Title

Priorities in the hierarchy of selenium homeostasis: brain region-specific uptake of selenium-75 and expression of selenoproteins in selenium deficient rats

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Abstract

Selenoproteins containing the essential trace element selenium play important roles in the central nervous system. The lack of selenium supply for the selenoprotein expression in the brain or the knock out of their genes leads to severe neurological phenotypes. Although this organ shows not the highest selenium concentration in the case of selenium sufficient supply like other organs it shows a high priority for selenium uptake in the case of dietary selenium deficiency. To characterize this hierarchy of selenium supply in the brain, in vivo radiotracer labeling with ^{75}Se of 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. Like the brain the testis shows a high priority in this selenium status dependent hierarchy and therefore was also investigated. This study shows significant differences in the uptake of ^{75}Se in the brain and testis between selenium deficient and selenium sufficient fed rats. A brain region-specific uptake pattern of the radiotracer ^{75}Se in the rats with the selenium deficient status could be shown. The hippocampus, parts of the cerebellum and the ventricles show a high uptake of the radiotracer.

Short Title: Selenium-75 in selenium deficient rat brains

Key words: autoradiography, brain, radiotracer, rat, selenium, selenoproteins

Introduction

The homeostasis of essential inorganic elements in the central nervous system shows certain differences in comparison with other organs (Spector 1989, Takeda 2004). The element selenium seems to be such an essential micronutrient with a brain specific physiology. But in disparity to most of those other trace elements, which are coordinated to protein ligands, selenium is covalently bound. As component of the amino acid selenocysteine it is cotranslationally incorporated in several selenoproteins which play important roles in brain development and metabolism as recently reviewed (Brauer & Savaskan 2004, Chen & Berry 2003, Schweizer *et al.* 2004a, Schweizer & Schomburg 2006, Whanger 2001). The lack of selenium supply for their expression in the brain, the knock out of their genes (Hill *et al.* 2003, Klivenyi *et al.* 2000, Schomburg *et al.* 2003) or knock down of a single selenoprotein (Savaskan *et al.* 2007) leads to severe neurological phenotypes. In two recent (Ashrafi *et al.* 2007a, Ashrafi *et al.* 2007b) and two former case reports about epilepsy it was shown that a significant reduction of plasma Se and of the activity of the selenoprotein GPx in the blood could be measured in children with intractable seizures (Weber *et al.* 1991) and that similar cases can be treated by Se supplementation (Ramaekers *et al.* 1994). Other rodent studies have shown an association of a low selenium diet with a drug-induced nigral neuronal loss (Kim *et al.* 1999, Kim *et al.* 2000) and susceptibility to glutamate-induced excitotoxicity (Savaskan *et al.* 2003). In contrast the supplementation with selenium results in protective effects against degeneration of neurons by iron (Rubin & Willmore 1980, Willmore & Rubin 1981) and other drug induced neurotoxicity (al-Deeb *et al.* 1995, Imam *et al.* 1999). A dose-dependent neuroprotective effect of selenium was also shown in a rat model of Parkinson's disease (Zafar *et al.* 2003). Like other micronutrients selenium is an important factor for the development of the brain (Georgieff 2007) and is under investigation for more than 30 years now (Kohrle 2000, Loflin *et al.* 2006, Mitchell *et al.* 1998, Prohaska & Ganther 1976).

The micronutrient selenium is ingested in form of four different chemically species (Andersen & Nielsen 1994): selenomethionine, selenocysteine, selenate and selenite (Fig. 1a). Its specific incorporation into the 21st amino acid selenocysteine is conserved in all three taxa of bacteria, archaea and eukaryotes and is encoded by a UGA codon, which normally specifies the termination of translation (Berry *et al.* 1991, Caban & Copeland 2006). A distinctive signal in the mRNA (SECIS for Selenocysteine Insertion Sequence) (Walczak *et al.* 1998) is a key feature in this process (Fig. 1b). Using this feature for bioinformatical screening of sequenced genomes (Castellano *et al.* 2001, Lescure *et al.* 1999) 25 human selenoproteins were detected *in silico* (Kryukov *et al.* 2003). The selenoproteome of the brain consists of several selenoproteins like the glutathione peroxidases (GPx), the deiodinases (D), the thioredoxin reductases (TR) and others. The glutathione peroxidases (GPx) reduce hydroperoxides and control via the glutathione system the cellular redox status (Aguirre *et al.* 2006, Dringen *et al.* 2005, Holmgren *et al.* 2005). The region specific-expression pattern of GPx in mouse brains, expressed in both neurons and glial cells, was proved by immunocytochemical localization (Trepanier *et al.* 1996). The homozygous null mutation of the cytosolic GPx (GPx1) in mice leads to an increased susceptibility to oxidative stress in the cortical neurons (de Haan *et al.* 1998) and a study with knocked out GPx1 in the same animal species showed stronger neurotoxic effects of mitochondrial toxins than in wildtype mice with the expressed antioxidant protein (Klivenyi et al. 2000). The expression of selenoproteins in the brain depends on the supply with the trace

element. One approach to lower this supply is the use of selenium deficient diets (Trapp & Millam 1975). Differences in a kind of hierarchy on the protein expression level of selenoproteins in dependence to the selenium status was found in the activity of GPx and the expression of the selenoprotein W in rat brains (Sun *et al.* 2001). Another approach to lower the brain supply with selenium is the knock out of one its key players. Two key players for the transport of selenium via the blood into the target cells of the brain are identified. One is the selenoprotein P (SePP), a glycoprotein named after its plasma location (Motsenbocker & Tappel 1984) and the first discovered selenoprotein containing more than one selenocysteine (Burk & Hill 2005). Because of the amount of 10 selenocysteines in the full length isoform of SePP in humans, 12 in bovine species (Saijoh *et al.* 1995) and 17 selenocysteines in zebrafish (Tujebajeva *et al.* 2000) the role of SePP for transport of selenium seemed evident from the beginning. *In vitro* studies with neurons (Yan & Barrett 1998) and astrocytes (Steinbrenner *et al.* 2006) as well as *in vivo* experiments with disruption of the encoding gene of SePP (Hill *et al.* 2004, Schomburg et al. 2003, Valentine *et al.* 2005) demonstrated the importance of this protein for the central nervous system. Several recent studies about the role of SePP for the selenium supply of the brain (Hill *et al.* 2007, Peters *et al.* 2006, Scharpf *et al.* 2007) show that there is a brain specific role of this protein. But there is evidence for one more supply route independent on plasma SePP (Schweizer *et al.* 2004b) but dependent on brain cell expressed SePP (Schweizer *et al.* 2005). A second key player for the uptake of selenium into the brain seems to be a receptor (Burk *et al.* 2007) which is also important for the uptake of selenium in testis (Andersen *et al.* 2003, Olson *et al.* 2007).

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 in the case of selenium sufficient supply in comparison 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 at the top of a tissue specific hierarchy in prolonged periods of selenium deficiency suggested especially important functions of selenium in this organ (Behne *et al.* 1988).

The presented work was based on a classic tool in selenium research, the radiotracer ^{75}Se. The application of a radiotracer offers diverse insights in trace element metabolism but has specific requirements and limitations also. Former investigations with the application of ^{75}Se (Pullen *et al.* 1995, Pullen *et al.* 1996, Trapp & Millam 1975) and recent own studies about the selenium uptake and homeostasis in the central nervous were based on manual separation of gross anatomical parts of the investigated brains. In our study we used autoradiographic imaging for the determination of the ^{75}Se-distribution in this organ, 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 of 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.

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 1.3×10^{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. In two experiments a total number of 12 adult male Wistar rats (Charles River, Sulzfeld, Germany) were used. In both experiments two treatment groups, each of three adult male, were kept in cages made of polycarbonate and under an artificial day:night rhythm of 12:12 hours. They were nourished with special Torula yeast-based diets and deionised water ad libitum. One group was fed a selenium-deficient diet containing a maximum of 2–5 µg Se/kg, and was the offspring of parents (preceding generation) who also received the same diet in the 23rd generation in the first and 4th generation in the second experiment. The other group received in both experiments a selenium-adequate diet, which consisted of the same basal diet with 300 µg Se/kg added as sodium selenite (MP Biomedicals, formerly ICN Biochemicals, Cleveland, Ohio, USA) (Behne *et al.* 1982). The analysis of the 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 with about 1 µg selenium. In experiment 1 the eight month old rats with body weights of 520-830 g received an activity of 16.7 MBq per animal. After the injection, three and seven days post injection (d.p.i.) the whole-body ^{75}Se activity was measured *in vivo*. After seven days they were anaesthetized with Isofluran (CuraMED Pharma GmbH, Karlsruhe, Germany) and sacrificed by injection of an overdose of Nembutal (Sanofi Pharma, Munich, Germany). The testes together with the epididymides and the brain were taken, immediately shock frozen in liquid nitrogen and stored at -80 °C. In experiment 2 six month old rats with body weights of 400-580 g received an activity of 7 MBq per animal and were killed five days after the injection.

For the investigations performed on brain and testis tissue, two samples from each animal 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 with cresyl violet was applied.

The tissue samples for the analysis by two-dimensional (2D) electrophoresis were homogenized in two steps at 4 °C by means of an electronically controlled handheld disperser (Polytron PT 1300, Kinematica AG, Littau-Luzern, Switzerland) and an 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 cytosolic 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 as described elsewhere (Bradford 1976) 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). In the 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) an the gels were dried between sheets of cellophane in a gel-drying device (Biometra biomedizinische Analytik GmbH, Göttingen, Germany).

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.

Autoradiography

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.

The 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.

The slides with the dried cryosections of both investigated tissues 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. With the system, based on the optical detection of scintillation signals by a CCD camera, an imaging area of 24 × 32 mm with a spatial resolution of 20 µm and a pixel size of 5 µm was obtained. In contrast to the methods mentioned above, the MicroImager is a real time system. 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 shown in the next paragraph amounted to 22 h and 144 h. The autoradiographic image was visualized with the ß-Vision software (Biospace).

Results

The two groups of rats ($n_{total} = 12$) fed with special Torula yeast-based diets, containing different amounts of sodium selenite, showed a significant difference in the retention of the injected ^{75}Se activity. In figure 2 the retention of the whole-body ^{75}Se activity, measured *in vivo* 3 and 7 days post injection in experiment 1 and 5 days post injection in experiment 2, is shown. The values are expressed as a percentage of the whole-body ^{75}Se activity directly after the intraperitoneal injection. Each bar represents three animals. The selenium deficient rats of both experiments kept back up to 90 % of the applied activity, while rats, fed with the selenium-adequate diet, excreted about 60 % in experiment 1 and about 35 % in experiment 2. In the selenium-deficient rats most of the metabolized selenium is not excreted but is reutilized and incorporated again into the selenoproteins (Behne & Hofer-Bosse 1984). ^{75}Se administered to the deficient animals, is distributed relatively fast in the same way as the native selenium among the selenoproteins.

Like the difference in retention between the two groups of animals, they showed also significant dissimilarity in the autoradiogaphic images of testis and brain as presented in figures 3-6. The autoradiograms of tissue sections from the animals fed with the selenium-adequate diet show ubiquitous distribution as well as less activity in contrast to the samples of the selenium deficient rats. The quantitative autoradiographic results of a 12 µm thick section of the testis and the epididymidis from a selenium deficient rat, labeled for seven days with ^{75}Se are presented in table 1. The values of the ^{75}Se activity per area show significant differences for the testis and the three main parts of the epididymidis. Considering the variations of the histological tissue structures, especially the different diameters of the tubules, the color coded autographic image in figure 3 has even more evidence for the higher activity of ^{75}Se in the testis in comparison with the epididymidis. The highest ^{75}Se activity per area was located in the seminiferous tubules. Within the same seminiferous tubule cross-section it was possible to differentiate between the lumen and the peripheral edge of the tubule, but due to the thickness of the sample the resulting resolution of the autoradiography was not sufficient enough to visualize single spermatozoae.

While the distribution of ^{75}Se in the testis shows no specific variations between different seminiferous tubules and can be described as homogeneously, the autoradiographic images of the ^{75}Se distribution in the brain sections show not only quantitative differences. The pattern of the distribution in the brain of the selenium deficient rat shows a brain region-specific presence of the radiotracer.

In figure 4 the autoradiograms of 12 µm thick brain sections from a rat fed with the selenium-adequate diet and a selenium deficient rat are illustrated in greyscale. Both rats were radiolabeled with ^{75}Se for seven days. The autoradiogram of the section from the selenium-adequate nourished rat provides proof for a lower uptake and a quite ubiquitous distribution of the radiotracer in comparison with the sample of the selenium deficient rat. Only the ventricles show higher ^{75}Se activity.

Focusing on the main finding of our study, the brain region-specific uptake of ^{75}Se in selenium deficient rats, a quantitative autoradiographic evaluation is presented in figure 5. The photomicrograph shows a Nissl stained horizontal brain section from a selenium deficient rat, labeled for seven days with ^{75}Se. The color coded autoradiogram of the same sample clarifies the specificity of the ^{75}Se uptake. The red color indicates regions with high ^{75}Se activity while pale blue to white encodes for a low activity of the radiotracer. In general shows white matter less presence of ^{75}Se than grey matter does. In the hippocampal formation a higher activity was present in the molecular layer of the dentate gyrus as well as in the cornu ammonis. The entorhinal cortex showed intermediate levels of activity. Very high ^{75}Se activity was located in the lateral ventricles were cerebrospinal fluid (CSF) was present, being the highest ^{75}Se activity per area measured. In the cerebellum high levels of activity were found with lower values in the lamina medullaris. The artifacts with high ^{75}Se activity per area caused by tissue wrinkling can be identified in the image of the Nissl staining.

The comparative evaluation of 11 specified brain regions in autoradiograms from in total 23 horizontal brain sections is summarized in the column diagram of figure 5. The highest ^{75}Se activity per area was measured in the ventricle. The brain sections from the selenium deficient rat show averaged 5.7 times as much uptake of ^{75}Se than the sections of the selenium-adequate rat, calculated by quantifying the blackening of the autoradiograms and subsequent normalizing to the background signals.

The results of the digital autoradiography with the MicroImager system are shown in figure 6. Samples were taken from two selenium deficient rats of experiment 2. Both were labeled for five days with ^{75}Se. The autoradiogram of the 10 µm thick sagittal brain section, which was exposed for 144 hours, confirms the results of experiment 1. The brain regions with high amounts of ^{75}Se, especially the hippocampus, distinct cell layers of the cerebellum and the ventricles filled with cerebrospinal fluid, are the preferential targets of the brain selenium supply in selenium deficient rats. Due to the shorter exposure time of 22 hours, the autoradiogram of the coronal brain section shows a weaker contrast. But the highest ^{75}Se activity can also be localized in the third ventricle and the lateral ventricles.

In order to obtain information about the differences of the selenoprotein expression pattern in the brain in dependence on the selenium status of the rats, we applied a non-equilibrium pH gradient gel electrophoresis (NEPHGE) in combination with SDS-PAGE for 2D electrophoresis. Rats with different selenium status of experiment 2, which were labeled for five days with ^{75}Se, were investigated. The figure 7 shows the autoradiograms of the labeled cytosolic brain proteins of a selenium deficient rat and a selenium-adequate nourished rat, following 2D electrophoresis. After visualizing weakly labeled protein spots by digital image processing, a total number of 33 labeled protein spots, marked with blue arrows, could be identified in the autoradiogram of the selenium deficient rat sample. In comparison the autoradiogram of the cytosolic brain sample from the rat fed with the selenium-adequate diet all ^{75}Se-labeled protein spots showed less intensity. After image processing, a total number of 12 labeled protein spots could be distinguished. The results of the quantitative evaluation of the ^{75}Se activity in these protein spots in both autoradiograms are listed in figure 8. The quantitative evaluation of the signal intensity of the protein spots was achieved with the densitometry module of the AIDA software. The relative radiotracer content of the labeled proteins in percent was estimated by the degree of blackening in the autoradiograms. Comparing the relative amount of the ^{75}Se activity in percent in each protein spot in both gels, no general disparity between the rats with different selenium status could be found. In both animals with different selenium status the protein spots 8 and 9 show the highest amount of ^{75}Se activity.

Discussion

Radiolabeling 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 very appropriate for such studies. 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). Although autoradiographic methods still do not reach the efficiency of a borehole detector, the technological progress for the spatial localization of radionuclides in the last decades was significant. The shown results of different autoradiographic methods for the localization of ^{75}Se will be discussed in the following regarding former life science related applications of ^{75}Se.

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 about the selenium metabolism, like in (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). Further research was focused on the selenium itself (McFarland *et al.* 1970). 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 was already established in the first half of the last century its application in selenium research was few in number. The presented application of three different methods demonstrates the progress of the autoradiographic imaging capabilities of radiotracers in the last years. Especially two aspects 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 photostimulable 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 the lack of spatial resolution restricted the outcome. Regarding the limited knowledge about selenoproteins at the time that work was published, the findings about the prompt uptake and retention of ^{75}Se by the brain of selenium deficient rats indicated already a special uptake mechanism of this trace element into the brain. Although using the same tool, the radiotracer ^{75}Se, the new findings of our work are based on the key feature of the applied autoradiographic techniques, the spatial resolution.

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) the samples were cryofixated and thaw mounted, so that the shown distribution of ^{75}Se should represent the 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

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. Like for other essential elements the dose concept of Paracelsus (Deichmann *et al.* 1986) applies also to the trace element selenium. The lack of selenium supply causes different severe neurological phenotypes and selenium over-supply leads to toxicity. The finding, that it takes 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. The selenium homeostasis is a complex issue and has been investigated with element analytical methods as well as with ^{75}Se. The selenium homeostasis is age dependent (Archimbaud *et al.* 1993, Ostadalova *et al.* 1982) and shows circadian oscillations (Eakins 1979). One approach is the investigation of the time dependent retention of the applied ^{75}Se in different tissues (Thomassen & Leicester 1964). Using a radiotracer, the method of application is of importance. Because the oral administering of ^{75}Se leads to a time delayed resorption in the gastrointestinal system, we used the intraperitoneal injection in order to get a faster uptake.

In confirmation with earlier studies (Burk *et al.* 1972) we could show that the uptaken 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. The new information presented here about the distribution of ^{75}Se in selenium deficient rats is the specificity of the uptake of different brain regions. The common finding about selenium physiology in the rat reported in other publications about the highest retention of ^{75}Se in the brain in comparison with other tissues (Brown & Burk 1972), about the maintained level of selenium in the brain in distinction to blood after feeding a selenium-deficient diet (Prohaska & Ganther 1976) and about the preferential supply of the brain at the top of a tissue specific hierarchy with this element in prolonged periods of selenium deficiency (Behne *et al.* 1988) is now specified and illustrated with a chemical image of the selenium physiology in the brain of this animal. Not the brain as a whole organ but distinct brain regions show this preferential uptake of selenium in case of selenium deficiency. Although differences in the selenium concentrations in diverse adult human brain regions (Demmel *et al.* 1980, Ejima *et al.* 1996, Hock *et al.* 1975) and some gross anatomical differences in the retention (Trapp & Millam 1975) and distribution (Prohaska & Ganther 1976) of ^{75}Se in the rat brain were already reported, indicating selenium content of the brain was highest in regions containing the most grey matter (Chen & Berry 2003), the outcome of our autoradiographic study is the visualization and quantitative evaluation of the selenium homeostasis in the brain at the histological level.

The investigation of the brain specific selenium uptake mechanism with transgenic animal models includes the SePP (Schweizer *et al.* 2004c) as well as the second key player necessary for this pathway, a receptor (Burk et al. 2007) which is also important for the uptake of selenium in testis (Andersen et al. 2003, Olson et al. 2007). The key players for selenium transport to different organs, SePP and its putative receptor, are in the focus of the selenium research for the last years. Therefore numerous findings of different studies have to be taken into account reflecting their findings with respect to the results of our autoradiographic imaging of the ^{75}Se

distribution in the rat brain. In conclusion some assumptions can be made. 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.

Due to the fact that our results present a snapshot of the selenium metabolism in the brain five days after radiolabeling the rats, the time frame covers the turnover rates of different selenoproteins. The plasma half life of ^{75}Se in SePP is 3 to 4 h, indicating a rapid turnover (Burk & Hill 1994). In order to investigate the uptake route of selenium into the brain via SePP in more detail, the time of radiolabeling in the follow up study has to be adapted. But 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.

One of our main findings is the significant role of the cerebrospinal fluid for the selenium homeostasis in the brain. Earlier studies about the selenium content in the CSF are mostly limited to its relation with neuronal diseases showing no correlation like Alzheimer (Meseguer *et al.* 1999), motor neurone disease (Mitchell *et al.* 1984), cerebral neoplasms (El-Yazigi *et al.* 1984) and other neuronal diseases of children (Haas *et al.* 1987) or showing increased selenium levels in non treated Parkinson patients (Aguilar *et al.* 1998, Qureshi *et al.* 2006).

The CSF is produced in the brain by modified ependymal cells in the choroid plexus. Therefore the choroid plexus will be investigated in relation to its role for selenium homeostasis. 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).

In figure 9 we reveal our proposed mechanism scheme of the selenium uptake into the brain. In order to understand the real neurochemical machinery behind the selenium physiology of the brain further aspects have to be addressed. Recent research findings confirm gender related differences for the uptake (Minami *et al.* 2002, Riese *et al.* 2006) and role of selenium in the brain: As a result of a study about the polymorphisms in the human selenoprotein P gene, a gender specific response of the expression of several important selenoproteins to selenium supplementation was found (Meplan *et al.* 2007). Therefore the role of sex and the related role of hormones will be investigated. Questions like: Is there a specific selenium receptor in the brain? Is the expression of such a receptor under hormonal control, signalling the selenium status and also linked with gender? might be answered with the use of the radiotracer ^{75}Se that is still not an old fashioned but valuable tool for selenium research.

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Tables

Tab. 1 Quantitative evaluation of the autoradiogram of a 12 µm thick section of a testis and the epididymidis from a selenium deficient rat, labeled for seven days with ^{75}Se. The values of the ^{75}Se activity per area show significant differences for the testis and the three main parts of the epididymidis.

Figure legends

Fig. 1 (a) Selenium is a rare essential trace element which is consumed in form of different chemical species. (b) The element is incorporated cotranslationally in the amino acid selenocysteine via a 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 shown schematic example for the cotranslational insertion of selenocysteine the UGA codon and the SECIS element of the mRNA encoding the selenoprotein M in mice are colored in yellow. The amino-terminal part up to the selenocysteine of the selenoprotein M, which is highly expressed in the mammalian brain (Korotkov *et al.* 2002), is visualized with Cn3D, a visualization tool for biomolecular structures. The protein structure data (Ferguson *et al.* 2006) were obtained from the Molecular Modeling Database (Chen *et al.* 2003).

Fig. 2 Whole-body ^{75}Se retention (mean ± SD) in male rats of two experiments fed a Torula yeast-based diet with low selenium (n = 6) or the same diet with added selenite (n = 6) measured 3 and 7 days post injection (d.p.i.) of 1.67 x 10^7 Bq in 500 µl [^{75}Se]-selenite solution with about 1 µg selenium in each animal of both groups in experiment 1 and 5 days post injection of about 7 x 10^6 Bq of the equivalent solution in each rat of experiment 2. Each bar represents three animals. The values are expressed as a percentage of the whole-body ^{75}Se activity directly after the intraperitoneal injection.

Fig. 3 Autoradiogram of a 12 µm thick section of a testis and the epididymidis from a selenium deficient rat, labeled for seven days with ^{75}Se. The color code bar illustrates the coding scheme for the ^{75}Se activity. The black bar represents 5 mm.

Fig. 4 Autoradiograms of 12 µm thick brain sections from a rat fed with the selenium-adequate diet and a selenium deficient rat. Both rats were radiolabeled with ^{75}Se for seven days. The black bar represents 5 mm.

Fig. 5 Quantitative autoradiographic evaluation of brain sections from a rat fed with the selenium-adequate diet and a selenium deficient rat: (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. The numbers indicate the quantitative evaluated brain regions. (b) Autoradiogram of the same sample. The color code bar illustrates the coding scheme for the ^{75}Se activity. The black bar represents 5 mm. (c) Column diagram of the ^{75}Se activity per area in the specified brain regions.

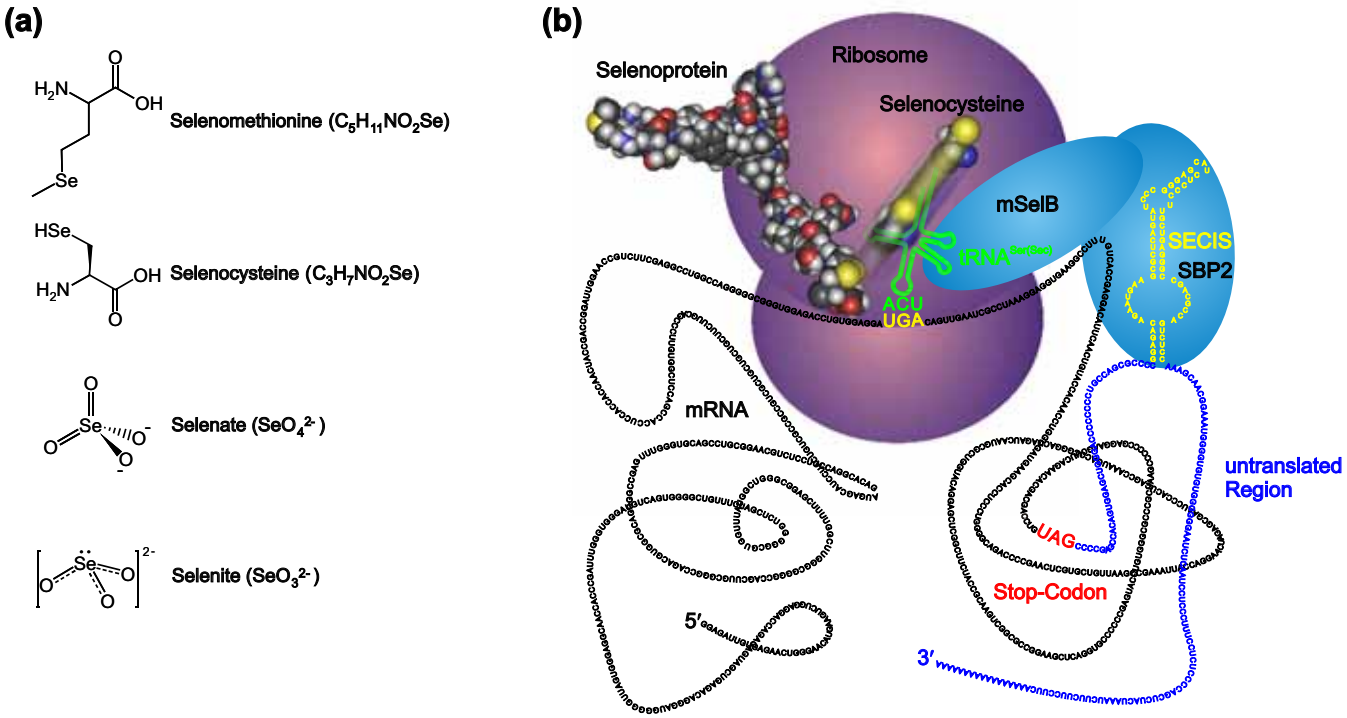
Fig. 6 Digital autoradiography with the MicroImager system (Biospace Mesures, Paris, France): Autoradiograms of 10 µm thick (a) sagittal brain section (144 h exposure time) and (b) coronal brain section (22 h exposure time) from a selenium deficient rat, labeled for five days with ^{75}Se. The color code bar illustrates the coding scheme for the ^{75}Se activity. The black bar represents 5 mm.

Fig. 7 Selenoprotein expression in rat brain determined autoradiographically by labeling rats *in vivo* with ^{75}Se for five days and two-dimensional protein separation of cytosolic proteins. (a) Selenoprotein pattern in a selenium deficient rat (Se (-)) and in a rat fed with the selenium-adequate diet (Se (+)). (b) Autoradiograms after application of digital filter processing. The red arrows indicate those protein spots labeled with ^{75}Se, which could be identified only in the brain cytosol of the selenium deficient rat.

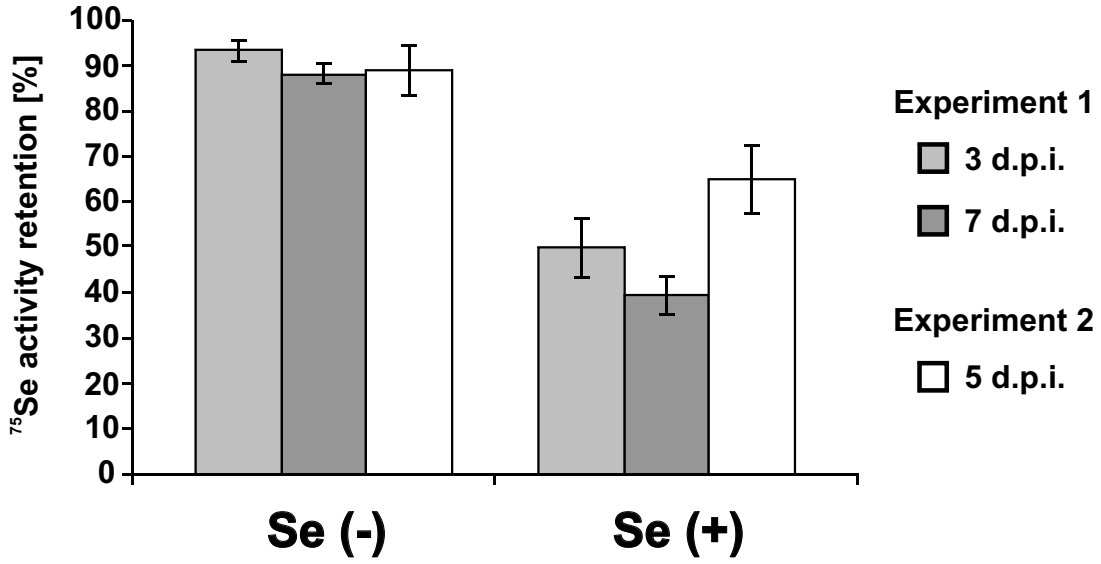
Fig. 8 Comparison of the quantitative expression patterns of selenoproteins identified in a rat fed with the selenium-adequate diet (indicated by blue numbers in figure 7) with their corresponding proteins in a selenium deficient rat. The relative radiotracer content of the labeled proteins in percent was estimated by the degree of blackening in the autoradiograms.

Fig. 9 Proposed scheme of the main physiological components involved in the brain specific uptake mechanism of selenium.

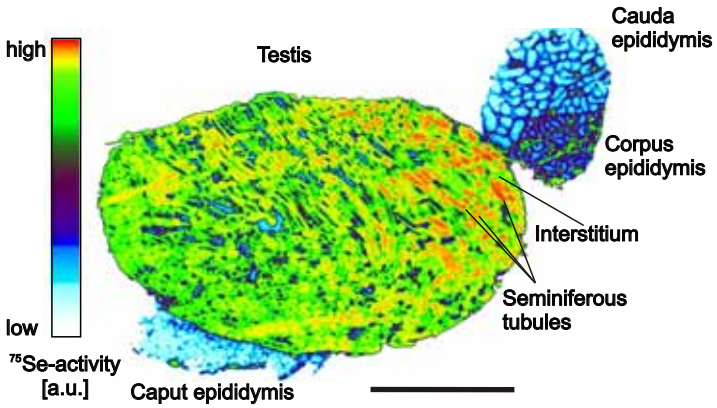
Kuehbacher et al., Fig. 1



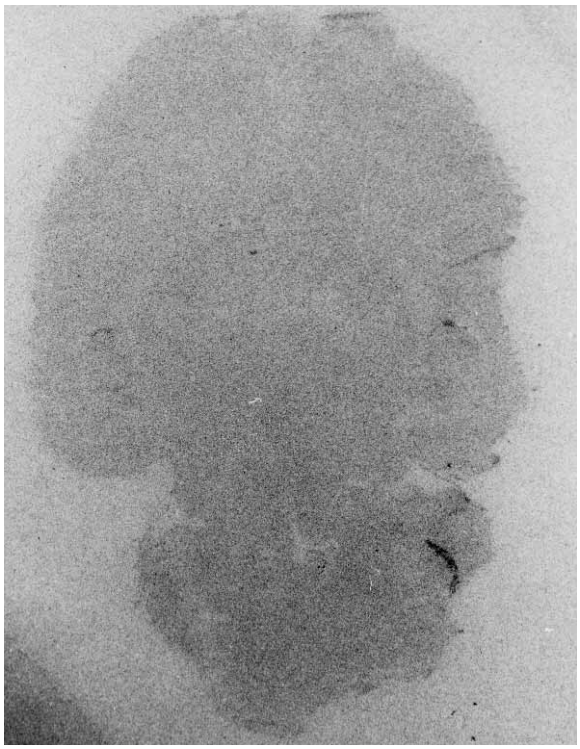
Kuehbacher et al., Fig. 2



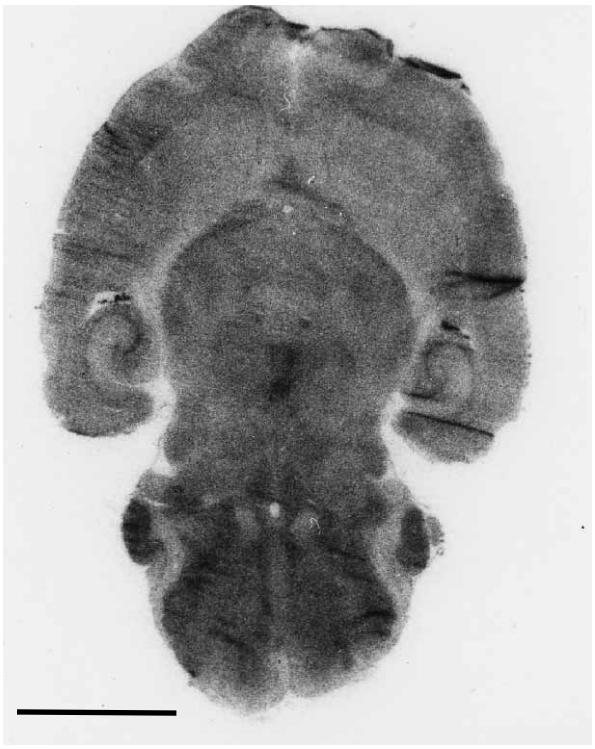
Kuehbacher et al., Fig. 3

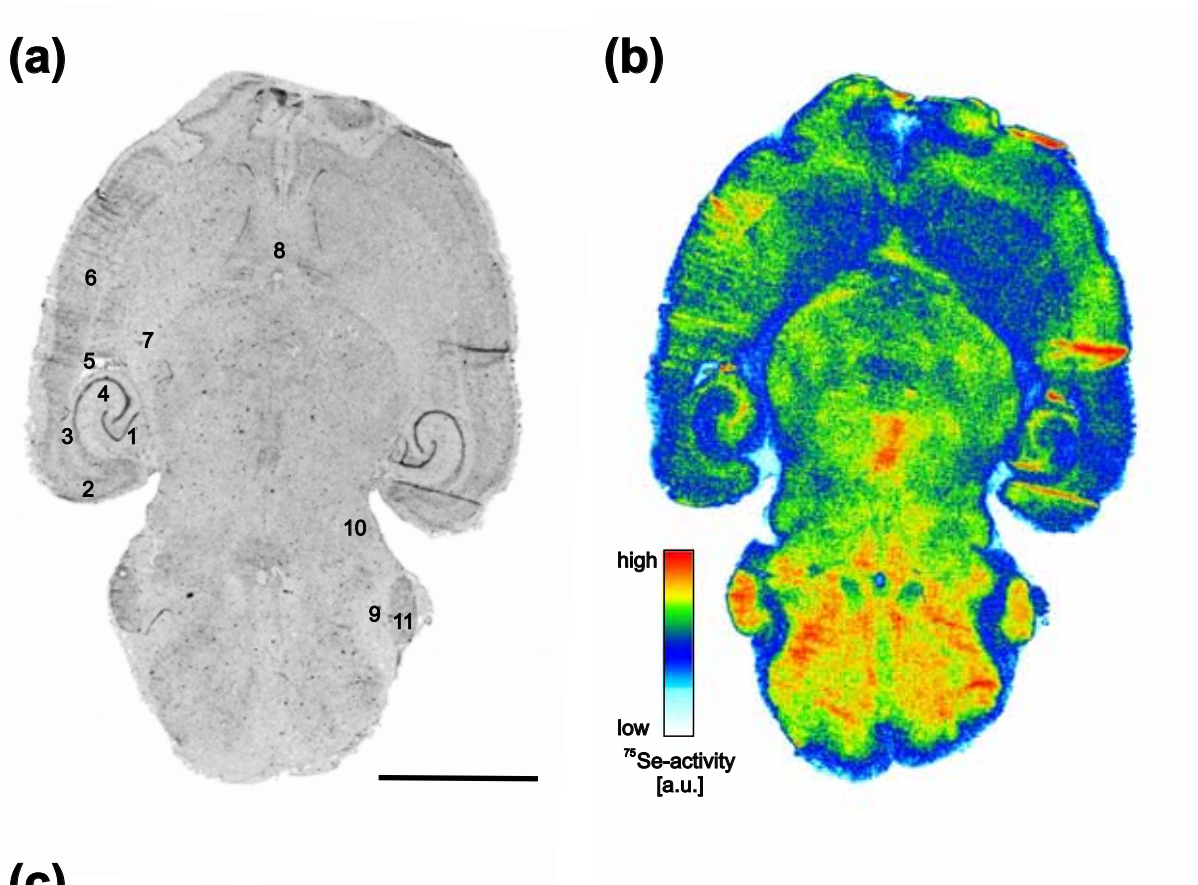


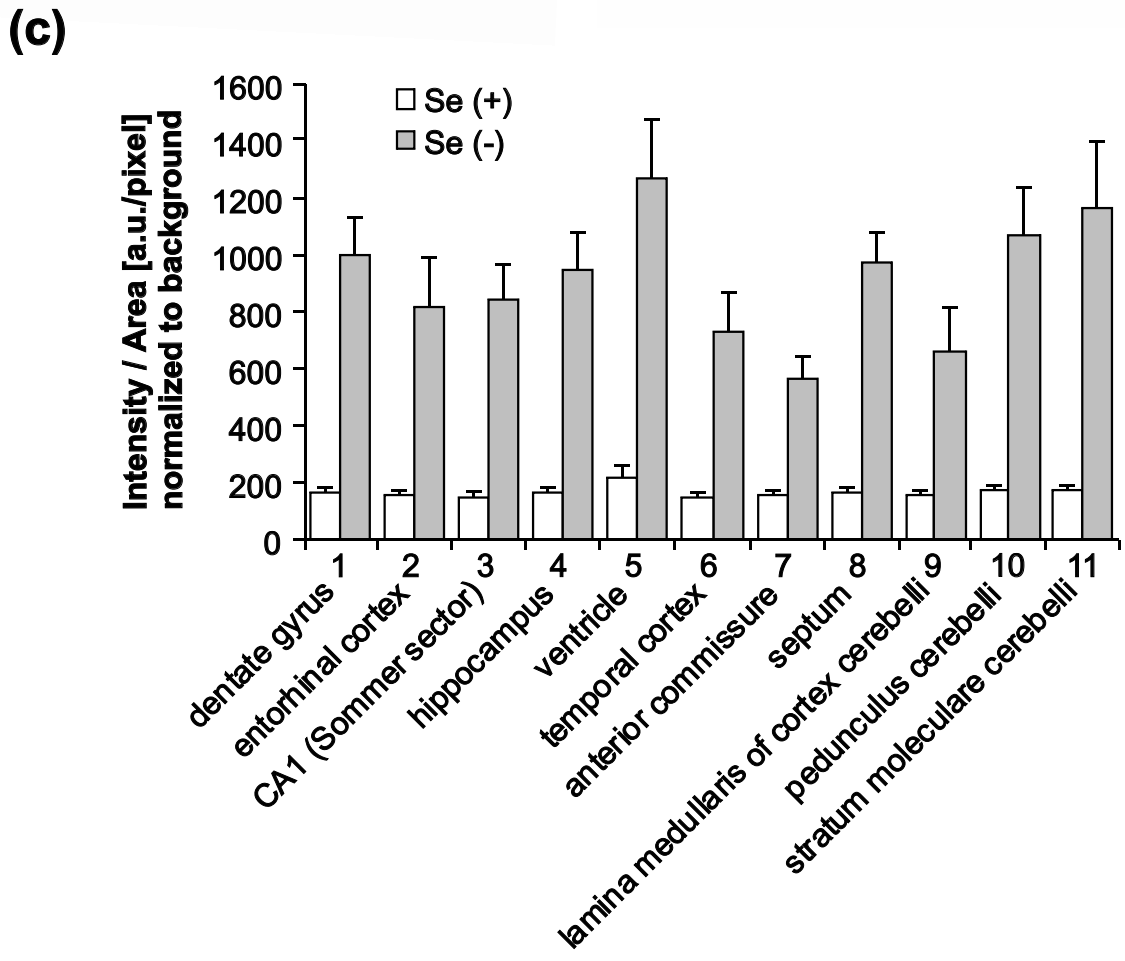
Se (+)

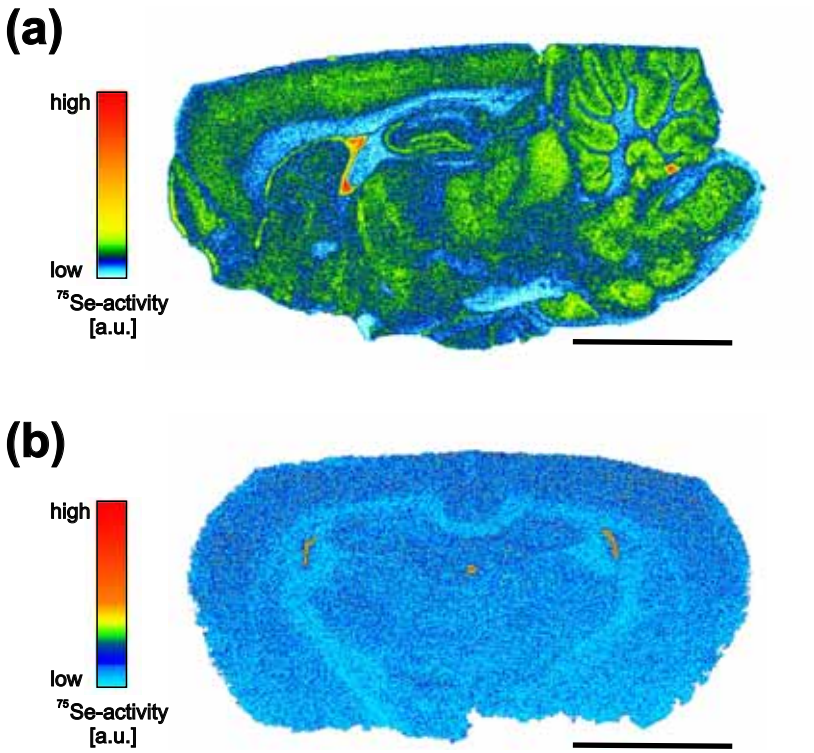


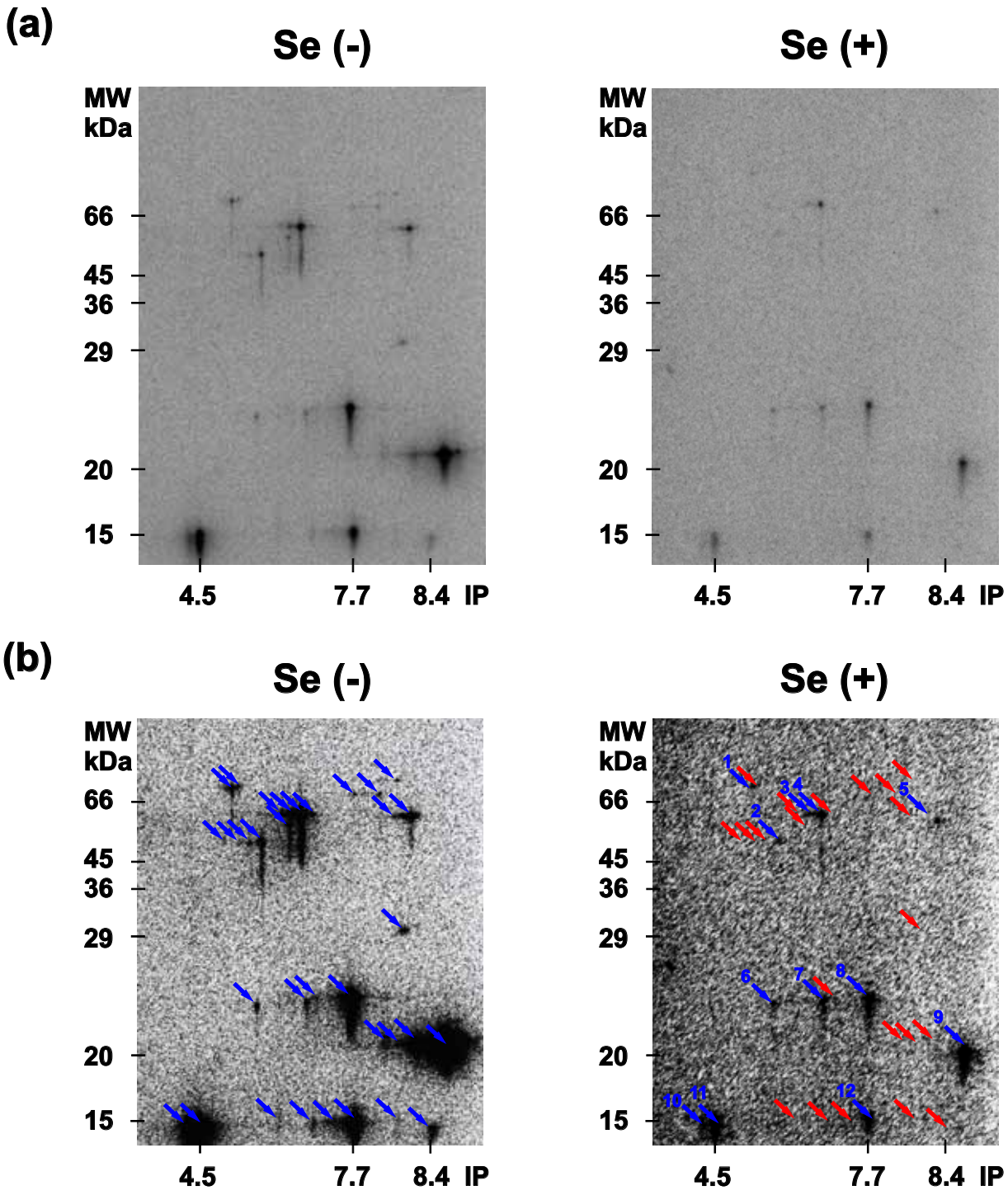
Se (-)



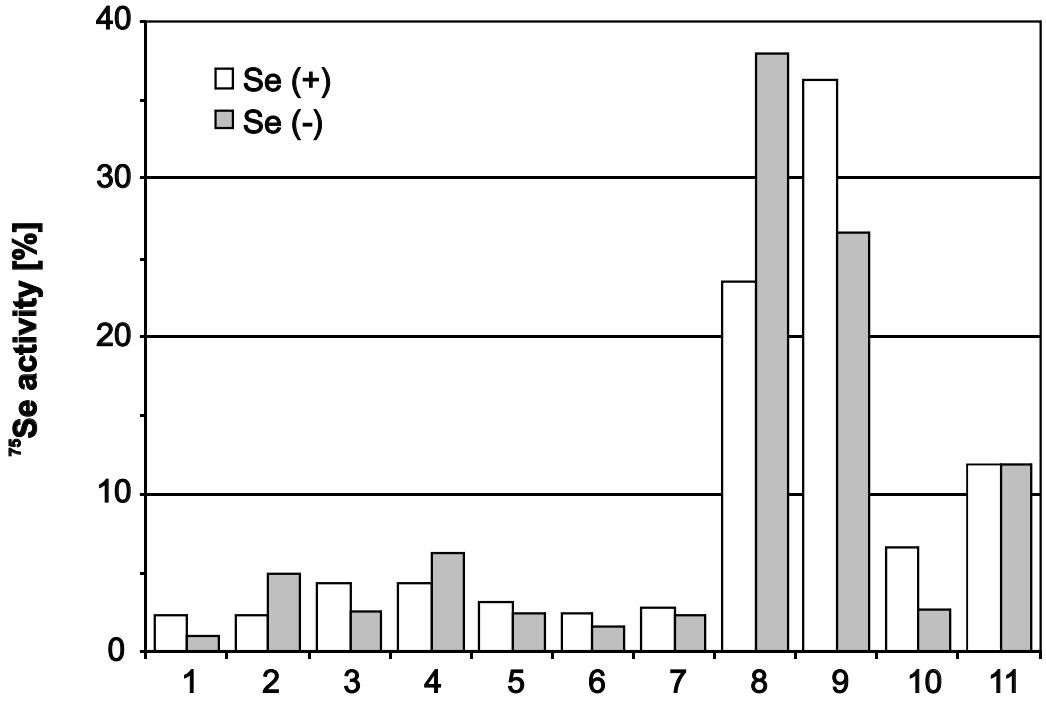

(a)
(b)
1
2
3
4
5
6
7
8
9
10
11
high
low
75Se-activity
[a.u.]


(c)
Intensity / Area [a.u./pixel]
normalized to background
Se (+)
Se (-)
1600
1400
1200
1000
800
600
400
200
0
1 dentate gyrus
2 entorhinal cortex
3 CA1 (Sommer sector)
4 hippocampus
5 ventricle
6 temporal cortex
7 anterior commissure
8 septum
9 lamina medullaris of cortex cerebelli
10 pedunculus cerebelli
11 stratum moleculare cerebelli

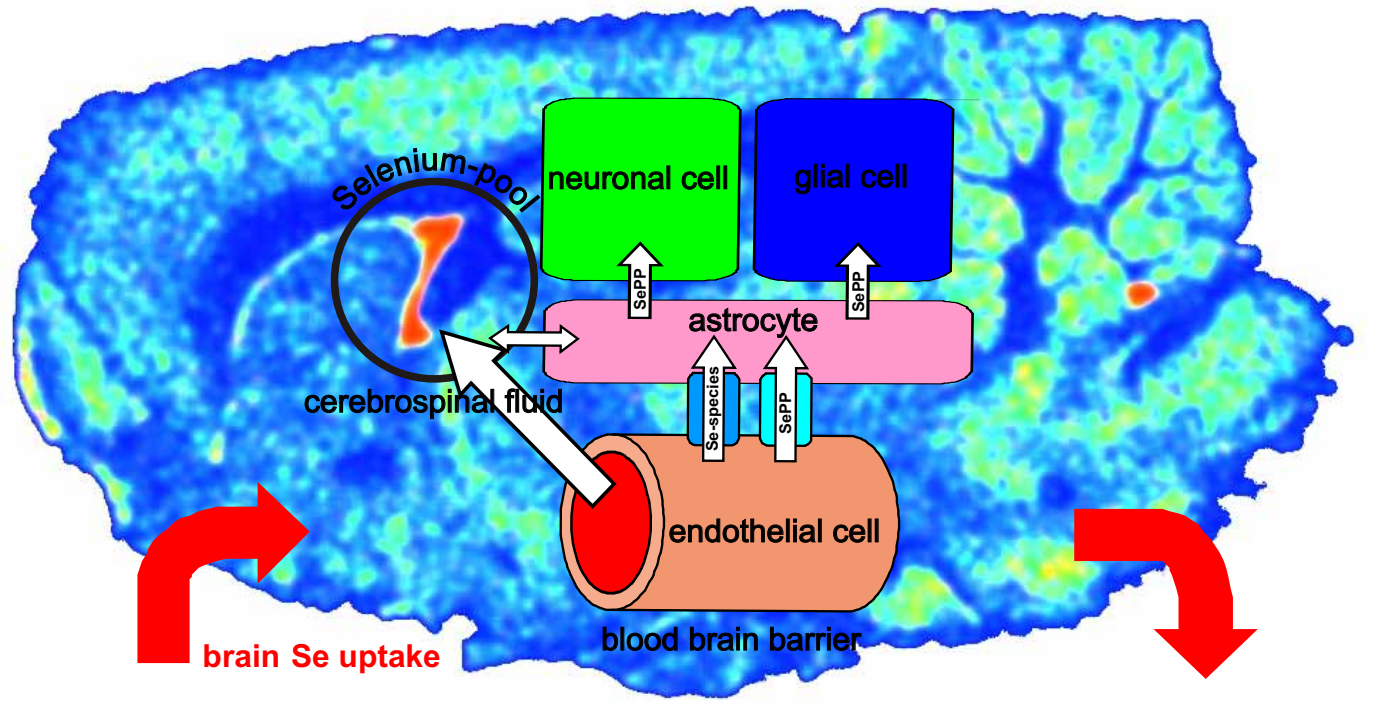

(a)
high
low
75Se-activity
[a.u.]
(b)
high
low
75Se-activity
[a.u.]

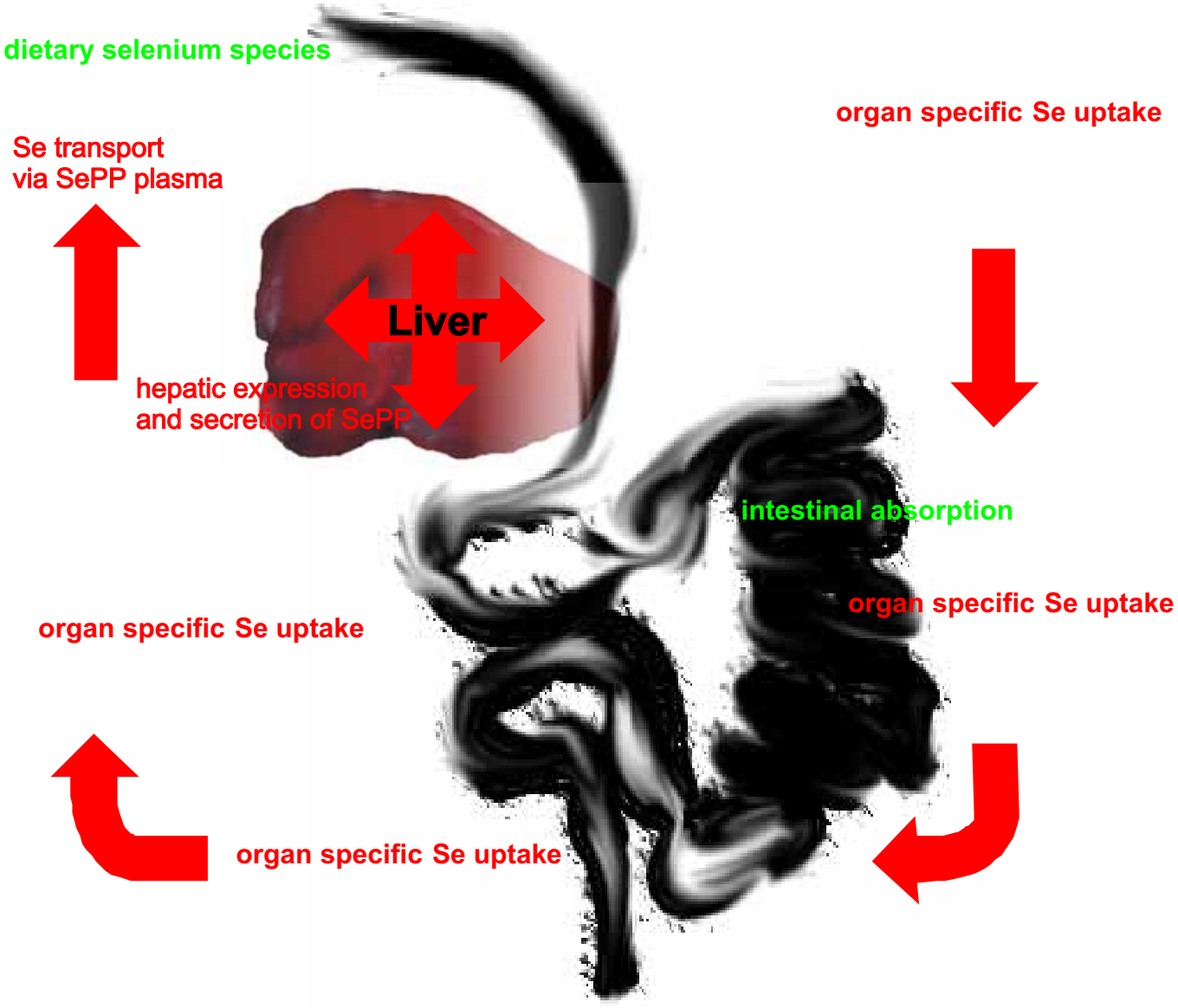

(a)
Se (-)
Se (+)
MW
kDa
66
45
36
29
20
15
4.5
7.7
8.4
IP
(b)
Se (-)
Se (+)
MW
kDa
66
45
36
29
20
15
1
2
3
4
5
6
7
8
9
10
11
12
4.5
7.7
8.4
IP

Kuehbacher et al., Fig. 8



Kuehbacher et al., Fig. 9





Kuehbacher et al., Table 1

	^{75}Se activity / Area [a.u./pixel]
Testis	0.79
Seminiferous tubules	0.94
Caput epididymis	0.17
Corpus epididymis	0.44
Cauda epididymis	0.23