Zinc enriched neurons influence early neuronal changes following traumatic brain injury

PhD Thesis

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Preface

The present PhD-thesis is based on the following two articles referred to in the text by their Roman numerals. The articles have never been part of a PhD or Doctoral thesis before.

List of papers:

I. Changes in the Vesicular Zinc Pattern Following Traumatic Brain Injury. Doering P, Danscher G, Larsen A, Bruhn M, Søndergaard C, Stoltenberg M.
Neuroscience. 2007 Nov 30;150(1):93-103. Epub 2007 Oct 9.

II. Chemical Blocking of Zinc Ions in CNS Increases Neuronal Damage Following Traumatic Brain Injury (TBI) in mice. Peter Doering, Meredin Stoltenberg, Milena Penkowa, Jørgen Rungby, Agnete Larsen and Gorm Danscher. PLoS One. 2010 Apr 9;5(4):e10131.

Hypotheses

The involvement of vesicular zinc in neuronal cell damage and cell death in an array of pathological scenarios including Traumatic Brain Injury, Seizure and Ischemia has been defined in the Translocation Theory; The theory states that a presynaptic release of vesicular zinc ions transcends the synaptic cleft and aggravate damage of the post-synaptic neurons (Frederickson et al., 1989; Koh et al., 1996; Sørensen et al., 1998; Suh et al., 2001) after the above mentioned pathological events. However, other studies, suggest that a decrease in the amount of zinc ions, established either by chelation or genetic manipulation, will increase the extent of brain damage following pathological circumstances (Yeiser et al., 2002; Takeda et al., 2005B-C; Li et al., 2010) and that the "toxic" transsynaptic zinc signal defined in the translocation theory could come from a non-vesicular zinc pool (Cole et al., 2000; Lee et al., 2002; Li et al., 2010). We speculate that this zinc signal can be traced with the $ZnSe^{AMG}$ method and that either genetic removal of vesicular zinc (using a KO model) or chemical binding of the vesicular zinc pool will have profound, and according to the translocation theory neuroprotective effects

Aim of the PhD-thesis

The thesis focuses on the acute relationship of the dynamic changes seen in the vesicular zinc pattern and the neuronal damage following traumatic brain injury (TBI). The studies were performed on zinc transporter 3 knockout (ZnT3-KO) mice, Balb/C mice and the ZnT3-KO mouse littermate control the Wild type (Wt) mouse (Wenzel et al., 1997; Linkous et al., 2008).

1. Specifically we wanted to trace the changes in the neuronal terminals to the ultrastructural level, in vivo, using the $ZnSe^{AMG}$ method and secondly, to trace the in the translocation theory hypothesised zinc signal.
2. We wanted to compare the morphological changes in mice with vesicular zinc (Balb/C and Wt mice) to a mouse without vesicular zinc (the ZnT3-KO mouse)
3. To investigate what effects genetic removal of vesicular zinc has for the TBI aftermath and to compare this with the post TBI events in normal mice
4. Furthermore we wanted to investigate the effect of zinc binding on the TBI aftermath in both mice containing vesicular zinc and in mice without vesicular zinc
5. Finally we wanted to couple the anatomical findings to the quantitative findings of our studies.

Two studies form part of the thesis: Paper I is based on study I and describes the dynamic changes seen in the vesicular zinc pattern 24 hours after TBI, and paper II is based on study II and aims at quantifying the cellular damage in the ZnT3-KO mouse and the Wt mouse and coupling this to the alterations seen in the zinc pattern after TBI.

Introduction

Traumatic brain injury (TBI) is one of the leading causes of disability and death among young people. It is estimated that every year approximately 500,000 individuals are affected by TBI (including minor head trauma) in the US alone (Levenson, 2005) and approximately 10,000 in DK (Danish Medical Bulletin, 2007). Although much is already known about brain injury and the secondary brain damage that follows the initial insult, the full range of brain tissue responses to TBI remains to be elucidated.

Brain diseases as diverse as seizure, ischemia and TBI are believed to cause the release of large amounts of zinc ions from the pre-synaptic terminals of zinc enriched (ZEN) neurons. These zinc ions are believed to enter the post-synaptic neurons and initiate a cascade of events that aggravate the damage (Frederickson et al., 1988, 1989, 2001; Koh et al., 1996; Myhrer et al., 2003; Sørensen et al., 1998; Suh et al., 1999, 2000A, 2001; Hellmich et al., 2004, 2007; Wei et al., 2004).

In the mammalian brain loosely bound or free zinc ions are all located to the presynaptic vesicles of ZEN neurons (Danscher et al., 1994, 1997). This pool of zinc comprises approximately 10-15% of total brain zinc (Wenzel et al., 1997; Takeda, 2001).

ZEN neurons exist throughout the mammalian CNS, but are particularly abundant in regions associated with higher cognitive functions such as hippocampus, isocortex and amygdala. Changes in the amount of zinc in these regions have been implicated in disorders as diverse as neurodegenerative diseases, developmental disorders, seizures, ischemia and TBI (Bancila et al., 2004; Capasso et al., 2005; Choi & Koh, 1998; Erickson et al., 1997; Frederickson et al., 2005A,B; Liguz-Lecznar et al., 2005; Stoltenberg et al., 2005, 2007).

The hippocampal formation contains the most intensively described ZEN systems (Frederickson et al., 1989; Slomianka, 1992). Early studies showed that free or loosely bound zinc ions are located to a pool of synaptic vesicles in the giant boutons of the hippocampal mossy fibres (Danscher, 1984; Perez-Clausell and Danscher, 1985) and are released during brain activity (Assaf & Chung, 1984; Howell et al., 1984) where they partake in synaptic transmission (Wall, 2005; Wang et al., 2002; Weiss & Sensi, 2000). The extent, complexity and beauty of the network of ZEN terminals that becomes visible by the autometallographic (AMG) technique imply that ZEN neurons are involved in several of the hippocampal functions. All ZEN neurons in hippocampus are glutamatergic, but it is not all of the glutamatergic neurons in the hippocampal formation that are ZEN neurons, i.e. ZEN neurons belong to a subgroup of glutamatergic neurons in the brain (Frederickson et al., 2000).

Other studies have concluded that all mammals utilize the same family of proteins for zinc ion homeostasis. Both import and export of zinc ions from cells are maintained by the SLC30 and SLC39 proteins (Palmiter et al., 2004; Liuzzi & Cousins, 2004).

In neuroscience especially the ZnT3 protein (the zinc transporter 3 protein found solely on the membranes of the synaptic vesicles and a member of the SLC30 group of proteins) has been examined. The ZnT3 protein is responsible for sequestering zinc ions into the vesicles of the ZEN terminals (Palmiter et al., 1996; Wenzel et al., 1997; Linkous et al., 2008).

Studies on the ZnT3-KO mouse (a mouse without the ZnT3 protein) have shown a lack of obvious phenotypic differences despite the complete lack of vesicular zinc. The result was that the ZnT3-KO mouse tends to be more seizure prone when treated with kainite acid. Interestingly, some of these studies revealed zinc ions in the neuronal somata of the ZnT3-KO mouse after seizure induction, and the authors hypothesized that these zinc ions had a non-synaptic vesicle origin (Cole et al., 2000; Lee et al., 2000, 2003). Accordingly, the translocation hypothesis has been modified in order to include this controversy (Frederickson et al., 2004A, B). Studies on the ZnT3-KO mouse have failed to show any obvious phenotypical differences compared to littermates (Cole et al., 2001; Lopantsev et al., 2003), which has even caused some researchers to question whether zinc is released from the presynaptic terminals (Kay, 2006; Kay & Toth, 2008).

Studies using divalent ion chelating agents like DEDTC, TPEN and CaEDTA have obtained diverse results when evaluating neuronal damage following brain injury. Some have concluded that zinc chelation is neuro-protective (Canzoniero et al., 2003; Frederickson et al., 2002A; Hellmich et al., 2004; Koh et al., 1996), partially neuro-protective (Lee et al., 2002) or harmful (Blasco-Ibanez et al., 2004; Dominguez et al., 2003B, 2006; Lees et al., 1998). In vitro (cell) studies have implicated zinc in potential cyto-toxicity (Kim et al., 1999A,B).

Arrays of studies have shown that zinc deficiency is associated with impaired cognitive functions, increased risk of developing neuropsychological symptoms and abnormal brain development (Mocchegiani et al., 2005; Saito et al., 2000), and neurodegenerative disorders (Frederickson et al., 2005A,B). Furthermore, zinc deficiency has been found to increase neuronal damage, especially in regions that are highly zinc enriched after insults as diverse as TBI, ischemia and seizure (Takeda et al., 2005B,C; Yeiser et al., 2002), and to reduce neurogenesis and proliferation of stem cells (Adamo et al., 2009; Suh et al., 2009).

A mechanism for this aggravated neuronal damage might be that zinc deficiency potentiates the excito-toxicity of glutamate in neurological disease (Pal et al., 2004; Takeda and Tamano, 2009).

This raises the following questions: Why do ZEN neurons harbour a potentially damaging agent in their pre-synaptic terminals? And what influence can alterations of this dynamic zinc pool have on the TBI (and other neurological disorders) aftermath?

Zinc homeostasis and vesicular zinc

In mammalian cells, the level of cytoplasmic zinc is maintained within a narrow range (Palmiter & Findley, 1995). Specialized mechanisms are required for both zinc uptake and release, since zinc cannot cross cell membranes by passive diffusion (McMahon & Cousins, 1998A, B). The transport of zinc has been investigated in a number of tissues and cell types, including ZEN neurons in the CNS (Colvin et al., 2000, 2003; Takeda, 2000, 2001). At the cellular level, there are specific membrane bound transporter proteins, including the ZnT family, that control the cell levels of free (ionic) zinc (Colvin et al., 2003, Takeda and Tamano, 2009). From an evolutionary point of view, it is interesting that all eukaryote organisms utilize the ZnT proteins to maintain a tight zinc homeostasis, ranging from the banana fly, *Drosofila melanogaster*, to *Homo sapiens* (Palmiter et al., 2003). In regard to neuroscience the most examined protein is Zinc Transporter 3 (ZnT3) which is predominantly found in the brain. The ZnT3 protein is responsible for sequestering zinc ions in synaptic vesicles in the ZEN neurons terminals. The ZnT3 (and the ZnT family in general) has been proposed to function by either facilitated diffusion or secondary active transport, as symporters or antiporters. The antiport mechanism has been described in detail on the *Bacillus subtillis* where zinc is exchanged for H^+ in the extracellular fluids (Palmiter et al., 2003).

Studies on ZnT3, mRNA and protein expression have verified that the zinc pattern and the ZnT3 protein/mRNA patterns coincide (Palmiter et al., 1996; Cole et al., 1999). Furthermore, it is generally accepted that the hippocampus and the isocortex contain a large population of ZEN neurons (Perez-Clausell & Danscher, 1985, 1986; Perez-Clausell, 1996; Slomianka L. 1992; Stoltenberg et al., 2007), and changes in the level of zinc ions in these parts of the brain are therefore believed to be especially prone to develop into an array of neurological deficits.

Although not well established it is hypothesized that the metallothionein 3 (MT3) protein is an integral part of transporting zinc ions from the neuronal somata to the synaptic vesicles in the ZEN terminals (Colvin et al., 2000, 2003; Knipp et al., 2005). MT3 belongs to a family of metalloproteins found throughout the organism. These are hypothesized to be part of the cellular defence against oxidative damage and are capable of binding heavy metals such as zinc and copper with high affinity. Four metallothioneins are present in rodents, MT1-4, of which only MT1-3 exist in the brain. Growing evidence indicates that MT1-3 are neuroprotective proteins and controllers of the reactive gliosis and regenerative processes seen after CNS lesions (Carrasco et al., 2003; Chung et al., 2003; Chung & West, 2004; Giralt et al., 2002; Penkowa et al., 1999B,C). MT3 was until recently called a groWth inhibitory factor and works in an inverse relationship with the MT1-2 proteins (Chung et al., 2003; Erickson et al., 1997). Overall, in the case of reduced dietary zinc, the MTs and especially MT3 have been shown to release zinc and in the case of prolonged deficiency to be down-regulated (Colvin et al., 2003).

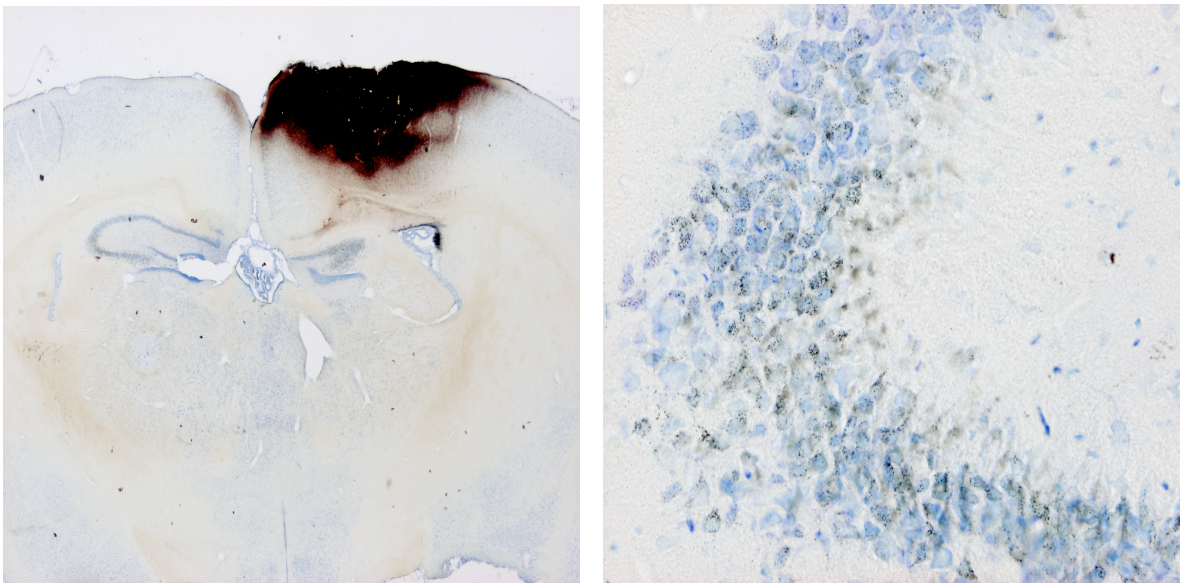
The important role of MT3 in vesicular zinc homeostasis and on the functions of the ZEN neuron is supported by the fact that it is predominantly found in the ZEN neurons (Chung et al., 2003). The ZnT3 and MT3 proteins work in the same pathways (Cole et al., 2000), MT3 deficient mice show reduced neuronal damage after insults (Lee et al., 2003), the MT family of proteins is the regulator and buffer of free (ionic) zinc in cells (Maret, 2000, 2003, 2006, 2009), and MT3 effectuates this in the ZEN neurons (Penkowa et al., 1999A; Suhy et al., 1999).

The $ZnSe^{AMG}$ method

The $ZnSe^{AMG}$ method is designed to capture zinc ions in vivo. During the process zinc-selenium (nano) crystals are being formed after an injection of either selenite (IP) or selenide (IC). These crystals can then be silver amplified, which makes them visible for studies at both light and the electron microscopic levels (Danscher, 1982, 1984; Slomianka et al., 1990; Danscher and Stoltenberg 2005, 2006). Apart from other autometallographic techniques a number of methods for zinc ion visualization in tissue sections exist, including use of fluorescent probes (Frederickson et al., 2004B, Zalewski et al. 2006).

The reasons for utilizing the $ZnSe^{AMG}$ method in studies of TBI are plentiful. First and foremost the $ZnSe^{AMG}$ method is **the only** in vivo method. It is suited for ultrastructural analysis and creates a stable product of ZnSe nanocrystals. Thus there is no time concern

when analysing specimens stained with this method (Danscher and Stoltenberg, 2006). Secondly, the ZnSe^{AMG} method is the only approach for mapping neuronal pathways and studying retrograde axonal transport of zinc ions (Brown & Dyck, 2004, 2005; Slomianka et al., 1990, 1992).



A **B**

Selenide injection into the isocortex showing applying the ZnSe^{AMG} method, a heavy AMG positive stain is seen in the selenide injected area (**A**). Retrograde accumulation of ZnSe nanocrystals in the Hippocampus formation is seen in (**B**) Example of retrograde tracing of ZEN neurons.

ZEN neurons and the isocortex

The rodent cortex can be subdivided into distinct parts; for practical purposes the cortex, has in this text been divided into only the isocortex and allocortex because of its complexity and subsequent tricky terminology. The isocortex represents the evolutionarily newest part of the cortex, showing a distinct six layered pattern while the allocortex has only one cell layer and represents the evolutionary oldest part of the cortex. In between these two pools there are a number of areas in the rodent brain showing characteristics of both the neo- and allocortex (Paxinos, 1995).

The isocortex has a lamination consisting of six more or less defined layers. Each layer is characteristic and differentiated, meaning that there are major morphological differences in the layers in between, based on a diverse topological placing of different subsets of neurons

(Paxinos, 1995). Neocortical layer 4 is of special interest in that it only contains very few ZEN neurons and borders layers 2-3 and 5 that both harbour a great amount of ZEN neurons.

The ZEN neurons combine into a delicate network with a distinct pattern within the six laminae of the isocortex. In rodents PAR1 contains a representation of the head and is dominated by the very big area devoted to the whiskers, also called the barrel field. In PAR1 the "barrel cortex" is defined in a special arrangement; in layer 4 of the isocortex, the vibrissae correspond to five rows of distinct cylindrical cellular aggregates of granule cells. Barrels are arranged in a pattern matching the vibrissae on the contralateral side of the "face" and consist of a cell sparse centre and a cell dense wall (Paxinos, 1995). The barrel cortex shows great differences in AMG positivity when comparing the individual layers to each other (Brown & Dyck, 2004, 2005), and the region contains lots of ZEN neurons, but still maintains a much differentiated AMG zinc stain (Brown & Dyck, 2004; Czupryn & Skangiel-Kramska, 1997). This makes it perfect for studying the impact of a knife lesion (TBI) on the isocortex including the inherent vesicular zinc pattern, and subsequently the ZEN neuronal behaviour in the TBI aftermath.

Materials and methods (for details please refer to studies I-II)

Experimental animals

The studies were undertaken in accordance with the Danish guidelines for animal welfare. Permission to perform these studies was obtained from the Danish committee for animal welfare.

Study I

A total of 129 mice were sacrificed during this study (n=129). Balb/C, Wt (wild type mice) and ZnT3-KO mice were used. Of these 108 were divided into three groups according to strain with 36 mice in each, which were then further divided into six subgroups with six mice in each (n=6).

21 mice served as controls and were divided into three groups with seven animals in each, also according to strain. This was in order to test for zinc ion specificity of the $ZnSe^{AMG}$ method.

Study II

A total of 58 mice were sacrificed during this study (n=58). Wt and ZnT3-KO mice were used. The mice were divided into two groups according to strain, with 25 mice in each. The mice were further subdivided into five groups of five (n=5) depending on the kind of analysis that was to be done. 8 mice served as controls to test for comparability between strains.

The stereotaxic cut lesion, studies I- II

The mice were deeply anaesthetized prior to the operation. The anaesthetic used was a combination of 2,0 ml (Ketamine) Ketaminol Vet. 50 mg/ml, 0.25 ml (Xylacine) Narcoxyl Vet. 20 mg/ml and 3.75 isotonic salt water. Of this solution 0.1 ml per 10 gram bodyweight of the animal was injected intraperitoneally (IP). Each animal was observed closely for withdrawal reflexes as a test of satisfactory anaesthesia. Then it was placed in a small animal stereotaxic instrument designed to keep the head of the animal securely fixed. The skin above the calvarium was cut mid-sagitally and kept aside by two clips. Under the operation microscope a 1 mm wide and 3.5 mm long furrow was drilled 2 mm lateral to bregma in the right parietal bone. A scalpel was then mounted in an electronic stereotaxic devise lowered to the drilled furrow and then further lowered 1 mm, penetrating the isocortex, and gently moved 3 mm caudally. The animals were after the operation kept wrapped in a paper towel under a heating lamp in order to avoid hypothermia, as it has been shown that hypothermia suppresses vesicular zinc release (Frederickson et al., 2000; Suh et al., 2006).
. The major advantage of this procedure is that it is highly reproducible and that our model of TBI allows us to specifically target areas with high amounts of ZEN terminals.

Chemical binding of zinc, study II

DEDTC is a zinc chelator that binds the endogenous free zinc pool, preventing creation of catalytic selenide nanocrystals and thereby autometallographic development (Danscher et al., 1973; Danscher & Stoltenberg, 2006). The zinc specificity was tested by intraperitoneal injection of 1000 mg DEDTC per kg bodyweight one hour before selenite or selenide exposure, followed by optimal zinc staining procedures (vide infra).

The choice of selenite as a “chelator” was due to the fact that we traced the ZEN terminals to the ultrastructurel level in study I, applying the $ZnSe^{AMG}$ method (Doering et al., 2007). This makes a direct coupling of the anatomical findings to the quantitative assessment of neuronal damage in study II possible. DEDTC was chosen as chelator, first and foremost

because it is the active component of disulfiram/antabuse which is commonly used in the treatment of alcoholism, and because alcohol consumption has been closely linked to an increased risk of TBI, rendering DEDTC clinically relevant. Secondly, because even at high dosages it is a relatively safe drug with a reversible block of the AMG staining pattern (Danscher et al., 1973). Finally, our department has great experience in utilizing these two drugs, with a minimum of harm done to the experimental animals.

Autometallographic development, studies I- II

Tissue preparation for light microscopy (LM)

Cryostat sections: The newly dissected brains were placed in a 30 % solution of sucrose until they sank to the bottom of the jar. The brains were then frozen with CO2 for a period of 2 minutes. The tissues were placed in a cryostat and allowed to increase in temperature to -17ºC. 30-µm thick sections were cut, placed on Farmer cleaned glass-slides and AMG developed (vide infra). After development the sections were counterstained with a 0.1 % aqueous toluidin blue solution (pH = 4), dehydrated in alcohol to xylene, and ultimately embedded in DEPEX and covered with a cover-glass.

The AMG development procedure, study I- II

All glass ware and tools used for the AMG development were rinsed in a 10 % Farmer solution. The silver lactate developer consists of:

Protective colloid: 60 ml gum arabic solution. Dissolve 1 kg of non-refined acacia resin in 2 l deionised water by intermittent stirring over 5 days at room temperature. Filter the solution through several layers of gauze to remove impurities and freeze suitable portions of the filtrate in plastic bottles.Citrate buffer (pH = 3.7): 10 ml sodium citrate buffer. Dissolve 25.5 g citric acid in 1l of H20 and 23.5 sodium citrate in 2 l of H20 in 100 ml distilled water.

Reducing agent: 15 ml reducing agent. 0.85 g hydroquinone dissolved in 15 ml distilled water at 40ºC.

Silver ion supply: 15 ml solution containing silver ions. 0.12 g silver lactate in 15 ml distilled water at 40ºC added immediately before use while the AMG solution is thoroughly stirred. The glass-slides were placed in Farmer cleaned jars, poured with the AMG developer and placed in a water bath at 26ºC on an electric devise that shakes the jars gently.

The entire setup was prepared in plain daylight on the lab bench, but covered with a dark hood throughout development. After 60 minutes the AMG development was stopped by

replacing the developer with a 5 % sodium thiosulfate solution for 10 minutes (designated AMG stop bath). The jars were then placed under running ion-exchange water for 5 minutes before being counterstained with toluidin blue and cover slipped (for a review see Danscher and Stoltenberg, 2006).

Histochemical procedures

Fluorojade B (FJB) staining, study II

The one major advantage of the FJB method is that it produces a high signal to noise ratio, making the identification of damaged neurons possible in severely distorted tissue.

The dye is anionic and binds to released nuclear parts from necrotic and apoptotic cells (Schmued & Hopkins. 2000A,B).

TUNEL staining, study II

We wanted insight into the number of degenerating cells (probably undergoing apoptosis) after TBI and influence of vesicular zinc on this process; subsequently we chose to apply TUNEL stains (for details see Larsen et al., 2008).

Sampled areas, study I

The area around the cut lesion was arbitrarily divided into 3 zones. This was in order to ensure a quantitative assessment of the vesicular zinc changes and hence to reduce the variation between animals by constructing ratios (see paragraph on densitometric analysis); zone I = the lesion tract, zone II = the peri-lesional area with an immediate increased AMG zinc pattern; zone III = the area surrounding zone II. These zones were used for pseudo-colouring and zinc quantification in the Balb/c and WT mice.

Sampled areas, study II

The cell counts were performed on five mice per group by the same investigator, who was blinded to the animals' identity and treatment. The quantifications were used for statistical comparisons.

Five sections per animal were collected from the lesioned hemisphere and counted. Every third section down through the lesion was collected, starting randomly on slides 1-3 until a

total of 5 sections was obtained from each animal using Systematic Uniform Random Sampling (SURS). A frame was superimposed on the border of the lesion tract ranging into the lesioned area where the damaged cells were counted.

Application of pseudo-colours, study I

Because of the different sizes of the AMG particles, they give rise to stains from black-brown to light yellow corresponding to the distribution and size of the AMG developed zinc-selenium nanocrystals (Danscher, 1982; Danscher and Stoltenberg, 2006). To enhance this contrast the pictures were given pseudo-colours and converted to 8-bit resolution.

Densitometric analysis, study I

Every third section containing zones I-III was photographed (magnification x 2) until a total of five pictures was collected from each animal. A frame sized 500 μm x 500 μm was loaded into each picture. The frame was lined up at respectively the medial and lateral border of zone I, engulfing all of zone II and parts of zone III. These measurements were then compared to two adjacent measurements located to zone III. This ratio represents the mean staining intensity of zone II relative to zone III and was chosen

1) in order to reduce the variation in zinc staining percentages between different groups (Brown & Dyck, 2003; Riba-Bosch & Perez-Clausell, 2004),
2) to reduce the effect of the irregular stain seen in some of the animals with short survival times.

Counting procedures, study II

Positively stained cells were defined as cells with positive staining of the soma except in the case of TUNEL staining, where the apoptotic cells were defined as those with nuclear staining (nuclear TUNEL).

The cell counts were performed on five mice per group by the same investigator, who was blinded to the animals' identity and treatment. The quantifications were used for statistical comparisons.

Five sections per animal were collected from the lesioned hemisphere and counted. Every third section down through the lesion was collected, starting randomly on slides 1-3 until a

total of 5 sections was obtained from each animal using the Cavalieri principle. A frame was superimposed on the border of the lesion tract ranging into the lesioned area where the damaged cells were counted.

Design, study II

Through a number of pilot studies and an extensive search through literature we ended up using a methodology that would give us exact estimates of differences between the groups (a discourse employed by other researchers in the field; Riba-Borsch & Perez-Clausell, 2004; Penkowa et al., 1999A,B and others). We found that this approach was sufficient to test our hypothesis and to generate data strong enough for statistical evaluation and to test the involvement of vesicular zinc in the aetiology of TBI and neuronal damage, even though our methodology did give less absolute numbers of dead neurons etc. than for example a pure stereological approach would.

Statistics

Where only two independent groups were compared we used the Students t-test. The groups used for multiple comparisons were analysed using analysis of variance (ANOVA). All values are given as means ± SEM (with a 95% confidence interval (ci)), and the significance level was set at $P < 0.05$. Application of non-parametric tests was considered. However, this would have omitted the confidence intervals and could subsequently have blurred the subtle changes seen both over time (study I) and after chelation therapy (study II).

Observations and comments

Study I (for details please refer to paper I)

Following TBI induction the entire area adjacent to the lesion tract transformed radically in terms of ZnSe AMG positivity. Almost immediately after TBI a dark AMG band discarded the normally segregation of the neocortical layers, reaching from the superficial layer one to the deepest part of layer six, including the normally very faintly stained laminae four and six. The increased AMG staining was most pronounced two hours after TBI and gradually diminished over the 24 hours.

When evaluating the semi-thin sections clear signs of morphological damage were seen, with vacuolation of the neuronal cytoplasma and with condensed, eccentrically placed and fragmented nucleii. However, the damaged cells did not contain any ZnSe nanocrystals in their somata and all the AMG grains were still confined to the neuropil. Examination at the electron microscopic level confirmed that the ZnSe nanocrystals were all to be found in the neuropil and that the neurons did not contain any AMG grains in the somata despite of the clear signs of damage.

We found a distinct increase of the AMG staining in layer four of the isocortex, and EM analysis confirmed that all the AMG reactivity was to be found in the neuropil. We applied pseudo-colours to enhance contrast, and in pilot experiments we used shorter development times in order to clearly observe the increased AMG stain around the lesion tract (see figure 1 in appendix).

After TBI the ZnT3-KO mouse contained a number of somata marked neurons in the immediate surroundings of the lesion tract (see figure 2 in appendix). The neuropil was continuously void of ZnSe nanocrystals. A similar picture was seen when evaluating the semi-thin section.

At the EM level we found that the somata markings were ZnSe containing lysosomal structures.

Study II (for details please refer to paper II)

In the ZnT3-KO mice the $ZnSe^{AMG}$ method revealed numerous neurons with silver enhanced zinc nanocrystals in their somata. After 24 hours the loaded neuronal somata were all found in the periphery of the lesion, marking the transition between TBI influenced isocortex and morphologically intact brain tissue.

Opposite to this characteristic ZnT3-KO TBI AMG pattern the WT control mice were completely void of stained neuronal somata although clear signs of morphological cell damage were conspicuous. LM analysis revealed that the ZnSe nanocrystals were confined only to the neuropil. These findings were a recapture of what was seen in study I, interestingly, the morphological changes were indicative of what the quantitative assessments also showed.

The ZnT3-KO mouse had numerous more damaged neurons after TBI (see table 1). However, this difference was annulled after application of zinc binding agents, either selenite or DEDTC (see table 2).

FluoroJade B staining 24 hours after TBI

Table 1
Students t-test.

TBI	n = 10 mean	95% ci
WT	n = 5 570.6	[348.16 793.04]
ZnT3	n = 5 983.0	[664.20 1301.80]
Diff	n = 5 −412.4	[−769.58 −55.22]*

FluoroJade B staining of cryo sections.
*** Indicates statistically significant difference, $p<0.05$.**

TUNEL staining 24 hours after TBI

Table 2

TBI	n = 30 mean	95% ci
WT o41–45	n = 5 241.2	[164.01 318.39]
ZnT3 o46–50	n = 5 478.8	[401.61 555.99]
WT + DEDTC o51–55	n = 5 532.2	[454.81 609.19]
ZnT3 + DEDTC o56–60	n = 5 590.4	[513.21 667.59]
WT + selenite o61–65	n = 5 588.3	[510.81 665.19]
ZnT3 + selenite o66–70	n = 5 465.8	[388.61 542.99]

TUNEL; The group o46-50 has significantly more apoptotic neurons than o41-45, this difference equalizes after chelator application [o51-55;o56-60], [o61-65;o66-70].

Discussion

Implications, studies I-II

Previous studies have speculated that excess zinc increases brain damage following lesions. The suggestions are based on cell culture (Kim et al., 1999 A, B; Cho et al., 2003) and in vivo studies (Frederickson et al., 1989; Koh et al., 1996; Sørensen et al., 1998; Suh et al., 2000A, B, 2001, 2004, 2006, 2009; Lee et al., 2002). We suggest that this potential cyto-toxicity is the primary reason for the initial local increase in the vesicular zinc pool following TBI and that the origin of this zinc could be the large intracellular pool of metallo-proteins. Especially the MT3 protein has

been implicated in the same pathways and cascades as vesicular zinc (Cole et al., 2000; Colvin et al., 2003; Lee et al., 2003). If MT3 is the source of the increased zinc seen it would also explain the somata stained neurons in our ZnT3-KO mouse because the mouse does still harbour MT3 (and other metallo-proteins).

Another potential source of zinc ions could be the (hypothesized) mitochondrial zinc pool (Colvin et al., 2003; Dineley et al., 2003; Sensi et al., 2003). This, however, would not explain the different stains in the ZnT3-KO and WT mouse.

One of the functions of the increased vesicular zinc pool after TBI could be that it stabilizes glutamate. This phenomenon is seen with insulin and zinc in the pancreas (Søndergaard et al., 2005). Furthermore, vesicular zinc has been shown to have an inhibitory effect on the post-synaptic NMDA receptor, and this could explain why the ZnT3-KO mice are more seizure prone during "normo" physiology (own observations; Cole et al., 2000), why zinc chelation can result in increased neuronal death (Dominguez et al., 2003B, 2006; Blasco-Ibanez et al., 2004), and why an increased damage is seen in the ZnT3-KO mice compared to littermates after administration of kainic acid (Cole et al., 2000). Most recently it has been suggested that zinc ions during an ischemic challenge can control glutamate release via ATP-sensitive potassium channels, thereby granting a protective effect (Bancila et al., 2004).

Furthermore, studies on zinc deficient and chelator treated animals have shown an increased neuronal cell death after seizure and TBI (Takeda et al., 2005A,B; Yeiser et al., 2002), also suggesting that one of the functions of zinc (and possibly vesicular zinc) is to control glutamate and hence pose protective qualities.

In study I we have used the $ZnSe^{AMG}$ protocol and have been able to trace the vesicular zinc alterations to the ultrastructural level and hence directly examine the translocation thesis, in vivo.

The combined findings of studies I-II strongly suggest that zinc ions have protective qualities to the TBI aftermath, including that removal/ binding of zinc ions in the TBI aftermath have deleterious effects, a conclusion recent studies on TBI also supports (Li et al., 2010)

We found that the ZnT3-KO mice compared to the WT mice had initially more damaged neurons, as demonstrated by FluoroJade B and TUNEL stains; this difference was equalized after chemical binding of the free or loosely bound zinc ions in the ZEN neurons of the WT mice.

Study I showed that mice with a functioning ZnT3 protein respond to TBI by increasing their terminal content of zinc within 0-8 hours after TBI and that the ZnT3-KO mice exhibit a number of somata marked neurons in the same period of time.

We speculate that it is this difference in the neuronal handling/ processing of zinc ions that is responsible for the increased number of damaged neurons seen in the ZnT3-KO mice. This hypothesis implies that having a functioning ZnT3 protein could be an integral part of neuroprotection and reduce brain damage after TBI.

The fact that genetic (ZnT3-KO mice) or chemical removal/ binding of vesicular zinc increase cell death after TBI could rely on the pro-oxidative effect of zinc removal/ zinc deficiency (Maret, 2009), where binding of vesicular zinc generates more oxidative stress, or perhaps on a direct pro-excitatory effect of zinc deficiency (Bitanihirwe & Cunningham, 2009) as well as the above mentioned stabilizing effect of zinc on glutamate.

In support of this, seizure studies have shown that pre-treatment of mice with DEDTC renders them susceptible to otherwise subconvulsive doses of kainite acid (Cote et al., 2005; Dominguez et al., 2006) and that the ZnT3-KO mice also have an increased susceptibility to seizures (Cole et al., 2000) although appearing phenotypically normal (Cole et al., 2001), suggesting that vesicular zinc during seizures acts as a neuro-protective agent. We speculate that the function of vesicular zinc in the TBI aftermath may resemble the above scheduled.

Furthermore, low zinc concentration in cells has been associated with pro-apoptotic mechanisms and increased cell death (Yeiser et al., 2002) whereas increased zinc levels appear to be anti-apoptotic by reducing oxidative stress (Maret, 2009; Bitanihirwe & Cunningham, 2009). In relation to neurodegenerative disorders, zinc deficiency has been shown to increase the plaque load in a model of Alzheimer's disease, supposedly also due to increased oxidative stress (Stoltenberg et al., 2007).

Comments on causality

The fact that TBI initially on ZnT3-KO mice causes more damaged neurons than on the WT mice and that this difference equalizes after chemical blocking of the vesicular zinc implies that vesicular zinc is not the aetiological agent causing neurological damage as suggested by earlier studies (Frederickson et al., 1988, 1989; Sørensen et al., 1998; Lee et al., 2002; Suh et al., 2000A, B, 2001, 2006).

Studies utilizing zinc chelators as neuroprotectants against the proposed neurotoxicity of vesicular zinc have revealed very mixed results. A study on ischemia using the MCA occlusion model found early neuroprotection when using the membrane impermeable chelators CaEDTA. However, this protective effect was lost when the occlusion time was raised from 30 to 60 minutes or if the infarction was measured at a later time point after reperfusion (Lee et al., 2002). Another study on ischemia also using the MCA model reached the opposite result, namely an aggravation of the ischemic insult following pre-treatment with CaEDTA (Kitamura et al., 2006).

CaEDTA has been shown to have a protective effect after TBI (Suh et al., 2000A, B) while studies of seizures using DEDTC reported an enhanced excitotoxicity following kainic acid injections (Dominguez et al., 2003A, 2006).

Even more controversial is the fact that intracellular zinc accumulations can be blocked/depleted by the use of extracellular chelators such as CaEDTA given after the neurons have become zinc positive (Frederickson et al., 2002B). This implies that zinc injured cells do still have a turn-over of zinc and most importantly that application of CaEDTA after a given insult is not a good way of differing between transcellular (translocation hypothesis) and intrinsic zinc signals (labile zinc from metallothioneins or mitochondria) and that the cause and effect relationship cannot be established with certainty.

Studies on neurodegenerative diseases have also raised concerns about the use of zinc chelators; studies on Clioquinol have revealed transient global amnesia and general neurodegenerative disorders in association with this zinc chelating agent (Ismail et al., 2008), and studies on Alzheimer diseased brains have shown a rather deleterious effect when removing zinc from the diet, with a subsequently increased plaque load especially in the isocortex which contains high amounts of ZEN neurons (Stoltenberg et al., 2007).

The very mixed results obtained from previous studies strongly imply that a uniform and standardized methodology must be applied in order to generate a generalized hypothesis on the functions of vesicular zinc. Accordingly, it is imperative to know which zinc pools are being studied in vivo, scrutinized by the novel findings in the ZnT3-KO mouse.

The established notion of causality in vesicular zinc relations to neuronal cell death is according to the translocation theory:

1) TBI initiates the release of vesicular zinc into the synaptic cleft

2) The “free zinc” acts as a transcellular signal and enters the post-synaptic neurons
3) The internalized zinc causes cell damage and can be visualised as somata marked neurons
4) The blocking of the transcellular zinc signal with zinc chelators is neuroprotective

Ad 1) After tracing of vesicular zinc to the ultrastructural level (study I) we demonstrated no zinc in the extra-cellular compartments which suggests that a substantial release of vesicular zinc is a part of the post TBI events in mice harbouring vesicular zinc.

Ad 2) A consequence of 1) is that if no vesicular zinc is released after TBI no transcellular zinc signal can be generated.

Ad 3) We only saw somata marked neurons in the ZnT3-KO mice after TBI. However, in pilot studies we were able to obtain a vivid somata stain if we pre-treated the brain slices with a base, pH=11, or if we did a traditional tracing of axonal pathways with the $ZnSe^{AMG}$ method. Furthermore, the literature cites a number of occasions where somata marked neurons can be encountered: During embryonic development (Lee et al., 2006), after deprivation of neuronal targets (Land & Aizenman. 2005), as a normal part of apoptosis (Cote et al., 2005) and after seizures in the ZnT3-KO mouse (Cole et al., 2000).

Ad 4) We found that the binding of ionic zinc with zinc chelators increased the neuronal damage after TBI. Accordingly, extensive studies on seizures have reached the same conclusion, i.e. that blocking of ionic zinc is deleterious (Cuajungco and Lees, 1998; Blasco-Ibanez et al., 2004; Dominguez et al., 2003A, 2006).

Vesicular zinc involvement in TBI meets none of the translocation theory criteria and causality cannot be established. Furthermore, vesicular zinc is neither a necessary cause for obtaining somata marked neurons nor is it a sufficient cause, and eventually the chain of causality is ultimately broken. Conclusively, at least for TBI, the translocation of pre-synaptic zinc into post-synaptic neurons must be ruled out as a significant contribution to neuronal damage.

We have confirmed that ZnT3-KO mice are void of histochemically reactive zinc ions in their “ZEN terminals”, during “normo-physiology”, but do contain a pool of zinc ions in their somata after “TBI like events”.

It is well established that zinc is a potent modulator of numerous transcription factors and integral to well above three hundred proteins, and especially the metallo-thionenin (MT) family of proteins is of interest to both the acute and the chronic response of neural tissues to TBI (Chung et al., 2003). MT1-2 are predominantly located to the neuronal glia cells and MT3 is located primarily to the ZEN neurons (and more speculatively only to the ZEN neuronal terminals in normal mice). The proteins work in an inverse relationship, where up-regulated MT1-2 mean down-regulated MT3 and vice versa. MT1-2 are proactive proteins that promote neuronal elongation and healing (Chung et al., 2003), whereas MT3 is thought of as a groWth inhibitory factor. The interplay between metallo-proteins, vesicular zinc and free "ionic" zinc is essential in sensing and transducing redox states in the cell (Maret, 2000, 2003, 2006) and zinc ion fluctuations have been linked to secondary damage after TBI (Li et al., 2010).

General comments on validity

Our approach for studying vesicular zinc and TBI with the application of the $ZnSe^{AMG}$ method resulted in consistent and very reproducible results. However, one could argue that more than one zinc staining methodology should be applied when studying vesicular zinc alterations after TBI.

The argument is valid but problematic; no other zinc staining method is suitable for zinc ion tracing at the ultrastructural level and works in vivo. In pilot studies we did apply fluorescent dyes such as TSQ and Zinpyr which revealed zinc staining in accordance with our published result (Theoret et al., 1988); however, only in vitro with no possibility for ultrastructural tracing and with no added information compared to the $ZnSe^{AMG}$ method.

The primary focus of my research has been to describe the involvement of vesicular zinc in the TBI pathology in a very well defined area of the brain and in a narrow time period. This makes it obvious that a general extrapolation to other parts of the brain, other periods of time and other diseases such as ischemia and seizure must be met with great caution and that much more research is warranted in the field.

Reconsidering the role of vesicular zinc, new frontiers

The findings of studies I-II make us hypothesize that the acute increase in zinc stain seen in the WT and Balb/c mice is a neuroprotective phenomenon, whereas it is a neurodegenerative phenomenon in the ZnT3-KO mice.

The ZnT3-KO mice stained positive for zinc with the $ZnSe^{AMG}$ method after trauma. The number of somata marked neurons was dynamic and peaked after two hours (study I). With prolonged survival times the stained neurons disappeared. This correlated with the progression of the neuronal degeneration seen in study II. The neuropil of the ZnT3 mice was continuously void of vesicular zinc and the traceable zinc was located to the somata of the damaged neurons. At the ultrastructural level the ZnSe crystals were traced to lysosome-like structures. This establishes that this zinc is of a non-vesicular origin and accumulates from a yet unknown zinc pool. Future studies utilizing the $ZnSe^{AMG}$ method will help to determine the exact source of this new non-vesicular zinc. Furthermore, our studies strongly imply that the increased zinc accumulations seen after neuro insults are not a cause of, but rather a consequence of pathological processes a notion that is supported by recent research (Sensi et al., 2009) .

Conclusion

We found that:

1) WT mice do not appear to release vesicular zinc following TBI.
2) ZnT3-KO mice are inherently more prone to developing substantial neuronal damage after TBI compared to the WT mouse.
3) The difference in neuronal damage between the WT and the ZnT3-KO mice could be cancelled by zinc binding agents.
4) Zinc binding agents increase neuronal damage in WT mice and have relatively inert effects on the ZnT3-KO mice.

It is concluded that vesicular zinc is not to be considered a neuro-toxic agent in the realm of TBI and that vesicular zinc may have neuroprotective qualities.

Summary, UK

The acute relationship between the dynamic changes in the vesicular zinc pattern and neuronal damage seen after traumatic brain injury (TBI) was examined. The translocation theory states that vesicular zinc transcends the synaptic cleft and enters the post-synaptic neurons in a number of pathological circumstances, including TBI, contributing significantly to neuronal damage. In order to obtain uniform and highly reproducible data a stereotaxic lesion model was applied to examine the response of specific **Z**inc **En**riched neuronal centres. Experiments on ZnT3-KO mice (a mouse lacking vesicular zinc) were included to further investigate the importance of vesicular zinc in the TBI aftermath. After studying the synaptic alterations in the vesicular zinc pool we found that zinc was not released from the pre-synaptic terminals after TBI in the wild type mice and hence did not pose a threat to the post-synaptic neurons. A new zinc pool, interpreted as an acute neurodegenerative phenomenon, was observed in the somata of the "ZEN neurons" in ZnT3-KO mice. AMG staining of ZEN somata was never observed in the WT mice, but paralleled in time the acute increase in the AMG staining seen close to the wound in these animals.

To elaborate on these findings neuronal damage was quantified after TBI and after removal of vesicular zinc either genetically (the ZnT3-KO mouse) or by chemical binding of the vesicular zinc (with chelator treatments).

Quantification of cell damage showed that the ZnT3-KO mouse was much more susceptible to neuronal damage than the WT control. The assessment of neuronal damage also revealed that removal of vesicular zinc by zinc binding had deleterious effects in the WT mouse and that the pattern of damage resembled that of the ZnT3-KO mouse.

It is concluded that translocation of vesicular zinc is not a contributor to neuronal damage after TBI and that the idea of reducing the amount of zinc ions during pathological events such as TBI must be reconsidered. Further studies are warranted to examine potential neuroprotective qualities of the vesicular zinc pool.

Summary, DK

Det akutte forhold mellem de dynamiske forandringer i det vesikulære zinkmønster og nerveskade efter traumatisk hjerneskade undersøgtes eksperimentelt. Translokationsteorien påstår, at vesikulært zink kan transcendere den synaptiske kløft og akkumuleres i det postsynaptiske neuron efter læsioner, f.eks. traumatisk hjerneskade, og derigennem bidrage substantielt til hjerneskade. Under anvendelse af en stereotaktisk læsionsmodel undersøgte vi zinkberigede (ZEN) hjernecentres reaktion på traumatisk hjerneskade. Til studierne anvendtes ZnT3-KO mus (en mus uden vesikulært zink) og de tilsvarende, genetisk normale, WT (wild type) mus. Studiet af de synaptiske ændringer i den vesikulære zink pool viste, at vesikulært zink ikke bliver frisat fra de præsynaptiske terminaler efter TBI, og at denne zink-pool derfor ikke kan fremkalde en forværring af læsionen.

Desuden fandt vi en ikke tidligere beskrevet zink-pool i ZnT3-KO musen lokaliseret til 'ZEN' neuronernes somata. Den observerede pool af zinkjoner tydes som et neuro-degenerativt fænomen, som ikke ses hos WT musene. Disse normale mus udviser derimod en tidsmæssigt sammenfaldende forøgelse af indholdet af zinkjoner i ZEN terminalerne omkring læsionen.

For yderligere at undersøge disse fænomener kvantificerede vi neuronskaden efter TBI og efter binding af den vesikulære zink pool med kelatorer.

Kvantificeringen af skaden viste, at ZnT3-KO musene var meget mere modtagelige for neuronskade efter TBI. Desuden viste studiet, at kemisk binding af den vesikulære zink pool yderligere forværrede hjerneskaderne i WT musene, på en måde der svarer til ZnT3-KO musenes skader.

Konkluderende kan siges, at translokation af vesikulært zink ikke bidrager observerbart til neuronskade efter TBI, hvorfor den foreslåede anvendelse af kelatorer til reduktion af den vesikulære zink pool efter TBI nøje bør overvejes, inden de forsøges anvendt i klinikken. Yderligere studier vil være påkrævet for at undersøge vesikulært zink neurons beskyttende egenskaber.

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Appendix

I: Changes in the Vesicular Zinc Pattern Following Traumatic Brain Injury

II: Chemical Blocking of Zinc Ions in CNS Increases Neuronal Damage Following Traumatic Brain Injury (TBI) in Mice

Figure 1

Figure 2