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STUDIES ON THE ROLE OF NEUROGENESIS IN BRAIN PLASTICITY

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I Zharkovsky T, Kaasik A, **Jaako K**, Zharkovsky A. (2003) Neurodegeneration and production of the new cells in the dentate gyrus of juvenile rat hippocampus after a single administration of ethanol. Brain Res 978; 115–23.

II **Jaako, K,** Zharkovsky T, Kaasik A and Zharkovski A. (2003) Ethanol intoxication reduces, whereas ethanol withdrawal transiently enhances, production of the neural progenitor cells in the adult mouse dentate gyrus. Neurosci Res Comm 33; 158–167.

III **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.

IV **Jaako-Movits K**, Zharkovsky A. (2005) Impaired fear memory and decreased hippocampal neurogenesis following olfactory bulbectomy in rats. Eur J Neurosci 22: 2871–8.

V **Jaako-Movits K**, Zharkovsky T, Pedersen MV, Zharkovsky A. (2006) Decreased hippocampal neurogenesis following olfactory bulbectomy is reversed by chronic citalopram administration. Cellular and Molecular Neurobiology (submitted)

ABBREVIATIONS

ANOVA	analysis of variance
5-HT	5 hydroxytryptamine
AMPA	5-methyl-4-isoxazole propionic acid
BDNF	brain derived neurotrophic factor
BrdU	5-bromodeoxyuridine
CNS	central nervous system
CPu	Caudate putamen
CREB	cAMP response element binding protein
DAB	diaminobenzidine
DG	dentate gyrus
DHEA	dehydroepiandrosterone
FAS	fetal alcohol syndrome
FGF	fibroplast growth factor
GCL	granule cell layer
GFAP	glial fibrillary acidic protein
Hil	hilus
IGF1	insulin-like-growth-factor
LV	Lateral ventricle
MAM	methylazoxymethanol
NMDA	N-methyl-D-aspartate
OB	olfactory bulbectomy
PND	postnatal day
PSA-NCAM	poly-sialyated-neural cell-adhesion molecule
RMS	rostral migratory pathway
SGZ	subgranular zone
SVZ	subventricular zone
TOAD 4	collaspin response-mediated protein 4
Tuj1	β-tubulin isoform III
TUNEL	terminal deoxyribonucleotidyl transferase mediated dUTP nick end labelling
VEGF	vascular endothelial growth factor

INTRODUCTION

The dentate gyrus of hippocampus differs from other brain regions by its ability to generate new neurons throughout the whole life (Altman and Das, 1965) and cells, which are produced in the dentate gyrus during adulthood, differentiate into mature functionally active granule neurons (Cameron *et al*., 1993). Several factors, like age, genetics, chronic stress, depressive state, injuries, drugs of abuse, antidepressants could affect hippocampal neurogenesis (Gould and Tanapat, 1997; Cameron and McKay, 1999; Gould and Tanapat, 1999; Eisch *et al.,* 2000; Malberg *et al*., 2000; Duman *et al*., 2003; Eisch, 2002; Jaako *et al*., 2003). It has been hypothesized that adult hippocampal neurogenesis exists as a substrate for neuronal plasticity and is related to the memory formation and emotions (Jacobs, 2000; Drapeau *et al*., 2003; Shors *et al.,* 2001, 2002, 2004). The disruption of hippocampal neurogenesis might diminish the plasticity of the hippocampus and finally enhance the likelihood of mood and memory disorders (Duman *et al*., 1999; Jacobs *et al*., 2000; Kempermann, 2002a).

In the present study we examined the effects of ethanol and lead administration in the different periods of brain development on emotional and cognitive functions and the hippocampal neurogenesis. According to the current hypothesis linking a depressive state with reduced neuronal plasticity we conducted a series of studies on the hippocampal neurogenesis following olfactory bulbectomy, an animal model of depression in rats. Also we studied the ability of the selective reuptake inhibitor citalopram to restore reduced hippocampal neurogenesis in bulbectomized rats.

REVIEW OF LITERATURE

1. Brain plasticity

During the whole life brain is exposed to many endogenous and environmental insults, which the brain needs react to. This ability of the brain to undergo functionally relevant adaptations following external and/or internal stimuli is generally referred to as neural plasticity, which is absolutely necessary for the adequate functioning of an individual in the continuously changing environment. Within different brain structures hippocampus is one of the most plastic structure, which has high capacity to adapt and change its structure or functions in response to alterations in the internal and/or external environment (McEwen, 2000; Duman, 1999). By now it has also become clear that such alterations can at least partially account for phenomena such as memory and learning (Shors, 2002). Better understanding of the regulatory mechanisms of brain plasticity might be important in assessing potential future therapies based on manipulations on factors modulating plastic reactions.

2. Hippocampus

Anatomically, hippocampus is a part of the temporal lobe of the cerebral cortex. It receives inputs from the entorhinal cortex, contralateral hippocampus, hypothalamus, and basal forebrain. Output fibers project to the entorhinal cortex and the contralateral hippocampus. The term hippocampal formation encompasses following regions: the dentate gyrus (DG), hippocampus proper, subiculum, presubiculum, parasubiculum and the entorhinal cortex. In rats, the hippocampus proper comprises of four parts: CA1, CA2, CA3 and CA4 (hilus). The intrahippocampal connections form a *trisynaptic loop*, which is composed of the cells of the dentate gyrus, CA3 and CA1 and their interconnections (Figure 1).

The first synaptic connections of the loop are formed between the entorhinal cortex and dentate gyrus. The cells in the superficial layers (mainly layer II) of the entorhinal cortex send their axons to the molecular layer of the dentate gyrus and this pathway is called the perforant pathway. Collaterals of the same axons form also connections with CA3 pyramidal cells.

The axons from the granule cells leave the hilar region and synapse on the proximal dendrites of the CA3 pyramidal cells, and this represents the second stage of the *trisynaptic loop*. These innervations are called *mossy fibers*.

The axons of the CA3 pyramidal cells form connections with the dendrites of the CA1 pyramidal cells and these axons (*Schaffer collaterals)* represent the third stage of the *trisynaptic loop*.

While the *trisynaptic loop* is the main circuit of the hippocampus, it is still only one part of the entire circuitry. There are also connections from the entorhinal cortex to the CA1 and the subiculum, connections between the two hippocampi via the commissures, and the subcortical connections via the fimbria/fornix, mostly with the septum (for rev. see Kadish, 2002).

The hippocampus is involved in episodic, declarative, contextual and spatial learning and memory, as well as being a component in the control of autonomic and vegetative functions, such as ACTH secretion (Becker *et al.,* 1980; Eichenbaum and Otto, 1992; Phillips and LeDoux, 1992). The hippocampus has been implicated in the mediation of several cognitive processes including the formation and utilization of spatial cognitive maps (McNaughton *et al.,* 1993; Morris *et al.,* 1982; Nadel, 1991). Spatial memory is dependent on the integrity of hippocampus and hippocampal damage results in an impaired ability to solve tasks that relay on spatial search strategies in a number of mammalian species including rat, and human (Morris *et al.,* 1990; Volpe and Hirst, 1983). Beside lesions, it has been also demonstrated that increased levels of glucocorticoids, neurotoxins, age and mental disorders could impair performance in many types of tasks that are dependent on the use of spatial information (Newcomer *et al.,* 1994; McEwen, 2000; Heffelfinger and Newcomer, 2001). These alterations in hippocampal-dependent tasks indicate that changes in hippocampal plasticity could be mediated via changed synaptic or neuronal reorganization.

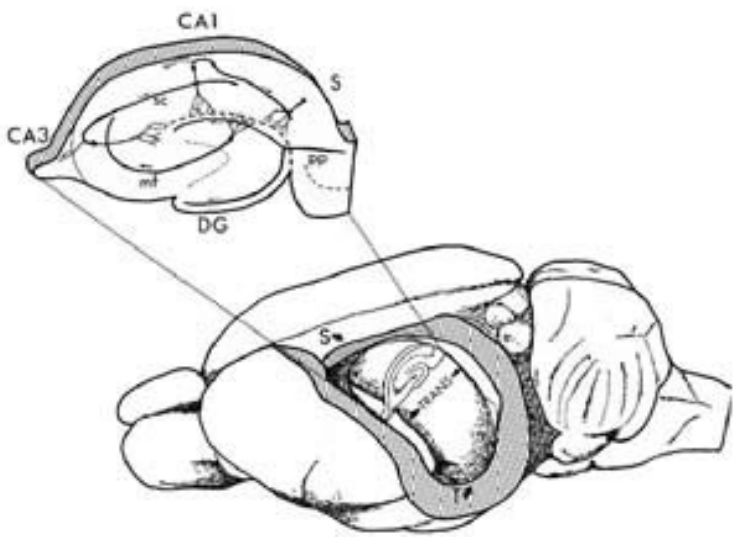

Figure 1. The upper plane is a slice, taken from across the hippocampus, revealing the *trisynaptic circuit*, in which the dentate gyrus (DG) projects to CA3, which projects to CA1, which projects to the subiculum. The lower plane is a diagram of a rat brain (Fuster, 1995).

3. Neurogenesis as a specific form of hippocampal plasticity

Contrary to previously held benefits about the static nature of the adult brain, in the late 1950s, a new method was developed to label dividing cells with [H^3]-thymidine, which incorporates into the replicating DNA during the S-phase of the cell cycle and can be detected with autoradiography (Sidman *et al.,* 1959). Altman and colleagues published a series of papers reporting [H^3]-thymidine evidence for new neurons in various regions of adult rats, including the dentate gyrus of the hippocampus, neocortex and olfactory bulb (Altman and Das, 1965; Altman 1969), but these new cells were considered to lack functional relevance.

In recent years, this view has been gradually revised based on various studies showing continuous neurogenesis in adulthood (Cameron *et al.,* 1993; Eriksson *et al*., 1998; Gould *et al.,* 1999b; Gross, 2000) and its possible functional significance in existing neuronal circuits and connections is subject to lifelong modifications and reorganizations (Kempermann, 2002b; Van Praag, 2002).

Adult neurogenesis has been demonstrated almost in all species including bird, rodent, monkey and humans (Eriksson *et al.,* 1998; Gould *et al.,* 1999a, b; Gross, 2000) and the conservation of adult neurogenesis across varied species including humans suggests that this process serves a significant biological function. In an adult mammalian brain, significant rates of adult neurogenesis are restricted to three brain regions: the subventricular zone (SVZ), hippocampus and cerebellum. The largest of these germinal regions in the adult brain is the SVZ, which lines the lateral walls of the lateral ventricles. Neural stem cells produce neuroblasts that migrate from the SVZ along a discrete pathway, the rostral migratory stream, into the olfactory bulb where they form mature neurons involved in the sense of smell (Peretto *et al.,* 1999) (Figure 2).

In the DG, progenitor cells are located in the subgranular zone (SGZ), which is the border between the granule cell layer and hilus (Figure 2). A subset of the new cells survives, migrates into the granule cell layer and differentiates into neurons or glial cells (van Praag *et al*., 2002). A recent detailed analysis of neurogenesis reports that in the adult rodent hippocampus there are approximately 9,000 new cells per day or 250,000 cells per month (Cameron and McKay, 2001). Approximately 50% of these cells differentiate and these maturated neurons represent about 6% of the total number of granule cells in the dentate gyrus. Newly born cells express cellular markers and acquire the characteristics of neurons, like extension of axons through the mossy fiber bundle (Hastings *et al.,* 2002), integration of dendrites into the molecular layer (Carlén *et al.,* 2002), expression of immediate-early gene proteins to a similar degree as mature neurons when appropriately stimulated (Jessberger and Kempermann, 2003) and having electrophysiological properties distinguishing granule cells (Van Praag *et al.,* 2002).

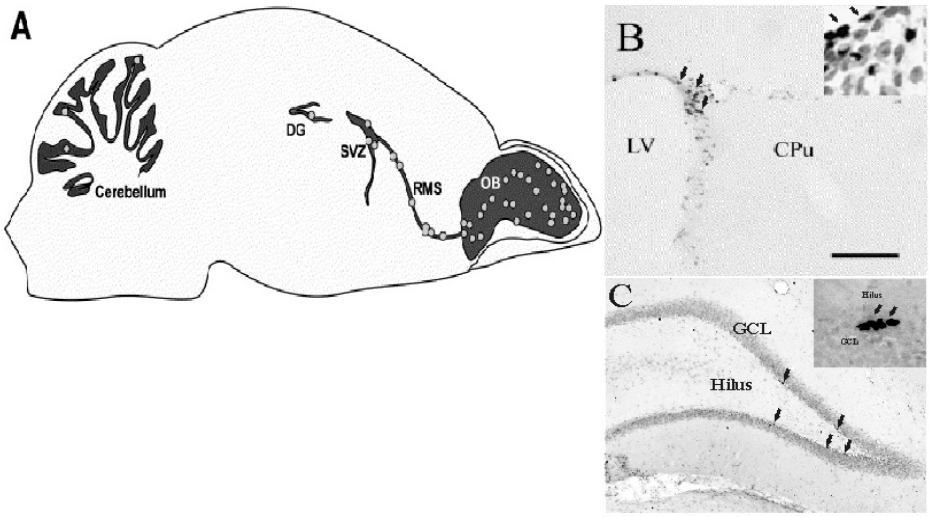

Figure 2. (A) Neurogenesis regions in the adult rat brain (Biebl *et al.*, 2000). Arrows show BrdU positive cells in the subventricular zone (B) and in the border between the granule cell layer and hilus of the hippocampus (C). Scale bar: 300 μm. Inserts show BrdU – positive cells at higher (x 1000) magnification. GCL – granule cell layer, CPu – Caudate putamen, LV – Lateral ventricle, RMS – rostral migratory pathway, OB – olfactory bulb, DG – dentate gyrus.

3.1. Functional significance of adult hippocampal neurogenesis

During recent years the functional significance of hippocampal neurogenesis has been a matter of speculations, since an increasing number of reports have provided evidence that adult hippocampal neurogenesis exists as a form of neuronal plasticity, which is related in learning and memory formation, emotions (Gould *et al.,* 1999a; Kempermann, 2002b) and the rate of neurogenesis correlates with certain learning abilities (Shors *et al.,* 2001, 2002; Monje and Palmer, 2002; Drapeau *et al*., 2003; Rola *et al.,* 2004).

The mechanisms how changes in adult hippocampal neurogenesis are relevant to hippocampal-dependent memory and learning remain to be elucidated. So far, technical barriers make it difficult to monitor the role of newly born cells in behaving animals. However, several mechanisms have been proposed how the new cells enhance learning: induction of synaptic disquietude (Teuchert-Noodt, 2000), adjustment to increased complexity at the "gate to memory" (Kempermann, 2002b), a lower threshold for long-term potentiation in new neurons (Van Praag *et al.,* 1999b) or keeping a network stable against outside influences (Lehman *et al.,* 2005).

The functional significance of adult hippocampal neurogenesis has been elucidated by disrupting or reducing the generation of newly born cells by using

toxins or irradiation. Long-lasting defects in cognition following disrupted neurogenesis should show up weeks after the defect, not immediately, and the potential contributions of adult neurogenesis to cognition most likely are long-term adaptations rather than acute effects (Rola *et al.,* 2004). Shors *et al*., (2001) demonstrated that administration of DNA methylating agent methylazoxymethanol (MAM), which is toxic for proliferating cells produced a dose-dependent inhibition of neurogenesis in the adult hippocampus that correlated with a disturbed hippocampus-dependent learning in the eye-blink conditioning test, whereas the effects of MAM were not detectable on a hippocampus-independent version of the same task and also in the Morris water maze.

Using hippocampal irradiation, an impaired learning process has been shown, and even three months after a single dose of irradiation 5 Gy, there was a persistent and significant decrease in the number of proliferating cells and immature neurons in the SGZ and a concomitant impairment of hippocampal-dependent cognitive function (Abayomi, 1996; Madsen *et al.,* 2003; Rola *et al.,* 2004).

Concerning the functionality of adult hippocampal neurogenesis an original hypothesis has been postulated, that persistent disruption of hippocampal neurogenesis might diminish the plasticity of the hippocampus and finally enhance the likelihood of addiction, mood and memory disorders (Duman, 1999; Jacobs *et al.,* 2000; Jacobs, 2002; Kempermann, 2002a).

This hypothesis links depression not only to changes in neurotransmitter concentrations and receptor activity levels, but also impairment of brain plasticity, tissue remodelling and alterations in neurogenesis (Duman, 1999; Jacobs *et al*., 2000) especially in the brain structures, which play a role in emotional behaviour and memory formation, like hippocampus, amygdala and prefrontal cortex. These regions demonstrate structural alterations in response to stress in animals and in patients with mood disorders (McEwen, 1997; Duman *et al.,* 1999; Sheline *et al*., 1999; Rajkowska, 2000; Villarreal *et al*., 2002; Radley and Morrison, 2005). The hippocampal atrophy (Sheline *et al.,* 1996, 1999) and deficits in hippocampal-related memory (Vythilingam *et al.,* 2004) have been demonstrated in depressive patients.

Recent animal studies have shown that prolonged stress reduced adult hippocampal neurogenesis and clinically active antidepressants induced an increase in the proliferation rate of the neuronal progenitors and enhanced their maturation into neurons in the dentate gyrus of the hippocampus after chronic administration (Duman *et al*., 1999; Gould and Tanapat, 1999; Malberg *et al*., 2000). Furthermore, recent studies undoubtedly demonstrated that hippocampal neurogenesis was required for the behavioural effects of antidepressants (Santarelli *et al*., 2003). On the basis of these findings, it has been proposed that the depressive state could be associated with altered neurogenesis. Until now, however, the precise role of adult hippocampal neurogenesis in depression remains unclear since the data indicating an impairment of neurogenesis in depression are missing due to the lack of an appropriate in vivo methodology

enabling the studying of this process in depressive patients, and therefore current research linking the depressive state with impaired neurogenesis has been focused on the available animal models of depression (Cźeh *et al*., 2001; Lee *et al*., 2001; Malberg and Duman, 2003). In rodents, one of the most validated behavioural models of depression is the behavioural syndrome, which develops following olfactory bulbectomy (OB) (Harkin *et al*., 2003). It has been shown in rodents that bilateral OB induces increased irritability, hyperactive responding in a novel, stressful environment, impaired passive avoidance behaviour and food-motivated behaviour (Kelly *et al.*, 1997). Several antidepressants, given repeatedly reversed OB-induced hyperactivity in the open field test and restored behaviour in the passive avoidance test and, therefore, OB was considered as a valuable behavioural model of depression (Kelly *et al.*, 1997; Harkin *et al*., 2003; Song and Leonard, 2005). It should be noted, however, that the definition of the OB-induced behavioural syndrome, which arises from a neurodegenerative process (Kelly *et al*., 1997; Harkin *et al*., 2003) as "depressive-like" is based exclusively on its sensitivity to antidepressants. To-date hippocampal neurogenesis has not been studied in OB rats.

3.2. Factors affecting neurogenesis

The possibility that neurogenesis plays a role in neural plasticity and remodelling is supported by reports where it has been shown that neurogenesis in the adult brain could be regulated in both positive and negative manners by internal and external factors (environmental or pharmacological stimuli) (Table 1).

The main internal factors affecting neurogenesis are genetics, age, gender, expression of growth factors, level of hormones and neurotransmitters.

One possible factor mediating the decrease of neurogenesis rate in the course of time and following stress is the level of glucocorticoids, produced by the adrenal glands, since adrenalectomized aged rats have levels of neurogenesis comparable to those of young adrenalectomized rats (Cameron and McKay, 1999).

Endogenous growth factors, like brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF), IGF-1 and hormones apart form glucocorticoids like dehydroepiandrosterone (DHEA) and oestrogen increase cell proliferation and differentiation (Tanapat *et al.,* 1999; Wagner *et al.,* 1999; Aberg, 2000; Karishma and Herbert, 2002; Sarainen *et al.,* 2005). The influence of neurotrasmitters on cell proliferation has been studied and every transmitter investigated so far has been shown to have an effect on the process of neurogenesis (Table 1).

Table 1. Summary of factors mediating adult hippocampal neurogenesis. Adapted from Lehman *et al*., 2005.

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

The environment has also been reported to modulate neurogenesis in the DG. For example exposure to an enriched environment, physical activity, spatial learning resulted in the significant increase in neurogenesis (Kempermann *et al.,* 1997; Gould, 1999a; Nilsson *et al.,* 1999). Social isolation, social status and early life experience such as maternal deprivation and prenatal stress decreased hippocampal neurogenesis (Kozorovitskiy and Gould, 2004; Mirescu *et al.,* 2004).

In pathological conditions, neurogenesis was adversely affected by stress, epilepsy, ishemia and strokes (Cameron and Gould, 1994; Gould and Tanapat, 1997; Parent *et al.,* 1997, 2002; Tureyen *et al.,* 2004). Stress, depression and experimentally induced diabetes decreased hippocampal neurogenesis, whereas following neurological diseases, such as stroke, epilepsy and traumatic brain injuries there was an increase in DG neurogenesis, which might be an adaptive process (Cameron and Gould, 1994; Liu *et al.,* 1998). However, the seizure-induced neurogenesis in the epileptic brain includes differentiation into granule neurons in ectopic locations in the hilus or molecular layers of the hippocampus and formation of aberrant connections to the inner molecular layer of the DG, in addition to the CA3 pyramidal cell region, which might contribute to the hippocampal kindling (Parent *et al.,* 1997, 2002).

Several abused drugs, such opiates, ethanol, nicotine or cannabinoids have been investigated to determine their influence on adult neurogenesis. It has been found that opiates including morphine and heroin inhibit the proliferation and differentiation rate of these newly born cells in the adult rat hippocampus (Eisch *et al.,* 2000) probably via the mu opioid receptor and by via activation of the HPA axis (Nestler, 2001). Chronic administration of opiates was necessary to decrease neurogenesis, since acute treatment had no effect (Eisch *et al.,* 2000).

A similar decrease in the rate of neurogenesis has been observed after nicotine (Abrous *et al.,* 2002), methamphetamine (Teuchert-Noodt *et al.,* 2000) and ethanol (Nixon and Crews, 2002; Jaako *et al.,* 2003) treatment.

4. Neurotoxins and brain plasticity

Neurotoxicity is generally defined as a structural change (molecular, cellular, neurochemical) or a functional alteration (changes in behaviour) of the nervous system, resulting from exposure to a chemical, biological or physical agent. At the neuroanatomical level, neurotoxins induce structural changes by mediating the dentritic branching and spine density and increased neuronal or glial cell death in many brain regions (Bull *et al*., 1983; Oberto *et al*., 1996; Ikonomidu *et al*., 2000). These alterations are believed to reflect some of the persistent behavioural sequela associated with neurotoxins exposition. Changes in behaviour usually appear following acute or chronic neurotoxin exposure in adulthood, but long-lasting alterations could be also mediated when neurotoxin was admi-

nistered in an early postnatal period in animals or during pregnancy in humans, when CNS is especially sensitive to toxins (Abel, 1996).

4.1. Susceptibility of brain to environmental insults by its developmental stage

Short- and long-term deleterious effects resulting from environmental insults during brain development or even in adulthood requires not only knowledge of the nature of the insult but also of the nature of the organ at the time of insult. An important period of brain development is the period of growth, which occurs in the first two postnatal weeks in the rat and in the third trimester of pregnancy and early infancy in humans (Abel, 1996).

Studies indicate that different brain areas develop at different times during gestation, and within a single brain region, subpopulations of neurons develop at different rates and at different times. For example, in the hippocampus, pyramidal cells in CA1 region are generated on gestational days 18–19 and in the CA3 region cells are generated on the gestational day 17 in rats, corresponding to the early gestational period in humans (Abel, 1996).

Many neurotoxins, which have free access to the developing brain due to the lack of a protective barrier, cause brain damage by interfering cell proliferation, and if the insult occurs during the stage of formation of a certain neuronal subpopulation, those cells will not be formed. Exposure to neurotoxins during the cell migration period, which is another important process during brain development, could affect neurons to reach their final location and contact between cells is important for the construction of complex circuits (Alfano *et al.,* 1983). Since neurogenesis persists during an extended period in several brain regions, the formation of normal neuronal network or migration could be affected following neurotoxin exposure in adulthood and the impairment is dependent on the duration and extent of the exposure.

4.2. Effects of lead on the developing brain

Lead is still widely distributed in the environment, and the consequences of chronic exposure to low levels of lead in childhood have been a matter for extensive research during recent years. The biggest concern among the effects of low-level Pb exposure is the occurrence of reduced cognitive capacity in children exposed early in life (Bellinger *et al.,* 1991). Exposure to low levels of lead, during early development, has been implicated in long-lasting behavioural abnormalities and cognitive deficits in children and experimental animals (Murphy and Reagan 1999; Moreira *et al.,* 2001; Canfield *et al.,* 2003). Animal studies have found observable behavioural and morphological effects at blood

lead levels of less than 15 μg/dl in primates (Rice and Karpinski, 1988) and less than 20 μg/dl in rats (Cory-Slechta *et al.,* 1985). Long-lasting cognitive deficits suggest that hippocampus, a critical neural structure for learning and memory, might be one region adversely affected during early life after lead administration.

Animal studies have shown an increase in the size and in numerical density of the mossy fibers and the commissural-associational area of the dentate molecular layer (Slomianka *et al.,* 1989) following lead administration. Lead has been shown to be neurotoxic during the period of neural differentiation and synaptogenesis (Bull *et al.,* 1983). Lead seems to have its greatest effects during the later stages of brain development, perhaps by affecting synaptogenesis (Oberto *et al.,* 1996). Lead can produce a significant decrease in the formation of myelin, particularly during late gestational development and during the postnatal period (Mendola *et al.,* 2002). Alterations have also been reported in the properties of glutamatergic, cholinergic, and dopaminergic neurotransmitter function and signal transduction (Cory-Slechta, 1995). Indeed, recent findings demonstrated that early lead exposure disrupted expression and phosphorylation of the cAMP-responsible element binding protein (CREB), a transcription factor directly related to the neuronal plasticity in the hippocampus of juvenile rats (Toscano *et al*., 2003). Furthermore, early lead exposure altered the N-methyl-D-aspartate receptor subunit composition in favor of the prevalence NR 2B receptor subunit and decreased expression of the NR 2A subunit, which might be important in hippocampal development and maturation (Toscano *et al.,* 2002). The possible reduction of neuronal plasticity, caused by lead exposure, is also reflected by the altered hippocampal long-term potentiation (Xu *et al.,* 1998). Thus, developmental lead exposure induces persistent alterations in the hippocampus, which could, at least partly contribute to the behavioural and cognitive impairments observed in adulthood.

4.3. Effects of ethanol on developing brain

Ethanol is a well-documented developmental toxin causing a wide range of physical and mental dysfunctions in children after prenatal exposure. Chronic maternal ethanol abuse during pregnancy is associated with important teratogenic effects on the offspring and alcohol is the leading cause of mental retardation and congenital malformation in humans (Abel, 1984). The abnormalities that have been characterized as Fetal Alcohol Syndrome (FAS) include retarded body growth, microcephaly, poor coordination, underdevelopment of the mid-facial region and minor joint abnormalities (Archibald *et al.,* 2001)

FAS is associated with reduced brain mass and a variety of neurobehavioural disturbances, ranging from attention-deficit hyperactivity disorder and mild to severe learning impairment in children, to a high incidence of major depression

or psychosis in adults (Famy *et al.,* 1998). Generally, the final outcome of ethanol exposure during brain development is the reduction of cell number in various structures of the brain (Barnes and Walker, 1981; Cragg and Phillips, 1984; Pentney and Miller, 1992; Miller, 1995b; Miller, 1986; Marcussen *et al.,* 1994; Maier *et al.,* 1996; Ikonomidou *et al.,* 2000). Not all structures in the developing brain, however, demonstrate neuronal loss following ethanol exposure. Dentate gyrus of the hippocampal formation is one exemption. Previous studies (Miller 1995a; West *et al;* 1986) have demonstrated that the administration of ethanol during the early postnatal period led to an increase in the number of cells in the dentate gyrus of the rat brain. Furthermore, ethanol given between postnatal days 4 and 12 induced an increase in the weight of the hippocampus and DNA content of 21-day-old rats (Miller, 1996). The reason for this paradoxical reaction in the dentate gyrus is not clear. Neurons in the GCL of the rat dentate gyrus are born over a protracted period, from gestational day 15 into adulthood (Altman and Das, 1965; Cameron and McKay, 2001; Gould *et al.,* 2001). During the first weeks after birth, the granule cells in the dentate gyrus undergo the process of active neurogenesis and differentiation. In experiments on mice or rats, the effects of ethanol, given during this period, correspond to those during the third trimester of pregnancy in humans. It might be hypothesized that, in contrast to other brain regions, administration of ethanol directly or indirectly led to the enhancement of neuronal and/or glial proliferation in the dentate gyrus.

4.4. Effects of ethanol on the adult brain

The early postnatal developmental stage is not the only period of life where brain structures are sensitive to ethanol consumption. During several decades the effects of ethanol consumption on adult behaviour and potential morphological and functional alterations in the hippocampus following acute or chronic administration have been studied. It has been shown in animal models that chronic ethanol treatment significantly impairs hippocampal long-term potentiation and produces progressive learning and memory deficits across a variety of behavioural tests, including active avoidance and spatial memory which indicate that hippocampal functioning could be affected (Roberto *et al*., 2002). Chronic ethanol administration induces neurodegeneration in various brain structures, including hippocampus (Paula-Barbarosa, 1993; Pawlak *et al*., 2002). Bengochea and Gonzalo (1990) found significant pyramidal cell losses in the CA2, CA3 and CA4 areas of human hippocampus, but not in the CA1 or dentate gyrus. The volume of the hippocampal white matter, but not the grey matter, was reduced in alcoholics (Harding *et al.,* 1997).

The effect of chronic ethanol on the rodent hippocampus has been shown with more consistent results. After 5 month of ethanol exposure, there was a

16% loss of pyramidal cells in the dorsal hippocampus (Walker *et al.,* 1980) and the number of pyramidal cells was reduced by 20–30% following 18 month of ethanol consumption (Paula-Barbarosa *et al.,* 1993). In addition, the dentate gyrus granule cell loss by 40% following 4 months of ethanol exposure was reported (Paula-Barbarosa *et al.,* 1993).

Recently, it was found that chronic ethanol administration might affect neurogenesis in the dentate gyrus of adult mice. In a recent publication, binge ethanol administration for 4 days reduced the neural progenitor cell proliferation and survival (Nixon and Crews, 2002). Opposite results were found in the experiments where repeated ethanol administration with a liquid diet induced an enhancement of neurogenesis in the mouse hippocampus (Pawlak *et al.,* 2002). In later experiments, animals were fed with a diet containing ethanol and, therefore, were exposed to the intermediate withdrawal episodes due to the daily fluctuations in the ethanol concentrations. It is possible that ethanol intoxication and ethanol withdrawal could differently affect the production of new cells in the dentate gyrus.

THE AIMS OF THE STUDY

The general goal of the study was to elucidate a possible role of hippocampal neurogenesis in the alterations of behaviour and brain remodelling following neurotoxin (lead, ethanol) exposure or following OB, an animal model of depression. More specifically, the questions were:

1. Does low-level lead exposure during the extended postnatal period induce emotional and cognitive dysfunctions and alterations in the neurogenesis of an adult rat?
2. Does a single administration of ethanol in the early postnatal period affect hippocampal neurogenesis?
3. Does continuous ethanol intoxication and withdrawal in adulthood affect hippocampal neurogenesis?
4. Does olfactory bulbectomy, an animal model of depression, induce impairment of hippocampal functions and neurogenesis?
5. Does the antidepressant citalopram, a selective serotonin reuptake inhibitor restore impaired hippocampal neurogenesis induced by OB?

MATERIALS AND METHODS

1. 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 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 22ºC, under a 12-h light-dark cycle and were mated with males of the same strain.

2) To study the effect of ethanol administration on the early postnatal period 10-days-old Wistar rats (30–40 g) bred from the stock of animals obtained from Kuopio Animal Research Center (Kuopio, Finland) were kept in their home cage with mothers at 22ºC, under a 12-h light-dark cycle.

3) To study the effect of chronic ethanol administration on adult hippocampal neurogenesis we used (6 months old) male mice, NIH strain weighing 30–35 g. Mice were obtained from the Kuopio National Animal Center (Kuopio, Finland) and were housed in plastic cages at 22ºC, under a 12-h light-dark cycle.

4) To study the effects of OB and chronic citalopram administration on hippocampal neurogenesis, male Wistar rats, at the age of 2 months, weighing 170–200 g at the time of surgery were used. Rats were group housed (five rats per cage) in plastic cages at 22º C, under a 12-h light-dark cycle.

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. Ethanol administration

Ethanol injection
Ethanol administration in early postnatal period was performed on the PND 10 when animals were given i.p. 1.5 and 3 g/kg ethanol (20% w/v) solution at 9.00 h. Control animals were given saline injections.

Ethanol inhalation
For chronic ethanol administration ethanol inhalation methodology was used. Mice were placed in a Plexiglas box, with standard laboratory food and water available *ad libitum*. Air was bubbled into an ethanol solution using an air pump, and the vapour, above the solution, was passed continuously through the chamber for 24 h. The concentration of ethanol solution was gradually raised from 14ml/400ml (day 1) to 60 ml/400ml (day 14) and remained constant at this level for the next two weeks. The ethanol solution was changed twice a day. Under these settings ethanol concentration in the air of the chamber ranged from 7 mg/ml (day 1) to 60 mg/ml (day 24). Since the inhalation methodology of ethanol intoxication could lead to the malnutrition of animals, careful monitoring of the weights of animals during the whole experiment was performed. If the reduction of weight of laboratory animals was observed, the concentration of ethanol was slightly reduced until the animals regained weight. Control mice were placed in the identical Plexiglas box and were subjected to the same handling/air bubbling procedure except ethanol solution was replaced by water. In all experiments matched control animals were processed simultaneously with ethanol intoxicated/withdrawn animals.

2.3. 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 –70ºC. 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.

2.4. Measurement of blood ethanol concentration

Blood ethanol concentrations in 10 days old animals were determined in the separate groups at various times after ethanol administration. At each time point after ethanol administration animals (n=3) were decapitated, the truncal blood was collected into a heparinized tube and stored at -20°C until assayed. In the ethanol inhalation experiments, at the end of ethanol inhalation mice (n=4) were killed by decapitation, and ethanol concentration in the truncal blood was measured by headspace gas chromatography with n-propanol as the internal standard. Ethanol concentration in the gas sample was measured using a Hewlett Packard Gas Chromatograph HP 4890D, Hewlett Packard Headspace Autosampler HP 7694E and HP-Blood Alcohol Analysis Column. Sample preparation and analysis were provided by the standard method of the Estonian Forensic Medicine Bureau.

3. OB and citalopram administration

OB was performed as previously described (Kelly *et al*., 1997). Animals were randomly selected into two groups for OB and sham-operation. Animals were deeply anaesthetized with chloral hydrate (350 mg/kg body weight, i.p.). The top of the scull was shaved and swabbed with an antiseptic, after which the animal was placed under a stereotaxic instrument and a midline frontal incision was made in the scalp, the skin being retracted bilaterally. The surgical procedure involved drilling two burr holes on either side 1 mm. from the midline of the frontal bone overlying the olfactory bulbs. The bulbs were aspirated with a needle attached to a water pump. The cavity was packed with surgical foam and the holes were covered with dental cement with the skin bring closed with surgical clips. Sham-operated rats were treated similarly, except that the olfactory bulbs were not removed. The animals were allowed to recover within warming to maintain body temperature. After the surgery animals were housed in groups of 4, with 2 sham-operated, and 2 OB rats in the cage. Animals were handled and weighed daily during a 14 days- recovery period.

Citalopram hydrobromide (a generous gift of H. Lundbeck A/S, Copenhagen, Denmark) was dissolved in saline (0.9% NaCl). Two weeks following surgery sham-operated or OB rats were randomly divided into two groups and were given i.p. either saline on citalopram in a daily dose of 10 mg/kg of citalopram for 28 consecutive days.

4. Behavioural testing

Behavioural 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) or 2 and 6 weeks following OB (open-field, passive avoidance test, fear conditioning).

4.1. General locomotor activity

General locomotor activity was determined in a rectangular wooden cage (50 x 50 x 50 cm) uniformly illuminated with dim lighting. The 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 analysed using VideoMot2 software (TSE Systems, Germany) during a 30-minute observation period.

4.2. Elevated plus maze

Anxiety was evaluated in the elevated plus maze test. The elevated plus maze apparatus consisted of two open and two enclosed arms of equal length and width (50 × 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 (sec) 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 % of entries in the open arms.

4.3. Fear conditioning

Two tasks: contextual fear conditioning (multimodal) and tone-dependent fear conditioning (unimodal) were employed in the study.

For the fear conditioning tasks the plexiglas conditioning chamber was used. The chamber was made of dark-brown plastic panels (27 x 25 x 45 cm). The apparatus included a steel-rod grid floor, which consisted of 40 parallel bars (0.3 cm in diameter, set 1.2 cm apart). Each rod of the floor was wired to a shock generator designed to deliver scrambled shocks.

On the first day (training), the animals (n=16 in sham-operated or OB groups) were individually placed in the apparatus, where they remained for 5

minutes. The behaviour of each animal was recorded continuously by measuring seconds it remained frozen (defined as complete immobility and absence of movements and sniffing). After that, in OB study, three auditory cues (conditioned stimulus, 5 sec, 85 dB, 1000 Hz) at 60-second intervals were delivered. The tone sounded for 5 seconds and in the last second a foot-shock (unconditioned stimulus, 1 mA, 1 sec) was delivered, which ended together with the tone. Rats remained in the chamber for an additional 5 min, after which they were removed and returned to their home cages.

In lead toxicity study, the animals were given 3 electric shocks (0.8 mA, 1 sec duration) on their feet, at 60 sec. intervals between each shock, and were removed from the apparatus 1 min. after the last shock to the feet.

In OB study, contextual conditioning tests were performed on the second day, 24 hours after training. Half of the animals (n=8) were placed in the same training context, no foot-shocks and tone were delivered and the freezing time was recorded for a 5-min period.

In the lead toxicity experiment contextual conditioning tests were performed 24 h., 48 h. and 72 h. after the 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 ± SEM time of freezing.

For the tone-dependent memory test, another half of sham-operated or OB animals (n=8) were placed into the novel context: a modified conditioning chamber, consisting of carton walls and a plastic floor for 5-min and baseline freezing was measured. After that, a tone (5 sec, 85 dB, 1000 Hz) was presented three times at 60-sec intervals, and the total duration of freezing episodes recorded during the following 5-min period.

4.4. Open-field test

The open-field test was performed as previously described (Kelly *et al*., 1997) and carried out after 2 and 6 weeks following OB. Open field activity was determined between 9.00 AM and 11.00 AM. The open-field apparatus consisted of 100 x 100 x 45 cm wooden box, which was covered inside with folium to increase the reflectivity of the walls. The floor of the box was divided into 16 squares. A 60 W light bulb was positioned 90 cm above the base of the apparatus, and was the only source of illumination in the room. Each animal was tested for a 5-min period. Animals (n=12 in each group) were placed in the centre of the apparatus and allowed to explore freely for 5 min. During the test time the number of passed squares and number of rearings were measured. After each animal, the test apparatus was cleaned with a 10% ethanol solution and water to remove any olfactory cues.

4.5. Passive avoidance test

The passive avoidance test was performed after 6 weeks following OB. A step-through passive avoidance apparatus was used. It consisted of a Plexiglas box divided into two compartments. One compartment (17 x 25 x 45 cm) is white and illuminated by a light fixture, featuring a 50 W bulb fastened to the compartment lid. The second compartment is dark and made of dark-brown plastic panels (27 x 25 x 45 cm). Two compartments are separated by a door. The apparatus included a steel-rod grid floor, which consisted of 40 parallel bars (0.3 cm in diameter, set 1.2 cm apart). 6 OB and 6 sham-operated animals were used in the passive avoidance test. The animal was placed in the light compartment and the latency to enter the dark compartment with all four feet was measured in seconds. After that, the door was closed and an electric shock (1 mA) was delivered for 1 sec. The animal was then returned to its home cage. After 24 h, the animal was placed in a light compartment and latency to enter the dark compartment was measured but electric shock was not delivered during this session.

5. Neurogenesis assay

5.1. BrdU administration

In experiments bromodeoxyuridine (BrdU, Boehringer Mannheim) in a total dose of 300 mg/kg, or 50 mg/kg i.p. in rats and 200 mg/kg i.p. in mice was used to evaluate the neurogenesis following lead or ethanol 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. 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.

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. 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.

To study the effect of ethanol administration on neurogenesis in the early postnatal period immediately after ethanol or saline injections, both control and

ethanol treated animals were administered BrdU (50 mg/kg) and 24 h after that group of control (n=6) or ethanol (n=6) treated animals were killed and their brains were taken for immunohistochemical detection of the newly born cells or neuronal death. To determine the survival and differentiation pattern of the newly born cells additional, groups of animals were administered a high dose of ethanol (3 g/kg) and BrdU and their brains were processed for immunohistochemical detection of BrdU label or double immunohistochemistry for BrdU and neuronal or glial markers at one (n=4) and three weeks (n=6) following ethanol and BrdU administration.

To assess the effect of ethanol intoxication on the generation of new cells in adult brain BrdU, 200 mg/kg, i.p. was administered to a group of mice (n=6) a day before the termination of ethanol inhalation. The ethanol inhalation was continued for additional 24 hours and after that animals were killed and their brains were processed for BrdU immunohistochemistry.

To assess the effect of ethanol intoxication on the survival and differentiation of the proliferating cells in adult hippocampus a group of mice (n=4) was given BrdU (200 mg/kg, i.p.) before the start of ethanol inhalation. Immediately after that the animals were placed into the inhalation chamber and were exposed to ethanol vapour for 4 weeks as described above. At the end of inhalation mice were processed either for BrdU immunohistochemistry or for double immunohistochemistry of BrdU with neuronal or glial markers.

To assess the effect of ethanol withdrawal on the generation of the new cells and their survival, differentiation animals exposed to the ethanol vapour for four weeks, were withdrawn from ethanol. Six hours later ethanol-withdrawn mice were administered BrdU (200 mg/kg, i.p.) and 24 hours (n=6) or 3 weeks (n=4) later were processed for BrdU immunohistochemistry or double immunohistochemistry with BrdU and neuronal or glial markers, respectively. Matched control groups of mice were also exposed to BrdU and processed simultaneously with ethanol-intoxicated and ethanol-withdrawn mice.

In the OB study, two protocols were used. First, to assess the differentiation, half of the OB (n=6) and sham-operated (n=6) animals received 2 weeks following OB three i.p. injections of BrdU (total dose: 300 mg/kg) with a 2 hour interval and were killed 4 weeks thereafter. The other half of the animals were kept for an additional 4 weeks in their home cages and after that were taken for a proliferation study. To assess the rate of proliferation, animals received the same dose of BrdU and were killed 24 hours later.

To study the effects of chronic citalopram administration on proliferation, OB and sham-operated animals received three i.p. injections of BrdU (100 mg/kg; total dose: 300 mg/kg) with two hours interval on the last day of citalopram administration. 24 hours later animals from each group were sacrificed in order to detect proliferation of new cells. For survival/ differentiation studies BrdU (total dose 300 mg/kg, i.p.) was given on the first day of citalopram administration, and the animals were sacrificed 24 hours after the last dose of antidepressant following four weeks.

5.2. BrdU immunohistochemistry and quantification of BrdU-positive cells

For 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 μm 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_2O_2, followed by incubation in a 0.1M TRIS HCl buffer, containing 0.025% trypsin and 0.1% $CaCl_2$, for 10 min. Thereafter, the sections were incubated in 2N HCl in PBS at 37 ºC 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 ºC 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 visualised 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.

5.3. 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 analysed 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 β-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 μm 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 staked images taken at 0.5 μm 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.

5.4. 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 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% $CaCl_2$, for 10 min followed by incubation in acid-alcohol (HCl 1% in 70% ethanol) solution for 10 sec. 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 (Nv) was calculated according to the formula $Nv= \Sigma Q/\Sigma v$ (dis), where ΣQ is the number of cells counted and Σv (dis) is the volume of disectors. The volume of the dentate gyrus was estimated according to Cavaleri`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 (Nv) of the cells multiplied by the reference volume (West, 1993).

6. Detection of neuronal death

6.1. TUNEL-labelling

Following ethanol administration in the early postnatal period, 2 to 3 sections from each animal (n=4) killed 24 hours after ethanol or saline and BrdU administration were processed for the detection of the neuronal death. Dentate gyri from each section were dissected out under the stereomicroscope and incubated for 5 min in absolute methanol in a 96-multiwell plate. Thereafter, gyri were preincubated with a 50 µl equilibration buffer for 10 min at room temperature followed by overnight incubation in a 50 µl reaction buffer containing digoxigenin-dUTP and terminal deoxynucleotidyltransferase (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Intergen Co, USA) at 37 ºC. After a 15 min incubation with 200 µl of stop/wash buffer at room temperature, the sections were rinsed 3 times in PBS-Triton X-100 and incubated for 3 h with sheep anti-digoxigenin antibody. This was followed by extensive PBS-Triton X-100 rinses and incubation with donkey antisheep-Alexa-488 (1:100, Molecular Probes) for 3 h. For the detection of morphology of the apoptotic cells, gyri were additionally incubated in 5 µg/ml propidium iodide (Sigma) for 15 min before final rinsing and mounting. As a positive control, sections were treated with DNAse solution (0.5 µg/ml for 10 min.) before assay. To determine the identity of TUNEL-positive cells, sections were subjected to NeuN immunohistochemistry (mouse anti-NeuN monoclonal antibody (1:100, Chemicon International, UK) after overnight incubation with deoxynucleotidyltransferase as described above. Gyri were then incubated with sheep digoxigenin antibody for 3 h followed by donkey anti-sheep-Alexa-488 (1:100) to visualize DNA strand breaks and for 1 h with goat anti-mouse-Alexa-594 (1:100) to detect NeuN.

TUNEL-positive cells were counted on the 3D reconstructed stacked images obtained using laser confocal microscopy within a sampling volume 270 x 270 x 40 µm and values were expressed as a Nv. In experiments with combined TUNEL- and NeuN immunochemistry assays, a percentage, of TUNEL-positive cells, which expressed NeuN marker, was calculated.

Following OB, a commercial apoptosis detection kit (ApopTag®Peroxidase *In situ* Apoptosis Detection Kit; Chemicon International) was used according to the method described in detail elsewhere (Gorter *et al*., 2003). Apoptotic nuclei were visualised by the peroxidase-diaminobenzidine (DAB) reaction. Sections were then counterstained with cresyl-violet. Every 20th section (40 µm thick) in the entire extent of the DG was taken for quantification of TUNEL-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 TUNEL-positive cells in a given region, the sum of

cell counts from 5 sections was then multiplied by 20. Left and right dentate gyri were analysed separately and the estimates were averaged for each animal.

6.2. Fluoro-Jade staining

Fluoro-Jade staining was used for the detection of the occurrence of the 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 μm 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 analysed separately and the estimates were averaged for each animal.

7. Data analysis

Data were expressed as mean ± SEM and statistical comparisons were made using either one-way or two-way ANOVA followed by Bonferroni test or Student's t-test where appropriate.

RESULTS

1. The effects of developmental lead exposure on behaviour and hippocampal neurogenesis in the adult rat brain

1.1. 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 ± 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 (μg/dl)	29.3 ± 5.0**	34.2 ± 5.8**	10.4 ± 3.2	6.5 ± 1.2	4.2 ± 1.7
Brain (ng/g)	456.0 ± 23.0 **	781.0 ± 87. 0**	20.0 ± 8.0*	6.0 ± 1.0	6.0 ± 2.0

1.2. Behaviour 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/sec) at any time point during a 30-min-observation period (Figure 3).

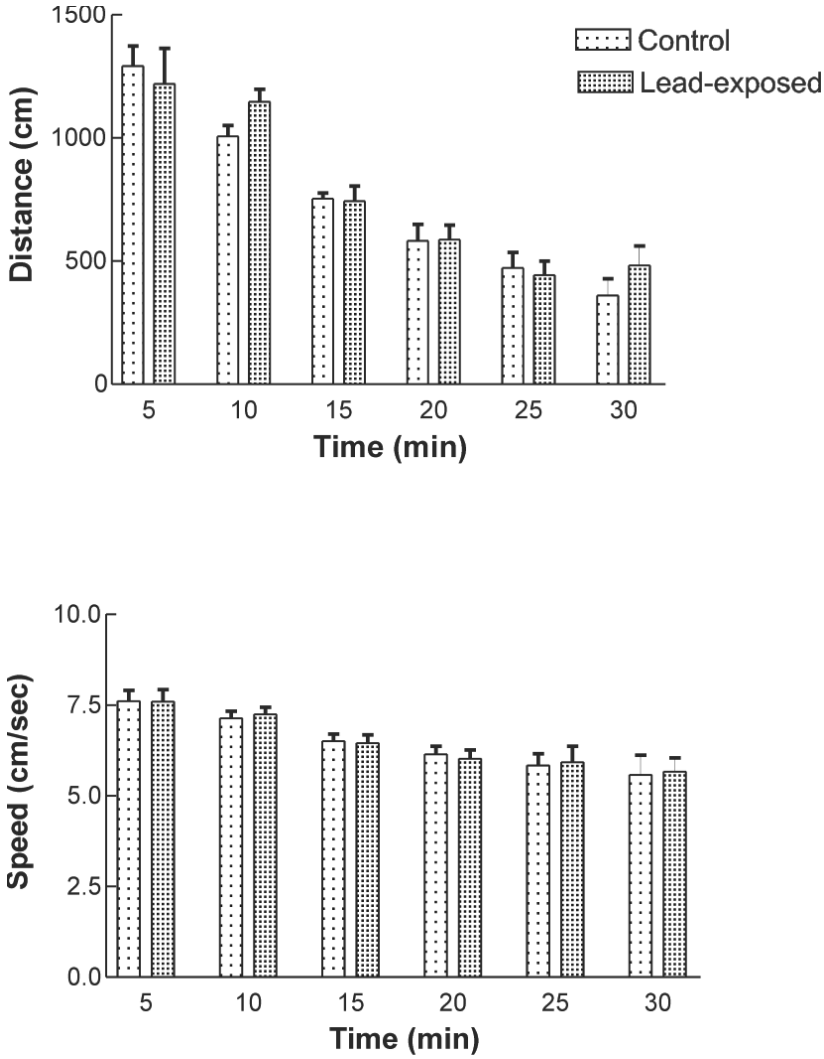

Figure 3. Locomotor activity of control and lead-exposed rats measured as distance (cm) travelled (upper panel) and mean speed (cm/sec, lower panel) during a 30-min-observation period. Each group consisted of 8 rats. (Student's t-test).

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 4). 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 4).

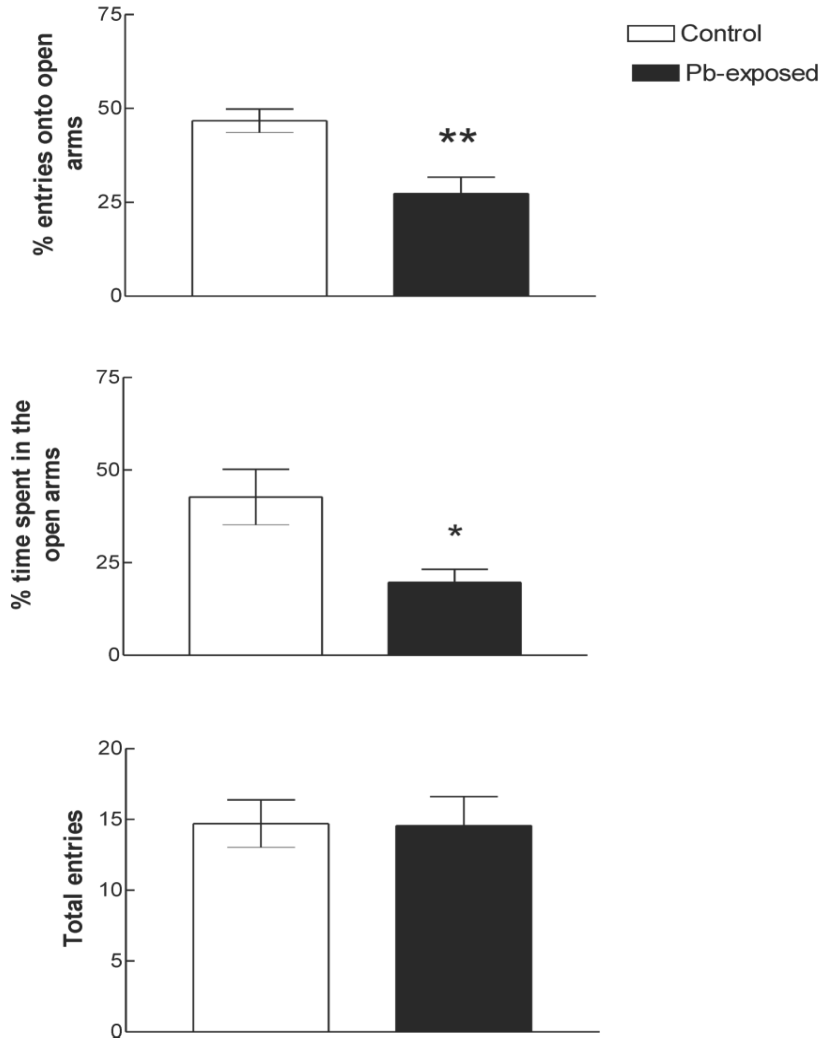

Figure 4. The effects of lead exposure on the rat behaviour in the plus-maze test. Upper panel: The % of entries onto open arms; middle panel: The % of time spent on the open arms; Lower panel: The total number of entries. Values are mean ± SEM from groups of 8 animals. *p<0.05, ** p<0.001 (Student's t-test).

In the contextual fear conditioning test the baseline freezing time, prior to conditioning in lead-exposed animals, was 35.7 ± 6.8 sec (n=9) and did not significantly differ from those found in control rats — 30.4 ± 4.6 sec (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 5).

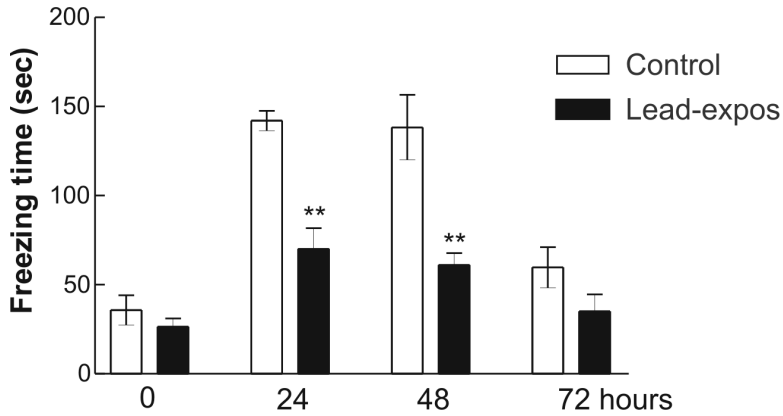

Figure 5. Contextual fear conditioning in control and lead-exposed rats. Freezing time in seconds measured during 5 min. of the context presentation prior and 24, 48 and 72 hours following training session. Values are mean ± SEM from groups of 8 animals. **p<0.01 (Bonferroni test).

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 6).

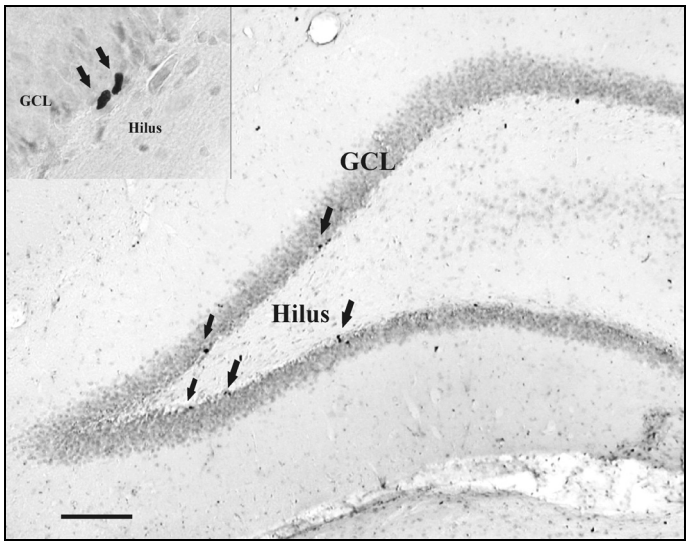

Figure 6. Representative microphotographs of BrdU-immunoreactive cells in the dentate gyrus of control rats. Arrows show BrdU-positive cells. Insets demonstrate cells taken from images acquired with x 60 objective. GCL – granule cell layer; Scale bar: 300 μm.

After 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 7). The reduced proliferative activity in the dentate gyrus has not been associated, however, with the changes of the volume of the dentate gyrus (Figure 7).

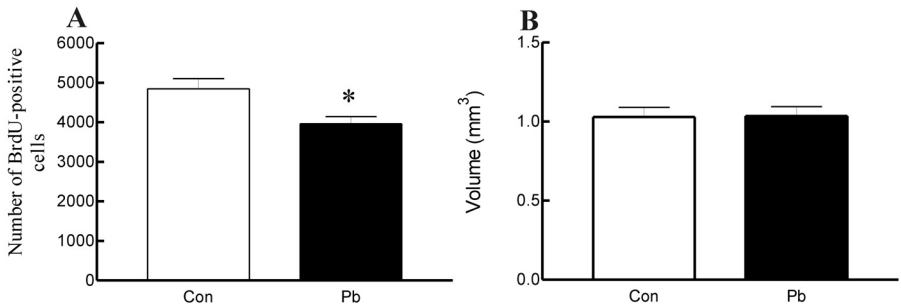

Figure 7. Production of new cells in the rat dentate gyrus following postnatal lead exposure. The number of BrdU-positive cells (A) and the volume of the dentate gyrus (B) in control and lead-exposed rats (n=6). *$p<0.05$ (Student's t-test).

To determine the 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).

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 8).

Table 3 shows that in control rats 40.6 ± 3.4% of survived BrdU positive cells expressed the marker of adult granule neurons calbindin, whereas in lead-exposed animals only 28.7 ± 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 ± 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 ± 133	11.3 ± 2.6	40.6 ± 3.4	11.9 ± 3.4
Lead-exposed	1562 ± 256*	30.1 ± 3.4**	28.7 ± 3.5*	35.4 ± 5.1*

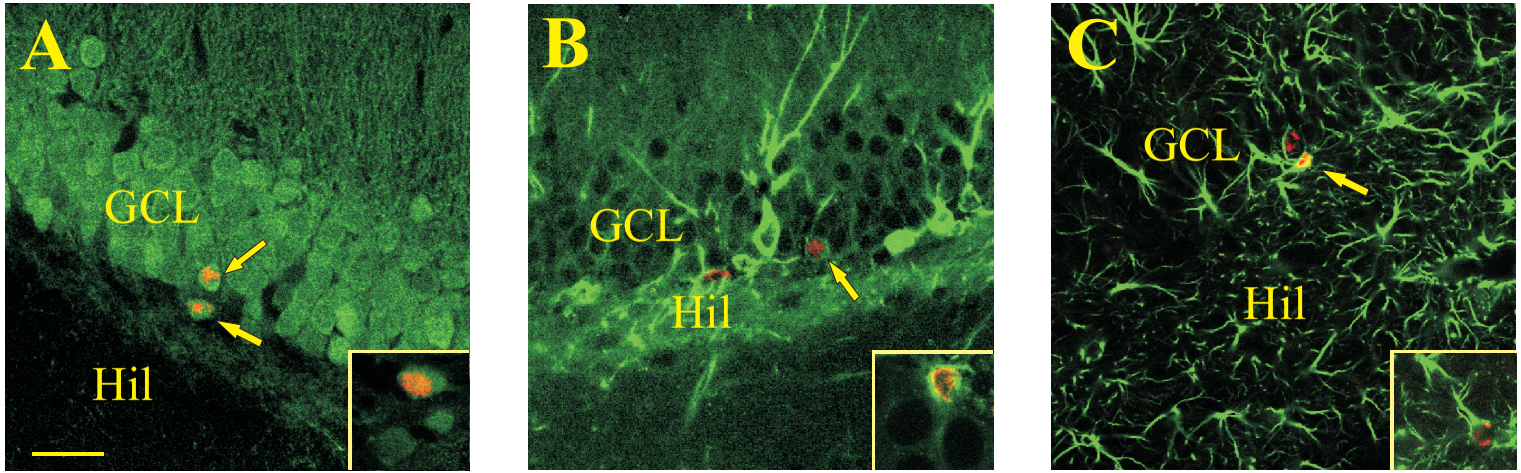

Figure 8. Confocal scanning laser images of sections double-labelled by BrdU (red) and (A) marker for mature granule neurons calbindin (green), (B) marker for post-mitotic young neurons PSA-NCAM (green) and (C) marker for astroglia GFAP (green). Arrows show co-localization of BrdU with neuronal or glial markers. Abbreviations: GCL – granule cell layer; Hil – hilus. Scale bar: 50 μm. Insets demonstrate cells with co-localization taken from images acquired with x 100 objective.

2. The effect of a single administration of ethanol on hippocampal neurodegeneration and neurogenesis in juvenile rats

2.1. Blood ethanol concentrations following a single administration of ethanol

Administration of ethanol resulted in a dose-dependent increase in the blood ethanol concentration with a peak one hour after administration (Figure 9). Some animals looked lethargic during the first two hours after highest dose (3 g/kg) of ethanol. All animals, however, survived after ethanol administration.

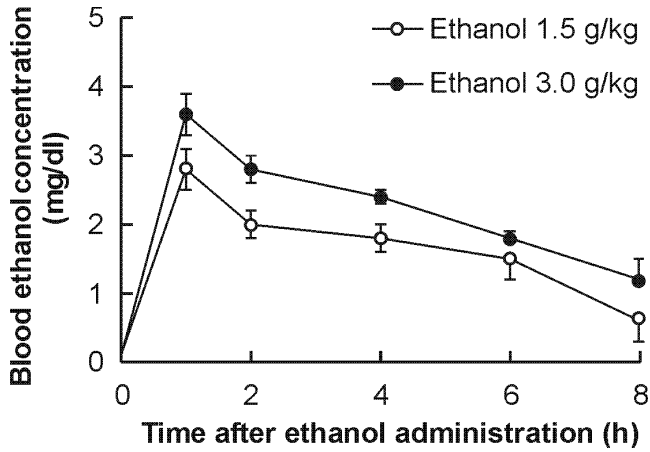

Figure 9. Blood ethanol concentrations at various times following ethanol administration to 10 days old rats. The data are mean ± SD (n=3).

2.2. Ethanol-induced neurodegeneration in the young rat hippocampus

24 hours after ethanol administration, a dose-dependent increase in the number of TUNEL-positive cells was observed (ANOVA: $F_{(2,21)}=34.4$, $p<0.0001$), with the maximum effect after higher dose of ethanol 3 g/kg (Figure 10).

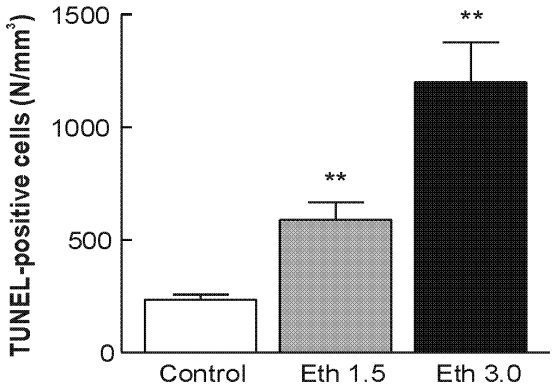

Figure 10. The effect of ethanol (1.5 and 3 g/kg) on the density (N/mm^3) of TUNEL-positive cells. TUNEL-positive cells were counted on the stacked images in sampling volume 270 x 270 x 50 µm. The densities obtained from 5–6 images for each dentate gyrus and mean ± SEM for group of animals was calculated (n=4),** $p<0.05$ (Bonferroni test).

2.3. Neurogenesis in the dentate gyrus of juvenile rat hippocampus following a single administration of ethanol

Twenty four hours after BrdU administration intense peroxidase-BrdU labelling of the proliferating cells was observed in the dentate gyrus of the 10 days old rats. After ethanol (1.5 g/kg) administration no changes in the number of BrdU-positive cells were observed in either GCL or hilus compared to control animals. In contrast, a higher dose of ethanol (3 g/kg) increased the number of BrdU-positive cells (Figure 11). This effect was seen only in GCL but not in hilus where at the 24 hours time point the number of BrdU-positive cells did not differ from control.

We further examined whether the survival of the BrdU-positive cells might be also affected by a high dose (3 g/kg) of ethanol. Survival of the dividing progenitor cells was addressed by staining of BrdU labelled cells following 1 and 3 weeks after ethanol (3 g/kg) or saline administration. Experiments demonstrated a time-dependent decline of the number of BrdU-positive cells in both GCL and hilus at one and three weeks time points in both control and ethanol (3 g/kg)-treated animals. Nevertheless, the number of the newly born cells in the GCL of ethanol-treated animals remained significantly higher than in the corresponding controls at these time points (Figure 11). In the GCL of control animals, 3 weeks after BrdU administration only 35% of the newborn cells survived whereas in the ethanol group the survival was 51%. A higher number of BrdU-positive cells 3 weeks after ethanol administration was also found in hilus (Figure 11). It should be noted that the BrdU label became

progressively fragmented with the time. This indicates dilution of the label due to the mitotic activity of the proliferating cells.

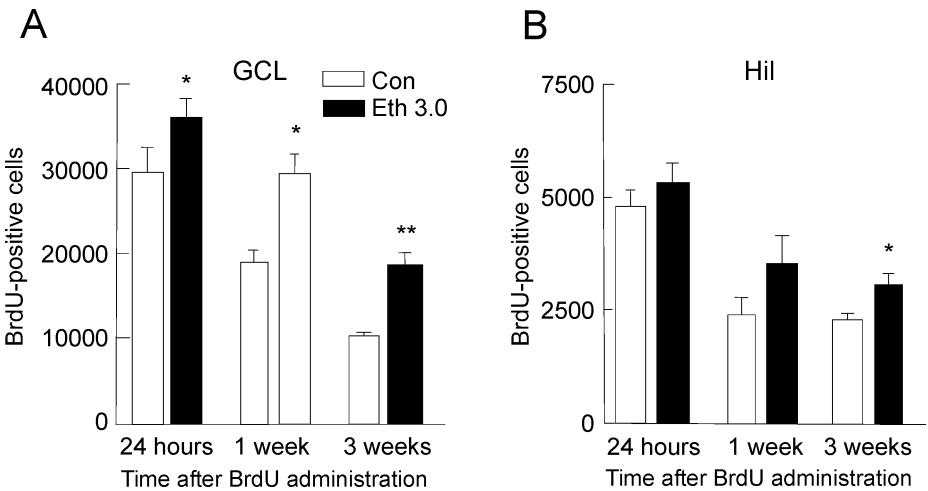

Figure 11. The effect of ethanol on the proliferation and survival of the new cells in the dentate gyrus of 10 days old rats. GCL – granule cells layer; Hil – hilus. The data are mean ± SEM. (n=6); *P<0.05 (Bonferroni test).

In order to explore whether ethanol (3 g/kg)-induced neuronal death and enhanced generation of new cells might have an impact on the neuronal populations in the different regions of the hippocampus we counted cells in the GCL and hilus three weeks after ethanol (3 g/kg) administration. For comparison the number of cells in the CA3 region of hippocampal formation was estimated at the same time point. Three weeks after ethanol administration there was an increase in the number of cells in GCL and a decrease in CA3 region, compared with control animals. No changes were observed in hilus (Table 4).

Table 4. Total number of cells in the granular cell layer (GCL), hilus (Hil) and CA3 region of the juvenile rat hippocampus 3 weeks following a single administration of ethanol (3 g/kg) to 10-day-old rats. The data are mean ± SEM (n=6), *p<0.05 (Student's t-test).

Group	GCL	Hil	CA3
Control	612 800 ± 22 040	46 400 ± 1881	442 900 ± 17 740
Ethanol 3 g/kg	762 700 ± 23 630*	50 030 ± 4030	386 600 ± 13 930*

Examination of the phenotypes into which BrdU-positive cells had differentiated also revealed differences between control and ethanol-treated animals. In control animals, one week after BrdU administration, 57.7% of newly born cells in the dentate gyrus co-expressed a marker for young neurons PSA-NCAM (Table 5). After three weeks a percentage of BrdU-positive cells co-expressing PSA-NCAM was reduced to 27.9%. Administration of ethanol resulted in a significantly higher proportion of BrdU-positive cells co-expressing PSA-NCAM, 77.5% and 54.3% at one and three weeks time points, respectively. Recent data have demonstrated that PSA-NCAM might be expressed not only in the neurons but also in astroglial cells and, therefore, enhanced PSA-NCAM expression might be attributed to both neuronal and glial proliferation. We, therefore, performed additional experiments using a more specific marker for immature postmitotic neurons Tuj1 (Hämmerle and Tejedor, 2002) and found a similar increase in the proportion of BrdU and Tuj1 co-expressing neurons 1 week after ethanol administration (Table 5).

One week after administration of saline or ethanol only a few BrdU-positive cells did express the marker for mature neurons calbindin. Most of the BrdU-positive cells co-expressing calbindin appeared after three weeks following BrdU administration (Table 5) and were found mostly in GCL. In control animals, at that time point one third of BrdU-positive cells co-expressed calbindin, whereas in the ethanol-treated group the percentage of BrdU-positive cells co-expressing calbindin was lower ($p<0.05$). Using the data obtained in stereological studies we re-calculated the percentages of BrdU/calbindin co-expressing cells into absolute numbers and found that the number of BrdU cells co-expressing calbindin in the ethanol group (3940 ± 215 cells/GCL) did not differ significantly from those obtained in control animals (3910 ± 339 cells/GCL, $p>0.05$; $n=6$).

Parallel to the increase in the proportion of immature neurons an enhancement of astroglial proliferation after ethanol treatment was also observed (Table 5). In control animals, the proportions of BrdU-positive cells, which have been differentiated into astrocytes were 1.3 ± 1.3% and 21.8 ± 6.4% at 1 and 3 weeks, respectively. In ethanol-treated animals the percentages of BrdU-positive cells co-expressing the astroglial marker GFAP were 10.3 ± 2.3% (1 week time point; $p<0.01$) and 39.7 ± 4.5% (3 weeks time point; $p<0.05$). Almost all BrdU-positive cells with the astroglial marker GFAP were found in the hilar region.

Table 5. Phenotypes of the BrdU-positive cells in the DG of juvenile rats 1 and 3 weeks following ethanol (3 g/kg) and BrdU (50 mg/kg) administration. The data are given as percentages of BrdU-positive cells co-expressing the markers for young immature neurons, PSA-NCAM or Tuj1, marker for mature granule cells, calbindin, and marker for astroglia, GFAP. The data are mean ± SEM obtained from group of 4 (one week) or 6 rats (3 weeks). The total number of cells analyzed varied between 25–50 per animal and 100–300 per group. *p<0.05; **p<0.01 (Student's t-test).

Group	1 week			
	% co-localization with PSA-NCAM	% co-localization with Tuj1	% co-localization with calbindin	% co-localization with GFAP
Control	57.7 ± 5.5	23.9 ± 4.7	1.0 ± 0.4	1.3 ± 1.3
Ethanol	77.5 ± 4.9*	45.1 ± 5.0**	1.8 ± 1.4	10.3 ± 2.3**

Group	3 weeks		
	% co-localization with PSA-NCAM	% co-localization with calbindin	% co-localization with GFAP
Control	27.9 ± 5.2	38.3 ± 2.1	21.8 ± 6.4
Ethanol	54.3 ± 6.3**	21.2 ± 1.3*	39.7 ± 4.5*

3. The effects of ethanol intoxication and withdrawal on hippocampal neurogenesis in the adult mouse brain

3.1. The effect of chronic ethanol administration on blood ethanol concentration and evaluation of the withdrawal

Exposure of mice to ethanol in the inhalation chamber resulted in the death of 3 animals at the time when air alcohol concentration was switched to the maximum (60 mg/ml). 24 animals survived ethanol exposure. After removal from the inhalation chamber mice looked slightly lethargic. The mean blood alcohol concentration at the end of the inhalation period was 353 ± 40.5 mg/dl (n=4). The estimated concentration of ethanol produces strong intoxication and lethargy in naive mice. Thus, it seemed that mice became tolerant to ethanol at the end of inhalation. Delivery of ethanol via the inhalation route often results in the caloric and nutrient restriction that might affect experimental data. To minimise this problem, the weights of mice in the control and experimental groups were monitored during the whole experiment and if a trend toward a decrease of weight of the experimental animals was observed then the concentration of ethanol was temporarily decreased. At the beginning of the experiments, the weights of control and ethanol exposed mice were 29.2 ± 0.5 g and 28.4 ± 0.8 g, respectively. At the end of ethanol inhalation, the weight of mice was 30.5 ± 0.7 g and did not differ significantly from the weight of control

animals — 32.4 ± 0.6 g (n=24). Withdrawal from ethanol resulted in the appearance of the signs of abstinence. The first signs of abstinence such as occasional myoclonic jerks and tremor appeared at 4 hours and reached a maximum at 6–8 hours and completely disappeared at 24 hours following the termination of ethanol inhalation. No spontaneous or handling-induced convulsions were observed in ethanol-withdrawn mice.

3.2. The effect of ethanol intoxication and withdrawal on hippocampal neurogenesis

To determine the effect of ethanol intoxication on the generation of new cells, a group of mice received BrdU 24 hours before the termination of ethanol inhalation and BrdU labelling was performed at the end of ethanol inhalation. Quantification of BrdU-positive cells revealed a significant decrease in the number of proliferating cells 24 hours following BrdU administration (Figure 12 A).

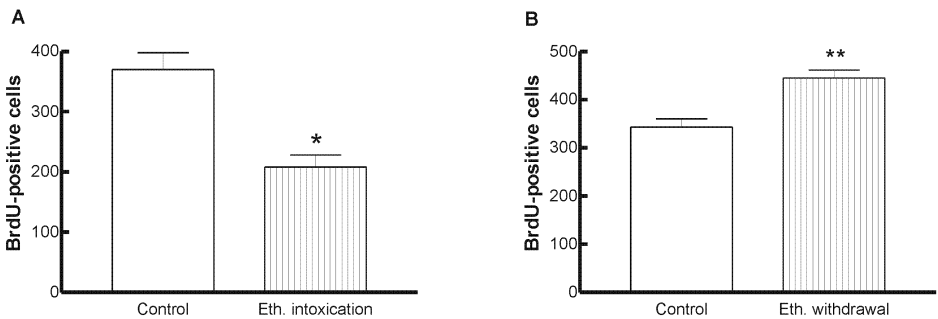

Figure 12. The number of BrdU-positive cells determined 24 hours after BrdU (200 mg/kg, i.p.) administration in (A) ethanol-intoxicated and (B) ethanol-withdrawn mice. The data are mean ± SEM (n=6), *p<0.05; **p<0.01 (Student's t-test).

When BrdU was administered at the beginning of ethanol exposure and BrdU-positive cells were determined at the end of ethanol inhalation e.g. 4 weeks later, the estimated number of BrdU-positive cells in the ethanol group did not differ from those in control animals (Table 6). It should be noted, however, that in both groups much lower amount of BrdU-positive cells were found. These data show that the newly born cells that have been labelled with BrdU at the beginning of ethanol administration were not affected by the chronic ethanol exposure. Ethanol changed the pattern of differentiation of the survived cells (Table 6). In control mice 49.6 ± 17.7% of BrdU labelled cells had differentiated into mature calbindin-positive neurons, while only 9.4 ± 2.4%

($p<0.01$) of BrdU-positive cells co-expressed calbindin in the ethanol group. No significant changes were observed in the proportion of BrdU-positive cells co-expressing the markers for immature neurons Tuj1 or the astroglial marker GFAP (Table 6).

Table 6. The effect of ethanol inhalation on the survival and differentiation of newly born cells in the adult mouse dentate gyrus. Mice were given proliferative marker BrdU (200 mg/kg i.p.) and after that were exposed to ethanol vapour for 4 weeks. Immunohistochemical detection of BrdU-positive cells was performed at the end of ethanol inhalation. The number of cells examined for co-localization of BrdU and neuronal or glial markers was 25–30 per dentate gyrus. The data are mean ± SEM (n=4). **$p<0.01$ (Student's t-test).

Group	Number of BrdU-positive cells	% co-localization with Tuj1	% co-localization with calbindin	% co-localization with GFAP
Control	186 ± 22	11.8 ± 4.5	49.6 ± 1.7	5.2 ± 2.7
Ethanol	148 ± 9	16.3 ± 9.4	9.4 ± 4.5**	11.4 ± 3.8

To assess the effect of ethanol withdrawal on the proliferative activity of the dentate gyrus, BrdU was administered to mice 6 hours after ethanol withdrawal and BrdU-positive cells were determined 24 hours later. Experiments revealed a significantly higher number of BrdU-positive cells in the dentate gyrus of ethanol-withdrawn mice compared with the control group (Figure 12). Three weeks later the numbers of BrdU-positive cells had decreased in both groups, whereas no differences were observed in the number of surviving cells between control and ethanol-withdrawn mice at this time point (Table 7). The data indicate that newly born cells that have been generated in excess during the withdrawal were eliminated at the later stages. Examination of the phenotypes (Table 7) into which BrdU-positive cells had differentiated revealed retardation of maturation in ethanol-withdrawn mice. In control animals, 53.9±4.3% of BrdU-positive cells were co-localized with the marker for mature neurons – calbindin, whereas a significantly smaller proportion of mature neurons (34.3 ± 7.1%, $p<0.05$) co-expressing BrdU and calbindin was found in the dentate gyrus of ethanol-withdrawn animals. No differences, were observed in the proportion of BrdU-positive cells that differentiated into astroglia.

Table 7. The effect of ethanol withdrawal on the survival and differentiation of the newly born cells in the adult mouse dentate gyrus. Mice were subjected to ethanol vapour for 4 weeks and after that were withdrawn from ethanol. 6 hours following the withdrawal animals were given proliferative marker BrdU (200 mg/kg, i.p.) and BrdU-positive cells were determined 3 weeks (effect on survival) later. The pattern of differentiation was determined 3 weeks later. The number of cells examined for co-localization of BrdU and neuronal or glial markers was 25–30 per dentate gyrus. The data are mean ± SEM (n=6). *p<0.05 (Student's t-test).

Group	Number of BrdU-positive cells	% co-localization with Tuj1	% co-localization with calbindin	% co-localization with GFAP
Control	213 ± 12	7.5 ± 3.7	53.9 ± 4.3	5.2 ± 2.7
Ethanol	228 ± 16	12.9 ± 4.6	34.3 ± 7.1*	9.4 ± 1.8

4. Behavioural alterations and changes in neurogenesis following OB in adult rats

4.1. OB-induced behavioural syndrome

In the open-field test, OB animals demonstrated a typical increase in activity. This effect was time-dependent being more pronounced 6 weeks following OB (Figure 13A). One-way ANOVA indicated significant differences in the number of passed squares among groups ($F_{2.28}$=19.2, p<0.001). Post-hoc analysis revealed an increase in the number of passed squares after 2 weeks (p<0.05) and this effect was more pronounced (p<0.001) 6 weeks following OB. Similar changes were observed in the vertical activity (number of rearings) 6 weeks after OB (p<0.001) (Figure 13B).

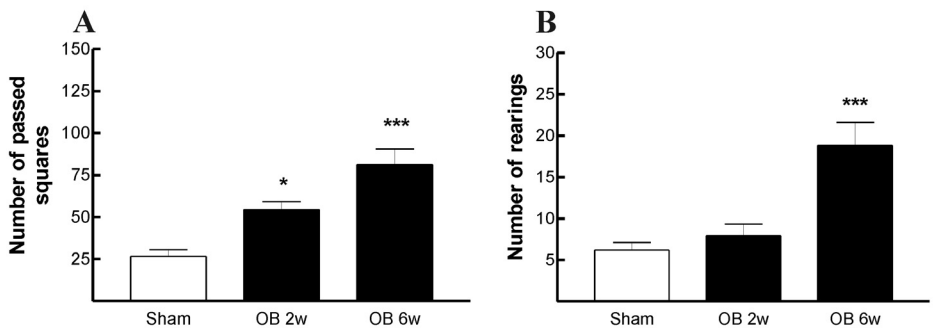

Figure 13. Measures of activity in the open-field test in OB (n=12) and sham-operated rats (n=12). (A): number of passed squares and (B): number of rearings during a 5-min observation period 2 weeks (OB 2w) and 6 weeks (OB 6w) following OB. * p<0.05, *** p<0.001. (Bonferroni multiple comparison test).

OB rats demonstrated an impairment in the passive avoidance behaviour. In the passive avoidance test OB animals demonstrated shorter latency time to enter the dark compartment than sham-operated animals (Figure 14). No differences in the baseline latency were found between sham-operated and OB animals (Figure 14).

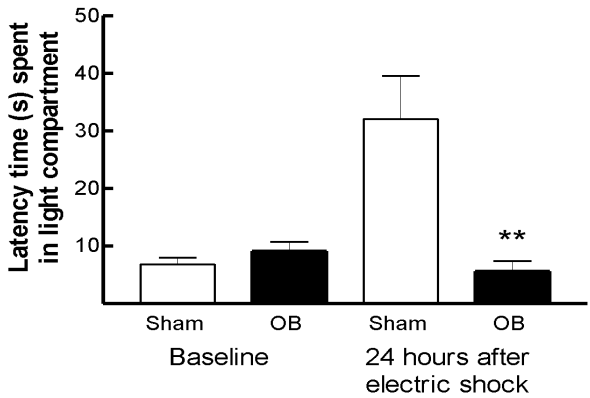

Figure 14. The effect of OB on long-term memory in the passive avoidance test 6 weeks following surgery in sham-operated (n=6) and in OB animals (n=6). Values represent mean ± SEM of the latency time to enter into the dark side of the passive avoidance apparatus before (baseline) and 24 hours after exposure to an electric shock. ** $p<0.01$ (Student's t-test).

The contextual conditioning test revealed that 24 hours following the context and foot-shock presentation sham-operated animals showed a robust freezing response in the training context, indicating a long-term recognition memory of the test chamber. OB animals demonstrated a shorter freezing time when they had been exposed to the same context 24 hours following training (Figure 15A). There were no differences in the baseline freezing on the training day, indicating that OB animals do not have an increased reactivity to the novel context.

In the tone-dependent fear memory test, 24 hours following training (tone and foot-shock) the animals were placed in the novel context and exposed to tone. A trend toward an increase in the freezing time was observed in OB rats compared to sham-operated animals (Figure 15B). No differences in the baseline freezing time were found.

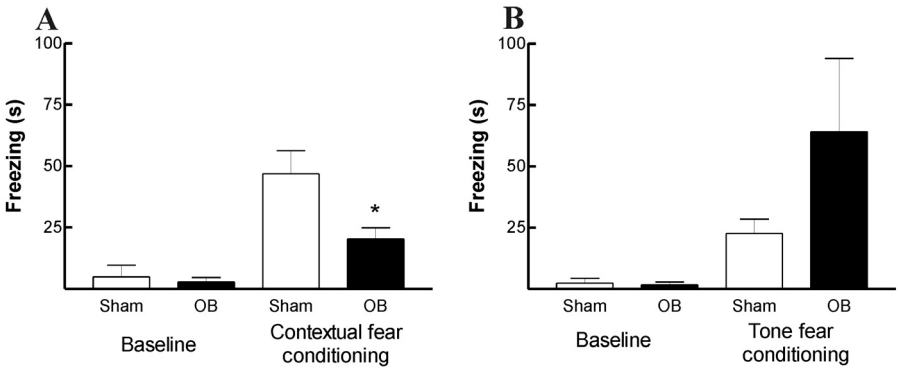

Figure 15. (A) Contextual- and (B) tone (cued) fear conditioning in sham-operated (n=8) and in OB-animals (n=8) 6 weeks following surgery. Values represent mean ± SEM of the freezing time (s) in the apparatus. *p<0.05 (Student's t-test).

4.2. Neurogenesis in OB rats

Estimation of BrdU-positive cells revealed that OB induced a significant decrease in the number of proliferating cells in the dentate gyrus (Figure 16). This was accompanied by a reduction in the total number of cells in the GCL of the DG and a tendency toward reduced volume of the dentate gyrus (Table 8).

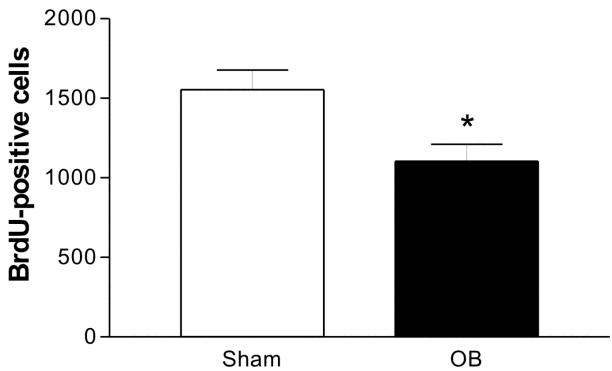

Figure 16. The number of BrdU-positive cells 6 weeks following surgery and 24 hours following BrdU (total dose 300 mg/kg) administration in sham-operated and OB animals DG. The data are mean ± SEM, (n=6). *p<0.05 (Student's t-test).

Table 8. The effect of OB on the volume of the dentate gyrus and on the total number of granule cells in the granule cell layer (GCL) 6 weeks following surgery. The data are mean ± SEM (n=5). *p<0.05 (Student's t-test).

Group	Volume of the dentate gyrus (mm^3)	Total number of granule cells in the GCL of dentate gyrus
Sham	1.16 ± 0.025	693 200 ± 72 640
OB	1.09 ± 0.023	514 900 ± 21 740*

To determine the effect of OB on the differentiation of the proliferating cells, rats were given BrdU and 4 weeks later the co-localization studies of BrdU with either neuronal or glial markers were performed. The co-localization experiments revealed that the pattern of differentiation of the survived cells was changed in the OB rats (Table 9). In sham-operated rats, 37.6 ± 2.1% of BrdU labelled cells differentiated into mature calbindin-positive granule neurons, while in OB animals only 16.7 ± 1.7% (p<0.001) of BrdU-positive cells co-expressed calbindin. No significant changes were observed in the proportion of BrdU-positive cells co-expressing the marker for young neurons PSA-NCAM (Table 9). The proportion of BrdU-positive cells co-expressing GFAP tended to decrease in OB rats (Table 9), the data, however, did not reach the level of significance.

Table 9. The effect of OB on the differentiation of newly born cells in the dentate gyrus of sham-operated and OB rats. The number of cells examined for co-localization of BrdU with neuronal or glial markers was 70–100 per animal. The data are expressed as a mean ± SEM percentage of BrdU-positive cells, which express neuronal or glial markers. *** p<0.001 (Student's t-test).

Group	% co-localization with PSA-NCAM	% co-localization with calbindin	% co-localization with GFAP
Sham	10.2 ± 1.5	37.6 ± 2.1	13.3 ± 3.1
OB	12.4 ± 2.2	16.7 ± 1.7 ***	7.4 ± 1.5

4.3. Cell death following OB

Only a few cells with the positive TUNEL staining were seen in the sections of the dentate gyri of sham-operated animals. Quantification of TUNEL-positive cells did not reveal any significant increase in the number of dying neurons in the dentate gyrus of OB rats 6 weeks following OB (Figure. 17).

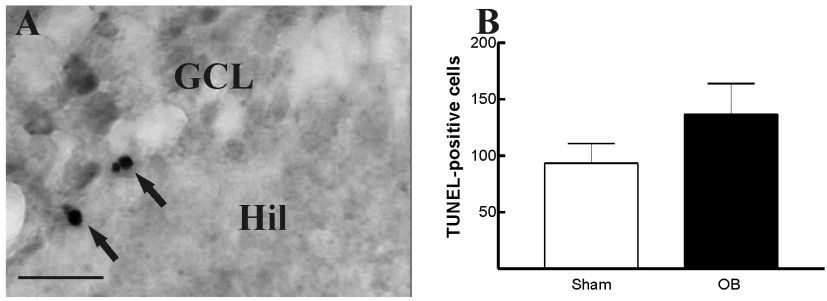

Figure 17. Representative microphotograph (A) of the TUNEL-positive apoptotic cells (arrows) in the granule cell layer of the dentate gyrus. B: Number of TUNEL-positive cells in the dentate gyrus of sham-operated and OB animals 6 weeks following OB. Scale bar: 15 μm.

5. The effect of chronic citalopram administration on hippocampal neurogenesis following OB

Estimation of BrdU-positive cells (two-way ANOVA) revealed a significant main effect for OB ($F_{3,27}$=14.9, p=0.001) and drug treatment ($F_{3,27}$=23.065, p=0.0001) on the proliferation of progenitor cells (Figure 18A) after 28-day citalopram administration in sham-operated and OB animals.

The production of progenitor cells was decreased by 25% in OB-animals as compared to sham-operated animals (p<0.01), whereas the citalopram treatment counteracted this effect of OB and restored the number of BrdU-positive cells to the control level (Figure 18A). In sham-operated animals, repeated citalopram treatment failed to affect proliferation of the progenitors and only a trend toward an increase of the newly born cells was observed (Figure 18A).

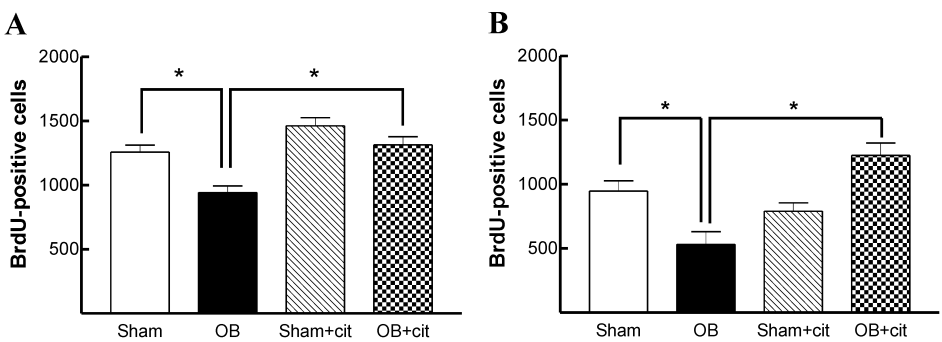

Figure 18. Effects of citalopram on the (A) proliferation and (B) survival of BrdU-immunoreactive cells in the rat dentate gyrus following OB. *p<0.05 (Bonferroni multiple comparisons test).

To determine the effect of repeated citalopram administration on the survival of the proliferating cells, BrdU was simultaneously given the groups of OB and sham-operated animals with the first injection of citalopram and the number of BrdU-positive cells was determined at the end of a 28-day treatment with citalopram. Estimation of the number of BrdU-positive cells in sham-operated and OB animals revealed that chronic citalopram treatment increased the survival of BrdU-positive cells in OB rats without any effect on the cell survival in sham operated rats (Figure 18 B).

Next, we studied the effects of citalopram on the differentiation pattern of the survived cells in sham-operated and OB animals. Two-way ANOVA analysis showed that both OB $F_{(3,11)}=5.73$ ($p=0.02$) and citalopram treatment $F_{(3,11)}=8.28$ ($p=0.02$) had an effect on the differentiation of BrdU-positive cells into mature calbindin-positive neurons. In sham-operated rats 39.7 ± 2.8% of BrdU labelled cells had differentiated into calbindin-positive granule neurons, whereas in OB animals only 19.0 ± 3.3% of BrdU-positive cells co-expressed calbindin (Table 10). Repeated citalopram administration in OB rats restored the percentage of BrdU-positive cells, which had been differentiated into mature neurons to the control levels without a significant effect on sham-operated animals. No changes were observed in the proportion of BrdU-positive cells co-expressing astroglial marker GFAP or marker for young neurons PSA-NCAM (Table 10).

Table 10. The effect of chronic citalopram treatment on the differentiation of newly born cells in the dentate gyrus of sham-operated and OB rats. The number of cells examined for co-localization of BrdU with neuronal or glial markers was 70–100 per animal. The data are mean ± SEM. (Two-way ANOVA). *$p<0.05$ as compared with sham-operated group; #$p<0.05$ as compared with the OB group.

Group	% co-localization with PSA-NCAM	% co-localization with calbindin	% co-localization with GFAP
Sham (n=5)	12.0 ± 13.4	39.7 ± 2.8	12.8 ± 4.2
OB (n=5)	19.0 ± 1.4	19.0 ± 3.3 *	15.5 ± 3.9
Sham+cit (n=5)	11.7 ± 1.5	40.8 ± 5.8	16.5 ± 1.7
OB+cit (n=5)	12.7 ± 4.6	41.7 ± 4.0 #	12.2 ± 2.4

DISCUSSION

1. The effects of low-level postnatal lead exposure on behaviour and hippocampal neurogenesis in adulthood

Our experiments demonstrated that postnatal lead exposure induced increased anxiety in the plus-maze test and disrupted contextual fear conditioning. The observed behavioural 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 induced long-lasting or even permanent alterations in brain functions. The observed behavioural syndrome in rats is very similar to those described by others (Salinas and Huff, 2002) and is in agreement with the observations on humans where children exposed to low levels of lead display attention deficit, increased emotional reactivity and impaired memory and learning (Fikelstein, 1998).

The observed behavioural alterations 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 *et al.,* 2005). Our study confirms very recent data, which demonstrated the inhibitory action of developmental lead exposure on hippocampal neurogenesis in vivo (Gilbert *et al*., 2005). In contrast, 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 labelled 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 a lower proportion of BrdU-positive cells differentiated into adult calbindin-positive neurons, whereas the proportion of young post-mitotic neurons expressing Tuj1 was significantly higher in lead-exposed rats than in control animals. Based on these data we propose that lead exposure retards the transition of young postmitotic neurons into adult neurons. 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 the 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 a generation of new cells in the dentate gyrus might have an importance for the hippocampal functions as the 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, a BDNF, 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 the serotoninergic neurotransmission (Widmer *et al*., 1991) and induce a 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.

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 behavioural impairment and reduced neurogenesis. Lesion studies show that the 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 the 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*., 1999a). A recent study (Schneider *et al*., 2001) showed that an enriched environment was also neuroprotective against lead-induced hippocampal memory deficits. On the other hand, previous studies, when inhibition of neurogenesis was induced by the administration of the 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 yet been established and further experiments are needed.

2. The effects of early postnatal ethanol administration on hippocampal neurodegeneration and neurogenesis

Our results demonstrated that a single administration of ethanol given during the early postnatal period (PND 10) was able to induce widespread neurodegeneration in the DG of the rat hippocampus 24 hours following administration. The neurotoxic effect of ethanol was dose-dependent and observed after both doses 1.5 and 3 g/kg of ethanol. Thus, it seems that the dentate gyrus does not differ in its sensitivity to the neurotoxic effects of ethanol from other structures in the juvenile brain, where widespread neuronal death was observed and deletion of neurons depended on the time and duration of treatment (Miller, 1995b; Napper and West, 1995; Miller, 1996; Ikonomidou *et al*., 2000).

There are conflicting data regarding the sensitivity of various neuronal populations to ethanol. Our results demonstrated that a majority (75%) of the TUNEL-positive cells co-localized with the neuronal postmitotic marker NeuN, indicating that ethanol largely affects postmitotic neurons. These data are in agreement with former reports (Marcussen *et al*., 1994; Ikonomidou *et al*., 2000) that demonstrated higher sensitivity of postmitotic, migratory or differentiating neurons to ethanol. We cannot exclude that some undifferentiated proliferating cells were killed by ethanol, since the phenotypes of the considerable proportion of TUNEL-positive cells (25%) were not identified. To detect neurodegeneration, we used in situ DNA nick-end labelling, TUNEL assay. TUNEL assay has been widely used for the detection of apoptotic death in brain tissues (Mahalik *et al*., 1997; Ikonomidou *et al*., 2000). Recent data demonstrated that TUNEL-positive labelling cannot be exclusively considered as a hallmark of apoptosis and might detect necrotic death as well (Van Lookeren-Campagne and Gill, 1996; Fujukawa *et al*., 2000). Therefore, we considered TUNEL-positive cells as dead cells without indication of the death mode.

Neuronal death after a high dose (3 g/kg) of ethanol was accompanied by an increase in the generation of new cells in the dentate gyrus. Although a lower dose (1.5 g/kg) of ethanol induced neuronal death, it did not affect the number of BrdU-positive cells 24 hours after BrdU administration. It remains to be determined whether enhanced cellular proliferation represents a reaction of the brain to the ethanol-induced damage or ethanol in high doses directly stimulates the proliferative activity in certain brain regions. The fact that a lower dose of ethanol was unable to enhance the generation of new cells in the dentate gyrus despite observed neurotoxicity in this region might indicate that ethanol itself exert direct stimulatory action on proliferation of the new cells.

Not only enhanced proliferation but also increased survival of the newly born cells at one and three weeks following ethanol (3 g/kg) administration was observed. This is reflected by the larger number of BrdU-positive cells found in the dentate gyrus of ethanol-treated rats as compared with corresponding controls at these time points. There is a possibility that ethanol administration enhanced the permeability of blood-brain barrier for BrdU and observed increase in the BrdU label was due to its higher accumulation in the brain of ethanol treated rats (Cameron and McKay, 2001). In our preliminary experiments when higher doses 100–200 mg/kg of BrdU were used in 10 days old rats (unpublished data) no further increase in the number of BrdU-positive cells was found as compared with the dose 50 mg/kg used here. It seems that in 10 days old rats even a relatively low dose of BrdU might saturate all dividing cells. It should be also noted that in the cells undergoing active proliferation, a rapid dilution of BrdU label might occur that would lead to the under-estimation of the newly generated cells. In order to ensure that the observed increase in the number of BrdU-positive cells indeed reflects an increased generation of new cells and is not related to the retardation of the mitotic activity we counted the cells in the selected regions of the rat hippocampal formation. Cell counting revealed an increase in the number of cells in the GCL of the ethanol-treated rats, which is in agreement with the data reported previously (West *et al*., 1986; Miller, 1995a). These data allowed us to propose that an increased cell number in GCL after ethanol administration is due to the enhanced production of new cells and suggest that this enhancement is able to compensate for the neuronal loss and even led to the increase in total cell number in this region. By contrast, the CA3 region, which is highly vulnerable to the toxic effects of ethanol (Barnes and Walker, 1981) and where no considerable postnatal neurogenesis has been observed, demonstrated a reduction in the total cell number that was evident even at three weeks time point after ethanol administration.

A single ethanol administration also affected the fate of the newly generated cells. Double immunohistochemical labelling for BrdU and the marker for young postmitotic neurons PSA-NCAM after one and three weeks following ethanol and BrdU administration revealed an increased proportion of BrdU-positive cells co-expressing this marker. Recent studies provide abundant evidence that PSA-NCAM plays an important role in the processes related to the neuronal migration, differentiation, axonal growth and synaptogenesis (Kiss *et al*., 2001). Furthermore, up-regulation of PSA-NCAM appears functionally important for the regeneration and recovery of the neuronal tissue following various lesions (Kiss *et al*., 2001). Not only neuronal cells, however, are able to express PSA-NCAM. Studies have demonstrated that in the neonatal brain PSA-NCAM might be expressed in the glial cells as well (Minana *et al*., 2000). To ensure that ethanol administration led to an increase in the proportion of young postmitotic neurons we performed additional experiments with another marker for young postmitotic neurons Tuj1 (Hämmerle and Tejedor, 2002) and

found a similar increase in the proportion of BrdU-positive neurons co-expressing Tuj1 at one week following ethanol administration.

Despite an increased proportion of young postmitotic neurons in the dentate gyrus, the proportion of fully differentiated neurons expressing calbindin was even decreased after ethanol administration. Using data obtained in the stereological estimation of the total number of BrdU-positive cells we re-calculated the proportions of BrdU-positive granule neurons expressing calbindin into absolute numbers and found no significant differences between ethanol-treated and control mice. Taken together, these data show that a single ethanol administration does not increase the number of mature calbindin-expressing cells but rather increases the population of immature neurons within the dentate gyrus.

A functional significance of the ethanol-induced increase in the number of the postmitotic immature neurons generated from the pool of proliferating precursors is not clear. Studies on the models of kainate- or pilocarpine-induced seizures or after ischemic damage in rats demonstrated an increased neurogenesis in the dentate gyrus (Van Lookeren-Campagne and Gill, 1996; Gray and Sundstrom, 1998; Takagi *et al*., 1999; Scharfman *et al*., 2000). This increase on some circumstances might lead to the appearance of the ectopic neurons and formation of the aberrant connections and to the enhanced excitation. It is not excluded that an ethanol-induced increase in the number of immature neurons undergoing a process of migration in the dentate gyrus of the juvenile rat might increase a risk of the ectopic neuronal migration, formation of aberrant connections, enhanced excitation and lead to the dysfunction of the hippocampus in the adulthood. Further studies are necessary to define the precise role of those newly generated neurons after ethanol treatment.

Parallel to the enhanced neurogenesis an increase in the proportion of BrdU-positive cells that have been differentiated into astroglia after administration of ethanol was found. Almost all BrdU-positive cells co-expressing the marker for astrocytes GFAP were found in Hil region. Astroglial proliferation probably represents a reaction to the ethanol-induced damage or might be a secondary process due to the increase in the number of neuronal population, which in turn, stimulates glial proliferation to retain the ratio between neurons and glia.

Based on the data obtained in our study we conclude that even a single high dose of ethanol is able to induce neuronal degeneration and enhance neuro- and gliogenesis in the dentate gyrus of the hippocampus of juvenile rats. Acute ethanol exposure to juvenile rats induced neuronal and glial reorganization of the dentate gyrus, which may be significant for its functions in adulthood.

4. Effects of ethanol intoxication and withdrawal on hippocampal neurogenesis in the adult brain

To elucidate the effects of ethanol intoxication and withdrawal on hippocampal neurogenesis we performed chronic ethanol administration via inhalation. The data obtained in the study demonstrated a significant decrease in the number of dividing cells at the end of chronic ethanol administration in the adult mouse dentate gyrus. It is not clear whether ethanol directly kills the dividing cells or retards their proliferation. Previous studies have demonstrated that ethanol induced a prolongation of the cell cycle and inhibits expression of cyclin-dependent kinase 2, a key protein that regulates the passage from G1 into S-phase and S phase progression (Liu *et al*., 1998). It seems, therefore, that the observed decrease in the BrdU-positive cells in the ethanol-intoxicated mice was due to the inhibition of mitotic activity of dividing precursors. When BrdU labelling was performed at the beginning of ethanol administration whereas the BrdU-positive cells were determined at the end of ethanol inhalation period no differences were observed in the number of BrdU-positive cells. The reason for the higher resistance of these cells is not clear. One plausible explanation might be that newly generated cells have already started to differentiate and therefore became more resistant to the toxic concentrations of ethanol. In addition, it should be noted that ethanol concentrations in the inhaled air have been gradually increased during the first two weeks of inhalation, and therefore differentiating cells might become tolerant to the high ethanol concentrations. Despite the lack of the observable effect on the numbers of BrdU-positive cells, ethanol administration affected the pattern of their differentiation. As revealed by co-localization experiments with the neuronal and glial markers, a smaller proportion of the BrdU-positive cells acquired the phenotype of the mature calbindin-positive granule neurons in the dentate gyrus of the ethanol-treated mice as compared with the control mice. It might be, therefore, proposed that chronic ethanol treatment either induces a retardation of the neuronal maturation or induces the death of mature granule cells. Indeed, previous in vivo as well as in vitro, experiments clearly demonstrated a retardation of maturation of several neuronal populations after ethanol administration (Cadete-Leite *et al*., 1998; Yanni and Lindsley, 2000).

In contrast to the ethanol-intoxication experiments, BrdU labelling of the dividing cells during ethanol withdrawal revealed an activation of proliferation of the precursors in the dentate gyrus. Our previous data, as well as data obtained by others (Brandao *et al*., 1992; Paula-Barbarosa *et al*., 1993; Vassiljev *et al*., 1998), have demonstrated that ethanol withdrawal exacerbates neuronal death in various brain structures including the hippocampus. Activation of neurogenesis often occurs as a response to the neuronal death following various neurotoxic insults (Gould and Tanapat, 1997; Gray and Sundstrom, 1998; Liu *et al*., 1998; Scott *et al*., 2000). It can be proposed that enhanced generation of the

new cells during ethanol withdrawal is a compensatory reaction and reflects the capacity of the dentate gyrus to restore the size of the pool of the neural cell precursors. The cells that have been generated in excess in ethanol-withdrawn animals do not, however, survive and are eliminated during 3 weeks following ethanol withdrawal. Therefore, their significance in the replacement of lost neurons in the hippocampus after ethanol exposure is questionable. Our data demonstrate that the differentiation of newly born neurons into mature, calbindin-positive granule cells is retarded during withdrawal. Thus, both ethanol intoxication and ethanol withdrawal exert similar inhibitory action on the differentiation of the newly born cells, which probably remain undifferentiated for a longer time during and after ethanol exposure.

In conclusion, the data obtained in the study demonstrate that ethanol intoxication inhibits whereas ethanol withdrawal enhances the proliferation of neuronal precursors in the dentate gyrus of the adult mouse brain. The data probably explain the conflicting results where either inhibition (Nixon and Crews, 2002) or activation of neurogenesis (Pawlak *et al*., 2002) after repeated ethanol administration was found. The cells formed in excess during ethanol withdrawal do not survive and are eliminated during 3 weeks. The functional significance of the ethanol-induced changes in the proliferation and maturation of neuronal and probably glial precursors remain unknown. Previous studies have demonstrated that hippocampal-dependent learning and experience–dependent activation of the hippocampus are preferentially disrupted by ethanol (Melia *et al.,* 1996). Recent studies also showed that newly born neurons in the dentate gyrus morphologically and functionally play more important role than old neurons in the hippocampal functions (Wang *et al.,* 2000; Shors *et al.,* 2001; Van Praag *et al.,* 2002). Whether the observed changes in the hippocampal neurogenesis are involved in the impairment of hippocampal functions following chronic ethanol administration remains to be elucidated.

5. Behavioural alterations following OB

Our study shows that OB animals demonstrated a characteristic syndrome consisting of increased activity in the open-field test and disrupted responding in the passive avoidance test. These behavioural changes are typical for OB and have been described by others (Kelly *et al*., 1997).

In addition, our experiments demonstrated a disruption of contextual fear conditioning in OB rats. This effect seems not only due to the enhanced emotional reactivity or increased aggressiveness because OB rats did not differ from sham-operated rats in regard to baseline freezing time in the conditioning box, but rather suggests selective disruption of contextual memory. The hippocampus is involved in spatial or topographical memory (O'Keefe and Nadel, 1978), which is consistent with the cognitive map theory. The cognitive map

theory proposes that the hippocampus of animals represents their environments, their locations within those environments, and their contents, thus providing the basis for flexible navigation (Burgess *et al.,* 2002). Furthermore, emerging evidence from the recordings of hippocampal neural activity shows that hippocampal networks encode episodic memories as sequences of events and places, where they occur (Eichenbaum, 2000). Previous studies have suggested that contextual memory is, at least in part, dependent on the hippocampal activity since destruction of the hippocampus impairs this form of memory (Maren *et al*., 1997; Corcoran and Maren, 2001; Mattus–Amat *et al*., 2004). It might be proposed, therefore, that OB might affect the normal acquisition of the context and formation of stable connections between contextual cues and an unconditioned stimulus or could decrease the accuracy of the processing of contextual details of memories. These data fit well with some clinical observations demonstrating declarative memory deficits in depressive patients (Vythilingam *et al.,* 2004; Kieseppa *et al*., 2005) due to hippocampal dysfunctions. On the other hand, cognitive theories of depression emphasise the vicious circle linking depressed mood and biased recall of negative information and therefore depressive patients are characterised by a selectively greater recall of fear (Bhagwagar *et al*., 2004; Bishop *et al*., 2004). Our data show that impairment of the contextual fear memory is not related to the global memory deficits in OB rats since tone-dependent fear memory was not affected by OB. Instead, a trend toward an increase of freezing in tone fear conditioning was found. It is not excluded, therefore, that the observed trend toward greater freezing in the tone-dependent fear memory task reflects such a biased recall of negative information in OB rats. The tone conditioning (unimodal) memory task, in contrast to contextual fear conditioning (multimodal memory task), is less dependent on hippocampal functions (Phillips and LeDoux, 1992) and therefore it might propose that OB mediates, at least partly, dysfunctions of the hippocampus.

6. Decreased hippocampal neurogenesis following OB is reversed by chronic citalopram administration

Our data demonstrate a reduced production of new cells in the dentate gyrus of OB rats. Furthermore, a retardation of the differentiation of the newly generated cells into mature calbindin-positive granule neurons was observed. To our knowledge this is the first report where a decrease in the hippocampal neurogenesis following OB is described. The observed decrease in the neurogenesis following OB fits well with the current hypothesis linking a depressive state with reduction of neurogenesis (Jacobs *et al.*, 2000; Malberg *et al.*, 2000; Czéh *et al*., 2001; Kempermann, 2002a; Duman, 2004) and suggests that OB might be a useful model for the study of hippocampal neurogenesis in depression. Furthermore, impaired neurogenesis following OB is reversed by chronic

treatment with citalopram, a selective serotonin re-uptake inhibitor. This was evidenced by an increased proliferation of neuronal progenitors in the dentate gyrus and accelerated differentiation of progenitors into adult granule neurons. No such effect of antidepressant was observed in sham-operated rats.

Although the dynamics of neurogenesis following OB have not been studied in the present study, a reduction of neurogenesis seems to be a long-lasting process, which might lead to the impairment of structural and functional plasticity of the hippocampus. The mechanisms underlying the decreased neurogenesis in OB rats are not clear.

It is obvious that olfactory sensory inputs have a major impact on the animals' general physiology and disruption of OB might produce stress for the animals, and, therefore stress, by itself, could be responsible for decreased neurogenesis. It has been shown that adult hippocampal neurogenesis is glucocorticosteroid-dependent and increased levels of glucocorticosteroids inhibit production of the new cells in the dentate gyrus of rodents and primates (Gould *et al*., 1997; Hellsten *et al*., 2002). The effects of OB, on the basal and stress-induced glucocorticosteroid levels, remain controversial. Whereas some studies have demonstrated raised levels of both basal and stress-induced glucocorticosteroids following OB (Marcilhac *et al*., 1999), other studies failed to observe any differences in the glucocorticosteroids secretion between sham-operated and OB animals (Kelly *et al*., 1997). Not only the increased levels of glucocorticosteroids might have an impact on the reduction of neurogenesis in OB rats. OB causes degeneration of neurons in various regions projecting to the olfactory bulb and normally receiving projections from the bulbs. OB induces neurodegeneration in the piriform cortex, amygdala (Capurso *et al*., 1997; Lopez-Mascaraque and Price, 1997) and reduces spine density in the CA1, CA3 regions and dentate gyrus of the hippocampus (Northolm and Ouimet, 2001). In addition, OB induces neurodegeneration in dorsal raphe nuclei and locus coeruleus (Nesterova *et al*., 1997).

Tissue remodelling following OB is accompanied by the multiple changes in neurotransmitter functioning. The most consistent changes have been found in serotoninergic neurotransmission. The neurodegeneration of serotoninergic neurons in dorsal raphe nucleus following OB results in the permanent deficits in secretion of serotonin in dorsal hippocampus and amygdala (van der Stelt *et al*., 2005), which in turn might induce denervation supersensitivity in several regions of the brain including cortex, amygdala and hippocampus and might be responsible for the depression-like syndrome observed in OB animals. There are several reports demonstrating that serotoninergic system exerts a positive effect on adult neurogenesis (Jacobs *et al*., 2000; Brezun and Daszuta 2000; Duman *et al*., 2001) and therefore it is plausible to propose that reduced serotoninergic output to the hippocampus following OB might contribute to the observed reduction in neurogenesis. Consequently, the restoration of neurogenesis in OB rats by chronic citalopram treatment might be explained by an enhancement of the serotoninergic neurotransmission in the dentate gyrus.

Not only serotoninergic neurotransmission is altered in OB animals but several other signalling molecules are also affected by OB, and these alterations might play a role in the observed reduction of neurogenesis and the actions of citalopram. It has been reported that OB alters expression of the BDNF gene (Sohrabji *et al*., 2000), enhances expression of the neuropeptide Y gene (Holmes *et al*., 1998), enhances glutamatergic neurotransmission (Ho *et al*., 2000) and reduces the levels of the neuroactive steroid allopregnenalone in the brain (Uzunova *et al*., 2004). It remains to be studied whether the above-mentioned changes also contribute to the reduction of neurogenesis and its restoration by citalopram treatment in OB animals.

Whether the reduced neurogenesis is causally linked to the observed deficits of contextual fear memory in OB rats, remains open. Previous studies did not find any changes in contextual fear conditioning in rats with reduced neurogenesis after treatment with the antimitotic agent methylazoxymethanol (Shors *et al*., 2002). Instead, an impairment in trace fear conditioning was detected. More recent studies, however, demonstrated that impaired contextual fear conditioning could be associated with reduced neurogenesis in the dentate gyrus of mice (Wang *et al*., 2004).

In addition to retarded neurogenesis we also observed a trend toward a decrease in the number of newly born cells in the dentate gyrus co-expressing marker for astrocytes GFAP. The reason for that is not clear and further experiments are needed. A stereological estimation of the total number of granule neurons, within the granule cell layer, revealed a reduction in the total number of cells and a trend toward reduction in the volume of the dentate gyrus. These findings are also in line with other studies where reductions in the cell number and volume of several regions of hippocampus were observed in a depressive state (Sheline *et al.*, 1996, 1999; Sheline, 2003). The observed reduction in the total cell number, within the granule cell layer, is not probably only due to the reduced neurogenesis. An increased neuronal death might also contribute to this. We were not able to demonstrate an increased neuronal death (TUNEL-labelling), neurodegeneration might occur during the first days after OB as demonstrated in the piriform cortex and in some other regions of the brain (Song and Leonard, 2005).

The data obtained in the study show that OB affects fear memories, which is accompanied by the reduced hippocampal neurogenesis. These results are in concordance with the current hypothesis linking a depressive state with reduced neurogenesis. The antidepressant citalopram reversed the OB induced decrease in neurogenesis, which could be one possible mechanism by which antidepressants alleviate OB-induced depressive-like behaviour.

Whether impaired hippocampal neurogenesis could impact the development of OB-induced “depressive-like” symptoms needs further studies.

CONCLUSIONS

1. Developmental lead exposure induced a persistent increase in the level of anxiety and inhibition of contextual fear conditioning and reduced generation of new cells in the dentate gyrus in adulthood. Lead administration altered the pattern of differentiation of BrdU-positive cells into mature neurons. In contrast, the proportion of young not fully differentiated neurons and the proportion of astroglial cells, generated from newly born cells, was increased in lead-exposed animals. Persistent inhibition of neurogenesis within the dentate gyrus could, at least partly, contribute the behavioural deficits in adulthood after postnatal lead administration.

2. A single ethanol administration in the early postnatal period increased the number of degenerating cells in the dentate gyrus of the hippocampus with maximum effect after the ethanol dose of 3 g/kg. Also the number of newly born cells following a single ethanol administration in GCL of the hippocampus was increased and a higher proportion of newly born cells had acquired a phenotype of immature postmitotic neurons. The proportion of astroglial cells was also increased following ethanol treatment. These changes in neurogenesis demonstrate that even a single high dose of ethanol in the early postnatal period is able to induce neuronal degeneration and neuronal and glial reorganization of the dentate gyrus, which might be significant for the functions of hippocampal structure in adulthood.

3. Continuous ethanol intoxication in adulthood inhibited the proliferating activity of the dentate gyrus. However, ethanol withdrawal following long-term ethanol intoxication enhanced the generation of new cells in the dentate gyrus of the hippocampus. Both ethanol intoxication and withdrawal in adulthood retarded differentiation of newly born cells into mature neurons, which might have an impact on the ethanol-induced impairment of hippocampal functions.

4. OB affected fear memories, which was accompanied by the reduced hippocampal neurogenesis. These results are in concordance with the current hypothesis linking a depressive state with reduced neurogenesis. Whether impaired hippocampal neurogenesis could impact the development of OB-induced “depressive-like” symptoms needs further studies.

5. Using OB, an animal model of depression, we demonstrate that the reduction of neurogenesis in OB rats was reversed by chronic citalopram administration. Thus, an antidepressant-induced increase in neurogenesis could be one possible mechanism by which antidepressants alleviate OB-induced depressive-like behaviour.

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SUMMARY IN ESTONIAN

Neurogeneesi roll aju plastilisuses

Kuni viimase ajani on aju remodelleerimis- ja regenereerumisvõimet peetud väheseks. Neuronaalseid tüvirakke, millest uued neuronid tekivad, on leitud põhiliselt kahes eesaju piirkonnas, subventrikulaarses tsoonis ning hipokampuse *gyrus dentatus'*es. *Gyrus dentatus'*es genereeritakse uusi rakke hiiluse ja granulaarrakkude kihi vahelises tsoonis, mida nimetatakse subgranulaarseks tsooniks. Eellasrakud migreeruvad seejärel granulaarrakkude kihti ning diferentseeruvad granulaarneuroniteks ja moodustavad sünaptilisi kontakte olemasolevate neuronitega (Cameron jt, 1993; Hastings ja Gould, 1999; Hastings jt, 2001). Uued rakud integreeruvad seejärel antud piirkonna närviringidesse. Uute genereeritavate neuronite arv *gyrus dentatus'*es (kuni 9000 päevas hiirel ja rotil) näib olevat võrreldes neuronite koguhulgaga granulaarrakkude kihis (1–2 miljonit) väike (Cameron ja McKay, 2001), kuid kogu eluea kohta arvestatuna moodustab see kuni 20% neuronite koguarvust. Hiljutised uuringud on näidanud rikastatud keskkonna, füüsilise aktiivsuse, samuti ruumilise õppimise soodustavat mõju neurogeneesile (Kempermann jt, 1997; Van Praag jt, 1999a; Gould, 1999a) ning viitavad selle rollile mälu kujunemisel. Stress ja/või kõrge glükokortikoidide tase näib samas neurogeneesi vähendavat (Cameron jt, 1995). Pikaajaline stress võib omakorda viia depressiooni tekkeni ja viimastel aastatel on pakutud üheks depressiooni tekke teooriaks ka pärsitud neurogeneesi teooria (Jacobs, 2000). Seda hüpoteesi kinnitavad mitmed eksperimendid, mille kohaselt suurendavad erineva toimemehhanismiga antidepressandid (tritsüklilised antidepressandid, selektiivsed serotoniini tagasihaarde inhibiitorid) kroonilise manustamise korral *gyrus dentatus*'es nii granulaarakkude proliferatsiooni kui ka elulemust (Malberg jt, 2000; Czéh jt, 2001). Samuti võivad hipokampaalset neurogeneesi mõjutada ka mitmed mürgid nagu plii, mis on levinud kõikjale elukeskkonda ja sõltuvust tekitavad ained nagu opiaadid ja etanool (Eicsh, 2002; Nixon ja Crews, 2002; Jaako jt, 2003; Gilbert jt, 2005; Jaako-Movits jt, 2005)

Töö eesmärgid

Neurogenees täiskasvanu ajus on üheks neuronaalse plastilisuse vormiks. Muutused nende protsesside kontrollimise mehhanismides võivad viia aju plastilisuse languseni ning patoloogiate tekkeni, mille tagajärjel võivad kujuneda muutused mälu- ja õppimisfunktsioonides. Lähtuvalt eelnevast oli meie töö eesmärgiks uurida aju plastilisuse ja käitumise muutusi etanooli ja plii kroonilise manustamise mõjul varajases sünnijärgses perioodis ja etanooli kroonilise manustamise mõju täiskasvanuea neurogeneesile. Samuti uurisime olfaktoorse

bulbektoomia (OB, depressiooni loommudel) järgselt katseloomade käitumist, kasutades erinevaid mäluteste ja muutusi hipokampuse neurogeneesis ning hindasime selektiivse serotoniini tagasihaarde inhibiitori tsitalopraami toimet bulbektoomia järgselt.

Töö tulemused ja järeldused

Töö tulemused näitavad, et plii krooniline manustamine varajases postnataalses perioodis põhjustab katseloomadel rahutust ja mäluhäireid ning viib hipokampaalse neurogeneesi vähenemiseni täiskasvanueas.

Etanooli ühekordse annuse (3 g/kg) manustamine varajases postnataalses perioodis kutsub esile rakkude degeneratsiooni ja suurendab uute tekkinud rakkude arvu ning nende diferentseerumist gliiarakkudeks, mis omakorda võib põhjustada olulisi häireid hipokampuse funktsioonis täiskasvanueas.

Etanooli krooniline manustamine täiskasvanueas inhibeerib proliferatiivset aktiivsust kuid abstinents suurendab uute rakkude teket hipokampuse *gyrus dentatus'*es. Nii etanooli krooniline manustamine kui ka abstinents aeglustavad uute tekkinud rakkude küpsemist neuroniteks, mis võib avaldada mõju hipokampuse funktsioonile.

Olfaktoorse bulbektoomia järgselt ilmnes, et OB põhjustab hüperaktiivset käitumist ja mõjutab negatiivsete assotsiatsioonidega seotud mälu ning vähendab hipokampuse neurogeneesi. Need tulemused on kooskõlas teooriaga, mis käsitleb depressiooni ühe tekkepõhjusena langenud neurogeneesi. Kas neurogeneesi vähenemine mõjutab ka OB-järgseid muutusi katseloomade käitumises vajab edasisi uuringuid.

Kasutades olfaktoorset bulbektoomiat näitasime, et OB-järgne vähenenud neurogenees taastus kroonilise tsitalopraami manustamise tagajärjel. Seega, antidepressantide poolt esile kutsutud neurogeneesi suurenemine võib olla mehhanismiks, mille kaudu antidepressandid mõjutavad OB-järgset käitumuslikku hüperaktiivsust.

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Teadustöö

Põhiliseks teadustöö suunaks neurogeneesi rolli uurimine aju plastilisuse muutuste korral.