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Differences in glutathione S-transferase pi expression in transgenic mice with symptoms of neurodegeneration

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Glutathione S-transferase pi (GST pi) is an enzyme involved in cell protection against toxic electrophiles and products of oxidative stress. GST pi expression was studied in transgenic mice hybrids (B6-C3H) with symptoms of neurodegeneration harboring SOD1G93A (SOD1/+), *Dync1h1* (Cra1/+) and double (Cra1/SOD1) mutations, at presymptomatic and symptomatic stages (age 70, 140, 365 days) using RT-PCR and Western blotting. The main changes in GST pi expression were observed in mice with the SODG93A mutation. In SOD1/+ and Cra1/SOD1 transgenics, with the exception of cerebellum, the changes in GST pi-mRNA accompanied those in GST pi protein. In brain cortex of both groups the expression was unchanged at the presymptomatic (age 70 days) but was lower at the symptomatic stage (age 140 days) and at both stages in hippocampus and spinal cord of SOD1/+ but not of Cra1/SOD1 mice compared to age-matched wild-type controls. In cerebellum of the presymptomatic and the symptomatic SOD1/+ mice and presymptomatic Cra1/SOD1 mice, the GST pi-mRNA was drastically elevated but the protein level remained unchanged. In Cra1/+ transgenics there were no changes in GST pi expression in any CNS region both on the mRNA and on the protein level. It can be concluded that the SOD1G93A but not the *Dync1h1* mutation significantly decreases detoxification efficiency of GST pi in CNS, however the *Dync1h1* mutation reduces the effects caused by the SOD1G93A mutation. Despite similarities in neurological symptoms, the differences in GST pi expression between SOD1/+ and Cra1/+ transgenics indicate a distinct pathogenic entity of these two conditions.

Keywords: glutathione S-transferase pi, central nervous system, transgenic mice, SOD1G93A mutation, *Dync1h1* mutation, motor neuron disease

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INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a multigene family of isoenzymes classified as phase II detoxification enzymes. They catalyze the nucleophilic attack of the sulfur atom of glutathione (GSH) on the electrophilic groups of electrophilic substrates (Jakoby, 1978; Mannervik & Danielson, 1988). Because of their low substrate specificity, GSTs play a critical role in protection of cells from injury by toxic chemicals including pesticides, carcinogens, drugs and products of oxidative stress (Hayes & Pulford, 1995; Barańczyk-Kuźma *et al*., 2004; Tew, 1994). Based on biochemical properties, the cytosolic GSTs are divided into 8 subclasses named alpha, kappa, mu, pi, sigma, theta, zeta, and omega (Mannervik *et al*., 1992). GST pi is most prevalent in mammalian cells, present at high level in many cancerous tissues and overexpressed in drug-resistant tumors (Ruzza *et al*., 2009; Inoue *et al*., 1995; Cookson *et al*., 1997; Laborde, 2010). It is expressed in the Central Nervous System, and has been the only subclass implicated in protection of cells from ROS-inducing agents (Awasthi *et al*., 1994; Beiswanger *et al*., 1995; Baez *et al*., 1997; Tew & Ronai, 1999). Like other GSTs, the isoform pi is a carrier protein which non-enzymatically binds and transports a wide group of ligands within the cells, including hormones and neurotransmitters (Listowsky *et al*., 1988; Sawicki *et al*., 2000; Hayes & Strange, 1995). Human brain GST pi can also bind antidepressants and seems to be responsible for the resistance of epileptic patients to antiepileptic drugs (Barańczyk-Kuźma *et al*., 2004; Shung *et al*., 2008).

Most GST classes show a high degree of polymorphism (Mo *et al*., 2009). *GSTP* polymorphisms affect substrate selectivity and increase the susceptibility to Parkinsonism-inducing effects of environmental toxins and late onset of Alzheimer's disease (AD) (Hayes & Strange, 2000; Bernardini *et al*., 2005). Reduced GST activity was found in multiple brain regions and ventricular cerebrospinal fluid in short *postmortem* interval in AD patients (Lovell, 1998). In our previous studies, we have observed significant decrease in GST pi expression in spinal cord, motor (but not sensory) brain cortex, as well as in peripheral blood mononuclear cells of patients with sporadic amyotrophic lateral sclerosis (Amyotrophic Lateral Sclerosis/Motor Neuron Disease, ALS/MND) (Usarek *et a*l., 2005; Kuźma *et al*., 2006). ALS/MND is the most devastating condition among neurodegenerative diseases. It is characterized by progressive degeneration and loss of motor neurons, causing skeletal muscle wasting, paralysis and death (Rowland, 1995). In about 90% of cases the origin of the disease is unknown (sporadic ALS). The toxicity of environmental factors, excitotoxicity, deprivation of neurotrophic factors, defects of RNA processing and oxidative stress, have been implicated in neuronal degeneration. However, factors underlying the neuronal loss remain unclear (Al-Chalabi *et al*., 1995; Terro *et al*., 1996; Kuźma-Kozakiewicz & Kwieciński, 2011). In 5–10% of cases the disease is genetically determined (familiar ALS), and in one fifth of these cases

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Abbreviations: GST pi, glutathione S-transferase pi; SOD1, superoxide dismutase 1; *Dync1h1,* dynein, cytoplasmic 1, heavy chain 1; Cra1, cramping 1; CNS, central nervous system**;** GSH, reduced glutathione; AD, Alzheimer's disease; ALS/MND, amyotrophic lateral sclerosis/motor neuron disease; MAPs, microtubule associated proteins; Loa, legs at odd angles; TDP-43, 43-kDa transactivating responsive sequence DNA-binding protein.

it is linked to mutations in the *SOD1* gene (Mulder *et al.*, 1986). Transgenic mice that express human *SOD1* gene with the G93A mutation develop ALS-like symptoms and are used as an animal model for ALS/MND studies (Dal Canto & Gurney, 1995; Gurney *et al.*, 1994; Tuy *et al.*, 1996). However, the symptoms of neurodegeneration such as progressive locomotor disorders, early muscle weakness, hyperactivity were also observed in mice with missense point mutations in the heavy chains of cytoplasmic dynein (*Dync1h1* gene), one of the microtubule associated proteins (MAPs) (Hafezparast *et al.*, 2003). Due to abnormal body twisting and cramping of the hindlimbs, when suspended by tail, the mutations were called "Legs at odd angles" (Loa) and "Cramping 1" (Cra1). The phenotype of Cra1 mice is similar (but milder) to that of mice with the SOD1G93A mutation. The life span of Cra1 heterozygotes remains almost the same as that of wild-type animals, but in double heterozygotes (Loa/SOD1 and Cra1/SOD1) the disease progression is slower down and the life span increases slightly compared to SOD1G93A mice (Hafezparast *et al.*, 2003; Kieran *et al.*, 2005; Teuchert *et al.*, 2006). Mice with mutations in the *Dync1h1* gene were considered as a new, promising model of motor neuron degeneration (Hafezparast *et al.*, 2003; Teuchert *et al.*, 2006). Despite the fact that the Cra1 mice suffer from motor disturbances throughout their whole life, there are studies indicating that they display sensory neuropathy rather than motor neuron disease (Chen *et al.*, 2007; Dupuis *et al.*, 2009; Braunstein *et al.,* 2010).

Due to the controversy about the involvement of *Dync1h1* mutations in the pathogenesis of the motor neuron disease we studied GST pi expression in various parts of the central nervous system obtained from transgenic hybrid mice with human SOD1G93A mutation (SOD/+), with *Dync1h1* mutation (Cra1/+) and with double *Dync1h1*/SOD1G93A mutations (Cra1/SOD1).

MATERIALS AND METHODS

Animals. The studies were conducted on mouse strain C57BL/6GJ-C3H/HeJ (B6-C3H) hybrids harboring human SOD1G93A amyotrophic lateral sclerosis-associated mutation (SOD1/+), dynein heavy chain 1 mutation (*Dync1h1*; so-called Cra1/+), double *Dync1h1*/SOD1G93A mutation (Cra1/SOD1), and on genetic background-matched wild-type controls (+/+). The SOD1G93A males (B6 background; Jackson Laboratories, Barr Harbor, ME, USA) were crossed with Cra1/+ heterozygote female mice (C3He background; Ingenium Pharmaceuticals AG, Martinsried, Germany), as described earlier by Teuchert and coworkers (2006). There were 6 males in each studied group. Transgenic mice were at the presymptomatic stage (age 70 days for SOD1/+ and Cra1/+SOD1, 70 and 140 days for Cra1/+) and at the symptomatic stage (age 140 days for SOD1/+, Cra1/SOD1, and 365 days for Cra1/+ mice). GST pi expression was determined in frontal cortex, hippocampus, spinal cord and cerebellum by RT-PCR and Western blotting.

Methods. Total RNA was isolated with the use of the NucleoSpin® RNAII kit (Macherey-Nagel) according to manufacturer's protocol followed by reverse transcription, polymerase chain reaction and DNA electrophoresis in agarose gel. Specific oligonucleotide primers for mice GST pi were as follows: 5'AGCCCACTTGTCTGTATGGG3' and 5'CAGGGCCTTCACGTAGTCAT3'. The GST pi-mRNA level was expressed as the ratio of the optical density of GST pi band to the optical density of 40 S ribosomal S12 protein RNA (housekeeping gene). Each assay was performed in duplicates and repeated 2 times.

Western blotting was performed after electrophoresis in 12% SDS-polyacrylamide gel according to Laemmli (1970), with rabbit anti-GST-pi polyclonal antibodies (Novocastra). GST pi from human placenta was used as a standard (Sigma). Blots were visualized using ECL plus Western Blotting Detection System (Amersham). The assay was repeated 3–6 times for each tissue. Actin (monoclonal Anti-beta-Actin, Sigma) was used as a protein loading control. System UVI-KS4000, Syngen Biotechnology was used for densitometric analysis of RT-PCR and Western blotting. Results were expressed as means ± S.D. and the data were analyzed by the two-way analysis of variance (ANOVA). Quantitative comparison between studied groups was performed by Student's t-test using Statistica 9.0 (StatSoft). Means were considered statistically significant at $p<0.05$.

The animal use was were approved by the Ethic Committee for Experiments on Animals at the Medical University of Warsaw, Poland (permission No. AOKEZ/622/3/2008).

RESULTS

GST pi-mRNA expression

GST pi-mRNA expression in frontal brain cortex of 70-day-old wild-type (+/+) hybrid mice accounted for 0.97 ± 0.2 and was similar as in spinal cord (0.89 ± 0.09), but significantly ($p < 0.0001$) higher than in hippocampus and cerebellum (0.68 ± 0.04 and 0.64 ± 0.14, respectively). In older mice, aged 140 and 365 days, the expression in brain cortex, hippocampus and spinal cord remained at the same level as in the younger group (aged 70 days). Only in cerebellum of 365 day-old mice it was almost 2-fold higher than in younger groups (1.15 ± 0.06, $p < 0.0001$) (Fig. 1). In brain cortex of SOD1/+ transgenics aged 70 days (presymptomatic stage) GST pi-mRNA expression did not differ from that in wild-type mice (0.97 ± 0.04), but in the 140-day-old group (symptomatic stage) it was decreased to 0.68 ± 0.15 (30% decrease, $p < 0.0001$) (Fig. 1). In hippocampus of each age group it was lower than in the age-matched control and accounted for 0.59 ± 0.05 ($p < 0.0001$) and 0.56 ± 0.06 ($p < 0.05$) in 70- and 140-day-old transgenics, respectively (Fig. 1). In spinal cord, GST pi-mRNA expression was significantly decreased in both age groups with $p < 0.001$ for the younger and $p < 0.05$ for the older one (Fig. 1). Differently than in other regions of CNS, in cerebellum of SOD1/+ mice GST pi expression was significantly higher at both the presymptomatic and symptomatic stages reaching about 150% and 180% of the age-matched controls, respectively (0.95 ± 0.1 in 70-day-old mice and 1.18 ± 0.15 in 140-day-old mice ($p < 0.001$) (Fig. 1). In Cra1/+ transgenics, GST pi-mRNA expression was unchanged in all investigated tissues at any studied age compared to the age-matched controls (Fig. 1). In Cra1/SOD1 double hybrids, GST pi-mRNA expression was changed similarly as in the SOD1/+ groups but only in brain cortex of the symptomatic and cerebellum of the presymptomatic mice. In brain cortex of 140-day-old hybrids it accounted for 0.67 ± 0.19 ($p < 0.0001$) and was about 30% lower than in the wild-type control, and in cerebellum it accounted for 0.91 ± 0.05 ($p < 0.0001$) and

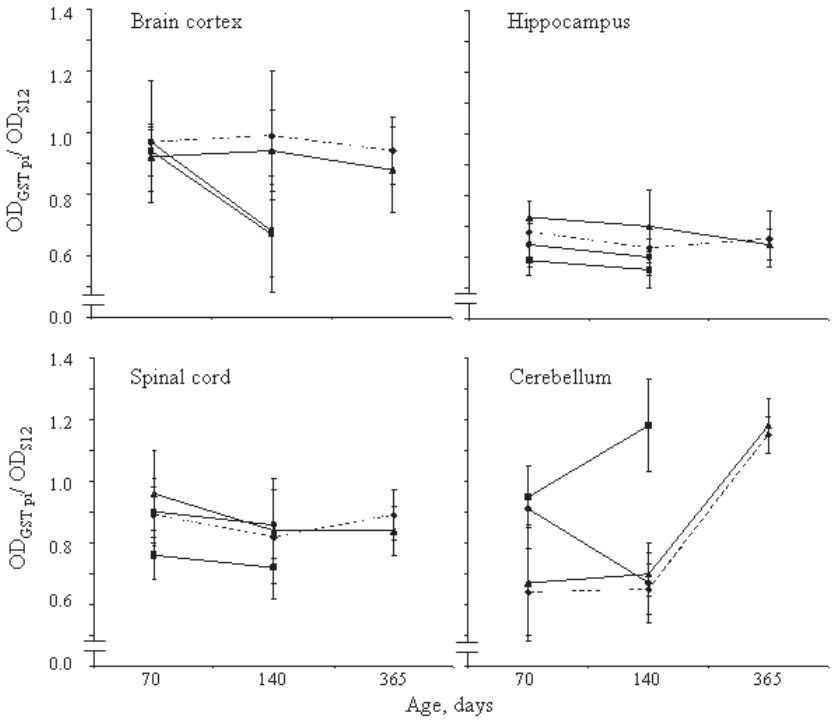

Figure 1. Age- and clinical stage-related GST pi-mRNA expression in CNS of transgenic mice.
GST pi-mRNA assessment was performed by RT-PCR and expressed as the ratio of optical density band of GST pi band to optical density of the 40 S ribosomal S12 protein RNA, as indicated in Materials and methods. ◆, dashed lines, wild-type controls (+/+); ■, SOD1/+ transgenics; ●, Cra1/SOD1 hybrides; ▲, Cra1/+ transgenics.

Table 1. Mean level of GST pi protein in various parts of CNS.

Part of CNS	GST pi protein in mice aged 70 days (ng/μg of total protein)			
	Wild-type (+/+)	SOD1/+	Cra1/+	Cra1/SOD1
Brain cortex	10.0 ± 3.7	11.5 ± 4.2	10.1 ± 2.2	8.3 ± 1.7
Hippocampus	6.6 ± 0.9	4.2 ± 1.0	6.1 ± 1.2	6.6 ± 2.2
Spinal cord	12.7 ± 4.9	9.4 ± 4.4	11.1 ± 3.3	12.9 ± 3.2
Cerebellum	8.6 ± 2.7	9.5 ± 3.9	8.8 ± 2.6	9.4 ± 3.3
Part of CNS	GST pi protein in mice aged 140 days (ng/μg of total protein)			
	Wild-type (+/+)	SOD1/+	Cra1/+	Cra1/SOD1
Brain cortex	10.5 ± 4.0	6.3 ± 1.4	10.0 ± 3.8	6.6 ± 3.0
Hippocampus	6.3 ± 1.0	4.3 ± 0.3	6.1 ± 0.8	6.0 ± 0.8
Spinal cord	10.2 ± 3.2	7.8 ± 3.6	10.5 ± 3.4	8.4 ± 2.6
Cerebellum	9.1 ± 2.9 13.3 ± 1.5*	10.2 ± 3.6 –	8.1 ± 2.4 13.2 ± 2.0*	8.3 ± 3.6 –

GST pi protein assessment was performed by Western blotting, as indicated in Materials and methods section. Each value is the mean ± S.D. from 3–6 experiments.* values for mice age 365 days.

was about 40% higher than in the age-matched control (Fig. 1). ANOVA analysis showed that GST pi-mRNA expression in brain cortex and cerebellum was dependent on the mutation and the age/clinical stage with F (4.12)=5.96, p=0.0002 and F (4.80)=11.2, p=0.00001, respectively. In hippocampus and spinal cord, statistical difference was only found between the GST pi-mRNA expression and the clinical stage (F=3.95, p<0.05 and F=11.02, p<0.001, respectively). The results also indicated that GST pi-mRNA expression was greatly related to the SOD1G93A but not *Dync1h1* mutation. In mice with double mutation (Cra1/SOD1), the expression in brain cortex was more like in SOD1/+ mice but in hippocampus, spinal cord and cerebellum (symptomatic stage) it was like in Cra1/+ mice.

GST pi protein expression

Mean values of GST pi protein expression are shown in Table 1. In wild-type mice aged 70 and 140 days, the level of GST pi protein in brain cortex, spinal cord and cerebellum was similar (close to 10 ng/μg of the total protein), but it was lower in hippocampus (about 6 ng/μg of the total protein) (Table 1). In cerebellum of older mice (age 365 days) it was almost 2-fold higher than in the younger groups and accounted for 13.50 ± 1.54 ng/μg of the total protein (Table 1). In SOD1/+ and Cra1/SOD1 transgenic mice, with the exception of cerebellum, the profile of GST pi protein accompanied that of GST pi-mRNA. In brain cortex of both groups it was unchanged at the presymptomatic (age 70 days) but was lower at the symptomatic stage (age 140 days) (Fig. 2A, B), and in hippocampus and spinal cord of SOD1/+ but not Cra1/SOD1 mice, at both stages when compared to age-matched wild-type controls (Table 1). In cerebellum of 70- and 140-day-old SOD1/+ and Cra1/SOD1 mice, GST pi protein was at the same level as in the control groups (Table 1, Fig. 2C, D). In Cra1/+ transgenics the expres-

sion was unchanged in all studied tissues, and in all age-groups (Table 1, Fig. 2).

DISCUSSION

Till now, the exact mechanisms underlying sporadic ALS/MND are not known, but also it is not clear how mutations in the *SOD1* gene contribute to pathogenesis of the disease (Kuźma-Kozakiewicz & Kwieciński, 2011). In our previous studies we have observed a significant decrease in glutathione S-transferase activity in peripheral blood mononuclear cells of patients with sporadic ALS (Kuźma *et al*., 2006). The decrease in activity was accompanied by the decrease in GST pi expression at both mRNA and protein levels. Since we also observed a decrease in GST pi expression in spinal cord and motor cortex of ALS cases, we concluded that the protective barrier formed by this enzyme was originally affected in sporadic ALS/MND. Thus, the subjects would be more sensitive to the toxicity of electrophilic compounds and organic peroxides (Usarek *et al*., 2005; Kuźma *et al*., 2006). In the present studies we observed similar changes in GST pi expression in brain cortex and spinal cord (about 30 and 15% decrease, respectively) obtained from mice harboring human SOD1G93A mutation (animal model for familial ALS). The decrease that we observed in SOD1/+ mice appeared in brain cortex at the symptomatic stage – age 140 days (not at the presymptomatic — age 70 days) and was more pronounced than in spinal cord, where it appeared earlier (prior to symptoms) but was independent of age and clinical stage. When we expanded our studies over the regions of CNS believed not to be affected in ALS/MND, we found a slight decrease in GST pi expression in hippocampus (on both mRNA and protein levels), and a high increase in GST pi-mRNA in cerebellum. GST pi-mRNA expression in cerebellum escalated with age/stage reaching about 150 and 180% compared to the age-matched controls. However, the changes in GST pi-mRNA expression were not accompanied by the changes in GST pi protein. Recently, studying tau expression in cerebellum of hybrid mice with human SODG93A mutation at presymptomatic and symptomatic stages, we observed a dramatic increase in the level of total tau-mRNA, in tau 0N, 1N, 2N isoform-mRNA but not in the tau protein (Kuźma-Kozakiewicz *et al*., 2011). Cerebellum is the structure usually not studied in motor neuron diseases, since cerebellar dysfunction is not a clinical feature of ALS. However, it is possible that gait disturbance observed at early stages of ALS, and usually explained by the instability of paretic muscles, may result from the primary involvement of cerebellum. Moreover, there are studies that indicate the disturbances in the expression of various proteins in this structure. The pathological 43-kDa transactivating responsive sequence DNA-binding protein (TDP-43) was identified as the major disease protein in many areas of the central nervous systems of ALS patients, including cerebellum (Geser *et al*., 2008). Chung and coworkers (2005, 2008) detected the presence of astrocytes immunoreactive to phosphorylated extracellular signal-regulated kinases and glycogen synthase kinase 3alpha involved in anti-apoptotic signal transduction pathways in spinal cord, brainstem, central gray and cerebellar nuclei of symptomatic SODG93A mice. Therefore, more studies are needed to explain why the increase in GST pi-mRNA is not accompanied by an increase in protein.

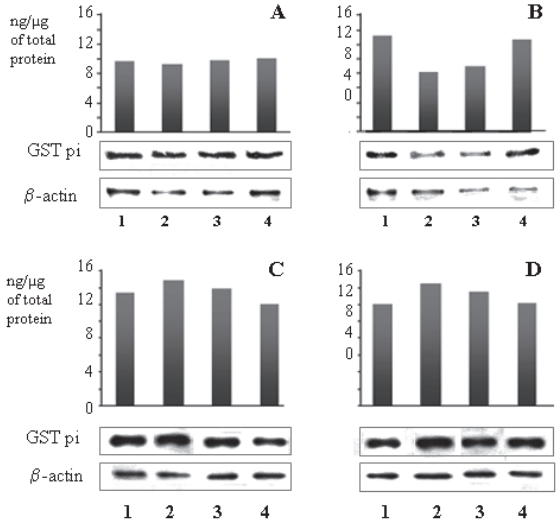

Figure 2. GST pi protein level in brain cortex and cerebellum. Protein was determined by Western blotting, as indicated in Materials and methods. Comparable amounts of protein (6 µg) were run in each lane. A and B, GST pi in brain cortex of mice aged 70 and 140 days, respectively; C and D, GST pi in cerebellum of mice aged 70 and 140 days, respectively. GST pi (100ng) from human placenta was used as a standard and β-actin as a protein loading control. A: Lane 1, wild-type (OD 212), lane 2, SOD1/+ (OD 191), lane 3, Cra1/SOD1 (OD 238), lane 4, Cra1/+ (OD 246); B: Lane 1, wild-type (OD 257), lane 2, SOD1/+ (OD 117), lane 3, Cra1/SOD1 (OD 129), lane 4, Cra1/+ (OD 244); C: Lane 1, wild-type (OD 252), lane 2, SOD1/+ (OD 279), lane 3, Cra1/SOD1 (OD 261), lane 4, Cra1/+ (OD 227); D: Lane 1, wild-type (OD 220), lane 2, SOD1/+ (OD 226), lane 3, Cra1/SOD1 (OD 249), lane 4, Cra1/+ (OD 224).

Intracellular transport along microtubules is especially important in motor neurons, the axons of which may reach the length of over one meter (El-Kadi *et al*., 2007). The defects in the retrograde axonal transport appear in motor neurons of SOD1G93A mice very early in the embryonic development (Kieran *et al*., 2005). An impaired retrograde axonal transport was also observed in genetic defects of the dynein complex, one of the microtubule associated proteins (MAPs) (Gibbons, 2005; Goldstein & Yang, 2000). The cytoplasmic dynein complex is formed by two identical heavy chains, a few intermediate, and several light chains (Samso *et al*., 1988). Heavy chains are encoded by a 78-exon gene, *Dync1h1*. They bind ATP, a source of energy for dynein movement along microtubules. Knocking out *Dync1h1* results in early embryonic lethality in mice (Harada *et al*., 1998). Mice with autosomal dominant missense point mutations in the *Dync1h1* gene show a progressive limb paresis. In Cra1 mice, the mutation (Cra1: 1051VWLQCQCLW1059; wild type: 1051VWLQYQCLW1059) within dynein heavy chains leads to the exchange of tyrosine by cysteine and subsequently to an age-related progressive degeneration of the motor neurons. On the molecular level Cra1 mice show defects in the dynein complex resulting in an impaired retrograde axonal transport (Hafezparast *et al*., 2003; Hrabe de Angelis *et al*., 2000; LaMonte *et al*., 2002). However, crossing SOD1G93A mice with Loa or Cra1 delays the disease progression and significantly increases the life span of SOD1 double heterozygotes (Loa/SOD1 and Cra1/SOD1) (Kieran *et al*., 2005). Thus, mutations in the *Dync1h1* gene appear to influence the progression of motor neuron degeneration and SOD1G93A-toxicity (Kieran *et al*., 2005; Teuchert *et al*., 2006). In our studies no changes in GST pi expression were observed either on the mRNA or on protein level in any studied part of CNS in Cra1/+ mice. In Cra1/SOD1 groups, GST pi expression was changed only in brain cortex, where

it was decreased identically as in the SOD1/+ symptomatic group.

Our results indicated that the protective barrier formed by GST pi is affected in CNS of mice with the SOD1G93A but not with the *Dync1h1* mutation. This may expose the brain and spinal cord to the toxicity of uncompletely inactivated toxic compounds including organic peroxides, and subsequently contribute to neurodegeneration. Despite similarities in neurological symptoms, the differences in GST pi expression between SOD1/+ and Cra1/+ transgenics indicate a distinct pathogenic entity of these two conditions. However, a mutation in the *Dync1h1* gene that impairs the physiological function of the dynein complex, seems to be beneficial for GST pi expression in SOD1-related ALS/MND. This is the first report concerning GST pi expression in various CNS regions of mice with the G93A mutation in human SOD1, mice with a mutation in the heavy chain of cytoplasmic dynein (*Dync1h1* gene), and mice with the double SOD1G93A/*Dync1h1* mutations. More studies are needed to explain the molecular mechanism of these findings.

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