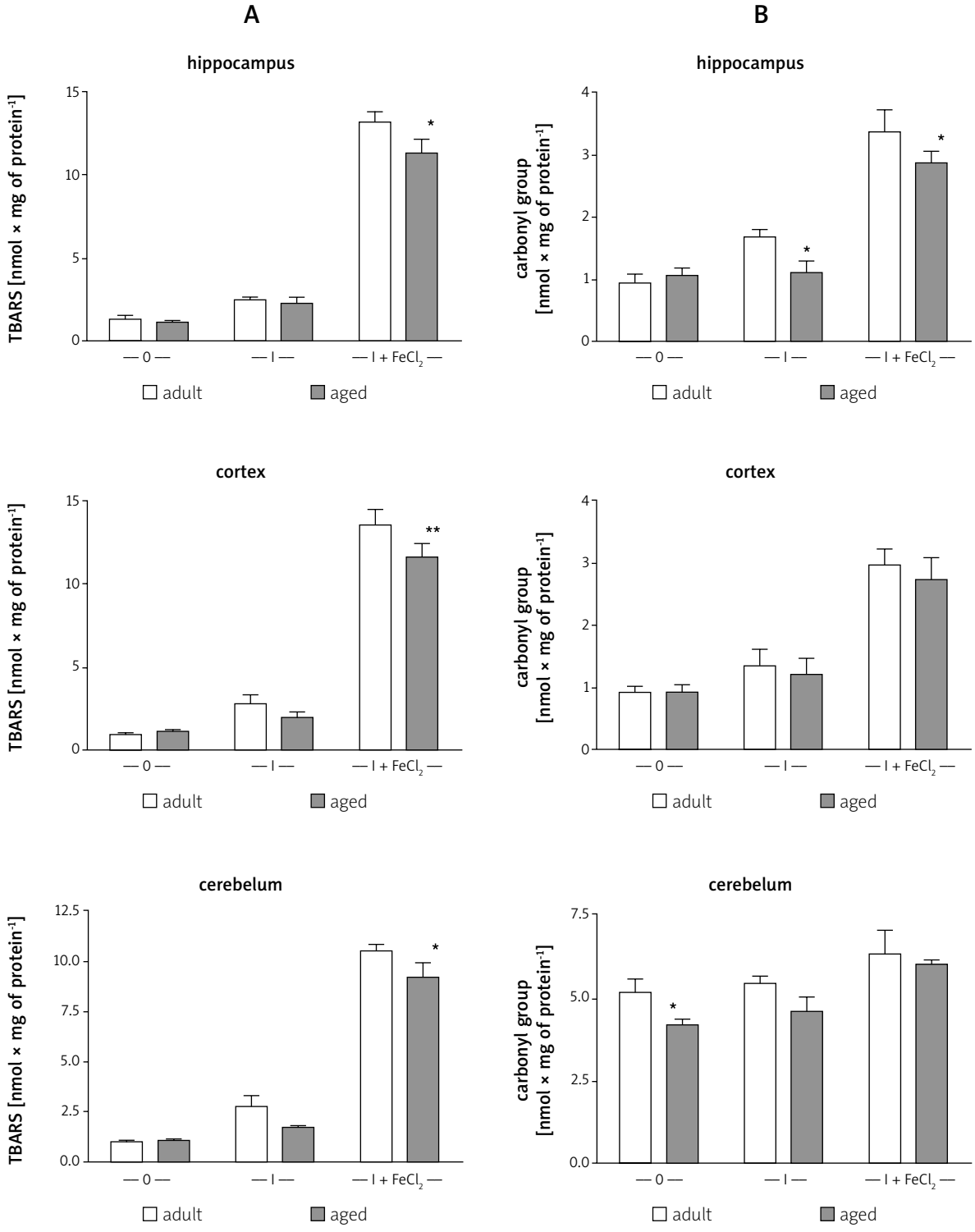

Fig. 2. The concentration of TBARS (A) and carbonyl groups (B) in different parts of adult and old brain. The homogenates of hippocampus, brain cortex and cerebellum were used directly for measurements (0) without incubation or after incubation for 15 min. at 37°C in control conditions (I) or in the presence of 25 μM $FeCl_2$ and 10 μM ascorbate. The values are means ± SEM from 4-6 independent experiments

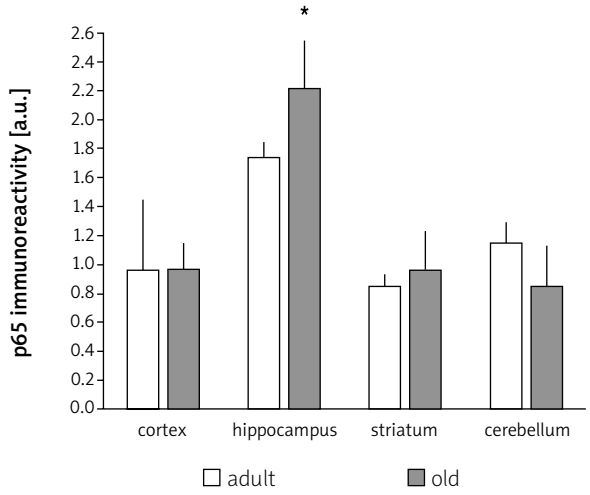

Fig. 3. Immunoreactivity of NF-κB p65 protein in nuclear fraction of brain parts Our Western--blot experiments demonstrated that NF-κB p65 subunit level considerably increased in aged hippocampus. Results are normalized to β-actin as loading control and are means ± SEM from six independent experiments. * $p<0.05$

Our results show that the free radical-dependent protein and lipid damage evaluated as the level of carbonyl groups and malondialdehyde, respectively, is not altered in aged brain parts as compared to adult. However, under excessive oxidative stress evoked by Fenton reaction the enhancement of the level of oxidative products was slightly but significantly lower in aged brain parts than in adult. The results suggest that this difference could be caused by altered availability of substrates for the oxidation in aged brain, rather than by lack of oxidative stress itself. The NF-κB transcription factor is one of the proteins that detect and react to oxidative stress. NF-κB regulates transcription of pro- and antiapoptotic proteins as well as antioxidant enzymes. It is also a key player in the regulation of inflammation, a process that can indirectly change the fate of neural cells.

In this study, a significantly higher level of immunoreactivity for the p65 subunit of NF-κB has been found in aged hippocampus nuclear fraction versus adult. These data confirm the occurrence of oxidative stress during aging. The intensity of the stress seems to be moderate and is not reflected in elevated TBARS or protein carbonyl level.

Free radicals exert numerous kinds of damage to DNA, including strand breaks. Many other lesions are also converted to single or double strand breaks in the course of DNA repair. Irrespectively of their origin, breaks are detected by PARP-1, a nuclear enzyme that utilizes βNAD$^+$ to post-translationally modify proteins related to DNA metabolism and to the regulation of cell survival [21 and 26]. PARP-1 is an early sensor of free radical-dependent DNA damage and its activation can be seen even before the damage itself reaches detectable levels [22]. Both p53 and NF-κB transcription factors bind to and are substrates for PARP-1. This fact can have an important significance for the outcome of oxidative stress and aging. PARP-1 inactivation inhibits NF-κB activation and tissue damage; PARP-1 can also cause cell death by itself, through depletion of βNAD$^+$ in conditions of massive DNA damage. However, in physiological conditions moderate level of PARP-1 activity is necessary for the homeostasis and defence against constantly occurring damage to DNA. Maximal PARP-1 metabolic capacity correlates with mean and maximal life span of mammals; it also correlates with human life span as judged from centenarian cell line research. We have found that in the course of pure physiological aging, basal activity of PARP-1 in pathogen-free (SPF) rat hippocampus and cerebral cortex increases, probably reflecting moderate increase of oxidative damage and DNA breaks (Fig. 7) [21].

Several studies have shown the importance of oxidative stress in the pathogenesis of neurodegenerative disorders [2]. The interaction between GSK3β activity and oxidative stress has recently been investigated (Fig. 8). The GSK3 pathway is involved in the control of many critical cell responses. Therefore, optimal inhibition of GSK-3β activity serves a crucial role in designing therapeutic strategies of neurodegenerative disease, diabetes and other disorders [17]. In our study we have investigated the effect of brain aging on GSK3β. GSK3β immunoreactivity has been measured in different parts of the adult and old brain. Our data indicate that GSK3β protein level is similar in the hippocampus, cerebellum, striatum and brain cortex and it does not change during aging. We observe that in the adult brain the active form of GSK3β (Tyr216) does not differ significantly in any investigated structures, but the aging process decreases the active form of GSK3β (Tyr216) in the striatum.

Both NF-κB and PARP-1 data suggest elevated oxidative stress in aged brain hippocampus and cortex. These data prompted us to investigate GSK3β in

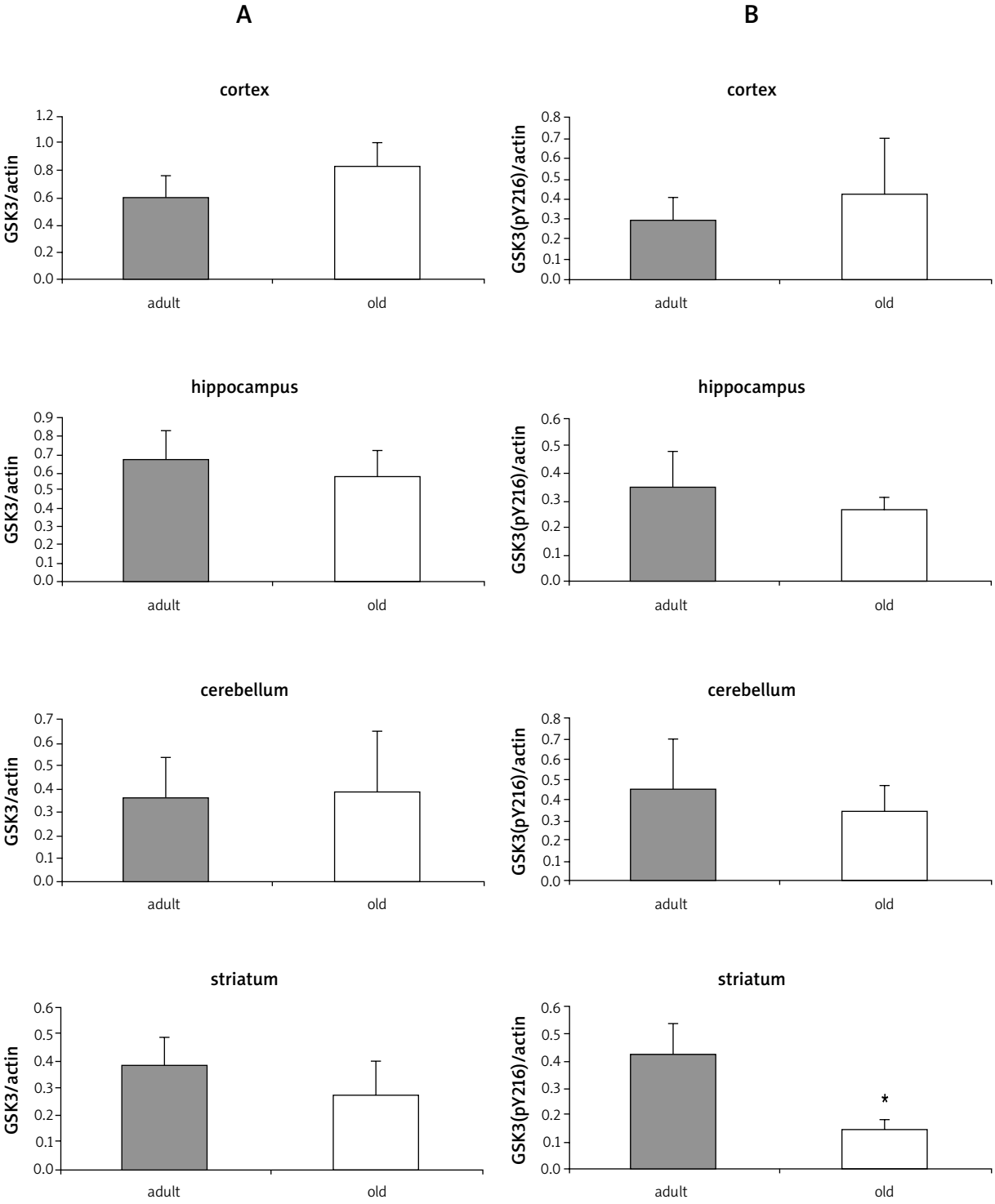

Fig. 4. GSK3β immunoreactivity in different parts of adult and old brain. Our data have shown that GSK3β protein level is not changed during aging (A). In adult brain (B) the active form of GSK3β(Tyr216) did not differ significantly between investigated structures with the exception of striatum. Results were normalized to β-actin as loading control and are means ± SEM from six independent experiments. pY216 stands for phosphorylated Tyr216. * $p<0.05$

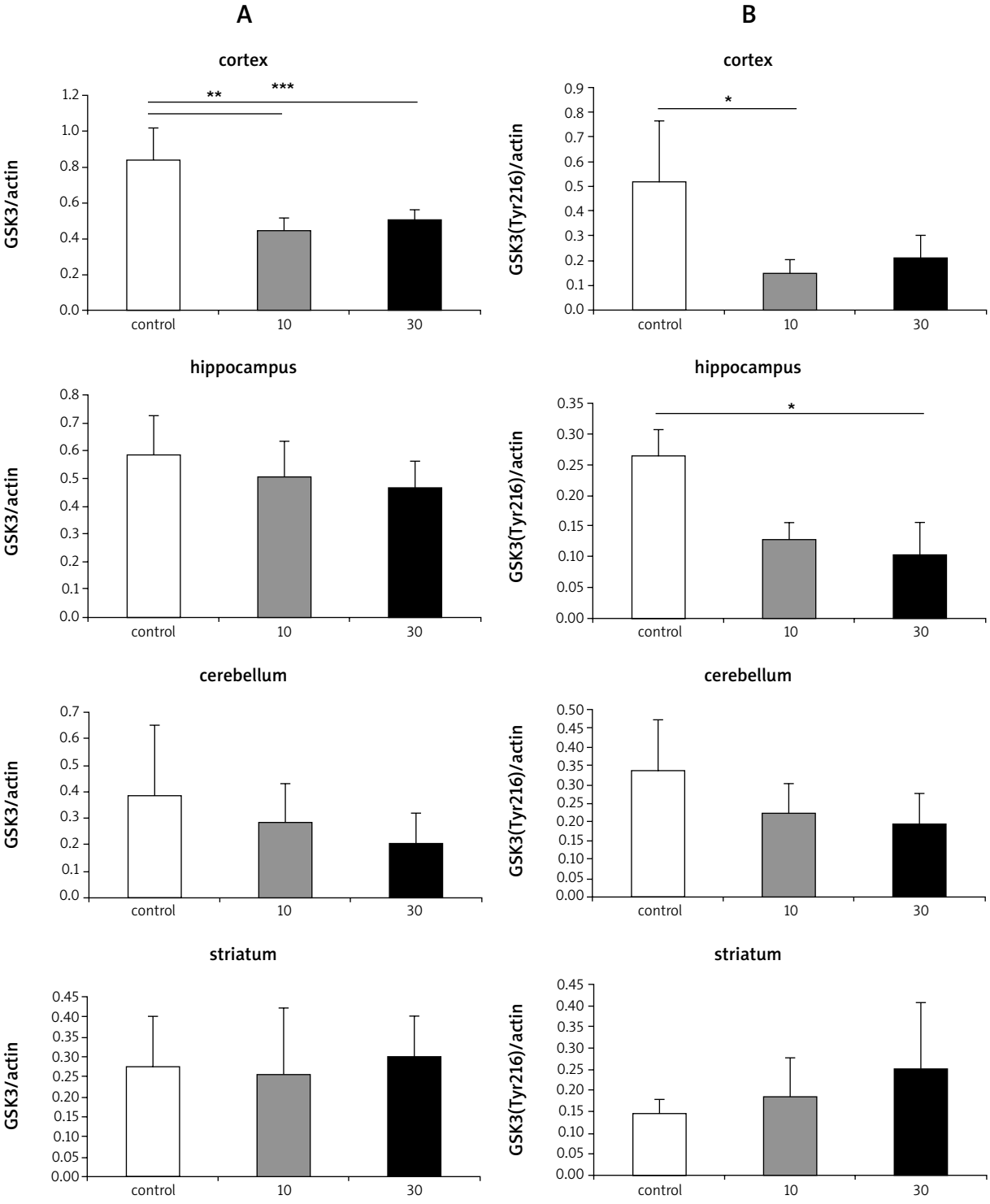

Fig. 5. Effect of 3-aminobenzamide (3-AB) on GSK3β immunoreactivity in different parts of old brain. Administration of PARP-1 inhibitor 3-aminobenzamide (10 and 30 mg/kg b.w.) caused a significant decline of GSK3β immunoreactivity in brain cortex compared to old, untreated control (A). A decline of active form of GSK3β was observed in brain cortex and hippocampus (B). Results were normalized to β-actin as loading control and are means ± SEM from six independent experiments. * $p<0.05$; ** $p<0.01$

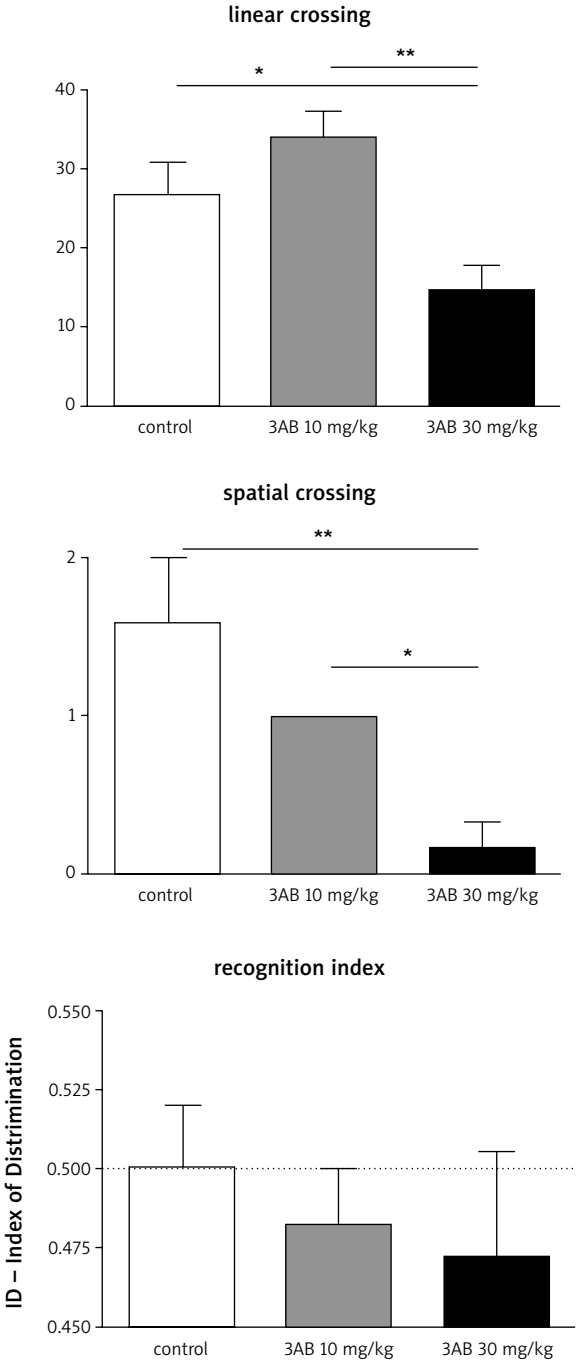

Fig. 6. The influence of 3-AB on locomotor activity and cognitive function in rats. Object memory acquisition and consolidation did not change in adult rats treated with 3-AB compared to control animals. However, locomotor activity was considerably decreased after treatment of aged animals with a dose of 30 mg/kg b.w. of 3-AB. Results are means ± SEM from six animals in each group

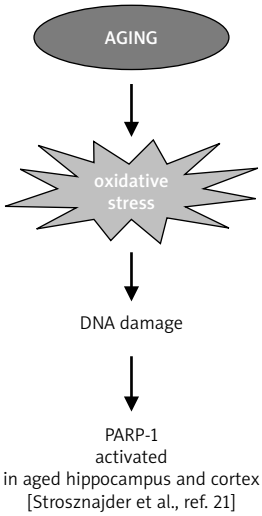

Fig. 7. PARP-1 activity in aging of the central nervous system

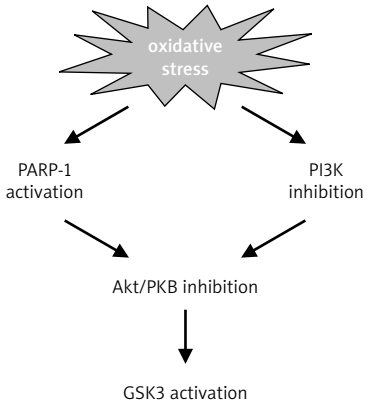

Fig. 8. Possible relationship between PARP-1 and GSK-3β in oxidative stress

animals treated with PARP-1 inhibitor (3-AB). The results show that PARP-1 inhibitor significantly decreases GSK3β activity exclusively in the hippocampus and brain cortex, and GSK3β protein level decreases only in the hippocampus. 3-AB does not change memory function but significantly decreases locomotor activity at high dose.

Recently, Lee et al. [16] have found a significant increase of GSK3β immunoreactivity in aged rat pyramidal neurons of cortical regions as well as in the pyramidal layer of CA1-CA3 hippocampal regions and in the granule cell layer of dentate gyrus. An expla-

nation for the discrepancy between the results of GSK-3β total protein level between our and Lee's experiments [16] may be the use of a different rat strain and different methodology (including use of pentobarbital anaesthesia).

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