Neurochemical imaging of selenium homeostasis and the selenoproteome in the brain under nutritional deficiency

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Abstract

Dietary selenium (Se) and selenoproteins play a pivotal role in the brain. Selenium deficiency with subsequent selenoprotein depletion or targeted gene disruption causes severe neurological phenotypes. Although not the highest Se content, in addition to testis the brain is a privileged organ with high priority for selenium uptake under low nutritional selenium supply. To address the critical privilege of selenium in the brain and characterize nutritional effects on the selenoproteome, we applied the radiotracer ^{75}Se *in vivo* and combined autoradiographic localization and 2D proteomic analysis. Selenium was region-specifically incorporated and in addition to choroid plexus neuron-rich areas such as hippocampus, olfactory bulb, cortex and cerebellar nuclei showed highest Se levels. In contrast, the Se privileged organ testis showed an even prenchymal Se incorporation. Low Se status led to increased selenium uptake in all regions of the grey matter as well as Se enrichment in choroid plexus. Proteomic analysis revealed 21 uniquely labeled selenoprotein spots solely present in selenium deficient brains and 4 unique selenoproteins which were mitigated under low selenium status. Immunoblotting further confirmed the down-regulation of GPx1 and GPx4 in neurons following Se deficiency. Thus, this study revealed a unique Se uptake pattern in the brain and identified candidate selenoproteins which are nutritionally regulated. We identified in particular specific brain areas such as hippocampus, cortex, olfactory bulb, cerebellum and choroid plexus as Se privileged regions which points most probably to the requirement of certain selenoproteins for proper brain function.

Key words: autoradiography, nutritional gene expression, radiotracer, GPx4/PHGPx, selenium, selenoproteins

Abbreviations: ApoER2, apolipoprotein E receptor 2; CSF, cerebrospinal fluid; Cx, cortex;GPx, glutathione peroxidase; SeP, selenoprotein P; Se, selenium;

Introduction

The homeostasis of essential inorganic elements in the central nervous system shows distinct differences in comparison with other organs (Spector 1989, Takeda 2004). In particular, the trace element selenium is an essential micronutrient required for normal functioning of the brain (Savaskan et al., 2003; Chen and Berry, 2003; Schweizer et al., 2004). The micronutrient selenium is ingested in form of four different chemically species (selenomethionine, selenocysteine, selenate and selenite) (Andersen & Nielsen 1994) (**Fig. 1A**). However, in disparity to most other trace elements, selenium occurs in proteins in the form of selenocysteine (Sec), the 21st amino acid (Berry et al., 1991; Hatfield and Gladyshev, 2002). Its specific insertion into selenoproteins is conserved in all three taxa of bacteria, archaea and eukaryotes and requires in-frame UGA codons, which normally specifies termination of translation (Berry *et al.* 1991, Caban & Copeland 2006) (**Fig. 1B**). Using this feature and its distinct Selenocysteine Insertion Sequence (SECIS) for bioinformatical screening of the genomes, 24 selenoproteins could be identified in animal kingdom and 25 genes in humans (Kryukov *et al.* 2003; Castellano *et al.* 2001; Lescure *et al.* 1999).

Recent findings showed that the Se status is critical for proper brain function and mitigated brain Se levels cause brain defects during development and various brain disorders (Ramaekers et al., 1994; Arthur et al., 1997; Benton, 2002; Savaskan et al., 2003). However, at the expense of other organs, brain and testis are two privileged organs which remain high Se levels even under selenium deficiency indicating its biological importance for the organism. The uptake mechanism of selenium into the central nervous system is closely linked with the whole body homeostasis of selenium. Although this organ shows not the highest selenium concentration compared to other organs it shows a high priority for selenium uptake and retention in the case of dietary selenium deficiency. The preferential supply of the brain with this element in prolonged periods of selenium deficiency suggests a specific Se uptake mechanism and storage in this organ (Schomburg et al., 2003; Hill et al., 2003) and one key factor for Se supply and storage has been suggested as selenoprotein P (SePP) (Burk and Hill, 2005; Renko et al., 2007). Importantly, the expression of selenoproteins in the brain depends on the supply with the trace element and thus, behavioural changes and

neuropathological alterations following Se deficiency are likely due to altered selenoprotein expression.

Knowledge on the selenoproteome distribution in the brain were limited and systematic expression data for selenoproteins were restricted to GPx1, SePP (Saijoh et al., 1995) and GPx4 (Borchert et al., 2006; Savaskan et al., 2007). Very recently, a comparative selenoproteome expression study including 24 selenoproteins have been performed showing that the whole selenoproteome is expressed in the adult mouse brain (Zhang et al., 2007).

The presented work was based on a classic tool in selenium research, utzilizing the radiotracer ^{75}Se combined with quantitative real time imaging systems. Former investigations with the application of ^{75}Se (Pullen *et al.* 1995, Pullen *et al.* 1996, Trapp & Millam 1975) and studies on selenium uptake and homeostasis in the central nervous were based on manual separation of gross anatomical parts which hindered spatial resolution at the cellular and regional level (Behne et al., 1982, 1988). In this work, we used autoradiographic imaging for the determination of the ^{75}Se-distribution in the brain at the cellular level, verifying recent findings and gaining new information about the priorities in the hierarchy of selenium homeostasis. To characterize the hierarchy of selenium supply in the brain, *in vivo* radiotracer labeling with ^{75}Se rats with different selenium status was combined with autoradiographic detection of ^{75}Se in brain tissue sections and ^{75}Se-labeled selenoproteins after protein separation by two-dimensional gel electrophoresis. We could show that selenium is incorporated in specific brain regions whereas testis, another Se privileged organ, shows an even parenchymal Se distribution. In addition, Se deficiency leads to increased selenium uptake in all grey matter regions as well as in the choroid plexus. Proteomic analysis further revealed candidate selenoproteins solely present in selenium deficient brains. Thus, this study revealed a unique Se uptake pattern in the brain and identified candidate selenoproteins which are nutritionally regulated. This study investigated the distribution of the trace element Se in the brain and their regulated selenoproteins under different nutritional conditions and thus provides important data on Se metabolism and its relation to for proper brain function.

Materials and Methods

Production of ^{75}Se

The radiotracer ^{75}Se was produced in the nuclear reactor BER II of the Hahn-Meitner-Institute (Berlin, Germany). ^{74}Se in elemental form, enriched from the natural abundance of 0.9 % to more than 99 %, was oxidized to ammonium selenite by dissolving in nitric acid and then irradiated for several months at a neutron flux density of 1.3 x 10^{14} $cm^{-2} \cdot s^{-1}$. The produced [^{75}Se]-selenite was diluted in physiological saline solution, neutralized with sodium hydroxide and sterilized by sterile filtration.

Animal treatment, radiolabeling and sample preparation

The animal experiments were carried out in accordance with the German legislation on animal protection, and approved by the local authorities. A total number of 12 adult male Wistar rats (Charles River, Sulzfeld, Germany) were used and were kept in cages made of polycarbonate under an artificial day/night rhythm of 12:12 hours. Rats were nourished with special Torula yeast-based diets and deionised water ad libitum. Selenium-deficient diet containing a maximum of 2–5 µg Se/kg, and preceeding offsprings up to the 23rd generation were kept under Se-deficient diet. Control group received a selenium-adequate diet according to RDA in humans, which consisted of the same basal diet with 300 µg Se/kg added as sodium selenite (MP Biomedicals, formerly ICN Biochemicals, Cleveland, Ohio, USA). Analysis of selenium concentrations in the diets was performed as formerly described (Behne *et al.* 1994). The animals were labeled *in vivo* by intraperitoneal injection of 0.5 ml of [^{75}Se]-selenite solution containing 1 µg selenium. Rats with body weights of 520-830 g received each an activity of 16.7 MBq. After injection, three and seven days post injection (d.p.i.) the whole-body ^{75}Se activity was measured *in vivo*. Anaesthesia was performed with Isofluran (CuraMED Pharma GmbH, Karlsruhe, Germany) and rats were sacrificed by injection of an overdose of Nembutal (Sanofi Pharma, Munich, Germany). Testis together with epididymides and brain were prepared, immediately shock frozen in liquid nitrogen and stored at -80 °C.

Behavioural and locomotion monitoring

Selenium deficient and adequate rats were video time lapse monitored overnight. For motorcooordination, time and accuracy on grid walk and narrow beam line was measured.

Neurochemical imaging and autoradiographic analysis

For the investigations performed on brain and testis tissue, samples from each group were prepared for autoradiographic imaging at -21 °C with a freezing microtome. Sagittal, coronal and horizontal cryosections of 10 µm and 12 µm were prepared and placed on SuperFrost® Plus glass slides (Menzel, Braunschweig, Germany). After autoradiographic exposure, histological Nissl staining was performed with cresyl violet. The radionuclide ^{75}Se has a half-life of 119.8 days (Unterweger *et al.* 1992) and decays by electron capture into stable ^{75}As. The emitted γ-spectrum ranges with the sum peak of the most intensive γ-lines at 136.0 keV and 264.7 keV up to 401 keV (Firestone & Shirley 1997). Three different methods were applied for the autoradiography of the radiotracer. Radiolabeled proteins were determined after 2D electrophoresis by exposing photostimulable storage phosphor imaging plates (BAS 1000, FUJIFILM Corporation, Tokyo, Japan) to the ionizing radiation caused by the ^{75}Se decay in the dried gels, followed by their readout using an imaging analyzer (FLA-3000, FUJIFILM Corporation, Tokyo, Japan). The spatial resolution of the imaging analyzer amounts to 50 x 50 micrometer^2. The quantitative evaluation was performed by means of the software AIDA 4.19 (raytest GmbH, Straubenhardt, Germany). For visualizing weakly labeled protein spots, digital image processing was applied, combining different filter algorithms. For the autoradiographic imaging of the ^{75}Se distribution in the tissue sections X-ray films, image plates and a recently developed digital autoradiographic system, the MicroImager (Biospace Mesures, Paris, France) were used. Slides were exposed to X-ray films (Kodak X-OMAT AR) for 40 days and developed according to the manufacturer's protocol. Cryosections from the same tissue samples were exposed for 7 days to image plates for comparison reasons. Despite the advantages of the image plates, a linear dynamic range covering five orders of magnitude and a sensitivity 10 to 250 times of film (Johnston *et al.* 1990), the quantitative evaluation was applied to the results of the X-ray films because of their higher spatial resolution. The quantitative analysis was performed by means of the software AIDA 4.19 (Raytest GmbH,

Straubenhardt, Germany). Additionally to these established methods of autoradiography we applied a digital autoradiography system called MicroImager (Biospace Mesures, Paris, France) for the acquisition of autoradiographic images of the histological samples. Based on the optical detection of scintillation signals by a CCD camera, an imaging area was obtained of 24 × 32 mm with a spatial resolution of 20 µm and a pixel size of 5 µm. In contrast to the methods mentioned above, the MicroImager functions in a live time modus. As a result of radiation-matter interaction processes within a thin foil of scintillating paper, light spot emissions were acquired by a CCD coupled to an image intensifier. During the measurement, results were displayed live on a computer. Filter processing of the detected pixel pattern from the acquired emission signals allowed the recognition of background radiation according to the sizes of the optical spots acquired by the CCD camera and therefore an improved signal to noise ratio. The acquisition time of the samples amounted to 22 h and 144 h. The autoradiographic image was visualized with the ß-Vision software (Biospace, Germany).

Proteomic analysis

Brain tissue was collected and homogenized on ice by means of an electronically controlled handheld disperser (Polytron PT 1300, Kinematica AG, Littau-Luzern, Switzerland) for the analysis by two-dimensional (2D) electrophoresis. Sonification was done with ultrasonic cell disruptor (Sonifier W-450, Branson Ultrasonics Corporation, Danbury, USA) in the threefold volume of a Tris/HCl buffer (25 mM, pH 7.4) containing a mixture of protease inhibitors (P8340, Sigma-Aldrich Chemie GmbH, Munich, Germany). The soluble protein fractions were separated by ultracentrifugation (Beckman Coulter, Fullerton, USA) at 4 °C with 160 000 x g for 60 minutes. Total protein contents were measured by the method of Bradford and then solubilised by adding 6 M urea, 3 M thio-urea and 70 mM DTT - given as final concentrations. For the separation of 300 µg of the cytosolic protein samples a non-equilibrium pH gradient gel electrophoresis (NEPHGE) was applied (Klose 1975, O'Farrell 1975). First dimension of the 2D electrophoresis the proteins were separated by isoelectric focusing (IEF) in a pH gradient (pH 3-10) that was formed by 2 % soluble carrier ampholytes (SERVALYT™ 3-10, SERVA Electrophoresis GmbH, Heidelberg, Germany) within tubing gels during electrophoresis. In the second dimension standard sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) adapted to the high resolution format of 22 x 30 cm was used to separate the proteins. Finally the proteins were visualized by silver staining according to the method of Blum (Blum *et al.* 1987) and the gels were dried between sheets of cellophane in a gel-drying device (Biometra biomedizinische Analytik GmbH, Göttingen, Germany). For immunoblotting, α-GPx1 (Lab Frontier) and α-GPx4 (in house made) antibodies were used. The equal amount of protein lysates and equal β-actin expression served as internal controls.

In vivo measurement of ^{75}Se

The retention of the whole-body ^{75}Se activity *in vivo* was measured by means of a 3 x 3" NaI(Tl)-detector connected to a single-channel analyzer, with the rats kept in a fixed position in a plastic container.

Results

Global analysis of selenium incorporation of low selenium diets.

First, we studied the total Se retention in rats (n_{total} = 12) fed with special Torula yeast-based diets, containing adequate levels of sodium selenite (Se+) equivalent to the RDA in humans and selenium-deficient levels based on a maximum of 2-5µg/kg. Both groups showed a significant difference in the retention of the injected ^{75}Se activity (**Fig. 2A**). The retention of the whole-body ^{75}Se activity was measured *in vivo* 3 and 7 days post injection Selenium deficient rats kept back up to 90 % of the applied total activity, while Se adequately fed rats excreted approx. 60 % of the injected Se content.

Region-specific distribution of Se in the Se-privileged brain.

We went on comparing Se incorporation in the brain under different nutritional status. Equal to the total retention profile between the two groups, both groups showed significant differences in Se incorporation in the brain (**Fig. 2B**). The autoradiograms of tissue sections from the animals fed with the selenium-adequate diet show ubiquitous distribution in white and grey matter regions as well as less activity in contrast to the brain sections of selenium deficient rats (**Fig. 2B**). However, choroid plexus solely gave strong signals Se adequate fed rats (**Fig. 2B**). In contrast, selenium deficient brains displayed a specific Se incorporation pattern with strongest signals in grey matter structures such as hippocampus (CA1-CA3 and dentate gyrus), cerebral and cerebellar cortex, whereas signals in white matter such as commissure anterior, corpus callosum or spinal cord were lower (**Fig. 2B; Fig. 3**). Quantitative analysis of Se incorporation was performed on color coded images. Red color indicates regions with high ^{75}Se activity while white encodes for a low radiotracer activity (**Fig. 3B**). In general, Se distribution was lower in white matter regions than in grey matter. However, distinct areas within the grey matter showed quantitatively higher Se activity, indicating that Se is specifically and quantitatively incorporated rather than ubiquitously in neuronal cells. The CA1-CA3 regions, dentate gyrus, cortex, granular cell layer of the cerebellum and choroid plexus gave highest Se signals (**Fig. 3C**). Interestingly, comparative evaluation of 11 distinct brain regions from a total of 23 horizontal brain sections revealed choroid plexus as highest Se containing region (**Fig. 4A, B**).

Region-specific distribution of Se in Se-privileged testis.

The values of the ^{75}Se activity per area showed significant differences between testis and the three main parts of the epididymidis (**Fig. 4C**). Considering the variations of the histological tissue structures, highest ^{75}Se activity was found in the testis compared to the epididymidis. Within testis, highest Se levels were located in the seminiferous tubules whereas the interstitium showed only moderate levels (**Fig. 4C**). Within seminiferous tubules it was possible to differentiate between the lumen and the peripheral edge of the tubule, but sufficient resolution for visualizing single spermatozoae was not reached.

Candidate selenoproteins in the brain under low nutritional selenium status.

In order to obtain information about the differences of the selenoprotein expression pattern in the brain in dependence of the nutritional status, we applied a non-equilibrium pH gradient gel electrophoresis (NEPHGE) in combination with SDS-PAGE for 2D electrophoresis. Rats with different selenium status were labeled for five days with ^{75}Se and autoradiograms of the labeled total protein fractions were developed (**Fig. 5**). After visualizing weakly labeled protein spots by digital image processing, a total number of 33 labeled protein spots could be identified in selenium deficient brains (**Fig. 5B**). Quantitative comparison of identical protein spots in brains selenium-adequate and selenium deficient rats distinguished in total 12 labeled protein spots (**Fig. 5C**). In both animals with different selenium status the protein spots 8 and 9 show the highest amount of ^{75}Se activity. Molecular weight and IP range indicated that spot 8 (22—25 kDa, IP: 5.5 – 7.8) allocated approx. the range of glutathione peroxidases (GPx1-3) whereas spot 9 (20 kDa, IP 7.7 – 8.8) migrates at the range of GPx4 (Scheerer et al., 2007). Since most of the nutritionally available Se is metabolized and incorporated in selenoproteins at least under low Se status, we hypothesized that prominent labeld protein spots found in 2D-PAGE are those, which are down-regulated under Se deficiency. As indicated for spot 8 and 9, we experimentally tested the expression levels of these candidate selenoproteins directly by immunoblotting. Both, GPx1 and GPx4 were diminished under low Se status and were present in high

amounts in control brains (**Fig. 6**). In former autoradiogram studies, we identified primarily distinct neuron-rich regions heavily labeled for Se, suggesting neurons as the main source for selenoproteins. To test this, we performed immunocytochemistry and subsequent confocal evaluation. One candidate selenoprotein, GPx4, has been therefore evaluated and immunostaining revealed that GPx4 is mainly distributed in cortical neurons and their dendrites (**Fig. 6C**).

Discussion

This study represents the first spatial analysis of the trace element Se in the brain under nutritional deficiency combined with a selenoproteome analysis. We showed that two Se privileged organs, brain and testis, incorporate Se in a region-specific manner. Furthermore, 33 selenoprotein spots could be identified in a proteomic approach, of which 11 spots were altered following low Se status.

Methodological considerations of neurochemical imaging with ^{75}Se

Since the first application of the tracer principle by George Hevesy for studying the solubility of lead (Georg V. Hevesy 1913) and the application of radionuclides in life science (Hevesy & Ottesen 1945, Hevesy 1948), the suitability of a radioisotope for *in vivo* tracer studies is determined by the physical properties of its radioactive decay. The half-life of 119.8 days and the properties of the emitted radiation of ^{75}Se are indeed very appropriate for such studies. First, the radioactive decay data of this radionuclide allows detection limits that cannot be reached with other analytical methods. Due to the high detection efficiency of radioanalytical methods, single atoms of radionuclides can be identified, whereas other element analytical methods often require a million or more atoms for detection (Adelstein & Manning 1995). The radionuclide ^{75}Se was used for *in vivo* experiments in rats (McConnell 1941, McConnell *et al.* 1959), dogs (McConnell & Cooper 1950, McConnell & Van Loon 1955, McConnell & Wabnitz 1957), mice (Anghileri 1966, Hansson & Jacobsson 1966), bovine (Campo *et al.* 1966), sheep (Muth *et al.* 1967), Escherichia coli (Tuve & Williams 1961), mumps virus (Jensik & Northrop 1971) and other living species. In humans, the application of ^{75}Se was mostly limited to cancer studies using this radiotracer as tumor marker in general (Cavalieri *et al.* 1966) and for the identification of neuroblastoma (D'Angio *et al.* 1969), but the radionuclide was also used in studies on selenium metabolism (Ben-Parath *et al.* 1968, Jereb *et al.* 1975). The use of stable selenium isotopes allowed a more intensive research with human subjects (Janghorbani *et al.* 1981, Janghorbani *et al.* 1982, Veillon *et al.* 1990). The application of ^{75}Se for neurochemical research started with ^{75}Se-labeled selenomethionine (Liwnicz 1968) especially as tool for the investigation of the blood brain barrier and associated questions (Oldendorf & Sisson 1970, Oldendorf *et al.*

1971b, Oldendorf *et al.* 1971a, Steinwall 1969). In most of these cited studies the analyzed physical dimension was the specific activity of the radionuclide in certain tissues, measured with energy dispersive detectors. The size of the typically investigated samples, whole organs, was limited upwards by the geometry requirements of the detector and downwards by the manual skills of the experimenter. In case of the central nervous system, this method allowed only the investigation of gross anatomical regions of the brain. In difference to such bulk analytical approaches autoradiographical methods enable a spatial resolution down to the cellular level.

Although the autoradiography is a well established technique its application in selenium research was mainly concentrated on fractionated protein samples. Here, we presented the application of three different methods (spatial autoradiography, real time autoradiography, selenoproteomic analysis) with especially two aspects which have been improved: The extension of the dynamic range and the reduction of the required exposure time. Whereas the dynamic range of film autoradiography is about 10^2 with only limited linear response, the dynamic range of imaging plates utilizing photoexcited luminescence is 10^4 to 10^5 with linearity over the entire range (Amemiya & Miyahara 1988, Hamaoka 1990, Sonoda *et al.* 1983). Despite the substantial reduction of the exposure time using imaging plates instead of films, both methods cannot facilitate the improvement of a real time system like the MicroImager. In all cases of autoradiographic detection the quantitative evaluation of the measured data linked to histology allows more specific information about the distribution of ^{75}Se than the other detection methods mentioned above. We could show a very specific uptake of selenium-75 in certain brain regions under selenium deficiency. In a comparable approach (Trapp & Millam 1975) the ^{75}Se distribution in the central nervous system was addressed but lack of spatial resolution restricted the measure.

Further important factors for probing the ^{75}Se distribution are the sample preparation procedures. Often overlooked, these steps are important for the correct histochemical localization of diffusible molecules. In order to prevent the spread out of diffusible species (Stumpf 1998) we cryofixated and thaw mounted brain samples, thereby preserving ^{75}Se distribution in a real *in situ* situation. Other aspects for the application of ^{75}Se for *in vivo* radiolabeling to reflect on, both from the radiochemical and biochemical

considerations are the used specific activity and the selenium status of the organism. In this study especially the different selenium status of both animal groups facilitates insights into the selenium homeostasis of the brain.

Selenium homeostasis in the brain is region-specific

The homeostasis of essential elements in the central nervous system is realized on different levels. Their concentrations are maintained on the level of binding proteins, organelles, cells, brain regions and via the bloodstream behind the blood brain barrier linked with the whole body. The deregulations of their concentrations are associated with a number of neurodegenerative diseases. Therefore the investigation of the physiological control of their concentrations is an issue of clinical relevance. Lack of selenium supply causes different severe neurological phenotypes and selenium over-supply leads to toxicity (Schweizer et al., 2004; Chen and Berry, 2003). The facts that it lasts at least three generations in rats fed with a selenium deficient diet to express the phenotype of selenium deficiency, demonstrates that the homeostasis includes also the level above the individual organism.

In confirmation with earlier studies (Burk *et al.* 1972) we could show that the absorbed amount of ^{75}Se into the brain depends on the selenium status of the rat and shows a significant higher quantity in selenium deficient rats. Here, we could show that Se is region-specifically distributed in the brain. There, Se was enriched in certain grey matter regions, in particular hippocampus, cortex, olfactory bulb and in granule cell layers of the cerebellum whereas other neuron-rich structures such as striatum or septum showed only low Se levels. Thus, our data indicate that Se uptake and incorporation follows a certain region specificity indicating a functional specificity of Se and selenoproteins in these brain regions. Recently, we revealed that these regions indeed express also most of the known selenoprotein mRNAs at high level (Zhang et al., 2007).

Remarkable high Se was found in choroid plexus irrespectively of the nutritional Se status. The CSF is produced in the brain by modified ependymal cells in the choroid plexus. A recent study about neuronal and ependymal expression of selenoprotein P (Scharpf et al. 2007) confirms this outcome of our work. The choroid plexus plays a

prominent role in regulation of the homeostasis of other trace elements like zinc (Wang *et al.* 2004) and manganese (Takeda *et al.* 1994). Firstly, the role of SePP for selenium homeostasis in the brain is multifarious. Secondly, there exists a route for the uptake of selenium into the central nervous system independent from SePP. Thirdly, the cerebrospinal fluid is part of the brain selenium homeostasis system (**Fig. 7**). SePP is also expressed in brain cells and the mRNA of SePP is expressed in all the areas of the brain but above all in the cerebellar cortex, hippocampus, and olfactory bulb (Saijoh et al. 1995). The importance of SePP for all regions of mouse brain (Nakayama *et al.* 2007) and hippocampus synaptic function was shown recently (Peters et al. 2006). In concordance with these findings our results confirm a high quantity of ^{75}Se in the hippocampus. The fact that the selenium in the hippocampus was lowered by deletion of SePP but not by selenium deficiency (Nakayama et al. 2007) and that the severe neurological phenotype of SePP deficient mice can be rescued by supplementing their nursing mothers with sodium selenite (Schweizer et al. 2004b) suggest that there is more than the one uptake route of selenium into the brain via the SePP although this selenoprotein plays an important role for maintaining selenium in the hippocampus.

In summary, we could show that Se is distributed in the brain in a region specific manner. We identified in particular the hippocampus, cortex, olfactory bulb, cerebellum and choroid plexus as Se privileged regions which points most probably to the requirement of certain selenoproteins for proper brain function. Under low Se status, increased selenium uptake occurred in all regions of the grey matter as well as Se enrichment in choroid plexus. Proteomic analysis revealed 21 uniquely labeled selenoprotein spots solely present in selenium deficient brains and 4 unique selenoproteins which were mitigated under low selenium status. We further confirmed the down-regulation of GPx1 and GPx4 in neurons following Se deficiency. Thus, this study revealed a unique Se uptake pattern in neurons and identified candidate selenoproteins which are nutritionally regulated.

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Figure legends

Figure 1: Cotranslational selenium incorporation into Selenoproteins. *A*, The trace element selenium is biologically available in form of organic and inorganic chemical species. ***B***, The element is incorporated cotranslationally in the amino acid selenocysteine via its specific tRNA. Selenocysteine is encoded by UGA, a codon acting usually as a stop signal, and by the presence of a stem-loop structure with a characteristic base-pairing pattern present in the 3′ untranslated region of eukaryotic selenoprotein-encoding mRNA, called SECIS element (SelenoCysteine Insertion Sequence). Also mandatory for the insertion of the selenocysteine loaded $tRNA^{Ser(Sec)}$ to the A site of the ribosome are the SECIS-binding protein SBP2 (Copeland *et al.* 2000) and the specific elongation factor mSelB (Fagegaltier *et al.* 2000). In the presented scheme the SECIS element of the mRNA encoding the murine Selenoprotein M (SelM) are colored in yellow. The amino-terminal part up to the selenocysteine of the selenoprotein M, which is highly expressed in the brain (Korotkov *et al.* 2002), is visualized with the biomolecular structure tool Cn3D. Data on protein structure (Ferguson *et al.* 2006) were obtained from the Molecular Modeling Database (Chen *et al.* 2003).

Figure 2: Effects of low selenium diet on total selenium retention and distribution in the brain. *A*, Whole-body ^{75}Se retention in male rats of fed a Torula yeast-based diet with low selenium (Se-) (n = 6) or the same diet with adequate selenium (Se+) (n = 6) measured 3 and 7 days post injection (d.p.i.). For injection, 1.67 x 10^7 Bq in 500 µl aqueous [^{75}Se]-selenite solution with about 1 µg selenium has been utilized. Each bar represents three animals. The values are expressed as a percentage of the whole-body ^{75}Se activity (mean ± SD) directly after the intraperitoneal injection. ***B***, Autoradiogram of representive brain sections from rats with the selenium-adequate diet (Se+) and selenium deficient diet (Se-). Both groups were radiolabeled with ^{75}Se for seven days. Scale bar represents 5 mm.

Figure 3: Neurochemical imaging and analysis of selenium distribution in the brain. Quantitative autoradiographic evaluation of brain sections from selenium-adequate (Se+) and selenium deficient rats (Se-). ***A***, Photomicrograph of a horizontal brain section from a selenium deficient rat, labeled for seven days with ^{75}Se. The Nissl staining was applied after the autoradiographic procedure. Numbers indicate areas of quantitative evaluation. ***B***, Autoradiogram of the same sample. The color code bar illustrates the coding scheme for the ^{75}Se activity. Scale bar represents 5 mm. ***C***, Quantitative analysis of the ^{75}Se activity per area in 11 distinct brain regions of selenium deficient and adequate rats. Note, that especially neuron rich areas (grey matter) such as CA1-3, septum and cortex are enriched in selenium accumulation. The values are expressed as a percentage of ^{75}Se activity/area (mean ± SD).

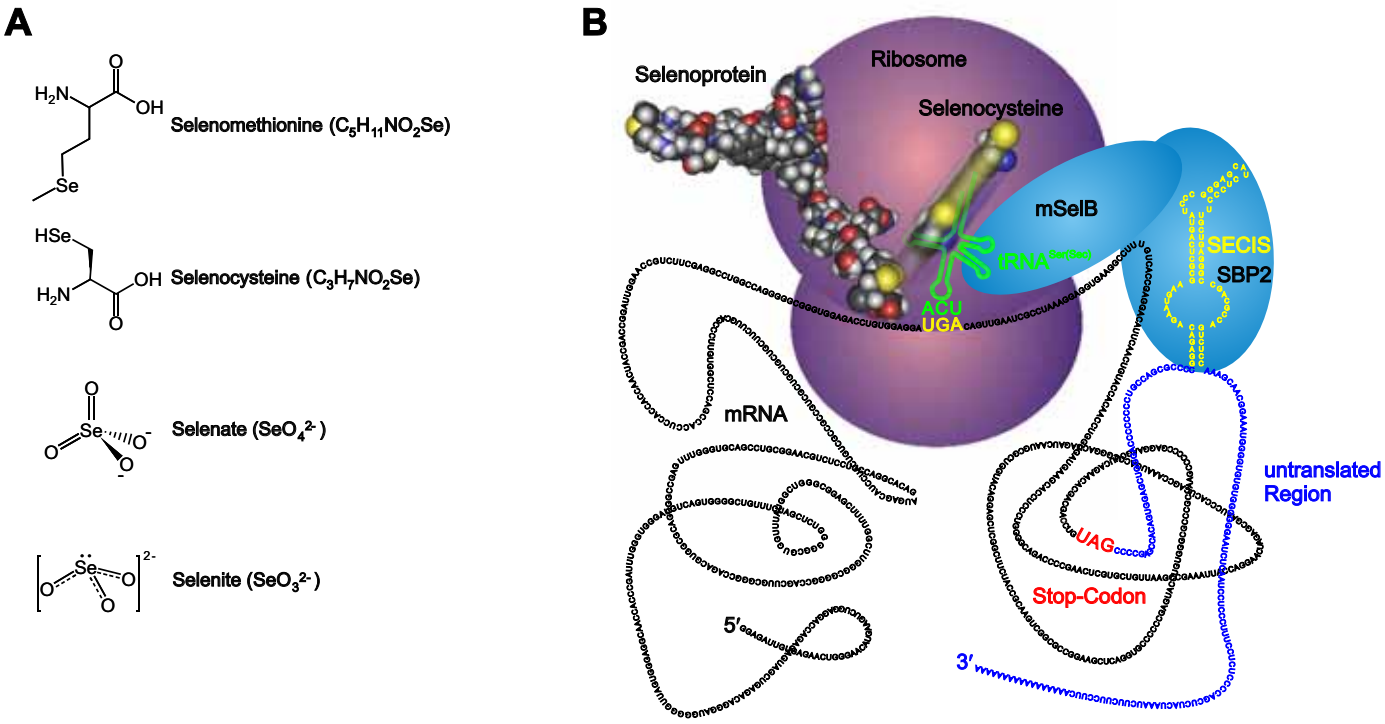
Figure 4: Neurochemical imaging of two selenium privileged organs. *A,* Digital autoradiography of 10 µm thick saggital brain section (144 h exposure time) and ***B***, a coronal brain section (22 h exposure time) analysis with MicroImager system. Note the enriched Se signal in plexus choroid and in neuronal cell layers. ***C***, Autoradiogram of a 12 µm thick section of testis and the epididymidis obtained from selenium deficient rats. The radiotracer ^{75}Se was applied seven days before tissue extraction. The color code bar illustrates the quantitative coding scheme for ^{75}Se activity. Scale bar represents 5 mm. ***D***, Quantitative analysis of the ^{75}Se activity per area in distinct areas of testis in selenium deficient rats. Note, that especially seminiferous tubules show enriched selenium accumulation. The values are expressed as a percentage of ^{75}Se activity/area of three different rats.

Figure 5: Analysis of the brain selenoproteome under low selenium status. *A*, Selenoprotein expression in rat brain determined by two-dimensional gel electrophoresis. After ^{75}Se tracing for five days, total protein separation has been performed and labeled proteins have been detetcted autoradiographically. Comparison of the selenoprotein pattern under selenium deficiency (Se-) and normal Se- status (Se +). ***B***, Analytical autoradiograms after digital filter processing. The blue arrows (numbered 1-12) indicate unique protein spots with are only present in selenium

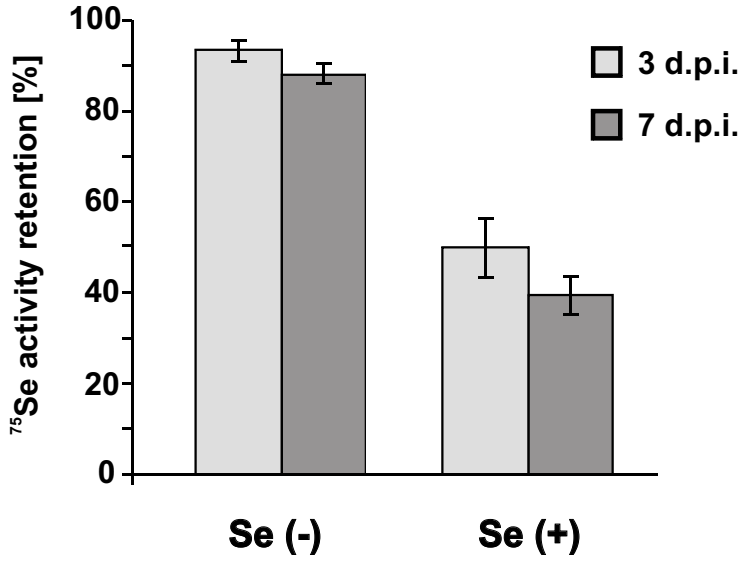
deficient brains. Protein spots which are identical in both groups independent of the selenium status are marked with red arrows. ***C***, Quantification of 12 unique protein spots. Note that protein spots 1, 9 and 10 are significantly decreased under low selenium status, whereas the other unique spots show equal or increased amounts compared to wild type controls.

Figure 6: Regulation of candidate selenoproteins under low selenium status. *A*, Representive immunoblot loaded with hippocampus total protein lysates from selenium deficient (Se-) and wild type selenium adequate rats (Se+) and blotted for selenoprotein glutathione peroxidase 1 (GPx1, 25 kDa). ***B***, Representive immunoblot loaded with hippocampus total protein lysates from selenium deficient (Se-) and wild type selenium adequate rats (Se+) and blotted for selenoprotein phospholipid hydroperoxide glutathione peroxidase 4 (phGPx/ GPx4; 20 kDa). β-Actin serves as a loading control. ***C***, GPx4 profile in the brain. GPx4 is neuron-specifically expressed in the brain. Confocal image of GPx4 immunostained rat brain section. Arrows mark immunopositive pyramidal neurons, arrow heads indicate dendrites. Scale bar represents 10 µm.

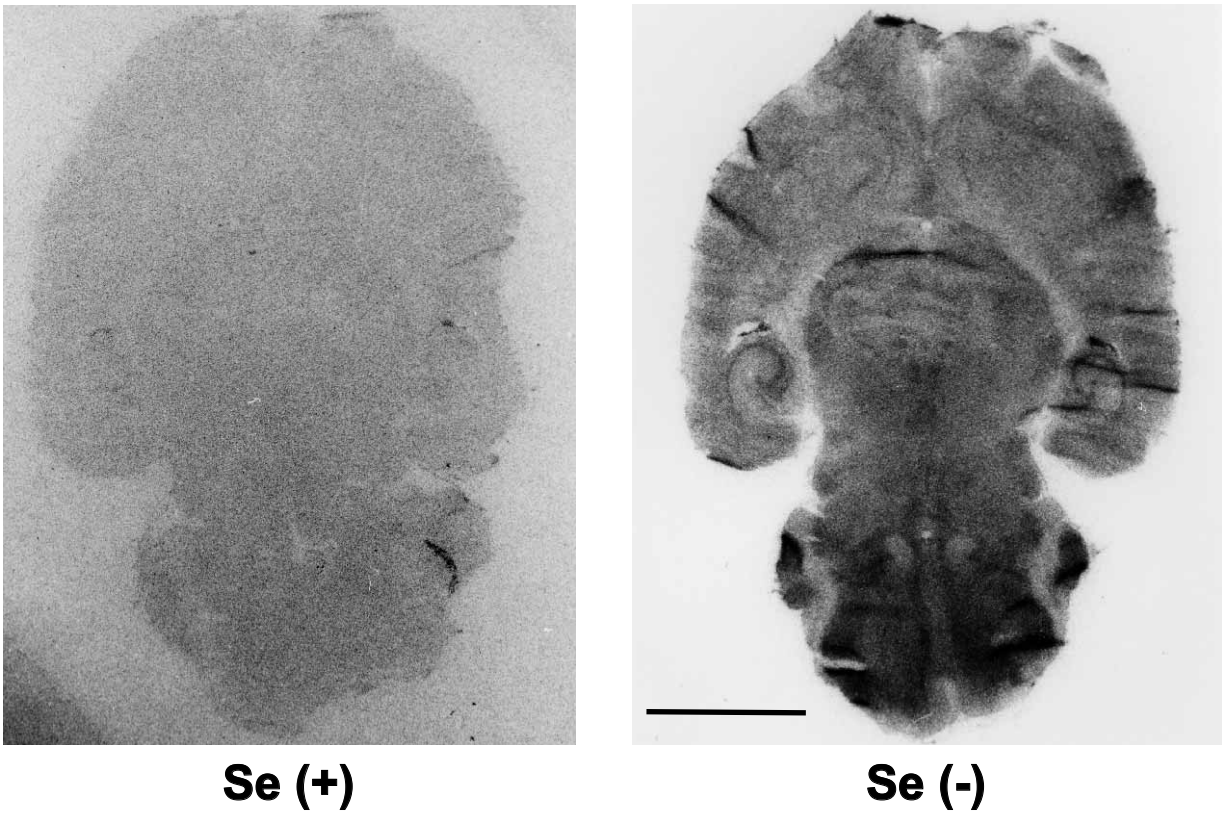
Figure 7: Current model of dietary intestinal selenium uptake, transport and brain uptake and storage. Proposed scheme of the main physiological components involved in the brain specific uptake mechanism of selenium. Dietary selenium compounds (organic as well as inorganic selenium forms) are taken up by intestinal resorption and are metabolized in the liver. There, selenoprotein synthesis such as plasma GPx and Selenoprotein P (SePP) takes place and is secreted into plasma. The brain takes up selenium in a receptor dependent mechanisms involving apoER2. In the choroid plexus, selenoprotein P is expressed and extracellularly supplied to other brain regions via the cerebrospinal fluid. In parallel, selenium is also locally stored in neurons, astrocytes and other glila cells (oligodendrocytes and microglia) in proteinergic forms such as selenoprotein P. In this way, the selenium privileged brain maintains its selenium pool independently of the periphery and retains expression of essential selenoproteins.

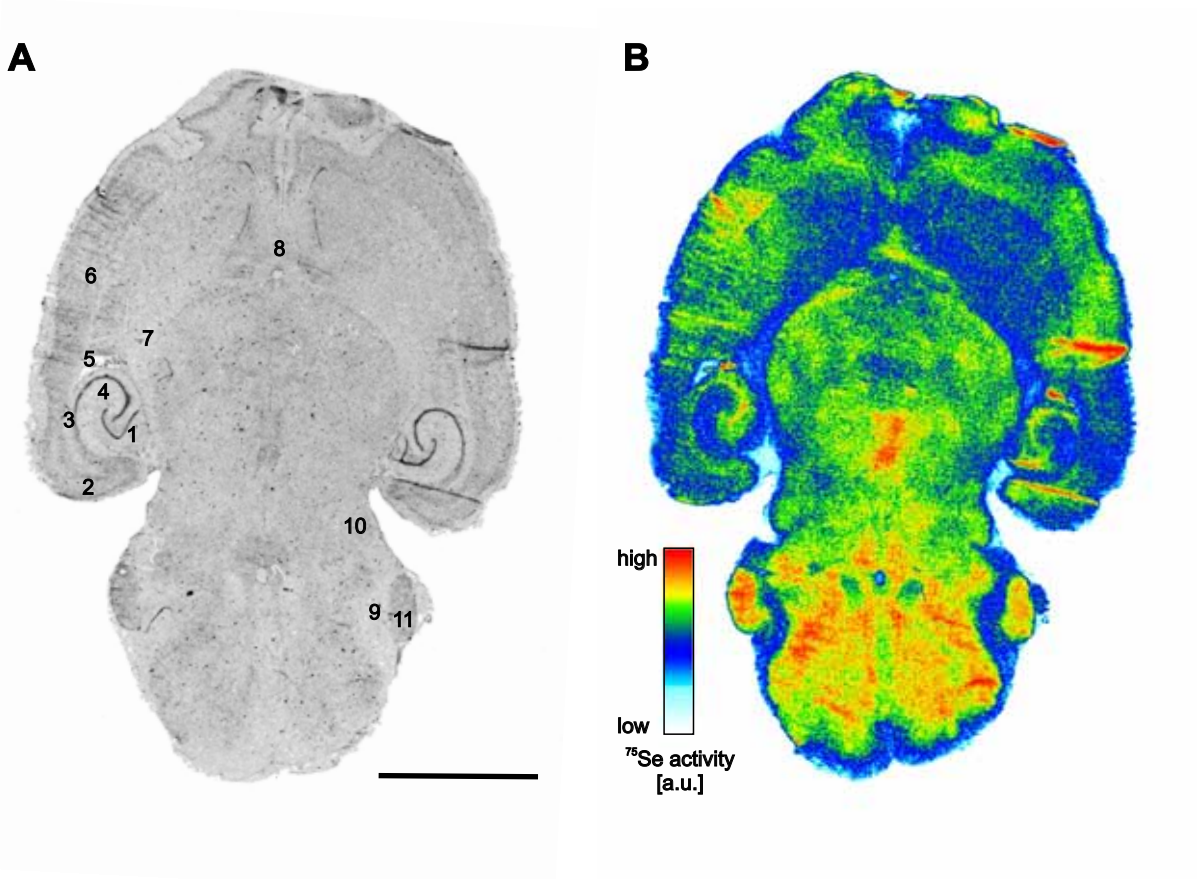

A
Selenomethionine ($C_5H_{11}NO_2Se$)
Selenocysteine ($C_3H_7NO_2Se$)
Selenate (SeO_4^{2-})
Selenite (SeO_3^{2-})
B
Selenoprotein
Ribosome
Selenocysteine
mSelB
tRNA
SECIS
SBP2
mRNA
untranslated Region
Stop-Codon
5′
3′

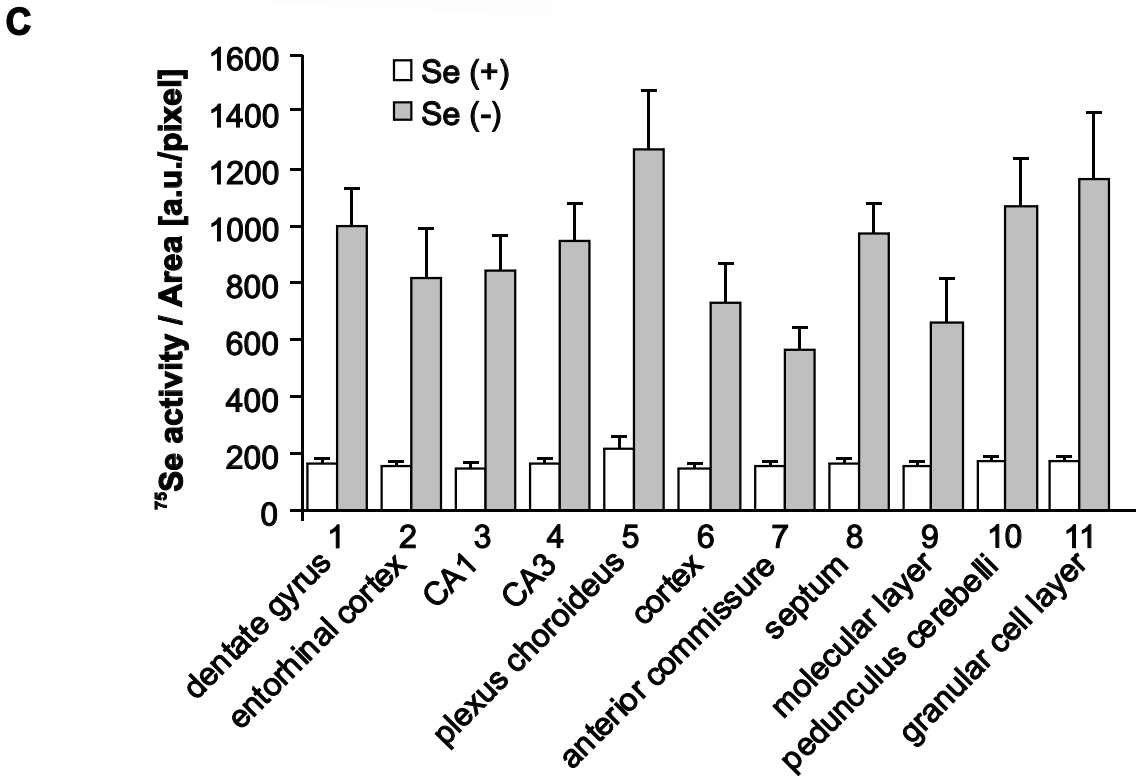
A

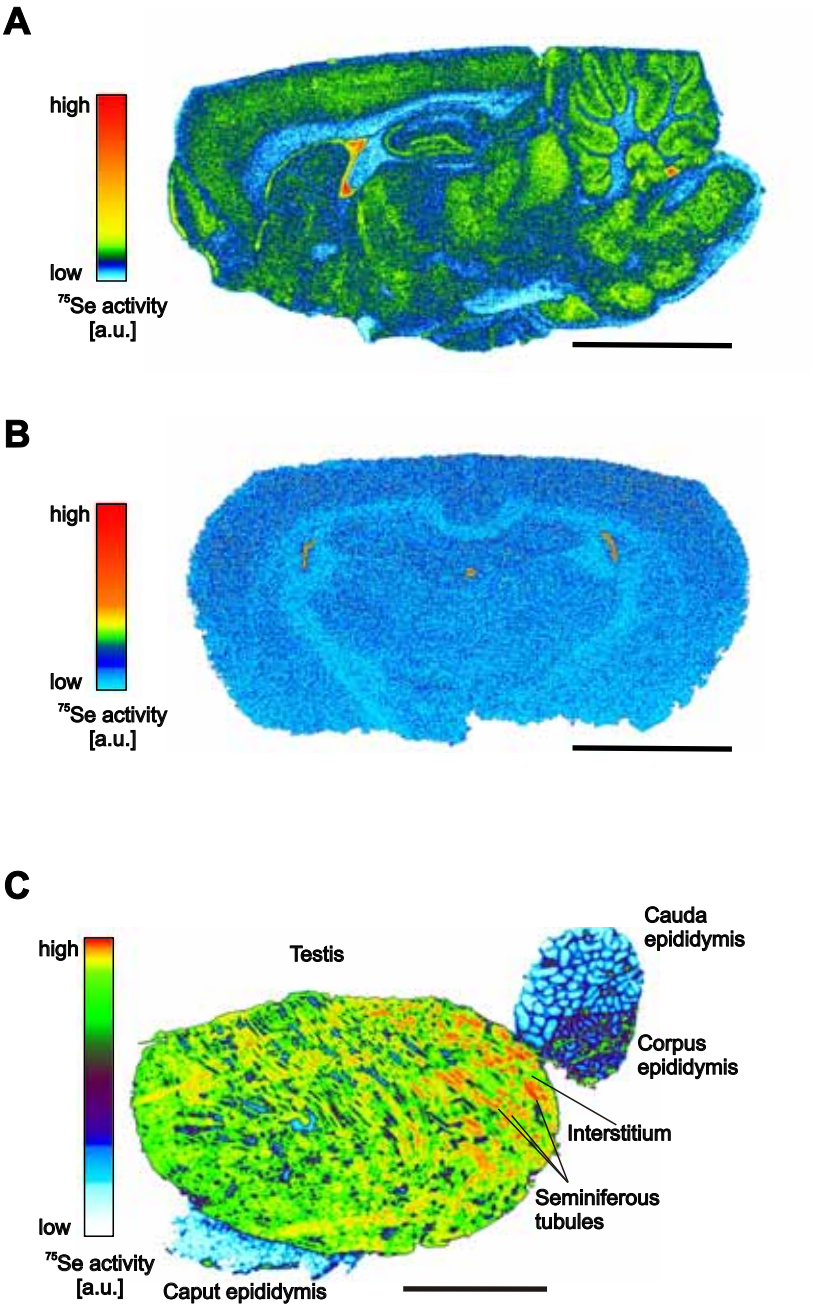


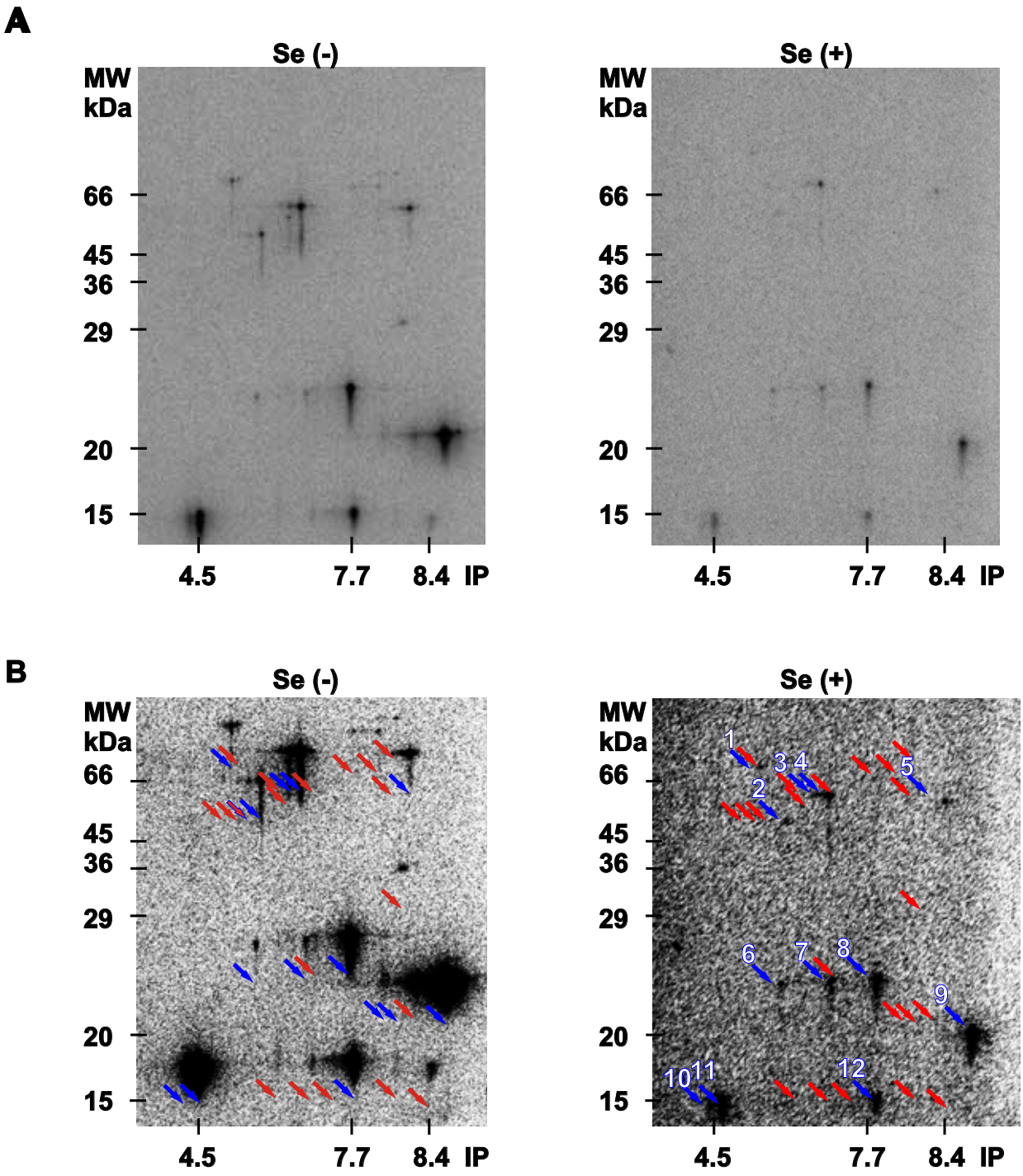
B



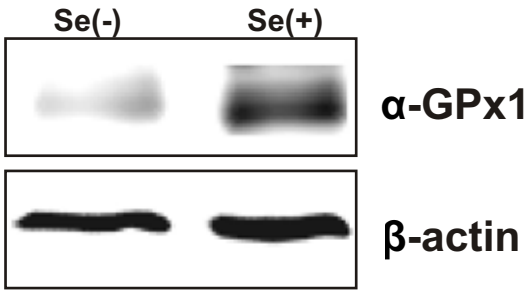

A
B
8
6
7
5
4
3
1
2
10
9
11
high
low
75Se activity
[a.u.]


C
75Se activity / Area [a.u./pixel]
1600
1400
1200
1000
800
600
400
200
0
Se (+)
Se (-)
1
2
3
4
5
6
7
8
9
10
11
dentate gyrus
entorhinal cortex
CA1
CA3
plexus choroideus
cortex
anterior commissure
septum
molecular layer
pedunculus cerebelli
granular cell layer

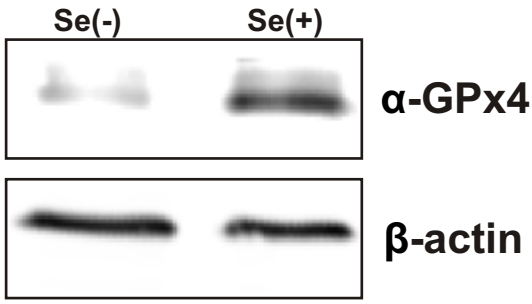

A
high
low
75Se activity
[a.u.]
B
high
low
75Se activity
[a.u.]
C
high
low
75Se activity
[a.u.]
Testis
Cauda
epididymis
Corpus
epididymis
Interstitium
Seminiferous
tubules
Caput epididymis
D
75Se activity / Area [a.u./pixel]
1
0.8
0.6
0.4
0.2
0
Testis
Seminiferous
tub lus
Carput
epididymis
Corpus
epididymis
Cauda
epididymis


A
Se (-)
Se (+)
MW
kDa
66
45
36
29
20
15
4.5
7.7
8.4
IP
B
Se (-)
Se (+)
1
2
3
4
5
6
7
8
9
10
11
12
C
Se (+)
Se (-)
75Se activity [%]
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12

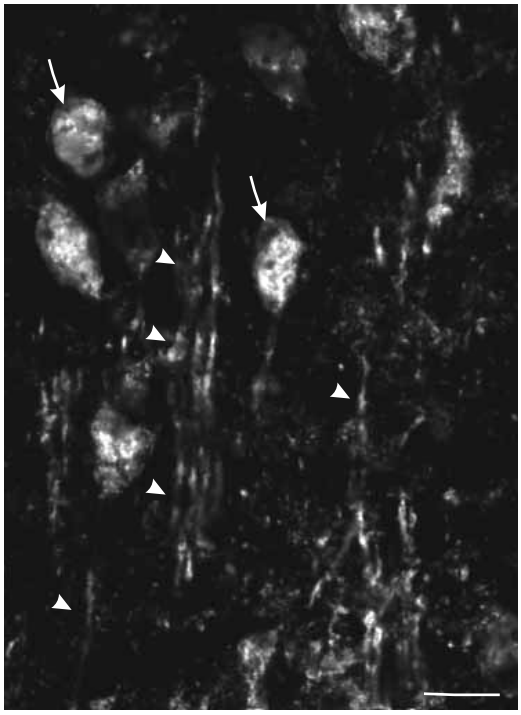
A



B



C



Kühbacher et al., Figure 7