Aus der Klinik für Neurologie des Fachbereichs Medizin

der Philipps-Universität Marburg

Kommissarischer Leiter: Prof. Dr. med. R. C. Dodel

Adult neurogenesis in the orexin/ataxin-3 mouse

Inaugural-Dissertation

zur Erlangung des Doktorgrades der gesamten Humanmedizin

dem Fachbereich Medizin der Philipps-Universität Marburg vorgelegt von

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Marburg an der Lahn 2016

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg
am: 24.03.2016

Gedruckt mit Genehmigung des Fachbereichs.

Dekan: Herr Prof. Dr. H. Schäfer
Referent: Herr Prof. Dr. G. Höglinger
1. Korreferentin: Frau PD Dr. R. Preisig-Müller

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1 SUMMARY

Introduction: The adult mammalian brain retains neural stem cells (NSCs) that continually generate new neurons within two restricted regions, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (HC). This process is called adult neurogenesis. Controlled stimulation of endogenous neurogenesis might be an elegant way to treat neurodegenerative diseases. It is therefore important to understand the molecular signals, which govern the proliferation, migration, and differentiation of endogenous NSCs in the neurogenic niches. Regulatory mechanisms in the so-called neurogenic niches have already been shown by in vivo studies for various factors, including numerous neurotransmitters, and behavioral and environmental factors. However, our knowledge is still insufficient to exploit adult neurogenesis for controlled brain repair or for stimulation of its physiological function.

The sleep disorder narcolepsy is considered to be a neurodegenerative disease because there is a massive progressive loss of neurons containing the neuropeptide orexin. Consequently narcoleptic patients have very low cerebrospinal fluid levels of orexin. Narcolepsy is defined as a sleep-wake disorder with REM and non-REM sleep associated symptoms existing longer than 6 months, such as daytime sleepiness, cataplexy, fractionated sleep at night and automatic behaviors. The postulated pathophysiology of human narcolepsy is mimicked very closely in a transgenic mouse model called orexin/ataxin-3 mouse*,* in which orexin-containing neurons are ablated progressively by specific overexpression of a truncated cytotoxic ataxin-3 gene product under the orexin-promoter.

Aim: We aimed to study the possible role of the protein orexin in the regulation of adult neurogenesis in the orexin/ataxin-3 mouse.

Study design: Adult neurogenesis in the SGZ, SVZ and rostral migratory stream (RMS), where the cells migrate from the SVZ to the olfactory bulb (OB), was studied by immunohistology. For the evaluation of the stem cell proliferation in SVZ and SGZ and migration in the RMS, orexin/ataxin-3 mice (n = 8) and wild type (WT) mice (n = 8) received a single intraperitoneal injection of 100 mg BrdU (bromodeoxyuridine)/kg body weight 2 hour prior to sacrifice. For the analysis of differentiation and survival of newly built cells, BrdU was administered intraperitoneally on 5 consecutive days once per day

to orexin/ataxin-3 mice (n = 8) and WT mice (n = 8) and they were sacrificed 30 days after the last injection.

Results: We found a significantly higher proliferation of stem/precursor cells in the orexin/ataxin-3 mice in both neurogenic regions, the SGZ and the SVZ. Also in the RMS, higher levels of newly built cells in the orexin/ataxin group were found, but these differences were not significant.
We were able to demonstrate a significantly higher survival of newly built cells in the granular zone, but not for the periglomerular zone of the OB in the orexin/ataxin group. A tendency for higher survival rates could be shown for the HC of the orexin/ataxin-3 mice (not significant).
By triple staining we could show that the proportion of newly born neurons relative to the total number of newly built cells in the HC was significantly higher with 90 % in the orexin-ataxin group compared to 83 % in the control group. In both the granular zone and the periglomerular zone of the OB, over 90 % of the total amount of new built cells differentiated into neurons in both groups. Also the rate of differentiation into a dopaminergic phenotype of the newly born neurons in the periglomerular zone of the OB was not significantly changed with 93 % in the orexin/ataxin-3 mice compared to 91 % in the WT mice.

Conclusion: In the absence of orexin in the adult mouse brain, the proliferation of adult neural stem/precursor cells was increased, the survival rate was significantly increased in the granular zone of the OB and a consistent not significant trend was seen in the HC. The proportion of newborn neurons among all newly born cells was higher in the HC, however, no significant differences in the differentiation of newly built cells could be found in the OB. Together, these observations lead to the assumption that orexin suppresses the proliferation of adult NSC, affects the survival rate in the OB negatively and hinders the differentiation of newly built cells into neurons in the HC.

2 ZUSAMMENFASSUNG

Einleitung: Im adulten Gehirn von Säugetieren gibt es zwei Hirnregionen, die subventrikuläre Zone (SVZ) des lateralen Ventrikels und die subgranuläre Zone (SGZ) im Gyrus dentatus des Hippocampus (HC), in denen durch Stammzellteilung ständig neue Nervenzellen entstehen. Dies wird als adulte Neurogenese bezeichnet. Die kontrollierte Stimulation der adulten Neurogenese könnte als ein endogener Reparaturmechanismus genutzt werden um neurodegenerative Erkrankungen zu behandeln. Es ist daher wichtig molekulare Signale, welche die Proliferation, Migration und Differenzierung endogener neuronaler Stammzellen in den neurogenen Zonen beeinflussen, besser zu verstehen. Regulationseffekte der sogenannten neurogenen Nische wurden bereits für verschiedene Faktoren, v.a. für zahlreiche Neurotransmitter nachgewiesen. Allerdings ist unser Wissen noch nicht ausreichend, um die adulte Neurogenese für die Reparation von degeneriertem Nervengewebe gezielt einzusetzen.
Narkolepsie gilt als eine neurodegenerative Erkrankung, die mit einem massiven Verlust von Orexinneuronen und einem stark vermindertem Orexin-Spiegel im Liquor einhergeht. Dies führt unter Anderem zu einer Störung der Schlaf-Wach-Regulation. Das hier verwendete transgene Orexin/Ataxin-3 Mausmodell kommt der vermuteten Pathophysiologie der Narkolepsie sehr nahe. Die spezifische Expression des zytotoxischen Ataxin-3 Genprodukts unter der Kontrolle des Orexin Promoters führt zu einem progredienten Untergang der orexinergen Neurone.

Ziel: Ziel der Arbeit war es, den Einfluss von Orexin auf die adulte Neurogenese in dem Orexin/Ataxin-3 Mausmodell zu untersuchen.

Versuchsanordnung: Die adulte Neurogenese wurde im Bulbus olfactorius (OB), dem rostralen migratorischen Strom (RMS), in der SVZ und im HC immunhistologisch untersucht. Für die Untersuchung der Stammzellproliferation in der SGZ und SVZ und der Migration im RMS erhielten gesunde Wildtyp Mäuse und Orexin/Ataxin-3 Mäuse (n=8 pro Gruppe) einmalig eine intraperitoneale Injektion von 100mg BrdU (Bromodeoxyuridine)/kg Körpergewicht 2 Stunden bevor sie getötet wurden. Zur Untersuchung der Differenzierung und der Überlebensrate der neu gebildeten Zellen wurde jeder Maus BrdU als intraperitoneale Injektion an 5 aufeinander folgenden Tagen verabreicht (n=8 pro Gruppe) und die Versuchstiere wurden 30 Tage nach der letzten Injektion getötet.

Ergebnisse: In den Orexin/Ataxin-3 Mäusen fanden wir eine signifikant höhere Proliferationsrate der Stammzellen in beiden neurogenen Zonen, der SGZ und der SVZ. Auch im RMS, in welchem die neu gebildeten Zellen aus der SVZ in den OB wandern, wurde eine tendenziell höhere Anzahl BrdU markierter Zellen in der Orexin/Ataxin-3 Gruppe gefunden (nicht signifikant). Zudem wurde im zweiten Versuchsteil eine tendenziell höhere Überlebensrate neu gebildeter Zellen im HC der Orexin/Ataxin-3 Mäuse sichtbar (nicht signifikant). Für die granuläre Zone des OB konnten wir dagegen eine signifikant höhere Überlebensrate in der Orexin/Ataxin-3 Gruppe zeigen. In der periglomerulären Zone des OB fanden wir keinen Unterschied in der Überlebensrate. Der Einfluss der Orexin-Defizienz auf die Differenzierung der Neurone wurde durch eine Fluoreszenz-markierte Dreifachfärbung untersucht. Hier konnten wir zeigen, dass die neuronale Differenzierung der neugeborenen Zellen im HC mit 90 % in der Orexin/Ataxin-3 Gruppe signifikant höher war als in der Kontrollgruppe mit nur 83 %. In der granulären und periglomerulären Zone des OB konnte kein signifikanter Unterschied in der neuronalen Differenzierungsrate gefunden werden (jeweils > 90 %). Die dopaminerge Differenzierungsrate in der periglomerulären Zone des OB zeigte sich mit 93 % in der Orexin/Ataxin-3 Gruppe gegenüber 91 % bei den Wildtyp-Mäusen ebenso nicht signifikant erhöht.

Interpretation: Hieraus kann geschlossen werden, dass im adulten Mäusegehirn in der Abwesenheit von Orexin die Proliferation der adulten neuronalen Stammzellen in der SVZ und SGZ gesteigert war. Zudem war die Überlebensrate der neugeborenen Zellen in der granulären Zone des OB signifikant erhöht. Im HC konnte dagegen kein signifikanter Unterschied in der Überlebensrate gezeigt werden. Für die neuronale Differenzierung konnte ein signifikanter Unterschied für den HC, nicht jedoch für den OB nachgewiesen werden. Die Ergebnisse führen zu der Annahme, dass Orexin die Proliferation der adulten neuronalen Stammzellen unterdrückt, sich auf die Überlebensrate der neu gebildeten Zellen im OB negativ auswirkt und die neuronale Differenzierung im HC erschwert.

3 INTRODUCTION

3.1 Adult neurogenesis

In the last century, research in the field of neuroscience was determined by a central paradigm on neurons in the central nervous system: *"Everything may die, nothing may be regenerated".* Ramon y Cajal postulated this phrase in his publication in 1928 "Degeneration and regeneration of the nervous system". This paradigm found great acceptance in the neuroscientific community for a long time. Thereafter, it was assumed that all neurons in the human central nervous system were generated during embryonic and early postnatal development. Due to its complexity and specialization, the neural network of the adult brain was thought to be a fixed structure and its function was not compatible with the idea of adult neurogenesis (Brandt and Storch, 2008). Early reports of neurogenesis in the adult central nervous system (Allen, 1912, Levi, 1898) were ignored, because technical capabilities of cell labeling to identify mitotic cells as neurons were not yet developed. Five decades later, the technical possibilities were extended, allowing Joseph Altman to demonstrate that cells of the hippocampus (HC) (Altman and Das, 1965) and in the olfactory bulb (OB) (Altman, 1969) of the rat incorporate radio-labeled thymidine. Thus, he provided first evidence of cell proliferation in this region. Kaplan and Hints (1977) could first show by electron microscopy that these newly built cells in the adult brain are actually neurons. Further support on the way to a paradigm shift and acceptance of the concept of adult neurogenesis in higher vertebrates yielded the discovery of neurogenesis in birds in connection with the occurrence of seasonal song (Nottebohm, 1989). The breakthrough and international acceptance of the phenomenon of adult neurogenesis occurred with the demonstration that newly derived neurons in the adult mammalian brain can migrate and integrate into existing circuitries (Gross, 2000). This possibility was given by the development of new methods, such as the exogenously supplied bromodeoxyuridine (BrdU). BrdU is a synthetic analogue of thymidine that is incorporated in the cellular DNA of proliferating cells in the S phase of mitosis and can be subsequently detected by immunohistochemistry. This was the first opportunity to undertake detailed quantitative and qualitative analyses of adult-generated cells in the brain (Gage, 2000, Kempermann et al., 2004b). Since then a lot of research was done concerning regulation and function of adult neurogenesis.

3.1.1 Regions of adult neurogenesis

There are two regions in the adult mammalian brain where adult neurogenesis occurs: in the subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the dentate gyrus in the HC. Progenitor cells in the SVZ proliferate and form neurons that migrate along the so-called rostral migratory stream (RMS) into the OB. New granule cells, formed in the SGZ in the HC, get functionally integrated into the existing hippocampal neuronal network.

Neural stem cells (NSC) or precursor cells, differentiating to glial cells and neurons in vitro or after transplantation could also be isolated from other areas of the adult brain (Palmer et al., 1995, Palmer et al., 1999, Hermann et al., 2006b). Under physiological conditions these NSC seem to rest, hindered from proliferation and differentiation by their microenvironment. These observations confirm the theory that the two neurogenic regions of the brain are distinguished from non-neurogenic regions by their stem and progenitor cell-friendly environment providing proliferation, differentiation and integration of new neurons (Alvarez-Buylla and Lim, 2004, Palmer et al., 2000).This specific microenvironment of SGZ and SVZ is also known as the “neurogenic niche” (Doetsch, 2003, Zhao et al., 2008).

The **SVZ** is localized along the lateral wall of the lateral ventricles and corresponds to a residual of the embryonic ganglionic eminence which retains the ability to reconstitute neuronal and glial cells until adolescence (Lois and Alvarez-Buylla, 1993, Parnavelas, 2000). This thin cell layer lies next to the ependyma and is thereby also called subependyma. Initially, the ependymal cells were thought to be adult NSC and thus responsible for adult neurogenesis in the SVZ (Johansson et al., 1999). Later it was shown that these cells do not possess the capabilities of NSC. Instead other cells, which have structural and molecular characteristics of astrocytes, such as expression of the glial fibrillary acid protein (GFAP), were shown to function as primary precursors (Doetsch et al., 1999a). According to a lineage model, these multipotent neural progenitors (***type B cells***) produce clusters of transit amplifying progenitor cells (***type C cells***), which in turn generate the neuronal progenitor cells also called neuroblasts (***type A cells***) by asymmetric cell division (Alvarez-Buylla and Lim, 2004, Doetsch et al., 1999a, Doetsch and Alvarez-Buylla, 1996). Because ***type B cells*** are mitotically relatively quiescent (Doetsch et al., 1999a), their identification was mainly based on morphological analysis

by electron microscopy. ***Type C and A cells*** in turn can also be identified by immunhistochemical methods such as cell labeling with BrdU (Zhao et al., 2008).

The neural progenitor cells, neuroblasts or ***type A cells,*** migrate along the RMS into the OB (**Fig 1**). Only after reaching their final destination, the precursor cells differentiate into neurons, more precisely into periglomerular or glomerular interneurons, and get functionally integrated (Carleton et al., 2003, Gritti et al., 2002, Doetsch et al., 1999b). The majority of new neurons is sorted out by apoptosis before their functional integration (Kaplan and Hinds, 1977). 99 % of the surviving cells migrate towards the granule cell layer, only 1 % into the glomerular layer. Almost all granule cells are GABAergic (gamma-aminobutyric acid = GABA) (Mugnaini et al., 1984). The percentage of new periglomerular cells turning into dopaminergic phenotype is about 30 % in the adult (Winner et al., 2002) compared to 10 % in the neonatal OB (Betarbet et al., 1996).

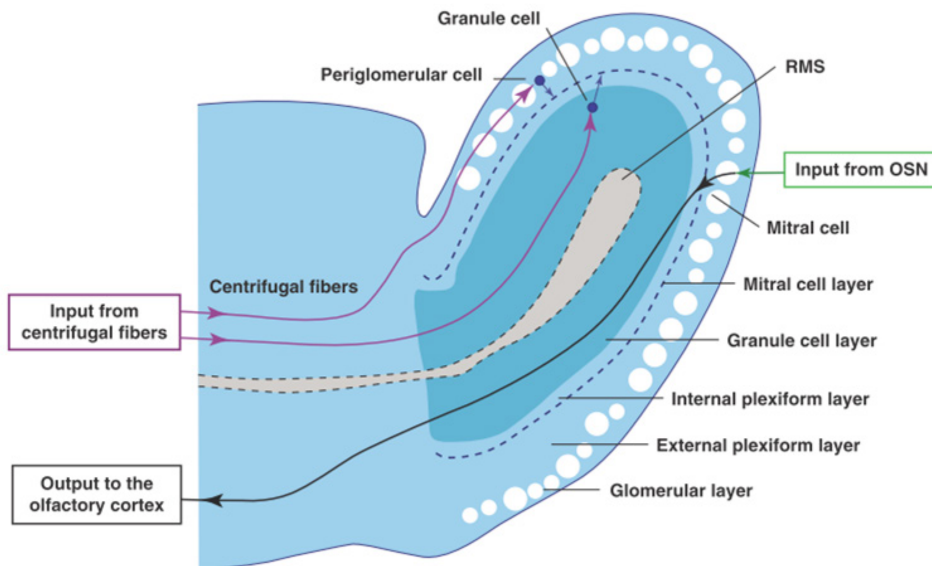


Fig. 1 : Schematic illustration of the rodent OB structure and its connections. The OB is divided into six main layers: **(i)** the glomerular layer, **(ii)** the external plexiform layer, **(iii)** the mitral cell layer, **(iv)** the internal plexiform cell layer, **(v)** the granule cell layer, and **(vi)** the RMS at the core. The OB receives inputs from olfactory sensory neurons (OSN) and from centrifugal fibers. The OB output is provided by mitral/tufted cells, and these project to several parts of the olfactory cortex. OSN project axons to glomeruli (white disks), where they activate mitral/tufted cells and periglomerular cells. Centrifugal inputs modulate periglomerular and granule cell activities, and these in turn modulate mitral cell activity. Taken from (Lazarini and Lledo, 2011).

Neurons born in the **SGZ** migrate into the granule cell layer of the dentate gyrus and become dentate granule cells (Zhao et al., 2008). As in the SVZ, GFAP-positive astrocytes are also seen as stem cells of adult hippocampal neurogenesis in the SGZ (Gage, 2000). Three subtypes of SGZ cells can be distinguished. According to their specific morphologies and molecular markers two types of neuronal progenitors could be identified. ***Type 1 cells*** are radial-glia like cells. They express the astrocytic marker GFAP and share several other astrocytic features such as vascular end-feet and typical electrophysiological properties (Kempermann et al., 2004a). Furthermore, they express nestin and the transcription factor Sox2 (Fukuda et al., 2003, Garcia et al., 2004, Suh et al., 2007). ***Type 2 cells*** do not express GFAP but Sox2 and were thought to be the transiently amplifying progenitor cells in the neuronal lineage and would arise from ***type 1 cells*** (Kempermann et al., 2004a). However Suh et al. demonstrated that ***type 2 cells*** itself also show stem cell characteristics like the capacity for self-renewal and ability to give rise to neurons and astrocytes (Suh et al., 2007) and thereby challenge the concept of a lineage relationship. The transcription factor Sox2, which is expressed by ***type 1*** and ***type 2 cells***, seems to be an essential property of neuronal stem cells (Zhao et al., 2008). GABAergic synaptic contacts reach ***type 2 cells***. GABA acts excitatory on these cells and stimulates the maturation (Tozuka et al., 2005, Wang et al., 2005). ***Type 3 cells*** correspond to the so-called neuroblasts, which have only a low proliferation rate. The exclusive expression of neuronal proteins and the absence of glial immunohistochemical characteristics suggest that these cells are already determined on a neuronal phenotype. This stage is characterized by morphological change due to the incipient arborisation and migration into the granule cell layer (Plumpe et al., 2006, Rao and Shetty, 2004). Similar to the embryonic and early postnatal neurogenesis much more immature neurons are formed during the proliferation phase, as ultimately will be integrated in the neural network (Kuhn et al., 2005, Biebl et al., 2000).

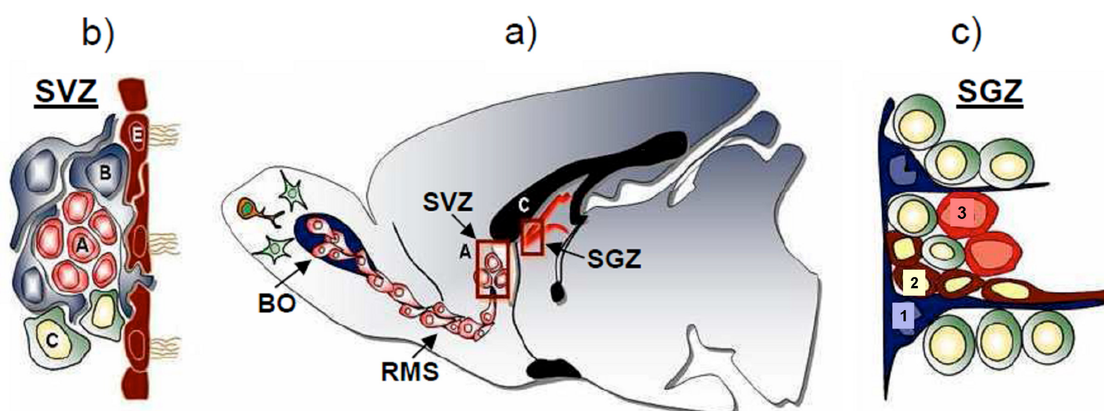


Fig. 2 Location and structure of neurogenic zones in the brain (with kind permission from Oscar Arias-Carrión).

a) Sagittal section through an adult mouse brain: The neuroblasts, built in the SVZ (box A) migrate along the RMS into the OB and differentiate there in interneurons. The newly built cells from the SGZ (box C) migrate only a short distance into the granular cell- layer of the dentate gyrus within the HC.

b) Cellular composition of the SVZ: The multipotent stem cells of the SVZ, called ***type B-cells*** (B) produce the highly proliferating ***type C-cells*** (C) from which the neuroblasts (***type A-cells***) emerge. The cells of the SVZ are demarcated by the ventricular system through the cells of the ependymal layer (***type E-cells***).

c) Cellular composition of the SGZ: From multipotent stem cells, called ***type 1 cells*** (1), arise ***type 2 cells*** (2). ***Type 3 cells*** (3) correspond to the so-called neuroblasts, which have only a low proliferation and differentiate into neurons of the granular cell layer.

3.1.2 Regulation of adult neurogenesis

The regulation of adult neurogenesis can be considered at different levels. First of all, the influence on adult neurogenesis by changes of behavior and environmental stimuli shall be displayed. This will be illustrated by means of examples like the influence of activity, sleep, stress and changes in odor or other environmental stimuli. Thereafter, on the level of microenvironment, also known as "the neurogenic niche", the role of cell-cell interaction between astrocytes and progenitor cells, selected molecular signals and the importance of structures such as blood vessels will be shown. Finally, the influence of neurotransmitters will be explained in detail. Since a lot of research concerning regulation of adult neurogenesis has been done in recent years, this chapter will only describe selected influences on the different levels.

Influence of behavior and environment

In 1997, Kempermann et al. have shown that significantly more new neurons exist in the dentate gyrus of mice exposed to an enriched environment (large cages equipped with paper tubes, nesting material, a rearrangeable set of plastic tubes, a running wheel etc.) compared to littermates housed in standard cages (Kempermann et al., 1997). However, environmental enrichment appears to have no effect on adult neurogenesis in the OB (Brown et al., 2003). But opposite results have been found for olfactory enrichment (Rochefort et al., 2002). Using a combination of immunohistological and behavioral approaches it was shown that an odor-enriched environment enhances the bulbar interneuron population and improves olfactory memory without up-regulating hippocampal neurogenesis. Different natural odors (lavender, garlic, paprika etc.) were used to enrich the olfactory environment. Experiencing an enriched environment can be seen as a form of continued learning. Implying, that complexity and novelty are key stimuli for adult neurogenesis (Kempermann, 2002, Kempermann et al., 2004b, Kempermann, 2011). Physical activity is a potent inducer of adult neurogenesis in particular. It leads to increased proliferation and differentiation of progenitor cells and prolonged survival of newly built cells (van Praag et al., 1999, van Praag et al., 2005, Kempermann, 2011).

Stress is a potent negative regulator on adult neurogenesis. In 1994 cortisol was already known as an important stress hormone, when Cameron and Gould showed that adrenalectomy leads to up-regulation, whereas exogenous glucocorticoid has down-regulation of adult hippocampal neurogenesis (Cameron and Gould, 1994). Prolonged sleep deprivation decreases adult neurogenesis in the HC and is considered to be stressful (Guzman-Marin et al., 2005, Mirescu et al., 2006). Indeed it was shown that this down-regulating effect of sleep-deprivation occurs during elevated levels of corticosterone and was prevented by clamping high levels of corticosterone (Mirescu et al., 2006). In contrast to effects of prolonged sleep deprivation, just one night sleep deprivation significantly increased cell proliferation and the total number of surviving cells in the hippocampal dentate gyrus (Grassi Zucconi et al., 2006).

Neurogenic niche

The neurogenic niche describes a special microenvironment in the neurogenic regions SGZ and SVZ, facilitating and regulating neurogenesis in the adult mammalian brain. In the SGZ, neuronal precursor cells lay close to a dense layer of granule cells containing

both mature and immature neurons. As mentioned above, astrocyte-like cells function as precursor cells of these regions. Apparently another specific population of astrocytes acts as regulator cells in the neurogenic niche. It was shown that these cells instruct the stem cells to adopt a neuronal fate and promote their proliferation (Song et al., 2002). In cell culture systems, this group demonstrated that these effects of astrocytes seem to be regionally specified. Astrocytes from the adult HC retain the ability to promote neurogenesis, whereas astrocytes of the spinal cord do not. In the regulation of adult neurogenesis by astrocytes, a cell-cell contact seems to be crucial, which was shown in vitro. Thus, SVZ cells proliferate rapidly on an astrocyte-monolayer in a culture system free of serum or exogenous growth factors (Lim and Alvarez-Buylla, 1999). Molecular signals released by astrocytes seem to be involved too. Transplantation of SGZ cells into the neocortex of adult mice promotes neurogenesis in this non-neurogenic region (Jiao and Chen, 2008). It was demonstrated that a significant proportion of radial glia-like cells (type 1 cells) in the dentate gyrus are coupled by gap junctions indicating a possible communication pathway (Kunze et al., 2009). Induction of virus-mediated ablation of connexins, key constituents of gap junctions, led to a dramatic decrease of proliferation and reduced numbers of radial glia-like cells and granule neurons in the adult dentate gyrus of the HC. Furthermore precursor cell activity seems to be calcium dependent. Excitation sensed via calcium channels on the proliferating precursors increases expression of NeuroD, a positive regulator of neuronal differentiation (Deisseroth et al., 2004). As described above, the precursor cells in the SVZ lay right next to the ependymal cell layer of the lateral ventricles. By expressing the protein Noggin, ependymal cells may promote SVZ neurogenesis. Noggin antagonizes the action of bone morphogenetic proteins, an inhibiting factor for adult neurogenesis, expressed by SVZ cells (Lim et al., 2000). Another factor derived from the ependymal cells is the pigment epithelium-derived factor which promotes self-renewal of adult NSCs (Ramirez-Castillejo et al., 2006). Important cellular components of the neurogenic niche are blood vessels (Palmer et al., 2000, Palmer, 2002). Endothelial cells secrete soluble factors which stimulate self-renewal and enhance neurogenesis of NSC in vitro (Shen et al., 2004). As in the regulation of vascularisation, the vascular endothelial growth factor (VEGF) also plays an important role in regulating neurogenesis (Cao et al., 2004, Jin et al., 2002). It even seems to be the crucial regulating factor in exercise-induced neurogenesis in adult hippocampal neurogenesis (Fabel et al., 2003). Fabel et al. demonstrated that peripheral blockade of the VEGF receptor abol-

ished running-induced neurogenesis but had no detectable effect on baseline neurogenesis in non-running animals.

Neurotransmitters

Neurotransmitters are frequently involved in cell-to-cell signaling. Axon terminals ending at the dentate gyrus of HC coming from a variety of brain regions distribute many different transmitters. The innervation of the SVZ seems to be less dense than that of the SGZ (Kempermann, 2011). Excitatory glutamatergic neurons project from the entorhinal cortex via the perforant pathway to the granule cells of the dentate gyrus. Effects by different ionotropic receptors, N-Methyl-D-Aspartat (NMDA), Kainate (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, are not homogenous but intricately balanced so that glutamate elicits a range of different responses in the same cells (Cameron et al., 1995, Gould et al., 1997, Kempermann, 2011). In summary, glutamatergic neurotransmission acts pro-neurogenic. In addition, SGZ and SVZ are innervated by cholinergic neurons coming from the nucleus basalis of Meynert and the septal region. Lesioning cholinergic neurons in the forebrain, reliable achieved with an immunotoxin, caused a reduction in adult neurogenesis in both the HC and the OB (Cooper-Kuhn et al., 2004). Hence, the cholinergic system seems to have a survival-promoting effect (Kempermann, 2011). The neurotransmitter serotonin plays a key role in brain development (Gaspar et al., 2003). Accordingly, it is plausible to attribute serotonin with the regulation of adult neurogenesis. Serotonergic afferents project from the raphe nuclei in the brain stem to the SGZ. Indeed, serotoninergic input was found to increase adult neurogenesis in the HC and the SVZ. This was demonstrated by inhibition of serotonin synthesis or selective lesions of serotonin neurons which led to a decrease in the number of newly generated cells in the dentate gyrus and in the SVZ (Brezun and Daszuta, 1999). Dopaminergic fibers coming from the midbrain also innervate the SVZ (Hoglinger et al., 2014). Höglinger et al. were able to demonstrate that type C cells in the SVZ express dopamine receptors (2004). Dopamine denervation decreased adult neurogenesis in the OB in vivo, whereas activation of the dopaminergic receptor D2 led to increased proliferation in vitro. Also extensive noradrenergic projections reach the dentate gyrus from the locus coeruleus (Loy et al., 1980). Results of one study indicate that the proliferation, but not the survival or differentiation of adult hippocampal granule cell progenitors is affected by noradrenalin depletion (Kulkarni et al., 2002). For the development of new neurons in the dentate gyrus, GABA plays an important role. It has been demonstrated, that GABAergic inputs

to hippocampal progenitor cells promote activity-dependent neuronal differentiation (Tozuka et al., 2005).

3.1.3 Adult neurogenesis in the human brain

In 1998 the Swedish neurologist Peter Eriksson histologically examined the brains of laryngeal cancer patients, who had received an injection of BrdU at lifetime for diagnostic purposes. Beside an enrichment of BrdU in cancer cells, he could also detect this proliferation marker in mitotically active neural precursor cells of the HC. This was the first evidence suggestive of adult neurogenesis in the human brain (Eriksson et al., 1998). As further evidence, the detection of BrdU-immunoreactive neurons in the human OB followed (Curtis et al., 2007). Human neurogenic regions presumably include, like in rodents, the SVZ and the OB because the expression of cell cycle-associated and cell-type-specific proteins suggestive of neurogenesis in human cells was shown in these regions (Sanai et al., 2004, Bedard and Parent, 2004, Liu and Martin, 2003). Moreover, cells with stem cell properties could be isolated out of the SVZ and the HC (Kukekov et al., 1999, Roy et al., 2000b, Roy et al., 2000a, Hermann et al., 2006a, Maisel et al., 2007). However, the existence of the RMS, important for migration of new born cells from the SVZ to the OB in rodents, is still much debated in humans (Curtis et al., 2007, Sanai et al., 2004, Sanai et al., 2007).

3.1.4 Functions of adult neurogenesis

3.1.4.1 Hippocampus

It proofs to be difficult to assess the precise functional relevance of adult neurogenesis in the HC, since the function of the HC in itself is not yet fully understood. A widely accepted idea is that the HC consolidates declarative memory contents, meaning that it processes information for long-term memory storage. It is supposed that the dorsal HC takes part in learning and memory, whereas the ventral HC participates in regulating affective behaviors (Bannerman et al., 2004). Morris showed that hippocampal lesions cause impaired place navigation in rats and demonstrated thereby, that spatial memory depends on hippocampal functions (Morris et al., 1982). Today, the Morris water maze is a common HC-dependent memory test investigating the spatial memory: Animals are placed in a pool of water that is colored opaque and have to find an escape platform hidden under the surface (Morris et al., 1982). This is studied repeatedly on successive

days. Rodents with unimpaired spatial-memory will find the platform more rapidly after each trial.

Kempermann and Gage (2002) found a significant correlation between the number of new neurons generated in the dentate gyrus and parameters describing the acquisition of the water maze task. Memory acquisition reflects how fast an animal learns the task. Conclusively, adult neurogenesis is involved in specific aspects of hippocampal function, particularly in the acquisition of new information. One group points out that spatial learning modifies neurogenesis by a selective cellular stabilization process (Dupret et al., 2007). They were able to demonstrate, that spatial learning promotes survival of relatively mature neurons, apoptosis of more immature cells, and finally, proliferation of neural precursors. The relevance of apoptosis in this process was shown by blocking apoptosis during the late phase of Morris water maze training, which led to impaired performance in rats. The conclusion of these observations was that during learning, neuronal networks are sculpted by a tightly regulated selection and suppression of different populations of newly born neurons (Dupret et al., 2007). To investigate the function of hippocampal neurogenesis in learning and memory, different experimental methods have been developed to decrease or even ablate SGZ neurogenesis in adult animals using drugs or radiation. In general, results of these studies led to the assumption that adult neurogenesis has a functional relevance for hippocampal-dependent cognitive performance (Shors et al., 2001, Shors et al., 2002, Snyder et al., 2005). In 2001, Shors et al. used a toxin killing proliferating cells, the DNA methylating agent methylazoxymethanol acetate, to diminish adult neurogenesis in dentate gyrus of rats. By performing different HC conditioning experiments they concluded that adult neurogenesis in the HC is involved in the formation of trace memories (Kempermann et al., 2004b, Shors et al., 2001). By using low-dose irradiation to inhibit adult hippocampal neurogenesis it has been shown that adult neurogenesis plays an important role in the formation and consolidation of long-term spatial memories (Snyder et al., 2005). A recent study confirms the role in long-term spatial memories and furthermore found an impairment of object recognition memory (Jessberger et al., 2009). A correlation between hippocampal neurogenesis and learning was observed in mice with different genetic background that influences the levels of cell proliferation, differentiation and survival (Zhao et al., 2008, Saxe et al., 2006). The group of Kempermann postulated, by analogy with a computer, addition of new neurons does not mean installing a new hard ware for more storage, but rather strategic changes in the network architecture

lead to an optimization of the performance (Biebl et al., 2000). According to the current concept, explicit memory contents have to be processed in the HC before its consolidation and long-term storage in cortical structures. One hippocampal particularity lies in a structural bottleneck in the neuronal network, which is exactly the position at which adult neurogenesis occurs. The bottleneck lies in the mossy fiber projection of the dentate gyrus into the much smaller region CA3. The new neurons contribute to the mossy fiber tract and thereby structurally counteract the narrowness of this spot (Kempermann, 2002, Kempermann, 2008, Brandt and Storch, 2008).

In recent years the role of adult neurogenesis in the HC in the context of depression has been studied extensively. Empirical and experimental data build a connection between alterations of cellular plasticity in the HC and the pathophysiology of depression (Brandt and Storch, 2008). On the empirical side, several studies demonstrate patients with known depression perform significantly worse in HC-dependent memory tests (Gould et al., 2007) and the HC has a lower volume than comparable control groups (Campbell et al., 2004, Videbech and Ravnkilde, 2004). Furthermore many antidepressants lead to a stimulation of the hippocampal neurogenesis (Malberg et al., 2000, Czeh et al., 2001, Santarelli et al., 2003). In addition, the effectiveness of antidepressants is abolished in the absence of adult neurogenesis (Santarelli et al., 2003, Duman and Monteggia, 2006, Jiang et al., 2005). As mentioned above, one night sleep deprivation significantly increased cell proliferation and the total number of surviving cells in the hippocampal dentate gyrus (Grassi Zucconi et al., 2006). This response could be partially responsible for the beneficial effect, elicited in depressed patients by one night sleep deprivation.

Taken together, these findings suggest that hippocampal neurogenesis depends on functional demands of the HC and some functions of the HC in return depend on neurogenesis.

3.1.4.2 Olfactory bulb

In order to interpret the behavioral experiments investigating the function of adult neurogenesis in the OB, the influence of the newly formed cells at the cellular level has to be illustrated. Imayoshi (2008) made an important contribution in understanding the function of adult neurogenesis in the OB. Using a genetic method for labeling NSC in adult mice, they could show that continuous neurogenesis results in the replacement of the majority of granule neurons in the OB. In addition, inhibition of adult neurogenesis resulted in a substantial reduction of the granule cell number. Due to these observations it was postulated, that continuous neurogenesis is required for the maintenance and reorganization of the whole interneuron system in the OB. Using a mouse model, lacking the neural cell adhesion molecule (NCAM), necessary for the migration of newly formed cells through the RMS, the anatomical and behavioral consequences of failed neurogenesis was further analyzed by Gheusi et al. (2000). They found disturbances in the discrimination between different odors and were able to correlate low performance in odor differentiation to a specific reduction in a newly generated interneuron population. In contrast, the detection limits for both odors and short-term olfactory memory were unaffected. Therefore they assumed that a critical number of bulbar granule cells is crucial for odor discrimination in particular, but not for general olfactory functions. Another group assumes that adult neurogenesis in the OB allows the constant adaption to new odor information from the environment (Lledo and Saghatelyan, 2005). Olfaction is the main sensory input for rodents. Several studies demonstrate that an odor-enriched environment results on the one hand in an increased neuron survival rate in the OB and on the other hand improves odor memory (Petreanu and Alvarez-Buylla, 2002, Yamaguchi and Mori, 2005, Rochefort et al., 2002). Therefore, the function of neurogenesis cannot be separated from its regulation.

3.1.5 Adult neurogenesis and neurodegenerative diseases

Interestingly, in different neurological disorders such as stroke, Huntington's disease and Alzheimer's disease, an increase in precursor cell proliferation and neurogenesis in these neurogenic niches have been observed (Curtis et al., 2003, Arvidsson et al., 2002, Perry et al., 2012). Arvidsson et al. (2002) showed, that stroke, caused by transient middle cerebral artery occlusion in adult rats, leads to a marked increase of cell proliferation in the SVZ. Moreover, they have shown that stroke-induced new neuroblasts are probably already formed before the insult, migrate into the severely dam-

aged area of the striatum, where they express markers of developing and mature, striatal medium-sized spiny neurons. Thus, stroke induces differentiation of new neurons into the phenotype of most of the neurons destroyed by the ischemic lesion. These findings indicate that ischemic neuronal death transmits signals to the new neurons to migrate into the damaged area of the striatum. A flow of new neurons with migratory morphologies could be traced back to the SVZ. These neurons sometimes formed aggregates, suggesting that some of them may reach the damaged area using chain migration, similar to neuronal precursors in the rostral migratory stream. In sharp contrast, there is a reduction in precursor cell proliferation in the SVZ of Parkinson's disease (PD) patients and corresponding experimental animal models (Hoglinger et al., 2004, Freundlieb et al., 2006). Höglinger et al. have shown that the generation of neuronal precursor cells is impaired in Parkinson disease as a consequence of dopaminergic denervation.

3.2 The orexin system

3.2.1 Orexin peptides and their receptors

In 1998 two scientific working groups, De Lecea et al. and Sakurai et al., could independently identify two similar and previously unknown neuropeptides. De Lecea et al. (1998) used directional tag PCR subtraction to identify 38 rat mRNAs selectively expressed within the hypothalamus. Hereunder they detected an mRNA, which was very similar to secretin, a peptide hormone produced in the S cells of the duodenum. Because of the homology to secretin and the location of its producing cells within the lateral and dorsal hypothalamic area, these hormones were named *hypocretin 1 and 2.* Sakurai et al. (1998) on the other hand detected the same neuropetides by chance, while they were searching for some ligands for orphan G-proteins. As they noted that the administration of these peptides to rats increased their food intake, they named the peptides *orexin A and B* after the Greek word for hunger (*Orexis),* and the two orphan G-protein receptors *orexin receptor 1 and 2.* In the literature, hypocretin and orexin are still used as synonyms. For better comprehension the term orexin will be used exclusively in the following text.

The orexins are split products of a common precursor peptide named prepro-orexin encoded by one gene located on chromosome 11. Orexin A is a 33-amino acid residue peptide with two intrachain disulfide bonds, while orexin B is a linear 28-residue pep-

tide. Both peptides are amidated at their C terminals. While the C-terminal shows great homology between the two peptides, the N-terminal differs largely in the amino acid sequence (Sakurai et al., 1998). The corresponding receptors are G-protein coupled and are termed orexin receptor 1 (OX_1R) and orexin receptor 2 (OX_2R). Although the receptors are very similar in structure, they exhibit a different affinity for the two neuropeptides. Orexin A and B bind equally well to OX_2R, however orexin A shows a higher affinity to OX_1R. OX_1R is an activating G-protein. OX_2R in contrast shows both, inhibitory and activating effects on its activation. Moreover, the two receptors show a different and basically complementary distribution within the brain, suggesting that they have distinct physiological functions (Sakurai, 2007, Kroeger and de Lecea, 2009, Peyron et al., 1998, de Lecea et al., 1998, Sakurai et al., 1998).

3.2.2 Neuroanatomy

The orexin-producing cells are located in the posterior hypothalamus, more precisely in its lateral and perifornical areas (Peyron et al., 1998). Up to 3200 of these cells, exclusively producing orexin, were found in mouse brains (Modirrousta et al., 2005). In healthy human brains approximately 50,000-90,000 orexin cells are present (Thannickal et al., 2000). The axonal projections of orexin neurons are widely distributed in the brain (Nambu et al., 1999). Orexin nerve terminals were observed throughout the hypothalamus, including the arcuate nucleus, the tuberomammillary nucleus and paraventricular hypothalamic nucleus. Outside the hypothalamus, orexin fibers were detected in the cerebral cortex, medial groups of the thalamus, and in circumventricular organs, such as the subfornical organ and the area postrema. Moreover the limbic system, more precisely HC, amygdala, and indusium griseum, is innervated by orexin. Furthermore fibers project to the brain stem, specifically to the locus coeruleus and the raphe nuclei. Less prominent projections were found in the OB (Peyron et al., 1998).

3.2.3 Physiological and pathophysiological functions

Regarding the widely spread projections throughout the brain, it can be assumed that orexin holds many different functions. The following chapter will illuminate the best characterized of the various known physiological and pathophysiological functions of orexin, starting with its role in the regulation of hunger and food intake. Thereafter, orexin's effects on the hypothalamus-pituitary-adrenal (HPA) axis, the sympathetic out-

flow and its role in the reward system will be described. Finally its influence on the regulation of the sleep-wake cycle and the sleep stages will be shown.

3.2.3.1 Feeding regulation and food intake

The lateral hypothalamic area (LHA) was already known as a classical region relevant for feeding regulation and energy homeostasis, when orexin neuropeptides were discovered. Several years before this, lesion- and electrical stimulation-studies gave ground-breaking information: animals with lesions of the LHA exhibit hypophagia and an increased metabolic rate, whereas acute electrical stimulation of the LHA causes hyperphagia and chronic stimulation causes even obesity (Danguir and Nicolaidis, 1980, Bernardis and Bellinger, 1996, Levitt and Teitelbaum, 1975). Since orexin neurons were specifically detected within and around the LHA, its role in feeding regulation has been analyzed in several studies. Sakurai et al. (1998), who consequently named the neuropeptide after this function, gave the first evidence for the role of orexin in feeding regulation. They showed that intracerebroventricular administration of orexin into the brain of rats leads to increased food consumption. This effect seems to be due to orexin A via the orexin receptor 1 (Haynes et al., 2000, Haynes et al., 2002). The admission of NPY-receptor antagonists reduces the effect of orexin A. Accordingly the NPY system is probably one of the downstream pathways by which orexin A induces feeding behavior (Yamanaka et al., 2000). In support to this assumption the arcuate nucleus (ARC), containing the NPY neurons, was shown to be densely innervated by fibers of orexin (Date et al., 1999, Peyron et al., 1998). Beside the effect via the NPY pathway, feeding regulation of orexin also includes the inhibition of glucoreceptive proopiomelanocorticotropin (POMC) expressing neurons. POMC neurons are located in the ARC as well. However their activation suppresses food intake. An opposing player of orexin is leptin, which plays a fundamental role in maintaining body weight homeostasis by reducing appetite (Zhang et al., 1994). The major mechanism by which leptin suppresses feeding is the inhibition of NPY-Neurons and excitation of POMC-Neurons (Hakansson and Meister, 1998, Elias et al., 1999). As leptin can only partly inhibit orexin-induced increase of food intake, orexin-induced feeding seems to involve both leptin-sensitive and -insensitive pathways (Zhu et al., 2002). This may suggest that one reason for leptin resistance in obese humans might be the activation of this leptin-insensitive pathway by orexin (Nishino and Sakurai, 2006). Yamanaka et al. (2003) have shown that the activity of isolated orexin neurons is inhibited by glucose and leptin and stimulated by ghrelin. Thereby, they function as indicators of the energy bal-

ance. Mice, in which orexin neurons were ablated, failed to respond to fasting with increased wakefulness and activity. This observation allows the assumption that orexin regulates arousal according to energy balance. Regarding body weight homeostasis, orexin has opposite impacts on feeding and metabolic rates. Hence, orexin increases food intake (Sakurai et al., 1998) and at the same time metabolic rate (Lubkin and Stricker-Krongrad, 1998).

3.2.3.2 Autonomic nervous system, neuroendocrine system and reward system

The emotional experience of fear leads to increased arousal and vigilance levels, accompanied by increased HPA axis activity and sympathetic outflow in animals. Orexin neurons are thought to be involved in the regulation of these responses to stressful situations (Mieda and Sakurai, 2009). The perifornical area, where orexin neurons are located, is known to function as the centre of defense responses, also known as the "fight or flight" response, which is characterized by an increase of arterial blood pressure, heart rate, and respiratory frequency (Jansen et al., 1995). In tracing studies the existence of afferents from several regions of the limbic system, responsible for the emotional experience of fear and other emotional states, to orexin neurons was shown. This included the amygdala, the infralimbic cortex, the nucleus accumbens, the lateral septum, and the brainstem (Sakurai et al., 2005, Yoshida et al., 2006).

A high density of orexin fibers and orexin-2-receptors (OX2R) has been identified in neurons of the hypothalamic paraventricular nucleus (PVN) (Sakurai et al., 1998, Trivedi et al., 1998, Date et al., 1999), which is thought to be involved in control of the autonomic nervous system, cardiovascular function and neuroendocrine system.

Several findings underline the neuroendocrine influence of orexin in the sense of an activation of the HPA axis. The HPA axis consists of corticotropin-releasing hormone (CRH) and arginin vasopressin neurons in the paraventricular nucleus (PVN) of the hypothalamus which regulates the secretion of ACTH in the anterior pituitary gland. ACTH in turn affects the adrenal cortical glucocorticoid secretion. Jaszberenyi et al. administered orexin intracerebroventricularly to adult male rats and demonstrated by measuring consecutively increased levels of plasma corticosterone an orexin-induced activation of the HPA axis. Pretreatment with the CRH antagonist alpha-helical CRH9-41 completely prevented the action of orexins, accordingly orexin effects on the HPA system are mediated by CRH (Jaszberenyi et al., 2000). These results suggesting

orexin participates in the stress response through effects on CRH in the PVN. The influence of orexin on the autonomic nervous system was also shown by intracerebroventricular administration. After injection increased mean arterial pressure, heart rate and renal sympathetic nerve activity in conscious rats was observed (Shirasaka et al., 1999).

Moreover, orexin seems to influence the reward system and drug addiction (Mieda and Sakurai, 2009). Dopaminergic projections from the ventral tegmental area to the nucleus accumbens are classically known as the “reward pathway”. Orexin neurons are interconnected with dopamine neurons in the ventral tegmental area and in the nucleus accumbens (Yoshida et al., 2006, Peyron et al., 1998, Fadel and Deutch, 2002). This pathway is also stimulated by drug abuse. Interestingly, patients with narcolepsy become rarely drug addicted, when they were treated with amphetamine-like stimulants against daytime-sleepiness (Guilleminault et al., 1974).

3.2.3.3 Influence on sleep and wakefulness and the sleep stages

The finding that orexin deficiency results in symptoms and pathophysiology of a disease called narcolepsy (Peyron et al., 2000, Chemelli et al., 1999, Lin et al., 1999, Thannickal et al., 2000) offered the possibility for a better understanding of its role in the regulation of sleep and wakefulness. Narcolepsy is characterized by the inability to maintain vigilance states, pathological intrusion of rapid eye movement (REM) sleep and/or non-REM (NREM) sleep into wakefulness and frequent transitions between the states of sleep and wakefulness. These symptoms indicate that orexin plays an important role in maintenance and stabilization of sleep and wakefulness. The clinical manifestation of narcolepsy will be further explained in detail in a separate chapter.

The brain circuitry that regulates our daily cycles of sleep and wakefulness includes various brain regions and neurotransmitters. Wakefulness and arousal depend on a network of cell groups, which is located in the brainstem and activates the thalamus and the cerebral cortex. They are functionally combined into the ascending reticular arousal system (ARAS) (Saper et al., 2001). The ARAS contains two pathways, one that innervates the thalamus and a second that extends into the posterior hypothalamus and forebrain (**Fig.3**).

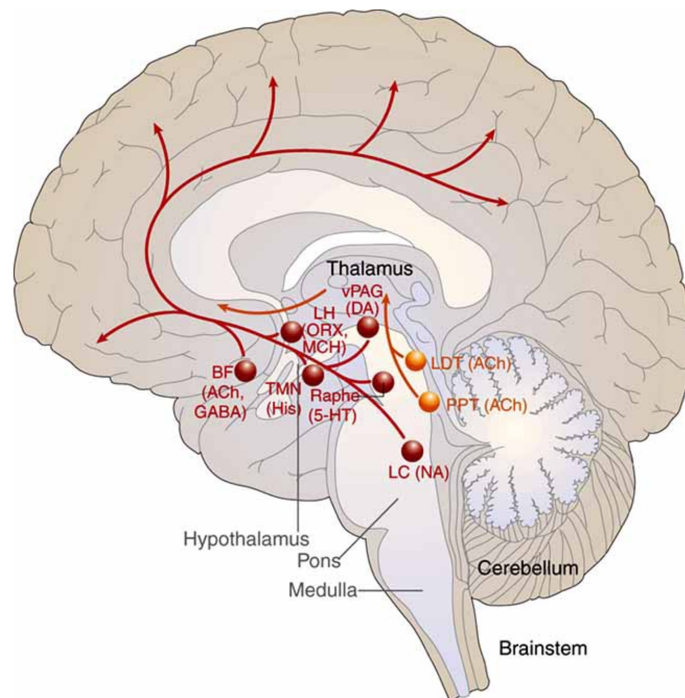


Fig. 3 A schematic drawing showing key components of the ascending arousal system (ARAS). Illustration taken from Saper et al. (2005).

The cholinergic pedunculopontine (PPT) and laterodorsal tegmental nuclei (LDT) project from the caudal midbrain and rostral pons to the thalamus and are thought to be essential in activating thalamocortical transmission (Hallanger et al., 1987). With different behavioral states PPT and LDT neurons change their activity. During wakefulness and REM-sleep many PPT and LDT neurons fire rapidly, whereas at sleep only few PPT and LDT neurons are active (Jasper and Tessier, 1971). The second branch of the ARAS projects into the lateral hypothalamus, basal forebrain, and the cerebral cortex (Saper, 1985, Jones, 2003). It comprises a number of monoaminergic cell populations, including noradrenergic neurons of the locus coeruleus, serotoninergic dorsal and median raphe nuclei, dopaminergic neurons of the ventral periaqueductal grey matter and the histaminergic tuberomammillary nucleus. They show the highest activity during wakefulness, slow down during NREM sleep, and are nearly inactive during REM sleep (Steininger et al., 1999, Aston-Jones and Bloom, 1981, Fuller et al., 2006).

A key switch in the hypothalamus shuts off this arousal system during sleep. This is done by neurons located in the ventrolateral preoptic nucleus (VLPO), which inhibits the ARAS by using the neurotransmitters GABA and galanin (**Fig.4**).

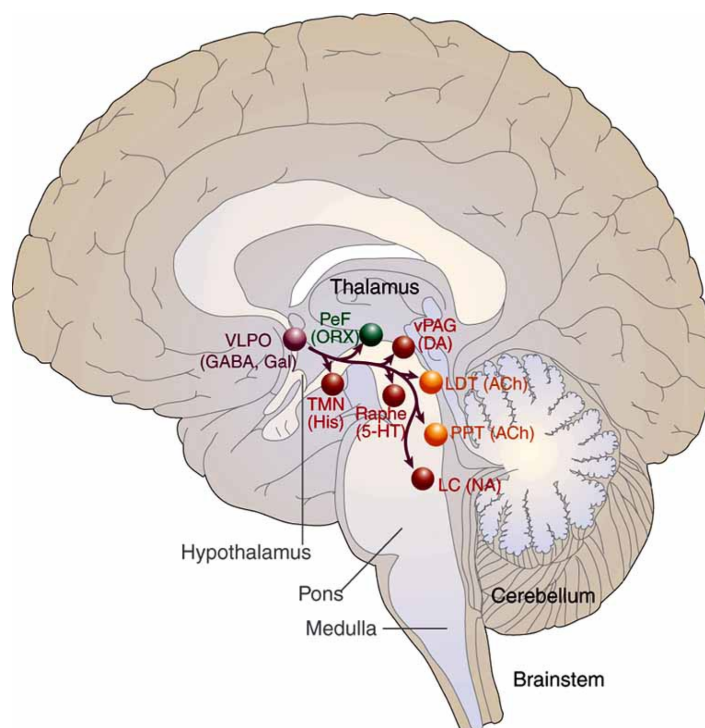


Fig. 4 A schematic drawing showing primary projections of the VLPO to the main components of the ascending arousal system. Illustration taken from Saper et al. (2005).

At the state of wakefulness the monoaminergic neurons of the ARAS inhibit the VLPO. This neuronal circuit is called flip-flop switch, a term originally used by engineers. The flip-flop switch sets up a self-reinforcing loop, where activity in one of the competing sides shuts down inhibitory inputs from the other side, and therefore disinhibits its own action. In this system the transitional state is very rare, because when either side begins to overcome the other, the switch 'flips' into the alternative state (Saper et al., 2001, Saper et al., 2005).

The orexin neurons stabilize the switch (**Fig 5**). Through excitatory projection to neurons in the locus coeruleus, tuberomammillary nucleus and ventral tegmental area (Hagan et al., 1999, Brown et al., 2001, Eriksson et al., 2001), orexin neurons reinforce the arousal system during wakefulness and are inhibited by the VLPO cells during sleep (Yoshida et al., 2006). Since VLPO neurons do not express orexin receptors (Marcus et al., 2001), there seems to be no direct but only indirect influence via the activation of the ARAS. By increasing the activity of the arousal regions and thereby indirectly inhibiting the sleep-promoting VLPO neurons, orexin gives the state of wakefulness more consistency, coordination and prevents inappropriate transitions into sleep. In narcolepsy the activating influence of orexin is reduced and the flip-flop circuit

becomes unstable and more vulnerable to small changes in homeostatic sleep drive, which refers to the desire to sleep that gradually increases with prolonged wakefulness (Mochizuki et al., 2004, Scammell, 2003, Nishino and Sakurai, 2006). Narcoleptic patients do not sleep more than normal individuals, but easily doze off during the day and wake up more frequently during night (Saper et al., 2005, Sakurai, 2007).

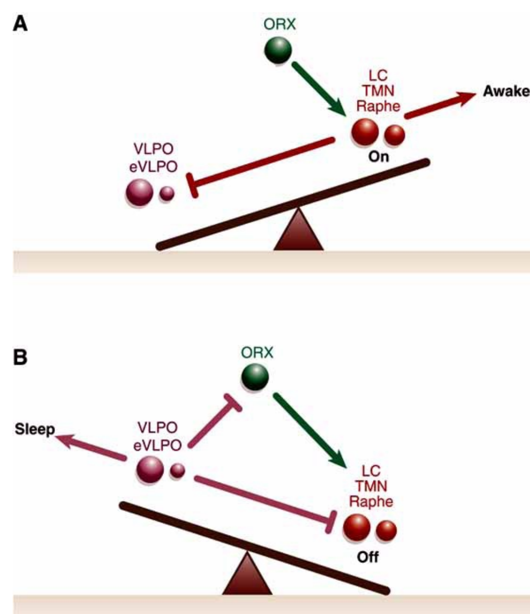


Fig. 5 A schematic diagram of the flip-flop switch model. Illustration taken from Saper et al. (2005).

This model could also explain the rapid transitions into REM sleep or fragments of REM sleep, which are seen in narcoleptics. As described above, orexin neurons send many fibers to the locus coeruleus and to the raphe nuclei. The locus coeruleus, tuberomammillary nucleus and raphe nuclei contain orexin receptors (Marcus et al., 2001) and all three groups inhibit REM sleep (Strecker et al., 2000). Bourgin at al. proposed, that orexin receptor 1 in the locus coeruleus is a key target for REM sleep regulation. Accordingly local administration of orexin A, but not of orexin B, in the locus coeruleus suppressed REM sleep in a dose-dependent manner and increased wakefulness at the expense of deep, slow-wave sleep (Bourgin et al., 2000). In addition intracerebroventricular administration of orexin A in rats, prepared for electroencephalography and electromyography recording, showed an increased latency in the onset of the first REM period (Smith et al., 2003). In the absence of an excitatory orexin input, the weakened arousal influence and increased activity of the extended VLPO would allow earlier and more frequent transitions to the REM state (Saper et al., 2001).

3.2.4 Narcolepsy

Narcolepsy was first described in 1880 by Gélineau. In the guidelines of the *Deutsche Gesellschaft für Neurologie (DGN),* narcolepsy is defined as a sleep-wake disorder with REM and non-REM sleep associated symptoms existing longer than 6 months, such as daytime sleepiness, cataplexy, fractionated sleep at night and automatic behavior. Reduced sleep latencies and early onset REM sleep can be observed in polysomnography (DGN, 2008). Excessive daytime sleepiness (EDS), a feeling of severe sleep-deprivation or chronic tiredness, the cardinal symptom of narcolepsy manifests in different ways (Nishino, 2007). Patients generally experience a permanent sleepiness, which is only shortly relieved by quick naps (15-30 min). In addition, they do suffer from sleep attacks (few seconds), which occur in very unusual situations such as in the middle of a meal and sometimes are followed by automatic behaviors. During such periods patients go on with their activity in a semi-conscious manner and might write or say incoherent things. Within five years after appearance of EDS, the pathognomonic symptom cataplexy occurs in two third of the cases. This phenomenon is defined as a sudden episode of muscle weakness triggered by emotional factors such as laughter. In the beginning the patients are awake but may have blurred vision or ptosis for a few seconds. It is a variable clinical symptom often occurring only mild with simple buckling of the knees, head dropping, facial muscle flickering, sagging of the jaw or weakness in the arms (Gelb et al., 1994). Between 2 and 7 years after the onset of EDS, 20-50 % of the patients develop sleep paralysis. This symptom appears in a brief inability to perform voluntary movements at the onset of sleep, upon wakening during the night or in the morning, which can be associated with frightening hallucinations. However, this phenomenon also occurs among 5-40 % of the general population. Hypnagogic and hypnopompic hallucinations are abnormal visual or auditory perceptions that occur while falling asleep (hypnagogic) or upon waking up (hypnopompic) and occur during REM-sleep (Nishino, 2007). Accompanying phenomena are obesity, memory and concentration problems, doze off related accidents, depression, erectile dysfunction, personality changes and headache (Sturzenegger and Bassetti, 2004). Narcolepsy often coexists with other sleep disorders, like obstructive sleep apnea syndrome, periodic limb movements in sleep, REM sleep behavior disorder, and nocturnal eating disorder (Sansa et al., 2010, Knudsen et al., 2010, Spaggiari et al., 1994).

The prevalence of narcolepsy ranges between 26-50/100000 (Hublin et al., 1994, Ohayon et al., 1996, Longstreth et al., 2007). The disease manifests mostly in the second decade before the age of 21 (Guilleminault and Pelayo, 1998). Mutations in orexin-related genes are rare in humans. Only a single case with early-onset narcolepsy (6 months of age) was found to be associated with a single-point mutation in the prepro-orexin gene (Peyron et al., 2000). Despite the lack of genetic abnormalities in the orexin system, it was found that human narcoleptics have an 85-95 % reduction in the number of orexin neurons (Thannickal et al., 2000). Moreover the presence of gliosis in the orexin cell region was shown, which is consistent with a degenerative process being the cause of the orexin cell loss in narcolepsy. However the pathogenesis seems to be multifactorial with decreased orexin containing neurons in the dorsolateral hypothalamus, as well as disturbances in the cholinergic and noradrenergic (NA) system (Lin et al., 1999). 98 % of Caucasian narcoleptic patients have the HLA DRB1 * 1501, DQB1 * 0602-type, making it the disease with the highest HLA association. This HLA type has a high sensitivity of 95 % but a low specificity, since it is detectable in 25-35 % of the general population (Mignot et al., 1994a, Mignot et al., 1999, Poirier et al., 1986). Against this background, an autoimmune cause for the destruction of orexin-producing neurons was postulated. However the failed search for neuron-specific and organ-specific auto-antibodies in the serum could not support the hypothesis (Black et al., 2002). Most cases of human narcolepsy are sporadic, a familial clustering accounts for only 10 % of the patients and only 25–31 % of monozygotic twins are reported to be concordant for disease (Mignot, 1998). Therefore a gene-environment interaction is highly likely (Longstreth et al., 2007, Ton et al., 2010).

One etiologic model for narcolepsy suggests that some environmental toxin selectively and irreversibly destroys orexin-producing cells in individuals with human leukocyte antigen (HLA) DQB1*0602 (Longstreth et al., 2007, Mignot, 1998). A highly sensitive and specific finding for narcolepsy is the reduction of orexin in the cerebrospinal fluid (CSF) below the detection limit. This is found in about 85 % of narcolepsy-cataplexy cases (Bassetti et al., 2003, Ripley et al., 2001, Nishino et al., 2000). In one study analyzing CSF orexin levels for the diagnosis of narcolepsy, most subjects with low levels were HLA-DQB1*0602-positive narcolepsy-cataplexy patients. But only a few HLA-DQB1*0602-positive subjects without cataplexy had low levels. Hence, cataplexy correlates highly to orexin deficiency, in contrast to other REM sleep-related phenomena in narcolepsy (Mignot et al., 2002). Occurrences of cataplexy are rare in acute sympto-

matic cases of EDS associated with a significant orexin deficiency, therefore it appears that a chronic and selective deficit of orexin neurotransmission may be required for the occurrence of cataplexy (Nishino, 2007). In 5 – 10 % of the narcolepsy cases, especially in hereditary/familial forms of the disease, the orexin values can lie within the normal range (Khatami et al., 2004).

3.2.4.1 Treatment

The treatment of narcolepsy is currently merely symptomatic and mostly requires a long-term drug therapy. Daytime sleepiness is treated in general with stimulating drugs. Previously used methylphenidate and amphetamine were replaced by modafinil as the first-line treatment for EDS and sleep attacks (Billiard, 2008). Modafinil is effective in 70-80 % of patients (Billiard et al., 2006). Compared to amphetamine, modafinil has an additional phenyl and an amid instead of an amine group, which suggests a distinct mechanism of action. However the mode of action is still unclear, even if a dopamine reuptake inhibition is likely to be involved (Mignot et al., 1994b, Nishino et al., 1998, Wisor et al., 2001). Another option is sodium oxybate, the sodium salt of gamma-hydroxybutyrate (GBH) which corresponds to a natural metabolite of the neurotransmitter GABA. Sodium oxybate has reductive effects on EDS, sleep attacks, cataplectic attacks and night time awakenings (US_Xyrem_Multicenter_Study_Group, 2003). The combination of modafinil and sodium oxibate shows an additive effect in treating EDS (Black and Houghton, 2006). In case patients become therapy-refractory to the first-line treatment against daytime sleepiness, alternative therapies are ephedrine, dextroamphetamine and MAO inhibitors. The treatment can be supported by non-pharmacological behavior-modifying measures, such as improving coping strategies, sleep hygiene and customized day-sleep-episodes. Cataplexy, sleep paralysis and hypnagogic hallucinations are also treated with sodium oxybate or new antidepressants specially venlafaxin or atomoxetine (DGN, 2008, Billiard, 2008). If these treatments are inefficient, tricyclic antidepressants or SSRIs can be used. Today the common assumption is that human narcolepsy is caused by orexin deficiency (Thannickal et al., 2000, Peyron et al., 2000). Consequently the natural treatment seems to be the replacement of orexin. However, research is still at the beginning. One group found normalization of sleep/wake patterns and behavioral arrest episodes (equivalent to cataplexy and REM sleep onset) in orexin-deficient mouse knockout models, supplemented by central administration of orexin (Mieda et al., 2004). Hence they could demonstrate that tempo-

rally regulated and spatially targeted secretion of orexin is not necessary to prevent narcoleptic symptoms. This encouraged research in orexin replacement therapies.

3.2.5 Animal models of narcolepsy

In 1973, non-human narcolepsy was first described in a cat and a dog (Knecht et al., 1973). At Stanford University, a colony of narcoleptic dogs was established in 1976 to study the pathophysiology of the disease (Cederberg et al., 1998). After the canine genomic bacterial artificial chromosome (BAC) library was built from a Doberman pinscher (Li et al., 1999), the positional cloning approach in the canine narcolepsy model led to the identification of mutations in the OX_2R gene (Lin et al., 1999). Hence, animal models gave the first indications that the orexin system seems to be involved in narcolepsy. As the two orexin peptides and their two receptors OX_1R and OX_2R were discovered, the possibility was given to create different types of knockout models in mice. Chemelli et al. developed an orexin knockout mouse, induced by targeted disruption of the prepro-orexin gene, which results in a phenotype strikingly similar to human narcolepsy patients. Hence these mice show symptoms like EDS, cataplexy and other REM sleep-related phenomena (Chemelli et al., 1999). OX_1R knockout mice seem to have no cataplectic attacks and only moderate sleep fragmentation (Willie et al., 2003). Orexin double receptor knockout mice have the same phenotype as prepro-orexin knockout mice (Chen et al., 2009). The use of constitutive knockout animal models is potentially confounded by the compensation of the orexin system during the embryonic brain development. A different opportunity exhibits chemical lesion, where orexin-B conjugated to the ribosome-inactivating toxic protein saporin is injected into the lateral hypothalamus. Consequently this method targets only orexin receptor-bearing neurons. Because orexin neurons and non-orexinergic neurons in the lateral hypothalamus express orexin receptors this method destroys both orexin and non-orexin cells. Treated rats showed narcoleptic symptoms like increased slow-wave sleep, REM sleep, and sleep-onset REM sleep periods (Gerashchenko et al., 2001). The establishment of antisense or RNA interference (RNAi) technology facilitates a transient inhibition of the gene expression of either orexin or its receptors. Animals treated either with the OxR2 antisense (Thakkar et al., 1999) or with short interfering RNAs (siRNA) targeting the prepo-orexin mRNA (Chen et al., 2006) showed an increase in total REM sleep and infrequent cataplectic attacks. Advantages of this model comprise its high selectivity, reversibility and fast onset. A method which can be used in all species is the use of

antagonists against both orexin receptors. Somnolence without cataplexy and a decrease of alertness was observed in rats, dogs and humans when the orexin receptor antagonists were given during the active phase of their circadian cycle (Brisbare-Roch et al., 2007). In rats, increases in electrophysiological markers of non-REM and REM sleep were shown additionally.

As mentioned above mutations in the prepro-orexin or orexin receptor genes seem to be unlikely the cause for human narcolepsy. Post-mortem studies indicate a neurodegenerative pathogenesis with a global loss of orexin neurons in human narcoleptics (Thannickal et al., 2000, Peyron et al., 2000). Thus, Hara et al. (2001) developed a mouse model called *orexin/ataxin-3 mouse,* which mimics the postulated pathophysiology of human narcolepsy very closely. They constructed a transgenic model in which orexin-containing neurons are ablated by orexinergic-specific expression of a truncated cytotoxic gene product. Therefore, they used a fragment of the 5'-upstream region of the prepo-orexin gene as a promoter, which was ligated to the N-terminally truncated cDNA for human ataxin-3, an expanded polyglutamine stretch known from the Machado-Joseph disease. The expression of this transgene induces apoptosis and results in postnatal chronic progressive loss of orexin-containing neurons exclusively. The group observed a 99 % loss of orexin cells after 12 weeks of age and could hardly detect any orexin cell in the lateral hypothalamic region after 15 weeks (Hara et al., 2001). It could be assumed that the phenotype of this mouse model differs from the others, because of the global cell loss. Orexin neurons produce also galanin, angiotensin and the endogenous opiate dynorphin (Chou et al., 2001), which may act synergistically with orexin to increase the activity of wake-promoting brain regions and influences feeding behavior (Walker et al., 1980). Furthermore orexin neurons contain the neuronal activity-regulated pentraxin (NARP) (Reti et al., 2002) which may contribute to the symptoms of human narcolepsy. Interestingly the difference between the actual narcoleptic phenotype of prepo-orexin knockout and orexin/ataxin-3 mice does not seem to be intense. Differences exist in the temporal appearance of abnormal behavior. Orexin/ataxin-3 mice become symptomatic after 6 weeks, whereas some of prepro-orexin mice already show symptoms at an age of less than 3 weeks. Usually narcolepsy patients show symptoms not until adolescence, thus postnatal loss of orexin neurons in the orexin/ataxin-3 model is more comparable to the typical human narcolepsy case. Concerning behavioral arrests, duration of episode and sleep state patterns no significant differences between prepo-orexin knockout and orexin/ataxin-3 mice could be observed.

However the duration of awakening state during the light phase, which equates sleep disruption in human narcolepsy at night, was significantly shorter in over 12 week old orexin/ataxin-3 mouse than in compared littermates. This difference could not be observed in prepro-orexin knockout mice in comparison to their littermates. In orexin/ataxin-3 mice, a metabolic abnormality characterized by reduced food consumption combined with decreased motor activity and lower basal metabolic rate results in late onset obesity, a symptom also noticed in human narcoleptic patients. In contrast prepro-orexin knockout mice display rather normal weight than obesity although they are also hypophagic. Reason for these fine metabolic differences might be the absence of orexin-cell-produced neuropeptides like dynorphin (Walker et al., 1980). Moreover the fact that postnatal orexin/ataxin-3 mice still produce orexin and are exposed to its modulatory effects, in contrast to prepo-orexin knockout mice, which are not able to produce any orexin at any time, could cause a crucial difference. However the fundamental similar symptoms of these two mouse models underline once more the important role of orexin in the regulation of sleep and wakefulness and eating behavior (Hara et al., 2001).

3.3 Orexinergic innervation of the neurogenic brain regions

A direct, topographically organized innervation of adult NSC and proliferating progenitor cells by orexin has been demonstrated in the SVZ, the SGZ, the RMS and the OB by our group (unpublished data). For localization of NSC and progenitor cells nestin-GFP transgenic mice were used. These are genetically modified mice in which the expression of nestin and the green fluorescent protein (GFP) were coupled together (Mignone et al., 2004). Nestin is an intermediate filament protein that is expressed in stem cells of the CNS but not in mature CNS cells (Reynolds and Weiss, 1992). DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA (Kapuscinski, 1995) and thus marks all DNA containing nuclei. By immunofluorescence labelling for orexin A and the OX_2R the co-localization of orexin neurons and nestin-GFP labeled adult NSC or proliferating progenitor cells could be shown using a confocal laser scanning microscope.

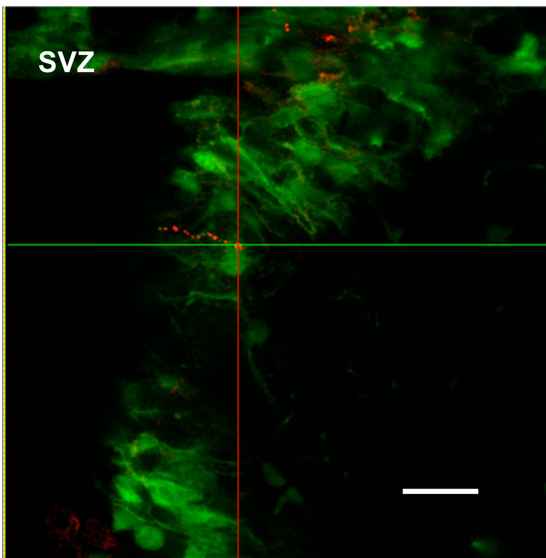


Fig. 6: **Orexinergic innervation of adult NSC in the SVZ.** The picture shows the contact of orexinergic dentrites with new built cells in the SVZ. Red: Orexin A; Green: adult NSC. *scale bar:* 10 µm (Image kindly provided by O. Arias-Carrion).

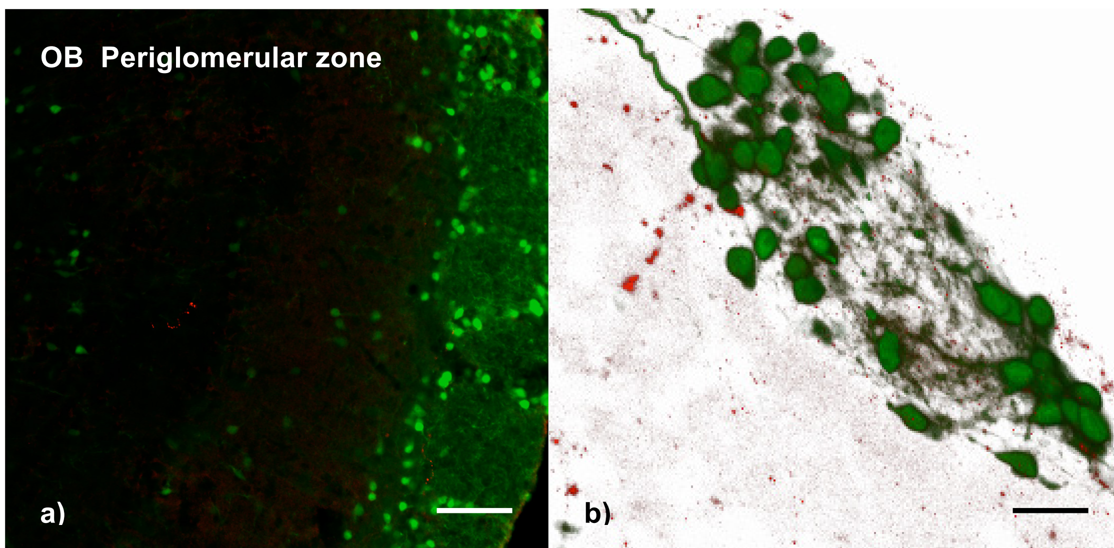


Fig. 7: Orexinergic innervation of new built cells in the OB. Left panel: Overview shows the colocalization of Orexin dentrites and new built cells in the periglomerular zone of the OB. Right panel: Shows the contact of orexinergic dentrites with new built cells in details. Red: Orexin A; Green: new built cells. *scale bars:* 100 µm (**a**); 10 µm (**b**) (Image kindly provided by O. Arias-Carrion).

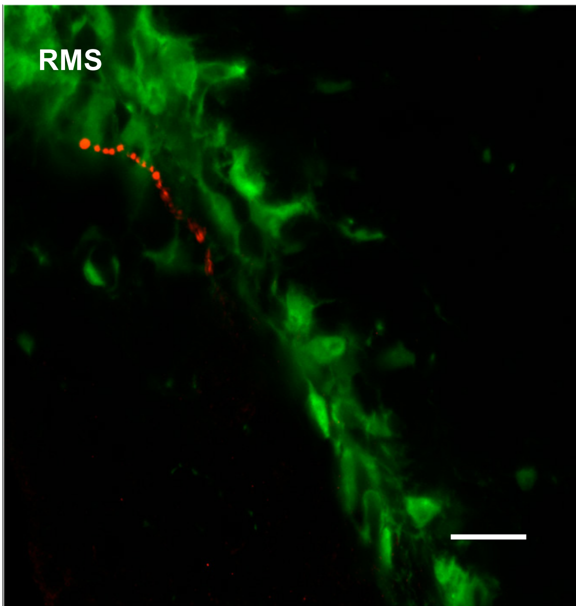


Fig. 8: Orexinergic innervation of new built cells in the RMS. The picture shows the contact of orexinergic dentrites with new built cells in the RMS. Red: Orexin A; Green: new built cells. *scale bar:* 10 µm (Image kindly provided by O. Arias-Carrion).

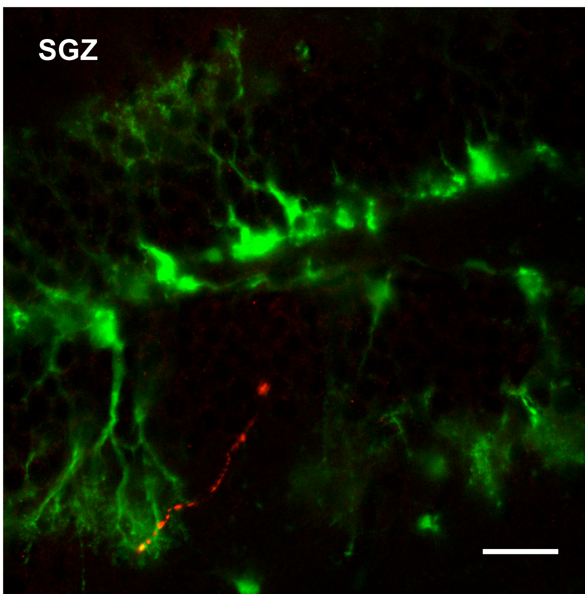


Fig. 9: **Orexinergic innervation of adult NSC in the SGZ.** The picture shows the contact of orexinergic dentrites with new built cells in the SGZ of the HC. Red: Orexin A; Green: new built cells. *scale bar:* 10 µm (Image kindly provided by O. Arias-Carrion).

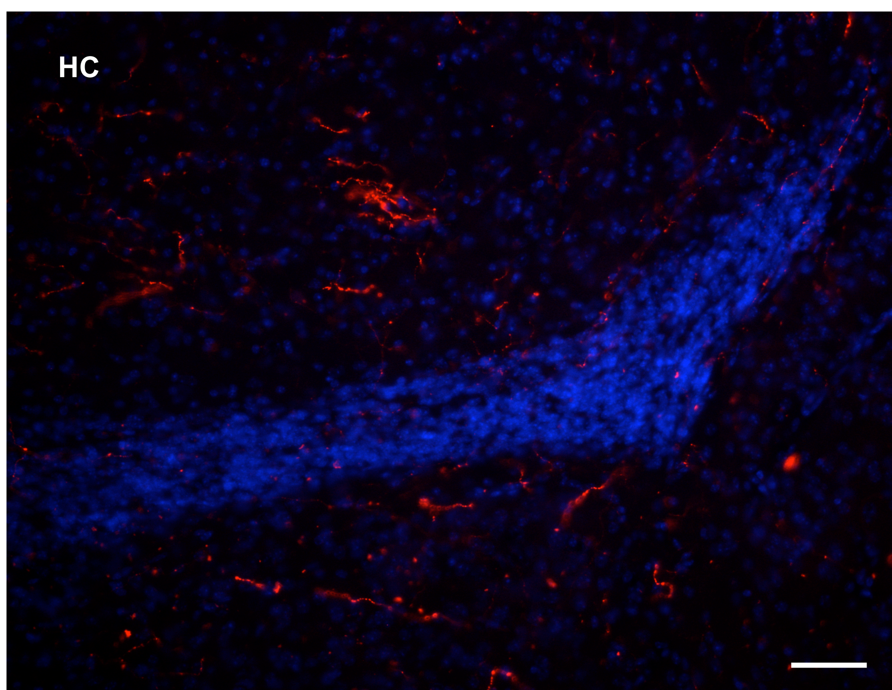


Fig. 10: Occurrence of OX_2R in the HC. Red: OX_2R; Blue: Cellular nuclei in the HC. *scale bar:* 100 µm (Image kindly provided by O. Arias-Carrion).

3.4 Aim of the study

In preliminary investigations of our working group, a high density of orexin afferents was found in the SVZ, RMS, OB, and SGZ (see introduction 3.3.). This suggests that orexin may contribute to regulation of the niches of adult neurogenesis.

The aim of this study was to evaluate the influence of the orexin system on adult neurogenesis. In this particular study the genetic ablation in the orexin-ataxin mouse was investigated.

The central hypothesis was that orexin, acting at the orexin receptors, suppress the proliferation of the adult NSC. If confirmed, *in vivo* modulation of the orexinergic neurotransmission might therefore offer novel options for the stimulation of adult neurogenesis with a therapeutic perspective for human patients with neurodegenerative disorders.

4 MATERIAL

4.1 Equipment

Material	Source
Tissue freezing medium	Leica Instruments GmbH – Nussloch, Germany
Glass carrier slide, 25*75*1.0 mm	Menzel-Gläser GmbH - Braunschweig, Germany
Cover slips, 24*50 mm	Menzel-Gläser GmbH - Braunschweig, Germany
Microtome-blades, type S35	Feather - Köln, Germany
Pipettes,10-100 ul/ 100-1000 ul/ 1000 ul	Eppendorf AG - Hamburg, Germany
Pipette tips	Eppendorf AG - Hamburg, Germany
Stereotaxie atlas for mice	The Mouse Brain in Stereotaxic Coordinates, 2nd edition; Paxinos & Franklin, 2001
Terralin-Liquid (disinfectant)	Schülke & Mayr - Norderstedt, Germany
Dry ice	Pharmacy, Philipps-Universität Marburg, Germany

4.2 Chemicals

5-Bromo-2´-Deoxyuridin (BrdU)	Vector Laboratories, Burlingame, CA, USA
Normal Goat Serum (NGS)	Sigma, St. Louis, MO, USA
Normal Horse Serum (NHS)	Sigma, St. Louis, MO, USA

4.3 Antibodies

Name	Factory no.:	Dilution	Company
Primary antibodies			
Rat anti-BrdU	OBT0030	1:500	AbD Serotec, Oxford, UK
Rabbit anti-TH (thy-rosine hydroxylase)	AB152	1:1000	Millipore Billerica, MA, USA
Mouse anti-NeuN (neuronal nuclear antigen)	MAB377B	1:1000	Millipore Billerica, MA, USA
Secondary antibodies			
Donkey anti-rat biotinylated	712-065-150	1:500	Jackson ImmunoResearch La-boratories, Inc. West Grove, PA, USA

Alexa Fluor 488-conjugated goat anti-rat IgG	A11006	1:1000	Invitrogen Carlsbad, Kalifornien, USA
Cy3-conjugated goat anti-mouse IgG	115-166-062	1:500	Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA
Cy5-conjugated donkey anti-rabbit IgG	711-175-152	1:500	Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA

4.4 Solutions

Antifreeze (1 l):	NaH_2PO_4	1.57 g
	Na_2HPO_4	5.18 g
	$H_2O_{dest.}$	400 ml
	ethylenglycol	300 ml
	glycerin	300 ml
Borat buffer (450 ml):	boric acid	3.09 g
	$H_2O_{dest.}$	450 ml
	adjust to pH 8,5 with 5 N NaOH	
DAB 3,3'-Diaminobenzidine (20 ml):	Tris	20 ml
	DAB	10 mg
	H_2O_2	2 µl
Gelatine-solution (700 ml):	gelatine	3.5 g

	Chromium potassium sulfate	0.35 g
	$H_2O_{dest.}$	700 ml
PB phosphate buffer (10 l):	$H_2O_{dest.}$	10 l
	$Na_2HPO_4*2H_2O$	230 g
	$NaH_2PO_4*H_2O$	52.44 g
PBS phosphate buffered saline (1 l):	0.2 M PB	50ml
	$H_2O_{dest.}$	950 ml
	NaCl	9 g
PFA Paraformaldehyde 4% (200 ml):	$H_2O_{dest.}$ (heat up to 60 °C)	50 ml
	PFA (add while stirring)	4 g
	NaOH 10 N (=> filtrate)	100 µl
	0.2 M PB	50 ml
Saccharose-solution 30% (400 ml):	saccharose	150 g
	0.1 M PB	400 ml
TBS Tris-buffered saline (2 l):	Tris 1 M	500 ml
	NaCl	17.6 g
	KCl	0.4 g
	H_2Odest.	2000 ml
Tris HCl-buffer (1 l):	Tris	121.1 g
	H_2Odest	1000 ml
	adjust to pH 7.5 with HCl	

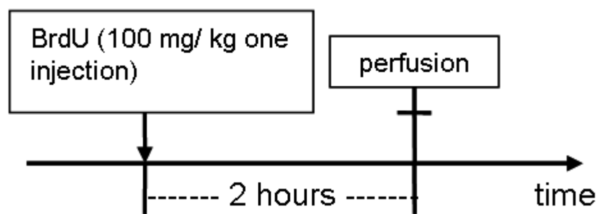
4.5 Experimental animals

Adult male and female littermates with (orexin/ataxin-3 mice, n = 16) and without (wildtype (WT), n = 16) the orexin/ataxin-3 transgene (Hara et al., 2001) were obtained from a congenic line and matched for age and body weight. All used mice were aged between 96 and 127 days and weighed between 20 and 35g at the start of treatment. Mice were kept under standard conditions (room temperature 23 °C ± 1 °C, humidity 55 ± 5 %, 12-hour light-dark cycle, lights on 6:30 p.m.). 1-5 mice were kept in one cage. The animals had standard food (Altromin) and water to drink at free disposal. All experimental procedures involving animals were approved by the *Regierungspräsidium Gießen* and were carried out in accordance with NIH guidelines.

5 METHODS

5.1 Experimental design

For the evaluation of stem cell proliferation in the SVZ and SGZ and migration in the RMS, mice (orexin/ataxin-3 mice n=8; WT n=8) received a single intraperitoneal injection of 100 mg BrdU/kg body weight 2 hour prior to sacrifice. For the analysis of survival and differentiation of newly build cells, BrdU was administered intraperitoneally for 5 consecutive days (one per day) to each mouse (orexin/ataxin-3 mice n=8; WT n=8). The last injection was made 30 days before the mice were sacrificed **(Fig. 11)**. Adult neurogenesis in the OB, RMS, SVZ and HC was studied by immunohistology as shown in **table 1**.



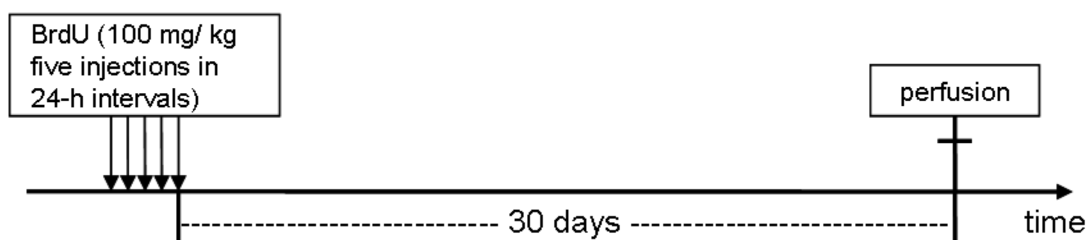


Fig. 11: Experimental design

Staining	Purpose of the staining	Regions in group no.1	Regions in group no.2
Cresyl violet	overview, cutting control	all brain regions	all brain regions
BrdU	quantification of proliferation and survival	HC, SVZ and RMS	HC, OB
Triple staining (BrdU/NeuN/TH)	differentiation of newly built cells into neurons/ dopaminergic neurons		HC, OB

Table 1: Overview of stainings used in this study

5.2 Bromodeoxyuridine treatment

BrdU as a synthetic analogue of thymidine, is incorporated in the cellular DNA of proliferating cells in the S phase of mitosis and can be subsequently detected by immunohistochemistry. For detection of adult neurogenesis, BrdU labeling is considered as the gold standard (Vega and Peterson, 2005). BrdU (Vector Laboratories, Burlingame) was dissolved in 0.9 % NaCl and filtered sterile. The mice received single intraperitoneal doses of 100 mg BrdU/kg body weight at a concentration of 10 mg/ml.

5.3 Perfusion

The purpose of the transcardial perfusion is to drain the blood out of the brain and fix the tissue with paraformaldehyde (PFA) to optimize preservation of cellular structures and antigens. For this procedure, a Masterflex pump controller (Masterflex, Gelsenkirchen, Deutschland), two perfusion fluids (phosphate-buffered saline (PBS, 0.1 M, pH 7.3), 4 % PFA in PBS), a scalpel, scissors, a cannula, and two large self-holding forceps were prepared.

After intraperitoneal injection of a lethal dose (40 mg) of pentobarbital, absence of the interdigital pain reflex was verified before continuing. The animals were placed on a polystyrene board and the body was pinned out using all four extremities. The thorax was opened by a median sternotomy. The access to the thoracic cavity was made by an incision in the diaphragm. The pericard was cautiously opened with scissors, an insert was made carefully into the left heart ventricle and the cannula was placed into it. Right after the pump system was turned on, a small incision in the right auricle was

done in order to drain the venous blood. The mice were perfused first with 0.1 M PBS followed by ice cold 4 % PFA. To ensure blood was fully drained from the brain, each solution was applied for the duration of 10 min with a speed of 15ml/min. Therefore, 150 ml per solution and 300ml fluid were used per brain which equates 4-5 times of the blood volume of a mouse.

5.4 Tissue preparation

After decapitation with a large, sharp pair of scissors, the brain was removed carefully. First the two flaps of skin and muscle were pulled back so that the skull underneath was exposed. Using a small, sharp pair of scissors, the skull bone was cut along the sagittal sutura before the skull was broken up. For post-fixation, brains were placed in a 4 % PFA solution and stored at 4°C until they sank to the bottom of the vessel, which took about 24 hours. To prevent tissue damage during freezing, the brains were dehydrated in 30 % sucrose solution for 72 hours at 4°C.

The brains were divided into two halves at the interhemispheric fissure, and placed for freezing into a box with dry ice for 5 min. *Jung Tissue freezing medium* was used to fixate each brain hemisphere with the help of the *Leica* cryostat onto an object holder. With the *Leica* cryostat coronary serial brain sections with 30 µm thickness were created from the right half of each brain. The left half of the brains was used for sagittal sections. The chamber and slide temperature was -20°C to -25°C. Coronary brain sections were collected from the following anatomical regions: OB, striatum, HC and substantia nigra. For each brain 10 series were sorted and stored in cryo-tubes in *antifreeze* solution at -20°C.

5.5 Tissue staining

5.5.1 Cresyl violet staining

The cresyl violet method uses a basic aniline dye to stain RNA blue, and is used to highlight important structural features of neurons. The *Nissl substance* (rough endoplasmic reticulum) appears dark blue due to the staining of ribosomal RNA, giving the cytoplasm a mottled appearance.

The fixed brain tissue sections were mounted onto gelatinized glass slides and dried for at least 12 hours. Then they were stained in 0.1% cresyl violet for 4 min. Afterwards a quick rinse with demineralized water was done, to remove excess stain. Subsequent-

ly sections were put into 70 % ethanol for 1 min and then for 1-2 min in glacial acetic acid (2 drops glacial acetic acid in 95 % ethanol). Then the slides underwent subsequent rinses in ethanol in ascending concentrations (70 %, 96 %, 100 %) each for 5 minutes. Finally, sections were washed twice in xylene for 5 min.

5.5.2 Immunhistochemical staining

The principle of the immunohistochemical staining is based on a specific antigen-antibody binding. In general, a primary antibody is used to detect its specific antigen in the tissue sample. Several options are possible to visualize bound antibodies for microscopy. Two different marker systems were used in this study.

In the fluorescent method, a secondary fluorochrome-labeled antibody was added, which is directed against the Fc-part of the first specific antibody. Fluorochromes absorb and emit light at defined wavelengths and can be visualized using a fluorescence microscope.

The 3,3'-diaminobenzidine (DAB) staining, however, is an enzyme-based method. In this method, the secondary antibody is biotinylated. A so-called tertiary reagent; “ABC”-(avidin-biotin-complex) is used. It contains biotinylated avidin. Because avidin has a high affinity for biotin (over one million times higher than antibodies for most antigens), the binding of avidin to biotin is essentially irreversible. In addition, avidin has four binding sites for biotin. These properties allow macromolecular complexes to be formed between avidin and biotinylated enzymes. The enzyme used to visualize bound antibodies is a peroxidase. The secondary biotinylated antibody serves as a bridge between the primary antibody and the avidin-biotin-enzyme complex. This binds to the biotin on the Fc part of the secondary antibody. DAB is a widely used chromogen for immunoperoxidase staining. The peroxidase reacts with 3 % hydrogen peroxide substrate to degrade it, which in turn reacts with DAB to precipitate it at the positive sites giving dark brown color. The result can be observed with a light microscope. In order to prevent false positive results in this enzyme-based staining the endogenous peroxidase was quenched. To this end, the tissues were incubated in a solution of 12.5 % methanol peroxidase.

All immunohistochemical stainings were performed by using the "free floating" technique. For this purpose multi-wells with mesh inserts were used. Incubation in the respective medium and washing were carried out using a shaker, which guaranteed a

constant movement of the sections in the liquid. In order to ensure adequate cleaning after each incubation step, the sections were washed five times for five minutes in 0.1 M PB solution between each incubation step.

5.5.2.1 Bromodeoxyuridine

Because the BrdU epitope is hidden within the DNA under non-denaturing conditions, DNA was denatured prior to immunostaining. For that purpose, the sections were incubated for 30 minutes in 2 N HCl at 37°C. Thereafter, slices were washed in 0.1 M PBS 5 times for five minutes. The primary antibody, rat anti-BrdU (OBT0030), was used in a concentration of 1 / 500, the secondary antibody, donkey anti-rat (711065152), in a concentration of 1 / 500.

Brain sections including the OB, striatum and HC were stained according to the following protocol:

Washing	4 x 5 min in 3 ml 0.1 M PB shaking
Quenching endogenous peroxidase activity in methanol peroxidase solution	15 min at RT in 3 ml of methanol peroxidase solution (e.g. 4 ml methanol 100 %, 4 ml H2O2 35 %, 34 ml 0.1 M PB)
Washing	4 x 5 min in 3 ml 0.1 M PB shaking
DNA denaturation	30 min in 2 N HCl in a 37 °C water bath
Washing in 0.1 M borate buffer, pH 8.5	2 x 5 min in 0.1 M sodium borate buffer (MW 381.4) 3.8 g + distilled water 100 ml, mix to dissolve and adjust pH to 8.5
Washing	4 x 5 min in 3 ml 0.1 M PB shaking
Blocking in 5 % Normal Goat Serum (NGS)	2 h at RT in 3 ml of 5 % NGS solution (9.5 ml 0.3 % Triton/0.1 M PB + 0.5 ml NGS)
Primary antibody: Rat anti-BrdU (AbD, Serotec OBT0030; 1:500)	Incubation overnight at 4 °C in 1 ml of antibody solution (1 ml PB/NGS + 2 µl primary antibody)

Washing	5 x 5 min in 3 ml 0.1 M PB shaking
Secondary antibody: Donkey anti-rat biotinylated, (Jackson ImmunoResearch, 712-065-150, 1:500)	Incubation for 60 min at RT in 1 ml of biotinylated secondary antibody solution (e.g. 10 ml 0.3 % Triton/0.1 M PB + 20 µl secondary antibody)
Incubate in ABC solution ~1:200	Incubation for 60 min at RT in 1 ml of ABC solution (e.g. 5 ml 0.1 M PB + reagent A 1-drop + reagent B 1-drop)
Washing	4 x 5 min in 3 ml 0.1 M PB shaking
Incubate in DAB	Incubation for 2 min in DAB solution (1 ml DAB, 9 ml 0.1 M PB, 0.2 ml 1 % H_2O_2 (50 µl sol 30 % H_2O_2, 1450 µl deionized water))
Mounting on slides, dehydration and coverslipping	

Table 2: BrdU staining protocol

5.5.2.2 BrdU/NeuN/TH triple staining

NeuN (neuronal nuclei) is a neuron-specific DNA-binding nuclear protein which functions as a marker for neurons. NeuN immunoreactivity is observed in post-mitotic neurons that are initiating cellular and morphological differentiation. Tyrosine hydroxylase (TH) is the rate limiting enzyme for the production of catecholamines, in particular for dopamine. Therefore it is suitable to mark dopaminergic cells.

Brain sections including the OB, striatum and HC were stained according to the following protocol:

Washing	4 x 5 min in 3 ml 0.1 M PB shaking
Quenching endogenous peroxidase activity in methanol peroxidise solution	15 min at RT in 3 ml of methanol peroxidase solution (e.g. 4 ml methanol 100 %, 4 ml H_2O_2 35 %, 34 ml 0.1 M PB)

Washing	4 x 5 min in 3 ml 0.1 M PB shaking
DNA Denaturation	30 min in 2 N HCl at 37 °C water bath
Washing in 0.1 M borate buffer, pH 8.5	2 x 5 min in 0.1 M sodium borate buffer (MW 381.4) 3.8 g + distilled water 100 ml, mix to dissolve and adjust pH to 8.5)
Washing	4 x 5 min in 3 ml 0.1 M PB shaking
Blocking in 5 % Normal Goat Serum (NGS)	2 h at RT in 3 ml of 5 % NGS solution (9.5 ml 0.3 % Triton/0.1 M PB + 0.5 ml NGS)
Primary antibody: BrdU Rat anti-BrdU (AbD, Serotec OBT0030, 1:500)	Incubation overnight at 4 °C in 1 ml of antibody solution (1 ml PB/NGS + 2 µl primary antibody)
Washing	5 x 5 min in 3 ml 0.1 M PB shaking
Secondary antibody goat anti-rat IgG (Alexa Fluor 488, Invitrogen, A11006, 1:1000)	Incubation for 2 h at RT in 1 ml of light sensitive secondary antibody solution (1 ml PB/NGS + 1 µl AB) (Staining was performed under light protection from here on)
Washing	5 x 5 min in 3 ml 0.1 M PB shaking
Primary antibody NeuN Mouse anti-NeuN (Millipore; MAB377B ; 1 :1000)	Incubation overnight at 4 °C in 1 ml of primary antibody solution (1 ml PB/NGS + 1 µl NeuN AB)
Washing	5 x 5 min in 3 ml 0.1 M PB shaking
Secondary antibody Goat anti-mouse Cy3 (Jackson Immunologicals PB/NGSX 115-166-062, 1:500,)	Incubation for 2 h at RT in 1 ml of light sensitive secondary antibody solution (1 ml PB/NGS + 2 µl AB)
Washing	5 x 5 min in 3 ml 0.1 M PB shaking

Primary antibody TH Rabbit anti-TH (Chemicon AB152, 1; 1000)	Incubation overnight at 4 °C in 1 ml of primary antibody solution (1 ml PB/NGS + 1 µl TH AB)
Secondary antibody Donkey anti-rabbit Cy5 (Jackson Immunologicals) PB/NGSX 1:500, 711-175-152	Incubation for 2 h at RT in 1 ml of light sensitive secondary antibody solution (1 ml PB/NGS + 2ul AB)
Washing	5 x 5 min in 3 ml 0.1 M PB shaking
Mounting in a 0.9 % saline pool; Coverslipping after 3-5 min with DABCO (1,4-Diazabicyclo-octane) mounting medium	

Table 3: Triple staining protocol

5.6 Microscopic analysis

5.6.1 Qualitative evaluation of cresyl violet

In order to evaluate the quality of the tissue sections, the cresyl violet stained sections were screened under a light microscope to assess, whether the perfusion was carried out properly.

5.6.2 Stereology

For the quantification of cells, the *Nikon Microphot FX* (Nikon Shinjuku, Tokyo, Japan) together with a semi-automatic stereology system *Stereoinvestigator* (MicroBrightField Bioscience, Williston, VT, USA) were used. The analysis was done by an observer blinded to the treatment of the animals. The counting of immunohistochemically stained cells in the DAB-staining technique was carried out on every 10th coronal section. The number of positively stained cells was estimated with an optical fractionator by using systematic random sampling of the sections of interest in order to yield the approximate number per anatomical region. The regions taken for counting were defined according to “The Mouse Brain in Stereotaxic Coordinates” (2nd edition, Paxinos and Franklin, 2001). The reference volume was defined by manual tracing of the regions of interest with the semiautomatic stereology system. The system works with a light microscope equipped with a motorized stage interfaced with a video camera and a PC. Regions to be counted are manually defined with a 10 x objective. The program then generates

unbiased counting grid of 100 x 100 µm with a 40 x objective. Positive cells within the frame are marked by the investigator manually. All positive cells in the accordant region were counted. A cell was defined as positive and counted when fulfilling the following criteria: evenly stained, complete, sharply bounded nuclei with a dark brown color. Positive profiles that intersected the uppermost focal plane (exclusion plane) or the lateral exclusion boundaries of the counting frame were not counted. All sections of one series were counted by the same investigator to minimize investigator-related-bias. When finished with counting the system automatically moves the total counting grid to the next frame so that the predefined traced regions are systematically screened. The estimated cell count is calculated by the system. Therefore the total counts of marked cells are multiplied by the ratio of reference volume and sampling volume and thereby the total cell number is mathematically estimated.

5.6.2.1 BrdU immunhistochemical staining

SVZ: For counting $BrdU^+$ cells in the SVZ coronal sections were analyzed. To define the SVZ-region, the atlas shown in **Fig. 12** was used. All $BrdU^+$ cells in this region were counted (**Fig. 13**).

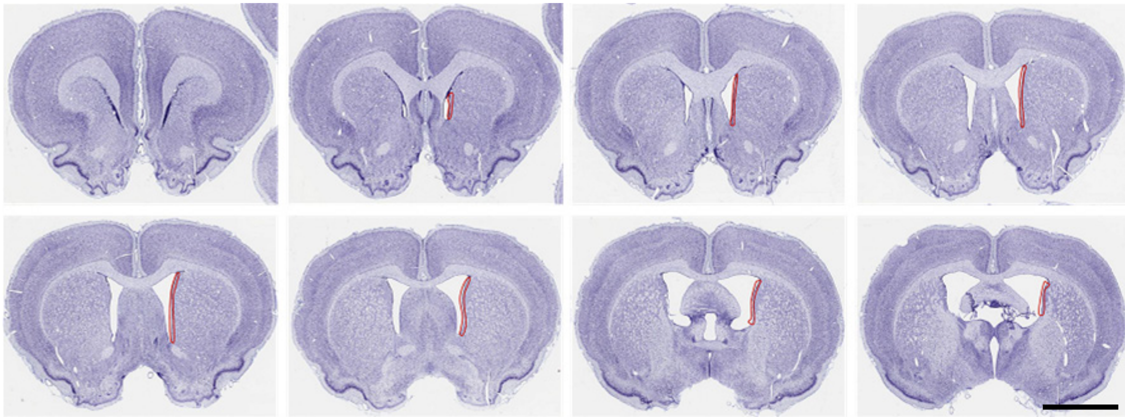


Fig. 12: Atlas of the SVZ. The SVZ is highlighted in red color. *scale bar:* 2 mm

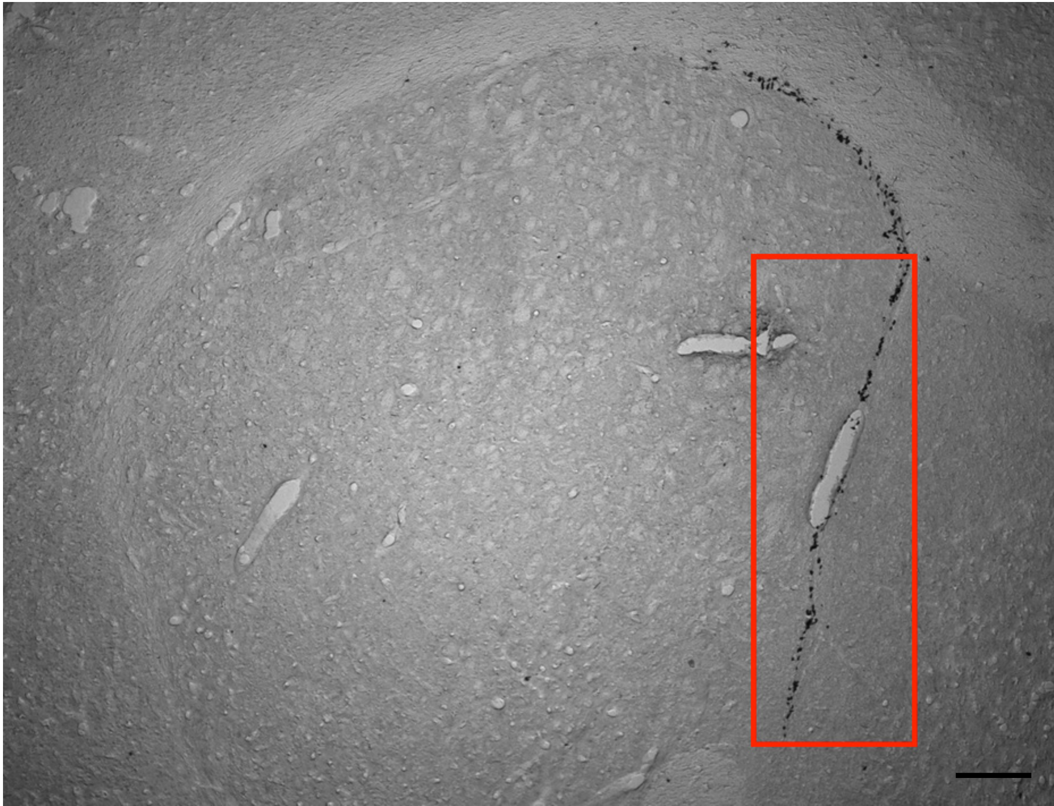


Fig. 13: SVZ in a BrdU-stained brain section of an experimental mouse. The SVZ is highlighted in the red panel. *scale bar:* 1 mm

SGZ: For counting BrdU$^+$ cells, the SGZ of the dentate gyrus of the HC was analyzed in coronal sections. Only cells in the subgranular layer, which is located at the very bottom of the granular layer, were counted. To define SVZ-region, the atlas shown in **Fig. 14** was used. **Fig. 15** shows on a BrdU-immunostained section, which cells belong to the SGZ.

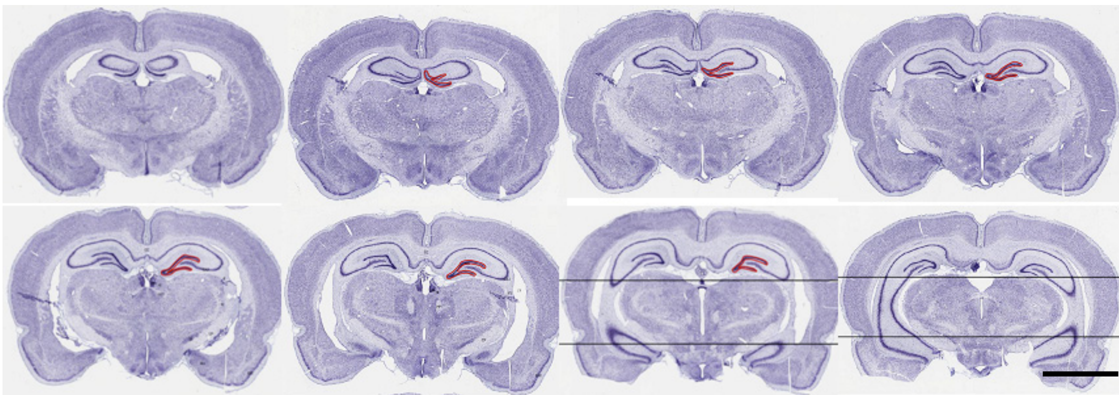


Fig. 14: Atlas of the SGZ in the dentate gyrus of the HC. The SGZ is highlighted in red color. *scale bar:* 2 mm

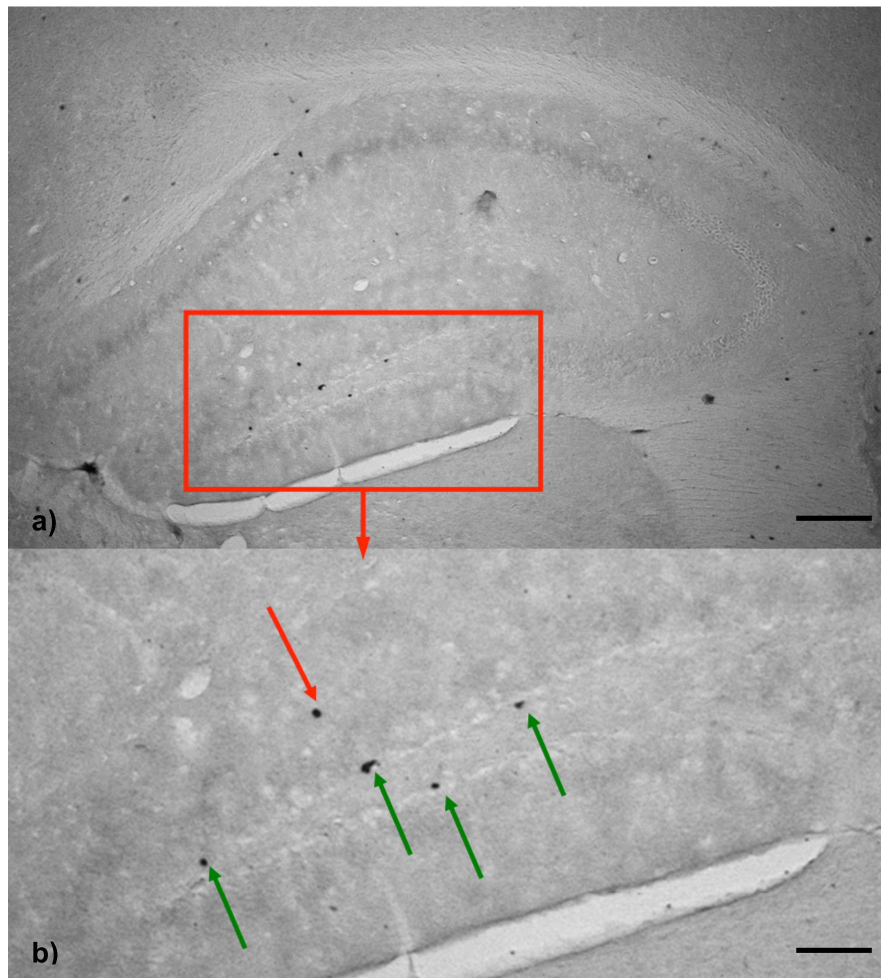


Fig. 15: Dentate gyrus of the HC in the brain slice of an experimental mouse. The green arrows point the stained cells in the subgranular layer. These were counted. The red arrows indicate a cell outside the subgranular layer (molecular layer). These were not counted. *scale bars:* a) 1 mm; b) 500 µm

<u>OB:</u> BrdU^{+} cells were counted in the granular and periglomerular region in the OB. This is illustrated in **Fig.16**.

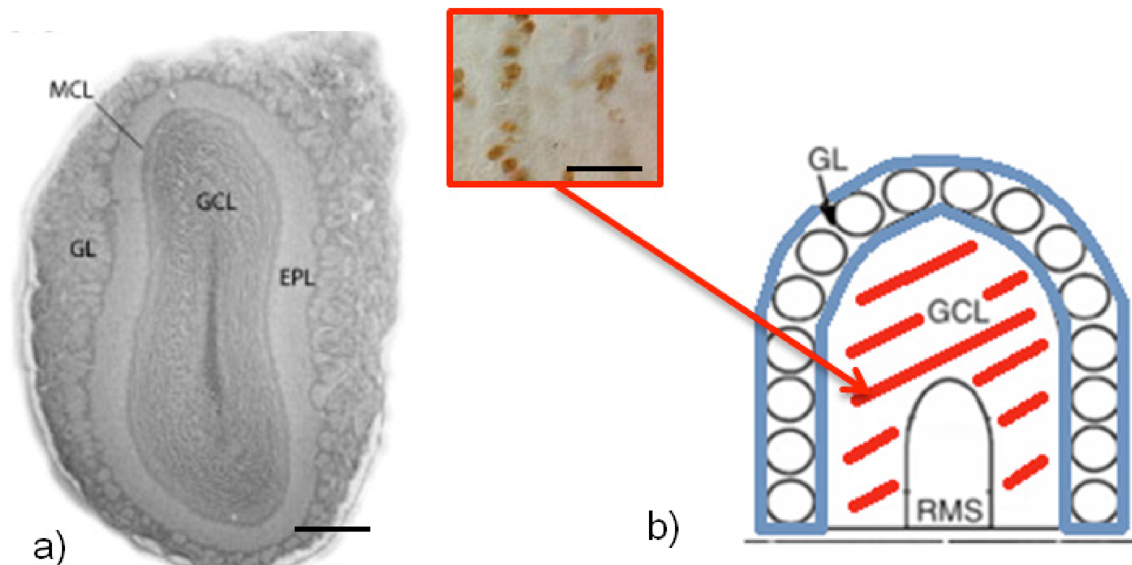


Fig. 16: Areas of quantification in the OB.

a) Overview over the OB. (GCL= Granular cell layer, GL= Glomerulus, MCL= Mitral cell layer, EPL= External plexiform layer) b) Schematic drawing of areas included into quantification of BrdU^{+} cells in the OB (RMS = rostral migratory stream, blue frame = quantification of peri-glomerular cells, red area = quantification of granular cells). Pictures modified according to (Hack et al., 2005, Saghatelyan et al., 2005) *scale bars:* a) 1 mm; b) 30 µm

5.6.2.2 Quantification of triple staining

The neuronal differentiation of newborn cells in the HC, the granule cell and peri-glomerular layer of the OB was assessed using triple immunofluorescence labelling for BrdU, NeuN and TH. The evaluation was performed on a confocal laser scanning microscope (Zeiss LSM 510, Oberkochen, Baden-Württemberg, Germany). Confocal microscopy enables the reconstruction of three-dimensional structures from the obtained images. It uses laser light to excite fluorescent dyes and generate digital microscopic images of high spatial resolution and strict separation of detection channels. This technique gives the opportunity to find out whether a newly built (BrdU^{+}) cell is a (NeuN^{+}) neuron and if it is from the dopaminergic (TH^{+}) phenotype or not. Coronary sections from the HC and the OB were analyzed.

5.7 Statistical analysis

Data are reported as the mean ± SEM. Normal parametric data were compared with a two-sided unpaired t-test calculated with IBM SPSS Statistics 20.0. A value of $p < 0.05$ was considered to be statistically significant.

6 RESULTS

6.1 Proliferation

For the evaluation of the stem cell proliferation in SVZ and SGZ, 3 months old orexin/ataxin-3 (n = 8) and WT mice (n = 8) received a single intraabdominal injection of 100 mg BrdU/kg body weight 2 h prior to sacrifice and perfusion. The BrdU-quantification was carried out with microscope using the semi-automatic stereology system.

6.1.1 Proliferation in the subgranular zone of the hippocampus

In the SGZ of the HC, the BrdU-quantification revealed a significantly higher number of newly built cells in the orexin/ataxin-3 mice (103 ± 6) than in WT mice (77 ± 5; p = 0.008) (**Fig. 17**).

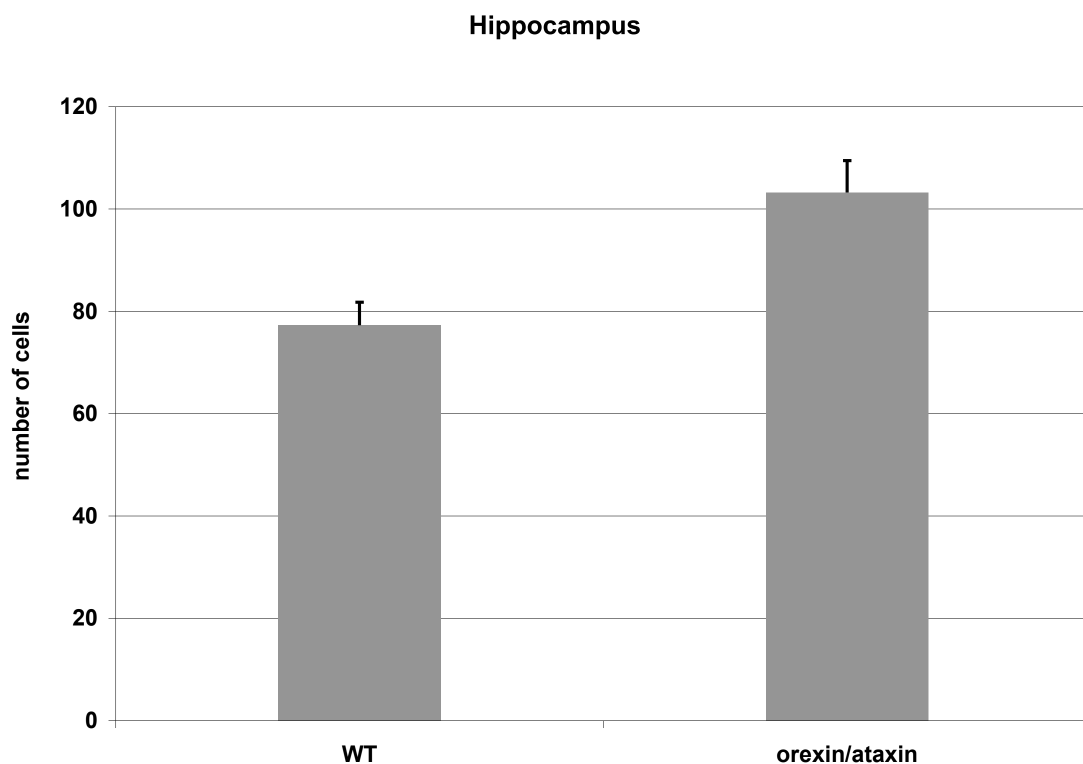


Fig. 17: Comparison of newly built $BrdU^+$ cells, labeled 2 hrs. before sacrifice, in 3 months old WT and orexin/ataxin-3 mice in the SGZ of the HC. The number of newly built cells in orexin/ataxin-3 mice was significantly higher than in WT mice (p = 0.008).

6.1.2 Proliferation in the subventricular zone

In the SVZ, the BrdU-quantification revealed a significantly higher number of new built cells in the orexin/ataxin-3 mice (5864 ± 115) than in WT mice (4951 ± 164; $p < 0.001$) **(Fig. 18)**.

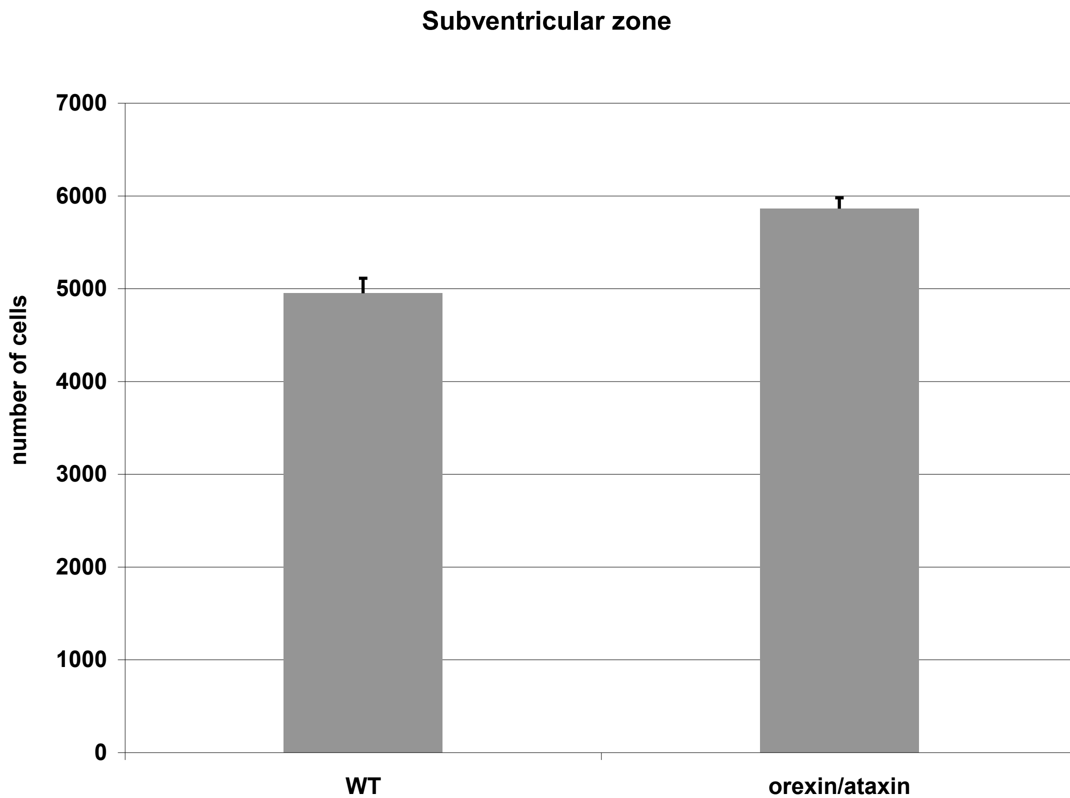


Fig. 18: Comparison of newly built BrdU$^+$ cells, labeled 2 hrs. before sacrifice, in 3 months old WT and orexin/ataxin-3 mice in the SVZ. The number of newly built cells in orexin/ataxin-3 mice was significantly higher than the number in WT mice ($p < 0.001$).

6.2 Migration

For the analysis of migrating cells in the RMS, $BrdU^+$ were counted 2 h after injection. BrdU-Quantification revealed a tendency for a higher number of newly built migrating cells in the RMS of orexin/ataxin-3 mice (817 ± 55) than in WT mice (678 ± 107), which did not reach statistical significance ($p = 0.221$) (**Fig. 19**).

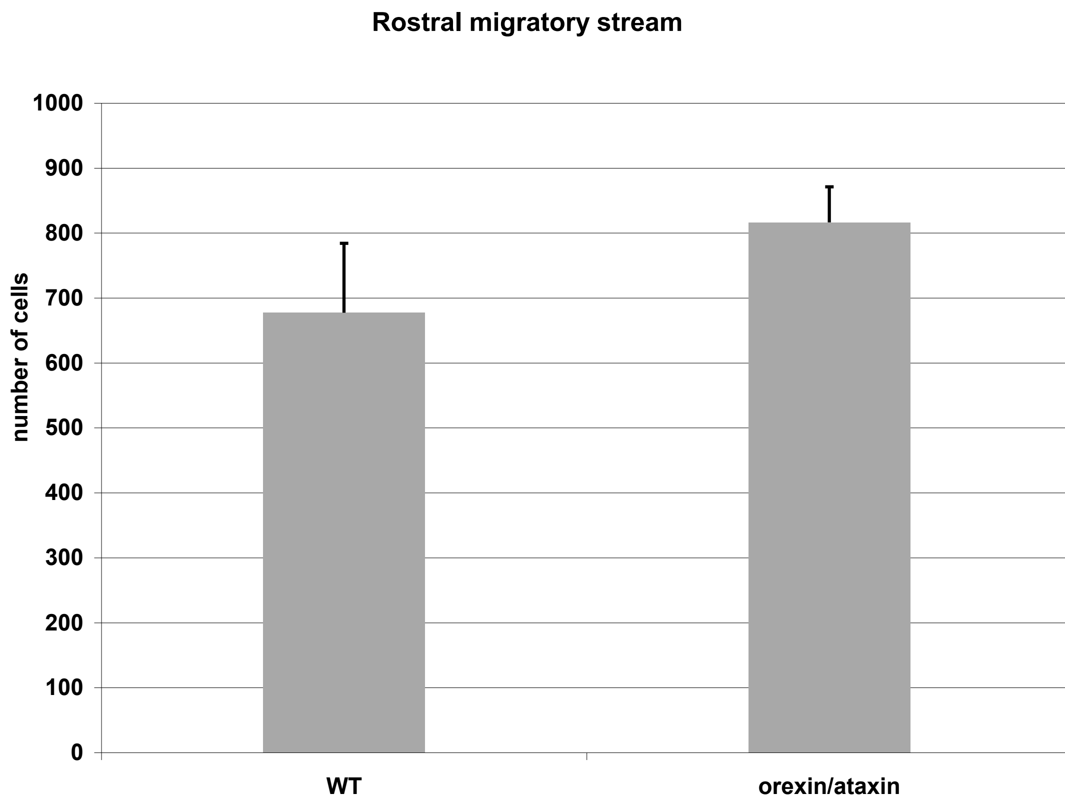


Fig. 19: Comparison of newly built $BrdU^+$ migrating cells, labeled 2 hrs. before sacrifice, in 3 months old WT and orexin/ataxin-3 mice in the RMS. The number of newly built cells in orexin/ataxin-3 mice (817 ± 55) was higher than the number in WT mice (678 ± 107), but did not reach statistical significance ($p = 0.221$).

6.3 Survival

For the illustration of survival of newly built neurons, BrdU was administered intraperitonially once per day for 5 consecutive days to 3 months old orexin/ataxin-3 (n = 8) and WT mice (n = 8). The last injection was made 30 days before the mice were sacrificed.

6.3.1 Survival in the hippocampus

Although the BrdU-quantification revealed tendency for a higher number of surviving $BrdU^+$ cells in the HC in orexin/ataxin-3 mice (399 ± 29) than in WT mice (311 ± 25), the difference was not significant (p = 0.058) (**Fig. 20**).

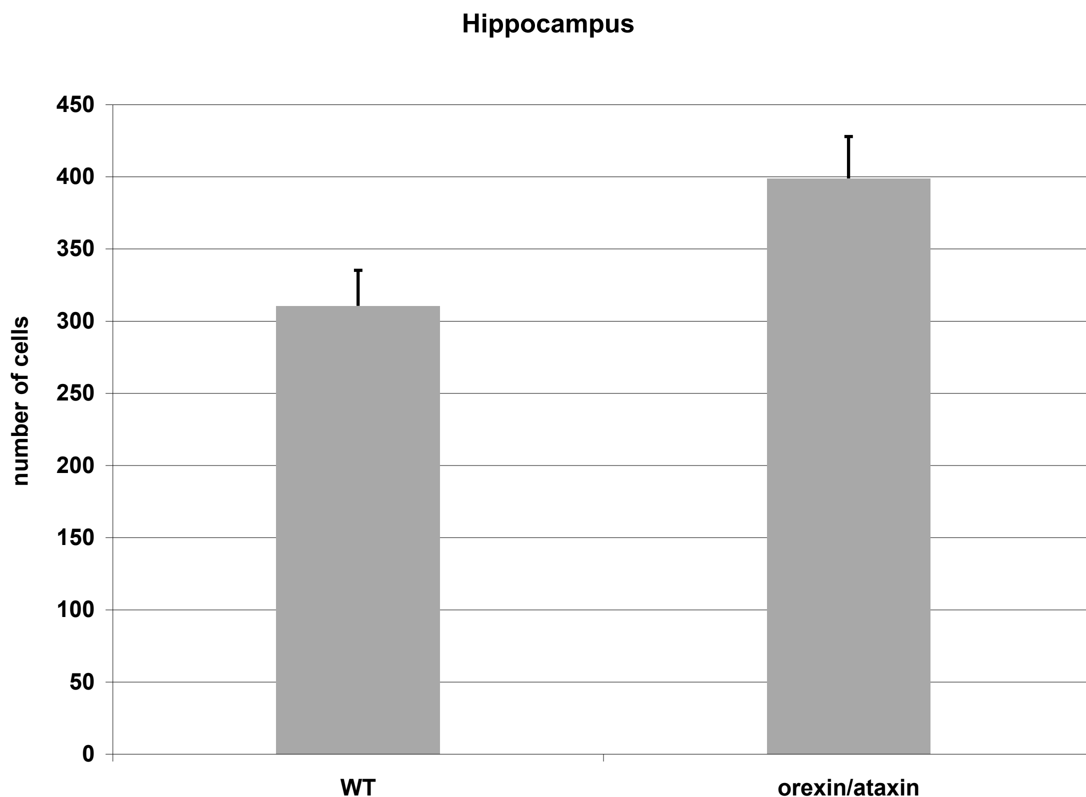


Fig. 20: Comparison of the surviving newly built $BrdU^+$ cells in the HC of WT and orexin/ataxin-3 mice, labeled at an age of 3 months on 5 consecutive days 30 days before sacrifice. The number of cells in orexin/ataxin-3 mice (399 ± 29) had a tendency for higher numbers than in WT mice (311 ± 25) without reaching significance (p = 0.058).

6.3.2 Survival in the granular zone of the olfactory bulb

In the granular zone of the OB, BrdU-quantification revealed a significantly higher number of newly built $BrdU^+$ cells surviving for 30 days in orexin/ataxin-3 mice (14716 ± 413) than in WT mice (11786 ± 385; $p < 0.001$) (**Fig. 21**).

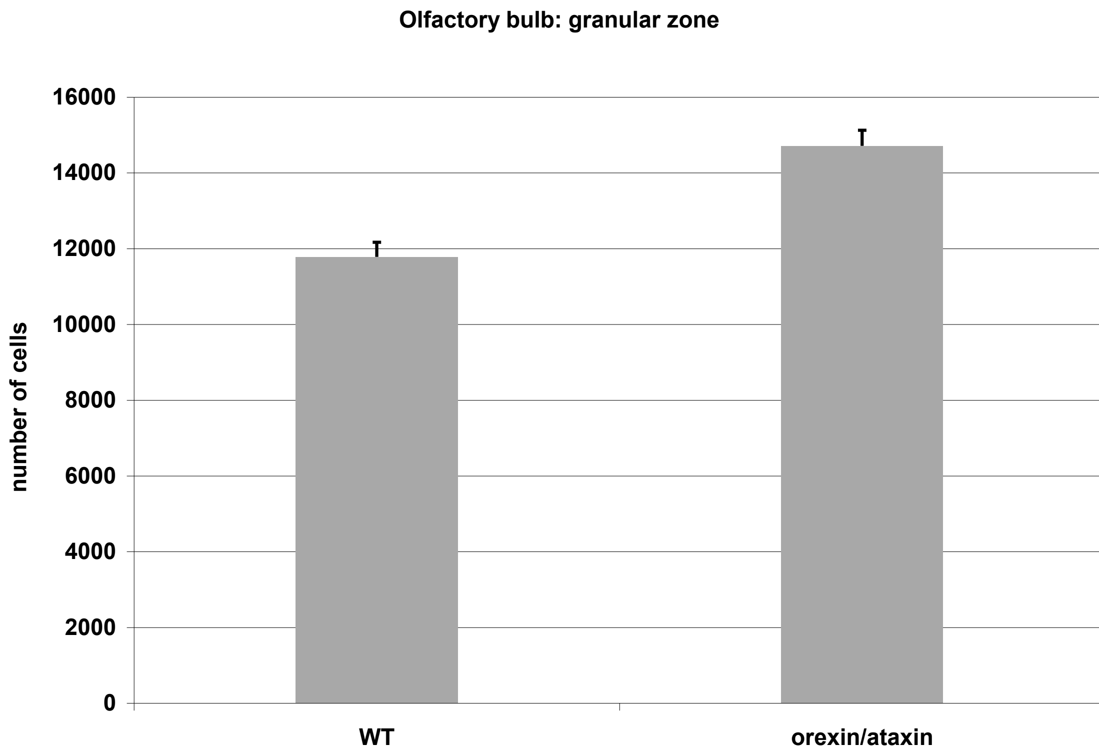


Fig. 21: Comparison of newly built $BrdU^+$ cells in the granular zone of the OB in WT and orexin/ataxin-3 mice, labeled at an age of 3 months on 5 consecutive days 30 days before sacrifice. The number of new built cells in orexin/ataxin-3 mice (14716 ± 413) was significant higher than in WT mice (11786 ± 385; $p < 0.001$).

6.3.3 Survival in the periglomerular zone of the olfactory bulb

The BrdU-quantification revealed no significant difference between the number of newly built BrdU^{+} cells surviving for 30 days in the periglomerular zone of the OB in orexin/ataxin-3 mice (2615 ± 76) and in WT mice (2617 ± 71; p = 0.894) (**Fig. 22**).

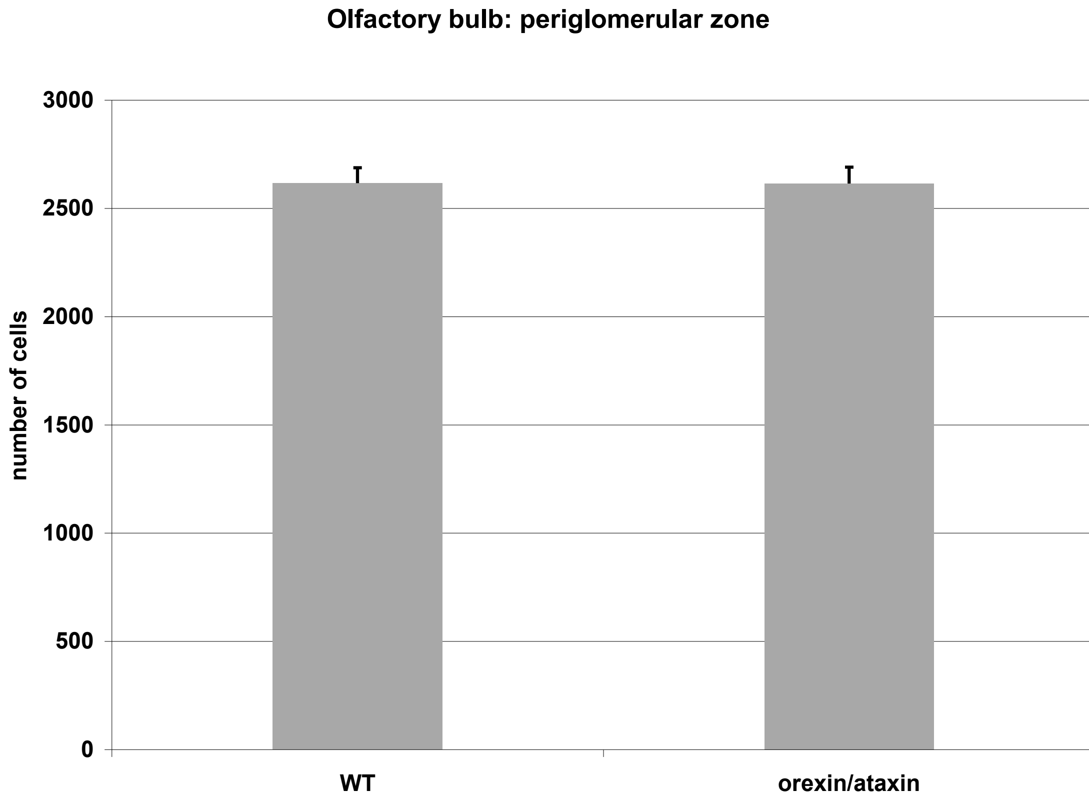


Fig. 22: Comparison of the surviving newly built BrdU^{+} cells in the periglomerular zone of the OB of WT and orexin/ataxin-3 mice mice, labeled at an age of 3 months on 5 consecutive days 30 days before sacrifice. No significant difference was found between the cell numbers in orexin/ataxin-3 mice (615 ± 76) and that in WT mice (2617 ± 71; p = 0.894).

6.4 Differentiation

Triple immunostaining (BrdU/NeuN/TH) was carried out to assess the percentage of newly built BrdU$^+$ cells, which differentiated within 30 days into neurons (NeuN$^+$) in general, or into a dopaminergic phenotype (TH$^+$) in particular.

6.4.1 Differentiation in the hippocampus

The differentiation of BrdU$^+$ cells into NeuN$^+$ neurons in the HC of orexin/ataxin-3 mice (89.50 % ± 1.12 %) was significantly higher than in the HC of WT mice (83.14 % ± 1.99 %; p = 0.020) (**Fig. 23**).

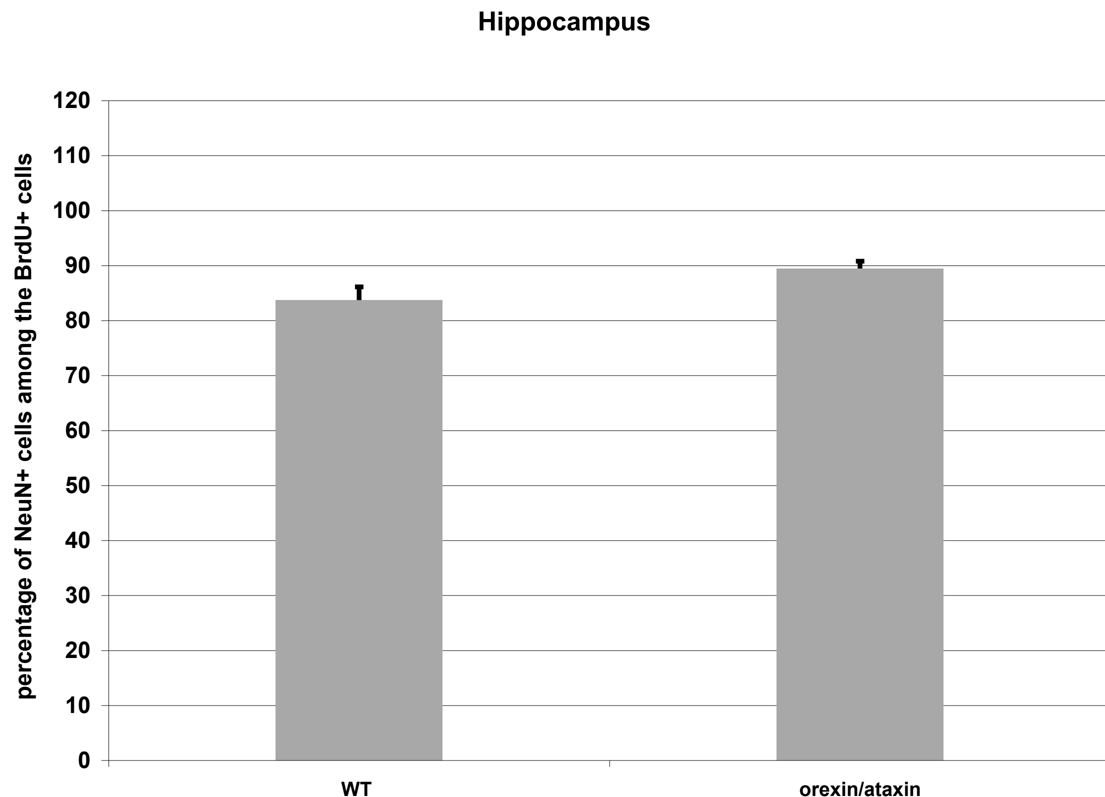


Fig. 23: Comparison of the percentage of newly built NeuN$^+$ neurons among the newborn BrdU$^+$ cells in the HC of WT and orexin/ataxin-3 mice, BrdU labeled at an age of 3 months on 5 consecutive days 30 days before sacrifice. The percentage of newly built NeuN$^+$ neurons of all newly built BrdU$^+$ cells in orexin/ataxin-3 mice (89.50 % ± 1.12 %) was significantly higher than in WT mice (83.14 % ± 1.99 %; p = 0.020).

6.4.2 Differentiation in the olfactory bulb

No significant difference could be found in the percentage of differentiated neurons ($NeuN^+$) and for the percentage of differentiated dopaminergic cells (TH^+). The quantification of BrdU labeled and BrdU/NeuN double labeled cells revealed, that the percentage of differentiated neurons of all new built cells in orexin/ataxin-3 mice (93.63 % ± 1.44 %) was not significantly higher than the proportion of WT mice (91.29 % ± 1.51 %; p = 0.445) (**Fig. 24**).

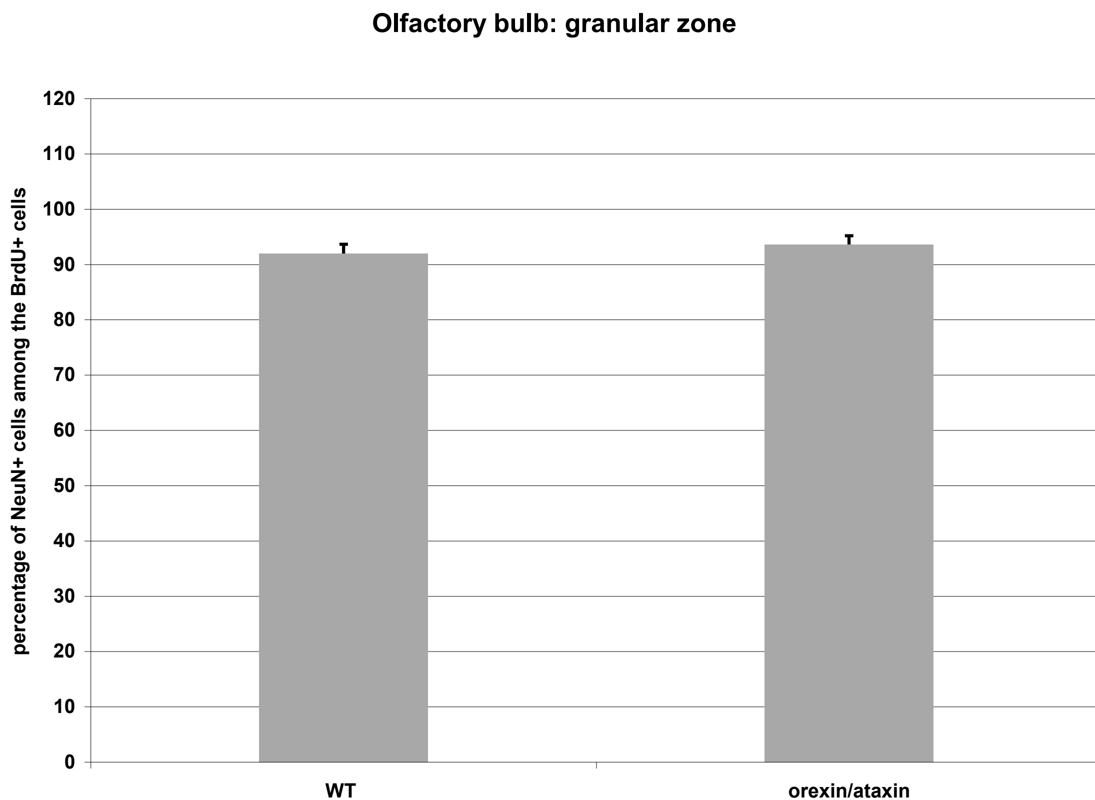


Fig. 24: Comparison of the percentage of differentiated $NeuN^+$ neurons of all newly built $BrdU^+$ cells in the granular zone of the OB in WT and orexin/ataxin-3 mice, labeled at an age of 3 months on 5 consecutive days 30 days before sacrifice. The percentage in orexin/ataxin-3 mice (93.63 % ± 1.44 %) was not significantly higher than in WT mice (91.29 % ± 1.51 %; p = 0.445).

The quantification of BrdU/TH double labeled cells revealed, that the percentage of dopaminergic cells of all newly built cells in orexin/ataxin-3 mice (86.38 % ± 1.36 %) in the periglomerular zone of the OB was not significant higher than the proportion in WT mice (81.29 % ± 1.71 %; p = 0.088) (**Fig. 25**).

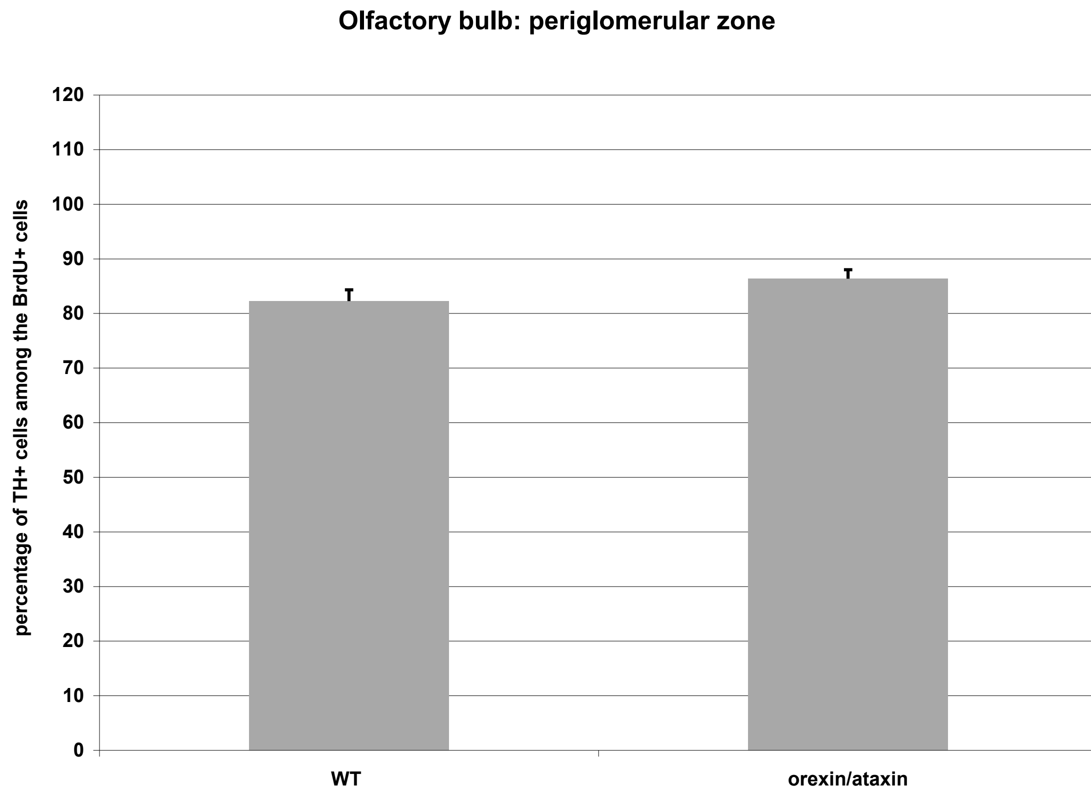


Fig. 25: Comparison of the percentage of dopaminergic TH^+ cells of all new built $BrdU^+$ cells in the periglomerular zone of the OB in WT and orexin/ataxin-3 mice, labeled at an age of 3 months on 5 consecutive days 30 days before sacrifice. The percentage in orexin/ataxin-3 mice (86.38 % ± 1.36 %) was not significantly higher than in WT mice (81.29 % ± 1.71 %; p = 0.088)

7 DISCUSSION

7.1 Summary and interpretation of the main results

In this study the regulative role of the neuropeptide orexin on adult neurogenesis was investigated. Neurogenesis in adult WT mice was compared to the orexin/ataxin-3 mouse model, showing a 99 % loss of orexin neurons at the time of investigation.

Mice of the **first group** were sacrificed 2 hours after a single BrdU injection. In this group, precursor cell proliferation in the SGZ and SVZ was significantly higher in the orexin/ataxin3 mouse. Also in the RMS, proliferation rates were slightly higher in the absence of orexin, but this difference did not reach statistical significance.

Mice of the **second group** were sacrificed 30 days after 5 consecutive daily BrdU injections to investigate survival rates of newly generated cells. A significant difference in the granular zone of the OB could be demonstrated. Again the number of newly built cells was higher in the orexin/ataxin group. However, this could not be shown for the periglomerular zone of the OB. Higher survival rates of new built cells were observed in the HC of orexin/ataxin-3 mice, closely missing statistical significance (p = 0.058). Results are limited by the low cell counts in the HC. In the HC, absolute $BrdU^+$ cell numbers range around 100 per HC cell layer, whereas in the granular zone of the OB, the $BrdU^+$ cell population ranges between 11.000-15.000 per OB cell layer.

After finding a higher proliferation and partly higher survival rates in the orexin/ataxin group, the influence of orexin on the differentiation of new built cells was investigated by triple staining. The proportion of neurons relative to the total number of new built cells in the HC was significantly higher with 90 % in the orexin/ataxin group compared to 83 % in the control group. Hence it may be concluded for the HC that in the absence of orexin a higher proportion of new built cells differentiates into neurons as the total number of new built cells was not influenced.

No significant difference (p = 0.445) in the number of newly born neurons relative to the total number of newly built cells could be found in the granular zone of the OB (94 % in orexin/ataxin-3 mice, 91 % in the WT group).

Since it is known, that almost all granule cells are GABAergic (Mugnaini et al., 1984), the proportion of dopaminergic cells was examined only for the periglomerular zone,

where a differentiation into both GABAergic and dopaminergic phenotypes is known. A higher trend of dopaminergic differentiation could be seen in the periplomerular zone of the OB of the orexin/ataxin-3 mice, but the proportion of dopaminergic cells of all new built cells in orexin/ataxin-3 mice was not significantly higher than in WT mice.

Taken these results together, it can be assumed that orexin suppresses the proliferation of the adult NSC, affects the survival rate in the OB negatively and hinders the differentiation of new built cells into neurons in the HC.

7.2 Discussion of methodological correctness

7.2.1 Study size

The biometric experimental design of this study is based on the experience of our group in this area (Hoglinger et al., 2004) and on the need to collect statistically relevant data. In both groups we studied n = 8 mice. Although an exclusion rate of 15 % was calculated previously in the given number, all mice could be successfully evaluated and included into analysis. With the given study size significant results turned out for the proliferation rate in the SVZ and SGZ, for the survival rate in the granular zone of the OB and for the differentiation in the HC. In the case of the survival rate in the periglomerular zone of the OB and the differentiation in the OB the study size was sufficient to identify differences, but too small to make clear statements for all of these parameters. For this purpose the study should be repeated with a larger population.

7.2.2 Suitability of the orexin/ataxin-3 mouse model

Is the orexin/ataxin-3 mouse a suitable model to investigate the influence of orexin on adult neurogenesis? Advantages and disadvantages compared to other mouse models will be addressed in the following section.

Advantages: The selected orexin/ataxin-3 mouse model does not require subsequent interventions, such as externally applied chemical or mechanical ablation of orexinergic neurons. Postnatal interventions often lead to a greater source of error than genetically modified mouse models. Consequently comparability to WT mice is often limited.
Compared to the knock-out mouse a decisive advantage exists for the orexin/ataxin-3 mouse model, which is due to the different development of the mice. In the knock-out mouse the entire embryonic brain development occurs without orexin. Thus, this organism may adapt to the lack of orexin and uncontrolled compensatory mechanisms may

emerge. Probably, this will not be without consequences for the regulatory mechanisms of the later adult neurogenesis.
Another thinkable way of determining the role of orexin on the adult neurogenesis is the use of an orexin receptor antagonist. However, if the orexin-effect is switched off by the use of antagonists, a compensatory up-regulation of the receptor numbers is likely to occur. Thus, this model would be less reliable in comparison.

Disadvantages: In contrast to the knock-out mouse, the orexin-ataxin-3 mouse loses all its orexin-containing neurons. Beside orexin these neurons produce galanin, angiotensin and the endogenous opiate dynorphin (Chou et al., 2001). Even if these peptides are still produced by other cells in the brain (Kaplan et al., 1988), their quantity and site of action is changed, which might have unknown effects neuronal homeostasis. Controversially to orexin, a promotional effect on neuronal differentiation in murine subventricular zone cell cultures could be shown for galanin (Agasse et al., 2013). It has been shown, that an increased concentrations of angiotensin in the systemic circulation during exercise may promote neurogenesis in the adult rat HC (Mukuda et al., 2014). Currently there are no scientific studies of dynorphin and adult neurogenesis. Thus it is not excluded that the reduced occurrence of galanin, angiotensin or dynorphin may bias the present results.
Since progressive loss of orexin neurons is due to apoptosis induced by a toxic gene product, the increased proliferation might also be a result of a general inflammatory reaction caused by the apoptotic process. This issue will be addressed further below.

7.2.3 Reliability of BrdU to label newborn cells

At present, essentially all in vivo studies on adult neurogenesis rely on the BrdU method for quantification of newly built cells. BrdU as a synthetic analogue of thymidine, is incorporated in the cellular DNA of proliferating cells in the S phase of mitosis and can be subsequently detected by immunohistochemistry. For detection of adult neurogenesis BrdU is considered as the gold standard (Vega and Peterson, 2005). The most critical aspect of using BrdU as a proliferation marker is the suspicion that it labels not only dividing cells but also marks dying or damaged cells. Cooper and Kuhn could show that BrdU may be incorporated not only in dividing cells, but also in any cell effecting DNA synthesis, including DNA repair. Several experimental data argue against the concern that the concentration, in which BrdU is commonly used, may be sufficient to detect cells undergoing DNA repair (Cooper-Kuhn and Kuhn, 2002). The group around

Cooper-Kuhn combined BrdU and labeling methods for apoptosis to argue against DNA repair being a major contribution of $BrdU^+$ cells. They could show that apoptotic cell death, which is accompanied by activation of DNA repair mechanisms, is not labeled by BrdU incorporation.

However, subsequent investigations showed contradictive results confirming that BrdU may label apoptotic cells as well (Hoglinger et al., 2007). Consequently, the number of apoptotic cells should be co-examined in the investigated areas, to ensure that the rate of apoptotic cells is negligible in relation to the newborn cells.

As an alternative method integration of a retroviral genome into the chromosomal DNA is used to detect cell proliferation. The stable integration of the retroviral genome into the chromosomal DNA can only occur after nuclear membrane breakdown. Expression of viral genes in infected cells is therefore only present in cells that have undergone mitosis. Retroviral labeling, therefore, distinguishes between cell division and DNA repair. In contrast to the BrdU labeling, the viral DNA gets transferred reliably to each daughter cell. But several disadvantages make retroviral infection less suitable for in vivo applications. Retrovirus particles have to be directly applied to the CNS region of interest, since the blood–brain barrier is an impermeable obstacle. Consequently, lesion-induced effects on neurogenesis have to be taken into account when studying stem cell activity in the intact brain. In addition, the method is more technically demanding and costly than BrdU labeling. Nevertheless it is a secure method to confirm results obtained with BrdU labeling (Cooper-Kuhn and Kuhn, 2002).

7.2.4 The first paradigm – proliferation of progenitor cells

For the evaluation of the stem cell proliferation in SVZ and SGZ, orexin/ataxin-3 mice and WT mice received a single intraperitoneal injection of 100 mg BrdU/kg body weight 2 hour prior to sacrifice. A crucial variable in the analysis of BrdU labeling studies is the time the tracer is injected in relation to the time of scarification (Prickaerts et al., 2004). In the first paradigm the selected moment for sacrifice after BrdU injection attempts to ensure that only proliferating progenitor cells are labeled. It has been shown, that proliferation should be measured using a protocol in which subjects are treated with a tracer and killed maximally within 24 h after injection (Hayes and Nowakowski, 2002). This is due to the fact, that BrdU has a bioavailable time for incorporation of only about 30–60 min, thus immediately after injection almost all S-phase cells are labeled

(Boswald et al., 1990). Each newly made cells can then either remain in proliferation mode and continue to divide or can migrate out of the proliferation zone and either differentiate or die. It is known, that in the days after a single BrdU administration, the numbers of $BrdU^+$ cells increase peaking at around 1 week post-injection (Gould et al., 1999). The total number of cells counted at any time point, except for 1–2 h after the label injection, is a summation of proliferation at the time of injection plus proliferation on each day after injection less the death rate of the newly made cells.
The selected dose of 100 mg BrdU/kg body weight has been chosen because BrdU doses of 50–100 mg/kg BrdU body weight are sufficient to label the vast majority of S-phase proliferative cells in mice (Burns and Kuan, 2005). It has been reported that single doses of BrdU 100, 50 and 25 mg/kg body weight labels 60 %, 45 % and 8 % of S-phase cells in adult rat DG, respectively. Doses greater than or equal to 300 mg/kg body weight are needed to label most S-phase cells, as the number of BrdU-labeled cells appeared to plateau (Cameron and McKay, 2001). Nevertheless BrdU is a toxic substance at much higher doses. It has been that the chosen dose of 100 mg/kg body weight is sufficient and safe to use in rodent for studying adult neurogenesis without inducing relevant toxic effects (Sekerkova et al., 2004).

7.2.5 The second paradigm – survival and differentiation of progenitor cells

For the analysis of differentiation and survival of newly built neurons, BrdU was administered intraperitoneally on 5 consecutive days once per day to each mouse. The mice were sacrificed 30 days after the last injection. The neuronal differentiation of newborn cells in the HC, the granular and periglomerular zone of the OB was assessed using triple immunofluorescence labeling for BrdU, NeuN and TH. Choosing the right time after the administration of BrdU is fundamentally important to draw a reliable conclusion for survival and differentiation rates of newly formed cells. It is apparent from quantitative studies that approximately 50 % of newly formed cells die within the first two weeks of life in both neurogenic zones (Petreanu and Alvarez-Buylla, 2002).
For the region of the HC, one study indicates that the quantification of $BrdU^+$ cells permits a reliable estimation of survival rate four weeks after administration of the proliferation marker (Kempermann et al., 2003). According to these results, the second paradigm is reliable to mark the survival rate of newly formed cells in the HC.

Moreover Winner and colleges (2002) examined the long-term effects of adult neurogenesis in the OB. These data show that a peak of $BrdU^+$ cells was reached in the olfactory bulb 1 month after BrdU injection, when all new cells have finished migrating from the ventricle wall. However, the number of $BrdU^+$ cells in the region of the OB no longer significantly changed from the end of the third month up to a period of 19 months after administration of BrdU (Winner et al., 2002). Moreover, dopaminergic differentiation in the periglomerular zone was first visualized one month after BrdU injection, however the peak of dopaminergic differentiation occurred three month after BrdU injection. Thus, the influence of orexin on the dopaminergic differentiation could be examined more reliable after three months. In the present study dopaminergic differentiation was investigated after one month. Thus, the rate of dopaminergic differentiation may have been underestimated and the differences between groups were not yet visible. For investigations in the OB the chosen date of sacrifice gives the advantage that due to the peak of new built cells after one month it is statistically more likely to measure a difference between the two experimental groups. This also applies to the neuronal differentiation, which reaches its peak at the same time (Winner et al., 2002). The set time point of investigation yields the disadvantages that no prediction can be made for long-term-survival of new built cells. For that propose, the examination was carried out to early.

Furthermore it should be taken to account, that the dying of progenitor cells was not analyzed in this work. It is known, that about 50 % of proliferating progenitor cells die. Especially young and immature cells are under pressure of elimination by programmed cell death (Biebl et al., 2000). It is therefore useful to measure apoptosis in order to determine to what extent changes in survival reflect differences in apoptotic rates (Young et al., 1999). Biebl and colleges analyzed programmed cell death using the TUNEL-assay, which labels fragmented DNA with tagged nucleotides. They conclude that programmed cell death may have an important regulatory function by eliminating supernumerous cells from neurogenic regions and may thus contribute to a self-renewal mechanism in the adult mammalian brain (Biebl et al., 2000).

7.3 Effect of orexin depletion in neurogenic regions

In the absence of orexin in the adult mouse brain, the proliferation of adult NSC was increased, the survival rate was significantly increased in the granular zone of the OB and a consistent not significant trend was seen in the HC.

Regarding the results of this study so far it is not possible to judge whether the influence of orexin on neurogenesis is due to either direct effects on neural adult stem cells or indirect effects by interacting with other neurotransmitters. In addition, the orexin/ataxin-3 mouse model mimics a neurodegenerative process. Therefore, changes of adult neurogenesis could be caused by mediators of neuroinflammation.

7.3.1 Direct effects of orexin on progenitor cells

Since a high density of orexin afferents in the SVZ has been shown previously (Alvarez-Buylla and Lim, 2004, Lledo et al., 2006) and a direct, topographically organized innervation of the SVZ and SGZ by orexin neurons and orexin receptor expression on adult NSC has been shown by Arias-Carrión (pictures above) a direct effect of orexin on the progenitor cells within the SGZ of the HC and SVZ appears to be likely.

7.3.2 Indirect effects of orexin on progenitor cells

Orexin cells widely project to various neurotransmitter systems, which also evidently influence adult neurogenesis. Therefore, indirect ways of orexinergic effects on neurogenesis have to be taken into account. Orexin fibers project to the brain stem, specifically to the noradrenergic locus coeruleus, the serotonergic raphe nuclei and the dopaminergic substantia nigra (Peyron et al., 1998). The dopaminergic VTA receives an input from orexin neurons (Korotkova et al., 2003). Orexin generally exerts excitatory actions (Nishino and Sakurai, 2006).

Extensive **noradrenergic** projections reach the dentate gyrus from the locus coeruleus (Loy et al., 1980). Previous results have indicated, that depletion of noradrenaline decreases the proliferation, but does not influence the survival or differentiation of adult hippocampal granule cell progenitors (Kulkarni et al., 2002). By identifying these relationships it becomes clear that adult neurogenesis in the HC may actually be enhanced by the excitatory effect of orexin on noradrenergic neurons.

Serotonergic afferents project from the raphe nuclei in the brain stem to the SGZ and serotoninergic input was found to increase adult neurogenesis in the HC and the SVZ (Brezun and Daszuta, 1999). Therefore, another indirect way of orexin on neurogenesis can be derived via serotonin. Orexin neurons send excitatory projections to serotonergic neurons in the raphe nuclei (Hagan et al., 1999, Brown et al., 2001). Consequently, one would expect via strengthening the action of serotonin, analogous to noradrenaline, a proliferative indirect effect by orexin.

Experimental depletion of **dopamine** in rodents decreases precursor cell proliferation in both the subependymal zone and the subgranular zone. Experiments with neural precursors from the adult subependymal zone grown as neurosphere cultures confirm that activation of D2-like receptors directly increases the proliferation of these precursors (Hoglinger et al., 2004). Orexin directly activates VTA dopaminergic neurons (Korotkova et al., 2003), this might enhance the proliferative effect of dopamine on precursor cells.

An indirect effect of orexin via the serotonergic and dopaminergic projections is not likely with the results of this study. However, an indirect effect via noradrenaline on precursor cell proliferation seems likely. Considering the complexity of the brain, additional indirect effects of orexin on adult neurogenesis by interaction of various neurotransmitters seem to be possible. Further studies are needed to give evidence for these mechanisms. For example, a study design with an experimental depletion of the noradrenergic, dopaminergic or serotonergic system in the orexin/ataxin-3 mice would be an interesting approach.

7.3.3 Role of inflammatory mediators on adult neurogenesis

The orexin/ataxin-3 mouse mimics a neurodegenerative condition. Since progressive loss of orexin neurons is due to apoptosis induced by a toxic gene product, the increased proliferation might also be a result of the general inflammatory reaction caused by the apoptotic process.

It could be shown for example that stroke caused by transient middle cerebral artery occlusion in adult rats, leads to a marked increase of cell proliferation in the SVZ (Arvidsson et al., 2002). In another study, SVZ neurospheres were maintained in the presence of diffusible signals derived from the non-neurogenic cerebral cortex either previously treated with the apoptosis inducer staurosporine or not. Increased cell prolif-

eration could be shown in the SVZ neurosphere which was maintained in the presence of factors derived from cortex with apoptotic damage (Agasse et al., 2004). One important component in these pathological environments is the process of inflammation (Kerschensteiner et al., 2009). With its diversity of cell types and activation states, the inflammatory reaction could be everything from detrimental to supportive for adult neurogenesis (Simard and Rivest, 2004). A major player is the innate microglia. Microglia activation, as an indicator of inflammation, is not pro- or antineurogenic per se but the net outcome is dependent on the balance between secreted molecules with pro- and antiinflammatory action (Ekdahl et al., 2009). It has been shown that transgenic overproduction of interleukin-6 by astroglia is sufficient to decrease overall neurogenesis by 63 % in the hippocampal dentate gyrus of young adult mice (Vallieres et al., 2002). Moreover, it could be demonstrated that lipopolysaccharide administration inhibits neurogenesis in the HC, which can be restored by a non-selective cyclooxygenase inhibitor (Monje et al., 2003). On the other hand, other inflammatory molecules have a pleiotropic role in neurogenesis, at least for neural progenitor cells prepared from embryonic rat HC (Harada et al., 2004). Thus, the innate immune response in the brain is able to positively or negatively modulate neurogenesis in the CNS depending on the cytokines and growth factors that are predominantly expressed in the cellular environment.

In order to assess, the impact of the apoptotic process on the present results the experimental setup has to be considered. In the used orexin/ataxin-3 mouse model a 99 % loss of orexin cells was observed after 12 weeks and any orexin cell in the lateral hypothalamic region could hardly be detected after 15 weeks (Hara et al., 2001). In the present study BrdU was injected when mice were aged between 96 and 127 days, at the chosen time the major part of the apoptotic process of orexin neurons was already over. However, differences in adult neurogenesis may be biased by a still ongoing inflammatory apoptotic process, which has not been controlled for.

7.4 Influence on differentiation in the olfactory bulb and in the hippocampus

Given that multipotent precursor cells can give rise to neuronal and glial cell types in a characteristic order of birth, it is clear that precursor cell proliferation must be precisely regulated. Several intrinsic factors expressed by stem cells and progenitors control both proliferation rates and the fate of newborn cells. In addition, external factors such as neurotransmitters, hormones and growth factors might cooperate with intrinsic programmes to modulate these processes (Lledo et al., 2006). In order to understand the regulatory mechanism underlying neuronal differentiation, it is important to determine the types of neural inputs on adult progenitor cells and their physiological effects.

By triple staining it could be demonstrated that neuronal differentiation in the HC was significantly higher with 90 % in the orexin-ataxin group compared to 83 % in the control group. Since there is a significantly higher neuronal differentiation in the absence of orexin, one can assume an inhibitory effect of orexin on neuronal differentiation. It seems reasonable to proof whether orexinergic neurons act directly on adult hippocampal progenitor cells. Little information exists regarding the neural connections between orexin cells and the hippocampal network. As mentioned above, a direct innervation of the SGZ in the HC has been indicated by our group (unpublished data see above). By immunofluorescence labeling for orexin A and the OX_2R the co-localization of orexin neurons and nestin-GFP labeled adult NSC or proliferating progenitor cells could be illustrated using a confocal laser scanning microscope.

So far research in the field of adult neurogenesis primarily focused on exogenous factors influencing proliferation, migration and survival. Little information exist for differentiation of new born cells in the HC. The best evidence as exogenous regulating neurotransmitter for the neuronal differentiation in the HC exists for GABA. It has been demonstrated, that GABAergic inputs to hippocampal progenitor cells promote activity-dependent neuronal differentiation. More precisely, excitation of adult hippocampal progenitor cells by activated GABA receptors initiated a calcium influx. This subsequently induced the accumulation of the neurogenic transcription factor, NeuroD. Indeed, NeuroD is required for differentiation of the granule cells in the dentate gyrus of the hippocampus (Liu et al., 2000). Moreover, a BrdU-pulse labeling study with GABA receptor agonists demonstrated the promotion of neuronal differentiation via GABAergic excitation (Tozuka et al., 2005). As there is no evidence for connections between

orexin and GABA, orexinergic influence on neuronal differentiation in the HC via GABAergic interneurons remains speculative.

In the granular zone and in the periglomerular zone of the OB over 90 % of the total amount of new built cells differentiated into neurons in both groups. There was no significant difference. The rate of dopaminergic differentiation in the periglomerular zone of the OB of the orexin/ataxin-3 mice compared to WT mice was also not significantly changed.

With regard to the regulation of neuronal and dopaminergic differentiation in SVZ, the given data are still sparse. It is assumed that, SVZ progenitors give rise to different OB interneuron types in accordance to their position along the ventricles (Merkle et al., 2007). However, it is still debated to which extent this co-relation between progenitor position and neuronal type generated is due to an internal program or distinct environmental factors impinging onto progenitors (Sequerra et al., 2013). Although we have some information on the internal programs of different interneuron subtypes and about their site and time of origin, little is known about external factors. It is already known, that SVZ neurogenesis is regulated by the olfactory experience of animals (Lledo et al., 2006). Moreover, development of OB dopaminergic phenotypic expression specifically required particular patterned activity from olfactory receptor cells (Baker and Farbman, 1993, Puche and Shipley, 1999). In the field of neurotransmitters, it could be already shown, that dopamine depletion impairs precursor cell proliferation (Baker et al., 2004, Hoglinger et al., 2004), but there is no evidence about influence on neuronal or dopaminergic differentiation.

First descriptions about the distribution of orexin fibers found less prominent orexinergic projections in the OB (Peyron et al., 1998). Our group could also illustrate orexin fibers and receptors in the SVZ, RMS and in the OB.

The present results indicate that in the absence of orexin the rate of dopaminergic differentiation is not changed. Still the orexinergic innervation of the SVZ, RMS and OB is evident. Understanding the mechanisms underlying the development of dopaminergic cells is relevant to the research of restorative therapy for Parkinson's disease (Baker et al., 2001). To prove the role of orexin in neuronal and dopaminergic differentiation further studies are needed.

7.5 Functional relevance of orexin on adult neurogenesis

To discuss the role of orexin for adult neurogenesis it is important to assess the meaning, possible benefit and biological relevance of new built cells. The common perception is that new neurons in the adult brain would be beneficial. However, during evolution, the amount of adult neurogenesis decreases with increasing brain complexity. Whereas lower vertebrates, such as lizards, can regenerate entire brain parts, neurogenesis in adult mammals is restricted to a few regions (Kempermann et al., 2004b). Thus, not only quantitative proliferation rates, but mainly functional integration of new built cells into existing brain circuitries appears to be relevant. Functional integration is considered to be a result of increased proliferation rate and a consequent impact on the behavioral or cognitive level. This can be addressed experimentally in form of behavioral experiments. An example to prove the functional integration of new built cells in the HC is shown by Kempermann and Gage (2002). They found a significant correlation between the number of new neurons generated in the dentate gyrus and parameters describing the acquisition of new memories in the water maze task. In order to evaluate the functional integration of the newly built cells in the ataxin/orexin-3 mouse, behavioral experiments of this kind have been conducted in a parallel study, the results of which are still under analysis.

7.5.1 The influence of orexin on adult neurogenesis and sleep regulation

Sleep is required for memory consolidation (Walker and Stickgold, 2004) and disturbed sleep results in cognitive deficits (Muzur et al., 2002). Sleep deprivation decreases adult neurogenesis (Guzman-Marin et al., 2005, Mirescu et al., 2006). Four days of selective REM sleep deprivation contributes to reduction of neurogenesis in the hippocampal dentate gyrus. Cell proliferation was reduced, when animals were woken up automatically when the EEG indicated REM sleep. These findings support a hypothesis that REM sleep-associated processes facilitate proliferation of granule cells in the adult hippocampal dentate gyrus (Guzman-Marin et al., 2008). Rapid transitions into REM sleep are seen in narcoleptics. This phenomenon occurs because, the weakened arousal influence and increased activity of the extended VLPO in the absence of an excitatory orexin input would allow earlier and more frequent transitions to the REM state (Saper et al., 2001). As the lack of orexin is associated with frequently occurring episodes of REM sleep it can be considered that proliferation of neural progenitors may be increased by yet unidentified mechanisms associated with this sleep stage.

Sleep displays a clear circadian or daily rhythm that is governed by an endogenous biological clock located in the suprachiasmatic nucleus of the hypothalamus (Mistlberger et al., 1983). Therefore, if sleep plays a direct role in adult neurogenesis, a daily rhythm in cell proliferation and expression of neurogenic markers that parallels the sleep–wake rhythm might be expected (Meerlo et al., 2009). Under the inhibitory influence of VLPO cells with a defined circadian rhythm it may be assumed that orexin neurons and thus adult neurogenesis show a circadian activity pattern as well. Consequently, one would expect increased neurogenesis at nighttime in phases of low orexin levels. A number of studies have dealt with the question whether a daily rhythm exists in hippocampal cell proliferation. Two studies have reported that under baseline conditions the number of proliferating cells in the SGZ of the dentate gyrus is independent of the time of day (Kochman et al., 2006, van der Borght et al., 2006). However, another study showing that restricted access to a running wheel for 3 h at different times of day increased cell proliferation, cell survival, and the total number of new neurons only in animals that had wheel access during the middle of the active phase (Holmes et al., 2004). These data suggest that the influence of exercise on cell proliferation and neurogenesis is modulated by both circadian phase and the amount of daily exercise. The question whether adult neurogenesis is under the influence of a circadian rhythm remains open. However, with regard to the effects of orexin on adult neurogenesis and sleep new research opportunities arise.

7.5.2 Implications of the current study results on clinical treatment with orexin antagonists

As mentioned above orexin plays a critical role in the maintenance of wakefulness by regulating function of monoaminergic and cholinergic neurons that are implicated in the regulation of sleep-wake-cycle. These findings have encouraged the pharmaceutical industry to develop drugs targeting orexin receptors as novel medications of sleep disorders, such as insomnia.

Suvorexant (Merck, Germany) is a potent non-selective (dual) antagonist for both orexin receptors (Cox et al., 2010). It has been shown, that patients taking the drug fell asleep faster and slept longer than those on placebo (Hopkins, 2012). It was approved for sale by the U.S. Food & Drug Administration in August 2014 for the treatment of primary insomnia. Thus, the discovery of the critical role of orexin in the regulation of sleep/wakefulness has opened the door for a new era of sleep medicine (Mieda and

Sakurai, 2013). Theoretical side effects can be considered with regard to the present results of the influence of orexin on the adult neurogenesis (see below).

Recent studies have shown that orexin is involved into modulation of motivated behavior for drugs of abuse as well as natural rewards. In self-administration studies, the orexin 1 receptor antagonist SB-334867 attenuated operant responding for high-fat pellets, sucrose pellets and ethanol, but not cocaine. Demonstrating that signaling at orexin receptors is necessary for reinforcement of specific rewards (Cason et al., 2010). Thus, it is expected that in the future, orexin antagonists may also be used in the therapy of addiction.

With regard to the present experiments with rodents, the influence of orexin on adult neurogenesis might also be relevant in humans. Our results indicate that increased adult neurogenesis could occur in patients taking continuously orexin antagonists. Considering the functions of adult neurogenesis described above, patients with short- to long-term treatment should be examined on whether they are less prone to depressive symptoms, develop a better sense of smell or improve pre-existing memory difficulties.

7.6 Outlook

The results of this study suggest a suppressive action of orexin on adult neural stem cell proliferation. It remains unclear if orexin acts directly on the progenitor cells within the HC and SVZ or indirectly through other neurotransmitters and their systems (e.g. noradrenalin, serotonin and dopamine). In addition, the influence via inflammatory mediators seems to be possible. Further studies have to be initiated to clarify the nature of this interaction.

Further research is needed to substantiate the supposed antiproliferative effect of orexin. To prove the hypothesis the effect of orexin on adult neurogenesis could be examined in a dose-dependent manner. Orexin/ataxin-3 mice should be investigated at different time points from birth on up to 12 weeks, when already 99 % of orexin neurons are lost. With increasing reduction of orexin neurons, a concomitant increase in adult neurogenesis could be expected. In an additional experiment orexin/ataxin-3 mice may be treated by administration of orexin agonists. If the anti-proliferative effect disappears, it is very likely that the effect is caused by orexin. A parallel study with orexin

knock out mice (unpublished data) might additionally support the evidence of an anti-proliferative effect of orexin.

Furthermore, it should be investigated whether the anti-proliferative effect of orexin has functional relevance on memory and behavior of the affected animals. Results of behavioral experiments with orexin/ataxin-3 mice in a parallel study might clarify this point. The relationship between orexin-mediated effects on sleep and the circadian rhythm on adult neurogenesis appears to be another interesting aspect of future research. The influence of orexin on the balance of newly born and dying progenitor cells should be also included in following studies. A study design with an experimental depletion of noradrenergic, serotonergic or dopaminergic systems in the orexin/ataxin-3 mice would be an interesting approach to make a statement about the significance of indirect effects mediated via these neurotransmitters.

7.7 Final summary

We found a significantly higher proliferation of stem/precursor cells in the orexin/ataxin-3 mice in both neurogenic regions, the SGZ and the SVZ. Also in the RMS, higher levels of newly built cells in the orexin/ataxin group were found, but these differences were not significant. We were able to demonstrate a significantly higher survival of newly built cells in the granular zone, but not for the periglomerular zone of the OB in the orexin/ataxin group. A tendency for higher survival rates could be shown for the HC of the orexin/ataxin-3 mice. By triple staining we could show that the proportion of newly born neurons relative to the total number of newly built cells in the HC was significantly higher with 90 % in the orexin-ataxin group compared to 83 % in the control group. In both the granular zone and the periglomerular zone of the OB, over 90 % of the total amount of new built cells differentiated into neurons in both groups. Also the rate of differentiation into a dopaminergic phenotype of the newly born neurons in the periglomerular zone of the OB was not significantly changed with 93 % in the orexin/ataxin-3 mice compared to 91 % in the WT mice. Together, these observations lead to the assumption that orexin suppresses the proliferation of adult NSC, affects the survival rate in the OB negatively and hinders the differentiation of newly built cells into neurons in the HC.

APPENDICES

8 REFERENCES

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9 LIST OD ABBREVIATIONS

ARAS	ascending reticular activating system
BrdU	5-bromo-2'-deoxyuridine
CRH	corticotrophin-releasing hormone
DAB	3,3′-Diaminobenzidine
DNA	deoxyribonucleic acid
EDS	excessive day time sleepiness
GABA	gamma aminobutyric acid
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HPA	hypothalamic pituitary adrenal
HC	hippocampus
KO	knock-out
LHA	lateral part of the hypothalamus
LDT	laterodorsal tegmental nuclei
NeuN	neuronal nuclear
NCAM	neural cell adhesion molecule
NDS	normal donkey serum
NGS	normal goat serum
NREM	non-rapid eye movement
NSC	neural stem cell
OB	olfactory bulb
OSN	olfactory sensory neuron
OX1R/ OX2R	orexin receptor
PB	phosphate buffer
PBS	phosphate buffered saline
REM	rapid eye movement

PFA	paraformaldehyd
POMC	proopiomelanocortin
PPT	pedunculopontine nuclei
PVN	paraventricular nucleus
RMS	rostral migratory stream
SCN	suprachiasmatic nucleus
SGZ	subgranular zone
SOREM	sleep-onset-REM
SN	substantia nigra
SVZ	subventricular zone
TBS	Tris-buffered saline
TH	tyrosine hydroxylase
TMN	tuberomammillary nucleus
VEGF	vascular endothelial growth factor
VLPO	ventrolateral preoptic nucleus
VTA	ventral tegmental area
WT	wild type

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11 DANKSAGUNG

Die vorliegende Arbeit wurde in der Klinik für Neurologie am Universitätsklinikum Gießen und Marburg, Standort Marburg, in der Arbeitsgruppe Experimentelle Neurologie unter Leitung von Herrn Prof. Dr. med. Günter Höglinger angefertigt.

Ich danke Herrn Prof. Dr. Günter Höglinger herzlich für seine ausgezeichnete wissenschaftliche Betreuung und kontinuierliche Motivation.

Meinem wissenschaftlichen Betreuer Prof. Dr. Oscar Arias-Carrión danke ich für die Einarbeitung in das wissenschaftliche Arbeiten im Labor und die aktive Mitwirkung bei der Immunhistologie und der Mikroskopie.

Allen Mitgliedern der Arbeitsgruppe möchte ich für ihre Unterstützung in der Durchführung der Immunhistologie und Gewebeaufarbeitung, sowie für die Bereitschaft mich von ihrem reichhaltigen Erfahrungsschatz profitieren zu lassen danken.

Zuletzt möchte ich meinem Mann, meiner Familie und meinen Freunden danken, welche mich in allen Phasen dieser Arbeit unterstützt und motiviert haben.

12 LISTE DER AKADEMISCHEN LEHRER

Meine akademischen LehrerInnen an der Philipps-Universität Marburg waren die Damen und Herren:

Adamkiewicz, Alter, Arabin, Aumüller, Barth, Bartsch, Basler, Bauer, Baum, Becker K., Becker S., Benes, Czubayko, Daut, Dodel, Donner-Banzhoff, Eggert, Eilers, Fendrich, Feuser, Fuchs-Winkelmann, Görg, Gress, Grzeschik, Hamer, Hassan, Hellmeyer, Hertl, Heverhagen, Höffken, Hofmann, Höglinger, Hoyer, Jansen, Kaltenborn, Kann, Kircher, Klose, Knake, König, Koolman, Kühnert, Leonhardt, Lill, Löffler, Lohoff, Maier, Maisch, Mandrek, Mennel, Michl, Moll, Moosdorf, Mueller, Mutters, Neubauer, Neumüller, Nimsky, Oertel, Pagenstecher, Plant, Printz, Renz, Richter, Riße, Röhm, Röper, Rothmund, Ruchholtz, Schäfer H., Schäfer J., Schmidt, Schneider, Schofer, Schrader, Sekundo, Tackenberg, Vogelmeier, Wagner, Waldegger, Weihe, Werner, Westermann und Wulf.