





**LENNE-TRIIN HEIDMETS**

The effects of neurotoxins on brain plasticity:  
focus on Neural Cell Adhesion Molecule



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*To my family*



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## LIST OF ORIGINAL PUBLICATIONS

**This dissertation is based on the following publications:**

- I. Jaako-Movits K, Zharkovsky T, Romantchik O, Jurgenson M, Merisalu E, **Heidmets LT**, Zharkovsky A. (2005) Developmental lead exposure impairs contextual fear conditioning and reduces adult hippocampal neurogenesis in the rat brain. *Int J Dev Neurosci* 23; 627–35.
- II. **Heidmets LT**, Zharkovsky T, Jurgenson M, Jaako-Movits K, Zharkovsky A. (2006) Early post-natal, low-level lead exposure increases the number of PSA-NCAM expressing cells in the dentate gyrus of adult rat hippocampus. *Neurotoxicology* 27; 39–43.
- III. **Heidmets LT**, Kalda A, Zharkovsky A. (2007) Acute amphetamine treatment decreases the expression of 180–200 kDa isoform of polysialic acid linked neural cell adhesion molecule in mouse hippocampus. *Brain Res* 24;1165:89–97.

**Author's contribution:**

- I. The author participated in behavioral experiments, in tissue processing for immunohistochemical studies and in manuscript writing.
- II. The author was the main person responsible for tissue processing for immunohistochemical experiments, data analyzing and writing the manuscript.
- III. The author was the main person responsible for Western blotting experiments, data analyzing and writing the manuscript. Participated in study design and behavioral experiments.

## ABBREVIATIONS

|                |                                                        |
|----------------|--------------------------------------------------------|
| AA             | arachidonic acid                                       |
| ABC            | avidin-biotin complex                                  |
| 2-AG           | 2-arachidonylglycerol                                  |
| ANOVA          | analysis of variance                                   |
| AMPA           | 5-methyl-4-isoxazole propionic acid                    |
| AMPH           | amphetamine                                            |
| Asn            | asparagin                                              |
| ATP            | adenosine triphosphate                                 |
| BDNF           | brain derived neurotrophic factor                      |
| BLL            | blood lead level                                       |
| BrdU           | 5-bromodeoxyuridine                                    |
| BS             | behavioral sensitization                               |
| CA             | <i>cornu ammonis</i> , Ammon's horn                    |
| CAM            | cell adhesion molecule                                 |
| CAMK-II        | type II Ca <sup>2+</sup> /CaM-dependent protein kinase |
| CNS            | central nervous system                                 |
| CSF            | cerebrospinal fluid                                    |
| DAB            | diaminobenzidine                                       |
| DAG            | diacylglycerol                                         |
| DG             | dentate gyrus                                          |
| ECL            | enhanced chemoluminescence                             |
| Endo-N         | endoneuraminidase N                                    |
| ER             | endoplasmic reticulum                                  |
| ERK            | extracellular signal-regulated kinase                  |
| FAK            | focal adhesion kinase                                  |
| FGFR           | fibroblast growth factor receptor                      |
| FGL            | FG loop protein                                        |
| FNIII          | fibronectin type III-like                              |
| GCL            | granule cell layer                                     |
| GDNF           | glial derived neurotrophic factor                      |
| GFAP           | glial fibrillary acidic protein                        |
| GFR $\alpha$ 1 | receptor for GDNF                                      |
| GPI            | glycosylphosphatidylinositol                           |
| Hil            | hilus                                                  |
| HRP            | horseradish peroxidase                                 |
| IgSF           | immunoglobulin superfamily                             |
| IP3            | inositol 1,4,5-trisphosphate                           |
| L1             | laminin- $\alpha$ 1                                    |
| LHRH           | luteinizing hormone releasing hormone                  |
| LTD            | long-term depression                                   |
| LTP            | long-term potentiation                                 |

|                |                                                      |
|----------------|------------------------------------------------------|
| MAM            | methylazoxymethanol                                  |
| MAP            | mitogen-activated protein                            |
| mRNA           | messenger ribonucleic acid                           |
| NCAM           | neural cell adhesion molecule                        |
| NMDA           | N-methyl-D-aspartate                                 |
| NMDAR          | NMDA subtype of excitatory amino acid receptors      |
| OD             | optical density                                      |
| PBS            | phosphate buffered saline                            |
| PFC            | prefrontal cortex                                    |
| PKC            | protein kinase C                                     |
| PLC            | phospholipase C                                      |
| PND            | postnatal day                                        |
| PPI            | prepulse inhibition                                  |
| PrP            | prion protein                                        |
| PSA            | polysialic acid                                      |
| PSA-NCAM       | polysialic acid linked neural cell adhesion molecule |
| RMS            | rostral migratory pathway                            |
| Sal            | saline                                               |
| SDS            | sodium dodecyl sulfate                               |
| SGZ            | subgranular zone                                     |
| SNP            | single nucleotide polymorphism                       |
| ST8SiaII (STX) | Golgi polysialyltransferase II                       |
| ST8SiaIV (PST) | Golgi polysialyltransferase IV                       |
| SVZ            | subventricular zone                                  |
| TAG-1          | transient axonal glycoprotein-1                      |
| TBS            | TRIS buffered saline                                 |
| Tuj1           | $\beta$ -tubulin isoform III                         |
| TRIS           | trishydroxymethylaminomethane                        |
| TRK-B          | tyrosine kinase receptor B                           |
| VASE           | variable alternative splice exon                     |

# INTRODUCTION

Two seemingly contradictory but essential challenges of the nervous system are the capacity to preserve the specificity/stability of the tissue and on the other hand, the ability to transmute. Denoting, while connectional, neurochemical and functional specificities are substantial properties of central nervous system (CNS) structure and functioning, the ability to change, *plasticity*, must be considered according to current knowledge as another fundamental attribute (Zilles, 1992; Bonfanti, 2006). Brain plasticity or neuronal plasticity derives from the Greek word “*plaistikos*” meaning “to form” and refers to the ability of brain tissue to adequately react and adapt to continuous endogenous and environmental changes which is undoubtedly an essential property of an individual to survive. Four types of stimuli have been described to which a brain responds with change: developmental, such as in the newly formed and evolving brain of a child; activity dependent, such as in cases of lost senses; learning and memory, in which the brain changes in response to a particular experience; and finally injury induced, resulting from damage in the brain (Lledo *et al.*, 2006).

Among different types of plasticity the present study will mainly explore the field of adult structural plasticity, which comprises different aspects of structural and shape changes of the nervous tissue, focusing on cell adhesion systems and on the phenomenon of neurogenesis. The continuous production of new neural cells (Altman and Das, 1965) basically comprises all types of structural plasticity, including cell proliferation, migration, axonal and dendritic growth, differentiation and integration into neuronal circuits (Lledo *et al.*, 2006). Cell adhesion systems are involved in different levels in most of the above-mentioned processes creating the foundation for dynamic modulation of intercellular contacts underlying any structural change.

Our work essays to widen the knowledge about the effects of still widespread neurotoxin lead on specific behavioral paradigms, further trying to link long-term cognitive dysfunction following developmental low-level lead exposure with possibly underlying alterations in brain plasticity. The second part of the work studies the effects of different amphetamine treatment regimens on the expression levels of neural cell adhesion molecules (NCAM) possessing a potential role to learning in the development of behavioral sensitization and considering the role of NCAM's in learning and memory formation.

Better understanding of the effects and mechanisms of different substances affecting processes involved in brain plasticity might be important in understanding the physiology and pathology of the above-mentioned phenomenon, which could be significant in the search for new therapeutic approaches.

# REVIEW OF LITERATURE

## I. Cell Adhesion Molecules

Cell Adhesion Molecules (CAM) are proteins located on the cell surface, required for continuous dynamic contacting of cells with each other or with the extracellular matrix in the process called cell adhesion, which forms the basis for tissue formation, specialization, maintenance and functioning during development as well as in adulthood. There exist hundreds of adhesion molecules that are so far classified into four main families: Ig (immunoglobulin) superfamily (IgSF CAMs), the integrins, the cadherins and the selectins, of which the immunoglobulin superfamily is in our interest. Proteins in the immunoglobulin superfamily are characterized by the presence of a motif that resembles immunoglobulin proteins, immunoglobulin-like domain (Brummen-dorf and Rathjen, 1995) and fall into different subfamilies depending on the number of Ig-like domains, the presence and number of fibronectin type III-like (FNIII) repeats (Cunningham, 1995), the mode of attachment on cell membrane and the presence of catalytic cytoplasmic domain (for review see Crossin and Krushel, 2000; Figure 1).

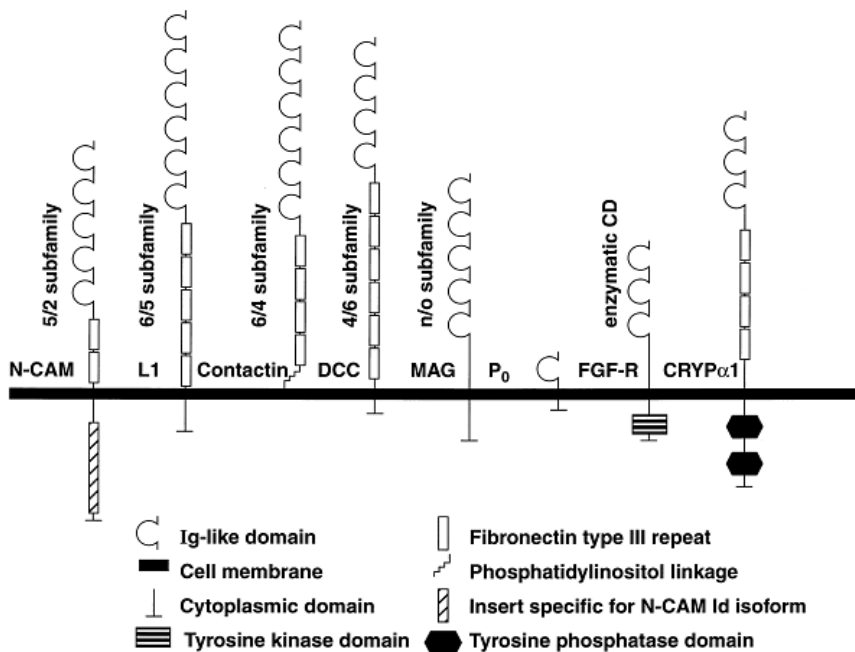


**Figure 1.** Immunoglobulin superfamily of CAMs falling into several subfamilies depending on the number of immunoglobulin-like domains and fibronectin repeats (from review of Crossin and Krushel, 2000).

## 1.1. Neural cell adhesion molecule

The neural cell adhesion molecule (NCAM, also N-CAM, CD56, D2) was the first isolated and characterized immunoglobulin-like cell adhesion molecule (Rutishauser *et al.*, 1976) that has been found in almost all tissues with the highest expression in the central and peripheral nervous tissue.

There exist several isoforms of NCAM that are generated via alternative splicing from a primary transcript of gene NCAM1 (Jorgensen and Bock, 1974) consisting of 20 major exons plus 6 additional small exons in mouse (Walmod *et al.*, 2004) that in man is located in chromosome 11, in mouse in chromosome 9 and in rat in chromosome 8 (Yasue *et al.*, 1992; D'Eustachio *et al.*, 1985; Nguyen *et al.*, 1986; Walsh *et al.*, 1986). Another gene, named NCAM2, has been described in 21q21 (Paoloni-Giacobino *et al.*, 1997), showing strong homology with members of the neural cell adhesion molecule family of genes from different species, the predicted polypeptide of the NCAM2 gene contains 837 amino acids and shows 62% similarity to the NCAM homologues. However, not much information has been accumulated related to its functions and signaling.

Three major isoforms of NCAM are named according to their approximate molecular weight (Figure 2) of which NCAM-180 (NCAM-A) is a transmembrane protein generated from exons 1–19, NCAM-140 (NCAM-B) is also a transmembrane protein with a shorter cytoplasmic domain and differs from NCAM-180 only in exon 18; NCAM-120 (NCAM-C) is a glycosylphosphatidylinositol (GPI) anchored protein generated from exons 0–15 (Gascon *et al.*, 2007). The extracellular region (N-terminal) of all isoforms of NCAM consists of five immunoglobulin-like modules and two-fibronectin type III modules.

Further variations are achieved by the inclusion of the variable alternative splice exon (VASE) in the original transcript, slightly modifying the extracellular part of NCAM (Doherty *et al.*, 1992). NCAM also exists in a secreted form produced when one small exon containing a stop codon between exons 12 and 13 is included in the mRNA giving rise to a truncated form of NCAM (Walmod *et al.*, 2004). Soluble forms of NCAM can also be generated by the enzymatic excision of NCAM-120 from the GPI anchor (He *et al.*, 1986) or by the proteolytic cleavage of the extracellular part of NCAM molecules (Hinkle *et al.*, 2006).

Within synaptic environments NCAM isoforms seem to have distinct expression profiles, NCAM-120 being mostly expressed on glial cells, NCAM-180 on the postsynaptic sides of neurons and NCAM-140 can be found both in glial and neural cells (Noble *et al.*, 1985).

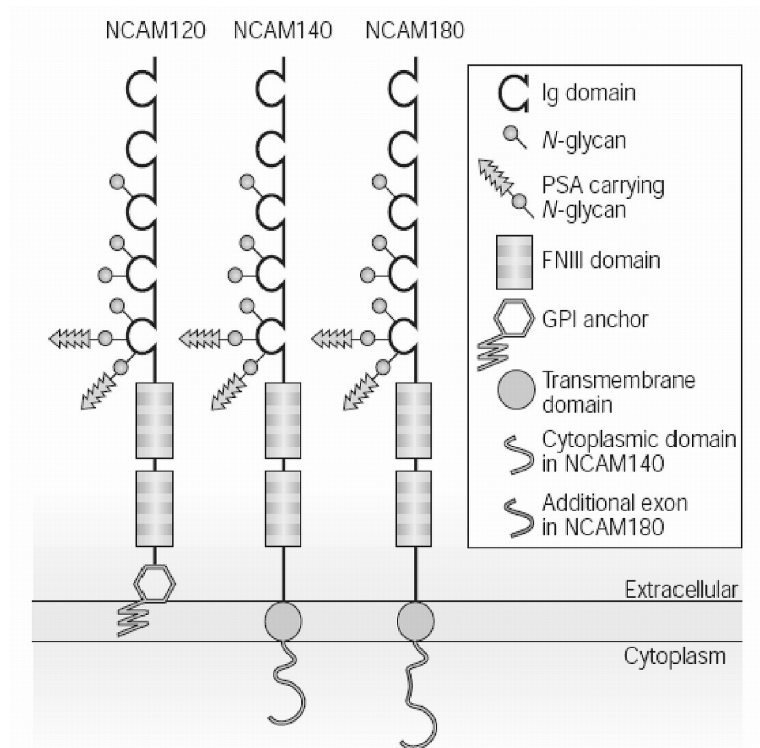


**Figure 2.** Structure of three main isoforms of NCAM named according to their approximate molecular weight (from review by Kleene and Schachner, 2004)

### 1.1.1. Homophilic and heterophilic NCAM interactions

All NCAM isoforms can mediate homophilic binding (NCAM to NCAM). According to current studies based on crystallography (Soroka *et al.*, 2003) and biological experiments it seems that dimerization of NCAM molecules on the same cell (*cis* interaction) requires aromatic residues located in the IgI module to interact with the hydrophobic pocket formed by the IgII module of another NCAM molecule bending over each other in a crosslike formation (Figure 3; Soroka *et al.*, 2003). Moreover, interaction between NCAM molecules on the surfaces of opposing cells (*trans*-interaction) requires first the formation of NCAM *cis*-dimers. Two kinds of *trans*-interactions have been proposed: the “flat-zipper” involves IgII to IgIII antiparallel binding (Figure 3) and the second, “compact zipper” requires interaction between IgI-IgIII and IgII-IgII (Soroka *et al.*, 2003; Walmod *et al.*, 2004). Combining both types of zippers could lead to the formation of two-dimensional zipper pattern (Kiselyov *et al.*, 2005), the presence of polysialic acid (PSA) moiety is proposed to prevent the formation of a strong two-dimensional zipper (Yang *et al.*, 1992).

NCAM has been shown to interact also in heterophilic manner with a wide range of molecules other than NCAM itself found on cell surface, more detailed description is in the section 1.3.

**Figure 3a**

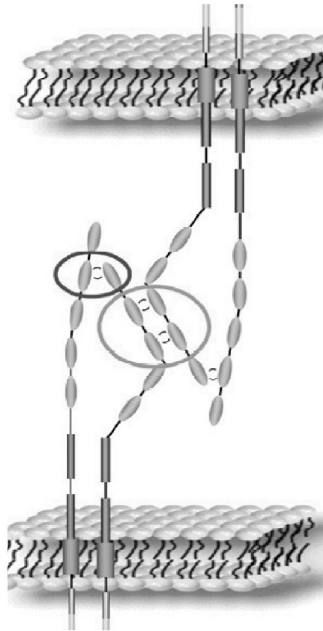


**Figure 3b**

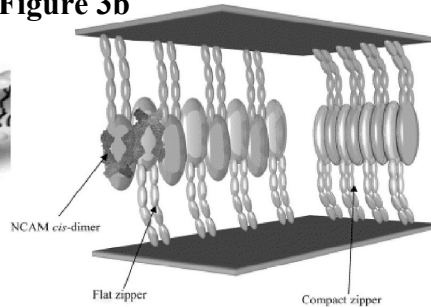


**Figure 3c**

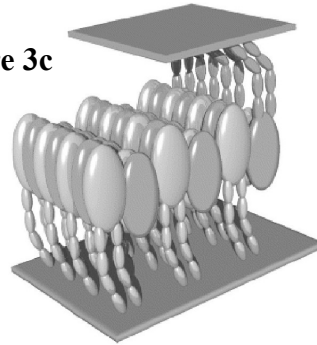


**Figure 3a.** The current model for NCAM interactions. NCAM *cis*-dimers involve the interaction between IgI and IgII (small circle). NCAM trans-interactions require the initial formation of *cis*-dimers. Then, two kinds of interactions between NCAM molecules on opposing cell membranes are possible: the “flat zipper” interaction, illustrated in the picture, involves IgII and IgIII domains (big circle) (from review of Gascon *et al.*, 2007). **3b.** The two N-terminal triple Ig modules shown in the left part are indicated by an ellipsoid. The other extracellular modules of NCAM are indicated individually by smaller circular ellipsoids, with a twofold smaller diameter. A flat one-dimensional zipper is shown in the left part and a compact one-dimensional zipper is shown in the right part. **3c.** A two-dimensional zipper, which is a combination of the flat and compact zippers (from the review of Kiselyov *et al.*, 2005).

## 1.2. Polysialic acid and polysialic acid linked neural cell adhesion molecule

NCAM protein is a subject to different posttranslational modifications. Among others by far the most interesting and functionally important modification is glycosylation having major impact on three-dimensional folding, stability and function of proteins (Varki, 1993). Among other carbohydrates, polysialic acid,

a linear homopolymer of  $\alpha$ 2,8-linked sialic acid, was found to be attached to NCAM in mammalian brains almost three decades ago by Jukka Finne who started the research on the interplay between carbohydrates and proteins in cell interactions in the nervous system (Finne, 1982; Finne *et al.*, 1983).

All main NCAM isoforms and their derivatives have been shown to carry on their 5<sup>th</sup> Ig-like domain a highly negatively charged carbohydrate polysialic acid (PSA) (Finne *et al.*, 1983), a linear homopolymer of  $\alpha$ 2,8-linked sialic acids which can be up to 100 residues long (Kiss and Rougon, 1997). The extracellular part of NCAM contains at least 6 N-glycosylation sites (Asn 203, 297, 329, 415, 441, 470 in mouse) that can be glycosylated in a dynamic spatial and temporal pattern (Albach *et al.*, 2004) in the endoplasmic reticulum (ER) or Golgi compartment (Kiss and Rougon, 1997). Attachment of PSA to NCAM generates a large hydration cloud around the core protein, which sterically modulates the homophilic binding of (the) NCAM molecule resulting in an opposite effect to NCAM – deadhesion (Figure 4; Cunningham *et al.*, 1983; Sadoul *et al.*, 1983; Johnson *et al.*, 2005), allowing cells to move towards each other and thereby, forming the basis for dynamic changes underlying structural plasticity.

It must be mentioned that only mammalian NCAMs carry sugar like PSA, in invertebrates the cycles of adhesion/deadhesion are regulated on the level of the expression of whole NCAM molecule (Mayford *et al.*, 1992; Fambrough *et al.*, 1996).

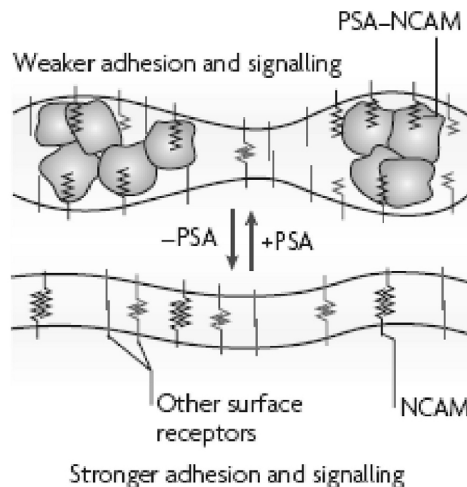


**Figure 4.** A schematic representation of molecular interactions during membrane-membrane contact. Note that although PSA is only attached to NCAM, its global effect on membrane-membrane apposition affects other cell-contact-dependent receptors, such as cadherins (from the review of Rutishauser, 2007).

### **1.2.1. The occurrence of polysialic acid**

The expression of PSA is abundant during developmental stage and is referred to as the embryonic form on NCAM. Conversion of the embryonic form to non-polysialylated NCAM, the adult form of NCAM, takes place during development after birth, resulting in the expression of polysialylated NCAM in the restricted areas of adult brain known to exhibit physiological plasticity or self-renewal (Theodosios *et al.*, 1991; Seki *et al.*, 1993): mouse olfactory bulb (Miragall *et al.*, 1988), rat piriform and entorhinal cortices (Seki and Arai, 1991a; O'Connell *et al.*, 1997), suprachiasmatic nucleus and hippocampus (Seki *et al.*, 1991b), hypothalamus-neurohypophyseal system (Theodosios *et al.*, 1991), mouse subventricular zone (SVZ) (Rousselot *et al.*, 1995), nuclei of spinal cord and the rostral migratory stream, a pathway by which precursor cells migrate throughout life from SVZ of the forebrain to olfactory bulbs (Chazal *et al.*, 2000). In the adult hippocampus, PSA-NCAM expression persists in the mossy fibers (axons of the granule neurons), in the hilus of dentate gyrus, in subgranular zone, in the CA3a and CA3b stratum lucidum (Seki *et al.*, 1991b, 1999), in the alveus and fimbria, above the hippocampal fissure on the molecular layer of CA1 (Seki *et al.*, 1991b), in the subiculum and in Schaffer collaterals and neurons in CA3 (O'Connell *et al.*, 1997).

### **1.2.2. The regulation of PSA expression**

The synthesis of PSA is catalyzed by two different Golgi polysialyltransferases, ST8SiaII (STX) and IV (PST) (Nakayama *et al.*, 1998) either of which is sufficient to the complete synthesis of PSA chains. Cells producing PSA express both STX and PST and it has been proposed that the enzymes might work cooperatively to produce higher levels and longer polymers of PSA (Angata *et al.*, 2002; Angata and Fukuda, 2003) as the degree of polysialylation by ST8SiaII is only about 40 sialic acid residues and by ST8SiaIV about 60 residues (Angata *et al.*, 2002). STX has been shown to be more active during development and in stem-cell derived immature granule cell neurons while PST is more involved in the polysialylation of NCAM in mature neurons.

Interestingly, when most carbohydrates are attached to a variety of proteins, polysialic acid is almost exclusively synthesized on the last of NCAM's five tandem amino terminal immunoglobulin domains and adjacent to the proteins two membrane proximal FNIII domains (Mendiratta *et al.*, 2005). Only voltage-sensitive sodium channels and neurophilin have been demonstrated to carry detectable levels of polysialylation (James and Agnew, 1987; Curreli *et al.*, 2007).

The regulatory mechanisms of the expression level of PSA are still poorly understood. There is evidence that both transcriptional and post-transcriptional mechanisms exist. In many situations, the level of NCAM polysialylation is

correlated with the amount of both ST8SiaII and ST8SiaIV transferase mRNA, however, independent transcriptional control of ST8SiaII and ST8SiaIV have been shown, demonstrating different enzymological activity, biological functions and expression in distinct vertebrates (Angata and Fukuda, 2003; Rutishauser, 2008). Also post-transcriptional regulatory mechanisms appear to operate in the context of specific developmental and physiological conditions (Bruses and Rutishauser, 1998). Pharmacological and biochemical analyses of PSA synthesis have suggested calcium-dependence of the polysialyltransferase activity (Bruses and Rutishauser, 1998). Proteolytic cleavage of the extracellular domain of NCAM from cell surface by metalloproteases has been demonstrated (Hinkle *et al.*, 2006) and the effect of N-methyl-D-aspartate (NMDA) receptor on the levels of PSA (Bouzioukh *et al.*, 2001). Alterations in the expression levels of PSA-NCAM on cell surface could reflect also differential delivery of PSA to the cell surface. Support to the hypothesis comes from the observation that in oligodendrocyte precursor cells NMDA can induce an influx of calcium that is proposed to enhance the transport of PSA to (the) cell surface (Wang *et al.*, 1996), the fusion of PSA containing granules to the plasma membrane in pancreatic cells can be induced by depolarizing the membrane (Kiss *et al.*, 1994).

An interesting and intriguing regulatory mechanism could be the removal of PSA from cell surface, which in physiological conditions could happen by the endocytosis of PSA-NCAM or the degradation of PSA, however, evidence supporting this mechanism to play an important role is still insufficient.

In 1985 Finne and Mäkelä demonstrated an enzyme isolated from bacteriophages, endoneuraminidase N (Endo-N) to specifically recognize the sialic acid polymers in  $\alpha$ -2,8-linkage and cleave units of eight sugar residues. Since then Endo-N has been extensively used *in vivo* and *in vitro* to study the roles of PSA under physiological and pathological conditions. The removal of PSA from NCAM by Endo-N has been shown to disrupt neuronal migration, axonal sprouting, branching and fasciculation, and synaptogenesis (for review see Rutishauser, 2008); Endo-N treatment prevents the induction of LTP and LTD in organotypic cultures (Muller *et al.*, 1996) and in hippocampal slices (Becker *et al.*, 1996); Endo-N injections into the rat hippocampus leads to significant impairments in the formation of spatial memory (Becker *et al.*, 1996).

### **1.2.3. The roles of PSA in developing and adult nervous system**

Possessing certain tools to qualitatively and quantitatively measure PSA content and also manipulate PSA expression has enabled to elucidate the role of PSA in the developing as well as adult brain. In general, PSA mediated attenuation of cell-interactions results in conditions that are permissive for changes in cell position and shape or, on the other hand, controls the overall ability of cells to interact. Participation of PSA in several other processes, like differentiation and

survival, has been discovered recently (Seidenfaden *et al.*, 2003; Seki *et al.*, 2007).

During nervous system development, PSA has been demonstrated to create “permissive” conditions for the postmitotic migration of precursor cells. Well documented example is the rostral migration of progenitor cells from their birthplace in SVZ to the olfactory bulb that is inhibited if the PSA is by different means eliminated from the cell surface (Ono *et al.*, 1994; Hu *et al.*, 1996). The role of PSA as a modulatory factor has been elucidated also in hippocampal granule cell translocation (Seki *et al.*, 2007), in the migration of luteinizing hormone releasing hormone (LHRH) neurons from the olfactory placodes into the developing forebrain (Murakami *et al.*, 2000) and oligodendrocyte precursor cell translocation (Barral-Moran *et al.*, 2003).

Axon growth and guidance is influenced by the presence of PSA, more specifically branching of motor neuron bundles require PSA for the axons to be able to separate from each other, which is important for the selective innervation of different muscles (Landmesser *et al.*, 1990). Doherty and collaborators (1990) demonstrated that NCAM-dependent neurite outgrowth of chicken retinal ganglion cells *in vitro* could be inhibited by the removal of PSA. Also, during axon pathfinding the removal of PSA causes aberrant and persistent innervation of the pyramidal cell layer by mossy fibers, including excessive collateral sprouting and/or defasciculation of these processes, as well as formation of ectopic mossy fiber synaptic boutons (Seki and Rutishauser, 1998) suggesting an insulative property of PSA during adequate targeting and innervating large leading mark regions.

One recent discovery is the role of PSA in the timing of cell differentiation. For example in the newly generated granule-cell precursors in hippocampus the abundant expression of PSA is associated with cell migration and the loss of polysialic acid coincides their differentiation into mature neurons expressing mature neuronal markers (Seki *et al.*, 2007). In addition, removal of PSA from cell surface from neuroblastoma cells in culture led to reduced proliferation and activated extracellular signal-regulated kinase (ERK), inducing enhanced survival and neuronal differentiation of neuroblastoma cells (Seidenfaden *et al.*, 2003). The role of PSA in the processes of myelination during development must be mentioned as it has been shown that down-regulation of PSA during oligodendrocyte differentiation is a prerequisite for efficient myelination by mature oligodendrocytes (Fewou *et al.*, 2007).

In spite of the fact that the expression of PSA is generally down-regulated in the adulthood it has been shown to remain prominent in some brain areas that exhibit physiological plasticity, as already described in section 1.2.1. Many of these structures, like hippocampus, piriform cortex, amygdala and neocortex are associated with memory formation and learning. Also, experimental manipulations altering the expression levels of PSA-NCAM change the ability of an animal to learn and even more interestingly, learning alters the expression of the molecule. One of the first insights into the role of PSA in cognitive functions

came from the work of Doyle and collaborators (1992), who demonstrated that the acquisition and consolidation of the passive avoidance response is associated with the transient increase in the sialylation of the NCAM180 in hippocampal membrane fractions. Further immunohistochemical experiments revealed the time dependent increase in the number of PSA-NCAM expressing cells in the border of the granule cell layer and the hilus of the dentate gyrus as early as 4 hours following training with the peak 12 hours after exposure to the paradigm and subsequent return to the starting level 24 hours following avoidance learning (Fox *et al.*, 1995). Similar time-dependent and transient changes have been observed also following spatial learning in Morris water-maze test (Murphy *et al.*, 1996) and in reward-based odor discrimination task providing information about the consolidation of olfactory memory in hippocampus (Foley *et al.*, 2003). Intraventricular injections of antibodies and synthetic peptide-ligands interacting with NCAM have been shown to affect exploratory behavior and memory in rodents (Hartz *et al.*, 2003), application of NCAM antibodies to hippocampal slice cultures impairs LTP (Lüthi *et al.*, 1994), NCAM-deficient mice show deficits in spatial learning and decayed LTP (Cremer *et al.*, 1994; Muller *et al.*, 1996), enzymatic removal of PSA results in decayed LTP in hippocampus that is associated with impaired acquisition and retention of spatial memory in Morris water maze (Becker *et al.*, 1996).

### **I.3. NCAM and PSA-NCAM mediated signalling**

In the last decade, knowledge about the functions and roles of NCAM and its polysialylated form has widened dramatically showing that adjacent to being a mediator of cell adhesion NCAM is also a versatile transduction molecule affecting a wide range of biological processes.

NCAM has been demonstrated to be involved in several homo- and heterophilic interactions resulting in the formation of intracellular signaling complexes (Figure 5). NCAM *per se* does not possess intracellular catalytic activity that would initiate intracellular signaling, however, NCAM activates signaling pathways by acting in concert with different extracellular or intracellular binding partners. Most of the evidence comes from *in vitro* studies on NCAM promoting axonal growth. Several approaches have been used to mimic NCAM homophilic binding *in vitro* to investigate downstream signaling events and other cellular effects of NCAM binding, of which the use mimetic peptides have been introduced recently and provide the most convenient means to stimulate NCAM (Berezin and Bock, 2004).

### 1.3.1. Extracellular NCAM interaction partners

NCAM has been shown to interact in heterophilic manner with a wide range of molecules other than NCAM itself found on cell surface, mentioning other CAM-s such as transient axonal glycoprotein-1 (TAG-1) and laminin- $\alpha$ 1 (L1) (Brummendorf and Rathjen, 1995), members of the extracellular matrix like heparin sulfate proteoglycans (Cole and Glaser, 1986), chondroitin sulfate proteoglycans (Grumet *et al.*, 1993), glycosaminoglycan heparin (Cole and Glaser, 1986). NCAM has been shown to interact with adenosine triphosphate (ATP) having ecto – ATPase activity (Dzhandzhugazyan and Bock, 1993, 1997) and also the prion protein (PrP) (Santucci *et al.*, 2005). In the following section we briefly overview the interactions of NCAM with several growth factor receptors and glutamate receptors.

**Fibroblast growth factor receptor (FGFR).** It is well established that NCAM homophilic binding induces dimerization and autophosphorylation of the FGFR and results in neurite outgrowth (Saffell *et al.*, 1997; Kiselyov *et al.*, 2003). Activation of FGFR results in the activation of phospholipase C (PLC) that generates second messengers inositol 1,4,5-trisphosphate (IP3) which induces calcium release from internal stores, and diacylglycerol (DAG) that gives rise to arachidonic acid (AA) and 2-arachidonylglycerol (2-AG). It is proposed that neurite outgrowth depends on the ability of 2-AG to activate cannabinoid receptors 1 and 2 (Williams *et al.*, 2003).

**Glial derived neurotrophic factor (GDNF).** It has been recently described by Paratcha and collaborators (2003) that NCAM also acts as a signaling receptor for members of the GDNF ligand family. Association of NCAM with GFR $\alpha$ 1 (receptor for GDNF) downregulates NCAM-mediated cell adhesion and promotes high-affinity binding of GDNF to NCAM, resulting in rapid activation of cytoplasmic protein tyrosine kinases Fyn and focal adhesion kinase (FAK) in cells lacking RET (known GDNF signaling receptor). The binding of GDNF is independent of the presence of the PSA on NCAM and does not interfere with the homophilic NCAM binding. GDNF stimulates Schwann cell migration and axonal growth in hippocampal and cortical neurons *via* binding to NCAM and activation of Fyn, but independently of RET (Paratcha *et al.*, 2003).

### 1.3.2. Intracellular NCAM interactions

**Spectrin.** Spectrin, an important cytoskeletal organizer of membrane and cytosolic protein macromolecular complexes, is a known NCAM interaction partner. It has been demonstrated that NCAM selectively interacts with NH2 terminus of spectrin and protein kinase C- $\beta$ 2 (PKC- $\beta$ 2) is recruited into these complexes. The formation of NCAM-spectrin-PKC complexes is required for the neuritogenic effect of NCAM (Leshchyn'ska *et al.*, 2003). NCAM-spectrin

associations have been reported to be important for the proper assembly, maintenance and activity-dependent remodeling of the postsynaptic signaling complexes in excitatory synapses (Sytnyk *et al.*, 2006).

NCAM has been shown to stimulate **mitogen-activated protein (MAP) kinase pathway** (Kolkova *et al.*, 2000), as NCAM-NCAM interaction induces the phosphorylation of Fyn, which leads to the recruitment and activation of FAK. Phosphorylated FAK interacts with several adaptor proteins, eventually recruiting and activating several elements of the MAP-kinase pathway including Ras and Raf (Zhang *et al.*, 2002).

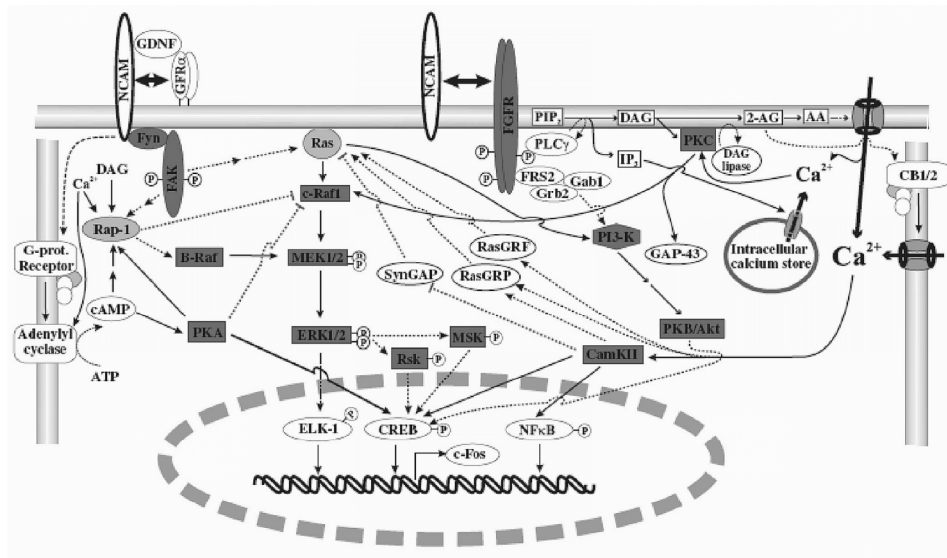


**Figure 5.** NCAM-mediated intracellular signal transduction pathways. The figure is highly schematic and does not necessarily reflect the correct cellular localization of the respective proteins. Pathways, whose role in NCAM-mediated signaling remains to be determined, are indicated with dashed lines. Kinases are shown in dark grey, Ser/Thr-kinases or dual-kinases as squares, Tyr-kinases as ellipses (from the review of Walmod *et al.*, 2004).

### 1.3.3. The role of PSA in NCAM mediated signaling

Considering the physical spacer function of PSA that reduces adhesion forces between cells and allows dynamic contacts between cells, the presence of PSA has been suggested to favor the association between NCAM with other receptors that is impeded in the presence of firm adhesion mediated by unpolysialylated NCAM, or, in other words, the expression of PSA on NCAM may function as a “switch” regulating whether NCAM is involved in adhesion

or signaling. However, the presence of PSA does not affect unimodally the interactions of NCAM with all signaling partners.

**Brain derived neurotrophic factor (BDNF).** In synaptic environments the interaction partner of PSA-NCAM has been shown to be BDNF receptor tyrosine kinase receptor B (TRK-B). It has been demonstrated that deficient LTP observed in freshly isolated hippocampal slices of NCAM-deficient mice or organotypic cultures treated with Endo-N could be recovered by the addition of BDNF (Muller *et al.*, 2000). Also BDNF signaling, as measured by the level of TRK-B phosphorylation, was found to be decreased in NCAM knock-out mice and Endo-N treated cultures suggesting the possibility that PSA could act by sensitizing pyramidal neurons to BDNF (Muller *et al.*, 2000). Additionally, Vutskits and collaborates (2001) demonstrated that the loss or inactivation of PSA tail on NCAM leads to reduced survival and differentiation, which could be prevented by adding exogenous BDNF. The above-mentioned data suggests that PSA tail on NCAM is required for adequate BDNF signaling and good survival response of neurons to BDNF.

**Fibroblast growth factor receptor (FGFR).** The role of PSA on NCAM has been proposed by Kiselyov and collaborates (2005) also in FGFR signaling. The expression of PSA has been demonstrated to favor the association between NCAM and FGFR predisposing the formation of loose clusters of one-dimensional zippers (Kiselyov *et al.*, 2005).

PSA has been demonstrated to increase cell responses also to several other growth factors like platelet-derived growth factor (PDGF; Zhang *et al.*, 2004) and ciliary neurotrophic factor (CNTF; Vutskits *et al.*, 2003).

**Glutamate receptors.** Recent reports suggest that PSA-NCAM can directly interact with  $\alpha$ -amino-5-methyl-4-isoxazole propionic acid (AMPA) and NMDA glutamate receptors (Vaithianathan, 2004; Hammond, 2006). PSA-NCAM inhibited NMDA receptor currents (Hammond, 2006), prolonged the open channel time of AMPA-receptor mediated currents and altered the bursting pattern of receptor channels (Vaithianathan, 2004). The above-mentioned data suggest that during developmental stage PSA NCAM could play a role in synapse formation regulating glutamate receptor activity.

#### **I.4. Mice deficient in NCAM or PSA**

Several NCAM-related transgenic mice lines have been generated, which has profoundly widened our knowledge about the roles and functions of NCAM and also its sialylated form. In 1994, Cremer and collaborates demonstrated that animals lacking all major isoforms of NCAM appear healthy and fertile presenting only minor defects in the brain development and behavior, like size-reduced olfactory bulbs, increased lateral ventricles, deficits in spatial learning and exploratory behavior. Further experiments revealed additional deficits in contextual and cued fear conditioning (Stork *et al.*, 2000), impaired LTP in CA1

and CA3 regions (Muller *et al.*, 1996) and abolished LTP *in vivo* in dentate gyrus (Stoenica *et al.*, 2006). These observations suggest the possible existence of a compensatory mechanism during development.

Further conversance of the functions of NCAM and PSA came from animals lacking ST8SiaII and -IV (Weinhold *et al.*, 2005), demonstrating a severe phenotype which includes beside features shared with NCAM-null animals specific brain wiring defects, progressive hydrocephalus, postnatal growth retardation, and precocious death. Strikingly, these defects were selectively rescued by an additional deletion of NCAM, demonstrating that they originate from a gain of NCAM functions because of PSA deficiency (Weinhold *et al.*, 2005).

### **1.5. The role of PSA-NCAM and NCAM in pathological conditions**

Considering the physiological functions of NCAM and PSA-NCAM, the role of the molecules in different pathological conditions has been investigated.

The changes in neuronal structure and connectivity, with at least partly underlying alterations in PSA-NCAM and NCAM expression or functioning, have been suggested in the molecular pathology underlying depression and also as possible target molecules for antidepressants (Varea *et al.*, 2007a, b), also in the development of mood and anxiety disorders following juvenile stress (Tsoory *et al.*, 2008). Chronic stress has been shown to induce biphasic PSA-NCAM expression in the adult rat dentate gyrus (Pham *et al.*, 2003).

An increased expression of PSA-NCAM has been found in patients with Alzheimer's disease, however, it is unclear whether the finding is an attempt of the brain tissue to try to restore its structure and function, or to compensate for the damage caused by the disease, or these changes are a part of the disease's pathologic cascade (Mikkonen *et al.*, 2001).

NCAM1 ranked fourth in a meta-analysis of schizophrenia susceptibility loci, also single nucleotide polymorphisms (SNPs) in STX and PST have been reported as schizophrenia susceptibility loci (for review see Maness *et al.*, 2008). PSA-NCAM levels are decreased in the hippocampuses of schizophrenia patients, however, the levels of soluble fragments consisting mostly of the extracellular domain of NCAM in the cerebrospinal fluid (CSF) and affected brain regions (prefrontal cortex (PFC), hippocampus) are increased, which has been also correlated with disease severity and duration. A mouse model with increased NCAM cleavage (NCAM-EC) that has also been observed in schizophrenic patients has been shown to display decreased emotional memory and prepulse inhibition (PPI), also increased hyperactivity and enhanced sensitivity to amphetamines, as well as stereotypy. These mice have been proposed to provide a model for novel treatment options, as blocking NCAM

cleavage could restore observed alterations at least partly (Barbeau *et al.*, 1995; Vincente *et al.*, 1997).

In 2003, Sato and collaborators demonstrated that the number of PSA-NCAM positive cells in the bilateral DG as well as marked extension of immunopositive dendrites to the molecular layer increased significantly after repeated exposure to amygdaloid kindled general seizures, which indicate that increased migration of newly generated cells as well as plastic changes of preexisting neural cells occur in response to recurrent GS. Observed alteration could contribute to an abnormal reconstruction of the synaptic network in the hippocampus and, thus, epileptogenicity from kindling (Sato *et al.*, 2003a). However, Pekcec and collaborators (2008) found that loss of PSA counteracted the status epilepticus-induced increase in neurogenesis and the effect of endoneuraminidase treatment on hippocampal neurogenesis did not impact the subsequent development of spontaneous seizures. In contrast, transient lack of PSA during status epilepticus and in the early phase of epileptogenesis exhibited a cognition sparing effect as revealed in the Morris water maze paradigm. In conclusion, the role of NCAM and its polyiallylated form in the pathogenesis of epilepsy needs further clarification.

Changes in the expression of NCAM and PSA-NCAM have been associated also with different malignant processes. In colon carcinoma, pancreatic cancer, and astrocytoma, NCAM expression is markedly down-regulated, and the loss of NCAM correlates with poor prognosis. In contrast, in neuroblastoma and certain neuroendocrine tumors, cancer progression correlates with increased NCAM expression (Crnic *et al.*, 2004). The results concerning the role of polysialic acid is more consistent demonstrating that PSA is re-expressed during the progression of several malignant human tumors, including small cell lung carcinoma, Wilms' tumor, neuroblastoma, and rhabdomyosarcoma. In these tumors, polysialylation of NCAM appears to increase the metastatic potential and has been correlated with tumor progression and a poor prognosis (Seidenfaden *et al.*, 2003).

## **1.6. The roles of PSA-NCAM and NCAM in neuroprotection**

The role of NCAM mediated signaling in neuroprotection has been recently demonstrated. The role of NCAM in the protection of neurons induced to undergo apoptosis was reported for the first time by Ditlevsen and colleagues (2003), who demonstrated that NCAM mimetic peptide C3 reduced the death of neurons in different *in vitro* models of apoptosis. The protective effect of C3 included an inhibition of both DNA-fragmentation and caspase-3 activation. Further, Skibo and collaborators (2005) showed that FGL, a NCAM-derived peptide binding induces phosphorylation of FGFR, acting neuroprotectively after an ischemic insult both *in vitro* and *in vivo*. Recently it has been demonstrated that signalling pathways underlying neural cell adhesion molecule-

mediated survival of dopaminergic neurons required signalling through Fyn, FGFR, MEK, PKA and PKC but, in contrast to NCAM-mediated neurite outgrowth, PLC and type II  $\text{Ca}^{2+}$ /CaM-dependent protein kinase (CaMKII) were not prerequisites for NCAM-mediated neuroprotection (Ditlevsen *et al.*, 2007).

## **2. Neurogenesis**

Among different types of structural plasticity the continuous production of neural cell precursors in certain so called neurogenic regions throughout the lifespan (Altman and Das, 1965) could be considered one of the most striking discoveries in the field of neuroscience in the 20th century and the concept of static nervous tissue has been disproved. Neurogenesis is a process that is generally believed to involve cell proliferation, migration, axonal and dendritic growth, differentiation and integration into neuronal circuits (Lledo *et al.*, 2006). Adult neurogenesis has been demonstrated almost in all species including bird, rodent, monkey as well as human (Gross, 2000) and two main neurogenic areas have been identified in mammalian brain: the subventricular zone (SVZ) of the forebrain and the dentate gyrus (DG) of hippocampus (Altman and Das, 1965; Gage, 2000), the focus of our interest in the present study. In DG the progenitor cells are located in the subgranular zone (SGZ; border between the granule cell layer and the hilus) from where a subset of newly born cells, arising from the proliferation of stem cells, survive, migrate into the granule cell layer and differentiate into glial cells or acquire the cellular markers and characteristics of neurons (van Praag *et al.*, 2002). Cameron and McKay (2001) have demonstrated that in adult rodent hippocampus approximately 9000 cells are generated daily, around 50% of these newly born cells differentiate eventually expressing neuron-specific markers. This number of new granule neurons generated each month is 6% of the total size of the granule cell population which supports the idea that these new neurons could play an important role in the hippocampal function.

### **2.1. The functional significance and factors affecting adult hippocampal neurogenesis**

The neurogenesis in adult brain could be regulated in positive as well as negative manners by various internal and external factors (Table 1). The main internal factors to be mentioned are gender, age and genetics, level of hormones and neurotransmitters, and expression of growth factors. Several environmental and pharmacological stimuli are the main external factors affecting adult hippocampal neurogenesis, thereby modulating neurogenesis mediated processes.

Apart from the fact that new cells are produced throughout the adult life also the functional significance of the phenomenon has been under investigation revealing that adult neurogenesis exists as a form of neuronal plasticity which is related to different cognitive processes like learning and memory formation as well as emotions (Gould *et al.*, 1999; Kempermann, 2002). It has been shown that various genetic and environmental factors affecting hippocampal neurogenesis cause corresponding changes in cognitive performance. Hippocampal neurogenesis is influenced by the genetic background of mice (Kempermann and Gage, 2002), further, mutant mice with decreased SGZ neurogenesis demonstrated impaired performance in hippocampus-dependent learning tasks in several experiments. Also environmental factors have major impact on SGZ neurogenesis (Olson *et al.*, 2006), as voluntary running increases SGZ cell proliferation and exposure to enriched environment promotes the survival of 1- to 3-week old immature neurons (Tashiro *et al.*, 2007) and both of previously mentioned improve the performance of young and aged mice in Morris water-maze.

SGZ neurogenesis has been shown to be enhanced only in hippocampus-dependent learning tasks like trace eyeblink conditioning and learning in the Morris water maze (for review see Leuner *et al.*, 2006). Moreover, a correlation between survival of 7-day-old neurons and learning and memory performance of the individual rats, despite the variable space effect during training, suggests the effect of learning *per se*, not the effect of training inducing the survival (Sisti *et al.*, 2007). Learning has been demonstrated to elicit divergent influence on neural precursors at different developmental stages, and distinct phases of learning have dissimilar impact on SGZ neurogenesis suggesting complex and complicated regulation of SGZ neurogenesis by hippocampus-dependent learning (for review see Zhao *et al.*, 2008).

Two major negative regulators on hippocampal neurogenesis are ageing and stress. Still, when the correlation between stress and cognition is controversial (Shors, 2004), aged animals have been demonstrated to consistently display impaired learning and memory in Morris water-maze and various other tasks (for review see Klempin and Kempermann, 2007).

The functional significance of adult hippocampal neurogenesis has also been investigated by disrupting or reducing the generation of new cells by low-dose irradiation, toxins and using genetically engineered mice to specifically eliminate neural progenitors (Saxe *et al.*, 2006). Also ageing has been used as a natural process to reduce neurogenesis (for review see Klempin and Kempermann, 2007).

In 2001, Shors and collaborates demonstrated that treatment with DNA methylating agent methylazoxymethanol (MAM), which is toxic for proliferating cells, produced dose dependent inhibition in the neurogenesis of adult hippocampus that correlated with disturbances in hippocampus dependent learning task eye-blink conditioning test, however, no disturbances were observed in the hippocampus independent version of the same task and also in

Morris water-maze. In contrast, Saxe and collaborators demonstrated that focal X-irradiation of the hippocampus or genetic ablation of glial fibrillary acidic protein-positive neural progenitor cells (GFAP-tk mice) impaired contextual fear conditioning but not cued conditioning, hippocampal-dependent spatial learning tasks such as the Morris water maze and Y maze were unaffected (Saxe *et al.*, 2006). Controversy of the previous results could arise partly from the different protocols and methods used and also from the fact, that MAM treatment targets only very young cells whereas also newly born neurons are affected by irradiation and in GFAP-tk animals, suggesting different roles of immature cells and newborn neurons in learning. Also the role of hippocampal neurogenesis in spatial learning and memory has been investigated (for review see Leuner *et al.*, 2006) and place and object recognition memories (Madsen *et al.*, 2003; Wincour *et al.*, 2006). Although partly controversial, probably due to different methodological and procedural differences, previously mentioned functional studies suggest the potential role of neurogenesis in cognition, however, definitive demonstrations need further investigations and approaches.

## **2.2. Neurogenesis and CNS disorders**

Different pathological conditions have been associated with the up- or down-regulation of neurogenesis (Table 1).

Seizure activity has been shown to influence neurogenesis in SGZ as the production of adult-born neurons increases in rodent models of temporal lobe epilepsy, and both newborn and pre-existing granule neurons contribute to aberrant axonal reorganization in the epileptic hippocampus which could eventually contribute to network hyperexcitability (for review see Parent, 2007). Similar findings of granule cell layer dispersion and ectopic granule neurons in humans suggest the possible role of neurogenesis in the pathogenesis of epilepsy and possibly in learning and memory disturbances in epileptic syndrome.

Focal as well as global ischemia in rodents has been shown to increase neurogenesis (Kokaia *et al.*, 2006), also newborn cells in the SVZ are able to migrate into the site of ischemic injury and start expressing markers of neurons which could be relevant to postischemic brain repair. The same process has been proposed to happen in human stroke patients (Zhao *et al.*, 2008).

Stress, as an important precipitating factor of depression, and glucocorticoids (stress-related hormones) decrease hippocampal neurogenesis, however, do not affect anyhow SVZ neurogenesis (Cameron and Gould, 1994; Pham *et al.*, 2003). One post-mortem study demonstrated that neurogenesis is not altered in depressed humans, however, the study included six patients only and most of them were on antidepressant treatment (Reif *et al.*, 2006). Further studies are needed to clarify the possible role of neurogenesis in humans in the pathogenesis on depression.

**Table 1.** Summary of factors mediating adult hippocampal neurogenesis (adapted from Jaako, 2006)

| <b>Adult neurogenesis is mediated by:</b>                                |        |                                                                 |
|--------------------------------------------------------------------------|--------|-----------------------------------------------------------------|
|                                                                          | Effect | Citations                                                       |
| <b>Transmitters</b>                                                      |        |                                                                 |
| Glutamate (NMDA)                                                         | ↓      | Cameron <i>et al.</i> , 1995                                    |
| Glutamate (AMPA)                                                         | ↑      | Bai <i>et al.</i> , 2003                                        |
| 5-HT (via 5-HT 1A, also antidepressants)                                 | ↑      | Malberg <i>et al.</i> , 2000                                    |
| Nitric oxide                                                             | ↓      | Moreno-López <i>et al.</i> , 2004                               |
| Endogenous opioids (via $\mu$ - and $\delta$ receptors)                  | ↓      | Eisch <i>et al.</i> , 2000                                      |
| <b>Hormones</b>                                                          |        |                                                                 |
| Adrenal steroids (via NMDA receptors or cytoplasmatic steroid receptors) | ↓      | Cameron <i>et al.</i> , 1995; Gould <i>et al.</i> , 1997        |
| Estrogen                                                                 | ↑      | Tanapat <i>et al.</i> , 1999                                    |
| <b>Growth factors</b>                                                    |        |                                                                 |
| BDNF                                                                     | ↑      | Lee <i>et al.</i> , 2002                                        |
| VEGF                                                                     | ↑      | Fabel <i>et al.</i> , 2003                                      |
| IGF-1                                                                    | ↑      | Aberg <i>et al.</i> , 2000                                      |
| <b>Environmental factors</b>                                             |        |                                                                 |
| Isolation                                                                | ↓      | Nilsson <i>et al.</i> , 1999                                    |
| Maternal care                                                            | ↑      | Bredy <i>et al.</i> , 2003                                      |
| Maternal separation                                                      | ↓      | Mirescu <i>et al.</i> , 2004                                    |
| Running                                                                  | ↓      | Van Praag <i>et al.</i> , 1999                                  |
| Stress                                                                   | ↓      | Gould <i>et al.</i> , 1997, Lemaire <i>et al.</i> , 2000        |
| Social domination                                                        | ↑      | Kosorovitskiy and Gould, 2004                                   |
| Sleep deprivation                                                        | ↓      | Hairston <i>et al.</i> , 2005                                   |
| <b>Other</b>                                                             |        |                                                                 |
| Epilepsy                                                                 | ↑      | Parent <i>et al.</i> , 2007                                     |
| Ishemia                                                                  | ↑      | Kokaia <i>et al.</i> , 2006, Zhao <i>et al.</i> , 2008          |
| Azheimer's disease (in humans)                                           | ↑      | Jin <i>et al.</i> , 2004                                        |
| x-ray irradiation                                                        | ↓      | Rola <i>et al.</i> , 2004, Madsen <i>et al.</i> , 2003          |
| Ethanol                                                                  | ↑↓     | Nixon and Crews 2002, Jaako <i>et al.</i> , 2003                |
| Lead                                                                     | ↓      | Gilbert <i>et al.</i> , 2005, Jaako-Movits <i>et al.</i> , 2005 |
| Schizophrenia                                                            | ↓      | Reif <i>et al.</i> , 2006                                       |
| Lithium                                                                  | ↑      | Chen <i>et al.</i> , 2000                                       |

The possible role of neurogenesis in neurodegenerative diseases has been studied revealing that cell proliferation is increased in the SGZ of postmortem Alzheimer's patients, decreased in the SGZ and SVZ of Parkinson's patients and increased in the SVZ of Huntington's patients (Jin *et al.*, 2004; Zhao *et al.*, 2008 and references therein). However, in animal studies the observations have been conflicting so far and the issue needs further experiments.

In postmortem studies of schizophrenic patients the neurogenesis of DG has been shown to be decreased (Reif *et al.*, 2006). Also after phencyclidine administration (an animal model of schizophrenia) the number of BrdU-positive cells decreased by 23% in the subgranular zone of the dentate gyrus 24 h after repeated injections, the decreased levels of BrdU-positive cells returned to control levels within 1 week. These data suggest the decrease in neurogenesis in schizophrenia patients.

The effects of several drugs of abuse on neurogenesis have been studied trying to elucidate the possible role of neurogenesis in the development of addictive behaviours. Drugs such as psychomotor stimulants, opioids, alcohol and psychedelic compounds have been shown to alter one or several aspects of adult neurogenesis, including the rate of progenitor proliferation, the survival of newly generated cells, and the maturation and acquisition of cellular phenotype (for review see Canales, 2007). However, the specific contribution of adult hippocampal neurogenesis in the process is yet to be determined as drug addiction is a complex recurrent process involving the acquisition and maintenance of drug taking, followed by detoxification, abstinence and eventual relapse to drug seeking and the results so far cannot be interpreted unimodally.

### **2.3. Neurogenesis and PSA-NCAM**

Considering the location of NCAM and PSA-NCAM expression (see section 1.2.1.) in adult mammal brain it could be seen that it coincides with the adult neurogenic sites (Seki and Arai 1991a, b; 1993; Rousselot *et al.*, 1995; Garcia-Verdugo *et al.*, 1998). Indeed, PSA-NCAM immunoreactivity has been linked to neurogenesis since some days after BrdU injection (bromodeoxyuridine; marker for newly generated cells) the BrdU-PSA-NCAM double-immunoreactive cells could be seen in the DG (Seki, 2002). Also most PSA-NCAM positive cells are positive for NeuroD, doublecortin and neuronal marker NeuN (Seki, 2002) suggesting that PSA-NCAM expression persists also in the later stages of neurogenesis. An increasing body of evidence suggests the role of NCAM and PSA-NCAM in several stages of the process of neurogenesis like neuronal precursor migration (Cremer *et al.*, 1994; Ono *et al.*, 1994; Muller *et al.*, 2000; Vutskits *et al.*, 2003), in neuronal precursor differentiation (Seidenfaden *et al.*, 2003; Petridis *et al.*, 2004) as well as survival of newly born cells (Ono *et al.*, 1994; Vutskits *et al.*, 2006; Gascon *et al.*, 2007). However, it should be noted that changes in hippocampal PSA-NCAM might not be correlated with

changes in neurogenesis considering different factors altering the expression level of PSA-NCAM.

### **3. Neurotoxins and brain plasticity**

The term neurotoxicity generally refers to the ability of a chemical, physical or biological agent to elicit anatomical or functional alterations in the nervous system. Toxic agents have been shown to affect different processes involved in structural plasticity, mentioning stem cell proliferation and migration, dendritic branching, spine density, increased neuronal cell death and glial reorganization (Oberto *et al.*, 1996; Olney *et al.*, 2000; Bingham *et al.*, 2004; Rubert *et al.*, 2006; White *et al.*, 2007).

Although neurotoxicity is known to occur mostly in adult individuals following acute or chronic exposure to the toxic agent, for example following psychostimulant abuse, a growing body of evidence indicates that exposure to certain chemicals during development (prenatally or early postnatally) can cause profound disruptions in the morphology of the CNS, developmental exposure to different exogenous substances is believed to be associated with several persistent cognitive and behavioral disturbances (for review see Williams and Ross, 2007).

In the present section we review the effects of lead administration and amphetamine sensitization in different periods of brain development on various cognitive functions as well as distinct cellular changes.

#### **3.1. Lead exposure and brain plasticity**

Lead ( $\text{Pb}^{2+}$ ) is undoubtedly one of the oldest occupational toxins that has been mined and used by mankind for at least 6000 years, the evidence of lead poisoning is dating back to Roman times (Hernberg, 2000; Gidlow, 2004). In spite of the fact that the use of lead in different aspects has been strictly regulated and the emission of lead has dropped significantly (from 220 000 tons a year in 1970 to 3000 tons a year in 2004 (U.S. EPA 2006); White *et al.*, 2007), lead toxicity is still considered as a major public health issue due to point sources like smelters, incinerators as well as poor areas where the lead-based paint is still a source of exposure.

The influence of lead exposure on human health could be generally divided into acute and long-term/chronic effects, the severity of which mainly depends on the magnitude of internal dose and developmental stage during exposure (for review see Gidlow, 2004). In the present study we focus on the long-term effect of developmental lead exposure.

For decades by now, a major concern has arisen from the observation that exposure to low levels of lead, during early development, can induce long-lasting behavioral abnormalities and cognitive deficits in children and experimental animals persisting into adulthood (Bourjeily and Suszkiw, 1997; Murphy and Regan, 1999; Finkelstein *et al.*, 1998; Moreira *et al.*, 2001; Canfield *et al.*, 2003). Epidemiological studies have revealed consistent alterations in attention, visual-motor reasoning skills, social behavior, mathematics and reading abilities in previously lead exposed children (Moreira *et al.*, 2001). The most recent evidence suggests that there is no proven threshold above which lead can be considered harmless (U.S. EPA. Air Quality Criteria for Lead (Final). U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-05/144aF-bF, 2006). Neurobehavioral effects have been observed in children with blood levels of less than 10  $\mu\text{l/dl}$ , far below doses that could produce any clinical symptoms (Canfield *et al.*, 2003). Animal studies have found observable behavioral effects at blood lead level (BLL) less than 15  $\mu\text{l/dl}$  in primates (Rice and Karpinski, 1988) and less than 20  $\mu\text{l/dl}$  in rats (Cory-Slechta *et al.*, 1985).

Possible mechanisms underlying lead long-term toxicity have been extensively studied using rodent and non-human primate models. In rodents different types of learning and memory formation impairments have been demonstrated in contextual and cued memory tasks following lead exposure (Winneke, 1996; Chen *et al.*, 1997; Salinas and Huff, 2002), data concerning the effects on spatial learning in Morris water maze is contradictory (Gilbert *et al.*, 2005; Chang *et al.*, 2006). However, the underlying mechanisms are still poorly understood.

Considering the role of hippocampus in contextual learning and memory, we have focused in our studies on the plastic changes within hippocampal formation. It has been demonstrated that low-level lead exposure reduced the generation of new cells in the dentate gyrus (Jaako-Movits *et al.*, 2005; Verina *et al.*, 2007) and altered the pattern of differentiation of BrdU-positive cells into mature neurons (Jaako-Movits *et al.*, 2005). Lead also affected synaptogenesis most probably interfering with trimming/pruning of synaptic connections, thereby affecting the number of synaptic connections (Bull *et al.*, 1986; Oberto *et al.*, 1996; Verina *et al.*, 2007). Verina and collaborators have demonstrated aberrant dendritic morphology in hippocampus following lead exposure (Verina *et al.*, 2007).

Recent experiments trying to identify the cellular targets of lead toxicity have accentuated the role of lead on LTP, a form of synaptic plasticity, which could serve as one possible link between lead exposure, impaired learning and memory and deviated neuronal development and plasticity (Xu *et al.*, 1998; Zhang *et al.*, 2001). The effects of  $\text{Pb}^{2+}$  on LTP have been attributed to the fact that lead is a selective and potent inhibitor of the NMDA subtype of excitatory amino acid receptors (NMDAR; Alkondon *et al.*, 1990; Gavazzo *et al.*, 2001) altering the expression of NMDAR subunits in the rat hippocampus (Guilarte

and McGlothan, 1998; Guilarte *et al.*, 2000), affecting NMDAR complex composition (Toscano *et al.*, 2002) and downstream calcium signaling (Toscano *et al.*, 2003).

The ability of lead to substitute for calcium which results in impaired regulatory action of calcium in cell functions and is a common factor for many of its toxic actions must be accentuated.

Subnanomolar concentrations of lead activate PKC, which results in the rise in intracellular free calcium (Bressler and Goldstein, 1991), and also induce mitochondrial release of calcium (Silbergeld, 1992) that could eventually initiate apoptosis.

A limited number of studies have investigated the effects of early post-natal low-level lead exposure on cell adhesion molecules. So far it has been found that lead chloride significantly stimulates Golgi sialyltransferase activity from PND 16 onwards *in vitro* (Breen and Regan, 1988) and chronic low-level lead exposure antagonizes the physiological decrease in the NCAM polysialylation state during brain maturation (Cookman *et al.*, 1987). On the other hand, it has been shown that that low-level lead exposure does not induce any significant change in the basal sialylation state in the dentate gyrus of hippocampus (Murphy *et al.*, 1995; Murphy and Regan, 1999).

Altogether, conducted experiments refer to the possibility that lead could elicit its long-term neurobehavioral effects, at least partly, affecting hippocampal plasticity, however, the issue needs further clarification.

In the present study we aimed to investigate whether low-level lead exposure, during the extended post-natal period, would induce deviations in learning and memory formation as well as emotional disturbances and further changes in the hippocampal neurogenesis and/or cell adhesion systems in adulthood as possible mechanisms underlying long-term cognitive dysfunction.

### **3.2 Amphetamine administration and brain plasticity**

Drug addiction is a chronic relapsing disorder characterized by compulsive pattern of drug-seeking and drug-taking behavior in spite of serious adverse consequences. Following primarily induced euphoria and relieved distress, continued use of addictive substance induces adaptive changes in the nervous system leading to tolerance, physical dependence, sensitization, craving and relapse.

In the present section we mainly focus on the phenomenon of behavioral sensitization (BS) following repeated psychostimulant (amphetamine) administration in rodents that provides a model for addictive behaviors such as those associated with craving and relapse, as well as psychotic complications following psychostimulant abuse (Robinson and Berridge, 1993, 2001; Badiani *et al.*, 1995). Repeated intermittent exposure to a variety of drugs of abuse, amphetamine among others, have been shown to produce hypersensitivity

(sensitization) to their psychomotor activating and incentive motivational effects that can persist for years after the termination of drug exposure and in animal experiments is characterized by enhanced behavioral response to subsequent drug exposure (Segal *et al.*, 1981; Robinson and Becker, 1986; Robinson and Berridge, 1993). The phenomenon has been extensively investigated for it is believed to manifest a compelling example of experience-dependent plasticity (Robinson and Kolb, 1997; Robinson and Becker, 1986) and because of the hypothesis that sensitization-related neuroplasticity may contribute to the development of addiction (Robinson and Berridge, 1993). The neurobiology of BS is believed to comprise cellular adaptations following repeated exposure to addictive substance (Robinson and Becker, 1986), however, under some circumstances contextual learning has been demonstrated to gain powerful control over the ability of the sensitized neural substrate to influence behavior (Stewart and Vezina, 1988; Anagnostara and Robinson, 1996).

Persistent alterations in the behavior of sensitized animals are believed to be mediated, at least partly, by structural modifications in neural circuitry, especially alterations in the patterns of synaptic connectivity (Kolb and Whishaw, 1998; Robinson and Kolb, 2004). Drugs of abuse have been shown to induce structural changes in the dendritic branching and spine density in medium spiny neurons of the nucleus accumbens and pyramidal cells of the medial prefrontal cortex, structures that are primarily involved in the reward circuitry (Robinson and Kolb, 2004) but also, as described recently, in the CA1 region of the hippocampus (Crombag *et al.*, 2005).

Among different molecules that could participate in the modulation of synaptic connectivity neuronal cell adhesion molecule must be accentuated.

Given a potential role to learning in the development of behavioral sensitization the purpose of our experiments was to investigate the possible role of PSA-NCAM and NCAM (as markers of neural plasticity) in the associative learning mechanisms related to behavioral sensitization. To obtain our goal we examined the effects of acute and repeated amphetamine administration to the expression level of PSA-NCAM and NCAM in mouse hippocampus, cortex and striatum in a context-specific behavioral sensitization model.

## **THE AIMS OF THE STUDY**

To assess whether low-level lead exposure during early postnatal period induces emotional and cognitive dysfunction and alterations in neurogenesis and cell maturation in adult rat

To evaluate the effects of early postnatal low-level lead exposure on the number of polysialic-acid linked neural cell adhesion molecule expressing cells and their phenotype in adult rat hippocampus

To study the effects of different amphetamine administration regimens on the expression levels of neural cell adhesion molecule and its polysialylated form in various brain regions of adult mouse

# **MATERIALS AND METHODS**

## **I. Animals**

All experiments were undertaken in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC). Experiments conformed to the local guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering.

- 1) To study the effect of low-level lead exposure during the extended postnatal period on emotional and cognitive functions and the effects on hippocampal neurogenesis and cell adhesion systems in adulthood, Wistar rats were obtained from Kuopio Animal Research Center (Finland) and used as the parent generation. Adult female rats (350–400 g) were individually housed in plastic cages at 22°C, under a 12-h light-dark cycle and were mated with males of the same strain.
- 2) To study the effects of amphetamine administration on cell adhesion molecules male C57BL/6 mice (weight 25–30 g) were obtained from the Scanbur BK (Sweden) and were maintained in temperature and humidity-controlled rooms with 12-h light-dark cycle (light from 7:00 am to 7:00 pm). Prior to behavioral testing (which was conducted during the light phase of the light-dark cycle), all mice were handled for 4 days.

## **2. Neurotoxin administration and concentration assays in blood**

### **2.1. Lead administration**

Adult female Wistar rats were individually housed in plastic cages and were mated with males of the same strain. Lead administration was performed according to the protocol described by Murphy and Regan (1999), with minor modifications. In short, one day after parturition, litters were culled to 8 pups. On the same day, water was replaced by a 0.2% solution of lead acetate. The treatment lasted during the whole lactation period until weaning. Pups were weaned at the age of 21 days and then were kept in a group of 5 males per cage. The animals continued to receive 0.2% lead acetate with drinking water until postnatal day 30. At postnatal day 30, lead was removed from drinking water, and the animals were allowed to attain adulthood (postnatal day 60). The control group dams and pups remained on tap water. Pup weights, maternal and pups' fluid and food consumption were measured on a weekly basis. During the whole experiment, animals were fed with regular laboratory foodstuff.

## **2.2. Measurement of lead concentration**

On days 15, 30, 60 and 80 after birth, separate groups of animals (3–4 per group) were taken for lead determination in blood and brain tissue. Animals were anaesthetized with chloral hydrate and blood was taken from the heart. Animals then were perfused with physiological saline and the brain was removed. The blood samples and brain tissue were immediately frozen at –70°C. Quantitative analysis of lead levels was performed in an independent State Environmental Laboratory using a Perkin-Elmer 1100B atomic adsorption spectrometer with a Philips HGA/P3105 graphite furnace and a deuterium background corrector.

## **2.3. Amphetamine administration and different treatment regimens: context-specific model**

The mice were injected intraperitoneally (i.p.; 0.1 ml/10 g body weight) with d-amphetamine sulfate (2.0 mg/kg) (Sigma, MO) or saline.

There were a total of six groups in these experiments, which came from two types of pretreatment groups: the Paired and Unpaired. Both pretreatment groups were divided into the following three subgroups: (1) saline (SAL), (2) amphetamine 2.0 mg/kg, i.p. (AMPH) and (3) saline control for Western blot (Cont). Animals in the Paired group were transported to the locomotor test environment, where they received the respective treatment. After the daily treatment the Paired mice were left in the locomotor test environment for 60 min and then returned to their home cages. Locomotor activity was recorded on the 1st and 8th day of treatment. Animals in the Unpaired group were transported from their home cages to the Third World environment, where they received treatments. We defined the Third World as standard polypropylene cages (15 × 25 cm) with wire lids, pine wood-shavings on the floor and located in a quiet room, distinct from the locomotor test environment. After treatment all mice returned to their home cages. These procedures for the Paired and Unpaired groups were repeated for 8 days, once a day.

Three days after the last pretreatment day (day 12) all the pretreatment groups were transported to the locomotor test environment, where they received a single saline i.p. injection (the saline challenge). Horizontal locomotor activity was recorded for 20 min after saline treatment. Then all of the mice received a subsequent injection of amphetamine (2.0 mg/kg i.p.) and locomotor activity was recorded for another 60 min (the amphetamine challenge). The Paired control group (Cont) for Western blot analysis received only saline injections during the 8-day induction period as well as in the expression phase on the 12th day in a test environment. The Unpaired control group for Western blot analysis received only saline injections during 8 days in the Third World and received saline in the expression phase on the 12th day in a test environment.

### **3. Behavioral testing**

#### **3.1. Behavioral testing following early postnatal lead administration**

Behavioral testing following early postnatal lead administration was performed on male pups at the age of 60 days (locomotor activity and anxiety testing) and the age of 80 days (contextual fear conditioning).

##### **3.1.1. General locomotor activity**

Open field activity tests are used to measure locomotor activity in rodents and can also serve as preliminary tests to determine motor deficits, anxiety and effects of drugs. Locomotor activity is measured by determining the amount of distance traveled and observations of various horizontal, vertical, and stereotyped behaviors are also measured. Anxiety is determined by the pattern of exploration in the open-field (center versus periphery). The test is sensitive to motor dysfunction as well as hippocampal and basal ganglia damage and is a useful test for initial screening during transgenic phenotyping.

In our studies the general locomotor activity was determined in a rectangular wooden cage ( $50 \times 50 \times 50$  cm) uniformly illuminated with dim lighting. A light sensitive video camera, connected to the computer, was mounted about 1 m above the observation cage and the locomotor activity of an animal was monitored and analyzed using VideoMot2 software (TSE Systems, Germany) during a 30-min observation period.

##### **3.1.2. Elevated plus maze**

Anxiety was evaluated in the elevated plus maze test, which is a widely used behavioral assay for rodents and has been validated to assess the anti-anxiety effects of pharmacological agents as well as to define brain regions and mechanisms underlying anxiety-related behavior. Unlike other behavioral assays used to assess anxiety responses that rely upon the presentation of noxious stimuli (i.e., electric shock, food/water deprivation, loud noises, etc.) that typically produce a conditioned response, the elevated plus maze relies upon rodents' proclivity toward dark, enclosed spaces (approach) and an unconditioned fear of heights/open spaces (avoidance) (Walf and Frye, 2007).

The elevated plus maze apparatus consisted of two open and two enclosed arms of equal length and width ( $50 \times 10$  cm). The enclosed arms were not entirely closed, with walls that extend 40 cm high. The plus maze was elevated 50 cm above the floor. Each rat was placed in the center of the elevated plus

maze facing one of the open arms, and the number of entries and time spent (s) in the open or closed arms were recorded during a 5-min test period. The elevated plus maze was carefully cleaned with 5% ethanol before each animal was introduced. Data was quantified and presented as a % of time spent in the open arms and a % of entries in the open arms.

### **3.1.3. Fear conditioning**

Associative fear conditioning is a form of learning, where an animal is exposed to an initially neutral conditioned stimulus (CS; generally a tone) temporally associated with an aversive unconditioned stimulus (US; usually an electrical shock). Subsequent presentations of the CS elicit an array of defensive responses, collectively referred to as conditioned fear. Behavioral freezing, defined as the absence of all non-respiratory movements, is perhaps the most widely studied conditioned fear response (Kopec *et al.*, 2007)

In our studies at post-natal day 80, control animals and lead-exposed animals were trained for the contextual fear conditioning. The study was performed between 09.30 a.m. and 14.00 p.m. The task was carried out during four consecutive days in the experimental chamber (220 mm × 160 mm × 160 mm; L × W × H). Three sides of the box were made of non-transparent plastic and the fourth side was made of transparent plastic. The floor was made of stainless steel rods connected to a shock delivery apparatus. Rats were placed in the experimental chamber for 5 min and allowed to explore. During this time, the duration of the freezing state (defined as complete immobility) and the number of freezing episodes, was measured by two independent observers. Next, the animals were given three electric shocks (0.8 mA, 1.0 s duration) on their feet, at 60 s intervals between each shock, and were removed from the apparatus 1 min after the last shock. Contextual conditioning tests were performed 24, 48 and 72 h after training. Animals were placed in the same context, and no foot shocks were delivered. The total duration of freezing episodes was recorded during 5 min. Data was quantified and presented as a mean time of freezing.

## **3.2. Behavioral testing following amphetamine administration**

### **3.2.1. Locomotor activity**

Horizontal locomotor activity was assessed in standard polypropylene cages (15 × 25 cm) uniformly illuminated with dim lighting. A light sensitive video camera, connected to the computer, was mounted about 1.5 m above the observation cage and locomotor activity of eight animals in time were monitored and analyzed using VideoMot2 software (TSE Systems, Germany) during a 60-min observation period.

## **4. Neurogenesis assay**

### **4.1. BrdU administration**

In experiments bromodeoxyuridine (BrdU, Boehringer Mannheim) was used to evaluate the neurogenesis following lead administration. BrdU is a thymidine analog, which is incorporated into DNA during the S phase of the cell cycle and widely used to label dividing cells. In lead-experiments at the age of 80 days, BrdU was administered both to control and lead-exposed pups in a total dose of 300 mg/kg, i.p. Due to limited BrdU dissolution, the dosage of BrdU was divided into three portions, and each portion (100 mg/kg) was given, with an interval of 2 hours, in a volume of 0.4 ml/ to 100 g body weight. The total dose BrdU, 300 mg/kg allowed maximum labelling of the dividing progenitors. For the proliferation study, control (n=6) or lead-exposed (n=6) rats were killed and their brains were taken for immunohistochemical detection of the newly born cells or neuronal death 24 hours after administration of BrdU. For survival/differentiation studies, following BrdU administration animals (n=6 in each group) were left in their home cages for additional 3 weeks and were processed for the immunohistochemical detection BrdU label (peroxidase method), or fluorescence double immunohistochemistry for BrdU and neuronal or glial markers.

### **4.2. BrdU immunohistochemistry and quantification of BrdU-positive cells**

Immunohistochemistry is the localization of antigens in tissue sections by the use of labeled antibody through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold. In our studies we used the immunohistochemistry technique to visualize the newly born, BrdU positive cells and to follow their destiny as well as to visualize PSA-NCAM expressing cells (see 4.3).

To perform immunohistochemistry, animals were deeply anaesthetized with chloral hydrate (350 mg/kg) and transcardially perfused with normal saline and then with 4% paraformaldehyde in a phosphate buffered saline (PBS, 0.1 M, pH=7.4), the brain was removed and post-fixed for an additional 24 hours in a paraformaldehyde solution. Our experience showed that animal perfusion, tissue fixation and following immunohistochemical detection procedures might greatly affect the estimates of BrdU-positive cells. Therefore animals from experimental and matched control groups were processed simultaneously.

After the post-fixation period, the brain was cut in coronal sections 40  $\mu$ m thick on a vibratome and then processed for immunohistochemistry. Immunohistochemical detection was performed on the free-floating sections.

For BrdU immunohistochemistry, the sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub>, followed by incubation in a 0.1M TRIS HCl buffer, containing 0.025% trypsin and 0.1% CaCl<sub>2</sub>, for 10 min. Thereafter, the sections were incubated in 2N HCl in PBS at 37°C for 30 min, then in 0.1 M boric acid in PBS for 15 min, and blocked in the mixture of 2% normal rabbit serum (DAKO, Denmark) in PBS containing 0.25% Triton X-100 for 1 h. This was followed by overnight incubation at 4°C with rat monoclonal antibody to BrdU (1:200, Accurate Chemicals, USA) followed by incubation in biotinylated rabbit anti-rat antibody (1:400, Vector Laboratories, UK) for 1 h. BrdU-positive cells were visualized using the peroxidase method (ABC system and diaminobenzidine as chromogen, Vector Laboratories). The slides were counter-stained using cresyl violet, dried, cleaned with xylol and cover-slipped with a mounting medium (Vector Laboratories).

All counting of the BrdU-positive nuclei was done according to the method described previously (Malberg and Duman, 2003). For each animal, BrdU-positive cells (peroxidase staining) were counted in one-in-ten sections (400 µm apart) within the dentate gyrus (granule cell layer and hilus) omitting cells in the outermost focal plane. All counts were performed using an Olympus BX-51 microscope equipped with x60 magnification (numerical aperture – 1.4) objective to achieve optimal optical sectioning of the tissue. An average of 10 sections was analysed from each animal. To estimate the total number of BrdU-positive cells in a given region the sum of cell counts from 10 sections was then multiplied by 10. Left and right dentate gyri were analysed separately and the estimates were averaged for each animal.

### **4.3. Determination of PSA-NCAM positive cells**

For PSA-NCAM immunohistochemistry animals were sacrificed and brain tissue processed on PND 80 as described in details in the previous section. PSA-NCAM immunohistochemistry was performed on free-floating coronal sections (40 µm). For PSA-NCAM immunohistochemistry, the sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> in PBS for 30 minutes and thereafter blocked in 3% normal goat serum in PBS containing 0.3% Triton X-100 for 1 h. This was followed by 48 h of incubation at 4 °C with mouse monoclonal antibodies against PSA-NCAM (1:400, AbCys, France) diluted in the blocking buffer. After being washed in PBS, sections were incubated in biotinylated goat anti-mouse antibody (1:50, Vector Laboratories, UK) diluted in the/a blocking buffer for 2h. PSA-NCAM positive cells were visualized using the standard immunoperoxidase method (ABC system and diaminobenzidine as chromogen, Vector Laboratories). The sections were dried, cleared with xylol and cover-slipped with mounting medium (Vector Laboratories).

The number of PSA-NCAM expressing cells was estimated using a modified version of the fractionator method (West, 1993; Madsen *et al.*, 2003), counting

all PSA-NCAM positive cells on every 20th section in a subgranular zone of the dentate gyrus, starting randomly and multiplied by 20 to get the total number of PSA-NCAM expressing cells per animal.

#### **4.4. Volume of the dentate gyrus and quantification of the total number of granule cells in the granule cell layer**

For quantification of the volume of the dentate gyrus and the total number of granule cells in the granule cell layer, every tenth section throughout the dentate gyrus was incubated in a 0.1M TRIS HCl buffer, containing 0.025% trypsin and 0.1% CaCl<sub>2</sub>, for 10 min followed by incubation in the acid-alcohol (HCl 1% in 70% ethanol) solution for 10 s. The slides were stained using haematoxylin-eosine, washed in PBS and cover-slipped with a water-based mounting medium (Vector Laboratories, UK).

Cell numbers were quantified according to the optical fractionation method (West, 1993). The stereology system consisted of an Olympus BX-51 microscope, a microcator (Heidenhain, DN 281) and the computer Assisted Stereological Toolbox (CAST-2)-Grid system (Olympus, Denmark). Numerical density ( $N_v$ ) was calculated according to the formula  $N_v = \Sigma Q / \Sigma v$  (dis), where  $\Sigma Q$  is the number of cells counted and  $\Sigma v$  (dis) is the volume of disectors. The volume of the dentate gyrus was estimated according to Cavalieri's principle by summing up the points falling on the cross sectional area of all sections and by multiplying the distance between two sections by the thickness of the section and by the area associated with each point. The total number of cells was calculated from the density ( $N_v$ ) of the cells multiplied by the reference volume (West, 1993).

#### **4.5. Determination of the phenotype of the BrdU-positive cells**

4 to 6 sections from control or experimental animals, surviving 4 weeks after the BrdU injection, were analyzed for co-expression of BrdU and neuronal or glial markers. For immunofluorescent double-labelling, sections were incubated with a mixture of rat anti-BrdU monoclonal antibody (1:100) and one of the following: monoclonal antibody against neuronal class III  $\beta$ -tubulin (Tuj1, marker for immature postmitotic neurons, Covance, USA); mouse monoclonal antibody against PSA-NCAM (marker for young neurons, AbCys, France); rabbit polyclonal anti-calbindin antibody (1:1000, marker for mature neurons, Chemicon International, UK) or rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:1000 marker for astrocytes, Dako, Denmark). Secondary antibodies were: goat anti-rat Alexa-594, goat anti-mouse Alexa-488

and goat anti-rabbit Alexa-488 (Molecular Probes, USA). Fluorescent signals were detected with a confocal microscope MRC-1024 (Olympus/Bio-Rad, Germany) equipped with an argon-krypton laser. 3D images were constructed from a series (12–15) of scans of the dentate gyri at 2  $\mu\text{m}$  intervals taken using x40 (water) objective and further analysed for the co-localization of the BrdU signal with the signals of neuronal or glial markers. In some cases, the analysis for co-localization was performed using x100 objective (oil) on stacked images taken at 0.5  $\mu\text{m}$  intervals. The data were expressed as a percentage of BrdU-positive cells that expressed phenotype marker- calbindin, Tuj1, PSA-NCAM or GFAP, in the dentate gyrus of control or neurotoxin treated animals.

#### **4.6. Determination of the phenotype of the PSA-NCAM-positive cells**

Double immunofluorescent labeling was performed using a mixture of mouse anti PSA-NCAM primary antibody with one of the following primary antibodies: rabbit anti-GFAP (1:1000, marker for astrocytes, DAKO, Denmark), mouse anti-Tuj1 (1:100, Covance, USA), rabbit-anti calbindin (1:1000, marker for mature neurons, Chemicon). Secondary antibodies were goat anti-mouse Texas Red (Jackson Im., USA) and goat anti-rabbit Alexa-488 (Molecular Probes). Between four to six sections from each animal were analyzed for co-expression of PSA-NCAM and neuronal or glial markers.

Fluorescent signals were detected with a confocal microscope MRC-1024 (Olympus/BioRad, Germany) equipped with an argon-krypton laser. 3D images were constructed from a series (12–15) of scans at 2  $\mu\text{m}$  intervals and further analyzed for co-localization signals.

The data were expressed as a percentage of PSA-NCAM-positive cells (80–100 PSA-NCAM expressing cells counted per animal) that expressed the phenotype marker Tuj1, calbindin or GFAP.

### **5. Detection of neuronal death**

#### **5.1. Fluoro-Jade staining**

Fluoro-Jade staining was used for the detection of the occurrence of neuronal death following postnatal low-level lead exposure. Brain sections were immersed in 100% ethanol for 3 min and de-hydrated through graduated alcohol solutions. Sections were then incubated in 0.06% potassium permanganate solution for 15 min, rinsed with distilled water, and incubated in a solution of 0.001% Fluoro-Jade (Histo-Chem, Jefferson, USA) in 0.1% acetic acid for 30 min. Sections were then rinsed in water, air-dried, cleared in xylene

coverslipped and examined with an epifluorescence microscope with the filter designed to detect fluorescein.

Every 20th section (40  $\mu\text{m}$  thick) in the entire extent of the DG was taken for quantification of Fluoro-Jade-positive cells in the dentate gyrus. The cells from 5 sections, per each dentate gyrus, were counted by using an Olympus BX-51 microscope at x60 magnification. To estimate the total number of degenerating cells in a given region, the sum of cell counts from 5 sections was then multiplied by 20. Left and right dentate gyri were analyzed separately and the estimates were averaged for each animal.

## **6. Western immunoblotting**

The western blot is a method to detect a specific protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide or by the 3-D structure of the protein. The proteins are then transferred to a membrane, where they are probed (detected) using antibodies specific to the target protein.

For western immunoblotting we sacrificed mice 2–3 h after the last injection of amphetamine or saline. Cortex, striatum and hippocampus were removed quickly in +4°C room, placed immediately into liquid nitrogen and stored at –80°C until processing. Tissues were lysed in 10 vol (w/vol) of lyses buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X 100 and protease inhibitors, homogenized with ultrasound homogenizer, incubated 1 h at +4°C and centrifuged ( $13\,000 \times g$  for 10 min at 4°C). The protein concentration was determined using RCDC Protein Assay (BioRad, Hercules, CA). The supernatants (50  $\mu\text{g}$  of protein) were resolved by electrophoresis on 8% SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes in 0.1 M Tris base, 0.192 M glycine and 20% (w/w) methanol using an electrophoretic transfer system. The membranes were blocked with 0.1% (w/w) Tween 20/TBS (T-TBS) containing 5% (w/w) nonfat dried milk at room temperature for 1 h. After blocking the membranes were incubated overnight with the anti-PSA-NCAM antibody (1:2000; AbCys, France) followed by incubation with biotinylated anti-mouse IgM secondary antibody (1:1000) for 2 h and ABC solution for 30 min. For the detection of NCAM expression we used anti-pan-NCAM antibody (1:1000; BD Biosciences, USA) followed by HRP-conjugated goat-anti-mouse secondary antibody (1:10000; Pierce, USA). The membranes were briefly incubated with an ECL detection reagent (ECL, Amersham, UK) to visualize proteins and exposed to X-ray film. To normalize PSA-NCAM and NCAM immunoreactivity,  $\beta$ -actin protein was measured on the same blot with a mouse monoclonal anti- $\beta$ -actin antibody (Sigma St. Louis, USA) as an internal control for loading and PSA-NCAM and NCAM/ $\beta$ -actin optical density ratio was calculated. To analyze the X-ray film we used

QuantityOne 710 System (BioRad) and the data are expressed as adjusted volume of optical density (OD)  $\times$  mm<sup>2</sup>, mean  $\pm$  S.E.M.

## **7. Data analysis**

Data were expressed as mean  $\pm$  SEM, statistical comparisons were made using either Student's t-test, one-way or two-way ANOVA or ANOVA for repeated measures followed by Bonferroni post-test, as appropriate. Significance was set at  $p < 0.05$ .

## RESULTS

### I. The effects of developmental lead exposure on behavior and hippocampal neurogenesis in the adult rat brain

#### I.1. The effects of early postnatal low-level lead exposure on lead concentrations in blood and in brain tissue

Table 2 shows the mean lead levels in the blood and brain of pups during the period of lead administration and afterwards. Lead exposure during the lactation period resulted in significantly high levels of lead in blood and brain tissue. These values corresponded to those observed by others (Salinas and Huff, 2002). After termination of lead administration, the levels of lead in blood decreased and, at postnatal days 60 and 80, did not differ significantly from the levels found in control rats. The levels of lead, in brain tissue, decreased to control levels by postnatal day 80. The lead administration protocol employed here did not affect the weight gain as compared to control (data not shown). Fluid consumption, however, was slightly reduced in lead-exposed animals during the whole period of lead administration.

**Table 2.** Lead concentrations in blood and brain tissues of pups at various times during (PND 15 and 30) and after (60 and 80 days) administration of lead acetate with drinking water. The data are mean  $\pm$  SEM of 3–6 experiments at each time point. \* $p < 0.01$ ; \*\* $p < 0.001$  (Student's t-test).

|                                   | Postnatal days        |                       |                  |               |               |
|-----------------------------------|-----------------------|-----------------------|------------------|---------------|---------------|
|                                   | 15                    | 30                    | 60               | 80            | Control       |
| Blood ( $\mu\text{g}/\text{dl}$ ) | $29.3 \pm 5.0^{**}$   | $34.2 \pm 5.8^{**}$   | $10.4 \pm 3.2$   | $6.5 \pm 1.2$ | $4.2 \pm 1.7$ |
| Brain ( $\text{ng}/\text{g}$ )    | $456.0 \pm 23.0^{**}$ | $781.0 \pm 87.0^{**}$ | $20.0 \pm 8.0^*$ | $6.0 \pm 1.0$ | $6.0 \pm 2.0$ |

#### I.2. Behavior of lead-exposed rats

The locomotor activity test did not reveal any differences between lead-exposed and control animals in the distance (cm) travelled or mean speed (cm/s) at any time point during a 30-min-observation period (Figure 6).

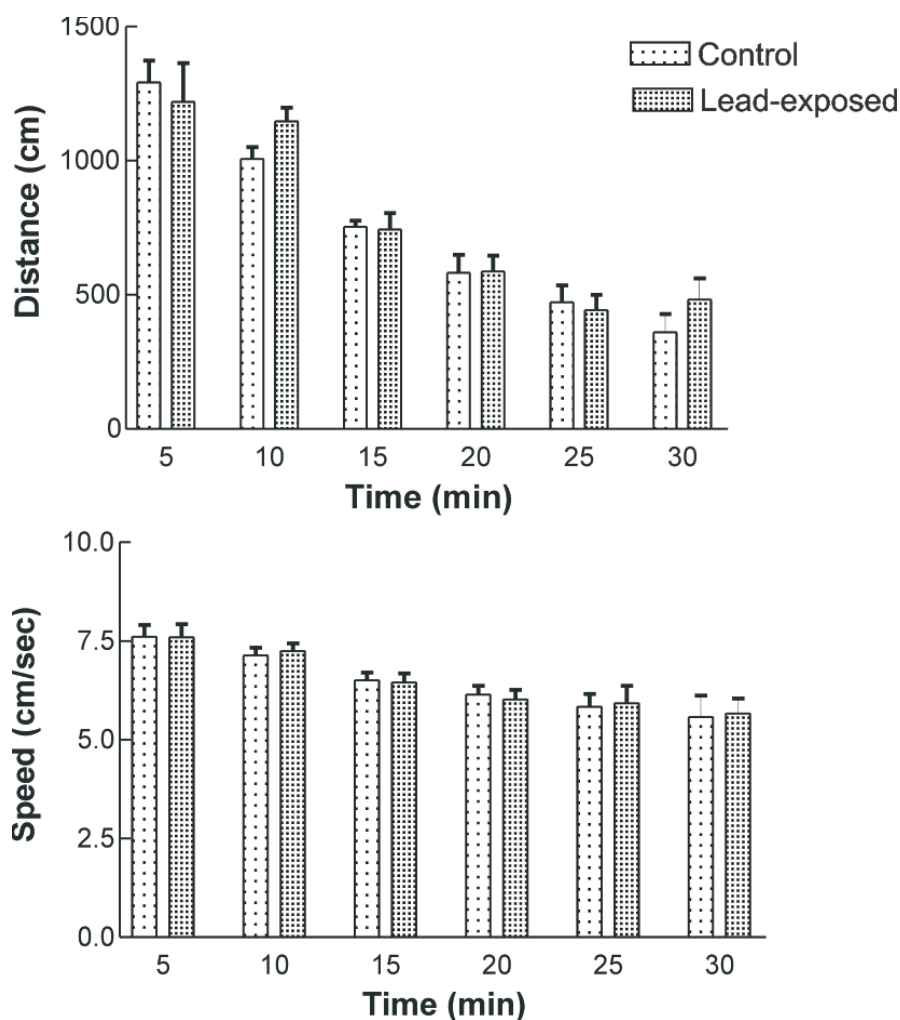


**Figure 6.** Locomotor activity of control and lead-exposed rats measured as distance (cm) travelled (upper panel) and mean speed (cm/sec, lower panel) during a 30-min-observation period. Each group consisted of 8 rats.

Lead-exposed animals demonstrated an increase in the level of anxiety in the plus-maze test as evidenced by a reduction % of entries onto and % of time spent in the open arms of the plus-maze (Figure 7). The observed reductions in the measures of anxiety were not attributed to the changes in the locomotor activity in the plus-maze since the total number of entries did not differ between control and lead-exposed rats (Figure 7).

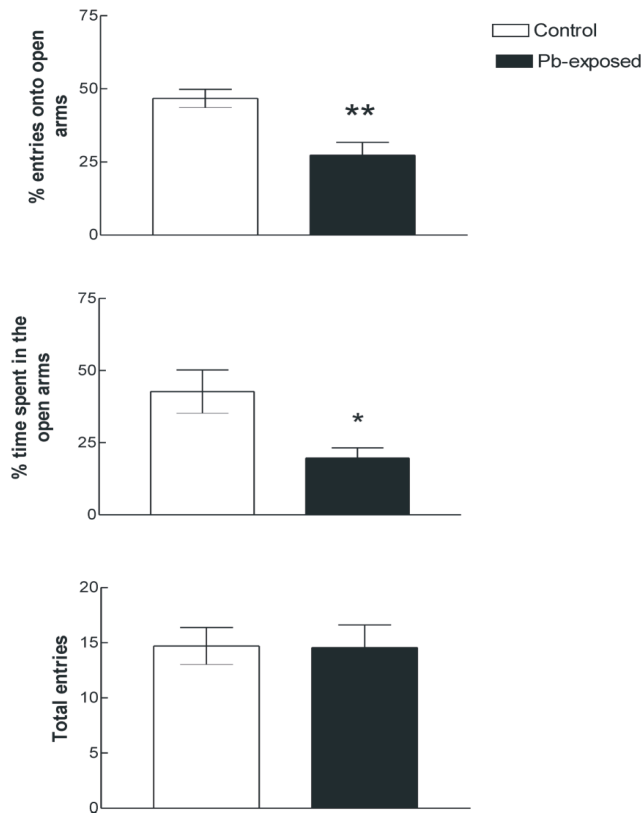


**Figure 7.** The effects of lead exposure on the rat behavior in the plus-maze test. Upper panel: The % of entries onto open arms; middle panel: The % of time spent on the open arms; Lower panel: The total number of entries. Values are mean  $\pm$  SEM from groups of 8 animals. \* $p < 0.05$ , \*\*  $p < 0.001$  (Student's t-test).

In the contextual fear conditioning test the baseline freezing time, prior to conditioning in lead-exposed animals, was  $35.7 \pm 6.8$  s ( $n=9$ ) and did not significantly differ from those found in control rats –  $30.4 \pm 4.6$  s ( $n=9$ ). For the assessment of retention and extinction of fear, animals were placed in the same context without presentation of a foot shock (unconditioned stimulus) at 24, 48 and 72 hours after the training session, and were scored for freezing during a 5 min observation period. One-way ANOVA for repeated measures demonstrated a significantly lower freezing time in lead-exposed rats (ANOVA;  $F(5, 41) = 14.47$ ,  $P < 0.01$ ). Post-hoc analysis revealed a significant reduction in freezing time at 24 and 48 but not at 72 hours following training, as compared with control pups (Figure 8).

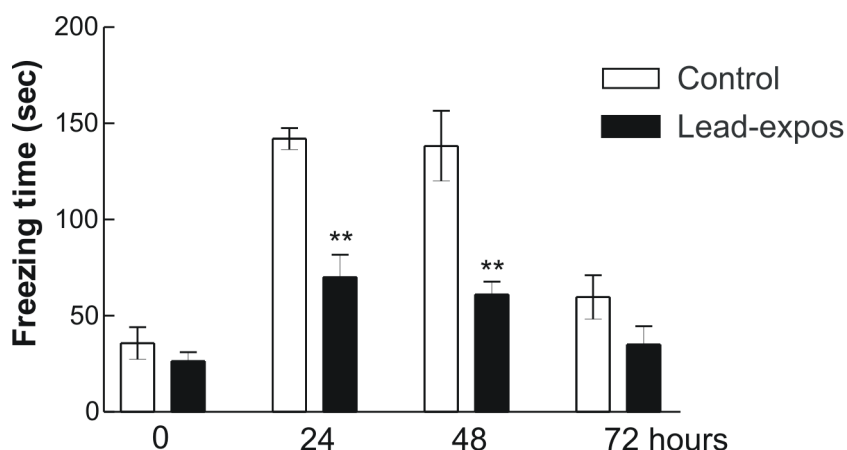


**Figure 8.** Contextual fear conditioning in control and lead-exposed rats. Freezing time in seconds measured during 5 min. of the context presentation prior and 24, 48 and 72 hours following training session. Values are mean  $\pm$  SEM from groups of 8 animals. \*\* $p < 0.01$  (Bonferroni test).

### **1.3. The effect of early postnatal low-level lead exposure on the adult hippocampal neurogenesis**

To examine the potential effect of lead on the proliferation of the neuronal and/or glial precursors in control and lead-exposed rats, animals were administered a proliferation marker BrdU and the number of BrdU-positive cells in the dentate gyrus was estimated 24 hours later. BrdU-positive nuclei of both control and neurotoxin exposed rats appeared as irregularly shaped clusters of 2–3 cells located in the border between the granule cell layer and hilus. Only a few BrdU-positive cells were found in the hilus (Figure 9).

The estimation of BrdU-positive cells revealed that the number of BrdU-labelled cells was significantly ( $p < 0.01$ ) lower in lead-exposed rats as compared to controls (Figure 6). The reduced proliferative activity in the dentate gyrus has not been associated, however, with the changes of the volume of the dentate gyrus (Figure 10).

To determine the survival of BrdU-positive cells after lead exposure, we counted the number of BrdU-positive cells in the dentate gyrus three weeks after BrdU administration. Experiments failed to demonstrate any differences in the survival rate of BrdU-positive cells between control and lead-exposed animals. The percentage of surviving cells in lead-exposed rats was 39.4% and did not differ significantly from those in control animals – 47.8% ( $n = 6$ ).

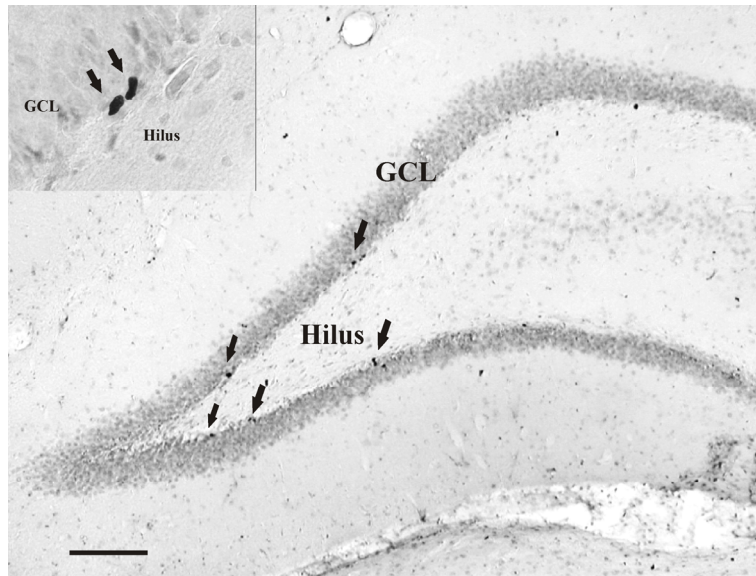


**Figure 9.** Representative microphotographs of BrdU-immunoreactive cells in the dentate gyrus of control rats. Arrows show BrdU-positive cells. Insets demonstrate cells taken from images acquired with x 60 objective. GCL-granule cell layer; Scale bar: 300  $\mu$ m.

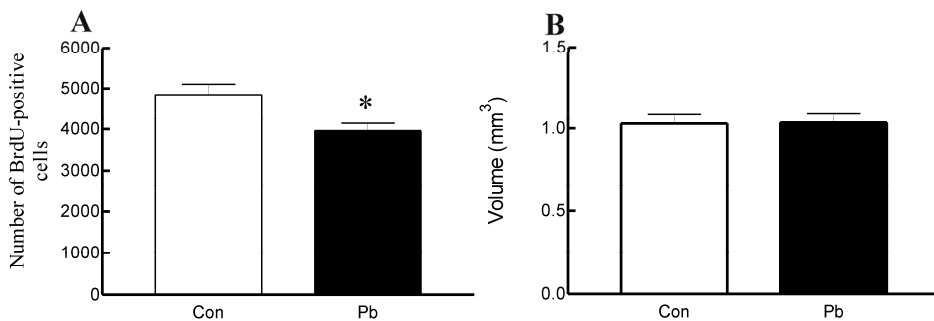


**Figure 10.** Production of new cells in the rat dentate gyrus following postnatal lead exposure. The number of BrdU-positive cells (A) and the volume of the dentate gyrus (B) in control and lead-exposed rats (n=6). \* $p < 0.05$  (Student's t-test).

To determine the phenotype of BrdU-positive cells, double immunohistochemical labelling for BrdU and for markers of neuronal or glial phenotypes was performed 3 weeks following BrdU administration. Marker for mature neurons was calbindin, marker for immature postmitotic neurons was Tuj 1, marker for young neurons was PSA-NCAM, and marker for astrocytes was anti-glial fibrillary acidic protein (GFAP) (Figure 11).

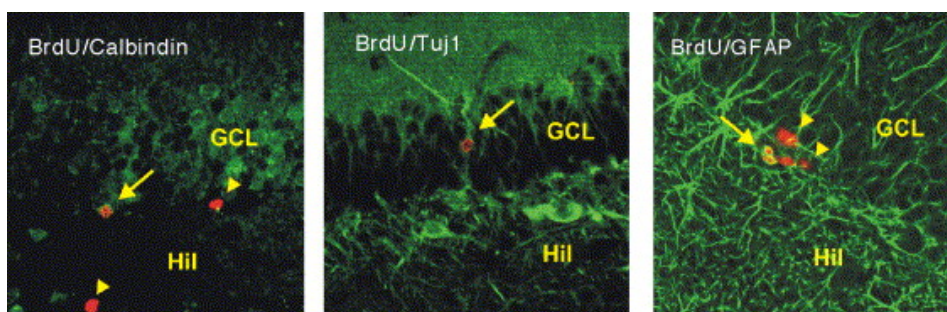


**Figure 11.** Confocal scanning laser images of sections double-labelled by BrdU (red) and (A) marker for mature granule neurons calbindin (green), (B) marker for post-mitotic young neurons PSA-NCAM (green) and (C) marker for astroglia GFAP (green). Arrows show co-localization of BrdU with neuronal or glial markers. Abbreviations: GCL- granule cell layer; Hil- hilus. Scale bar: 50  $\mu$ m. Insets demonstrate cells with co-localization taken from images acquired with x 100 objective.

Table 3 shows that in control rats  $40.6 \pm 3.4\%$  of survived BrdU positive cells expressed the marker of adult granule neurons calbindin, whereas in lead-exposed animals only  $28.7 \pm 3.5\%$  of cells became adult calbindin-positive neurons ( $p < 0.05$ ). By contrast, the proportion of young post-mitotic neurons expressing Tuj1 was significantly higher ( $p < 0.01$ ) in lead-exposed rats as compared with those in the control animals. In addition, a significantly higher proportion of BrdU-positive cells in the dentate gyrus of lead-exposed rats had differentiated into astroglia compared with control rats (Table 3).

**Table 3.** The effect of postnatal lead exposure on the survival and differentiation of newly born cells in the rat dentate gyrus. Rats were given BrdU in a total dose of 300 mg/kg (i.p.) and three weeks later BrdU-positive cells were examined for co-localization with neuronal or glial markers. The number of BrdU-positive cells examined per animal was 50–100. The data are mean  $\pm$  SEM ( $n=6$ ). \* $p < 0.05$ ; \*\* $p < 0.01$  (Student's t-test).

| Group        | Number of BrdU-positive cells | % co-localization with Tuj1 | % co-localization with calbindin | % co-localization with GFAP |
|--------------|-------------------------------|-----------------------------|----------------------------------|-----------------------------|
| Control      | $2320 \pm 133$                | $11.3 \pm 2.6$              | $40.6 \pm 3.4$                   | $11.9 \pm 3.4$              |
| Lead-exposed | $1562 \pm 256^*$              | $30.1 \pm 3.4^{**}$         | $28.7 \pm 3.5^*$                 | $35.4 \pm 5.1^*$            |

## 2. The effects of developmental low-level lead exposure on the number of polysialic-acid linked neural cell adhesion molecule expressing cells and their phenotype in adult rat hippocampus

### 2.1. The effects of developmental low-level lead exposure on the number of polysialic-acid linked neural cell adhesion molecule (PSA-NCAM) expressing cells in adult rat hippocampus

PSA-NCAM immunohistochemistry exteriorized a population of cells preferably located in the subgranular zone of the dentate gyrus, with occasional cells presented in the granular cell layer and hilus. PSA-NCAM positive cells extended neurites towards the GCL region. Almost no PSA-NCAM positive cells were found in CA1 and CA3 regions of the hippocampal formation. Quantitative analysis revealed that chronic post-natal low-level lead exposure induced a significant mean increase in the number of PSA-NCAM immunoreactive cells in the subgranular zone of the hippocampus compared to the control group. In control animals the number of PSA-NCAM positive cells was  $27\,540 \pm 3024$  and in lead-exposed animals  $47\,540 \pm 3575$  ( $t = 4.271$ ; d.f. = 8;  $p < 0.01$ ; Figure 12), respectively.

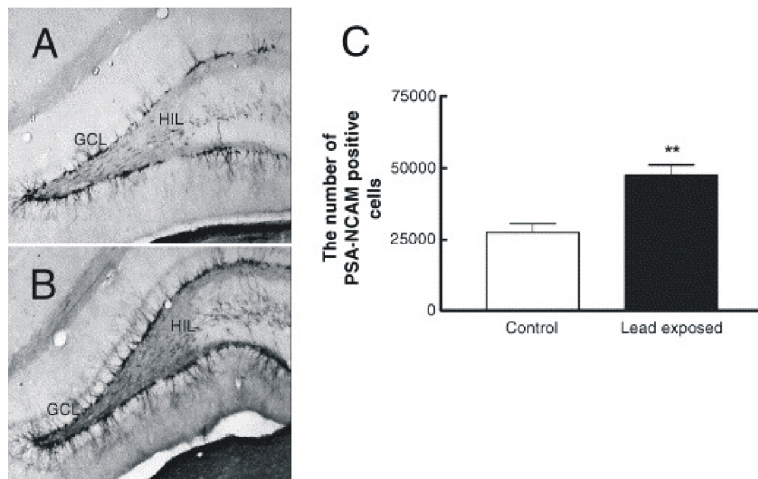


**Figure 12.** PSA-NCAM immunoreactive cells in the subgranular zone of the dentate gyrus of control (A) and lead exposed (B) animal. (C) The number of PSA-NCAM expressing cells in lead exposed and control animals. The data are mean  $\pm$  S.E.M;  $t = 4.271$ ; d.f. = 8; \* $p < 0.01$  (Student's  $t$ -test). Each group consists of five animals. Hil – hilus; GCL – granular cell layer.

## 2.2. The effects of developmental low-level lead exposure on the phenotype of PSA-NCAM expressing cells

To obtain more information about the phenotype of PSA-NCAM expressing cells we performed double immunohistochemistry using antibodies against PSA-NCAM and neuronal or glial markers. Double immunofluorescence labeling of PSA-NCAM and a marker for young immature neurons, TuJ1, revealed that most of PSA-NCAM positive cells co-localized with TuJ1. The proportion of cells co-expressing PSA-NCAM and TuJ1 did not differ, however, between lead-exposed and control animals. Double-labeling immunofluorescence, using antibodies against GFAP and PSA-NCAM or calbindin and PSA-NCAM, showed that both markers never co-localized within the same cell in this region (Table 4; Figure 13).

**Table 4.** The effect of early postnatal low-level lead exposure on the percentages of PSA-NCAM-positive cells that expressed phenotype markers TuJ1 or calbindin or GFAP. The data are expressed as a percentage of PSA-NCAM-positive cells that expressed phenotype marker TuJ1, calbindin or GFAP (co-localizations). The data are mean  $\pm$  S.E.M. Each group consists of four animals. 80–100 PSA-NCAM positive cells were counted per animal.

|              | Percent of colocalization of PSA-NCAM and TuJ1 | Percent of colocalization of PSA-NCAM and GFAP | Percent of colocalization of PSA-NCAM and calbindin |
|--------------|------------------------------------------------|------------------------------------------------|-----------------------------------------------------|
| Control      | 93.43 $\pm$ 1.24                               | 0                                              | 0                                                   |
| Lead exposed | 96.73 $\pm$ 1.24                               | 0                                              | 0                                                   |

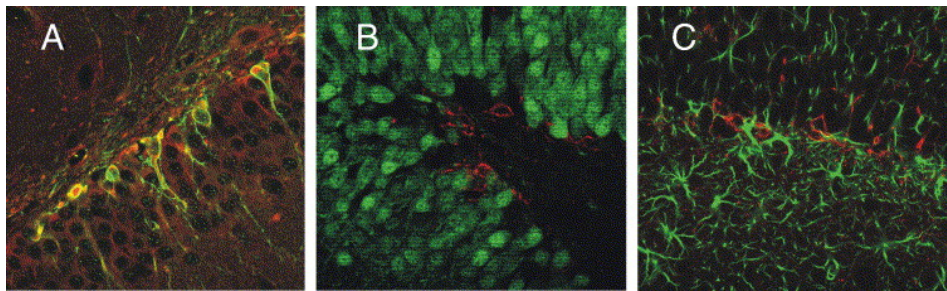


**Figure 13.** Confocal scanning laser images of sections double labeled by PSA-NCAM and calbindin (marker for mature granular neurons) or GFAP (marker for astroglia) or TuJ1 (marker for postmitotic young neurons) in the subgranular zone of the dentate gyrus. (A) Co-localization of PSA-NCAM (green) and TuJ1 (red); (B) Distribution pattern of PSA-NCAM (red) and calbindin (green) expressing cells; (C) Distribution pattern of PSA-NCAM (red) and GFAP (green) expressing cells. PSA-NCAM never co-localized with calbindin or GFAP.

### 3. The effects of different amphetamine administration regimens on the expression levels of PSA-NCAM and NCAM

#### 3.1. Induction of behavioral sensitization

Figure 14 demonstrates locomotor activity on the 1st and 8th day of the Paired (context associated) saline (SAL) and amphetamine treatment group (AMPH, 2.0 mg/kg i.p.). The Paired AMPH treated mice had substantially more locomotor activity on the 1st day than SAL group. Following daily injections of amphetamine for 7 days, mice displayed nearly 3-fold increase in responding across days (repeated measures ANOVA,  $F(1,35) = 8.39$ ,  $p < 0.0064$ , followed by Bonferroni post-test, 1st day vs. 8th day,  $p < 0.001$ ) indicating robust sensitization. Locomotor activity of the saline-treated mice did not change across the induction of sensitization (Bonferroni post-test, 1st day vs. 8th day,  $p > 0.05$ ). The Unpaired mice also received saline or amphetamine treatment, but in a different, Third World environment and their behavior was not recorded during the induction of sensitization. These results demonstrate that repeated treatment with amphetamine induced the development of amphetamine sensitization in mice.

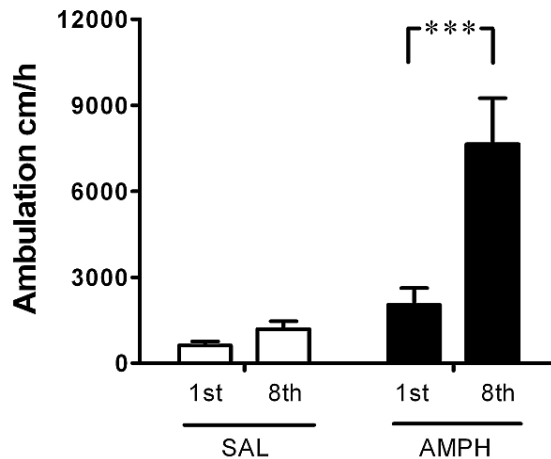


**Figure 14.** The induction of behavioral sensitization. Mice in the Paired groups were treated with amphetamine (AMPH, 2.0 mg/kg, i.p.) or saline (SAL) daily for 8 days and locomotor response of the Paired groups was monitored on the 1st and 8th day. Data expressed as mean  $\pm$  S.E.M., AMPH  $n = 10$  and SAL  $n = 8$ . ANOVA for repeated measure followed by Bonferroni post-test, AMPH 1st day vs. 8th day \*\*\* $p < 0.001$ .

## **3.2. Expression of behavioral sensitization**

### **3.2.1. Saline challenge**

To study a facilitatory conditioned response we evaluated a saline challenge response to the locomotor testing environment of the Paired and Unpaired groups. After the induction of amphetamine sensitization for 8 days, both the Paired and Unpaired groups underwent a washout period for 3 days. This was followed (on the 12th day) by a 20-min saline challenge test in the locomotor test environment, which was novel to the Unpaired groups but not to the Paired groups. Figure 15A shows that, after the saline challenge, there was a significant difference in the locomotor activities between the Paired and Unpaired groups (two-way ANOVA; context  $F(1,63) = 6.08$ ,  $p = 0.0113$ , treatment  $F(1,63) = 4.04$ ,  $p = 0.039$ , and interaction  $F(1,63) = 5.33$ ,  $p = 0.024$ ). The Paired AMPH mice exhibited substantially more locomotor activities in response to saline injection in the test environment than the Unpaired AMPH group (Bonferroni post-tests  $p < 0.01$ ). Thus, the facilitatory conditional response was detected in the Paired AMPH mice but not in the Unpaired AMPH group after the saline challenge.

### **2.2.2. Amphetamine challenge**

An occasion-setting mechanism can block the expression of neural sensitization in contexts where a drug of abuse is not expected and can enhance the expression of neuronal sensitization in contexts where the drug is expected (Anagnostaras and Robinson, 1996). To evaluate the effect of occasion-setting mechanism, we compared the Paired and Unpaired mice after an amphetamine challenge. Immediately after saline test, all the Paired and Unpaired mice were given a challenge dose of AMPH (2.0 mg/kg) in the locomotor test environment and ambulation was recorded for 60 min. We found that the challenge dose of AMPH given to the Paired and Unpaired animals elicited robust sensitization (Figure 15B; two-way ANOVA, interaction  $F(1,63) = 3.02$ ,  $p = 0.0869$ ; treatment  $F(1,63) = 39.25$ ,  $p < 0.0001$ ; and context  $F(1,63) = 0.91$ ,  $p = 0.345$ , followed by Bonferroni post-test, the Unpaired SAL vs. AMPH,  $p < 0.01$  and the Paired SAL vs. AMPH,  $p < 0.001$ ). Thus, amphetamine challenge induced robust sensitization in both the Paired and Unpaired groups, however there was no significant difference of sensitization responses between the Unpaired and Paired AMPH mice.

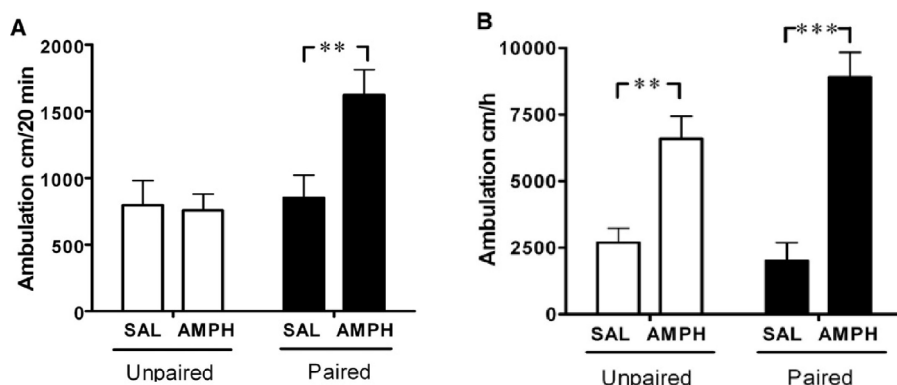


**Figure 15.** (A, B) Saline and amphetamine challenge test. Both the Unpaired and Paired mice received saline treatment, and the locomotor activity was measured for 20 min in the locomotor test environment (A). Immediately after the saline challenge all mice received amphetamine (2.0 mg/kg i.p.) and ambulation was recorded for 60 min (B). Data expressed as mean  $\pm$  S.E.M., SAL  $n = 8$  and AMPH  $n = 10$  both in the Paired and Unpaired groups. Two-way ANOVA followed by Bonferroni post-test,  $**p < 0.01$  the Paired and Unpaired SAL vs. AMPH and  $***p < 0.001$  the Paired SAL vs. AMPH.

### 3.3. Effects of amphetamine administration on the expression levels of PSA-NCAM in hippocampus, cortex and striatum

To evaluate whether synaptic plasticity could participate in the amphetamine-associated learning processes, we studied PSA-NCAM and NCAM expression levels after acute amphetamine administration and in a context-specific model. For Western blot analysis all the Paired and Unpaired mice were divided into three subgroups: (i) control group (Cont, received saline for 8 days followed by washout period for 3 days and challenged with saline on the day 12); (ii) acute amphetamine group (Ac, treated 8 days with saline, then the amphetamine challenge on the day 12); and (iii) behavioral sensitization group (BS, received amphetamine for 8 days, followed by three-day washout period and challenged with amphetamine on day 12).

Immunoblotting analysis of hippocampus, cortex and striatum revealed that PSA-NCAM appears as one broad band around 180–200 kDa (Figure 16A). It is noteworthy that whatever experimental condition used, we always detected the bands around 130 and 70 kDa, which we believe are the products of degradation of PSA-NCAM or the non-specific bands.

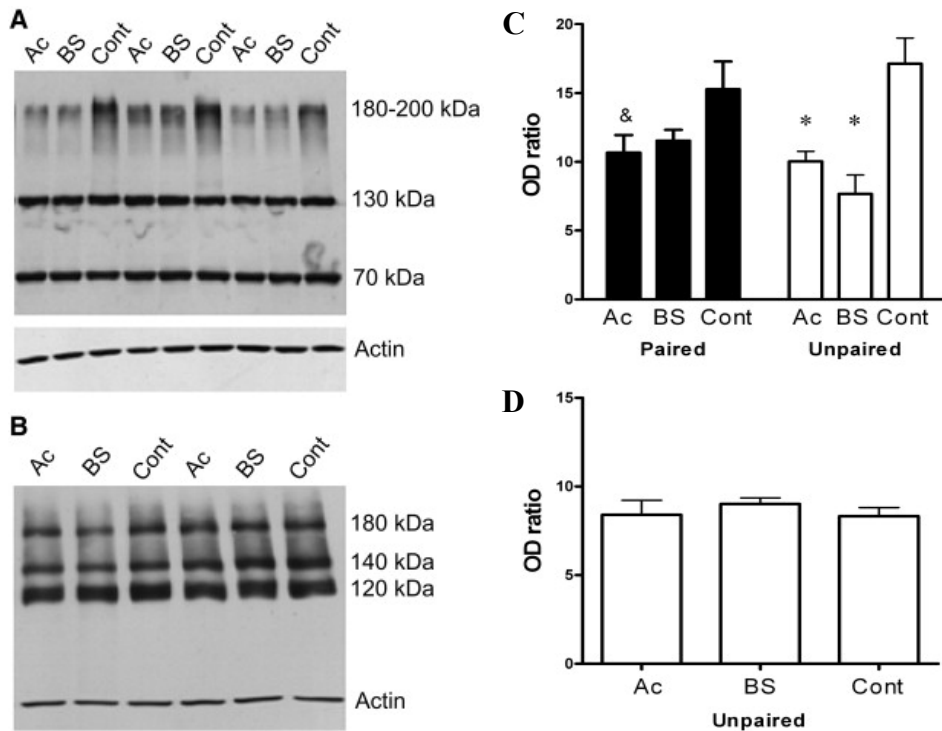


**Figure 16.** (A, B) The effects of amphetamine challenge on the expression level of PSA-NCAM and NCAM in mouse hippocampus. Representative Western blots of PSA-NCAM (A) and NCAM (B) from hippocampus isolated 2–3 h after the amphetamine challenge. Control group (Cont) received only saline treatment, a single dose of amphetamine to the saline pretreated mice (Ac) and amphetamine treatment to the sensitized mice (BS). Each line represents different animal.  $\beta$ -Actin was used as a loading control.

(C, D) Immunoblotting analysis of the expression level of 180–200 kDa isoform of PSA-NCAM in mouse hippocampus. Hippocampus was isolated 2–3 h after the amphetamine challenge (C) or 24 h (D). Control group (Cont) received only saline treatment, a single dose of amphetamine to the saline pretreated mice (Ac) and amphetamine administration to the sensitized mice (BS). Data expressed as mean  $\pm$  S.E.M. Cont  $n=6$ , Ac  $n=5$  and BS  $n=5$  both in the Paired and Unpaired groups. OD – optical density. Two-way ANOVA followed by Bonferroni post-test, \* $p < 0.05$  the Unpaired Ac or BS vs. Cont and  $p < 0.05$  the Paired Cont vs. Ac.

We found that both in the Paired as well as the Unpaired groups' amphetamine treatment decreased the expression level of 180–200 kDa isoform of PSA-NCAM in hippocampus (Figure 16C, two-way ANOVA, interaction  $F(2,26) = 1.80$ ,  $p = 0.186$ ; treatment  $F(2,26) = 12.33$ ,  $p = 0.0002$ ; and context  $F(1,26) = 0.56$ ,  $p = 0.462$ ). There were significant differences between Cont and Ac both in the Paired as well as Unpaired groups 3 h after challenge (respectively

Bonferroni post-test,  $p < 0.05$  and  $p < 0.01$ ). However, between Cont and BS there was significant difference only in the Unpaired groups (Bonferroni post-test,  $p < 0.001$ ). Our data also showed that amphetamine effect on 180 – 200 kDa isoform of PSA-NCAM expression level was transitory because there was no difference between treatment groups 24 h after the challenge (Figure 16D).

The expression levels of other isoforms of PSA-NCAM in hippocampus were unaffected after the amphetamine challenge (Table 5). We could not detect any significant difference in the expression levels of PSA-NCAM in striatum and cortex (Table 5).

**Table 5.** Immunoblotting analysis of the expression level of PSA-NCAM and NCAM in mouse hippocampus striatum and cortex following amphetamine challenge. Hippocampus, frontal cortex and striatum of the Unpaired mice were isolated 2–3 h after the amphetamine challenge. Control group (Cont) received only saline treatment, a single dose of amphetamine to the saline pretreated mice (Acute) and amphetamine treatment to the sensitized mice (BS). Data (optical density of adjusted volume) expressed as mean  $\pm$  S.E.M. One-way ANOVA followed by Bonferroni post-test, the Unpaired Acute or BS vs. Cont. \* $p < 0.001$ .

|                       | Control |      |   | Acute  |      |   | BS    |      |   |
|-----------------------|---------|------|---|--------|------|---|-------|------|---|
|                       | Mean    | SEM  | n | Mean   | SEM  | n | Mean  | SEM  | n |
| <b>Hippocampus</b>    |         |      |   |        |      |   |       |      |   |
| PSA-NCAM 180kDa       | 17.13   | 1.86 | 6 | 10.03* | 1.81 | 5 | 7.66* | 1.38 | 5 |
| PSA-NCAM 130kDa       | 26.56   | 4.20 | 4 | 23.42  | 0.86 | 4 | 29.01 | 2.07 | 4 |
| PSA-NCAM 70kDa        | 16.56   | 2.43 | 4 | 14.72  | 0.67 | 4 | 17.19 | 0.52 | 4 |
| NCAM 180kDa           | 13.56   | 0.93 | 4 | 15.07  | 1.37 | 4 | 16.45 | 0.74 | 4 |
| NCAM 140kDa           | 16.36   | 1.42 | 4 | 14.90  | 1.03 | 4 | 18.92 | 1.73 | 4 |
| NCAM 120kDa           | 26.62   | 1.57 | 4 | 21.67  | 1.93 | 4 | 29.59 | 2.71 | 4 |
| <b>Frontal cortex</b> |         |      |   |        |      |   |       |      |   |
| PSA-NCAM 180kDa       | 13.37   | 4.36 | 4 | 14.72  | 3.60 | 4 | 16.64 | 3.06 | 4 |
| PSA-NCAM 130kDa       | 18.97   | 2.62 | 4 | 16.89  | 0.81 | 4 | 17.46 | 1.16 | 4 |
| PSA-NCAM 70kDa        | 14.16   | 2.46 | 4 | 12.84  | 1.05 | 4 | 13.31 | 1.18 | 4 |
| NCAM 180kDa           | 13.42   | 2.48 | 4 | 11.46  | 0.89 | 4 | 12.94 | 0.94 | 4 |
| NCAM 140kDa           | 18.96   | 2.17 | 4 | 17.60  | 1.55 | 4 | 17.70 | 0.63 | 4 |
| NCAM 120kDa           | 16.75   | 1.54 | 4 | 16.66  | 1.81 | 4 | 16.82 | 0.65 | 4 |
| <b>Striatum</b>       |         |      |   |        |      |   |       |      |   |
| PSA-NCAM 180kDa       | 3.76    | 0.86 | 4 | 3.41   | 0.84 | 4 | 5.88  | 0.60 | 4 |
| PSA-NCAM 130kDa       | 4.51    | 0.77 | 4 | 5.55   | 0.57 | 4 | 6.44  | 1.48 | 4 |
| PSA-NCAM 70kDa        | 5.39    | 0.98 | 4 | 6.16   | 0.59 | 4 | 6.96  | 1.34 | 4 |

### **3.4. Effects of amphetamine administration on the expression levels of NCAM in hippocampus, cortex and striatum**

We also investigated the expression levels of all isoforms of NCAM (120, 140 and 180 kDa) in all above-mentioned structures following different amphetamine administration regimens, but no significant difference was observed (Table 5, Figure 17B). Also neuraminidase treatment (neuraminidase from *Clostridium perfringens*) or using crude synaptosomal pellets instead of whole brain homogenate did not change our results (data not shown).

## **DISCUSSION**

### **I. Effects of lead exposure on animal behavior, neurogenesis and PSA-NCAM expression level**

#### **I.1. Effects of early post-natal low-level lead administration on animal behavior in adulthood**

Our results demonstrate that post-natal lead exposure induces increased anxiety in the plus-maze test and disrupted contextual fear conditioning. The observed behavioral alterations persisted long after the termination of lead administration. At that time, the blood lead levels returned to those seen in control animals and therefore we propose that lead exposure induces long-lasting or even permanent alterations in brain functions. The observed behavioral syndrome in rats is very similar to those described by others (Salinas and Nuff, 2002) and are in agreement with observations on humans where children exposed to low levels of lead display attention deficit, increased emotional reactivity and impaired memory and learning (Finkelstein, 1998).

#### **I.2. Effects of early post-natal low-level lead administration on neurogenesis and differentiation in adult animal**

The observed behavioral alterations, following lead exposure, were accompanied by the inhibition of the production of new cells in the dentate gyrus of the hippocampus. These data are consistent with recently found lead-induced retardation of proliferation of the neural stem cells derived from embryonic brain *in vitro* (Huang and Schneider, 2004) and *in vivo* following lead exposure in adult animals (Schneider and Anderson, 2005). Our study confirms very recent data, which demonstrates the inhibitory action of developmental lead exposure on hippocampal neurogenesis *in vivo* (Gilbert *et al.*, 2005). In contrast to our study, Gilbert *et al.*, (2005) failed to demonstrate the reduction in BrdU-positive proliferating cells when measured 24 hours after the last administration of BrdU in lead-exposed rats. It should be noted however, that in the study of Gilbert *et al.*, (2005) BrdU was given repeatedly during 12 days. Repeated daily administration of BrdU does not allow an exact estimation of proliferating cells, since, in the case of multiple BrdU injections over several days, cells are labeled over several cell cycles, and thus the total cell numbers will be influenced not only by the rate of proliferation but also by the differentiation and survival (Eisch, 2002). Our studies also show that not only proliferation of neuronal precursors was retarded, but lead exposure also affected maturation of the newly born cells into adult neurons. Indeed, our study shows that 3 weeks after BrdU administration lower proportion of BrdU-positive cells differentiated into

adult calbindin-positive neurons, whereas the proportion of young post-mitotic neurons expressing Tuj1 is significantly higher in lead-exposed rats than in control animals. On the basis of these data we propose that lead exposure retards transition of young postmitotic neurons into adult neurons. Our data also shows that observed alterations in neurogenesis are accompanied by an enhanced gliogenesis since a higher proportion of astroglial cells were generated from BrdU-positive cells. Activation of astroglia and enhanced expression of astroglial protein GFAP, after lead exposure, has also been described by others (Selvin-Testa *et al.*, 1994; Struzinska *et al.*, 2001). How the observed changes in the differentiation of newly generated cells affect hippocampal functions, remains largely unknown. All current evidence suggests that the generation of new cells in the dentate gyrus might have an importance for the hippocampal functions as newly generated cells differentiate into mature neurons, establish synaptic contacts and become integrated into the hippocampal circuitry, thereby providing a greater degree of plasticity to the mature brain (Snyder, *et al.*, 2001; Van Praag *et al.*, 2002). The reduction in the number of mature granule neurons might result in the decreased level of neuronal plasticity of the brain. On the other hand, the accumulation of immature neurons in the dentate gyrus might be also relevant since immature neurons depict an increased risk of inappropriate migration, establishment of aberrant synaptic contacts, formation of aberrant neural networks within hippocampal formation, which might affect hippocampal functions (Scharfman *et al.*, 2002). The mechanisms, by which lead exposure can affect neurogenesis, remain largely unknown. There are some data demonstrating that serotonin, neurotrophins, such as an insulin-like growth factor, brain derived neurotrophic factor, or cAMP stimulate hippocampal neurogenesis, whereas glucocorticosteroids or NMDA receptor agonists inhibit it (for ref. see Duman *et al.*, 2001). Previous studies have demonstrated that developmental lead exposure can damage serotonergic neurotransmission (Widmer *et al.*, 1991) and induce long-lasting decrease in the expression of neurotrophic factors (Schneider *et al.*, 2001), which might have negative consequences on hippocampal neurogenesis. On the other hand, alterations in the glutamatergic neurotransmission at the level of receptors (Toscano *et al.*, 2003) might have negative consequences on both memory functions and hippocampal neurogenesis.

### **1.3. Possible links between behavioral impairment and reduced neurogenesis**

Until the present date it is impossible to establish a direct link between the observed increase in anxiety, impaired contextual fear conditioning and alterations in neurogenesis seen in lead-exposed rats. Only some indirect correlations could be provided to support a possible link between behavioral impairment and reduced neurogenesis. Lesion studies show that CA3, CA1 regions

and the dentate gyrus are critically involved in the acquisition and retrieval of contextual fear memories (Lee and Kesner, 2004). Animals with the mutation in presenilin 1 gene, responsible for the development of familial Alzheimer's disease, demonstrate deficits in contextual fear memory and reduced neurogenesis (Wang *et al.*, 2004) similar to those observed in our experiments. When animals are grown in an enriched environment they learn better and demonstrate increased hippocampal neurogenesis (Van Praag *et al.*, 1999). The recent study (Schneider *et al.*, 2001) showed that an enriched environment is also neuro-protective against lead-induced hippocampal memory deficits. On the other hand, previous studies, when inhibition of neurogenesis was induced by the administration of antimitotic agent methylazoxymethanol, failed to affect contextual fear conditioning or spatial navigation learning in the Morris water maze (Shors *et al.*, 2002). Thus, the direct link between contextual fear memory formation and hippocampal neurogenesis has not been established yet and further experiments are needed.

#### **I.4. Effects of early post-natal low-level lead administration on PSA-NCAM expression and co-localization in adult animal**

We also found that early post-natal low-level lead exposure induces a significant increase in the number of PSA-NCAM immunoreactive cells in the granular cell layer of the adult rat dentate gyrus. Our results are in a logical correlation with some previous reports (Cookman *et al.*, 1987; Breen and Regan, 1988).

It should be emphasized that the observed increase in the number of PSA-NCAM-positive cells was found in 80-days-old animals, whereas the lead exposure was terminated at PND 30. At this age, the blood lead level did not differ from those found in control animals. This suggests that early postnatal lead exposure induces profound perturbations in the brain, which persist in adulthood and are reflected by the accumulation of PSA-NCAM positive cells. In our experiments PSA-NCAM-positive cells were found exclusively within the border between the GCL and hilus. This region is known to be able to produce neuronal precursors throughout the whole life (Altman and Das, 1965; for review see Mackowiak *et al.*, 2004). A limited proportion of newly generated cells survives and differentiates into adult granule neurons (Gould and Tanapat, 1997). Previous studies have demonstrated that newly generated cells transiently express PSA-NCAM (Seki, 2002), which allows them to migrate and form synaptic contacts with other neurons (Rutishauser, 1996). It is reasonable to propose that PSA-NCAM-positive cells originate from newly born cells within this region. Indeed our experiments demonstrated that the majority of PSA-NCAM positive cells co-express markers for young postmitotic neurons. On the other hand, recent *in vivo* studies have demonstrated that lead exposure results in the inhibition of neurogenesis (Schneider *et al.*, 2005) and therefore a

decrease in the PSA-NCAM-positive cells instead of their increase could be expected in lead-exposed animals. Since newly generated cells start to express PSA-NCAM soon after they become postmitotic, and the expression sharply reduces when they become adult neurons, we propose that early lead exposure retards the maturation of newly generated cells into adult neurons. Our recent studies demonstrate that the differentiation of bromodeoxyuridine (BrdU) positive cells into mature calbindin positive cells was greatly retarded following early post-natal low-level lead exposure (Jaako-Movits *et al.*, 2005). It is possible to hypothesize that the increase in the number of PSA-NCAM expressing cells following early post-natal low-level lead exposure could be the result of perturbations in the differentiation processes that results in the accumulation of not fully differentiated neurons in the dentate gyrus despite the reduced generation of the precursors.

It is not excluded, however, that adult neurons start to express PSA-NCAM and the observed increase in the number of these cells is due to the de-differentiation of the neurons as it is observed in Alzheimer's disease (Arendt, 2000). Although not fully excluded, such a possibility seems unlikely, because none of the PSA-NCAM-positive cells did express the marker of mature granule neurons, calbindin. Furthermore, almost all cells were found within the inner part of GCL, which contains mainly neuronal precursors and young postmitotic neurons.

We cannot exclude the possible effect of low-level lead exposure on the cell cycle that could lead to the situation that the maturation of cells is arrested in a certain phase where the expression of PSA-NCAM is high. In fact, it has been shown that lead may contribute to the overall lengthening of the cell cycle (Cookman and Regan, 1991).

It might be proposed that an increased number of PSA-NCAM expressing cells could be partly the basis of impairments in the learning and memory formation following low-level lead exposure as an excessive synthesis of PSA on NCAM, or inhibited physiological desialylation (Edelman and Chuong, 1982; Cookman *et al.*, 1987), could affect/decrease functional synaptic stabilization after remodeling of synapses during memory consolidation and learning processes through its anti-adhesive properties. It has been shown that redundant sialylation of NCAM causes a decrease in cell-cell aggregation and an increase in heterophilic binding to extracellular matrix proteoglycans (Mayanil *et al.*, 2000).

## **1.5 Conclusions**

Taken together our data shows that developmental lead exposure induces inhibition of neurogenesis and at the same time increases the number of young, PSA-NCAM-positive cells in subgranular zone, which suggests the possibility of a perturbed differentiation process resulting in the accumulation of not fully

differentiated neurons in the dentate gyrus. Observed alterations could, at least partly, contribute to specific behavioral deficits observed in adulthood following early post-natal low level lead exposure.

## **2. Effects of amphetamine administration on animal behaviour and the expression levels of PSA-NCAM and NCAM**

In our experiments we found that (i) amphetamine (2.0 mg/kg, i.p.) induces robust behavioral sensitization and the expression of sensitization after the saline challenge is context-dependent; (ii) immunoblotting analysis demonstrates that acute amphetamine treatment time-dependently decreased only PSA-NCAM isoform 180–200 kDa expression level in hippocampus; (iii) PSA-NCAM isoform 180–200 kDa expression level of sensitized mice is decreased in the *Unpaired*, but not in the *Paired* group after amphetamine challenge; (iv) there are no changes in PSA-NCAM expression level in striatum and a frontal cortex, and NCAM expression levels in hippocampus, striatum and the frontal cortex after the amphetamine challenge.

### **2.1. Effects of amphetamine administration on animal behavior: associative learning processes governing amphetamine sensitization**

Behavioral sensitization is the manifestation of neuronal plasticity that occurs as a consequence of repeated exposure to drug of abuse. Under some circumstances, a contextual stimulus can gain powerful control over the ability of the sensitized neural substrate to influence behavior (Anagnostaras *et al.*, 2002; Stewart and Vezina, 1988). It has been proposed that a neural sensitization is modulated by two associative mechanisms. One of these is a conditioned facilitation, resulting from the Pavlovian pairing of the context and drug, which produces a conditional response to drug-associated cues. This can add to the sensitized response in the drug-paired context (Tilson and Rech, 1973; Tirelli and Terry, 1998). The other is an occasion-setting mechanism that prevents the expression of sensitization in the environment where the drug is not expected but enhances the expression of sensitization in the context where the drug is expected (Anagnostaras and Robinson, 1996; Stewart and Vezina, 1988).

In the present experiments, the facilitatory conditional response was clearly observed in the *Paired* AMPH mice, when they were given saline and placed in the drug-associated environment. However, the *Unpaired* AMPH mice, which had the same treatment but were placed in the *Third World* environment, did not demonstrate the facilitatory conditional response.

It has been proposed that an occasion-setting mechanism, acting via conditional facilitation or inhibition, can gate the expression of the sensitized neural substrate (Anagnostaras and Robinson, 1996). If animals receive amphetamine only in one distinctive environment they form an expectation that they would receive the drug only in that environment. This expectation can serve as a facilitatory occasion-setter in the treatment-associated context and as an inhibitory occasion-setter in a different context (Anagnostaras *et al.*, 2002). Our data demonstrated that both the *Paired* and the *Unpaired* AMPH mice expressed significantly higher locomotor activity compared to SAL groups after the amphetamine challenge, but there was no difference between these AMPH groups. We speculate that lack of a significant difference between the *Paired* and *Unpaired* AMPH groups could be due to a too high amphetamine challenge dose. Thus, the challenge data suggested that there was a significant context effect after the saline challenge, but the context effect did not reach significant difference after the amphetamine challenge.

## **2.2. The effects of amphetamine treatment on the expression levels of PSA-NCAM in hippocampus**

To investigate the possible roles of PSA-NCAM and NCAM in the amphetamine-induced associative learning mechanism, we measured the expression levels of different isoforms of PSA-NCAM and NCAM proteins in different brain regions after amphetamine treatment. We have chosen the regions, which are primarily involved in the reward circuitry – striatum, prefrontal cortex (see review Robinson and Kolb, 2004) and hippocampus (Crombag *et al.*, 2005).

Our results demonstrate that amphetamine administration selectively decreases the expression level of 180–200 kDa isoform of PSA-NCAM in mouse hippocampus. We were unable to detect any significant change in striatum or prefrontal cortex in any isoforms of PSA-NCAM. As we found the decrease of PSA-NCAM expression level 2–3 h after a single dose of amphetamine, but not 24 h after treatment, it could be concluded that amphetamine most probably has only an acute transitory effect on the expression of PSA-NCAM. The conclusion is also supported by the fact that we found similar decrease after the amphetamine challenge in different treatment groups (non-sensitized and sensitized mice).

The decrease in hippocampal PSA-NCAM induced by amphetamine might have important functional consequences. Having in mind the physiological role of PSA-NCAM, which is to confer the anti-adhesive properties of NCAM and allow neurons to reshape their morphology, elongate dendrites and rearranged synaptic connections – decreased expression of PSA-NCAM reduces plasticity, stiffens neuronal shape and arrests synaptic reorganization (Durbec and Cremer, 2001), thereby affecting the processes that require the previously mentioned arrangements – including learning and memory formation.

One main goal of our study was to investigate the possible role of PSA-NCAM in the amphetamine-induced associated learning mechanisms. Unfortunately, our results demonstrated that there were no statistically significant differences between the *Paired* and *Unpaired* sensitized mice after amphetamine challenge. However, we detected a trend towards increased expression of PSA-NCAM in the context-associated group (a significant PSA-NCAM expression difference between the *Unpaired* Cont and BS groups but no difference between the *Paired* ones). As PSA-NCAM is transiently upregulated in hippocampus during the consolidation of spatial information (Fox *et al.*, 1995; Murphy *et al.*, 1996; Murphy and Regan, 1999) and considering the fact that we were able to detect the significant change in PSA-NCAM isoform 180–200 kDa expression only in hippocampus – a structure which is primarily involved in spatial learning – we hypothesize that the phenomenon could be related to an associative learning and/or memory consolidation mechanisms. However, the suggestion needs future investigations to clarify PSA-NCAM role in a context-dependent learning mechanisms.

In conclusion, we have found that amphetamine induces a remarkably selective effect on PSA-NCAM expression in mouse hippocampus, which could be relevant in different synaptic plasticity associated processes. We also speculate that amphetamine treatment and an associative learning may alter differently PSA-NCAM expression level in hippocampus – acute amphetamine treatment non-associatively decreases PSA-NCAM expression level in hippocampus, but amphetamine-induced associative learning may increase PSA-NCAM expression level.

### **2.3. Possible mechanisms underlying amphetamine-induced decrease of expression level of 180–200 kDa PSA-NCAM in hippocampus**

The possibility that the reduction of PSA-NCAM expression level was the result of a decrease of NCAM level was not supported by our findings as we were unable to detect any significant difference in any major isoforms of NCAM in hippocampal formation. Thus, it is more likely that alteration of the expression level of PSA-NCAM reflects a changed state of sialylation of NCAM.

Our results join recent findings highlighting a potential role of hippocampal plasticity in the acute and chronic effects of several drugs of abuse. Indeed, it has been shown that nicotine self-administration profoundly decreased expression of PSA-NCAM as well as neurogenesis in the dentate gyrus of the hippocampus (Abrous *et al.*, 2002) and nicotine decreased the number of PSA-NCAM expressing cells dose-dependently (Shingo and Kito, 2005). Morphine treatment induced decrease in a cell proliferation paralleled with a decrease in the number of PSA-NCAM immunoreactive granule cells in the dentate gyrus (Kahn *et al.*, 2005), and a single injection of cocaine (15 mg/kg i.p.) induced a

time-dependent decrease in the number of PSA-NCAM positive cells in dentate gyrus and lasted 4 days after treatment (Mackowiak *et al.*, 2005). Our findings do not support the early study of Mackowiak *et al.* (2005) as we found that there were no differences between SAL and Ac groups 24 h after treatment. We speculate that this discrepancy between two studies reflects the differences between the methods (Western blot vs. immunohistochemistry), strain (rat vs. mouse) and area of study (Mackowiak *et al.* studied the dentate gyrus, we evaluated PSA-NCAM expression from a lysate of all hippocampus).

In spite of extensive work on the field, molecular mechanisms possibly related to the decreased expression of PSA-NCAM following administration of different drugs of abuse are not known. However, some common molecular targets have been suggested, like dopamine and glutamate. D-amphetamine has been shown to significantly increase the spontaneous release of aspartate and glutamate (Labarca *et al.*, 1995). Then, it follows that over-activity of glutamate induced by amphetamine treatment would decrease PSA-NCAM expression level, which refers to the possibility that *N*-methyl-D-aspartate (NMDA) receptor activity may be involved in the regulation of PSA expression following amphetamine administration. This hypothesis is supported by the study of Bouzioukh *et al.* (2001), which shows that activity-linked stimulation of the NMDA-nitric oxide-cGMP pathway induced a rapid decrease of PSA-NCAM expression in adult brainstem synapses.

Another possibility is to affect the removal of PSA from the cell surface, either by endocytosis of PSA-NCAM or by degradation of PSA. Endocytosis is believed to be the mechanism which dynamically controls the level of PSA-NCAM expression on cell surface and may play an important role in rapid changes occurring under physiological and pathological conditions (Minana *et al.*, 2001). However, if that regulatory mechanism is involved, we should see changes also in NCAM expression. No evidence has been obtained so far to suggest that surface degradation of PSA plays a regulatory role. Still, Manzi *et al.* (1994) have shown that a single internal cleavage in tertiary structure formed by PSA would be sufficient to cause an irreversible release of a large portion of a long chain. These intermolecular cleavages of the glycosidic bonds preferably form under mildly acidic conditions that can be encountered in various physiological and pathological conditions. However, the exact role of amphetamine in the regulation on PSA biosynthesis remains to be elucidated.

Also alterations in neurogenesis following amphetamine administration could result in the observed reduction in PSA-NCAM levels. Newly born cells in the adult dentate gyrus transiently express the high levels of PSA-NCAM (Seki, 2002) and the decreased PSA-NCAM expression might be the result of decreased neurogenesis. However, there is no direct evidence of the effects of d-amphetamine on neurogenesis. Some studies have been conducted using methamphetamine to study the effects of psychostimulants on cell proliferation. It has been shown that treatment with methamphetamine transiently decreases dentate granule cell proliferation in adulthood (Teuchert-Noodt *et al.*, 2000). It

could be hypothesized that it is one possible mechanism of the decreased PSA level following amphetamine treatment. However, if the decrease in PSA is associated with decreased neurogenesis we should also be able to find changes in NCAM expression, which we did not observe. Abrous *et al.* (2002) have demonstrated that PSA-NCAM is significantly decreased for a dose of nicotine lower than necessary for decreasing neurogenesis, which indicates that changes in PSA-NCAM could be independent to the alterations in neurogenesis.

## **2.4. Conclusions**

In conclusion, we found that amphetamine produced robust behavioral sensitization and the expression of sensitization after the saline challenge was context-dependent. The immunoblotting analysis demonstrated that acute administration of amphetamine selectively and time-dependently decreases the expression of 180–200 kDa isoform of PSA-NCAM in hippocampus in both the context-associated (the *Paired*) as well as the context non-associated (the *Unpaired*) groups. The present results suggest that PSA-NCAM is not involved in the amphetamine-induced associated learning mechanisms.

## CONCLUSIONS

Early post-natal low-level lead exposure induced persistent increase in the level of anxiety and inhibited contextual fear conditioning in adulthood. Developmental lead exposure also reduced the generation of new cells in the dentate gyrus of adult animal and altered the pattern of differentiation of BrdU-positive cells into mature, calbindin-positive neurons. The proportion of young not fully differentiated neurons and the proportion of astroglial cells were increased following lead exposure. Observed alteration in neurogenesis could, at least partly, account for the cognitive impairments in adulthood following developmental lead exposure.

Early post-natal low-level lead exposure induced significant increase in the total number of PSA-NCAM expressing cells and did not alter the proportion of cells co-expressing PSA-NCAM with glial or neuronal markers calbindin, TujI and GFAP. The observed increase in the number of PSA-NCAM expressing cells following developmental lead exposure could attribute to the characteristic cognitive dysfunction in adulthood, which could be the results of perturbation in the differentiation process that results in the accumulation of not fully differentiated neurons in the dentate gyrus despite the reduced generation of the precursors, considering our previous results.

Chronic amphetamine administration produced robust behavioral sensitization and the expression of sensitization after the saline challenge was context-dependent. The immunoblotting analysis demonstrated that acute administration of amphetamine selectively and time-dependently decreases the expression of 180–200 kDa isoform of PSA-NCAM in hippocampus in both context associated (the Paired) as well as context non-associated (the Unpaired) groups. There were no changes in PSA-NCAM expression levels in striatum and frontal cortex, and NCAM expression levels in hippocampus, striatum and frontal cortex after amphetamine challenge. The observed selective decrease following amphetamine administration on PSA-NCAM 180–200 kD isoform could be relevant in different synaptic-plasticity associated processes, however, PSA-NCAM is not involved in the amphetamine-induced associated learning mechanism.

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## SUMMARY IN ESTONIAN

### Närviraku adhesioonimolekuli roll neurotoksiinide toimes aju plastilisusele

Ajukoe kaks näiliselt vastukäivat, kuid säilimise ja adekvaatse toimimise seisukohalt olulist ülesannet on säilitada närvisüsteemi spetsiifilisus ja stabiilsus ning samas reageerida nii organismi sisekeskkonnast kui ka väliskeskkonnast tulevatele stiimulitele kohaste adaptatiivsete muutustega, ehk olla *plastiline*. Käesolev töö uurib täiskasvanuea struktuurset plastilisust, mis hõlmab närvikoe erinevaid morfoloogilisi muutusi, keskendudes neurogeneesi fenomenile ja raku adhesioonisüsteemide rollile aju plastilisuses. Täiskasvanueas pidev uute närvirakkude teke ehk neurogenees hõlmab mitmeid struktuurse plastilisuse aspekte, sealhulgas tüvirakkude proliferatiooni, rakkude migreerumist sihtpunkti, aksonite ja dendriitide kasvu, diferentseerumist ning lülitumist uutesse närviringetesse (Gage *et al.*, 2000; Lledo *et al.*, 2006). Adhesioonimolekulid loovad aluse rakkudevaheliseks dünaamiliseks seostumiseks. Meie töö uurib peamiselt immunoglobuliinide superperekonda kuuluva närviraku adhesioonimolekuli (NCAM) ning selle polüsialüülitud vormi (PSA-NCAM) ekspressiooni muutusi plii ja amfetamiini manustamise tagajärjel. NCAM-i põhiliseks rolliks on närvirakkude vaheliste stabiilsete kontaktide loomine ning tema ekspressioon on kõrge nii arenevas kui täiskasvanud ajukoos (Rutishauser *et al.*, 1976; Jorgensen and Bock, 1974; Soroka *et al.*, 2003). Polüsiaalhappe süntees NCAM-i külge omab seevastu adhesioonivastast toimet ning on rakkudevahelise struktuurse dünaamilisuse oluline vahendaja. PSA-NCAM ekspressioon on kõrge arenevas närvikoes, täiskasvanud imetaja ajus on ekspressioon kõrge ja funktsionaalselt oluline ainult subventrikulaartsoonis ning hipokampuse subgranulaarrakkude kihis (Rutishauser, 2008). On leitud, et NCAM ja tema polüsialüülitud vorm osalevad õppimises ja mälu formeerumises (Doyle *et al.*, 1992; Murphy *et al.*, 1996; Becker *et al.*, 1996), samuti on näidatud PSA-NCAM-i ja NCAM-i ekspressiooni muutusi erinevate haigusseisundite korral (Varea *et al.*, 2007; Mikkonen *et al.*, 2001; Barbeau *et al.*, 1997; Pekcec *et al.*, 2008; Seidenfaden *et al.*, 2003).

### Töö eesmärgid

Täiskasvanueas aju plastilisuses toimuvad muutused moodustavad olulise osa närvikoe füsioloogilistest adaptatsioonimehhanismidest. Endogeensete või eksogeensete faktorite poolt esile kutsutud mittefunktsionaalsed muutused aju plastilisuses võivad osaleda erinevate haiguslike seisundite patogeneesis, muuhulgas kognitiivse düsfunktsiooni tekkes.

Meie töö eesmärgiks oli uurida plii varajase sünnijärgse manustamise mõju täiskasvanud roti mälule ja õppimisvõimele, samuti muutusi neurogeneesis ja närviraku adhesioonimolekuli ekspressiooni tasemes hipokampuse hammastuumas. Samuti oli eesmärgiks uurida psühhostimulaatori amfetamiini erinevate manustamisviiside toimet hiirte käitumisele ja närviraku adhesioonimolekuli ekspressiooni tasemele aju erinevates piirkondades.

## Töö tulemused

Töö tulemused näitasid, et madalalävine plii manustamine varajasel sünnijärgsel perioodil põhjustas katseloomadel täiskasvanueas ärevuse tõusu ja mäluhäireid. Immunohistokeemilisel uuringul leidsime neurogeneesi languse hipokampuses ning häired tekkinud rakkude diferentseerumises küpseteks neuroniteks.

Samas suurendas madalalävine plii ekspositsioon märkimisväärselt PSA-NCAM- positiivsete rakkude arvu hipokampuse hammastuumas subgranulaarrakkude kihis, mõjutamata oluliselt PSA-NCAM- positiivsete rakkude fenotüüpi.

Eeltoodud muutused nii neurogeneesis kui ka PSA-NCAM ekspressiooni tasemes võivad osaleda varajase plii ekspositsiooni kaugtagajärjena tekkivate kognitiivsete häirete kujunemisel. Kuigi langenud neurogeneesi korral võiks eeldada langust PSA-NCAM ekspressioonitasemes, mida käsitletakse noorte veel lõplikult diferentseerumata rakkude markerina ja samuti struktuurse dünaamilisuse olulise vahendajana, viitavad meie tulemused võimalusele, et uute rakkude vähenenud tekke ja tekkinud rakkude küpsemise häirumise korral kujuvad diferentseerumisjärgus ebaküpsed PSA-NCAM-positiivsed rakud.

Selleks, et uurida PSA-NCAM-i rolli ravimsõltuvusega seotud plastilisuse mehhanismides, kasutasime kontekstiga seotud ja mitteseotud amfetamiini poolt indutseeritud käitumusliku sensitisatsiooni mudelit. Uurides psühhostimulaatori amfetamiini akuutset toimet PSA-NCAM ekspressioonile leidsime, et akuutne amfetamiini manustamine langetab selektiivselt ja ajast sõltuvalt 180–200 kD molekulmassiga PSA-NCAM-i isovormi hipokampuses nii keskkonnaga assotsieeritud (*Paired*) kui ka keskkonnaga assotsieerimata (*Unpaired*) loomade gruppis, mis võib olla oluline amfetamiini manustamise kaugtagajärjena tekkiva kognitiivse düsfunktsiooni tekkes. Krooniline amfetamiini manustamine kutsus esile märkimisväärse käitumusliku sensitisatsiooni, kuid ei mõjutanud PSA-NCAM ekspressiooni aju erinevates struktuurides, millest võib järeldada, et PSA-NCAM ei osale sensitisatsiooni välja kujunemisel.

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## **PUBLICATIONS**





Jaako-Movits K, Zharkovsky T, Romantchik O, Jurgenson M, Merisalu E, **Heidmets LT**, Zharkovsky A. (2005) Developmental lead exposure impairs contextual fear conditioning and reduces adult hippocampal neurogenesis in the rat brain. *Int J Dev Neurosci* 23; 627–35.

## II

**Heidmets LT**, Zharkovsky T, Jurgenson M,  
Jaako-Movits K, Zharkovsky A. (2006)  
Early post-natal, low-level lead exposure increases  
the number of PSA-NCAM expressing cells in the dentate gyrus  
of adult rat hippocampus. *Neurotoxicology* 27; 39–43.

### III

**Heidmets LT**, Kalda A, Zharkovsky A. (2007)  
Acute amphetamine treatment decreases the expression  
of 180–200 kDa isoform of polysialic acid linked neural  
cell adhesion molecule in mouse hippocampus.  
Brain Res 24;1165:89–97.

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### **Teadustöö**

Peamiseks teadustöö valdkonnaks on olnud aju plastilisuse uurimine, keskendudes adhesioonimolekulide rollile närvikoe struktuurse plastilisuse kontekstis. Kokku on ilmunud seitse artiklit eelretsenseeritavates väljaannetes.

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