



**Developmental neuroplasticity
in the hippocampus –
an interplay of prefrontal-limbic circuits**

Dissertation

zur Erlangung des
Doktorgrades *rerum naturalium*
der Fakultät für Biologie
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τῆς μέντοι φρονήσιος οὐδετέρῳ μέτεστιν, ἀλλὰ
πάντων τούτων αἴτιος ὁ ἐγκέφαλός ἐστιν.

Neither [the heart nor the diaphragm] have anything to do, however,
with the operations of the understanding, but of all these the brain is
the cause.

(Hippocrates, 460 – 370 BC)

ABSTRACT

Neuroplasticity, the brain's ability to respond to environmental and intrinsic stimulation, is a prerequisite for an adaptive structural development. One of its most extraordinary examples is neurogenesis – the generation of new neurons – in the adult hippocampus. The examination of the postnatal development of neuroplasticity in the hippocampus and its dependence on environmental stimulation was the aim of the present work. The main focus was on the regulatory mechanisms on both the local level and the level of the whole prefrontal-limbic system.

First, we found that CD1 mice reared under deprivation of a stimulating social and physical environment did not differ in rates of hippocampal cell proliferation or cell survival from their enriched-reared littermates. However, only in deprived-reared animals a wheel-running challenge led to a significant increase in cell proliferation and cell survival (project I). This different reactivity was tried to explain by local (neurotrophic and growth factors, neurotransmitter influences) as well as systemic mechanisms, and a hypothetical model was proposed (project II) involving the whole prefrontal-limbic system. Like neurogenesis, synaptic remodeling (project III) was not affected by social and physical deprivation during rearing. However, wheel running affected synaptic remodeling independent of the rearing conditions by leveling layer-specific distribution patterns.

To test the hypothesis that the maturation of the prefrontal cortex is jointly responsible for regulating adult hippocampal neuroplasticity, we used an approach with wheel running as a stimulus of cell proliferation and, afterwards, environmental enrichment or a prefrontal-cortex dependent working-memory task in an automated T-maze to enhance prefrontal activity (project IV). We could, indeed, demonstrate that the prefrontal cortex is causally involved in the regulation of cell proliferation in the dentate gyrus.

Finally, a strain comparison within the species *Mus musculus domesticus* (project V) revealed that environmentally-dependent plasticity is similar between outbred CD1 and inbred C57Bl/6 mice. Contrastingly, an effect of neither environmental deprivation during adolescence nor adult wheel running on cell proliferation was found in wild house mice.

The present work provides evidence that environmental stimulation during juvenile brain development determines neuroplastic potentials at least in the adult hippocampus of domesticated mouse strains. However, the reactivity of neuroplasticity seems to be a consequence of genetic modifications by domestication and even in domesticated mice an outcome of deprivation from natural environmental stimulation during juvenile brain development. The prefrontal cortex might be causally involved in the regulation of cell proliferation in the hippocampal dentate gyrus. These findings draw attention to a more systemic view of the regulation of neuroplasticity in the hippocampus as an interplay of limbic-prefrontal circuits.

ZUSAMMENFASSUNG (deutsch)

Neuroplastizität, die Fähigkeit des Gehirns auf umweltbedingte und intrinsische Stimulation zu reagieren, ist eine Grundvoraussetzung für eine adaptive Strukturentwicklung. Eines ihrer ungewöhnlichsten Beispiele ist die Neurogenese - die Bildung neuer Nervenzellen - im adulten Hippokampus. Die Untersuchung der postnatalen Entwicklung der Neuroplastizität im Hippokampus und ihrer Abhängigkeit von einer stimulierenden Umwelt war das Ziel der vorliegenden Arbeit. Das Hauptaugenmerk lag dabei auf den regulatorischen Mechanismen sowohl auf der lokalen Ebene als auch auf der Ebene des gesamten limbo-präfrontalen Systems.

Als erstes fanden wir heraus, dass CD1-Mäuse, die unter Deprivation von einer stimulierenden, sozialen und physikalischen Umwelt aufgezogen wurden, sich in ihren Zellproliferations- und Zellüberlebensraten nicht von ihren angereichert aufgezogenen Artgenossen unterschieden. Jedoch führte eine Laufradintervention nur in depriviert aufgezogenen Tieren zu einem signifikanten Anstieg der Zellproliferation und des Zellüberlebens (Projekt I). Diese unterschiedliche Reaktivität der Neurogenese wurde durch lokale (neurotrophe Faktoren und Wachstumsfaktoren, Transmittereinflüsse) sowie systemische Mechanismen zu erklären versucht, und es wurde ein hypothetisches Modell unter Einbeziehung des gesamten limbo-präfrontalen Systems vorgeschlagen (Projekt II). Wie die Neurogenese, so wurde auch der synaptische Umbau (Projekt III) nicht durch soziale und physische Deprivation während der Aufzucht beeinträchtigt. Laufradlaufen beeinflusste den synaptischen Umbau jedoch unabhängig von den Aufzuchtbedingungen, indem es die lamina-spezifische Verteilung einebnete.

Um die Hypothese zu überprüfen, dass die Reifung des präfrontalen Kortex für die veränderte Neuroplastizität mitverantwortlich ist, wurde ein Ansatz mit Laufradlaufen als einem Stimulus der Zellproliferation und anschließender Umweltanreicherung oder einer Präfrontalkortex abhängigen Arbeitsgedächtnisaufgabe in einem automatisierten T-Labyrinth eingesetzt, um die Aktivität im präfrontalen Kortex zu erhöhen (Projekt IV). Wir konnten in der Tat zeigen, dass der präfrontale Kortex kausal an der Regulation der Zellproliferation im Gyrus dentatus beteiligt ist.

Schließlich zeigte ein Stammesvergleich innerhalb der Spezies *Mus musculus domesticus* (Projekt V), dass die umweltbedingte Plastizität zwischen Auszucht-CD1-Mäusen und Inzucht-C57Bl/6-Mäusen ähnlich ist. Im Gegenteil dazu wurde in wilden Hausmäusen weder ein Effekt der Umweltdeprivation während der juvenilen Gehirnentwicklung noch des Laufradlaufens im Erwachsenenalter auf die Zellproliferation gefunden.

Die vorliegende Arbeit liefert Anhaltspunkte dafür, dass Umweltstimulation während der jugendlichen Gehirnentwicklung die neuroplastischen Potentiale zumindest im adulten Hippokampus domestizierter Mausstämme determiniert. Die Reaktivität der Neuroplastizität scheint jedoch eine Folge von genetischen Modifikationen durch Domestikation und darüber hinaus auch in domestizierten Mäusen eine Folge der Deprivation von natürlicher Umweltstimulation während der jugendlichen Gehirnentwicklung zu sein. Der präfrontale Kortex scheint kausal an der Regulation der Zellproliferation im hippocampalen Gyrus dentatus beteiligt zu sein. Diese Befunde lenken die Aufmerksamkeit auf einen systemischeren Blick der Regulation der Neuroplastizität im Hippokampus als einem Zusammenspiel von limbo-präfrontalen Schaltkreisen.

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1. GENERAL INTRODUCTION

Neuroplasticity is the brain's ability to change itself in response to environmental and intrinsic stimulation. It grants the compensation for injury and disease and the adaptation to new situations or changes in the environment. In the last decades, since its potential to generate new neurons was discovered in 1965 (Altman & Das, 1965), the hippocampus has gained a lot of attention. Indeed, neurogenesis is one of the most extraordinary examples of neuroplasticity. It holds the hippocampus in a state of lifelong development. Lifelong development bears high plastic capacities but makes also susceptible to external influences.

The development of the individual brain is a continuous process which is defined by genetic and epigenetic factors. The characteristic of the phenotype is determined by the interaction of the individual with its environment and is, thus, the outcome of an adaptive structural development (Wolff, 1982a). Hence, an environment rich in physical structure and populated with conspecifics and so providing stimuli for various sensory, motoric and associative systems, is crucial for the neural developmental program (Würbel, 2001).

The central aim of the present work was to examine whether and how the environment during juvenile development determines neuroplastic potentials in the hippocampus not only for the short term, but even in adulthood. The main focus was, thereby, on the biological mechanisms mediating these influences. It has been suggested that it is a network of regulatory pathways whose activation over many intermediate steps, supposedly in many different brain regions, ultimately leads to a particular change in the cellular niche of the precursor cell (Kempermann, 2006). For that reason, attention was not only paid to the local regulatory mechanisms, but a systemic approach was chosen involving the whole prefrontal-limbic system.

This approach was based on previous work on environmental influences on developmental plasticity mainly in gerbils (*Meriones unguiculatus*). In gerbils, deprivation of “natural” environmental stimulation during brain development leads to severe alterations in the maturation of the prefrontal cortex, i.e., in dopaminergic, serotonergic and GABAergic innervation (Winterfeld et al., 1998; Neddens et al., 2001, 2003), and its efferents to caudal limbic areas (Bagorda et al., 2006). In contrast, neurotransmitter innervation is enhanced in limbic circuits (Neddens et al., 2002; Lehmann et al., 2003; Busche et al., 2004, 2006; Lesting et al., 2005) leading to a dysbalance between these systems and a “dysconnection” of the prefrontal cortex (Bagorda et al., 2006). Numerous findings in other rodents support the assumption of a severely impacted prefrontal cortex after deprivation during brain development (Dalley et al., 2002; Ago & Matsuda, 2003; Melendez et al., 2004; Leng et al.,

2004; Gregory & Szumlanski, 2008; Bloomfield et al., 2008). Furthermore, in gerbils, cell proliferation in the hippocampal dentate gyrus is enhanced after deprived (impoverished) rearing, while synaptic-remodeling rates are decreased (Hildebrandt, 1999; Keller et al., 2000; Butz et al., 2008). This effect could not be confirmed in other rodents (Kempermann et al., 1997a, 1998; Nilsson et al., 1999), probably because of differences in study design, e.g., the environmental set-up or the use of proliferation markers. The aim of the present projects was now to examine developmental neuroplasticity in the hippocampus in the interplay of prefrontal-limbic circuits in the widely-used mouse model (*Mus musculus domesticus*).

1.1. THE HIPPOCAMPAL FORMATION

The hippocampal formation is a prominent structure in the rodent brain. It appears as a drawn-out C-structure, whose longitudinal axis, also called septo-temporal axis, reaches from the septal nucleus (septal pole lying rostral/medial) to the basal forebrain (temporal pole lying caudal lateral) (Fig 1.1). It is composed of several related brain regions, the hippocampus proper, which is further divided into CA1, CA2 and CA3 (CA, cornu ammonis), the dentate gyrus, subiculum, pre- and parasubiculum and the entorhinal cortex (Amaral & Witter, 1995; Amaral & Lavanex, 2006; Brummelte et al., 2007).

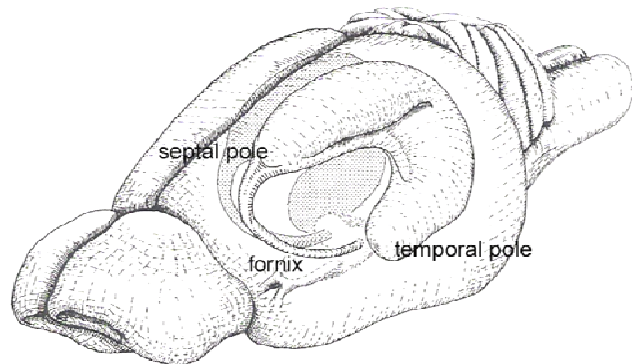


Fig. 1-1: The hippocampal formation in the rodent brain (Amaral & Witter, 1995).

As part of the Papez circuit and limbic system (see chapter 1.2) the hippocampus is involved in memory consolidation (Kandel et al., 2000; Rager et al., 2000), i.e., the transfer of declarative memory contents from short- to long-term memory (Squire et al., 1993; Rager et al., 2000). In this context, it is – especially in rodents – responsible for the representation of places ('place cells'; O'Keefe & Nadel, 1978) and formation of spatial memory (Eichenbaum, 1999; Rager et al., 2000). Due to its numerous connections with the amygdala (Pitkanen et al., 2000) and hypothalamus (Amaral & Witter, 1995; Amaral & Lavanex, 2006), the hippocampal system is also implicated in emotional and motivational functions. There is even evidence that functional dissociations exist along the septo-temporal axis of the hippocampal formation, namely that the septal or dorsal hippocampus is more important in spatial learning, while the temporal or ventral hippocampus is rather involved in emotional processes (Moser et al., 1993; Bannerman et al., 1999, 2003, 2004). At last, also the detection of novelty and

coupled attentional processes seem to be tied to the hippocampal formation, especially driven by its cholinergic and GABAergic septal inputs, which create a signal of presence (Buzsaki, 2002, 2005) in the so called theta-rhythm (4-7 hertz; Smythe et al., 1992; Lee et al., 1994). This facilitates the registration and integration of simultaneously converging information and is, therefore, also a prerequisite for memory processing (Thomas et al., 1994; Edwards, 1995; Kandel et al., 2000).

One of the most intriguing features of the hippocampal formation is its relatively simple organization of its principal cell layers together with its highly organized laminar distribution of many of its extrinsic and intrinsic connections.

The dentate gyrus is composed of three layers: the granule cell layer (stratum granulare) formed by densely packed granule cells, the relatively soma free molecular layer (stratum moleculare), and the polymorphic layer or hilus, which is passed through by the axons of the granule cells, the mossy fibers. The molecular layer can be further subdivided based on its inputs: the outer molecular layer receives extrinsic glutamatergic inputs from the lateral entorhinal cortex via the lateral perforant path, and the middle molecular layer from the medial entorhinal cortex via the medial perforant path (Van Groen et al., 2002). Via the perforant path, the dentate gyrus receives highly preprocessed information (Knowles, 1992; Amaral & Witter, 1995). In contrast, in the inner molecular, primarily associational and commissural fibers from mossy cells and interneuronal feedback and feed-forward loops terminate as well as modulatory inputs from the septum (Amaral & Witter, 1995; Amaral & Lavanex, 2006). The so called germinative zone, in which the precursor cells are located and give birth to new neurons, lies between the granule cell and polymorphic layer and is, thus, also named subgranular zone. This zone receives modulatory transmitter influences from the locus coeruleus (norepinephrine), the raphe (serotonin) and the ventral tegmental area (dopamine; Amaral & Witter, 1995; Vizi & Kiss, 1998; Amaral & Lavanex, 2006). Topographically, the dentate gyrus can be divided into a suprapyramidal and an infrapyramidal blade, which join in the crest.

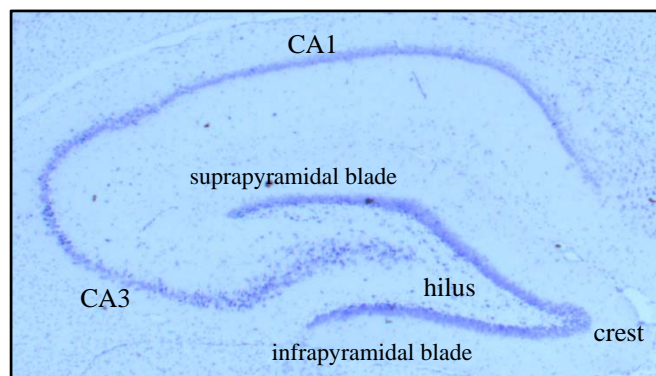


Fig. 1-2: Nissl-stained sagittal section of the rat hippocampus (100-fold). Indicated are the CA3 and CA1 region of the hippocampus proper, the supra- and infrapyramidal blade, the crest and the hilus of the dentate gyrus.

The principal cell layer of the hippocampus proper is the pyramidal cell layer. Deep to it, the relatively cell-free stratum oriens is located and contains the basal dendrites of the pyramidal cells. In CA3, this layer also contains commissural and associative fibers (Amaral & Witter, 1995; Vizi & Kiss, 1998; Amaral & Lavanex, 2006). In CA3, but not in CA2 or CA1, a narrow acellular zone, the stratum lucidum, is located just above the pyramidal cell layer and contains the terminals of the mossy fibers. Superficial to the stratum lucidum in CA3 and immediately above the stratum pyramidale in CA2 and CA1 the stratum radiatum, one finds the stratum radiatum, in which the commissural and associational connections as well as the CA3 to CA1 Schaffer collaterals terminate. The most superficial layer is the stratum lacunosum moleculare, in which fibers from the entorhinal cortex terminate. In CA1, this layer also receives inputs from the thalamus and perirhinal cortex. Inputs from the septum and the amygdala terminate in the stratum radiatum and stratum oriens. Serotonergic inputs from the raphe are distributed throughout all layers, while noradrenergic fibers only terminate in the stratum lacunosum moleculare and stratum lucidum (Vizi & Kiss, 1998). In general, CA1 receives much lighter monoaminergic innervation than CA3 (Amaral & Lavanex, 2006). CA3 and CA1 differ in their intrinsic and extrinsic connectivity pattern underlying different functions (Amaral & Witter, 1989; Leutgeb et al., 2004). CA3 is characterized by a recurrent network of densely interconnected pyramidal cells, while CA1 constitutes a feed-forward network with almost no intrinsic excitatory connections.

The entorhinal cortex is called the entry port of the hippocampal formation. It can be subdivided into two areas: the lateral entorhinal cortex and the medial entorhinal cortex (Amaral & Witter, 1995; Van Groen et al., 2002; Amaral & Lavanex, 2006). The entorhinal cortex is composed of six layers: four cellular (layer II, III, V, VI) and two acellular layers (Peretto et al., 1999). Layer II and III receive the main inputs; in the lateral entorhinal cortex especially from sensory modalities, the amygdala, the prefrontal cortex and the perirhinal cortex, which itself receives similar inputs as the lateral entorhinal cortex; in the medial entorhinal cortex especially visual and visual spatial inputs converge as well as inputs from the postrhinal cortex, which itself gets similar inputs like the medial entorhinal cortex. The layer II and III pyramidal cells convey this topographical distribution to the dentate gyrus via the perforant path (Van Groen et al., 2002). Fibers from the lateral entorhinal cortex terminate as lateral perforant path in the outer molecular layer, while fibers from the medial entorhinal cortex terminate as medial perforant path in the middle molecular layer. Furthermore, the perforant path also terminates in CA3, CA1 and the subiculum. The layer V cells of the entorhinal cortex receive one of the main outputs of the hippocampus and reciprocate connections

from other cortical areas that project to the superficial entorhinal cortex (Amaral & Lavanex, 2006).

The subiculum is the transition region between the hippocampus proper and the entorhinal cortex and, thus, gradually changes from three to six layers (Amaral & Witter, 1995). Its principal cell layer contains pyramidal cells that are the main output targets of the CA1 pyramidal cells. As the main output region of the hippocampal formation it projects to the neocortex, the amygdala, the entorhinal cortex and the mammillar bodies of the hypothalamus. Figure 1-3 summarizes the main connections within the hippocampal formation.

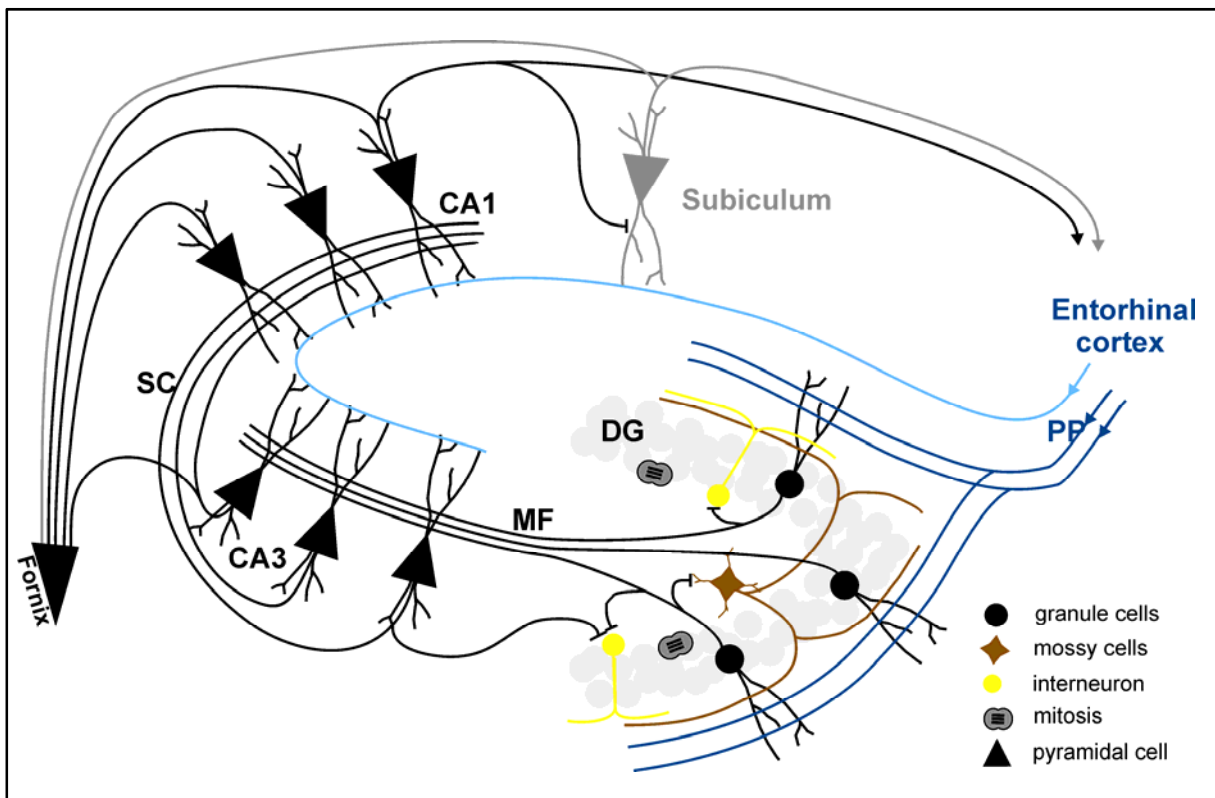


Fig. 1-3: Main connections within the hippocampal formation.

The input to the hippocampal formation converges in the entorhinal cortex. The entorhinal cortex projects via the perforant path (PP) to the outer and middle molecular layer of the dentate gyrus (DG) as well as to CA1 and CA3 of the hippocampus proper. The granule cells project as mossy fibers (MF) to CA3. Collaterals innervate the mossy cells and hilar interneurons which project as commissural and associative fibers back to the inner molecular layer. The axons of the CA3 pyramidal cells innervate as Schaffer collaterals (SC) the pyramidal cells of CA1. Collaterals of CA3 pyramidal axons project back to the polymorphic layer of the DG innervating hilar interneurons (Amaral & Witter, 1995; Wu et al., 1998). CA1 pyramidal cells project to the subiculum as well as to the entorhinal cortex. CA3, CA1 and subiculum project via the fornix to the hypothalamus (cf. Papez circuit; for further details and references see main text; modified according to documents of Teuchert-Noodt).

1.2. THE PREFRONTAL-LIMBIC SYSTEM

The limbic system is a functional unit of the brain which is implicated in the processing of emotion, motivation and memory. It was first described by Paul Broca as the «grand lobe limbique» (1878). Its central part is the Papez circuit (Papez, 1937), a neuronal circuit involving the hippocampus, the fornix projecting to the mammillar bodies of the hypothalamus, the projection from there to the cingulate gyrus and back to the hippocampal formation. This circuit was first believed to play a central role in the processing of emotion. Meanwhile, however, it is clear that it rather takes part in the consolidation of memory. Today, further structures have been added to the limbic system like the amygdala, the nucleus accumbens and the monoaminergic nuclei in the brain stem, which are all functionally and anatomically connected (Krettek & Price, 1977; Cassell & Wright, 1986; Le Moal & Simon, 1991; Totterdell & Meredith, 1997; Pikkarainen et al., 1999; Pitkanen et al., 2000; Pikkarainen & Pitkanen, 2001).

After all, the numerous reciprocal connections of the limbic system with the prefrontal cortex (Swanson, 1981; Jay & Witter, 1991; Sesack & Pickel, 1992; Mc Donald et al., 1996; Rosenkranz & Grace, 2001, 2002) led to the broader description of a prefrontal-limbic system.

The prefrontal cortex is defined as the region lying rostrally to the motor and premotor cortex, where the cortical projection of the mediodorsal thalamus and the dopaminergic projections form the ventral tegmental area overlap (Ray & Price, 1992; Divac et al., 1993). As a higher associational area (Kolb & Wishaw, 1990), the prefrontal cortex is connected with diverse parts of the brain like the thalamus, sensory and motor areas, limbic circuits, hypothalamus and the brain stem (Öngur & Price, 2000). It is mostly responsible for executive functions, for attentional processes, inhibitory control, social behavior, working memory and action planning (Fuster, 1979).

Especially the two cortical structures, the prefrontal cortex and the hippocampus, seem to be essential integration centers of the prefrontal-limbic system. They receive multimodal afferences from associative areas as well as from subcortical nuclei and project back to various regions of the brain (Fuster, 1979; Sesack et al., 1989; Amaral & Witter, 1995; Insausti et al., 1997). They are implicated in higher cognitive functions, which are characterized by long developmental periods. The prefrontal cortex, due to the slowly developing mesoprefrontal dopamine projection (Kalsbeek et al., 1988; Dawirs et al., 1993; Rosenberg & Lewis, 1995), does not reach maturation until young adulthood (Mrzljak et al., 1990; Casey et al., 2000), whereas the hippocampus, due to its lifelong neurogenesis in the dentate gyrus

(Altman & Das, 1965; Kaplan & Bell, 1984; Eriksson et al., 1998), remains in a state of continuous development. These developmental patterns leading to the high plastic capacities of these regions predict, on the one hand high learning and memory capabilities, but on the other hand, enhanced vulnerability to environmental influences and disturbances. Furthermore, both structures influence each other in their development insofar that disturbances in one of these areas lead to adaptive alterations in the other (Schroeder et al., 1999; O'Donnell et al., 2002; Lipska et al., 2002) or even in the whole prefrontal-limbic system (Friston, 1998; Lipska et al., 2000).

1.3. NEUROPLASTICITY

Neuronal connections are not fixed and invariable. They can change in response to lesions as well as functional events like learning (Kampfhammer, 2001). In the latter case one can say that form follows function in the brain (Kempermann, 2006).

Neuroplasticity involves several levels. On the level of the synapse, changes in the strength of synaptic transmission can take place (*synaptic plasticity*). This involves, on the one hand, *functional plasticity* without anatomical alterations, meaning changes in the quantity of transmitter release (presynaptic events) or in receptor densities (postsynaptic events), and, on the other hand, *structural plasticity* meaning anatomical changes like the enlargement or reduction of the synaptic contact area and the remodeling of whole synapses. Structural plasticity can even go beyond the synapse. Complete axons or dendrites can be retracted or extended and the number of branches or spines can be altered. Moreover, the generation of new neurons, *neurogenesis*, also constitutes a form of structural plasticity.

1.3.1. NEUROGENESIS

Neurogenesis is functionally defined as the generation of new neurons from undifferentiated precursor cells (stem cells or progenitor cells). Two regions in the mammalian brain were found, where neurogenesis takes place even in the adult; the subgranular layer of the hippocampal dentate gyrus (Altman & Das, 1965) and the subventricular zone from where the newborn neurons migrate via the rostromigratory stream to the olfactory bulb (Kaplan & Hinds, 1977). Neurogenesis is more than an event of cell division. It is a process involving cell proliferation, migration, differentiation and integration, with the latter determining cell survival (Fig. 1-4). For the dentate gyrus this means that after the completion of the last cell cycle, the cells start to extend their dendrites towards the molecular layer (Zhao et al., 2006) and their axons through the hilus towards CA3 (Hastings & Gould, 1999; Zhao et al., 2006).

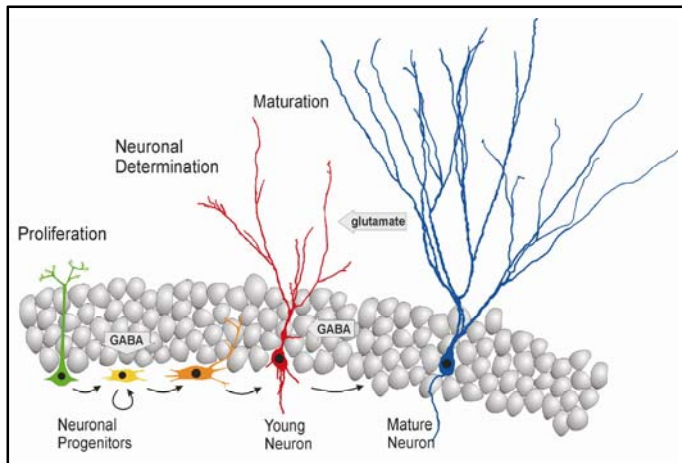


Fig. 1-4: Development of newly generated granule cells in the adult dentate gyrus. Neural progenitors in the subgranular zone give birth to young neurons. While a young neuron matures, extends dendrites and its axon, it migrates into the granule cell layer (cf. text; modified according to Bischofberger 2007).

Meanwhile, the cell migrates into the granule cell layer (Fig. 1-4; Zhao et al., 2006). Already after about one week, the newborn cells receive the first GABAergic inputs, which, however, are excitatory for the young cells. One week later, these inputs are replaced by glutamatergic ones (Esposito et al., 2005; Ge et al., 2006). The axons of the maturing neurons reach their targets in the stratum lucidum of CA3 4-10 days after division (Hastings & Gould, 1999; Zhao et al., 2006). Although the cells begin to express mature neuronal markers like NeuN already three weeks after becoming postmitotic (Kempermann et al., 2004; Fig. 1-5), it takes them up to 4-7 weeks to become undistinguishable from mature granule cells (van Praag et al., 2002; Jessberger & Kempermann, 2003).

Neurogenesis can be visualized and detected by labeling the dividing cells with exogenous markers like tritiated ($[^3\text{H}]$) thymidine and 5-bromo-2-deoxyuridine (BrdU; Fig. 1-5) or the detection of endogenous markers (e.g., Ki67) and proteins expressed by immature neurons (e.g., doublecortin, PSA-NCAM). Meanwhile the fluorescence labeling (green fluorescence protein, GFP) of newborn neurons using retroviral vectors allows even the observation of neurons during their course of maturation (cf. van Praag et al., 2002).

But for all that, the regulation of the different processes of neurogenesis is still rather unknown. A lot of factors were identified that exert an influence on cell proliferation or cell survival, but often it remains undecided whether influence actually reflects regulation (Kempermann, 2006). Neurogenesis is highest after birth and declines with age (Kuhn et al., 1996; Cameron & McKay, 1999; Dawirs et al., 2000). Thus, aging seems to be an important

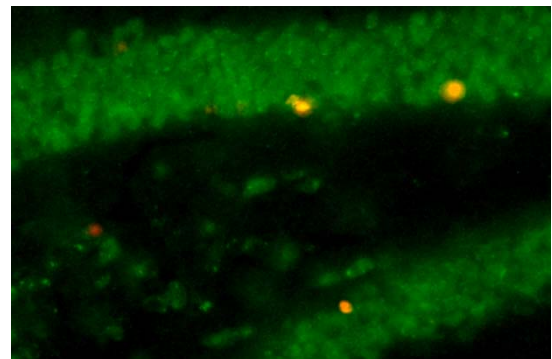


Fig. 1-5: Double immunofluorescence labeling for mature neurons (NeuN, green) and BrdU (red) in the mouse dentate gyrus 21 days after BrdU-injection. Double labeled cells (yellow) indicate newborn neurons.

regulating factor. Stress decreases neurogenesis (Gould et al., 1998), most likely by increasing glucocorticoid levels, which in turn suppress cell proliferation (Gould et al., 1992; Cameron & Gould, 1994). In contrast, mild and challenging stressors like enriched environment or running seem to increase neurogenesis (Kempermann et al., 1997a; van Praag et al., 1999a, b; Nilsson et al., 1999; Uda et al., 2006). However, how these effects are mediated is still subject to current research. It seems that neurotrophic factors, like brain-derived neurotrophic factor, vascular endothelial growth factor or insulin-like growth factor 1 (Wagner et al., 1999; Aberg et al., 2000; O'Kusky et al., 2000; Trejo et al., 2001; Fabel et al., 2003; Cao et al., 2004; Scharfman et al., 2005), neurotransmitters (Hildebrandt et al., 1999; Brezun & Daszuta, 1999; Teuchert-Noodt et al., 2000; Malberg et al., 2000; Kulkarni et al., 2002; O'Keefe et al., 2009), and NMDA-receptor activation (Gould et al., 1994; Cameron et al., 1995; McEwen, 1996) are involved.

Probably regulation is coupled to function. Since neurogenesis takes place in a region which is involved in learning, it could be assumed that neurogenesis plays an essential role in learning as well. Indeed, blocking neurogenesis leads to impairments in hippocampus-dependent but not hippocampus-independent learning tasks (Shors et al., 2001; Madsen et al., 2003; Rola et al., 2004) and in turn hippocampus-dependent learning enhances the survival of newly generated granule cells (Gould et al., 1999; Ambrogini et al., 2000; Döbrössy et al., 2003; Leuner et al., 2004; Olariu et al., 2005). However, not all studies could support this view and the functional implication of neurogenesis in learning is still a matter of debate (rev. in Leuner et al., 2006).

With the findings that depressed patients show hippocampal symptoms and hippocampal atrophy (Sheline, 1996; Stockmeier et al., 2004; Greenberg et al., 2008) and that a decreased hippocampal neurogenesis can be restored by antidepressant drugs like selective serotonin reuptake inhibitors or tricyclic antidepressants in the animal model (Malberg et al., 2000; Santarelli et al., 2003; Malberg & Duman, 2003), the hypothesis came up that a failure of neurogenesis could (at least partially) underlie the pathogenesis of major depression. This putative link between new neurons and emotional behavior is particularly interesting, because it widens the discussion beyond reductionistic learning paradigms (Kempermann, 2006). Moreover, the assumption of an implication of neurogenesis in both hippocampus-dependent learning and emotional processes fits to the probable functional dissociation of the hippocampal formation as a whole (cf. chapter 1.1).

1.3.2. STRUCTURAL PLASTICITY BEYOND NEUROGENESIS

Already in 1911, Ramon y Cajal postulated that the extension of axons and the formation of new synapses is an underlying mechanism of learning (Ramón y Cajal, 1911). Based on this, Hebb formulated his learning theory on the level of synapses that, if activity in two cells was closely enough related in time, this activity would strengthen their connection (Hebb, 1949). First experimental evidence for this theory came from Changeux and Danchin (1976), who could demonstrate that instable primary contacts are selectively strengthened or weakened depending on the activity pattern. Today it is known that this strengthening or weakening is achieved by progressive (e.g. addition of synapses) as well as regressive events (degeneration and degradation of synapses). Thus, neuronal networks mature through continuous remodeling of their synaptic contacts (Wolff, 1982b; Wolff & Wagner, 1983; Wolff et al., 1989). Moreover, they do so not only during the course of development, but also in adulthood as an adaptive process permitting the individual to learn and to cope with new situations (rev. in Morris et al., 2003).

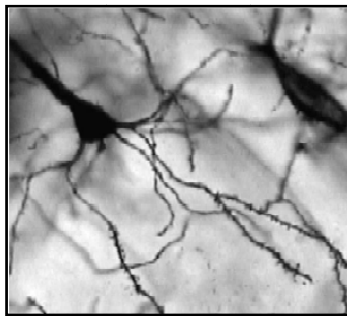


Fig. 1-6: Golgi-Cox impregnation of a pyramidal neuron in the CA1 region of the rat (Silva-Gómez et al., 2003).

Processes of structural plasticity can be visualized by histological as well as immunohistochemical methods. With selective silver staining methods (e.g., Golgi-Cox impregnation; Fig. 1-6) dendritic and axonal branching as well as spine densities can be made quantifiable. Synaptic marker proteins like synapsin, synaptophysin or postsynaptic-density-95 can be detected immunohistochemically and taken as an indicator of synaptic densities. In axon terminals, synaptic turnover can be visualized on the basis of silver impregnation of secondary lysosomes and their

accumulation in the course of degradation (Gallyas et al., 1980; Wolff et al., 1989; Wolff & Missler, 1993; Fig. 1-7). Meanwhile, it is even possible to observe synapses during the synaptic remodeling processes with life-imaging techniques.

With the help of these techniques it was discovered that nearly the same conditions influencing adult neurogenesis in the hippocampal dentate gyrus also exert an influence on the other forms of structural plasticity. Stress and glucocorticoids reduce dendritic

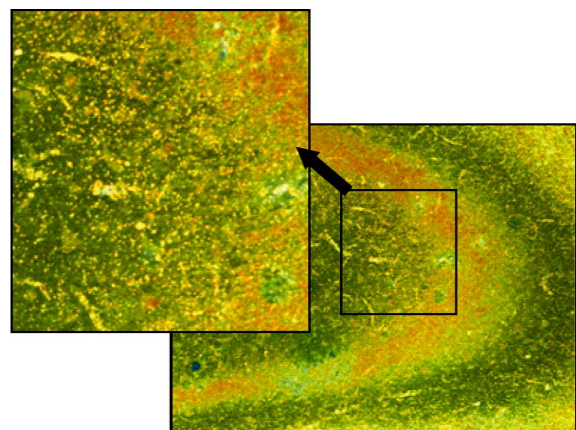


Fig. 1-7: Gallyas silver staining against lysosomal accumulations indicating axon terminal degradation in the mouse CA3 stratum lucidum.

size and complexity (Woolley et al., 1990; Watanabe et al., 1992; McEwen, 1996; Magarinos et al., 1996), while living in enriched environments enhances synaptophysin and psd-95 levels (Frick & Fernandez, 2003; Nithianantharajah et al., 2004), synaptic densities (Briones et al., 2005), dendritic branching and spine growth (Green et al., 1983; Juraska et al., 1985; Faherty et al., 2003) compared to isolated housing (Silva-Gómez et al., 2003) as well as axonal remodeling (Keller et al., 2000; Butz et al., 2008). Similar but less clear effects are seen concerning physical activity (but also see Faherty et al., 2003; Eadie et al., 2005; Stranahan et al., 2007). It is plausible that also the underlying regulating mechanisms of neurogenesis and other forms of structural plasticity are similar: involved are especially neurotrophic factors (McAllister et al., 1996; O'Kusky et al., 2000; Tolwani et al., 2002; Tyler & Pozzo-Miller, 2003) as well as the activity state of the system (rev. in Butz et al., 2009).

1.3.3. NEUROGENESIS AND STRUCTURAL PLASTICITY

Newborn neurons are a considerable source of neuroplasticity. They demand integration and, thus, induce a cascade of structural changes (rev. in Lehmann et al., 2005; Gould, 2006). Newborn neurons are more excitable than mature ones and long-term potentiation as a form of synaptic plasticity can be more easily evoked in them (Schmidt-Hieber et al., 2004; rev. in Bischofberger, 2007). At last, neurogenesis induces neuronal turnover; since the dentate gyrus and especially the number of granule cells does not substantially grow in the course of life (Heine et al., 2004), not even after treatments which enhance neurogenesis substantially (Nilsson et al., 1999; Kitamura & Sugiyama, 2006), a lot of newborn as well as mature granule cells must undergo apoptosis. They leave free synapses which have to degrade and form new contacts with other cells.

Although the relationship between neurogenesis and other forms of structural plasticity is obvious, the correlation between them seems to be a negative one. Studies in gerbils as well as theoretical models suggest that a moderate number of newborn cells is a prerequisite for a fully functional structural plasticity, since manipulatively enhanced rates of cell proliferation lead to a decrease of synaptic remodeling rates measured by the amount of degrading synapses (Keller et al., 2000; Butz et al., 2008). This at first sight counterintuitive inverse relationship can be explained by an overstressed plasticity when too many newborn neurons demand integration (Teuchert-Noodt, 2000; Butz et al., 2008). This assumption finds support by behavioral studies showing that moderate proliferating activities in the dentate gyrus are conducive to or coincident with more efficient memory processing in the hippocampus (Epp & Galea, 2009).

1.4. AIMS AND APPROACH

The aim of the present work was to investigate developmental neuroplasticity in the hippocampus in the interplay of prefrontal-limbic circuits with the widely-used mouse model (*Mus musculus domesticus*). This aim was approached with four experimental studies and a theoretical work on a hypothetical model based on the experimental findings and the current literature:

Project I) In case that it is a general principle that environmental stimulation during juvenile brain development is so crucial that it determines neuroplasticity in the hippocampus even in the adult, it should be detectable also in the widely-used mouse model. To follow this issue, female CD1 mice were reared under social and physical deprivation and exposed to wheel running in young adulthood. We wanted to know, whether baseline neurogenesis or the reactivity of neurogenesis in the adult dentate gyrus is affected by deprivation of “natural” environmental stimulation during juvenile brain development.

Project II) Based on the findings of project I and a review of the current literature, a hypothetical model was proposed to follow the question, how the environment during juvenile development determines the response of neurogenesis to wheel running in adulthood.

Project III) Neurogenesis induces structural changes, neuronal and synaptic turnover. We wanted to know, if and how far a relationship between neurogenesis and synaptic remodeling exists in the mouse dentate gyrus and even beyond in the entire hippocampal formation. We exposed male CD1 mice to the same treatments as in project I, and examined the effects on synaptic remodeling in the hippocampal formation.

Project IV) In the theoretical project II, it was hypothesized that beside local mechanisms the prefrontal cortex is causally involved in the regulation of cell proliferation in the hippocampal dentate gyrus. To test this hypothesis, CD1 mice were reared under social and physical deprivation, exposed to wheel-running in young adulthood and, afterwards, to either environmental enrichment or a prefrontal-cortex-dependent working-memory task as stimuli for prefrontal activity. The question was, whether an enhancement of prefrontal activity can suppress the increased cell proliferation after wheel running.

Project V) Deprivation of environmental stimulation during juvenile brain development affects neuroplasticity in the hippocampus in CD1 mice in a qualitatively similar manner as in gerbils (project I, project II). However, the strength of the deprivation effect is less pronounced. The questions remained, what determines the strength of the deprivation effect, and whether differences exist even within the species *Mus musculus domesticus*. To answer these questions, a strain comparison on the effects of deprivation during juvenile brain development was conducted including domesticated (outbred C57Bl/6, inbred CD1 in project I) as well as undomesticated mouse strains (wild-caught house mice).

2. MATERIAL & METHODS

2.1. ANIMALS

All experiments were performed with domesticated and wild mouse strains of the species *Mus musculus domesticus*, the western house mouse. This species belongs to the order *rodentia* and the family *muridae*.

Most of the experiments were carried out with CD1 mice (CrI:CD1(ICR); Fig. 2-1). CD1 is an outbred laboratory strain, which was derived in 1926 from a small inbred stock in Switzerland (synonym “Swiss mice”) and bred by Charles River since 1959 (Volstroff, 2008). The albino strain is characterized as docile and easy to handle. It shows a rather average learning performance (Dellu et al., 2000; Adams et al., 2002). Its cell proliferation in the adult dentate gyrus is moderate, but the survival rate after four weeks is relatively high with 1 out of 1.3

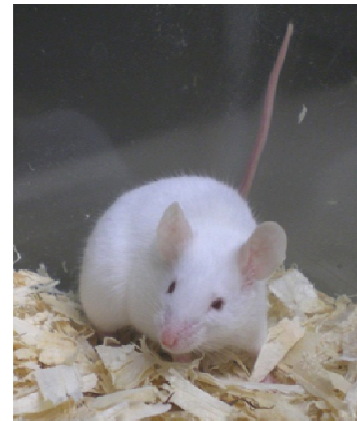


Fig. 2-1: CD1 mouse.

newborn cells surviving (Kempermann et al., 1997b). For our experiments, we purchased either pregnant females from Charles River Laboratories (Sulzfeld, Germany) giving birth to the experimental animals in our animal facility or young females at the age of weaning (postnatal day (pd) 21).

C57Bl/6 (Fig. 2-2) is an inbred mouse strain. It was created in 1921 and introduced to the Jackson Laboratory in 1948 (synonym “Jax® Mice”) Today, it is the most widely used



Fig. 2-2: C57Bl/6 mouse.

laboratory mouse strain. It shows very good learning performance in spatial as well as operant tasks (Crawley et al., 1997; Owen et al., 1997; Baron & Meltzer, 2001). Its cell proliferation rate is rather high (1.5 fold higher than CD1), but cell survival is with 1 of 3 lower than in CD1 mice (Kempermann et al., 1997b). Both strains do not differ in phenotypic differentiation; about 60% of the surviving cells have a neuronal phenotype. For our

experiments, C57Bl/6 mice were bred in the central animal facility of Bielefeld University and delivered to us on the day of weaning, pd 21. Experiments were carried out in our own animal facility.

Wild house mice (Fig. 2-3) were obtained from the Institute of Zoology, Animal Behavior in Zurich. The working group captures wild house mice near Zurich and uses them for breeding and behavioral experiments. They provided us with 12 females and five males of

their F1 generation, which we brought to Bielefeld by car. After one week of recovery from transport, we started breeding in our own animal facility. All experiments were carried out with females of the F2 generation. In contrast to the laboratory mouse strains, these animals are very difficult to handle; they are very active, run, climb and jump reaching jump heights of up to 70 cm. Moreover, they show a strong desire to escape.

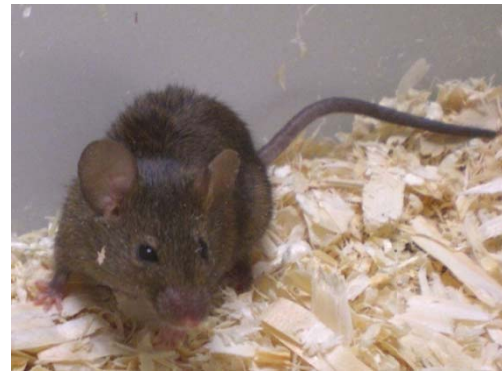


Fig. 2-3: Wild house mouse.

2.2. REARING CONDITIONS

Depending on the experiments, animals were assigned to two different rearing conditions on the day of weaning, postnatal day (pd) 21; enriched (ER_{SPW}-) or socially and physically deprived rearing (DR_{SPW}-), both without running wheels (w-). These rearing conditions were defined relative to three parameters: the social environment (single or group housed) denoted by *s*, the physical environment (small or large space and presence or absence of environmental objects such as tunnels, tubes, and solid hiding places) denoted by *p*, and the absence

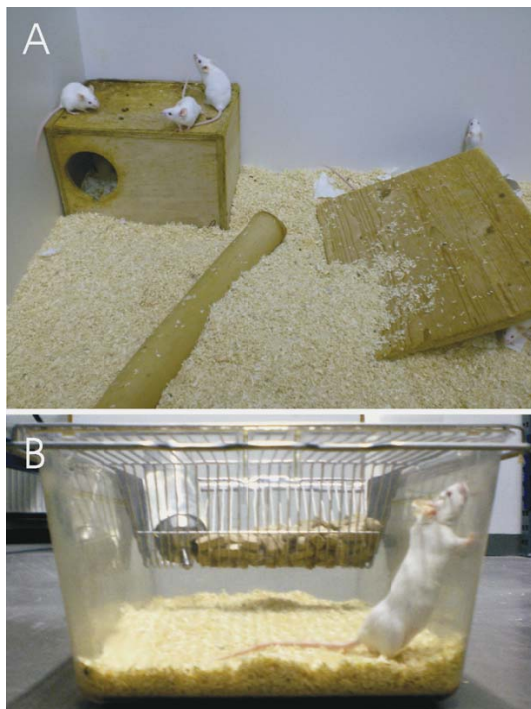


Fig. 2-4: Rearing conditions. **A)** CD1 mice in the socially and physically enriched-rearing condition; **B)** CD1 mouse in a cage of the socially and physically deprived-rearing condition.

of a running wheel under all conditions denoted by w-. Accordingly, ER_{SPW}- animals were reared in groups of six to 15 individuals in a large enclosure (100 x 100 x 50 cm) that in addition to animal bedding contained items such as tunnels, tubes, and solid hiding places (Fig. 2-4, A). DR_{SPW}- animals were reared individually in standard type III cages (42.5 x 26.5 x 18 cm) that contained no other materials than animal bedding and some sheets of toilet paper (Fig. 2-4, B). All animals were on a 12-h-light-dark schedule and received laboratory animal feed (Höveler, Dormagen) and water *ad libitum*. Animals were kept under their respective experimental rearing conditions until they reached young adulthood, pd 66 to 90 depending on the experiment.

2.3. WHEEL-RUNNING CHALLENGE

In project I, III and V, half of the animals received an additional wheel-running challenge in young adulthood. The rearing conditions ended and all individuals from the previous experimental groups (ER_{SPW} and DR_{SPW}) were united and two mixed groups were formed (identification of animals by earmarks). Each group was placed into a large enclosure (100 x 100 x 50 cm) that contained animal bedding, food and water *ad libitum* as well as a solid hiding place. One of the mixed groups had access to running wheels from pd 70 to pd 74. For this, one running wheel (circumference 46 cm) per animal was placed into the respective enclosure cage (Fig. 2-5, A). The other mixed group received no further treatment (Fig. 2-5, B).

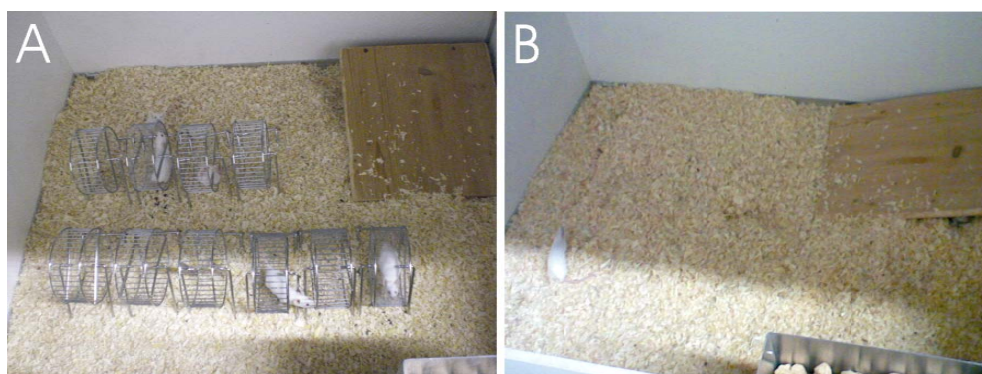


Fig. 2-5: Wheel-running challenge. A) Access to running wheels for one mixed group, one wheel per animal; B) The other mixed group remained without further treatment.

2.4. TRANSPONDER TAGGING

For individual identification of the animals in project IV, animals were equipped with glass-covered RFID transponders (glass tag unique, 12.1 x 2.12 mm, 125 kHz, Sokymat, Wetzikon, Switzerland). Animals were shortly anaesthetized with diethyl ether and the transponder was injected from behind under the skin in the neck. One day and one week later, animals were checked with a handheld scanner for retention of the transponders and if necessary the transponder was reinjected.

2.5. BEHAVIORAL EXPERIMENTS

2.5.1. AUTOMATED RUNNING WHEELS

To document, how much animals from different strains and different rearing conditions run in running wheels, running activity was measured using automated running wheels.

Six running wheels (circumference 46 cm) were mounted side by side on a metal rod with a distance of 6 cm between wheels and hung up in a wooden rack (Fig. 2-6, A). For the experiments, the rack was placed into an enclosure. Each running wheel was equipped with a magnet on one of its spokes. A reed switch was placed close to the wheel, so that the magnet would pass close by the switch on every turn of the wheel (like with a cyclometer; Fig. 2-6, C). The reed switches were connected with a computer via a digital I/O device (USB, National Instruments, Munich, Germany). Both reed switches and connection cables were protected from mice by thin stainless steel tubes which were screwed onto the rod and led the cables out of the enclosure (Fig. 2-6, A, B). The connected laptop computer (IBM, think pad T41) registered the closing of the switches and, thus, the wheel revolutions.

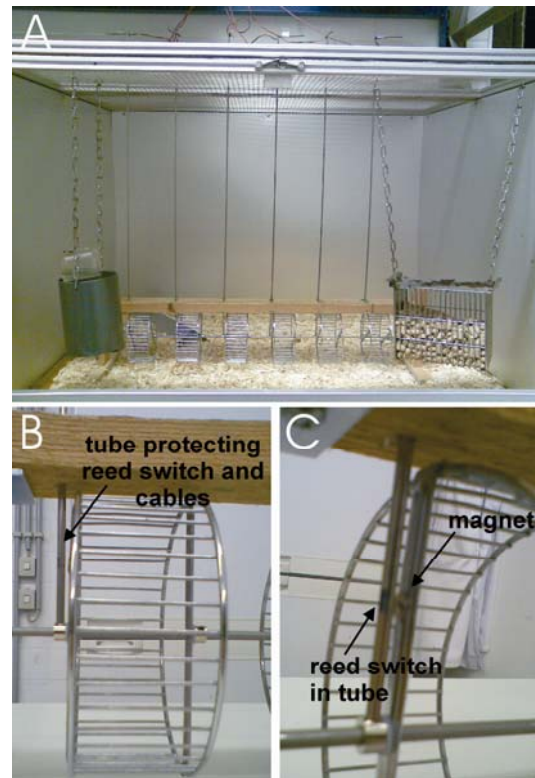


Fig. 2-6: A) Automated running wheels in an enclosure; B) Example of a running wheel; C) Magnet close to a reed switch.

2.5.2. AUTOMATED T-MAZE

For the spatial delayed-alternation task in project IV, an automated T-Maze was constructed. The delayed-alternation task using a T-Maze is a widely used experiment to investigate working memory in rodents (Olton, 1979; Morris et al., 1986; Zahrt et al., 1997; Baeg et al., 2003; see also Dalley et al., 2004; Dudchenko, 2004; Clinton et al., 2006). In the first phase, the animal is placed at the base of the T and required to run up the stem and enter one arm, which is open, while the other one is closed. A reward is available at the end of the arm. Then, the animal runs back or is placed back to the start position and is required to enter the arm opposite to the previously visited arm. The correct choice results in a reward, whereas the incorrect choice is not rewarded. A delay period can be introduced between first and second run, in which the animal is prevented from responding until a certain time period has elapsed. By extending or shortening the delay period one can vary the working-memory load (or task difficulty; Pedigo et al., 2006).

Manually-operated or semi-automated T-mazes have the disadvantage that the experimenter can introduce variability and that frequent handling of the animals can influence their

attention, emotion and behavior. To circumvent these unintentional effects on the outcome of the experiment, we constructed an automated T-maze, which the animals can enter voluntarily and without any handling. To ensure that only one animal at a time could enter the T-maze, a sorting system was inserted between the home cage and the T-maze.

2.5.2.1. Apparatus

Sorting system

The sorting system consisted of a pipe (plexiglas for stability and wire mesh, because animals dislike to rest in wire mesh; diameter 40 mm), through which the animal could go from the home cage to the maze, three transponder readers (Fig. 2-7, A,B: T1-T3; 684-52 REV6 OEM proximity reader, Aceprox, Helpsen, Germany) and two computer-controlled gates (Fig. 2-7, A,B: gate 1 and 2). Each gate was operated by means of a servomotor (Top-line Servo RS-2; Modelcraft, Blaine, WA, USA) with a catch so that it could either be opened in the direction left-to-right (towards the T-maze), right-to-left (towards the home cage), in both directions or completely closed by the experimental computer. The transponder readers were situated directly adjacent to the pipes of the sorting system; one between the home cage and the first gate (T1), the other two between the gates, one close to the first (T2), the other close to the second gate (T3). When an animal entered the pipe leading from the home cage to the sorting system, it was registered by the first transponder reader (T1). Upon this registration, the first gate opened towards the T-maze to let the animal into the sorting system. When the animal passed through the pipe and reached the second gate, it was registered by the second (T2) and third transponder reader (T3), which caused the computer to close the first gate. Now the animal was confined in the wire-mesh pipe between the two gates for 30 sec. Meanwhile, the computer “checked” that no other animal was in the sorting system, i.e. that no other animal was registered by transponder reader T2 or T3. If this was the case, the second gate opened towards the T-maze and the sorted animal could enter the maze. Otherwise the first gate was opened again to release all animals into the home cage and the sorting had to start over.

T-maze

The T-Maze consisted of plexiglas pipes, PVC connectors and wire mesh (see Fig. 2-7, C, diameter ca. 40 mm). The central T-pipe had a transponder reader at its base (T4; Fig. 2-7, A, C). Two feeders equipped with light-barriers delivered water as reward at the end of each arm. The animals could return to the base of the T through two return pipes that formed a figure 8 together with the T. Despite the fact that this maze looked like a figure 8 maze from above,

the functionality is that of a T-maze. Water was provided by a 50 ml syringe operated by a computer-controlled step-motor pump (stepping motor and threaded spindle, Nanotec, Landsham, Germany, resolution 0.0254 mm/step). The two feeders were equipped with a computer-controlled valve each (PS-1615NC, Takasago Electric, Nagoya, Japan). Depending on which valve was opened, the water reward was given either at the one or the other feeder. The pipes of the T-maze were a one-way-street system due to four one-way flaps that allowed passage in one direction only (Fig. 2-7, A, C).

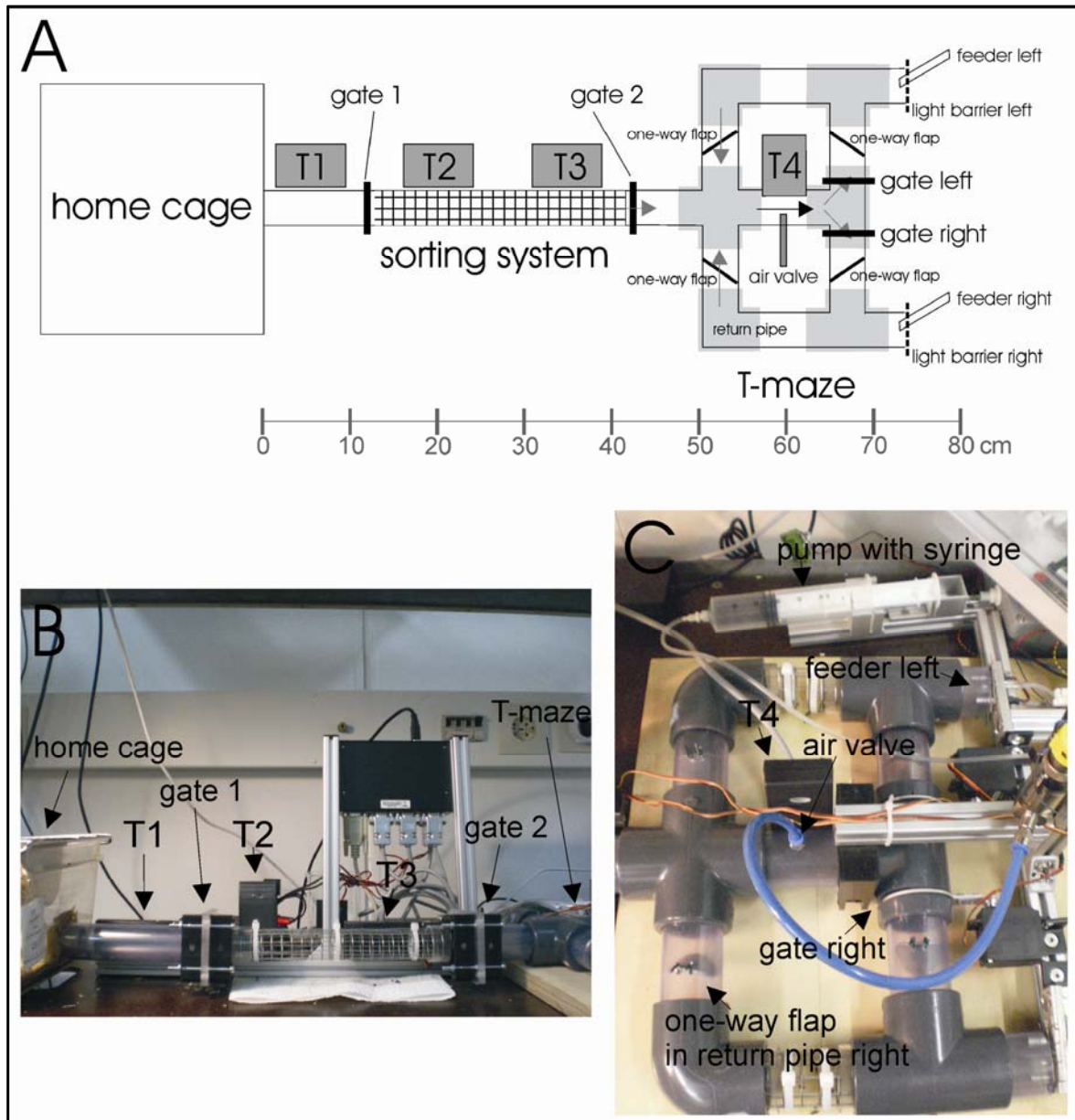


Fig. 2-7: Experimental setup. **A)** To scale scheme of the apparatus, including home cage, sorting system and T-maze; **B)** Photo of the sorting system with indication of the main components; **C)** Photo of the T-maze with indication of the main components; T, transponder reader.

Like in a manually operated T-maze, guillotine gates controlled the access to the arms, and thus, the initiation of *forced* or *choice runs* and delay periods, (see chapter 2.5.2.2). These were actuated as well by servomotors and controlled by the computer program (Fig. 2-7, A, C). An air valve (EV-3M-24 VDC with electronic valve booster EVB-3, Clippard, Cincinnati OH, USA) was installed in the stem of the T (Fig. 2-7, A,C) to enable the system to chase the animal out, if it was inactive for a certain time, e.g., if it wanted to sleep or groom in the maze, thereby blocking it for other animals.

Computer

The sorting system and the T-Maze were controlled by a laptop computer (IBM, think pad T41) which was connected to the system via an I/O device (IO-warrior24 Starterkit, Code Mercenaries, Schönefeld, Germany). The computer was equipped with a self-developed computer program. The different experimental parameters could be adjusted in parameter files. Moreover, the computer recorded the movements of the animals (by registrations at the transponder readers and light barriers) as well as delivered rewards.

2.5.2.2. Protocol and training phases

The performance of a delayed-alternation task requires certain habituation and training phases to introduce the animals to the task step-by-step. The protocol used here was developed on the base of the protocols for manually-operated T- or Y-mazes used by Winterfeld and colleagues (1998) and Ivkovich and Stanton (2001). It included two *habituation* phases, a *Pretraining* phase, in which the animals learned to alternate in the maze, a *Training phase without delay*, in which they learned the actual alternation task, and the *Training with delay*, which constituted the actual delayed alternation und thus the working-memory task. Beside this protocol leading to the working-memory task, further programs (*PretrainingRandom* and *TrainingRandom*) were developed to allow the examination of control animals without alternation and working-memory load to check for unspecific effects of the exposure to the apparatus and the protocol.

Habituation Phase I

During this phase, the animals were habituated to the apparatus for the first time. All gates were open, and animals were allowed to explore the maze together. They could receive 20 μ l of water every 5 sec. when breaking the light barrier of a feeder.

Habituation Phase 2

During this phase the sorting system allowed only one animal to enter the maze in the way described above (cf. chapter 2.5.2.1). In the maze, both guillotine gates were open, and animals could receive 20 µl of water every 5 sec. by breaking the light barrier of a feeder. After 5 min., the door of the sorting system opened, and the feeders did not give reward any more, marking that the animal should leave the maze and make room for others. If an animal remained in the maze for more than 1 hour after opening of the door (e.g., sleeping, grooming), it was chased out by activation of the air puff from the air valve.

Pretraining

During this phase, the sorting system allowed only one animal to enter the maze, while both gates within the maze were closed. When an animal reached transponder reader T4, one of the two gates opened allowing the animal to enter this arm and get a single water reward of 20 µl at the respective feeder. Induced by the breaking of the light barrier of the feeder, the gate of the arm was closed again. When the animal returned to reader T4 via the return pipe, the other gate opened and so on and so forth. Thereby, the animals were trained to alternate in the maze. After 10 single runs, the guillotine gates closed, while the sorting system opened to direct the animal back to the home cage. When an animal remained inactive between the runs for more than 3 min. (inactive time, meaning that it was not registered by a reader or a light barrier), the gates of the maze did not open either but the gate of the sorting system opened towards the home cage. When an animal did not reach reader T3 of the sorting system within 2 min. after opening of the gate, it was chased out by activation of the air puff from the air valve.

Training without delay

During the training period, the sorting system allowed only one animal to enter the maze, while both gates within the maze were closed. When an animal reached reader T4, the *forced run* was started, meaning that one gate opened randomly allowing the animal to enter the respective arm and get a single water reward of 20 µl at the feeder. Induced by the breaking of the light barrier, the gate of the arm was closed. When the animal returned to reader T4 via the return pipe, the animal was confronted with the *choice run*, meaning that the gates of both arms opened, but only the arm opposite to the previously visited arm delivered a reward, when the animal broke the light barrier of the feeder. If the animal chose the same arm as before, it was not rewarded. Induced by the breaking of a light barrier (never mind which

one), both gates were closed again. After the animal had returned to reader T4, it had to wait for 30 sec., before the next *forced run* was started. After 5 single runs, the guillotine gates closed, while the sorting system opened to direct the animal back to the home cage. The inactive time was again 3 min. and 2 min. after the opening of the sorting system.

Training with delay

The procedure was the same as in the *training with delay*, but between the *forced* and the *choice run* a delay of 15 sec. was introduced, in which both gates remained closed. This phase presented the actual working-memory load.

PretrainingRandom

This program was used with the control animals and was similar to the *Pretraining* with the exception that the gates did not open in an alternating but in a random fashion. Thus, the control animals were not trained to alternate.

TrainingRandom

The *TrainingRandom* was also used with the control animals and was similar to the *Training*, but no delay was introduced between the *forced* and the *choice run*. Furthermore, in the *choice run*, the animals were not rewarded depending on their correct choice, but at both feeders with a certain probability. This probability could be adjusted at the performance of the experimental animals (e.g., 80%) to confront the control animals with the same frustration as the experimental animals but without any working-memory load.

2.6. NEUROGENESIS

For quantification of proliferating and surviving cells in the dentate gyrus (project I, IV, V) the *in vivo* labeling with the DNA-synthesis marker 5-bromo-2-deoxyuridine (BrdU) was used. BrdU is a thymidine analog which is incorporated into the DNA of dividing cells during the S-phase of the cell cycle. After systemic injection, BrdU is available in the adult organism for at least two hours (Packard et al., 1973; Hayes & Nowakowski, 2000). Thus, it marks only a fraction of newborn cells, but it allows rather precise birth dating of cells and the observation of cell survival.

2.6.1. BRDU-LABELING

In all experiments examining neurogenesis, a dose of 50 mg/kg BrdU (Sigma-Aldrich, Steinheim, Germany) was used. The animals were weighed prior to the injection, and the adequate amount of BrdU was dissolved in 0.9% NaCl. The solution was injected intraperitoneally (i.p.). For this the animal was removed from its cage or enclosure and placed on a grid, so that it could grab hold of it. The tail and rump were lifted, and the injection was made from behind into the abdominal cavity. The procedure lasted about 20-30 sec., and all efforts were made to minimize stress for the animals.

2.6.2. PERFUSION AND TISSUE PREPARATION

Perfusion

After a certain survival time after the BrdU-injections (depending on the experiment), animals were perfused with 4% paraformaldehyde (solved in 0.1M phosphate buffer). Each animal was deeply anaesthetized by an overdose of diethyl ether. As soon as it ceased to breath, the abdomen was opened by an abdominal incision. The thorax was opened, and a cannula was inserted into the left heart ventricle. Additionally, the right atrium and the *vena cava* were incised to ensure the drainage of blood and perfusion solution. With a tube pump (Cyclo II, Carl Roth, Karlsruhe, Germany) the perfusion solution was pumped through the body loop of the blood circulation and left it trough the right incised ventricle and *vena cava*. The perfusion was carried out with 150-200 ml 4% paraformaldehyde in about 15-20 min.

Dissection

Immediately after the perfusion, the brain was dissected. Therefore, the head was cut off from the body. The scalp, neck and temporal muscles were removed, till the cranial bone was exposed. The bone and the meninges were carefully removed with tweezers beginning caudally at the brain stem and cerebellum and operating rostrally. Last, the ossicles and the bones covering the olfactory bulb were cracked. Then, the brain could be released from the base of the skull with concomitant transection of the brain nerves. It was put into 4% paraformaldehyde for postfixation over night at 4°C.

Brain sectioning

On the next day, brains were transferred to a 30% sucrose solution in 0.1M phosphate buffer for cryoprotection for at least 24 hours. Then, the brains were taken out and the hemispheres were transected with a razor blade. Cerebellum and olfactory bulb were cut off, and the re-

maining part was placed with the rostral part onto the plate of the freezing microtome (Frigomobil, Reichert-Jung, Vienna, Austria). 40 μ m coronar sections were made throughout the septotemporal axis of the hippocampus and every third section was collected in a cryoprotectant solution (8.56 g Sucrose, 0.14 g magnesium chloride hexahydrate ($\text{MgCl}_2 \times 6\text{H}_2\text{O}$), 50 ml 99% glycerol, 50 ml 0.1M phosphate buffer). Then, sections were stored at -20°C until further processing.

2.6.3. IMMUNOHISTOCHEMISTRY

The immunohistochemical detection of BrdU-labeled cells was based on the two-step indirect ABC-(Avidin-Biotin-Complex-) method involving an unlabeled primary antibody (anti-BrdU) and a biotinylated secondary antibody that reacts with the anti-BrdU.

After defrosting, the sections were rinsed in phosphate-buffered saline (10mM PBS, pH=7.4) containing 0.1% Triton X-100, pretreated in 0.6% H_2O_2 for 30 min. to block endogenous peroxidases, rinsed again in PBS and denaturated at 37°C in 2N HCl for 60 min. The acid was neutralized by 0.1M Borate buffer, pH 8.5. After rinsing, the sections were incubated in M.O.M. blocking reagent (Vector Laboratories, Burlingame, USA) for 60 min., rinsed again in PBS and M.O.M. diluent (8% M.O.M. Protein concentrate (Vector Laboratories, Burlingame, USA) in PBS/T) and then incubated in the primary antibody (mouse anti-BrdU, Roche Molecular Biochemicals, Indianapolis, USA) over night at 4°C . After rinsing, the sections were incubated in the secondary antibodies, (M.O.M. anti-mouse reagent, Vector Laboratories, Burlingame, USA, and biotinylated goat anti-mouse, Vector Laboratories, Burlingame, USA) dissolved in M.O.M. diluent, for 2 hours at room temperature. Then, after another rinsing step, the sections were transferred to the Avidin-Biotin-Complex (Vectastain Elite ABC-Kit, Vector Laboratories, Burlingame, USA) and incubated for 1 hour at room temperature. The DAB reaction was conducted using 25 mg diaminobenzidine (DAB) dissolved in 50 ml PBS, filtered and supplemented with 0.5 ml of 1% H_2O_2 . The sections were incubated in DAB for about 4 min., depending on the intensity of the staining. The sections were mounted on slides in a rostro-caudal order, air-dried over night and dehydrated on one of the consecutive days in an ascending series of alcohol. After replacing the alcohol by immersion in xylene, the slides were cover-slipped with the xylene-soluble mounting medium DePeX (Serva, Heidelberg, Germany) and allowed to harden for at least 1 day (staining protocol, appendix IV).

2.6.4. QUANTITATIVE EVALUATION/STEREOLOGY

The number of BrdU-positive cells was estimated by counts made systematically on every third consecutive section along the rostrocaudal axis of the hippocampal formation. Counting was started when both blades of the dentate gyrus fully appear (Bregma -1.34 mm; Fig. 2-8, A) and ended just before the blades ventrally start to form a circle (Bregma -3.40 mm; Fig. 2-8, B; following the Mouse brain atlas of Franklin & Paxinos, 2008). On each section, BrdU-positive cells were counted in the granule cell and subgranular layer using a light microscope (400-fold magnification, Olympus BX41, Olympus Europa Holding GmbH, Hamburg, Germany).

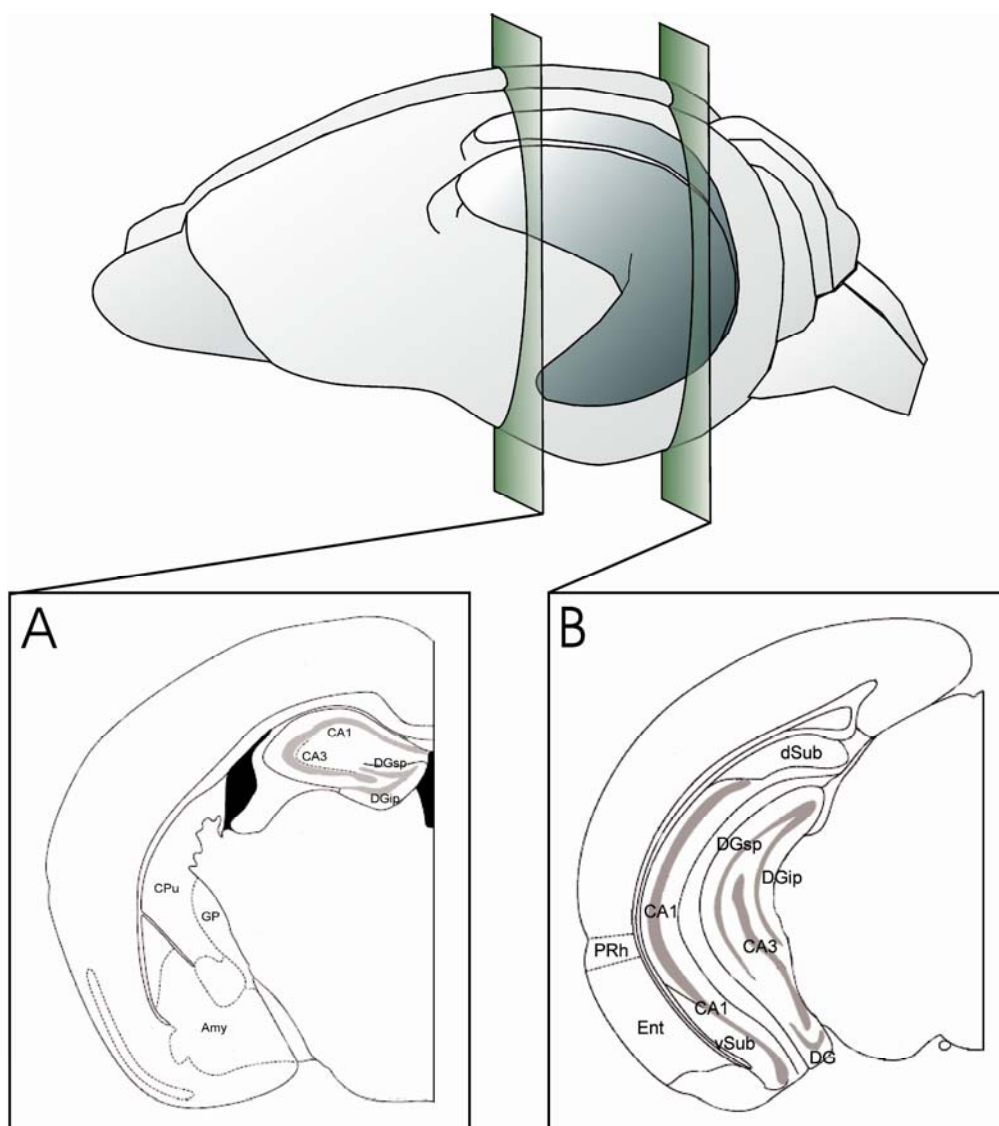


Fig. 2-8: First and last evaluated section.

Counting was started when both blades of the dentate gyrus appear (A) Bregma - 1.34 mm) and ended just before the blades start to form a circle (B) Bregma -3.40 mm). Counts were made systematically on every third section.

Additionally the volume of the granule and subgranular layer was measured using a light microscope (Olympus BX61, Olympus Europa Holding GmbH, Hamburg, Germany), a digital camera (Color View, Soft Imaging Systems GmbH, Münster, Germany) and software for image analysis (Cell*, Olympus Soft Imaging Solutions, Münster, Germany). Therefore, a picture was taken at 100-fold magnification, and the granule and subgranular layer was circumscribed on the picture (Fig. 2-9, A). For some studies, the dentate gyrus was subdivided into supra- and infrapyramidal blade and the area of the hilus was additionally measured (Fig. 2-9, B). The data were provided in a table by the program, and then transferred to Excel (Microsoft Office 2003) for further analyses.

The reference volume was estimated according to the Cavalieri principle: $V_{ref} = T \times \Sigma A \times 1/ssf$, where T is the thickness of the section, A is the area of the granule cell layer and 1/ssf is the inverse of the section sampling fraction (1/ssf=3). The number of BrdU-positive cells was then related to the granule cell layer sectional volume to obtain the density of BrdU-positive cells per mm^3 . Additionally, the number of BrdU-positive cells was multiplied by the reference volume to estimate the total number of BrdU-positive cells per dentate gyrus.

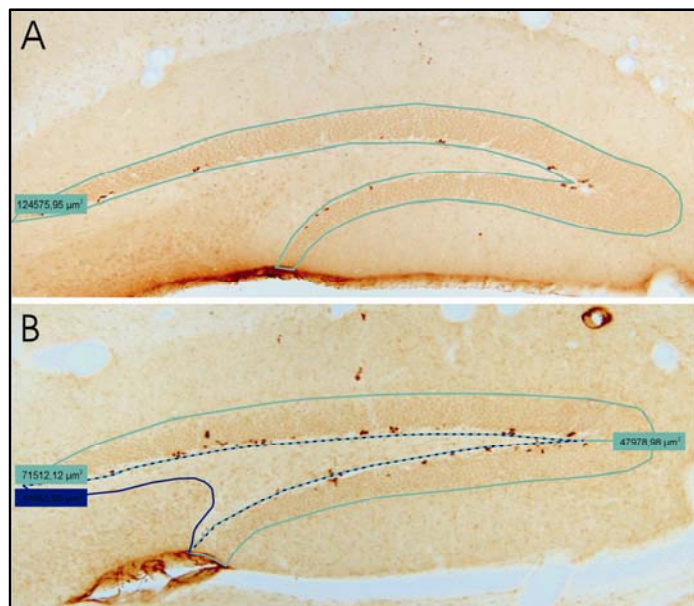


Fig. 2-9: Measuring fields. A) Measuring field around the dentate gyrus (green); B) Measurement of supra- and infrapyramidal blade separately (green) and of the hilus (blue; wild house mouse).

In some studies it was additionally tested, whether the effects were equally distributed along the septotemporal axis of the dentate gyrus. Therefore, three different regions, a septal, a temporal and an intermediate region, were defined and compared to each other. Within these regions, the average densities of BrdU-positive cells over two sections were calculated.

2.6.5. IMMUNOFLUORESCENCE

In the projects involving the investigation of cell survival, a triple fluorescence staining was additionally performed to get a qualitative overview over the phenotype of the surviving cells. Therefore, antibodies against BrdU, NeuN (a marker of mature neurons), and GFAP, (glial fibrillary acidic protein, a marker for glia cells) were used.

After defrosting, the sections were rinsed in phosphate-buffered saline (10mM PBS, pH=7.4), and DNA denaturation was carry out at room temperature in 2N HCl for 60 min. The acid was neutralized by 0.1M Borate buffer, pH 8.5. After rinsing, the sections were incubated in 5% donkey serum in PBS containing 0.1% Triton X-100 (PBS/T) for 60 min. Afterwards, sections were rinsed again in PBS and incubated in the primary antibody cocktail solved in 5% donkey serum in PBS/T for 45 min., rinsed again and incubated in the secondary antibodies solved in 2% bovine serum albumin in PBS for 45 min. The antibodies used were rat anti-BrdU (AbD Serotec, Kidlington, Oxford, UK), mouse anti-Neun (Millipore, Schwalbach/Ts., Germany), rabbit anti-GFAP (dianova, Hamburg, Germany), donkey anti-rat Rhodamin Red-X (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK), donkey anti-mouse FITC (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK), and donkey anti-rabbit AMCA (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK). After the antibodies were rinsed out, the sections were transferred to distilled water and therefrom mounted on slides. Slides were dried on a hot plate at 30°C, cover-slipped with Aqua-Poly/Mount (Polysciences Inc., Eppelheim, Germany) and allowed to harden for at least one day in the dark (staining protocol, appendix IV). Pictures for qualitative evaluation were taken with a fluorescence microscope (Olympus BX61, Olympus Europa Holding GmbH, Hamburg, Germany) at magnifications of 100-600-fold.

2.7. GALLYAS SILVER IMPREGNATION

Nondegenerative processes of presynaptic degradation as a prominent form of synaptic remodeling are characterized by a transient accumulation of secondary lysosomes and degenerating organelles in the degrading axonal terminal (Dean, 1978; Wolff et al., 1989; Wolff & Missler, 1993). Electron microscopy and ultrathin section analyses of silver impregnated material have proven that the Gallyas silver impregnation (Gallyas et al., 1980) selectively stains secondary lysosomes, degrading mitochondria, lamellar bodies and multivesicular bodies in remodeling axonal terminals (Wolff et al., 1989; Teuchert-Noodt et al., 1991; Dawirs et al., 2000). Even to date, the Gallyas silver impregnation seems to be the best method to specifically detect synaptic remodeling by staining the transient phase of presynaptic lysosomal accumulation (for further explanation and validation of this method cf. Butz et al., 2008; Neufeld et al., 2009). We used the Gallyas silver impregnation of lysosomal accumulations to quantify presynaptic degradation in different regions of the hippocampal formation.

2.7.1. PERFUSION AND TISSUE PREPARATION

Animals were perfused according to the procedure described in chapter 2.6.2 with ca. 200 ml of 5% formaldehyde. Immediately after the perfusion, the brain was dissected as described in chapter 2.6.2 and the brain was stored in 5% formaldehyde at 4°C until further processing. 60 µm coronar sections were made throughout the septotemporal axis of the hippocampus (see chapter 2.6.2) with a freezing microtome (Frigomobil, Reichert-Jung, Vienna, Austria). Every slice was collected in distilled water.

2.7.2. SILVER IMPREGNATION

According to the procedure described by Gallyas et al. (Gallyas et al., 1980), the free floating sections were prepared in an alkaline acid (pH 13) containing 9% sodium hydroxide and 1% ammonium nitrate and, subsequently, silver impregnated by a silver-nitrate solution containing 9% sodium hydroxide, 16% ammonium nitrate and 50% silver nitrate. The optimal silver concentration was estimated by examining stained test-sections by light microscopy. After impregnation, the sections were rinsed three times in changing rinsing solutions (solution: 30% ethyl-alcohol with 0.5 g sodium carbonate mixed with 1% ammonium nitrate). The developer contained 15 ml 40% formalin and 0.5% citric acid in 1000 ml 10% ethyl alcohol. After another rinsing step, the sections were air-dried, mounted on slides and embedded in DePeX (Serva, Heidelberg, Germany; staining protocol, appendix IV (German version)).

2.7.3. MEASURING FIELDS AND COMPUTERIZED ASSESSMENT OF LYSOSOMAL ACCUMULATIONS

Silver impregnated lysosomal accumulations can be viewed and counted in the dark field at 100-fold magnification. For computer-assisted quantification of the number of these lysosomal accumulations, pictures were taken from the dentate gyrus, CA1, CA3, subiculum and entorhinal cortex using a light microscope (Olympus BX41, Olympus Europa Holding GmbH, Hamburg, Germany), a digital camera (Color View, Soft Imaging Systems GmbH, Münster, Germany) and software for image analysis (Cell*, Olympus Soft Imaging Solutions, Münster, Germany). Within the respective areas, well-defined measuring fields consisting of three joined rectangular-shaped sub-fields (200 x 50 pixels, sized 600 x 50 pixel in total) were set to estimate the numbers of lysosomal accumulations per layer. The assessment was carried out by a self-developed classification algorithm implemented in MATLAB Vers. 6 (cf. Butz et al., 2008) that assesses the average number of lysosomal accumulations in every measured layer.

2.8. TIMM STAINING

For morphometric analyses of the dentate gyrus, the different layers were visualized with a method modified after Timm (1962). This method is based on the conversion of available heavy metals in the tissue into heavy metal sulphide, upon which silver is deposited, when the tissue is placed in a solution containing gum arabic, citrate buffer, hydroquinone and silver nitrate (Danscher & Zimmer, 1978). Zinc is a modulatory substance in the vesicles of mossy fibers in the dentate gyrus (Haug, 1967). Thus, by detecting zinc, one can visualize granule cells and mossy fibers as well as the termination fields in the dentate gyrus (Fig. 2-10; 7-5).

2.8.1. PERFUSION AND TISSUE PREPARATION

Perfusion and dissection

Animals were anaesthetized, and the needle was positioned in the left heart ventricle as described in chapter 2.6.2. The perfusion was performed in three steps. It was initiated with 150 ml of 1.17% sodium sulphide in phosphate buffer (pH 7.35) and continued with 400 ml of fixation solution composed of 3% glutaraldehyde in 0.15M Sørensen buffer (1.47 g potassiumhydrogenphosphate and 7.57 g disodiumhydrogenphosphate, pH 7.35). With 250 ml of the initial sulphide solution the perfusion was terminated. Immediately after the perfusion, the brain was dissected as described in chapter 2.6.2 and stored in the fixation solution containing additionally 20% sucrose at 4°C for at least 24 hours.

Brain sectioning

Brains were taken out, and the hemispheres were transected with a razor blade. Cerebellum and olfactory bulb were cut off, the remaining part of the left hemisphere was placed with the rostral part onto the plate of the cryostat (Frigocut 2700, Reichert-Jung, Wien, Austria) embedded with TissueTek (Sakura, Tokyo, Japan). 20 µm coronar sections were made throughout the septotemporal axis of the hippocampus, and every third section was mounted on slides. Slides were stored in the dark at room temperature until further processing.

2.8.2. SILVER IMPREGNATION

Preparation of solutions

Due to the sensitivity of the Timm reaction, all vessels had to be incubated in 10% RBS for at least 24 hours, rinsed in tap and distilled water afterwards, and allowed to dry in the hot cupboard. The Timm solution was prepared out of gum arabic solution (50 g in 100 ml distilled water), citrate buffer (5.1 g citric acid and 4.7 g sodium citrate in 20 ml distilled water), hy-

droquinone solution (3 g hydroquinone in 60 ml distilled water) and 0.5 ml of 17% silver-nitrate solution, while the hydroquinone solution and the citrate buffer were mixed first, before the gum arabic solution was added. This solution was incubated in the hot cupboard for 30 min., before the silver nitrate solution was added.

Staining procedure

The slides were placed in vessels containing the Timm solution. For incubation, the vessels were placed in the hot cupboard (Adams et al., 2002) at 30°C. The incubation time was varied and tested to obtain the optimal staining of the layers of the dentate gyrus (Coleman et al., 1987). The reaction was stopped by rinsing slides under running tap water followed by rinsing in tap water for 10 min. The sections were dehydrated in ethanol (96%, 100%). The alcohol was replaced by immersion in xylene, and the slides were coverslipped with Eukitt (Kindler, Freiburg, Germany; staining protocol, appendix IV (German version)).

2.8.3. QUANTITATIVE EVALUATION

The quantitative evaluation of the different layers was carried out according to descriptions of Hildebrandt (1999) on every third section along the septotemporal axis of the hippocampus. As start point the slice was taken, on which both blades of the dentate gyrus first appear, and as endpoint the last slice, before the blades start to form a circle (cf. Fig. 2-8). For each animal 14-19 sections were analyzed. Pictures were taken of the dentate gyrus at 100-fold magnification using a light microscope (Olympus BX61, Olympus Europa Holding GmbH, Hamburg, Germany), a digital camera (Color View, Soft Imaging Systems GmbH, Münster, Germany) and software for image analysis (Cell*, Olympus Soft Imaging Solutions, Münster, Germany).

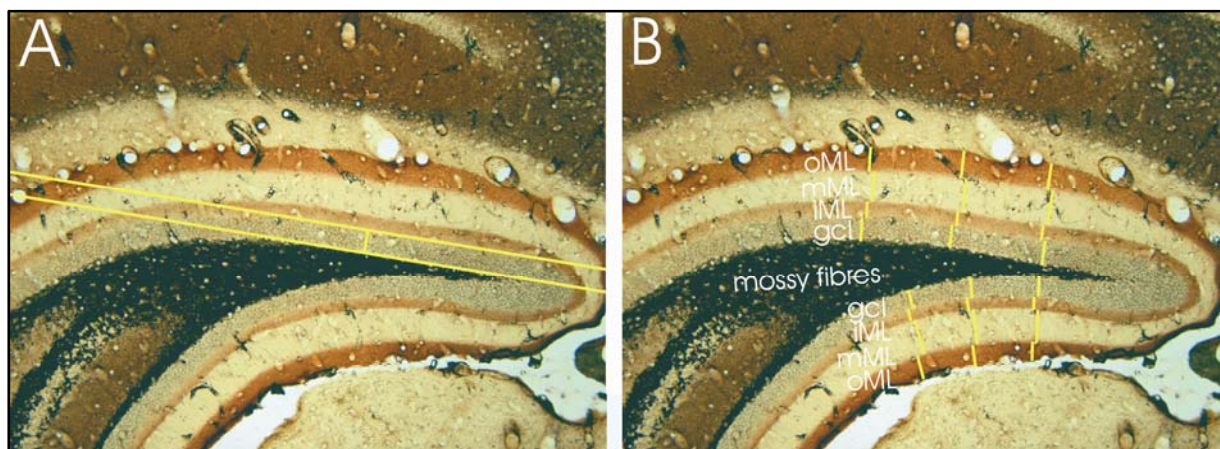


Fig. 2-10: Measurements on Timm-stained sections of the dentate gyrus (100-fold, wild house mouse).

A) Dimension line with rectangular reference lines in the granule cell layer; **B)** dimension lines in the granule cell (gcl), inner (iML), middle (mML) and outer molecular layer (oML) of the supra- and infrapyramidal blade of the dentate gyrus.

With help of dimension lines between rectangular reference lines (Fig. 2-10, A), the height of the granule cell, inner, middle and outer molecular layer in each blade was measured at three different points (Fig. 2-10, B). All data were provided in a table and transferred to an Excel sheet (Microsoft Office 2003). The mean over the three measurements and the sections were taken to get a value for every layer.

2.8.4. IMMUNOFLUORESCENCE

A fluorescence staining against the zinc transporter 3 (ZnT 3) was additionally performed to get a qualitative validation of the zinc content of the different dentate layers.

Paraformaldehyde fixated tissue was used (for perfusion and tissue preparation see chapter 2.6.2). After defrosting, the 40 µm sections were rinsed in phosphate-buffered saline (10mM PBS, pH=7.4) and incubated in 5% donkey serum in PBS containing 0.1% Triton X-100 (PBS/T) for 60 min. Afterwards, sections were rinsed again in PBS and incubated in the primary antibody (rabbit anti-ZnT 3, Synaptic Systems, Göttingen, Germany) solved in 5% donkey serum in PBS/T for 45 min., rinsed again and incubated in the secondary antibody (donkey anti-rabbit AMCA, Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK) solved in 2% bovine serum albumin in PBS for 45 min. After the secondary antibody was rinsed out, the sections were transferred to distilled water and therefrom mounted on slides. Slides were dried on a hot plate at 30°C, cover-slipped with Aqua-Poly/Mount (Polysciences Inc., Eppelheim, Germany) and allowed to harden for at least one day in the dark (staining protocol, appendix IV). Pictures for qualitative evaluation were taken with a fluorescence microscope (Olympus BX61, Olympus Europa Holding GmbH, Hamburg, Germany) at magnifications of 100-200-fold.

3. PROJECT I:

DISTINCT EFFECTS OF REARING CONDITIONS AND WHEEL RUNNING ON NEUROGENESIS IN ADULT CD1 MICE

3.1. INTRODUCTION

The development of the brain is strongly influenced by environmental conditions. Especially in the phase after weaning, when an individual begins to independently interact with its social and physical environment, this development is under considerable influence of external factors. The natural environment of an animal in this phase is both rich in physical structure and populated with conspecifics providing necessary input for neural development. Deprivation or impoverishment of such social and physical stimulation can lead to differences in behavioral and neuroplastic development (Olsson & Dahlborn, 2002). In gerbils, (*Meriones unguiculatus*) social and physical deprivation (impoverishment) during juvenile development leads to a reduced dopamine innervation of the prefrontal cortex (Winterfeld et al., 1998; Neddens et al., 2001) and reduced maturation of both prefrontal layer II/III and layer V/VI pyramidal efferents to frontal, parietal and limbic areas (Bagorda et al., 2006; Witte et al., 2007). In contrast, dopaminergic fibers in limbic structures mature excessively under deprived conditions (Neddens et al., 2002; Busche et al., 2004; Lesting et al., 2005) and further neurotransmitters like serotonin and acetylcholine are also altered in these structures.

This dysbalance and reduced prefronto-cortico-limbic connectivity have been postulated to be causally involved in deprivation-induced effects on neuroplastic potentials in the hippocampus (Keller et al., 2000; Teuchert-Noodt, 2000; Butz, 2006). On the one hand, juvenile deprivation in gerbils leads to an increased adult hippocampal cell proliferation (Hildebrandt, 1999; Keller et al., 2000) while at the same time synaptic-remodeling rates in the dentate gyrus are decreased (Hildebrandt, 1999; Butz et al., 2008).

However, other studies in mice and rats found diminished neurogenesis after juvenile physical (not social) deprivation (Kempermann et al., 1997a; Young et al., 1999; Nilsson et al., 1999; Iso et al., 2007). These contradictory results could be due to different designs of the experimental conditions. The control condition, the so called semi-natural or enriched environment confounds animate (social group) and inanimate factors (tunnels, hiding places, running wheels...), although, e.g., wheel running on its own is sufficient to enhance neuroge-

neogenesis at least in adult individuals (van Praag et al., 1999a, b; Holmes et al., 2004; Uda et al., 2006).

The present study was conducted to examine to which extent the findings on the epigenetic effects of juvenile environmental stimulation for gerbils are also valid for the mouse model. We focused on measuring adult neurogenesis, i.e., cell proliferation and cell survival in the dentate gyrus, as a representative parameter for the epigenetic effects of environmental deprivation/stimulation on juvenile neural development (weaning, postnatal day (pd) 21 to young adulthood, pd 66). We applied the same experimental treatment as in the gerbil studies, and, in contrast to former studies in mice and rats, excluded running wheels from the enriched environment. Additionally, we employed a wheel-running challenge in young adulthood (pd 70-74) to examine the reactivity of neuroplasticity to external neurogenic stimulation.

3.2. MATERIAL & METHODS

3.2.1. ANIMALS AND REARING CONDITIONS

49 female CD1 mice were bred in our facility in standard type III cages (42.5 x 26.5 x 18 cm) with one litter per cage. At weaning (postnatal day (pd) 21), animals were randomly assigned to one of two rearing conditions, enriched (ER_{SPW.}) or socially (s) and physically (p) deprived (DR_{SPW.}) rearing without running wheels (w.; rearing conditions are specifically described in chapter 2.2). ER_{SPW.} animals were reared in two groups of 15 and 10 individuals in large enclosures (200 x 100 x 50 cm) that contained items such as tunnels, tubes, and solid hiding places (Fig. 2-4, A). DR_{SPW.} animals were reared individually in standard type III cages (Fig. 2-4, B). Animals were kept under their respective experimental rearing conditions for 45 days from pd 21 to pd 66 (Fig. 3-1).

3.2.2. WHEEL-RUNNING CHALLENGE

On pd 66, all individuals from the previous two experimental groups were now united, and two mixed groups were formed (n=24 and n=25). Each group was placed into a large enclosure (100 x 100 x 50 cm). One of the mixed groups had access to running wheels (as described in chapter 2.3) from pd 70 to pd 74 (Fig. 2-5; 3-1).

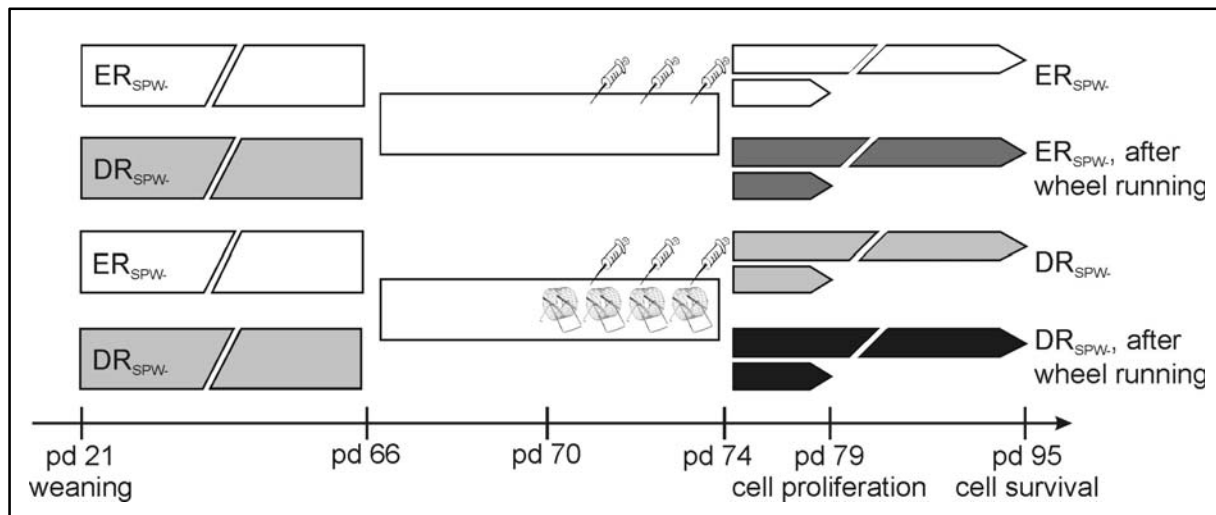


Fig. 3-1: Experimental design with time axis showing animal age in postnatal days (pd); pd 21-66, rearing phase under socially and physically enriched conditions (ER_{SPW.}) or socially and physically deprived conditions (DR_{SPW.}); pd 66, forming of the wheel-running and non-wheel-running group; pd 70-74, 4-day wheel-running challenge for one of the groups; pd 71-74, BrdU-injections for all animals; The resultant four groups were split in half for sampling of cell proliferation (pd 79) or cell survival (pd 95).

3.2.3. BRDU-LABELING, PERFUSION, IMMUNOHISTOCHEMISTRY

All animals received injections of the DNA synthesis marker 5-bromo-2-deoxyuridine (BrdU) on pd 71 to pd 73 at 5 p.m., thus, just before the dark period and the onset of the active phase of the animals (see chapter 2.6.1). After a survival time of 5 days (for detecting cell proliferation) or 21 days (for detecting cell survival), the animals were deeply anaesthetized by an overdose of diethyl ether and transcardially perfused with 200 ml of 4% paraformaldehyde solution (for details see chapter 2.6.2). The brains were immediately dissected and stored overnight in 4% PFA at 4°C. On the next day, brains were transferred to a 30% sucrose solution for cryoprotection for at least 24 hours. Then, the left hemispheres of the brains were freeze-sectioned with a Frigomobil. 40 µm coronar sections were made throughout the septotemporal axis of the hippocampus (see chapter 2.6.2), collected in a cryoprotectant solution and stored at -20°C until further processing.

The immunohistochemical detection of BrdU-labeled cells was based on the two-step indirect ABC-(Avidin-Biotin-Complex-) method involving an unlabeled primary antibody (mouse anti-BrdU, Roche Molecular Biochemicals, Indianapolis, USA) and a biotinylated secondary antibody (M.O.M. anti-mouse reagent, Vector Laboratories, Burlingame, USA, and biotinylated goat anti-mouse, Vector Laboratories, Burlingame, USA; for further explanations see chapter 2.6.3). To get a qualitative overview over the phenotype of the BrdU labeled cells, on some sections a triple fluorescence staining was additionally performed involving the antibodies mouse anti-BrdU (AbD Serotec, Kidlington, Oxford, UK), anti-Neun

(Millipore, Schwalbach/Ts., Germany), rabbit anti-GFAP (dianova, Hamburg, Germany), donkey anti-rat Rhodamin Red-X (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK), donkey anti-mouse FITC (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK), and donkey anti-rabbit AMCA (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK; for further explanations see chapter 2.6.5)

3.2.4. QUANTITATIVE EVALUATION/STEREOLOGY

The slides were coded prior to quantitative analysis and the code was not broken, until the analysis was completed. The number of BrdU-positive cells was estimated by counts made systematically on every third consecutive section along the rostrocaudal axis of the hippocampal formation (see chapter 2.6.4). On each section, BrdU-positive cells were counted in the granule cell layer and subgranular zone under the light microscope at 400-fold magnification. Additionally the volume of the granule cell and subgranular layer was measured at 100-fold magnification (see chapter 2.6.4). The reference volume was estimated according to the Cavalieri principle: $V_{ref}=T \times \Sigma A \times 1/ssf$, where T is the thickness of the section, A is the area of the granule cell layer and 1/ssf is the inverse of the section sampling fraction (1/ssf=3).

The number of BrdU-positive cells was then related to the granule cell layer sectional volume to obtain the density of BrdU-positive cells per mm³. Additionally, the number of BrdU-positive cells was multiplied by the reference volume to estimate the total number of BrdU-positive cells per dentate gyrus.

For testing, whether the effects were equally distributed along the septotemporal axis of the dentate gyrus, three different regions, a septal, a temporal and an intermediate region, were compared to each other. Within these regions, the average densities of BrdU-positive cells over two sections were calculated.

3.2.5. STATISTICAL ANALYSES

The data were analyzed by ANOVA. Specific comparisons were made with Tukey HSD and Unequal N HSD post-hoc tests. Data analyses were performed with Excel (Microsoft Office 2007), statistical analyses with Statistica 6.0 (StatSoft, Tulsa, USA).

3.3. RESULTS

Cell proliferation was assessed by BrdU labeling of dividing cells over three days and immunohistochemical detection five days after the last injection. For assessing cell survival, BrdU-positive cells were detected after 21 days of survival.

3.3.1. QUALITATIVE RESULTS

The immunohistochemical detection of cell proliferation and cell survival revealed BrdU-positive cells in all animals. BrdU-positive cells appear as brown to dark brown precipitates either in the whole cell or in parts of it. The majority of BrdU-positive cells resides in the subgranular zone and granule cell layer. Five days after the last BrdU injection, nearly all BrdU-positive cells are located in the subgranular zone (Fig. 3-2, A-D), while 21 days after a large part of the cells can also be seen in the granule cell layer indicating migrating cells (Fig. 3-2 E-H). Triple stainings against BrdU, NeuN, a marker for mature neurons, and GFAP, a marker for glia cells, demonstrate that most of the BrdU-positive cells after a survival time of 21 days are neurons (Fig. 3-3).

Photomicrographs of animals from socially and physically enriched and deprived rearing without and after the adult wheel-running challenge exemplary illustrate the distribution of BrdU-positive cells in the dentate gyrus (Fig. 3-2). Qualitatively, there appears hardly no difference between deprived (Fig. 3-2, B,F) and enriched-reared animals (Fig. 3-2, A,E) as well as enriched-reared animals after wheel running (Fig. 3-2, C,G). However, it is apparent that the number of BrdU-positive cells is higher in deprived-reared animals after wheel running (Fig. 3-2, D,H).

3.3.2. QUANTITATIVE RESULTS

In the following the statistical data for the total number of BrdU-positive cells per dentate gyrus are given. Similar results were seen for density of BrdU-positive cells per mm³ and can be drawn from table 3-1 and Fig. 3-4, B,D).

3.3.2.1. Effect of the rearing conditions

Cell proliferation in the dentate gyrus was not affected by the conditions of social and physical deprivation or enrichment during rearing alone (ER_{SPW} . 2193 vs. DR_{SPW} . 2364 cells per dentate gyrus; ANOVA, rearing condition $F_{1,21}=13.65$, $p < 0.01$; Post-hoc Unequal N HSD, $p > 0.05$) Fig. 3-4, A). The number of surviving cells was also not affected by the rearing conditions (ER_{SPW} . 1250 vs. DR_{SPW} . 1407 cells per dentate gyrus; ANOVA, rearing conditions $F_{1,20}=9.47$, $p < 0.01$; Post-hoc Tukey HSD, $p > 0.05$, Fig. 3-4, C).

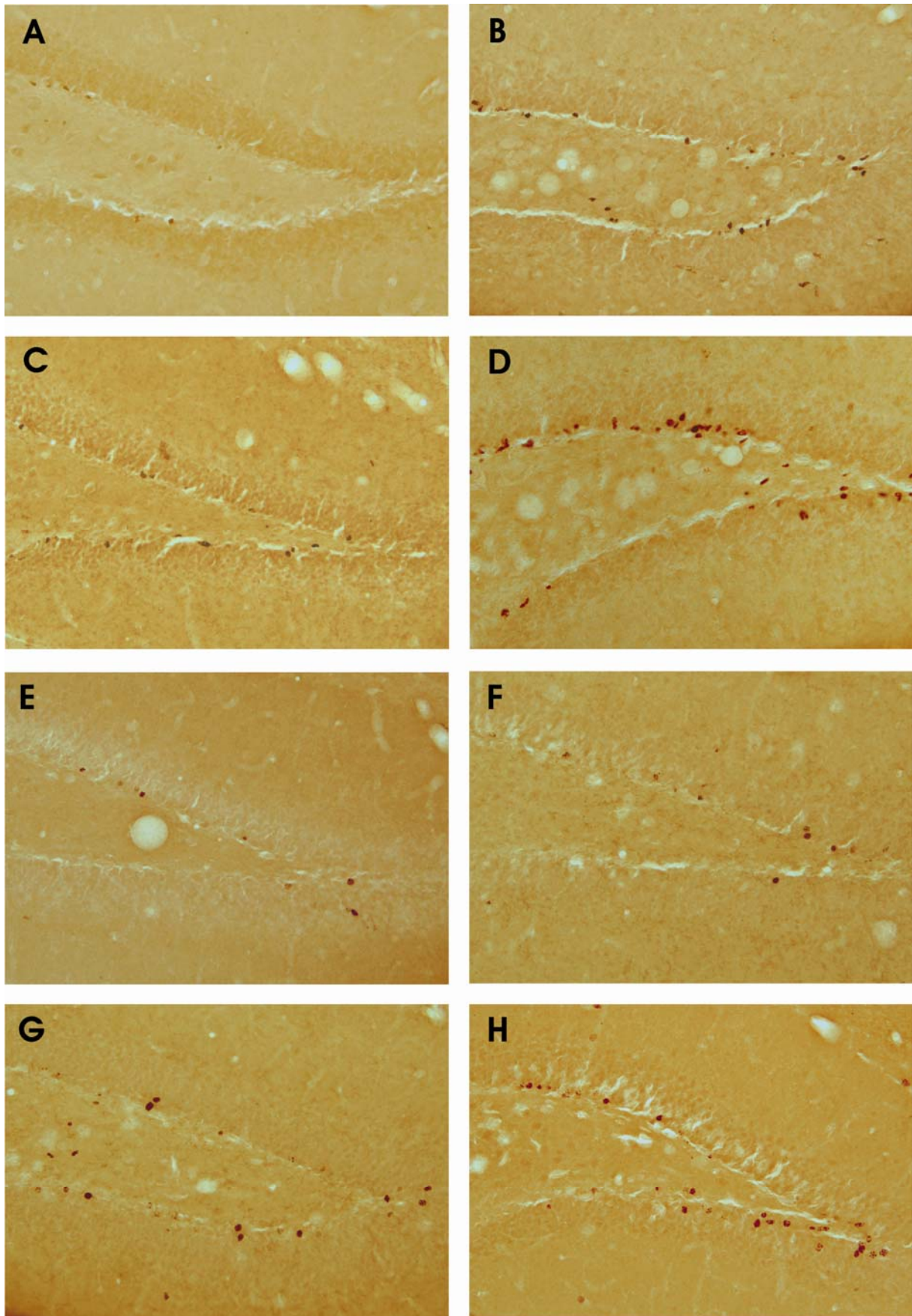


Fig. 3-2: Photomicrographs of BrdU-positive cells (200-fold magnification) 5 days (**A-D**) and 21 days (**E-H**) after the last injection in socially and physically enriched-reared animals without (**A+E**) and after wheel running (**C+G**), socially and physically deprived-reared animals without (**B+F**) and after wheel running (**D+H**).

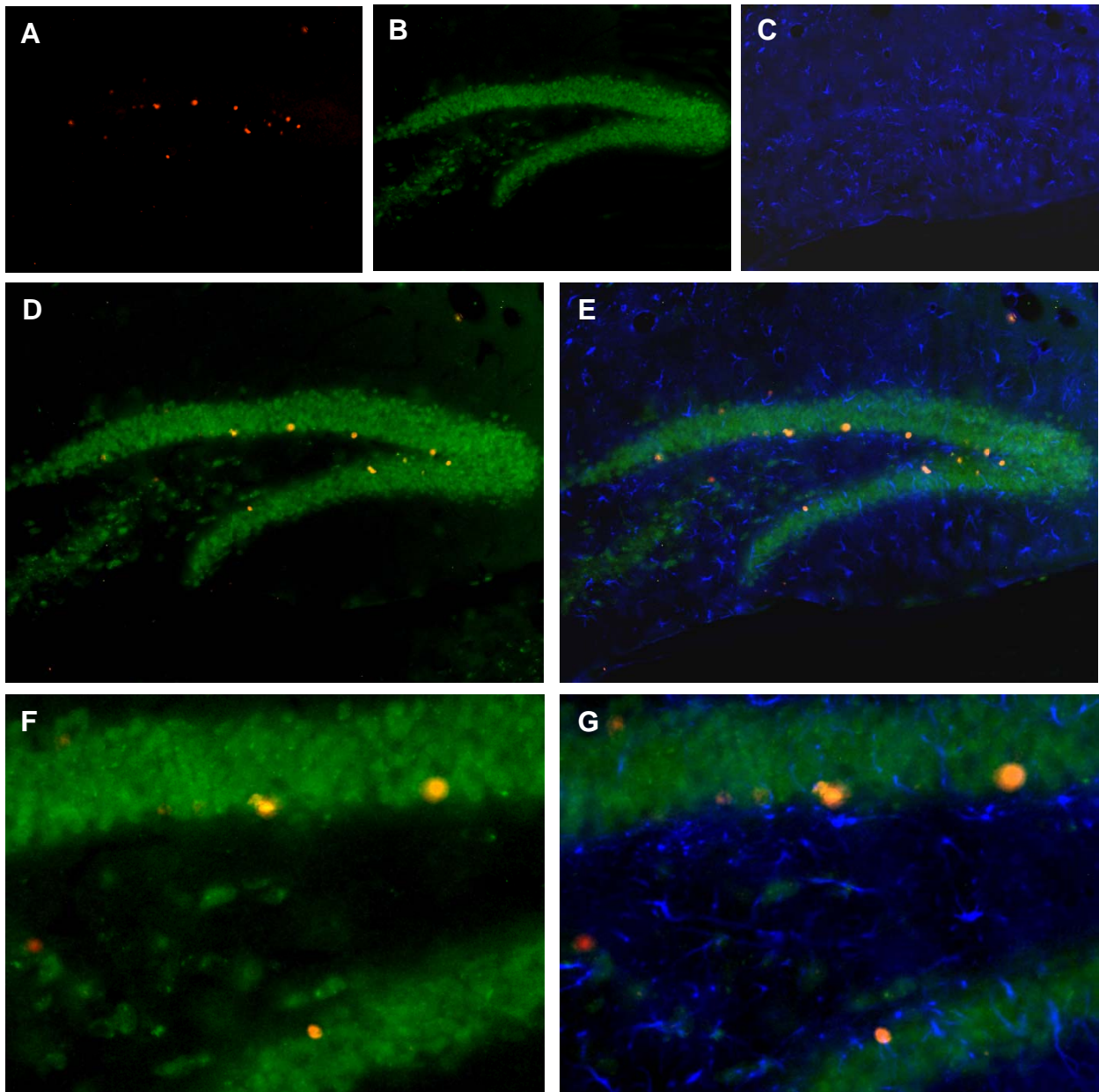


Fig. 3-3: Phenotypical examination of BrdU-positive cells in the dentate gyrus.

A) BrdU-positive cells (red); **B)** mature neurons (NeuN, green); **C)** astrocytes (GFAP, blue); merged pictures (**D-G**) reveal the phenotype of the BrdU-positive cells: double-labeled BrdU-positive neurons appear in yellow. Original magnification 100-fold (**A-E**) and 400-fold (**F-G**).

3.3.2.2. Effect of wheel-running challenge depends on the rearing history

The effect of wheel running on cell proliferation was significantly dependent on the previous rearing history of the animals (ANOVA, wheel running $F_{1,21}=24.0$, $p < 0.0001$; interaction effect rearing condition*wheel running $F_{1,21}=10.7$, $p < 0.01$). Wheel running caused an increase in cell proliferation only in DR_{SPW-} animals (+140% compared to DR_{SPW+} ; +99% compared to ER_{SPW-} , after wheel running; Post-hoc Unequal N HSD, $p < 0.001$). Surprisingly, for individuals reared under enrichment the four days of wheel-running challenge did not lead to

any significant effect on cell proliferation (ER_{SPW-} , after wheel running 2852 cells per dentate gyrus; Post-hoc Unequal N HSD, $p > 0.05$; Fig. 3-4, A).

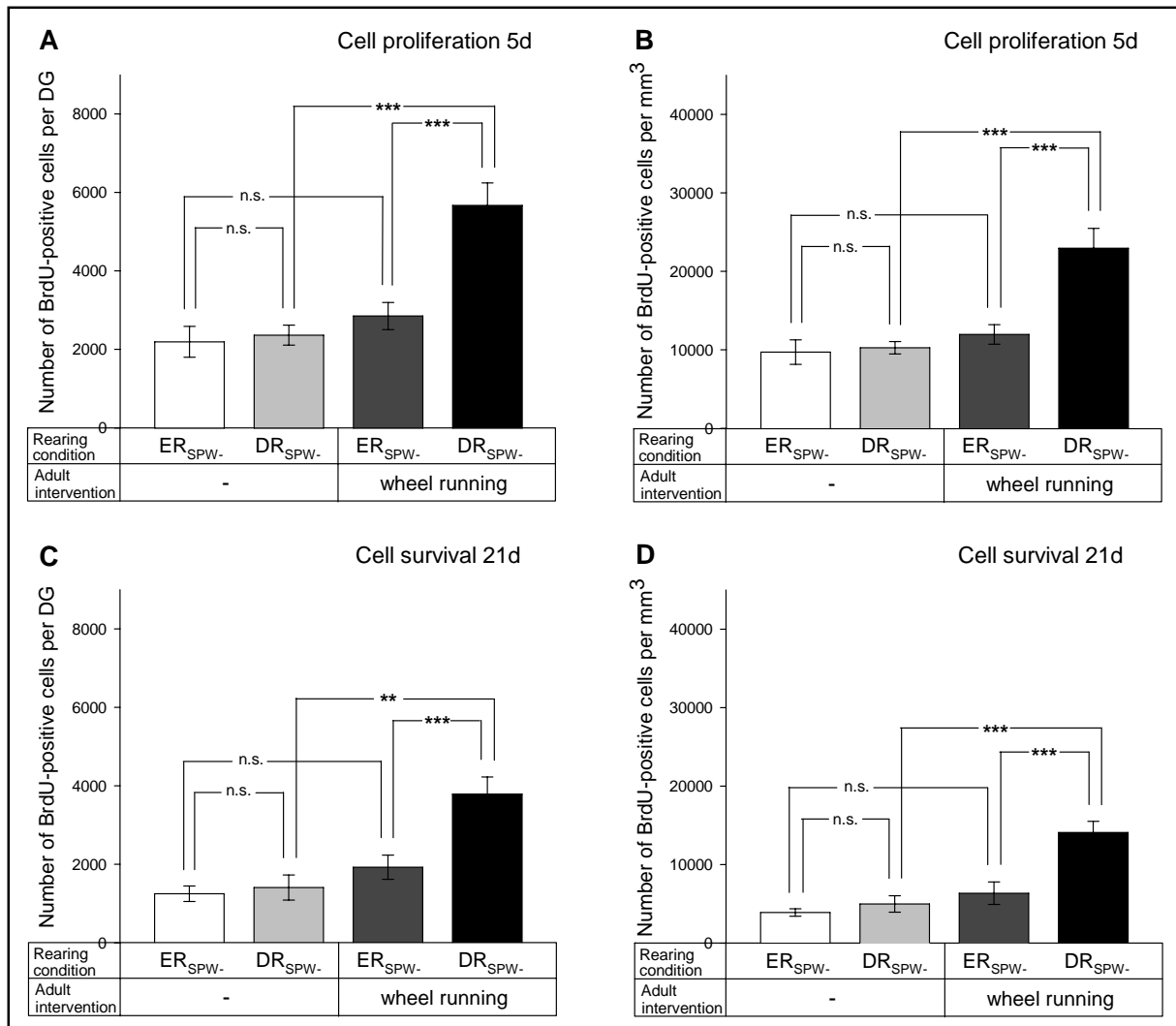


Fig. 3-4: **A)** Total number of BrdU-positive cells in the granule cell layer 5 days after BrdU-injection; **B)** Density of BrdU-positive cells per mm³ in the granule cell layer 5 days after BrdU injection; **C)** Total number of BrdU-positive cells in the granule cell layer 21 days after BrdU-injection; **D)** Density of BrdU-positive cells per mm³ in the granule cell layer 21 days after BrdU-injection. ER_{SPW-} , socially and physically enriched-reared animals; DR_{SPW-} , socially and physically deprived-reared animals; wheel running, additional wheel running challenge (pd 70-74); data given as means \pm S.E.M.

The general effect of our experimental treatment on cell proliferation was also found for cell survival. Wheel running only led to an increase in the number of surviving cells when animals had been reared under deprivation. For animals reared under enriched conditions, four days of wheel running had no effect on the rates of cell proliferation or cell survival (Fig. 3-4, C; Tab. 3-1).

Table 3-1 indicates the mean total number and the mean density of BrdU-counts for cell proliferation and cell survival for the four different groups as well as significant differences between the groups.

| | | ER _{SPW-} | DR _{SPW-} | | ER _{SPW-} | ER _{SPW-} after wheel running | |
|----|--------------|--------------------|--------------------|------|--------------------|---|------|
| | | value | value | p | value | value | p |
| CP | Total number | 2193 | 2364 | n.s. | 2193 | 2852 | n.s. |
| | Density | 9699 | 10251 | n.s. | 9699 | 11965 | n.s. |
| CS | Total number | 1250 | 1407 | n.s. | 1250 | 1926 | n.s. |
| | Density | 3894 | 4987 | n.s. | 3894 | 6360 | n.s. |

| | | DR _{SPW-} (100%) | DR _{SPW-} after wheel running | | | ER _{SPW-} after wheel running (100%) | DR _{SPW-} after wheel running | | |
|----|--------------|------------------------------|---|------------|--------|---|---|------------|--------|
| | | value | value | diff. in % | p | value | value | diff. in % | p |
| CP | Total number | 2364 | 5671 | +140 | <0.001 | 2852 | 5671 | +99 | <0.001 |
| | Density | 10251 | 22933 | +124 | <0.001 | 11965 | 22933 | +92 | <0.001 |
| CS | Total number | 1407 | 3785 | +169 | <0.001 | 1926 | 3785 | +97 | <0.01 |
| | Density | 4987 | 14096 | +183 | <0.001 | 6360 | 14096 | +122 | <0.001 |

Tab. 3-1: Group comparison of total numbers and densities of BrdU-positive cells

ER_{SPW-}, socially and physically enriched-reared animals; DR_{SPW-}, socially and physically deprived-reared animals; after wheel-running, after an additional wheel running challenge in adulthood (pd 70-74); CP, cell proliferation; CS, cell survival; total number of BrdU-positive cells per dentate gyrus; density of BrdU-positive cells per mm³; given are absolute values (means), differences between the groups if significant, and p-values.

3.3.2.3. Cell proliferation and cell survival along the septotemporal axis

Three different regions within the dentate gyrus, a septal, a temporal and an intermediate region, were defined and compared to each other to test, whether the effects were equally distributed along the septotemporal axis of the dentate gyrus. As expected, the analysis of this subset of already analyzed count data revealed the same effects for the rearing conditions and the wheel-running challenge. All these effects were equally distributed along the septotem-

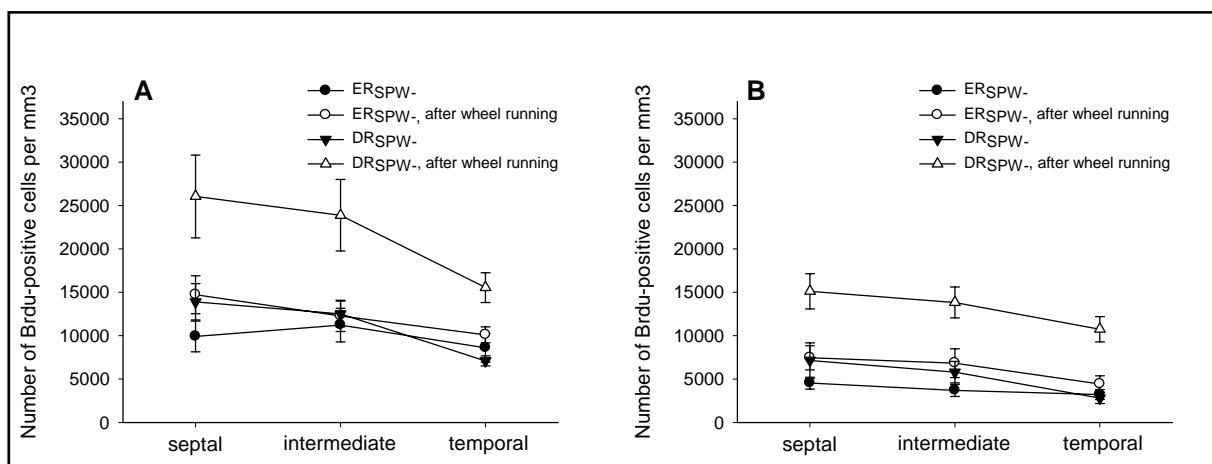


Fig. 3-5: Rates of cell proliferation A) and cell survival B) along the septotemporal axis of the dentate gyrus. ER_{SPW-}, socially and physically enriched-reared animals; DR_{SPW-}, socially and physically deprived-reared animals; wheel running, additional wheel running challenge (pd 70-74); data given as means \pm S.E.M.

poral axis. Neither the regions within the groups showed different numbers of cell proliferation or cell survival, nor did differences along the septotemporal axis vary between the four groups (Post-hoc Unequal N HSD or Tukey HSD, $p > 0.05$; Fig. 3-5).

3.3.2.4. Effect of rearing conditions and wheel running on the reference volume of the granule cell layer

The reference volume comprising the granule cell layer and subgranular zone was not affected by the rearing conditions or the wheel-running challenge neither when it was measured together with cell proliferation (5 days after the last BrdU injection; ANOVA rearing condition $F_{1,21}=4.01$, $p > 0.05$; wheel running $F_{1,21}=1.14$, $p > 0.05$; interaction effect of rearing condition*wheel running $F_{1,21}=0.04$, $p > 0.05$) nor when it was measured together with cell survival (21 days after the last BrdU injection; ANOVA rearing condition $F_{1,20}=2.07$, $p > 0.05$; wheel running $F_{1,20}=0.35$, $p > 0.05$; interaction effect of rearing condition*wheel running $F_{1,20}=0.32$, $p > 0.05$; Fig. 3-6). Interestingly, when the groups were taken together and the reference volume of animals 5 days after the last BrdU injection, thus 79 days old, and 21 days after, thus 95 days old, were compared, a significant increase in the reference volume (Welch two-sample t-test, $t=-3.62$, $df=47$, $p < 0.001$) was found.

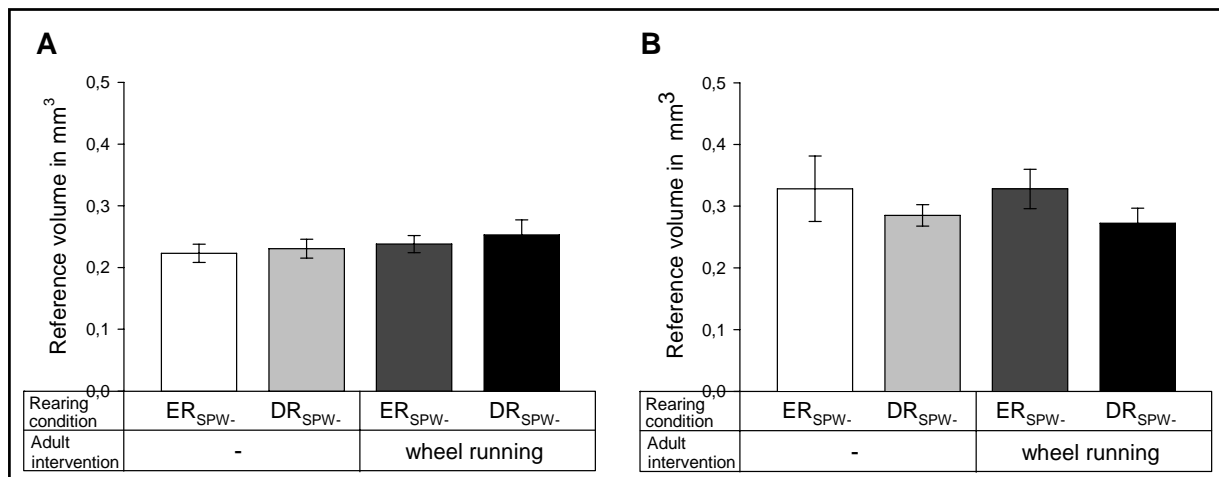


Fig. 3-6: Reference volume of the granule cell layer and subgranular zone **A**) 5 days (pd 79) and **B**) 21 days after BrdU-injection (pd 95); ER_{SPW-}, socially and physically enriched-reared animals; DR_{SPW-}, socially and physically deprived-reared animals; wheel running, additional wheel running challenge (pd 70-74); data given as means \pm S.E.M.

3.4. DISCUSSION

Our results demonstrate that brain maturation in CD1 mice is susceptible to environmental deprivation during rearing. However, this effect is subtle: social and physical deprivation during rearing did, at first sight, not cause different rates of cell proliferation or cell survival

in young-adult animals if compared to enriched rearing. This result stands in contrast to other studies. In mice, individuals from standard rearing had lower rates of cell survival than individuals reared under environmental enrichment including running wheels (Kempermann et al., 1997a; van Praag et al., 1999b; Brown et al., 2003). In contrast, in gerbils, deprived-reared individuals - treated similarly to the animals from this study - had higher rates of cell proliferation than individuals reared under enriched conditions without running wheels (Keller et al., 2000; Butz et al., 2008). In line with this latter finding, our further analyses did show that also our deprived-reared CD1 mice were affected in their neuroplastic development with consequences on the regulation of neurogenesis in adulthood. Social and physical deprivation (DR_{SPW}-) led to a significant increase in cell proliferation and cell survival as compared to enrichment (ER_{SPW}-), when young adult individuals received a neurogenic stimulation through a 4-day exposure to running wheels.

All these effects were equally distributed along the septotemporal gradient of the hippocampus notwithstanding the different connectivities and functional embeddings of these two hippocampal subregions (Bannerman et al., 1999, 2004). Both treatments had no effect on the reference volume of the granule cell layer and subgranular zone neither 5 nor 21 days after the last BrdU injection, so after the wheel-running challenge. This result is in line with other findings that adult neurogenesis does not alter the volume of the dentate gyrus (Kitamura & Sugiyama, 2006). However, surprisingly, we found an increase in the reference volume in all groups between the 5th and the 21th day after the last BrdU injection and thus between pd 79 and pd 95. Hence, it seems that in CD1 mice, unlike in C57Bl/6 mice (Kitamura & Sugiyama, 2006), even in this age the granule cell layer still grows.

In three aspects our experimental results regarding neurogenesis differed in positive or negative direction from previous results and from what we have expected: First, in gerbils, post-weaning rearing under social and physical deprivation by itself leads to a significant increase in cell proliferation, when compared to enriched rearing (Keller et al., 2000; Butz et al., 2008). Such an effect was not seen in our CD1 mice. The reason for this difference may stem from a species-dependent effect. Or it could be due to different levels of domestication, with the consequence that environmentally-dependent neuroplasticity is generally lower in CD1 mice than in gerbils.

Secondly, our rearing under environmental deprivation did not lead to lower rates of cell survival in young-adult animals compared to our individuals from enriched rearing without running wheels. The effect of reduced cell survival could have been expected considering other studies which, however, included a running wheel as a component of enrichment

(Kempermann et al., 1997a; van Praag et al., 1999b; Brown et al., 2003). This difference in results could be due to the different reference groups. We compared deprivation versus enrichment, while others compared standard housing versus enrichment. Alternatively, the absence (in our case) or presence of a running wheel (in other studies) as part of environmental enrichment during rearing might have exerted this effect.

Last, the effects of the adult wheel-running challenge were weaker than might have been expected. Our individuals from enriched rearing did not respond to wheel-running activity with any changes in cell proliferation or cell survival. This seems surprising if one assumes that it is the wheel-running stimulation that is mostly responsible for the effects of enrichment found in other studies (Kempermann et al., 1997a; van Praag et al., 1999b; Brown et al., 2003). One reason for this could be the time point, duration and intensity of the wheel-running activity. The long-term exposure to a single running wheel included in an enriched environment during development could lead to long-lasting structural alterations. In contrast, our wheel-running exposure of four days during young adulthood with one running wheel per animal could lead to strong, but only short-term effects, which – as we observed – depend on the rearing history of the animals. Unfortunately, the available studies on the effects of wheel running on neurogenesis have been performed with animals reared and housed under standard conditions so that a comparison to enriched or deprived-reared individuals is not possible.

The reactivity of neurogenesis was significantly higher in socially and physically deprived-reared animals as we could demonstrate by our adult wheel-running challenge. This raises the question, how deprivation affects the maturing brain during juvenile development. In gerbils, deprived rearing causes a dysbalance in the dopamine system with a dopamine hypofunction in frontal and a hyperfunction in limbic areas (Winterfeld et al., 1998; Busche et al., 2004). It has been argued that this dysbalance is causally involved in the mechanism underlying enhanced cell proliferation after deprived rearing observed in this species (Keller et al., 2000; Teuchert-Noodt, 2000). Unfortunately, comparable data for mice are missing. In order to help understand the underlying mechanisms that may lead to the higher neurogenic reactivity after deprivation, we analyze and compare in the following available findings about the effects of environmental conditions and wheel running on the developing rodent brain.

Stress and corticosterone are negative regulators of neurogenesis (Gould et al., 1992; Cameron & Gould, 1994) and can delay the effects of wheel running on neurogenesis in the adult (Stranahan et al., 2006). However, deprived and enriched-reared animals have the same basal corticosterone levels, and it is the deprived-reared animals, which show higher corti-

costerone levels in response to stress (Shrijver et al., 2002; Bartolomucci et al., 2003; Stranahan et al., 2006). Thus, a corticosterone effect is unlikely to play a role in this case, as the deprived-reared animals from our study responded with higher not lower rates of neurogenesis to wheel running.

Neurotrophic and other growth factors play a major role in neuroplasticity, especially in the upregulation of neurogenesis (Wagner et al., 1999; O'Kusky et al., 2000; Aberg et al., 2000; Cao et al., 2004; Scharfman et al., 2005). They are found at higher levels in enriched-reared animals (with or without wheel running; Torasdotter et al., 1998; Young et al., 1999; Ickes et al., 2000; During & Cao, 2006; Lehmann et al., 2007) and also after wheel running (Trejo et al., 2001; Fabel et al., 2003; Vaynman et al., 2004a, b). Furthermore, cholinergic activity from the medial septum increases neurotrophic factors in the hippocampus (Lapchak et al., 1993; Knipper et al., 1994; Berchtold et al., 2002) and upregulates neurogenesis directly via granule cells (Cooper-Kuhn et al., 2004; Mohapel et al., 2005). In gerbils, acetylcholine is enhanced in deprived-reared individuals (Lehmann et al., 2004; Busche et al., 2006), and in rats, adult wheel running increases acetylcholine (Dudar et al., 1979) and activates the septo-hippocampal axis (Lawson & Bland, 1993). Thus, one interpretation might be that, if deprived rearing already enhances acetylcholine, a running-induced additional increase could lead to stronger effects on neurogenesis in these animals either directly or indirectly via neurotrophins.

Wheel running is rewarding (Iversen, 1993; Brene et al., 2007) and shares characteristics of stereotypies like high repetition, self reinforcement and the lack of any obvious function or goal (rev. in Sherwin, 1998). However, in contrast to stereotypies, which can be environmentally-induced (rev. in Cabib, 1993), wheel running occurs irrespective of the environment (de Kock & Rohn, 1971; Roper & Polioudakis, 1977; Sherwin & Nicol, 1996). Also in our experiments, deprived-reared animals ran the same distance and made the same number of running bouts as enriched-reared animals (supplemental experiment II, chapter 3.6; Haupt & Schaefer, *accepted*). Thus, the amount and frequency of wheel running does not seem to be responsible for the enhanced neurogenesis in deprived-reared animals. Neuronal substrates for these rewarding and reinforcing aspects of wheel running are related to the mesolimbic dopamine-opioid system (Werme et al., 2000, 2003; Lett et al., 2002; Vargas-Perez et al., 2004). Administration of opioids or dopamine agonists decreases cell proliferation in the dentate gyrus (Hildebrandt et al., 1999; Teuchert-Noodt et al., 2000; Eisch et al., 2000; Yamaguchi et al., 2004). Though, opioids influence neurogenesis via local mechanisms in the dentate gyrus (Drake & Milner, 2002; Persson et al., 2003), while dopamine exerts its effects

mainly indirectly via modulation of other inputs to the dentate gyrus, of neurotransmitters and opioids. Wheel running enhances e.g. beta-endorphin, which is required for the effects of wheel running on cell proliferation (Colt et al., 1981; Goldfarb & Jamurtas, 1997). The influence of wheel running via dopamine has to be described rather on a systemic level: running-induced beta-endorphin binds to GABA in the VTA, attenuates the inhibition and leads to a higher dopamine release in Ncl. accumbens and caudate putamen (Johnson & North, 1992; Spanagel et al., 1992). Additionally, wheel running leads to an increase in dopamine synthesis and a reduction of inhibition in the substantia nigra (Foley & Fleshner, 2008). Thus, dopamine is enhanced in motor circuits as a response to wheel running. This could lead to a diminished control of the prefrontal cortex and hippocampus, the structures which normally control the reward system (rev. in White, 2002) and inhibit stereotypies (Whishaw & Mittleman, 1991; Whishaw et al., 1992; Schaub et al., 1997). A lack of cortical input and thus NMDA-receptor activation in the hippocampus was shown to increase cell proliferation (Cameron et al., 1995; Bernabeu & Sharp, 2000; Nacher et al., 2001). Thus, a possible mechanism might be that differences in the dopamine-opioid system of socially and physically deprived-reared animals compared to their enriched-reared littermates are responsible for the different neuroplastic response to the wheel-running challenge in young adulthood.

This interpretation finds support, since data from gerbils suggest a differentially matured dopamine-system in socially and physically deprived-reared individuals. Deprived-reared gerbils show severely reduced maturation patterns of both prefrontal layer II/III and layer V/VI pyramidal efferents to frontal, parietal and limbic areas (Bagorda et al., 2006; Witte et al., 2007) as a consequence of diminished dopamine maturation (Winterfeld et al., 1998). This reduced prefronto-cortico-limbic connectivity may be an underlying cause of the increase in cell proliferation in deprived-reared gerbils (Keller et al., 2000; Teuchert-Noodt, 2000). Since socially and physically deprived-reared mice responded with a qualitatively similar increase in neurogenesis to wheel-running stimulation, the development of their mesolimbocortical dopamine system may have been similarly affected.

Social and physical deprivation during rearing led to changes in neuroplastic development of the mouse brain such as observed for the gerbil brain. Differences in the development of neurotransmitter systems and in the regulation and expression of neurotrophic factors may account for this enhanced reactivity of a brain developed under deprived rearing. Since wheel running affected cell proliferation and cell survival only under specific conditions of a deprived rearing history, this study also points out that the effects of wheel running have to be observed more specifically.

3.5. SUPPLEMENTAL EXPERIMENT I: THE INFLUENCE OF CAGE SIZE IN DEPRIVED REARING

The present study was conducted to examine to which extent the findings on the epigenetic effects of juvenile environmental stimulation for gerbils are also valid for the mouse model. For this, the same rearing conditions as in the gerbil studies were applied. Beside the omission of running wheels in the enrichment, this referred also to the cage size. We used standard type III cages with a size of 42.5 x 26.5 x 18 cm, while it is more usual to keep mice matched to their smaller body size in smaller cages, e.g., standard type II cages with a size of 26.5 x 20.5 x 14 cm.

The effect of cage size on neuroplasticity is not well examined, but there are indications that at least adult behavior is influenced by cage size during development: rats, e.g., remain similar explorative as their non-deprived conspecifics when physical deprivation involves only minimal decreases in cage size (Zhu et al., 2006), but are clearly less explorative when it is restricted drastically (Joseph & Gallagher, 1980). Also mice seem to be affected by cage-size restriction, since they are willing to work for additional space (Sherwin & Nicol, 1997), although in a recent study with C57Bl/6Tac mice, no direct relationship between cage size and behavioral performance in adulthood could be proven (Whitaker et al., 2009).

It seems possible that the difference in the strength of the deprivation effect on cell proliferation between gerbils and mice could be an outcome of the - relatively to the body size - bigger cage size used in mice than in gerbils.

To examine, if cage size played a role in the outcome of the present study, we additionally reared a group of female CD1 mice (n=8) individually in standard type II cages (26.5 x 20.5 x 14 cm) from weaning (pd 21) to young adulthood (pd 66). Except cage size, the other components of the social and physical deprivation remained unchanged (for a detailed description see chapter 2.2; 3.2.1). Like the other groups, the animals were united on pd 66 and placed into a large enclosure. From pd 71 to pd 73, animals received BrdU injections of 50mg/kg i.p. at 5 p.m. and were sacrificed 5 days later for examining cell proliferation (for details see chapters 3.2.3). The immunohistochemical detection of BrdU-labeled cells and the quantification of BrdU-positive cells were performed as described earlier (chapter 2.6; 3.2.3). The different rearing groups (ER_{SPW}-, DR_{SPW}- type III from the main study and DR_{SPW}- type II) were statistically compared with one-way ANOVA.

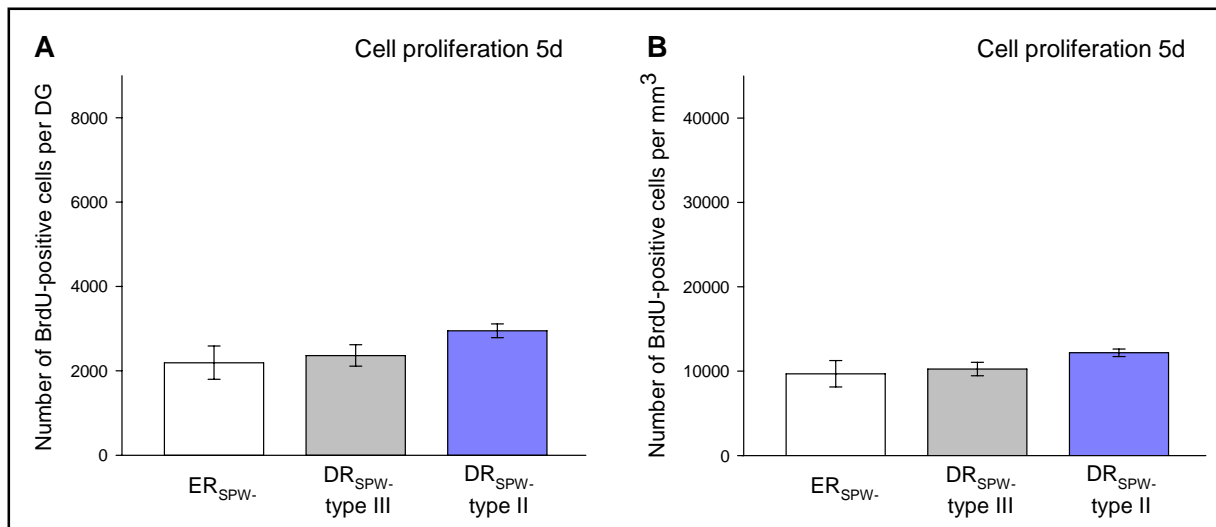


Fig. 3-7: **A)** Total number of BrdU-positive cells in the granule cell layer 5 days after BrdU-injection; **B)** Density of BrdU-positive cells per mm³ in the granule cell layer 5 days after BrdU injection; ER_{SPW-}, socially and physically enriched-reared animals; DR_{SPW-} type III, in type III cages socially and physically deprived-reared animals; DR_{SPW-} type II, in type II cages socially and physically deprived-reared animals; data given as means \pm S.E.M.

Although socially and physically deprived-reared individuals from type II cages seemed to have slightly more BrdU-positive cells (Fig. 3-7), this difference failed to be significant (ANOVA cells per DG: rearing condition $F_{2,17}=2.38$, $p = 0.12$; ANOVA cells per mm³: rearing condition $F_{2,17}=2.0$, $p = 0.17$).

These findings argue in favor, that cage size differences in this scale do not play a decisive role for the different strength of the effects of socially and physically deprived rearing on cell proliferation in adult gerbils and adult mice.

3.6. SUPPLEMENTAL EXPERIMENT II: EFFECTS OF REARING CONDITIONS ON WHEEL-RUNNING ACTIVITY

Four days of wheel running in young adulthood enhanced hippocampal cell proliferation and cell survival only in CD1 mice which were reared under social and physical deprivation, but not in their enriched-reared littermates. An obvious question is whether differences in wheel-running activity may account for the different rates in neurogenesis.

To answer this question, we measured wheel-running activity of enriched and deprived-reared animals (each group separately) over four days in young adulthood with automated running wheels. Two additional groups of female CD1 mice ($n=6$) were reared either under enriched or socially and physically deprived conditions as described before (see chapter 2.2 and 3.2.1). In young adulthood (ER_{SPW-} pd 62; DR_{SPW-} pd 66), animals were habituated to an enclosure which contained nothing than animal bedding, food and water *ad libitum* as well

as a solid hiding place. These conditions equated the habituation conditions in the main experiment (see chapter 3.2.2) except that the rearing groups were not mixed. Four days later (ER_{SPW}- pd 66; DR_{SPW}- pd 70) the automated running wheels were introduced into the enclosure. One running wheel per animal was provided, which was connected to a computer measuring the wheel revolutions (for detailed description of the apparatus see chapter 2.5.1).

Running analysis was started at the end of the first dark phase to get three complete 24-h cycles and include the three dark phases in which the BrdU labeling was performed in the main experiment. We included only long-term running events (>10 sec.) and excluded short-term running events, which often had purely investigative character (sniffing, outer touches, wheel crossings). Number of long-term running events and total distance covered in 24 hours were scored. As individual data points for number of events and distance we took days. These values were divided by the number of individuals (n=6). Normally distributed data (Shapiro Wilks test for normality) were analyzed using Welch two-sample t-test.

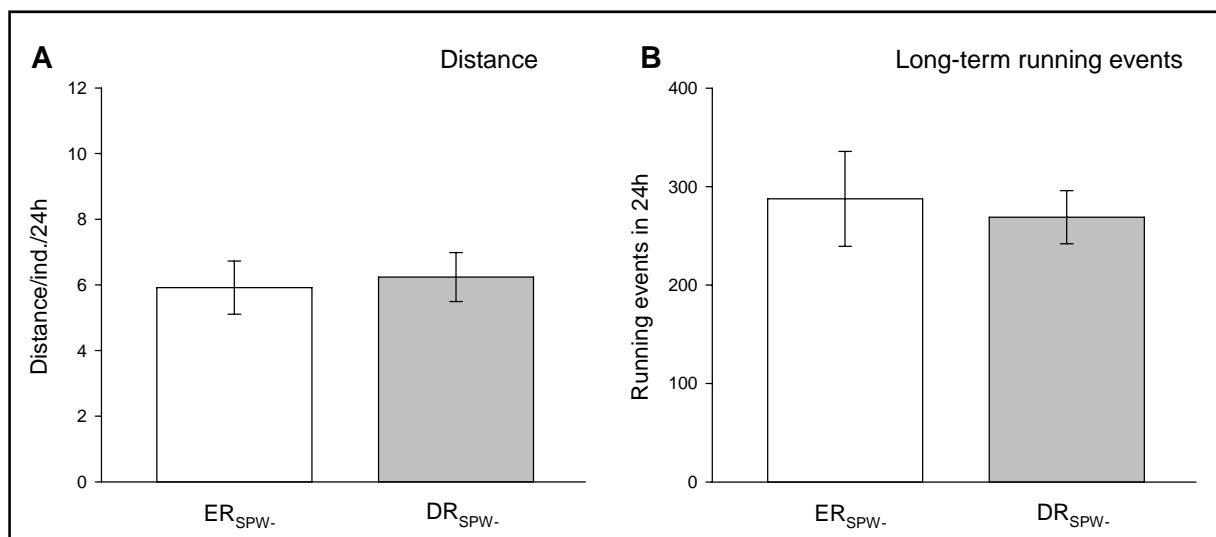


Fig. 3-8: Wheel running activity of enriched (ER_{SPW}-) and deprived-reared CD1 mice (DR_{SPW}-).

A) Running distance covered in 24 hours; **B)** Long-term running events (>10 sec.) in 24 hours; data given as means ± S.E.M.

Differences were found neither in total running distance (ER_{SPW}- 5.9 ± 0.47 km, DR_{SPW}- 6.2 ± 0.43 km) nor in the number of daily long-term events (ER_{SPW}- 287 ± 27, DR_{SPW}- 269 ± 16) between the two treatment groups (Welch two-sample t-tests, $p > 0.05$; Fig. 3-8).

Hence, enriched and socially and physically deprived-reared CD1 mice did not differ in their wheel-running activity in a 4-day wheel-running challenge in young adulthood. This indicates that differences in wheel-running activity cannot account for the different neuroplastic responses to wheel running.

4. PROJECT II:

A REVIEW AND HYPOTHETICAL MODEL OF THE EFFECTS OF WHEEL RUNNING ON NEUROGENESIS IN MICE WITH DIFFERENT REARING HISTORIES

4.1. INTRODUCTION

Environmental conditions especially during development as well as wheel running exert strong effects on hippocampal neurogenesis. Thus, they provide a good method to manipulate ontogenic plasticity and investigate biological mechanisms of neuroplastic differentiation.

Nevertheless, results concerning the effects of different rearing environments on neurogenesis are contradictory: studies in mice and rats using the experimental setup of an enriched environment with running wheels observed a reduced cell survival of adult born neurons in animals reared under standard-housing conditions compared to their enriched-reared littermates (Kempermann et al., 1997a; Nilsson et al., 1999). In contrast, studies in gerbils without running wheels in the enrichment found an enhanced cell proliferation in the dentate gyrus after socially and physically deprived rearing compared to enriched rearing (Hildebrandt, 1999; Keller et al., 2000).

Thus, the different designs of the environmental set-ups of enriched, deprived or standard housing create problems in interpreting the data. Concerning the enriched environment especially the running wheel is a matter of debate. Some authors even state that wheel running is essential to environmental complexity-related hippocampal neurogenesis (van Praag et al., 2000; Lewis, 2004).

Recently, we could support this by demonstrating that, in CD1 mice, enriched rearing without wheel running or socially and physically deprived rearing do not cause different rates neither of cell proliferation nor of cell survival. However, neuroplasticity in adulthood was influenced by our rearing conditions: neurogenic stimulation by an adult wheel-running challenge (postnatal day 70-74) enhanced cell proliferation and cell survival only in socially and physically deprived-reared animals (project I, chapter 3).

Hence, it is postulated that the effects of individual components of the environmental set-ups have to be examined more specifically (Shrijver et al., 2002; Lewis, 2004). In this review, this is tried to realize for the effects of wheel running. Studies on wheel running and its effects on neurogenesis are normally performed under deprived or standard-housing con-

ditions in animals which were also reared under these conditions (van Praag et al., 1999a, b; Holmes et al., 2004; Uda et al., 2006). In all these studies a strong cell proliferation enhancing effect of wheel running was found. However, most of the newborn cells do not reach maturation but undergo apoptosis before. Thus, enriched rearing including wheel running and wheel running itself under standard-housing conditions have nearly the same net effect on neurogenesis: while wheel running on its own enhances cell proliferation, enriched rearing including wheel running enhances cell survival. Thus, both experimental manipulations seem to affect differentially mechanisms that are involved in distinct phases in the process of neurogenesis (Olson et al., 2006).

Before the main issue of this review can be addressed, viz., how wheel running affects neuroplasticity in under different rearing environments grown up individuals, it should be dipped into the question, what wheel running actually is for the rodent brain. At the end, a hypothetical model is to be proposed of the underlying mechanisms mediating the distinct neuroplastic responses of animals with different rearing histories to wheel running.

4.2. WHAT IS WHEEL RUNNING? WHAT DOES IT DO?

The significance of wheel running and its effects on the brain are a matter of debate: Some positive effects of wheel running on the brain were shown like improved learning, better mental performance and enhanced brain plasticity (Cotman & Berchtold, 2002; Ehninger & Kempermann, 2003; van Praag et al., 2005). Nevertheless, wheel running is also discussed under the aspects of stereotypy and reinforcing behavior. Wheel running is rewarding (Iversen, 1993; Brene et al., 2007) and animals are willing to work for it, e.g., unlock wheels (Tepper & Weiss, 1986; Collier et al., 1990; Iversen, 1993; Belke & Heyman, 1994) or work for access to areas containing wheels (Collier et al., 1989; Sherwin & Nicol, 1996; Sherwin, 1996). It may be addictive (Belke, 1996) and self-reinforcing, as animals disregard hunger and thirst (Melcer & Timberlake, 1986; Looy & Eikelboom, 1989; Bauman, 1992). Food deprived animals run even more (Routtenberg & Kuznesof, 1967; Roper, 1976), and animals continue to run even when they are injured (Richards, 1966; de Kock & Rohn, 1971; Sherwin, 1998). Moreover, similar behavioral effects of wheel running as of stimulant drugs like amphetamine were described (Lett et al., 2002).

On the other hand, wheel running shares characteristics of stereotypies like high repetition, invariance and the lack of any obvious function or goal (Sherwin, 1998). Self reinforcement is also a typical feature of stereotypies (Fox, 1965, 1971; rev. in Robbins et al., 1990; Mason, 1991), and it seems that beginning stereotypies are even rewarding (Robbins et al.,

1990). Both wheel running and stereotypies can serve the coping with stress and show an inverse relationship to corticosterone (Mittleman et al., 1991; Würbel & Stauffacher, 1996). However, there exist also clear differences between wheel running and stereotypies: Wheel running can take very complex forms, when the animals perform acrobatics, probably to increase stimulation, such as running on the outside or clinging to the wheel and allowing themselves to be carried around (Sherwin, 1998). While stereotypies can be environmentally-induced (rev. in Cabib, 1993), wheel running occurs irrespective of the environment (Brant & Kavanau, 1964; de Kock & Rohn, 1971; Roper & Polioudakis, 1977; Sherwin & Nicol, 1996; Sherwin, 1998). Also in our experiments, socially and physically deprived-reared laboratory mice ran the same distance and showed the same number of running bouts as enriched-reared animals notwithstanding whether they ran individually or in a group (Haupt & Schaefer, *accepted*; cf. chapter 3.6). Thus, the amount and frequency of wheel running cannot account for the distinct neuroplastic responses of differently reared animals to wheel running.

4.3. WHEEL RUNNING AND NEUROGENESIS IN MICE WITH DIFFERENT REARING HISTORIES

Wheel running as a strong physical exercise with features of rewarding as well as of stereotypical behavior leads to numerous changes in the brain, namely in neurotrophic and other growth factors, endogenous opioids, neurotransmitters and especially in the mesolimbic dopamine-opioid-system (Werme et al., 2000, 2003; Lett et al., 2002; Vargas-Perez et al., 2004). Hence, some questions come up: Do socially and physically deprived-reared animals potentially differ in one or more of these systems? And if so, could these differences account for the different neuroplastic responses to the neurogenic stimulation by wheel running in adulthood?

It is known that the effects of wheel running are delayed by stressors which enhance corticosterone levels (Stranahan et al., 2006), because corticosterone suppresses neurogenesis (Gould et al., 1992; Cameron & Gould, 1994). In this regard, it is astonishing that wheel running itself increases corticosterone levels (Droste et al., 2003), what does not seem to interfere with the neurogenesis-stimulating effect of wheel running. Nevertheless, it could be argued that higher stress levels in enriched-reared animals could prevent an increase in neurogenesis after wheel-running (project I, chapter 3). However, deprived and enriched-reared animals have the same basal corticosterone levels, and it is the deprived-reared animals, which show higher corticosterone levels in response to stress (Shrijver et al., 2002; Bartolomucci et al., 2003; Marashi et al., 2004). Thus, if corticosterone determined the effects

of wheel running, it should have delayed the effects of wheel running in deprived-reared and not in enriched-reared individuals (cf. Stranahan et al., 2006).

Neurotrophic and other growth factors play a major role in the upregulation of neurogenesis (Wagner et al., 1999; Aberg et al., 2000; O'Kusky et al., 2000; Trejo et al., 2001; Kitamura et al., 2003; Fabel et al., 2003; Cao et al., 2004; Scharfman et al., 2005) and most of them, including brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and insulin-like growth factor 1 (IGF-1), are also enhanced after wheel running in the rodent dentate gyrus (Gomez-Pinilla et al., 1997; Oliff et al., 1998; Trejo et al., 2001; Fabel et al., 2003; Farmer et al., 2004; Vaynman et al., 2004a, b). Most studies revealing an increase in neurotrophic or growth factors after enrichment, included wheel running in the enriched condition (Falkenberg et al., 1992; Young et al., 1999; Pham et al., 1999; Ickes et al., 2000; During & Cao, 2006). These studies could be misleading, because the observed effects could be due to wheel running in the enrichment. However, studies in which the enriched condition was designed without wheel running in rats (Torasdotter et al., 1998; Stranahan et al., 2007) and gerbils (Lehmann et al., 2007) found the same effect for NGF and neurotrophin as well as BDNF, namely that deprived-reared animals show lower levels of these neurotrophic factors than their enriched-reared littermates.

For VEGF and IGF-1 it was even shown that they are necessary for the running-induced increase in neurogenesis (Trejo et al., 2001; Fabel et al., 2003). However, blocking VEGF returned neurogenesis to baseline but not below suggesting that central regulators of baseline neurogenesis are not influenced by running (Fabel et al., 2003). Thus, it could be hypothesized that factors regulating running-induced neurogenesis are affected by rearing conditions without any impact on baseline neurogenesis.

Acetylcholine is a neurotransmitter which exerts influence on BDNF. Cholinergic activity especially from the medial septum increases BDNF gene expression in the hippocampus (Lapchak et al., 1993; Knipper et al., 1994; French et al., 1999; Berchtold et al., 2002). Wheel running activates the septo-hippocampal axis (Lawson & Bland, 1993; Lee et al., 1994), which in turn activates the hippocampus by way of theta rhythms (Smythe et al., 1992; Lawson & Bland, 1993; Lee et al., 1994), and increases acetylcholine in the hippocampus (Dudar et al., 1979). Unfortunately, studies on the effects of rearing conditions without wheel running in the enrichment on the levels of neurotrophic and growth factors are rare. We know from studies in gerbils that acetylcholine is enhanced in deprived-reared individuals (Lehmann et al., 2004; Busche et al., 2006). In the case that this also holds true for other rodents, it might be reasonable that, if acetylcholine is already enhanced in deprived-reared

animals, a running-induced increase in acetylcholine could lead to stronger effects on neurogenesis than in enriched-reared animals either directly or indirectly via BDNF.

Serotonin is another neurotransmitter, which exerts an enhancing effect on neurogenesis in the dentate gyrus (Jacobs & Fornal, 1997; Brezun & Daszuta, 1999, 2000; Malberg et al., 2000; Wakade et al., 2002), while it is more instrumental in cell proliferation than in cell survival (Olson et al., 2006). Results concerning the effects of exercise on serotonin levels are fragmentary and partly contradictory. In one case treadmill running decreased serotonin in limbic structures including the hippocampus (Chen et al., 2008). In other studies, treadmill running increased serotonin in the rat brain (Chaouloff et al., 1985) including the cortex and hippocampus (Gomez-Merino et al., 2001; Bequet et al., 2001). Data for the effects of rearing conditions on serotonergic fiber distribution or serotonin levels are also rare. In gerbils, deprived rearing increased serotonergic fiber densities in caudate putamen, amygdala (Lehmann et al., 2003), insular and entorhinal cortex (Neddens et al., 2003). Thus, it could only be speculated that the effects of serotonin are similar to those of acetylcholine, viz., that serotonin levels are enhanced in deprived-reared animals and undergo a further increase after wheel running leading to the strong neurogenic response. However, since clear results concerning the effects of rearing conditions and wheel running on serotonin are missing, this has to remain speculation.

As aforementioned, wheel running has rewarding and reinforcing aspects. These are related to the mesolimbic dopamine-opioid system (Werme et al., 2000, 2003; Lett et al., 2002; Vargas-Perez et al., 2004). Furthermore, dopamine function is altered in mice bred for high wheel-running activity (Rhodes et al., 2001; Rhodes & Garland, 2003).

Administration of dopamine as well as of opioids decrease cell proliferation in the dentate gyrus (Dawirs et al., 1998; Hildebrandt et al., 1999; Eisch et al., 2000; Teuchert-Noodt et al., 2000; Persson et al., 2003; Yamaguchi et al., 2004). Dopamine and endogenous opioids influence each other via connections between the striatum and the ventral tegmental area (VTA): dynorphin coexpressing cells in the striatum coexpress D1-receptors and project to the substantia nigra and the ventral tegmental area (Gerfen et al., 1991; Le Moine et al., 1991). Beta-endorphin from the arcuate nucleus projects also to the ventral tegmental area and the nucleus accumbens (Khachaturian et al., 1985). Dopamine in turn regulates the expression of dynorphin and enkephalin in the striatum (Gerfen et al., 1991; Hurd & Herkenham, 1992; Lindfors, 1992; Spangler et al., 1993).

Opioids can influence neurogenesis either directly via acting on μ -receptor-expressing progenitors in the dentate gyrus (Persson et al., 2003) or indirectly via μ -receptor-expressing

GABAergic interneurons (Drake & Milner, 2002). In contrast, dopamine from the ventral tegmental area innervates the dentate gyrus only very sparsely (Swanson, 1982) and mainly in the polymorphic layer (Amaral & Lavanex, 2006). Thus, dopamine exerts its effects mainly indirectly via inputs to other structures projecting to the dentate gyrus as well as modulation of other neurotransmitters and opioids.

Wheel running enhances, e.g., beta-endorphin, which is required for the effects of wheel running on cell proliferation (Colt et al., 1981; Hoffmann et al., 1990; Goldfarb & Jamurtas, 1997). The influence of wheel running via dopamine has to be described on a more systemic level: running-induced beta-endorphin binds to GABA in the VTA, attenuates the inhibition and leads to more dopamine release in the nucleus accumbens and caudate putamen (Johnson & North, 1992; Spanagel et al., 1992). Additionally, wheel running leads to an increase in dopamine synthesis and a reduction of inhibition in the substantia nigra (Foley & Fleshner, 2008). Thus, as a response to wheel running, there is an increase of dopamine in motor circuits. This may diminish the control of the prefrontal cortex (PFC) and hippocampus, which normally control the reward system (rev. in White, 2002) and inhibit stereotypies (Whishaw & Mittleman, 1991; Whishaw et al., 1992; Lipska et al., 1995; Schaub et al., 1997). A lack of cortical input and thus entorhino-dentate N-methyl-D-aspartate (NMDA) receptor activation increases cell proliferation in the dentate gyrus (Cameron et al., 1995; Bernabeu & Sharp, 2000; Nacher et al., 2001). Thus, differences in the dopamine-opioid system between socially and physically deprived and enriched-reared animals might present a reason for the different neuroplastic response to wheel running.

Indeed, a differently matured dopamine system after deprived rearing has been demonstrated at least in gerbils. Deprived-reared gerbils show an impaired dopamine maturation into the prefrontal cortex with subsequent impairments in PFC-dependent tasks (Winterfeld et al., 1998) and reduced maturation patterns of both prefrontal layer II/III and layer V/VI pyramidal efferents to frontal, parietal and limbic areas (Bagorda et al., 2006; Witte et al., 2007). In contrast, dopamine fibers in limbic structures mature excessively (Neddens et al., 2002; Busche et al., 2004; Lesting et al., 2005). This leads to a dysconnectivity of the PFC with caudal limbic areas as well as deficient connections with the corticostriatal circuit (Bagorda et al., 2006). Diminished PFC activity to the limbic system was assumed to cause an inefficient entorhino-dentate NMDA-receptor activation and thus an increase in cell proliferation in these animals (Hildebrandt, 1999; Keller et al., 2000; Teuchert-Noodt et al., 2000). In our experiments with CD1 mice, socially and physically deprived rearing did not cause increased rates of cell proliferation in adulthood (project I, chapter 3) suggesting that mice experience

less strong impacts after deprivation. This would correspond to observations that mice do not show so many and not so various forms of stereotypies compared to gerbils (Würbel & Stauffacher, 1996; Wiedenmayer, 1996; own observations). Nevertheless, socially and physically deprived-reared mice underwent a different neuroplastic development compared to their enriched-reared littermates, which was revealed by the adult wheel-running challenge. Only deprived-reared, but not enriched-reared individuals responded to the wheel-running challenge with a significant increase in neurogenesis. This suggests perhaps not a dysbalance, but at least an enhanced vulnerability to external influences that can easily lead to disturbances in the interplay of prefrontal and caudal limbic areas.

4.4. SUMMARY AND PROPOSAL OF A HYPOTHETICAL MODEL

Taking all these factors together, this leads to the proposal of a hypothetical model of maturation-determined differences in the prefrontal-limbic system of socially and physically deprived and enriched-reared mice which – concerning neurogenesis – can be buffered under sedentary conditions, but become evident in adulthood after additional stimulation with a wheel-running challenge (Fig. 4-1).

During wheel running, subcortical motor circuits between the nucleus accumbens, the striatum and dopaminergic nuclei in the substantia nigra and VTA undergo stronger activation. The septo-hippocampal axis is activated and amplifies the cholinergic input to the dentate gyrus. On the local level, neurotrophic and growth factors, perhaps additionally incited by the cholinergic activity, are enhanced. In the brains of enriched-reared animals these activations remain without any impact on the PFC-hippocampus-interaction and neurogenic activity can be stabilized (Fig. 4-1, A). In contrast, in socially and physically deprived-reared mice, which already show higher cholinergic activity and a disturbed interplay in prefrontal-limbic circuits, the alterations induced by wheel running cannot be compensated. The already enhanced cholinergic activity found in these animals is additionally amplified by wheel running. Furthermore, the strong activation in extrapyramidal motor circuits could have far-reaching consequences on the whole prefrontal-limbic system. It leads to a shift of dopaminergic activity from the PFC to the motor circuits. As a consequence glutamatergic activity from the PFC to other cortical and limbic areas is lowered. The lack of prefrontal control over corticostriatal motor circuits allows them to further reinforce, what, in turn, leads to more dopaminergic activity in motor circuits, but less in the PFC. Hence, the balance shifts from the PFC-striatum-circuit to the motor circuits. This results in further changes in the interplay with other neurotransmitter systems and a lack of activity from the

PFC to the entorhinal cortex and perforant path. The consequential reduced NMDA-receptor-activation leads - together with the other afore mentioned factors - to an enhancement of proliferating activity in the dentate gyrus (Fig. 4-1, B).

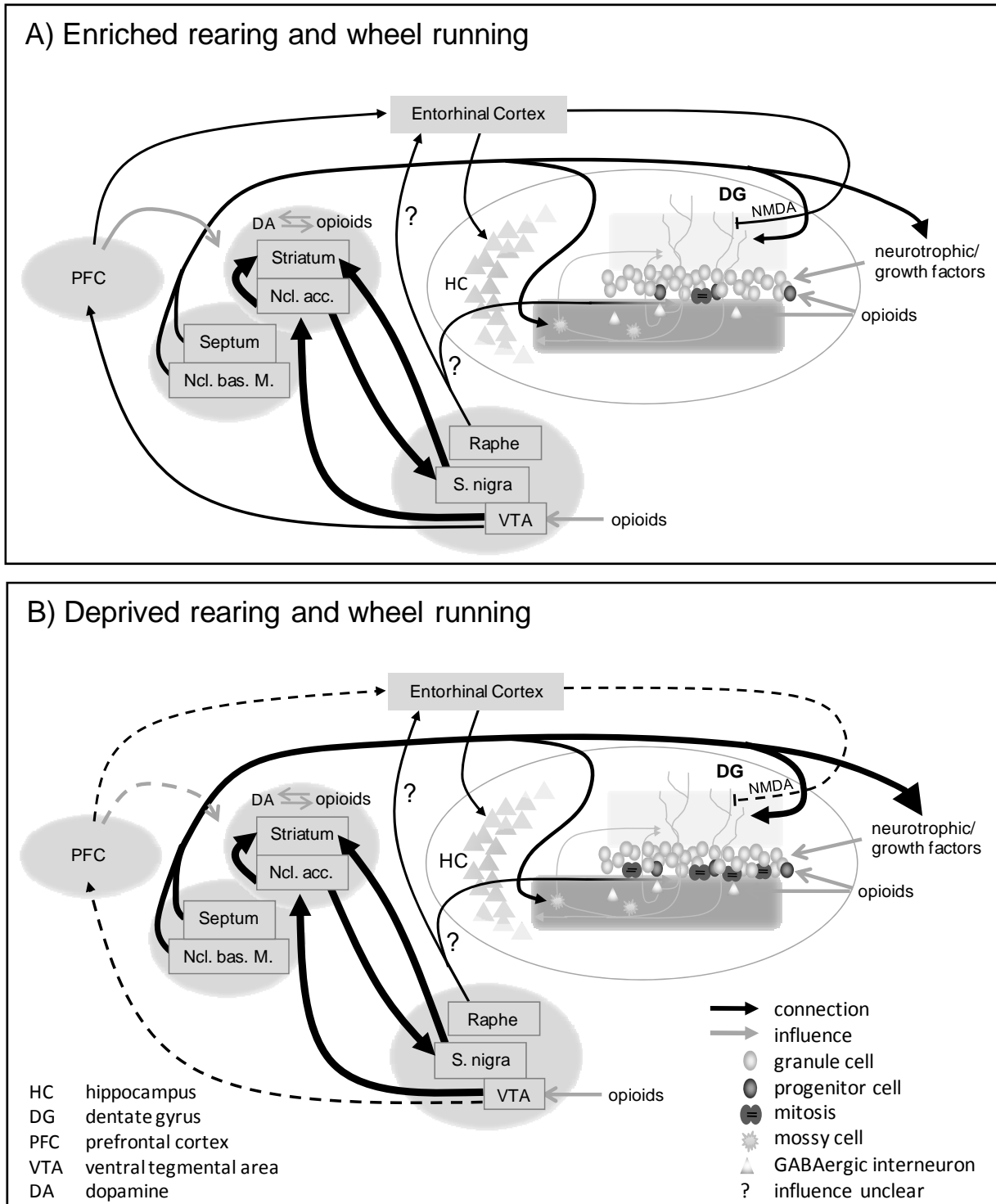


Fig. 4-1: A hypothetical model of the effects of wheel running on neurogenesis in mice reared **A)** under social and physical enrichment, **B)** under social and physical deprivation (explanation in the main text).

5. PROJECT III:

SYNAPTIC REMODELING IN THE DENTATE GYRUS, CA3 AND CA1 IS NOT AFFECTED BY SOCIALLY OR PHYSICALLY DEPRIVED REARING BUT BY WHEEL RUNNING IN ADULT CD1 MICE

5.1. INTRODUCTION

Synaptic plasticity in higher brain regions of mammals is not restricted to prenatal ontogenesis, but occurs also postnatally during development and even in the adult brain. Beside *reactive synaptogenesis* following lesions and deafferentiation (Holzgraefe et al., 1981; Merzenich et al., 1984; Steward et al., 1988), synaptic turnover in axon terminals is a *naturally-occurring process* of structural plasticity in the adult rodent brain (Dawirs et al., 1992; Keller et al., 2000; Dawirs et al., 2000; Neufeld et al., 2009). These nondegenerative synaptic degradation events of remodeling synaptic connections or revision of transmitter release (Wolff et al., 1989; Teuchert-Noodt, 2000) are activity-dependent processes (Wolff & Wagner, 1983; van Ooyen & van Pelt, 1994; Dawirs et al., 2000; Nägerl et al., 2004; Tailby et al., 2005; Fox & Wong, 2005; Hua et al., 2005; Butz et al., 2008) permitting the animal to learn and to adapt to environmental changes (Black et al., 1990; rev. in Morris et al., 2003).

Within the hippocampal formation, the generation of new neurons (neurogenesis) adds a further level of complexity of structural plasticity to the preexisting neuronal network even in the mature brain (rev. in Teuchert-Noodt, 2000; Lehmann et al., 2005; Gould, 2006). There are strong indications that neurogenesis is interrelated to synaptic remodeling, since new neurons demand synaptic integration (rev. in Teuchert-Noodt, 2000; Lehmann et al., 2005) and their survival depends on their functional integration into the existing neuronal network (van Praag et al., 2002; Overstreet et al., 2004; Kempermann et al., 2004). Both processes, neurogenesis and synaptic remodeling, are susceptible to environmental manipulations (Turner & Greenough, 1985; Kempermann et al., 1997a, 1998; Keller et al., 2000; Faherty et al., 2003; Briones et al., 2005; Iso et al., 2007) and voluntary physical activity (van Praag et al., 1999a, b; Eadie et al., 2005; Redila et al., 2006; Stranahan et al., 2006, 2007). Up to now, an interrelation of both processes and their susceptibility to environmental conditions has only been shown in gerbils. Intriguingly, an inverse relationship between adult hippocampal cell proliferation and synaptic remodeling in the inner molecular layer of the dentate gyrus was

found under different rearing conditions: Individuals reared under social and physical deprivation (impoverishment) had higher rates of adult hippocampal cell proliferation than enriched-reared animals while synaptic-turnover rates in the inner molecular layer were reduced (Hildebrandt et al., 1999; Keller et al., 2000, see also Butz et al., 2008 for a theoretical model). In CD1 mice, rates of cell proliferation and cell survival were not different between socially and physically deprived and enriched-reared individuals per se. However, only deprived-reared animals responded to a wheel-running challenge in adulthood with a significant increase in neurogenesis. Thus, conditions during rearing do affect the neuroplastic potential of the adult animals (project I, chapter 3).

The present study examined, whether CD1 mice reared under different environmental conditions have different rates of synaptic turnover in adulthood either under sedentary conditions or after wheel running. Specifically, we tested the prediction of an inverse relationship between adult hippocampal cell proliferation and synaptic turnover in the inner molecular layer as suggested by the model of Teuchert-Noodt and coworkers (Keller et al., 2000; Teuchert-Noodt, 2000; Butz et al., 2008). To obtain a comprehensive picture of the effects in the regions down- and upstream of the neurogenesis site, we included the CA3 and CA1 regions of the hippocampus proper and the subiculum and entorhinal cortex in our analyses. We applied the same experimental treatment as in the gerbil studies which, in contrast to most studies in mice and rats, excluded running wheels from the enriched environment during rearing. As an indicator of synaptic turnover we stained lysosomal accumulations with the Gallyas silver impregnation and quantified silver-stained precipitates in the dentate gyrus, CA3, CA1, subiculum and entorhinal cortex.

5.2. MATERIAL & METHODS

5.2.1. ANIMALS AND REARING CONDITIONS

25 male CD1 mice were bred in our facility in standard type III cages (42 x 26 x 18 cm) with one litter per cage. At weaning (postnatal day (pd) 21), animals were transferred to one of the two rearing conditions, enriched (ER_{SPW.}) or socially (s) and physically (p) deprived (DR_{SPW.}) rearing, both without running wheels (w-). The particular parameters of the rearing conditions were described previously (chapter 2.2). The ER_{SPW.} animals (n=13) were kept in one group in a large enclosure (200 x 100 x 50 cm). DR_{SPW.} animals (n=12) were kept individually in standard type III cages. All animals were on a 12-h-light-dark schedule and received laboratory animal feed (Höveler, Dormagen, Germany) and water *ad libitum*. Animals were kept under their respective rearing conditions till pd 74.

5.2.2. WHEEL-RUNNING CHALLENGE

On pd 74, the enrichment (tunnels, toys etc.) was removed from the enclosure of the ER_{SPW-} animals and the enclosure was divided by a wooden wall. Half of the group got access to running wheels (one per animal; n=6). Accordingly, half of the DR_{SPW-} animals received a running wheel into their cages (n=5). The wheel-running challenge lasted 4 days from pd 74 to pd 78. After the wheel-running challenge the animals remained under the respective conditions without running wheels for 3-7 days.

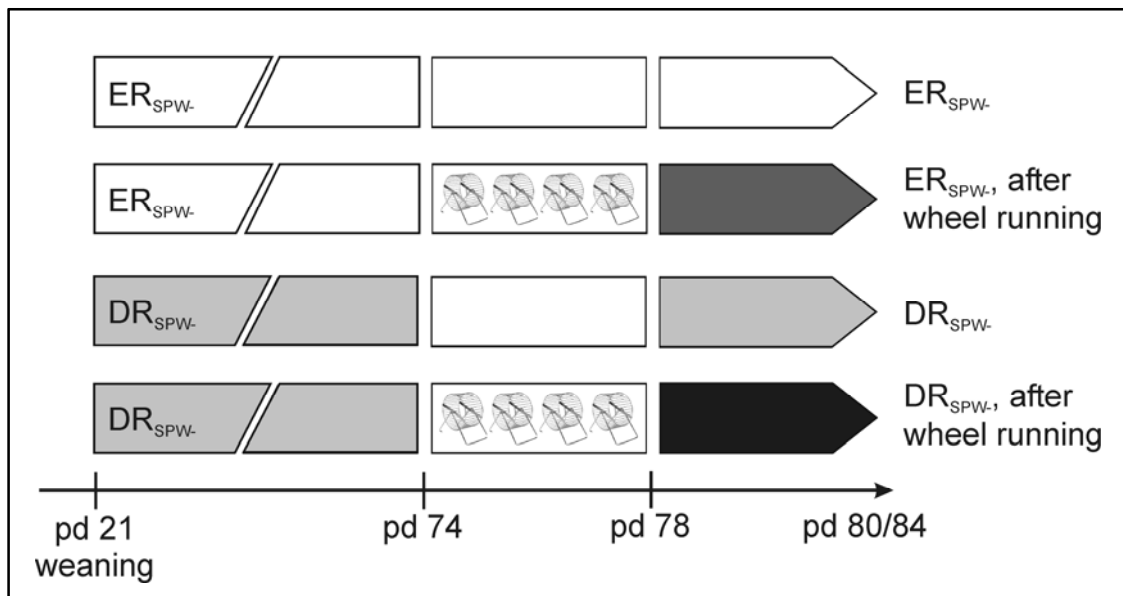


Fig. 5-1: Experimental design with time axis showing animal age in postnatal days (pd); pd 21-74, rearing phase under socially and physically enriched conditions (ER_{SPW-}) or socially and physically deprived conditions (DR_{SPW-}); pd 74-78, 4-day wheel-running challenge for the half of each rearing group; synaptic-remodeling rates were assessed between pd 80-84.

5.2.3. TISSUE PREPARATION AND STAINING

Animals were deeply anaesthetized by an overdose of diethyl ether and transcardially perfused with 5% formalin. The brains were subsequently kept in formalin for at least two weeks at 4°C. The left hemispheres were freeze-sectioned into 60 µm thick coronar sections and collected in distilled water (for tissue preparation see chapter 2.7.1). Every second section from the septal pole to the temporal pole of the hippocampus was used for staining. According to the procedure described by Gallyas and colleagues (Gallyas et al., 1980), the sections were silver impregnated as described in chapter 2.7.2.

5.2.4. MEASURING FIELDS AND COMPUTERIZED ASSESSMENT OF LYSOSOMAL ACCUMULATIONS

Silver-impregnated lysosomal accumulations can be viewed and counted in the dark field at 100-fold magnification. For computer-assisted quantification of precipitates, pictures were taken from the dentate gyrus, CA1, CA3, subiculum and entorhinal cortex (for details see chapter 2.7.3). For the subiculum and entorhinal cortex 6-8 sections per animal, for the dentate gyrus, CA1 and CA3 14-20 sections per animal were taken for quantification. Within the respective areas, well-defined measuring fields were set to estimate the numbers of precipitates per layer by a self-developed classification algorithm implemented in MATLAB Vers. 6 (cf. Butz et al., 2008; for details see chapter 2.7.3). Within the dentate gyrus, measuring fields were set in the outer, intermediate and inner molecular layer, in the granule cell layer and subgranular layer. Within the CA3 region, fields were set in the stratum oriens, stratum pyramidale, stratum lucidum and stratum radiatum. Since the stratum radiatum and stratum lacunosum-moleculare in CA3 were not distinguishable in this staining, only a measuring window in stratum radiatum could be set. Within the CA1 region measuring fields were set in the stratum oriens, stratum pyramidale, stratum radiatum and Stratum lacunosum-moleculare. Within the subiculum, fields were set in layer III, within the entorhinal cortex in layer II and III (Fig. 5-2). Measuring fields consisted of three joined rectangular-shaped sub-fields (200 x 50 pixels) sized 600 x 50 pixel in total. Pictures and measurements were performed by a person blind to the group membership of the individuals.

5.2.5. STATISTICAL ANALYSIS

According to the high connectivity of the layers within the different hippocampal regions, statistical analyses were conducted in each of the different regions comprising all layers respectively. Thus, factorial analyses of variance (ANOVA) with the dependent variable “precipitates” and the categorical predictors “rearing condition”, “wheel running” and “layer” were performed in all examined regions separately. Subsequent specific comparisons were made with Unequal N HSD post-hoc test. All statistical analyses were computed with Statistica 6 (StatSoft, Tulsa, USA).

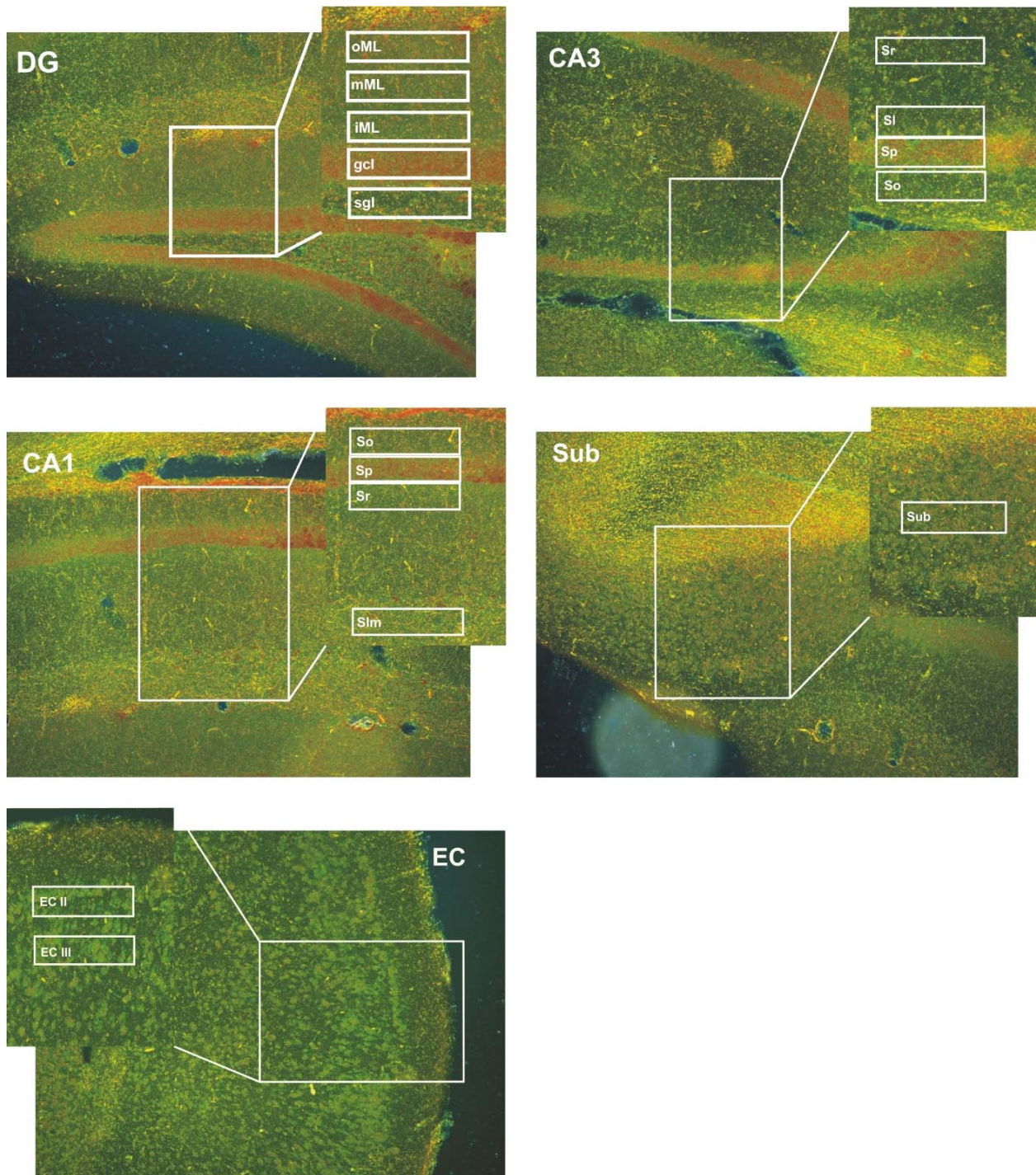


Fig. 5-2: Dark field light microscopic images of the dentate gyrus (DG), CA3, CA1, subiculum (Sub), and entorhinal cortex (EC) with silver-impregnated lysosomal accumulations appearing as bright precipitates. In the dentate gyrus, five measuring fields were positioned in the outer (oML), intermediate (mML) and inner molecular layer (iML) as well as in the granule cell (gcl) and subgranular layer (sgl) of both the suprapyramidal and infrapyramidal blade (not indicated). In CA3, measuring fields were positioned in the stratum radiatum (Sr), stratum lucidum (Sl), stratum pyramidale (Sp) and stratum oriens (So). In CA1, an additional field was set in the stratum lacunosum moleculare (SIm). One measuring field was positioned in the subiculum, and two in the entorhinal cortex in lamina II (ECII) and III (EC III), respectively.

5.3. RESULTS

5.3.1. QUALITATIVE RESULTS

The qualitative evaluation of the Gallyas silver impregnation in the dark field (100-200-fold magnifications) revealed precipitates throughout the hippocampal formation in all treatment groups. The staining appears as a fine granulation of silver precipitates of a size between 0.02-0.05 μm (cf. Gallyas et al., 1980). Region and layer-specific differences are visible (Fig. 5-2), whereas no differences appear among the treatment groups.

5.3.2. QUANTITATIVE RESULTS

The amount of synaptic turnover in axon terminals was assessed by quantification of silver impregnated lysosomal accumulations in the dark field (100-fold, magnification; Fig. 5-2). In the following the statistical data for the mean total number of precipitates in the measuring fields of each layer are given.

5.3.2.1. Layer-specific differences in the number of precipitates in the dentate gyrus and hippocampus proper

In our socially and physically enriched-reared control animals significant differences appeared in the number of precipitates between the layers of the dentate gyrus (ANOVA, layer $F_{9,21}=35.37$, $p < 0.0001$), CA3 (ANOVA, layer $F_{3,84}=31.87$, $p < 0.0001$) and CA1 (ANOVA, layer $F_{3,84}=82.33$, $p < 0.0001$).

In the dentate gyrus, post-hoc tests revealed that these differences in the number of precipitates were mainly present between the granule cell layer with a rather low density (92 in the suprapyramidal, and 109 in the infrapyramidal blade) and the molecular layers (Post hoc Unequal N HSD, $p < 0.01 - 0.001$) as well as the subgranular layer (Post-hoc Unequal N HSD, $p < 0.001$), while the subgranular layer showed the highest amount of precipitates (292 in the suprapyramidal, and 344 in the infrapyramidal blade) followed by the inner molecular layer (270 in the suprapyramidal, and 258 in the infrapyramidal blade).

In CA3, a similar pattern could be observed with most significant differences between the rather acellular layers and the pyramidal cell layer with the lowest number of precipitates (91; Post-hoc Unequal N HSD, $p < 0.001$). However, there was also a trend towards a difference between the stratum radiatum with the highest number of precipitates (333) and the stratum oriens (230; Post-hoc Unequal N HSD, $p = 0.07$).

CA1 resembled the distribution of CA3 with the highest differences between the rather acellular layers and the pyramidal cell layer with the lowest amount of precipitates (57; Post-hoc

Unequal N HSD, $p < 0.001$). However, in CA1, the stratum lacunosum moleculare appeared with the highest number of precipitates (335), although the difference to the stratum oriens or stratum radiatum was not significant.

5.3.2.2. No differences in the distribution of precipitates between socially and physically enriched and deprived-reared animals

Synaptic-turnover rates indicated by the number of silver impregnated lysosomal accumulations were neither in the dentate gyrus nor in CA3 or CA1 affected by the rearing conditions of social and physical deprivation or enrichment alone. Although there were significant main effects of rearing in the dentate gyrus (ANOVA, rearing $F_{1,21}=9.85$, $p < 0.01$) and CA3 (ANOVA, rearing $F_{1,84}=10.36$, $p < 0.01$), post-hoc tests did not reveal any significant differences between the two rearing groups (Tab. 5-1), and also the pattern of distribution of turnover processes between the layers appeared to be similar (Fig. 5-3).

5.3.2.3. Leveling of layer-specific differences after adult wheel running

Wheel running exerted strong effects on synaptic-turnover rates in the dentate gyrus (ANOVA, wheel running, $F_{1,21}=50.06$, $p < 0.0001$), CA3 (ANOVA, wheel running $F_{1,84}=21.2$, $p < 0.0001$; interaction effect wheel running*layer $F_{3,84}=14.71$, $p < 0.0001$) and CA1 ($F_{1,84}=54.57$, $p < 0.0001$; wheel running*layer $F_{3,84}=6.91$, $p < 0.001$).

It obviously leveled the afore seen differences in synaptic-turnover rates between the layers in both enriched and deprived-reared individuals mainly by decreasing turnover rates in all layers except the granule or pyramidal cell layer (Fig. 5-3). Surprisingly, this decrease was especially significant in CA1 in the stratum radiatum of enriched-reared (Post-hoc Unequal N HSD, $p < 0.05$) and the stratum lacunosum moleculare of deprived-reared individuals (Post-hoc Unequal N HSD, $p < 0.05$).

5.3.2.4. No effects beyond the dentate gyrus and hippocampus proper

No effects of either treatment were found beyond the dentate gyrus and hippocampus proper in the entorhinal cortex or subiculum (Tab. 5-1; Fig. 5-3).

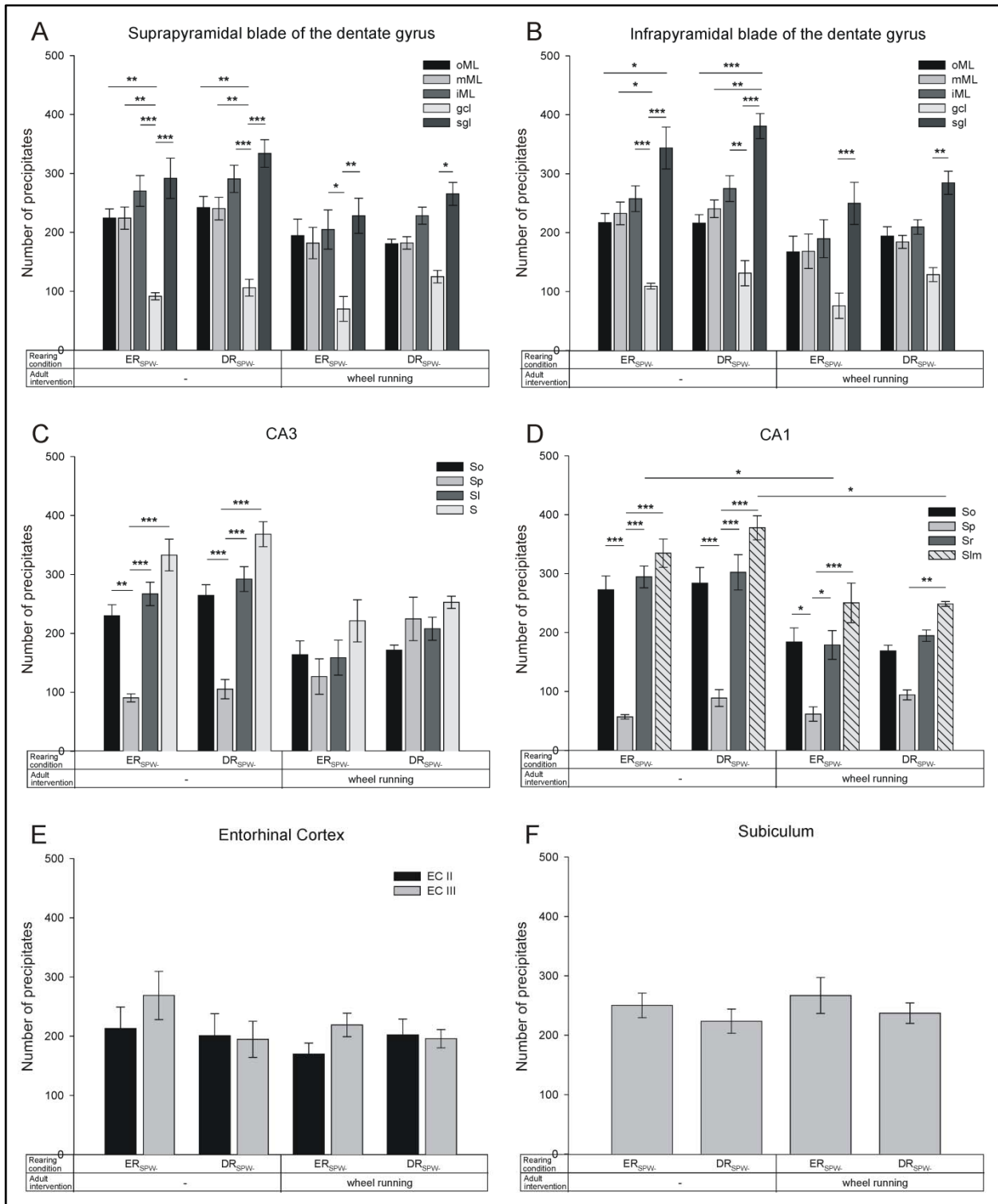


Fig. 5-3: Number of precipitates for each layer in the A) suprapyramidal and B) infrapyramidal blade of the dentate gyrus, in the C) CA3, D) CA1, E) entorhinal cortex and F) subiculum for the different treatment groups; ER_{SPW-}, socially and physically enriched-reared animals; DR_{SPW-}, socially and physically deprived-reared animals; wheel running, additional wheel-running challenge (pd 74-78); oML, outer molecular layer; mML, intermediate molecular layer; iML, inner molecular layer; gcl, granule cell layer; sgl, subgranular layer; spb, suprapyramidal blade; ipb, infrapyramidal blade; So, stratum oriens, Sp, stratum pyramidale; Sl, stratum lucidum; Sr, stratum radiatum; Slm, stratum lacunosum moleculare; EC II, entorhinal cortex lamina II; EC III, entorhinal cortex lamina III; data given as means ± S.E.M.

| measuring field | | | Number of precipitates | | | | | | | | | | |
|-----------------|-------|-----|------------------------|-------------|--|------|-------------|--|------|-------------|--|------|--|
| | | | ER _{SPW-} | | DR _{SPW-} | | | ER _{SPW-} after wheel running | | | DR _{SPW-} after wheel running | | |
| | | | value (abs) | value (abs) | difference ER _{SPW-} - DR _{SPW-} (%) | p | value (abs) | difference ER _{SPW-} - ER _{SPW-} after wheel running (%) | p | value (abs) | difference DR _{SPW-} - DR _{SPW-} after wheel running (%) | p | |
| dentate gyrus | oML | spb | 224 | 242 | 8 | n.s. | 195 | -13 | n.s. | 181 | -25 | n.s. | |
| | | ipb | 217 | 216 | -1 | n.s. | 167 | -23 | n.s. | 194 | -10 | n.s. | |
| | mML | spb | 224 | 240 | 7 | n.s. | 182 | -19 | n.s. | 182 | -24 | n.s. | |
| | | ipb | 233 | 240 | 3 | n.s. | 168 | -28 | n.s. | 184 | -23 | n.s. | |
| | iML | spb | 270 | 291 | 8 | n.s. | 205 | -24 | n.s. | 228 | -21 | n.s. | |
| | | ipb | 258 | 275 | 7 | n.s. | 190 | -26 | n.s. | 209 | -24 | n.s. | |
| | gcl | spb | 92 | 106 | 16 | n.s. | 70 | -23 | n.s. | 125 | 18 | n.s. | |
| | | ipb | 109 | 131 | 20 | n.s. | 76 | -31 | n.s. | 129 | -2 | n.s. | |
| | sgl | spb | 292 | 334 | 14 | n.s. | 228 | -22 | n.s. | 265 | -21 | n.s. | |
| | | ipb | 344 | 381 | 11 | n.s. | 250 | -27 | n.s. | 284 | -25 | n.s. | |
| | CA3 | So | 230 | 265 | 15 | n.s. | 164 | -29 | n.s. | 171 | -35 | n.s. | |
| | | Sp | 91 | 105 | 16 | n.s. | 127 | 40 | n.s. | 224 | 113 | 0.09 | |
| Sl | | 267 | 292 | 9 | n.s. | 159 | -41 | 0.09 | 208 | -29 | n.s. | | |
| Sr | | 333 | 368 | 11 | n.s. | 221 | -34 | 0.07 | 253 | -31 | n.s. | | |
| CA1 | So | 273 | 284 | 4 | n.s. | 184 | -33 | n.s. | 169 | -40 | 0.06 | | |
| | Sp | 57 | 89 | 56 | n.s. | 62 | 8 | n.s. | 94 | 6 | n.s. | | |
| | Sr | 294 | 302 | 3 | n.s. | 179 | -39 | <0.05 | 195 | -36 | n.s. | | |
| | Slm | 335 | 378 | 13 | n.s. | 250 | -25 | n.s. | 249 | -34 | <0.05 | | |
| EC | L II | 213 | 170 | -20 | n.s. | 201 | -6 | n.s. | 202 | 19 | n.s. | | |
| | L III | 269 | 219 | -18 | n.s. | 195 | -28 | n.s. | 196 | -11 | n.s. | | |
| Subiculum | | 250 | 267 | 7 | n.s. | 224 | -11 | n.s. | 237 | -11 | n.s. | | |

Tab. 5-1: Absolute numbers of silver-stained precipitates in equally-sized measuring fields in different hippocampal regions and laminae.

ER_{SPW-}, socially and physically enriched-reared animals; DR_{SPW-}, socially and physically deprived-reared animals; after wheel running, additional wheel-running challenge in adulthood (pd 70-74); oML, outer molecular layer; mML, intermediate molecular layer; iML, inner molecular layer; gcl, granule cell layer; sgl, subgranular layer; spb, suprapyramidal blade; ipb, infrapyramidal blade; So, stratum oriens, Sp, stratum pyramidale; Sl, stratum lucidum; Sr, stratum radiatum; Slm, stratum lacunosum moleculare; EC, entorhinal cortex; L, lamina; statistical differences between laminae only shown in Fig. 5-3.

5.4. DISCUSSION

We quantified naturally-occurring presynaptic turnover rates in the hippocampal formation. Synaptic remodeling is a naturally-occurring process of structural plasticity in the juvenile and adult brain that occurs in two major forms: structural modification and synapse elimination. Within the synapse, this process is accompanied by autophagy and lysosomal degradation of presynaptic components (Wolff et al., 1989).

Through the method of silver impregnation of lysosomal accumulations (Gallyas et al., 1980) the transient accumulations can be visualized and quantified (Wolff et al., 1989; Teuchert-Noodt et al., 1991; Wolff and Missler, 1993).

We specifically investigated whether being deprived of rich social and physical stimuli during adolescent brain development (pd 21-74) leads to alterations in synaptic turnover rates in young adult animals either under sedentary conditions and/or after additional wheel running.

Significant layer-specific differences in the amount of synaptic turnover were found both in the dentate gyrus and in the CA3 and CA1 region of the hippocampus proper of our enriched-reared control animals. The same pattern of a layer-specific distribution was seen in socially and physically deprived-reared animals. Thus, unlike the finding of much stronger effects in gerbils (Keller et al., 2000; Butz et al., 2008), social and physical deprivation during rearing seemed to have less effect on synaptic remodeling in CD1 mice. However, four days of subsequent wheel running exerted strong effects, both in enriched and deprived-reared individuals. It led to a leveling of layer-specific differences. This was mainly by decreasing synaptic-turnover rates in all layers except the granule cell and pyramidal cell layer. These effects were limited to the dentate gyrus and hippocampus proper. No effects of our treatment conditions were found for the entorhinal cortex and subiculum.

According to these findings, three main questions will be discussed in the following: Since noteworthy layer-specific differences in synaptic-turnover rates occurred, we will first address the issue what may determine these layer-specific differences in enriched-reared individuals. We expect this group to exhibit rather natural, intact plastic capacities within the hippocampal formation.

Secondly, we will follow the question why social and physical deprivation during rearing did not affect these capacities in CD1 mice, such as was found in gerbils.

Finally, we try to explain how wheel running might affect synaptic turnover in both enriched and deprived-reared individuals, and what the potential relationship may be between the stimulation of cell proliferation and the leveling of layer-specific differences of synaptic-turnover rates.

Synaptic remodeling in the hippocampal formation of enriched-reared CD1 mice

The dentate gyrus as well as the CA3 and CA1 region of the hippocampus proper displayed high amounts of synaptic turnover, indicating a high dynamic in these structures. In the dentate gyrus this is conceivable, since especially the inner molecular layer shows high plastic

capacities. Following lesions of the perforant path that terminates in the outer molecular layer, commissural and associative fibers normally terminating as recurrents in the inner molecular layer are able to sprout and make contacts with targets in the outer molecular layer, a phenomenon which is not seen *vice versa* (reactive synaptogenesis; Lynch et al., 1973; Cotman & Lynch, 1978; Frotscher et al., 1995). Beside the recurrent commissural and associative fibers, hilar commissural-associational pathway-related cells (HICAP cells), a class of interneurons located in the subgranular layer, innervate the proximal dendrites of granule cells in conjunction with commissural-associational afferents (Han et al., 1993; Sik et al., 1997), all together forming a highly plastic canonical microcircuit (Teuchert-Noodt, 2000; Fig. 5-4). Additional inputs from the septum are also open to shaping or tuning processes (rev. in Frotscher et al., 1996). These plastic processes in the inner molecular layer are susceptible to environmental manipulations (naturally-occurring synaptic remodeling; Hildebrandt, 1999; Butz et al., 2008).

The subgranular layer showed similar if not higher amounts of synaptic turnover especially in the infrapyramidal blade. In the subgranular layer, granule cells form synapses with mossy cells as well as interneurons like basket cells, the hilar commissural-associational path-

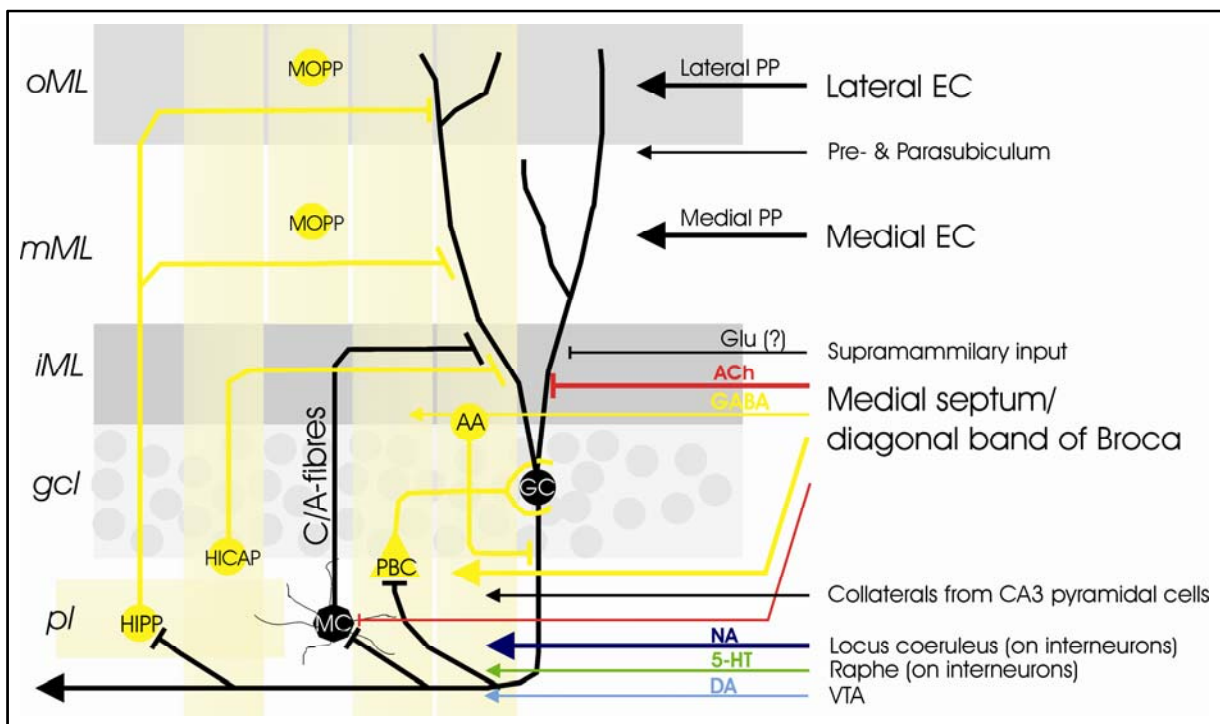


Fig. 5-4: Layer-specific connectivity patterns of the dentate gyrus.

oML, outer molecular layer; mML, middle molecular layer; iML, inner molecular layer; gcl, granule cell layer; pl, polymorphic layer; EC, entorhinal cortex; PP, perforant path; Glu, glutamate; ACh, acetylcholine; NA, noradrenaline; DA, dopamine; VTA, ventral tegmental area; GC, granule cell; MC, mossy cell; PBC, pyramidal basket cell; AA, axo-axonic cell; MOPP, HICAP, HIPP, further interneurons; C/A-fibres, commissural-associative fibres; (for further explanations of the connectivity see text).

way-related cells and hilar perforant path-associated cells (Freund & Buzsaki, 1996; for details see Amaral & Lavanex, 2006; Fig. 5-4). In addition, the subgranular layer - like the inner molecular layer - receives inputs from the septum, and further neurotransmitters like dopamine, serotonin and norepinephrine act in a modulatory way (rev. in Vizi & Kiss, 1998). As the only layer of the dentate gyrus, the subgranular layer receives collaterals of especially proximally located CA3-pyramidal cells (Amaral & Lavanex, 2006). Thus, both the inner molecular layer and the subgranular layer have in common to be the target of feed-back and feed-forward loops and modulatory transmitter influences (Fig. 5-4).

Furthermore, adult neurogenesis adds a further source of structural plasticity to the dentate gyrus, since thousands of new neurons are added to the granule cell layer every day and demand synaptic integration (for reviews see Teuchert-Noodt, 2000; Lehmann et al., 2005; Gould, 2006). The inner molecular and the subgranular layer are the first layers, in which dendrites (inner molecular) and axons (subgranular layer) of newborn neurons grow in, express neurotrophins (Seki & Arai, 1993; Cameron et al., 1993) and demand integration (Kaplan & Bell, 1983; Cameron et al., 1993; Kempermann et al., 2004). Thereby, they first receive GABAergic innervation, which is later partially replaced by glutamate (Ge et al.,

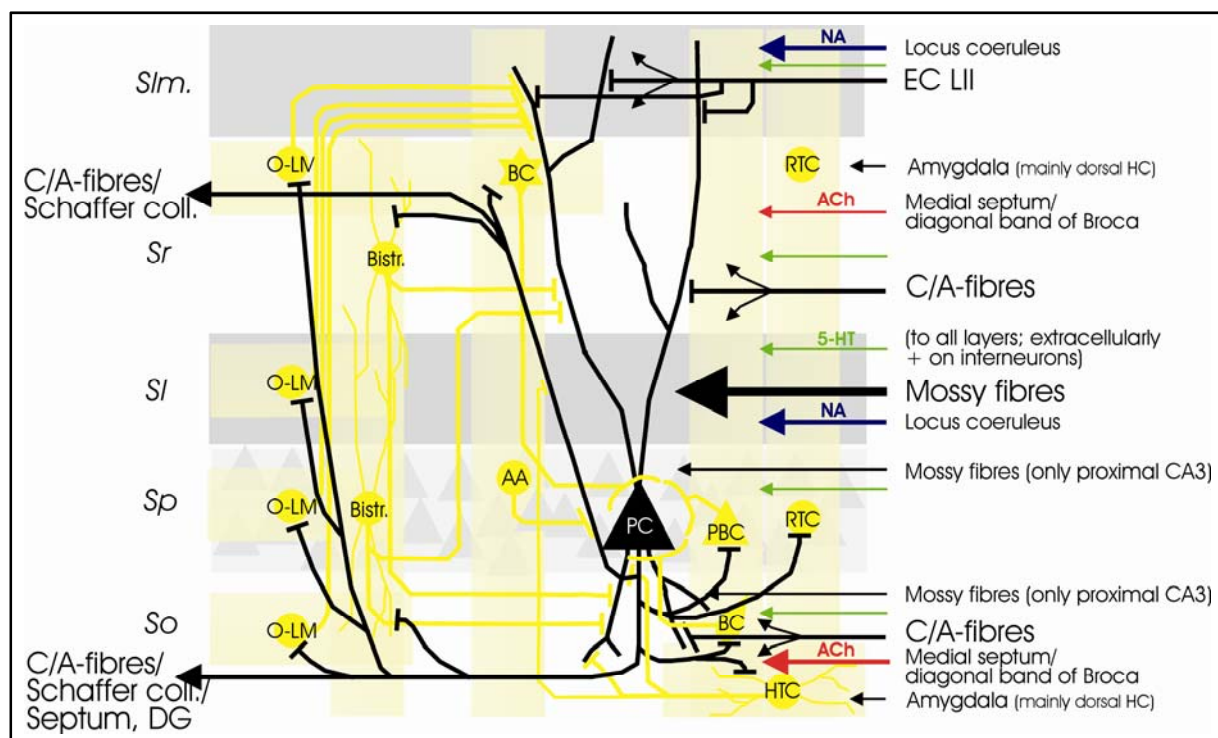


Fig. 5-5: Layer-specific connectivity patterns of the CA3 region.

Slm, stratum lacunosum moleculare; Sr, stratum radiatum; Sl, stratum lucidum; Sp, stratum pyramidale; So, stratum oriens; EC, entorhinal cortex; ACh, acetylcholine; NA, noradrenaline; PC, pyramidal cell; PBC, pyramidal basket cell; BC, basket cell; AA, axo-axonic cell; RTC, HTC, O-LM, Bistr., further interneurons; C/A-fibres, commissural-associative fibres; DG, dentate gyrus; HC, hippocampus (for further explanations of the connectivity see text).

2006). Both features, the specific connectivity and the challenge to integrate new neurons, may account for the high rates of naturally-occurring synaptic turnover in these layers.

A similar layer-specific distribution of synaptic turnover can be seen in CA3 and CA1 and also in these regions this distribution can be explained by innervation patterns. Like in the dentate gyrus, the layers with commissural and associative and collateral fibers as well as interneuronal modulation targets show the highest amount of synaptic turnover. This is the stratum radiatum of CA3, in which - like in the dentate gyrus - commissural and associative fibers as well as inputs from the septum terminate and most of the interneurons synapse on the pyramidal dendrites (Freund & Buzsaki, 1996; Amaral & Lavanex, 2006; Fig. 5-5).

Also the stratum radiatum of CA1 is the target of collaterals (Schaffer collaterals) and commissural projections as well as septal inputs and interneuronal modulation (Freund & Buzsaki, 1996; Amaral & Lavanex, 2006; Fig. 5-6). This innervation pattern is similar to that of the stratum oriens in CA1, which receives as intensive Schaffer collateral innervation as the stratum radiatum, and thus could explain the rather identical synaptic-turnover rates. Interestingly, the stratum oriens in CA3, although it is also target of commissural and associative fibers and septal inputs (Amaral & Lavanex, 2006), strikes with its rather few synaptic turnover. One reason could be that in CA3 this layer receives rather few commissural and associative fibers compared to the massive innervation of Schaffer collaterals in the CA1 stratum oriens.

Another interesting result is that in the stratum lacunosum moleculare of CA1, even higher synaptic-turnover rates occurred than in the stratum radiatum and oriens of this region. It is the target of perforant path fibers mainly from layer III of the entorhinal cortex (and to lesser extent also from layer II) and gets modulatory influence from the locus coeruleus (Kulkarni et al., 2002), perirhinal cortex and thalamus (Amaral & Lavanex, 2006; Fig. 5-6). Perhaps, especially the modulatory influences may account for the high naturally-occurring structural plasticity of this layer.

Astonishingly, the mossy fiber targets in the stratum lucidum in CA3 (Fig. 5-5) were not the site of the highest amounts of synaptic turnover. The extent of the mossy fiber targets is known to be correlated to spatial learning abilities in rats and mice (Lipp et al., 1987; Schwegler & Crusio, 1995). However, an effect of learning on their extent was only proven in rats (Holahan et al., 2006). Mice do not show mossy fiber sprouting in response to learning and thus seem to have rather low plastic potentials in mossy fiber targets (Rekart et al., 2007). This may explain why we did not find higher rates of synaptic turnover in the stratum lucidum than in the other molecular layers of CA3.

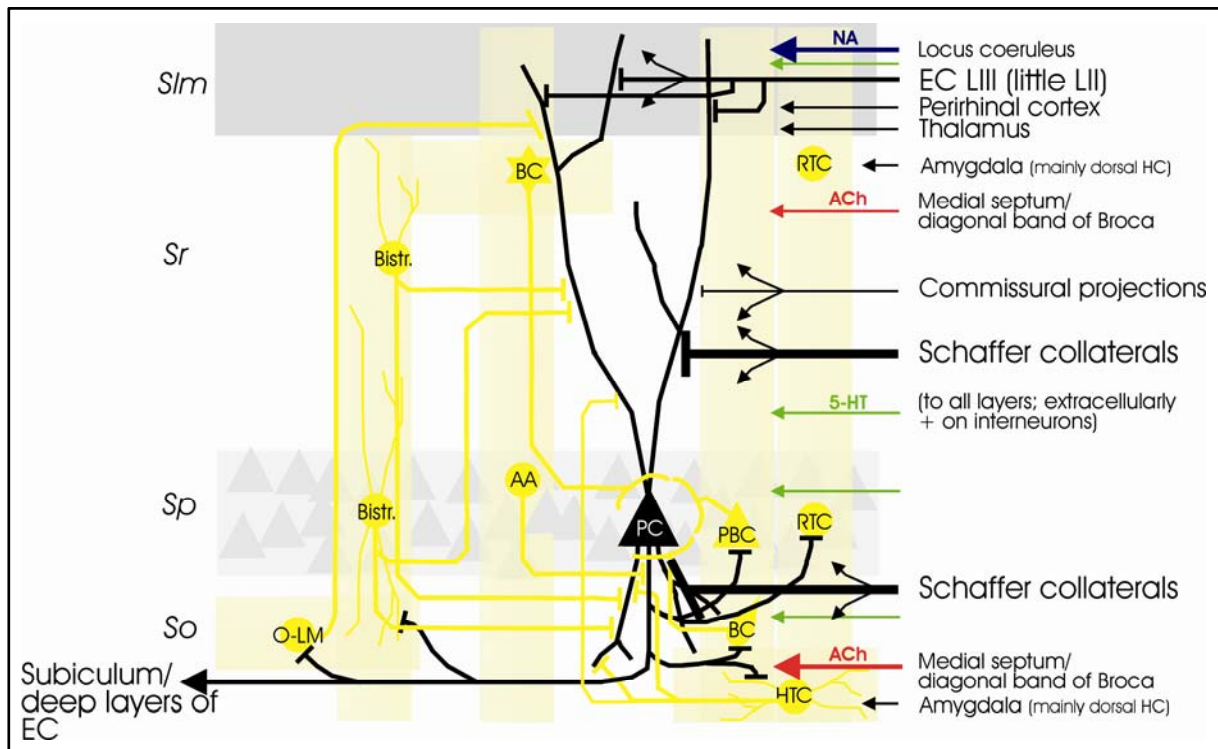


Fig. 5-6: Layer-specific connectivity patterns of the CA1 region.

Slm, stratum lacunosum moleculare; Sr, stratum radiatum; Sp, stratum pyramidale; So, stratum oriens; EC, entorhinal cortex; ACh, acetylcholine; NA, noradrenaline; PC, pyramidal cell; PBC, pyramidal basket cell; BC, basket cell; AA, axo-axonic cell; RTC, HTC, Bistr., further interneurons; HC, hippocampus; EC, entorhinal cortex; L, lamina (for further explanations of the connectivity see text).

Compared with the dentate gyrus and hippocampus proper, the subiculum and entorhinal cortex layer II and III displayed lesser and more equal synaptic remodeling. In the subiculum, we could only set one measuring window and in the entorhinal cortex we choose the main output layers for analysis. Perhaps, one would get a different pattern of synaptic remodeling, if one compares these output layers with the layers where inputs and modulatory transmitter influences converge. However, as we wanted to focus on the significance of neurogenesis for synaptic remodeling, we constrained our observations to the layers giving the main input to the dentate gyrus.

Synaptic remodeling in mice reared under social and physical deprivation

Remarkably, in CD1 mice, no statistically significant effects of social and physical deprivation during rearing could be seen on synaptic remodeling, neither in the dentate gyrus nor in the hippocampus proper, subiculum or entorhinal cortex. By contrast, in adult gerbils, synaptic-turnover rates decrease in the inner molecular layer, as well as in the intermediate and outer molecular layer, in response to deprived rearing. In the left hemisphere, further decreases are apparent in the subgranular and even in the granule cell layer (Butz et al.,

2008). However, the gerbil also shows an increase in cell proliferation after deprived rearing (Hildebrandt, 1999; Keller et al., 2000). At first sight, this inverse relationship is surprising, since newborn neurons constitute a further source of structural plasticity, and one would expect that more newborn neurons should lead to more synaptic remodeling. In fact, it seems that an exhaustion of available synapses is the consequence, if too many newborn neurons demand integration, making the inverse relationship between neurogenesis and synaptic remodeling feasible (Keller et al., 2000; Teuchert-Noodt, 2000; Butz et al., 2008). Since we did not find an effect of deprived rearing on cell proliferation in CD1 mice (project I, chapter 3), it is conceivable that also no alterations in synaptic-remodeling rates occurred in these animals.

Synaptic remodeling after wheel running

Four days of wheel running in young adulthood exerted strong effects on synaptic remodeling in the dentate gyrus and hippocampus proper of both socially and physically deprived and enriched-reared CD1 mice. It led to a leveling of the layer-specific differences mainly by decreasing synaptic-turnover rates in all layers except the granule and pyramidal cell layer. These effects did not extend to the subiculum or entorhinal cortex, indicating a lower dynamic and sensitivity to external influences in these regions.

Socially and physically deprived-reared CD1 mice showed the same inverse relationship between cell proliferation and synaptic remodeling in the dentate gyrus as found in gerbils. No enhancement of cell proliferation was found after wheel running in these animals (project I, chapter 3), and here we now found a strong effect on synaptic turnover rates, too. However, in enriched-reared CD1 mice, an effect of wheel running on cell proliferation was not found (project I, chapter 3), while synaptic turnover rates were, nevertheless, affected. Hence, factors other than the enhancement of cell proliferation played a role in affecting synaptic-turnover rates after the wheel-running challenge. A crucial point may be the duration of the intervention and the resulting response of cell-proliferating activity. Socially and physically deprived rearing is a chronic challenge during brain development leading to chronically enhanced cell proliferation in adult gerbils. In contrast, our only four days lasting adult wheel-running challenge enhanced cell proliferation rates acutely and only in deprived-reared individuals (project I, chapter 3). Three to six days after the challenge, cell proliferation rates reach a normal level again (Santoso, 2009). Thus, the time window, in which enhanced cell proliferation rates can affect synaptic remodeling, may

be narrow. It is likely, that other factors mediated the effects of wheel running on synaptic turnover.

In fact, wheel running seems to enhance long-term potentiation (van Praag et al., 1999a), dendritic growth and complexity as well as spine densities (Eadie et al., 2005; Stranahan et al., 2007), although not all studies could confirm this (Faherty et al., 2003; Zhao et al., 2006). These latter postsynaptic processes can be directly influenced by, e.g., neurotrophic factors like brain derived neurotrophic factor (McAllister et al., 1996; Tolwani et al., 2002; Tyler & Pozzo-Miller, 2003), which is enhanced in the dentate gyrus and hippocampus proper in response to wheel running (Neeper et al., 1995, 1996; Farmer et al., 2004). Thus, it seems feasible, that neurotrophic factors might also affect presynaptic remodeling processes.

The assumption that other, perhaps intrinsic influences, must account for the effects of adult wheel running on synaptic remodeling is supported by the fact that altered synaptic-turnover rates did not only occur in the dentate gyrus, but also in CA3 and CA1. Axons of newborn neurons reach CA3 already 4-10 days after birth (Hastings & Gould, 1999; Zhao et al., 2006), thus, in the time window in which we analyzed synaptic turnover after wheel running. However, if alterations in the number of newborn neurons already affected synaptic plasticity in the hippocampus proper, one would expect the greatest effects in the mossy-fiber termination field, the stratum lucidum of CA3, and not in CA1. It seems in fact that in CD1 mice, CA3 and CA1 exhibit also intrinsic plasticity. This assumption finds support in recent findings of a systematic analysis of synaptic-turnover rates in diverse cortical areas of the gerbil, showing that also associative cortical areas display synaptic remodeling and region- as well as layer-specific differences which are mainly determined by local and distant associative connections (Neufeld et al., 2009). These layer-specific differences were leveled after environmental deprivation during rearing, an effect that parallels our findings after wheel running in CD1 mice.

Taken together, our results concerning synaptic remodeling in CD1 mice confirm our former hypothesis that environmentally-dependent plasticity in the hippocampal formation is generally lower in CD1 mice than in gerbils, perhaps as a consequence of domestication or as a species-dependent effect. Furthermore, they provide more support for a relationship between neurogenesis and synaptic remodeling in the rodent brain.

6. PROJECT IV:

PREFRONTAL ACTIVITY REGULATES CELL PROLIFERATION IN THE ADULT MOUSE DENTATE GYRUS

6.1. INTRODUCTION

Many local mechanisms have been demonstrated to regulate cell proliferation and neurogenesis in the adult dentate gyrus. These include neurotrophic and other growth factors (Wagner et al., 1999; Aberg et al., 2000; O'Kusky et al., 2000; Trejo et al., 2001; Fabel et al., 2003; Cao et al., 2004; Scharfman et al., 2005), local transmitter influences (Jacobs & Fornal, 1997; Brezun & Daszuta, 1999, 2000; Malberg et al., 2000; Kulkarni et al., 2002; Wakade et al., 2002; Cooper-Kuhn et al., 2004; Mohapel et al., 2005), glucocorticoids (Gould et al., 1992; Cameron & Gould, 1994) and opioids (Eisch et al., 2000; Persson et al., 2003).

However, external influences like deprivation of natural environmental stimulation during brain development impact not only neuroplasticity in the hippocampus but have far-reaching consequences on the whole brain. Social and physical deprivation during juvenile development leads to reduced dopaminergic innervation of the prefrontal cortex (Winterfeld et al., 1998; Neddens et al., 2001), and abnormalities in transmission and receptor expression of neurotransmitters (Dalley et al., 2002; Ago & Matsuda, 2003; Melendez et al., 2004; Leng et al., 2004; Brummelte et al., 2007; Gregory & Szumlinski, 2008; Bloomfield et al., 2008). Moreover, maturation of prefrontal pyramids (Pascual et al., 2007) and their efferents to frontal, parietal and limbic areas is reduced (Bagorda et al., 2006; Witte et al., 2007). In contrast, transmitter innervation and activity is enhanced in limbic circuits (Neddens et al., 2002, 2003; Lehmann et al., 2003, 2004; Busche et al., 2004, 2006; Lesting et al., 2005; Galani et al., 2007).

It has often been suggested that these dysbalances in the prefrontal-limbic system impact neuroplasticity in the adult hippocampus (Teuchert-Noodt, 2000; Keller et al., 2000; Butz et al., 2006; project I, chapter 3; project II, chapter 4). Nevertheless, there is so far no examination of the particular involvement of the prefrontal cortex in the regulation of cell proliferation in the hippocampus. We proposed a hypothetical model integrating local as well as systemic mechanisms involved in the regulation of adult hippocampal cell proliferation and drew particular attention to the prefrontal cortex (project II, chapter 4). We hypothesized that

activity from the prefrontal cortex is a negative regulator of cell proliferation via activation of the NMDA-receptor system at the entorhino-dentate interface.

The present study was conducted to test the hypothesis that the prefrontal cortex is causally involved in the regulation of cell proliferation. In a first step, we employed stimulation of cell proliferation and, in a second step, stimulation of prefrontal activity. Female CD1 mice were reared under socially and physically deprived conditions and exposed to a 4-day wheel-running challenge in young adulthood. Wheel running is a common stimulator of cell proliferation in deprived-reared CD1 mice (project I, chapter 3). Furthermore, it was demonstrated that the pro-proliferating effect of running in these animals persists for at least three days after withdrawal of the running wheel (Santoso, 2009). Afterwards we examined, whether stimulation of prefrontal activity by either environmental enrichment and/or a prefrontal-cortex-dependent (PFC-dependent) behavioral task (Dudchenko, 2004) during this short time window can decrease cell proliferation compared to control conditions, i.e., continued deprived housing or an unspecific behavioral control task. As working-memory task we chose a spatial-delayed alternation task, because it is an established task undoubtedly involving and activating the prefrontal cortex (Wikmark et al., 1973; Larsen & Divac, 1978; Nonneman & Kolb, 1979; Thomas & Brito, 1980; Brito et al., 1982; Silva et al., 1986; de Brabander et al., 1991; Baeg et al., 2003, 2007). Since stress is a negative regulator of cell proliferation (Gould et al., 1992; Cameron & Gould, 1994), our main concern was to minimize stress by using an automated spatial-delayed alternation task in a T-maze, instead of a manually-operated which would involve frequent handling of animals.

6.2. MATERIAL & METHODS

6.2.1. ANIMALS AND REARING CONDITIONS

34 female CD1 mice at the age of postnatal (pd) 21 were obtained from Charles River Laboratories (Sulzfeld, Germany) and reared in our animal facility under socially and physically deprived conditions (see chapter 2.2) until young adulthood. They received food and water *ad libitum* and were on a 12h-light-dark schedule.

6.2.2. TRANSPONDER TAGGING

For individual identification, all animals were equipped with transponders on pd 50 (for details see chapter 2.4). After 24 hours and again after one week, all animals were checked for retention of transponders, and if necessary the transponder was re-injected.

6.2.3. EXPERIMENTAL TREATMENT IN ADULTHOOD

Four different groups were defined on the basis of the treatment following a 4-day wheel-running challenge in young adulthood: socially and physically deprived (DC) or enriched-housing conditions (EC), a PFC-dependent working-memory task in an automated T-maze (PFC) or a T-maze control task (TC; Fig. 6-1). At the beginning of the wheel-running challenge, animals were 10-13 weeks old. Since the exposure to tasks in the automated T-maze required previous habituation of the animals to the apparatus, the wheel-running challenge had to take place in a home cage already connected to the apparatus. This led to a slightly different protocol for PFC, TC than for DC and EC animals. The different wheel-running environment was supposed not to cause differences in running activity, since wheel running occurs irrespective of the environment (Brant & Kavanau, 1964; de Kock & Rohn, 1971; Roper & Polioudakis, 1977; Sherwin & Nicol, 1996; Sherwin, 1998), what has recently been shown also for female CD1 mice (Haupt & Schäfers, *accepted*).

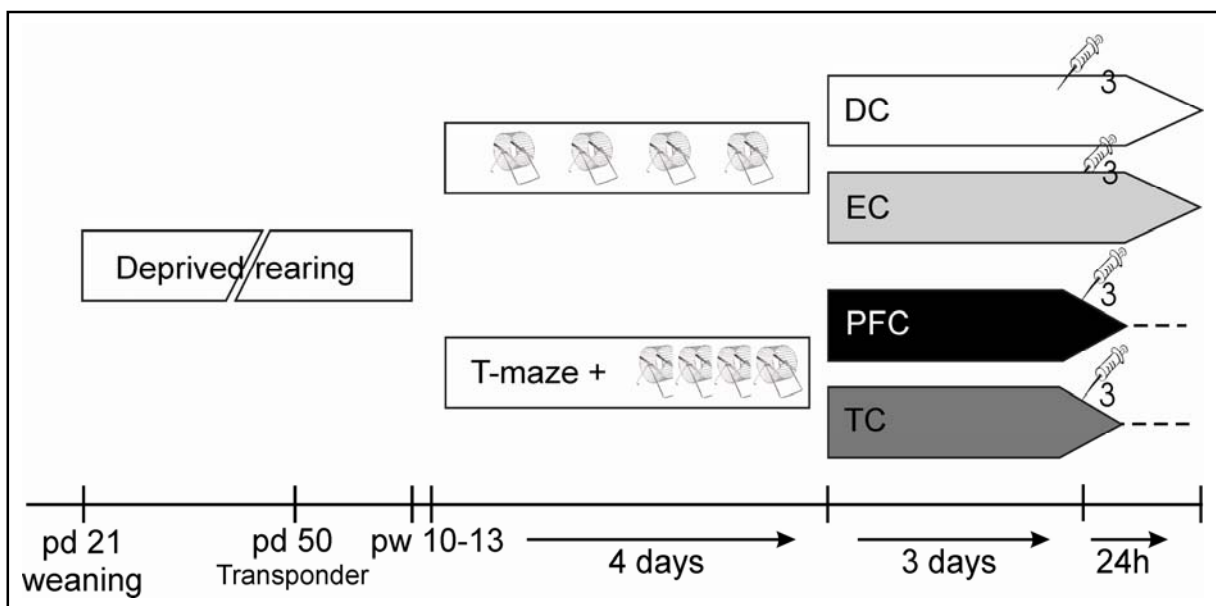


Fig. 6-1: Experimental design with time axis showing animal age in postnatal days (pd) and weeks (pw); from pd 21, rearing under socially and physically deprived conditions; on pd 50, all animals were equipped with transponders; in pw 10-13, wheel-running challenge for four days; animals assigned to deprived (DC) or enriched housing (EC) after wheel running ran individually in their home cages; animals assigned to the PFC (PFC) or T-maze control task (TC) ran in groups of four in the home cage connected to the T-maze; all animals received three BrdU-injections in the last dark phase of the experiment and survived for 24 hours.

6.2.3.1. DC and EC group

At the beginning of the experimental treatment in adulthood, DC (n=7) and EC animals (n=7) were weighed 2 hours prior to the onset of the dark phase. 4 hours later, individual cages were equipped with a running wheel (Fig. 6-2, A). After the fourth dark phase, the wheels were removed. The DC-animals remained under socially and physically deprived conditions. The EC-animals were united and transferred to an enclosure (100 x 100 x 50 cm) that contained enrichment items but no running wheels (for details of the enrichment see chapter 2.2). Both groups received food and water *ad libitum* (Fig. 6-3, column DC and EC).

6.2.3.2. PFC and TC group

PFC (n=8) and TC animals (n=8) were divided into two groups of four animals each and were exposed to the apparatus and the respective procedure successively. At the beginning of the experimental treatment in adulthood, each group was weighed 2 hours prior to the onset

of the dark phase and transferred to a type IV cage, which was connected to the automated T-maze (for detailed description of the T-maze see chapter 2.5.2) to allow the animals to habituate to the apparatus (start of *Habituation Phase 1*). 4 hours later, the running wheels were mounted into the cage (Fig. 6-2, B). The habituation phase of 4 hours without access to running wheels was necessary, because in a preliminary experiment, in which animals had access to running wheels directly after introduction to the apparatus, animals preferred running over exploring the maze and thus did not receive water. Also in the main experiment, some animals disregarded thirst, although they had learned before where to get water (cf. Melcer & Timberlake, 1986; Looy & Eikelboom, 1989; Bauman, 1992), and had to be watered additionally. They were briefly taken out of the home cage, transferred to an individual cage, and received water *ad libitum* for 10 min. Then, they were set back into the

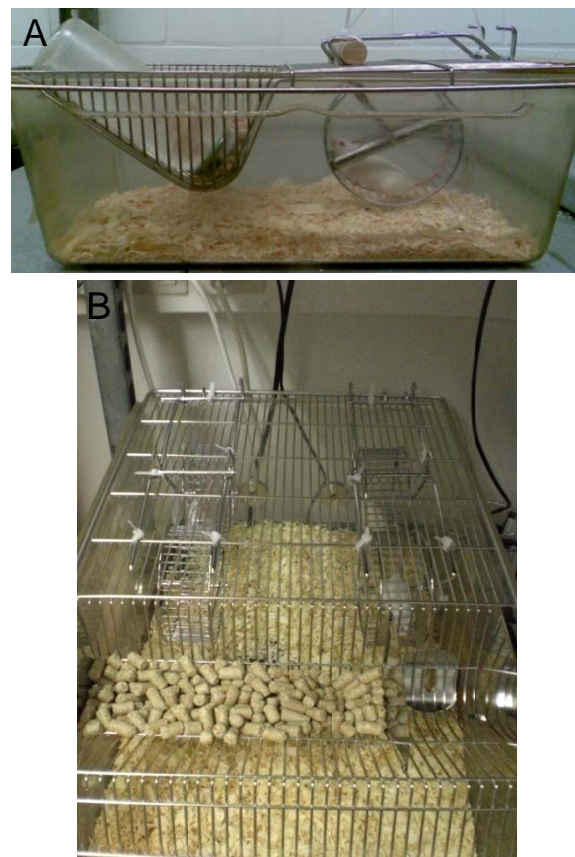


Fig. 6-2: Wheel-running challenge

A) Animals assigned to be kept in deprived or enriched conditions after wheel running ran individually in their home cages. **B)** Animals assigned to perform a PFC- or T-maze control task after wheel running ran in groups of four in a home cage connected to the automated T-maze. In both cases one wheel per animal was provided.

home cage. This additional watering was counted and later correlated to the number of BrdU-positive cells to check for an unwanted side effect).

Habituation Phase 1, in which the animals could explore the apparatus all together without restraints, lasted 24 hours. During the subsequent *Habituation Phase 2*, which lasted 72 hours, the interposed sorting system (see chapter 2.5.2.1) allowed only one animal at a time to enter the automated T-maze. After the running wheels were removed from the cage at the end of the fourth dark phase and the animals were transferred to a type II cage, *Habituation Phase 2* still continued for 10 hours (see Fig. 6-3, column for PFC and TC animals).

After habituation, PFC animals were trained successively on a PFC-dependent working-memory task, i.e., spatial-delayed alternation task (for details concerning the task and the use of a T-maze see chapter 2.5.2). First, animals were trained to alternate in the T-maze during the *Pretraining*, which lasted 24 hours. Afterwards, during the *Training without delay*, which lasted 10 hours, the animals learned in *forced* and subsequent *choice runs* to keep alternating, even if they were allowed to choose the arm in the second run voluntary. The last phase, the *Training with delay*, in which a delay of 15 seconds was interposed between the *forced* and the *choice run*, during which the animal had to remember which arm it visited before, constituted the actual working-memory load and lasted 37 hours.

The TC animals performed a control task in the T-maze without alternation or working-memory load. Accordingly, after *Habituation Phase 2*, during the *PretrainingRandom*, TC animals were rewarded not in an alternating but in a random fashion, so that they were not trained to alternate. Also during the subsequent *TrainingRandom*, the

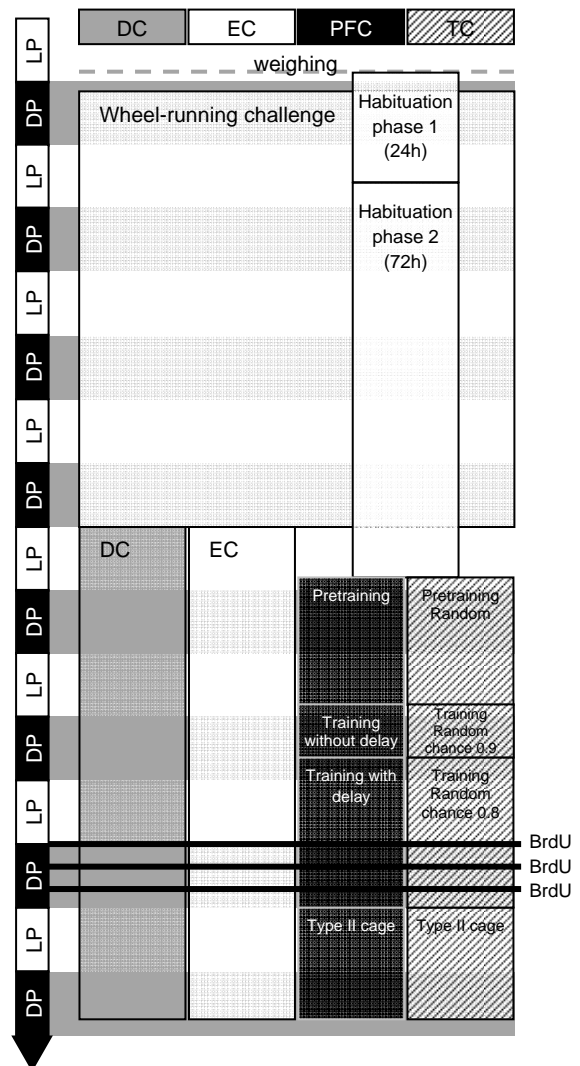


Fig. 6-3: Procedure of experimental treatment in adulthood with arrow on the left indicating the alternation of light (LP) and dark phases (DP) and columns showing the sequence of treatments for each group: DC, animals assigned for deprived housing after wheel running; EC, animals assigned for enriched housing after wheel running; PFC, animals assigned for the PFC-dependent task; TC, animals assigned for the T-maze control task (for further explanations see main text).

animals were rewarded in the *choice run* not depending on their choice, but randomly with a probability of 90% (adapted at the percentage of correct choices of the PFC animals in the respective phase). In the last phase, no delay was interposed between the *forced* and the *choice run* to avoid any working-memory load for animals of this group. However, the reward probability was adapted at 80%, i.e., at the percentage of correct choices of the PFC animals in this phase (for detailed descriptions of the individual protocols see chapter 2.5.2.2).

During all phases, PFC and TC animals received food *ad libitum* in the home cage and water in the T-maze with 20 μ l as a single reward. At the end of the experiment, the T-maze was disconnected and animals remained in their home cage with food and water *ad libitum* until sacrifice.

6.2.4. BRDU-LABELING, PERFUSION, IMMUNOHISTOCHEMISTRY

All animals received three injections of the DNA synthesis marker 5-bromo-2-deoxyuridine (BrdU; chapter 2.6.1) in the last dark phase of their respective housing or training condition beginning at the onset of the dark phase and followed by further injections in 4-hour intervals. 24 hours after the last injection, animals were deeply anaesthetized by an overdose of diethyl ether and transcardially perfused with 200 ml of 4% paraformaldehyde solution (for details see chapter 2.6.2). The brains were immediately dissected and stored over night in 4% PFA at 4°C. On the next day, brains were transferred to a 30% sucrose solution for cryoprotection for at least 24 hours. Then, the left hemispheres of the brains were freeze-sectioned with a Frigomobil. 40 μ m coronar sections were made throughout the septotemporal axis of the hippocampus (see chapter 2.6.2), collected in a cryoprotectant solution and stored at -20°C until further processing.

The immunohistochemical detection of BrdU-labeled cells was based on the two-step indirect ABC-(Avidin-Biotin-Complex-) method involving an unlabeled primary antibody (mouse anti-BrdU, Roche Molecular Biochemicals, Indianapolis, USA) and a biotinylated secondary antibody (M.O.M. anti-mouse reagent, Vector Laboratories, Burlingame, USA, and biotinylated goat anti-mouse, Vector Laboratories, Burlingame, USA; for further explanations see chapter 2.6.3).

6.2.5. QUANTITATIVE EVALUATION/STEREOLOGY

The slides were coded prior to quantitative analysis and the code was not broken until the analysis was completed. The number of BrdU-positive cells was estimated by counts made systematically on every third consecutive section along the rostrocaudal axis of the hippo-

campal formation (see chapter 2.6.4). On each section, BrdU-positive cells were counted in the granule cell layer and subgranular zone as well as in the hilus separately under the light microscope at 400-fold magnification. Additionally the volume of the granule cell and subgranular layer as well as the hilus was measured at 100-fold magnification (see chapter 2.6.4). The reference volume was estimated according to the Cavalieri principle: $V_{ref}=T \times \Sigma A \times 1/ssf$, where T is the thickness of the section, A is the area of the granule cell layer and 1/ssf is the inverse of the section sampling fraction (1/ssf=3).

The number of BrdU-positive cells was then related to the granule cell layer sectional volume to obtain the density of BrdU-positive cells per mm^3 . Additionally, the number of BrdU-positive cells was multiplied by the reference volume to estimate the total number of BrdU-positive cells per dentate gyrus.

6.2.6. DATA ANALYSES

During all phases of pretraining and training, the amount of runs, choices, correct (PFC animals) respectively rewarded choices (TC animals), and water intake of the individual animals were recorded and analyzed. Additionally, weight loss or gain of all animals during experimental treatment in adulthood as an indicator of, e.g., water deprivation (Bing & Mendel, 1931) or stress (Wallace, 1976), were documented, as well as necessary additional watering during the habituation/wheel-running of PFC and TC animals as another potential stressor.

In order to assess activity patterns of animals in the automated T-maze, the number of runs in 24 hours of *Pretraining* and *Training with delay/TrainingRandom* with 80% probability were calculated for dark and light phases.

One-way ANOVA was conducted over normal distributed data (Shapiro Wilks test for normality) with dependent variables of total number of BrdU-positive cells and density of BrdU-positive cells in the granule cell layer and hilus separately. Specific comparisons were made with Unequal N HSD post-hoc test. Pearson product-moment correlations were performed on total numbers and densities of BrdU-labeled cells in the granule cell layer of PFC and TC animals with weight loss or gain and water intake/24 h. Further correlations were made in PFC animals with number of correct choices during the *Training with delay*.

Data analyses were performed with Excel (Microsoft Office 2007), statistical analyses with Statistica 6.0 (StatSoft, Tulsa, USA).

6.3. RESULTS

6.3.1. QUALITATIVE RESULTS

All animals were visually checked for their wheel-running activity and could all be observed while running in a wheel. PFC, TC and EC animals were united with conspecifics for the treatments after the wheel-running challenge. No aggressive interactions were observed between animals in any of the groups. Contrastingly, social interactions like reciprocal grooming and sleeping together could be noticed already in the first hours.

All PFC and TC animals explored the automated T-maze during *Habituation Phase 1* and learned where to get water. However, during access to running wheels, most of them preferred running over entering the maze and drinking, so that water intake dropped rapidly. For some animals additional watering was even necessary.

Interestingly, although TC animals were not trained to alternate during the *PretrainingRandom*, they showed alternation behavior in the subsequent *TrainingRandom* phases.

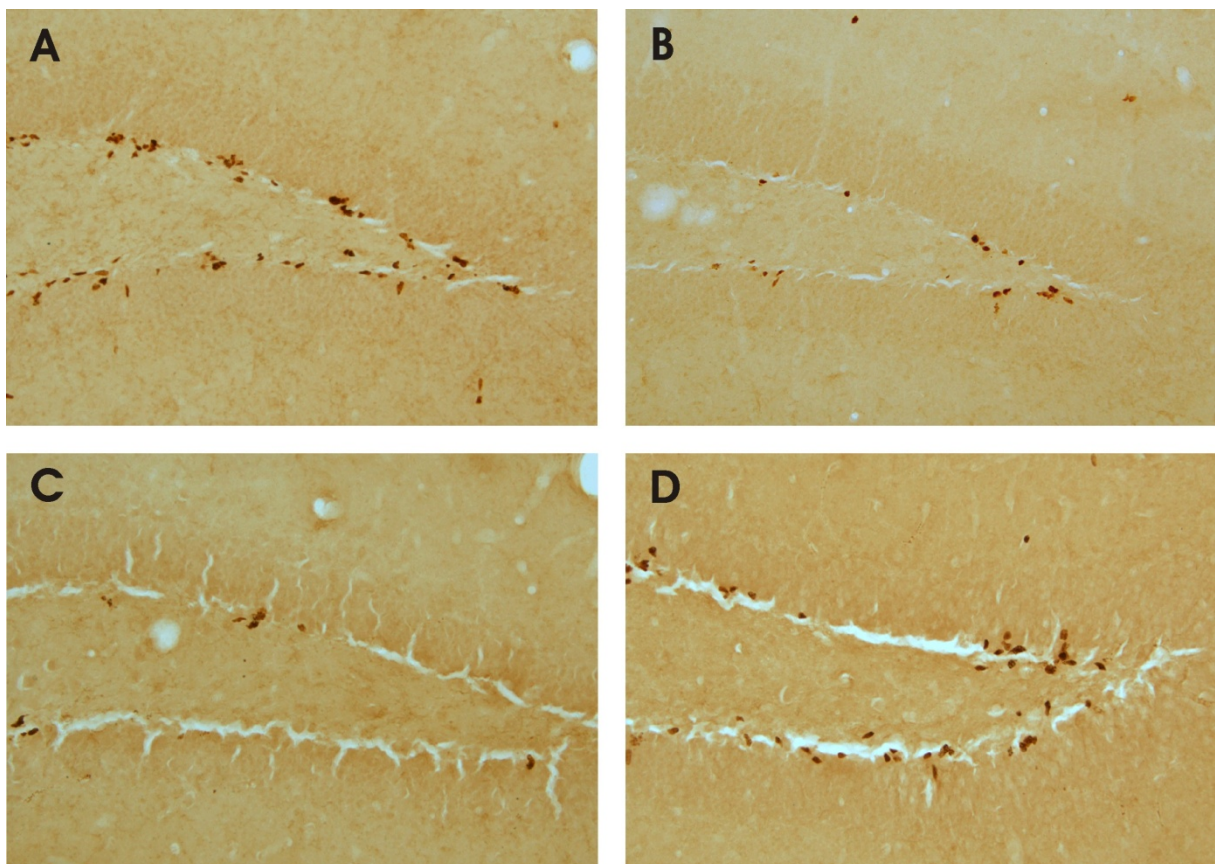


Fig. 6-4: Photomicrographs of BrdU-positive cells (200-fold magnification) 24 hours after the last BrdU injection in socially and physically deprived-reared CD1 mice after a 4-day wheel running challenge in young adulthood and subsequent **A)** socially and physically deprived or **B)** enriched housing, **C)** a PFC-dependent or **D)** a T-maze control task.

The immunohistochemical detection of cell proliferation revealed BrdU-positive cells indicated as brown to dark brown precipitates in all animals. The majority of BrdU-positive cells resides in the subgranular zone (Fig. 6-4). Photomicrographs of the dentate gyrus of DC, EC, PFC and TC animals exemplary illustrate the distribution of BrdU-positive cells (Fig. 6-4). Qualitatively, it is apparent that the number of BrdU-positive cells in the granule cell layer is lower in EC and PFC than in DC and TC animals. No differences are apparent concerning BrdU-positive cells in the hilus.

6.3.2. QUANTITATIVE RESULTS

In the following the statistical data for the total number of BrdU-positive cells per dentate gyrus are given. Similar results were seen for density of BrdU-positive cells per mm^3 and can be drawn from tables 6-1 and 6-2 and Fig. 6-5 and 6-6.

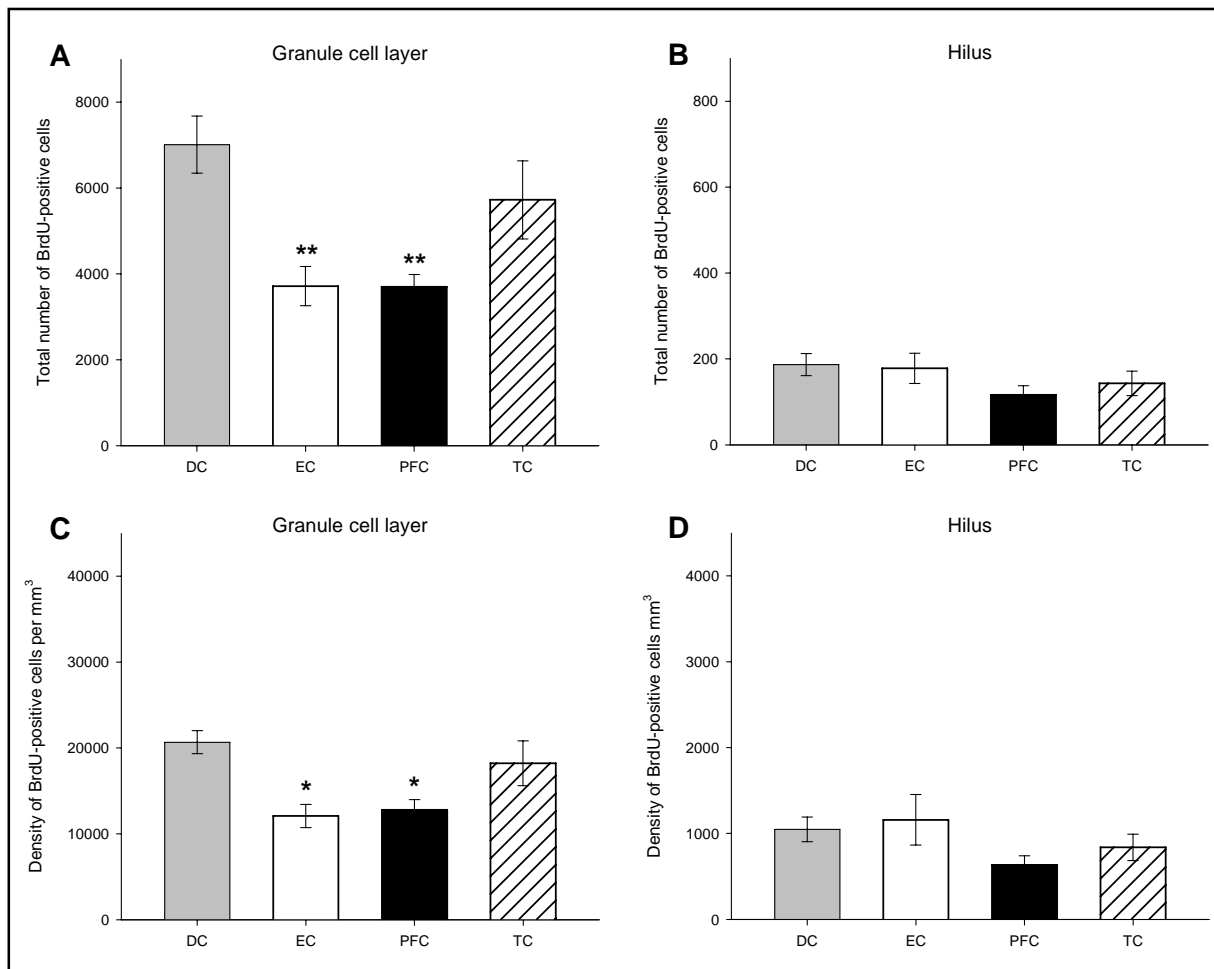


Fig. 6-5: A) Total number and C) density of BrdU-positive cells in the granule cell layer 24 hours after the last BrdU-injection; B) total number and D) density of BrdU-positive cells in the hilus 24 hours after the last BrdU-injection; DC, animals housed under socially and physically deprived conditions after wheel running; EC, animals housed under socially and physically enriched conditions after wheel running; PFC, animals which performed a PFC-dependent task after wheel running; TC, animals which performed a T-maze control task after wheel running; data given as means \pm S.E.M.

6.3.2.1. Enriched housing or performing a PFC-dependent task decreased cell proliferation in the granule cell layer after wheel running

Cell proliferation in the granule cell layer of the dentate gyrus was stimulated by a wheel-running challenge in young adulthood. Subsequent housing or training conditions had a strong impact on the total number of BrdU-positive cells (ANOVA, $F_{3,26}=6.39$, $p < 0.01$). Housing in an enriched environment for three days reduced the number of BrdU-positive cells significantly compared to control animals housed under continued deprivation (DC 7010 vs. EC 3716 cells per dentate gyrus; Post-hoc Unequal N HSD, $p < 0.01$) Fig. 6-5, Tab. 6-1). An equally strong effect was seen after the PFC-dependent task (PFC 3701 cells per dentate gyrus; Post-hoc Unequal N HSD, $p < 0.01$), but not after the T-maze control task (Post-hoc Unequal N HSD, $p > 0.05$).

Cell proliferation in the hilus was affected by neither enriched housing nor PFC-dependent nor T-maze control task after wheel running (ANOVA, $F_{3,26}=1.39$, $p > 0.05$; Fig. 6-5; Tab. 6-1).

| | | DC (100%) | EC | | | PFC | | | TC | | |
|-------|--------------|--------------|-------|------------|--------|-------|------------|--------|-------|------------|-------|
| | | value | value | diff. in % | p | value | diff. in % | p | value | diff. in % | p |
| gcl | Total number | 7010 | 3716 | -47 | < 0.01 | 3701 | -47 | < 0.05 | 5725 | -18 | > 0.5 |
| | Density | 20681 | 12086 | -42 | < 0.01 | 12801 | -38 | < 0.05 | 18226 | -12 | > 0.7 |
| hilus | Total number | 187 | 178 | - | n.s. | 116 | - | n.s. | 143 | - | n.s. |
| | Density | 1049 | 1159 | - | n.s. | 636 | - | n.s. | 839 | - | n.s. |

Tab. 6-1: Group comparison of total numbers and densities of BrdU-positive cells

DC, animals housed under socially and physically deprived conditions after wheel running; EC, animals housed under socially and physically enriched conditions after wheel running; PFC, animals which performed a PFC-dependent task after wheel running; TC, animals which performed a T-maze control task after wheel running; gcl, granule cell layer; total number of BrdU-positive cells per dentate gyrus; density of BrdU-positive cells per mm^3 ; given are absolute values (means), differences between the groups if significant, and p-values.

6.3.2.2. PFC and TC animals displayed most activity in dark phases

With the use an automated instead of a manually-operated T-maze we intended to enable the animals to display their main activity in the dark, i.e., their natural active phase. Accordingly, PFC as well as TC animals displayed most of their activity during the dark phases as indicated by the number of runs performed in 24 hours *Pretraining* (73% during the dark phase) and *Training with delay*, respectively *TrainingRandom* with 80% reward probability (75% of the runs during the dark phase).

6.3.2.3. Water deprivation or handling during additional watering does not account for the decrease in cell proliferation

All animals were weighed prior to the onset of experimental treatment in adulthood and at the end of the experiment to check for weight loss or gain as an indicator of, e.g., water deprivation or other stressors. No significant differences were found in weight loss or gain between the four experimental groups (ANOVA $F_{3,26}=2.9$, $p > 0.05$).

PFC animals made about 50 decisions in the *Training with delay* constituting a working-memory load during 50 delays. In doing so, they made $83 \pm 3\%$ correct choices. Due to the lack of a delay in the last training phase (*TrainingRandom*), TC animals could perform more runs, viz., about 100 decisions with a preset reward probability of 80% (according to the percentage of correct choices of the PFC animals). The resultant difference in water intake as well as interindividual drinking differences could have affected cell proliferation. However, no significant correlation was found between water intake in 24 hours of PFC and TC animals as well as weight loss or gain as another indicator of water deprivation with the number of proliferating cells in the granule cell layer (Tab. 6-2).

Some animals needed additional watering during the habituation phases, since they preferred wheel running over entering the maze for drinking. This involved short handling of the animals as a potential stressor. However, no correlation was found between additional watering and cell proliferation (Tab. 6-2).

| | Total number of BrdU-positive cells | | Density of BrdU-positive cells per mm ³ | |
|---------------------|-------------------------------------|--------|--|--------|
| | r | p | r | p |
| Additional watering | -0.13 | > 0.05 | -0.25 | > 0.05 |
| Weight gain or loss | 0.22 | > 0.05 | 0.18 | > 0.05 |
| Water intake/24 h | 0.29 | > 0.05 | 0.29 | > 0.05 |

Tab. 6-2: Correlation of total numbers and densities of BrdU-positive cells with additional watering in the habituation phases, weight gain or loss and water intake/24 h during *Pretraining/PretrainingRandom* and *Training with delay/TrainingRandom* (reward probability 80%) for PFC (performed PFC-dependent task) and TC animals (performed T-maze control task); Pearson product-moment correlation; given are r-and p-values.

6.3.2.4. Performing a PFC-dependent task affects cell proliferation independently of the number of correct choices

The number of BrdU-positive cells was not significantly correlated with the number of correct choices during the *Training with delay* in PFC animals (Pearson product-moment correlation $r = -0.58$, $p \geq 0.13$).

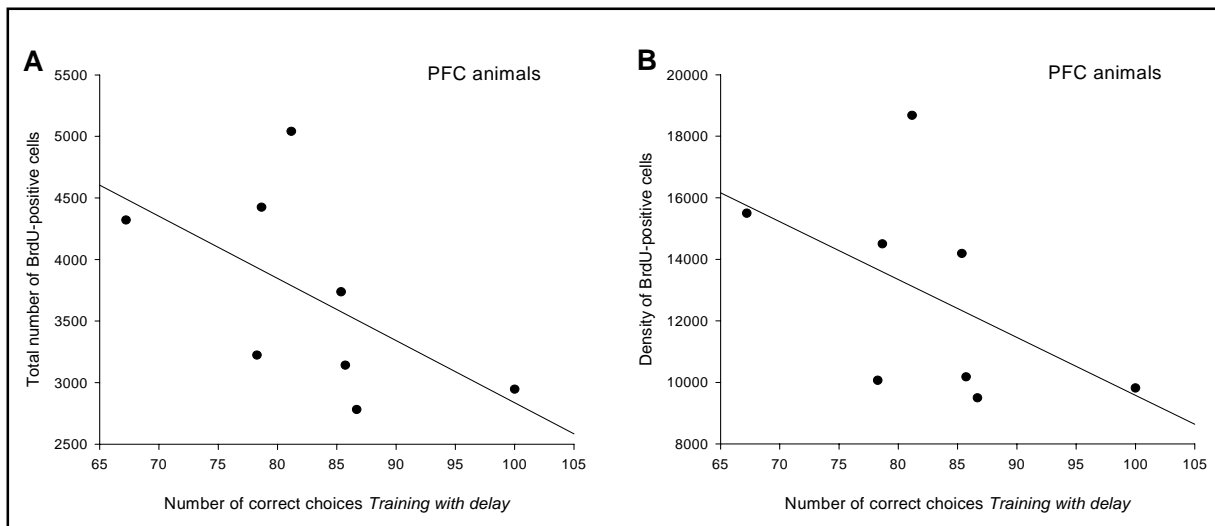


Fig. 6-6: Correlations between BrdU-positive cells in the granule cell layer and number of correct choices during *Training with delay* for PFC animals. There is no significant relationship between **A)** the total number of BrdU-positive cells or **B)** the density of BrdU-positive cells in the granule cell layer with the number of correct choices.

6.4. DISCUSSION

The aim of the present study was to investigate the role of the prefrontal cortex in the regulation of cell proliferation in the dentate gyrus. We hypothesized that activity from the prefrontal cortex is a negative regulator of cell proliferation via activation of the NMDA-receptor system at the entorhino-dentate interface (project II, chapter 4). This hypothesis was based on previous results that the reactivity of cell proliferation is higher in socially and physically deprived-reared mice (project I, chapter 3) and the assumption that a disturbed maturation of the prefrontal cortex and its efferents to caudal limbic areas (e.g., Winterfeld et al., 1998; Ago & Matsuda, 2003; Melendez et al., 2004; Bagorda et al., 2006) determines this different reactivity (project I, chapter 3; project II, chapter 4).

In the present study we enhanced cell proliferation by an adult wheel-running challenge in deprived-reared CD1 mice and strived to decrease it afterwards by stimulating prefrontal activity either by environmental enrichment or a PFC-dependent behavioral task. Indeed, three days of stimulation by environmental enrichment were sufficient to suppress cell proliferation after wheel running, while cell proliferation in control animals which were housed under continued deprivation, remained at a high level. These results correspond to other findings in so far that environmental enrichment even in adulthood and even if brief can exert strong effects on the brain, e.g., on cortical gene expression (Ferchmin et al., 1970; Ferchmin & Eterovic, 1986; Rampon et al., 2000), synaptophysin levels (Frick et al., 2003) and on neurogenesis in the dentate gyrus (Bruehl-Jungerman et al., 2005; Iso et al., 2007).

Recently, it was demonstrated that already 24 hours of exposure to environmental enrichment activate the medial prefrontal cortex as well as the hippocampus (Ali et al., 2009).

Short-term exposure to an enriched environment also involves exposure to novelty and thus mingles these effects. This was not necessarily undesirable in our study, since also novelty represents an environmental stimulus activating the prefrontal cortex (Wirtshafter, 2005; Ali et al., 2009) and enhancing catecholamine signaling (Rebec et al., 1997; Pudovkina et al., 2001). Hence, short-term environmental enrichment providing stimulation by novelty, contact to conspecifics and rich physical structure is able to suppress enhanced cell proliferation after wheel running. It can be assumed that the prefrontal cortex is causally involved in mediating these effects. However, environmental enrichment has various consequences not only for the prefrontal cortex but for the whole brain, so that further regulatory mechanisms could be implicated as well. Also stress effects due to exposure to novelty or sudden contact to conspecifics - even if we did not observe any aggressive interactions among individuals - cannot be ruled out.

To examine the contribution of the prefrontal cortex more specifically, we also applied stimulation of prefrontal activity by a PFC-dependent behavioral task, i.e., a spatial-delayed alternation task requiring working memory, and, to control for unspecific side effects of the procedure and the apparatus, compared it to a control task without working-memory load. To minimize stress by handling or shift in activity (manually-operated tasks are normally performed during the light phase, thus the inactive phase of the animals), we used an automated T-maze, which the animals entered voluntarily and - as intended - primarily during their active phase.

We found, that the PFC-dependent but not the control task decreased cell proliferation in the dentate gyrus significantly. This demonstrates that, indeed, the continuous working-memory load of the spatial-delayed alternation task and not stress or unspecific factors of the procedure or the apparatus caused the significant decrease in cell proliferation. That stress was not primarily responsible for the effect, gets further support, as there was no relationship between the rate of cell proliferation and stress indicators like weight loss, necessary additional watering in the habituation phases or water intake per 24 hours.

Interestingly, we did not find a significant relationship between the percentage of rewarded decisions and the rate of cell proliferation. Hence, it seems that not the performance in the working-memory task played the decisive role but the working-memory load *per se*. Since working memory - like it is required for spatial-delayed alternation in a T- or figure-8-maze - undoubtedly depends on the prefrontal cortex (Wikmark et al., 1973; Larsen & Divac, 1978;

Nonneman & Kolb, 1979; Thomas & Brito, 1980; Simon et al., 1980; Brito et al., 1982; Silva et al., 1986; Stam et al., 1989; Bubser & Schmidt, 1990; de Brabander et al., 1991; Murphy et al., 1996; Zahrt et al., 1997; Clinton et al., 2006) and activates it (Baeg et al., 2003; Baeg et al., 2007), we can conclude that enhanced activity in the prefrontal cortex was relevant for the decrease in cell proliferation in the dentate gyrus.

However, there is evidence that beside the prefrontal cortex also the hippocampus plays a role in spatial-delayed alternation mainly in the reference-memory component (rev. Dudchenko, 2004; Yoon et al., 2008), and in working memory during long delays (> 1 min.; Steele & Morris, 1999; Maruki et al., 2001; Lee & Kesner, 2003). To minimize an involvement of the hippocampus in the present task, we chose a short delay of 15 seconds. Moreover, hippocampus-dependent learning exerts only an effect on cell survival but not on cell proliferation, and this effect is even a positive and not a negative one (Gould et al., 1999; Ambrogini et al., 2000; Döbrössy et al., 2003; Leuner et al., 2004; Olariu et al., 2005; rev. in Leuner et al., 2006). Since we found lower and not higher rates of cell proliferation after the spatial-delayed alternation task, we can conclude that not the hippocampus, but activation of the prefrontal cortex due to working-memory requirements caused the decrease in cell proliferation in the dentate gyrus.

The prefrontal cortex is connected to the dentate gyrus mainly indirectly via the entorhinal cortex, the so called entry port of the hippocampal formation. Most prefrontal afferents terminate in the lateral entorhinal cortex (Burwell & Amaral, 1998; Kerr et al., 2007), from where the lateral perforant path conveys the input to the outer molecular layer of the dentate gyrus (Van Groen et al., 2002). The outer molecular layer contains a huge amount of N-methyl-aspartate (NMDA) receptors (Knowles, 1992; Kandel et al., 2000), through which the granule cells receive their major excitatory input from the entorhinal cortex (Collingridge, 1989). NMDA receptors are strongly involved in the regulation of cell proliferation. Activation of the NMDA-receptor system and thus excitation decreases cell proliferation, while deactivation increases it (Cameron et al., 1995; Bernabeu & Sharp, 2000; Nacher et al., 2001). Thus, the NMDA-receptor system at the entorhino-dentate interface provides a key mechanism through which the prefrontal cortex can exert its control over cell proliferation in the dentate gyrus.

Taken together, our results reveal that activation of the prefrontal cortex by either environmental enrichment or a PFC-dependent behavioral task can decrease cell proliferation after an increase evoked by socially and physically deprived-rearing and a subsequent wheel-running challenge. This demonstrates for the first time that the prefrontal cortex is causally involved in the regulation of cell proliferation in the dentate gyrus.

7. PROJECT V:

HIPPOCAMPAL CELL PROLIFERATION IS ONLY AFFECTED BY ENVIRONMENTAL MANIPULATIONS IN DOMESTICATED BUT NOT IN WILD HOUSE MICE (*MUS MUSCULUS DOMESTICUS*)

7.1. INTRODUCTION

Developmental neuroplasticity is highly susceptible to environmental influences. Rodents reared under deprivation (or impoverishment) of their natural social and physical environment show differences in neuronal development especially in the prefrontal-limbic system. This comprises a reduced dopaminergic innervation of the prefrontal cortex (Winterfeld et al., 1998; Neddens et al., 2001), abnormalities in transmission of neurotransmitters and in receptor expression (Dalley et al., 2002; Ago & Matsuda, 2003; Melendez et al., 2004; Leng et al., 2004; Brummelte et al., 2007; Gregory & Szumlinski, 2008; Bloomfield et al., 2008) as well as reduced maturation of prefrontal pyramids (Pascual et al., 2007) and their efferents to frontal, parietal and limbic areas (Bagorda et al., 2006; Witte et al., 2007). In contrast, dopaminergic, cholinergic, and serotonergic innervation and activity is enhanced in limbic circuits (Neddens et al., 2002, 2003; Lehmann et al., 2003, 2004; Busche et al., 2004, 2006; Lesting et al., 2005; Galani et al., 2007). Taken together, this leads to a dysbalance and a shift from prefrontal to limbic activities.

Such severe alterations impact neuroplasticity even in the adult. Effects on the regulation of neurogenesis in the hippocampal dentate gyrus, e.g., were demonstrated in different rodents. Nevertheless, the results remain inconsistent.

In gerbils, socially and physically deprived rearing causes increased rates of hippocampal cell proliferation in adulthood compared to enriched rearing (Hildebrandt, 1999; Keller et al., 2000).

In contrast, in C57Bl/6 and 129/SvJ mice, physical (but not social) deprivation during rearing compared to enrichment including wheel running has no effect on cell proliferation but leads to a decrease in cell survival (Kempermann et al., 1997a, 1998). Even a decrease in cell proliferation was found in C57Bl/6 mice, when social and physical deprivation was compared to enrichment with wheel running (Iso et al., 2007).

In a recent study, in which we applied the same rearing conditions as in the gerbil studies, i.e., excluded running wheels from the enrichment, we found no *per se* effect of the rearing conditions on neither cell proliferation nor cell survival in adult CD1 mice (project I, chapter 3). Nevertheless, the deprived-reared individuals were affected in their neuroplastic development, as only these animals responded to an adult wheel-running challenge with a significant increase in cell proliferation and cell survival revealing a higher reactivity of neurogenesis in these animals. This demonstrates that deprivation affects developmental neuroplasticity in a similar manner in CD1 mice, but that the strength of the deprivation effect is less pronounced than in gerbils.

An important question to address is therefore, what determines the strength of this deprivation effect. Is it species dependent and are the differences based on the different genetic backgrounds of *Mus musculus* and *Meriones unguiculatus*? Or is it domestication dependent? CD1 as well as C57Bl/6 mice are highly domesticated mouse strains and a lot of genetic modifications were performed to achieve these strains adapted at specific needs of research experiments (cf. chapter 2.1). On the other hand, despite decades of domestication, laboratory gerbils retained most behavioral traits and behavioral regulation mechanisms of their wild ancestors (Wiedenmayer, 1995; Stuermer, 1998; Waiblinger, 2002).

Another important issue is, for what reason the results within the species *Mus musculus* are so divergent. Do there strain or breeding-dependent differences exist, since C57Bl/6 mice are bred for high learning performance (Crawley et al., 1997; Owen et al., 1997; Baron & Meltzer, 2001), while CD1 mice show rather average learning abilities (Dellu et al., 2000; Adams et al., 2002)? Or are the divergent results just a matter of the study design, e.g., the use or omission of running wheels in the enrichment or the BrdU-labeling protocol?

To address these questions we examined the effects of social and physical deprivation and wheel running in young adulthood on hippocampal cell proliferation in adults of C57Bl/6 and the F2 generation of wild-caught house mice. Comparing deprivation effects in mouse strains of the same species (*Mus musculus domesticus*), but with different domestication background, enables us to investigate, whether the variability of neuronal plasticity in response to environmental stimulation is a unique feature in mice or if genetic background plays a role, too.

7.2. MATERIAL & METHODS

7.2.1. ANIMALS AND REARING CONDITIONS

27 female C57Bl/6 mice were bred in the central animal facility of Bielefeld University in Makrolon® type II cages (42.5 x 26.6 x 18.5 cm) and transferred on postnatal day (pd) 21 to our own facility. Wild house mice (F1 generation born in captivity) were obtained from the Institute of Zoology, Animal Behavior in Zurich for breeding. 31 female individuals of the next generation (F2 generation) were bred in our facility in Makrolon type III cages with one litter per cage (for further information see chapter 2.1). The entire experiment including rearing and adult intervention was performed with individuals of each strain separately.

According to the experiment performed with CD1 mice, C57Bl/6 and wild house mice were randomly assigned to one of two rearing conditions, enriched (ER_{SPW-}) or socially (s) and physically (p) deprived (DR_{SPW-}) rearing without running wheels (w-; rearing conditions are specifically described in chapter 2.2) at the day of weaning (postnatal (pd)). C57Bl/6 ER_{SPW-} animals were reared in a group of 11, wild caught ER_{SPW-} animals in three groups of six individuals each in large enclosures (100 x 100 x 50 cm) that contained items such as tunnels, tubes, and solid hiding places (Fig. 2-4, A). DR_{SPW-} animals were reared individually in standard type III cages (Fig. 2-4, B). All animals were kept under their respective experimental rearing conditions for 45 days from pd 21 to pd 66.

7.2.2. WHEEL-RUNNING CHALLENGE

On pd 66, within each strain, two mixed groups of ER_{SPW-} and DR_{SPW-} animals were formed. Each group was placed into a large enclosure (100 x 100 x 50 cm) and one of them had access to running wheels (as described in chapter 2.3) from pd 70 to pd 74, while the other received no further treatment. Wheel-running activity was checked by visual observations of the animals, while rearing-group members could be identified by earmarks.

7.2.3. BRDU-LABELING, PERFUSION, IMMUNOHISTOCHEMISTRY

All animals received injections of the DNA-synthesis marker 5-bromo-2-deoxyuridine (BrdU) on pd 71 to pd 73 at 5 p.m., thus, just before the dark period and the onset of the active phase of the animals (see chapter 2.6.1 and 3.2.3). 24 hours later, the animals were deeply anaesthetized by an overdose of diethyl ether and transcardially perfused with 200 ml of 4% paraformaldehyde solution (for details see chapter 2.6.2). The brains were immediately

dissected and stored over night in 4% PFA at 4°C. On the next day, brains were transferred to a 30% sucrose solution for cryoprotection for at least 24 hours. Then, the left hemispheres of the brains were freeze-sectioned with a Frigomobil. 40 µm coronar sections were made throughout the septotemporal axis of the hippocampus (see chapter 2.6.2), collected in a cryoprotectant solution and stored at -20°C until further processing.

The immunohistochemical detection of BrdU-labeled cells was based on the two-step indirect ABC-(Avidin-Biotin-Complex-) method involving an unlabeled primary antibody (mouse anti-BrdU, Roche Molecular Biochemicals, Indianapolis, USA) and a biotinylated secondary antibody (M.O.M. anti-mouse reagent, Vector Laboratories, Burlingame, USA, and biotinylated goat anti-mouse, Vector Laboratories, Burlingame, USA; for further explanations see chapter 2.6.3).

7.2.4. QUANTITATIVE EVALUATION/STEREOLOGY

All slides were coded prior to quantitative analysis and the code was not broken until the analysis was completed. The number of BrdU-positive cells was estimated by counts made systematically on every third consecutive section along the rostrocaudal axis of the hippocampal formation (see chapter 2.6.4). On each section, BrdU-positive cells were counted in the granule cell layer and subgranular zone under the light microscope at 400-fold magnification. Additionally the volume of the granule cell and subgranular layer was measured at 100-fold magnification (see chapter 2.6.4). The reference volume was estimated according to the Cavalieri principle: $V_{ref}=T \times \Sigma A \times 1/ssf$, where T is the thickness of the section, A is the area of the granule cell layer and 1/ssf is the inverse of the section sampling fraction (1/ssf=3).

The number of BrdU-positive cells was then related to the granule cell layer sectional volume to obtain the density of BrdU-positive cells per mm³. Additionally, the number of BrdU-positive cells was multiplied by the reference volume to estimate the total number of BrdU-positive cells per dentate gyrus.

7.2.5. STATISTICAL ANALYSES

The data were analyzed by ANOVA. Specific comparisons were made with Unequal N HSD post-hoc tests. Data analyses were performed with Excel (Microsoft Office 2007), statistical analyses with Statistica 6.0 (StatSoft, Tulsa, USA).

Two wild mice escaped during transposition or injections (cf. description of mouse strains in chapter 2.1), could be trapped later, but had to be excluded from the sample due to ‘undesired housing conditions’ during the escape period.

7.3. RESULTS

7.3.1 QUALITATIVE RESULTS

In both C57Bl/6 and wild house mice, members of both rearing groups could be observed frequently while running in wheels (visual observations).

The immunohistochemical detection of cell proliferation revealed BrdU-positive cells indicated as brown to dark brown precipitates in all animals. The majority of BrdU-positive cells resides in the subgranular zone (Fig. 7-1). Photomicrographs of C57Bl/6 and wild house mice from both socially and physically enriched and deprived rearing without and after the adult wheel-running challenge exemplarily illustrate the distribution of BrdU-positive cells in the dentate gyrus (Fig. 7-1). Qualitatively, there appears hardly difference between deprived and enriched-reared as well as enriched-reared C57Bl/6 mice after wheel running. Only deprived-reared C57Bl/6 mice after wheel running show an apparent increase in the number of BrdU-positive cells (Fig. 7-1, G). No difference is observable in wild house mice after the different experimental treatments.

7.3.2. QUANTITATIVE RESULTS

7.3.2.1. Effects of rearing conditions and wheel-running challenge in C57Bl/6 mice

The total number of BrdU-positive cells in dentate gyrus of C57Bl/6 mice was not affected by the conditions of social and physical deprivation or enrichment during rearing alone (ER_{SPW}- 5045 vs. DR_{SPW}- 4883 cells per dentate gyrus; ANOVA, rearing condition $F_{1,23}=2.36$, $p > 0.05$; Post-hoc Unequal N HSD, $p > 0.05$) Fig. 7-2, A). Similar effects were found for densities of BrdU-positive cells (ER_{SPW}- 22691 vs. DR_{SPW}- 24012 cells per mm³; ANOVA, rearing condition $F_{1,23}=9.98$, $p < 0.01$; Post-hoc Unequal N HSD, $p > 0.05$) Fig. 7-2, B).

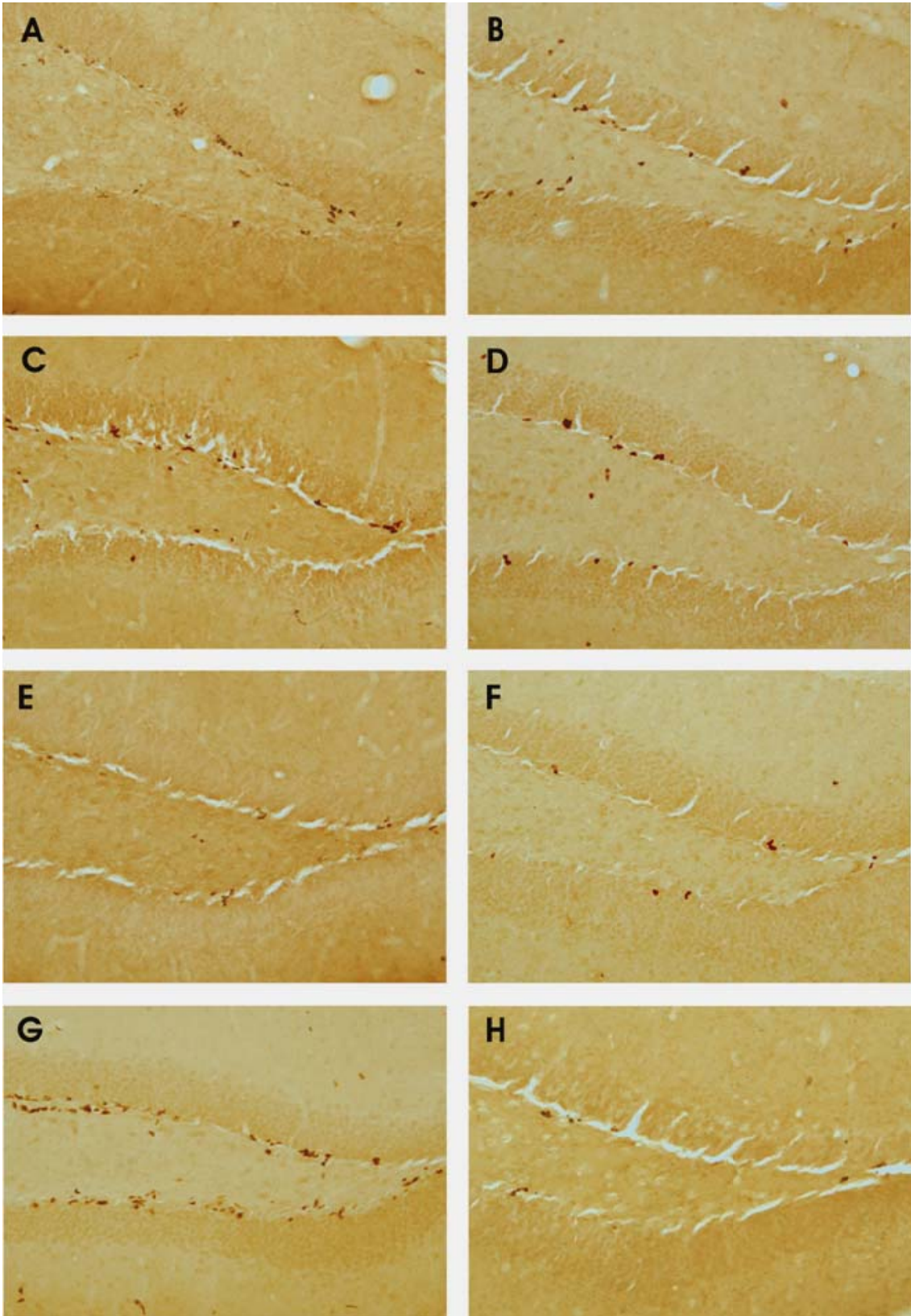


Fig. 7-1: Photomicrographs of BrdU-positive cells (200-fold magnification) 24 hours after the last BrdU-injection in socially and physically enriched-reared C57Bl/6 mice without (A+C) and after wheel running (E+G) as well as socially and physically deprived-reared wild house mice without (B+D) and after wheel running (F+H).

However, the effect of wheel running on the total number of BrdU-positive cells was significantly dependent on the previous rearing history of the animals (ANOVA, wheel running $F_{1,23}=13.96$, $p < 0.01$; interaction effect rearing condition*wheel running $F_{1,23}=3.54$, $p = 0.07$). Wheel running caused an increase in the total number of BrdU-positive cells in DR_{SPW-} animals (+54%; Post-hoc Unequal N HSD, $p < 0.01$), but not in ER_{SPW-} animals (ER_{SPW-}, after wheel running 5910 cells per dentate gyrus; Post-hoc Unequal N HSD, $p > 0.05$; Fig. 7-2, A). These effects were even stronger for density of BrdU-positive cells (ANOVA, wheel running $F_{1,23}=14.9$, $p < 0.001$; interaction effect rearing condition*wheel running $F_{1,23}=5.68$, $p < 0.05$). Wheel running caused an increase in the density of BrdU-positive cells in DR_{SPW-} animals (+44 %; Post-hoc Unequal N HSD, $p < 0.001$), which was even significant against the density found in ER_{SPW-} animals after wheel running (+38 %; Post-hoc Unequal N HSD $p < 0.05$; Fig. 7-2, B)

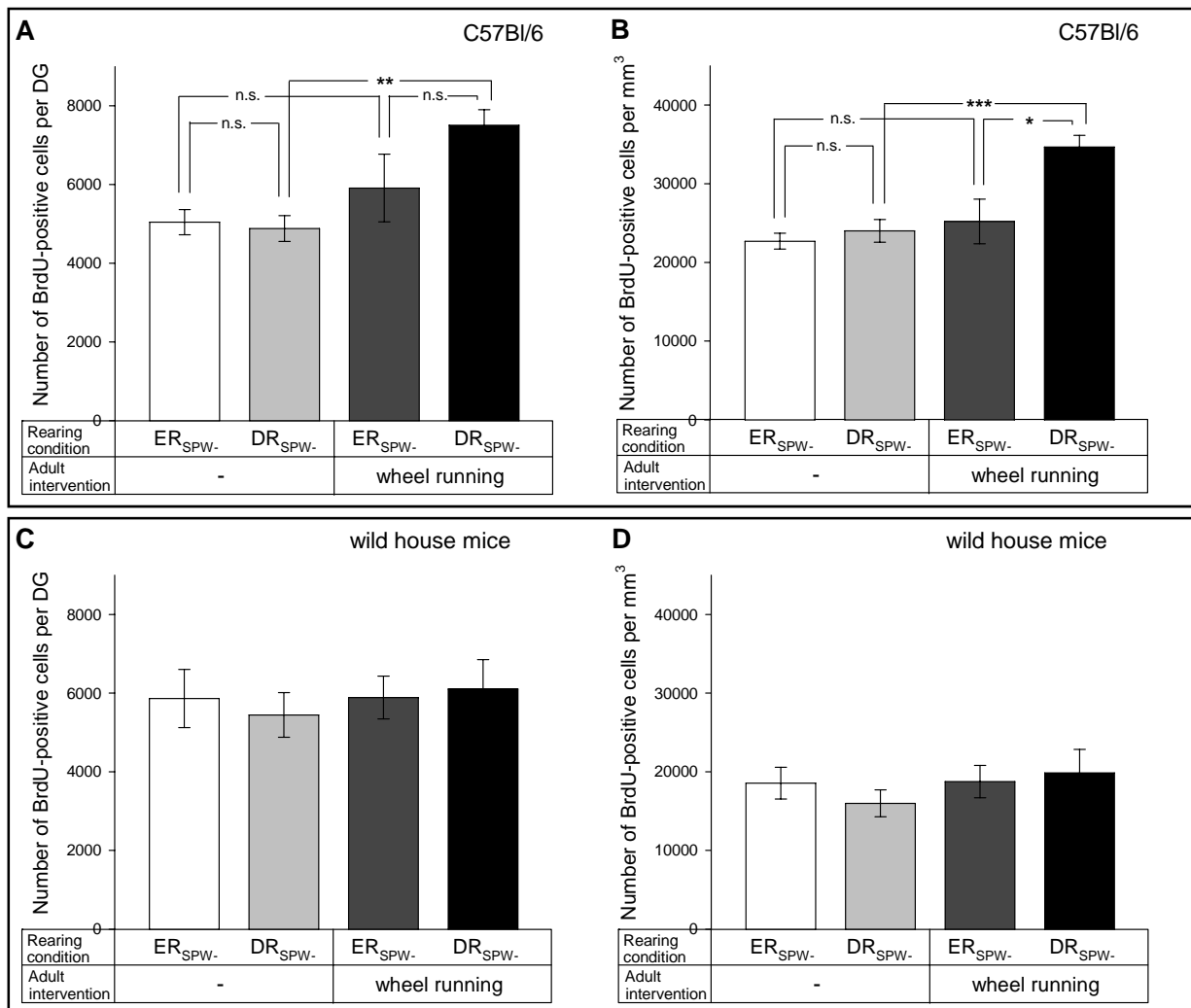


Fig. 7-2: **A)** Total number of BrdU-positive cells in the granule cell layer of C57Bl/6 mice; **B)** Density of BrdU-positive cells per mm³ in the granule cell layer of C57Bl/6 mice; **C)** Total number of BrdU-positive cells in the granule cell layer of wild house mice; **D)** Density of BrdU-positive cells per mm³ in the granule cell layer of wild house mice; ER_{SPW-}, socially and physically enriched-reared animals; DR_{SPW-}, socially and physically deprived-reared animals; wheel running, additional wheel running challenge (pd 70-74); data given as means \pm S.E.M.

7.3.2.2. Effects of rearing conditions and wheel-running challenge in wild house mice

Cell proliferation in the dentate gyrus of wild house mice was not affected neither by socially and physically deprived rearing (ANOVA, rearing condition, cells per dentate gyrus $F_{1,27}=0.02$, $p > 0.05$; density of cells $F_{1,27}=0.1$, $p > 0.05$) Fig. 7-2, C, D) nor by the wheel running challenge in young adulthood (ANOVA, wheel running, cells per dentate gyrus $F_{1,27}=0.26$, $p > 0.05$; density of cells $F_{1,27}=0.78$, $p > 0.05$; interaction effect rearing condition*wheel running, cells per dentate gyrus $F_{1,27}=0.23$, $p > 0.05$; density of cells $F_{1,27}=0.64$, $p > 0.05$; Fig. 7-2, C, D)

Table 7-1 indicates the mean total number and the mean density of BrdU-counts for cell proliferation and cell survival for the four different groups as well as significant differences between the groups.

| | | ER _{SPW.} | DR _{SPW.} | | ER _{SPW.} | ER _{SPW.} after wheel running | |
|------------------|--------------|--------------------|--------------------|------|--------------------|---|------|
| | | value | value | p | value | value | p |
| C57Bl/6 | Total number | 5045 | 4883 | n.s. | 5045 | 5910 | n.s. |
| | Density | 22691 | 24012 | n.s. | 22691 | 25207 | n.s. |
| Wild house mouse | Total number | 5865 | 5444 | n.s. | 5865 | 5888 | n.s. |
| | Density | 18660 | 16000 | n.s. | 18660 | 18758 | n.s. |

| | | DR _{SPW.} (100%) | DR _{SPW.} after wheel running | | | ER _{SPW.} after wheel running (100%) | DR _{SPW.} after wheel running | | |
|------------------|--------------|------------------------------|---|------------|------------------|---|---|------------|-----------------|
| | | value | value | diff. in % | p | value | value | diff. in % | p |
| C57Bl/6 | Total number | 4883 | 7505 | +54 | <0.01 | 5910 | 7505 | +27 | n.s. |
| | Density | 24012 | 34653 | +44 | <0.001 | 25207 | 34653 | +38 | <0.05 |
| wild house mouse | Total number | 5444 | 6104 | - | n.s. | 5888 | 6104 | - | n.s. |
| | Density | 16000 | 19851 | - | n.s. | 18758 | 19851 | - | n.s. |

Tab. 7-1: Group comparison of total numbers and densities of BrdU-positive cells

ER_{SPW.}, reared under socially and physically enriched conditions; DR_{SPW.}, reared under socially and physically deprived conditions; ER_{SPW.} after wheel running, reared under socially and physically enriched conditions with an additional wheel-running challenge in adulthood (pd70-74); DR_{SPW.}, reared under socially and physically deprived conditions with an additional wheel-running challenge in adulthood; total number of BrdU-positive cells per dentate gyrus; density of BrdU-positive cells per mm³; given are absolute values (means), differences between the groups if significant, and p-values.

7.3.2.3. Effects of rearing conditions, wheel running or domestication background on the reference volume of the granule cell layer

The reference volume comprising the granule cell layer and subgranular zone was not affected by the rearing conditions or the wheel-running challenge neither in C57Bl/6 (ANOVA rearing condition $F_{1,23}=1.571$, $p > 0.05$; wheel running $F_{1,23}=0.61$, $p > 0.05$; interaction effect of rearing condition*wheel running $F_{1,23}=0.005$, $p > 0.05$) nor in wild house mice (ANOVA rearing condition $F_{1,27}=0.53$, $p > 0.05$; wheel running $F_{1,27}=0.16$, $p > 0.05$; interaction effect of rearing condition*wheel running $F_{1,27}=0.64$, $p > 0.05$). However, taking the treatment groups together and comparing the reference volumes between the strains by Welch two-sample t-test revealed a significantly greater volume in wild house mice (C57Bl/6 $0.218 \pm 0.006 \text{ mm}^3$, wild house mice $0.325 \pm 0.01 \text{ mm}^3$, $p < 0.0001$; Fig. 7-3).

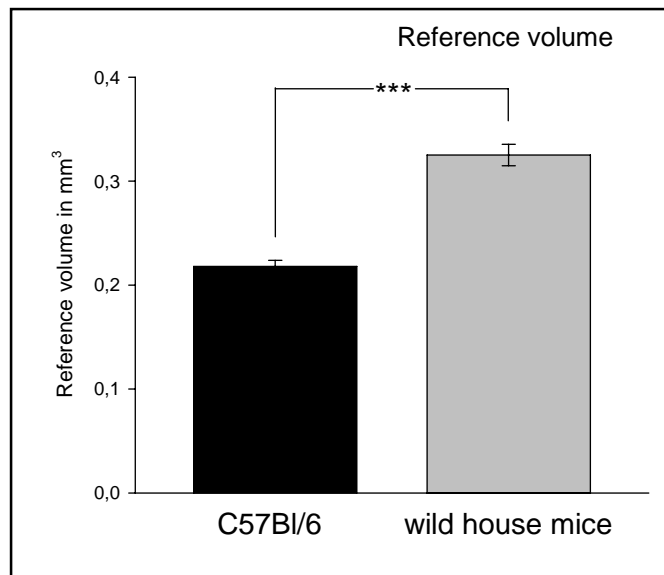


Fig. 7-3: Reference volume comprising the granule cell layer and subgranular zone in C57Bl/6 and wild house mice; data given as means \pm S.E.M.

7.4. DISCUSSION

The effects of our treatments on neuroplasticity in C57Bl/6 mice were similar to those in CD1 mice: social and physical deprivation during rearing did not cause different rates of hippocampal cell proliferation in young-adult animals if compared to enrichment without wheel running. However, also deprived-reared C57Bl/6 mice were affected in their neuroplastic development with consequences on the regulation of neurogenesis in adulthood. A neurogenic stimulation through a 4-day wheel-running challenge caused an increase in hippocampal cell proliferation only in deprived-reared, but not in enriched-reared individuals. These effects were somewhat less pronounced than in CD1 mice, probably because baseline cell proliferation is already higher in C57Bl/6 mice (cf. also Kempermann et al., 1997b), and after an additional stimulation, the maximum of newborn cells per dentate gyrus is reached.

In contrast, the effects found in the progeny of wild house mice were completely unexpected. Neither the rearing conditions nor the wheel-running challenge exerted an effect on cell proliferation in the adult dentate gyrus of these animals. This argues in favor that the reactivity of cell proliferation is lower in wild house mice than in laboratory mice.

The reference volume of the granule cell layer and subgranular zone was not affected neither by the rearing conditions nor the wheel-running challenge in any of the strains. However, a comparison of the reference volume between the strains revealed a significantly greater volume in wild house mice than in C57Bl/6, while the latter exhibited a volume similar to that of CD1 mice. Further morphometric analyses revealed that it is, indeed, only the granule cell layer - especially in the suprapyramidal blade - which has a greater extension in wild than in the laboratory strains (cf. supplemental experiment II, chapter 7.6). A larger granule cell layer was also found in other wild-living rodents compared to laboratory mice (Amrein et al., 2004), and we could demonstrate this now even within one and the same species.

The effect of social and physical deprivation during brain development depends on the domestication background

For two reasons the present results demonstrate that the effects of social and physical deprivation during brain development in mice strongly depend on genetic modifications by domestication. First, outbred C57Bl/6 mice responded to social and physical deprivation during rearing with similar alterations in hippocampal cell proliferation as inbred CD1 mice (project I, chapter 3), although both strains show different (spatial) learning abilities (Crawley et al., 1997; Owen et al., 1997; Dellu et al., 2000; Baron & Meltzer, 2001; Adams et al., 2002), and even significantly differ in their baseline cell proliferation and neurogenesis. In this way, our results correspond to others that cell proliferation in C57Bl/6 mice is about 1.5 fold higher than in CD1 mice (Kempermann et al., 1997b). However, our findings stand in contrast to the effects of environmental stimulation or deprivation during rearing found in C57Bl/6 mice so far, namely that deprived rearing decreased hippocampal cell proliferation in the adult (Iso et al., 2007). The different findings may be due to differences in the study design, e.g., the use or omission of running wheels in the enrichment, the BrdU-labeling protocol or the survival time after BrdU injections. Since, we applied the same experimental treatment to C57Bl/6 mice in the present study as previously to CD1 mice, and found similar effects on cell proliferation in adulthood, we can conclude that deprivation affects the regu-

lation of cell proliferation in the adult dentate gyrus equally in these two laboratory strains, and that there are no strain- or breeding-dependent differences.

Secondly, undomesticated wild house mice did not respond neither to deprivation during rearing nor to the wheel-running challenge with any significant differences in hippocampal cell proliferation. This result stands in contrast to expectations, since with regard to the findings from gerbils (Hildebrandt, 1999; Keller et al., 2000) we expected a higher and not a lower neuroplastic response in wild house mice. Herefrom, we cannot exclude that the strength of the deprivation effect is also subject to species-specific differences in the regulation of neuroplasticity between *Meriones unguiculatus* and *Mus musculus domesticus*. But it is also feasible that differences are due to the study design, e.g., the BrdU-labeling protocol or the survival time after the labeling. In this case, it might be that the effect of domestication in laboratory gerbils has been underestimated so far.

The missing effect of wheel running in deprived-reared wild house mice is even more surprising, since these animals ran twice as much in the wheels as their enriched-reared littermates, whose running distance was in the range of C57Bl/6 and CD1 mice (cf. supplemental experiment I, chapter 7.5). This argues in favor that adult hippocampal cell proliferation is only in domesticated mouse strains susceptible to environmental manipulations in adolescence and young adulthood, and that this susceptibility is a consequence of genetic modification by domestication. This assumption gets support from a recent study in wild-caught wood mice (*Apodemus sylvaticus*, Hauser et al., 2009). Also in these animals, which were in contrast to ours caught as adults and directly exposed to either deprived housing or wheel running, no effect of housing or running on neurogenesis was found. Although it concerns another species and does not regard developmental effects, these results together with ours indicate that undomesticated mice are more robust in their neurogenic regulation against environmental influences than domesticated mice.

Why are wild house mice more robust in their neurogenic regulation against environmental influences?

It can be assumed that in individuals of wild-living strains the reactivity of cell proliferation is buffered by mechanisms which are not effective in domesticated mice anymore. Indeed, there are indications that the developmental timing of neurochemical and neuroendocrinological mechanisms change in the route of domestication (Trut, 1999). Furthermore, melatonin is a key candidate to be involved. Melatonin influences proliferative and differentiative activity of

neural stem cells in the dentate gyrus (Moriya et al., 2007; Ramirez-Rodriguez et al., 2009). While wild mice synthesize pineal melatonin with a circadian rhythmicity (Ebihara et al., 1986, 1987), most domesticated mouse strains including C57Bl/6 have more or less no melatonin in their pineal glands (Ebihara et al., 1986, 1987; Goto et al., 1989; Conti & Maestroni, 1996; Vivien-Roels et al., 1998).

Finally, the maturation of the prefrontal cortex is severely affected by social and physical deprivation during rearing in gerbils (Winterfeld et al., 1998; Neddens et al., 2001; Bagorda et al., 2006; Witte et al., 2007; Brummelte et al., 2007). This coincides with an enhanced cell proliferation in these animals (Hildebrandt, 1999; Keller et al., 2000). A similar effect of deprived rearing was now demonstrated in the domesticated mouse strains CD1 and C57Bl/6, although the effects were less pronounced and more subtle. Therefore, we suggested that the maturation of the prefrontal cortex and its interaction with limbic structures is also in domesticated mice causally involved in the regulation of proliferating activity in the dentate gyrus (project I, chapter 3, project II, chapter 4; an involvement of the prefrontal cortex in the regulation of cell proliferation was demonstrated in project IV, chapter 6). Since wild house mice irrespective of their rearing history - like the enriched-reared domesticated mice - did not exhibit enhanced cell proliferation after wheel running, it might be assumed that in undomesticated animals the mesoprefrontal-limbic axis can buffer not only short lasting manipulations like a wheel-running challenge but even long-lasting deprivation of environmental stimulation during development.

Conclusion

This is the first study to demonstrate that cell proliferation in wild house mice cannot be manipulated by social and physical deprivation during adolescence or a 4-day wheel-running challenge, thus, by stimuli which are sufficient to alter cell proliferation rates in domesticated mice of the same species (*Mus musculus domesticus*). The investigation of the exact mechanisms determining the reactivity or stability of proliferating activity in the adult dentate gyrus is an important issue for future research, since they may provide a key to a better understanding of the regulation of hippocampal cell proliferation in the intact and disturbed brain.

7.5. SUPPLEMENTAL EXPERIMENT I: EFFECTS OF REARING CONDITIONS ON WHEEL-RUNNING ACTIVITY IN C57BL/6 AND WILD HOUSE MICE

Four days of wheel running in young adulthood enhanced hippocampal cell proliferation only in C57Bl/6 mice that were reared under socially and physically deprived conditions, but not in their enriched-reared littermates. Similar results were seen in CD1 mice (project I, chapter 3). Although enriched and deprived-reared CD1 mice did not differ in their wheel-running activity (cf. chapter 3.6; Haupt & Schaefer, *accepted*) and members of both rearing groups of C57Bl/6 could also be observed while running in the wheels, it is still not quantitatively substantiated that enriched and deprived-reared C57Bl/6 mice really show the same running activity. Furthermore, in neither enriched nor deprived-reared wild house mice the wheel-running challenge affected adult cell proliferation in the dentate gyrus. Thus, it has to be clarified if wild house mice, although they could be watched running in the wheels, really show similar running activity as individuals of the two laboratory strains.

To address these issues, we measured wheel-running activity of enriched and deprived-reared C57Bl/6 and wild house mice (each group separately) over four days in young adulthood with automated running wheels. Additional groups of female C57Bl/6 and wild house mice were reared either under enriched (ER_{SPW-}, n=6) or socially and physically deprived conditions (DR_{SPW-}, n=6) as described before (see chapter 2.2 & 7.2.1). In young adulthood (C57Bl/6 ER_{SPW-} pd 63; DR_{SPW-} pd 67, wild house mice ER_{SPW-} pd 62; DR_{SPW-} pd 66), animals were habituated to an enclosure which contained nothing than animal bedding, food and water *ad libitum* as well as a solid hiding place. These conditions equated the habituation conditions in the main experiment (see chapter 7.2.2) except that the rearing groups were not mixed. Four days later (C57Bl/6 ER_{SPW-} pd 67; DR_{SPW-} pd 71, wild house mice ER_{SPW-} pd 66; DR_{SPW-} pd 70) the automated running wheels were introduced into the enclosure. One running wheel per animal was provided, which was connected to a computer measuring the wheel revolutions (for detailed description of the apparatus see chapter 2.5.1).

Running analysis was started at the end of the first dark cycle to get three complete 24-h cycles and include the three dark phases in which the BrdU labeling was performed in the main experiment. We included only long-term running events (>10 sec.) and excluded short-term running events, which often had purely investigative character (sniffing, outer touches, wheel crossings). Number of long-term running events, and total distance covered in 24 hours were scored. As individual data points for number of events and distance we took

days. These values were divided by the number of individuals ($n=6$). Normally distributed data (Shapiro Wilks test for normality) were analyzed using Welch two-sample t-test.

One group of enriched-reared C57Bl/6 showed hardly running activity. Since visual observations had proven that ER_{SPW-} animals showed running activity in the main experiment (see chapter 7.3.1), the measurement was repeated with two additional groups of female C57Bl/6 ER_{SPW-} animals (2 x $n=6$). Both groups showed a running activity measured by running distance and number of long-term running events per 24 h, which was not significantly different from that of DR_{SPW-} animals (Welch two-sample t-tests, $p < 0.05$). Data of all three groups are shown in table 7-2 in comparison to C57Bl/6 DR_{SPW-} animals.

| | ER _{SPW-1} | ER _{SPW-2} | ER _{SPW-3} | DR _{SPW-} |
|----------------------------------|---------------------|---------------------|---------------------|--------------------|
| distance covered in 24 h | 0.36 ± 0.11 km | 4.33 ± 0.9 km | 5.42 ± 0.15 km | 5 ± 0.24 km |
| long-term running events in 24 h | 31 ± 8 | 159 ± 21 | 159 ± 5 | 169 ± 1 |

Tab. 7-2: Wheel-running activity of enriched (ER_{SPW-}) and deprived reared (DR_{SPW-}) C57Bl/6 mice.
Data given as means ± S.E.M.

Wild house mice of both rearing groups disliked the apparatus and even avoided exploring the automated running wheels for to date unexplained reasons. After introduction of the individual wheels which were used in the main experiment (cf. chapter 2.3 and 7.2.2), they started running immediately (own observations) and afterwards ran also in the automated running wheels, so that the measurements could be achieved at a later time point. Thus, data starting at the end of the 6th dark phase after the introduction of the apparatus were taken for comparison.

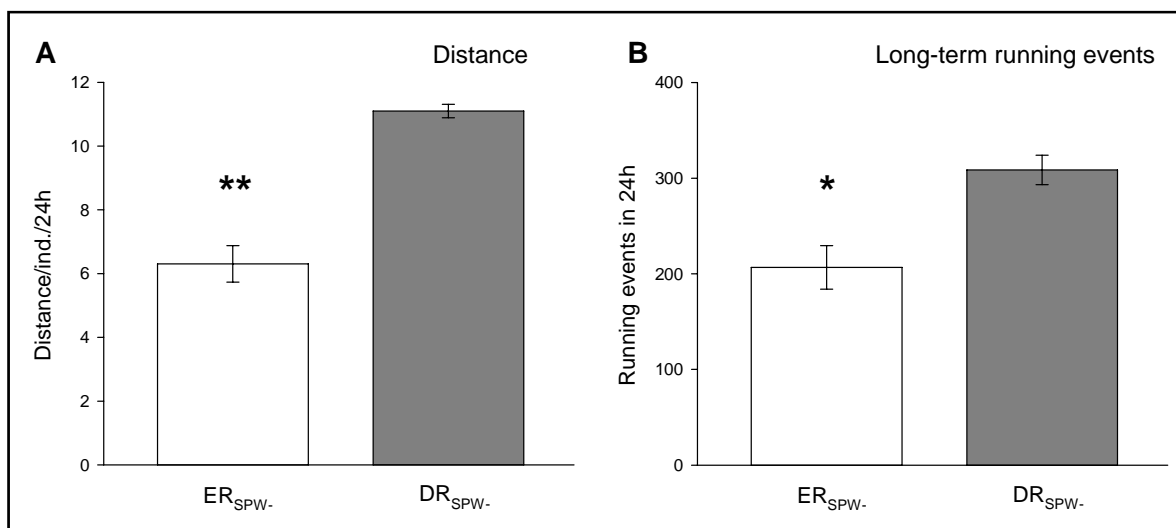


Fig. 7-4: Wheel running activity of enriched (ER_{SPW-}) and deprived reared wild house mice (DR_{SPW-}).
A) Running distance covered in 24 hours; B) Long-term running events (>10 sec.) in 24 hours; data given as means ± S.E.M.

Within this time, enriched-reared wild house mice showed a running activity in the range of the domesticated mouse strains (ER_{SPW} - 6.3 ± 0.57 km, 207 ± 23 long-term running events), whereas deprived-reared wild house mice covered a significantly larger distance each night (DR_{SPW} - 11.1 ± 0.21 km, Welch two-sample t-test, $p < 0.01$) and also showed significantly more long-term running events (DR_{SPW} - 309 ± 15 , Welch two-sample t-test, $p < 0.05$).

These results together with the afore made visual observations (see chapter 7.3.1) suggest that also socially and physically deprived-reared C57Bl/6 mice do not differ from their enriched-reared littermates in a 4-day wheel-running challenge in young adulthood. Thus, differences in wheel-running activity cannot account for the different neuroplastic responses to wheel running. Interestingly, the large running distance covered by deprived-reared wild house mice seemed to have, nevertheless, no impact on cell proliferation in these animals.

7.6. SUPPLEMENTAL EXPERIMENT II: MORPHOMETRIC ANALYSIS OF THE DENTATE GYRUS – WILD HOUSE MICE HAVE A LARGER GRANULE CELL LAYER THAN DOMESTICATED MICE

Domestication seems to affect the volume of the hippocampus and its subregions. In rats domestication is accompanied by a reduction of the hippocampus of about 10% (Kruska, 1975). Also in mice, a reduced volume of the granule cell layer was found in domesticated compared to wild mice (Amrein et al., 2004). This corresponds to our results of a bigger reference volume comprising the granule cell and adjacent subgranular zone in wild house mice (see chapter 7.3.2.3). It is, therefore, feasible that beside the granule cell layer also other layers of the dentate gyrus are affected by domestication. It is known that in rats, strain-specific differences exist in the occurrence of the distinct layers of the dentate gyrus (Lipp et al., 1987). In rats and mice, the extension of the mossy fiber bundle in different domesticated and wild strains is correlated with performance in spatial learning tasks (Crusio et al., 1987; Lipp et al., 1988; Schwegler & Crusio, 1995). Especially the inner molecular layer bears high plastic capacities (for detailed explanations see chapter 5.4). In gerbils, it covers about one third of the whole molecular layer and its extension was shown to be sensitive to environmental deprivation during rearing (Hildebrandt, 1999).

Hence, two interesting questions emerge: first, whether domestication led to a reduction of the whole dentate gyrus or only of the granule cell layer within the species *Mus musculus domesticus*; and second, whether other layers of the dentate gyrus also exhibit domestication- or strain-dependent differences in their extension.

To answer these questions, male CD1 (n=4), C57Bl/6 (n=7) and wild house mice (n=5) were reared under socially and physically deprived conditions from the day of weaning (pd 21; for more details see chapter 2.2). Adult animals were deeply anaesthetized by an overdose of diethyl ether and transcardially perfused with sodium sulphide solution and 3% glutaraldehyde (for detailed description see chapter 2.8.1). Brains were immediately dissected and stored over night in 3% glutaraldehyde and 20% sucrose. 20 μm coronar sections were made throughout the septotemporal axis of the hippocampus with a FrigoCut and every third section was mounted on slides (see chapter 2.8.1). Sections were stained with the Timm silver impregnation as described in chapter 2.8.2. Incubation times were adjusted to obtain the optimal staining of the layers of the dentate gyrus (Coleman et al., 1987) leading to a certain overstaining of the mossy fiber bundle, hippocampus proper and other cortical areas (Fig. 7-5, A).

Morphometric measurements were made on photomicrographs (100-fold magnification) of every section with the help of dimension lines between rectangular reference lines. The mean over three measurements and the sections were taken to get a value for every layer (for details see chapter 2.8.3).

To get a qualitative comparison of the Timm staining with a selective immunohistochemical staining against zinc containing fibers, a fluorescence staining against the zinc transporter 3 (ZnT 3) was additionally performed on paraformaldehyde fixated 40 μm sections (for perfusion and tissue preparation see chapter 2.6.2) of socially and physically deprived-reared females of each strain. Therefore, a primary antibody against ZnT 3 (rabbit anti-ZnT 3, Synaptic Systems, Göttingen, Germany) and a secondary antibody (donkey anti-rabbit AMCA, Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK) were used (for staining protocol see chapter 2.8.4).

Examination of Timm impregnated sections under light microscopy revealed a clearly distinguishable lamination in the dentate gyrus as well as a black-brown staining of the mossy-fiber bundle reaching from the dentate gyrus to CA3 of the hippocampus proper. The granule cell layer appears relatively light and somata of individual granule cells are visible.

Upside the granule cell layer, the inner molecular appears as a darker band, which can be easily distinguished from the adjacent lighter middle molecular layer. Directly below the hippocampal fissure in the supra- and directly above the border of the lateral ventricle in the infrapyramidal blade, a dark-brown staining marks the outer molecular layer. The intensity of the Timm staining is similar in all groups, although the color can vary among individuals from reddish brown to grey brown. Differences among the strains in the extension of the individual layers are not obvious (Fig. 7-5, B, C, D).

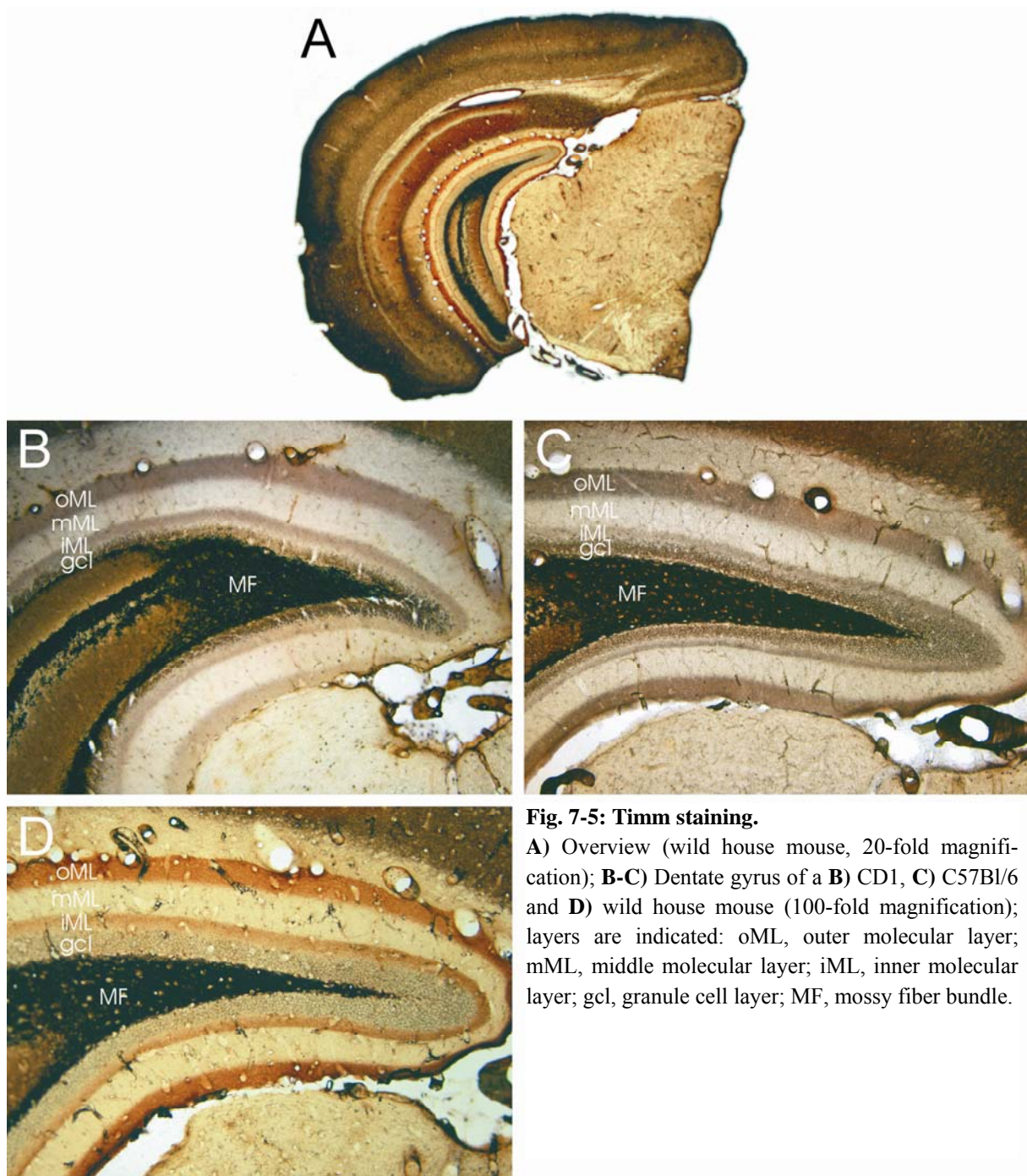


Fig. 7-5: Timm staining.

A) Overview (wild house mouse, 20-fold magnification); **B-C)** Dentate gyrus of a **B)** CD1, **C)** C57Bl/6 and **D)** wild house mouse (100-fold magnification); layers are indicated: oML, outer molecular layer; mML, middle molecular layer; iML, inner molecular layer; gcl, granule cell layer; MF, mossy fiber bundle.

The comparative immunohistochemical staining against ZnT 3 revealed a similar but inverted picture. The mossy fiber bundle shows the strongest fluorescence indicating the highest content of ZnT 3. Granule cell and middle molecular layer appear relatively dark, whereas the inner and outer molecular layer can be easily distinguished from them because of their higher ZnT 3 content and thus stronger fluorescence (Fig. 7-6).

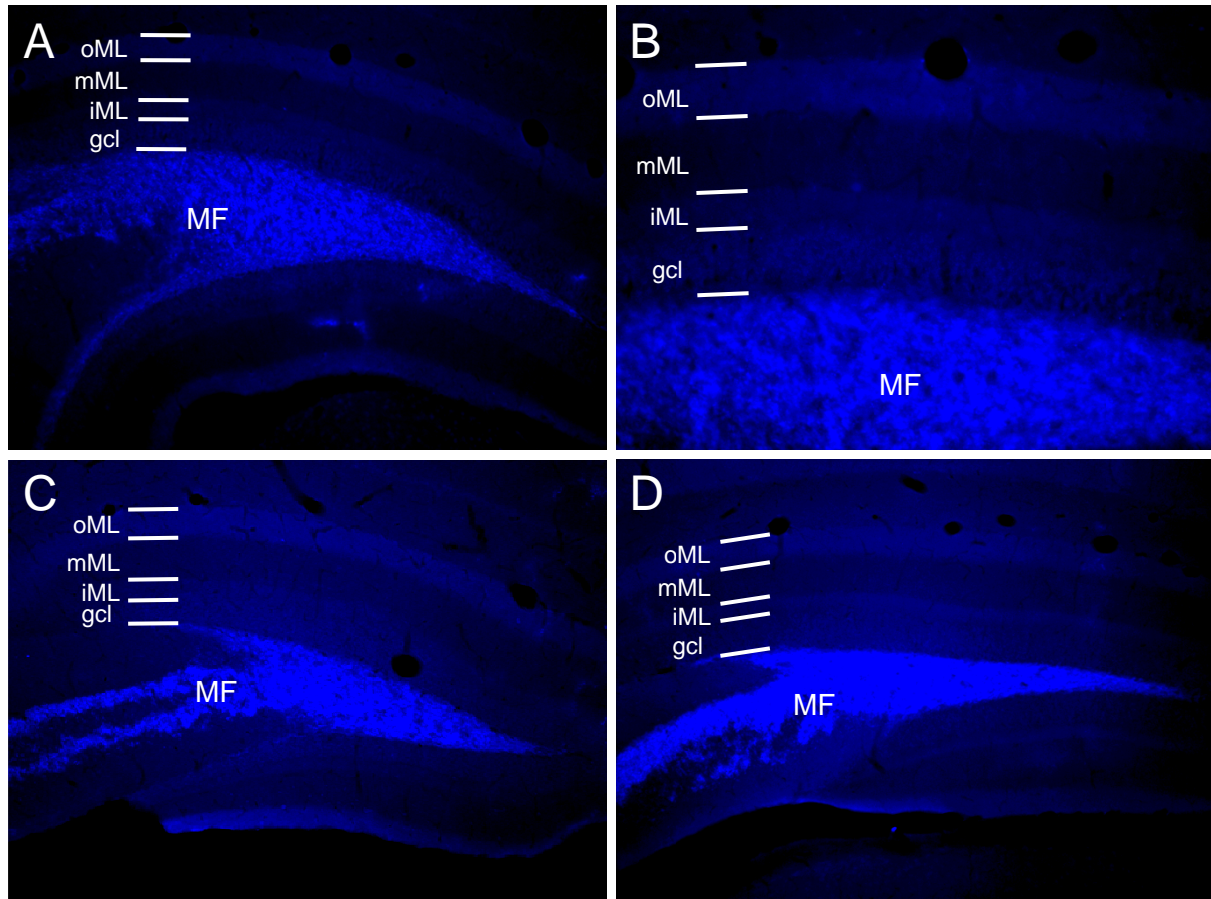


Fig. 7-6: Immunofluorescence staining against ZnT 3 in the dentate gyrus of a **A+B** CD1, **C** C57Bl/6 and **D** wild house mouse (100-fold (**A, C, D**) and 200-fold (**B**) magnification); lines mark the borders of the layers: oML, outer molecular layer; mML, middle molecular layer; iML, inner molecular layer; gcl, granule cell layer; MF, mossy fiber bundle.

For comparison of the absolute thickness of the dentate gyrus among the strains, thickness of the individual layers within one blade were taken together and compared by ANOVA. No significant differences in the absolute thickness of the dentate gyrus were found among the strains (ANOVA, strain $F_{2,26}=0.78$, $p > 0.5$) However, in all strains the infrapyramidal blade appeared to be thinner than the suprapyramidal, although this turned out to be significant only in C57Bl/6 mice (ANOVA, blade $F_{1,26}=20.63$, $p < 0.001$, interaction effect strain*blade $F_{2,26}=0.5$, $p > 0.5$, Post-hoc Unequal N HSD C57Bl/6 $p < 0.01$; Fig. 7-7).

Further analyses of the relative thickness of the individual layers within the dentate gyrus revealed a significantly thicker granule cell layer in the suprapyramidal blade of wild house mice than in the domesticated mouse strains (ANOVA interaction effect strain*layer $F_{14,1}=6.6$, $p < 0.001$, Post-hoc Unequal N HSD wild house mice vs. CD1 $p < 0.05$, vs. C57Bl/6 $p < 0.001$), although the other layers, except the middle molecular layer of

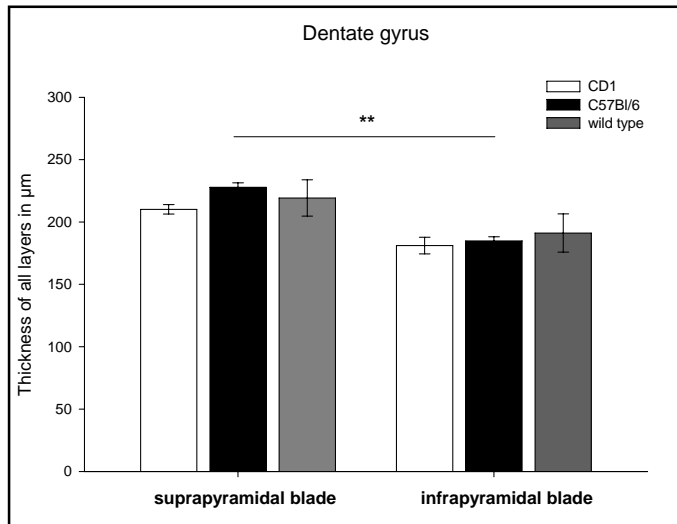


Fig. 7-7: Absolute thickness of the dentate gyrus in different mouse strains. Data given as means ± S.E.M.

the infrapyramidal blade (Post-hoc Unequal N HSD wild house mice vs. CD1 $p < 0.01$, vs. C57Bl/6 $p < 0.05$), were not subject to significant reductions (Fig. 7-8).

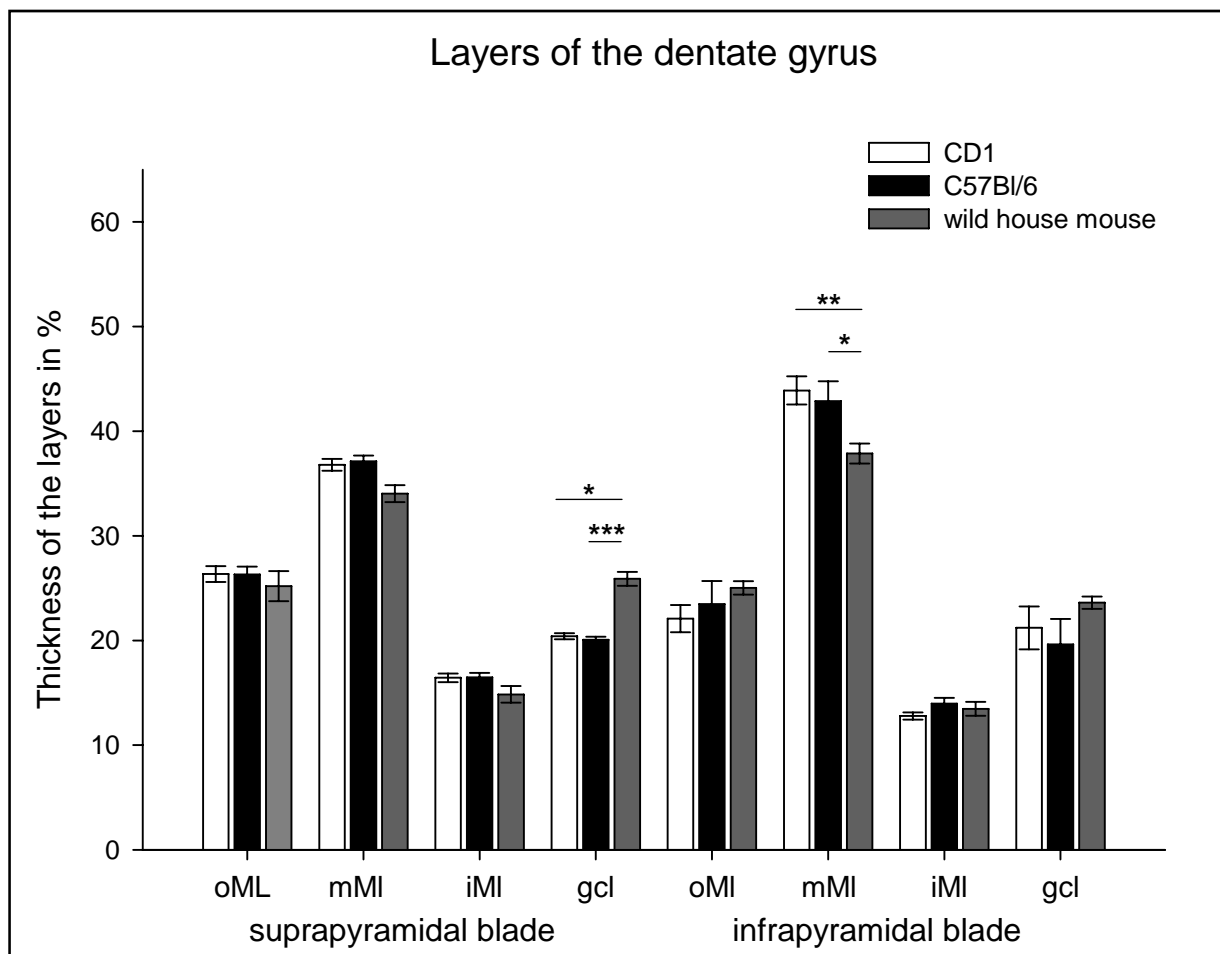


Fig. 7-8: Relative thickness of the layers of the dentate gyrus within one blade of different mouse strains. oML, outer molecular layer; mMI, middle molecular layer; iMI, inner molecular layer; gcl, granule cell layer; data given as means ± S.E.M.; all layers of one blade = 100%.

In all strains the inner molecular was found to cover about 16% (infrapyramidal blade) to 20% (suprasyramidal blade) of the whole molecular layer, while it was significantly thinner in the infrapyramidal blade of CD1 as well as of C57Bl/6 mice (Post-hoc Unequal N HSD $p < 0.05$; Fig. 7-9).

These results indicate that it is, indeed, the granule cell layer - especially in the suprasyramidal blade - which has a greater extension in wild than in the domesticated mouse strains CD1 and C57Bl/6. This corresponds to other findings of a larger granule cell layer in wild mice (cf. chapter 7.3.2.3; Amrein et al., 2004). A reduction in the thickness of the complete dentate gyrus in the course of domestication in *Mus musculus domesticus* was not found. However, in the Timm staining only the thickness of the layers was measured, so that a potential reduction of the volume of the dentate gyrus e.g. by a decrease in its rostrocaudal extension cannot be ruled out.

The infrapyramidal blade seemed to be thinner in all mouse strains, although this turned out to be significant only in C57Bl/6 mice. This finding corresponds to the fact that during ontogeny the suprasyramidal blade starts its differentiation before the infrapyramidal blade (Altman & Bayer, 1990).

Surprisingly, the inner molecular layer covered only about 16-20% of the whole molecular layer in all examined mouse strains without any significant differences among the strains. In contrast, in the gerbil, the inner molecular layer represents one third of the molecular layer (Hildebrandt, 1999). Therefrom, we expected a more prominent inner molecular layer at least in wild house mice. However, these results suggest that the ratio of the inner molecular layer is species-specific in *Mus musculus domesticus* and *Meriones unguiculatus* and that, at least in mice, it has not been reduced in the course of domestication.

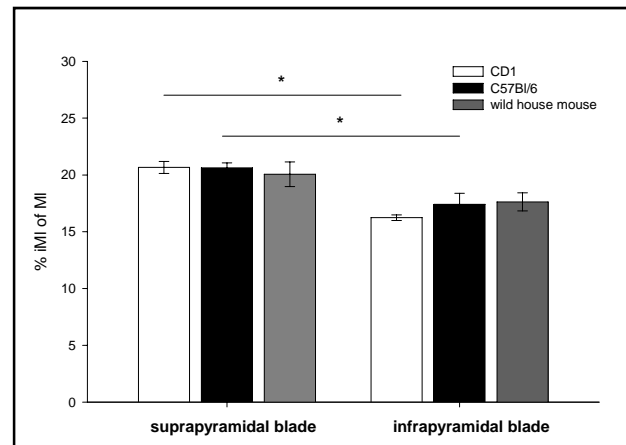


Fig. 7-9: Relative thickness of the inner molecular layer of the whole molecular layer within each blade. iMI, inner molecular layer, MI, molecular layer taken as 100% within each blade, data given as means \pm S.E.M.

8. CONCLUDING DISCUSSION

The aim of the present work was to investigate developmental neuroplasticity in the hippocampus in the interplay of prefrontal-limbic circuits with the widely-used mouse model (*Mus musculus domesticus*). The main focus was on whether and how the environment during juvenile brain development determines neuroplastic potentials in the hippocampus even in adulthood. Four experimental studies were carried out and a hypothetical model was developed integrating local as well as potential systemic mechanisms.

We first found out (project I) that adult neurogenesis indicated by the rates of cell proliferation and cell survival in the adult dentate gyrus was, at first sight, not affected by deprivation of “natural” environmental stimulation during juvenile brain development in CD1 mice, when wheel running was excluded from the enriched-environmental setup. Nevertheless, socially and physically deprived-reared CD1 mice were affected in their neuroplastic development as was revealed by an adult wheel-running challenge. Only deprived-reared individuals responded to the wheel-running challenge with a significant increase in cell proliferation and cell survival indicating a higher reactivity of neurogenesis in these animals. Since wheel-running activity measured by the running distance covered and running bouts made per night did not differ between rearing groups, it cannot be responsible for this effect. Hence, it can be concluded that developmental neuroplasticity in CD1 mice is susceptible to environmental deprivation during brain development, although it seems to be lower in CD1 mice than in gerbils.

The mechanisms underlying the different neuroplastic response to wheel running might lie on the local level: deprivation leads to alterations in the level of neurotrophic and growth factors (Falkenberg et al., 1992; Pham et al., 1999; Ickes et al., 2000; During & Cao, 2006; Lehmann et al., 2007), opioids (Eisch et al., 2000; Persson et al., 2003) and neurotransmitters (Busche et al., 2002, 2004, 2006) in the hippocampal formation. Wheel running affects these levels as well (e.g., Dudar et al., 1979; Colt et al., 1981; Goldfarb & Jamurtas, 1997; Gomez-Pinilla et al., 1997; Oliff et al., 1998; Trejo et al., 2001; Fabel et al., 2003; Vaynman et al., 2004a, b), probably leading to a greater shift in deprived-reared than in enriched-reared individuals. Furthermore, the maturation of the mesolimbocortical dopamine system, which was demonstrated to be severely affected by deprivation in gerbils (e.g., Winterfeld et al., 1998; Busche et al., 2004), might be affected in CD1 mice as well. A hypothetical model (project II) was proposed integrating these local and systemic mechanisms. It was proposed that, beside local effects, the maturation of the prefrontal cortex and its efferents and, thus, its control function over motor circuits and limbic areas including

the hippocampus might be affected by deprivation. A resulting lack of glutamatergic excitation at the NMDA-receptor system at the entorhino-dentate interface might cause the increase in cell proliferation in deprived-reared mice after wheel running.

Neurogenesis induces structural changes, neuronal and synaptic turnover. Nevertheless, in gerbils an inverse relationship between the rate of neurogenesis and the rate of synaptic remodeling in the dentate gyrus was found (Hildebrandt, 1999; Butz et al., 2008). In CD1 mice, we extended our observations even beyond the dentate gyrus to the entire hippocampal formation (project III). Like on neurogenesis, we found no effect of deprivation from environmental stimulation during brain development on synaptic remodeling in any region of the hippocampal formation. Both enriched and deprived-reared mice displayed high layer-specific differences in the rates of synaptic remodeling in the dentate gyrus, CA3 and CA1, which were equal in amount and distribution between the rearing groups. These layer-specific rates can be explained on the basis of the layer-specific connectivities as well as especially in the dentate gyrus on the background of the challenge to integrate new neurons into existing networks. Thus, these results emphasize a relationship between neurogenesis and synaptic remodeling in the hippocampal formation of the mouse. Surprisingly, a wheel-running challenge in young adulthood leveled the layer-specific differences in both deprived and enriched-reared individuals in the dentate gyrus, CA3 and CA1. The effects did not extend to the entorhinal cortex or subiculum. These findings argue in favor that beside neurogenesis further mechanisms mediate the effects of wheel running on synaptic turnover in the hippocampal formation and that beside the dentate gyrus, also CA3 and CA1 possess high plastic capacities.

Although the findings on neurogenesis and synaptic remodeling indicate that environmentally-dependent plasticity in the hippocampal formation is generally lower in CD1 mice than in gerbils, they confirm, nevertheless, that also in CD1 mice the environment during juvenile brain development determines plastic potentials even in the adult.

It was hypothesized that the prefrontal cortex respectively its maturation is jointly responsible for this altered neuroplasticity. It was, moreover, proposed that it constitutes a negative regulator of cell proliferation via excitation of the NMDA-receptor system at the entorhino-dentate interface. However, there was so far no evidence for an involvement of the prefrontal cortex in the regulation of neurogenesis in the dentate gyrus at all. We enhanced cell proliferation in CD1 mice reared under social and physical deprivation by adult wheel running. Afterwards we exposed the animals to either environmental enrichment or a prefrontal-cortex dependent working memory task to stimulate prefrontal activity (project IV).

Both environmental enrichment and the working-memory task decreased the enhanced cell proliferation compared to continued deprivation after wheel running. In contrast, a control task without working-memory requirements, which we applied to observe unspecific side effects of the behavioral task and the apparatus, did not lead to a significant effect on cell proliferation. This demonstrates, indeed, that the stimulation of the prefrontal cortex by a continuous working-memory load exerted the effect on cell proliferation. This provides evidence that the prefrontal cortex is causally involved in the regulation of cell proliferation in the dentate gyrus.

Finally, the questions remained why environmentally-dependent plasticity is lower in CD1 mice than in gerbils, concretely, why the effect of deprivation of environmental stimulation during brain development is less pronounced in these animals, and, furthermore, whether differences might exist even within the species *Mus musculus domesticus*. Comparing deprivation effects in mouse strains of the same species (*Mus musculus domesticus*), but with different domestication background (project V), enabled us to investigate, whether the variability of neuronal plasticity in response to environmental stimulation is a unique feature in mice or if genetic background plays a role, too. The effects of social and physical deprivation and subsequent wheel running in young adulthood were similar in inbred C57Bl/6 as in outbred CD1 mice. Deprived rearing did not cause different rates of cell proliferation in young-adult animals compared to enriched rearing, but led to a different neuroplastic development in so far that only deprived-reared individuals responded to the wheel-running challenge with a significant increase in cell proliferation. Astonishingly, in wild house mice, neither the rearing conditions nor the wheel-running challenge exerted an effect on cell proliferation in the adult dentate gyrus. Interestingly, whereas C57Bl/6 independently of the rearing history displayed wheel-running activity in the range of CD1 mice, deprived-reared wild house mice ran twice as much as their enriched-reared littermates. Nevertheless, this huge amount of running remained without impact on hippocampal cell proliferation. It can be assumed that in wild house mice the reactivity of cell proliferation is buffered by mechanisms which are not effective in domesticated mice anymore. Furthermore, in the course of domestication, anatomical modifications in the dentate gyrus occurred, that became manifest especially in a reduction of the granule cell layer.

The present work demonstrated that environmental stimulation during juvenile brain development determines neuroplastic potentials in the adult hippocampus of the domesticated, laboratory mouse strains CD1 and C57Bl/6. However, the reactivity of neuroplasticity seems to be a consequence of genetic modifications by domestication, since it was not found in wild house mice of the same species. Furthermore, evidence could be given that it is even in domesticated mice an outcome of deprivation from natural environmental stimulation during juvenile brain development. As a key candidate to be affected by environmental deprivation during juvenile brain development and to be involved in the regulation of neuroplasticity in the hippocampus, the prefrontal cortex could be expounded. This draws attention to a more systemic view of the regulation of neuroplasticity in the hippocampus as an interplay of limbic-prefrontal circuits.

The investigation of the exact mechanisms determining its reactivity or stability is an important issue for future research, since they may provide a key to a better understanding of the regulation of neuroplasticity in the intact and disturbed brain.

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PUBLICATIONS

Schaefer ATU, Teuchert-Noodt G, Bagorda F, Brummelte S (2009): Effect of post natal methamphetamine trauma and adolescent methylphenidate treatment on adult hippocampal neurogenesis in gerbils. *Eur J Pharmacol* 616(1-3): 86-90.

Haupt M, **Schaefer ATU**: Effects of post-weaning social and physical deprivation on locomotor activity patterns and explorative behaviour in female CD-1 mice. *Dev Psychobiol*, *accepted*.

Schaefer ATU, Grafen K, Teuchert-Noodt G, Winter Y: Synaptic remodeling in the dentate gyrus, CA3 and CA1, subiculum and entorhinal cortex of mice: effects of deprived rearing and voluntary running, *submitted*.

Further publications emanating from this work:

Schaefer ATU, Winter Y: Developmental epigenetic effects on neurogenic plasticity depend on mouse strain and are absent in wild house mice (*Mus musculus domesticus*), *in prep*.

Schaefer ATU, Winter Y: A review and hypothetical model on the effects of wheel running on neurogenesis in mice with different rearing histories. Review, *in prep*.

Winter Y, **Schaefer ATU**: An automated T-maze for working-memory tests in rodents. Method paper, *in prep*.

Posters and Presentations:

Haupt M, **Schäfers A**, Winter Y: Effects of post-weaning environment on adult motor activity and exploration of CD-1 mice, Proceedings of the International Ethological Conference 2009, Rennes.

Schäfers A, Grafen K, Winter Y: Distinct effects of post-weaning environmental enrichment and adult wheel running on neurogenesis and synaptic turnover in the hippocampus, subiculum and entorhinal cortex of mice, 8th Göttingen Meeting of the German Neuroscience Society, Abstr. Poster T1-7B.

Schäfers A, Yu F, Winter Y, Grafen K: Voluntary running affects hippocampal neurogenesis and synaptic rewiring in the hippocampus, subiculum and entorhinal cortex differentially in enriched and deprived reared mice, 6th FENS Forum 2008, Genf, Abstr. Poster 175.7.

Schäfers A, Bagorda F, Winter Y: Hidden effect of post-weaning social and physical deprivation on hippocampal neurogenesis uncovered by a wheel running challenge, Science Fair 2007, Bielefeld.

Schäfers A, Bagorda F, Winter Y: Hidden effect of post-weaning social and physical deprivation on hippocampal neurogenesis uncovered by a wheel running challenge, Adult Neurogenesis Symposium 2007, Dresden, Abstr. Poster 37.

Hsiao L, **Schäfers A**: Volumetric analysis of the dentate molecular layers in CD1- and C57Bl/6-mice, Adult Neurogenesis Symposium 2007, Dresden.

Awards:

Travel grant for the poster: **Schäfers A**, Grafen K, Winter Y: Distinct effects of post-weaning environmental enrichment and adult wheel running on neurogenesis and synaptic turnover in the hippocampus, subiculum and entorhinal cortex of mice, 8th Göttingen Meeting of the German Neuroscience Society.

3rd price for the presentation of: **Schäfers A**, Bagorda F, Winter Y: Hidden effect of post-weaning social and physical deprivation on hippocampal neurogenesis uncovered by a wheel running challenge, Science Fair 2007, Bielefeld.

SUPERVISED STUDENTS/ STUDENTS WHO PARTICIPATED IN THIS WORK

Katja Schneider: Establishment of a delayed alternation task protocol in manually-operated T- and Y-mazes for different mouse strains. Research Module Master of Brain & Behavior, 2008/2009.

Katja Schneider: Metric analysis of the dentate gyrus in mice. Research Module Master of Brain & Behavior, 2008/2009.

Vivian H. Lee, RISE – Research Internship in Science and Engineering of the DAAD, German Academic Exchange Service, 05-08/2008 (including financial support).

Lennie Hsiao, RISE - Research Internship in Science and Engineering of the DAAD, German Academic Exchange Service, 05-08/2007.

Fei Yu : The effect of rearing environment and physical activity on synaptic rewiring in the hippocampus, subiculum and entorhinal cortex. Master thesis, Brain & Behavior, 2008.

ANIMALS, GROUPS, TREATMENTS

| Project I: Neurogenesis in CD1 mice | | | | | | | | | | | | |
|--------------------------------------|-------------|-----|--|----------|--|-------------------|---------------------------------|-------------------------|------------------------------------|-----------------------|--------------------------|------------------------------------|
| project | strain | sex | anima-ID | origin | group | rearing condition | group size in rearing condition | wheel-running challenge | age (pd at start of wheel running) | BrdU: duration (days) | BrdU: injections per day | survival time after last injection |
| cell proliferation and cell survival | CD1 | f | Geh 4 | AF | ER _{SPW-} | enriched | 15 | - | 70 | 3 | 1 | 5d |
| | | f | Geh 7 | AF | ER _{SPW-} | enriched | 15 | - | 70 | 3 | 1 | 5d |
| | | f | Geh 16 | AF | ER _{SPW-} | enriched | 15 | - | 70 | 3 | 1 | 5d |
| | | f | Geh 22 | AF | ER _{SPW-} | enriched | 15 | - | 70 | 3 | 1 | 5d |
| | | f | Geh 116 | AF | ER _{SPW-} | enriched | 10 | - | 70 | 3 | 1 | 5d |
| | | f | Geh 217 | AF | ER _{SPW-} | enriched | 10 | - | 70 | 3 | 1 | 5d |
| | | f | Geh 19 run | AF | ER _{SPW-} , after wheel running | enriched | 15 | x | 70 | 3 | 1 | 5d |
| | | f | Geh 33 run | AF | ER _{SPW-} , after wheel running | enriched | 15 | x | 70 | 3 | 1 | 5d |
| | | f | Geh 35 run | AF | ER _{SPW-} , after wheel running | enriched | 15 | x | 70 | 3 | 1 | 5d |
| | | f | Geh 37 run | AF | ER _{SPW-} , after wheel running | enriched | 15 | x | 70 | 3 | 1 | 5d |
| | | f | Geh 111 run | AF | ER _{SPW-} , after wheel running | enriched | 10 | x | 70 | 3 | 1 | 5d |
| | | f | Geh 112 run | AF | ER _{SPW-} , after wheel running | enriched | 10 | x | 70 | 3 | 1 | 5d |
| | | f | Geh 213 run | AF | ER _{SPW-} , after wheel running | enriched | 10 | x | 70 | 3 | 1 | 5d |
| | | f | Box 60 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 5d |
| | | f | Box 37 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 5d |
| | | f | Box 27 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 5d |
| | | f | Box 25 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 5d |
| | | f | Box 106 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 5d |
| | | f | Box 207 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 5d |
| | | f | Box 4 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 5d |
| | | f | Box 1 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 5d |
| | | f | Box 34 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 5d |
| | | f | Box 69 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 5d |
| | | f | Box 101 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 5d |
| | | f | Box 202 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 5d |
| | | f | Geh 25 | AF | ER _{SPW-} | enriched | 15 | - | 70 | 3 | 1 | 21d |
| | | f | Geh 17 | AF | ER _{SPW-} | enriched | 15 | - | 70 | 3 | 1 | 21d |
| | | f | Geh 38 | AF | ER _{SPW-} | enriched | 15 | - | 70 | 3 | 1 | 21d |
| | | f | Geh 118 | AF | ER _{SPW-} | enriched | 10 | - | 70 | 3 | 1 | 21d |
| | | f | Geh 119 | AF | ER _{SPW-} | enriched | 10 | - | 70 | 3 | 1 | 21d |
| | | f | Geh 220 | AF | ER _{SPW-} | enriched | 10 | - | 70 | 3 | 1 | 21d |
| | | f | Geh 23 run | AF | ER _{SPW-} , after wheel running | enriched | 15 | x | 70 | 3 | 1 | 21d |
| | | f | Geh 13 run | AF | ER _{SPW-} , after wheel running | enriched | 15 | x | 70 | 3 | 1 | 21d |
| | | f | Geh 34 run | AF | ER _{SPW-} , after wheel running | enriched | 15 | x | 70 | 3 | 1 | 21d |
| | | f | Geh 31 run | AF | ER _{SPW-} , after wheel running | enriched | 15 | x | 70 | 3 | 1 | 21d |
| | | f | Geh 114 run | AF | ER _{SPW-} , after wheel running | enriched | 10 | x | 70 | 3 | 1 | 21d |
| | | f | Geh 115 run | AF | ER _{SPW-} , after wheel running | enriched | 10 | x | 70 | 3 | 1 | 21d |
| | | f | Box 47 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 21d |
| | | f | Box 2 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 21d |
| | | f | Box 35 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 21d |
| | | f | Box 108 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 21d |
| | | f | Box 109 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 21d |
| | | f | Box 110 | AF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 21d |
| | | f | Box 52 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 21d |
| | | f | Box 53 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 21d |
| f | Box 75 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 21d | | |
| f | Box 103 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 21d | | |
| f | Box 104 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 21d | | |
| f | Box 205 run | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 21d | | |

| Project I: Neurogenesis in CD1 mice | | | | | | | | | | | | |
|-------------------------------------|--------|-----|--------------------|--------------------|--------------------|--------------------|---------------------------------|-------------------------|-------------------------------------|-----------------------|--------------------------|------------------------------------|
| project | strain | sex | animal-ID | origin | group | rearing condition | group size in rearing condition | wheel-running challenge | age (at start of wheel running; pd) | BrdU: duration (days) | BrdU: injections per day | survival time after last injection |
| Effect of cage size in deprivation | CD1 | f | K2 | CR | DR _{SPW-} | deprived (type II) | 1 | - | 70 | 3 | 1 | 5d |
| | | f | K3 | CR | DR _{SPW-} | deprived (type II) | 1 | - | 70 | 3 | 1 | 5d |
| | | f | K4 | CR | DR _{SPW-} | deprived (type II) | 1 | - | 70 | 3 | 1 | 5d |
| | | f | K6 | CR | DR _{SPW-} | deprived (type II) | 1 | - | 70 | 3 | 1 | 5d |
| | | f | K7 | CR | DR _{SPW-} | deprived (type II) | 1 | - | 70 | 3 | 1 | 5d |
| | | f | K8 | CR | DR _{SPW-} | deprived (type II) | 1 | - | 70 | 3 | 1 | 5d |
| | | f | K11 | CR | DR _{SPW-} | deprived (type II) | 1 | - | 70 | 3 | 1 | 5d |
| f | K12 | CR | DR _{SPW-} | deprived (type II) | 1 | - | 70 | 3 | 1 | 5d | | |

| project | strain | sex | animal-ID | origin | group | rearing condition | group size in rearing condition | wheel-running challenge | age (at start of wheel running; pd) | BrdU: duration (days) | BrdU: injections per day | survival time after last injection |
|------------------------|--------|-----|-----------|--------|--------------------|-------------------|---------------------------------|-------------------------|-------------------------------------|-----------------------|--------------------------|------------------------------------|
| Wheel-running activity | CD1 | f | Geh 1 | CR | ER _{SPW-} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 2 | CR | ER _{SPW-} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 3 | CR | ER _{SPW-} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 4 | CR | ER _{SPW-} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 5 | CR | ER _{SPW-} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 6 | CR | ER _{SPW-} | enriched | 6 | x | 66 | - | - | - |
| | | f | Box 1 | CR | DR _{SPW-} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 2 | CR | DR _{SPW-} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 3 | CR | DR _{SPW-} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 4 | CR | DR _{SPW-} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 5 | CR | DR _{SPW-} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 6 | CR | DR _{SPW-} | deprived | 1 | x | 70 | - | - | - |

| Project III: Synaptic remodelling in CD1 mice | | | | | | | | | | | | |
|---|--------|-----|-----------|--------|--|-------------------|---------------------------------|-------------------------|------------------------------------|-----------------------|--------------------------|-----------------------------------|
| project | strain | sex | animal-ID | origin | group | rearing condition | group size in rearing condition | wheel-running challenge | age (pd at start of wheel running) | BrdU: duration (days) | BrdU: injections per day | survival time after wheel running |
| synaptic remodelling | CD1 | m | C13 | AF | ER _{SPW-} | enriched | 13 | - | 74 | - | - | 7 |
| | | m | C15 | AF | ER _{SPW-} | enriched | 13 | - | 74 | - | - | 7 |
| | | m | C16 | AF | ER _{SPW-} | enriched | 13 | - | 74 | - | - | 7 |
| | | m | C17 | AF | ER _{SPW-} | enriched | 13 | - | 74 | - | - | 7 |
| | | m | C19 | AF | ER _{SPW-} | enriched | 13 | - | 74 | - | - | 7 |
| | | m | C20 | AF | ER _{SPW-} | enriched | 13 | - | 74 | - | - | 7 |
| | | m | C21 | AF | ER _{SPW-} | enriched | 13 | - | 74 | - | - | 7 |
| | | m | C27 | AF | ER _{SPW-} , after wheel running | enriched | 13 | x | 74 | - | - | 3 |
| | | m | C28 | AF | ER _{SPW-} , after wheel running | enriched | 13 | x | 74 | - | - | 3 |
| | | m | C29 | AF | ER _{SPW-} , after wheel running | enriched | 13 | x | 74 | - | - | 3 |
| | | m | C30 | AF | ER _{SPW-} , after wheel running | enriched | 13 | x | 74 | - | - | 3 |
| | | m | C31 | AF | ER _{SPW-} , after wheel running | enriched | 13 | x | 74 | - | - | 3 |
| | | m | C32 | AF | ER _{SPW-} , after wheel running | enriched | 13 | x | 74 | - | - | 3 |
| | | m | C1 | AF | DR _{SPW-} | deprived | 1 | - | 74 | - | - | 7 |
| | | m | C3 | AF | DR _{SPW-} | deprived | 1 | - | 74 | - | - | 7 |
| | | m | C4 | AF | DR _{SPW-} | deprived | 1 | - | 74 | - | - | 7 |
| | | m | C5 | AF | DR _{SPW-} | deprived | 1 | - | 74 | - | - | 7 |
| | | m | C7 | AF | DR _{SPW-} | deprived | 1 | - | 74 | - | - | 7 |
| | | m | C8 | AF | DR _{SPW-} | deprived | 1 | - | 74 | - | - | 7 |
| | | m | C9 | AF | DR _{SPW-} | deprived | 1 | - | 74 | - | - | 7 |
| | | m | C10 | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 74 | - | - | 3 |
| | | m | C22 | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 74 | - | - | 3 |
| | | m | C23 | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 74 | - | - | 3 |
| | | m | C24 | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 74 | - | - | 3 |
| | | m | C25 | AF | DR _{SPW-} , after wheel running | deprived | 1 | x | 74 | - | - | 3 |

| Project IV: PFC and hippocampal cell proliferation | | | | | | | | | | | | | | | | |
|--|--------|-----|-----------|----------|---------------------------------------|-------------------|--------------------|--------------------------|-------------------------|------------------------------------|------------------------------------|---------------------------------|-----------------------|--------------------------|------------------------------------|-------------------------------|
| project | strain | sex | animal-ID | origin | group/treatment (after wheel running) | rearing condition | transponder number | transponder tagging (pd) | wheel-running challenge | age (pd at start of wheel running) | weight (at start of wheel running) | group size during wheel running | BrdU: duration (days) | BrdU: injections per day | survival time after last injection | weight (at end of experiment) |
| PFC activity | CD1 | f | DC18 | CR | DC | deprived | 0416ECEEEE | 50; RT 56 | x | 79 | 29.4 | 1 | 1 | 3 | 24h | 27.8 |
| | | f | DC19 | CR | DC | deprived | 0416ECE006 | 50 | x | 79 | 27.0 | 1 | 1 | 3 | 24h | 28.0 |
| | | f | DC21 | CR | DC | deprived | 0416ECDD8B | 50 | x | 79 | 30.4 | 1 | 1 | 3 | 24h | 30.9 |
| | | f | DC22 | CR | DC | deprived | 0416ECE638 | 50 | x | 79 | 24.5 | 1 | 1 | 3 | 24h | 23.8 |
| | | f | DC25 | CR | DC | deprived | 0416ECE680 | 50 | x | 79 | 30.2 | 1 | 1 | 3 | 24h | 29.5 |
| | | f | DC30 | CR | DC | deprived | 0416ECD2AB | 50 | x | 79 | 26.5 | 1 | 1 | 3 | 24h | 26.8 |
| | | f | DC35 | CR | DC | deprived | 0416ECC45B | 50; RT 56 | x | 79 | 26.1 | 1 | 1 | 3 | 24h | 26.2 |
| | | f | EC2 | CR | EC | deprived | 0416ECEEF0 | 50 | x | 79 | 33.5 | 1 | 1 | 3 | 24h | 30.5 |
| | | f | EC4 | CR | EC | deprived | 0416ECE203 | 50 | x | 79 | 28.4 | 1 | 1 | 3 | 24h | 27.0 |
| | | f | EC10 | CR | EC | deprived | 0416ECD414 | 50 | x | 79 | 29.9 | 1 | 1 | 3 | 24h | 27.5 |
| | | f | EC11 | CR | EC | deprived | 0416ECCF05 | 50 | x | 79 | 29.7 | 1 | 1 | 3 | 24h | 30.4 |
| | | f | EC17 | CR | EC | deprived | 0416ECD147 | 50; RT 56 | x | 79 | 26.0 | 1 | 1 | 3 | 24h | 25.4 |
| | | f | EC24 | CR | EC | deprived | 0416ECCC21 | 50 | x | 79 | 27.7 | 1 | 1 | 3 | 24h | 26.2 |
| | | f | EC33 | CR | EC | deprived | 0416ECE65E | 50 | x | 79 | 30.5 | 1 | 1 | 3 | 24h | 29.3 |
| | | f | P5 | CR | PFCII | deprived | 0416ECDA8D | 50 | x | 72 | 26.5 | 4 | 1 | 3 | 24h | 29.6 |
| | | f | P8 | CR | PFCII | deprived | 0416ECDB0C | 50; RT 56 | x | 72 | 29.0 | 4 | 1 | 3 | 24h | 27.6 |
| | | f | P16 | CR | PFCII | deprived | 0416ECC45E | 50; RT 56 | x | 72 | 27.5 | 4 | 1 | 3 | 24h | 30.5 |
| | | f | P31 | CR | PFCII | deprived | 0416ECD76F | 50; RT 56 | x | 72 | 30.3 | 4 | 1 | 3 | 24h | 31.0 |
| | | f | P7 | CR | PFCIII | deprived | 0416ECE680 | 50; RT 56 | x | 86 | 30.6 | 4 | 1 | 3 | 24h | 29.0 |
| | | f | P9 | CR | PFCIII | deprived | 0416ECCF05 | 50; RT 56 | x | 86 | 31.7 | 4 | 1 | 3 | 24h | 28.2 |
| | | f | P12 | CR | PFCIII | deprived | 0416EC D8AF | 50; RT 56 | x | 86 | 32.0 | 4 | 1 | 3 | 24h | 29.5 |
| | | f | P34 | CR | PFCIII | deprived | 0416ECE52D | 50; RT 56 | x | 86 | 26.7 | 4 | 1 | 3 | 24h | 27.2 |
| | | f | T14 | CR | TCI | deprived | 0416ECD3ED | 50; RT 56 | x | 79 | 28.2 | 4 | 1 | 3 | 24h | 28.1 |
| | | f | T26 | CR | TCI | deprived | 0416ECEA33 | 50 | x | 79 | 32.5 | 4 | 1 | 3 | 24h | 29.6 |
| | | f | T27 | CR | TCI | deprived | 0416ECD15F | 50 | x | 79 | 30.5 | 4 | 1 | 3 | 24h | 31.5 |
| | | f | T28 | CR | TCI | deprived | 0416ECEAEF | 50 | x | 79 | 29.0 | 4 | 1 | 3 | 24h | 32.0 |
| f | T15 | CR | TCII | deprived | 0416ECC2DC | 50; RT 56 | x | 93 | 26.1 | 4 | 1 | 3 | 24h | 27.2 | | |
| f | T20 | CR | TCII | deprived | 0416ECD85C | 50 | x | 93 | 29.2 | 4 | 1 | 3 | 24h | 31.4 | | |
| f | T29 | CR | TCII | deprived | 0416ECE46E | 50 | x | 93 | 28.4 | 4 | 1 | 3 | 24h | 32.0 | | |
| f | T32 | CR | TCII | deprived | 0416ECDFAF | 50 | x | 93 | 29.4 | 4 | 1 | 3 | 24h | 3.0 | | |

| Project V: Strain comparison | | | | | | | | | | | | | |
|------------------------------|---------|-----|--------------------|----------|--|-------------------|---------------------------------|-------------------------|------------------------------------|-----------------------|--------------------------|------------------------------------|-------------------------------|
| project | strain | sex | animal-ID | origin | group | rearing condition | group size in rearing condition | wheel-running challenge | age (pd at start of wheel running) | BrdU: duration (days) | BrdU: injections per day | survival time after last injection | weight (at end of experiment) |
| cell proliferation | C57Bl/6 | f | Geh 1 | CAF | ER _{SPW-} | enriched | 11 | - | 70 | 3 | 1 | 24h | |
| | | f | Geh 2 | CAF | ER _{SPW-} | enriched | 11 | - | 70 | 3 | 1 | 24h | |
| | | f | Geh 3 | CAF | ER _{SPW-} | enriched | 11 | - | 70 | 3 | 1 | 24h | |
| | | f | Geh 4 | CAF | ER _{SPW-} | enriched | 11 | - | 70 | 3 | 1 | 24h | |
| | | f | Geh 5 | CAF | ER _{SPW-} | enriched | 11 | - | 70 | 3 | 1 | 24h | |
| | | f | Geh 6 | CAF | ER _{SPW-} | enriched | 11 | - | 70 | 3 | 1 | 24h | |
| | | f | Geh run 15 | CAF | ER _{SPW-} , after wheel running | enriched | 11 | x | 70 | 3 | 1 | 24h | |
| | | f | Geh run 16 | CAF | ER _{SPW-} , after wheel running | enriched | 11 | x | 70 | 3 | 1 | 24h | |
| | | f | Geh run 17 | CAF | ER _{SPW-} , after wheel running | enriched | 11 | x | 70 | 3 | 1 | 24h | |
| | | f | Geh run 18 | CAF | ER _{SPW-} , after wheel running | enriched | 11 | x | 70 | 3 | 1 | 24h | |
| | | f | Geh run 19 | CAF | ER _{SPW-} , after wheel running | enriched | 11 | x | 70 | 3 | 1 | 24h | |
| | | f | Box 7 | CAF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 24h | |
| | | f | Box 8 | CAF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 24h | |
| | | f | Box 9 | CAF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 24h | |
| | | f | Box 10 | CAF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 24h | |
| | | f | Box 11 | CAF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 24h | |
| | | f | Box 12 | CAF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 24h | |
| | | f | Box 13 | CAF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 24h | |
| f | Box 14 | CAF | DR _{SPW-} | deprived | 1 | - | 70 | 3 | 1 | 24h | | | |

| Project V: Strain comparison | | | | | | | | | | | | |
|------------------------------|-----------------|------|---|----------|---|-------------------|---------------------------------|-------------------------|------------------------------------|-----------------------|--------------------------|------------------------------------|
| project | strain | sex | animal-ID | origin | group | rearing condition | group size in rearing condition | wheel-running challenge | age (pd at start of wheel running) | BrDU: duration (days) | BrDU: injections per day | survival time after last injection |
| cell proliferation | C57Bl/6 | f | Box run 21 | CAF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box run 22 | CAF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box run 23 | CAF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box run 24 | CAF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box run 25 | CAF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box run 26 | CAF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box run 27 | CAF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box run 28 | CAF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | wild house mice | f | Geh 7 | Z-AF | ER _{SPW} | enriched | 6 | - | 70 | 3 | 1 | 24h |
| | | f | Geh 8 | Z-AF | ER _{SPW} | enriched | 6 | - | 70 | 3 | 1 | 24h |
| | | f | Geh 19 | Z-AF | ER _{SPW} | enriched | 6 | - | 70 | 3 | 1 | 24h |
| | | f | Geh 20 | Z-AF | ER _{SPW} | enriched | 6 | - | 70 | 3 | 1 | 24h |
| | | f | Geh 21 | Z-AF | ER _{SPW} | enriched | 6 | - | 70 | 3 | 1 | 24h |
| | | f | Geh 119 | Z-AF | ER _{SPW} | enriched | 6 | - | 70 | 3 | 1 | 24h |
| | | f | Geh 120 | Z-AF | ER _{SPW} | enriched | 6 | - | 70 | 3 | 1 | 24h |
| | | f | Geh 121 | Z-AF | ER _{SPW} | enriched | 6 | - | 70 | 3 | 1 | 24h |
| | | f | Geh 4 run | Z-AF | ER _{SPW} , after wheel running | enriched | 6 | x | 70 | 3 | 1 | 24h |
| | | f | Geh 5 run | Z-AF | ER _{SPW} , after wheel running | enriched | 6 | x | 70 | 3 | 1 | 24h |
| | | f | Geh 22 run | Z-AF | ER _{SPW} , after wheel running | enriched | 6 | x | 70 | 3 | 1 | 24h |
| | | f | Geh 23 run | Z-AF | ER _{SPW} , after wheel running | enriched | 6 | x | 70 | 3 | 1 | 24h |
| | | f | Geh 24 run | Z-AF | ER _{SPW} , after wheel running | enriched | 6 | x | 70 | 3 | 1 | 24h |
| | | f | Geh 123 run | Z-AF | ER _{SPW} , after wheel running | enriched | 6 | x | 70 | 3 | 1 | 24h |
| | | f | Geh 125 run | Z-AF | ER _{SPW} , after wheel running | enriched | 6 | x | 70 | 3 | 1 | 24h |
| | | f | Geh 124 run | Z-AF | ER _{SPW} , after wheel running | enriched | 6 | x | 70 | 3 | 1 | 24h |
| | | f | Box2 | Z-AF | DR _{SPW} | deprived | 1 | - | 70 | 3 | 1 | 24h |
| | | f | Box 12 | Z-AF | DR _{SPW} | deprived | 1 | - | 70 | 3 | 1 | 24h |
| | | f | Box 13 | Z-AF | DR _{SPW} | deprived | 1 | - | 70 | 3 | 1 | 24h |
| | | f | Box 14 | Z-AF | DR _{SPW} | deprived | 1 | - | 70 | 3 | 1 | 24h |
| | | f | Box 101 | Z-AF | DR _{SPW} | deprived | 1 | - | 70 | 3 | 1 | 24h |
| | | f | Box 102 | Z-AF | DR _{SPW} | deprived | 1 | - | 70 | 3 | 1 | 24h |
| | | f | Box 104 | Z-AF | DR _{SPW} | deprived | 1 | - | 70 | 3 | 1 | 24h |
| | | f | Box 1 run | Z-AF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box 15 run | Z-AF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| | | f | Box 16 run | Z-AF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h |
| f | Box 18 run | Z-AF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h | | |
| f | Box 105 run | Z-AF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h | | |
| f | Box 106 run | Z-AF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h | | |
| f | Box 107 run | Z-AF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h | | |
| f | Box 108 run | Z-AF | DR _{SPW} , after wheel running | deprived | 1 | x | 70 | 3 | 1 | 24h | | |

| Project V: Strain comparison | | | | | | | | | | | | |
|------------------------------|-----------------|---|-----------|--------|-------------------|-------------------|---------------------------------|-------------------------|------------------------------------|-----------------------|--------------------------|------------------------------------|
| project | strain | | animal-ID | origin | group | rearing condition | group size in rearing condition | wheel-running challenge | age (pd at start of wheel running) | BrdU: duration (days) | BrdU: injections per day | survival time after last injection |
| Wheel-running activity | C57Bl/6 | f | Geh 1 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 2 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 3 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 4 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 5 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 6 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 21 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 22 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 23 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 24 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 25 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 26 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 31 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 32 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 33 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 34 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 35 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Geh 36 | CAF | ER _{SPW} | enriched | 6 | x | 67 | - | - | - |
| | | f | Box 1 | CAF | DR _{SPW} | deprived | 1 | x | 71 | - | - | - |
| | | f | Box 2 | CAF | DR _{SPW} | deprived | 1 | x | 71 | - | - | - |
| | | f | Box 3 | CAF | DR _{SPW} | deprived | 1 | x | 71 | - | - | - |
| | | f | Box 4 | CAF | DR _{SPW} | deprived | 1 | x | 71 | - | - | - |
| | | f | Box 5 | CAF | DR _{SPW} | deprived | 1 | x | 71 | - | - | - |
| | | f | Box 6 | CAF | DR _{SPW} | deprived | 1 | x | 71 | - | - | - |
| | wild house mice | f | Geh 1 | Z-AF | ER _{SPW} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 2 | Z-AF | ER _{SPW} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 3 | Z-AF | ER _{SPW} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 4 | Z-AF | ER _{SPW} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 5 | Z-AF | ER _{SPW} | enriched | 6 | x | 66 | - | - | - |
| | | f | Geh 6 | Z-AF | ER _{SPW} | enriched | 6 | x | 66 | - | - | - |
| | | f | Box 1 | Z-AF | DR _{SPW} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 2 | Z-AF | DR _{SPW} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 3 | Z-AF | DR _{SPW} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 4 | Z-AF | DR _{SPW} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 5 | Z-AF | DR _{SPW} | deprived | 1 | x | 70 | - | - | - |
| | | f | Box 6 | Z-AF | DR _{SPW} | deprived | 1 | x | 70 | - | - | - |

| Project V: Strain comparison | | | | | | | | | |
|--|-----------------|---|-----------|--------|--------------------|-------------------|---------------------------------|-------------------------|----------------------|
| project | strain | | animal-ID | origin | group | rearing condition | group size in rearing condition | wheel-running challenge | age (pd at analysis) |
| Morphometric analysis of the dentate gyrus | CD1 | m | C3 | AF | DR _{SPW-} | deprived | 1 | - | 63 |
| | | m | C4 | AF | DR _{SPW-} | deprived | 1 | - | 63 |
| | | m | C5 | AF | DR _{SPW-} | deprived | 1 | - | 63 |
| | | m | C6 | AF | DR _{SPW-} | deprived | 1 | - | 63 |
| | C57Bl/6 | m | B1 | CAF | DR _{SPW-} | deprived | 1 | - | 82 |
| | | m | B2 | CAF | DR _{SPW-} | deprived | 1 | - | 75 |
| | | m | B3 | CAF | DR _{SPW-} | deprived | 1 | - | 77 |
| | | m | B4 | CAF | DR _{SPW-} | deprived | 1 | - | 81 |
| | | m | B5 | CAF | DR _{SPW-} | deprived | 1 | - | 81 |
| | | m | B6 | CAF | DR _{SPW-} | deprived | 1 | - | 81 |
| | | m | B7 | CAF | DR _{SPW-} | deprived | 1 | - | 75 |
| | wild house mice | m | W1 | Z-AF | DR _{SPW-} | deprived | 6 | - | 59 |
| | | m | W2 | Z-AF | DR _{SPW-} | deprived | 6 | - | 59 |
| | | m | W3 | Z-AF | DR _{SPW-} | deprived | 6 | - | 59 |
| | | m | W4 | Z-AF | DR _{SPW-} | deprived | 6 | - | 59 |
| | | m | W6 | Z-AF | DR _{SPW-} | deprived | 6 | - | 64 |

f, female; m, male; AF, own animal facility; CAF, central animal facility of Bielefeld University; Z-AF, own animal facility, parents obtained from Zürich; CR, Charles River; pd, postnatal day; DC, deprived condition; EC, enriched condition; PFC, PFC-task; TC, T-maze, control (for more explanations see detailed descriptions of the projects)

STAINING PROTOCOLS

BRDU-DAB

Labelling with BrdU

BrdU solution (e.g. 50mg/kg dose \Rightarrow 5mg for 100g of mice)
(prepare just before use)

- 5mg BrdU (from freezer) in a centrifuge tube (2ml capacity)
- ad 1ml of saline and vortex

Inject 1ml per 100g, e.g. a 30g mouse would receive 0,3 ml of solution.

Preparation

Day 1

Preperfusion buffer, pH 7.4, = PB 0,1M (see stock solutions)

Perfusion solution

(prepare just before use)

8% Paraformaldehyde solution (PFA)

- 80g Paraformaldehyde (CH₂O)_n
- fill wiht A. dest. to 1000ml

\Rightarrow Heat solution to 60°C (not above). Turn off heat source.

\Rightarrow Add some drops of NaOH until the solution becomes clear (~10ml). Allow PFA to cool to RT. Filter the solution. Dilute 8% PFA to 4% PFA as described below.

4% Paraformaldehyde in 0.1M PB

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

After the perfusion brains are carefully removed and stored overnight in 4% (PFA).

Day 2

Then they are transferred to a 30% succrose solution for cryprotection for another 24 hours.

Cryoprotectant 1 : 30 % Succrose, 0.1M phosphat buffer (PB)

- 30g Succrose
- In 100ml PB (see stock solutions)

Day 3

Brains were cut with the FrigoMobil into 40 μ m coronar sections and collected in:

Cryoprotectant 2: 8.6% Succrose, 7mM/l Magnesium chloride, 0.05M PB, 44% glycerol

- 8.56g Succrose

- 0.14g Magnesium chloride hexahydrate ($MgCl_2 \times 6H_2O$)
- 50ml 0.1M PB
- 50ml 99% Glycerol

Sections can then be stored at $-20^\circ C$ until further processing.

Immunohistochemistry:

Slowly defrost sections at $4^\circ C$ for **several minutes** followed by half an hour at room temperature

| Treatment | Substance | Dilution | Incubation |
|--|---|--|-------------|
| Rinse | 10 mM PBS/T | (see stock solutions) | 5x5 |
| H ₂ O ₂ -treatment | 0.6% H ₂ O ₂ in 10 mM PBS | e.g. 2,94ml PBS + 0.06ml H ₂ O ₂ (30%ige)= 3ml (0.6%) | 30' |
| Rinse | 10 mM PBS | | 5 x 5' |
| DNA-denaturation | 2 N HCL | 1ml/cavity | 60', 37°C |
| Neutralization | 0.1 M Borate buffer, pH 8.5 | 3.09 g Boric acid (H ₃ BO ₃) per 500 ml A. dest., pH 8.5 | 2 x 5' |
| Rinse | 10 mM PBS | | 3 x 5' |
| Block | M.O.M. Blocking reagent | e.g. 2 drops to 2.5ml PBS/T per 3 cav. | 60' |
| Rinse | 10 mM PBS | | 2 x 5' |
| M.O.M Protein concentrate | M.O.M. Diluent | 200µl of Proteinconcentrate to 2.5ml PBS ⇔ 80µl/ml (0.83ml/cav.) | 5' |
| Primary Antibody Anti-BrdU (mouse) 1:400 | M.O.M. Diluent | See above for M.O.M. diluent, 6µl of AB /2,4ml (0.8ml/cav; aliquots) | ≈ 20 h, 4°C |

Day 4

| | | | |
|---|---|---|---------|
| Rinse | 10 mM PBS | | 5 x 5' |
| Secondary Antibody M.O.M. anti-mouse reagent + biotin. goat anti-mouse 1:266 | M.O.M. Diluent | 10µl of M.O.M. anti-mouse reagent to 2.5ml of M.O.M.™ Diluent + 12,5µl biotin. goat anti-mouse to 2.5ml M.O.M. Diluent incl. anti-mouse reagent (as described above) | 2 h |
| Rinse | 10 mM PBS | | 5 x 5' |
| Avidin Biotin Complex | ABC-Kit in 10mM PBS | 1drop sol. A + 1drop sol. B / 2,5ml PBS (mix 30min. before use!!) | 1 h |
| Rinse | 10mM PBS | | 2 x 5' |
| DAB-reaction | DAB-solution + 1% H ₂ O ₂ | 25mg DAB in 50ml PBS, filtrieren (just prepare before use !), + 0,5ml 1% H ₂ O ₂ (1% H ₂ O ₂ : 2,9ml PBS + 0,1ml H ₂ O ₂ (30%)) | ~ 4min. |
| Rinse | 10mM PBS | | 3 x 5' |
| Rinse | 10mM PBS/T | (It is easier to mount out of PBS/T) | 2 x 5' |

After rinsing, mount the sections on slides. The mounted sections should be air-dried over night.

Day 5

The slides should be **dehydrated** through an ascending series of **alcohol**:

2 × 5min. in 70%

2 × 5min. in 96%

2 × 5min. 100%

The alcohol must be replaced by immersion of the slides 3 × 5min. in **xylene** and then be cover-slipped with the xylene-soluble mounting medium **DePeX** (Serva) and allow them to harden for at least 3 days.

Stock solutions

0.4M Phosphate buffer, pH 7.4 (PB)

- 114g Di-sodium hydrogenphosphate-dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)
- 24,8g Sodium dihydrogenphosphate-dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$)
- fill with A. dest. to 1800ml
- adjust pH to 7.4 (with 1N NaOH)
- fill with A. dest. to 2000ml

⇒ store at RT

0.1M PB, pH 7.4

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

⇒ store in frigide

10mM Phosphate buffered saline, pH 7.4 (PBS): 0.15M Sodium chloride (2 liter)

- 50ml 0.4M PB
- 1950ml A. dest.
- 17g NaCl

10mM Phosphate buffered saline, pH 7.4 (PBS/T): 0.15M Sodium chloride, 0.1% Triton (1l.)

- 1000ml 10mM PBS
- 1ml Triton X-100

Stock solutions for M.O.M. kit

M.O.M.TM Mouse Ig Blocking Reagent:

- 2 drops (2 × 45µl) of stock solution to
- 2.5ml of PBS/T (10mM)

M.O.M.TM Diluent:

- 600µl of Protein Concentrate stock solution to
- 7.5ml of PBS

LIST OF REAGENTS AND CHEMICALS FOR BRDU-DAB-STAINING IN MICE

| Name | Product number | Supplier | Amount (Price) |
|---|-----------------------|----------------------------------|-----------------------|
| BrdU: 5-Bromo-2'-deoxyuridine | B5002 | Sigma-Aldrich | 500mg (38.20€) |
| Diethylether | 03024 | Chemical store University | |
| Paraformaldehyde (CH ₂ O) _n | 00074 | Chemical store University | |
| Sucrose | 107687 | Merck | 1kg (in request) |
| Magnesium chloride hexahydrate (MgCl ₂ ×6H ₂ O) | 00187 | Chemical store University | |
| Glycerol min. 99% | G5516 | Sigma-Aldrich | 500ml (63.60) |
| H ₂ O ₂ 30% p.A. | 00091 | Chemical store University | |
| 2 N HCL | T134.1 | Carl Roth | 1l (10.45€) |
| Boric acid (H ₃ BO ₃) p.A. | 00060 | Chemical store University | |
| Anti-bromodeoxyuridine | 11170376 | Roche Diagnostics | 50µg (341.50€) |
| Vector M.O.M. Immunodetection Kit | BMK-2202 | Vector Laboratories over Linaris | Kit (252€-10%) |
| biotinylated goat anti-mouse IgG | BA-9200 | Vector Laboratories over Linaris | 1.5mg (131€-10%) |
| Vectastain Elite ABC-Kit | PK-6100 | Vector Laboratories over Linaris | Kit (260€-10%) |
| Triton X-100 | 93443 | Fluka (Sigma-Aldrich) | 500ml (158€) |
| Diaminobenzidine | D5637 | Sigma-Aldrich | 10g (135€) |
| Di-sodium hydrogenphosphate-dihydrate (Na ₂ HPO ₄ *2H ₂ O) | 106580 | Merck | 1-5kg (in request) |
| Sodium dihydrogenphosphate-dihydrate (NaH ₂ PO ₄ *2H ₂ O) | 106345 | Merck | 1-5kg (in request) |
| Sodium chloride (NaCl) | 221 | Chemical store University | |
| Xylol Isomerengemisch technisch | 00095 | Chemical store University | |
| DePex | 18243 | Serva | 500ml (in request) |

TRIPLE LABELING IMMUNOFLOUORESCENCE (BRDU, NEUN, GFAP)

Defrost the slices (paraformaldehyde fixated)

| Treatment | Substance | Dilution | Incubation |
|-------------------|--|--|----------------------|
| Washing | 10 mM PBS | (s. stock sol.) | 4x5' |
| DNA-Denaturation* | 2 N HCl | 1 ml/ cavity | 60' |
| Neutralization* | 0.1 M Borate buffer, pH 8.5 | 3.09 g Boric acid (H3BO3) 500 ml A. dest., pH 8.5 | 2 x 5' |
| Washing* | 10 mM PBS | (s. stock sol.) | 2x5' |
| Blocking | 5% Donkey Serum | 0,15ml DS in 2,85ml PBS/T (1ml/cavity.; 3 cav./animal) | 60' |
| 1st Antibodies | Antibodies in 5% DS-PBS/T Anti-BrdU: 1:200 Anti-NeuN: 1:500 Anti-GFAP: 1:50 | Per cav.: Anti-BrdU: 4µl Anti-NeuN: 1.6µl Anti-GFAP: 16µl | Over night; RT |
| Washing | 10 mM PBS | (s. Stock sol.) | 4x5' |
| 2nd Antibodies | Antibodies in 2%BSA-PBS 1:50 (possilbe 1:50/1:100/1:200) | (60mg BSA in 2,94ml PBS) 16µl, 16µl, 16µl + 0,704µl in 2% BSA- PBS/ cav. (AB solved 1 :1 in glycerol ⇒ total volume 32µl each) | 45' |
| Washing | 10 mM PBS | (s. Stock sol.) | 3x5' |
| Washing | A. dest. | - | 1x5' |

Mount on slides and dry on heater; coverslip with AquaPolyMount. Store in the dark.

Antibodies

Rat anti-BrdU: AbD Serotec, Kidlington, Oxford UK (OBT0030G), 1:200
 Mouse anti-NeuN: Millipore, Schwalbach/Ts., Germany (MAB377), 1:500
 Rabbit anti-GFAP: dianova, Hamburg, Germany (PA1-23702), 1:50

Donkey anti-rat Rodamin Red-X: Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK (712-295-153), 1:50

Donkey anti-mouse FITC: Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK (715-095-151), 1:50

Donkey anti-rabbit AMCA: Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK (711-155-152), 1:50

Stock solutions

0.4M Phosphate buffer, pH 7.4 (PB)

114g Dinatriumhydrogenphosphatdihydrat (Na₂HPO₄*2H₂O)

24,8g Natriumdihydrogenphosphatdihydrat (NaH₂PO₄*2H₂O)

Fill with A. dest to 1800ml

Adjust pH 7.4 (with 1N NaOH)

Fill with A. dest to 2000ml

Store at RT.

10mM Phosphate buffer Saline, pH 7.4 (PBS/T): 0.15M Natriumchlorid, 0.1% Triton
 50ml 0.4M PB
 1950ml A. dest.
 17g NaCl
 2 ml Triton X-100
 Store at 4°C.

* Only necessary if used for BrdU.

LIST OF REAGENTS AND CHEMICALS USED FOR TRIPLE FLUORESCENCE

| Name | Product number | Supplier | Amount (Price) |
|---|----------------|---------------------------|--------------------|
| BrdU: 5-Bromo-2'-deoxyuridine | B5002 | Sigma-Aldrich | 500mg (38.20€) |
| Diethylether | 03024 | Chemical store University | |
| Paraformaldehyde (CH ₂ O) _n | 00074 | Chemical store University | |
| Succrose | 107687 | Merck | 1kg (in request) |
| Magnesium chloride hexahydrate (MgCl ₂ ×6H ₂ O) | 00187 | Chemical store University | |
| Glycerol min. 99% | G5516 | Sigma-Aldrich | 500ml (63.60) |
| 2 N HCL | T134.1 | Carl Roth | 1l (10.45€) |
| Boric acid (H ₃ BO ₃) p.A. | 00060 | Chemical store University | |
| Bovine Serum Albumin | A4503 | Sigma-Aldrich | 50g |
| Normal Donkey Serum | S30 | Chemicon (Millipore) | 100ml (79€) |
| Mouse Anti-NeuN | MAB377 | Chemicon (Millipore) | 500µg (312€) |
| Rat-Anti-BrdU | OBT0030G | AbD Serotec | 250µg (299) |
| Rabbit-Anti-GFAP | PA1-23702 | Dianova | 500µg (262€) |
| Rhodamine Red-X-conjugated Donkey Anti-Rat IgG | 712-295-153 | Jackson ImmunoResearch | 500µg (120€) |
| FITC-conjugated Donkey Anti-Mouse IgG | 715-095-151 | Jackson ImmunoResearch | 500µg (120€) |
| AMCA-conjugated Donkey Anti-Rabbit IgG | 711-155-152 | Jackson ImmunoResearch | 500µg (125€) |
| Di-sodium hydrogenphosphate-dihydrate (Na ₂ HPO ₄ *2H ₂ O) | 106580 | Merck | 1-5kg (in request) |
| Sodium dihydrogenphosphate-dihydrate (NaH ₂ PO ₄ *2H ₂ O) | 106345 | Merck | 1-5kg (in request) |
| Sodium chloride (NaCl) | 221 | Chemical store University | |
| Triton X-100 | 93443 | Fluka (Sigma-Aldrich) | 500ml (158€) |
| Aqua-Poly/Mount | 18606 | Polysciences | 20ml (45.50€) |

GALLYAS SILVER IMPREGNATION (GERMAN VERSION)DARSTELLUNG VON DEGENERIERTEN NERVENENDIGUNGEN

GALLYAS

Methode IIMaterial und Fixierung

Ganz sichere Ergebnisse bisher nur an Rattengehirnen.

Routinemäßig erprobte Fixierungen sind Formol (5-10%) und 4% (5%) Paraformaldehyd in 0,1m Cacodylatpuffer.

Gefrierschnitte von maximal 40 μ Dicke.

Alle Stammlösungen dieser Methode sind verschlossen über Wochen haltbar, die Konzentrationen wurden so ausgetestet, daß die Zeiten für Vorbehandlung, Imprägnation und Waschen nicht verändert werden sollten.

Vorbehandlung

9% Natronlauge

20 ml

1% Ammoniumnitrat

20 ml

werden 1:1 gemischt (pH 13), Dauer 2x5Minuten

1x wechseln
Auswaschen

Durch diese Vorbehandlung wird die Imprägnation von normalen terminals verhindert. 10 min

Imprägnation

9% Natronlauge

20 ml

16% Ammoniumnitrat 20 ml pH 13, 10 min.

50% Silbernitrat

ca 8-10 Tropfen

Natronlauge und Ammoniumnitrat werden 1:1 gemischt, der

Anteil des Silbernitrat wird in einem Vorversuch festge-

stellt, dieses ist die einzige Variable dieser Imprägnation.

deg.Ter. 2

Es empfiehlt sich eine Reihe, z.B.

0,05 / 0,1 / 0,15 / 0,2 ml des 50% Silbernitrats pro 10ml Gemisch.

Die optimale Silbernitratkonzentration ist abhängig bes.
vom Alter der einzelnen Lösungen, deren Temperatur, vom
Alter des Versuchstieres, der Dauer der Fixierung und der
Schnittdicke.

Die Imprägnationslösung kann innerhalb von 2-3 Stunden
mehrfach benutzt werden, sie ist nicht mehr brauchbar, wenn
sich ein Niederschlag bildet.

Die Schnitte sollten während der Imprägnationszeit gelegent-
lich leicht bewegt werden, um ein Zusammenkleben oder Haften
am Boden der Petrischale zu vermeiden.

Tropfenzahl testen

Waschen

0,5% Natriumcarbonat anhydr. in 30% Äthanol

1% Ammoniumnitrat

Mischung 100:1, 3 x 1 Minute, zusammen max. 6 Minuten

Das Waschen entfernt den Überschuß an Silbernitrat aus
den Schnitten, dauert es zu lange, allerdings auch spezi-
fische Anlagerungen.

Die Schnitte sollen auch in dieser Lösung bewegt werden
und möglichst nicht ~~an~~ ^{auf} der Oberfläche schwimmen. Spätestens
in der dritten Waschlösung sollen sie glatt entfaltet sein
und so mit einem Glasstaken oder einem Nylonnetz in die
Entwicklerlösung transportiert werden.

Die Waschlösungen sollten nicht länger als 2 Std. benutzt werden.

*40ml
+ 0,1ml
40ml
0,1ml*

Entwicklung

in 1000 ml 10% Äthanol:

15 ml 40% Formol

0,5 g Citronensäure

einige ml Merck Universalindikator flüssig pH 4-10

dann solange 9% NaOH zutropfen, bis die Lösung zitronengelb wird, ca. 2,2 ml, das entspricht pH 5 - pH 5,5

Zu dieser Stammlösung kommt vor Gebrauch 1% Ammoniumnitrat 100:1, die Lösung sollte mindestens 25°C warm sein.

40ml Entwickler
0,4ml 1% Ammoniumnitrat

Für die Entwicklerlösung und auch die Waschlösung sollte Äthanol (verg.) benutzt werden, andere Alkohole wie Methanol oder Propanol sind nicht geeignet.

Der Entwicklungsprozess läuft sehr schnell ab, (1 - 2 sec.), mindestens so lange müssen die Schnitte möglichst glatt unter der Oberfläche bewegt werden.

Je nach Silbernitratkonzentration in der Imprägnationslösung werden die Schnitte von zart gelbbraun bis dunkelbraun. Optimal ist ein heller Untergrund für den Kontrast im Durchlicht und im Dunkelfeld.

Je dunkler die Schnitte werden, desto ausgeprägter ist die Zellfärbung. Diese Methode ist also auch als Übersichtsfärbung geeignet, gelingt aber nur schwimmend an Gefrierschnitten.

In diesem Entwickler können die Schnitte längere Zeit verbleiben, ein weiteres Nachdunkeln wurde nicht beobachtet. Entwicklerlös. am besten nur 1x beutzen, Limit: Indikator wird grünlich.

Waschen

mit 0,5% Essigsäure mindestens 10 Minuten

Da die Schnitte in der Essigsäure leicht zerreißen, außerdem erheblich schrumpfen, sind wir dazu übergegangen, aus der Entwicklerlösung aufzuziehen und nach dem Trocknen mit Essigsäure zu spülen.

Dann A. dest., trocknen und eindecken mit Kanadabalsam.

eindecken mit Dekt und. über Nacht trocknen

Auch diese Präparate dunkeln bei längerem Lagern mit Lichteinwirkung etwas nach, das Bild wird allerdings nicht verändert.

TIMM STAINING (GERMAN VERSION)

Wichtig: Alle für diesen Versuch verwendeten Gefäße müssen mindestens 24h in 10% RBS eingelegt und 10x mit warmen, 10x mit kaltem Wasser und 10x mit Aqua dest. gewaschen und im Wärmeschrank getrocknet werden.

Timm Färbung

Schwegler and Lipp 1983, Behav. Brain Res.7, 1-38

Narkose:

Äther

Alle Angaben für 2 Gehirne (die der Versuchsleiter ausgeschlossen)

Perfusion:

1. Vorspülen: 1.17% Na⁺-Sulfid gepuffert (pH 7.35)
3 min
2. Perfusion: 3% Glutaraldehyd in 0.15M Sörensen's
Phosphatpuffer (pH 7.35)
5 min
3. Nachspülen: 1.17% Na⁺-Sulfid gepuffert
(pH 7.35)
7 min

Natriumsulfid gepuffert:

33,42g Na-sulfid (Na_2S 7-9 H_2O) und **11,9g** Na-hydrogen phosphat ($\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$) in 800ml Aqua dest. lösen, pH 7,35 einstellen, und auf 1l auffüllen.

Nachfix

ganze Gehirne über Nacht in Glutaraldehyd (s.o) mit 20% Saccharose

Stammlösung A:

9.078g Kaliumhydrogenphosphat (KH_2PO_4) in 1000 ml Aqua dest. lösen.

Stammlösung B:

11.876g Di-natriumphosphat ($\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$) in 1000 ml Aqua dest lösen.

Glutaraldehyd in Sörensen's Puffer (für 800 ml)

638ml der Lsg B und 162 ml der Lsg A und 96 ml 25%iges GA mischen und pH 7,35 einstellen. 100 ml davon für Nachfix in 20% Saccharose geben.

Schneiden

Gefriermikrotom

20 µm Horizontalschnitte auf Objektträger

Akaziengummi-Lösung (50%ig)

500g Acacia in 1000ml A.dest lösen (Wasser langsam dazu geben). Etwa 1 Woche bei RT rühren lassen. Dann (abfiltrieren) und in 60ml Portionen einfrieren.

Timm's Lösung

180 ml gefilterte 50%ige Akaziengummi-Lsg.
+ 30 ml Zitrat-Puffer
+ 90 ml 5%ige Hydrochinon-Lsg
+ 0,75 ml 17%iges AgNO_3

Zitrat-Puffer (immer frisch ansetzen)

7,65 g Zitronensäure (1 H_2O) und 7,05g Natriumcitrat (2 H_2O) mit Aqua dest auf 30 ml auffüllen.

Ansetzen der TIMM-Lösung

Hydrochinonlsg. und Zitratpuffer gut mischen. 50%-ige Akaziengummi-Lsg. dazu geben (WICHTIG: Reihenfolge beachten!) und gründlichst mischen (alles bei RT, es darf kein Raumtemperaturgradient entstehen!!) Diese Lsg 15 min. bei 28 °C warm stellen. Erst kurz vor der Reaktion die Silbernitrat-Lösung zugeben. Wenn ein Niederschlag entsteht, Lösung nicht mehr verwenden und neu ansetzen. 200 ml Färbelösung reichen für 4 hohe Färbeküvetten (= 2 Gehirne à 12 Objektträger

Silbernitrat-Lsg

0,85g Silbernitrat (AgNO_3) in 5ml Aqua dest. lösen. In Alufolie aufbewahren.

Hydrochinon-Lsg

4,5g Hydrochinon mit Aqua dest auf 90ml auffüllen. Die Lsg kann bei längerer Lagerung gelblich werden. Hat jedoch keinen Einfluß auf die Qualität.

Entwicklung

60-120 min im Dunkeln im Wärmeschrank bei 30°C inkubieren lassen
Prüfen ob sich innere von äußerer Molekularschicht abgrenzt
Schnitte einmal unter fließendes Leitungswasser halten
Anschließend 10 min im selbigen spülen
Entwässern (3x5min 96% EtOH, 3x5 100% EtOH, 3x5 min Xylol),Eindeckeln

IMMUNOFLUORESCENCE AGAINST ZNT3

Defrost the slices (paraformaldehyde fixated)

| Treatment | Substance | Dilution | Incubation |
|----------------|--|---|----------------------|
| Washing | 10 mM PBS | (s. stock sol.) | 4x5' |
| Blocking | 5% Donkey Serum | 0,15ml DS in 2,85ml PBS/T (1ml/cavity.; 3 cav./animal) | 60' |
| 1st Antibodies | Antibody rabbit anti-ZnT3 1:500 in 5% DS-PBS/T | Per cav.: 1,6µl to 788,4µl 5% DS-PBS/T | Over night; RT |
| Washing | 10 mM PBS | (s. Stock sol.) | 4x5' |
| 2nd Antibodies | Antibody donkey anti-rabbit AMCA in 2% BSA-PBS 1:50 | (60mg BSA in 2,94ml PBS) 16µl, 768µl in 2% BSA-PBS/ cav. (AB solved 1 :1 in glycerol ⇒ total volume 32µl) | 45' |
| Washing | 10 mM PBS | (s. Stock sol.) | 3x5' |
| Washing | A. dest. | - | 1x5' |

Mount on slides and dry on heater; coverslip with AquaPolyMount. Store in the dark.

Antibodies

Rabbit anti-ZnT3: Synaptic Systems, Göttingen, Germany, 1:500

Donkey anti-rabbit AMCA: Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK
(711-155-152), 1:50

Stock solutions

0.4M Phosphate buffer, pH 7.4 (PB)

- 114g Dinatriumhydrogenphosphatdihydrat ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)
- 24,8g Natriumdihydrogenphosphatdihydrat ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$)
- Fill with A. dest to 1800ml
- Adjust pH 7.4 (with 1N NaOH)
- Fill with A. dest to 2000ml

⇒ Store at RT.

10mM Phosphate buffer Saline, pH 7.4 (PBS/T): 0.15M Natriumchlorid, 0.1% Triton

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

⇒ Store at 4°C.

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass ich diese Arbeit selbstständig erstellt und nur die angegebenen Hilfsmittel und Quellen verwendet habe. Ferner erkläre ich hiermit, dass es sich um meinen ersten Promotionsversuch handelt.

Bielefeld, Dezember 2009

(Andrea T.U. Schäfers)

