Cell proliferation and cell survival in the dentate gyrus of adult mice under naturalistic conditions

Dissertation

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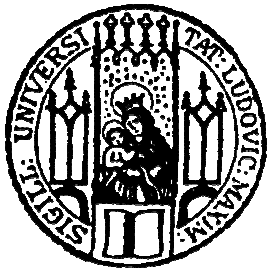

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Abbreviations

App	Appendix
BrdU	5-bromo-2'-deoxyuridine
BW	Body weight
CCD	Charge-coupled device
dd	Dorsodorsal blade of DG
dv	Dorsoventral blade of DG
DG	Dentate gyrus
EB	Eye blink conditioning
GCL	Granule cell layer
hi	Hilus
IMA	Integrated morphometry analysis
i.d.	Inner diameter
i.p.	Intraperitoneal
LD	light-dark
MWM	Morris water maze
n	Sample size
o.d.	Outer diameter
pAB	Primary antibody
PIT	Passive Integrated Transponder
PT	Post-treatment time
RFID	Radio frequency identification
RT	Room temperature
sAB	Secondary antibody
SD	Standard deviation
SEM	Standard error of the mean
SGZ	Subgranular zone
ve	Ventral DG

Summary

Throughout life, new cells are generated in the mammalian brain and incorporated as functional neurons in the networks of the olfactory bulb and the dentate gyrus (DG) of the hippocampal formation. So far proliferation and survival rates of newly generated cells in the adult DG have been investigated in commonly used and rather simple behavioral experiments like the Morris water maze, fear and trace conditioning, a running wheel and small enriched environments. Some of these studies gave evidence for an influence of single factors on neurogenesis, like physical activity, complexity of environment or associative learning. Results from laboratory experiments cannot directly be translated into the natural situation, because the relevance of these factors for animals in the wild is different from that for animals under laboratory conditions. Additionally, naturally an animal lives under a combination of several factors. Hence, we cannot derive the relevance of adult neurogenesis for wild-living animals from these studies.

The aim of this study was to examine neuronal plasticity in a naturalistic environment with respect to factors that have the capability to influence neurogenesis separately and under laboratory conditions.

Therefore, I compared cell proliferation and survival of newborn cells in DG of adult mice at different complexity levels of a naturalistic environment. Large enclosures equipped with computer-controlled water dispensers represented an environment near to nature, in which physical activity and exploration were possible and required. Foraging behavior was the basis for the investigation of the role of associative learning under naturalistic conditions. The extensive automation of the setup allowed for maximum avoidance of disruptions and interference of mouse behavior by the experimenter. With respect to this aspect, a new method for oral application of the proliferation marker BrdU via computer-controlled dispensers was established.

In a naturalistic environment, mice expressed distinct exploratory behavior and optimized their foraging following the variation of water dispenser qualities. Surprisingly, neither exploring novel water resources nor spatial learning of positions of profitable resources lead to a change in the rate of neurogenesis. From the finding, that running induced a marked increase of proliferation rate when performed in a running wheel but not when performed in a naturalistic environment, the question arose if the type physical activity is critical.

The comparison of running in a wheel with running in plane showed that the proliferation rate is independent from type of locomotion but strongly correlates with the

extent of running activity. The pro-proliferative effect of running occurs acute and persists for at least 3, but not more than 5 days.

Wheel running acts as a reliable promoter of cell proliferation in mice, but also represents a rather unnatural form of physical activity. Motivation for exercise as well as extent of exercise differ substantially between running wheel and natural locomotion. The results of this work indicate that the relevance of adult neurogenesis for natural behavior should be valuated with caution. In everyday life, the lifelong production of new cells in DG seems to function for the maintenance of a certain amount of neuronal resources rather than for the situational production of new neurons.

Zusammenfassung

Im Säugetiergehirn werden lebenslang neue Zellen gebildet, die als funktionelle Neurone hauptsächlich in das Netzwerk des Riechhirns und des Gyrus dentatus (dentate gyrus, DG) der Hippokampusformation eingebaut werden. Bisher umfassten Studien über Zellproliferation und Überlebensrate der neugebildeten Zellen im adulten DG meist gebräuchliche und einfache Verhaltensexperimente unter Standard-Laborbedingungen wie das Morris Wasser Labyrinth, Angst- und Spurenkonditionierung, Laufradaktivität oder angereicherte Käfighaltung. Der Einfluss einzelner Faktoren, wie physische Aktivität, Komplexität der Umwelt oder assoziatives Lernen auf die adulte Neurogenese wurde in einigen dieser Untersuchungen nachgewiesen. Die Ergebnisse aus Laboruntersuchungen können aber nicht direkt auf die Situation in der Natur übertragen werden, da diese Faktoren in der Natur eine andere Relevanz für die Tiere haben als unter Laborbedingungen. Zusätzlichen wirkt natürlicherweise eine Kombination mehrerer Faktoren auf das Tier. Die Relevanz der adulten Neurogenese für wildlebende Tiere kann also aus diesen Studien nicht direkt abgeleitet werden.

Das Ziel dieser Arbeit war es, die neuronale Plastizität in einer naturgetreuen Umgebung zu untersuchen unter Berücksichtigung der Faktoren, die einzeln und unter Laborbedingungen Neurogenese beeinflussen können.

Dazu wurden Zellvermehrung und –überlebensrate im DG adulter Mäuse bei verschiedenen Stufen von reizarmen und naturnahen Umgebungsbedingungen verglichen. Große Gehege, ausgestattet mit computergesteuerten Wasserspendern, repräsentierten eine naturalistische Umgebung, in der physische Aktivität und Explorationsverhalten möglich und gefordert waren. Das Nahrungssuchverhalten war die Grundlage für die Erforschung der Rolle von assoziativem Lernen unter naturgetreuen Bedingungen. Die weitgehende Automatisierung des Setups ermöglichte dabei in hohem Maße, Störungen und Beeinflussungen des Verhaltens der Mäuse durch den Experimentator zu vermeiden. In diesem Zusammenhang wurde auch eine neue Methode zur oralen Gabe des Proliferationsmarkers BrdU mithilfe computergesteuerter Tränken etabliert.

In der naturalistischen Umgebung zeigten die Mäuse ausgeprägtes Explorationsverhalten und optimierten ihr Nahrungssuchverhalten, wenn die Qualität der Wasserspender variiert wurde. Erstaunlicherweise führten weder die Erkundung neuer Wasserressourcen noch das Positionslernen rentabler Ressourcen zu einer Veränderung der Neurogeneserate. Die Feststellung, dass Laufen zwar im Laufrad, aber nicht in naturnaher

Umgebung eine deutliche Zunahme der Proliferationsrate verursacht, führte zu der Frage, ob die Art und Weise der physischen Aktivität dafür entscheidend ist.

Bei dem Vergleich von Laufen im Laufrad mit Laufen in einer Ebene zeigte sich, dass die Rate der Zellproliferation unabhängig ist von der Art des Laufens, aber stark korreliert mit dem Ausmaß der Laufaktivität. Der proliferationsfördernde Effekt des Laufens tritt akut auf und hält nach Laufradentzug mindestens 3, aber nicht länger als 5 Tage an.

Das Laufradlaufen ist für Mäuse zwar ein zuverlässiger Proliferationspromotor, stellt aber eine unnatürliche Variante physischer Aktivität dar. Sowohl die Motivation für die Bewegung, als auch deren Ausmaß unterscheiden sich im Laufrad erheblich von natürlichem Laufen. Die Ergebnisse dieser Arbeit deuten darauf hin, dass die Relevanz der adulten Neurogenese für natürliches Verhalten vorsichtig bewertet werden sollte. Im Alltag erfüllt die Bildung neuer Zellen im DG demnach vermutlich eher die Funktion, stets ein gewisses Maß an neuronalen Ressourcen aufrechtzuerhalten, als situationsbedingt neue Neurone zu produzieren.

General Introduction

History of adult neurogenesis

Traditionally, the brain has been thought of as a structure with limited regenerative potential. It was believed that new cells are produced only during embryonic stages and that structural changes in the adult central nervous system (CNS) are limited to the loss of neurons.

In the 1960s, first doubts arose about the total lack of regeneration capacity in the adult brain. Joseph Altman demonstrated post-natal genesis of brain cells in the rat (Altman, Das 1965). He used tritiated thymidine ([^{3}H]-thymidine), a radiochemical that is incorporated into newly formed DNA, to label proliferating cells and autoradiographic techniques to visualize the labeled cells. This new method allowed for labeling of proliferating cells and their progeny as well as for determination of their time and place of birth. Altman's concept of adult neurogenesis has been ignored or dismissed for several years. One reason for the disregard of Altman's findings might be that the available methods were not adequate to prove that newborn cells are neurons rather than glia cells. With the implementation of electron microscopy, Kaplan and Hinds (1977) could confirm that those [^{3}H]-thymidine labeled cells in the brain actually exhibit characteristics of neurons like dendrites and synapses. Later on, Nottebohm and colleagues showed that neurogenesis in the dorsomedial striatum of adult canaries correlates with song learning (Goldman and Nottebohm, 1983). The rediscovered theory of adult neurogenesis disclosed a substantial range of research and was subject of a growing amount of studies during the following years. Demonstrations of adult neurogenesis in non-mammalian vertebrates like fish, lizards or birds (Zupanc and Zupanc, 1992; Lopez-Garcia et al., 1988; Nottebohm, 1985) were readily accepted, but the relevance for the mammalian brain remained questionable. Further methodical developments contributed to the final establishment of neurogenesis on the adult mammalian brain.

The implementation of the synthetic thymidine analogue 5-bromo-3'-deoxyuridine (BrdU) was an important advancement in neurogenesis research. Similar to [^{3}H]-thymidine, BrdU is incorporated into the DNA of proliferating cells during mitosis. In contrast to [^{3}H]-thymidine, autoradiography is redundant because BrdU labeled cells can be visualized using immunohistochemical techniques. Another advantage of BrdU is that labeled cells can be accurately assessed for quantity and quality. A quantitative analysis of newly generated cells can be done using stereological counting technique. To define the

phenotypes newborn cells, confocal microscopy can be used to show unequivocal double labeling of BrdU-labeled cells with cell type-specific markers (Fig. 1). Gage and his colleagues were the first to use BrdU labeling and confocal microscopy in a study of adult neurogenesis in rodents (Kuhn et al., 1996; Kempermann et al., 1997). To this day, labeling of dividing cells using BrdU and immunohistochemistry is the prevalent method for the visualization and investigation of adult neurogenesis.

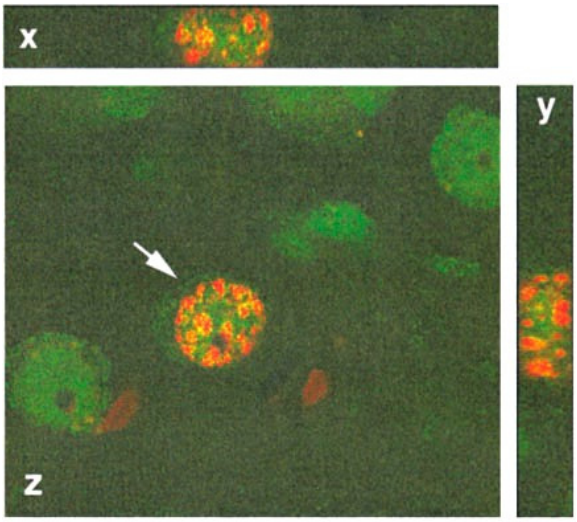

Figure 1: Some new cells in the adult rodent and primate brain have neuronal characteristics. Top panel, Confocal image of a cell (arrow) in the anterior cortex of an adult rat double-labeled with NeuN (green nuclear and cytoplasmic stain; a marker for mature neurons) and BrdU (red nuclear stain; a marker of DNA synthesis). The image is rotated in orthogonal planes (x, y, z) to verify double labeling throughout its extent. The rat was perfused 3 weeks after the BrdU injection. From Gould and Gross (2002).

Currently, it has become generally accepted that new neurons are produced in the brain of adult mammals, and adult neurogenesis has been identified in a variety of species including rats (Kaplan and Hinds, 1977), mice (Kempermann et al., 1998), hamsters (Huang et al. 1998), voles (Amrein et al., 2004), squirrels (Barker et al., 2005), tree shrews (Gould et al., 1997), New World primates (Marmoset monkeys, Gould et al., 1998), Old World primates (Macaques, Gould et al., 1999) and humans (Eriksson et al., 1998).

Cell proliferation in the mammalian CNS

In invertebrates, proliferative activity has been found in one of the main associative centers of several species of Orthoptera and Coleoptera, the mushroom bodies, and in structures of the olfactory pathway of decapod crustaceans. In non-mammalian vertebrates, proliferative areas have been identified at or near the surface of the ventricular and paraventricular systems, in the optical tectum of goldfish and in the telencephalon of lizards and turtles. High rates of cell proliferation in the lizard brain have been found in the olfactory bulb, the nucleus spericus and in the medial cortex that has homology with the

dentate gyrus (DG) of mammals (for review see Cayre et al., 2001). In birds, discrete regions have been identified in the walls of the lateral ventricles where new cells are generated, which then migrate into several areas of the telencephalon, especially in the high vocal center, a nucleus involved in song production (Goldman and Nottebohm, 1983).

In mammals, adult neurogenesis occurs in two distinct brain regions: in the olfactory bulb (OB) and in the dentate gyrus (DG) of the hippocampal formation. The origin of the newborn cells in the OB are neural progenitor cells in the anterior part of the subventricular zone (SVZ). The SVZ is a narrow zone of tissue within the wall of the lateral ventricles in the forebrain. The progeny of the proliferating SVZ cells migrate along the so-called rostral migratory stream (RMS) into the OB. There, the new cells differentiate into periglomerular interneurons and granule cells (Lois and Alvarez-Buylla 1993) that become synaptically integrated into the existing circuit (Carleton 2003).

In adult hippocampal neurogenesis, neural progenitors are located in the subgranular zone (SGZ) that is a small ribbon along the border between the granule cell layer of the DG and the hilus. In the SGZ these progenitor cells proliferate and give rise to immature neurons. Many of these newly generated cells die between the first and second week after they are born. The surviving neurons then migrate into the molecular layer (Kempermann et al., 2003). Within four weeks, they send axons to the CA3 region to form mossy fibers and project dendrites to the outer molecular layer (Hastings and Gould, 1999; Seri et al., 2001; van Praag et al., 2002). During this period, the newly generated neurons become electrically active and capable of firing action potentials. Electrophysiological studies have shown that these newborn granule neurons start to receive synaptic inputs from the cortex within four to six weeks after birth, appearing to become functionally integrated in the circuit (van Praag et al., 2002). The complexity and density of their dendritic spines continue to grow for at least several months.

The formation of new neurons in the dentate gyrus of the hippocampus throughout adulthood has not only been demonstrated in rodents but also in humans (Erikkson et al., 1998). As many as 250,000 new neurons are incorporated into the rodent dentate gyrus every month, that corresponds to 6% of its total cell number (Cameron and McKay, 2001).

Neurogenesis: A multi-step process

The generation of neurons during adulthood differs from embryonic neurogenesis. In the adult brain, new cells of all developmental stages are found. In contrast to the systematic and highly organized process during development, neurogenesis in the adult is a

flexible and individualized progression. To become a new neuron in the adult brain four main steps have to be passed:

1) cell proliferation
2) migration
3) differentiation
4) survival

An increase or decrease in cell proliferation does not necessarily mean that the number of neurons is increases or decreased. The migration of a new cell from its site of division to its target area is also an important part of the process, because only when located at the right place the cell can be properly integrated into the existing network. Until this time point it is not certain that the cell becomes a neuron at all. A newborn cell can either become a neuron or an astrocyte and the fate determination decides on the final function of the cell. Last, but not least, the survival or the elimination of newly formed cells ascertain the overall outcome of this process.

The mechanisms that regulate each step and the whole progression are just beginning to be elucidated. The prevailing view is that adult neuronal stem cells are astrocyte-like cells, characterized by expression of the glial fibrillary acidic protein (GFAP, Doetsch et al., 1999; Seri et al., 2001). These nesting-positive stem cells probably divide asymmetrically producing one stem cell and one proliferative neuroblast, also termed progenitor cell (Kempermann et al., 2004). The finding that these nestin-positive but GFAP-negative progenitor cells occur in two subtypes, positive or negative for immature neuronal marker doublecortin (DCX), indicates a lineage determination at this stage of neurogenesis (Kronenberg et al., 2005). The progenitor cells migrate a long or short distance towards their target area. Finally, the progenitor cells exit the cell cycle and reach a transient postmitotic stage in which cells are selected for long-term survival, the terminal maturation takes place and network connections are established. Those cells that mature to neurons are characterized by the expression of the neuron-specific nuclear protein (NeuN).

Not all newly produced cells become neurons. A part of them becomes glia cells and another substantial dies before complete maturation. The proportions of newborn cells that had become neurons and glia cells, respectively, cannot be generally specified because these rates depend on the methods used, like specificity of markers or age of cells. However, neurons account for the most part of surviving newborn cells with proportions given between 60 % (Kempermann et al., 1997a) and 93 % (Biebl. et al., 2000). The amount of matured cells that can finally be integrated into the network is strongly dependent on the survival rate of new cells. A substantial part of cells dies after birth and

before maturation, as indicated by an increased amount of apoptotic cells in proliferative brain regions (Biebl et al. 2000) and the definite decrease of BrdU-labeled with time. Kempermann et al. (2003) have shown that the number of BrdU-labeled cells considerably decreases within 4 weeks after division and remains stable thereafter. Cell death affects the amount of cells available for full maturation and consequently, cell survival is a decisive aspect of neurogenesis.

In the multi-step process of neurogenesis, each of these steps has to be passed through to result in functional and integrated new neurons. As each of these steps comprises a complex subset of factors, processes and interactions to work, it is obvious that neurogenesis is regulated and can be influenced by a variety of factors.

Regulation of neurogenesis

Since it is accepted that the adult brain is capable of renewing cells, together with the understanding of the process itself the investigation of the factors that regulate neurogenesis are of great value. The following section will give an overview of factors that have been found to influence the rate of neuron production. This list is not intended to be exhaustive, but it implies that is difficult to deeply understand all correlations that might contribute to adult neurogenesis. However, some of these factors led to the design of the experiments described in this dissertation and will be discussed in more detail in the discussion section of Chapter 2.

Molecular regulators

Growth factors are proteins that stimulate cell proliferation and differentiation. Consequently, a variety of growth factors were studied and found to be involved in the regulation of adult neurogenesis. Epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) are only examples for the regulators in this class (for review see Hagg 2005 and Lehmann et al. 2005). An integrative study of Cao and colleagues (Cao et al. 2004) showed that the vascular endothelial growth factor (VEGF) not only stimulated neurogenesis in the adult rat DG but can also be associated with improved cognition in standard learning tasks. Furthermore, the data show that the increase on cell proliferation induced by an enriched environment can be completely blocked by inhibiting VEGF expression.

Neurotransmitter like glutamate, serotonine, acetylcholine and nitric oxide appear be also able to influence cell proliferation and differentiation. Cooper et al. (2004) showed

that the rate of neuron production in the rat DG declined following lesion of the cholinergic forebrain which projects to the DG. In contrast, unilateral lesion of the entorhinal cortex, the major glutamatergic input into the hippocampus, resulted in an increased number of newborn cells in the DG on the lesioned side compared to the control side (Cameron et al. 1995). The assumption of transmitter system involvement in cell production is further supported by studies that showed alteration in the cell proliferation rate following treatment with NMDA (N-Methyl-d-Aspartate) or NMDA receptor antagonists (Cameron et al. 1995 and 1998, Gould et al. 1997, Nacher et al. 2001).

Hormones have been shown to interact with cell proliferation for both adrenal steroids and estrogen. Estrogen acts as stimulator on proliferation. The effect of estrogen removal by ovariectomy is a decrease of proliferating cells in the rat DG and can be reversed by estrogen replacement. Although female rats temporary produced more cells than males during proestrus, this difference between genders did not persist in the long-term (Tanapat et al. 1999). Corticosteroids act as suppressor on proliferation. Removal of circulating corticosteroids by adrenalectomy results not only in an increased number of newborn cells (Cameron & Gould 1994) but also in an increased number of new neurons (Gould et al. 1992). Since corticosteroid levels influence the rate of adult neurogenesis, it has been considered that they might play an important role in the age- and stress-related reduction of cell production (see respective sections below). However, the circadian fluctuations of corticosterone levels have been shown to have no effect on proliferation rate (Ambrogini et al. 2002).

Systemic and environmental regulators

The **genetic background** has been shown to contribute to differences in proliferative activity in adult brains. The baseline level of cell proliferation varies between different species of wild-living rodents (Amrein et al. 2004a) as well as between different inbred strains of laboratory mice (Kempermann & Gage 2002).

With age, neurogenic activity declines but still persists as shown for rats (Kuhn et al., 1996), mice (Kempermann et al., 1998), wild-living rodents (Amrein et al. 2004), monkeys (Gould et al., 1999a) and humans (Erikkson et al. 1998). Since aging is characterized by increasing levels of corticosteroids, it has been suggested that these hormones might contribute to the age-related decline of neurogenesis. Further support of this assumption was provided by Cameron & McKay (1999) who showed that the decline can be prevented by reducing the corticosteroid levels in aged rats.

Stress can suppress cell production and neurogenesis. The impact of stress has been investigated in studies using various types of stressful experiences, including male-male encounters in tree shrews (Gould et al. 1997), resident-intruder model in marmoset monkeys (Gould et al. 1998), immobilization of rats in restraining tubes (Vollmayr et at. 2003, Bain et al. 2004) or exposure of rats to predator, i.e. fox, odor (Tanapat et al., 2001). All types of stressful experiences lead to an decreased number of newborn cells in the adult DG.

Stressful experiences are known to activate the hypothalamic-pituitary adrenal (HPA) axis, thus leading to an increased level of circulating glucocorticoids, which have a suppressing effect on cell proliferation and neurogenesis.

Enrichment of environment seems to influence proliferation and neurogenesis rate. "Enriched environments" are used to provide more natural housing conditions and are implemented by adding various things like toys, tunnels and running wheels to enlarged laboratory cages for rats and mice. Most of the studies report enhanced survival of newborn cells and increased neurogenesis in animals that lived in an enriched environment (Kempermann et al., 1997b; Kempermann et al., 1998b; Ehninger & Kempermann, 2003; Brown et al., 2003; Nilsson et al., 1999), but regarding the rate cell proliferation there are contradicting reports. However, some studies could not find an increased survival rate following enriched housing (Kempermann et al. 2002, Kozorovitskiy & Gould, 2004).

Physical activity is a strong stimulator for cell proliferation in the DG. Most of the studies screened, reported that cell proliferation and survival are significantly increased in the DG of rats and mice that voluntarily used a running wheel (e.g. Eadie et al., 2005; Kronenberg et al., 2005; van Praag et al., 1999a). Even physical exercise that is considered to be less voluntary for rodents, like treadmill running, lead to an increased proliferation rate (Kim et al. 2002, Ra et al. 2002), whereas swimming lead to increased proliferation in rats (Ra et al. 2002) but had no effect in mice (Van Praag et al 1999a).

Learning and memory processes might potentially be associated with adult neurogenesis. Although a causality remains to be proven, several correlations support the view that neurogenesis might be involved in specific cognitive functions.

The hippocampus is one of the two prominent brain regions where adult neurogenesis takes place and this region is well defined in the context of learning and memory. Likewise, in the second prominent region with marked neurogenesis, the olfactory bulb, it has been shown that in mice living in an odor-enriched environment (containing several different odors), the amount of newborn neurons is significantly increased and, furthermore, this increase is accompanied by a longer and stronger olfactory memory

(Reochefort et al. 2002). In consideration of the decline of cognitive abilities with age, it is noteworthy that likewise hippocampal neurogenesis decreases with age (Kuhn et al. 1996, Amrein et al. 2004b). Furthermore, indications for the age-dependent cognitive decline can be reduced by restoring neurogenesis in older animals (Cameron et al. 1999, Kempermann et al. 1997, Kempermann et al. 2002b). Kempermann et al. (1997) showed that living in an enriched environment lead to increased neurogenesis and improved spatial learning performance in the Morris water maze task. Specific learning tasks can enhance hippocampal neurogenesis (Gould et al. 1999b) and toxicologic blocking of cell proliferation has been observed to impair performance in a hippocampal-dependent learning task (Shors et al. 2001).

The causal and functional relationship between learning and neurogenesis is not yet evidenced and there are some studies that, moreover, demonstrate a lack of relationship. Seasonal variations in spatial memory processing do not correlate with cell proliferation or total number of neurons in the DG of grey squirrels (Lavenex et al. 2000). Two studies in rats confirmed the age-dependent reduction of cell proliferation, but showed that this reduction does not predict the age-related impairment in a spatial learning task (Merrill et al., 2003; Bizon & Gallagher, 2003).

The last-named factors, environmental enrichment, physical activity and learning play a major role in the experiment described in Chapter 2 of this dissertation and are discussed in more detail in the corresponding section.

Aims of this Thesis

Overall goal

Summarizing previous findings, cell proliferation in the adult brain is influenced by a complex array of factors. However, the functional significance of the ability to produce new neurons throughout life remains unclear. Obviously, adult neurogenesis is linked to behavioral patterns. The fundamental question is when and to which extent is this process relevant for animal and hence, possibly human nature? We know that environment, physical activity and learning can correlate with adult neurogenesis under standardized laboratory conditions. But since we are not yet able to quantify newborn cells in living animals, we cannot observe neurogenesis under real natural conditions.

Therefore, the approach of the present study was to provide both, conditions that reflect more natural behavior and methods that result in reliable neurobiological data. Thus, the aim of this study was to investigate adult neurogenesis in mice living in a naturalistic environment and facing naturalistic conditions involving exploration, learning and physical activity.

Specific aims

Identification and quantification of newborn cells requires labeling of these cells. The proliferation marker 5-bromo-2'-deoxyuridine (BrdU) is well established and used in the majority of neurogenesis studies. BrdU is commonly administered via intraperitoneal or intravenous injections. The procedure, including catching, retaining and injecting, does not only mean intervention but also might cause stress in animals. Taking into account that stress is one of the factors that can diminish cell proliferation, it is worthwhile to look for a method that reduces stress to a minimum.

As a first step, I aimed in the development of a method of BrdU administration that provides at once

1) a minimum of intervention by the experimenter
2) the opportunity to apply precise dosages to individuals of a group
3) reliable BrdU labeling of proliferating cells
4) reproducible numbers of labeled cells.

The computer-controlled artificial flowers with RFID device, constructed and well established in the laboratory of York Winter for studies in nectar-feeding bats (see Winter

and Stich, 2005), provided a useful basis to this approach described in Chapter 1.

Since many studies have shown that the production of new cells in the mammalian hippocampus is influenced by a variety of conditions created in the laboratory, I wanted to ask the question whether these findings are transferable to the natural behavior of mice. Environmental enrichment, physical activity and learning play a role in adult neurogenesis, at least when considered separately under laboratory conditions. All of these factors are naturally part of an animal's life and I was interested whether these factors affect cell proliferation in the adult brain when considered from a more integrated point of view.

The approach was to digress from the standard methods of behavioral experiments and to construct conditions in the laboratory that allow for reproducible investigation of natural-like behavior. Chapter 2 describes the experiments that examined the extent of cell production in mice

1) living in a naturalistic environment, experiencing explorative and spatial learning tasks
2) living in a frugal, but spacious environment compared to mice living in standard laboratory cages.

As a continuation of these experiments, we wanted to go into further detail regarding the role of physical activity as a pro-proliferative factor. Running in a running wheel has an indisputable positive impact on cell production in rodents. But does wheel running reflect natural movement and is the effect on cell proliferation comparable? In the second set of experiments, described in Chapter 2, I investigated two types of physical activity in mice, running in a wheel and running on flat ground, and their influence on the rate of cell production.

The experiments conducted within this thesis were not only based on rarely explored hypotheses. We also used methods that lacked established experience in their application in the given context. Along with the new method of oral application of BrdU, which is described in detail in Chapter 1, the setup of the semi-natural environment including the automated water dispensers were applied to rodents for the first time and also physical activity in mice on flat ground has not yet been investigated to this extent. Chapter 3 describes a set of additional experiments that were designed in order to, firstly, clarify some fundamental questions in the run-up to the experiments of Chapter 1 and 2 and, secondly, confirm reliability of our results achieved in these Chapters.

Chapter 1

Individually dosed oral drug administration to socially-living transponder-tagged mice by a water dispenser under RFID control

published in: *Journal of Neuroscience Methods* 153, 208-213 (2006).

1 Abstract

The sensitivity of behavioral and physiological parameters to even mildly stressful experiences such as drug injections creates a need for alternative methods. We have established a method of stress-free administration of drugs via drinking water that allows multiple, individually specific and exact dosages, even for socially housed animals. The drug solution is supplied by a dispenser with automated volume control. Animals are PIT microchip-tagged with RFID transponders and identified in realtime at the water port. Computer control permits preprogramming of individual reward quantities so that drug administration is terminated after an individual has collected its daily dose. For our experiments, the substance 5-bromo-2'-deoxyuridine (BrdU) was given as a marker of proliferating cells that we quantified in the hippocampus of adult mice. Experimental groups received BrdU either via intraperitoneal injections or orally via the water dispensers. Immunohistochemical staining of BrdU-positive cells was of the same quality after oral administration as after injection. BrdU-positive cells did not differ statistically in cell numbers. Thus, water dispensers under transponder control allow the individual and stress-free application of drugs even to group-living animals without disturbing their behavior. This is useful where a complex temporal protocol of application is required, and for phenotyping experiments combining behavioral tests with neural, cellular or molecular analyses.

Keywords: Neurogenesis; RFID; Microchip; Transponder; PIT tag; Injection; Oral application; Drinking water

2 Introduction

Behavioral experiments ideally should have the human factor replaced by machine control. This is one consequence of the carefully standardized three-sites study of genetically identical mice by Crabbe and co-workers (Crabbe et al., 1999; Wahlsten et al., 2003c) where significant variation in behavioral responses of experimental groups under identical protocol was attributed to uncontrolled effects of human interaction. One type of human–animal interaction are drug injections that are potentially stressful. In this report, we describe an alternative to injection that is based on the oral application of drugs by a computer-operated system. We developed this method for the study of neurogenesis, where the extended labeling of cells may require multiple injections. Adult neurogenesis in the dentate gyrus of the hippocampal formation is often investigated with respect to behavioral parameters, because it is supposed to depend on, among other things, learning (Gould et al., 1999; Shors et al., 2002) and social interactions (Gould et al., 1998). One approach to the investigation of adult neurogenesis requires labeling of dividing cells with a proliferation marker, such as 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog. In contrast to KI-67, an endogenous proliferation marker, the effect of BrdU is transient, which allows the evaluation of the response of proliferating cells to stimuli in the context of cognition or learning (Kee et al., 2002). Application is usually done by one or several intraperitoneal injections. Although proliferation rate is not diminished by injection procedure (Kee et al., 2002), an alternative for experiments involving behavioral tests and learning experiments would be highly desirable. One approach suggested for animal models of medical disorders (Schleimer et al., 2005) is oral intake. However, a major difficulty of oral drug application is controlling dosage. Dosage may be estimated in retrospect from oral uptake after ad libitum presentation of the drug solution (Jecker et al., 1997; Bennewith et al., 2002). This, however, is unsuitable in cases where predefined individual doses are required, e.g. for quantification of labeled cells, and especially where animals are kept together as a social group within their home-cage. Another alternative, capsule application (Pasloske et al., 1999) is unsuitable for small rodents. Hand-feeding provides controlled dosage for each individual of a group, but significant time has to be spent in training of animals and in the administration procedure (Schleimer et al., 2005). Since liquid delivery with precise reward control is possible (Mitz, 2005) we developed a method that combines both controlled liquid delivery and drug administration. It is based on three elements: the drug is supplied as an aqueous solution from a computerized liquid dispenser providing small amounts of liquid each time the animal comes to drink.

Secondly, animals are microchip-tagged with RFID transponders or PIT (Passive Integrated Transponder) tags which allows individual identification during visits at the feeder equipped with a radio-frequency identification device (RFID). Thirdly, computer control of the liquid dispenser permits preprogramming of individual delivery quantities. This allows drug administration to be terminated after an individual has collected its daily dose. Thus, precise dosages of drugs can be applied, the method is stress-free by avoiding handling and surgery, and it leaves social interactions undisturbed by permitting individual application even to individuals living socially in a group, through transponder discrimination. In the following, we describe the method and provide an example demonstrating that detection of newborn cells in the dentate gyrus of the hippocampus of mice, after BrdU-labeling through water uptake or i.p. injections, is unaffected by the method of application

3 Methods

3.1 Animals and housing

Adult female CD-1 mice (n = 10) from the departmental breeding colony (Biocenter, University of Munich, Germany) were used in the experiment. The animals were housed in standard laboratory cages either separately (n = 4) or in groups of three individuals per cage (n = 6) with free access to food in a 12:12 h light:dark cycle. At the beginning of the experiments, the mice were between 9 and 11 weeks old, weighing 31.5 ± 2 g. One week before BrdU application, sterilized industrial transponders encapsulated in biocompatible glass (2.1 mm × 12 mm, 0.09 g, Unique, Sokymat) were injected under isoflurane anesthetic subcutaneously ventral to the left shoulder using an injector (Trovan) with self-inserted transponders.

3.2 Computer-controlled water dispenser

The liquid dispenser that we used is suitable for any solution. It functions by a computer-switched solenoid pinch valve (AscoJoucomatic W295A112-12VDC, Rueil Malmaison Cedex, France) for flow control through a 55-shore silicone tube measuring 3.2 mm o.d. and 1.6 mm i.d. (Fig. 1A, see Winter and Stich, 2005). By use of a pinch valve the drug remains within the tube and does not contaminate the interior of the valve. This permits the exchange of solution and tubes without the risk of carrying over drugs between different applications. Access to the water port was restricted to a single mouse by a

Perspex entry cylinder of 35 mm inner diameter and 60 mm in length (Fig. 1). Each visit at the dispenser opening was recorded by an infra-red photosensor. A circular transponder antenna placed around this cylinder permitted individual identification while a transponder-tagged mouse was at the water port. We used a generic, industry standard radio frequency identification (RFID) reader suitable for Unique/EM4102-compatible transponders. We can easily adapt the system for Trovan, Destron, Biomedic, Datamarsor ISO transponders. Communication of the reader with the host computer was via an RS232 serial connection. The settings of the computer program allowed us to control single-release volumes of liquid as well as the total number of such single releases available to any individual. The amount of liquid dispensed depended on the opening duration of the magnetic pinch valve, which was calibrated beforehand. To avoid the continuous release of rewards, an individual had to leave the water dispenser for at least ten seconds before a subsequent visit was rewarded.

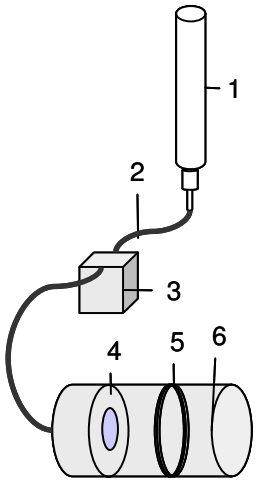

Figure 1: Computer-controlled water dispenser for water supply and BrdU administration: (1) liquid reservoir (luer-lock syringe), (2) silicone tube, (3) solenoid pinch valve, (4) liquid port with infrared photosensor, (5) transponder antenna and (6) Perspex cylinder.

3.3 BrdU application

Animals received 100 µg 5-bromo-2'-deoxyuridine (BrdU, Sigma, Germany) per gram body weight daily for four consecutive days. One group (injection group, n = 4) was injected with BrdU dissolved in 0.9% NaCl solution (10 mg/ml), the second group (oral group, n = 6) received BrdU dissolved in drinking water (2.2 mg/ml). Both treatments with BrdU started with the beginning of the activity phase after lights off. To facilitate acceptance of the BrdU-water solution this was sweetened (1.6% Cyclamate/Saccharine, Huxol, Germany) and, in addition, the oral group was water-deprived 2 h prior to BrdU application. BrdU solution was released in amounts of 140 or 160 µl per single visit. To

obtain a total volume of 1.4 ml one mouse had to visit the dispenser 9 or 10 times. Once an animal had reached this number, further visits did not lead to any more liquid release. However, the dispenser continued to operate until each animal of the group had been given its daily dose. Thereafter, the BrdU solution was replaced with regular, unsweetened water released in smaller amounts of 100 µl per single visit. The injection group always had free access to a water bottle.

3.4 Tissue preparation

One day after final BrdU administration the animals were deeply anesthetized with pentobarbital (160 µg/g body weight, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed, postfixed over night in the fixative and immersed in 30% sucrose in 0.1 M phosphate buffer for at least 24 h for cryoprotection. After shock-freezing in Isopentane at -55 .C brains were cut into 40 µm-thick coronal sections on a cryostat (Leica). The sections were stored at -20 .C in cryoprotectant containing 44% glycerol, 8.6% sucrose, 6.9 mM $MgCl_2 \cdot 6H_2O$ in 0.05 M phosphate buffer.

3.5 Immunohistochemistry

Free-floating sections were incubated in 0.6% H_2O_2 in 10 mM phosphate-buffered saline solution containing 0.1% Triton X-100 (PBS/T) at room temperature (RT) for 30 min. to block endogenous peroxidase. For DNA-denaturation, free-floating sections were incubated in 2 N HCl at 37°C for 1 h followed by several rinse-steps in 0.1 M boric acid buffer (pH 8.5). Sections were incubated with the primary antibody, a monoclonal mouse anti-BrdU (1:400, Roche Diagnostics) overnight at 4°C, then for 2 h at RT with biotinylated goat anti-mouse secondary antibody, followed by incubation with avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories) at RT for 1 h. For staining reaction, sections were treated with 0.03% Diaminobenzidine (DAB) as chromogen, 100 mM $NiSO_4 \times 6H_2O$ and 0.003% H_2O_2. Between all steps, sections were rinsed several times in PBS/T. Additionally a mouse-on-mouse kit (M.O.M., Vector Laboratories) was used to reduce background staining. Sections were mounted on gelatin-coated slides, air-dried and cover slipped with DePeX (Serva, Germany).

3.6 Quantification

The number of BrdU-positive cells was assessed 1 day after the last BrdU administration. BrdU-positive cells were counted in every sixth 40 µm-thick brain section (240 µm interval) throughout the granule cell layer of the dentate gyrus in one randomly chosen hemisphere. Hemispheres had previously been shown not to differ in cell count (Mann–Whitney, $T_{39} = 1565$, $p = 0.97$, unpublished). Photomicrographs were taken with a CCD-camera (SPOT RT, Diagnostic Instruments) via a microscope (Axioskop, Zeiss) and analyzed using MetaMorph software (Visitron). Single pictures were combined digitally using a computer-controlled XY-translation stage (Märzhäuser, Germany) together with an image-stitching software module (Meta-Morph) to obtain a composite picture of the structure. Regions of interest were outlined by tracing the well-defined border between granule cell layer (GCL) and hilus according to a stereotaxic mouse atlas (Paxinos and Franklin, 2001). The subgranular zone (SGZ) was defined as a 20 µm ribbon at the hilus side of the border line. Points within the GCL, SGZ and hilus that conformed to predefined criteria, i.e. minimum/maximum area, were counted, subject to manually set gray level thresholds. The number of counted cells was multiplied by 6 to provide an estimate for the total number of BrdU-positive cells per dentate gyrus. All data shown are mean values ± S.E.M.

4 Results

4.1 Integration of oral BrdU application into natural behavior

Behavioral analysis was performed with data from one group of our socially-housed mice with oral BrdU administration ($n = 3$) using visits at the water dispenser as an indicator of activity. Mice ingested 9.2 ± 0.7 ml of water daily from visiting the water dispenser 85 ± 7 times, as measured for 3 days prior to BrdU administration (control condition). Activity started shortly after lights off and consumption rose to a peak of 0.9 ml/h during the first half of the dark phase (Fig. 2A). During the dark phase, water uptake was significantly higher than during the light phase (paired t-test, $t = 4.92$, d.f. = 8, $P < 0.05$). Small standard errors indicate that mice living in a group synchronized their activity phases.

The procedure for one BrdU injection took about half a minute per animal, whereas BrdU treatment via drinking water lasted several minutes to hours (see Fig. 3). On average, a single individual in the oral group needed 166 ± 22 min (range 25–295 min, Fig. 3) to

ingest 1.4 ml BrdU solution from nine visits at the dispenser. As with regular water uptake, mice started drinking BrdU solution shortly after lights off (Fig. 2). There was no difference between control and BrdU condition in the oral group with regard to general behavior pattern, for example activity distribution over light and dark phases (ANOVA, $F_{(1,10)} = 0.23$, $p = 0.64$), or between the total amount of daily water uptake between control conditions (9.2 ± 0.7 ml) and BrdU administration (9.7 ± 0.7 ml, ANOVA, $F(1,13) = 0.28$, $p = 0.6$).

While it needed a fraction of a second to release one drop of solution through the valve to the feeder opening, the mice stayed on average for 19.1 s (±4.5 s at 160 µl) with their heads within the water dispenser opening. This extended time spent at the feeder port makes it highly likely that all solution released was quantitatively licked up and thus imbibed by the animals.

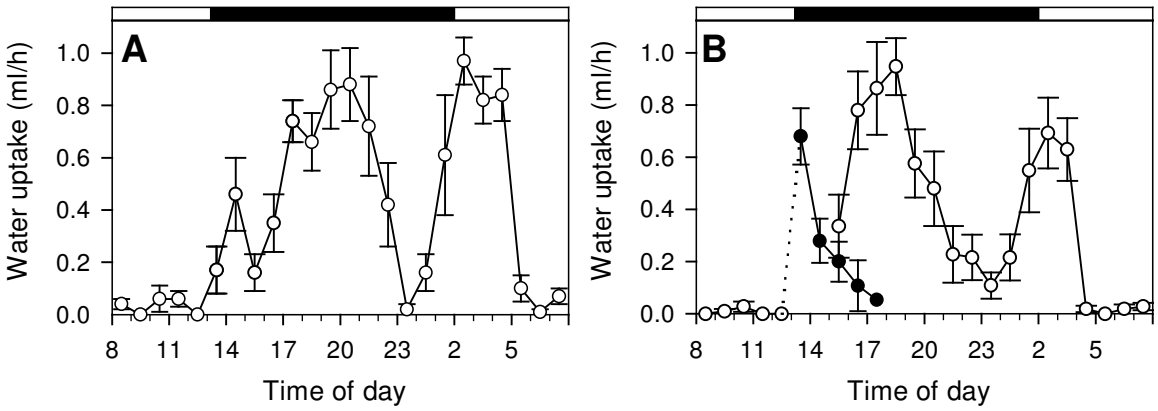

Figure 2: Daily water uptake by mice. (A) Water uptake during regular maintenance (data from n = 3 individuals over 3 days ± S.E.M.). (B) Uptake of BrdU solution (black symbols) from the beginning of dark phase (13:00 h) and water uptake (white symbols) before and after BrdU administration. Regular drinking water availability in B commenced after all individuals had ingested their daily doses of BrdU (means ± S.E.M. from three mice over 4 days). Top bar indicates experimental light (white) and dark phases (black).

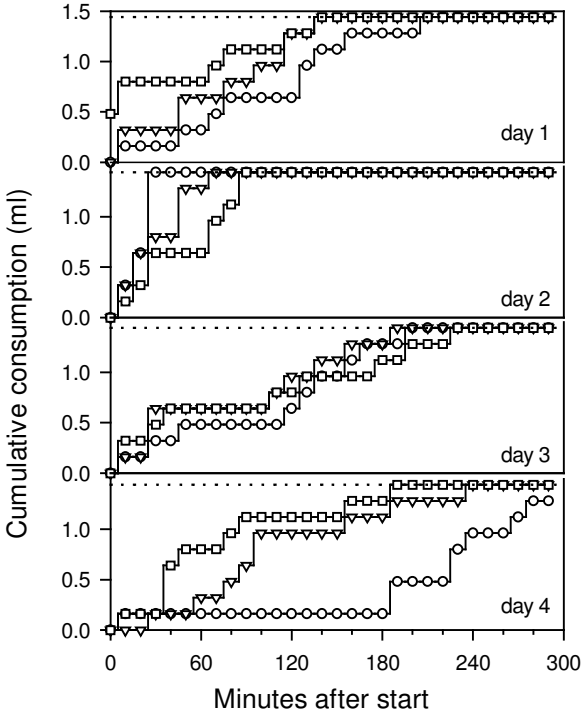

Figure 3: Time course of cumulative uptake of BrdU solution from automatic dispenser by three transponder-tagged individuals on days 1–4 of BrdU treatment. Symbols designate three different individuals, dashed line indicates the cumulative limit of daily dose (1.4 ml BrdU solution).

4.2 Effect of BrdU treatment on BrdU-positive cells

Histological appearance of the sections (Fig. 4A and B) and single cells (Fig. 4C and D) did not differ between oral group and injection group. In addition, intensity of DAB-stained cells, measured as ratio of cell average gray value to threshold value, was not different between groups (ANOVA, $F_{(1,4)} = 2.409$, $p = 0.2$). The estimated total numbers of BrdU-positive cells per dentate gyrus hemisphere averaged across animals were 1371 ± 165 (injected) and 1107 ± 156 (oral), which did not differ statistically between treatments (t-test, $t = 1.13$, d.f. = 8, $P = 0.29$; Levene test for equal variance, $P = 0.891$).

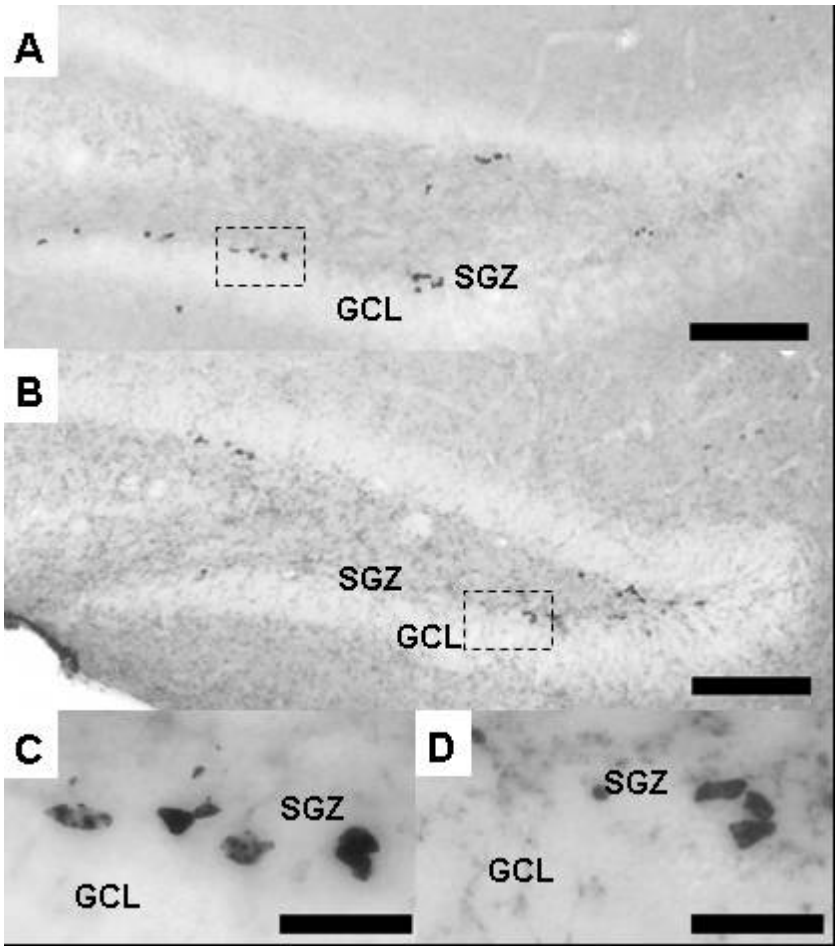

Figure 4: Photomicrographs of BrdU-positive cells in the dentate gyrus of mice after BrdU injections (A, C) or oral BrdU administration (B, D). C and D show details from A and B (rectangles). GCL: granule cell layer, SGZ: subgranular zone. Scale bar = 100 µm (A, B) and 20 µm (C, D).

5 Discussion

5.1 Functionality of oral BrdU administration

Histological results show that oral application of BrdU is highly suitable for the study of cell proliferation in laboratory animals. Overall histological appearance of sections and single cells, and also quantified intensities of DAB-stained cells, did not differ between oral group and injected group. The equality of variance between groups indicates the same reliability of quantification after both treatments. It is well known that numbers of labeled BrdU cells depend on the dose administered (Cameron and McKay, 2001). Such dosage, of course, can be adapted to experimental requirements because comparability and relative changes of cell counts within a study are often more important than absolute numbers. In our results, cell counts appeared to be slightly higher in mice after injection as compared to oral BrdU administration. However, even this difference, which may easily be caused by differences in unknown details of uptake kinetics, was not statistically

significant. The time needed for BrdU to reach detectability is about 10 min; rapid DNA labeling continues for 40 min but then continued incorporation at low levels persists only for 4–8 h after a single injection (Nowakowski et al., 2002). This short duration of activity is often countered by applying BrdU several times during a single day or over several consecutive days (Kempermann et al., 2003; Lu et al., 2003; Holmes et al., 2004). Larger numbers of cells are thereby labeled, or the effect of cyclic events such as the female estrogen cycle is eliminated. Attempting to provide such extended labeling via drinking water, however, is not easy. BrdU can be toxic and different strains of mice may respond differently to the same dosage. A two-week ad libitum administration of BrdU in drinking water at 20 mg per day (as compared to the 3 mg used here), for example, proved lethal to B6 mice (Jecker et al., 1997). For such requirements, the application of BrdU by an automated water dispenser provides an obvious advantage. Defined and limited doses of BrdU can be applied within predetermined time windows within a day and this can be repeated over several day-periods. For extended applications, a BrdU dispenser can be programmed to alternate with a second dispenser providing regular water. No experimenter intervention is required, even for more complicated protocols of drug application. Thus, the extended duration of oral BrdU administration without animal handling should facilitate the labeling of a larger cohort of dividing cells over a longer time interval.

5.2 Oral administration of drugs with individual dosage even in socially-living animals

Computer control of liquid release permits the experimental variation of parameters such as delivery volume per visit, timing of liquid availability, or total quantity released. In combination with individual transponder-tagging, each parameter can be specified on an individual basis even for a whole group sharing a single dispenser. Behavior of animals as recorded by the computer through visits at the dispenser was undisturbed during the whole experimental episode. Administration of BrdU via the dispenser had no influence on behavioral patterns, measured as activity over the day. The controlled water dispenser can be integrated into behavioral experiments without itself affecting the animals. With two dispensers, any temporal schedule of regular water versus drug administration can be realized on an individual basis.

One remaining concern may be precision of dosage, as this method lacks the certainty of a clean injection. However, we do not think this to be critical for many applications (see also Mitz, 2005). We provided only very small droplets of drug solution

during each drinking event, 140 or 160 µl. From our experience the volume of a single bolus could be reduced even further to a droplet of 20 µl. Mice made considerable efforts to imbibe all solution by licking the water port for 10 s or longer. This makes it unlikely that spillage of drug solution will be a serious source of error. The high degree of automation reduces the need for an experimenter to be present. Behavioral experiments run undisrupted because handling of animals is not required. We consider this feature to be highly relevant in view of the troubling findings of the carefully standardized three-sites study by Crabbe and co-workers (Crabbe et al., 1999; Wahlsten et al., 2003c). Here, matched groups of genetically identical mice subjected to identical procedures produced large variation in the results of several behavioral assays. Differences in animal handling by the staff and movements of the workers are among the potential variables held responsible for this outcome (Wahlsten et al., 2003c; Lathe et al., 2004). A machine-controlled environment circumvents the problematic human factor.

Our system can be adapted to various experimental designs and animal species. We have successfully used multiple dispensers within a single home-cage, or distributed within a larger experimental arena representing an artificial habitat for mice. This highly automated system is suitable for any experiments with liquids used as reward or in experiments in which drug application is required. For phenotyping experiments combining near-natural behavioral tests with neural, cellular, or molecular analyses this method should prove to be highly useful.

Appendix A. Contact information for suppliers

Complete units can be obtained through Animal Cognition Systems
http://www.animal-cognition-systems.com.

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Chapter 2

Neurogenesis in adult mice is not enhanced in a naturalistic environment but only by unnatural locomotion

1 Abstract

Throughout life, new cells are generated in the mammalian brain and incorporated as functional neurons in the networks of the olfactory bulb and the dentate gyrus (DG) of the hippocampal formation. So far, proliferation and survival rates of newly generated cells in the adult DG have been investigated in rather simple behavioral experiments like the Morris water maze, fear and trace conditioning, a running wheel and small enriched environments. In the present approach, cell proliferation and survival in the DG of adult mice were compared under different levels of poor and naturalistic conditions: 1) living in a large room (11.5 m^2) poor in stimuli with one computer-controlled water dispenser, 2) exploring a large (15.9 m^2), novel and complex room equipped with 11 of these dispensers, 3) spatial learning in this complex room, 4) running in a running wheel and 5) running in plane implemented by a tube system. Mice in large rooms lived socially in groups and received water exclusively via computer-controlled dispensers. In order to keep animals undisturbed as far as possible, administration of the proliferation marker BrdU was accomplished orally via one of the dispensers dissolved in drinking water (see Santoso et al., J Neurosci Meth 2006). The results showed that mice in our naturalistic environment optimize their feeding behavior when dispenser qualities are changed and therefore learned and remembered the locations of high quality dispensers. However, cell proliferation and survival in the DG of adult mice were unaffected by i) living in a room poor in stimuli with much space for exercise, ii) exploring a novel complex naturalistic environment and iii) associative learning in a naturalistic environment. The only condition that led to a considerable increase in the number of newly generated cells was the running wheel condition. Additionally, the present results indicate that the influence of physical activity on cell proliferation is independent from locomotion type when comparing wheel and in plane running, but strongly correlates with the amount of running activity. Furthermore, our results showed that the pro-proliferating effect of running persists for at least 3 but not longer than 5 days after withdrawal of the running wheel. Thus, we suggest that cell

proliferation in the adult DG is strongly correlated to running activity and not correlated to the complexity of associative learning in a naturalistic environment.

2 Introduction

The production of new cells in the mammalian brain throughout life has been documented in a variety of species, including rodents (Gould et al., 1992; Luskin, 1993; Kempermann et al., 1997a), primates (Gould et al., 1999c; Bernier et al., 2002) and humans (Eriksson et al., 1998). Newborn cells in limited numbers have been detected in several brain regions, for example in the amygdala (Bernier et al., 2002), hypothalamus (Fowler et al., 2002) and neocortex (Gould et al., 1999c), but their origin is still unknown. Only two regions of the adult brain are known to act as proliferative zones that sustain producing new cells. Progenitor cells of the subventricular zone (SVZ), a narrow tissue zone within the walls of the lateral ventricles, generate cells that migrate along the so-called rostral migratory stream into the olfactory bulb (Luskin, 1993). The second neurogenic region is the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampal formation, where new cells are produced that partly become functional neurons in the granule cell layer (GCL) of the DG (van Praag et al., 2002; Carlen et al., 2002). A part of the newborn cells undergoes apoptosis shortly after birth (Biebl et al., 2000) and not all of the surviving cells express neuronal markers, i.e. differentiate into neurons. The different stages of this process, proliferation, survival and differentiation, are not implicitly dependent from each other and, therefore, can be regulated separately (see Prickaerts et al., 2004). Adult neurogenesis in the DG is influenced by a variety of factors (reviewed in Lehmann et al., 2005). Several intrinsic factors, including growth factors, neurotransmitters (Cameron et al., 1998) and hormones (Gould et al., 1992; Ambrogini et al., 2002; Tanapat et al., 1999) are supposed to influence cell proliferation and neurogenesis in the adult brain. Genetic background and age also play a role in baseline hippocampal neurogenesis (Bizon & Gallagher, 2003; Kempermann et al., 1997a) and, furthermore, determine the sensibility of neurogenesis to environmental challenges (Kempermann et al., 1998a; Kronenberg et al., 2005; van Praag et al., 2005).

Concerning the functional relevance of adult neurogenesis, external regulators are of great importance. A great deal of research has been done on the response of hippocampal plasticity following different behavioral features. Stressful experiences can diminish cell proliferation regulated by NMDA-receptor activation and elevated glucocorticoid levels (for review see Gould & Tanapat, 1999). In particular, severe stress induced according to a resident-intruder model in marmoset monkeys (Gould et al., 1998), by male-male

encounters in tree shrews (Gould et al., 1997) or by exposure of rats to fox odor (Tanapat et al., 2001) resulted in a rapid decrease of cell production in the DG. Beneficial effects on adult neurogenesis have been reported for the enrichment of housing but, unfortunately, not all studies investigating this condition agree with each other (Table 1). The majority of studies reported an increased number of surviving cells, but no difference in cell proliferation in the DG of animals living in an enriched environment compared to the DG of controls (Kempermann et al., 1997b; Kempermann et al., 1998b; Ehninger & Kempermann, 2003; Brown et al., 2003; Nilsson et al., 1999). However, environmental enrichment was associated with an increase of hippocampal cell proliferation in the 129/SvJ mouse strain (Kempermann et al., 1998a) or after long-term enrichment in old mice (Kempermann et al., 2002). A higher number of new neurons without a concomitant increase of proliferation rate reveals an enhanced survival rate and, hence, a survival promoting effect of environmental enrichment. But interestingly, Kempermann et al. (2002) and Kozorovitskiy and Gould (2004) did not find an increased number of surviving new cells at all in enriched living animals compared to control animals.

Table 1: Overview of reported effects of environmental enrichment on cell proliferation and survival of newborn cells in the dentate gyrus (DG). Enriched environments could include a running wheel (wheel +) and additional food (add. food +) or not (-). Duration specifies the time period that animals (species) lived in their respective environment. In some studies, animals were trained in the Morris water maze task (MWM) following BrdU treatment.

Enrichment (wheel/ add. food)	Duration	Species	First BrdU-injection	Effect on proliferation	Effect on survival	Tests	Reference
Large cage (+/-)	12 and 42 days	Mouse (C57BL/6)	Day 1	→	↑	-	(van Praag et al., 1999b)
Large cage (+/+)	40 and 68 days	Mouse (C57BL/6)	Day 40	→	↑	MWM	(Kempermann et al., 1997; 1998b)
Large cage (+/+)	6 month	Mouse (C57BL/6)	Month 5	→	↑	MWM	(Kempermann & Gage, 1999)
	3 month			↑	↑		
Large cage (-/+)	4 and 8 weeks	Rat (Sprague-Dawley)	Day 26	→	↑	MWM	(Nilsson et al., 1999)
Large cage (+/-)	12 and 43 days	Mouse (C57BL/6)	Day 1	→	↑	-	(Brown et al., 2003)
Large cage (-/-)	40 days	Mouse (C57BL/6)	Day 30	→	↑	-	(Ehninger & Kempermann, 2003)
Large cage (+/+)	40 days	Mouse (129/SvJ)	Day 28	↑	↑	MWM	(Kempermann et al., 1998a)
Large cage (+/n.s.)	105 days	Mouse (129/SvJ)	Day 102	↑		-	(Meshi et al., 2006)
Large cage (+/-)	10 month	Mouse (C57BL/6)	Month 9	↑	→	-	(Kempermann et al., 2002)
Preweaning Small box	14 days, 15-60 min/d	Mouse pups (C57BL/6)	Month 3	→	→	-	(Kohl et al., 2002)
Visible borrow system (-/-)	21 days	Rat (Sprague-Dawley)	Day 4		→	-	(Kozorovitskiy & Gould, 2004)

The role of the hippocampus in learning and memory processing together with the occurrence of neurogenesis in this specified region suggests an active role of new neurons

in memory formation. Indeed, some factors regulating neurogenesis in a positive (running, environmental enrichment) or a negative (stress, age) manner, in parallel enhance or impair learning performance, respectively (see Gould et al., 1999d). Commonly used behavioral tests for the investigation of learning in the laboratory are, among others, the Morris water maze and contextual fear conditioning. In the water maze task, rodents have to find a submerged platform in a water basin. The reports of adult neurogenesis following water maze training vary from increased (Gould et al., 1999a) to unaffected (van Praag et al., 1999b) and even decreased (Ambrogini et al., 2004) amounts of newborn cells (Table 2). Gould et al. (1999a) found an enhancement of plasticity in the DG specific to hippocampus-dependent learning. Training in hippocampal-dependent tasks in a Morris water maze and in trace eyeblink conditioning led to an increased number of surviving cells, whereas non-hippocampal-dependent tasks did not. In contrast, Van Praag et al. (1999b) observed equal numbers of newborn and surviving cells in mice trained in a water maze task compared to control mice. Yet, Ambrogini et al. (2004) reported a reduction of surviving cells after water maze training. In the fear conditioning paradigm, animals received an aversive unconditioned stimulus (foot shock) and expressed conditioned fear responses when placed in the conditioning chamber, because the chamber acts as conditioned stimulus. This hippocampus-dependent paradigm has no effect on the survival rate of newborn cells and, furthermore, diminishes the production of new cells in the DG of rats (Pham et al., 2005). In turn, the formation of new neurons is not essential for accomplishment of the water maze task and the fear conditioning process, because specific blocking of cell proliferation with the toxin methylazoxymethanolacetate (MAM) did not impair the performance in these tasks (Shors et al., 2002).

Table 2: Overview of reported effects of learning on cell proliferation and survival of newborn cells in the dentate gyrus (DG). Morris water maze task (MWM), eyeblink conditioning (EB), fear conditioning or social transmission of food preference were applied as learning tasks under hippocampus-dependent (+) or –independent (-) conditions. The time point of BrdU treatment is given with respect to the training period.

Learning task	Hippocampus-dependency	Species	BrdU-injections	Effect on proliferation	Effect on survival	Reference
MWM place EB trace MWM cue EB delay	+ + - -	Rat (Sprague-Dawley)	7 days before		↑ ↑ → →	(Gould et al., 1999)
MWM	+	Mouse (C57BL/6)	at start	→	→	(van Praag et al., 1999c)
MWM	+	Rat (Fisher)	direct after		→	(Merrill et al., 2003)
MWM place MWM cue	+ -	Rat (Sprague-Dawley)	8 days before		↓ →	(Ambrogini et al., 2004)
contextual fear conditioning	+	Rat (Sprague-Dawley)	direct after or 10 days before	↓	→	(Pham et al., 2005)
Social transmission of food preference	+	Rat (Long Evans)	8-13 days before	→	↑ (1 d training) ↓ (2 d training)	(Olariu et al., 2005)

Learning task	Hippocampus-dependency	Species	BrdU-injections		Effect on learning performance	Reference
EB trace EB delay	+ -	Rat (Sprague-Dawley)	2-6 days before	Proliferation blocked	↓ →	(Shors et al., 2001)
MWM Context. fear Trace fear	+ + +	Rat (Sprague-Dawley)	2-6 days before		→ → ↓	(Shors et al., 2002)

Indisputable is the influence of physical activity on cell proliferation and neurogenesis, because the literature agrees about the substantial pro-proliferating effect of running in a running wheel in rodents (Eadie et al., 2005; Kronenberg et al., 2005; van Praag et al., 1999a; 2005, Table 3). Therefore, it has to be mentioned that physical activity is a factor that is integrated in environmental enrichment as well as in maze learning tasks. Literally, this is the case if running wheels are a part of the enriched environment (Kempermann et al., 1997b; 1998a; 1998b; Brown et al., 2003; van Praag et al., 1999b).

Table 3: Overview of reported effects of physical activity on cell proliferation and survival of newborn cells in the dentate gyrus (DG). The time point of BrdU treatment is given with respect to the training period (Duration). In some studies animals had to perform the Morris water maze task (MWM) during the training period and Stranahan et al. (2006) tested mice living socially in groups and housed individually.

Locomotion type	Duration	Species	BrdU-injections	Effect on proliferation	Effect on survival	Tests	Reference
Wheel	2-3 weeks	Rat (Sprague-Dawley)	end	↑		-	(Eadie et al., 2005)
Wheel	3 days 10 days 32 days	Mouse (C57BL/6)	end	↑ ↑ →		-	(Kronenberg et al., 2005)
Wheel	9 days	Mouse (C57BL/6)	none (Ki-67)	↑		-	(van der Borght et al., 2006)
Wheel	1.5-4 month	Mouse (C57BL/6)	start		↑	MWM	(van Praag et al., 1999a; 2005)
Wheel	21 days	Mouse (C57BL/6)	end	↑	↑	-	(Kitamura et al., 2003)
Wheel	7-28 days	Mouse (C57BL/6)	light phase lights off dark phase	→ → ↑	→ → ↑	-	(Holmes et al., 2004)
Wheel	40 days	Mouse (Hsd:ICR)	start		↑	MWM	(Rhodes et al., 2003)
Wheel	11 days 11 days 3-24 days 48 days	Rat (Sprague-Dawley)	start start end end	↑ ↓ → ↑		grouped individually individually individually	(Stranahan et al., 2006)
Wheel	9 days 24 days	Rat (SHR)	end	↑ ↓		-	(Naylor et al., 2005)
Treadmill	7 days	Rat (Sprague-Dawley)	start	↑		-	(Kim et al., 2002)
Treadmill Swimming	4 days	Rat (Sprague-Dawley)	end	↑ ↑		-	(Ra et al., 2002)
Wheel Swimming	12-40 days	Mouse (C57BL/6)	start	↑ →	↑ →	-	(van Praag et al., 1999b)

The literature provides some evidence that cell proliferation and neurogenesis in the mammalian DG are a form of plasticity that contributes to cognitive function and is sensitive to a variety of internal and external factors. Conflicting results, even when standardized methods were used, reflect the difficulty of making general predictions about the functional role of neurogenesis, because results strongly depend on the specific condition or task and its execution. However, the investigation of specific features is important for understanding the mechanisms of adult neurogenesis, but does not conclusively give evidence about the involvement of cell production in the context of natural behavior.

Our approach concerned the interaction of three of the previously named factors as they occur in a natural context. Naturally, behavior involves physical activity, exploration of novel objects or environments and associative learning for remembering profitable feeding locations. Therefore, we enunciated the following hypotheses on the effect of these factors on cell proliferation and survival:

I) Cell proliferation and survival is stimulated solely by physical activity. Physical activity plays a major role in the behavior of animals. Adult neurogenesis increases with

voluntary exercise and in an enriched environment, which includes the facility for high levels of activity.

II) Neurogenesis is involved in the processing of new, spatial information. The increase of cell proliferation following running in a running wheel (e.g. van Praag et al., 1999b) is not caused by physical activity per se, but rather by actualization of the cognitive map in the brain. Running induces the need for map actualization because usually locomotion is associated with cognitive information that is processed with involvement of neuronal plasticity in DG (see Jacobs & Schenk, 2003). Hence, adult hippocampal neurogenesis is influenced by the processing of new spatial information that is required, exemplarily, for exploring a novel environment.

III) Neurogenesis occurs in association with hippocampus-dependent learning and is amplified when associative learning is required. Cell proliferation and neurogenesis serve as neuronal substrate for learning and memory processes in hippocampal-dependent learning tasks (see Gould et al., 1999a).

In order to test these hypotheses with respect to a natural context, we designed experiments to be conducted in a semi-natural environment. This computer-controlled environment provided much space to move, social interaction and undisturbed behavior. By confronting mice with this condition as a novel environment and the challenge of adopting foraging behavior by varying dispenser qualities, we emulated situations that mice have to cope with in nature. The aim of this study was to investigate the response of cell proliferation and survival rate to natural-like situations in a semi-naturalistic environment. Therefore, groups of mice lived in a large impoverished enclosure with one computer-controlled water dispenser or a naturalistic environment represented by a large enclosure containing obstacles and 10 computer-controlled water dispensers. This naturalistic environment either was new to animals or challenged optimal foraging behavior. Hypothesis I would be corroborated if proliferation increases in all conditions compared to controls, because physical activity occurs irrespective of environmental complexity or behavioral task. Hypothesis II would be corroborated if enhancement of neurogenesis is highest in mice exploring the novel complex environment. In this case, the impoverished environment would have no effect on proliferation and survival rate and associative learning in the complex environment would have no or only a small effect due to the processing of spatial information about changing locations of profitable water dispensers. Hypothesis III would be corroborated if the highest increase of neurogenesis occurs in mice expressing associative learning by memorizing profitable water locations in the complex environment. In this case, the impoverished environment would again have no

effect on proliferation and survival rate. Exploring the novel environment would have no or only a small effect due to the processing of spatial information of dispenser locations in the novel environment.

Additionally, we further examined the role physical activity on cell proliferation in order to associate this prominent feature with behavior in a naturalistic environment. Therefore, we investigated the duration of persistence of the enhanced proliferation rate induced by running wheel activity and controlled for the influence of locomotion type by opposing wheel running, a rather unnatural form of locomotion, and running in plane as natural form.

All mice received the proliferation marker 5-bromo-2'-deoxyuridine (BrdU) to evaluate the number of newborn cells in DG.

3 Materials and methods

3.1 General methods

Experiments were conducted to examine the effect of different environmental and behavioral conditions on cell proliferation and survival of newborn cells in the hippocampus. Therefore, the following general methods were applied to all experimental groups. Exceptions and additional methods are described for each experiment below.

3.1.1 Animals

All subjects of this study were mice from the outbred stock CD-1. We used only females, because male mice show territorial behavior. This, on the one hand could have affected learning experiments that were conducted in grouped living mice. On the other hand, stress in subordinate males decreases adult hippocampal neurogenesis (Gould et al., 1997). At the beginning of experiments, mice were between 8 and 11 weeks old, weighing 27.3 g (± 2.9 SD). During all experiments, mice had free access to food. Animals sharing one or more computer controlled water dispensers were labeled with transponders for individual identification. Therefore, sterilized transponders encapsulated in biocompatible glass (2.1 mm × 12 mm, 0.09 g, Unique, Sokymat) were injected under isoflurane anesthetic subcutaneously between the shoulder blades using an injector (Trovan).

For detailed information about animals and general animal treatment see Appendix (A.1).

3.1.2 Water dispensers

Computer-controlled water dispensers were designed for the controlled delivery of water to individuals living together in a group (see Santoso et al., 2006). The main components of the dispenser were a photoelectric barrier, a transponder-reading device and a tube system for liquid conduction (see Figure 1). When entering the dispenser, the transponder tagged mouse interrupted the photoelectric barrier. Followed by identification of the individual by the reading device, opening of the magnetic pinch valve that controlled the silicon tube was actuated. The water released (20 - 160 µl single volume) was licked off the liquid port. The opening duration of the valve (40 - 600 ms) determined the volume of water released and was predefined according to the experimental protocol for each specific dispenser. Dispensers were blocked (10 to 60 sec.) for subsequent visits of the same individual at the same dispenser to avoid permanent water release. Input parameters, i.e. interruptions of photoelectric barrier and transponder-IDs were recorded and output parameters, for example the time point of visit and opening duration of valves, were computer-controlled using a self-written program (Versuch by Y. Winter). For technical details of computer-controlled dispensers see Winter & Stich (2005).

For cage experiments, one or two dispensers were mounted to the cage and directly accessible to mice. For experiments in large enclosures (see 3.1.3), water dispensers were fixed to the ceiling of the room about 80 cm above the floor. Each dispenser was accessible for mice by a 1 m long rope that was fixed to the floor at one end and to the dispenser entry at the other end.

3.1.3 Naturalistic and simple environment

The naturalistic environment was designed to provide much space for locomotion, environmental enrichment and the facility to investigate and use mouse behavior based on foraging activity. Therefore, a large enclosure with an area of 15.9 m^2 (5.92×2.81 m with a 1.64×0.47 m recess) was bounded by a 72 cm high metal wall. This complex room (Figure 5, right) contained 11 water dispensers and 11 obstacles in terms of opaque, 20 cm high plastic plates of different lengths (20, 40 and 80 cm).

The simple room (Figure 5, left) was designed to provide much space for locomotion similar to the complex room, but in contrast, this room was poor in stimuli. Therefore, a second large enclosure with an area of 11.5 m^2 (5.0×2.3 m), bounded by a 72 cm high metal wall, contained only one dispenser in the middle of the room.

In both rooms, a standard laboratory cage without cover served as home cage and was freely accessible for mice via ramps and holes in the cage wall. Cleaning papers, paper rolls and cartons were provided as nest-building material.

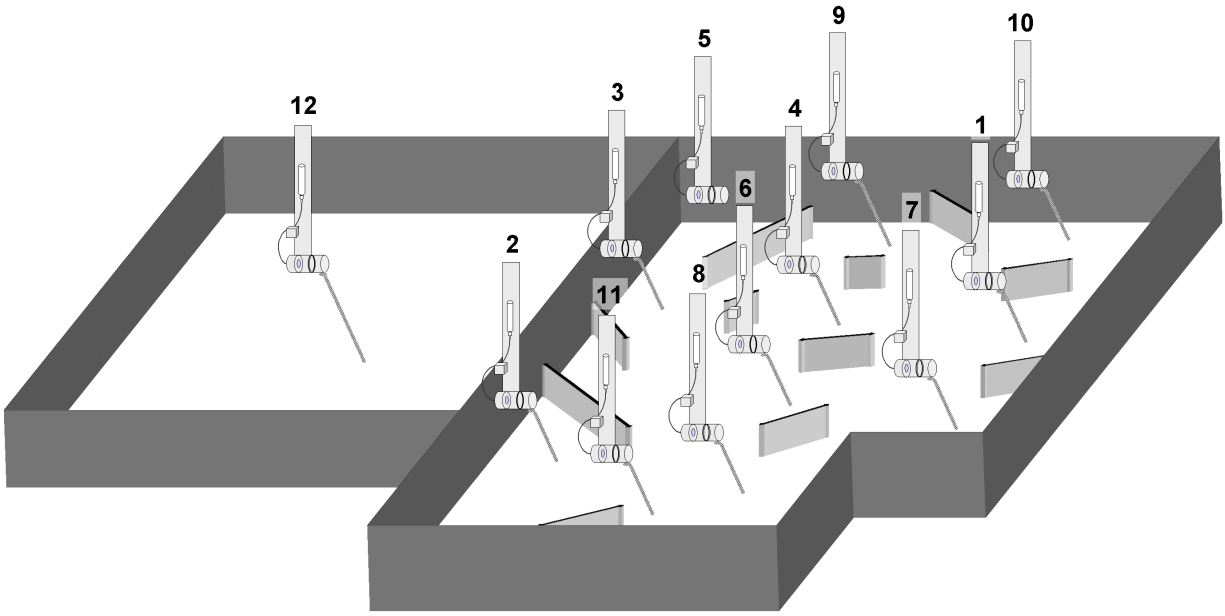

Figure 5: Schematic of the simple (left) and complex (right) room. A 72 cm high metal wall bound the rooms. Dispensers 1 to 11 were distributed over the complex room (15.9 m^2) and dispenser 12 was placed in the middle of the simple room (11.5 m^2). 11 opaque plastic plates of different lengths (20, 40 or 80 cm) and 20 cm in height served as obstacles in the complex room.

3.1.4 Running wheels and running tubes

A running wheel with a diameter of 14.5 cm was fixed up to the wire mesh lid of a standard laboratory cage. Recording of wheel revolutions by a photoelectric barrier allowed for the measure of distance and velocity of running. Furthermore, the maximum running distance could be limited through computer-controlled activation of a motorized lock mechanism (for details see app. A.2.2).

For the investigation of running in plane, one cage was equipped with two U-shaped tubes, each 4.3 m in length. One end of both tubes was fixed to the cage and a swinging wicket combined with a photoelectric barrier recorded every pass from cage to tube and vice versa. A dispenser at the other end of each tube provided the water delivery. The two dispensers released water alternating, i.e. once a mouse had received water at one dispenser it had to run 8.6 m through both tubes to obtain water at the other dispenser (for detail see Appendix A.2.1).

3.1.5 BrdU

5-bromo-2'-deoxyuridine (BrdU, Sigma, Germany) is a cell proliferation marker that incorporates into DNA during cell cycle. BrdU treatment always started at the beginning of the dark phase, i.e. the activity phase of the animals.

For oral application, BrdU was dissolved in sweetened (1.6 % Cyclamate/Saccharine, Huxol, Germany) drinking water (2.2 mg/ml). Oral administration of BrdU was applied to avoid disruption of animals as far as possible and was previously described in Santoso et al. (2006). Animals received daily doses of 100 µg BrdU per g body weight (BW) on 4 consecutive days via one automatic water dispenser. During BrdU treatment, no other water source was available. Depending on the different tube systems in the experiments, a release volume of 110 to 160 µl per single visit required 9 to 13 visits at the dispenser to ingest the daily total volume of 1.3 to 1.6 ml BrdU solution. Once an animal had reached its cumulative dose, following visits did not lead to further liquid release. As soon as all individuals had ingested their daily dose of BrdU conditions of the respective experimental group applied. The dispensers for BrdU administration were the same that delivered regular drinking water during experiments (except for the complex room, in which the BrdU dispenser was excluded from the experiment). Prior to BrdU treatment, water was removed from the tube system and the tubes were filled with BrdU solution. When BrdU treatment was completed, BrdU solution was replaced by drinking water after thorough rinsing of the tubes with water. Animals were killed one day after BrdU treatment to investigate cell proliferation (post-treatment time PT = 1) or 28 days after BrdU treatment for the investigation of cell survival (PT = 28).

For intraperitoneal injections, BrdU was dissolved in 0.9 % NaCl (10 mg/ml) and filtered sterile at 22 µm. Mice in the complex environment and the associated control mice were injected with single doses of 50 µg BrdU/g BW on 4 consecutive days once a day and were killed 28 days after the last day of BrdU treatment (PT = 28). Mice in cages with running tubes or wheels and their associated control mice (3.2.2) received 3 BrdU injections per day (50 µg/g BW per single dose) with 4-hour intervals between injections. Repeated injections were applied to circumvent potential fluctuations of the number of proliferating cells due to circadian rhythm or different distribution of running activity over the day. These mice were perfused one day after the last day of BrdU treatment (PT = 1). To determine the duration of the persistence of enhanced cell proliferation (3.2.3), mice received one BrdU injection (100 µg/g BW) and were killed 24 hours later (PT = 1).

3.1.6 Tissue preparation

Animals were deeply anesthetized by an intraperitoneal injection of pentobarbital (160 µg/g BW) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (see app. A.4.1). Brains were removed, postfixed over night in the fixative and immersed in 30 % sucrose in 0.1 M phosphate buffer for at least 24 h for cryoprotection. After embedding in 15 % gelatin, brains were cut into 40 µm-thick coronal sections (see app. A.4.2) on a cryostat (Leica). Brain sections were stored at −20°C in cryoprotectant containing 44 % glycerol, 8.6 % sucrose and 6.9 mM $MgCl_2 \times 6\ H_2O$ in 0.05 M phosphate buffer until further processing.

3.1.7 Immunohistochemistry

Free-floating sections were incubated in 0.6 % H_2O_2 in 10 mM phosphate-buffered saline solution containing 0.1 % Triton X-100 (PBS/T) at room temperature (RT) for 30 min. to block endogenous peroxidase. For DNA-denaturation, free-floating sections were incubated in 2 N HCl at 37°C for 30 min. followed by several rinse-steps in 0.1 M boric acid buffer (pH 8.5). Sections were incubated with the primary antibody, a monoclonal mouse anti-BrdU (1:400, Roche Diagnostics) overnight at 4°C, then for 2 h at RT with biotinylated goat anti-mouse secondary antibody, followed by incubation with an avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories) at RT for 1 h. For the staining reaction, sections were treated with 0.03 % Diaminobenzidine (DAB) as chromogen, 100 mM $NiSO_4 \times 6H_2O$ and 0.003 % H_2O_2. Between all steps, sections were rinsed several times in PBS/T. Additionally, a mouse-on-mouse kit (M.O.M, Vector Laboratories) was used to reduce background staining. Sections were mounted on gelatin-coated slides, air-dried and cover slipped with DePeX (Serva, Germany). The immunohistochemical procedure is described in detail in the Appendix (A.4.3).

3.1.8 Data analysis

BrdU-positive cells were counted in every sixth 40 µm-thick brain section (240 µm interval) throughout the dentate gyrus (DG) in one randomly chosen hemisphere. Photomicrographs were taken with a CCD-camera (SPOT RT, Diagnostic Instruments) via a microscope (Axioskop, Zeiss) and analyzed using MetaMorph software (Visitron). Single pictures were combined digitally using a computer-controlled XY-translation stage (Märzhäuser, Germany) together with an image-stitching software module (MetaMorph) to obtain a composite picture of the structure. Regions of interest within DG were outlined by tracing the well-defined border between granule cell layer (GCL) and hilus according to a

stereotaxic mouse atlas (Paxinos & Franklin, 2001). The subgranular zone (SGZ) was defined as 20 µm wide ribbon at the hilus side of the borderline. Points within the GCL and SGZ that conformed to predefined, standardized criteria, i.e. minimum/maximum area, were counted, subject to manually set gray level thresholds. The number of counted cells was multiplied by 6 to provide an estimate for the total number of BrdU-positive cells per DG. For a complete description of this quantification method, refer to App. A.5.

3.1.9 Statistical analysis

All data shown are means ± SEM. Statistical tests were performed using SigmaStat (Systat Software, Inc.). According to experimental design one-, two- or three-way ANOVAs with Fisher's LSD for *post-hoc* tests were performed.

3.2 Experimental design

3.2.1 Simple environment and exploration and learning in a complex environment

This experiment was conducted to investigate cell proliferation and survival in the dentate gyrus of adult mice under different environmental conditions. Beyond classical setups for an enriched environment (Kempermann et al., 1997b; van Praag et al., 1999b), our experimental setup consisted of large enclosures containing one or more automatic dispensers as described above. As main advantages of this setup, animals had much space for free and voluntary movements and experiments ran computer controlled to a great extent. Hence, stressful disturbance by the experimenter, due to handling for example, were reduced to a minimum. A stress free social environment was provided using groups of 3 to 8 individuals in each experiment. Overall, 39 female CD-1 mice from the departmental breeding colony (Dept. of Biology, University of Munich, Germany) were used in this experiment. Animals were divided into 5 groups and subjected to the following experimental treatments (Table 4, Figure 6).

3.2.1.1 Simple environment (SIM)

Group SIM (n = 8) lived socially together in the simple environment described above, which was characterized by much space for voluntary movements but poorness in stimuli. Mice in the simple environment group were allowed to move freely in this large enclosure with a frugal equipment including only a freely accessible home cage and one dispenser in the center of the room (for dispenser attributes refer to app. A.3.1). A water bottle was available in addition to the dispenser at start of the experiment and was removed on day 3. BrdU treatment started on day 6 and was applied on 4 consecutive days. 3 mice

were sacrificed on day 10 (PT = 1) and the 5 remaining animals lived in the room for additional 4 weeks (PT = 28).

3.2.1.2 Exploration in the complex environment (EXP)

The task of group EXP was to explore a novel complex environment. Therefore, this group (n = 8) was placed in the complex environment described above. All 10 dispensers were equal in quality, i.e. released the same amount of water (see app. A.3.2). Prior to the start of the experiment in the complex room, the environment was unknown to the animals. For the first 6 days, mice were housed in a special cage containing one dispenser accessible via a rope to accustom animals to the functioning of the water dispenser. The first 4 days in the novel environment were concomitantly the 4 days of BrdU application. 3 mice were sacrificed on day 10 (PT = 1) and the 5 remaining animals lived in the complex room under constant conditions for additional 4 weeks (PT = 28).

3.2.1.3 Learning in the complex environment (LRN)

A central aspect of group LRN was the challenge of learning and remembering locations in a complex environment. This group (n = 8) lived in the same complex environment as group EXP. In the learning task, mice were confronted with varying combinations of dispensers with changing qualities. One criterion of differentiation was the amount of water per visit. Therefore, one half of the dispensers was of high quality, i.e. released the twofold amount of water per visit as the low quality dispensers. Another criterion was the steadiness of water release. Therefore, one half of the dispensers released constantly the same amount of water per visit, whereas the remaining dispensers randomly varied between high and low amounts. The mean value of water released at variable dispensers equaled the water amount at constant dispensers. The distribution pattern of different dispenser attributes was varied at regular intervals to keep animals at challenge (see app. A.3.3). These conditions were applied to separate groups, one group of 6 mice with PT = 28 (MG 2) and one group of 3 mice with PT = 1 (MG 2-1d).

Table 4: Experimental groups, their conditions (task and environment) and number of individuals (N). All mice received BrdU (100 µg/g BW) orally on 4 days and were killed one (PT = 1) or 28 (PT = 28) days after the last dose of BrdU.

Group	Task	Environment	Posttreatment time (PT)	N
SIM	simple environment	11.5 m^2 room, 1 feeder	1 d	3
			28 d	5
EXP	exploration in complex environment	15.9 m^2 room, 11 feeders, obstacles	1 d	3
			28 d	5
LRN	learning in complex environment	15.9 m^2 room, 11 feeders, obstacles	1 d	3
			28 d	5
CON	control	standard cage	1 d	5
			28 d	6
RUN	positive control, running	standard cage with running wheel	1 d	4

3.2.1.4 Standard laboratory conditions (CON)

The control group (CON, n = 11) was housed in groups of 3 or 5 individuals together in a standard laboratory cage containing one water dispenser. Water delivery via one computer-controlled dispenser was provided *ad libitum*.

3.2.1.5 Running in a running wheel ad libitum (RUN)

As running in a running wheel has a well-documented pro-proliferative effect (e.g. Brown et al., 2003; van Praag et al., 1999b), this condition served as a positive control. The appropriate group RUN (n = 4) was placed socially together in one standard laboratory cage containing one automatic dispenser and one running wheel. Mice were allowed to use the running wheel unrestricted during the experiment.

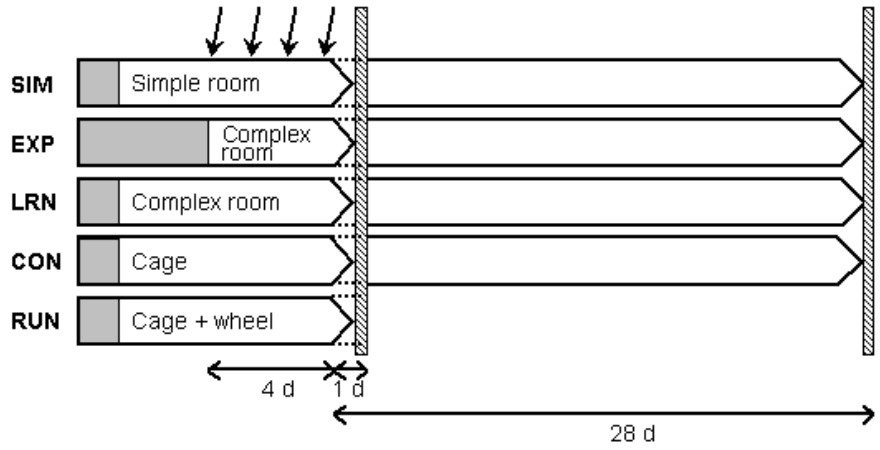

Figure 6: Experimental design for the investigation of the role of different environmental conditions on cell proliferation and survival. Grey areas indicate phases of accustoming animals to automatic feeders prior to start of experimental conditions (white areas). Control group lived in a standard cage (CON), positive control group in a cage containing a running wheel (RUN), simple environment group in an impoverished enclosure (11.5 m^2) with one automatic dispenser (SIM), exploration group in an enclosure (15.9 m^2) with 11 automatic dispensers as novel complex environment (EXP) and learning group in the complex environment with learning tasks (LRN). Mice received BrdU (100 µg/g BW) orally on 4 consecutive days. Arrows indicate BrdU treatment and hatched bars the time point of perfusion.

3.2.2 Locomotion types: wheel versus plane

The aim of this experiment was to investigate the effect two different types of locomotion on hippocampal cell proliferation in a quantitative manner. The two locomotion types were represented by physical activity either in a running wheel or in running tubes. Physical activity was not permitted *ad libitum*, but limited to fixed running distances, determined prior to experiments according to protocol. 30 female CD-1 mice (Charles River Laboratories, Germany) were used in this experiment. Single mice were kept individually in the experimental cages. In each cage, two automatic dispensers provided water supply in an alternating manner. Animals were randomly assigned to running wheel exercise group WHE (n = 18) or running tube exercise group TUB (n = 6). WHE mice were kept in a cage equipped with a running wheel (see app. A.2.2). TUB mice were kept in a cage connected to its two dispensers through two tubes, each 4.3 m in length (see app. A.2.1). Control group (CON) consisted of 6 mice in standard cages without running wheel (n = 3) or containing an immobilized wheel (n = 3). With respect to the fact that the presence of a locked running wheel has no effect on the number of new born cells (see app. 2.7), CON was treated as consistent group. Running distances were limited to values with respect to the lowest individual *ad libitum* distances measured in a pre-experiment (3365 m for WHE and 1006 m for TUB, see app. 2.6). Daily running distances were limited to 250, 500, 1000, 2000 or 4000 m in the running wheel (n = 3 for each

distance) and 400 m (n = 3) or 800 m (n = 2) in the running tubes. Once a mouse had covered its pre-defined distance, the wheel was automatically locked and the tube entries were blocked, respectively. One group of WHE mice was allowed to run *ad libitum* (n = 3) in a permanently unlocked running wheel.

3.2.3 Persistence of enhanced cell proliferation following running activity

In order to examine if and how long the enhancement of cell proliferation induced by running wheel activity persists, mice had free access to a running wheel on 4 consecutive days. At the beginning of the dark phase on day 5, the running wheel was completely removed. Cell proliferation then was analyzed at different time points with respect to removal of the running wheel. 23 female CD-1 mice (Charles River Laboratories, Germany) were used in this experiment. Mice were separately placed in a cage containing a running wheel and had free access to food and water from a bottle. Animals were intraperitoneally injected with one single dose of BrdU (100 µg/g BW). Time point of injection was either on the last day of wheel running (t = 0) or on day 1 (t = 1), day 3 (t = 3) or day 6 (t = 6) after removal of the running wheel (Figure 7). Animals were perfused 1 day after BrdU injection.

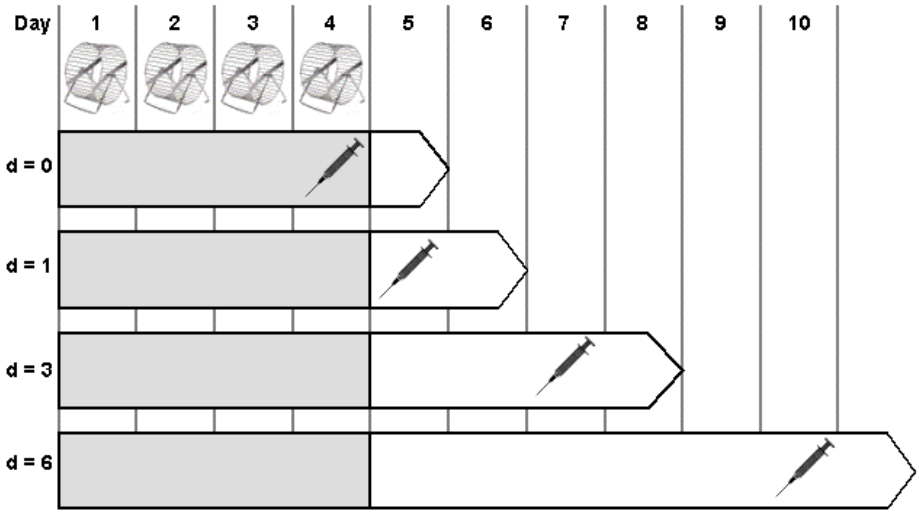

Figure 7: Experimental design for the investigation of the persistence of enhanced cell proliferation induced by running wheel activity. Running wheels indicate the 4 days with free access to a running wheel. Syringes indicate the different time points of BrdU injection, on the last day with access to running wheel (d = 0) or 1 (d = 1), 3 (d = 3) and 6 (d = 6) days after withdrawal of running wheel, respectively. Animals were killed one day after the BrdU injection (100 µg/g BW).

4 Results

4.1 Behavior

4.1.1 Exploration and learning

The assumption for this experiment was that two experimental groups, EXP and LRN, attended tasks that possibly affect cell proliferation in the adult dentate gyrus. Group EXP had to explore a novel complex environment and group LRN had to learn and memorize dispenser locations. Computer recorded events of dispenser visits were used to analyze the behavior of individuals of the groups. Behavior of mice during the 4 days of BrdU treatment was of special interest because proliferating cells were labeled within this period.

In group EXP, BrdU was applied on the first 4 days in the novel complex environment to detect proliferating cells during explorative behavior. On day 1 in the novel environment, animals had visited 6 dispensers and gained a number of 9 out of 10 dispensers found until day 4 (Figure 8A). On the 3rd and 4th day, mice visited significantly more dispensers than on day 1 (RM-ANOVA with post-hoc, $p < 0.001$).

Mice in group LRN were already accustomed to the complex environment at the time of BrdU application and had to find and remember positions of high quality dispensers. The learning curve (Figure 8B) shows the portion of visits at high quality dispensers from the first 20 dispenser visits of each day. Choices of high quality dispensers rose from 59 ± 4 % on day 1 to 75 ± 4 % on day 4 ($F_{(3,15)} = 5.8$, $p < 0.01$). On day 3 and 4, the portion of visits at high quality dispensers was significantly greater than on day 1 and significantly above the 50 % chance level (RM-ANOVA with post-hoc, $p < 0.01$). Behavioral analysis of experimental days without BrdU treatment, including reactivity of mice to changes of high and low dispenser locations and risk sensitivity, is described in the Appendix (A.2.4).

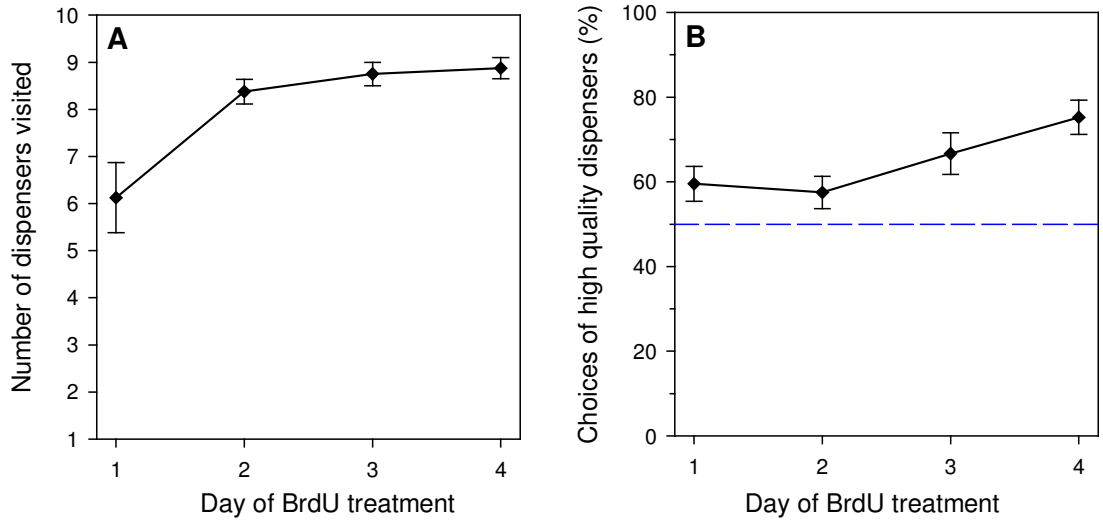

Figure 8: Dispenser visits of mice in the complex environment during the 4 days of BrdU treatment. A) Cumulative number of dispensers visited by mice of group EXP. The first 4 days in the complex environment represent the phase of exploration. Data contain a total of 1710 visits of 8 mice (mean ± SEM). B) Learning curve of group LRN (n = 5). Percentage of visits at high quality dispensers from the first 20 feeder visits of each day. One half of the 10 dispensers provided 60 µl water per single visit (high quality), the other half only 20 µl per single visit (low quality). Distribution of high and low quality dispensers was new to animals at day 1.

4.1.2 Locomotion activity

This experiment was designed to investigate potential differences between two different types of locomotion in mice, running in a running wheel (WHE) and running in plane (i.e. in tubes, TUB). The velocity and the time spent in running for each predefined distance were used to characterize both types of locomotion. WHE mice that were allowed to run *ad libitum* in the running wheel covered a distance of 9668 ± 371 m per day. They spent 8.0 ± 0.6 hours per day in the running wheel.

Table 5: Locomotion activity of mice running either in running tubes (TUB) or in a running wheel (WHE) and numbers of BrdU-positive cells per dentate gyrus in these groups and the control group in the standard cage (CON). Mice received 3 BrdU injections per day (50 µg/g BW each) on 4 consecutive days. Data are means with SEM in parenthesizes.

Treatment	Distance (m)	Velocity (m/min)	Duration (min)	BrdU-positive cells
TUB	400	17.0 (2.8)	32 (5)	1947 (446)
	800	19.7 (2.9)	47 (11)	1911 (429)
WHE	250	15.0 (1.5)	15 (1)	2004 (493)
	500	17.0 (0.6)	27 (2)	2226 (609)
	1000	18.2 (0.3)	50 (1)	1792 (488)
	2000	17.9 (1.9)	92 (7)	2522 (528)
	4000	20.1 (1.4)	174 (12)	3442 (689)«
	ad libitum	16.0 (1.0)	477 (34)	4298 (618)«
CON				1302 (322)

« significant difference ($p<0.01$) from control group CON

Table 5 summarizes the results of this experiment. The time animals spent running, i.e. the duration of running, was correlated to the distance run in both groups, strongly in WHE ($r = 0.97$, $p < 0.001$, Figure 9A) and weakly in TUB ($r = 32$, $p < 0.05$). The velocity of locomotion (Figure 9B) was irrespective of the distance run in both groups, TUB (18.8 ± 1.5 m/min, $p > 0.5$) and WHE mice (18.3 ± 1.1 m/min, $p > 0.3$). Comparison of WHE and TUB showed that these treatments did not differ in the characteristics of locomotion. There was no difference between WHE and TUB mice in running duration ($F_{(1,17)} = 0.009$; $p > 0.9$) or velocity of running ($F_{(1,17)} = 0.11$; $p > 0.7$).

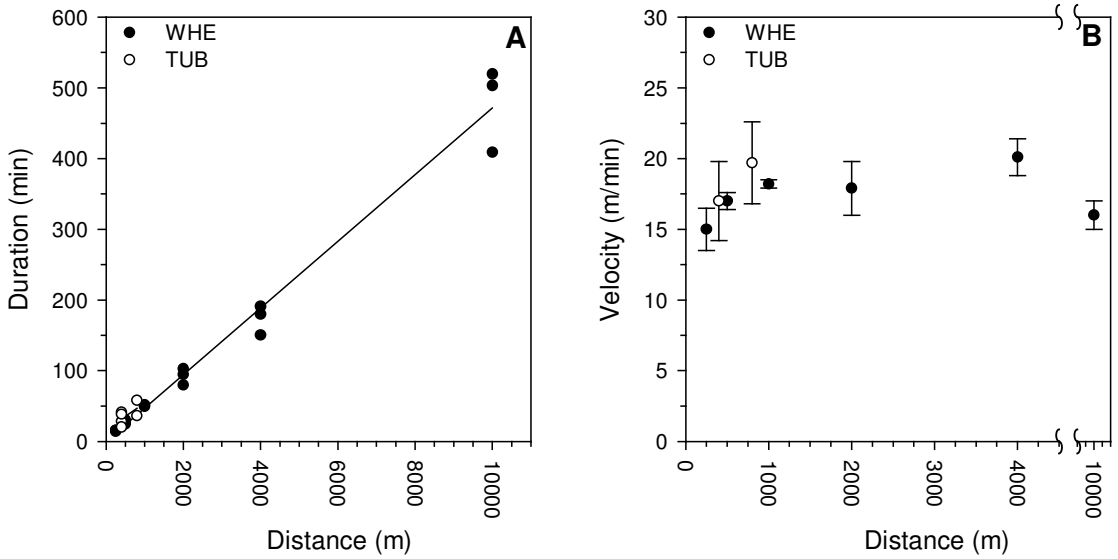

Figure 9: Characterization of exercise in running wheels (WHE) and running tubes (TUB) in association with running distance. A) The running duration, i.e. the time mice spent in running, could be predicted by the distance in WHE ($p < 0.01$) and TUB mice ($p < 0.05$). B) The running velocity was independent from locomotion type (WHE or TUB, $p > 0.7$) and distance ($p > 0.3$). Data are means ± SEM.

4.2 BrdU-positive cells

4.2.1 Simple environment, exploration and learning

The number of surviving newborn cells (PT = 28 d) was significantly lower than the number of proliferating cells (PT = 1 d, Three-way ANOVA, $F_{(1,30)} = 5.27$; $p < 0.05$), independently from the environmental condition (group × PT, $F_{(2,30)} = 1.75$, $p > 0.15$). In SGZ, significantly more BrdU-positive cells were present compared to GCL ($F_{(1,30)} = 8.1$; $p < 0.01$). After a post-treatment time of 28 days, the ratio of cells in SGZ to cells in GCL on average was 49:51 %, compared to 71:29 % after 1 d (see Table 6). Hence, the distribution of newborn cells among SGZ and GCL depends on post-treatment time (PT) as indicated by a strong interaction between these factors (layer × PT, $F_{(1,30)} = 13.68$; $p < 0.001$, see also Chapter 3, 2.3).

Table 6: Numbers of BrdU-positive cells per dentate gyrus (DG) and percentages of BrdU-positive cells in subgranular zone (SGZ) and granule cell layer (GCL) of DG for each group and their post-treatment times (PT). Data are means with SEM in parenthesizes.

Group name	PT	No. of cells DG total	% of cells SGZ	% of cells GCL
SIM	1 d	490 (173)	78.5 (3.0)	21.5 (3.0)
	28 d	513 (202)	53.7 (5.1)	46.3 (5.1)
EXP	1 d	708 (118)	77.2 (0.5)	22.8 (0.5)
	28 d	486 (143)	54.2 (3.5)	45.8 (3.5)
LRN	1 d	922 (92)	65.1 (1.7)	34.9 (1.7)
	28 d	485 (69)	40.3 (4.3)	59.7 (4.3)
CON	1 d	904 (113)	63.8 (1.3)	36.2 (1.3)
	28 d	585 (200)	n/a	n/a
RUN	1 d	5268 (429) «	78.0 (1.2)	22.0 (1.2)

« significant difference ($p<0.001$) from all other groups

Cell counts were not significantly different between experimental groups SIM, EXP and LRN ($F_{(3,23)} = 1.1$; $p > 0.36$, Figure 10B and Table 6). Furthermore, numbers of BrdU-positive cells in all experimental groups were statistically similar compared to control group in the standard cage (CON). In the positive control group, mice in cages with access to a running wheel (RUN), the amount of newly generated cells was approximately 6-fold higher compared to mice in standard cages (CON) and also considerably higher compared to all other groups ($F_{(4,29)} = 106$; $p < 0.001$, Figure 10A and Table 6).

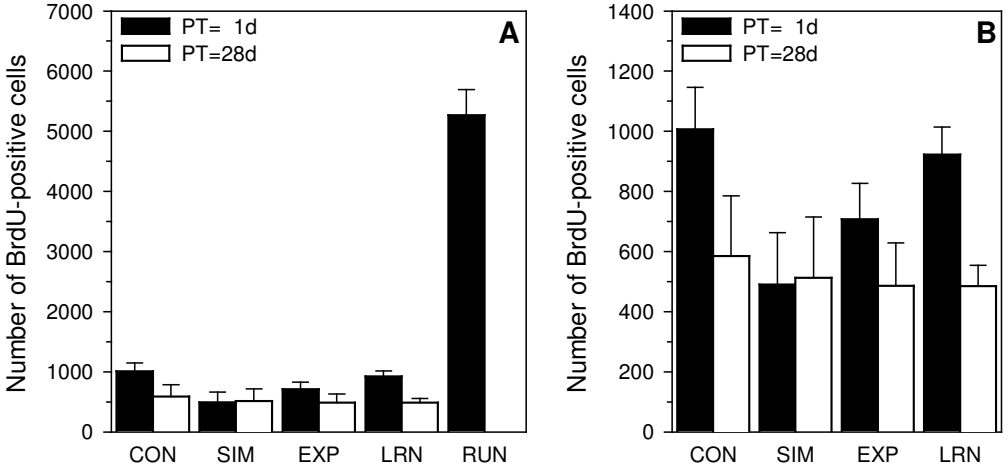

Figure 10: Numbers of BrdU-positive cells per dentate gyrus one day (black bars) and 28 days (white bars) after BrdU administration for the different treatments: control group (CON), simple environment group (SIM), exploration group (EXP), learning group (LRN) and positive control in a cage containing a running wheel (RUN). Mice received BrdU (100µg/g BW) orally on 4 consecutive days. Bars shown in B are the same as in A but with RUN excluded. Data are means ± SEM.

4.2.2 Locomotion types: Wheel versus plane

The amount of BrdU-positive cells was not different between mice running in wheels (WHE) and mice running in tubes (TUB, two-way ANOVA, $F_{(1,22)} = 0.68$, $p > 0.5$). With respect to running distance, cell counts were significantly different ($p < 0.05$). Covering a distance of 4000 m or greater, when running ad libitum, lead to a significant increase of BrdU-labeled cells compared to control mice (CON, $p < 0.01$, Table 5). An interaction between the factors (distance × locomotion type) could not be analyzed because of insufficient data for TUB at greater distances. However, the number of newborn cells in the dentate gyrus was strongly correlated to the distance run ($F_{(1,28)} = 29.5$; $p < 0.001$, Figure 11).

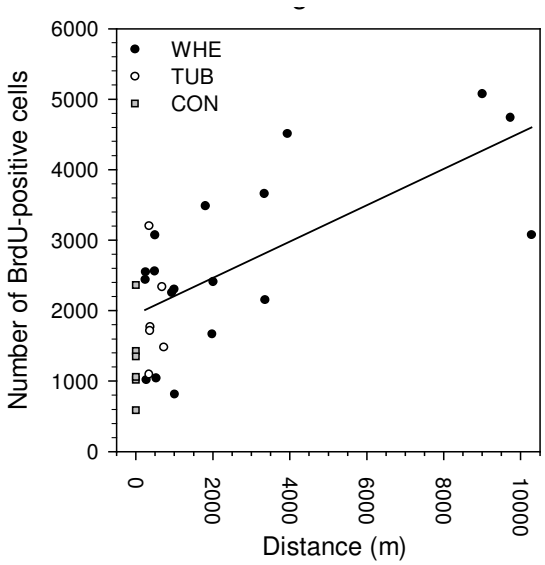

Figure 11: Scatter plot of BrdU-positive cells in dependence from running distance. Mice ran manually limited distances in a running wheel (WHE) or in running tubes (TUB). Control mice (CON) had no facility for locomotion. The number of newborn cells was significantly correlated with the distance run ($p < 0.001$).

4.2.3 Persistence of enhanced cell proliferation following running activity

The aim of this experiment was to identify the duration of the persistence of enhanced cell proliferation caused by wheel running. Therefore, the running wheel was removed following 4 days of free access to the wheel. The number of newborn cells was acquired on the last day of the running period (d = 0) as well as 1, 3 and 6 days after wheel withdrawal. As indicated in Figure 12, by the 4th day of voluntary wheel running (d = 0) the number of BrdU-positive cells was 2.5-fold higher than in mice without access to a running wheel (CON, $p < 0.01$). Following removal of the running wheel, the number of newborn cells was increased after 1 day ($p < 0.05$) and still elevated after 3 days ($p < 0.001$) compared to CON. After 6 days without access to a running wheel, the level of

cell proliferation was significantly lower than on day 0 and day 3 ($p < 0.05$). Additionally, the level of cell proliferation on day 6 after wheel withdrawal was not different from control condition ($p > 0.2$).

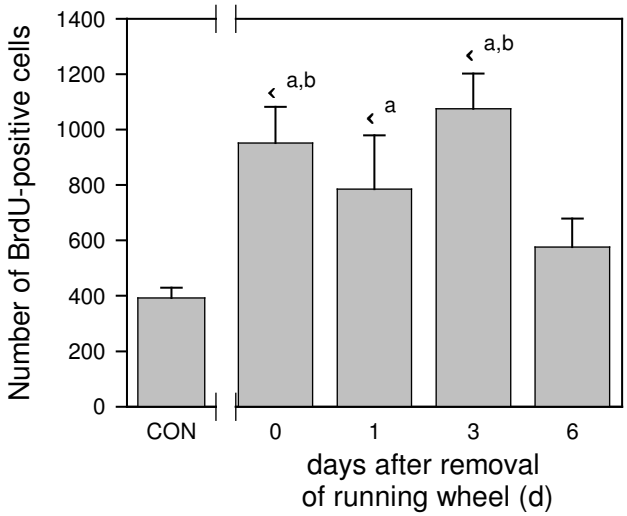

Figure 12: Numbers of BrdU-positive cells in DG of control mice (CON) and mice with free access to a running wheel on 4 consecutive days. The running wheel was removed on day 5 (d = 0). BrdU (100 µg/g BW) was applied on the same day (d = 0), day 1 (d = 1), day 3 (d = 3) or day 6 (d = 6) after removal of the running wheel, respectively. ‹ indicates significant difference ($p < 0.05$) compared to CON (a) and to d = 6 (b).

5 Discussion

The hypotheses, stated above, about the influence of physical activity, exploration and associative learning on cell proliferation and survival of newborn cells in the context of a naturalistic environment, have been rebutted by the results of the present experiment. Physical activity had no effect on neurogenesis under naturalistic conditions regardless of the level of environmental complexity (hypothesis I). Processing new spatial information as it is required when exploring a novel, complex environment had no influence on cell proliferation or survival rate (hypothesis II). Furthermore, associative learning in terms of optimizing foraging behavior by remembering profitable water locations had no effect on the formation or preservation of new cells (hypothesis III). The present data indicate that enhancement of the proliferation rate in the DG is solely affected by running in a running wheel. This effect is dose-dependent, occurs rapidly after stimulation and persists for a relatively short period of 3 to 6 days after stimulus (wheel) withdrawal. The assumption is that the pro-proliferative effect of locomotion in a running wheel results from a type of exercise that differs from any behavior occurring in nature. The strong correlation between activity level, i.e. the running distance, and proliferation rate indicates that the amount of physical activity is the most influential feature for hippocampal cell proliferation.

5.1 Cell proliferation and survival are affected in an enriched, but not in a naturalistic environment

The complex, naturalistic environment in which mice of group EXP and LRN lived the present experiment did not promote cell proliferation or survival of newborn cells in the DG. Assuming that our conditions represent a kind of enrichment, we have to discuss this negative result with respect to the characteristics of an enriched environment. Per definition, an enriched environment is a combination of complex inanimate and social stimulation (see van Praag et al., 2000). An enlarged cage containing various objects like tunnels, toys and nesting materials, commonly represents the implementation of these requirements. Additionally, these objects are changed or switched regularly to challenge exploration and spatial navigation and mice or rats are housed in groups together to allow for social interactions. Thus, our naturalistic environment, in terms of grouped-living mice in a large enclosure containing dispensers, obstacles, a home cage and nesting material, accomplished the crucial characteristics of an enriched environment.

Mice in the complex environment of the present study showed exploratory behavior and learned positions of high quality dispensers. However, neither of these behavioral patterns led to a change in cell proliferation or survival rate in the DG. Regarding our complex environment as an enriched environment, this is in contrast to several studies reporting a promoting effect of an enriched environment on cell proliferation and survival in the hippocampus, although the results of previous studies were not consistent (van Praag et al., 2000). A great deal of studies indicates that environmental enrichment can influence different aspects of adult neurogenesis. Therefore, the assumption is that changes in cell proliferation and neurogenesis result from other factors than environmental enrichment itself. Physical activity and learning, both part of an enriched environment, are factors that have been shown to influence separately cell proliferation and survival (see below). Interestingly, in many studies the enriched environment contained a running wheel (Brown et al., 2003; Kempermann et al., 1997b; Kempermann et al., 1998a; 1998b; van Praag et al., 1999b) and in some studies animals were trained on a learning task during or after enrichment (Kempermann et al., 1997b; 1998a; 1998b; Kempermann & Gage, 1999; Nilsson et al., 1999; Bruel-Jungerman et al., 2005). The paradigm of our experiment differs from previous studies in at least 4 fundamental aspects: i) We used the outbred strain CD-1 in contrast to the most commonly used inbred mouse strains C57BL/6 or 129/Sv. ii) We exposed laboratory mice to a large room in contrast to an enlarged cage and we demanded foraging behavior. These conditions might have induced stress in mice. iii) We considered

foraging at dispensers of different quality as a spatial learning task in contrast to standardized learning tasks like water maze or fear conditioning. iv) Physical activity in our naturalistic environment took place in the context of exploration and foraging rather than to the treat of exercise like in a running wheel. The question that prompted the present study was, whether an environment that provides the opportunity for more natural-like behavior would yield a similar effect on hippocampal cell proliferation as enrichment (e.g. Kempermann et al., 1997b), physical activity (e.g. van Praag et al., 1999b) and learning (e.g. Gould et al., 1999a, b) have been shown to. Surprisingly, the answer to this question was no. Therefore, in the following paragraphs we are going to discuss, how this result can be interpreted with regard to the differences mentioned above.

5.1.1 The role of genetic background

Genetic effects on mouse behaviors like locomotor activity, anxiety and spatial navigation are well documented (see Crabbe et al., 1999). The outbred strain CD-1 exhibits comparatively higher levels of novelty exploration in a Y-maze than inbred strains BALB/c, DBA/2 and C57BL/6 (Dellu et al., 2000). The investigation of exploratory behavior in an open-field paradigm supported this result and, additionally, showed that female CD-1 mice reacted more aggressive towards conspecific intruders and expressed lower levels of anxiety (Parmigiani et al., 1999). Therefore, regarding the behavioral tasks of our experiment, CD-1 mice were suitable subjects. To the best of our knowledge, so far no experiments have been done using CD-1 outbred mice with respect to adult neurogenesis, except for one. Kempermann et al. (1997a) included CD-1 mice in their comparative study on hippocampal cell proliferation and neurogenesis, which revealed significant differences between 4 mouse strains. The volume of the granule cell layer (GCL) as well as the total number of granule cells of the adult DG was higher in CD-1 mice compared to C57BL/6, 129/SvJ and BALB/c mice. Whereas the portion of surviving cells that differentiate into neurons was similar for all strains (~60%), strains differed in proliferation and survival rates. In the DG of CD-1 mice, the proliferation rate was lower than in C57BL/6 mice, but the survival rate was higher than in 129/SvJ mice. In particular, the two latter strains are interesting because it turned out that an enriched environment is associated with increased cell proliferation in 129/Sv mice (129/SvEv: Meshi et al., 2006; 129/SvJ: Kempermann et al., 1998a), but not in C57BL/6 (Kempermann et al., 1997a; 1998b; Brown et al., 2003), whereas survival rate was enhanced by enrichment in both strains. However, the response of hippocampal plasticity to enrichment could not be predicted by CGL volume or by baseline proliferation (Kempermann et al., 1998a).

Although inheritable traits seem to influence the dynamic changes in the hippocampus, it is unlikely that they are causal for the negative results of the present experiment, because others features, like running in a running wheel, affected cell proliferation in CD-1 mice to a comparable extent or even stronger than in C57BL/6 mice (van Praag et al., 1999b). Experiments that test CD-1 mice in commonly used setups, i.e. a large cage with toys or in a water maze learning task, are required to link this strain to the reported environmental effects on adult neurogenesis in other strains.

5.1.2 The role of stress

In order to prevent social stress, we used only females in our experiments, because they show less territorial behavior and are less aggressive than males (Parmigiani et al., 1999). Generally, we designed our experiment with a focus on avoidance of stress for the animals. Therefore, we reduced interruptions by the experimenter to a minimum by using an automated, computer-controlled setup. Additionally, by applying BrdU orally via drinking water, stressful handling of animals was unnecessary at all, except for the capture immediately before sacrifice. However, it might be possible that the experimental apparatus we used in the present experiment induced stress in mice due to size or novelty of the rooms. In this case, a pro-proliferative effect might have been suppressed by stress. Particularly, the simple impoverished environment (SIM) might represent an unpleasant situation for mice. A free accessible home cage was provided in one edge of the large enclosure (11.5 m^2) to avoid stress due to permanent exposure to an open field. Yet, mice were obliged to cross an open field from their cage to the only water source in the middle of room. However, the amount of daily water uptake and body weight at the end of the experiment were similar between group SIM (5.0 ± 0.3 ml/d and 29.7 ± 1.8 g, respectively) and the other groups (ANOVA, $F_{(3,212)} = 1.6$; $p > 0.18$ and $F_{(3,8)} = 1.7$; $p > 0.24$, respectively). These are gross measures of bodily adaptation, but also indicators for stress (Magarinos et al., 1996). However, the number of BrdU-positive cells in group SIM one day after BrdU administration was half the amount of newborn cells in the control group. Although this difference was not significant, this indicates that living in a large exposed room that lacks further stimuli diminishes cell proliferation. In this case, it is notable that this trend was not present at 4 weeks after BrdU application, indicating that this stress-induced effect affects cell proliferation rather than the survival of newborn cells. A trend for a reduction of the number of proliferating cells was not observed in groups EXP and LRN that lived in the naturalistic environment. This environment per se is unlikely to induce stress in mice and behavioral observation revealed no indicators for a stress

response. At the 4 days of BrdU administration, group LRN was already habituated to the environment as indicated by stable body weight, daily water consumption and running distance. During this critical period, mice of group EXP explored the novel naturalistic environment. The novelty of the situation may serve as a stressful experience, but we assume that this had been a moderate form of stress, because exploration behavior was voluntary and not forced. Again, observation of behavior as well as recorded data of dispenser visits indicated that mice were habituated to the environment on the second day of the experiment.

Stress that resulted in a rapid decrease of proliferating cells in previous studies was induced by rather severe stressing experiences (Tanapat et al., 2001; Gould et al., 1997; 1998; Mitra et al., 2006). In contrast, Kozorovitskiy and Gould (2004) allowed rats to establish a dominance hierarchy in a semi-naturalistic environment for 3 days that led to an increased number of newborn cells in the DG of dominant animals. However, a stress-induced reduction of cell proliferation in subordinate rats was not observed. This indicates that adult neurogenesis is influenced by acute and severe stress, but not implicitly by all stressful experiences an animals has to cope with. Therefore, the absence of an effect on cell proliferation and survival in the naturalistic environment should not be interpreted a stress-induced reduction or suppression of cell proliferation.

5.1.3 The role of learning

The results of the present experiment indicate that learning in terms of discovering and remembering the spatial distribution of high quality dispensers does not affect cell proliferation or survival in the DG of mice. After training in a Morris water maze neurogenesis has been shown to increase (Gould et al., 1999a), decrease (Ambrogini et al., 2004) or to remain unchanged (van Praag et al., 1999b). These contradicting results are certainly due to methodical discrepancies between the studies. The measure of BrdU-labeled cells in association with learning is highly sensitive to the timing and duration of BrdU application as well as to the learning paradigm (see Prickaerts et al., 2004). Regarding the time point and duration of BrdU administration, we followed a paradigm that should have detected a change in cell proliferation or survival. Firstly, mice received daily BrdU doses just before the learning phase in the complex environment and were killed 24 hours after the last dose of BrdU. This manner is comparable to Lemaire et al. (2000), who applied BrdU on 3 days just before water maze training and therewith found an increased cell proliferation in trained rats compared to controls. Secondly, a survival promoting effect of learning is assumed to be detected if BrdU is applied before rather than

during water maze training (Gould et al., 1999a). In the present study, mice received BrdU during their learning period, but we kept at challenging spatial learning in mice by changing the locations of high quality dispensers repeatedly during the survival period. Thus, if we failed to detect changes in the survival rate due to learning during BrdU treatment, then we at least should have detected changes due to the learning phases in the subsequent weeks. The more important consideration refers to the learning task is itself. The task in our naturalistic environment is based on the demand of spatial cognition to optimize foraging behavior. Thus, mice were confronted with a task that similarly occurs in a natural environment. The importance of considering the ecological relevance of learning tasks has been mentioned elsewhere (Gerlai & Clayton, 1999; Vyssotski et al., 2002) and one should keep this in mind when interpreting data from laboratory experiments.

In addition to the methodical discrepancies that might have contributed to contradicting results in learning tasks, one should take into account factors that might interfere with each other and with learning. Stress acts as a negative regulator of hippocampal cell proliferation (Gould et al., 1997; Tanapat et al., 2001) and might be induced by the confrontation with foot shocks in the conditioning chamber and with water in the water maze, which is an aversive component for mice. Physical activity acts as a positive regulator of cell production (Ra et al., 2002; van Praag et al., 1999b) and is also part of the water maze task. Thus, learning can hardly be measured in isolation of other factors. Another problem regarding these specific learning tasks is that different training paradigms lead to different results. One explanation for the results of Van Praag et al. (1999b), who found no effect of water maze training on the survival of new neurons, was that probably too few learning trials were given to animals with two instead of four (Gould et al., 1999b) trials per day. Olariu et al. (2005) applied a more natural form associative learning task (social transmission of food preference, STFP) that resulted in an increase of BrdU-labeled cells following one day of training, but a decrease following two days of training. Training, in terms of trials per day, were not part of our learning task, because animals were allowed to explore and remember the places of high quality dispensers for several days before switching the positions. Therefore, the present results support the interpretation that continued repetition of similar training events produce no or a negative effect on cell survival (Olariu et al., 2005). However, many studies demonstrate that conclusions about a correlation between learning and neurogenesis should be drawn with respect to the specific paradigm that has been tested. The focus in our experiment was to a smaller extent spatial learning per se. Our aim was to provide a naturalistic environment

including the demand for exploiting resources, which includes spatial navigation and learning. The present results indicate that this natural form of learning in the context of a naturalistic environment is not associated with adult neurogenesis.

5.1.4 The role of physical activity

The present result of the positive control group (RUN) further substantiates the tremendous pro-proliferative effect of running in a running wheel. We also demonstrated that the exercise-induced increase of cell proliferation is an acute and transient effect. 4 days of voluntary wheel running are sufficient for a more than two-fold increase in cell proliferation that persists for at least 3 days and is finally lost after 6 days. An earlier study found that the pro-proliferative effect is lost after 3 weeks (Kitamura et al., 2003). Thus, we narrowed the time window of this decrease process. Furthermore, our data indicate that this process is rather rapid (3 to 6 days) than gradually and not similar to the neurogenic response to seizures that persist for up to 2 weeks (Parent et al., 1997). One may argue that an increase of BrdU-labeled cells might result from an enhanced efficiency of BrdU incorporation due to the exercise-induced increase of cerebral blood flow, cerebral blood volume or permeability of the blood-brain-barrier. Invalidating this concern, immunostaining of the endogenous cell cycle marker Ki-67 for additional identification of proliferating cells revealed that a running-induced increase of Ki-67-stained cells occurs similar to that observed by BrdU-labeling (Eadie et al., 2005; Stranahan et al., 2006). Physical activity played a major role in the complex environment used our experiment, because the large enclosure provided much space for exercise and, furthermore, exercise was required to imbibe a sufficient amount of water. The recording of dispenser visits enabled us to calculate the distance one mouse had covered minimally per day. This is only a rough estimate of exercise, because mice moved much more in the complex environment than only from dispenser to dispenser. However, or just because of that, a calculated daily distance of 137.7 ± 58.7 m (Mean ± SD) supports the personal observation that mice were highly active in the naturalistic environment. Considering that exercise in groups EXP and LRN was considerably more intensive compared to control mice in a laboratory cage, it was surprising that not even this aspect led to an increase of cell proliferation. The question arose whether the type of locomotion may be crucial for the impact of physical activity on cell proliferation, i.e. is running in a wheel different from running on flat ground? The result of our experiments indicates that this is not true. More precisely, the crucial feature for increasing the rate of cell proliferation is the amount of exercise in terms of running distance (or duration). Additionally, we showed that the increase in new born cells is not

associated with the intensity of running in terms of running velocity, because velocity was independent from running type (TUB or WHE) and distance. The distance run and the number of BrdU-positive were strongly correlated when mice ran in a wheel but no correlation was observed in mice running in tubes. This difference could be due to the few data available for tube running mice. Unfortunately, we were not able to collect data from mice running greater distances in running tubes, because even if unrestricted, mice did rarely succeed in running more than 1500 m per day. However, considering both, tube and wheel running, the comparison between comparable categories of running distance, i.e. below 2000 m, revealed no difference in cell counts between distances or type of running. This indicates that even in the running wheel a minimal distance has to be covered to induce an increase in cell proliferation. We are not supposed to declare a precise value, because of the small sample size in this experiment. However, the conclusion is that mice running in plane, i.e. in tubes, a naturalistic environment or probably in their natural habitat, do not reach activity levels of mice running in a running wheel and therefore do not show an activity-induced increase of cell production.

5.2 Methodical considerations

5-bromo-2'-deoxyuridine (BrdU) is a well-established marker of cell proliferation and incorporates into DNA during the S-Phase of the cell cycle. More precisely, BrdU incorporates into any cell that synthesizes DNA and therefore it cannot be excluded, that also cells undergoing DNA-repair appear as BrdU-positive cells (Nowakowski & Hayes, 2000). However, Cooper-Kuhn and Kuhn (2002) argue that development of BrdU-labeled cells can be traced by double labeling with markers for immature or mature neurons and irradiation drastically reduces the number BrdU-positive cells although DNA-repair is increased. Thus, the authors conclude that DNA-repair does not represent a major source of BrdU labeling. One concern that has to be mentioned is the potential toxicity of BrdU to cells and animals (see Jecker et al., 1997). However, in adult rodents neither daily doses of up to 600 µg BrdU per gram body weight (Cameron & McKay, 2001) nor long-term oral administration of BrdU (Jecker et al., 1997) affect birth or survival of cells and the behavior or health of animals. In the present study, we used a daily dose of 100 µg BrdU per gram body weight, which is assumed to be non-toxic. Cameron and McKay (2001) mentioned that this dose yields an underestimation of the real number of proliferating cells in the dentate gyrus (DG), because at this dosage only a portion of cells in S-phase are detected. However, this issue is unlikely to be a problem for studies comparing different

groups of animals that were given the same doses of BrdU as it is the case in the present study.

Within the scope of this thesis we did not further investigate the development and differentiation of newborn cells as it is commonly done in the literature. By double-labeling of BrdU-positive cells with neuron and glia specific markers (e.g. NeuN and GFAP, respectively) the phenotype of newborn cells can be identified and cell types can be quantified using stereological analysis. In the literature the percentage values for new neurons and glia cells vary between studies. Kempermann et al. (1998b) for example reported that from surviving (4 weeks) newborn cells 41 % were neurons and 16 % were glia cells. Van Praag et al. (1999a) found 89 % neurons and only 3 % glia cells. However, the data in the literature are consistent in the finding that more newborn cells in the DG differentiate into neurons than into glia cells. Furthermore, it is suggested that neuron to glia ratio does not considerably change depending on conditions applied as it has been shown for environmental enrichment compared to control conditions (Kempermann et al., 1998b). Therefore, we assume that the surviving cells (4 weeks after BrdU) are neurons to a greater part than glia in all groups investigated indicating the formation of new neurons. However, since we did not identify cell types by specific labeling our results here refer to survival of newborn cells and are not conclusively applicable to neurogenesis.

We found no differences in the number of BrdU-positive cells between mice living in a standard laboratory cage (CON), a frugal large enclosure (SIM) or in a complex, naturalistic environment (EXP and LRN). One may argue that application of BrdU via the drinking water could be an inappropriate method for this purpose, because of uncontrolled uptake of BrdU solution or insufficient incorporation of BrdU. To treat of this issue, we demonstrated in a previous study that computer-controlled oral administration of BrdU is a reliable alternative to BrdU injections (Santoso et al., 2006; Chapter 1). Furthermore, in the positive control group of this experiment (mice with access to a running wheel, RUN), the number of proliferating cells was dramatically increased.

5.3 Conclusions

Under naturalistic conditions cell proliferation and survival are unaffected by exploration behavior, learning and physical activity. The present study opens up a novel and important perspective for the question of the functional relevance of adult neurogenesis. We are far from understanding the benefits of producing new neurons throughout life. The fact that this process is sensitive to various factors, at least in the

laboratory, and the assumption that hippocampal cell loss in association with diseases like depression and Alzheimer could be prevented or adjusted by adult neurogenesis, necessitate further investigation of this topic. However, adult neurogenesis in healthy subjects living in their natural environment perhaps should be considered as a stable process that is relatively insensitive for the impacts of "everyday life".

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Chapter 3

Cell proliferation and behavior: Insights

1 Introduction

Considering adult neurogenesis under naturalistic conditions means to carefully keep in mind both, the neurobiological and the behavioral aspects of an experiment. Hence, before and during conducting the experiments described in Chapter 2 several questions arose. Some of these questions were crucial for the reliability of the results, others concerned details that might be marginal for the experiment itself but nevertheless were of interest for as additional details supporting an integrated view on the topic.

One fundamental question was whether the administration of BrdU via the drinking water, described in Chapter 1, was a proper method for labeling cells in the brains of mice even when living in a semi-natural environment. Accurate dosing and the time point of BrdU application are crucial aspects for cell labeling. In contrast to the use of BrdU injections, it was a challenge to warrant both for each individual of a mouse group freely moving in a naturalistic environment. To show the achievement of this objective, we compared in experiment 2.1 our results of Chapter 24.2.1) with BrdU labeling using BrdU injections instead of oral application under similar conditions.

The new method we established in Chapter 1 of this thesis would become even more important if we could show that it is not restricted to the use in laboratory mice but is also applicable for different animal species. The nectar-feeding bat *Glossophaga soricina* (Phyllostomidae) is a routinely used subject in the ecological studies in the laboratory of York Winter. The specialized cognitive abilities of these flower bats, that are necessary to locate and remember profitable resources, indicate that spatial learning and memory play an important role these animals (Winter & Stich, 2005). Furthermore, the hippocampus, a brain region involved in spatial memory formation, is substantially larger in size in nectar-feeding bats than in allied insectivorous species (Baron et al., 1996). Therefore, knowing the role of adult neurogenesis in these animals could be an important step to come up to the still unanswered question of the functionality of this phenomenon. Within the scope of this thesis, in experiment 2.2 we addressed the question whether our method of automated oral BrdU administration is in principle also applicable to this animal species.

In experiment 2.3 we analyzed the characteristics of the BrdU labeled cell in terms of distribution within the DG and clustering. This information allowed us to further

accommodate our results to the well-established data of cell proliferation and migration from the literature.

A few standard tests are commonly used in the literature to investigate learning behavior and its impact on adult neurogenesis (see Chapter 2, tab.2). Our semi-natural setup was a new approach to this topic. So far, there was no experience on learning based on foraging behavior of mice in this environment. Therefore, it was essential to first observe and evaluate the behavior of mice in this setup before analyzing the cell counts in relation to the behavior. The aim of experiment 2.4 was to first quantify general behavior patterns of mice in the simple and semi-natural environment (see Chapter 2) in terms of activity by analyzing dispenser visits. Secondly, and more important, we wanted to show that learning and exploration occur in this environment in a way that qualifies for being a reliable basis for the related neurobiological analysis.

An incidental concern arose when moving the mice from the departmental animal husbandry to the experimental setup since we thereby shifted the light-dark (LD) cycle. A day-night shift of several hours results in a variety of physiological symptoms, also known as jet lag, and has been linked to changes of gene expression in the suprachiasmatic nucleus (Nagano et al., 2003; Reddy et al., 2002). Hippocampal cell proliferation is constant over the day (Ambrogini et al., 2002) and unaffected by sleep deprivation (van der Borght et al., 2006), but whether a shift of the LD cycle affects cell proliferation has not been investigated so far. In order to prevent potential jet lag effects, we observed and analyzed in experiment 2.5. the activity pattern of mice following a LD shift of 7 hours. The results of this experiment gave an estimation of the time needed for mice to accustom to the new LD, which consequently should be provided for acclimatization before starting studies in a new environment.

The running wheel is commonly used in rodents to investigate divers effects of physical activity. The assumption that wheel running has to be seen as an unnatural type of locomotion with self-rewarding and addictive effects (see Sherwin, 1998) led to further questions we wanted to address. Firstly, to compare the proliferative effects of two types of running, i.e. in a wheel versus in plane, we analyzed beforehand the activity under both conditions (experiment 2.6). Secondly, because of the prominent effect of wheel running on cell proliferation, we wanted to know whether simply the presence of a running wheel might influence the number of newborn cells (experiment 2.7).

Since the aims and types of these experiments are multifaceted, in the following section each of these issues is addressed separately by enunciating the purpose of the question, describing the methodical approach and finalizing with results and conclusions.

2 Experiments

2.1 Cell proliferation in the naturalistic environment with BrdU injections

Purpose

The most commonly used method for investigating the response of the cell proliferation and survival rate to behavioral or environmental changes is by injecting BrdU intraperitoneally (Gould & Gross, 2002). We demonstrated in a previous study that the administration of BrdU via the drinking water in computer-controlled dispensers is a suitable alternative to BrdU injections (Santoso et al., 2006). However, the procedure of BrdU uptake by oral application extends to several hours compared to injections that are completed within a few seconds. In particular, the effects of living in an enriched environment or learning might depend on this discrepancy. Since there are no experience values with this method, the aim of the following analysis was to get an indication for the functionality of oral BrdU application in our naturalistic setup. We therefore repeated parts of the experiment described in Chapter 2 (3.2.1), but used BrdU injections instead of oral BrdU application.

Methods

The conditions of this experiment correspond to the groups CON and LRN in Chapter 2 (3.1.3), except that mice received BrdU not orally but via i.p. injections. 12 female CD-1 mice were divided into two groups. 6 mice were housed in a standard laboratory cage (CON) and 6 mice lived in a large enclosure (15.9 m^2) containing a home cage, 11 automatic dispensers and 11 obstacles of different lengths (LRN). The dispenser qualities (amount of water released), were changed every 5-7 days to provide animals a variable distribution of different resources. Mice received one intraperitoneal injection of BrdU dissolved in 0.9 % NaCl (10mg/ml) on 4 consecutive days (50 µg BrdU/g BW) at the beginning of the dark phase. 28 days after the last dose of BrdU animals were transcardially perfused with 4 % paraformaldehyde. Because a part of the animals was used for different analyses, only 2 mice from CON and LRN were used for counting BrdU-labeled cells in the dentate gyrus.

Result and conclusions

Four weeks after the BrdU injections, the numbers of BrdU-labeled cells in the DG of mice were not significantly different between mice housed in a standard cage (CON) and mice living in the complex environment (LRN; Two-way ANOVA, $F_{(1,9)} = 0.05$;

$p > 0.8$, see Fig. 1). Furthermore, there was no statistical difference in cell counts after BrdU injections compared to oral BrdU administration ($F_{(1,9)} = 0.69$; $p > 0.4$).

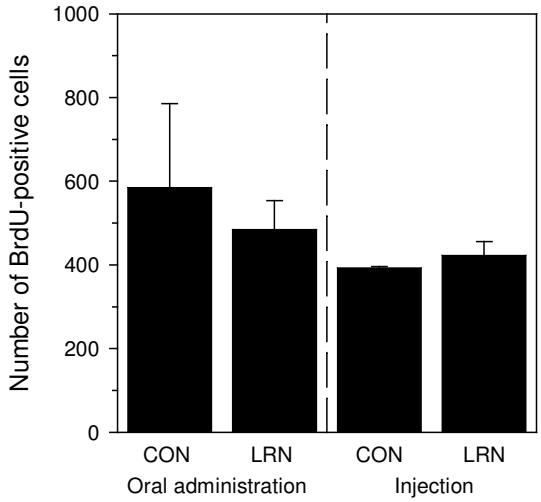

Figure 13: Numbers of BrdU-labeled cells in the dentate gyrus of mice housed in standard cages (CON) or in a complex naturalistic environment (LRN). Mice received BrdU orally via the drinking water (oral administration, data from 5.2.1) or via i.p. injections (50 µg BrdU/g BW) and were killed 4 weeks later. Data are means ± SEM.

Environmental enrichment, in terms of an enlarged cage containing running wheels, toys and tunnels, induces an increase in the number of surviving new cells but not in the number of proliferating cells compared to control conditions (Brown et al., 2003; Kempermann et al., 1997). Therefore, we analyzed only the number of cells that survived for 28 days and did not compare the number of proliferating cells (i.e. 1 day after BrdU application). Although the sample size is very small, the insignificant dispersion of the values in mice injected with BrdU points to reproducibility of this result. The present result confirms our previous finding that oral BrdU application yields reliable labeling of new cells with results comparable to BrdU injections (Santoso et al., 2006). Furthermore, the data verify that living in a naturalistic environment does not affect adult neurogenesis.

2.2 Oral application of BrdU in a nectar-feeding bat (Glossophaga soricina)

Purpose

The administration of BrdU in exact doses via drinking water by computer-controlled dispensers is a suitable method to detect proliferating cells in mice without disturbing their behavior (Santoso et al., 2006). In order to test the applicability of this method to other species, we applied it here to the nectar-feeding bat *Glossophaga soricina* (Phyllostomidae). The following experiment was designed to investigate whether a flower

bat would accept a BrdU-solution for the stress-free labeling of proliferating cells via oral application.

Methods

Subject of this experiment was one adult male long-tongued bat *Glossophaga soricina* (Phyllostomidae) that was bred in captivity. The climatic conditions of the housing and experimental room were approximately 23°C and 60 % humidity. The diet consisted of dry pollen and 1.5 ml of Nektar Plus (Nekton, Pforzheim, Germany) and NutriComp (B. Braun, Melsungen, Germany) dissolved in water. The experimental cage is described in detail in Winter & Stich (2005). In brief, the cage (w×h×d: $0.7 \times 2.0 \times 1.5$ m) was equipped with a basket attached to the ceiling for clinging and two computer-controlled dispensers attached to the rearward wall. The water dispensers were similar to those described before (Chapter 2, 3.1.2) except that they contained no transponder reading device. The dispensers released 45 µl of a 17 % sugar solution (fructose, glucose and saccharose in equal parts) per visit. During 11 days of acclimation, the bat had additionally free access to a small bowl of 17 % honey water. BrdU was dissolved in the sugar solution (0.23 mg BrdU/ml) and presented in one of the two dispensers for 8 consecutive days, whereas the other dispenser released pure sugar water. BrdU solution was swapped between the two dispensers every other day. To ingest the daily dose of 50 µg BrdU/g body weight the bat (body weight: 11.5 g) had to visit the BrdU-dispenser 46 times. Daily BrdU treatment started at the beginning of the active phase of the bat, i.e. at lights off (12:12 h light:dark cycle). The bat was sacrificed 59 days after the last dose of BrdU and the brain processed similar to the description in the Appendix (A.4).

Result and conclusion

During the whole experiment in the cage, the bat visited the two dispensers on average 943 (± 328 SD) times per day receiving 14.1 (± 3.4 SD) ml of liquid. During BrdU treatment, the dispenser visits were not evenly distributed over the two dispensers. A two-way ANOVA revealed a difference in the number of dispenser visits according to dispenser contents (sugar water vs. BrdU solution; $F_{(1,12)} = 12.3$; $p < 0.01$) and the dispenser itself (dispenser 1 vs. dispenser 2; $F_{(1,12)} = 7.7$; $p < 0.05$, see Fig. 2A). When both dispensers released sugar water to similar amounts, the bat visited dispenser 2 more often than dispenser 1 (t-test; $p < 0.01$, see Fig. 2B). From this data, we can assume that the bat developed a preference for one of the two dispensers regardless of the presence of BrdU.

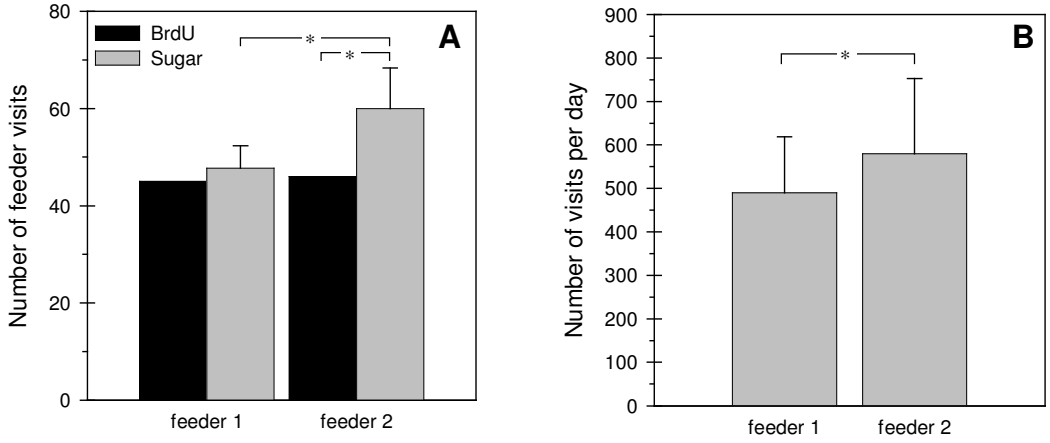

Figure 14: Numbers of visits of one bat at the two dispensers in the experimental cage. A) Dispenser visits during BrdU treatment. BrdU solution (black bars) was provided daily rotational by one of the dispensers while the other one released pure sugar water (gray bars). B) Dispenser visits during 48 days with pure sugar water provided by both dispensers to equal amounts (20 µl per visit). Data are means ± SD and * indicate significant differences ($p < 0.05$).

The bat distinguished between pure sugar water and sugar water containing BrdU independently from a dispenser preference, indicated by the lack of interaction between contents and dispenser (contents × dispenser; $p > 0.7$). However, the bat was allowed to freely choose a dispenser and nevertheless ingested the daily dose of BrdU within a few hours. Therefore, the bat seemed to perceive the taste of BrdU but, hence, does not avoid it. As conclusion, the computer-controlled dispensers are a suitable apparatus for oral application of drugs even in nectar-feeding bats.

We found no BrdU-labeled cells in the dentate gyrus (DG) of the bat.

2.3 Classification and distribution of BrdU-labeled cells

Purpose

Within the scope of this thesis, the differentiation of newborn cells was not analyzed since we concentrated on the quantification of proliferating and surviving cells. However, labeling those cells allowed for collecting additional information about the distribution and classification of newborn cells. Proliferating cells commonly appear in clusters (Ambrogini et al., 2002; Auvergne et al., 2002; Nixon & Crews, 2004; Tada et al., 2000) and surviving cells partly migrate from the subgranular zone (SGZ) into the granule cell layer (GCL, see Kempermann et al. 2003). Appearance of cell clusters and the distribution of BrdU labeled cells within the DG allowed us to link our data to the process of adult neurogenesis without verifying neurons per se with neuronal markers.

Methods

The integrated morphometry analysis (IMA) function in MetaMorph recorded for each point several attributes (see Table 16). The data gave information about shape, size, position and gray shade of the cells. Additionally, in the course of quantification (see App. A.5) information about the position of each cell within the DG was retained. In order to describe the attributes of newborn cells, all cells recorded from 89 animals were analyzed according to their characteristics ascertained by IMA.

Results and conclusion

A total of 27775 cells were included in this analysis. Figure 15 shows the distribution of new born cells 1 day and 4 weeks after BrdU administration with respect to DG region, i.e. dorsodorsal (dd) blade, dorsoventral (dv) blade, ventral DG (ve) and hilus (hi) and DG layer, i.e. granule cell layer (GCL) and subgranular zone (SGZ). The majority (87.9 %) of BrdU-labeled cells was located in the dorsal part of DG with slightly more cells in the dd blade (47,6 %) than in the dv blade (41.4 %). A small portion of new cells was found in the hilus (12.2 %) and in the ventral part of the DG (3.0 %). The portions of cells were significantly different between the regions (Two-way ANOVA, $F_{(3,424)} = 929.1$, $p < 0.001$) and were also different comparing 1 day post-treatment time (PT) to 4 weeks PT ($F_{(4,424)} = 4.2$, $p < 0.05$). A significant interaction between region and PT ($F_{(3,424)} = 13.4$, $p < 0.001$) indicates that the distribution of cells over DG regions changed in dependence of PT. In the dd blade of DG, the portion of BrdU-positive was independent from PT, whereas in the dv blade of the DG the portion of cells that survived 4 weeks was smaller than the portion of newborn cells in this region ($p < 0.001$). In exchange, more cells were found in the ventral DG and hilus after 4 weeks than after 1 day ($p < 0.001$). However, the main region of adult neurogenesis is surely the molecular layer including the dd and dv blade of DG. BrdU-labeled cells were significantly unequally distributed among DG layers GCL and SGZ (Two-way ANOVA, $F_{(1,194)} = 155.1$, $p < 0.001$). The interaction layer × PT ($F_{(1.194)} = 194.5$, $p < 0.001$) indicates that cell portions in GCL and SGZ are dependent from the time of survival after BrdU treatment. Proliferating cells at 1 day PT were located mostly in the SGZ, whereas after 4 weeks surviving cells were evenly distributed among SGZ and GCL.

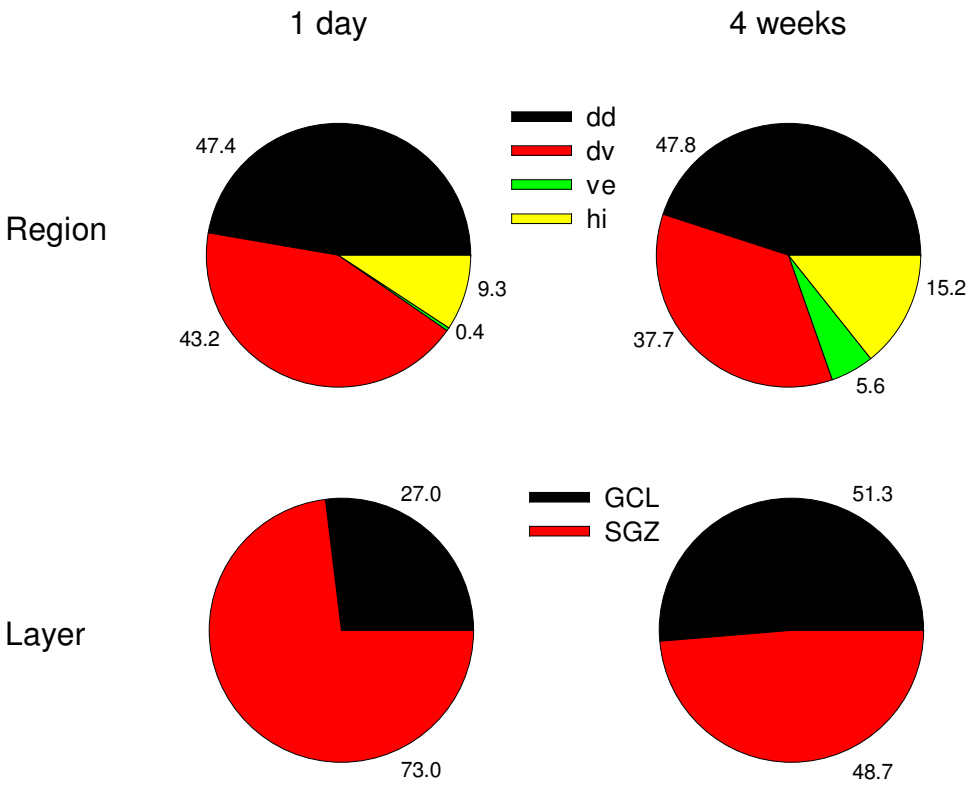

Figure 15: Distribution of BrdU-positive cells within the dentate gyrus (DG) 1 day (left charts) and 4 weeks (right charts) after the last dose of BrdU. Upper charts show portions of cells (%) distributed among DG regions, the dorsodorsal (dd) and dorsoventral (dv) blade, the ventral DG (ve) and the hilus (hi). Lower charts show cell distribution among DG layers, the granule cell layer (GCL) and the subgranular zone (SGZ).

BrdU-positive cells were on average 22.2 μm^2 ($\pm$ 8.6 SD) in size. The comparison of cell sizes between the regions of DG showed that cells in hi were significantly smaller than cells in the dorsal DG (dd and dv, $p < 0.001$). 85 % of the objects were single cells and 15 % were cell clusters of 2 or more cells indicated by a standard area count >1. These clusters were located to the major part in SGZ (78 %) and only 3.7 % were found in the hilus. Clusters were composed of more cells when located in SGZ compared to GCL ($p < 0.001$) and at PT = 1 compared to PT = 28 ($p < 0.001$, Figure 4). The interaction between these two parameters (layer $\times$ PT, $F_{(2,27768)} = 13.5$, $p > 0.001$) indicates that the difference in cluster size between DG layers changes with time elapsed after BrdU treatment.

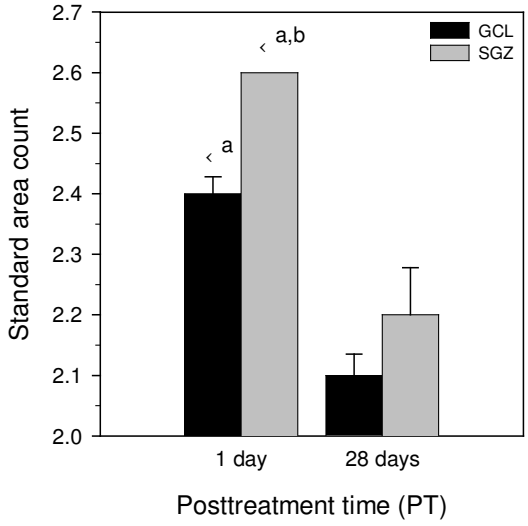

Figure 16: Cluster size in granule cell layer (GCL) and subgranular zone (SGZ) 1 day and 28 days after BrdU treatment, respectively. A cluster corresponds to objects with a standard area count > 2. ‹ indicate significance ($p < 0.001$) compared to same layer at PT = 28 days (a) and compared to GCL at PT = 1 day (b). Data are means ± SEM.

Our findings fit quite well into the few previous descriptions of clustering and distribution of newborn cells. As demonstrated in this experiment, during proliferation (i.e. 1 day after BrdU treatment) labeled cells often appear in clusters of 2-3 cells (Tada et al., 2000). Clusters are predominantly located in the SGZ of the DG (Auvergne et al., 2002) and no clusters have been found in the hilus (Ambrogini et al., 2002).

New cells in the hilus seem to play a minor role because former studies have shown that factors influencing cell proliferation had no effect on the numbers of new cells in this region (Bizon & Gallagher, 2003; Merrill et al., 2003; Nilsson et al., 1999). The ventral part of DG is difficult to acquire because of its small size. The probability that the ventral DG appears in a series of every 6th brain section is relatively small. The change of distribution of BrdU-positive cells in the dd and dv blades among post treatment times has been stated to be of limited relevance by Kempermann et al. (2003) because of its high variability over different time points. However, this publication shows, in agreement to our results, a tendency of more labeled cells in the dorsal blade compared to the ventral blade. Another consent is the proportion of labeled cells in the SGZ (more than 70 % at 1 day after BrdU treatment) that becomes smaller with time while more labeled cells are located in GCL (4 weeks compared to 1 day after BrdU treatment), reflecting the migration of maturating cells. We conclude that the pool of newborn cells labeled in our studies did undergo the well-established process of cell proliferation and neurogenesis and we therefore can make at least cautious assumptions on neurogenesis without having visualized neurons.

2.4 Behavior in the complex environment

Purpose

Learning and exploration are complex behaviors that are expedient to be investigated using standardized test for reproducibility. However, we approached this topic from a different perspective that focused on close-to-nature conditions rather than on standardization since there is no experience with rodents in this type of experiment. Nevertheless, we claimed from our data to be reliable and reproducible as well and therefore wanted to describe the behavior, in general, but particularly concerning learning and exploration, of mice in our experimental setup in more detail.

Methods

Therefore, the behavior of groups of mice (SIM, EXP and LRN) was analyzed in terms of dispenser visits when mice were introduced to experimental setup in order to give an estimate about the reactivity to the novel environment. Group LRN (two separate groups of mice: MG 2 and MG 2-1d; see Chapter 2, 3.2.1.3) challenged two different learning tasks based on foraging behavior in the naturalistic environment. Firstly, mice had to distinguish between dispensers of high an low quality. The 10 dispensers were randomly split into two groups of 5 with high and low quality, respectively. The amount of water released at high quality dispensers was at least twice the amount of low quality dispensers. This distribution pattern was inverted every few days or daily (see App. A.3). Secondly, mice could choose between dispensers releasing reliably a constant amount of water (constant dispensers) and dispensers releasing either a high or a low amount with a 50 % probability each (variable dispensers). The average amount of water released at variable dispensers was similar to the release volume of constant dispensers.

Results and conclusions

In the simple, impoverished environment (SIM) mice visited the dispenser in the middle of the room on average 35 hours following the introduction to the room (range: 21-71 hours, Figure 17). Mice in group EXP visited the first dispenser in the novel environment on average 6 hours after setting them into the room (range: 3 - 9 hours). In group LRN mice needed on average 15 hours until the first dispenser visit following the setting into the naturalistic environment (range: 1 - 54 hours). There are big individual differences in the latency for the first dispenser visit. The smallest variance has been observed in group EXP (SD = 0.7 hours). One explanation is that group EXP lived in a special cage outside the room to accustom to the function of water dispensers prior to start

of the experiment. At the time of release into the naturalistic environment, all of the animals were already aware of the relevance and handling of dispensers. This could also be the reason for the short latencies to the first dispenser visit. In group SIM, mice needed significantly longer to visit the dispenser for the first time than mice in groups EXP and LRN (ANOVA with post-hoc, $p < 0.01$). Group SIM was exposed to a large empty room with only one dispenser in the middle. This environment seems to represent a situation that induces anxiety in mice. The measure of latency to enter an exposed area and the duration spent in its center is a commonly used method to investigate anxiety-related behavior (Binder et al., 2004). Therefore, the long latencies for the first dispenser visit in group SIM could be an expression of the natural reaction to an unfamiliar large and exposed room.

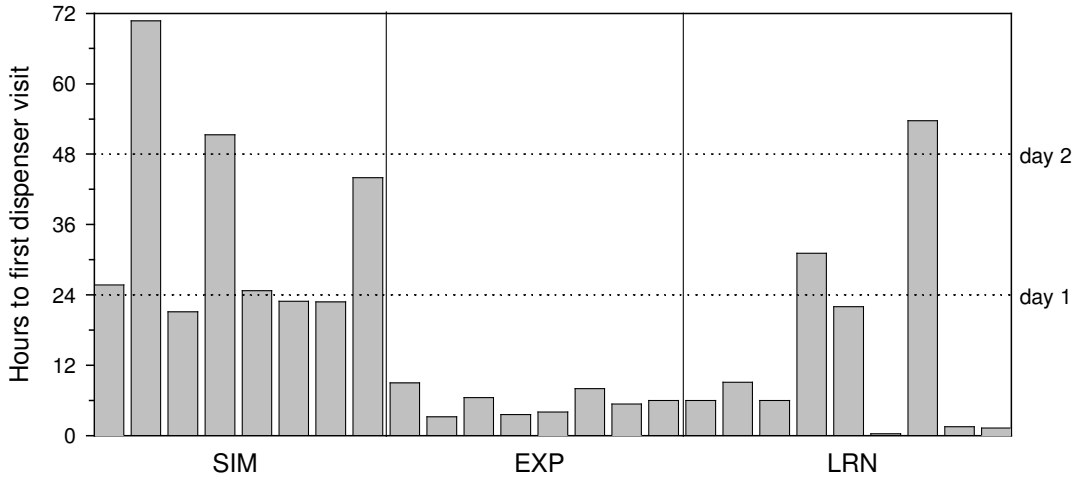

Figure 17: Time to the first visit of each mouse at any dispenser at the start of the respective experiment. SIM = simple environment, EXP = exploration in the complex environment, LRN = learning in the complex environment.

Mice living in the complex environment that contained 10 dispensers covered a minimal distance of 146 m (± 64 SD) and 112 m (± 28 SD) in the LRN and EXP condition, respectively. On average mice visited dispensers 108 (± 64 SD) times per day (maximum 343 times).

The first learning task in group LRN challenged mice to find and remember profitable water dispensers (high quality) that provide twice the amount of water per visit than the less profitable dispensers (low quality). Within the first 4 days, mice increasingly visited high quality dispensers more frequently than low quality dispensers (Figure 18, see also Chapter 2, 4.1.1). In both LRN groups (MG 2, Figure 18A and MG 2-1d, Figure 18B), the portion of visits at high quality dispensers was significantly different from chance level (50 %) on day 3 and 4 (and day 2 for MG 2-1d) in contrast to the first days (Bonferroni, $p < 0.05$). The changing of dispenser qualities, i.e. inverting of the distribution pattern of high and low quality dispensers, lead to an abrupt impairment of performance. In MG 2,

the portion of choices of high quality dispensers decreased to 48 % following pattern inverting (Figure 18, day 56) and was significantly smaller compared to the earlier days (RM-ANOVA with post hoc, $p < 0.001$). During 6 days of daily pattern change mice exceeded chance level only on two days to 72 and 64 % choices of high quality dispensers (day 59 and 61, Figure 18A). In MG 2-1d the performance of mice was not impaired after the first pattern inverting on day 15, but significantly impaired to 47 % choices of high quality dispensers after the second one on day 20 ($p < 0.001$, Figure 18B). The day before the first inverting, the portion of choices of high quality dispensers was smaller than the first 4 days for unknown reasons. This might be an explanation for the lack of a performance impairment in this case. However, performance improved significantly on the second day after inverting dispenser qualities ($p < 0.05$).

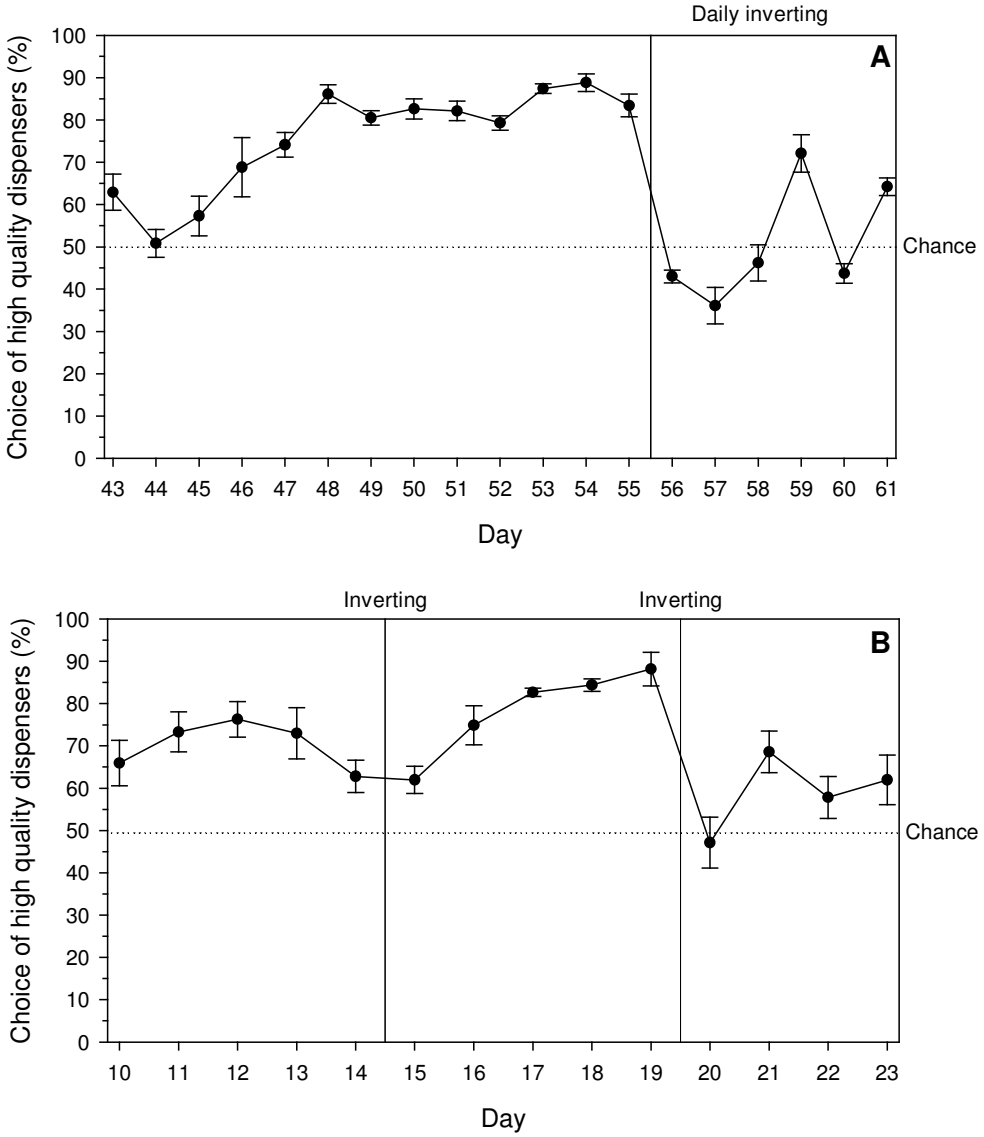

Figure 18: Optimal foraging in the naturalistic environment. Portions of choices of high quality dispensers of all visits in group LRN. One half of the 10 dispensers in the room released twice the amount of water per visits (high quality) than the other half (low quality). A) MG 2 (n = 6). From experimental day 56 on the distribution pattern of high and low quality dispensers was inverted daily. B) MG 2-1d (n = 3). The pattern was inverted on day 15 and 20.

In the second learning task, mice could choose between constant dispensers that released a fix amount of water per visit and variable dispensers that released a high or low amount of water in irregular rotation. In group MG 2-1d mice visited constant and variable dispensers by chance during the 5 days of this condition (Figure 19B). In contrast, mice in MG 2 showed a preference for variable dispensers (Figure 19A). Choices for variable dispensers were significantly different from chance level at all days except for day 66 and 67 ($p < 0.05$). The present data can not give evidence about risk sensitivity of mice because of the different result in the two groups tested. However, risk avoidance as would be expected when animals face variation in food amounts delivered (see Kacelnik & Brito e

Abreu, 1998) was not clearly observed here. In contrast, one group was risk prone for the amount of water released.

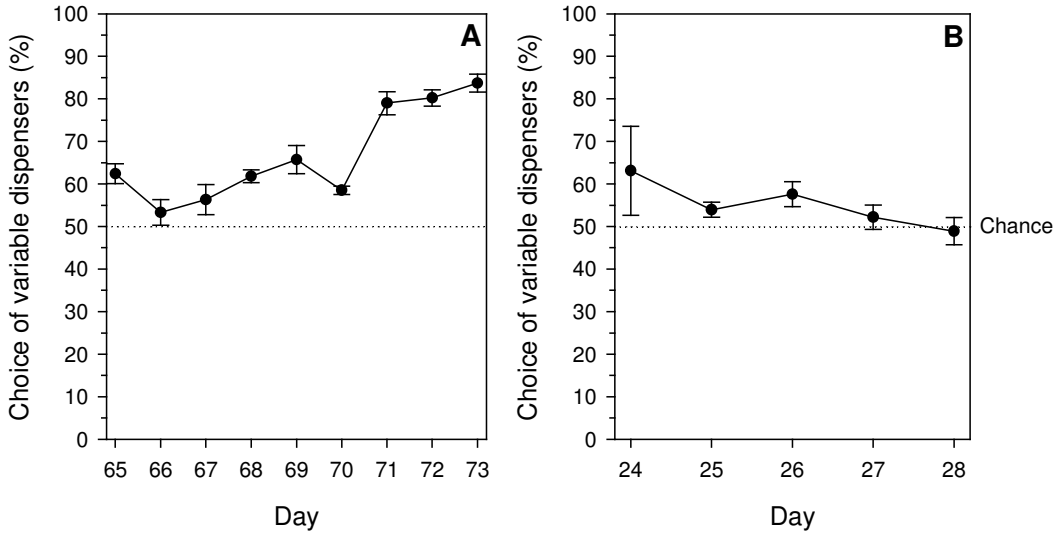

Figure 19: Risky foraging behavior in the naturalistic environment. Portions of choices of variable dispensers from all visits in group LRN. One half of the 10 dispensers in the room released a constant amount of water per visit (constant) and the other half released randomly a high or low amount (variable). A) MG 2 (n = 6). B) MG 2-1d (n = 3).

The present results indicate that foraging behavior of mice in terms of optimal foraging at water dispensers reflects a suitable basis for studies of learning tasks, provided that appropriate dispenser attributes are chosen carefully.

2.5 Recovery from a 7 hour shift of light-dark cycle

Purpose

The 12:12h light-dark (LD) cycle in the laboratories in which experiments took place was different from the circadian rhythm at the animal husbandry (lights off at 19:00h). We used a nocturnal species and therefore shifted the experimental light-dark cycle backwards in order to fit the activity phase of mice to that of humans. In the present experiments, lights were turned off at 10:00 h (MG 7-, 8-,9-, 10-, 11-, 12, 13-, 14-1d), 12:00 h (MG 1, 2), 13:00 h (MG 2-1d, 6, 6-1d) or 16:00 h (MG 4, 4-1d, 5, 5-1d), corresponding to a 9, 7, 6 and 3 hour delay of the LD cycle, respectively. Exemplarily, behavioral data of one group of mice during the first days under new LD conditions were analyzed with respect to general activity in terms of dispenser visits to get an estimate of the duration of the recovery from a jet lag.

Methods

Mice of MG 2 (LRN, n = 6) were transponder tagged on the day of arrival from the animal husbandry (see Table 7). The very next day mice were placed in the complex environment containing 10 water dispensers (see Chapter 2, 3.1.3, Fig. 5). The number of visits at the dispensers, summed up for all individuals, was used as a measure for general activity during 8 consecutive days. The standard designation for LD cycles refers to the Zeitgeber time (ZT), where ZT = 0 corresponds to the time point of lights on. In the present experiment lights off (ZT = 12) was at 12:00 h, which represents a 7 hour delay of LD cycle. Because mice had also to accustom to the novel form of water delivery via dispensers, they had access to a regular water bottle during the first night.

Result and conclusion

The total number of dispenser visits rose from 6 ± 3 on day 1 to a maximum of 111 ± 15 on day 6. The numbers of visits were significantly different between the days (Two-way RM-ANOVA, $F_{(7,35)} = 52.6$, $p < 0.001$). A strong interaction of day $\times$ light phase ($F_{(7,35)} = 11.7$, $p < 0.001$) indicates that the distribution of visits over dark and light period changes over the days. There was no difference in the frequency of dispenser visits between light and dark phase during the first 4 days (Fisher's LSD post-hoc, $p > 0.08$). From day 5 on, mice were significantly more active during the dark period than during the light phase ($p < 0.01$, Figure 20). These data indicate that mice steadily adapt to a 7 hour delaying shift of LD cycle and adjust their active phase to the new cycle. This process seems to require at least 5 days.

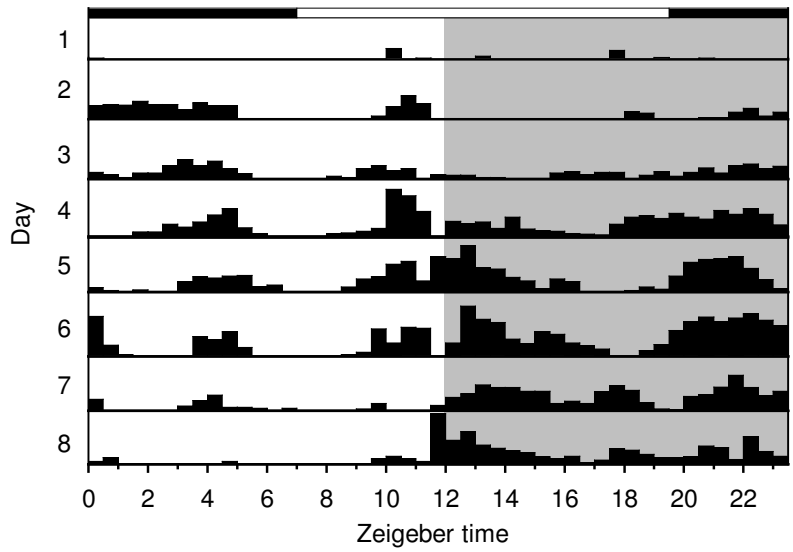

Figure 20: Activity during the first 8 days following a 7 hour delaying shift of light-dark cycle. Black bars represent the numbers of dispenser visits of 6 mice measured in 30 min. intervals. Zeitgeber time (ZT) 0 denotes the time point of lights on (24:00 h) in the new cycle. White background represents light phase and the dark phase is highlighted in gray. The upper horizontal bar shows the light (white) and dark (black) phases of the original cycle (lights on at 7:00 h).

In conclusion of this analysis all animals in the following experiments were allowed to accustom to changing clocks for at least one week (7 days) before starting behavioral testing. Consequently, it is assumed that any effect of jet lag on cell proliferation, if existent, can be excluded for the subjects in the present studies.

2.6 Running in wheels and tubes ad libitum and limited to 1000 m

Purpose

The investigation of cell proliferation in association with two different locomotion types, running in a running wheel and in plane (Chapter 2, 4.2.2), was conducted by limiting running distances to several values. In order to define appropriate distances for mice it was necessary to know their running behavior under unrestricted conditions.

Methods

11 CD-1 mice were divided into 3 groups. Group WHE (n = 4, MG 8-1d) ran in running wheels, TUB (n = 4, MG 9-1d) ran in running tubes and control group CON (n = 3, MG 10-1d) lived in a standard laboratory cage without running opportunity. Single mice were placed in their respective cage containing a running wheel (WHE, see App. A.2.2) and two dispensers or running tubes with two dispensers at the tube ends (TUB, see App. A.2.1) or nothing but one dispenser (CON). During the first days in the

experimental cages, mice were allowed to accustom to the apparatus and the alternating water release at the two dispensers (see App. A.2). Running wheels were present right from start of the experiment, whereas running tubes were attached to the cage of TUB mice when animals coped with visiting dispensers alternating (after 2-4days). All mice were allowed to run unlimited in their respective apparatus for at least 4 days, except for one WHE mouse that was limited to a distance of 2000 m during this period. Following this, running distances were limited to 1000 m per day for both groups. Because TUB mice needed more time to cover this distance than WHE mice and activity should be similarly distributed over the day, the 1000 m were allocated in three portions (250 + 250 + 500 m) for WHE mice. Therefore, wheels were unlocked at 10:00 h (beginning of the dark phase), locked when 250 m were achieved, unlocked at 13:00 h, locked when another 250 m were achieved and unlocked at 18:00 h until final locking after additional 500 m. On the last 4 days of the experiment (total duration 10 – 18 days), mice received BrdU orally via the dispensers with a daily dose of 100 µg/g bodyweight under maintenance of limited running conditions.

Results and conclusions

Mice running ad libitum in a running wheel covered on average a distance of 8325 ± 870 m per day during the first 7 days in their apparatus (range: 197 - 16656 m). The distances were not statistically different between the days (RM ANOVA, $F_{(6,12)} = 2.85$, $p = 0.06$), but tended to increase from day 1 to day 5 (Figure 21A). Mice running unlimited in running tubes covered on average a distance of 1608 ± 139 m per day during the first 5 days (range: 347 - 2512 m). The distance run was significantly shorter on day 1 than on each of the following days (RM ANOVA with post hoc, $p < 0.01$).

The first day with the respective locomotion facility seemed to be needed for familiarization, because running distances were greater and leveled off from day 2 on in WHE and TUB mice, although this is statistically confirmed only for running tubes. The minima of running distance in both conditions occurred on the first day. With respect to running distances from day 2 on, mice ran at least 3365 m in wheels and 1006 m in tubes. These values were used as reference distances for the investigation of activity dependent cell proliferation in the two locomotion types (Chapter 2, 3.2.2).

The amount of proliferating cells was statistically not different between groups (One-way ANOVA, $p = 0.06$). Data showed a trend for cell numbers in TUB mice to be similar to control mice, whereas the number of BrdU-labeled cells (Figure 21B) was higher in DG

of mice running 1000 m in running wheels than in mice running the same distance in tubes and control mice.

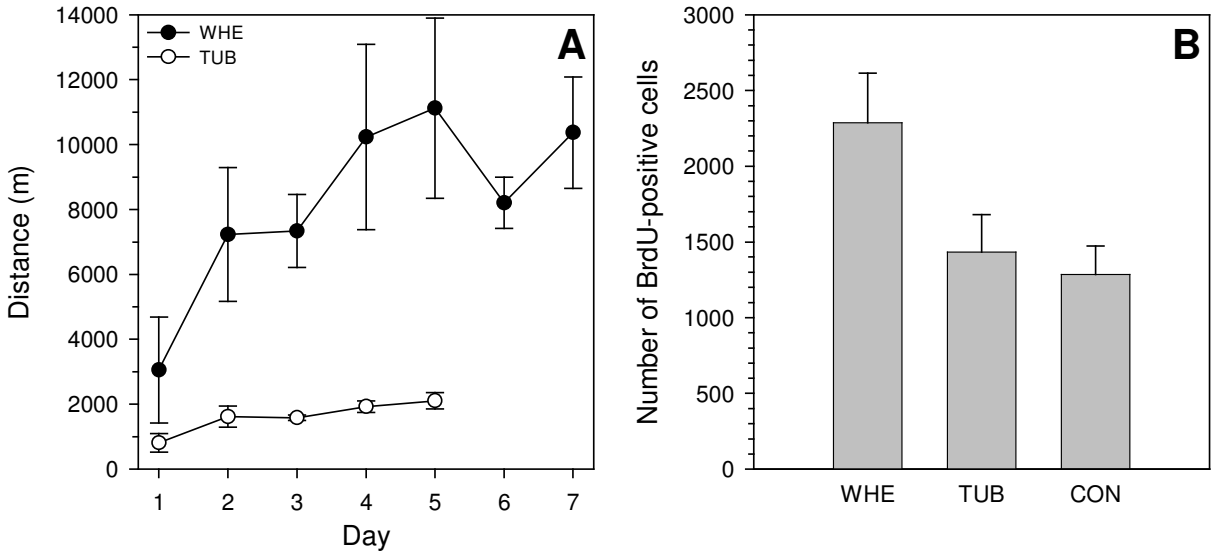

Figure 21: Results of pre-experiment running wheels (WHE) vs. running tubes (TUB). A) Running distances during the first days under the respective conditions. Mice were allowed to run ad libitum. B) Numbers of proliferating cells in mice running in wheels (WHE) or tubes (TUB) and in control mice without locomotion (CON). During BrdU treatment, the running distance was limited to 1000 m per day. Data are means ± SEM.

These data indicate that wheel running itself affects hippocampal cell proliferation rather than the higher level of activity that occurs in wheels compared to locomotion in plane. This is in contrast to our results of Chapter 2 (4.2.2) and to previous reports (Holmes et al. 2004). The time course of this experiment held the risk, that the period of ad libitum locomotion before limiting the daily distance to 1000 m, could have influenced the cell proliferation rate. This assumption is supported by the results of the experiment concerning the persistence of increased cell proliferation following wheel running in the present study (Chapter 2, 4.2.3). We suggest that the days of ad libitum exercise before limiting the distance to 1000 m led to an increase of cell proliferation rate that overlapped the proliferation rate during the following days of limited exercise.

2.7 Presence of an immobilized running wheel

Purpose

The running wheel takes an exceptional position because proofs of its pro-proliferative effect are numerous and consistent. On the other side wheel running has to be seen as an unnatural type of locomotion with self-rewarding and addictive effects (see Sherwin, 1998). In association with the comparison of locomotion in wheels vs. tubes (2.6

and Chapter 2, 3.2.2) the question arose whether simply the presence of a running wheel without exercise in it could alter the rate of cell proliferation.

Methods

The control group of the experiment comparing activity dependent cell proliferation in wheel and tube running mice (Chapter 2, 3.2.2) was divided into two subgroups. In one group (CON-, n = 3), mice were individually placed in standard cages, whereas mice of the second control group (CON+, n = 3) were placed individually in cages containing an immobilized running wheel. Mice lived in the respective cages for 8 days and were injected with BrdU (50 mg/g BW) during the last 4 days with 3 injections per day in 4 hour intervals between injections (see Chapter 2, 3.1.5).

Result and conclusion

The number of BrdU-labeled cells in the DG (1170 ± 130 SEM for CON- and 1434 ± 514 SEM for CON+) was not different between control mice with and without a running wheel (t-test, $p = 0.65$). These data indicate that the presence of a locked running wheel that cannot be used for running is inconsequential for cell proliferation. Although mice used the immobilized wheel for climbing, this kind of enhanced physical activity is assumed to have no effect on the cell proliferation rate.

3 Discussion

The experiments described in this chapter were conducted to endorse that our experimental methods are reproducible and that our results in the previous chapters are in general comparable to data in the literature. That the method of oral BrdU administration is a reliable and useful method for labeling proliferating cells has been illustrated in Chapter 1. This was further verified by the results of experiment 2.1. where we used BrdU injections instead of oral administration in mice living in the naturalistic environment facing the learning task. Furthermore, those results support the conclusion made in Chapter 2 of this thesis, that mice living in the naturalistic environment do not produce more new cells than mice under standard laboratory conditions, in fact independently from the method of BrdU administration.

By applying this methodology to a nectar-feeding bat in experiment 2.2 we demonstrated that oral BrdU administration, as well as the automated setup used, are easily transferable to a wide range of subject species. This method might open novel possibilities for experimental designs in behavioral studies involving drug application. Since only one

subject was used in this experiment, we cannot present universally valid statements. However, our bat showed a preference for one of two dispensers and this species has been demonstrated to have preferences for specific corners of an more complex array of 64 dispensers (Winter & Stich, 2005). The observation of a complete lack of BrdU-labeled cells in the bat's dentate gyrus what somewhat surprising. But the lack of adult neurogenesis in the DG of *Glossophaga soricina* was also reported by Kaiser (2005) and Lipp (personal communication) who applied also BrdU injections and, additionally, endogenous proliferation markers. The assumption is that this species does not or only at low rates produce new granule cells, in contrast to all mammalian species investigated so far, including laboratory rodents (Nilsson et al., 1999; van Praag et al., 1999), wild-living rodents (Amrein et al., 2004), even primates (Gould et al., 1999a) and humans (Eriksson et al., 1998).

The detailed analysis of the distribution of new born cells, described in experiment 2.3 does not yield new findings. But that the distribution and clustering of the BrdU labeled cells in our study are comparable to other published data showed that labeling, counting and analyzing procedures coincide with the methods used in other laboratories.

The aim of experiment 2.4 was to verify the behavioral tests applied to the subjects in the semi-natural environment with respect to learning and memory process. Here, the paradigm of risky foraging seems to be less solid since the results were inconsistent with one another. Further verification is needed to show how far the theory of risky foraging behavior (see Kacelnik & Brito e Abreu, 1998) is applicable to mice since the group size of the present experiment was too small to make definite conclusions. In contrast, the dispenser quality, i.e. amount of water released, has been shown to be a reliable attribute to induce optimal foraging behavior in mice. Experiments in rodents, using a similar paradigm that is based on locations of water dispensers, revealed this task as a proper method for investigation cognitive behavior including hippocampus-dependent spatial learning (Galsworthy et al., 2005). The setup used in this publication was a standard rat cage containing two computer-controlled water dispensers in each corner of the cage and the subjects had to distinguish between corners with water-releasing and disabled dispensers, respectively. This setup differs from the experimental design of the present theses in two major aspects: the environment is rather homecage-like than semi-natural and the learning task is simplified in terms of 4 corners for the choice between correct (water) or incorrect (no water). We aimed at more naturalistic conditions by applying a spacious naturalistic distribution of dispensers and demanding optimization of foraging behavior rather than correct choices. However, both setups are, in principle, based on a similar

concept (location of water dispensers) and we therefore assume that both are similarly implementable to experiments investigating spatial learning and memory tasks.

Before starting our experiments, we applied a minimum acclimatization period of one week in our experimental room to all subjects after relocation from the animal husbandry. The results from experiment 2.5 brought us to this precaution, since this time was required for mice to adopt their circadian rhythm to the new light-dark cycle as indicated by the dispenser visit activity. A jet lag might cause not only behavioral but also physiological changes and, additionally, there is a possibility that cell proliferation rates might vary with circadian phases (Holmes et al. 2004). Considering this, it was important for us, and should be considered in general, to assure exclusion of potential jet leg effects on both, behavior and number of newborn cells.

The objective of experiments 2.6 and 2.7 was to characterize the properties of a running wheel in order to precise our statements on physical activity in general as well as with respect to the naturalistic environment. Our results support the assumption that wheel running reflects a special rather than a natural type of locomotion in rodents (see Sherwin, 1998). Although only the presence of a running wheel itself does not play a role, as shown in experiment 2.7, the extent of activity in a wheel is substantially higher in a wheel than under different conditions like on a treadmill (Ra et al., 2002; Kim et al., 2002), in tunnel systems (Eayrs, 1954) or in tubes (experiment 2.6 of the present study). Our results indicate that the intensity of exercise is the decisive factor for an increase of the cell proliferation rate. Consequently, the strong pro-proliferative effect of physical activity, as demonstrated in numerous studies including the present work, results from the high activity level in the running wheels used in those experiments. Nevertheless, running wheels are an important and effective tool for investigating physical activity and its effects. But once more, our results call attention to potential discrepancies between laboratory conditions and their transferability to the real-life situation.

In summary, this chapter provides a few approaches that should be further investigated in order to consolidate the data given. Nevertheless, they point to important aspects to consider in behavioral experiments, particularly in the context of adult neurogenesis, including the method of drug application and the type of behavioral conditions (learning and running). Furthermore, this chapter might give new impulses to future research with respect to animal behavior as well as neurobiological processes, in particular when combining both in neuroecological studies.

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General discussion

This thesis aimed at the investigation of adult neurogenesis in mice under natural-like conditions in order to get an idea of the functional relevance of this phenomenon. The focus was on the development and application of a novel approach to the question whether and to which extent behavior is related to changes in cell production rates in the adult mouse brain.

Oral BrdU administration is a suitable alternative to BrdU injections

As a first step, we developed a method, which allows for reliable labeling of proliferating cells in the mouse brain while reducing disruption of animal behavior to a minimum. Chapter 1 demonstrates that the use of automated liquid dispensers is particularly suitable for the application of the proliferation marker BrdU. Furthermore, we showed that this method of oral BrdU administration yields appropriate results that are comparable to the results following the commonly used method of BrdU injections.

The preference for administration via injections in almost all studies so far surely results from the fast and simple application of this method. If the time point of BrdU incorporation is crucial, e.g. when investigating the time course (e.g. Hayes & Nowakowsky, 2002) or time points (e.g. Chapter 2, 3.2.3) of proliferation, injections are still the method of choice because oral uptake can hardly be limited in time. However, oral BrdU application can still be optimized in order to solve this disadvantage. Further experiments using different doses of BrdU and alternative solution liquids could help to enhance the flexibility, precision and applicability of this method.

Cell proliferation in a naturalistic environment is not enhanced by exploration or spatial learning

We adopted and applied the novel method of oral BrdU administration to the semi-natural environment and thereby demonstrated in Chapter 2 (4.2.1) and 3 (2.1) its suitability for complex behavioral studies. Under naturalistic conditions, reflected by social living in groups, voluntary movements in a spacious room, there was no impact of physical activity, explorative behavior or learning on cell proliferation. Neither exploration of a novel complex environment nor spatial learning in this environment led to a change of proliferation rate in the dentate gyrus of mice. This finding was confirmed by applying BrdU via both routes, orally and by injections. Paradigms of explorative and learning behavior have been shown to be accepted and implemented by animals as the behavioral analyzes in Chapters 2 (4.1.1) and 3 (2.4) revealed. Not even the higher level of physical

activity that was provided and excited by this environment induced an increase of cell production. Thus, we can conclude that the naturalistic environment had no influence as a whole.

The imitation of natural conditions in the laboratory is difficult because it is a matter of definition what factors are the determining ones for "natural". Of course, our naturalistic setup cannot reflect a natural habitat, but some, to our definition, crucial factors were implemented. These were: much space for voluntary movement, enrichment by obstacles, ropes and cages, social interaction as well as common behavior like nest building and foraging. The one condition that is named naturalistic environment here, combines several aspects that have been shown to influence adult neurogenesis when considered separately. But even when considered as separate factors, the paradigms "environmental enrichment" and "learning" led to results that could not give a conclusive picture of their impact. Table 1 illustrates the contradicting results summarized over publications where similar enriched conditions (enlarged cage containing toys and tunnels) caused an increase in cell proliferation and survival (Kempermann et al., 1998a), only in proliferation (Kempermann et al., 2002; Meshi et al., 2006) or only in survival (e.g. Kempermann et al., 1997b; Brown et al., 2003). No change in neurogenesis could be found in one of the rare studies using different conditions like a visible borrow system (Kozorovitskiy & Gould, 2004). To establish a relation between adult neurogenesis and learning, seems to be even more difficult as the overview of previous results in table 2 shows. With regards to important aspects like hippocampus-dependency or type of learning task, there seems to be no explanatory pattern in the findings of increased (e.g. Gould et al., 1999), unchanged (e.g. Merrill et al., 2003) or even decreased (e.g. Ambrogini et al., 2004) numbers of surviving newborn cells in correlation with learning.

As with all essentially negative results, we cannot draw any strong conclusions about why no effects were seen and whether under different circumstances an effect might have been found. On the other hand, one might also learn from negative results. Possibly, the lack of effect here might be due to the interaction of conditions, which separately influence cell proliferation in opposed ways. For example, competition between socially living animals or the exposed situation in our spacious environments could have had a stress related diminishing effect (see Kozorovitskiy & Gould, 2004), while the physical activity and learning task per se would have induced an increase in proliferation.

The present experiments are hardly comparable to previous studies in this field because the environment, as well as the learning task applied here, is quite different and was not used in this context before. Additionally, the small sample size of animals used in

this study does not allow for conclusive interpretation of the results. However, we do not doubt that we at least opened a new perspective on adult neurogenesis that is worthy of attention and further examination.

Pro-proliferative effect of physical activity is transient and strongly depends on activity level

The present results of Chapter 2 (4.2.2, 4.2.3) and 3 (2.6, 2.7) are consistent with all previous reports of the remarkable pro-proliferative effect of physical activity in a running wheel (see table 3). However, our further investigations on this issue indicate that this effect is transient and strongly depends on the amount of activity. The activity level that is needed to induce an increase of cell production is easily reached in a running wheel, but seems not to be achieved in different types of locomotion. Neither verifiable activity in a tube system nor in the spacious complex environment led to an increased number of newborn cells.

The running wheel is the most commonly used apparatus for studying physical activity and its use is reasonable for investigating exercise-induced effects and their mechanisms. However, one should keep in mind that wheel running is a distinct behavior that is not directly comparable to normal locomotion (reviewed in Sherwin, 1998). The high motivation for using a running wheel (Sherwin, 1996) and the self-reinforcing virtue of wheel running (Sherwin, 1998) are characteristics that stand out from other types of locomotion. Additionally, consistent with the present data, former studies reported that distances covered in a running wheel are conspicuously higher than distances run on a treadmill (Collier & Hirsch, 1971) and in a tunnel system (Eayrs, 1954). Therefore, wheel running is no substitute for planar locomotion and conclusions from wheel running-induced effects should be drawn carefully referring to activity under natural conditions. Yet, only few studies investigated the association between exercise and hippocampal cell proliferation using other apparatuses than running wheels. Rats running on a treadmill had more newborn cells than sedentary controls (Ra et al., 2002; Kim et al., 2002). The calculation of distances covered by rats in these studies resulted in amounts (200 - 540 m) that had no effect on proliferation in our experiments. Two aspects could be responsible for the discrepancy between these results. Firstly, the results might be due to species-specific differences of plasticity. Restraint stress, for example, induced a decrease in the number of newborn cells in rats, but an increase in mice (Bain et al., 2004). Swimming, as another type of locomotion, led to an increased proliferation rate in rats (Ra et al., 2002), but not in mice (van Praag et al., 1999b). Secondly, although running velocity is not associated with

cell proliferation in a running wheel, as demonstrated in the present study, it might play a role in planar locomotion. The treadmill exercise-induced increase of new born cells became smaller with increasing intensity in terms of predefined velocities (Ra et al., 2002). When rats ran at the maximum of 22 m/min on the treadmill, cell proliferation rate was equal to controls. The average velocity performed by mice in the present study (17.6 ± 0.6 m/min) fits well into that category.

Conclusion

We do not want to contradict previously published studies since each of the factors investigated, including enriched environment, learning and locomotion, has been shown to have some impact on cell proliferation and neurogenesis, at least if considered separately under standardized laboratory conditions. It is worth knowing how cell production can be stimulated, especially when considering the possible use of single factors for medical purposes in the field of neurodegenerative diseases. Nevertheless, our results show that one should not directly conclude from those experiments to the functional relevance of naturally occurring neurogenesis.

Outlook

We are far from understanding the regulatory factors and processes of adult neurogenesis in every detail and the functional relevance of this phenomenon still remains unclear.

Physical activity seems to play a major role as pro-proliferating factor and it would be of high interest whether activity induces an increase in cell proliferation also in other species than rodents. Studies with controlled activity in other mammals like cats, dogs or primates could uncover whether the prominent effect found in wheel-running in rodents is really dependent on the level of activity. Accurate tracing of animal movements by e.g. video analysis could give more detailed information about the characteristics of activity outside running wheels and therefore provide for deeper insights into the effects of natural motion on cell proliferation and neurogenesis.

Of special interest for the relevance of adult neurogenesis in real live are studies in wild-living animals. The possibilities of such studies are presently limited by methodical requirements, e.g. the need for sacrificing animals. A comparison of net neurogenesis rates between individuals classified according to pre-defined characteristics like age or sex could bring more insight into effects of e.g. corticosteroids and hormones. Furthermore, comparing individuals showing different levels of physical activity in their natural

environment could answer the question whether cell proliferation is still activity dependent in natural movement.

To get an idea of the importance of neurogenesis for spatial memory performance, more inter-species comparisons would be helpful. In case neurogenesis is correlated to hippocampal-dependent learning and memory, this should be reflected in a difference in neurogenesis rates of related species with differing needs for spatial memory, due to e.g. varied foraging strategies or different availability of resources.

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Appendices

Appendix A Methodical details

A.1 Animals and treatments

All subjects used in this work were female CD-1 mice. This outbred stock origins from a group of Swiss mice that served as progenitors of this stock. Two male and seven female albino mice derived from a non-inbred stock in the laboratory of the Centre Anticancereux Romand, Lausanne, Switzerland were brought to the United states by Clara Lynch in 1926 and arrived at Charles River laboratories in 1959 (Rice & O'Brien, 1980). In contrast to inbred mouse strains, CD-1 mice are genetically heterogeneous and the genetic variation is very similar to estimates from wild populations.

Animals were obtained from the departmental breeding colony (LMU Biocenter, Munich, Germany) or from Charles River Laboratories (Sulzheim, Germany). At the animal husbandry of LMU Biocenter (Munich, Germany), mice were housed in standard cages type III (see A.2) with maximum 7 mice per cage. Following monogamous mating (one male and one female) mothers and their litter changed cages with maximum 2 mothers per cage. 3 weeks after birth, young mice were weaned and siblings housed together in one cage. At Charles River Laboratories mice were kept in standard cages type III with maximum 30 mice per cage. For mating males and females were put together in a mating ratio of 1:10 for 3 weeks and subsequently pregnant females changed cages (8 per cage). 4 mothers lived together with 50 pups until weaning after 3 weeks.

On arrival, mice were placed in cages within the experimental room to habituate to the light:dark cycle obtained during the following experiments. During the acclimatization period, the groups of mice were placed in standard laboratory cages (type III) for small groups of 3 to 6 individuals or in larger cages (type IV, $59.5 \times 38 \times 20$ cm, area 1820 cm^2) for groups of 8 to 12 individuals (MG 11-, 12-, 13-1d) with free access to food and water. The duration of acclimatization time varied between 4 and 28 days (see Table 7, 'Start of experiment'). For experiments with grouped-living mice that shared water dispensers, individuals were labeled by injecting transponders during acclimatization period (Table 7, 'Transponder injection'). Therefore, mice were anesthetized with isoflurane and sterilized transponders encapsulated in biocompatible glass (2.1×12 mm, 0.09 g, Unique, Sykomat) were injected subcutaneously between the shoulder blades using an injector (Trovan). This procedure was completed within approximately 1 minute and mice recovered rapidly from

anesthesia and surgery. In all experiments with separately housed mice, we forbore transponder tagging.

Mice had always free access to food (compressed pellets, complete diet for rat and mouse husbandry, ssniff GmbH, Soest, Germany). Access to drinking water via a water bottle or dispensers was free except for mice with oral BrdU administration. During the days of BrdU treatment, those mice were water deprived for 2 to 6 hours prior to the start of BrdU delivery. Drinking water in bottles and dispenser reservoirs was refreshed every 3 days.

The first day in the respective experimental environment, e.g. the complex room or a cage containing a running wheel, is called 'start of experiment' and 'end of experiment' is the day of perfusion (Table 7). Mice were on average 10 weeks old (± 2 SD, range: 7-16 weeks), weighing 28.2 g (± 3.9 SD, range: 20.8 - 41.6 g) at start of experiment and 13 weeks old (± 4 SD, range: 8-31 weeks) weighing 28.3 g (± 4.4 SD, range: 20 - 39.9 g) at the end of experiment.

Table 7: Overview of animal treatments. The nomenclature of individuals (**animal-ID**) contained species abbreviation (MM = *Mus musculus*), year and sequential number. **Group-ID** is the sequential number of experimental groups including the suffix "-1d" for groups with a **post treatment time (PT)** of one day (i.e. killed one day after the last dose of BrdU) in contrast to groups without suffix with a PT of 28 days. Individuals lived in their experimental housing condition (for details see 4.1.3) separately or in groups of several **individuals simultaneously**. CD-1 mice were born in and obtained from the departmental breading colony of the LMU (Biocenter, Munich) or Charles River Laboratories. Day 0 represents the **date of arrival** at the experimental room for acclimatization to light:dark cycle. Acclimatization period ended at start of experiment, i.e. the first day under experimental conditions. Days of **transponder injection**, **start** and **end of experiment** and **start of BrdU treatment** are given with respect to day 0. **Age** and **body weight** of mice are given for the first (start) and last (end) day of experiment, respectively. Mice received **BrdU** via intraperitoneal injections (i.p.) or orally via drinking water (oral) with the given single doses on one day or on 4 consecutive days. From a total of 127 animals, 5 mice were **excluded** from analysis of BrdU-positive cells because histological staining procedure failed to label cells (*). 6 mice were used for other analyses than BrdU labeling (§) and the BrdU cell count of one mouse was excluded as outlier (#).

Animal-ID	Group-ID	Group name	Condition	Experimental housing	Individuals simultaneous	Date of birth	Origin	Date of arrival (=day 0)	Transponder injection (day x)	Start of experiment (day x)	Start of BrdU treatment (day x)	Posttreatment time (PT)	End of experiement (day x)	Duration of experiment (days)	Age at start (weeks)	Age at end (weeks)	Body weight at start (g)	Body weight at end (g)	BrdU: application	BrdU: single dose (µg/g BW)	BrdU: duration (days)	BrdU: doses per day	Excluded
MM 03-25																	38						§
MM 03-26																	35.7						§
MM 03-27																	32.4						§
MM 03-28	MG 0	CON	control	cage	6	01.06.03	LMU	08.09.03	--	7	7	28	39	32	15	19	41.6	35	i.p.	50	4	1	
MM 03-29																	34.3	32.7					
MM 03-30																	35	35					*
MM 03-05						01.02.03		08.05.03	0	0	95	28	127	127	16	31	29.3						§
MM 03-08																	32.5						§
MM 03-09	MG 1	LRN	learning	complex room	6		LMU										29.5		i.p.	50	4	1	§
MM 03-10						01.04.03		19.05.03	7	7	76	28	108	107	8	23	27.5	32.3					
MM 03-11																	30.2	30.5					
MM 03-12																	27.5	29					*
MM 03-19																	28.4	33.5					
MM 03-20																	27.2	33.4					*
MM 03-21	MG 2	LRN	learning	complex room	6	15.07.03	LMU	15.09.03	0	1	49	28	81	75	9	19	28	35	oral	100	4	1	
MM 03-22																	28.9	34.8					
MM 03-23																	27.7	34					
MM 03-24																	26	34.4					
MM 04-27																	31.4	29.5					
MM 04-28	MG 2-1d	LRN	learning	complex room	3	03.02.04	LMU	13.04.04	2	6	41	1	46	40	11	16	27.5	25.3	oral	100	4	1	
MM 04-29																	27.7	25.7					
MM 03-33																	40.2	39.6					
MM 03-34																	32.7	35.7					
MM 03-35	MG 3	CON	control	cage	6	01.10.03	LMU	01.12.03	0	9	14	28	46	37	10	15	33.5	35.4	oral	100	4	1	
MM 03-36																	33.2	35.6					
MM 03-37																	35.3	38.4					
MM 03-38																	40	39.9					
MM 04-05																	29.8	30.5					
MM 04-06																	26.7	29.5					
MM 04-07	MG 4	EXP	exploration	complex room	8/5	10.11.03	LMU	20.01.04	0	6	11	28	43	37	11	16	27.4	29.8	oral	100	4	1	
MM 04-08																	26.8	29					
MM 04-09																	26.9	26.9					*
MM 04-10																	26.4	24					
MM 04-11	MG 4-1d	EXP	exploration	complex room	8	10.11.03	LMU	20.01.04	0	6	11	1	16	10	11	12	29.2	28.2	oral	100	4	1	
MM 04-12																	28.3	28.5					
MM 04-13																	29.3	29.6					
MM 04-14																	31.8	32.7					
MM 04-15	MG 5	SIM	poor in stimuli	simple room	8/5	10.12.03	LMU	18.02.04	7	7	13	28	45	38	11	16	24.7	24.8	oral	100	4	1	
MM 04-16																	27.8	30.4					*
MM 04-17																	29.5	32.2					
MM 04-18																	26.2	25					
MM 04-19	MG 5-1d	SIM	poor in stimuli	simple room	8	10.12.03	LMU	18.02.04	7	7	13	1	18	11	11	12	32.5	31	oral	100	4	1	
MM 04-20																	29.2	32.2					
MM 04-22																	29.3	30.3					
MM 04-23																	25.7	27.8					
MM 04-24	MG 6	CON	control	cage	5	03.02.04	LMU	13.04.04	2	3	7	28	39	36	10	15	30.1	30.8	oral	100	4	1	
MM 04-25																	27.3	28					#
MM 04-26																	30.7	32.5					
MM 04-40																	34.5	35					
MM 04-41						10.03.04		28.05.04	4	4	7		12	8	11	12	28.5	27.8					
MM 04-42	MG 6-1d	CON	control	cage	3		LMU					1					32.6	32.7	oral	100	4	1	
MM 05-42																	30						
MM 05-43						05.09.05		28.10.05	4	4	9		14	10	8	10	28.6						
MM 05-44																	27.7						
MM 05-09																	30.3	30.6					
MM 05-10	MG 7-1d	RUN	running wheel ad lib.	cage + wheel	4	12.12.04	LMU	16.02.05	7	7	21	1	26	19	10	12	31.2	31.5	oral	100	4	1	
MM 05-11																	29.9	31.1					
MM 05-12																	31.9	32					

Animal-ID	Group-ID	Group name	Condition	Experimental housing	Individuals simultaneous	Date of birth	Origin	Date of arrival (=day 0)	Transponder injection (day x)	Start of experiment (day x)	Start of BrdU treatment (day x)	Posttreatment time (PT)	End of experiement (day x)	Duration of experiment (days)	Age at start (weeks)	Age at end (weeks)	Body weight at start (g)	Body weight at end (g)	BrdU: application	BrdU: single dose (µg/g BW)	BrdU: duration (days)	BrdU: doses per day	Excluded
MM 05-27						28.03.05		24.05.05		20	33		38	18	10	13	32.3	33.1					
MM 05-30	MG 8-1d	WHE	running wheel 1000m	cage + wheel	1	18.04.05	LMU	23.06.05	--	8	18	1	23	15	10	12	32.2	31.4	oral	100	4	1	
MM 05-33						19.04.05		02.07.05		13	23		28	15	12	14	32.5	38.8					
MM 05-39						10.08.05		30.09.05		4	10		15	11	8	9	27.3	28					
MM 05-28						28.03.05		24.05.05		21	33		38	17	10	13	32.2	29.9					
MM 05-31	MG 9-1d	TUB	running tubes 1000m	cage + tubes	1	18.04.05	LMU	23.06.05	--	8	18	1	23	15	10	12	28.8	28.8	oral	100	4	1	
MM 05-34						19.04.05		02.07.05		13	23		28	15	12	14	30.9	30.6					
MM 05-40						10.08.05		30.09.05		4	10		15	11	8	9	26.8	30.5					
MM 05-29										28	33		38	10	11	13	32.5	29.8					
MM 05-32	MG 10-1d	CON	control	cage	1	28.03.05	LMU	24.05.05	--	8	18	1	23	15	10	12	29.5	29.5	oral	100	4	1	
MM 05-35										13	23		28	15	12	14	34.4	34.8					
MM 05-45			running wheel 500m														22.8	26.3					
MM 05-46			running wheel 1000m			13.09.05				5	18		23	18	8	10	23.7	25.6					
MM 05-47			running wheel 2000m														24.4	24					
MM 05-48			running wheel 4000m					03.11.05									25	25.5					
MM 05-50			running wheel 0m														25.4	24.3					
MM 05-51			running wheel 250 m			20.09.05				22	25		30	8	9	10	24.5	25					
MM 05-52			running wheel 500 m														23.9	24.4					
MM 05-53			running wheel ad lib.														23.7	24					
MM 05-55			running wheel ad lib.														22.9	23.7					
MM 05-56			running wheel 4000m			12.10.05				8	11		16	8	7	8	23.2	23.8					
MM 05-57			running wheel 1000m				Charles River Laboratories										23	21.2					
MM 05-58	MG 11-1d	WHE	running wheel 250m	cage + wheel	1			24.11.05	--			1					22.6	22.7	i.p.	50	4	3	
MM 05-60			running wheel 2000m														21.4	22					
MM 05-61			running wheel 500m			19.10.05				15	18		23	8	7	8	22.8	20.2					
MM 05-62			running wheel 0m														22.9	21.9					
MM 05-63			running wheel 1000m														20.8	20					
MM 05-65			running wheel 250m														28.4	26.1					
MM 05-66			running wheel ad lib.							7	10		15	8	8	9	29	27					
MM 05-67			running wheel 4000m														28.5	25.4					
MM 05-68			running wheel 2000m					09.12.05									27.6	26.5					
MM 05-70			without running wheel														25.4	24					
MM 05-71			without running wheel			26.10.05				14	18		23	9	8	9	29.6	27.4					
MM 05-72			without running wheel														28	27					
MM 05-73			running wheel 0m														27.2	25.7					
MM 05-49			running tubes 400m			13.09.05		03.11.05		5	18		23	18	8	10	24.3	24.3					
MM 05-54			running tubes 800m			20.09.05	Charles River Laboratories			22	25		30	8	9	10	25.4	22.6					
MM 05-59	MG 12-1d	TUB	running tubes 400m	cage + tubes	1	12.10.05		24.11.05	--	8	11	1	16	8	7	8	22.5	22.6	i.p.	50	4	3	
MM 05-64			running tubes 400m			19.10.05				15	18		23	8	7	8	23.4	22.4					
MM 05-69			running tubes 400m			19.10.05		09.12.05		7	10		15	8	8	9	29.3	26.5					
MM 05-74			running tubes 800m			26.10.05				14	18		23	9	8	9	27	25.4					
MM 06-01													18	11	8	9	26.3	24.6					
MM 06-02										7	13		18	11	8	9	27.4	26					
MM 06-03													18	11	8	9	26.8	23.4					
MM 06-04													18	11	8	9	26	22.8					
MM 06-06													25	14	9	10	26.2	24.4					
MM 06-07						11.12.05		31.01.06		11	20		25	14	9	9	26.7	24.5					
MM 06-08													25	14	9	9	24.7	25					
MM 06-09													19	8	9	9	28.2	26.6					
MM 06-11													24	9	9	10	29.2	27.8					
MM 06-12							Charles River Laboratories			15	19		24	9	9	10	27.2	26.5					
MM 06-13	MG 13-1d	PER	running wheel ad lib.	cage + wheel	1				--			1	24	9	9	10	25.2	24.2	i.p.	100	1	1	
MM 06-14													23	8	9	10	26.7	27.5					
MM 06-16								31.01.06		19	22		27	8	10	10	23.2	23.5					
MM 06-23										5	14		19	14	9	10	26						
MM 06-24													19	14	9	10	25.1						
MM 06-25											11		16	11	9	10	23.4	22					
MM 06-26						02.02.06		28.03.06					16	11	9	10	27.5	25.4					
MM 06-27										9	12		17	8	9	10	26.8	25.1					
MM 06-28													17	8	9	10	26.4	27.3					
MM 06-29											13		18	9	9	10	24.6						
MM 06-30													18	9	9	10	23.6						
MM 06-05										7	13		18	11	8	9	25.9	25.5					
MM 06-10						11.12.05	Charles River Laboratories	31.01.06		11	16		21	10	9	9	28.2	26.3					
MM 06-15	MG 14-1d	CON	control	cage	1				--	15	21	1	26	11	9	10	23.8	24	i.p.	100	1	1	
MM 06-31						02.02.06		28.03.06		5	11		16	11	9	10	26.3	26					
MM 06-32										9	14		19	10	9	10	25.4						

A.2 Cages

All cages used in the experiments were standard laboratory cages type III ($42.5 \times 26.6 \times 15$ cm, area 810 cm^2) covered with stainless wire lids (7 mm wire spacing). Water bottle and food were placed on the lid outside the cage, but accessible for mice from inside. The cage bottoms were covered with wooden shavings as bedding material (Dehner, Germany) and cleaning papers, paper rolls and cartons were added as nest-building material.

A.2.1 Cage with running tubes

In order to investigate the role of locomotion type, running in a running wheel was opposed to running in plane. For the latter type of locomotion, a standard cage was equipped with tubes in which a mouse could move from one tube end to the other end (Figure 22). The motivation to run through the tubes was provided by water delivery at the tube ends. Therefore, two holes (about 5 cm in diameter) were cut next to each other into the short wall of the cage. A 2 m long polypropylene high-temperature resistant (HT)-pipe DN 50 (50 mm i.d.) was mounted to each of the holes. To this pipe, a second HT-pipe, 2 m in length, was connected via two orthogonal plug-in sockets resulting in a U-shaped tube with a total length of 4.3 m that started from each of the holes. At the end of each tube, a water dispenser was fixed by clamps. Lockable swinging doors with light barriers connected the cage and each of the tubes. Locking worked by a motorized lever arm fixed above the door and allowed passing from tube to cage, but inhibited entering the tube from the cage. Once the tube entries and consequently access to water were blocked, a third dispenser, fixed to the sidewall of the cage, was activated and provided further water delivery.

Figure 22: Cage with running tubes.

The registration of each door pass and dispenser visit allowed for the recording and control of the distance covered by mice in this tube system. By numeration door 1 corresponded to the tube leading to dispenser 4 and door 2 to the tube leading to dispenser 3.

The two water dispensers at the tube ends released water in a alternating manner, i.e. once a mouse had received water at one dispenser, it had to run 8.6 m (2×4.3 m) through

both tubes to get water at the other dispenser. Thus, the running distance d_t in m was calculated from

d_t = number of dispenser visits × 8.6 m.

For analysis of running velocity, events were assessed as relevant if a tube entry event (result sheet "Nr_d_Einheit" = 1 or 2, Table 8) was directly followed by a dispenser visit (result sheet "Nr_d_Einheit" = 4 or 3, respectively) or vice versa. The duration between door and dispenser event represents the time needed to get from one tube end to the other one. Thus, the duration in seconds (result sheet "Tür2_Tränke 3 (s)" and "Tür1_Tränke4 (s)") was calculated by subtracting the corresponding time points of the respective events (result sheet "Uhrzeit"). The running velocity v_t in m/s (result sheet "Geschwindigkeit2") for each of the two tubes was calculated from

v_t = 4.3/duration.

Because mice did not always run straight from one tube end to the other, but also stayed within a tube for a while, events were only included in velocity calculation if the duration was less than 60 seconds.

Table 8: Exemplary extract from a result sheet of an experiment with running tubes (MM 05-54, 12-1d). "Vers-Tag" is the day of experiment, "Rechnerzeit" shows the date/time information in excel format, "Datumzeit" is the respective date/time information with the associated milliseconds in "Datumzeit_ms", "Funktionstyp" indicates the activated unit (1=dispenser, 17=door) with the specification of unit number in "Nr_d_Einheit" (dispenser 3 and 4, door 1 and 2), "Info_Code"=1 stands for input information, "Wert1_Dauer" is the duration of the event in ms, "Wert2" shows the opening duration of dispenser valves in ms (output), "Uhrzeit" is the time of day extracted from "Datumzeit", "Tür2_Tränke3 (s)" is the duration in s the mouse needed from door 2 to dispenser 3 and vice versa, "Tür1_Tränke4" shows the same for door 1 and dispenser 4 and "Geschwindigkeit2" is the calculated running velocity in m/s. Columns that have been calculated manually are highlighted in gray in contrast to data created automatically (white).

Vers-Tag	Rechner-zeit	Datumzeit	Datum-zeit_ms	Funktions-typ	Nr_d_ Einheit	Info_ Code	Wert1_ Dauer	Wert2	Uhrzeit	Tür2_ Tränke3 (s)	Tür1_ Tränke4 (s)	Geschwin-digkeit2
2	40930040	26.11.05 11:22:10	40	1	4	1	174	0				
2	40951950	26.11.05 11:22:31	950	17	1	1	3083	0	11:22:31		21	0.20
2	40978462	26.11.05 11:22:58	462	17	2	1	1449	0	11:22:58			
2	40993960	26.11.05 11:23:13	960	1	3	1	200	300	11:23:13	15		0.29
2	41058936	26.11.05 11:24:18	936	17	1	1	2205	0	11:24:18			
2	41083640	26.11.05 11:24:43	640	1	4	1	4672	300	11:24:43		25	0.17
2	41095527	26.11.05 11:24:55	527	1	4	1	282	0	11:24:55			
2	41114886	26.11.05 11:25:14	886	1	4	1	497	0	11:25:14			
2	41116996	26.11.05 11:25:16	996	1	4	1	361	0	11:25:16			
2	41282544	26.11.05 11:28:02	544	17	1	1	42456	0	11:28:02		166	
2	41285796	26.11.05 11:28:05	796	17	1	1	1889	0	11:28:05			
2	41296321	26.11.05 11:28:16	321	1	4	1	874	0	11:28:16		11	0.39
2	41347198	26.11.05 11:29:07	198	17	1	1	2855	0	11:29:07		51	0.08
2	41349003	26.11.05 11:29:09	3	17	2	1	304044	0	11:29:09			
2	41396309	26.11.05 11:29:56	309	1	3	1	189	300	11:29:56	47		0.09
2	41467126	26.11.05 11:31:07	126	17	2	1	3760	0	11:31:07	71		
2	41810433	26.11.05 11:36:50	433	17	1	1	1799	0	11:36:50			
2	41824954	26.11.05 11:37:04	954	1	4	1	5255	300	11:37:04		14	0.31

A.2.2 Cage with running wheel

One metal running wheel (14.5 cm in diameter) per cage was fixed up to the wire mesh lid. In group RUN (MG 7-1d) 4 mice shared one running wheel and one water dispenser. Transponder tagged individuals were identified when visiting the dispenser, but there was no control for the use of the running wheel in terms of individual identification. The dispenser was plugged through a matching hole in the cage wall and therefore was easily accessible for animals.

For the rest of experiments including a running wheel (MG 8-, 11-, 13-1d), a device for recording and controlling wheel revolutions was added. Therefore, a photoelectric barrier was fixed to the side of the wire mesh that recorded every passing of the wheel brace. Hence, wheel revolutions and consequently running distance as well as running velocity could be calculated. The running wheel was controllable by a motorized lever arm fixed up above the wheel at the wire mesh lid (Figure 23). Once the number of wheel revolutions reached a predefined value, the lever arm lowered between the wheel wires and thereby locked the wheel.

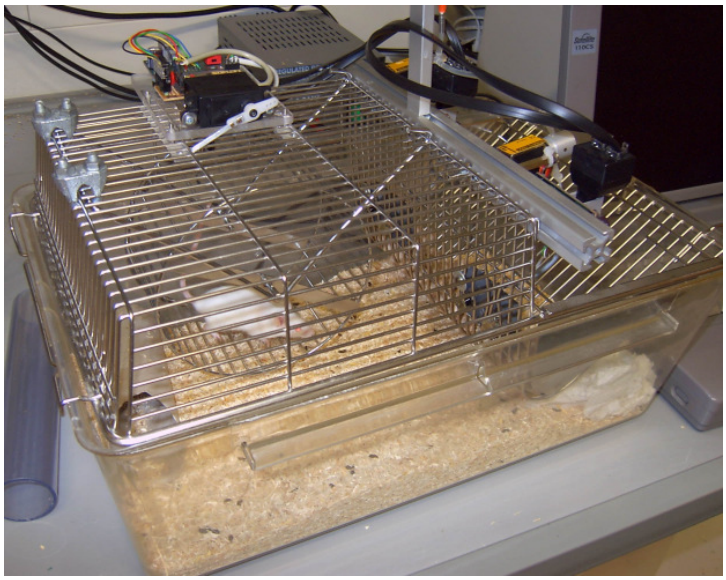

Figure 23: Cage with running wheel.

One wheel revolution corresponds to two interruptions of the light barrier (brace on both sides of the wheel center). Sensor signals were counted and merged to one event if the time interval between two signals did not exceed 500 ms (result sheet 'Wert2', Table 9). The number of revolutions of one event results from the number of sensor signals divided by 2. For analysis of running distance and velocity, only events containing more than 5 signals were included in order to compensate for signals elicited by swinging of the wheel rather than by running activity. The running distance d_w in m was calculated from

d_w = number of revolutions × 2πr = number of revolutions × 0.455 m

with r = wheel radius (0.0725 m).

The running velocity in revolutions per second (result sheet "Umdrehungen/s) for each event was calculated by dividing the number of revolutions by the duration of the event (result sheet 'Wert1_Dauer'). The velocity v_w in m/s (result sheet "Geschwindigkeit (m/s)") was calculated from

v_w = revolutions/s × 2πr = revolutions/s × 0.455 m.

Summing up the event durations (Result sheet "Wert2") gave an estimate of the time spent in running per day. Drinking water was delivered by a water bottle (MG 13-1d) or via two dispensers (MG 8-, 11-1d). In the latter case, the two dispensers released water in an alternating manner in order to provide comparable conditions for tube and wheel running mice.

Table 9: Exemplary extract from a result sheet of an experiment with running wheels (MM 05-60 to 63, 11-1d). Three cages, each containing one wheel and two dispensers, were controlled simultaneously. Columns equal those from tube experiments (Table 8) with the following exceptions. In "Funktionstyp" unit 17 (= door) was replaced by 16 (= wheel), in "Nr_d_Einheit" units are numbered 1,2 and 3 (wheels) and 1,2,3,4,5 and 6 (dispensers) and "Wert2" additionally indicates the number of wheel sensor signals. Manually calculated values (highlighted in gray) were "Umdrehungen/s" (revolutions per s = "Wert2"/2), "Geschwindigkeit (m/s)" ("Wert1_Dauer"/revolutions per s) and "Tiernummer" which results from unit numbers ("Nr_d_Einheit").

Vers-Tag	Rechner-zeit	Datumzeit	Datum-zeit_ms	Funktions-typ	Nr_d_ Einheit	Info_ Code	Wert1_ Dauer	Wert2	Uhrzeit	Umdre-hungen/s	Geschwin-digkeit (m/s)	Tier-nummer
6	37227699	14.12.05 10:20:27	699	1	5	1	192	350	10:20:27			3
6	37230810	14.12.05 10:20:30	810	1	5	1	9819	0	10:20:30			3
6	37293412	14.12.05 10:21:33	412	16	2	1	71306	89	10:21:33	0.62	0.28	2
6	37368639	14.12.05 10:22:48	639	16	2	1	110433	162	10:22:48	0.73	0.33	2
6	37400841	14.12.05 10:23:20	841	16	1	1	45947	51	10:23:20	0.55	0.25	1
6	37448180	14.12.05 10:24:08	180	16	1	1	2509	2	10:24:08	0.40		1
6	37451029	14.12.05 10:24:11	29	16	1	1	31093	38	10:24:11	0.61	0.28	1
6	37479562	14.12.05 10:24:39	562	16	2	1	45175	67	10:24:39	0.74	0.34	2
6	37482435	14.12.05 10:24:42	435	16	1	1	26883	34	10:24:42	0.63	0.29	1
6	37509469	14.12.05 10:25:09	469	16	1	1	7891	10	10:25:09	0.63	0.29	1
6	37517933	14.12.05 10:25:17	933	16	1	1	15240	18	10:25:17	0.59	0.27	1
6	37534280	14.12.05 10:25:34	280	16	2	1	23752	31	10:25:34	0.65	0.30	2
6	37539987	14.12.05 10:25:39	987	16	1	1	15647	21	10:25:39	0.67	0.31	1
6	37556521	14.12.05 10:25:56	521	16	1	1	10016	12	10:25:56	0.60	0.27	1
6	37558078	14.12.05 10:25:58	78	16	2	1	8896	13	10:25:58	0.73	0.33	2
6	37566725	14.12.05 10:26:06	725	16	1	1	89907	129	10:26:06	0.72	0.33	1
6	37567211	14.12.05 10:26:07	211	16	2	1	13492	20	10:26:07	0.74	0.34	2
6	37581664	14.12.05 10:26:21	664	16	2	1	6556	8	10:26:21	0.61	0.28	2

A.3 Treatments and parameters in the simple and complex environment

A.3.1 Group SIM – simple environment (MG 5/MG 5-1d)

In the simple environment on single water dispenser was placed in the middle of the room. The amount water of water released per single visit was 144 µl during the first 5 days of the experiment and reduced to 35 µl for the remaining period (Table 10). The dispenser was locked for subsequent visits of the same individual for 5 seconds during the first 5 days and for 1 minute during the rest of the experiment.

Table 10: Dispenser attributes for group SIM in the simple environment containing one dispenser.

From day	To day	Dispenser attributes	
1	5	Dispenser number	12
		release volume (µl)	144
		opening duration (ms)	200
		lock duration [s]	5
6	37	Dispenser number	12
		release volume (µl)	35
		opening duration (ms)	100
		lock duration [s]	60

Oral BrdU administration from day 6 to 9 (dispenser 12, 144 µl, 200 ms, 5s)

A.3.2 Group EXP – exploration in the complex environment (MG 4/MG4-1d)

In order to present the complex room as novel environment, mice of group EXP were allowed to accustom to the function of water dispensers in a special cage prior to start of the experiment. This cage contained one dispenser attached to the wall and raised about 80 cm above the ground of the cage. Mice reached the dispenser via a rope fixed in the middle of the cage floor. During the 6 day in this cage water release volume was successively reduced from 85 µl to 35 µl per visit with a lock duration of 10 seconds for visits of the same individual (Table 11). In the complex environment, the 10 dispensers were of same quality, releasing about 50 µl per visit with 1 minute lock duration between visits of the same individual. Dispenser no. 5 was used for oral BrdU application and therefore excluded from the complex environment.

Table 11: Dispenser attributes for group EXP in the complex environment containing 11 dispensers. On days 1 to 6 mice were accustomed to the function of the water dispensers in a special cage containing 1 dispenser accessible via a rope. Day 7 was the first day in the complex environment with 10 dispensers of equal quality. Dispenser 5 was used exclusively for BrdU administration.

from day	to day	Dispenser attributes											
1	2	Dispenser number	12	Special cage with one dispenser and rope									
		release volume (µl)	85										
		opening duration (ms)	200										
		lock duration [s]	10										
3	5	Dispenser number	12										
		release volume (µl)	65										
		opening duration (ms)	150										
		lock duration [s]	10										
5	6	Dispenser number	12										
		release volume (µl)	35										
		opening duration (ms)	100										
		lock duration [s]	10										
7	38	Dispenser number	1	2	3	4	5	6	7	8	9	10	11
		release volume (µl)	52	48	50	52		46	52	52	40	56	48
		opening duration (ms)	130	100	120	120		110	100	100	110	110	100
		lock duration [s]	60	60	60	60		60	60	60	60	60	60

Oral BrdU administration from day 7 to 10 (dispenser 5, 160 µl, 250 ms, 5 s)

A.3.3 Group LRN – learning in the complex environment

Mice in the complex environment facing a learning task were two temporally separated groups, one with a post-treatment time (PT) of 1 day (MG 2-1d) and one with a PT of 28 days (MG 2). The dispenser attributes for both groups were as follows:

MG 2

During the first days in the complex environment all dispensers were of equal quality, releasing about 120, 50 or 25 µl per visit with equal amounts at a time and 30 seconds lock duration for subsequent visits of the same individual at the respective dispenser (Table 12). On day 43 the first learning task started. Dispensers were divided into two groups characterized by distinct qualities (20 µl for low quality and 60 ml for high quality). Following 13 days under this condition the pattern of high and low quality dispensers was inverted, i.e. dispensers that were previously of high quality (Pattern A) were now of low quality (Pattern B) and vice versa. The pattern was inverted daily for 6 days followed by two days of pattern inverting every 3 hours. On day 65 the second learning task was introduced in which mice had to distinguish between constant and variable dispensers for 8 days. Therefore, one half of the dispensers released a unvarying amount of water (40 µl per visit, constant), whereas in the other half each dispenser released high (70 µl) and low (20 µl) amount in irregular sequence (variable). During the last 2 days of this period dispenser no. 5 was added to the room again and released sugar water instead of pure water in order to induce competition between individuals for this attractive dispenser. Result of behavioral analysis are described in app. 2.4.

Table 12: Dispenser attributes for group LRN (PT = 28) in the complex environment containing 11 dispensers. Until day 42, all dispensers were of equal quality. From day 43, the first day of BrdU treatment, there were 2 types of dispensers: high (italicized) and low quality (day 43 - 64) or risky (italicized) and constant (day 65 - 73). Dispenser 5 was used for BrdU administration (day 43 - 46) and as competition dispenser (day 71 - 73), but excluded from all other conditions.

from day	to day	Dispenser attributes												Dispenser quality
1	3	Dispenser number	1	2	3	4	5	6	7	8	9	10	11	
8	10	release volume (µl)	130	120	118	144	128	130	126	132	120	116	128	equal quality
14	16	opening duration (ms)	250	200	200	250	200	200	200	200	200	200	200	
21	26	lock duration [s]	30	30	30	30	30	30	30	30	30	30	30	
28	31													
35	38													
4	7	Dispenser number	1	2	3	4	5	6	7	8	9	10	11	
11	13	release volume (µl)	52	48	50	52	48	46	52	52	40	56	48	equal quality
17	20	opening duration (ms)	130	90	110	120	100	100	100	100	100	110	90	
32	34	lock duration [s]	30	30	30	30	30	30	30	30	30	30	30	
39	42	Dispenser number	1	2	3	4	5	6	7	8	9	10	11	
		release volume (µl)	24	26	24	24	24	24	26	24	22	26	22	equal quality
		opening duration (ms)	70	90	100	70	70	60	90	80	60	100	50	
		lock duration [s]	30	30	30	30	30	30	30	30	30	30	30	
43	55	Dispenser number	1	*2*	*3*	4	5	6	*7*	*8*	9	*10*	11	
		release volume (µl)	23	*64*	*61*	24		24	*64*	*71*	26	*51*	23	*high* - low quality
		opening duration (ms)	70	*120*	*130*	70		60	*120*	*110*	60	*120*	50	Pattern A
		lock duration [s]	90	*90*	*90*	90		90	*90*	*90*	90	*90*	90	
56	57	Dispenser number	*1*	2	3	*4*	5	*6*	7	8	*9*	10	*11*	
		release volume (µl)	*60*	20	20	*60*		*60*	20	20	*60*	20	*60*	*high* - low quality
		opening duration (ms)	*140*	60	70	*130*		*110*	60	50	*130*	60	*110*	Pattern B
		lock duration [s]	*90*	90	90	*90*		*90*	90	90	*90*	90	*90*	
57	61	Pattern inverting every day												Pattern A <-> Pattern B
62	64	Pattern inverting every 3 hours												Pattern A <-> Pattern B
65	73	Dispenser number	*1*	*2*	*3*	4	5	6	7	8	*9*	10	*11*	
		release volume (µl)	*10*	*10*	*10*	40		40	40	40	*10*	40	*10*	*risky* - constant
			70	*70*	*70*						*70*		*70*	
		opening duration (ms)	*50*	*40*	*40*	100		95	90	80	*40*	100	*30*	
			150	*140*	*140*						*140*		*140*	
		lock duration [s]	*90*	*90*	*90*	90		90	90	90	*90*	90	*90*	
71	73	Dispenser number	*1*	*2*	*3*	4	5	6	7	8	*9*	10	*11*	
		release volume (µl)	*10*	*10*	*10*	40	10	40	40	40	*10*	40	*10*	*risky* - constant
			70	*70*	*70*						*70*		*70*	
		opening duration (ms)	*50*	*40*	*40*	100	30	95	90	80	*40*	100	*30*	+ competition dispenser
			150	*140*	*140*						*140*		*140*	filled with sugar water
		lock duration [s]	*90*	*90*	*90*	90	30	90	90	90	*90*	90	*90*	

Oral BrdU administration from day 43 to 46 (dispenser 5, 154 µl, 250 ms, 5 s)

MG 2-1d

The principle of learning task in the complex environment in this group was similar to the conditions in group MG 2 described above. During the first 9 days, the 10 dispensers were of equal quality (60 µl per visit), followed by a period with 5 high (80 µl) and 5 low (20 µl) quality dispensers for 4 days and pattern inverting (Pattern A and B) two times after 4 days. Inverting of the high-low quality pattern daily and every 3 hours was not applied to this group. Dispenser no. 5 was added as competition dispenser releasing sugar water for the last 3 days of the experiment.

Table 13: Dispenser attributes for group LRN (PT = 1) in the complex environment containing 11 dispensers. Until day 9, all dispensers were of equal quality. From day 10 there were 2 types of dispensers: high (italicized) and low quality (day 10 - 23) or risky (italicized) and constant (day 24 - 39). Dispenser 5 was used for BrdU administration and as competition dispenser (day 36 - 39), but excluded from the rest of conditions.

from day	to day	Dispenser attributes												Dispenser quality
1	9	Dispenser number	1	2	3	4	5	6	7	8	9	10	11	
		release volume (µl)	61	61	58	65		56	50	69	71	50	65	equal quality
		opening duration (ms)	120	110	130	110		130	110	120	130	110	120	
		lock duration [s]	10	10	10	10		10	10	10	10	10	10	
10	10	Dispenser number	1	2	3	4	5	6	*7*	*8*	*9*	*10*	*11*	
		release volume (µl)	32	33	34	35		34	*50*	*69*	*71*	*64*	*65*	*high* - low quality
		opening duration (ms)	80	70	90	70		90	*110*	*120*	*130*	*130*	*120*	
		lock duration [s]	60	60	60	60		60	*60*	*60*	*60*	*60*	*60*	
11	14	Dispenser number	1	2	3	4	5	6	*7*	*8*	*9*	*10*	*11*	
		release volume (µl)	22	25	24	19		23	*71*	*86*	*83*	*76*	*87*	*high* - low quality
		opening duration (ms)	60	60	70	50		70	*150*	*150*	*150*	*150*	*150*	Pattern A
		lock duration [s]	90	90	90	90		90	*90*	*90*	*90*	*90*	*90*	
15	19	Dispenser number	*1*	*2*	*3*	*4*	5	*6*	7	8	9	10	11	
		release volume (µl)	*82*	*77*	*70*	*80*		*100*	20	19	22	19	25	*high* - low quality
		opening duration (ms)	*150*	*130*	*150*	*130*		*200*	60	50	60	60	60	Pattern B
		lock duration [s]	*90*	*90*	*90*	*90*		*90*	90	90	90	90	90	
20	23	Pattern inverting												Pattern A
24	28	Dispenser number	1	*2*	3	*4*	5	*6*	7	*8*	9	*10*	11	
		release volume (µl)	50	*25*	50	*19*		*23*	50	*19*	48	*20*	46	
				92		*80*		*100*		*86*		*80*		*risky* - constant
		opening duration (ms)	100	*60*	110	*50*		*70*	110	*50*	100	*50*	90	
				150		*130*		*200*		*150*		*120*		
		lock duration [s]	90	*90*	90	*90*		*90*	90	*90*	90	*90*	90	
29	39	Dispenser number	1	2	3	4	5	6	7	8	9	10	11	
		release volume (µl)	*22*	47	*24*	49		49	*20*	48	*21*	50	*25*	
			82	92	*106*	80		100	*80*	86	*83*		*87*	all risky
		opening duration (ms)	*60*	90	*70*	90		120	*60*	90	*30*	110	*60*	
			150	150	*200*	130		200	*150*	150	*150*		*150*	
		lock duration [s]	*90*	90	*90*	90		90	*90*	90	*90*	90	*90*	
36	39	Dispenser number	1	2	3	4	5	6	7	8	9	10	11	
		release volume (µl)	50	*25*	50	*19*	50	*23*	50	*19*	48	*20*	46	
				92		*80*		*100*		*86*		*80*		all risky
		opening duration (ms)	100	*60*	110	*50*		*70*	110	*50*	100	*50*	90	+ competition dispenser
				150		*130*		*200*		*150*		*120*		filled with sugar water
		lock duration [s]	90	*90*	90	*90*	90	*90*	90	*90*	90	*90*	90	

Oral BrdU administration from day 36 to 39 (dispenser 5, 160 µl, 250 ms, 5 s)

A.4 The histological procedure

All buffers and solutions used are listed below (A.4.4).

A.4.1 Perfusion

The perfusion is the method of choice to fix tissue with minimal damage or loss of cells. During transcardially perfusion, the cardiovascular system is used to rinse out blood by an isotonic NaCl solution and then flush the circuit with the fixative. The perfusion apparatus basically consisted of a flexible-tube pump and a tube with a canula at the delivery end. Three-way stopcocks allowed for the control which of the solutions flowed

from their bin to the pump. Precisely, a lethal dose of pentobarbital (160 µg/g body weight) was injected intraperitoneally to euthanize the animal. Within approximately 3 minutes the mouse was pain-free and fixed to the plate of the perfusion apparatus by plasticine. The thorax was opened and after removing the pericardium, the left ventricle was penetrated with the canula. Immediately after opening the right atrium by a cut, the pump was turned on. First, the mouse was perfused with a 0.9 % NaCl solution (containing sodium nitrite for inhibition of coagulation) for 3 to 4 minutes to remove blood from the system. Subsequently the tissue was fixed by perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 30 to 49 minutes. A successful fixation was identifiable already during this process by stiffening of the body. The animal was decapitated and the brain was warily removed from the skull. For the following immersion fixation (postfix), the meninges were detached and the brain was put into the fixative for 4 to 24 hours. In order to prevent the tissue from damage due to freezing (cryoprotection), the brain was then immersed in 30 % sucrose in 0.1 M PB for at least 24 hours. During this cryoprotection, liquid is withdrawn from the tissue that would produce freezing artifacts.

A.4.2 Sectioning

The brain was embedded into a block of gelatin to allow for sectioning in a straight plane. For this purpose the brain was placed in a special provision, a perplex box, and aligned by small pins through the bottom of the box. When the brain laid in the right position, it was fixed by cover piece with another pin. The pins did not prick into the tissue, but clamped the brain in the right position within the box. The box was then filled with warm, molten gelatin and put into icebox for a few minutes until the gelatin hardened. After unhinging the block from the box it was quick-frozen in isopentane (2-methylbutane) at –55°C for 90 seconds and then, with the caudal plain down, stuck to the object holder of the cryostat (Leica) using Tissue-tek® compound at the freezing station of the cryostat. At a object temperature of –25 to –18°C (chamber temperature: -18 to –15°C) the block was firstly trimmed to the rostral end of the olfactory bulbs and from there cut into 40 µm thick coronal sections up to the caudal end of the cortex. All sections were collected and transferred into vials containing 0.1 M PB according to the following allocation sequence: Each single section was placed in one vial, beginning from the upper left vial of a 24-vial plate in horizontal direction to the lower right vial and from there starting again at the upper left vial of the same plate. Consequently, the 4 vials of each column of the plate contained a set of every 6th brain section. The sections were either instantly processed or stored in cryoprotectant at –20°C.

A.4.3 Immunostaining of BrdU

Theoretical background of the immunohistochemical procedure

The immunohistochemical detection of BrdU-labeled cells was based on a two-step indirect method. This method involves an unlabeled primary antibody (pAB) that reacts with the tissue antigen and a labeled secondary antibody (sAB) that reacts with the pAB. The advantage of this method is the sensitivity due to signal amplification because several sAB can react with different antigenic sites on the pAB. For BrdU labeling, we used a monoclonal mouse anti-BrdU as pAB. This antibody was produced from a single clone of cells and reacts with only a single antigenic determinant of the antigen (monoclonal) and origins from mouse cells. The sAB was made in goat, reactive to heavy and light chains of mouse IgG and conjugated with biotin (biotinylated goat anti-mouse). The biotin conjugation is essential for the enzymatic staining reaction we used, the ABC (Avidin-Biotin Complex) method. This method is based on the very high affinity of avidin for biotin and the enzymatic oxidation of Diaminobenzidine (DAB). More precisely, the enzyme horseradish peroxidase (HRP) is conjugated with biotin that, in turn, forms an irreversible complex with avidin. When this avidin-biotin-enzyme complex is added to the tissue, it binds with its free avidin binding sites to the biotin of the sAB. The enzymatic staining reaction is the catalysis of the substrate hydrogen peroxide (H_2O_2) in the presence of the electron donor DAB via the peroxidase activity of HRP. DAB is called chromogen, because it coverts by oxidation into a brown colored product that is insoluble in organic solvents.

The original procedure of BrdU-staining

Free-floating sections were rinsed in 10 mM phosphate buffered saline containing 0.1 % Triton (PBS/T) two times for 5 min. each (2×5'). Triton® X-100 is a detergent used for permeabilizing cell membranes. If stored in cryoprotectant, the sections previously had to be slowly defrosted at 4°C for several minutes, followed by half an hour at room temperature (RT) and were then rinsed 5×5' in PBS/T. In order to block endogenous peroxidase, which could interfere with the enzymatic staining reaction, sections were incubated in 0.3 % hydrogen peroxide (H_2O_2) in PBS/T at RT. This process is based on the inactivation of an enzyme by the excess of substrate in absence of an electron donor (chromogen). After rinsing in PBS/T (5×5'), the sections were incubated at 37°C (warming cupboard) for 30 min. in 2 N hydrochloric acid (HCl, pH 1) for antigen retrieval, i.e. DNA-denaturation. Two rinse steps (2×5') with 0.1 M borate buffer (pH 8.5) for the neutralization of the acid were followed by further rinsing with PBS/T (3×5'). The

incubation of sections in a blocking solution is required in order to prevent unspecific background staining that is caused by unspecific binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections. These sites were blocked by the proteins of normal serum from the same species the sAB stems from (normal goat serum, NGS). Bovine serum albumin (BSA) served as additional protein block. Therefore, sections were incubated in blocking solution containing 3 % NGS and 3 % BSA in PBS/T for one hour at RT. Following 2×5' rinsing sections were incubated with the pAB, a mouse anti-BrdU antibody. The required antibody dilution of 1:400 was obtained by pipetting 2.5 µl mouse anti-BrdU (Roche Diagnostics) per 1 ml 3% NGS in PBS/T. The incubation continued over night (approx. 20 hours) at 4°C. Following 5×5' rinsing in PBS/T sections were incubated with the sAB for 2h at RT. Therefore, one ml of 3% NGS in PBS/T contained 5 µl biotinylated goat anti-mouse (1:200, Vector Laboratories). Before expiry of incubation time, the avidin-biotin complex (ABC) had to be prepared following the instructions of the package insert of ABC-kit (Vector Laboratories), i.e. adding 5 µl of solution A and solution B to each ml PBS/T. Sections were rinsed 5×5' in PBS/T followed by incubation in ABC solution at RT for 1 hour. Rinsing steps of 5 min. were done two times in PBS/T followed by 3 times in acetate-imidazole buffer (pH 7.2). Sections then were pre-incubated in 0.03 % DAB in acetate-imidazole buffer. This chromogen solution contained in addition 100mM Nickel(II)-sulfate ($NiSO_4$) for enhancement and bluish coloring of the signal. The enzymatic staining reaction started immediately after adding 0.003 % H_2O_2 (10 µl of 30 % H_2O_2 per 100 ml) to the chromogen solution and was stopped by transferring the sections into acetate-imidazole buffer. The time point of stopping the reaction had to be determined by observation according to the appropriate staining intensity (max. 5 min.). After thorough rinsing in acetate-imidazole buffer (2×5') and PBS/T (5×5') sections were mounted on slides. The slides were previously coated with gelatin for a better adhesion of the sections on the slide. The mounted sections air-dried over night and then dehydrated through an ascending series of alcohol (70 %, 2 × 96 %, 2 × 100 % ethyl alcohol) by immersing slides at each step for 2 min. The alcohol was replaced by immersion of slides 3×3 min. in xylene. Finally, the slides were cover-slipped with the xylene-soluble mounting medium DePeX (Serva) and allowed to harden for at least 3 days.

Optimizing the histological procedure

The original procedure was used according to commonly described methods in the literature (e.g. Gould et al., 1999a). However, this protocol led to unsatisfactory results

with string background staining and weakly stained cells. We therefore applied a set of variations to the procedure that were assumed to reduce background and enhance BrdU-labeling (Table 14). The procedures were similar to that described above except for the stated additions or modifications.

The first assumption was that the strong background staining resulted from insufficient blocking of endogenous peroxidase activity. Therefore, the sections were incubated in cold methanol at 4°C for 15 min. for additional peroxidase blocking (Table 14, A). This treatment had no effect on background or intensity of the BrdU-staining.

Table 14: List of variations applied to the original immunohistochemical procedure. Reduced background and/or enhanced cell labeling were rated as positive result (+), whereas no change or impaired staining were rated as negative result (-). The methodical modifications that resulted in enhanced staining quality were included in the standard protocol of immunohistochemical BrdU-staining.

Method	**Result**
A) Additional pre-treatment with cold methanol (15 Min.) for blocking endogenous peroxidase	-
B) Tris-buffered saline (TBS) instead of PBS	-
C) Increased H_2O_2 concentration from 0,3% to 0,6% for blocking endogenous peroxidase	+
D) Rat anti-BrdU instead of mouse anti-BrdU	-
E) 50% formamide/2xSSC (sodium chloride, sodium citrate) for DNA-denaturation	-
F) Increased incubation time of 2N HCl from 30 to 60 Min. for DNA-denaturation	+
G) Sheep anti-BrdU with rabbit anti-sheep instead of mouse and goat anti-mouse	-
H) Method according to Hierck et al. (1994): Complex of primary and secondary antibody instead of two-step antibody reaction	-
I) $F(ab)_2$-fragment secondary antibody, specific binding to F_c-fragment of the primary mouse anti-BrdU	-
J1) Mouse-on-mouse Kit (M.O.M., Vector) according to fabricator instructions and J2) diluted	+ +

The second test included 4 columns of one BrdU-injected mouse. For all columns Tris-buffered saline (TBS) was used instead of PBS (B) and the concentration of H_2O_2 for blocking endogenous peroxidase activity was doubled, from 0.3% to 0.6% (C). Two columns were incubated with mouse anti-BrdU (Roche) as primary antibody and two with rat anti-BrdU (Accurate Chemical, D). One column of each antibody type was treated with 50% formamide/2×SSC (Sodium chloride + Sodium citrate) for 2 hours at 65°C in the warming cupboard (E). This treatment was used for DNA-denaturation in addition to HCl treatment. The comparison of the columns showed that rat pAB revealed no better results than mouse pAB and that there is no difference between the columns depending on formamide/SSC treatment. Generally, in all columns, background staining was still present but BrdU-labeled cells were well identifiable. That this improvement was rather due to the

increased H_2O_2 concentration than to the use of TBS has been confirmed in subsequent experiments in which PBS was replaced TBS again. Therefore, to block endogenous peroxidase activity a concentration of 0.6% H_2O_2 was used in all subsequent histological procedures.

In another test condition, DNA-denaturation was further modified by extending the incubation time of HCl treatment from 30 to 60 minutes (F). This variation led to a more intense staining of BrdU-labeled cells and was therefore integrated in the standard staining protocol.

The assumption that severe background and insufficient BrdU-staining was due to improper antibodies or the problem of using mouse antibody on mouse tissue led to the following four approaches.

Firstly, we applied a pAB raised in sheep instead of mice (sheep anti-BrdU; DPC Biermann GmbH, Germany) followed by incubation with a sAB raised in rabbit (biotinylated rabbit anti-sheep; Vector laboratories) in order to avoid using mouse anti-BrdU on mouse tissue (G). In sections treated with this combination, background staining was almost absent but cell staining was unspecific, because all cell bodies of the DG were stained. This was probably due to unspecific labeling of the pAB as totally absent staining in the negative control (without pAB) indicated.

Secondly, Hierck et al. (1994) presented a modification of the indirect detection method for the use of mouse antibodies on murine tissue that should completely eliminate background staining. According to the methods described by the authors, we allowed the pAB (mouse anti-BrdU) to complex with the sAB (biotinylated goat anti-mouse) over night at 4°C. Therefore, we prepared an antibody solution containing 1 µl pAB, 1 µl sAB, 20 µl NGS and 10 µl normal mouse serum (MGS) per 1 ml PBS/T the day before starting the histochemical procedure (H). Standard immunohistochemical protocol was then applied with incubation of sections in the antibody solution over night at 4°C. In contrast to Hierck et al. (1994), this method did not work at all for staining BrdU-labeled cells, because sections were totally unstained.

Thirdly, Lu and Partridge (1998) demonstrated that background staining can be reduced by using only $F(ab')_2$ fragments as sAB because an elevated background is, at least partly, due to binding of Fc fragments of the sAB to endogenous Fc receptors or other tissue components. Following this approach, we replaced the standard sAB by biotinylated $F(ab')_2$ fragments of anti-mouse IgG that specifically binds to mouse IgG Fc fragments (Biomol, Germany; I). To different columns of the same BrdU-injected animal, we applied the Mouse-on-mouse kit (M.O.M., Vector laboratories) to circumvent the problem of using

mouse antibody on mouse tissue (J). M.O.M. was applied following the instructions of the fabricator (J1) and by integrating M.O.M. in the standard immuno protocol pure or diluted (J2). The $F(ab')_2$ fragments used as sAB resulted in reduced background but labeled cells were not identifiable. In contrast, in all sections treated with M.O.M., background was moderate and labeled cells were clearly visible except for the 1:4 dilution of M.O.M.

The ultimate protocol for immunostaining of BrdU (Table 15) included all modifications, which led to a positive result in terms of reduced background staining and optimal perceptibility of BrdU labeling. In summary, these were the incubation in 0.6% H_2O_2, the incubation in HCl for 60 min. and the M.O.M. kit diluted at a ratio of 1:3. To compensate for the dilution of M.O.M. sAB concentration biotinylated goat anti-mouse (1:266) was added to the sAB solution.

Table 15: Schedule of the immunohistological procedure for staining BrdU-labeled cells.

Treatment	**Substance**	**Incubation**
H_2O_2-treatment	0.6% H_2O_2 in 10 mM PBS/T	30´
Rinse	10 mM PBS/T	5 x 5´
DNA-denaturation	2 N HCL	60´, 37°C
Neutralization	0.1 M Borate buffer, pH 8.5	2 x 5´
Rinse	10 mM PBS/T	3 x 5´
Block	M.O.M. Blocking reagent	60'
Rinse	10 mM PBS/T	2 x 5´
M.O.M Protein concentrate	M.O.M. Diluent	5´
Primary Antibody Anti-BrdU (mouse) 1:400	M.O.M. Diluent	≈ 20 h, 4°C
Rinse	10 Mm PBS/T	5 x 5´
Secondary Antibody M.O.M. anti-mouse reagent + biotin. goat anti-mouse 1:266	M.O.M. Diluent	2 h
Rinse	10 mM PBS/T	5 x 5´
Avidin Biotin Complex	ABC-Kit in 10mM PBS/T	1 h
Rinse	10mM PBS/T	2 x 5´
Rinse	Acetate-Imidazole buffer/T	3 x 5´
DAB-preincubation	0.03% DAB/Acetate/Imidazole/Triton/Ni	10´
DAB-reaction	+ 0.003% H_2O_2	
Rinse	Acetate-Imidazole buffer/T	2 x 5´
Rinse	10mM PBS/T	5 x 5´

*A.4.4 **Buffers and solutions***

Rinsing solution for perfusion, pH 7.4 (0.07 M $NaNO_2$, 0.150 M NaCl)

- 5 g Sodium nitrite ($NaNO_2$)
- 8.5 g Sodium chloride (NaCl)
- 1000 ml A. dest.

8 % Paraformaldehyde solution

- 80 g Paraformaldehyde $(CH_2O)_n$
- 1000 ml A. dest.

4 % Paraformaldehyde in 0.1 M PB

- 500 ml 8% Paraformaldehyde solution
- 250 ml 0.4M PB
- 250 ml A. dest.

Gelatin for embedding (15 % Gelatin, 30 % Saccharose)

- 15 g Gelatin (baker-grade)
- 30 g Saccharose
- 100 ml A. dest.

Gelatin for coating slides (0.4 % Gelatin, 2 mM $CrK(SO_4)_2$)

- 0.88 g Gelatin Bloom 300 (gel strength)
- 0.106 g Potassium chromium(III) sulfate ($CrK(SO_4)_2$)
- 200 ml H_2O

Cryoprotectant (8.6 % Saccharose, 7 mM Magnesium chloride, 0.05 M PB, 44 % glycerol)

- 8.56 g Saccharose
- 0.14 g Magnesium chloride hexahydrate ($MgCl_2 \times 6H_2O$)
- 50 ml 0.1 M PB
- 50 ml 87 % Glycerol

*0.4 M Phosphate buffer (**PB**), pH 7.4*

- 368 ml Solution A: 27.38 g Potassium-di-hydrogenphosphate (KH_2PO_4) in 500 ml A.dest.
- 1400 ml Solution B: 85.17 g Di-sodium hydrogenphosphate (Na_2HPO_4) in 1500 ml A. dest.

0.1 M PB, pH 7.4

- 250 ml 0.4 M PB
- 750 ml A. dest.

*10 mM Phosphate buffered saline (**PBS/T**; 0.15 M Sodium chloride, 0.1 % Triton), pH 7.4*

- 50 ml 0.4 M PB
- 1950 ml A. dest.
- 17 g NaCl
- 2 ml Triton X-100

0.1 M Borate buffer, pH 8.5

- 3.09 g Boric acid (H_3BO_3)
- 500 ml A. dest.
- NaOH for pH adjustment

0.2 M Imidazole, pH 9.2

- 13.6 g Imidazole ($C_3H_4N_2$)
- 1000 ml A. dest.

1 M Sodium acetate, pH 7.2

- 82 g Sodium acetate (41 g $NaCH_3COO$)
- 1000 ml A. dest.

Acetate-imidazole buffer (0.175 M Sodium acetate, 0.01 M Imidazole, 0.05 % Triton), pH 7.2

- 175 ml 1 M Sodium acetate
- 50 ml 0.2 M Imidazole
- 500 µl Triton X-100
- 775 ml A. dest.
- Pure acetic acid for pH adjustment

DAB solution (0.03 % DAB, 0.125 M Sodium acetate, 0.01 M Imidazole, 0.1 M Nickel sulfate, 0.05 % Triton, 0.003 % H_2O_2), pH 6.5

- 30 mg DAB
  (3,3'-diaminobenzidine tetrahydrochloride, $(NH_2)_2C_6H_3C_6H_3(NH_2)_2 \times 4HCl$)
- 2.63 g Nickel sulfate ($NiSO_4$)
- 12.5 ml 1 M Sodium acetate
- 5 ml 0.2 M Imidazole
- 50 µl Triton X-100
- 100 ml A. dest.

0.1 M Tris-buffered saline (0.15 M NaCl), pH 7.4

- 12.1 g Trizma
- 8.5 g NaCl
- 1000 ml A. dest.
- HCl for pH adjustment

50 % formamide / 2 × SSC (0.3 M NaCl, 0.03 M $Na_3C_6H_5O_7$)

- 50 ml 99 % Formamide
- 50 ml 2 × SSC (17.5 g NaCl and 8.8 g Sodium citrate ($Na_3C_6H_5O_7$) in 1000 ml A. dest.)

A.5 Quantification of BrdU-labeled cells

The DAB-stained sections were analyzed with a light-optical microscope (Axioskop2, Zeiss) extended by a CCD (charge-coupled device)-camera (Spot RT color, Diagnostic instruments) and a XYZ-automated stage (Märzhäuser) that was controlled by a modular control device (Ludl Electronic Products). Together with Metamorph software (Visitron), this setup allowed for generating a composite picture of a structure or whole brain section by stitching single pictures. The region of interest, the dentate gyrus (DG), was identified according to a stereotaxic mouse atlas (Paxinos & Franklin, 2001). BrdU-labeled cells were counted in a series of every 6th brain section throughout the DG in one randomly chosen hemisphere or in both hemispheres. In the latter case, data from one of the hemispheres were chosen at random after counting to be factored into analysis.

A.5.1 Photographing brain sections

At first, the microscope was adjusted to the Köhler illumination in order to prevent diffused light and to illuminate the preparation brightly, evenly and only within the

required area. Therefore, the object slide was placed on the stage and focused using the 10× or 20× objective lens at maximum illumination, i.e. with the aperture diaphragm quite open and the condenser at the upper stop. When the object was in focus, the field diaphragm was closed and the condenser moved down until the edge of the field diaphragm appears as sharp image. After centering the field diaphragm, it was opened until the edge was just outside the field of view.

All photographs were taken as 12-bit monochrome (grayscale) images. A flatfield correction was performed prior to taking pictures to assure an even background. Therefore, this process was proceeded at a clean position of the slide without tissue to define the white background.

At a position where the region of interest was in the field of view, exposure times of the camera were adjusted. Modification of illumination intensity and, if required, application of a neutral gray filter were used to achieve an exposure time that should not exceed 100 ms. Once the illumination and filter settings were appropriate, the optimal exposure time was computed by MetaMorph for each section to compensate for different levels of brightness among sections. All images were taken using the auto-focus function in order to avoid differences between images due to subjective defined focus. Therefore, cells within the DG were roughly focused and the z-value memorized as point of origin. The auto-focus proceeded by taking several (2 to 6) pictures in each z-direction from the origin with 2 µm intervals and selecting automatically the sharpest one.

For composed pictures, multiple images were acquired automatically with auto-focus. The step size in µm and the direction (+ or -) the motorized stage had to move was predefined depending on the objective used (5×: x/y = 2131/-1598 µm; 10×: 1066/-799 µm; 20×: 533/-499 µm). The step size included a 10 % overlap of adjacent images for accurate stitching of the single images. Following the definition of step size and picture size (n columns × n rows of single images), the single images were photographed automatically in horizontal zigzag order beginning at the upper left image (start point). The resulting stack of single images was calibrated according to the objective used and automatically stitched together by MetaMorph stitch stack or montage function. The nomenclature of the pictures, for example MM 05-09_S2207_HipL, was composed of the species (MM, *mus musculus*), year of processing (2005), serial number of the animal (9), column number (S2 = "Säule" 2), number of slide (2), number of section on the slide (7) and region of interest (HipL = left DG).

A.5.2 Counting BrdU-labeled cells

BrdU-labeled cells were counted semi-automatically, i.e. following the manual marking of regions in which to count and manual setting of definitions what to count, the MetaMorph analyzing tool counted cells automatically. In each picture, regardless if single or stitched image, the regions of interest were manually outlined. The aim of this outlining was to get information from counted cells about their location within the DG. Therefore, a line (multi-line tool) was drawn along the well-defined border between granule cell layer (GCL) and hilus at each, the dorsodorsal (dd) and dorsoventral (dv), blade of the DG (Figure 24A). Rectangular segments of 100 µm length were created along the lines. Rectangles in GCL direction were 50 µm wide, to cover the area of GCL and rectangles in hilus direction were 20 µm wide to cover the area of the subgranular zone (SGZ, Figure 24B and C). The SGZ was defined as a ribbon along the borderline between GCL and hilus with a width corresponding to two cell body diameters (approx. 20 µm). GCL segments were corrected for the actual extent of the GCL and the hilus was traced along the inner border of SGZ segments (trace-region tool, Figure 24D). Therefore, counted cells were automatically assigned to layer (GCL, SGZ or hilus) and blade (dd or dv).

Cell counting based on the detection of areas characterized by a certain shape, size and minimum gray value. The minimum gray value (threshold) depended on intensity of background and BrdU-staining and was therefore defined for each section separately. The criterion for setting the threshold was to cover a maximal area of labeled cells and concomitant a minimal area of background staining (Figure 24D). The threshold gray value finally applied to the image was logged for each section.

Areas beyond the threshold were stated as cells if they were at least 20 μm^2 in size and had a shape factor > 0.6. The shape factor, calculated by $4 \times \pi \times \text{area}/(\text{perimeter})^2$, has a value between 0 and 1 that represents how closely the shape is to a circle (1 = perfect circle). The predefined standard area of a single cell (30 μm^2) was used by MetaMorph to determine the number of single cells contained in a cluster of cells recorded as one large area (standard area count).

The Integrated Morphometry Analysis (IMA) tool automatically recorded all areas according to gray threshold and morphological criteria within the predefined segments and logged the data to a respective excel sheet (see Table 16).

Table 16: Data logged by IMA (Integrated morphometry analysis) for each recorded object. Description of the column titles (Measure) of the IMA LOG-files.

Measure	Description
Image Name	name of image file
Region Name	location of the object, e.g. dd(SGZ)
Object #	serial number of this object
Area	area of the object in μm^2
Standard area count	amount of standard areas (30 μm^2) that fit into the area of the object, integer
Centroid X	x-coordinate of the centroid of the object
Centroid Y	y-coordinate of the centroid of the object
Shape factor	values 0-1, roundness of the object
Mean radius	average distance from centroid to all points along the edge of the object
Average gray value	average of the pixel grayscale values that are contained in the object
Total gray value	sum of the pixel grayscale values that are contained in the object

A.5.3 Analysis of the measurements

Raw data files containing the IMA measurement data (*_LOG_Measure.xls) were edited to analyze numbers of BrdU-labeled cells and saved separately (*_Ergebnistabelle.xls). An excel macro extracted the information about animal, hemisphere and region from the columns "Image name" and "Region name". Numerical region names were renamed as "Hi" (hilus), because the trace-line that outlined the hilus had no name and occurred as number in "Region names". Each row in the LOG file represented one object. If the area of an object was equal or smaller than the predefined standard area (standard area count = 1), this object corresponds to one cell. If the area of an object was larger than the standard area (standard area count ≥ 2) the object was assumed as cell cluster and multiplied by the value of the standard area count. Additionally, morphological data were corrected for the single cell analysis by dividing area and mean radius, each by the standard area count (columns "Area_one" and "Radius_one"). The analysis of cell numbers was accomplished by an excel macro (verteil.xlm) and results were multiplied by 6 to provide an estimate of the total number of BrdU-positive cells per DG.

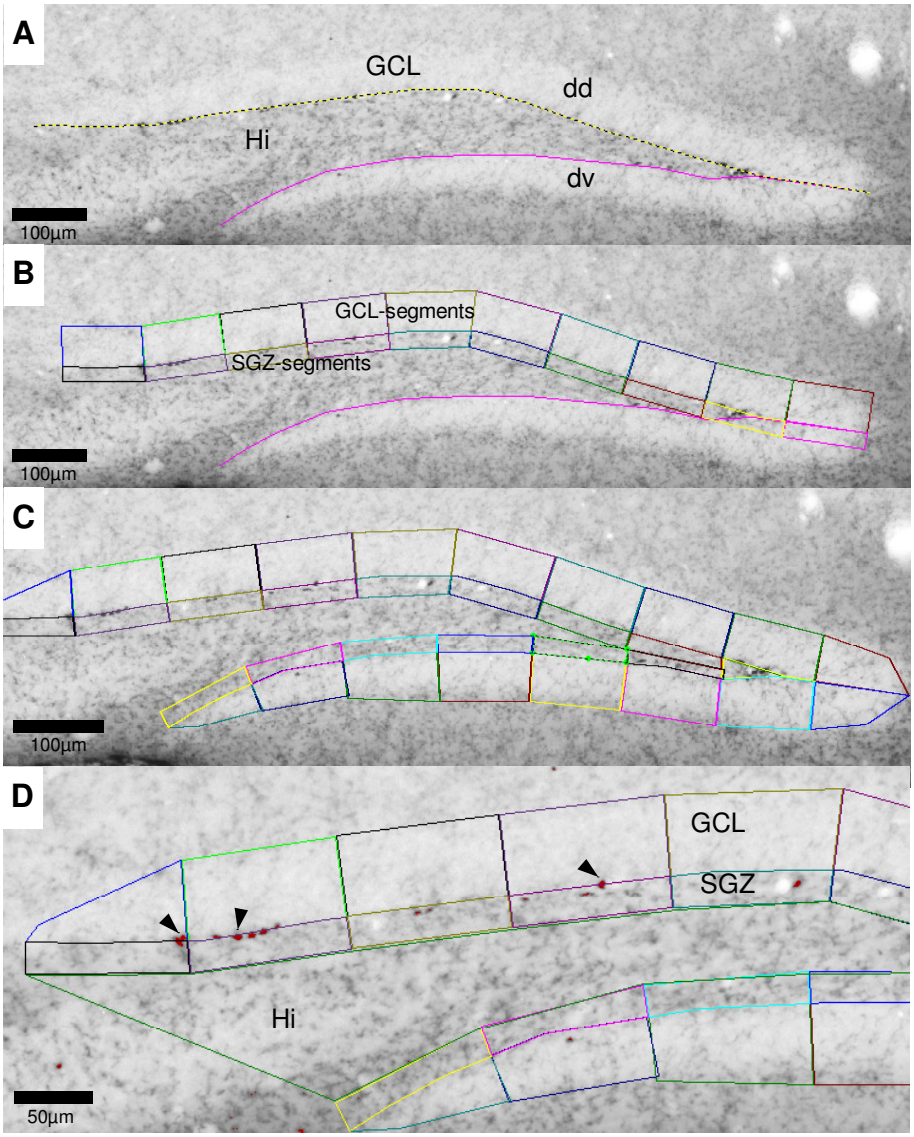

Figure 24: Processing images for the semi-automatical counting of BrdU-labeled cells in the dentate gyrus (DG). A) Trace lines along the border between granule cell layer (GCL) and hilus (Hi) at the dorsodorsal (dd) and dorsoventral (dv) blade of the DG. B) Rectangular segments (100 µm in length) created along the trace lines in both directions cover the area of GCL (50 µm wide) and SGZ (20 µm wide), respectively. C) Segments corrected for the actual extent of the GCL. D) In the thresholded image, red areas represent objects with a gray value above threshold. Arrows indicate objects above gray threshold that were counted as cells according to their size and shape.

A.6 References

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Appendix B Figures and tables

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Figure 3	Time curse of cumulative BrdU uptake	Trinkverhalten MG 6-d.jnb	Auswertung_MG 6-1d.xls	Daten\Auswertung\Verhaltensdaten\Plots\
Figure 4	Photomicrographs of BrdU positive cells in the dentate gyrus	DG_BrdU cells.jpg	MM 04-40_S2205xx.tif MM 05-01_S2210xx.tif	Bilder\
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Figure 6	Experimental design for the investigation of the role of different environmental conditions on cell proliferation and survival	Methods_1.gif		Bilder\
Figure 7	Experimental design for the investigation of the persistence of enhanced cell proliferation induced by running wheel activity.	Methods_3.gif		Bilder\
Figure 8	Dispenser visits of mice inthe complex environment during BrdU treatment: exploration and learning	Verhalten_11tr.jnb	Auswertung_MG 4.xls Auswertung_MG 2.xls	Daten\Auswertung\Verhaltensdaten\Plots\
Figure 9	Characterization of exercise in running wheels and tubes	WHE-TUB.jnb	Auswertung_MG 11-1d_12-1d (WHE-TUB).xls	Daten\Auswertung\Verhaltensdaten\Plots\
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Figure 11	Correlation between BrdU-positive cells and running distance	WHE-TUB.jnb	Zusammenfassung_Zellzahlen.xls	Daten\Auswertung\Zellzahlen\Plots\
Figure 12	Numbers of BrdU-positive cells at different time points after running wheel withdrawal	Zellzahlen_Persistence.jnb	Zusammenfassung_Zellzahlen.xls	Daten\Auswertung\Zellzahlen\Plots\
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Originaldateien\		
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LOG-Dateien\	MG *******_LOG.xls	Raw data from MetaMorph (thresholds and IMA-measures)
Tagesdaten\	Tagesdaten_MG***.xls	Summaries of parameters and daily behavioral analyses
	Tagesauswertung_11Tränken_8Mäuse.	Template sheet of daily behavioral analysis
Makros\	HistoDaten.xlm	Macro for compilation of LOG-files
	Univeral.xls	Macro for compilation of csv-files
	Verteil.xlm	Macro for analysis
Auswertung\		
Verhaltensdaten\	Auswertung_MG****.xls	Behavioral analyses of mouse groups
	Alle Gruppen_Auswertung Verhalten.xls	Summary of behavioral data from all groups
	Fledermaus_BrdU.xls	Behavioral analysis of the bat
	Statistik_Verhalten.doc	Statistical analyses of behavior: learning, exploration,
Zellzahlen\	MG *******_Ergebnistabelle.xls	Analyses of cell counts of mouse groups
	Zelleigenschaften.xls	Analyses of cell characteristics
	Zufallszahlen.xls	Definition of randomly chosen hemispheres
	Zusammenfassung_Zellzahlen.xls	Summary of cell counts from all groups
	Statistik_Zellen.doc	Statistical analyses of cell counts, characteristics and distribution
Dokumente\	Auswertung_MetaMorph***.doc	Manual for cell quantification and screen shots
	Immunoprotokoll_BrdU.doc	Standard protocol for BrdU staining
Plots\	Bat_Plots.jnb	Bat experiment: dispenser visits
	Jetlag.JNB	MG 2: First days after shift of light:dark cycle, dispenser
	Trinkverhalten MG 6-1d.JNB	MG 6-1d: Dispenser visits over the day (with/without BrdU)
	Verhalten_11tr.JNB	MG 2, 4, 5: Time to first dispenser visit, learning, exploration
	WHE-TUB 1.JNB	MG 8-, 9-1d: Running distance in wheel and tubes (ad lib.)
	WHE-TUB.jnb	MG 11-, 12-, 13-1d: Running distance, velocity and cell
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	Schnitt-Präparate.xls	List of histological preparations (brain sections)
	Tiere.xls	List of mouse groups
	Übersicht Verhalten.xls	Overview of experimental design and parameters

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Curriculum vitae

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PUBLICATIONS

Santoso, A., Kaiser, A., & Winter, Y. (2006) Individually dosed oral drug administration to socially-living transponder-tagged mice by a water dispenser under RFID control. Journal of Neuroscience Methods, 153, 208-213.

ABSTRACTS AND POSTERS

Santoso, A., Kaiser, A. and Winter, Y. (2005): Stress-free oral administration of drugs in group-living mice through a transponder-controlled water dispenser. In: Proceedings of the 6th Meeting of the German Neuroscience Society/30th Göttingen Neurobiology Conference 2005. #PSA35,#465B.

Santoso, A., Kaiser, A. and Winter, Y. (2006): Increase of cell proliferation in the DG of adult mice in poor and naturalistic environments is only affected by running wheel activity. In: FENS Abstr., vol 3, A089.25.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Die vorliegende Dissertation wurde in der jetzigen oder ähnlichen Form bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

_____ (Ort, Datum)

_____ Ariane Santoso