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On Regulation of Hippocampal Neurogenesis

Roles of Ethanol Intake, Physical Activity and Environment

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ABSTRACT

The addiction research field struggles with the question of how long-term memories associated with addictions, which are likely to have a role in the relapse phenomenon, are formed. The work in this thesis has focused on structural adaptations and changes in plasticity genes in hippocampus, formed by both naturally awarding and drug-induced reward-seeking behaviors. Changes in the hippocampal neural network are further investigated in relation to social and environmental interactions. Specifically, analysis of formation, migration and differentiation of new cells, primarily neurons, in the dentate gyrus of hippocampus was performed. Mice and rats were studied in two putatively reward-generating behaviors, the two-bottle free-choice model of ethanol consumption, and voluntary wheel-running. Animals were also exposed to different environmental conditions, standard and enhanced, and altered social contacts.

Papers I and II describe effects of ethanol on hippocampal neurogenesis. Mice offered 10% ethanol in one of the two bottles in the free-choice model for short or long periods consumed ≈ 6 g ethanol/kg/day. These mice displayed increased cell proliferation and neurogenesis in the dentate gyrus. However, rats offered a lower ethanol concentration (5% v/v) consumed less ethanol (1.8 g/kg/day). Interestingly, this level of ethanol intake did not affect cell proliferation and neurogensis. When rats voluntarily consuming ethanol were subjected to repeated irregular withdrawal phases, ethanol intake decreased and they gained 30% in weight compared to continuously drinking animals or water-consuming controls. These animals also had decreased hippocampal neurogenesis. An irregular, and for the animals unpredictable and thus hypothetically stressful ethanol intake also decreased expression of the Nogo-receptor in hippocampus, while constant ethanol exposure did not. In conclusion, constant low ethanol intake does not affect hippocampal neurogenesis, while an irregular, presumably stressful intake does. Hypothetically, the increased number of new neurons detected after high ethanol intake could be involved in the formation of ethanol-associated memories, and ultimately in cue-induced relapse.

Papers III-VI address the effects of wheel running on cell proliferation, cell survival and neurogenesis and on plasticity genes in hippocampus. The adaptations found in hippocampus varied with running periods, social contacts and environment. In summary, long-term running increased neurogenesis in hippocampus whereas intermittent access to the running wheels did not induce any changes in hippocampal cell survival. Intermittent housing in enhanced environments, however, increased survival of newly formed cells compared to standard cage conditions. Cell proliferation in the dentate gyrus was increased more after 1 week than 4 weeks of running. In addition, the Nogo-receptor was found to be down-regulated at one week but not after four weeks of running. BDNF mRNA levels were increased after both one and four weeks of running. This suggests that the time frame for learning a new motor skill or to learn to appreciate the rewarding properties of a motor behavior, in this case the running in running-wheels, is associated with the highest hippocampal cell proliferation and the lowest levels of the Nogo-receptor. In support of this hypothesis that the Nogo receptor is always down-regulated in situations of long-term learning, it was found that Nogo-receptor overexpressing mice did not learn to develop an excessive running behavior during a five-week trial period.

Taken together, both natural and drug-induced reward-generating behaviors induce changes of hippocampal neurogenesis and transcription of plasticity associated genes. These adaptations are likely to be linked to associative and motor learning of the two behaviors and can therefore function as key elements in the establishment of addictions and in relapse.

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Table of Contents

List of Papers

This thesis is based on the following papers, which are referred to by their Roman numerals:

I. **Elin Åberg,** Christoph P. Hofstetter, Lars Olson and Stefan Brené. Moderate ethanol consumption increases hippocampal cell proliferation and neurogenesis in the adult mouse. International Journal of *Neuropsychopharmacology* 8: 557-567, 2005

II. **Elin Åberg** and Stefan Brené. Ethanol consumption with unpredictable withdrawal episodes, but not constant access, decreases hippocampal neurogenesis in adult rats. *Manuscript*

III. **Elin Åberg,** Alexandra Karlén, Anna Josephson, Lars Olson and Stefan Brené. Voluntary running is Nogo receptor-sensitive and associated with changes of plasticity genes and neurogenesis in hippocampus. *Manuscript*

IV. **Elin Åberg,** Therese M. Pham, Mieke Zwart, Vera Baumans and Stefan Brené. Intermittent individual housing increases survival of newly proliferated cells. *NeuroReport* 16: 1419-1422, 2005

V. Shun-Wei Zhu, Therese M. Pham, **Elin Åberg,** Stefan Brené, Bengt Winblad, Abdul H. Mohammed and Vera Baumans. Neurotrophin levels and behaviour in BALB/c mice: Impact of intermittent exposure to individual housing and wheel running. *Behavioural Brain Research* 167: 1–8, 2006

VI. **Elin Åberg,** Thomas Perlmann, Lars Olson and Stefan Brené. Running increases neurogenesis without retinoic acid receptor activation in the adult mouse dentate gyrus. *Manuscript*

On Regulation of Hippocampal Neurogenesis
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INTRODUCTION

Hippocampal functions are crucial for daily life, with an important role in learning and memory and also in the control of mood. Experimental studies in adult animals suggest that the formation of new neurons in this area contributes to the ability of the brain to handle and adapt to new situations. New neurons are continuously formed in the dentate gyrus throughout life in a highly regulated manner. Physical activity is known to be beneficial for health and promotes learning and memory. However, committed runners can develop an addictive-like behavior with withdrawal symptoms when deprived of the activity. In this thesis we investigate the effects on neurogenesis of voluntary running in running-wheels in relation to different environmental conditions and plasticity genes in hippocampus. High alcohol intake during longer periods is associated with general brain damage. However, analysis of post-mortem brains of human alcoholics cannot document neuronal cell loss in hippocampus. Hypothetically, the establishment of new neurons in hippocampus during alcohol intake could be important for the formation of new alcohol-associated memories. These memories, in turn, could increase risk of relapse in non-drinking alcoholics when specific cues activate these memories and trigger craving for alcohol. In this thesis we investigate the effects of both high and low ethanol intake during different intake patterns on the formation of new neurons in hippocampus.

An overall aim has been to advance understanding of hippocampal plasticity and neurogenesis in relation to ethanol consumption and excessive running. Because addictive behavior can also be influenced by alterations of social contacts and other environmental factors, we also analyzed how these factors affect neurogenesis and behavior.

The Hippocampal Formation

Neuroanatomy

The hippocampal formation is a large structure in the rat, spanning a substantial part of the brain (Fig. 1) and has extensive connections with many other brain areas. The hippocampal formation is divided into six distinct regions: the dentate gyrus, hippocampus proper (CA1, CA2 and CA3), subiculum, presubiculum, parasubiculum and entorhinal cortex (Amaral and Witter, 1995). These regions are connected through the unidirectional

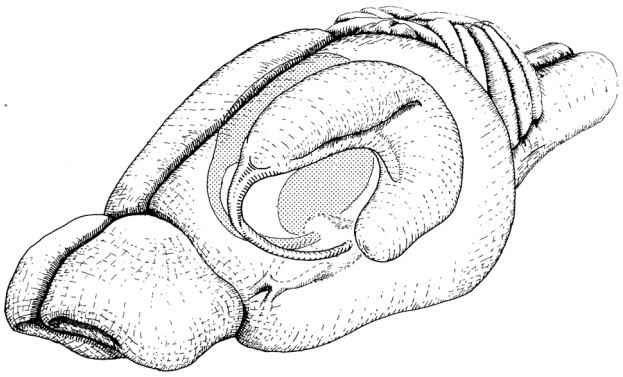

Figure 1. A three-dimensional drawing of the rat brain. The large, C-shaped structure is the hippocampus (Amaral and Witter, 1995).

tri-synaptic pathway, which has its main input and also output through the entorhinal cortex. The perforant path projects from the entorhinal cortex to the dentate gyrus granule cells. The CA3 field then gets its main input from the dentate gyrus via the mossy fiber pathway. Axons from the pyramidal cells in CA3 form the Schaffer collaterals, which synapse on CA1 pyramidal neurons, which further project to subiculum. Subiculum, in turn, connects the tri-synaptic pathway with the entorhinal cortex and other cortical areas such as the perirhinal, frontal and temporal cortex. These cortical areas are also the main input cortical areas. Many subcortical areas also have connections with the hippocampal formation. Some of them are amygdala, the ventral tegmental area (VTA), the raphe nucleus, locus coeruleus, nucleus accumbens and thalamus. There are also direct connections from the entorhinal cortex to CA3 and CA1 (Amaral and Witter, 1989, 1995). Most cells in the hippocampal formation are glutamatergic (pyramidal and granule cells), but there are also modulatory GABA-ergic interneurons both in the dentate gyrus and hippocampus proper.

The dentate gyrus consists of three layers: the low cell density molecular layer, the granule cell layer with highly packed granule cells, and the polymorphic layer (hilus) (Amaral and Witter, 1995). The main input signals to the dentate gyrus arrive via the perforant path, which makes synapses on dendrites of the granule cells in the molecular layer. The signals in the activated granule cells are modulated by a number of interneurons both in the granule cell layer and hilus, before the signals reach the CA3 field (Fig. 2). The mossy fibers give rise to several collaterals within the hilus region, which make contacts with cells in this area, for instance the mossy cells (glutamatergic) and with pyramidal basket cell dendrites (GABA-ergic) (Halasy and Somogyi, 1993). In addition, mossy cells synapse on granule and basket cell dendrites, and basket cells connect granule cell soma and dendrites. The mossy cells also receive non-hippocampal input from the dopamine cells of the ventral tegmental area, and noradrenergic input from locus coeruleus (Gasbarri et al., 1997; Vizi and Kiss, 1998). The CA3 area also has a connection with mossy cells in the hilus through feed-back glutamatergic connections (Lisman, 1999). Thus, modulation of the granule cell activity is high.

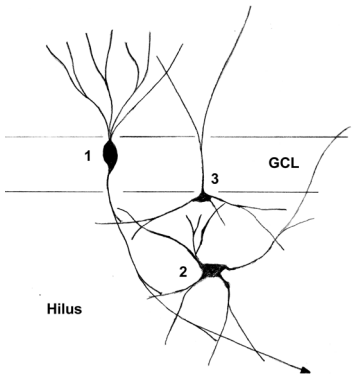

Figure 2. A schematic illustration of different cell types in the dentate gyrus of hippocampus. Granule cells (1) are connected with mossy cells (2) and dentate pyramidal basket cells (3). See text for more details. GCL = granule cell layer (modified from Amaral and Witter, 1995).

Hippocampal function

The main function of the hippocampal formation relates to learning and memory (Lisman 1999; Riedel et al., 1999) and regulation of mood (Phillips et al., 2003). Learning and memory can be defined as processes where an individual acquires knowledge, skills and experiences which are retained and modified by the brain. Underlying mechanisms for learning and memory are activity-dependent adaptive changes at synaptic and network levels, modified by experience (Kandel, 2001). Hippocampus, being a part of the limbic system, is also highly involved in modulation of mood; for instance, depressed patients have decreased hippocampal volume (Bremner et al., 2000; Videbech and Ravnkilde, 2004).

Hippocampal plasticity

Synaptic plasticity

Hippocampus is a highly adaptable structure, as it constantly reorganizes its functional network, both at synaptic and neuronal network levels in response to changing inputs. Important for learning and memory are long-term potentiation (LTP) and long-term depression (LTD), two forms of long-term synaptic plasticity characterized by an increase or decrease in synaptic strength driven by neuronal activity, respectively (Bliss and Collingridge, 1993; Frey and Morris, 1998; Martin and Kandel, 1996; Martin and Morris, 2002; Silva, 2003; Wittenberg and Tsien, 2002).

Neurogenesis

A central dogma in neuroscience for over 100 years was that new nerve cells are not formed in the adult mammalian brain. Although Altman and colleagues published several papers in the early 1960s to demonstrate the formation of new neurons in hippocampus, neocortex and the olfactory bulb (Altman, 1962; Altman and Das, 1965, 1967), it took many years until there was consensus about this form of plasticity in the adult brain. During the 1960s Nottebohm also demonstrated formation of new nerve cells

in the brain of adult birds (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984). The discovery of the formation of new neurons in the brains of adult humans in the late 1990s established adult neurogenesis as a major research field (Eriksson et al., 1998). At that time, new techniques, other than radiolabelled thymidine which requires autoradiography, were used. The introduction of the synthetic thymidine analogue BrdU (bromo-deoxy-uridine), which can be detected by immunohistochemistry and analyzed with stereology, was a key development for the findings of new neurons the adult brain (Kempermann et al., 1997; Kuhn et al., 1996).

Adult neurogenesis takes place in two main regions in the brain, the subventricular zone of the lateral ventricles in the forebrain (Alvarez-Buylla and Garcia-Verdugo, 2002) and in the subgranular zone of the dentate gyrus hippocampus. Neurogenesis has now been described in several species and continues throughout life, even though the formation of new cells decreases with age. The formation of new adult nerve cells is a complex process that requires a series of developmental steps beginning with the proliferation of a neuronal stem cell followed by the migration, differentiation, and functional integration of mature cells in neuronal networks (Duman et al., 2001; Jessberger and Kempermann, 2003; Kempermann et al., 2003; Stanfield and Trice, 1988; Zhao et al., 2006). The rate of formation of new cells in the rodent dentate gyrus appears to be relatively high, even though the actual or true number of newborn neurons has been difficult to establish, because of the response to environmental influences. An estimated 100–150 new granule cells are formed each day (Kempermann et al., 1997; van Praag et al., 1999b). This number could be even higher (Cameron and McKay, 2001). Within 1–2 weeks, the cell numbers are reduced by half (Biebl et al., 2000; Gould et al., 1999; Levison et al., 2000; Young et al., 1999) and this death of newly formed cells continues for about one month. Cells that survive the initial month survive long term (Kempermann et al., 2003) and have differentiated into mature neurons, although not fully integrated into the hippocampal system. Maturation and integration of the newly formed neurons into a functional network are crucial for their long-term survival (Linden, 1994). The roles for hippocampal neurogenesis are however not well understood. Formation of new neurons in hippocampus could allow better processing of novel and complex situations (Kempermann, 2002). Since new neurons are integrated into hippocampal networks (van Praag et al., 2002), they are probably also involved in hippocampal functions such as learning and memory (Gould et al., 1999; Schinder and Gage, 2004; Shors et al., 2001, 2002), and the in control of mood and treatment of mood disorders (Malberg et al., 2000; McEwen, 1999).

Stages of neuronal development

The formation of a new fully integrated mature neuron from that of a progenitor cell in the subgranular zone takes several months, and is a complex process with many developmental steps sensitive to different regulatory influences (Fig. 3). Developmental stages

can be revealed by detection of specific expression markers and morphology. An asymmetric cell division of a neural stem cell (radial glia-like cells that express markers for nestin and GFAP) is the first step in the neural development in the subgranular zone (SGZ) (Gage, 2000; Gage et al., 1998; Kempermann et al., 2004; Palmer et al., 1997; Rietze et al., 2001). After some days, one daughter progenitor cell becomes lineage-specific and expresses the immature neuronal marker doublecortin (DCX). After additional days of maturation, the neuroblast starts to migrate into the granule cell layer on its way to become a mature granule cell. Between one to three weeks after the first cell division, the immature neurons express both DCX and NeuN (Brown et al., 2003). In the maturation progress, dendrites and axon are formed and importantly, the mature neuron establishes synaptic contacts with other cells. The mature neurons express the mature neuronal marker NeuN. If the cells do not make synaptic contacts, they are lost. Functional integration into the hippocampal neuronal network takes one to two months from the first division. Around 60% till almost 100% of the newly formed cells in the dentate gyrus are neurons, the rest are glial cells (astrocytes) and some cells are also self-renewing stem cells, although the number varies depending on strain, species and gender (Kempermann and Gage, 2002; Perfilieva et al., 2001).

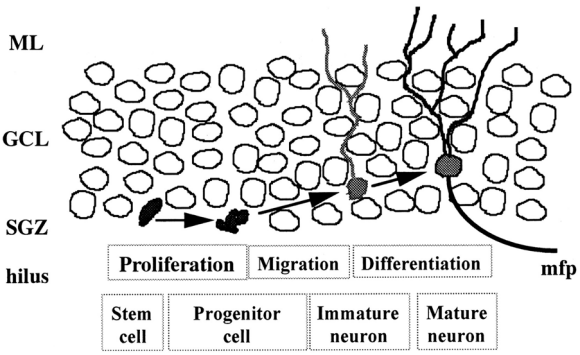

Figure 3. Neurogenesis in the dentate gyrus of hippocampus. The formation of new neurons can be divided into three major steps; proliferation of a neuronal stem cell in the subgranular zone (SGZ), migration into deeper granule cell layers (GCL) and differentiation, where the mature neuron send out dendrites into the molecular layer (ML) and the axon (mossy fiber) through the hilus to the CA3 field. mfp = mossy fiber pathway (modified from Duman et al., 2001).

Regulation Of Neurogenesis

The focus of this thesis is how ethanol intake, running and environment influence the hippocampal plasticity, and the formation, migration and differentiation of new neurons and also on the modulation of other plasticity genes.

Ethanol consumption

Until recently, ethanol was believed to produce global depression of brain function and to give rise to general effects on cognition and behavior. It was believed that ethanol interacted in a non-specific manner with neuronal membranes. However, it is now clear that ethanol influences cellular activity at different specific levels and also has different effects in different brain regions. Two brain systems that are highly affected by ethanol intake are the mesolimbic dopamine system (Di Chiara and Imperato, 1988) and hip-

pocampus. Activation of the mesolimbic dopamine system is associated with motivation and reward while activation of the hippocampal neural network is involved in memory and emotional processing.

Ethanol and the mesolimbic dopamine system

Dopamine neurons projecting from the ventral tegmental area (VTA) to nucleus accumbens are referred to as the mesolimbic dopamine pathway (Ungerstedt, 1971). Dopamine release in nucleus accumbens (Ungerstedt, 1971) is essential for motivation and reward (Di Chiara and Imperato, 1988; Koob et al., 1998; Wise, 1998). The mesolimbic pathway is, of course, also activated by natural behaviors, such as eating, drinking, mating and physical activity. Chronic treatment with drugs of abuse induces several morphological and biochemical changes in the mesolimbic pathway (motivation and reward), hippocampus (spatial and emotional memory), amygdala (affective states), caudate-putamen (motor memory), prefrontal cortex and other (integrating and controlling) cortical areas (Ortiz et al., 1995; Robinson and Kolb, 1999). All these areas are tightly functionally linked and together they are responsible for the mediation of motivation, reward, emotional processing and the formation of associative memories. Adaptive changes in these areas caused by ethanol consumption can however lead to life-long vulnerability to relapse. The relapse phenomenon can be investigated in animal models, for instance in the ethanol deprivation model. Animals in this model increase their ethanol intake when ethanol is reintroduced after a period of deprivation, demonstrating craving and possibly an experience of negative effects of ethanol withdrawal (Fig. 4). This animal model can also be used to investigate drug-memories (more discussed in next section) (Spanagel and Holter, 1999).

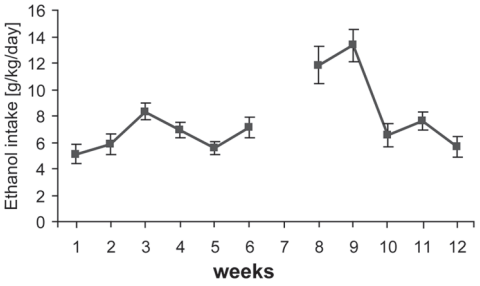

Figure 4. The ethanol deprivation model. Mice consume higher levels of ethanol when the bottles are reintroduced after one week of ethanol withdrawal. Ongoing experiment.

Ethanol and hippocampus

Ethanol affects hippocampus differently depending on dose and intake pattern. Human imaging studies have demonstrated that the hippocampal volume is decreased in alcoholics (Harding et al., 1997; Harper, 1998; Harper and Kril, 1989, 1991; Kril and Halliday, 1999, Kril et al. 1997). The hippocampal shrinkage is largely accounted for by loss of white matter, while neuronal loss appears insignificant in alcoholics (Harding et al., 1997;

Jensen and Pakkenberg, 1993; Kril et al., 1997), even though conflicting data have also been published (Bengochea and Gonzalo, 1990). Animal studies concerning the effects of ethanol on hippocampal neurons are diverse, reflecting the variation in ethanol effect depending on administration pattern and dose. Forced administration of high and/or toxic levels of ethanol has revealed impairment of hippocampal neurogenesis (Nixon and Crews, 2002). On the other hand, voluntary ethanol intake has been shown to have less dramatic effects on the formation of new cells and on learning and memory in hippocampus (Fadda et al., 1999; Kalev-Zylinska and During, 2007).

Long-term plasticity underlying addiction

Long-term brain adaptations may underlie addictive behavior and may be responsible for the establishment of a life-long risk for relapse. Molecular changes have been found primarily in the ventral tegmental area and nucleus accumbens (Hyman, 2005). However, these changes, for instance in CREB and ΔFosB levels, are transient and cannot *per se* be responsible for the life-long changes found in the brain (Nestler, 2002). They could possibly induce changes of other genes leading to lasting effects. Changes at synaptic levels in these areas are also evident after drugs of abuse (Hyman et al., 2006). It is suggested that relapses among drug-addicted individuals involve associative learning (Berke and Hyman, 2000). Animals, for instance, can learn to associate a specific drug or reward to a defined environment or cue (Lett et al., 2000). The persistence of drug abuse may thus reflect the persistence of normal memories. Relapses often occur when addicts meet people, visit places or are exposed to other cues associated with their prior drug use (Berke and Hyman, 2000). These associations are often conditioned emotional responses that can occur many years after taking drugs, thus long after cessation of physical withdrawal symptoms. The persistence of these kinds of memories could hypothetically involve the formation and modulation of new neurons in the dentate gyrus of hippocampus. While the long-lasting drug (and non-drug) memories presumably reside in cortex cerebri, alterations in hippocampus may influence the intensity with which the memory-forming input reaches cortex, and thereby the strength of the engrams, as well as the effectiveness of recall of specific memories. This thesis investigates the association between ethanol consumption and the formation of new neurons in hippocampus and also between ethanol intake and key plasticity genes in the hippocampus.

Physical activity

Physical activity, such as running, has positive effects on general health. It also increases hippocampal neurogenesis, promotes learning and memory and has an antidepressant effect (Bjornebekk et al., 2005; Greist et al., 1979; Morgan, 1985; van Praag et al., 1999a).

Single-housed rats and mice develop a remarkably high running activity over time; rats typically run as much as 10 km/day after approximately ten days of free access to the running wheel (Werme et al., 1999, 2000, 2002). Mice and rats that run these distances have changed levels of neuropeptides and ΔFosB in striatum and nucleus accumbens, and thus show changes similar to those seen following chronic cocaine administration (Werme et al., 2000, 2002). Moreover, rats can be trained to lever-press to get access to running wheels (Iversen, 1993). Animals that run in cages equipped with running wheels are conditioned to prefer an environment that they associate to the aftermath of running (Lett et al., 2000). Such conditioning to the running wheel-associated environment is blocked by the administration of an opioid receptor antagonist (Lett et al., 2001), which suggests that endogenous opioids are involved in conditioning to the running wheels. Taken together, these facts argue that voluntary wheel running can be seen as a model of behavioral addiction (Brené et al., 2007).

Running is not only rewarding and addictive, it can also have antidepressant effects both in humans (Babyak et al., 2000; Martinsen et al., 1985, 1989; Strawbridge et al., 2002) and in rodent models (Brené et al., 2007). In addition, running promotes formation of new cells in hippocampus (van Praag et al., 1999b). However, there are also situations were neurogenesis is unchanged or decreased after running (Bjornebekk et al., 2005; Naylor et al., 2005; Stranahan et al., 2006). In the light of wheel running as a model of a behavioral addiction that also increases hippocampal neurogenesis and improves memory, it was of interest to compare aspects of wheel running with voluntary ethanol consumption with respect to regulation of hippocampal structure and plasticity molecules such as BDNF and components of the Nogo-receptor complex.

Environment

Housing conditions, including interactions with other individuals and objects, have a strong impact on animal condition and behavior (Renner and Rosenzweig, 1987; Wurbel, 2001). In the 1940s Hebb described that rodents in an enriched housing environment performed better in learning and memory tests (Hebb, 1947). In the last decade many reports, in line with Hebb, describe improved learning and memory in enriched rodent environments and also increased cell proliferation and neurogenesis (Kempermann and Gage, 1999; Kempermann et al., 1997; Nilsson et al., 1999; van Praag et al., 1999b, 2000). Deprived environments and social interactions influence animals in different ways depending on context, gender and stress level. Social isolation can be experienced as stressful, particularly in male rodents (Bowman et al., 2001; Conrad et al., 2003; Weiss et al., 2004). The effects of social isolation on neurogenesis also differ between females and males, different test conditions and stress levels (Shors et al., 2007; Weiss et al., 2004; Westenbroek et al., 2003).

Neurotrophins

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) comprise the neurotrophin family (Barde et al., 1982; Cohen, 1960; Ernfors et al., 1990; Hallbook, 1991, 1999). Neurotrophins mediate signaling through the low-affinity receptor p75 (Johnson et al., 1986) and the high-affinity receptors TrkA (NGF) (Martin-Zanca et al., 1986), TrkB (BDNF and NT4) (Klein et al., 1989) and TrkC (NT3) (Lamballe et al., 1991) and they are all important for neuronal growth, survival and differentiation. BDNF is more highly expressed and widely distributed than NGF in the CNS, but both of these and their receptors are expressed in hippocampal neurons (Korsching et al., 1985; Wetmore et al., 1990). Neurotrophic factors have recently been described to be associated with mood disorders (Duman, 2004). In addition, BDNF has also been associated with hippocampal adaptations after antidepressant treatments and physical exercise (Bjornebekk et al., 2005; Neeper et al., 1996; Nibuya et al., 1995). This thesis investigates the modulation of BDNF and NGF in hippocampus after housing in different environments, after physical activity and in correlation to other key plasticity genes, such as the Nogo-receptor.

The Nogo Signalling System

Synaptic plasticity allows the brain to adapt to new situations and to acquire new skills. The Nogo-signaling system plays an important role in controlling axonal growth (Schwab et al., 1993) via the ligands NogoA (Schwab and Bartholdi, 1996), MAG (Lai et al., 1987) and OMgp (Wang et al., 2002b) situated on myelinating oligodendrocytes and neurons (NogoA) and a receptor complex consisting of the Nogo receptor (NgR1-3) and the coreceptor Lingo-1 (Mi et al., 2004) together with either the low-affinity NGF receptor p75 (Watkins and Barres, 2002), or Troy (Fig. 5) (Park et al., 2005). NogoA, MAG and OMgp can all bind to the NgR and thereby activate two different receptor complexes, NgR/Lingo-1/Troy or NgR/Lingo-1/p75NTR in the axon membrane (McKerracher et al., 1994; Schwab et al., 2006; Wang et al., 2002a). Activation of the receptor complex triggers intracellular RhoA kinases lead-

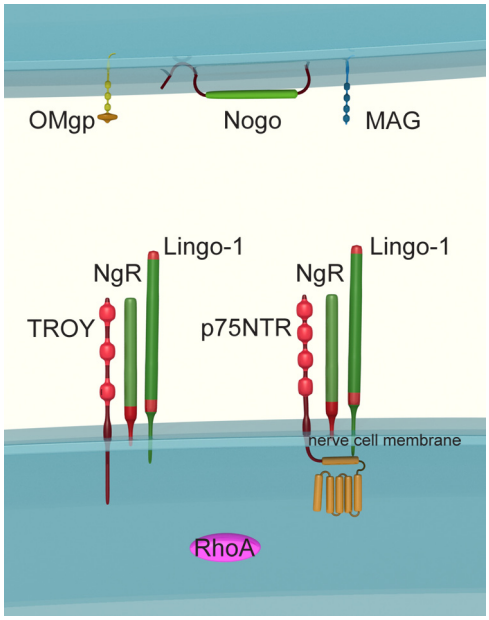

Figure 5. The Nogo-signalling system. Interactions of the myelin-associated inhibitors (Nogo, MAG and OMgp) with either the TROY/NgR/Lingo-1 or p75NTR/NgR/Lingo-1 complex activates RhoA causing growth cone collapse. (Illustration: Mattias Karlén)

ing to growth cone collapse and inhibition of axonal growth (He and Koprivica, 2004; McKerracher and Winton, 2002). NgR-1, co-receptors and ligands are expressed in brain regions displaying a high degree of plasticity such as cerebral cortex and hippocampus (Josephson et al., 2002; Karlén et al., 2007; Lauren et al., 2007; Trifunovski et al., 2004). These regions undergo structural changes at the synaptic level in response to neural activity (Martin and Kandel, 1996). The Nogo-signaling system can be involved in control of axonal outgrowth but also in the regulation of the fine tuning and structural stability of neural networks and synaptic connections (Endo et al., 2007; Josephson et al., 2003; Karlén et al., 2007; McGee et al., 2005; Trifunovski et al., 2004).

The Retinoid Signalling System

The essential role of vitamin A during development is well established and the understanding of the physiological role of retinoid signaling in the adult central nervous system is increasing. Retinoid receptors belong to the nuclear hormone receptor superfamily, which comprises more than sixty members. The retinoic acid receptors (RARα, β, γ) and the retinoid X receptors (RXRα, β, γ) are ligand-activated transcription factors that mediate physiological effects of retinoids (Chambon, 1994; Krezel et al., 1999; Leid et al., 1993; Mangelsdorf et al., 1990; Zetterstrom et al., 1994, 1999). The retinoic acid receptor has two ligands, the all-trans and 9-cis retinoic acid, whereas 9-cis retinoic acid and docosahexaenoic acid are ligands to the retinoic X receptor. RAR heterodimerizes with RXR and binds to specific DNA sites, while RXR also can bind DNA as a homodimer (Chambon, 1996; Kastner et al., 1997). In addition, RXR can heterodimerize with other transcription factors in the nuclear receptor superfamily and thereby couple retinoid signaling with other signaling pathways (Mangelsdorf and Evans, 1995). The wide spectrum of abnormalities caused by vitamin A deficiency is indicative of the broad involvement of retinoids in development and adult physiology. Blindness, infertility and immunological disorders are just some examples of vitamin A deficiency syndromes (Bates, 1995). Functional roles of retinoid signaling have also been demonstrated in the mesolimbic dopamine system (Krezel et al., 1998) and in hippocampus (see Lane and Bailey, 2005). Retinoid signaling is also implicated in disorders of the nervous system such as schizophrenia (Goodman, 1998) and depression (Crandall et al., 2004; Wysowski et al., 2001a, b). Dysfunctional retinoid signaling can trigger cognitive impairments (McCaffery et al., 2003). For instance, LTP and LTD are dependent on retinoid signaling (Chiang et al., 1998; Misner et al., 2001) and vitamin A deficiency causes impaired learning and memory (Cocco et al., 2002; Crandall et al., 2004; Etchamendy et al., 2003). RAR and RXR as well as cellular retinoid-binding proteins and ligands are located in hippocampus (Zetterstrom et al., 1994, 1999) and it has also been demonstrated that they are associated with the formation of new neurons in the dentate gyrus (Crandall et al., 2004; Jacobs et al., 2006; Sakai et al., 2004).

AIMS

The overall aim of this thesis was to increase knowledge about hippocampal plasticity and neurogenesis in relation to both natural and drug-induced reward-generating behaviors. Because addictive behaviors also can be influenced by alterations of social contacts and other environmental factors, we also analyzed how these factors affect neurogenesis and behavior. More specifically, the aims were to:

Investigate how "high" levels of voluntary ethanol intake affects cell proliferation, migration and differentiation in the dentate gyrus of adult mice.

Analyze how "low" constant versus irregular voluntary ethanol intake affects hippocampal neurogenesis.

Analyze the impact of voluntary wheel running on hippocampal neurogenesis and how a putative regulation of neurogenesis associates to expression levels of the plasticity-associated molecules BDNF and Nogo-signaling components.

Investigate if forced forebrain Nogo-receptor overexpression affects the development of a running behavior.

Analyze how intermittent changing from group to individual housing, in cages with or without running wheels, affects hippocampal cell survival and BDNF levels.

Investigate whether running, which increases hippocampal neurogenesis, is associated with changes in retinoid receptor activation in hippocampus.

MATERIALS AND METHODS

Animals

Adult female C57Bl/6 mice were used in paper I and female Balb/C mice were used in papers IV and V. In papers III and VI bitransgenic Nogo-receptor 1-overexpressing mice and transgenic retinoid reporter mice were used, respectively. Adult female Wistar rats were used in paper II. For details about the animals such as weight and age, see the individual papers. All experiments were approved by the local ethical committee for animal research in Stockholm.

Breeding

The retinoid reporter mouse (paper VI)

Retinoid reporter mice (C57Bl/6-129 background) were in paper VI backcrossed with wt C57Bl/6 mice. Tail tips were cut and lysed and DNA was extracted. DNA was amplified using PCR and genotyping was carried out with specific primers.

These mice have an inducible feedback expression system based on the detection of a reporter gene induced by an effector protein (Mata De Urquiza et al., 1999). The effector protein consists of a fusion between the DNA-binding domain of GAL4 and the ligand-binding domain of RAR or RXR. The reporter gene lacZ is combined with the effector gene in a single plasmid cassette. The effector-reporter genes are expressed from the same promoter elements consisting of GAL4 binding sites and minimal hsp promoters. Detection of activation is possible in all brain areas since the promoter is ubiquitously expressed (Mata De Urquiza et al., 1999; Misner et al., 2001; Solomin et al., 1998). The basal expression of the effector-reporter genes is low, but in the presence of ligand an autoregulatory feedback loop develops. The ligand-activated effector proteins will then upregulate its own expression and also the reporter gene expression. Thus, these mice will only express β-galactosidase when the RAR or RXR ligand-binding domain is activated by ligands.

The nogo overexpressing mouse (paper III)

A DNA fragment (5.2 kB) from a pTRE-NgR plasmid was microinjected into pronuclei of fertilized mouse eggs from C57Bl/6 (MouseCamp, Stockholm, Sweden). CamKII-tTA mice (The Jackson Laboratory) were crossbred with pTRE-NgR mice to obtain bitransgenic mice with tissue-specific overexpression of NgR-1 in forebrain neurons (Karlén et al., 2007).

Housing conditions

All animals in this thesis were kept alone in their cages, except for the group-housed control animals in papers IV and V. The reason for individual housing in the ethanol con-

sumption experiments is that the animals consume more ethanol if they are kept one to a cage (Deatherage, 1972; Schenk et al., 1990; Wolffgramm, 1990; Yanai and Ginsburg, 1976) and the ease of measuring individual ethanol consumption. Likewise, wheel runners were kept individually in order to obtain individual running data. In papers IV and V it was investigated how an intermittent housing regime, in which mice were single-housed with or without running wheels every second day, and every other second day social-housed (4 mice/cage) affects brain neurotrophins, corticosteroids levels as well as cell proliferation. It was also analyzed how constant social or individual housing affects cell survival of newly proliferated hippocampal cells (manuscript in preparation).

Standard environment

The standard environment consisted of a Macrolon cage (40.5 × 25.5 × 14.5 cm) with 50 ml of wood chips bedding (Fig. 6).

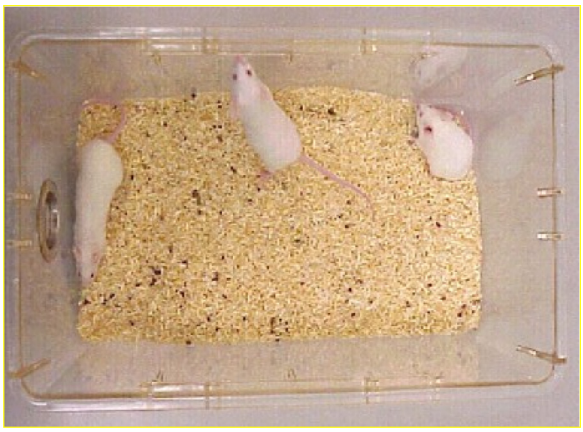

Figure 6. Standard environment

Enhanced environment

The enhanced cage environment consisted of a standard cage plus an egg-box carton, two Kleenex® tissues and two gnawing sticks. The mice had free access to food and tap water and the cage materials were renewed when necessary. For more details, see papers IV and V (Fig. 7).

Figure 7. Enhanced environment

Ethanol administration paradigms

Two different ethanol administration protocols were used in this thesis, the two-bottle free-choice model during shorter (4 and 14 days) and longer (2 months) continuous periods and also experiments in which withdrawal phases were introduced (papers I and II). Female individually housed mice and rats were used. Female mice and rats drink more ethanol than male mice and rats (unpublished results; Middaugh et al., 1999) and individually housed animals drink more than group housed animals (Deatherage, 1972; Schenk et al., 1990; Wolffgramm, 1990; Yanai and Ginsburg, 1976). Lights were completely on at 7 a.m. and completely of at 8 p.m., preceded by one hour of dawn and one hour of twilight, respectively. The animals had free access to food.

The two-bottle free-choice model

The two-bottle free-choice model allows the animals to choose to drink ethanol or water (Fig. 8). Such voluntary ethanol intake may possibly reflect human consumption patterns better than forced ethanol delivery protocols. Forced ethanol models are by definition involuntarily and reflect the effects of ethanol *per se* but also imposes stress on the animal. In the model used in this thesis, there are two bottles in each cage, one with ethanol (10% v/v, paper I, or 5% v/v, paper II) and one with tap water. The weight of the bottles was recorded at least three times a week and the positions of the bottles were changed at every weighing occasion to avoid side conditioning. New ethanol (99.9% ethanol and tap water) solutions were prepared once a week.

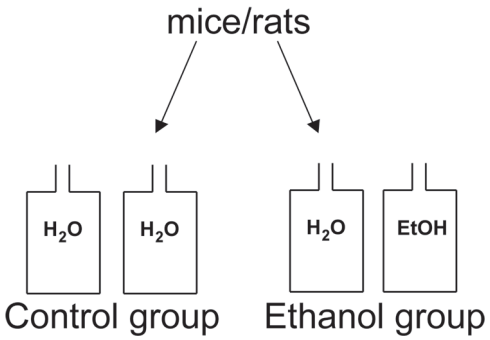

Figure 8. A schematic illustration of the two-bottle free-choice model. The animals in the ethanol group could voluntarily choose to drink from a water bottle or an ethanol bottle. The control animals had two bottles of water.

Ethanol withdrawal

In paper I, one ethanol withdrawal episode was introduced three days before sacrifice to investigate how it influenced cell proliferation. In paper II, four repeated irregular ethanol withdrawal phases, each phase consisting of three days, were introduced during a two-month voluntary ethanol consumption experiment in the two-bottle free-choice model.

Blood alcohol concentration

To determine blood ethanol concentrations (paper I), when the animals were sacrificed, trunk blood was taken and collected in heparinized tubes. The tubes were centrifuged at 3200 rpm for 10 minutes and serum was assayed for ethanol using the NAD:NADH enzyme spectrophotometric method (Sigma Diagnostics, MO, USA).

Plasma corticosterone analyses

Trunk blood was collected from each animal and centrifuged for plasma. Plasma samples were stored at -20°C until analyses for corticosterone concentration using a commercially available radioimmunoassay kit (Coat-A-Count Rat Corticosterone, Diagnostic Products Corporation, Belgium) (paper IV).

Wheel running

Interactions between wheel-running, cell proliferation and neurogenesis as well as correlations between running and brain neurotrophins, the retinoid system and the Nogo-signalling system were investigated in papers III–VI. Different running periods, from one week to two months, and access to the wheels were investigated in papers III–VI.

Mice were placed in individual cages (22 × 16 × 14 cm) with free access to running wheels (12.4 cm in circumference, one revolution corresponding to 39 cm; Fig. 9). Running data were collected every 30 minutes using customized software. Lights were completely on at 7 a.m. and completely of at 8 p.m., preceded by one hour dawn and one hour twilight, respectively. The animals had free access to food and water.

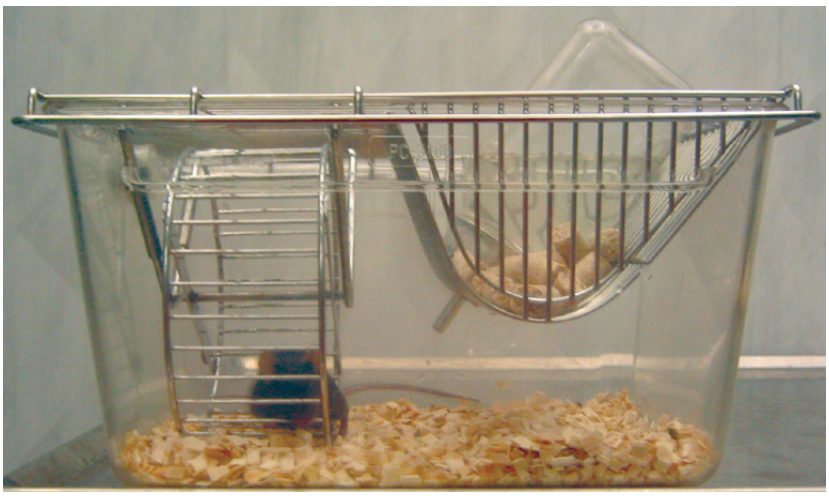

Figure 9. The running-wheel set-up. The individually housed mice had free access to the running wheel.

LABORAS

A behavioral profile of mice was obtained in paper V using the LABORAS system (Laboratory Animal Behaviour Observation Registration and Analysis System, Metris, Netherlands). Locomotion, velocity and total distance moved were registered. Briefly, mice were individually placed in clean cages with bedding, food and water on sensing platforms for 30 min. The animal movements were automatically registered.

Elevated-plus maze

The apparatus (made of dark grey polyvinyl chloride) consists of four arms (30 × 5 cm) extending from the central area (5 × 5 cm) and elevated 1 m above the floor (TSE systems, Bad Homburg, Germany). Ten-centimeter-high walls enclose two opposing arms, whereas the other two arms have no walls. The animals were tested in the elevated plus-maze between 9–12 a.m. during the light period. Before testing, animals were brought to the experimental room and allowed to habituate to the new environment for at least 60 minutes prior to testing. Animals were placed in the central area facing one open arm and monitored for 5 minutes by a video-tracking system (TSE systems). The plus-maze was carefully cleaned with 70% ethanol between each animal. Time spent in closed arms, open arms and in the central area was recorded as well as total distance traveled during the experiment (locomotion index) and the number of entries into closed and open arms (exploration index).

Cell proliferation, migration and neurogenesis detection

There are several different strategies for detection of newly proliferated cells, cell distribution, cell survival and neurogenesis (Altman and Das, 1967; Brown et al., 2003; Cameron and McKay, 2001; Duman et al., 2001; Hastings and Gould, 1999; Kempermann et al., 2004; Kornack and Rakic, 1999; Nakagawa et al., 2002). Some of these were used in this thesis.

BrdU labeling

The thymidine analogue Bromo-deoxy-uridine (BrdU), which is incorporated during the S-phase of DNA synthesis, was administered (i.p. injections) according to different regimes to detect cell proliferation, cell distribution, cell survival and neurogenesis (papers I, III and IV).

Two BrdU injections (i.p.) were administered three days before sacrifice to detect cell proliferation (papers I and III).

BrdU was also administered around two weeks from sacrifice to detect newly formed cells that had survived for two weeks and also for detection of neurogenesis (papers I and IV).

Ki-67 labeling

Another strategy for detection of newly proliferated cells is to label them with a Ki-67 antibody, which binds to a large nuclear protein expressed during all active phases of the cell cycle (G_1, S, G_2 and M phase). Ki-67 immunoreactivity thus reflects the presence of mitotic cells at the time of sacrifice (paper II).

Doublecortin labeling

Doublecortin is expressed in young immature neurons and doublecortin immunoreactivity at the time of sacrifice, and therefore reflects the presence of newly formed immature neurons (papers II and VI).

Sample preparation

Brains from animals used in this thesis were dissected out and frozen instantly, or obtained after formalin perfusion.

Fresh frozen tissue

At the end of the experiments, the animals were killed by decapitation and the brain was removed and rapidly placed on ice before being stored at -80°C for subsequent histological analyses.

Perfusion of tissue

Animals were deeply anesthetized with pentobarbital in the morning and perfused intracardially with 50 ml Ca^2-free Tyrode's solution including 0.1 ml heparin. This was followed by 50 ml of fixative (4% paraformaldehyde and 0.4% picric acid in 0.16 M PBS, pH 7.4) at forced pressure, followed by 250 ml of the same fixative over a period of 15 min. Brains were dissected, divided and postfixed in the same fixative for 1 hr at room temperature and subsequently rinsed in 0.1 M PBS with 10% sucrose and 0.1% sodium azide several times during 48 hr. The brains were stored in the last-mentioned solution at 4°C before cryosectioning.

Immunohistochemistry

Serial coronal 30-µm sections were cut on a cryostat through the entire hippocampus beginning at -0.94 mm from bregma and ending at –3.88 mm from bregma (Franklin and Paxinos, 1997). Every tenth section (papers I, II, III and IV) or every fifth section (paper VI) was processed for immunohistochemistry.

BrdU immunohistochemistry (papers I, III, IV)

For BrdU immunohistochemistry, sections were post-fixed in 4% formaldehyde in PBS for 10 minutes, rinsed in PBS followed by incubation in 2 M HCl for 30 min at 37°C to increase exposure of intranuclear BrdU epitopes to the primary antibody. After washing

in PBS, sections were blocked in a blocking solution (10% goat serum in 0.1 % Tween in PBS) overnight at 4°C. Sections were then incubated for 90 min at room temperature with the primary antibody against BrdU (rat anti-BrdU, Harlan Seralab, 1:100) diluted in the blocking solution, followed by 3 × 30 min 0.1 % Tween PBS rinses. Sections were next incubated with biotinylated goat anti-rat biotin (Vector Laboratories, 1:200) in the blocking solution for 60 min at room temperature. After another series of rinses (0.1 % Tween PBS, 90 min), avidin-biotin (Vector Laboratories) was administered for 40 min followed by rinses in PBS alone for 1 hour, and visualization using DAB (Sigma). The sections were counterstained with hematoxyline (Vector Laboratories) and mounted.

Doublecortin immunohistochemistry (papers II and VI)

Doublecortin immunohistochemistry was performed to detect newly formed cells with a phenotype of young neurons in the dentate gyrus. The sections were incubated with a blocking solution (1.5 % horse serum) 1 hour at room temperature. After washing in PBS the primary antibody (goat anti-doublecortin, Santa Cruz Biotechnology, Inc., 1:50) was applied for 48 hours at 4°C. After another series of rinses with PBS, sections were incubated with a secondary antibody (biotinylated anti-goat IgG, Vector Laboratories, 1:200). This was followed by rinses in PBS, incubation with avidin-biotin (Vector Laboratories) for 60 min and DAB (Sigma) staining.

Ki-67 immunohistochemistry (paper II)

Ki-67 immunohistochemistry was used to evaluate cell proliferation in the subgranular zone of the dentate gyrus. Sections were pretreated in 10 mM citrate buffert (10 mM Na-citrate buffer/0.05% Tween, pH 6.0) for 30 min at 95°C to increase exposure of Ki-67 epitopes in nuclei to the primary antibody. After washing in PBS, sections were blocked in a blocking solution (10% goat serum in 0.6% triton/PBS) for 1 hour at room temperature. Sections were then incubated with the primary antibody (rabbit anti-Ki-67, Dako-Cytomation, Denmark, 1:300, diluted in blocking solution for 48 hours at 4°C. After rinses in PBS, sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, 1:200) in blocking solution for 60 min at room temperature. After another series of rinses in PBS, avidin-biotin (Vector Laboratories) was applied for 40 min followed by rinses in PBS for 1 hour, and visualization using DAB (Sigma). Sections were counterstained with hematoxyline (Vector Laboratories) and mounted.

BrdU/NeuN and BrdU/S100β double labeling (paper I)

For immunofluorescence double-labeling of BrdU/NeuN and BrdU/S100β, where NeuN is a marker for neurons and S100β a marker for glial cells, the same protocol as described for BrdU immunohistochemistry was used, adding one antibody at a time. Primary antibodies used for immunofluorescence were: NeuN (mouse anti-NeuN, Chemicon, 1:1500) and S100β (rabbit anti-S100β, Swant, Bellizona, Switzerland, 1:100). Second-

ary antibodies were: FITC (donkey anti-rat FITC, Jackson, 1:200), Cy3 (donkey anti-mouse Cy3, Jackson, 1:800 or donkey anti-rabbit Cy3, Jackson, 1:400). Co-localizations of BrdU/NeuN and BrdU/S100β were detected on two sections/animal (-2.06 mm from bregma – -2.30 mm from bregma (Franklin and Paxinos, 1997) where all cells on one side were investigated for co-localization using a number-weighted sample of BrdU-immunoreactive cells and confocal microscopy (Zeiss).

β-galactosidase and β-galactosidase/Nurr1 immunohistochemistry (paper VI)

Serial coronal 14-μm sections were cut on a cryostat starting at –1.58 mm from bregma and ending at at –2.30 from bregma (Franklin and Paxinos, 1997). Immunohistochemistry was performed on every fifth section to detect RAR/RXR activation or co-localization between RXR activation (β-galactosidase-immunoreactivity) and Nurr1-immunoreactivity (paper IV).

Briefly, sections were incubated with the primary β-galactosidase antibody or a mixture of β-galactosidase antibody and Nurr1 antibody (chicken anti-β-galactosidase, Chemicon, 1:2000 and rabbit anti-Nurr1, E-20: sc 990, Santa Cruz Biotechnology, Inc., 1:100) 72 hours at 4°C. Sections were then incubated with biotinylated anti-chicken antibodies or a mixture of anti-chicken antibodies and anti-rabbit antibodies (Jackson Immunoresearch, 1:500 or donkey anti-chicken CY3, Jackson, 1:400 and donkey anti-rabbit CY2, Jackson, 1:200) for 60 min at room temperature. After a series of rinses in PBS, avidin-biotin (Vector Laboratories) was administered for 60 min followed by rinses in PBS and visualization using DAB (Sigma).

TUNEL staining

Detection of apoptotic cells (paper I) was made on two sections from every individual (-2.06 mm from bregma – -2.30 mm from bregma (Franklin and Paxinos, 1997)) using an in situ TUNEL cell death detection kit (Roche Diagnostics GmbH, Germany). TUNEL detects free 3' DNA ends, which is indicative of a DNA fragmentation. Briefly, sections were fixed in 4% formaldehyde for 20 minutes followed by incubation in a blocking solution (0.3% H_2O_2 in methanol) for 30 minutes. The sections were then incubated in a permeabilization solution (0.1% triton, 0.1% sodium citrate) at room temperature for 30 minutes followed by the TUNEL reaction for 1 hour at 37°C.

ELISA

BDNF and NGF protein levels were investigated in paper V in different brain areas using an ELISA assay kit (Promega). Briefly, 96-well ELISA plates were incubated overnight at 4°C with the neurotrophin antibody of interest followed by a second incubation with a secondary antibody and later with a species-specific antibody conjugated with horseradish peroxidase. A TMB solution was used to develop color in the wells. The absorbance

was recorded at 450 nm. The neurotrophin values were evaluated by comparisons to standard curves. Using this kit, the BDNF and NGF protein levels could be quantified in the range of 7.8–500 pg/ml.

Stereology

The "optical fractionator" was used to count BrdU-immunoreactive cells in the dentate gyrus (West and Gundersen, 1990; West et al., 1991). Briefly, every tenth section was systematically sampled (section sampling fraction [ssf] = 1/10) after randomly selecting the first section within the first interval. An unbiased counting frame with a known area was superimposed on the field of view by appropriate software (Stereologer®, SPA inc.). Counting frames were systematically distributed with known x and y steps throughout the marked region from a random starting point. The area of the counting frame relative to the area associated with the x and y steps gives the second fraction (area sampling fraction [asf]). The height of the optical dissector relative to the thickness of the section results in the third fraction (height [h] / thickness [t]). The total number of neurons is given by

$$N_{total} = \sum Q \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{h}{t}$$

where $\sum Q$ is the number of neurons counted in the dissectors.

Optical fractionator estimates are free of assumptions about cellular shape and size and are unaffected by tissue shrinkage. The dentate gyrus including an area exceeding the subgranular zone by two cell diameters and an area exceeding the molecular layer by one cell diameter was manually outlined using a 10× lens. Cell counts were performed with a 60× lens (numerical aperture = 1.4). The rostral and caudal half of the systematic random sample of sections were defined as the rostral or caudal part of the dentate gyrus. The separation between the rostral and caudal part was made at –2.41 mm from bregma (Franklin and Paxinos, 1997).

In situ hybridization

High stringency in situ hybridization (Dagerlind et al, 1992) was performed on 30-µm coronal sections thawed onto glass slides after cryosectioning. In situ hybridization was carried out with radioactive oligonucleotide DNA probes (for more information about the probes used, see papers II, III, and V). The probes were 3'-end labeled with α-^{33}[P]-dATP (Dupont NEN, Wilmington, DE, USA) using terminal deoxynucleotidyl transferase (Gibco) to a specific activity of approximately 1×10^9 c.p.m./mg. Hybridization was performed for 18 h in a humidified chamber at 42°C. The hybridization cocktail contained 50% formamide, 4 × SSC (1 × SSC is, in **M**, NaCl, 0.15; sodium citrate, 0.015, pH 7.0), 1 × Denhardt's solution, 1% Sarcosyl, 0.02 M Na_3PO_4, pH 7.0, 10% dextransulphate, 0.06 M dithiothreitol and 0.1 mg/mL sheared salmon sperm DNA. Follow-

ing hybridization, sections were rinsed 4 × 20 min in 1 × SSC at 60°C. Finally, sections were rinsed in autoclaved water for 10 s, dehydrated in alcohol and air-dried. Thereafter, the slides were exposed for X-ray film (Kodak Biomax MR film, Kodak, Rochester, NY, USA) and developed for quantification (for exposure times, see papers II, III, and V). Films were scanned and optical density values of regions of interest were quantified using appropriate software (Image J v. 1.32j, http://rsb.info.nih.gov/ij/). A ^{14} [C] step standard (Amersham, Buckinghamshire, UK) was included to calibrate optical density readings and convert measured values to nCi/g. Measurements were performed on two sections per animal and on both hemispheres and a mean value was calculated for each animal. Data were expressed as means ± SEM.

RESULTS AND DISCUSSION

The results of papers I–VI are summarized and discussed below. For detailed descriptions, see appended publications and manuscripts.

Voluntary ethanol intake affects hippocampal plasticity (papers I and II)

In papers I and II the effects of voluntary ethanol consumption on hippocampal plasticity were investigated. In particular, we studied the effects of ethanol on cell proliferation, migration and differentiation. Ethanol was made available during either short or long periods with constant or irregular exposure patterns. We also analyzed the effect of ethanol on the Nogo-signaling system in hippocampus.

Ethanol in our experiments is always available together with water. Animals can therefore always choose to drink water or ethanol. This two-bottle choice protocol is an attempt to mimic human alcohol consumption patterns. We varied ethanol concentrations, exposure time and withdrawal periods. Voluntary ethanol consumption is a very complex behavior. The animals will learn to consume ethanol and to associate the physiologic response to the consumption. Animals presumably develop an emotional relation to the effects of ethanol that includes the wanting, the reward, the withdrawal and other effects of ethanol that will engage different brain structures. Forced ethanol intake, including injections of ethanol, to rats and mice does not readily resemble these aspects of human ethanol consumption. One should keep this in mind, because forced and voluntary ethanol intake protocols can lead to markedly different results in animal experiments, and the forced intake protocols may not always be good models of alcoholism.

Ethanol intake is associated with changes of cell proliferation and neurogenesis

In paper I we investigated whether voluntary moderate to high levels of ethanol intake in single-housed female C57BL/6 mice could influence cell proliferation, migration and differentiation in the dentate gyrus. We also analyzed if ethanol withdrawal influenced cell proliferation. In paper II we further investigated cell proliferation and neurogenesis after two months of continuous voluntary low ethanol intake with or without four unpredictable withdrawal periods within the same time frame.

Two months of voluntary consumption of ethanol offered at a relatively high concentration (10% v/v) resulted in moderate-high ethanol intake (6 g/kg/day) in adult C57Bl/6 mice, and caused increased cell proliferation and neurogenesis in the dentate gyrus. Voluntary consumption of a lower concentration of ethanol (5% v/v) resulted in lower total ethanol intake levels (1.8 g/kg/day) and no change in cell proliferation or neurogenesis in adult Wistar rats. Interestingly, one withdrawal phase introduced after continuous voluntary high ethanol intake in mice resulted in a decrease of cell proliferation down to con-

trol levels. In parallel, decreased neurogenesis was also detected in Wistar rats subjected to four irregular withdrawal phases during low ethanol consumption in the two-bottle free-choice model.

In paper I, cell proliferation and cell survival was detected in animals by administration of the tymidine analogue BrdU, which is incorporated in DNA during the S-phase of the cell cycle. In paper II we used another strategy to detect newly formed cells and young neurons based on specific antibodies to detect Ki-67, which is expressed in all newly proliferated cells, and DCX, which is expressed in young immature neurons (maximum two-three weeks old). BrdU injections three days before sacrifice labels cells that were born close to the time point of injection such that the BrdU-immunoreactive cells found at sacrifice reflect cells born the last three days before sacrifice. Injection of BrdU two weeks before sacrifice gives the opportunity after sacrifice to visualize BrdU-immunoreactive cells that have survived for two weeks. Co-localiszation of BrdU-immunoreactivity with neuronal or glial markers allows phenotypic characterization of newly formed neurons and glial cells.

To investigate if the ethanol-induced increased neurogenesis was a compensatory response to increased cell death in hippocampus after ethanol (Pawlak et al., 2002; Zharkovsky et al., 2003) we performed TUNEL staining for detection of apoptotic cells. There was no difference in number of apoptotic cells between ethanol and control groups. We also analyzed if newly formed cells were particularly sensitive to ethanol by administering BrdU one day before the ethanol bottles were put into the cages, and then sacrificing the animals after four days of voluntary ethanol consumption. Again, there was no group difference. We thus found no evidence for increased cell death in the dentate gyrus in the two-bottle free-choice ethanol model. In paper II we also allowed animals to consume ethanol in the two-bottle free-choice model for a shorter period of time (14 days). In line with paper I, there was no difference between the two groups in cell proliferation or neurogenesis. Thus, moderate ethanol consumption during shorter periods is not neurogenic, nor does it increase death of cells in the dentate gyrus in adult mice.

Exposure to ethanol could hypothetically change the relative proportion of newly formed neurons versus glial cells. We investigated this possibility by phenotypic characterization of BrdU-positive cells using NeuN and S100β-immunoreactivity to label neurons and glia respectively. There was no difference between the relative numbers of newly formed neurons and glial cells after two months of ethanol consumption. Finally, we examined whether the distribution of newly formed cells in the dentate granule cell layers differed between the two groups. We then divided the granule cell layer into three defined areas (see paper I) and analyzed the cell distribution. Again, there was no difference in distribution of the newly formed cells in the dentate granule cell layers between the groups. Taken together, these experiments do not indicate any ethanol-induced disturbances of

survival, location or fate of newly formed cells, suggesting that it is the rate of cell formation *per se* that was influenced.

The dose and administration pattern is important for how ethanol affects hippocampal cell proliferation and neurogenesis. Previous studies have shown that toxic levels of ethanol decrease cell proliferation and neurogenesis in hippocampus (Nixon and Crews, 2002). Also important is how ethanol is administered, if the animal is forced to drink/eat ethanol/ethanol pellets or if ethanol is injected or given orally via gavage. We found that the formation and survival of new cells is unchanged if Wistar rats voluntarily consume low levels of ethanol on a daily basis. There are also other experiments describing that administration of constant moderate levels of ethanol in the diet does not affect neurogenesis (Kalev-Zylinska and During, 2007). In addition, studies on human alcoholics demonstrate that even though they consumed high levels of ethanol, there were no neuronal losses in hippocampus (Harding et al., 1997; Jensen and Pakkenberg, 1993; Korbo, 1999; Kril et al., 1997). Here we demonstrate in both mice and rats and at two different voluntary ethanol consumption doses (6 g/kg/day and 1.8 g/kg/day) that there is no decrease in neurogenesis after constant voluntary ethanol intake. Further, no increase in cell death was found in mice consuming relatively high levels of ethanol.

Our data suggest that moderate voluntary ethanol intake, during shorter or longer periods, does not cause any marked decrease of hippocampal cell proliferation or neurogenesis, nor does it alter migration or differentiation of newly formed cells. In fact, under certain circumstances ethanol consumption can lead to increased cell proliferation and neurogenesis as demonstrated in paper I. We suggest that one key effect of moderate voluntary ethanol intake may be to act as an antidepressant. If animals are kept under circumstances that decrease mood, such as single-housing conditions, then sufficient amounts of ethanol may increase mood and hence neurogenesis. If decreased mood is less likely, or the doses of ethanol are too low, then neurogenesis is not affected. Finally, if ethanol doses are very high, as can be the case in forced ethanol administration models, then direct effects of ethanol may instead depress neurogenesis

Decreased numbers of DCX-immunoreactive young nerve cells in Wistar rats and of newly proliferated BrdU-positive cells in C57BL/6 mice were detected when the continuous voluntary ethanol access was interrupted. In the mice, it was a single three-day ethanol withdrawal, and in rats four irregular three-days withdrawal phases. The animals exposed to repeated withdrawal phases decreased ethanol intake successively with withdrawal phases. These animals and the animals that were exposed to one withdrawal phase could possibly experience the withdrawal phase/phases as stressful, as the ethanol bottles were withdrawn in an unpredictable pattern. Hypothetically, such stress could trigger a stress-induced decrease of neurogenesis in the dentate gyrus. Our data suggest that it is not the amount of alcohol consumed *per se* that determines the rate of neurogenesis, but

rather the availability and the stress of irregular access to ethanol that matters. Weight is a good overall measure of animal well-being and we found that animals exposed to irregular access to ethanol gained almost 30% more weight compared to animals with constant access and animals without any access to ethanol during the whole experiment. Chronic mild stress is often associated with decreased body weight (Harris et al., 1997; Kubera et al., 1998; Levine and Morley, 1981; Matthews et al., 1995; Zelena et al., 1999), but increased body weight is seen under certain circumstances of mild stress (Nagaraja and Jeganathan, 2002; Tonissaar et al., 2007; Zelena et al., 1999). In humans, depression tends to lead to altered body weight with either gain or loss of weight (Dragan and Akhtar-Danesh, 2007; Gecici et al., 2005).

Stress is a factor that can induce depression, anxiety and very reproducibly decreases hippocampal neurogenesis (Czeh et al., 2001; Gould et al., 1997; McEwen, 1999). If irregular withdrawal phases are a form of mild stress, it is possible that the observed decreased neurogenesis in hippocampus is a result of such stress.

The decreased neurogenesis found after irregular ethanol intake might also be related to the high caloric intake, since there are links between caloric intake, body weight and neurogenesis. Decreased caloric intake increases neurogenesis while high caloric intake decreases neurogenesis (Mattson et al., 2003). Interaction between mild stress and high caloric intake may have caused the decreased neurogenesis in the Wistar rats.

Modulations of cell proliferation and neurogenesis were also found after relatively high ethanol intake (6 g/kg/day) in C57BL/6 mice. Daily ethanol intake doses like these are considered high (Spanagel, 2003) and correspond to approximately 6 bottles of wine every day in humans. However, the absorbance, metabolism and clearance in rodents is rapid and almost six times as high as in humans (Adalsteinsson et al., 2006; Wartburg, 1976). We speculate that the formation of new neurons during high ethanol intake could be involved in long-term adaptive changes of the hippocampal network.

We conclude that shorter or longer periods of low ethanol consumption can influence cell proliferation and neurogenesis. However, it is likely that it is rather the context in which the ethanol is consumed and not necessarily the dose of ethanol consumed that predicts how hippocampal neurogenesis will be affected by alcohol. To speculate, high ethanol intake could possibly result in adaptive changes of hippocampal networks that ultimately code for drug memories that can be involved in the recognition of different cues that associates to ethanol and that can trigger to craving and relapse conditions.

Ethanol intake down-regulates Nogo-receptor component in hippocampus (paper II)

The Nogo-signaling system is involved in inhibition of axonal growth, restricts axonal plasticity and is important for synaptic plasticity and thus rearrangements of neuronal networks (Chivatakarn et al., 2007; Endo et al., 2007; Josephson et al., 2003; Karlén et

al., 2007; Lavebratt et al., 2006; McGee et al., 2005; Schwab et al., 1993; Trifunovski et al., 2004).

In paper II we investigated the association between ethanol intake and the Nogo-system. We found that ethanol intake with irregular ethanol access down-regulates the Nogo-receptor 1 (NgR-1) in CA3 of hippocampus and that uninterrupted ethanol intake was associated with a trend in the same direction. Lingo-1 also appeared down-regulated in CA3, although not significantly so, after irregular and constant low ethanol intake experiments. It is tempting to speculate that down-regulation of Nogo-receptor components reflect ongoing hippocampal synaptic structural plasticity, since NgR-1 has previously been shown to be down-regulated in situations of increased neuronal activity and plasticity (Endo et al., 2007; Josephson et al., 2002, 2003).

Running is associated with hippocampal plasticity

All animals in the wheel-running experiments were single-housed and had free access to a running wheel during the whole experimental period and could thus run on a voluntary basis. Control animals were housed in the same type of cages, but without running wheels.

Wheel running influences cell proliferation and neurogenesis in hippocampus (papers III-VI)

Male mice in paper III had access to running wheels during one or four weeks. Interestingly, running for both one and four weeks increased cell proliferation in the dentate gyrus. Running for one week induced a stronger increase in cell proliferation compared to running for four weeks. Running during longer periods, in this case two months (paper VI) also enhanced hippocampal reorganization. A two-fold increase in neurogenesis was found in male mice after two months of constant access to running wheels and the increase in neurogenesis was positively correlated to running distance. It appears, however, that access to a running wheel in the cage does not always predict an increase in cell proliferation and neurogenesis. An example that we provide for this statement in paper IV is that when animals are group-housed in a standard environment every second day and housed in a cage containing a running wheel every other day, there is no increase in the number of newly proliferated cells that survive for at least 14 days. Instead there is an increased number of surviving cells in animals that were group-housed every second day in a standard environment and single-housed in cages without running wheels every other day. To further increase the complexity, when mice are group-housed in an enhanced environment every second day and housed in a cage with a running wheel every other day, wheel runners do indeed have an increased survival of BrdU-positive cells in the dentate gyrus compared to intermittent runners under standard housing conditions. Thus, as with the ethanol consumption models in papers I and II, it is evident that it is

the circumstances around the putatively rewarding behavior, be it ethanol consumption or wheel running, that determine whether there will be a change in hippocampal cell proliferation or neurogenesis.

In line with previous observations (van Praag et al., 1999b) we found that running can increase cell proliferation and neurogenesis in dentate gyrus. However, our mice were single-housed, while the mice in the van Praag study were group-housed. Single-housed Sprague-Dawley rats were recently found not to increase neurogenesis in response to running, while group-housed rats did, after 12 days of running (Stranahan et al., 2006). However, 2 months of running increased neurogenesis in both single-housed and group-housed animals (Stranahan et al., 2007). We show that male single-housed C57BL/6 mice do increase cell proliferation and neurogenesis in response to running both shorter and longer periods. The female BalbC mice in paper IV increased the running distance per day if they were group-housed every second day in enhanced environment compared to runners every second day housed in standard conditions.

Hence, our results together with those of others support the notion that many different factors, such as gender, strain, species, housing conditions and time of access to the rewarding activity, determines whether the behavior will lead to a change of hippocampal cell proliferation/neurogenesis or not (Bjornebekk et al., 2005; Holmes et al., 2004; Naylor et al., 2005; Stranahan et al. 2007).

Wheel running, BDNF, NGF, the Nogo-system and neurogenesis (papers III, IV and V)

Increases of neurotrophin expressions were found after both short- and long-term running in paper III, where also an increase in cell proliferation was detected. Wheel running one week and four weeks induced higher levels of BDNF mRNA in hippocampus compared to controls. Intermittent running in papers IV and V also increased BDNF mRNA and in addition NGF mRNA levels as well as the protein levels of both BDNF and NGF in hippocampus (papers IV and V). Thus, an increased neuronal activity in hippocampus was accompanied by robust increased neutrophin activity. However, all different conditions causing increased BDNF levels associated with running were not always associated with increased hippocampal cell proliferation and neurogenesis. Thus, there is no absolute correlation between increased BDNF levels and increased hippocampal cell proliferation and neurogenesis.

The higher degree of cell proliferation found after one week compared to four weeks of running suggests that the cellular hippocampal network reorganizes the most at the beginning of a new situation, in this case running in running wheels for the first time. The Nogo-system has previously been shown to be important for inhibition of axonal growth, axonal plasticity and most likely also learning and memory (Chivatakarn et al., 2007; Endo et al., 2007; Josephson et al., 2003; Karlén et al., 2007; Lavebratt et al.,

2006; McGee et al., 2005; Schwab et al., 1993; Trifunovski et al., 2004). Nogo-receptor 1 was down-regulated after one week, but not after four weeks of running, suggesting that the Nogo-receptor 1 is involved in the adaptive changes associated with learning a new skill. Down-regulation of Nogo-receptor 1 presumably facilitates synaptic reorganisation in hippocampus (Endo et al., 2007; Karlén et al., 2007). We used Nogo-receptor 1-overexpressing mice to investigate how the Nogo-signaling system might be involved in the establishment of a running behavior. The Nogo-receptor 1-overexpressing mouse was developed to investigate the role of Nogo-receptor regulation in memory formation (Karlén et al., 2007). Here we investigated if running behavior was altered in NgR-1-overexpressing mice, where the receptor thus could not be down-regulated, which we have hypothesized is important for plasticity, in a situation when both hippocampal cell proliferation and BDNF levels are increased. Interestingly, animals with an overexpressed NgR-1 did not develop a running behavior like control animals did. Control animals increased the daily running from week one to week two whereas transgenic animals did not. The running behavior in control animals declined after week two, even though there was no significant decrease. The transgenic animals, on the other hand, decreased running distance per day after week two until the end of the experiment. It thus seems as if overexpression of the NgR-1 has a large impact on running behavior, especially the first two weeks, when the running behavior becomes established as a learned behavior and neuronal adaptations occur.

In summary, voluntary running behavior in naïve mice could be associated with alterations in the Nogo-signaling system and neurotrophins, genes that are important for brain plasticity. Interestingly, these alterations occur when reorganizations, cell proliferation and neurogenesis, all induced by running, are high. This suggests that cell proliferation may be influenced by the Nogo-system. A further indication of a link between running and the Nogo-system was the demonstration that NgR-1-overexpressing mice were unable to develop a normal running behavior, which suggests impaired capacity to learn the excessive running behavior.

Intermittent changing from group to single housing is associated with increased cell proliferation and neurogenesis (papers IV and V)

Housing is one factor known to affect hippocampal neurogenesis. In papers IV and V we analyzed female Balb/c mice that were group-housed in two different conditions, enhanced or standard housing. Every second day the animals were single-housed in cages with or without a running wheel (papers IV and V). Cell survival in group versus individually housed C57BL/6 mice was investigated in another set of experiments (manuscript in preparation). These mice were also tested for anxiety-like behavior in the elevated-plus maze.

Intermittent individual housing increased the number newly formed cells that survived for 14 days as compared to constant social housing. Surprisingly, intermittent individual housing with a running wheel counteracted the increased cell survival. Constant individual housing did not affect cell survival compared to constant social housing. The increase in surviving BrdU-positive cells in intermittent individually housed mice was associated with an increase of BDNF mRNA and protein levels in hippocampus (paper V).

Mice that were intermittently individually housed were more active than group-housed control animals. Animal locomotion was evenly distributed in the open-field arena (data not shown). Normally, anxious animals tend to spend more time in shielded areas next to the walls. Our mice spent as much time in the middle of the arena as did controls, suggesting that the mice were not more stressed/anxious than the controls. Corticosterone levels were also the same between groups. Anxiety, stress and corticosterone have previously been shown to be associated with neurogenesis in hippocampus (Gould and Tanapat, 1999; Malberg et al., 2000; McEwen, 1999). A higher activity in the open-field arena could perhaps be explained by a stronger tendency of individually housed animals to explore a novel environment and move around in a relatively large area, compared to the continuously group-housed mice or mice that had access to a running wheels. The higher activity found in intermittent individually housed mice was counteracted by running every second day. Wheel runners did not explore new environments as much as the ones without access to running wheels, as indicated in the behavioral tests. A running wheel in the cage could possibly serve as a substitute for the cage mates, and these animals were focused on the wheel and not the environment around the wheel, leading to a lower degree of exploration in the cage and thus also in a new environment such as the open-field arena.

Mice from the constantly individually housed group were investigated in the elevated plus-maze to reveal whether these animals were more stressed/anxious than socially housed mice. Interestingly, there was no difference in anxiety between these groups. Further, cell survival was investigated by injections of BrdU three weeks before sacrifice. No change in cell survival could be detected after five weeks of constant individual housing compared to group-housed animals (data not shown, manuscript in preparation).

Changing environments and cage mates in a regular way is likely to keep the animals more alert by providing changing sensory inputs. Interestingly, mice that were group-housed with enhanced housing and single-housed in cages without running wheels had the highest cell survival levels. This group of animals was also more active in the different locomotor tests. The high locomotion is likely to be coupled to higher neuronal activity, which might promote survival of newly proliferated cells. The animals that were constantly individually housed lacked that sensory input. This could be one reason for the lack of change in cell survival when comparing the two different individual groups. It is

also possible that responses to housing conditions could be strain or be gender-specific (Stranahan et al., 2006; Weiss et al., 2004; Westenbroek et al., 2004). We suggest that the increased survival of newly proliferated cells could be due to enhanced environmental stimulation and the challenge of cage changes.

The retinoid system, running and neurogenesis (paper VI)

It is well established that retinoids are important for a normal development, and during the last decade evidence for its importance in the adult brain has increased (Crandall et al., 2004; Goodman, 1998; Krezel et al., 1998; Lane and Bailey, 2005; Wysowski et al., 2001b). For instance, retinoids are involved in cognitive systems and important for learning and memory. Retinoid receptors, binding proteins and ligands are all present in hippocampus and it is also documented that retinoids have the potential to modulate survival and differentiation of newly formed cells and neurogenesis (Crandall et al., 2004; Jacobs et al., 2006; Sakai et al., 2004). Because new neurons are formed in hippocampus and become functionally integrated in hippocampal networks (Zhao et al., 2006), it is tempting to speculate that there is a link between hippocampal neurogenesis, the retinoid system and hippocampal function. For instance, administration of the retinoic acid receptor agonist 13-cis retinoic acid (Accutane) for treatment of severe acne can cause severe depression and suicidal ideation as the worst outcome (Josefson, 1998; Scheinman et al., 1990; Wysowski et al., 2001b). Decreased neurogenesis and increased depression-like behavior have been noted when 13-cis retinoic acid were administered to animals (O'Reilly et al., 2006).

As running increases neurogenesis and has positive effects on general health (Bjornebekk et al., 2005; Greist et al., 1979; Morgan, 1985; van Praag et al., 1999b), we speculated that running might reduce activity in the retinoid system by modulating endogenous ligand-activation of the retinoic acid receptor in hippocampus. We took advantage of a transgenic mouse (Mata De Urquiza et al., 1999) to detect ligand-activation of retinoic acid receptor (RAR) and retinoid X receptor (RXR) in the adult mouse brain after running.

We found a robust increase in neurogenesis after running, but this increased neurogenesis was not correlated with RAR/RXR activation, nor was activation of RAR/RXR correlated with running. This result was surprising, in the light of previous studies correlating retinoid activation with decreased formation of new cells (Crandall et al., 2004). Our study suggests that there is no correlation between increased hippocampal neurogenesis and changes in ligand-mediated RAR activation after running using the reporter mice system. However, given the limitations of our methods, a putative correlation between hippocampal neurogenesis, the retinoid system, and running cannot be ruled out.

GENERAL CONCLUSIONS

The present work contributes to the understanding of how neurogenesis in hippocampus is associated with natural and drug-induced addictive behaviors.

Voluntary low ethanol consumption during short or longer periods does not affect hippocampal neuronal networks or key plasticity genes. However, higher ethanol intake, as well as irregular, unpredictable consumption, changes the formation and survival of new neurons in hippocampus. The context around the ethanol intake is thus important for how it effects the formation of new neurons and plasticity genes in hippocampus. Natural behavioral addictions such as running influence neurogenesis and plasticity genes in hippocampus differently depending on the running period, social and environmental context.

Taken together, both natural and drug-induced reward-generating behaviors induce changes of hippocampal neurogenesis and transcription of plasticity associated genes. These adaptations are likely to be linked to associative and motor learning of the two behaviors and can therefore function as key elements in the establishment of addictions and in relapse.

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