SUMMARY

Glycogen synthase kinase 3 (GSK3), also known as tau protein kinase-1 (TPK1), is highly conserved, multi-functional serine-threonine kinase, which in mammals exists in two isoforms: GSK3α and GSK3β. In my study I have focused on GSK3β, which is a single 47 kDa polypeptide consisting of 433 amino acids. It has the highest abundance in the brain in comparison to other organs [Takashima 2006, Perez-Costas et al, 2010]. Expression of this enzyme is higher in neurons than in glial cells [Takahashi et al, 2000]. GSK3β is predominantly a cytosolic enzyme, but its presence was also documented for nuclear and mitochondrial fractions [Franca-Koh et al, 2002, Fujimuro and Hayward, 2003, Meares and Jope, 2007, Songin et al, 2011ab, Pajak, et al, 2009]. GSK3β was originally identified as a key regulator of glycogen metabolism, but until now many other functions of this protein were discovered, e.g. regulation of transcription factors and signalling proteins, involvement in cell architecture or apoptosis. GSK3β also plays an important role in protein synthesis, cell proliferation and differentiation, microtubule dynamics, cell motility. GSK3β influences these processes by phosphorylation of various transcription factors, initiation factors involved in cell cycle as well as proteins involved in microtubule function and cell adhesion. Ultimately, this kinase is also responsible for phosphorylation of tau. In summary, GSK3β regulates the function of cytoskeletal proteins as well as cell survival and death mechanisms. GSK3β has two phosphorylation sites at Ser9 and Thr216 that influence its catalytic activity. Thr216 is located in the activation domain of this kinase and its phosphorylation increases GSK3β activity. The mechanism of this regulation is yet not well understood [Wang et al, 1994]. Some data underline the importance of auto-phosphorylation [Cole et al, 2004], while others indicate the role of other tyrosine kinases [Hughes et al, 1993, Kim et al, 1999, Lesort et al, 1999, Wang et al, 2003] in the regulation of GSK3β activity. Mechanisms of GSK3β inhibition, depending from Ser9 phosporylation, are better known. Phosphorylation on this site induces conformational changes that result in the blockade of substrate binding and therefore inhibit GSK3β activity.

Previous data also indicated the importance of GSK3β in the pathogenesis of Parkinson's disease (PD) and Alzheimer's disease (AD). It is estimated that nowadays about 400 million people suffer from degenerative diseases of the central nervous system, mainly the AD and

PD, but the aetiology of these diseases is not yet fully understood. The factors considered to be potentially involved in neurodegenerative process are oxidative stress and neurotixicity evoked by pesticides, herbicides and industrial chemicals. Environmental toxins may increase the sensitivity of neurons and cause their degeneration. Moreover they may induce brain blood vessels damage that is commonly spotted in neurodegenerative diseases [Miller et al, 2002]. One of the substances, which environmental abundance positively correlates with the incidence of PD morbidity, is a commonly used herbicide - paraquat (PQ) [Hertzman et al, 1990, Liou et al, 1997].

The chemical structure of PQ is similar to 1-methyl-4-phenylpyridinine (MPTP), but its neurotoxicity is much smaller than induced by MPTP or 6 - hydroksydopamine (6-OHDA) because of the poor blood-brain barrier (BBB) transport of this herbicide [Corasaniti and Nistico, 1993, Shimizu et al, 2001, Barlow et al, 2003, Ossowska et al, 2005, Prasad et al , 2007]. In a situation of no disturbances of BBB only the small part of PQ is transferred to brain by a transporter for neutral amino acids [Shimizu et al, 2001]. The toxicity of PQ is mediated by oxidative stress and mitochondria damage [Drechsel and Patel, 2008] that are the main features of the pathogenesis of neurodegenerative diseases [Lin and Beal, 2006; Pagani and Eckert, 2011]. Therefore using PQ as the experimental model may be useful in studies of the role of mitochondria dysfunction in the patomechanisms of both PD and AD.

It is well known that in AD pathology Aβ peptides disturb mitochondrial function [Sultana and Butterfield, 2009]. However it was recently demonstrated by Chen et al, 2011 that mitochondrial damage induced by PQ may be a phenomenon occurring prior to the Aβ aggregation.

The aim of this paper was to investigate the effect of PQ on the expression and protein level of GSK3β in the selected *in vivo* and *in vitro* models. In the experiments performed *in vivo* on male Wistar rats, the effect of single intraperitoneal 40 mg/kg PQ injection was investigated. For the further analysis brains were taken 3 and 24 hours after PQ injection. This experimental model was used to study the effect of PQ on the gene expression and protein level of total GSK3β and its active form phosphorylated on tyrosine 216 (GSK3β(pY216)) and on the expression of GSK3β, TNF-α, iNOS, COX-2 proteins in rat midbrain or striatum. We also examined long-term effect of PQ, by its weekly administration at a dose of 10 mg/kg for 4 and 37 weeks. The doses of PQ were determined experimentally or were chosen according

to the previously published studies [Ossowska et al, 2005a, b, Ossowska et al, 2006, Cutter et al, 2007, Shimizu et al, 2003]. In an experimental model of long-term PQ administration I examined protein levels of GSK3β and GSK3β(pY216) in the rat midbrain, striatum, and the sub-cellular fractions. *In vitro* studies were conducted on rat pheochromocytoma (PC12) cells of the adrenal medulla stably transfected with human amyloid precursor protein (APP) gene with Swedish double mutation (APPsw, K670M/N671L) or with empty vector in control cells. In APPsw cells the level of released Aβ peptides is a 4.8 - fold higher comparing to control PC12 cells [Chalimoniuk et al, 2007].

In these cells the effect of PQ (1 mM) and endogenously liberated Aβ peptides on protein level of total GSK3β and its phosphorylated forms: GSK3β(pY216) and GSK3β(pS9) was examined. *In vitro* studies were also concentrated on the measurement of intracellular free radicals level as well as on analysing cellular sensitivity to oxidative stress and cell viability.

In the *in vivo* studies, short-term administration of PQ resulted in the increase of expression of inducible isoform of NOS (iNOS) and cyclooxygenase 2 (COX-2). These enzymes were previously documented to be involved in inflammatory processes, and their hyperactivity can initiate cytotoxicity and neurodegeneration process. Three and twenty four hours after PQ injection I have noticed an increase in iNOS mRNA levels in the striatum and midbrain as well as increased levels of free radicals and oxidative stress, that can initiate cell death. Moreover, 3 hours after PQ injection, the increased level of COX-2 mRNA in the midbrain was observed. However in these brain structures there were no changes in the GSK3β expression either 3 or 24 hours after PQ administration. This phenomenon can be caused by too short PQ action or result from the age of experimental rats.

However long-term administration of PQ induced changes in GSK3β and GSK3β(pY216) protein level depending on brain structure. This herbicide caused a significant decrease of GSK3β in striatum with a simultaneous increase of this protein in midbrain. The prevalence of two GSK3β forms in different cell compartments is another very interesting phenomenon. PQ significantly decreased the total levels of GSK3β in isolated crude nuclear (P1), mitochondrial (P2), and cytosolic fractions (S2) in the striatum. The content of the active form of GSK3β (pY216) dropped significantly in the mitochondrial and cytosolic fractions and a trend in the same direction in the nuclear fraction was noted. On the other hand, paraquat significantly increased the total level of GSK3β immunoreactivity in the nuclear,

mitochondrial, and cytosolic fractions of the midbrain with pons. The level of the GSK3β (pY216) was also significantly elevated in all the above-mentioned fractions of this region.

It is likely that reduced levels of GSK3β in the striatum is associated with the impaired axonal transport of this protein from midbrain to striatum and results in increased accumulation of GSK3β in the midbrain. The increase in GSK3β protein level in midbrain by chronic PQ administration may cause excessive phosphorylation of proteins, that are associated with microtubules leading to the destabilization of cytoskeleton [Jope and Johnson, 2004]. However, the direct effect of PQ on striatal neurons cannot be excluded.

On the other hand aging process itself may induce changes in GSK3β level in the brain. I have observed selective reduction in the level GSK3β and its active form in the striatum of old rats comapring to young mature animals.

The *in vitro* data conducted in dopaminergic PC12 cells confirmed PQ toxicity. It was found that 24 hours of incubation with 1 mM PQ resulted in the cell death of approximately 50% of the cell population. In this study we also found a significant reduction in total GSK3β and phosphorylated GSK3β(pY216) immunoreactivity. The decrease of the active form of the enzyme may be a defensive mechanism against toxic effects of PQ. At the same time PQ had no effect on GSK3β(pS9) level. The results also indicated that the cell death induced by PQ is caused by an increase of free radicals level.

In my studies I have also investigated whether GSK3β phosphorylation and intracellular localization are affected by the cytotoxicity of Aβ. The enhancement of endogenous Aβ peptides release resulted in increased level of GSK3β(pS9) in APPsw cells, however it did not affect the levels of GSK3β and GSK3β(pY216) immunoreactivity. To emphasize the relationship between Aβ and enhancement of GSK3β activity I examined GSK3β-dependent tau phosphorylation on Ser396 in APPsw cells. It was found that Aβ-induced increase in GSK3β activity resulted in the enhancement of phosphorylated tau protein level. These may be responsible for cytoskeletal dysfunction in APPsw cells compared to control PC12 cells.