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Thioredoxin and glutaredoxin system proteins—immunolocalization in the rat central nervous system ☆

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ABSTRACT

Background: The oxidoreductases of the thioredoxin (Trx) family of proteins play a major role in the cellular response to oxidative stress. Redox imbalance is a major feature of brain damage. For instance, neuronal damage and glial reaction induced by a hypoxic–ischemic episode is highly related to glutamate excitotoxicity, oxidative stress and mitochondrial dysfunction. Most animal models of hypoxia–ischemia in the central nervous system (CNS) use rats to study the mechanisms involved in neuronal cell death, however, no comprehensive study on the localization of the redox proteins in the rat CNS was available.
Methods: The aim of this work was to study the distribution of the following proteins of the thioredoxin and glutathione/glutaredoxin (Grx) systems in the rat CNS by immunohistochemistry: Trx1, Trx2, TrxR1, TrxR2, Txnip, Grx1, Grx2, Grx3, Grx5, and γ-GCS, peroxiredoxin 1 (Prx1), Prx2, Prx3, Prx4, Prx5, and Prx6. We have focused on areas most sensitive to a hypoxia–ischemic insult: Cerebellum, striatum, hippocampus, spinal cord, substantia nigra, cortex and retina.
Results and conclusions: Previous studies implied that these redox proteins may be distributed in most cell types and regions of the CNS. Here, we have observed several remarkable differences in both abundance and regional distribution that point to a complex interplay and crosstalk between the proteins of this family.
General significance: We think that these data might be helpful to reveal new insights into the role of thiol redox pathways in the pathogenesis of hypoxia–ischemia insults and other disorders of the CNS.
This article is part of a Special Issue entitled Human and Murine Redox Protein Atlases.

1. Introduction

The Thioredoxin family of proteins is a class of enzymes that utilize the thiol groups of cysteinyl residues for the catalysis of thiol–disulfide exchange and peroxidatic reactions. This family includes the thioredoxins (Trxs), glutaredoxins (Grxs), and peroxiredoxins (Prxs) [1–3], which are all characterized by a common structural motif known as the thioredoxin fold [4].

Trx was first described as hydrogen donor for ribonucleotide reductase from *Escherichia coli* [5]. However, during the previous decade these proteins were recognized as central regulators of cellular functions in the response to redox signals and stress, for instance by the modulation of various signaling pathways, transcription factors, and the immune response [3]. Although the Trx family includes more than ten proteins, the major Trx isoforms are the cytosolic Trx1 and the mitochondrial Trx2. Trxs are thought to be tightly connected to thioredoxin reductases (TrxR). TrxR is a NADPH-dependent homodimer with one FAD cofactor per subunit and a cysteine-selenocysteine active site which reduces the disulfide in the active site of oxidized Trx [6,7] In addition, TrxR1 can directly reduce a number of substrates, in particular lipid hydroperoxides, H_2O_2, dehydroascorbate and lipoic acid [8–11]. Another protein that is discussed to be a regulator of Trx function is Trx interacting protein (Txnip). This protein, originally identified as a vitamin D up-regulated protein 1 (VDUP1) in HL-60 cells treated with 1,25-dihydroxyvitamin D-42, binds to reduced Trx but not to oxidized Trx and is thought to be a negative regulator of Trx [12].

Glutathione (GSH) constitutes the major intracellular redox buffer in the cells, but also functions as an antioxidant in xenobiotic metabolism and is considered a site for cysteine storage [13]. GSH is synthesized in the cytosol in two steps. First, the enzyme γ-glutamylcysteine synthetase (γ-GCS) catalyzes the formation of L-γ-

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glutamyl-L-cysteine [14]. The glycine residue of the GSH tripeptide is added in the second step by glutathione synthetase. Cellular GSH exists predominantly in a reduced form, but small amounts of the oxidized disulfide form GSSG can also be detected. GSSG is reduced by glutathione reductase (GR) at the expense of NADPH. The GSH/GSSG ratio is often taken as an indicator of the cellular redox status. Changes in this ratio have been associated with the modulation of transcription of a wide variety of genes implied in multiple cellular processes such as growth, differentiation and cell death. As of today, four Grx isoforms have been found in mammals [15]. The cytosolic dithiol Grx1, the mainly mitochondrial Grx2 (Grx2a, testicular cells and some cancer cells express two additional cytosolic/nuclear isoforms of the protein, Grx2b and Grx2c derived from alternative transcription initiation and splicing), the cytosolic multidomain monothiol Grx3, and the mitochondrial monothiol Grx5. Unlike in the Trx system, electrons in the Grx system are transferred from NADPH-dependent glutathione reductase to glutathione (GSH) which, in turn, reduces oxidized glutaredoxin. Grx reduces both protein disulfides and mixed disulfides with glutathione using two or one cysteinyl residues in their active site, respectively [15].

Prxs are a heterogeneous family of thiol-dependent peroxidases present in all kingdoms of life. Peroxiredoxins execute enzymatic degradation of H_2O_2, organic hydroperoxides, and peroxynitrite [16]. Unlike Trxs that possess the active double cysteine region forming an intramolecular disulfide bond when oxidized, Prxs can form intermolecular disulfide bonds. By the number of active Cys residues, mammalian peroxiredoxins fall into three groups: typical two-cysteine Prxs (Prx1–Prx4), which contain both, N- and C-terminal Cys residues, atypical two-cysteine Prxs (Prx5) that contain only the N-terminal conserved Cys but require an additional Cys for their peroxidase activity, and single-cysteine Prxs (Prx6) that contain only the N-terminal Cys [17,18]. Prxs are present in all subcellular compartments; Prx1, Prx2 and Prx5 are found in the cytoplasm and nuclei, Prx4 and Prx6 in cytoplasm and secreted, and Prx3 and Prx5 in mitochondria [18]. In mammals, two Cys-Prxs are believed to use exclusively Trxs as cofactors, 1-Cys-Prxs require other electron donors, for instance GSH [16,17].

The brain is more susceptible to oxidative damage compared to most other organs because of its high oxygen utilization, high iron content, presence of unsaturated fatty acids, and decreased activities of detoxifying enzymes such as superoxide dismutase (SOD), catalase, and GR [19–22]. Molecular oxygen is the central component in energy production in mammalians and the drop in oxygen supply to neuronal tissue has serious consequences for cell fate and survival. Ischemia-reperfusion injury induces serious oxidative stress and, as consequence, a multitude of spatially and temporally regulated responses, ranging from changes in the gene expression pattern to biochemical alterations and, ultimately, cell death. The disturbance of redox homeostasis, low levels of GSH, and an increased production of reactive oxygen species (ROS) and peroxynitrite have been described for a number of CNS disorders, for instance perinatal asphyxia [22,23], stroke [24], focal traumatic brain injury [25], and numerous neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amythrophic lateral sclerosis [26,27].

Ischemic brain injuries, resulting either from global or focal decreases in perfusion, are among the most common and important causes of disability and death worldwide after heart infarction and cancer [28]. Many experimental models of hypoxia and ischemia have identified cerebellum, hippocampus, neostriatum, cortex, substantia nigra, spinal cord, and retina as vulnerable targets of oxidative stress [28,29]. The reason of this selectivity remains unclear. Rat models have been extensively used to study the ischemia/hypoxia-reperfusion insult. Trxs, Grxs, Prxs, and functionally connected proteins are involved in various steps during the oxidative stress response. Thus, the main objective of this paper was to systematically describe the cellular localization of these redox proteins in the central nervous system (CNS). The results presented here revealed some striking differences in the localization of these proteins that might be helpful to increase our understanding of the mechanisms involved in the pathogenesis and the differential vulnerability of different cells of the CNS following the ischemia/hypoxia-reperfusion insult.

2. Materials and methods

2.1. Animal experiments

One month old adult male rats ($n=4$), were anesthetized with 28% (w/v) chloral hydrate, 0.1 ml/100 g of body weight, and perfused with 4% paraformaldehyde in phosphate buffer 0.1 M, pH 7.4 through the abdominal aorta. Brains were dissected and post-fixed in the same solution during 2 hours, and then immersed overnight in phosphate buffer 0.1 M, pH 7.4 containing 5% sucrose. Coronal brain sections containing the striatum (40 μm thick) were cut on an Oxford vibratome and then recovered for light microscopic studies. Some sections were stained with cresyl violet according to the procedures described in [30]. For retina studies, we have enucleated the eye, dissected the retina, and embedded it in paraffin. Then, sections of 4 microns were cut and mounted on silanized slices. All animal experiments and care were approved by the Ethical Board of the School of Medicine, University of Buenos Aires, Buenos Aires, Argentina.

2.2. Antibodies

The following antibodies were purchased from commercial vendors: γ-GCS (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-22755, 1:100), Prx2 (Santa Cruz Biotechnology, sc-33572, 1:200) Prx4 (Abcam, Cambridge, UK, ab59542, 1:500), Prx6 (Abcam, ab59543, 1:500), TrxR2 (Santa Cruz Biotechnology, sc-67127, 1:200), Txnip (Abcam, sc-33098, 1:200). The following antibodies were produced in the Lillig laboratory, their generation and evaluation for mouse tissues was described in detail in the accompanying paper: "Redox atlas of the mouse", published in this issue of Biochimica et Biophysica Acta—General Subjects: Grx1 (1:200, human antigen), Grx2 (1:500, mouse antigen), Grx3 (1:25, affinity purified, mouse antigen), Grx5 (1:1000, human antigen), Prx1 (1:500, human antigen), Prx3 (1:500, human antigen), Prx5 (1:500, human antigen), Trx1 (1:1000, mouse antigen), Trx2 (1:1000, human antigen), and TrxR1 (1:100, affinity purified, rat antigen). The specificity of these antibodies for the rat antigens was confirmed by performing Western blots on extracts of the rat (pancreas-derived) cell line INS-1.

2.3. Immunocytochemistry

Brain sections were incubated overnight with the primary antibodies in the dilutions stated above. Following several wash steps with PBS, striatal sections were incubated with the proper secondary antibodies (Vector Laboratories Inc., Burlingame, CA, USA) and followed by incubation with a biotin streptavidin complex (HRP Histo Mark, Caramillo, CA USA). After washing in PBS, sections were developed with AEC substrate kit (Invitrogen Gaithersburg, MD, USA) until staining was optimal as examined by light microscopy using Leica Microscopes. Images were collected using a CCD video Camera CU-M50 (Sony Inc.). Pictures were analyzed and compiled using Adobe Photoshop 8.0 CS3.

3. Results

3.1. Cerebellum

All redox proteins analyzed in this study were detected in cerebellum, however, with differences in intensities between the different layers and cell types that are part of the cerebellar cortex. Purkinje neuronal cytoplasm and the apical dendrites were intensely labeled

only with Trx1, TrxR2, and Prx2 antibodies. TrxR2 immunoglobulins stained the apical dendrites and the several branches of the dendritic tree very consistently (Table 1, Fig. 1). In addition, Purkinje nuclei showed strong immunostaining with Grx1 and Grx2 antibodies (Table 1, Fig. 1). In the molecular layer, neurons and some axonal fibers were stained for Trx1, TrxR2, Prx1, Prx2, Prx3, and Prx4. Trx2, TrxR1, Txnip, Grx1 Grx2, Grx5, γ-GCS, Prx5, and Prx6 staining was weak (Table 1, Figs. 1 and 2). The granular layer showed consistent staining of neuronal bodies for Prx1, Prx2, Prx3, and Prx4. Staining for the other proteins resulted in only weak staining (Table 1, Fig. 2).

Although neuroglia staining was observed for many of the Trx and Grx system proteins (see Table 1, e.g. TrxR2, Grx1, Grx2, and Prx4), two of them yielded strong and specific staining patterns: Trx2 and Prx6 (Figs. 1 and 2). The morphologic characteristics of these cells suggested that they were astrocytes.

3.2. Hippocampus

We did not see clear differences in the immunostaining between the areas in which the hippocampus is classically divided (CA1, CA2, CA3, CA4, dentate gyrus). However, we did record some differences in cellular localization and immunostaining intensity. Txnip, Trx1, and Grx3 showed weak staining in the neuronal cell bodies (pyramidal and granular cells), apical dendrites, and neuroglia (Table 1; Figs. 3 and 4). The intensity of the immunostaining was more consistent for Trx2, TrxR1 (including the first portion of the apical dendrite), TrxR2, Grx2, Grx5, γ-GCS, Prx1, and Prx3 (Table 1; Figs. 3 and 4). The most intense staining was observed with Grx1, Prx2, Prx4, Prx5 and Prx6 immunoglobulins (Table 1; Figs. 3 and 4). Immunolabeling for these proteins was mainly located in the cell bodies of neurons, in particular in the apical dendrite. Moreover, robust staining was seen in neuroglia in sections stained for Grx1, Prx2, and Prx6. The morphologic characteristics of these cells suggest that they were astroglia.

3.3. Striatum

In striatum, positive staining was observed for all of the redox proteins, however, with broad variations in localization and intensities for both neuronal cell bodies and neuroglia. Weak staining was observed in neurons or neuroglia for TrxR2 and Txnip (Table 1; Figs. 5 and 6). Trx1, TrxR1, Grx1, γ-GCS, Prx1, Prx2, Prx3, Prx4, and Prx5 antibodies stained the neuronal cell bodies. The staining in dendrites, on the other hand, was not consistent (Table 1; Figs. 5 and 6). Clear immunolabeling in both neuronal cell bodies and dendrites was observed in tissue stained for Trx2, Grx2, and Grx5 (Fig. 5). Prx6 displayed weak staining in the neuronal cell bodies and dendrites, but neuroglia were labeled strongly. Prx6 staining of neuroglia cell projections attached to the blood vessels was observed that might correspond to astroglial feet (glia limitant), a part of the blood–brain barrier (Fig. 6).

The striatum is crossed by several axonal packages that connect diverse areas in the CNS. Weak staining in these fibres was observed for Trx1 and Grx3 (Fig. 6). Trx1, Trx2, and Prx6 immunopositive neuroglia were detected in the corpus callosum structure located above the striatum (Figs. 5 and 6).

3.4. Cortex

We did not observe specific differences in the immunostaining of the histological layers of the cortex. In general, all of the histological components were stained, however, with some variation in intensity. As described for other layers of the brain, strong staining for Prx6 was observed in neuroglia cells, presumably astroglia. The other members of the Trx family were primarily detected in neurons. Txnip, TrxR1, Grx3, and Grx5 displayed faint staining; neuronal cell bodies were stained for Trx2, γ-GCS, TrxR2, Grx2, Prx5, and Prx6. In addition, Grx1, Trx1, Prx1, Prx2, Prx3, and Prx4 displayed staining in the first segment of the apical dendrites. By morphology, these neurons may be classified as projecting pyramidal neurons (Figs. 7 and 8).

3.5. Substantia nigra

Two areas were studied in the substantia nigra: pars compacta (PC), rich in dopaminergic neurons, and pars reticulata (PR) that is rich in gabaergic neurons and dendrites of neurons of the PC. Hardly any neuronal staining was observed in the PR, however, Trx2 and Prx6 labeling resulted in strong neuroglial staining. By morphology these may be classified as astroglia (Figs. 9 and 10).

In the dopaminergic neurons of the PC intense immunolabeling was observed for Trx1, Grx2, Grx5, Prx2, Prx4, and Prx5 (Figs. 9 and 10); both the neuronal cytoplasm and the proximal part of the dendrites were labeled. Weaker but also consistent staining was observed for TrxR2, Grx1, Grx3, Prx1, Prx3, and γ-GCS (Figs. 9 and 10). TrxR1 and Txnip immunoglobulins weakly stained dopaminergic neurons and the staining was scattered (Figs. 9 and 10).

3.6. Spinal cord

Trx1, Grx1, Grx2, Prx4, and Prx5 have been detected in neuroglia, neuronal cell bodies, ependymal cells, and blood vessels (Figs. 11

Table 1
Comparative analysis of the staining intensities of the thioredoxin and glutaredoxin system proteins in the different areas of the rat CNS.

CNS region							
Protein	Cerebellum	Hippocampus	Striatum	Cortex	Substantia nigra	Spinal cord	Retina
Trx1	++	+/−	+ (axons)	++	++	++	++
Trx2	+	+	++	+	++	+	+
TrxR1	+	+	+	+/−	+/−	−	+/−
TrxR2	++	+	+/−	+	+	+	• (nucleus)
Txnip	+/−	+/−	+/−	+/−	+/−	−	+
Grx1	+ (nucleus)	++	+	++	+	++ (nucleus)	+ (nucleus)
Grx2	+ (nucleus)	+	++ (nucleus)	+	++	++	+
Grx3	+	+	++ (axons)	+	+	+ (nucleus)	+/− (nucleus)
Grx5	+	+	++	+	++	+ (nucleus)	+
GSC	+	+	+	+	+	+	+/−
Prx1	+	+	+	++	+	+ (nucleus)	+/−
Prx2	++	++	+	++	+	+	++
Prx3	+	+	+	++		+	+ (nucleus)
Prx4	+	++	+	++	+	++	+
Prx5	+	++	+	+	+	++ (nucleus)	+ (nucleus)
Prx6	++	++	+ +	++	++	+	+

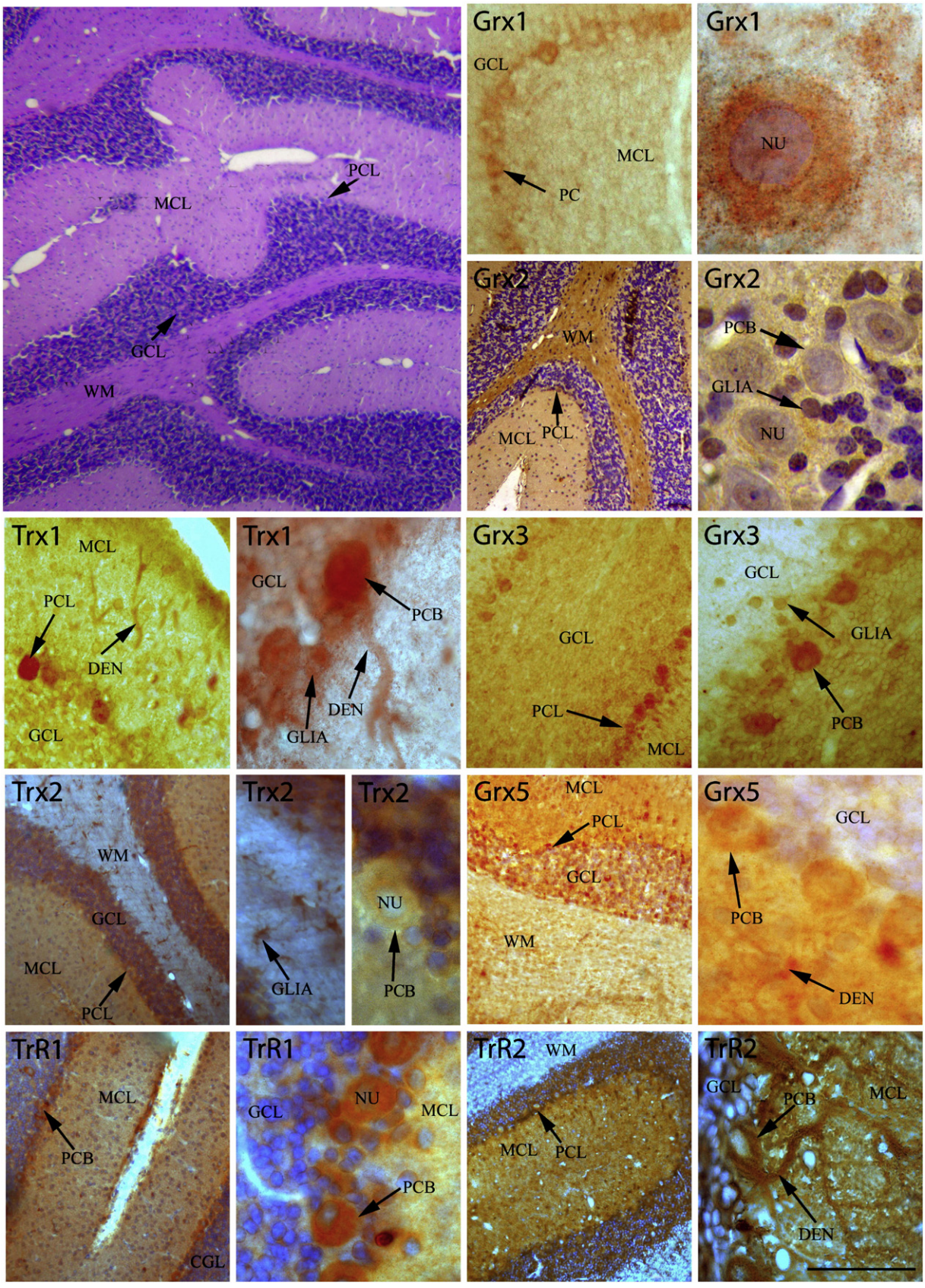

Fig. 1. Immunohistochemical localization of Trx1, Trx2, TrxR1, TrxR2, Grx1, Grx2, Grx3, and Grx5 in the different layers of rat cerebellar cortex. The figure located at the upper left shows a hematoxilin–eosin stained slice and the defined layers of the cerebellum cortex. Strong staining was observed for most cells in Purkinje cells and in the cell bodies of astrocytes. Trx1 was also labeled in the main dendrites of the Purkinje cells. Grx1 and Grx2 displayed a nuclear staining pattern. Trx2 showed a specific and intense staining in glia cells. Txnip showed the weakest staining. GCL: granular cell layer; MCL: molecular cell layer; PCL: Purkinje cell layer PCB: Purkinje cell body; DEN: dendrite, NU: nucleus; WM: white matter. Scale bar: 40 μm.

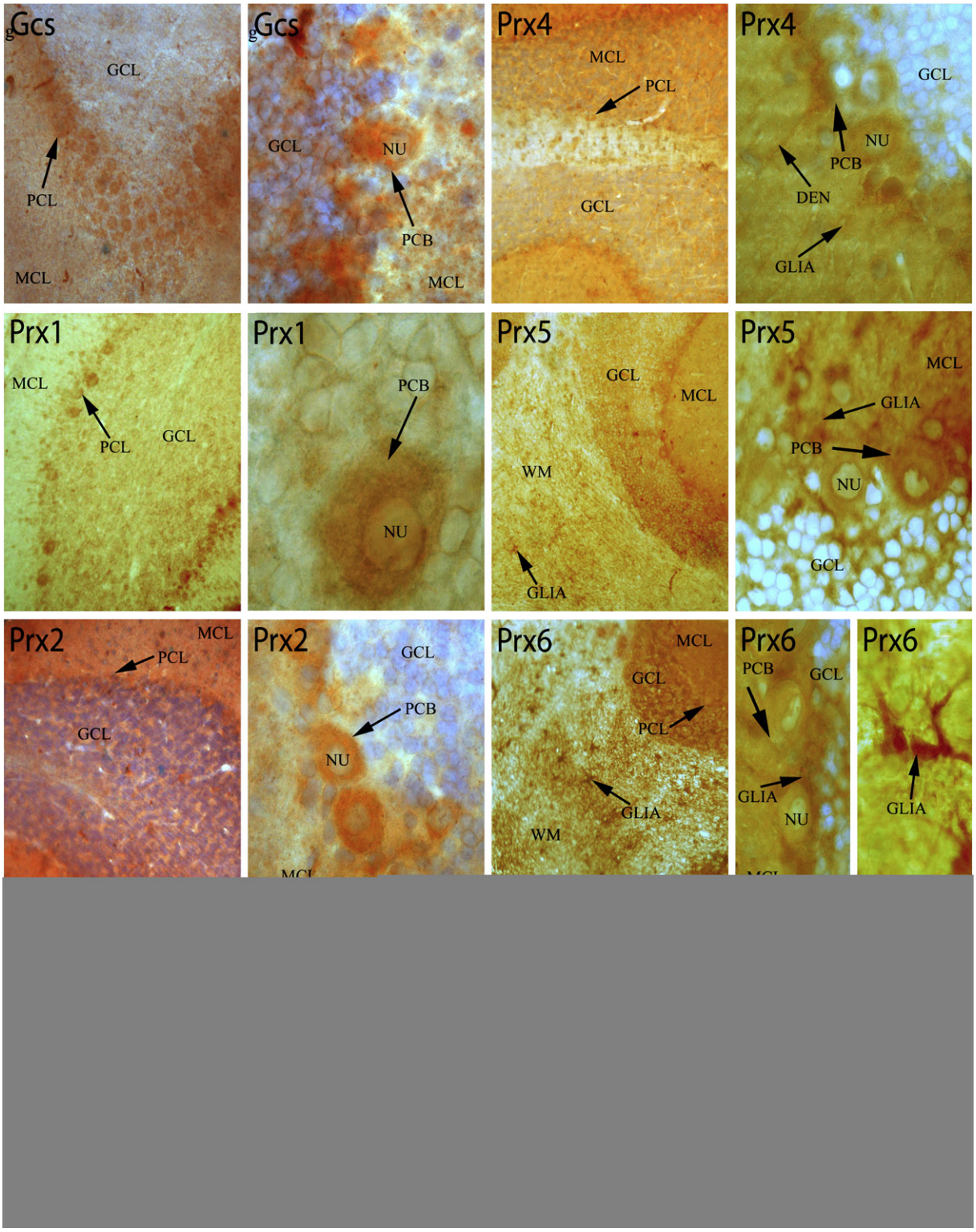

Fig. 2. Distribution of γ-GCS, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, and Txnip in the rat cerebellar cortex. The most striking feature was the staining for Prx6 in glia cells. By morphology these glia cells correspond to astrocytes. GCL: granular cell layer; MCL: molecular cell layer; PCL: Purkinje cell layer PCB: Purkinje cell body; DEN: dendrite; NU: nucleus; WM: white matter. Scale bar: 40 μm.

and 12). TrxR2, Grx3, Grx5, γ-GCS, Prx1, Prx2, Prx3, and Prx6 were not detected in the ependymal cells (Figs. 11 and 12). TrxR2, Trx2, Prx1 and, Prx2 were not present around the blood vessels (Figs. 11 and 12). Essentially no immunostaining was observed for TrxR1 and Txnip (Figs. 11 and 12). A nuclear staining pattern was observed in neurons stained for Grx1, Grx3 Grx5, Prx1, and Prx5.

3.7. Retina

The most intense immunostaining was observed in the retina stained for Prx2 including all layers from the ganglionar cells to the pigment epithelium; the most intense labeling was recorded for ganglionar cells (Table 1, Fig. 14). Following in the grade of intensity,

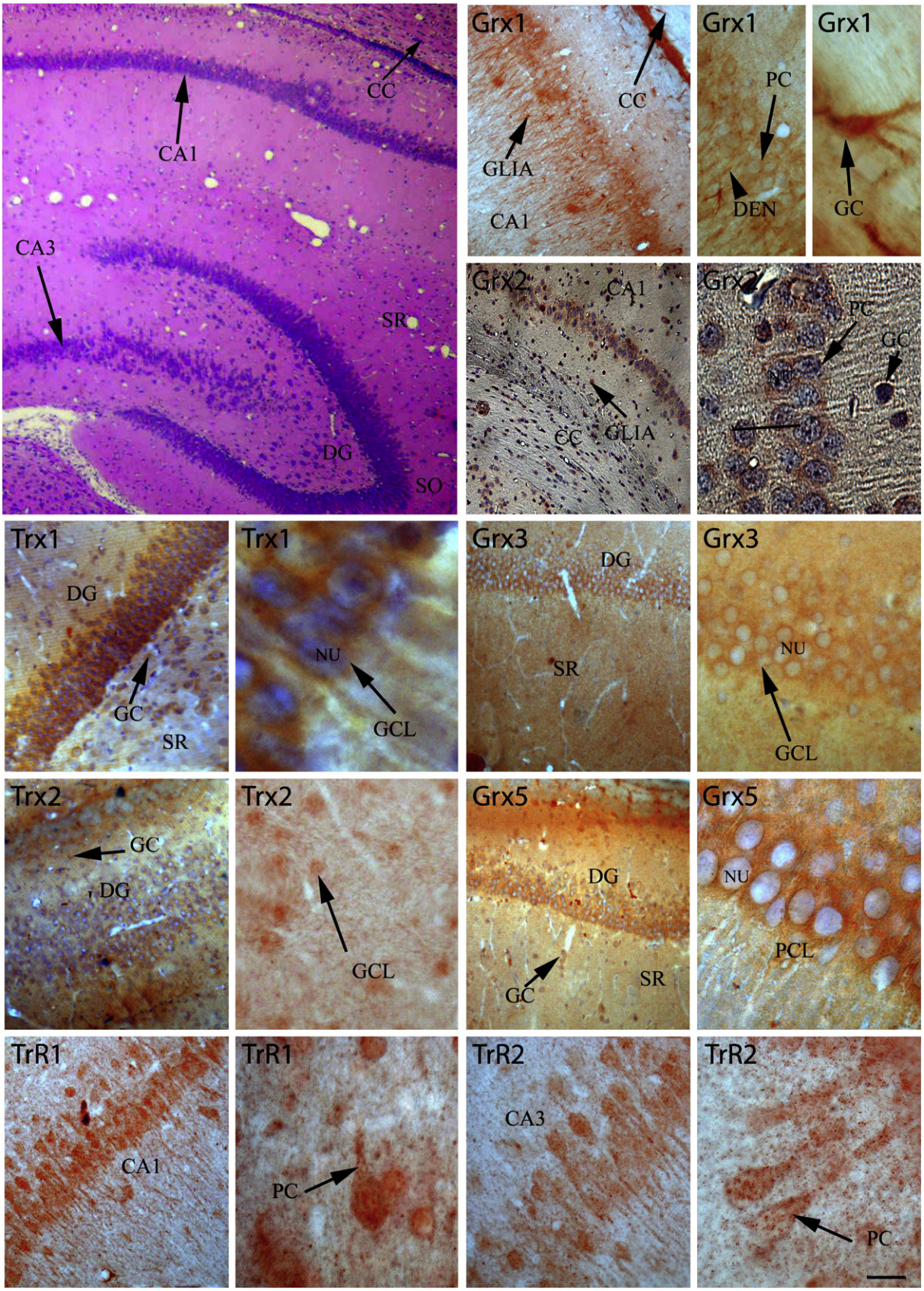

Fig. 3. Distribution of Trx1, Trx2, TrxR1, TrxR2, Grx1, Grx2, Grx3, and Grx5 in the different layers of rat hippocampus. The figure located at the upper left shows a hematoxilin–eosin stained sample and the definition of the regions. We found differences in the intensity of the immunostaining for the different Trxs. Grx2 showed a weak, presumably nuclear, staining. In addition Grx1 immunostaining was localized in the glia cells. CC: corpus callosum; DG: dentate gyrus; PC: pyramidal cell; GC: glial cell; GCL: granular cell layer. DEN: dendrites; NU: nucleus. Scale bar: 10 μm.

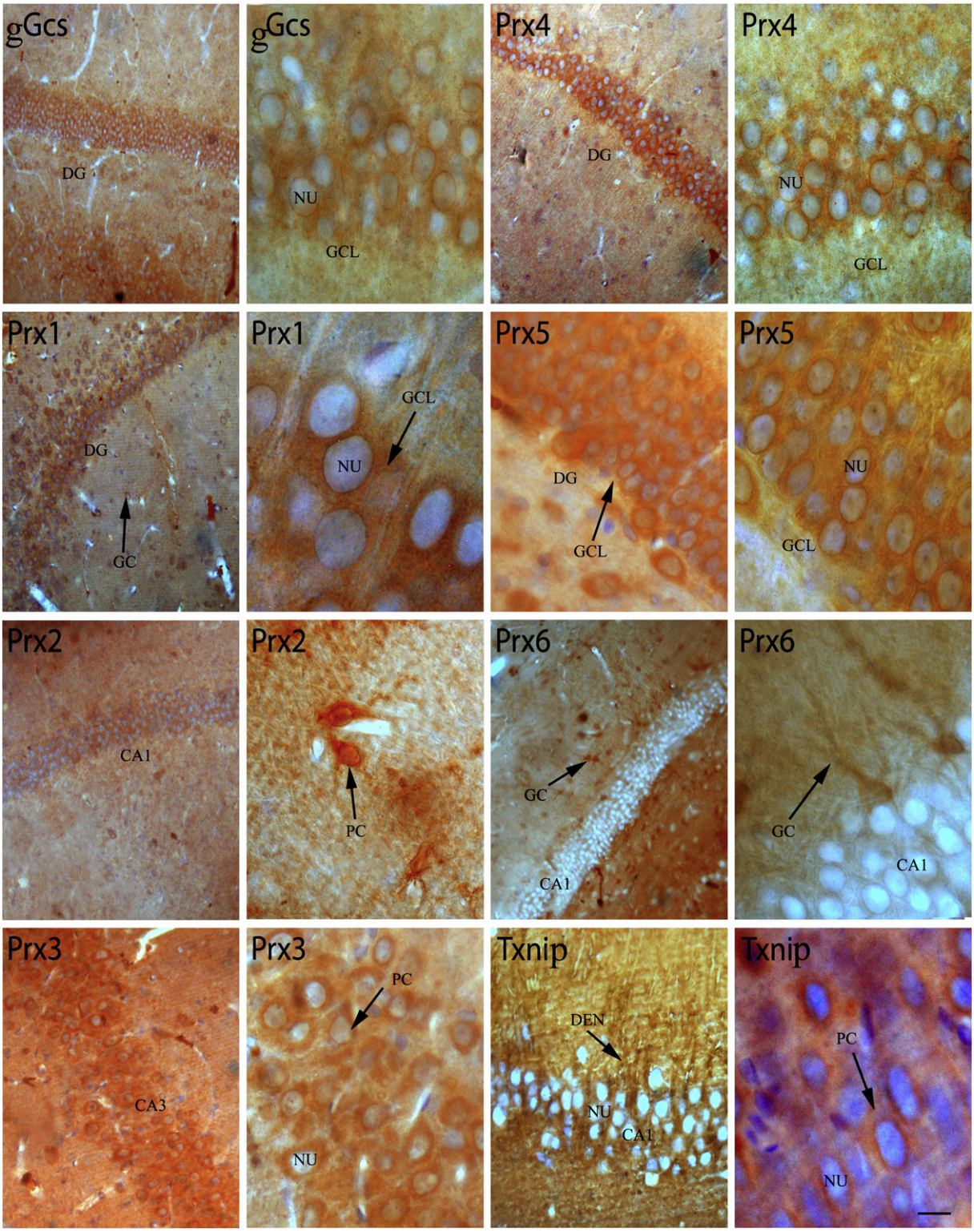

Fig. 4. Immunodetection of γ-GCS, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, and Txnip in the rat hippocampus. The most interesting features were the neuroglia stainings for Trx2 and Prx6. CC: corpus callosum; DG: dentate gyrus; PC: pyramidal cell; GC: glial cell; GCL: granular cell layer; DEN: dendrites; NU: nucleus. Scale bar: 10 μm.

Trx1 was detected in the outer plexiform layer, the inner plexiform layer and with high intensity in the ganglionar cell layer (Fig. 13). Faint staining for Trx1 was observed in the photoreceptors, pigment epithelium and choroids. A similar staining distribution was observed following staining for Trx2, TrxR2, Txnip, Grx1, Grx5, Prx3, Prx4, Prx5, and Prx6 (Figs. 13 and 14).

With respect to cellular localization, TrxR1 was primarily observed in the photoreceptor cells (Fig. 13), the other layers showed essentially no staining. Grx2 staining resulted in a high intensity of labeling in ganglionar cells and the inner and outer plexiform layers (Fig. 13). Essentially no staining for Grx2 was observed in photoreceptors. Grx3 immunoglobulins consistently labeled the inner

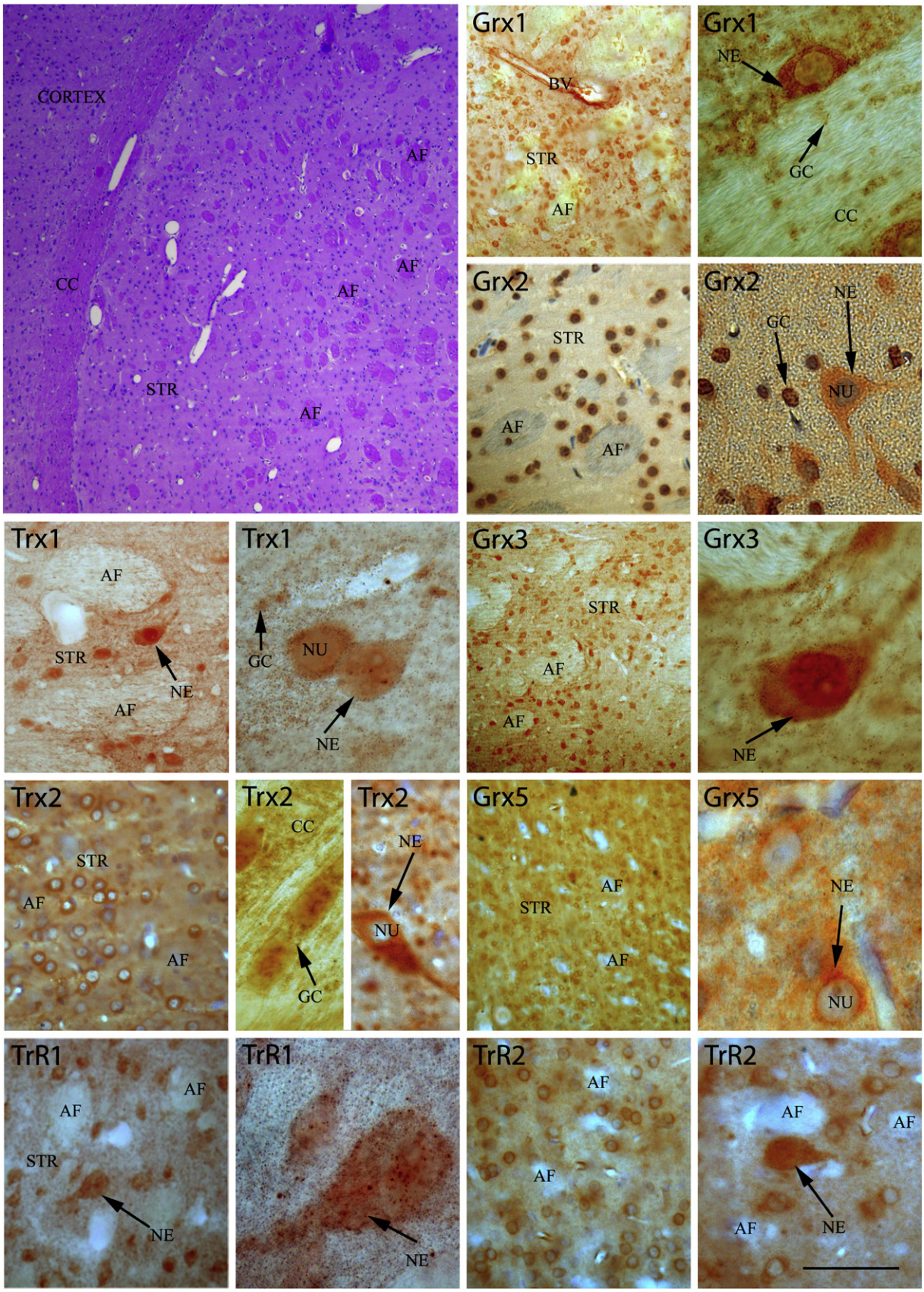

Fig. 5. Distribution of Trx1, Trx2, TrxR1, TrxR2, Grx1, Grx2, Grx3, and Grx5 in the rat striatum. The figure located at the upper left shows a hematoxilin–eosin stained slice of striatum. Trx2 immunoglobulins stained dendrites and astrocytes in the corpus callosum. Grx1 was stained in astrocytes but not dendrites. Nuclei were stained for Grx2. Grx3 stained very consistently the axon fascicles. STR: striatum. AF: axonal fascicles; NU: nucleus; NE: neuron; GC: glia cells. Scale bar: 10 μm.

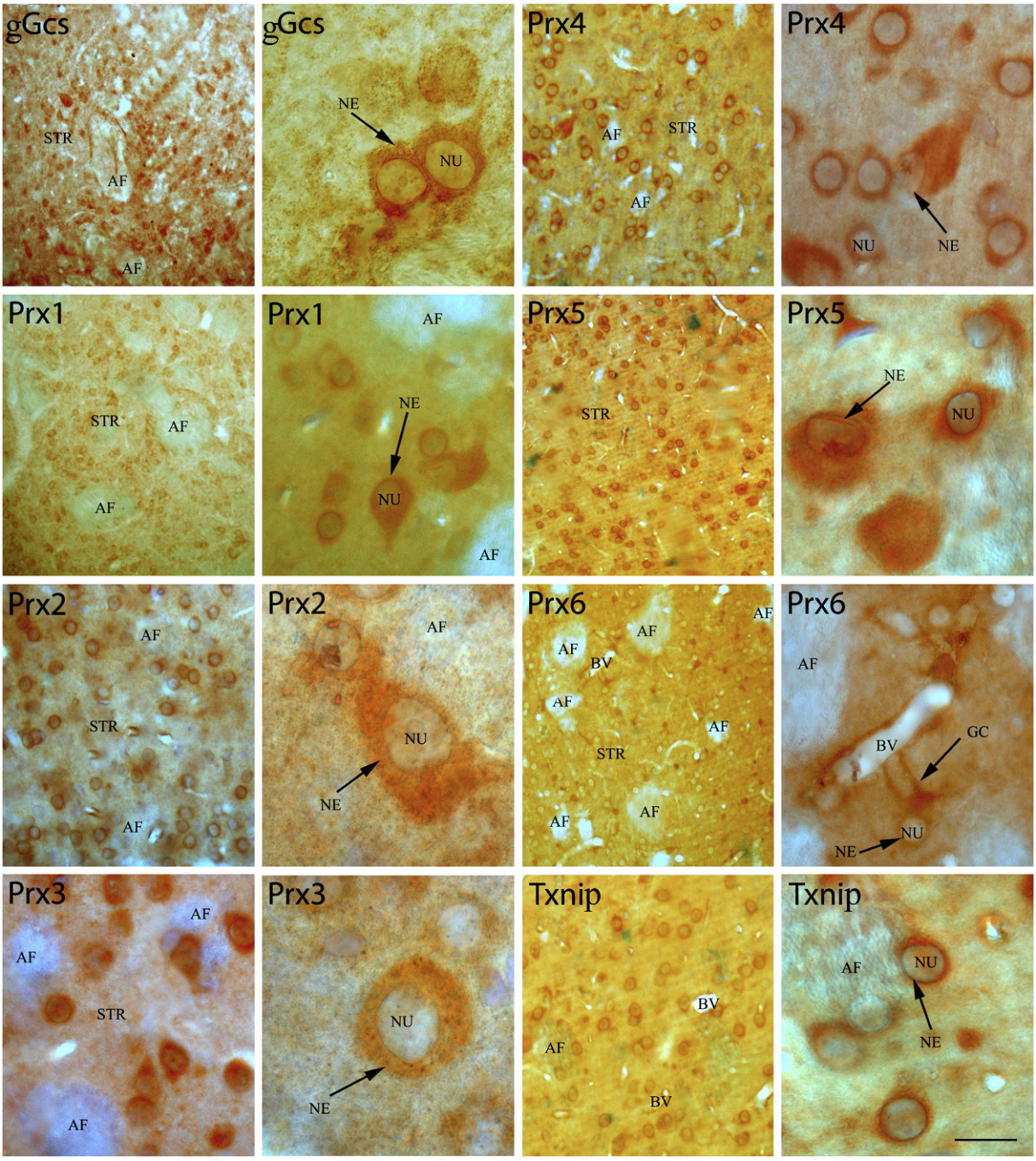

Fig. 6. Localization of γ-GCS, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, and Txnip in the rat striatum. Prx6 showed a strong staining in neuroglio reactivity was observed with TR from bacteria, yeast or rat and only a slight reaction was obtained with TR from horse. Immunoaffinity purified anti-thioredoxin and anti-glutaredoxin antibodies were used to develop competitive indirect ELISA assays that were validated giving very good linearity, reproducibility, sensitivity and parallelism. The glutaredoxin (Grx) immunoassay is the first quantitative method described to measure the protein. When applied to a battery of calf tissues the contents of Grx varied from 7 to 120 μg per gram of fresh tissue. Skeletala. Only weak staining was detected for Txnip. STR: striatum; AF: axonal fascicles; NU: nucleus; NE: neuron; GC: glia cells; STR: Striatum; AF: axonal fascicles; NU: nucleus; NE: neuron; GC: glia cells. Scale bar: 10 μm.

plexiform and the ganglionar cell layers, while only weak staining was seen in the outer plexiform, and outer and inner nuclear layers. No Grx3 staining was detected in photoreceptors and the pigment epithelium. A similar staining pattern was observed for Prx1 (Fig. 14). γ-GCS immunostaining was weak in all of the layers of the retina and negative in the outer nuclear layer (Fig. 14). Prx1 was strongly labeled in the ganglionar cell layer (Fig. 14). The other layers were labeled weakly for Prx1, the outer nuclear layer was negative. Most of the redox proteins were detected in what likely corresponds to the cytoplasm of the cells. In addition, a staining pattern that could point to nuclear localization was observed for proteins present in the ganglionar cells. Trx2, Grx1, Grx3, Prx3 and Prx5 displayed this nuclear labeling also in the inner nuclear layer (Figs. 13 and 14).

4. Discussion

To the best of our knowledge, this study is the first attempt to systematically describe the precise localization of the proteins from the Trx and Grx systems in the CNS of the rat using immunohistochemistry. Before discussing the details of our findings, however, we

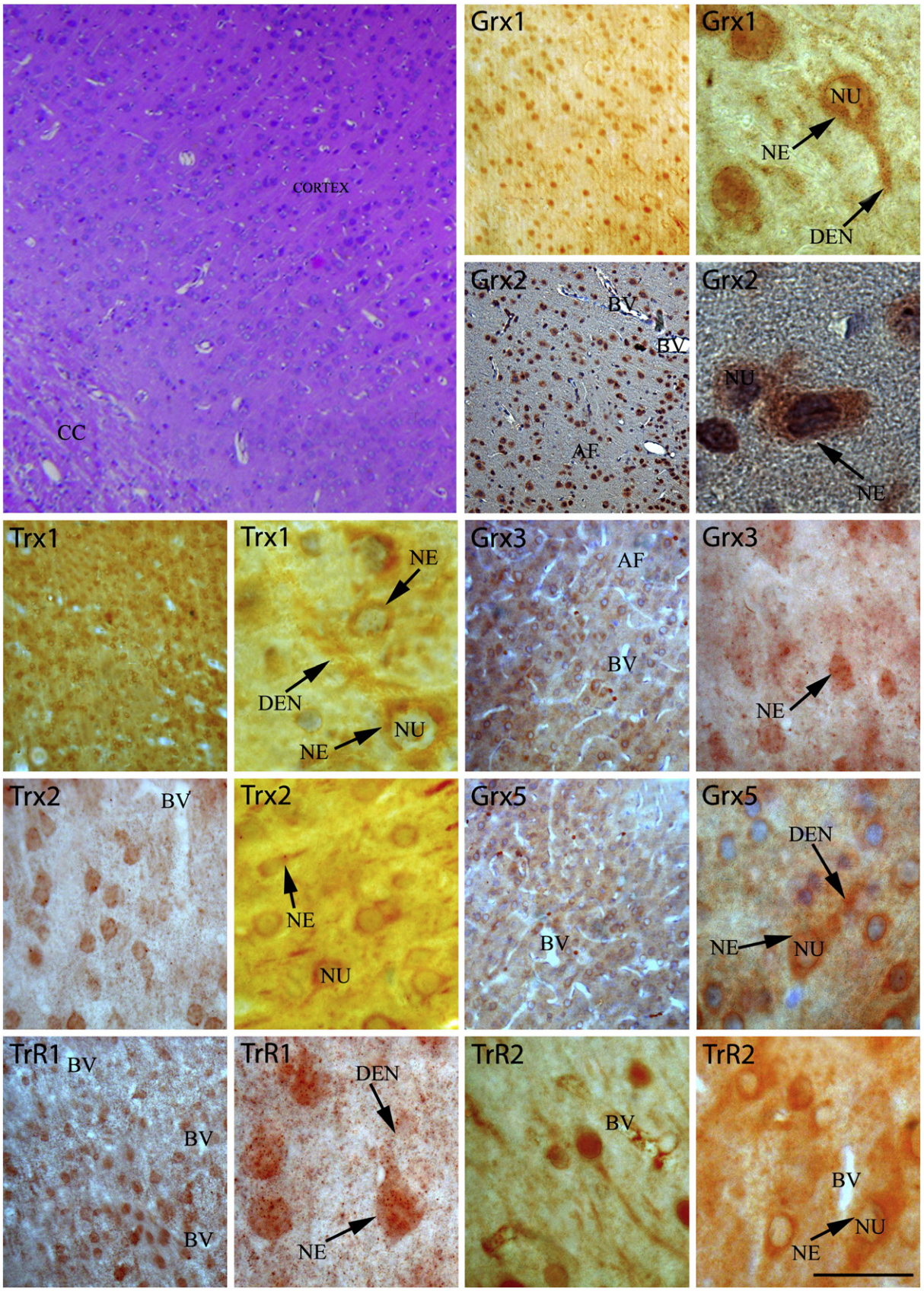

Fig. 7. Distribution of Trx1, Trx2, TrxR1, TrxR2, Grx1, Grx2, Grx3, and Grx5 in the rat cortex. The figure located at the upper left shows a slice of hematoxilin–eosin stained cortex. Most of the staining was concentrated in the neuronal cell bodies. CC: corpus callosum; BV: blood vessels; NE: neuron; NU: nucleus; DEN: dendrites. Scale bar: 10 μm.

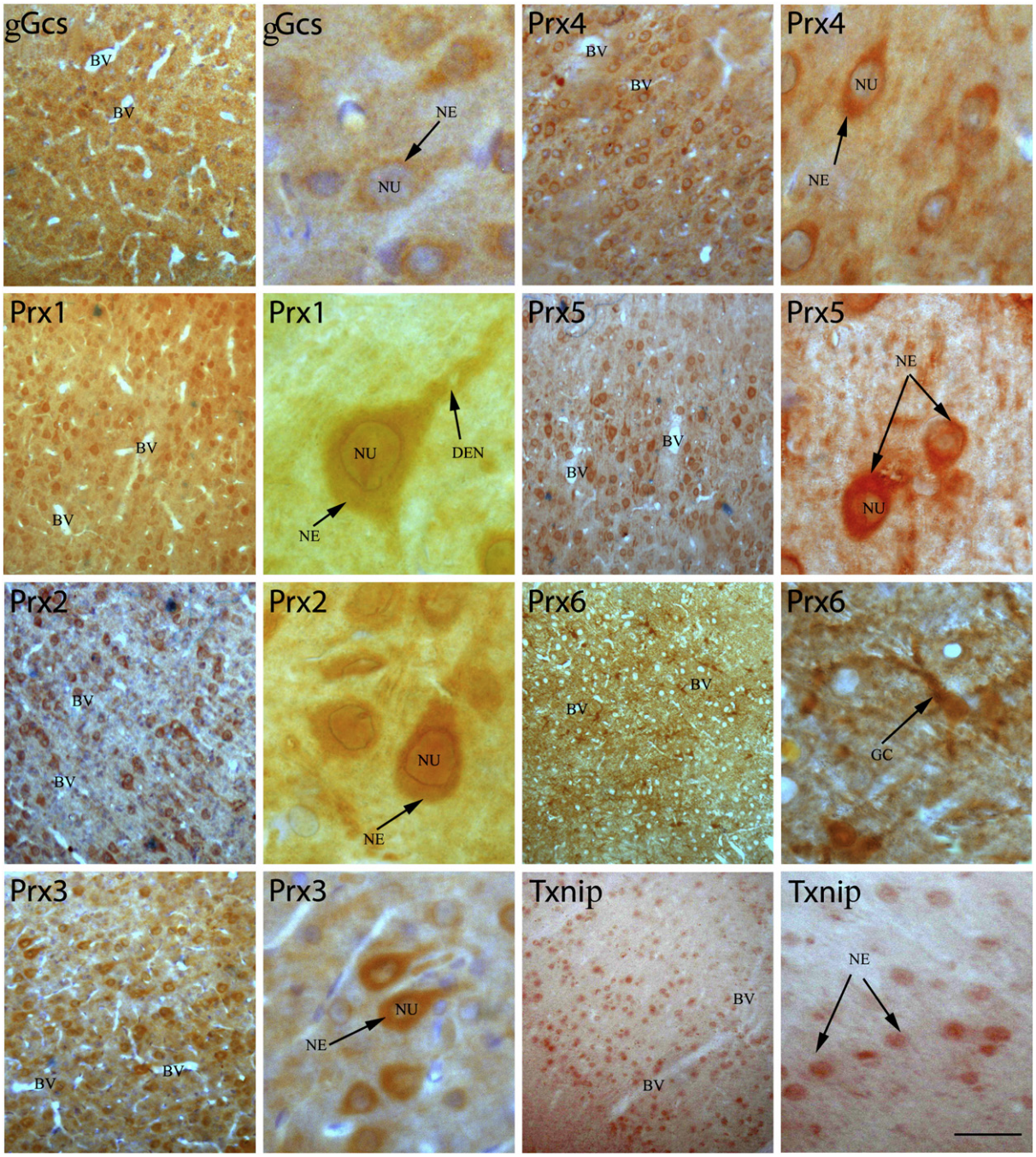

Fig. 8. Distribution of γ-GCS, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, and Txnip in rat cortex. Neuron cell bodies showed strong and consistant staining for many proteins. Prx1 was also detected in the apical dendrite. Consistent with the other areas of the CNS, Prx6 showed a strong neuroglia immunostaining. CC: corpus callosum; BV: blood vessels; NE: neuron; NU: nucleus; DEN: dendrites. Scale bar: 10 μm.

would like to point out potential problems with the interpretation of immunohistochemistry stainings in general. The availability of specific antibodies is essential for any attempt to describe the tissue distribution of a protein. The antibodies used here have been extensively validated for mouse tissues, please see the accompanying contribution "Redox atlas of the mouse" by Godoy et al. in this issue of Biochimica et Biophysica Acta—General Subjects. We have confirmed the specificity of these antibodies in rat by Western blotting and yielded, in all cases, specific bands of the expected sizes with essentially no additional bands present (see supplementary Fig. 1). Despite this validation, one must still consider the risk of unspecific staining not related to the antigen and the risk of false-negative staining due to low accessibility of the antigen in the processed tissue. These problems, and the different sensitivities of the immunoglobulins used, do not allow a quantitative comparison between different proteins. The results presented here may well represent the distribution of the proteins from the Trx and Grx systems in the rat CNS, however all stainings should be inspected with the necessary caution.

In general, all redox proteins investigated were detected throughout the rat CNS. Golgi I type neurons showed the most consistent immunostaining in all areas analyzed. Because of the great number of

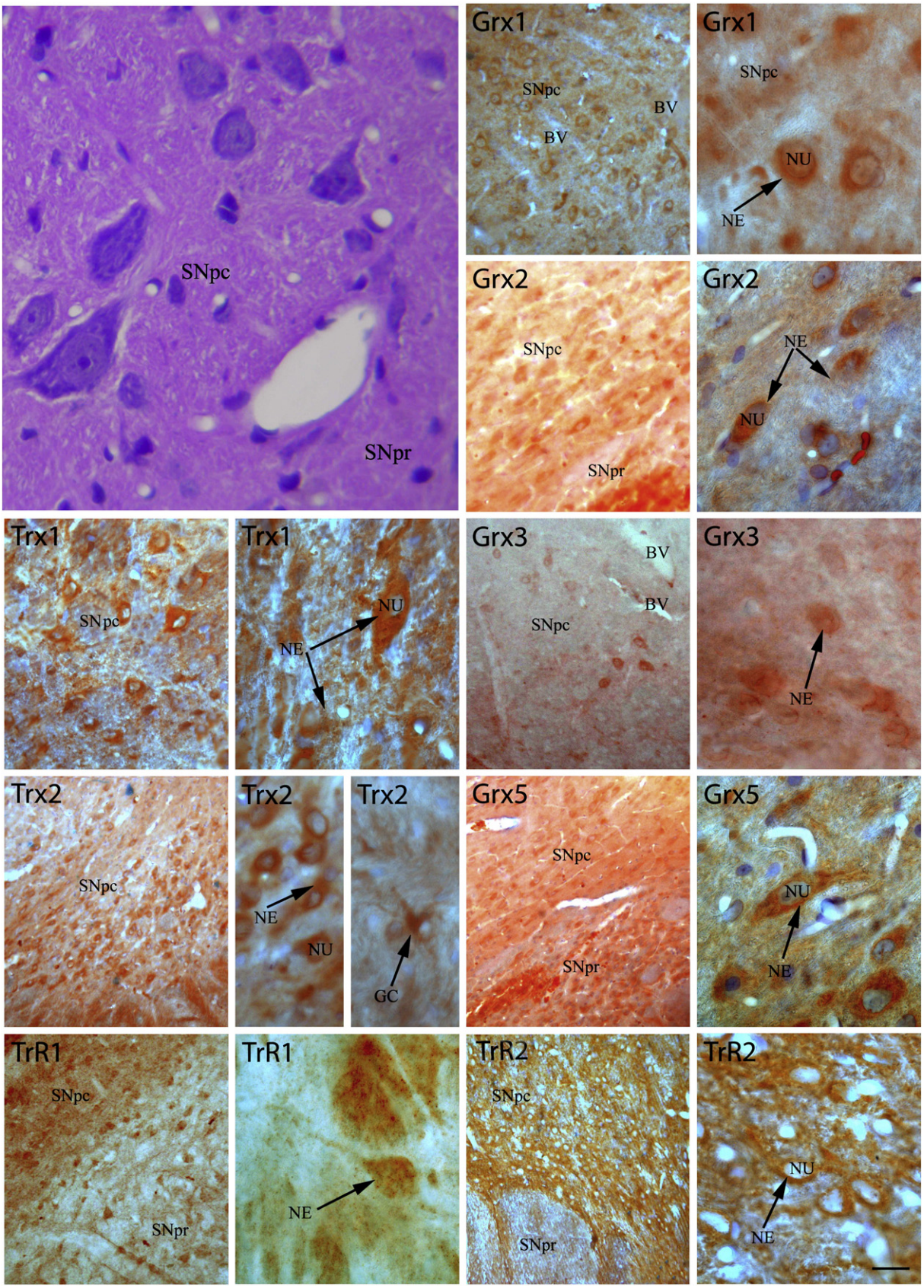

Fig. 9. Immunodetection of Trx1, Trx2, TrxR1, TrxR2, Grx1, Grx2, Grx3, and Grx5 in the rat substantia nigra. The figure located at the upper left shows a slice of substantia nigra stained with hematoxilin–eosin and points out the histological areas. Dopaminergic neurons showed strong staining for Grx2, Grx5 and Trx2. Intense staining for Trx2 in neuroglia was seen in the pars reticulata. Txnip staining was very weak. SNpc: substantia nigra pars compacta; SNpr: substantia nigra pars reticulate; NU: nucleus; NE: neuron. Scale bar: 5 μm.

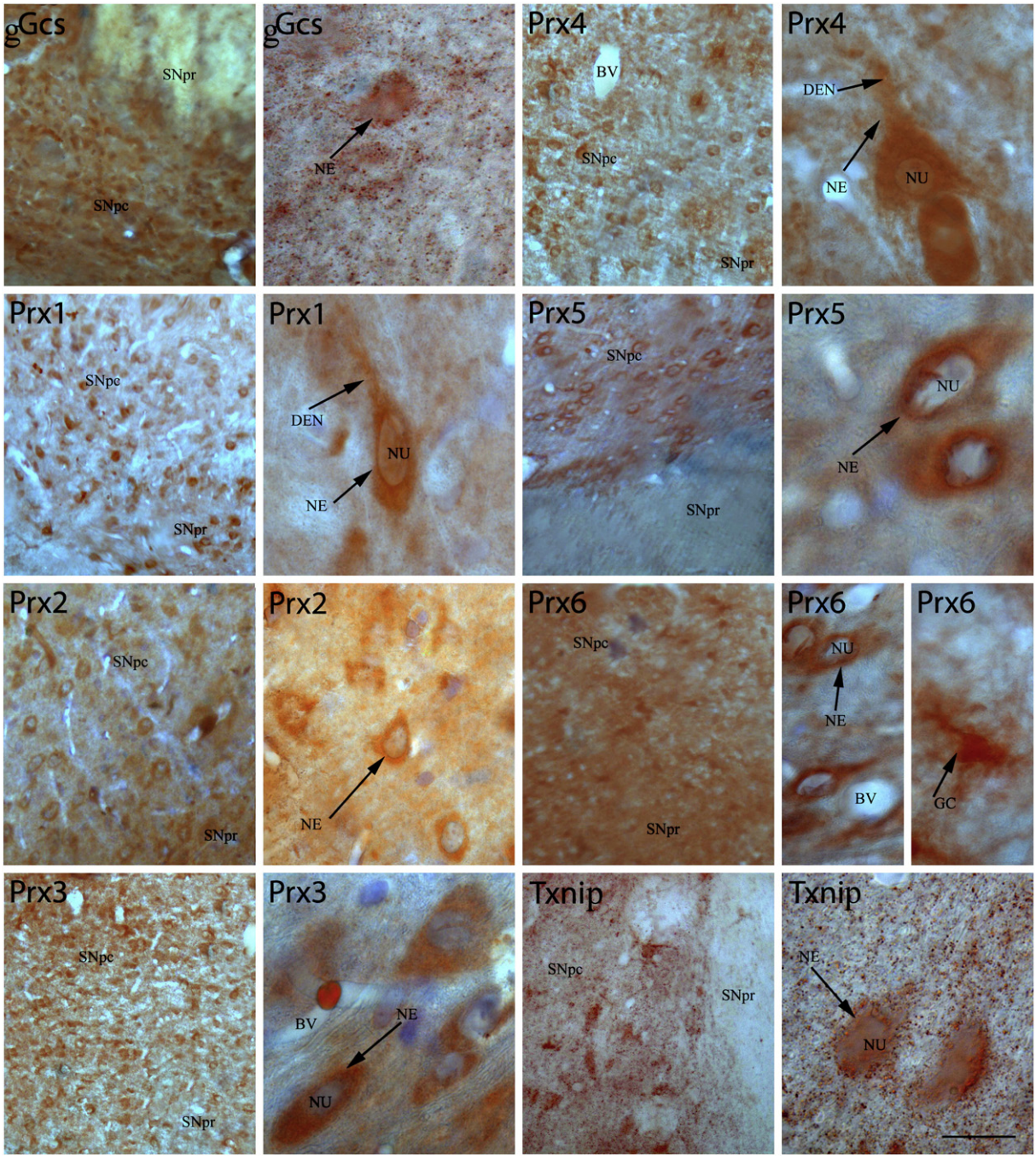

Fig. 10. Distribution of γ-GCS, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, and Txnip in the different regions of the substantia nigra. Most proteins were detected in dopaminergic neurons of the pars compacta. In addition, Prx6 showed intense staining in the neuroglia. SNpc: substantia nigra pars compacta; SNpr: substantia nigra pars reticulate; NU: nucleus; NE: neuron. Scale bar: 5 μm.

neurons and glial cells that are part of the CNS, we were not able to distinguish between the different cell types, even though some of them can be identified by morphologic features.

4.1. Thioredoxin and thioredoxin reductase system

In agreement with several reports [31–35], the Trx1 and Trx2 immunostaining observed here was intense in most regions of the CNS for both neurons and neuroglia. Trxs have been implied in neuroprotection during a hypoxic/ischemic insult in the CNS [36–40], and in Alzheimer's disease [40].

The intensity of Trx1 and Trx2 staining was low in the hippocampus, a well known target of oxidative stress [33,34]. This may explain, at least in part, the vulnerability of this region to stress situations such as ischemia-reperfusion. Intravenous administration of recombinant human Trx1 in mice was reported to decrease the hippocampal brain damage following transient focal cerebral ischemia. The administration of Trx1 reduced protein carbonyl content and attenuated the activation of the stress-related MAP kinase p38 in response to the ischemic insult [41]. Trx2 is the mitochondrial Trx and an important factor for cell viability. Fibroblasts of Trx2 knockout embryos undergo extensive apoptosis [42]. Trx2 can interact with mitochondrial

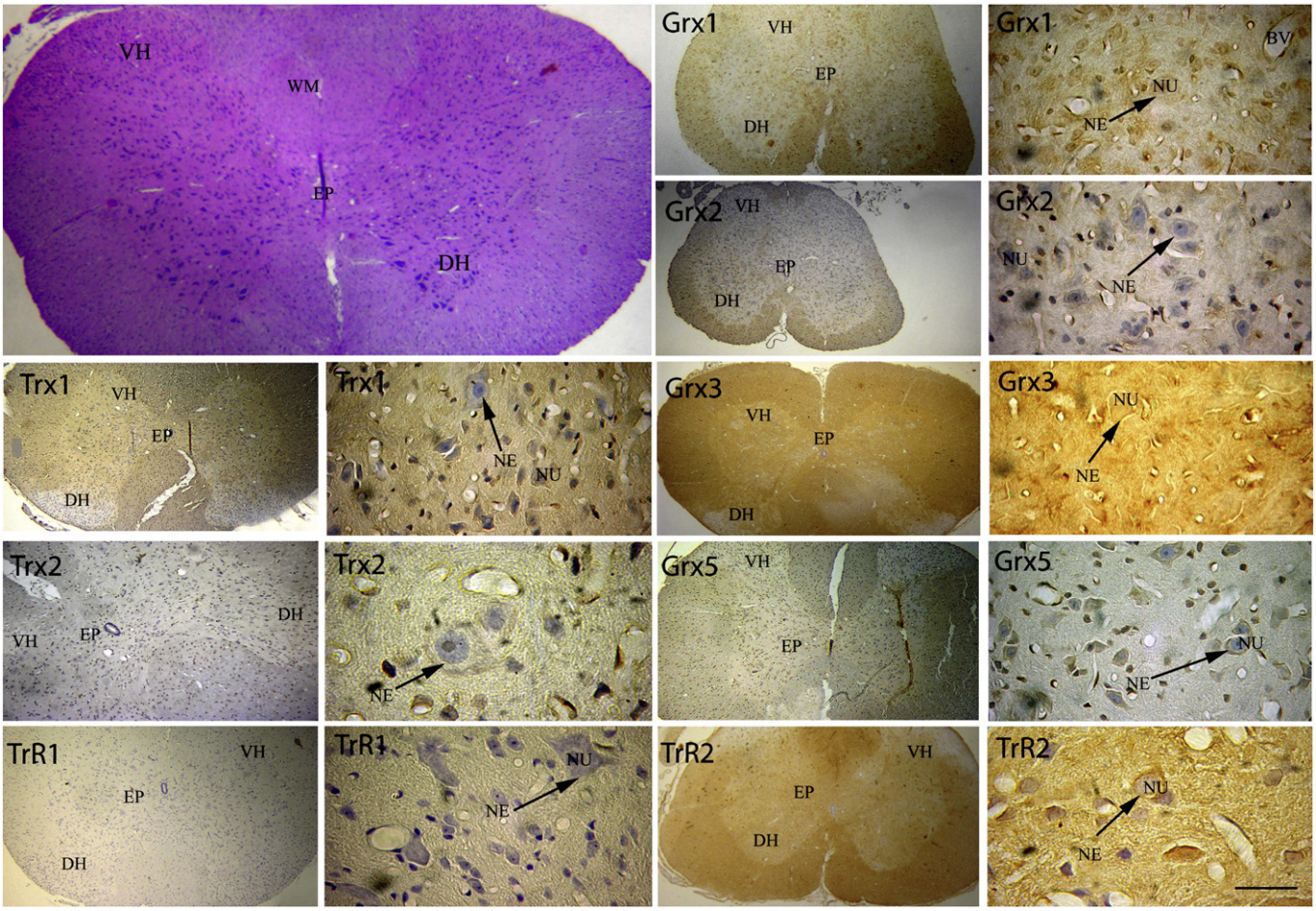

Fig. 11. Distribution of Trx1, Trx2, TrxR1, TrxR2, Grx1, Grx2, Grx3, and Grx5 in the rat spinal cord. The figure located at the upper left displays spinal cord stained with hematoxilin–eosin highlighting the histological organization. In general, the staining was weak, however, neurons were stained well. Grx1 and Grx3 displayed potentially nuclear staining. No astrocytes were observed. DH: dorsal horn; VH: ventral horn; NE: neuron; NU: nucleus; EP: ependymus. Scale bar: 40 μm.

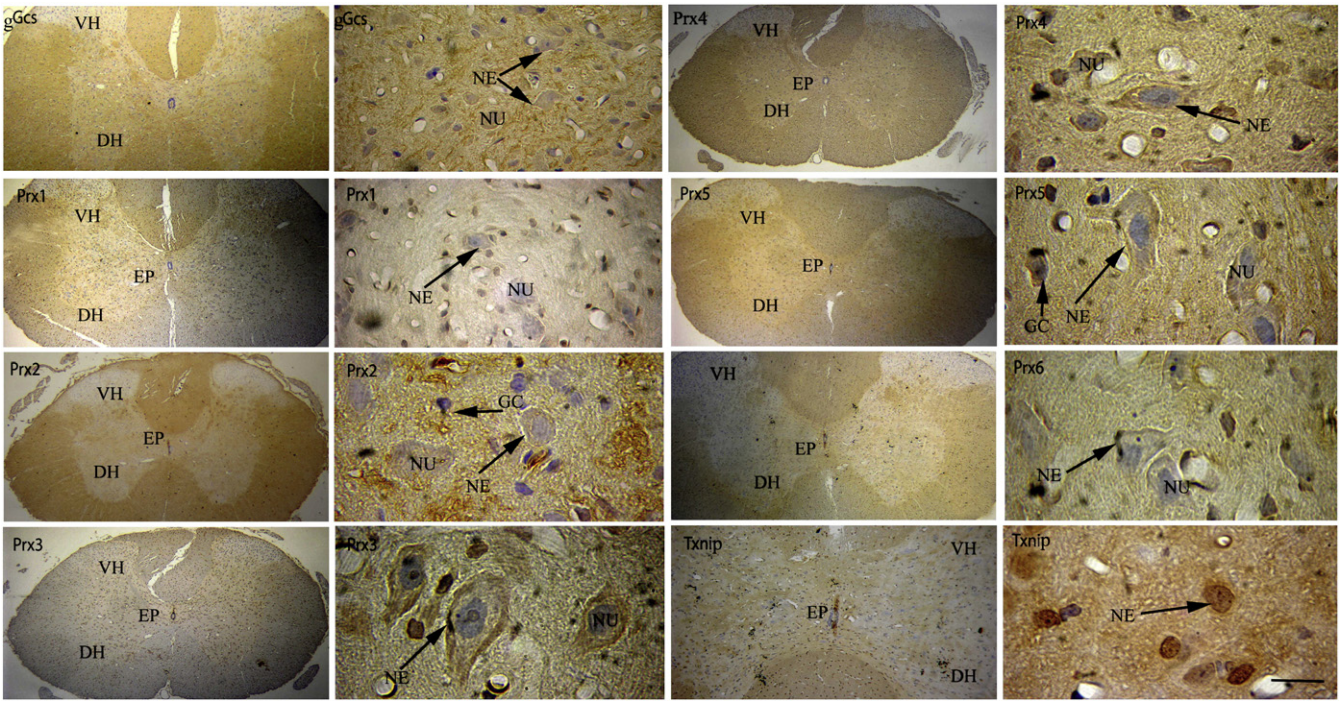

Fig. 12. Immunolocalization of γ-GCS, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, and Txnip in the rat spinal cord. Neurons and ependymal cells were stained well for most proteins. Prx1 and Prx5 display potentially nuclear staining. No astrocytes were stained. Txnip staining could not be observed. DH: dorsal horn; VH: ventral horn; NE: neuron; NU: nucleus; EP: ependymus. Scale bar: 40 μm.

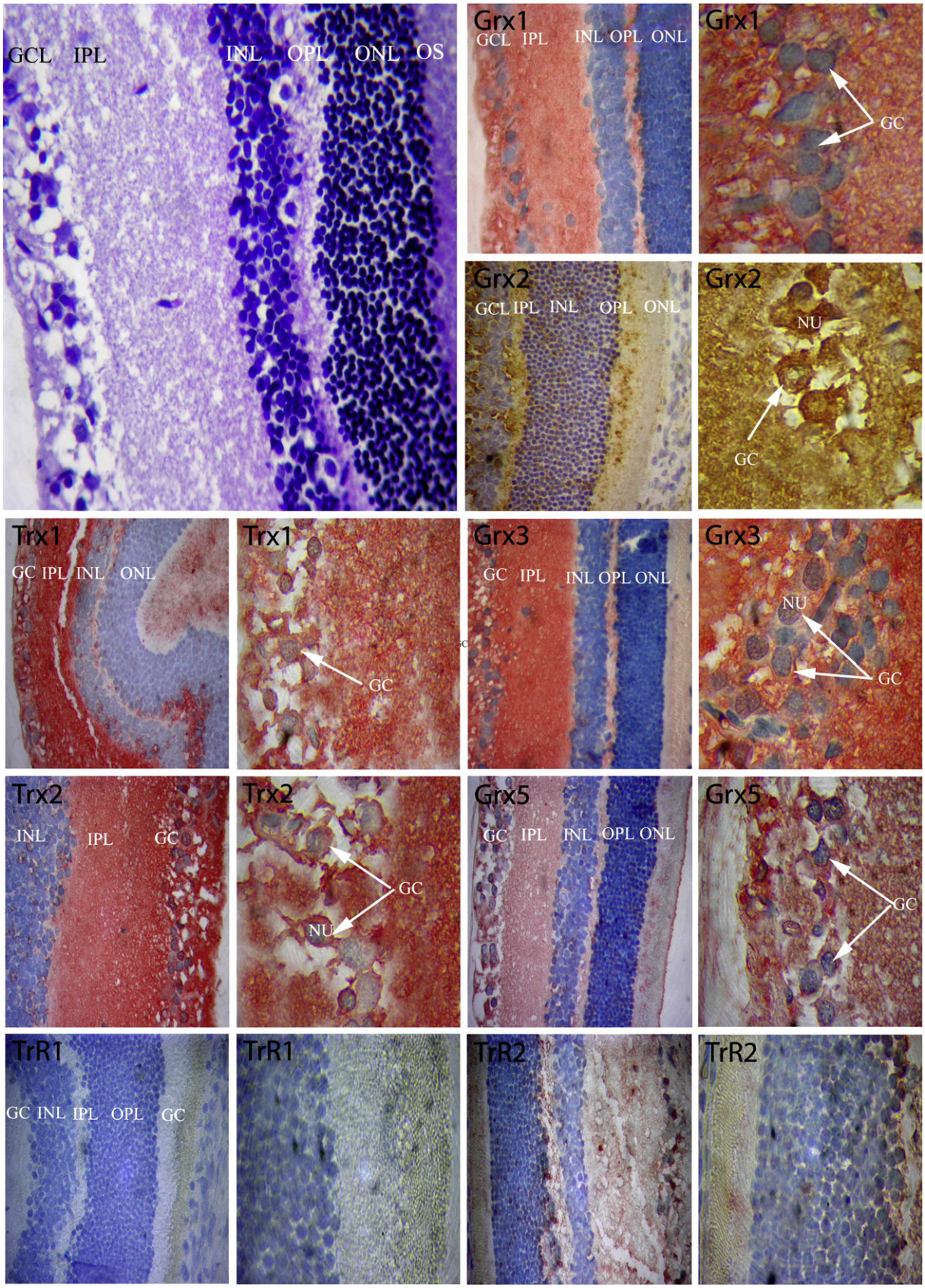

Fig. 13. Distribution of Trx1, Trx2, TrxR1, TrxR2, Grx1, Grx2, Grx3, and Grx5 in the different layers of the rat retina. The figure located at the upper left shows a slice of retina stained with hematoxilin–eosin highlighting the different layers of the retina. GCL: ganglionar cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; ONL: outer nuclear layer; OPL: outer plexiform layer. OS: stratum opticum. Scale bar: 30 μm.

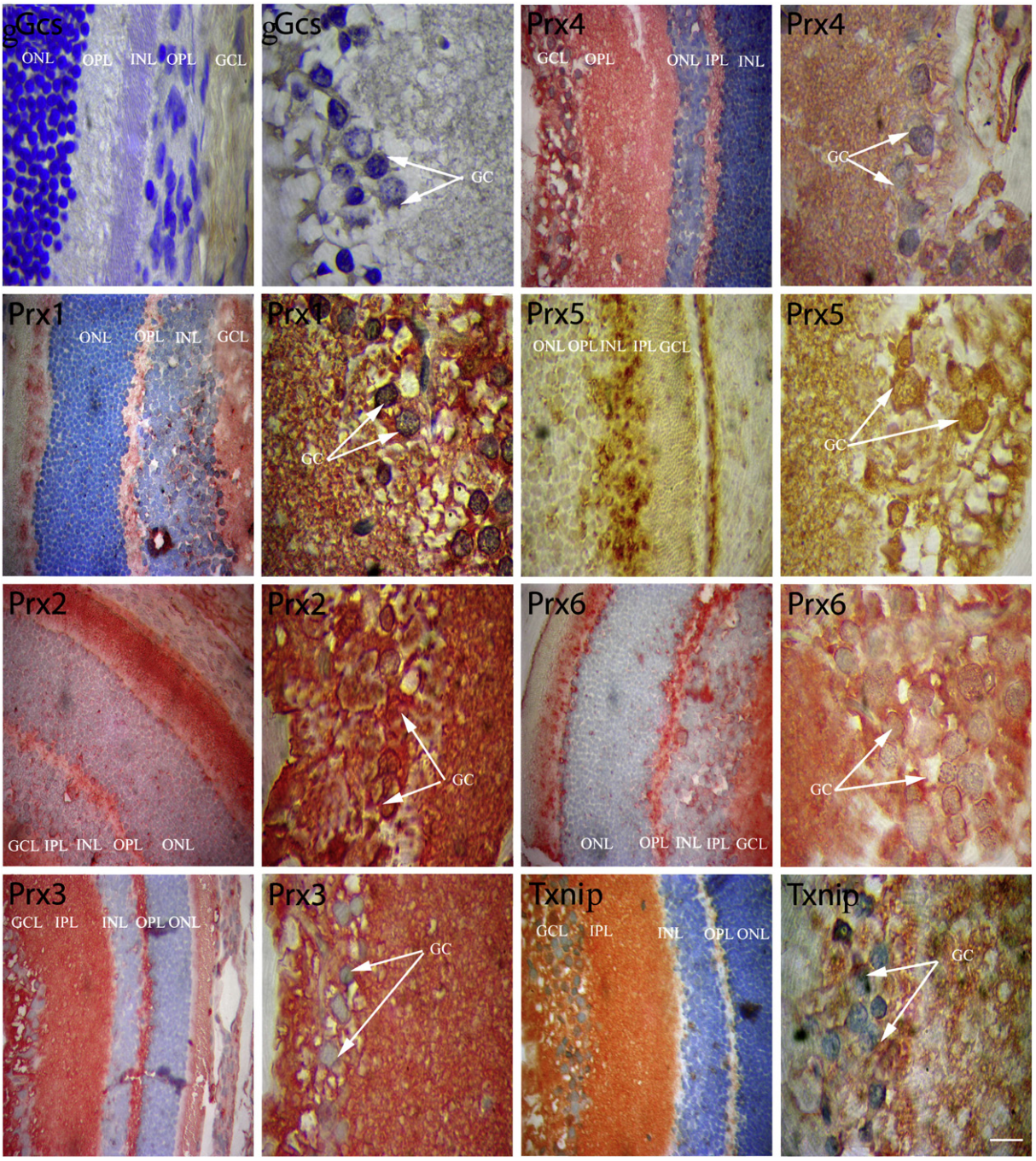

Fig. 14. Immunolocalization of γ-GCS, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, and Txnip in the different layers of the rat retina. For most proteins, the most consistent staining was seen in the ganglionar cell layer. TrxR2 and Grx3 displayed potentially nuclear staining. GCL: ganglionar cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; ONL: outer nuclear layer; OPL: outer plexiform layer; OS: stratum opticum. Scale bar: 30 μm.

respiratory chain components, effecting the mitochondrial membrane potential and apoptosis [43–45].

The distributions of TrxR1 and TrxR2 were fairly consistent with the localization of Trx1 and Trx2, respectively, with the remarkable exception of TrxR1 in the spinal cord. Some differences between Trx and TrxR localization could be attributed to the fact that both TrxR1 and TrxR2 are important selenoproteins and linked not only to Trxs, but also to other antioxidant functions and to the delivery of selenium to the brain [46].

Txnip was suggested to be an endogenous inhibitor of Trx1 function [12]. The Txnip immunoglobulins used in this study resulted in only weak staining in all areas analyzed. Although Trx1 and Txnip immunostainings were quite coincident, the spinal cord did not show labeling for Txnip.

4.2. The glutathione–glutaredoxin systems

The Grx1 staining pattern described here is in good agreement with the previously published expression patterns that resulted from both *in situ* hybridization and immunohistochemistry [47,48] (see Table 1 and Figs. 1–14). In addition, we observed a staining that may point to nuclear localization of the protein in Purkinje cells and spinal cord motoneurons. Induction of cerebral ischemia in rat by occlusion of the middle cerebral artery led to a reduction in Grx1 expression levels in hippocampus, however, the potentially protective function of Grx1 is still controversially discussed [47–52]. We observed a strinkingly intense immunostaining for Grx1 in glial cells in striatum radiatum. Failure of any of the supportive functions of the glial cells

for neurons will constitute a threat for neuronal survival [53,54]. The strong expression of Grx1 in these cells may point to a potential role of the protein in these supportive functions.

In agreement with a previous report in which the distribution of Grx2 in mouse brain was analyzed using fluorescent in situ hybridization [55], Grx2 staining was strong in type I neurons and glia cells of all areas investigated. In addition, we were able to see what might correspond to nuclear staining in Purkinje cells in cerebellum and Golgi type I neurons in neostriatum. The presence of cytosolic and nuclear Grx2 isoforms has been demonstrated in human and mouse before [56,57]. Grx2 protects cells from oxidative stress-induced cell death [58]. The presence of Grx2 in Golgi type I neurons may thus help these cells to cope with the damaging effects of an ischemia/hypoxia-reperfusion insult.

Grx3 was identified as regulator of protein kinase C function, Grx5 is important for the synthesis of iron-sulfur cluster cofactors in mitochondria [15]. In general the staining of Grx3 and Grx5 was weaker compared to Grx1 and Grx2. Grx3 and Grx5 displayed a staining pattern in neurons of the spinal cord that could be interpreted as nuclear localization, however, neither for Grx3 (cytosolic), nor for Grx5 (mitochondrial) nuclear localization or isoforms have been described. Potentially nuclear staining for Grx3 was also observed in retina (Figs. 11 and 12).

γ-GCS is the enzyme that catalyses the first and rate-limiting step in the synthesis of glutathione. To our knowledge this is the first report that systematically describes the distribution of γ-GCS in the rat brain by immunohistochemistry, although some functional changes in the activity of γ-GCS have been described before [59,60]. In contrast to a study in the mouse brain [61] that described a strong immunostaining in neurons and glia cells in hippocampus and cerebellum and weak staining in neostriatum and substantia nigra, we observed a broad distribution of γ-GCS in neurons and weak staining in neuroglia in all of the areas studied. Several reports have linked the vulnerability of the brain to oxidative stress to decreased activities of GR [19,20]. Disturbance of GSH synthesis as well as GSSG export from brain cells during oxidative stress contribute to the reduced GSH levels of diseased brains. Thus, the distribution of γ-GCS could help to understand how subtypes of neurons are affected in different brain diseases.

4.3. Peroxiredoxins

The expression of Prxs has been studied in human, murine, and (in case of Prx1) rat brain [62–64]. The immunohistochemical analysis in rat brain showed Prx1 immunostaining in many glial cells, particularly oligodendrocytes, while most neurons showed faint or negative staining [63]. In contrast, we have found an intense staining in neurons and only weak labelling of astrocytes. Consistent with other reports addressing human brains, we have also observed strong staining in projection neurons for Prx2 [64]. The same pattern of labelling was observed in Golgi type I neurons for Prx3, Prx4, Prx5, and Prx6, that was not reported before. In agreement with previous studies in mouse, we have observed the neuroglial localization of Prx6 [62]. The staining was very strong in cell bodies and neuroglia branches suggesting a great level of specificity for this type of cell.

A number of studies have shown that several Prxs isoforms can be induced in the brain in response to various insults, and suggested neuroprotective functions for these proteins in the CNS. For instance, Prx1 levels were increased specifically in glial cells in response to hemorrhagic and excitotoxic stress [65]. A proteomic comparison of old versus young human brain samples revealed decreased Prx2 protein levels in old individuals [66]. These apparent deficits in Prx antioxidant defenses might contribute to increased oxidative stress in the aging brain. Prx3 expression was also reported to be decreased in brain regions known to be specifically affected in Alzheimer's disease, Down's syndrome, and Parkinson's disease [67,68].

5. Conclusions

Overall, in this work we present the distribution of many members of the Trx and Grx systems in the rat brain. Since the role of these proteins in the regulation or removal of reactive oxygen species and oxidative stress is not well understood in the CNS, this work could help to explore new functions of these proteins in response to brain damage.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbagen.2010.06.011.

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