Cerebellar Hypoplasia in Mice Lacking Selenoprotein Biosynthesis in Neurons

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Abstract Selenium exerts many, if not most, of its physiological functions as a selenocysteine moiety in proteins. Selenoproteins are involved in many biochemical processes including regulation of cellular redox state, calcium homeostasis, protein biosynthesis, and degradation. A neurodevelopmental syndrome called progressive cerebellocortical atrophy (PCCA) is caused by mutations in the selenocysteine synthase gene, SEPSECS, demonstrating that selenoproteins are essential for human brain development. While we have shown that selenoproteins are required for correct hippocampal and cortical interneuron development, little is known about the functions of selenoproteins in the cerebellum. Therefore, we have abrogated neuronal selenoprotein biosynthesis by conditional deletion of the gene encoding selenocysteyl tRNA[^Ser][^Sec] (gene symbol Trsp).

Enzymatic activity of cellular glutathione peroxidase and cytosolic thioredoxin reductase is reduced in cerebellar extracts from Trsp-mutant mice. These mice grow slowly and fail to gain postural control or to coordinate their movements. Histological analysis reveals marked cerebellar hypoplasia, associated with Purkinje cell death and decreased granule cell proliferation. Purkinje cell death occurs along parasagittal stripes as observed in other models of Purkinje cell loss. Neuron-specific inactivation of glutathione peroxidase 4 (Gpx4) used the same Cre driver phenocopies tRNA[^Ser][^Sec] mutants in several aspects: cerebellar hypoplasia, stripe-like Purkinje cell loss, and reduced granule cell proliferation. Parvalbumin-expressing GABAergic interneurons (stellate and/or basket cells) are virtually absent in tRNA[^Ser][^Sec]-mutant mice, while some remained in Gpx4-mutant mice. Our data show that selenoproteins are specifically required in postmitotic neurons of the developing cerebellum, thus providing a rational explanation for cerebellar hypoplasia as occurring in PCCA patients.

Keywords Selenium · Brain · Gpx4 · Cell death · Proliferation

Introduction

Selenoproteins are proteins, which specifically have the rare amino acid, selenocysteine (Sec), incorporated in response to UGA codons in their mRNAs. The mouse and human genomes contain 24 and 25 genes encoding selenoproteins [1]. The mouse serves as a good model organism for mammalian brain development, and manipulation of its genome allows for easy studies of the physiological roles of selenoproteins. Most selenoproteins are expressed in the brain, particularly in neurons of the olfactory bulb, cortex, hippocampus, and cerebellum [2]. While dietary selenium (Se) deficiency is not known
to induce direct neurological disease or neurodevelopmental delay in mammals [3], inactivation of the plasma Se transport protein, selenoprotein P (SePP), does lead to a neurological disorder which is caused by reduced brain Se content and selenoenzyme activity [4, 5]. Similarly, inactivation of apolipoprotein E receptor 2 (ApoER2, Lrp8), which serves as a SePP receptor in the brain, mimics the phenotype of SePP-KO mice [6, 7].

Selenoprotein biosynthesis relies entirely on the presence of tRNA^{[Ser]Sec} because Sec is synthetized attached to its tRNA, and this tRNA is also required for Sec incorporation by the ribosome [8]. Previously, we took an advantage of a tRNA, and this tRNA is also required for Sec incorporation. Phenotypes observed through the loss of tRNA^{Ser}Sec [12, 14].

Reduced forebrain Gpx4 protein by at least 50 % and was produced forebrain Gpx4 protein by at least 50 % and was sufficient to disrupt interneuron development and mimic most neuronal expression of many selenoproteins. An interesting observation was the specific developmental defect of a certain subgroup of GABAAergic interneurons [12], while Se and vitamin E deficiency in vitro also affected principal, glutamatergic neurons [13]. Neuron-specific deletion of vitamin E receptor 2 (ApoER2, Lrp8), which serves as a SePP receptor in the brain, mimics the phenotype of SePP-KO mice [6, 7].

Immunohistochemistry Brains from mouse pups were immediately fixed after dissection in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, or the mice were perfused transcardially with the same fixative after a washing step with 0.1 M PB. After postfixation at 4 °C over night, brains were cryoprotected in 30 % sucrose in 0.1 M PB for 2 days and frozen at −80 °C. Sections were cut on a cryostat at 20–50 μm. Free floating sections were stained with the indicated antibodies at dilutions of 1:1,000–1:5,000 at 4 °C over night. Polyclonal rabbit α-calbindin, monoclonal mouse α-calbindin, and rabbit α-parvalbumin antibodies were from Swant, Bellinzona, Switzerland, guinea pig monoclonal α-GLAST was from Chemicon, and mouse monoclonal α-glial fibrillary acidic protein (GFAP) antibody was from Sigma. Pre-absorbed secondary Cy2- and Cy3-conjugated antibodies were from Jackson ImmunoResearch. Horseradish peroxidase and diaminobezidine or NovaRed substrate were used in conjunction with the Vectastain ABC kit (Vector, Burlingame, VT). Whole-mount immunohistochemistry was performed as described [19]. Photomicrographs were taken using a Zeiss Axioskop II equipped with AxioCam MRc and operated with AxioVision software. Other photomicrographs were taken using a Leica confocal microscope.

Methods

Animals Mice were maintained according to local regulations as described for the SePP-deficient mice generated in our laboratory [5]. All animal experiments were approved by the local authorities in Berlin and Munich. Conditional Trsp knockout mice (Trspfl/fl) have been described [9]. These mice were crossed with transgenic mice expressing Cre recombinase under control of the tubulinα1 promoter [18], yielding mice deficient in neuronal selenoprotein biosynthesis, Tα1-Cre/Trspfl/fl. Mutant mice and littermate controls were analyzed between P3 (not shown, no defects observed) and P15 (histology).

Enzyme Assays Cerebella were freshly dissected from post-natal mice and immediately frozen on dry ice. Brain tissue was powdered under liquid nitrogen using a dismembrator (Braun Melsungen), and aliquots were homogenized in buffer as described [5]. Activities of selenoenzymes Gpx and Txnrd were measured in cytosolic cerebellar homogenates as described with tBuOOH and bisdithionitrobenzene (DTNB) as substrates, respectively [5].

Terminal UTP-Nick End Labeling and Phosphorylated Histone H3 Assay Terminal UTP-nick end labeling (TUNEL) immunohistochemistry was performed on serial 5-μm paraffin sections as described [20]. Using AxioVision software, TUNEL + cells were counted on at least 10 fields (0.35 mm²) for each genotype. Phosphorylated histone H3 (pH3) + cells were counted in serial sections and the numbers expressed per 1-mm external germinal layer (egl). For each measurement, at least 10 sections from at least three animals per genotype were evaluated. Antibodies against phosphorylated (Ser10) histone H3 were from Cell Signalling.

Nissl Staining Cresyl violet staining was performed on frozen 20-μm sections as described recently [21] and combined with α-calbindin immunostaining.

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Results

Neuron-Specific Disruption of Selenoprotein Biosynthesis Reduces Selenoenzyme Activity

Selenoprotein expression was specifically abrogated in neurons by conditional inactivation of tRNA[^Ser]^Sec (Trsp) based on neuron-specific expression of Cre recombinase (Fig. 1a). Neuron-specific Trsp-knockout mice (Ta1-Cre/Trsp^fl/fl) failed to gain weight after the first week of life and rarely survived beyond P12 (Fig. 1b). Since the knockout animals did not gain postural control, we suspected a cerebellar defect and determined selenoprotein activities in cytosolic extracts of cerebella on P9. Glutathione peroxidase activity was reduced by 30% (p<0.01, Student’s t test; Fig. 1c), and cytosolic thioredoxin reductase was reduced by 60% (p>0.001; Student’s t test; Fig. 1d). Remaining selenoenzyme activities are likely derived from nonneuronal cells like glia and endothelial cells, which are not targeted by our approach as previously observed in the cerebral cortex [12].

Cerebellar Hypoplasia and Stripe-Like Loss of Purkinje Cells

The most striking anatomical phenotype of Ta1-Cre/Trsp^fl/fl mice was marked as cerebellar hypoplasia associated with immature foliation (Fig. 2(a)). Staining for Purkinje cells with antibodies against either calbindin or parvalbumin showed a significant loss of Purkinje cells in most folia on P7 and later stages. No Purkinje cell loss or size difference was apparent on P3 (not shown). Purkinje cell dendrites were clearly underdeveloped in Ta1-Cre/Trsp^fl/fl cerebella on P12 (Fig. 2a, insets), and occasionally, Purkinje cells were ectopically distributed within deep layers of the white matter. We noted that the degree of Purkinje cell loss varied among parasagittal sections from the same animal. Since Purkinje cell death is known to occur along parasagittal stripes in several models of Purkinje cell degeneration [22], we performed whole-mount immunohistochemistry using calbindin as a marker for Purkinje cells. Calbindin staining revealed a stripe-like pattern of Purkinje cell loss (Fig. 2(b)) that was readily apparent in a camera lucida representation (Fig. 2(c)). In order to assess whether Purkinje cell loss coincided with the pattern of expression of zebrin II/aldolase C, we stained cerebellar sections with calbindin and zebrin II antibodies (Fig. 2(d)). In the primary fissure, surviving Purkinje cells were zebrin II + on P11.

Cell Proliferation and Cell Death

TUNEL staining demonstrated that cell death was significantly increased in Ta1-Cre/Trsp^fl/fl cerebella on P6 and P8. Increased TUNEL labeling was not limited to the Purkinje cell layer but was also found in the external germinal layer and internal granule cell layer (Fig. 2(e)). The massive increase in size of the cerebellum during postnatal development is largely caused by the proliferation of granule cell precursors in the secondary neuroepithelium of the external germinal layer. In

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Fig. 1 Neuron-specific inactivation of selenoprotein expression. a Schematic drawing shows dependence of selenoprotein synthesis on selenocysteine tRNA in selenocysteine tRNA. b Body weight in postnatal Ta1-Cre/Trsp^fl/fl mice and controls. c Cytosolic glutathione peroxidase activity was determined with tert-butyl hydroperoxide as a substrate in P9 cerebella. d Cytosolic thioredoxin reductase activity was determined with the DTNB assay in P9 cerebella. n=5–7, **p<0.01; ***p<0.001, Student’s t test
Inactivation of Gpx4 Recapitulates the Cerebellar Phenotype

The strong cerebellar phenotype of Tg1-Cre/Trspfl/fl mice directly prompted the question whether a single selenoprotein could be identified which is responsible for the observed cell death and impaired cell proliferation. We therefore crossed Tg1-Cre with conditional glutathione peroxidase 4 (Gpx4fl/fl) mice. These mice showed the same growth phenotype as Tg1-Cre/Trspfl/fl mice and did not survive beyond P13. More interestingly, Tg1-Cre/Gpx4fl/fl mice phenocopied the cerebellar phenotype with respect to cerebellar hypoplasia and stunted growth of Purkinje cell dendrites or Purkinje cell loss (Fig. 3a). Purkinje cell loss again occurred along parasagittal stripes in Tg1-Cre/Gpx4fl/fl mice (Fig. 3b). Since parvalbumin-positive GABAergic interneurons were the most affected cortical neurons, we asked whether interneurons in the cerebellum are also affected in our mutants. Purkinje cells (which are also GABAergic) express both calbindin and parvalbumin. Co-labeling of calbindin (red) and parvalbumin (green) in fluorescence microscopy therefore allows visualizing parvalbumin-positive interneurons (green) on a yellow background of Purkinje cells. As shown in Fig. 3c, parvalbumin-positive interneurons, presumably basket and/or stellate cells, are completely absent in Tg1-Cre/Trspfl/fl mice, while they partly remain in Tg1-Cre/Gpx4fl/fl mice.

Bergmann Glia

Staining of radial glia with GFAP as a marker did not reveal a reduction of Bergmann fiber density or radial glia morphology. Even in areas where Purkinje cell dendrites have...
degenerated, there is no indication of structural changes of Bergmann glial fibers (Fig. 4).

Discussion

Initial observations in SePP−/− mice and later in Apoer2−/− mice established that selenoprotein expression in the mammalian brain is essential [4–7, 23–25]. Among the neurological phenotypes of affected mice were epilepsy/hyperexcitability and movement disorders. However, initial studies of SePP−/− mice did not demonstrate a clear pathology of the cerebellum, despite an abnormal gait of the mice [21, 23, 24]. In order to elicit the maximal phenotype associated with a lack of selenoprotein expression, we abrogated selenoprotein biosynthesis by inactivating Trsp, specifically in neurons using pan-neuronal Tα1-Cre (including cerebellum) and CamK-Cre (forebrain-specific, excluding cerebellum). In the cerebral cortex and hippocampus, parvalbumin-expressing interneurons did not develop at all, while parvalbumin-expressing cells in the globus pallidus were still detectable [12]. Most importantly, neuron-specific CamK-Cre/Trspfl/fl mice exhibited hyperexcitability. Here, we describe that Tα1-Cre/Trspfl/fl mice, in which cerebellar neurons are affected, show cerebellar hypoplasia. Epilepsy and cerebellar hypoplasia are among the phenotypes of children afflicted with mutations in PCCA due to mutations in the SEPSECS gene [16]. Therefore, the essential role of selenoproteins in the mammalian brain can now be considered firmly established.

What could be the specific roles of selenoproteins in the brain? We show here that inactivation of Gpx4 alone already mimics a loss of all selenoproteins when cerebellar hypoplasia and Purkinje cell death are chosen as parameters. This finding is reminiscent of findings in forebrain interneurons and cultured cortical neurons (mostly glutamatergic neurons) [12]. However, when comparing Tα1-Cre/Trspfl/fl mice and Tα1-Cre/Gpx4fl/fl mice, it was noticed that loss of Gpx4 yields a slightly milder phenotype, indicating that besides Gpx4, a yet to be identified selenoprotein may contribute to the more severe pathology as observed in Tα1-Cre/Trspfl/fl mice.

Thioredoxin reductases are essential selenoproteins, but their inactivation in neurons using Tα1-Cre was shown not to cause a cerebellar phenotype [15].

Cerebellar development represents a special paradigm since a secondary proliferating neuroepithelium is established in the course of its development. The first neurons to develop in the primary neuroepithelium of the cerebellar primordium are principal neurons of the deep nuclei and, most importantly, the Purkinje cells [26]. In a second step, neuronal precursors migrate through the Purkinje cell layer and form the external germinal layer where the precursors proliferate postnatally. The newly developed neurons, granule cells, then migrate along Bergmann glia processes inward through the Purkinje...
cell layer and establish the internal granule cell layer. Cerebellar hypoplasia is usually associated with a failure of the secondary neuroepithelium to produce the huge numbers of granule cells, which make up most of the cerebellar volume. Finally, GABAergic interneuron precursors are born in the primary neuroepithelium and migrate outwards where basket cells mature close to Purkinje cells and stellate cells migrate into the molecular layer and reside among the Purkinje cell dendrites. In our \( T\alpha1-Cre \)/Trspfl/fl mouse model, Purkinje cell death was observed at a stage when the Purkinje cell layer is already formed; hence, we find gaps in the layer. The \( T\alpha1-Cre \) transgene is only expressed in neurons, not neuronal precursors consistent with the loss of postmitotic Purkinje cells. Why Purkinje cell degeneration occurs often along parasagittal stripes (e.g., preferentially in aldolase C-negative cells in our model) is not known but may be associated with the functional patterning of the cerebellum [22]. Purkinje cells secrete along their dendrite factors stimulating granule cell proliferation, including sonic hedgehog and others [27]. A reduced number of Purkinje cells may thus entail reduced promitotic stimulation of granule cell precursors in the external germinal layer. Migration of granule cells along Bergmann fibers may occur normally, since we did not find evidence of radial glia defects. Targeting neural precursors and Bergmann fibers, nestin-Cre; Trnp1fl/fl mice also suffer cerebellar hypoplasia associated with defective foliation/migration disorganized Bergmann fibers [15]. This may bear some relevance in the case of global selenoprotein deficiency as in PCCA. Of note, our \( T\alpha1-Cre \) transgene targets only postmitotic neurons and not glia. We find, however, TUNEL-positive cells mostly in the internal granule cell layer, suggesting that death of granule cells contributes to the observed cerebellar hypoplasia. Whether granule cells degenerate in a cell autonomous process or because their synaptic targets, Purkinje cells, degenerate is not known. We observed that GABAergic interneurons, which express parvalbumin once they reach their destination in the cerebellar cortex, are virtually absent in \( T\alpha1-Cre \)/Trspfl/fl mice. Their numbers are also reduced in Gpx4 mutants, but occasionally, we observed few of these neurons in the Purkinje cell layer. It is interesting to note that the neurons most severely affected by the lack of selenoprotein expression (or Gpx4 alone) are GABAergic neurons that also express the calcium-binding protein parvalbumin: cortical interneurons, cerebellar interneurons, and Purkinje cells. Cortical GABAergic neurons expressing calbindin or calretinin are less affected [12]. However, neurons in the globus pallidus are also GABAergic and express parvalbumin and are not affected, or at least, they do not degenerate up to the latest time points we have studied them. Therefore, further studies are needed to define the mechanism of how neurons are damaged by the lack of selenoproteins. While “oxidative stress” is often invoked and massive oxidation of cell constituents is assumed, we speculate that specific developmental
mechanisms are targeted. Dysregulations of redox-sensitive pathways are our prime suspects for further study, because oxidative stress may not be very cell-specific at all. For example, signaling by receptor tyrosine kinases often entails transient oxidation of protein phosphatases. It has been shown that lipid hydroperoxides generated enzymatically by 12/15-lipoxygenase efficiently inactivate transient oxidation of protein phosphatases. It entails transient oxidation of protein phosphatases. It has been shown that lipid hydroperoxides generated enzymatically by 12/15-lipoxygenase efficiently inactivate transient oxidation of protein phosphatases. It has been shown that lipid hydroperoxides generated enzymatically by 12/15-lipoxygenase efficiently inactivate transient oxidation of protein phosphatases. It has been shown that lipid hydroperoxides generated enzymatically by 12/15-lipoxygenase efficiently inactivate transient oxidation of protein phosphatases.

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