Adult Brain Plasticity:
Serotonin receptor subtypes mediate opposing effects on adult hippocampal neurogenesis

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Für Mutti & Papi

"The problem, if you love it, is as beautiful as the sunset."
Jiddu Krishnamurti

Zusammenfassung

Der Hippocampus ist eine von zwei Gehirnregionen, in der zeitlebens kontinuierlich neue Nervenzellen gebildet werden. Er spielt eine wichtige Rolle bei der Gedächtniskonsolidierung und wird mit der funktionellen Entstehung neurodegenerativer Erkrankungen in Verbindung gebracht. Strukturveränderungen im erwachsenen Gehirn, die mit einer Depression einhergehen, sind laut Literatur auf einen geringen Serotoninspiegel und reduzierte hippocampale Neurogenese zurückzuführen. Selektive Serotonin-Wiederaufnahmehemmer (SSRI) erhöhen die Serotoninkonzentration im synaptischen Spalt und üben einen positiven Effekt auf die adulte Neurogenese aus. In der vorliegenden Arbeit wird untersucht, wie Veränderungen in der Serotonin (5-HT)-Neurotransmission durch einmalige oder chronische Gaben von Fluoxetin und speziellen Agonisten und Antagonisten für die Serotoninrezeptoren 5-HT1a und 5-HT2 in der erwachsenen Maus die Proliferation und Differenzierung von neugebildeten Nervenzellen im Gyrus dentatus beeinflussen. Die Ergebnisse zeigen, dass ein konträres Agieren beider Rezeptoren zu einem ausgewogenen Serotoninspiegel führt. 5-HT1a- und 5-HT2c-Rezeptoren haben einen Einfluss auf das Überleben neugebildeter Nervenzellen, wobei sie unterschiedliche Entwicklungsstadien innerhalb der adulten Neurogenese kontrollieren. Die vorliegende Arbeit bekräftigt außerdem, dass die chronische Gabe von Fluoxetin die adulte Neurogenese steigert.

Schlagwörter:
Adulte Neurogenese
Serotoninrezeptoren
Hippocampus
Gyrus dentatus
Fluoxetin

Abstract

The hippocampus as one region with ongoing neurogenesis throughout life contributes to the formation of long-term memory and has also been implicated in the pathology of major depression. Studies suggest that depression might be due to decreased levels of serotonin and reduced neurogenesis in the adult brain and that the beneficial effects of selective serotonin reuptake inhibitors would require adult hippocampal neurogenesis. Here, I investigated how modulation of serotonergic neurotransmission by acute and chronic treatment with the antidepressant fluoxetine, and selective serotonin receptor agonists and antagonists in adult mice influences precursor cell activity during development. I focused on 5-HT1a and 5-HT2 receptors as major mediators of serotonin action. The present findings suggest that an opposed action of 5-HT1a and 5-HT2c receptor subtypes result in a balanced regulation of serotonin levels in the dentate gyrus. Both receptors differentially affect intermediate cell stages in adult hippocampal neurogenesis and play an important role in the survival of newly generated neurons. Furthermore, this study confirms that chronic fluoxetine treatment increases adult neurogenesis. In conclusion, the latency of onset of fluoxetine action can be explained by a balanced interplay of 5-HT1a and 5-HT2c receptor subtypes.

Keywords:
adult neurogenesis
serotonin receptors
hippocampus
dentate gyrus
fluoxetine

Contents

Introduction

Modern living conditions including healthy diet, environmental awareness, and advanced medical services result in an extended lifespan, and are also altering the perception of the general public to scientific progress. Demographic changes toward an increasingly aged society stress the need for research on successful physical and cognitive aging, since aging also constitutes a risk factor for diseases such as cancer, and for the central nervous system, most notable neurodegenerative disorders. The discovery, that in the adult brain neurons are continuously generated opens a novel and interesting field of neuroscientific research. New perspectives on brain cell genesis deepen our understanding of brain biology, function, and particularly structural brain plasticity. The hippocampus as one of two brain areas with ongoing neurogenesis throughout life is a highly plastic brain region and involved in the formation of long-term memory. It has also been implicated in the pathology of depression. Inhibition of serotonin reuptake is an important pharmacological principle of antidepressant action and a provocative link to adult hippocampal neurogenesis has emerged.

Here, I focused on the regulation of sequential stages of adult neurogenesis by serotonin and its various receptor subtypes. Chapter 1 and 2 are a detailed introduction into the biology of adult stem cells, hippocampal neurogenesis, and the connected role of serotonin.

1 Newly Generated Cells in the Adult Brain

1.1 Neural stem cells in the adult Central Nervous System

The current public debate about stem cells, especially embryonic stem cells, distracts from the appreciation of adult neural stem cell biology. Whereas pluripotent stem cells are usually derived from embryonic tissue in development, multipotent neural stem cells (NSC)

reside in the adult central nervous system (CNS). It is known that bone marrow, skin and intestines renew continuously. In the 1990's the existence of precursor cells in the CNS became accepted. That suggested continuing neurogenesis in adulthood in higher vertebrates including birds (Goldman and Nottebohm 1983), rodents (Altman and Das 1965a), primates (Gould et al. 1999b; Kornack and Rakic 1999) and humans (Eriksson et al. 1998). Evidence for newly generated neurons has been confined to two brain structures in mammals, the olfactory bulb and the hippocampus (Altman 1969; Kaplan and Hinds 1977; Cameron et al. 1993; Luskin 1993).

The small number of highly plastic NSCs though restricted in their differentiation profile, maintain the potential to generate the three major cell types of the brain; namely neurons, astrocytes, and oligodendrocytes (Palmer and Bizios 1997). Neural stem cells have the ability to self-renew and reside in a particular niche formed by a microenvironment that promotes neuronal development. The adult brain is composed of many specialized glial and other non-neuronal cells including oligodendrocytes, endothelial cells (Palmer et al. 2000), pericytes, microglia, and astrocytes. Astrocytes and endothelial cells may play fundamental roles in the neurogenic niche. For example, astrocytes instruct NSCs to become neurons by establishing neurogenic permissiveness (Song et al. 2002), endothelial cells secret factors animate astrocytes to renew themselves (Shen et al. 2004). Glia cells are critical for the proper development of progenitor migration, neural differentiation, and synaptogenesis and they are surprisingly understudied in the dentate gyrus. The dominant hypothesis is that in adult neurogenic brain regions stem cells are a subset of glia cells expressing the glial fibrillary acidic protein (GFAP) and transform into slowly dividing radial glia-like precursor cells with astrocytic properties (Alvarez-Buylla et al. 2001; Seri et al. 2001).

Apart from the neurogenic brain regions hippocampus and subventricular zone/olfactory bulb system, neural stem cells are also concentrated in cerebellum and cerebral cortex. The presence of low neurogenic activity in regions classically considered as non-neurogenic is controversial. There are adult brain regions lacking neurogenesis that seem not to have the ability to generate new neurons under physiological conditions, although neurogenesis could occur in an induced germinative niche (Magavi et al. 2000). Progenitor cells in these areas derive from the subventricular germinal zone or local parenchymal progenitors. In the cortex precursor cells give rise to glia cells and not to neurons (Kornack and Rakic 2001; Rakic

2002). Whereas Gould suggested the existence of a migrating stream of new cortical neurons in the adult primates brain (Gould et al. 1999a), Rakic demonstrated that neurons in areas such as the cortex or cerebellum are a remnant of embryonic or early postnatal neurogenesis (Rakic, 2002; Kornack and Rakic, 2001). Hence, cortical neurogenesis appears to be confined to the developmental period.

Furthermore, endogenous stem or progenitor cells that proliferate throughout life and mostly differentiate into glia cells have been identified around the central canal of the adult spinal cord (Horner et al. 2000), in the hypothalamus (Markakis et al. 2004), in the optic nerve (Palmer et al. 1999), and in the substantia nigra (Lie et al. 2002; Zhao et al. 2003). The major fraction expresses the proteoglycan NG2 (Neuron-Glia2), which appears to be the common characteristic of precursor cells outside neurogenic zones. Surprisingly little is known regarding the role of newborn glia or gliogenesis in the process of adult hippocampal neurogenesis. NG2 cells can be found in the subgranular zone of the adult dentate gyrus, but their proliferation rate is low (Steiner et al. 2004).

Adult neurogenesis occurs throughout life in the olfactory bulb (OB) and the hippocampal dentate gyrus (DG, Fig. 1). Hippocampal neurogenesis is the topic of my thesis and will be discussed in detail below.

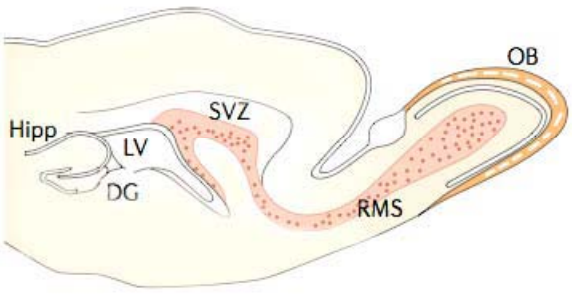


Fig. 1. Adult neurogenesis in the subventricular zone (SVZ)/olfactory bulb (OB) system. Stem cells in the lateral ventricle (LV) proliferate into amplifying cells which give rise to neuroblasts. Newly generated precursor cells migrate along the rostral migratory stream (RMS) to their final denstination in the OB where they differentiate into granule and periglomerular inhibitory interneurons. Furthermore, adult neurogenesis occurs in the hippoampal (Hipp) dentate gyrus (DG). *Modified from (Lledo et al. 2006)*

In the olfactory bulb two types of interneurons are generated from a dividing precursor cell population in the subventricular zone (SVZ, Fig. 1), which is a remnant of the embryonic neurogenesis (Altman and Das 1965b; Corotto et al. 1993; Luskin 1993; Winner et al. 2002). Cell proliferation takes place in the lateral wall of the lateral ventricle, neuroblast migration

in the rostral migratory stream (RMS), and neuronal differentiation in the olfactory bulb. Neuronal precursor cells form long chains and migrate without the guidance of axons or radial glia along the RMS to reach the olfactory bulb. They turn radially and differentiate into granule and periglomerular inhibitory interneurons. The continual addition of interneurons, which modulate spatial and temporal coding of olfactory information, might provide a substrate for adapting to environmental changes (Cecchi et al. 2001; Doetsch and Hen 2005).

Adult neurogenesis is a complex process, starting with the division of a precursor cell leading to the functional integration of newly born neurons into a preexisting circuitry. Neural stem cells are the focus of interest as therapeutic agents to treat cognitive impairments, thus adult neurogenesis may provide a capacity for endogenous brain repair. Adult stem and progenitor cell biology could help elucidate the complex events that occur during neurodegeneration in disease and aging, as well as promote studies in developmental neurobiology. Stem cell biology has raised new hope for Regenerative Medicine, but there is only limited evidence that the complex process of "adult neurogenesis" would primarily contribute to regeneration, although adult neurogenesis responds to a wide range of pathologies (reviewed in (Dietrich and Kempermann 2006)).

The following chapters introduce the morphology and functional relevance of the hippocampal formation and ongoing neurogenesis in the adult dentate gyrus. The different cell stages of newly born cells are characterized as well as their association with proliferative activity and their modulation by various intrinsic and extrinsic factors.

1.2 The hippocampal formation

The hippocampal formation is part of the limbic system and plays a key role in learning and memory. It contributes to synaptic plasticity and provides the foundation for cognitive abilities. There are three kinds of memory including semantic (e.g., facts like that the English word "green" is "grün" in German), procedural (e.g., learning how to surf or dance which would be subconscious) and episodic (e.g., personally remembered events and feelings such as the first kiss). The hippocampus has the capacity for transient memory storage to prepare contents for long-term storage in the cortex. The connections within the hippocampus

generally follow defined laminar structures and are largely unidirectional. A transverse brain section cut reveals a strong afferent set of three connected pathways known as the tri-synaptic circuitry, in which the perforant path forms the main input to the hippocampus (Fig. 2, (Anderson and Lomo 1966; Swanson et al. 1978; Witter et al. 1989). The input fibers mostly arise in the entorhinal cortex and innervate granule cell dendrites in the molecular layer (De Foubert et al.) of the dentate gyrus. Granule cells send axons to large pyramidal neurons of CA3 that constitute the mossy fibers. Mossy fiber synapses are large aggregations of termini, with multiple transmitter release sites and post-synaptic densities. The mossy fiber pathway is extensively studied as a model for the functional role of kainate receptors in synaptic plasticity (Contractor et al. 2000; Lauri et al. 2001; Feng et al. 2003), and the narrowest part within the hippocampal circuitry. Schaffer collaterals are the efferent projections of CA3 pyramidal neurons to CA1 pyramidal neurons, which in turn project to the subiculum, the main output of the hippocampus. The axons either come from CA3 neurons ipsilateral (the same hippocampus) or contralateral (from the opposite hemisphere). It was in the schaffer collateral fiber pathway (CA1) that long-term potentiation (LTP) was first discovered (Bliss and Lomo 1973).

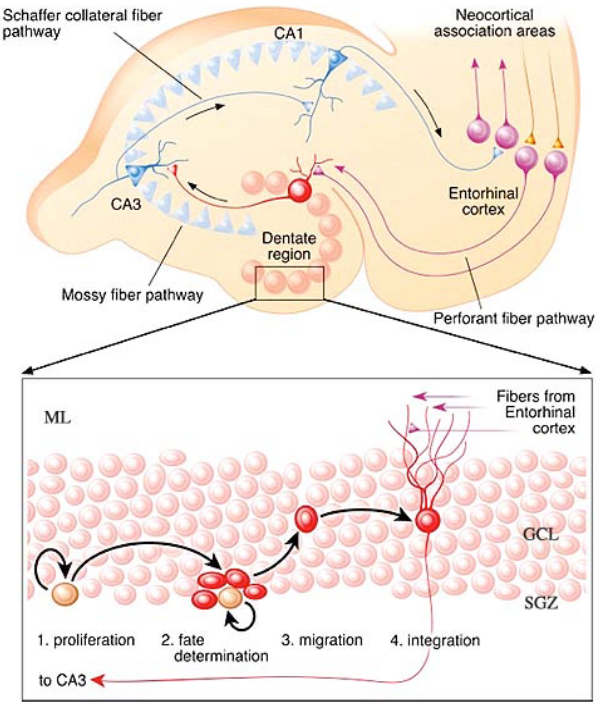


Fig. 2. The hippocampal formation and adult hippocampal neurogenesis in the mammalian brain. The transverse brain section shows the known tri-synaptic circuitry in the hippocampus, which contribute to process information and maintain neuroplasticity: perforant path fibers arise in the entorhinal cortex and innervate granule dendrites in the molecular layer (De Foubert et al.). Granule cell axons synapse with CA3 pyramidal neurons and constitute the mossy fiber. Efferent schaffer collateral fibers of CA3 neurons project to CA1 pyramidal neurons, which in turn project to the subiculum and entorhinal cortex.
1.-3. stem cells in the subgranular zone (SGZ) give rise to transient amplifying progenitor cells which differentiate into immature neurons and migrate into the granule cell layer (GCL). 4. newly generated cells get structurally and functionally integrated into the network: excitatory projections from the entorhinal cortex reaching the dendrites of granule cells in the molecular layer, which extend axons to target cells in the CA3 region. *(Modified from (Lie et al. 2004))*

1.3 Adult hippocampal neurogenesis

In the dentate gyrus of the hippocampus, new granule cells are continuously generated from precursor cells in the subgranular zone (SGZ, Fig. 2) (Altman and Das 1965a; Kaplan and Hinds 1977; Cameron et al. 1993; Kuhn et al. 1996). Stem or neural progenitor cells in the subgranular zone, the border between the granule cell layer (GCL) and the hilus, give rise to transient amplifying precursor cells that differentiate into immature neurons and migrate into the granule cell layer (Fig. 2, 1. -3.). Progenitor cells have two potential destinies, either to remain at the niche of origin (where they will eventually expire) or to migrate away in order to cultivate the cues of the neurophil and subsequently take on new life. Many of these newly generated cells die between the first and second week after they are born. Surviving cells mature into neurons, which are structurally integrated into a preexisting network and they become functional. This can be verified by the development of synaptic inputs from local interneurons (inhibitory afferents) and from the entorhinal cortex (excitatory projections) reaching the dendrites of the granule cells as well as by the extension of axons to target cells in the CA3 region (Fig. 2, 4.).

Newly generated cells receive neural input from the surrounding circuitry and exhibit electrophysiological properties (Ambrogini et al. 2004; Jonas et al. 2004; Schmidt-Hieber et al. 2004). Sequential recordings from adult neighboring neurons revealed glutamatergic, thus excitatory projections from the entorhinal cortex. However, intermediate precursor cells establish early GABAergic innervation, which acts as an excitatory transmitter at this developmental step (Wang et al. 2005). Glutamatergic input develops subsequently (Ben-Ari 2001, 2002) but this newly generated granule cell population expresses different properties than mature granule cells (Wang and Lambert 2000; Schmidt-Hieber et al. 2004), e.g. an increased level of synaptic plasticity is associated with a lower threshold for the induction of LTP.

The dentate gyrus obviously presents a “bottleneck” in processing information (Kempermann 2002), and acts as a “gateway to memory“: a comparatively small number of highly plastic cells arise in the dentate gyrus of the hippocampus, through which all information must pass before it can be memorized. Hippocampus-dependent learning and experience-dependent activation of the hippocampus are important cognitive functions and

maintain neuroplasticity in the adult brain. Kempermann hypothesizes that adult neurogenesis adds to the neuronal network in the dentate gyrus, in order to optimize hippocampal function by allowing a demand-driven optimization of the mossy fiber connection between the dentate gyrus and CA3 (Kempermann et al. 2002). This process is modulated according to the levels of complexity and novelty experienced by the individual (Kempermann et al. 2004b; Wiskott et al. 2006).

1.3.1 Six developmental steps

Adult hippocampal neurogenesis includes six developmental steps from a presumably bipotent radial glia-like stem cell with astrocytic properties (type-1) to transiently amplifying lineage-determined progenitor cells (type-2 and type-3) to early postmitotic and mature neurons (Fig. 3; (Brandt et al. 2003; Filippov et al. 2003; Kempermann et al. 2004a; Steiner et al. 2004)).

Adult neurogenesis is detected by incorporation of bromodeoxyuridine (BrdU) into dividing cells during S-phase and by co-labeling BrdU-positive cells with markers for mature neurons. Over time the distribution of BrdU-labeling shifts through the four cell types (type-1, type-2a, 2b and type-3). In a series of stages associated with proliferative activity, an intermediate precursor cell type, called type-2, is the most proliferative. Following a single BrdU-injection the number of labeled cells doubles within 24 hours. As previously described, the largest expansion of newly generated cells occurs within the first three days after BrdU-injection (Kronenberg et al. 2003). Further experiments confirmed the maximum of newly born cells at day two (Kirste et al., pers. comm., unpublished). During the first two days BrdU-labeled cells express nestin and GFAP, markers for radial glia-like stem cells with a characteristic morphology (Fig. 3) (Seri et al. 2001; Filippov et al. 2003; Kempermann et al. 2004a). Prox1, the transcription factor NeuroD, DCX (doublecortin), or PSA-NCAM (polysialylated neural cell adhesion molecule) immunoreactivity generally characterizes proliferative cell stages up to 4 weeks after their generation. Immature postmitotic granule cells express the calcium-binding protein Calretinin and the neuronal marker NeuN (Fig. 3).

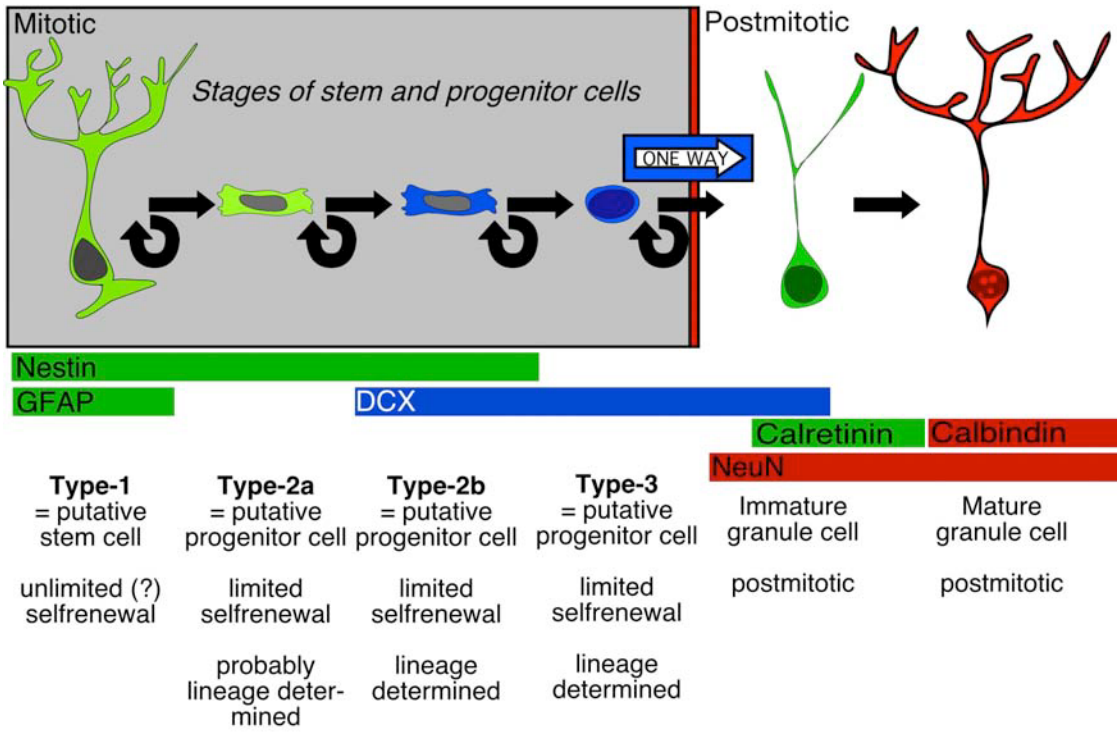


Fig. 3. Model of six proposed developmental steps in adult hippocampal neurogenesis, identified by cell morphology, proliferative ability and marker expression. A presumably bipotent putative radial glia-like stem cell with astrocytic properties (type-1, stage1, expression of nestin and the glial fibrillary acidic protein GFAP) give rise to three stages of transient amplifying lineage-determined progenitor cells (type 2a, still expression of nestin, but not GFAP; type 2b, nestin and doublecortin (DCX) expression; type-3, DCX expression, no nestin, stage2-4) to early postmitotic immature granule cells (stage5, expression of the nuclear marker NeuN and transient Calretinin) and mature neurons (stage6 NeuN and Calbindin expression). *(Kempermann et al. 2004a)*

1.3.2 Type-2 cells as an intermediate precursor cell stage

Newly born cells pass through different stages associated with proliferative activity. Presumably quiescent type-1 cells, identified by GFAP expression and morphological criteria, are rarely dividing (Filippov et al. 2003) and give rise to highly proliferative type-2 cells. Stem and progenitor cell stages were initially identified in nestin-GFP reporter gene mice thus enabled to distinguish subpopulations. Morphological and electrophysiological properties of type-2 cells appear versatile, including astrocytic features like type-1 cells, an early "complex" phenotype initially described for oligodendrocyte precursor cells and some of the type-2 cells showing sodium currents, which revealed that functional neuronal differentiation

could start at the level of type-2 cells (Fukuda et al. 2003). The large population of nestin-GFP-expressing type 2 cells could be subclassified by the absence (type 2a) or presence (type 2b) of early neuronal marker co-expression. The putative precursor cell subpopulation type-2a is the largest proliferative population and is characterized by features of stem cells as well as radial glia-like cells and expresses markers such as BLBP and Sox2. Type-2b cells are also positive for the immature neuronal marker DCX and characterized by an early "complex" phenotype described for neural progenitor cells. Furthermore, Kronenberg et al. (2003) documented the effect of physical activity on cell proliferation in the hippocampal dentate gyrus, and revealed an increase on the level of type-2b cells.

A part of this thesis was to describe the intermediate progenitor cell stages type-2a and type-2b detected by their morphology and marker expression. Here, on the basis of previous studies (Steiner et al. 2004) the proliferative activity was characterized more intensely as well as the electrophysiological properties of type-2 cells (Steiner et al. 2006). The data (shown in chapter 4.1) indicates that type-2 cells mark a transition between glial and neuronal lineage noticeable on the basis of marker expression, morphology and electrophysiological properties.

1.3.3 The Doublecortin-expressing cell stages

Early during the course of adult hippocampal neurogenesis at the stage of type 2b cells, DCX expression signals neuronal fate choice (Fig. 3, (Kempermann et al. 2004a). Newly born granule cells express a series of transient markers, such as the microtubule associated protein DCX, PSA-NCAM (Seki and Arai 1993), and Calretinin (Seki and Arai 1993; Brandt et al. 2003). DCX has been linked to neural structural plasticity and morphological changes associated with migration, axonal guidance and dendrite sprouting (Brown et al. 2003b; Kronenberg et al. 2003; Deuel et al. 2006). During development, DCX is expressed in radially and tangentially migrating neuroblasts of the cerebral cortex, subventricular zone, and in granule and Purkinje cells in the developing cerebellum (Gleeson et al. 1999; Nacher et al. 2001). During cortical development DCX is required for precursor cell migration probably through its interaction with microtubule networks (Francis et al. 1999; Tanaka et al. 2004).

In the adult dentate gyrus, DCX is expressed in precursor cells across a time span that extends from a proliferative progenitor cell stage (type 2b and 3) to a postmitotic phase (immature neurons) whereas DCX expression is absent from mature granule cells (Fig. 3). Transient DCX expression in proliferating neuronal progenitors and immature postmitotic neurons decreases below level of detection when mature neuronal markers (Tuc4, Calbindin) are expressed (Brown et al. 2003b). DCX immunoreactivity is shown in the somata and dendritic trees of granule cells entering the molecular layer of the dentate gyrus (Fig. 4 A). DCX-expressing cells in the hippocampus receive their first synaptic GABAergic input (Tozuka et al. 2005; Wang et al. 2005) and migrate into the inner third layer of the granule cell layer (Fig. 4 B). Dendritic development and radial migration into the inner granule cell layer on average lasts approximately 3 weeks (van Praag et al. 2002; Plumpe et al. 2006). DCX is not expressed in multipotent neural stem cells or glial cells, and constitute a population distinct from GFAP- (type-1) or nestin-expression (type-2a) (Kempermann et al. 2004a; Steiner et al. 2004; Plumpe et al. 2006). Only 20% of the actively dividing cell population type-2b, and more than 70% of type-3 cells and early postmitotic neurons (overlap with Calretinin) express DCX. Type-3 cells exit the cell cycle and differentiate into granule cells.

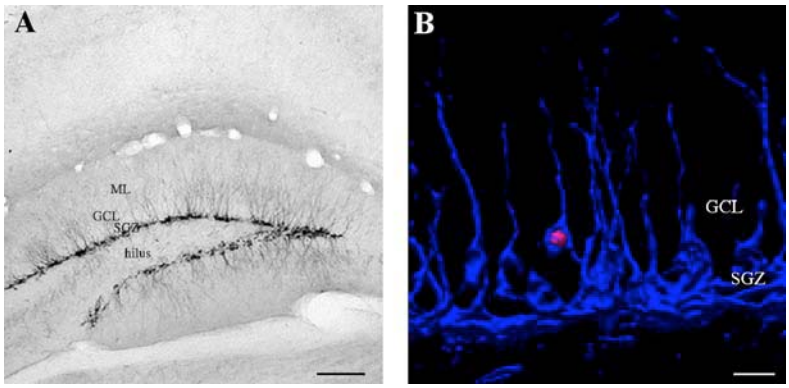


Fig. 4. DCX expression in the adult dentate gyrus. A. DCX immunoreactivity is shown in the somata and dendritic trees of granule cells in the subgranular zone (SGZ) entering the molecular layer (ML, peroxidase immunostaining, Scale bar 200 µm). **B.** 3-D reconstruction of confocal image stacks: DCX (blue) is expressed in proliferating precursor cells that migrate into the inner granule cell layer (GCL, BrdU in red). Scale bar 20 µm; SGZ, subgranular zone; *B is published in (Plumpe et al. 2006)*

However, in the adult rat and mouse brain DCX expression is not limited to the hippocampal dentate gyrus. As described previously, DCX is expressed in neuronal populations in the subventricular zone, rostral migratory stream, olfactory bulb, and also rarely in non-neurogenic regions like corpus callosum, the piriform cortex, and striatum (Nacher et al. 2001; Brown et al. 2003b; Kronenberg et al. 2007). Neurons continue to express the transient markers DCX and PSA-NCAM in the layer II and III of the adult piriform cortex, which receives a large number of olfactory fibers from the olfactory bulb.

The population of transient amplifying progenitor cells in the hippocampus that expresses DCX (type-2b, type-3 and early postmitotic neurons) represents a stage of great morphological and functional changes, and of synaptic plasticity in neuronal development. Thus it is likely to be an important phase to study stimulatory effects by microenvironmental factors such as serotonin. Another part of the thesis was to study the DCX-EGFP transgenic mice model and to characterize DCX-expressing cells in the adult dentate gyrus as compared to the non-neurogenic piriform cortex.

1.4 Activity-dependent regulation of adult hippocampal neurogenesis

Adult hippocampal neurogenesis is hypothesized to contribute to the processing of new information, especially information with greater levels of complexity. Furthermore, its activity-dependent regulation is influenced by certain environmental cues. Environmental factors and voluntary physical activity as well as pathological disorders and stress affect hippocampal neurogenesis. An enriched situation is characterized by sensorial stimulations, social experiences, and physical and cognitive exercise.

The process of differentiation in the adult dentate gyrus can be regulated by physical exercise (RUN) and environmental enrichment (ENR), and as we described earlier running primarily increases cell proliferation (Kempermann et al. 1998b; van Praag et al. 1999, 2000), whereas living in an enriched environment primarily promotes survival of newly generated cells (Kempermann and Gage 1999; Nilsson et al. 1999). Pro-proliferative stimuli in running conditions primarily affect early progenitor type-2 cells in the dentate gyrus (Kronenberg et al. 2003; Plumpe et al. 2006). An activity-depending survival effect of newborn cells primarily occurs on later precursor cells in the type-3 cell population that express DCX. The

stimulatory effects of running have been suggested to be mediated by the vascular endothelial growth factor (VEGF) (Fabel et al. 2003) or the insulin-like growth factor 1 (IGF1) (Trejo et al. 2001).

Increased cell proliferation might also reflect a preceding survival-promoting effect on proliferating stem or progenitor cells themselves (Kempermann 2002), whereas enriched housing conditions rather have a survival-promoting effect on postmitotic neurons (Brandt et al. 2003). As shown in Fig. 3 the transient marker Calretinin is expressed in early postmitotic immature neurons. Here, Calretinin-positive neurons are likely to represent the phase of greatest synaptic plasticity in the course of neuronal development in the adult dentate gyrus. Furthermore, the survival of adult-generated granule cells can be enhanced in response to conditioning tasks (Shors et al. 2001) and spatial learning such as water maze task (Shors et al. 2002); nonetheless physical activity causes an increase in proliferation, which might contribute to improved performances in learning tasks (Kempermann et al. 1998a; van Praag et al. 1999). But the data remains somehow ambiguous since Ehninger & Kempermann showed a reduced number of Calretinin-positive cells that might be due to stress influenced by the water maze task (Ehninger and Kempermann 2006). This process of differentiation influenced in such a way appears to run unidirectional and never backwards suggesting that stimulating tasks cannot redefine NSCs.

Whereas physical activity has a strong upregulating effect on adult hippocampal neurogenesis, neurogenesis in the subventricular zone/olfactory bulb is unaffected by environmental enrichment and physical exercise (Brown et al. 2003a). The findings demonstrate that learning and memory tasks as well as living in an enriched environment and physical activity conditions selectively increase hippocampal neurogenesis. This unique response of the hippocampus to these stimuli suggests an important role of newly generated neurons in the context of hippocampal function and plasticity (Kempermann et al. 2000).

Beside activity-dependent regulation, neurogenesis requires a specific microenvironment, which provides necessary signals such as growth factors, hormones or neurotransmitters to regulate cell proliferation and differentiation. Serotonin is one of the important neurotransmitter and neuromodulator in the brain. A depletion or low supply of serotonin directly effects the regulation of mood. Disorders in serotonergic activity could contribute to symptoms of major depression due to impaired serotonin synthesis, release, reuptake, or

serotonin receptor abnormalities. Studies have shown that medications such as antidepressants to treat depression can cause an increase in the number of newly generated neurons in the hippocampal dentate gyrus (Malberg et al. 2000; Czeh et al. 2001; Santarelli et al. 2003).

In the next chapter the role of serotonin on regulation of adult hippocampal neurogenesis is discussed more in detail including receptor function, drug mechanisms and clinical relevance.

2 Role of Serotonin on Regulation of Adult Hippocampal Neurogenesis

Serotonin is an evolutionarily conserved signaling molecule that plays many roles in various animal species and various tissues (Whitaker-Azmitia 1991; Turlejski 1996). In the CNS it participates in the regulation of many important brain functions like thermoregulation (Feldberg and Myers 1964), sleep (Jouvet et al. 1967), aggression (Sheard 1969) and feeding. Disturbed serotonergic signaling, e.g. decreased levels of serotonergic neurotransmission are associated with several disorders such as major depression, and correlate with increased anxiety and aggressive behaviors (Holmes et al. 2003a; Holmes et al. 2003b; Iritani et al. 2006). Extensive studies also suggest a role of serotonin in learning and memory, and in such a way, the serotonergic system is able to modulate cognitive processes (Wolff et al. 2003; Sanberg et al. 2006).

Chapter 2 gives in more depth a review of the serotonergic system and its role in adult hippocampal neurogenesis. It thereby focuses on the molecular basis of the neurotransmitter action and describes serotonin receptor function. The second part links the effect of serotonin on regulation of neurogenesis with the relevance of antidepressant action.

2.1 The serotonergic system

Serotonergic neurons are mainly located in the brainstem dorsal and median raphe nuclei. They are grouped into pairs and distributed along the entire length of the brain stem. The fibers terminate in the limbic system and spinal cord and extend through the medial forebrain bundle in the olfactory bulb, striatum and neocortex (Fig. 5, (Parent 1981; Jacobs and Azmitia 1992; Leger et al. 2001). The dentate gyrus of the hippocampus has a very dense plexus of serotonergic fibers, where it synapses on principle neurons and interneurons. One fiber population originates from the dorsal raphe nucleus, enter the entorhinal cortex and projects to the molecular layer, another arises from the median raphe nucleus and projects mainly to the

hilus. Serotonin has long been implicated in several neurodevelopmental processes including adult neurogenesis and dendritic maturation. It has been reported that serotonin has a positive regulating effect on adult neurogenesis in raphe lesions and transplantation studies (Brezun and Daszuta 1999, 2000), as well as in serotonin receptor knockout mice models (Zhuang et al. 1999; Santarelli et al. 2003). In addition, the well-known report by Santarelli indicates that the beneficial effects of selective serotonin reuptake inhibitors (SSRIs) in depression might require adult hippocampal neurogenesis (Santarelli et al. 2003).

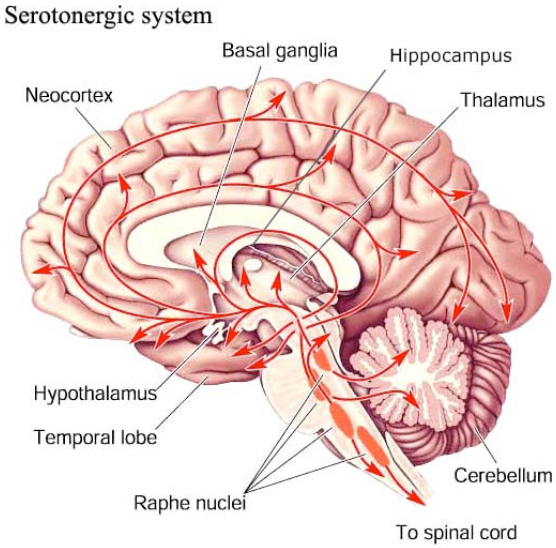


Fig. 5. Serotonergic system in the mammalian brain. Serotonergic neurons are mainly located in the dorsal and median raphe nuclei. The fibers terminate the limbic system (hippocampus, hypothalamus, thalamus) and spinal cord and extend into the olfactory bulb and neocortex. *(Modified from „Dongguk University Kyongju, Korea“).*

Lesions of serotonergic neurons in the raphe nuclei by injections of the neurotoxin 5.7-dihydroxytryptamine (5.7-DHT) have a long-term effect of hippocampal serotonergic denervation. Studies by Brezun & Daszuta describe a decrease in 5-HT fiber density in the dentate gyrus associated with a decreased number of new neurons expressing BrdU and PSA-NCAM after 8 days (Brezun and Daszuta 1999). In addition, they demonstrate that a fetal serotonergic neuron grafted to the adult rat dentate gyrus survived, develop 5-HT innervation, and restore a serotonergic control of granule cell proliferation previously deprived of their 5-HT input, and so reverse the changes after depletion (Brezun and Daszuta 2000).

2.2 The neurotransmitter serotonin

Serotonin (5-HT, 5-Hydroxytryptamine) is an important neuromodulatory monoamine neurotransmitter in both the central and peripheral nervous system of mammals. It is also synthesized extensively in the enterochromaffin cells of the gastrointestinal tract. The major storage place in non-neuronal tissue is platelets of the blood stream. In the developing brain, serotonin is produced by raphe neurons and is captured by thalamic axons, which store it in presynaptic vesicles (reviewed in (Gaspar et al. 2003)). Serotonin is formed by hydroxylation and decarboxylation of the amino acid L-Tryptophan. The tryptophan hydroxylase (THP) mediated reaction is the limiting step in the pathway, followed by the amino acid decarboxylase (DDC) step (Fig. 6). THP has been shown to exist in two isoforms, THP1 was found in several tissues, whereas THP2 occurs in the brain. There is evidence that the genetic polymorphisms in both subtypes influence susceptibility to anxiety and depression (Nash and Nutt 2005; Zhang and Rudnick 2005).

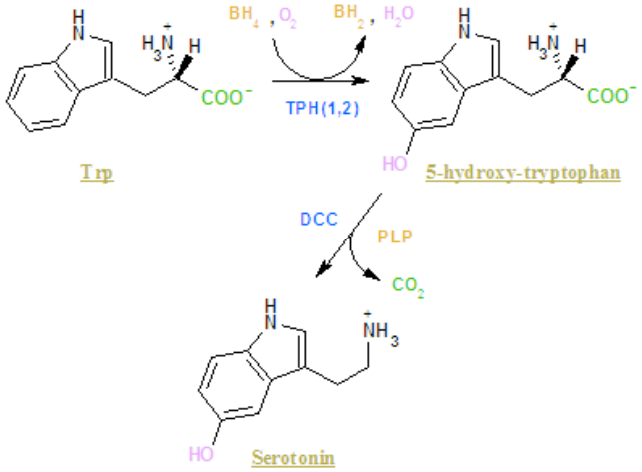


Fig. 6. Pathway of serotonin synthesis from Tryptophan. In the body, serotonin is synthesized from the amino acis tryptophan by a short metabolic pathway consisting of the enzymes – tryptophan hydrolase (TPH) and amino acid decarboxylase (DDC) (Images, Borislav Mitev)

A limited number of neurons in the CNS are specified to become serotonergic during development. This involves a sequence of transcription factors, such as Nkx2.2, Lmx and Pet-1. Pet-1 is expressed in the raphe, it directly controls the gene transcription and enzyme encoding of 5-HT, tryptophan hydrolase, as well as the serotonin plasma membrane transporter (reviewed in (Gaspar et al. 2003)).

Serotonin is released by serotonergic vesicles (mediated through increased intracellular Ca^{2+}-level) swelling along the axons. It activates 5-HT receptors located on the dendrites, cell bodies and presynaptic terminals of adjacent neurons. 5-HT receptors are located on the membrane of nerve cells and other cells and mediate the effects of serotonin as the endogenous ligand and of several pharmacological components. Serotonergic action is limited by reuptake of 5-HT from the synaptic cleft through specific 5-HT transporters on the presynaptic neuron. Several agents or drugs can inhibit serotonin reuptake including Methylenedioxymethamphetamine (MDMA or ecstasy), amphetamine, cocaine, dextrometorphan, tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs).

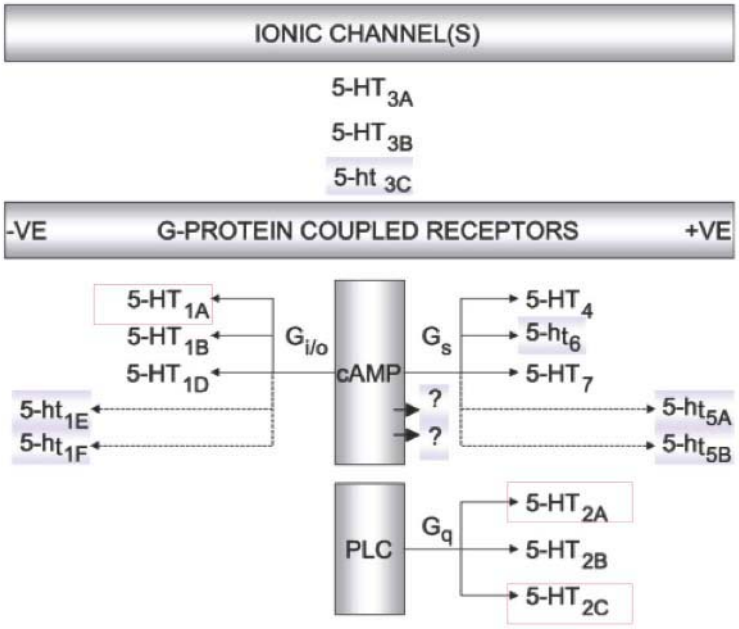
Several studies describe the regulation of adult hippocampal neurogenesis by serotonin and focus on intracellular signal transduction pathways. Palmer reported the role of the cAMP-CREB cascade (cyclic adenosine monophosphate-response element binding protein) a second messenger pathway in adult neurogenesis, *in vitro* (Palmer et al. 1997). This is of particular interest since Nibuya (Nibuya et al. 1996) and the group of Duman (Duman et al. 1997; Duman et al. 2000; Nakagawa et al. 2002) demonstrated that antidepressant treatment up-regulates this pathway *in vivo*. CREB is a transcription factor, activated by phosphorylation on protein kinase A (PKA) as well as by Ca^{2+}-calmodulin-dependent protein kinases (CaMKs). Furthermore, they suggest a prominent expression of phospho-CREB in cells within and in proximity to the subgranular zone of the dentate gyrus. Chronic administration of several antidepressants increases the expression of the brain-derived neurotrophic factor (BDNF), VEGF (Warner-Schmidt and Duman 2007) and Trk mRNA in the hippocampus, which in turn up-regulate the cAMP-CREB cascade.

2.2.1 Serotonin receptor subtypes

The effects of the monoamine neurotransmitter serotonin are subserved through a variety of membrane-bound receptors. 15 different transmembrane receptors have been classified into seven groups (5HT1 to 5HT7) largely based on their structural and functional characteristics (review in (Hoyer et al. 2002)). This physical diversity underscores the physiological importance of serotonin and indicates that different 5-HT receptors control different

developmental processes, such as axon branching, cell survival or adult neurogenesis. Table 1 shows the current classification of 5-HT receptors. With one exception, they belong to the G-protein-coupled super family. The 5HT3 receptors are ligand-gated ion channels and their activation results in direct depolarization of neurons (Tecott et al. 1993; Tecott et al. 1995a).

Table 1. Current classification of serotonin (5-HT) receptors. They belong to a G-protein coupled family with one exception; the 5-HT3 receptors are ligand-gated ion channels. Receptor subtypes in shaded boxes and lower-case designate receptors have not been demonstrated in definitively function. This study focuses on regulation of adult hippocampal neurogenesis by 5-HT1a, and 2 receptor subtypes (red squares). (–ve, negative; +ve, positive; cAMP, cyclic adenosine monophosphate; PLC, phospholipase C; G, guanine nucleotide binding proteins) *(Hoyer et al. 2002)*



Almost all of these receptors are expressed in the dentate gyrus. Table 2 summarizes the signal transduction pathways and general actions of the different receptor subtypes, which are expressed in the hippocampus. Once the neurotransmitter binds to the receptor, the nerve cell becomes activated through a G-protein induced confirmation of the receptor. This exchange activates different second-messenger cascades and effector-proteins, while the receptor is able to activate the next G protein. The enzyme adenylate cyclase is activated by G_s-GTP and synthesizes the second messenger cAMP from ATP. Phospholipase C (PLC) is activated by G_q or G_{11} G-protein subunits and is a key enzyme in phosphatidylinositol-4.5-bisphosphonat

(PIP2) metabolism. It hydrolyzes PIP2 into two second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG), which then go on to modulate the activity of downstream proteins during cellular signalling, e.g. calcium channels and protein kinase C.

The 5-HT1 receptor class compromises the subtypes 5-HT1a, b, d, e and f. Activation of 5-HT1 receptors mainly opens K+-channels causing hyperpolarization due to G_i/G_O mediated cellular effects, resulting in decreased cellular levels of cAMP. The 5-HT1a, b, f receptor subtypes also show a hydrolysis of PIP2, which causes a split into the second-messengers inositol triphosphate (IP3) and diacylglycol (DAG). This stimulates phospholipase C (PLC) and leads in turn to a cell depolarization. 5-HT1a receptors are widely distributed within the CNS. They are expressed early in embryonic life, mainly in the raphe nuclei and hippocampus, and also in motor neurons and cerebellum after birth (reviewed in (Gaspar et al. 2003). Presynaptic autoreceptors are present on serotonergic neurons in the raphe nuclei and provide a mechanism for feedback inhibition of the 5-HT system, whereas postsynaptic 5-HT1a receptors are found on cells in hippocampus and cortex (Hoyer et al. 2002) especially on GABAergic pyramidal neurons and interneurons in the hilus (Tecott et al. 1993; Matsuyama et al. 1997). Activation results in a suppression of 5-HT release of serotonergic neurons and a decreased firing rate of postsynaptic cells. The proposed role of 5-HT1a receptors is to modulate anxiety-related behavior, which was studied in 5-HT1a receptor knockout mice by Santarelli (Santarelli et al. 2003), and also reported by Radley and Jacobs (Radley and Jacobs 2002), and Malberg and Duman (Malberg and Duman 2003). Furthermore, data suggest that 5-HT1a receptors in the dentate gyrus play a crucial role in LTP induction (Sanberg et al. 2006). One aim of my study was to indicate whether precursor cells themselves express the receptor in the adult dentate gyrus or the 5-HT1a receptors exert an indirect effect through other cell types like glia cells or interneurons.

The IP3 cascade is mainly stimulated through the 5-HT2 receptor family activation. These receptors with the subtypes 5-HT2a, b, and c are G_q/G_{11} coupled, mediating cellular effects through increasing cellular levels of IP3 and DAG. 5-HT2a receptors are widely expressed in the cortex (Hoyer et al. 1986) as well as on GABAergic neurons in the hippocampus. They are expressed late in development, which indicates an important role of this receptor in cell differentiation and maturation (reviewed in (Azmitia 2001)). Mice that lack 5-HT2c receptors have deficits in long-term potentiation in the hippocampus (Tecott et al. 1995a) and show

decreased levels of anxiety. The neurotrophic factor BDNF is involved in neuronal survival and synaptic plasticity in the CNS. As previously mentioned, studies showed that several antidepressants increase the expression of BDNF in the brain (De Foubert et al. 2004; Russo-Neustadt et al. 2004; Larsen et al. 2007). In contrast, chronic treatment with 5-HT2a or 5-HT2c receptor agonists caused a downregulation of BDNF mRNA expression in the dentate gyrus (Vaidya et al. 1997).

The 5-HT3 receptor subtypes a, b, and c are found among others on neurons in CA1 pyramidal cell layer in the hippocampus, where they trigger rapid plasma membrane depolarization due to transient currents via ligand-gated Na^+ and K^+-ion channels. 5-HT3 mRNA expression was seen in interneurons of the dentate gyrus, which suggests that 5-HT3 receptors might mediate serotonergic inhibition of CA3 pyramidal cells via excitation of inhibitory interneurons (Tecott et al. 1993). Furthermore, investigations on 5-HT3a receptor knockout mice revealed a potential role of this receptor subtype in regulation of the hypothalamic-pituitary-adrenal (HPA) response to acute stress (Bhatnagar et al. 2004).

The 5-HT4 and 5-HT6 receptors are G_s-protein coupled, mediating cellular effects through increasing cellular levels of cAMP, which induces cell depolarization.

The 5-HT1 and 5-HT2 receptors appear to be of particular importance in regulation of hippocampal neurogenesis and anxiety behavior. Several studies indicate a role of serotonin in the pathogenesis of major depression thus serotonin action is a mechanism of antidepressant drugs. The following paragraphs review and discuss the results of serotonin research in the context of stress, depression and the hippocampus in more detail. Thematic priority is on the relevance of antidepressants, which positively influence adult neurogenesis as well as on new insights into serotonin receptor function.

Table 2. Serotonin receptor subtypes in the hippocampus. Summary of serotonin receptor signaling pathways in the hippocampus and its various developmental and behavioral effects. (Gi/s inhibition or stimulation of cAMP, G_q, stimulation of phospholipase C)

Receptor subtypes	Signal transduction pathways in the hippocampus	Developmental and behavioral effects
5-HT1a 5-HT1f	G_i/G_O mediated cellular effects through decreasing cellular levels of cAMP, opening of K^+ channels, hyperpolarization Weak stimulation of phosphatidylinositol-4.5-bisphosphonat (PIP_2)	Pre- and postsynaptic neuronal inhibition (Tecott 1993); 5-HT1a activation stimulates neurogenesis in DG and SVZ (Santarelli 2003, Banasr 2004); 5-HT1a blockade attenuated LTP (Sanberg 2006); behavioral effects (sleep, feeding, thermoregulation, aggression, anxiety; Santarelli 2003, Jacobs 2002, Malberg&Duman 2003)
5-HT2a 5-HT2c	G_q/G_{11} mediated cellular effects through stimulation of phospholipase C (PLC), increasing cellular levels of inositol trisphosphate (IP3) and diacylglycerol (DAG)	Presynaptic inhibition and role in LTP (Tecott 1995); synaptic plasticity and dendritic maturation (Azmitia 2001); regulation of BDNF mRNA (Vaidya 1997); behavioral effects
5-HT3	Ligand-gated Na^+ and K^+ ion channel, resulting in a direct plasma membrane depolarization	Neuronal excitation, inhibition of CA3 pyramidal cells via excitation of inhibitory interneurons (Tecott 1993); anxiety, role in HPA activity (Bhathagar 2004),
5-HT4	Gs mediated cellular effects through increasing cellular levels of cAMP, resulting in depolarization and permanent excitation	Neuronal excitation
5-HT6	Gs mediated cellular effects through increasing cellular levels of cAMP	Unknown

2.3 Stress, depression and the hippocampus

Beside activity-depending effects on neurogenesis, stress or corticosterone administration also induce structural changes in the hippocampus, and modulate functions of the highly plastic limbic system. The hippocampus has been demonstrated to be a site of serotonergic innervation associated with CNS control of the hypothalamic-pituitary-adrenal (HPA)-axis. The hippocampal formation is particularly sensitive to stress hormones due to a high concentration of corticosteroid receptors (Magri et al. 2006). Indeed, Glucocorticoids, produced by the stress-responsive HPA-axis, modulate hippocampal plasticity, acting on excitability and long-term potentiation or depression (Heffelfinger and Newcomer 2001; Magri et al. 2006). Stressful experiences, which elevate the levels of glucocorticoids and stimulate hippocampal glutamate release, inhibit precursor cell proliferation in the dentate gyrus (Gould and Tanapat 1999). Experiments show that removing a rat's adrenal glands

increased neurogenesis in the adult dentate gyrus. Adrenalectomy reversed that effect. The circulating glucocorticoids apparently suppressed the birth of neurons in the dentate gyrus under normal conditions (Cameron and Gould 1994).

For the brain, acute stress enhances the memory of events, whereas chronic stress causes adaptive plasticity in the brain due to local neurotransmitters as well as hormones, which produce structural and functional changes. These changes affect neurogenesis in the dentate gyrus, and in such a way, neuronal networks are remodeled, and a modification of dendritic spines on CA3 pyramidal neurons occurred (McEwen 2000). Chronic psychosocial stress in adult rodents which increased the level of stress hormones in turn reduced the proliferation rate and survival of newly generated granule cells in the hippocampus (Czeh et al. 2002). Additional studies done in the lab of Daniel Peterson indicate an influence of acute psychosocial stress on short-term survival of newly generated cells in the dentate gyrus (Thomas et al. 2007).

A stress-induced decrease in neurogenesis might be an important factor in depression. Many studies debate the hypothesis, which has linked the pathogenesis of major depression to changes in adult hippocampal neurogenesis. Although they acknowledged a significant hippocampal volume loss in depressed patients, histopathological studies afterwards failed to confirm a suppression of dentate neurogenesis (Czeh and Lucassen 2007). Furthermore, studies presumed the disorder might be due to decreased levels of serotonin in the adult brain. They mentioned that drugs increasing serotonergic neurotransmission are currently the most effective treatment for depression. These antidepressants cause an increase in the number of newly generated neurons in the hippocampal dentate gyrus (Malberg et al. 2000; Czeh et al. 2001; Santarelli et al. 2003). This in turn may suggest that a dysfunction of adult hippocampal neurogenesis could be implicated in depression, and that patients suffering from depression could be helped by treatments that increase the production of new neurons in the hippocampus. The precise mechanism that causes major depression is unknown. However, suppressed neurogenesis might not be essential for inducing depression, whereas stimulation of neurogenesis by serotonin might be necessary for antidepressant action.

2.3.1 Fluoxetine increases synaptic serotonin levels

Chronic stress causes neuronal remodeling in the hippocampal formation which is reversed by antidepressant treatments in animals (Daszuta et al. 2005). Therefore a serotonin-induced increase in adult neurogenesis might promote recovery from depression. Depression is treated with antidepressants that affect norepinephrine and serotonin levels in the brain. A variety of psychiatric medications affect serotonin levels, including the monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), atypical antipsychotics, SSRIs, and amphetamines.

Neurons in the brain communicate with each other by secreting chemical messengers. These cross the synaptic cleft and bind to receptors on neighboring nerve cell membranes. Once the neurotransmitter binds to the receptor, the nerve cell becomes activated. Drugs that enhance the binding of serotonin to its receptors work effectively to treat anxiety and depression, suggesting a role in regulation by this neurotransmitter. Therefore, SSRIs might act to enhance the degree of activity of various serotonin receptor subtypes. After its release, serotonin is normally reabsorbed by an uptake-pump on the presynaptic cell (SERT, Fig. 7; (Lesch et al. 1993; Emslie et al. 2002). Fluoxetine (N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]-propan-1-amine) is a selective serotonin reuptake inhibitor, which appears to stabilize the level of serotonin in the synaptic cleft. The enhanced neurotransmitter increases the amount of active serotonin that can be delivered to the postsynaptic nerve cell. Furthermore, in receptor binding studies fluoxetine was shown to have a weak affinity to 5-HT1a receptors, whereas it acts directly on 5-HT2c receptor subtypes in the brain. In a very high dose, fluoxetine can also inhibit the noradrenalin reuptake. The half-life of this SSRI after a single dose is in a range of 1 to 4 days (acute), and after a long-term treatment 4 to 6 days (chronic). The peak plasma concentration is reached in 6 to 8 hours, the steady state plasma levels are attained after 4 to 5 weeks of continuous administration.

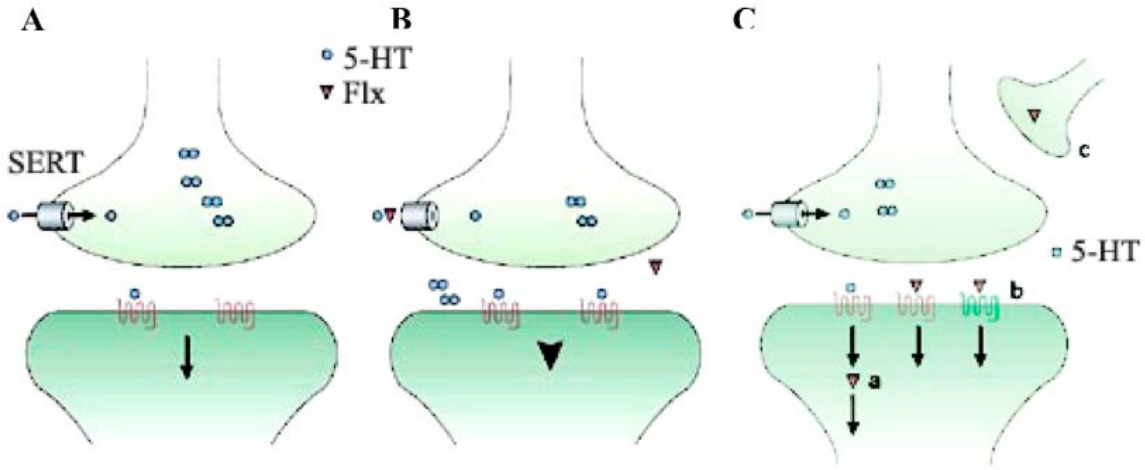


Fig. 7. Chemical stimulation of serotonin levels by fluoxetine (Flx) on the synaptic cleft. A. Serotonin (5-HT) reuptake in the presynaptic cell by serotonin transporters (Shapiro et al.) **B.** Fluoxetine blocks the serotonin reuptake transporters and thereby increases the serotonin level in the synaptic cleft. Enhanced serotonin activity stimulates adult hippocampal neurogenesis. **C**. Fluoxetine could increase synaptic transmission by a number of additional interactions: it can interact with downstream components in the postsynaptic cell (a), modulate activity of serotonin receptors or other receptors (b), or modulate presynaptic targets (c). (*Kaletta et al., 2006 Nature Reviews)*

The effects of antidepressants on newly generated granule cells in the hippocampus have been reported in rodents (Malberg et al. 2000; Malberg and Duman 2003; Santarelli et al. 2003) and tree shrews (Czeh et al. 2001). Recently, Perera examined whether adult neurogenesis was increased after antidepressant treatments in non-human primates (Perera et al. 2007). Pharmacological studies on adult rats (Malberg et al. 2000; Santarelli et al. 2003) have shown that the beneficial effect of antidepressants requires hippocampal neurogenesis. Thereby, it depends on the time course of the administration to see an effect: the number of BrdU-labeled cells is increased after chronic, but not acute, administration of fluoxetine, consistent with the time delay required for the therapeutic action of antidepressants. For the first experiment, rats were treated with fluoxetine for 1, 5, 14 and 28 days and were then given BrdU to analyze the effect on cell proliferation (Malberg et al. 2000). An increase in the number of proliferating cells was observed in response to drug administration after 14 and 28 days indicating that upregulated neurogenesis may be an action of antidepressants (Fig. 8).

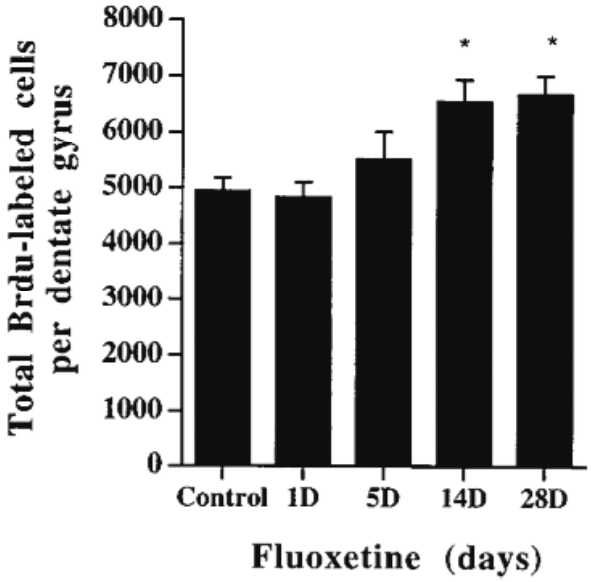


Fig. 8. Analysis of cell proliferation in the adult dentate gyrus after acute and chronic fluoxetine administration. The number of BrdU-labeled cells is increased after chronic, but not acute fluoxetine treatment. Rats were administered with the antidepressant for 1, 5 14 and 28 days followed by BrdU. The number of proliferating cells was increased in response to drug administration after 14 and 28 days. *(Malberg et al. 2000)*

To determine the influence of antidepressant treatment on cell fate, the number and phenotype of BrdU-positive cells was determined after another 28 days. The amount of BrdU-positive cells was significantly increased indicating that proliferating cells are still surviving 28 days later. Furthermore, they examined in a 3rd experiment the influence of antidepressant treatment on cell survival by giving BrdU 1 day before initiation of fluoxetine for 14 days. There was no difference in the number of BrdU-positive cells in the hippocampus 28 days after BrdU-administration (Malberg et al. 2000). Also Santarelli published that fluoxetine caused a 60% increase in the number of BrdU-positive cells in the dentate gyrus after 11 and 28 days of drug treatment, but that it had no effect after administration for 5 days (Santarelli et al. 2003). In summary, antidepressants like fluoxetine show no acute effect of serotonin on adult hippocampal neurogenesis, but a delayed increase in cell division ending up producing more neurons.

In animal models, a decrease in cell proliferation and adult neurogenesis is reversed by chronic antidepressant treatment. In addition, clinical studies indicate that escape deficiencies due to exposure to inescapable shock can be reversed by subchronic administration of fluoxetine (Malberg and Duman 2003). Furthermore, a reduction in the proliferation rate of precursor cells in the subgranular zone in chronic psychosocially stressed animals can be

prevented with tianeptine treatment (Czeh et al. 2001). A recently published study investigated the effect of chronic fluoxetine treatment within the developmental steps in adult hippocampal neurogenesis (Encinas et al. 2006). On the basis of seven-months old nestin-GFPnuc (cyan fluorescent protein, which is fused to a nuclear localization signal) reporter mice, this group presented a quantitative approach to show that fluoxetine targets amplify neural progenitor cells and increases the rate of their symmetric division.

In summary, several reports show that serotonin has an overall positive regulating effect both on neural precursor cells and survival of newly generated neurons. The results seem somehow controversial considering a long-lasting effect of hippocampal serotonergic denervation after raphe lesions (see chapter 2.1), whereas pharmacological studies showed no acute effect of serotonin, but a delayed increase. It is remarkable that the authors give no explanation concerning the latency period of antidepressant action. A central theory in my thesis is that this discrepancy might probably be explained by the activation of different serotonin receptors.

2.3.2 Serotonin receptor subtypes contribute to the action of antidepressants

The dentate gyrus has an extremely dense concentration of 5-HT1a receptors (Azmitia et al. 1996), which get most attention by scientists mainly because of their implication in anxiety-like behavior. Several studies have been described that 5-HT1a receptors contribute to the modulation of mood as well as to hippocampal neurogenesis (Table 3). In relation to stress and depression, Gould proposed that 5-HT1a receptor subtypes could probably prevent the effects of stressfully experiences on neurogenesis (Gould 1999). Furthermore, Santarelli compared the effect of increased serotonin levels for antidepressants delivered to wild-type mice and 5-HT1a receptor knockout mice (Santarelli et al. 2003). The results indicate that fluoxetine had no effect in knockout mice, which in turn suggested that 5-HT1a receptors are required for fluoxetine-induced neurogenesis.

Furthermore, it has been proposed that 5-HT1a agonists stimulate presynaptic receptors in the raphe nuclei, which inhibit the firing rate of serotonergic neurons and consequently reduce the signal at target postsynaptic receptors such as in the hippocampus. This in turn results in an anxiolytic-like effect. The delayed therapeutic activity is believed to result from increased

activation of postmitotic receptors after serotonergic raphe neurons regain their normal firing activity (Blier and Ward 2003).

Recently the group of Azmitia studied the plasticity of postsynaptic 5-HT1a receptors in presence of the antagonist WAY100635 (Abbas et al. 2007). They suggest an antagonist-induced increase in 5-HT1a receptor expression in the adult hippocampus and cortex. In the brain, antagonistic action naturally leads to more receptor expression as a compensating effect. Animal studies by Radley & Jacobs analyzed, that a short-term 5HT1a receptor antagonist treatment decreased the number of BrdU-labeled cells in the hippocampus (Radley and Jacobs 2002), whereas acute stimulation with the selective agonist 8-OH-DPAT significantly increased the proliferation rate of precursor cells in the dentate gyrus (Banasr et al. 2004). The group of Annie Daszuta analyzed the role of 5-HT1a, 5-HT2a and 2c receptor subtypes in depression. They showed a significant increase in the number of BrdU-positive cells in the dentate gyrus and olfactory bulb after acute (4 hours to 48 hours) and chronic (14 days) administration of the 5-HT1a receptor agonist 8-OH-DPAT. Four weeks after the last agonist treatment, BrdU-positive cells are still surviving. Acute 5-HT2a receptor blocking led to a decrease in the number of BrdU-labeled cells in the subgranular zone, whereas chronic 5-HT2c receptor stimulation led to an increase in cell proliferation in the subventricular zone (Banasr et al. 2004).

Concerning depression and hippocampal remodeling, glucocorticoids are known to decrease neurogenesis, and thereby stimulate BDNF mRNA expression. Chronic treatment with 5-HT2a or 5-HT2c receptor agonists caused a downregulation of BDNF mRNA expression in the dentate gyrus (Vaidya et al. 1997). Interestingly, an increase in BDNF expression due to 5-HT depletion might lead to a decrease in the number of newly generated granule cells, which suggests a role of BDNF in mediating serotonin effects.

Table 3. Overview of key studies of serotonergic activity in the brain, emphasizing the hippocampus. (DG, dentate gyrus; SGZ, subgranular zone; SVZ/OB, subventricular zone/olfactory bulb, IS, inescapable shock, ECS, electroconvulsive therapy)

Author	Animals	
Brezun & Daszuta 1999	Rats	Depletion of serotonergic neurons in the raphe nuclei decreased 5-HT fiber density in the DG associated with decreased number of neurons in GCL and SVZ
Brezun & Daszuta 2000	Rats	Raphe grafts restore serotonergic control on precursor cell proliferation and reverse lesion-induced decrease in PSA-NCAM-labeling
Malberg et al., 2000	Rats	Pharmacological studies with SSRIs indicated no acute effect of serotonin, but a delayed increase of the number of proliferating precursor cells in the DG after 14 days
Santarelli et al., 2003	Mice	Beneficial effects of SSRIs require hippocampal neurogenesis / 60% more BrdU+ cells in the DG after 11 days of fluoxetine treatment 5-HT1a receptor knockout mice: 5-HT1a receptors are required for fluoxetine-induced neurogenesis
Malberg & Duman 2003	Rats	Fluoxetine administration reversed (IS)-induced decrease in cell proliferation in a model of depression
Czéh et al., 2001	Tree shrews	Antidepressant treatment blocks the downregulation of adult neurogenesis as a result of exposure to stress
Perera et al., 2007	Non-human primates	ECS treated group showed increased cell proliferation after 4 weeks in the DG without altering maturational fates
Abbas et al., 2007	Rats	Antagonist-induced increase in 5-HT1a receptor expression in hippocampus and cortex
Radley & Jacobs 2002	Rats	Short-term 5-HT1a receptor blockade decreased number of proliferating cells in the SGZ; acute activation increased number of BrdU-positive cells
Banasr et al., 2004	Rats	5-HT1a receptor activation increased neurogenesis in the DG and SVZ/OB; 5-HT2a receptor blockade decreased cell proliferation in the DG; 5-HT2c receptor stimulation increased cell proliferation in the SVZ
Vaidya et al., 1997	Rats	Chronic treatment with 5-HT2a or 5-HT2c receptor agonists caused a downregulation of BDNF mRNA expression in the dentate gyrus

2.4 Hypothesis

The addition of newly generated neurons constitutes an adaptive response to internal and external changes; new cell-cell contacts represent molecular, synaptic, or morphological alterations in individual cells affecting the preexisting circuitry. Both forms of adult neurogenesis originate from different precursor cells, are independently regulated and serve different functions. The highly plastic hippocampus might contribute to a neural reserve,

which allows compensation in situations of functional loss. Adult hippocampal neurogenesis is induced by physical activity and environmental factors. When adult neurogenesis in the dentate gyrus is stimulated, the population of putative transient amplifying progenitor cells is affected. These cells are still able to divide and undergo morphological changes during the phase of DCX expression such as dendritic development, axon extension, and radial migration combined with vertical orientation. DCX-expressing cells in the hippocampus receive their first synaptic input.

Cell genesis in the hippocampal dentate gyrus is also affected by aging (Kuhn et al. 1996; Gould et al. 1999a) stress (Gould et al. 1997; Gould and Tanapat 1999), high levels of corticosteroids (Cameron and Gould 1994), and kainate induced seizure (Parent et al. 1997; Wenzel et al. 2000). These listed factors also affect the density of 5-HT1a receptors in the dentate gyrus as a determinant in serotonin action. Mental disorders, including depression and anxiety, are common diseases in the 21st century. Alterations in the neurotransmitter serotonin have been linked to the pathology of these psychiatric disorders. Multiple studies showed the role of SSRIs in the treatment of depression and anxiety disorders, and the enhanced degree of activity of various 5-HT receptor subtypes. New results in serotonin and depression research suggest a role of neurotrophic factors, e.g. VEGF and BDNF in the behavioral responses to antidepressants.

The aim of my study was to connect principles of neural stem cell biology in the adult hippocampus with mechanisms of serotonergic signaling. Thus, a detailed characterization of the developmental stages of type-2 and type-3 cells in hippocampal neurogenesis is given in the first part of the thesis. In the second part, I will focus on the 5-HT1a and 5-HT2c receptor subtypes as major determinants of serotonin action in the hippocampus. Might the difference of acute and chronic effects on neurogenesis be explained by the activation of different serotonin receptor subtypes? My hypothesis is that adult hippocampal neurogenesis is regulated by the action of serotonin on specific 5-HT receptor subtypes which in turn modulate the activity of precursor cells during their development into granule cells. The expression of these receptors in the adult dentate gyrus, and the interaction and different regulatory effects on proliferation and differentiation of precursor cells has now been investigated. Fluoxetine and specific agonists and antagonists for the 5-HT1a and 5-HT2 receptors were employed to dissect the interplay of these various receptors in regulation of adult hippocampal neurogenesis.

3 Materials and Methods

3.1 Animals and housing conditions

3.1.1 Transgenic mice strains

Three different strains of transgenic mice expressing GFP (green fluorescent protein) were used to investigate various developmental steps (type-1, type-2 and type-3 cells as well as early immature postmitotic neurons) in adult hippocampal neurogenesis. GFP is easily visible in living tissues with fluorescent microscopy, and diffuses freely within the cytoplasm so that the entire cell shape including long processes can be visualized. The generation of transgenic mice expressing GFP driven by regulatory elements of the nestin gene was first described by Yamaguchi et al. (Yamaguchi et al. 2000). Nestin is a class IV intermediate filament expressed in neural progenitor cells (Lendahl et al. 1990), and the gene has been successful used for making transgenic mice. We have previously used this approach to characterize nestin-expressing cells in the dentate gyrus (Kronenberg et al. 2003).

The generation of mice expressing enhanced green fluorescent protein (EGFP) under the DCX promoter has been described previously (Gong et al. 2003). Briefly, in the bacterial artificial chromosome (BAC) transgenic vector, endogenous protein coding sequences have been replaced by sequences encoding the EGFP reporter gene at the transcription part of DCX. The BAC transgenic mouse line was developed within the Gene Expression Nervous System Atlas (GENSAT) BAC Transgenic Project. DCX is a microtubule binding protein, which is used as a transient marker of the neuronal lineage. Breeding with FVBN mice at the animal facility of the MDC (Max-Delbrück-Centrum, Berlin) generated the transgenic offspring.

Glia-like stem cells with astrocytic properties were identified by their expression of GFAP and morphological criteria. GFAP-GFP transgenic mice allow a subtle estimation of astrocytic features as compared to the detection of the GFAP protein, such as radial glia morphology, vascular end-feet and for some cells delicate horizontal processes.

Six to eight weeks-old female C57Bl/6 mice were purchased from Charles River.

3.1.2 Housing conditions

The mice strains were bred at the animal facility of the MDC. Animals were seven to eight weeks old and weighed 18-22g at beginning of the experiments. All experimental animals were female. They were held five per cage under standard laboratory housing conditions with a light/dark cycle of 12 hours each and free access to food and water. All experiments were performed according to national and institutional guidelines and were approved by an official committee (LaGeSo, Berlin, Germany).

Experiment I: Characterization of type-2 and type-3 cells

Twelve nestin-GFP animals were randomly assigned to two experimental groups to allow the investigation of proliferating cells at two different time points after BrdU-labeling (see next chapter). Three adult transgenic GFAP-GFP mice were used and held under standard laboratory conditions. Five DCX-EGFP-expressing animals were used to characterize morphological criteria and precise location of these cells in dentate gyrus and piriform cortex. Nine DCX-EGFP mice were randomly distributed into three groups to investigate the proliferative activity of DCX-EGFP-expressing cells and their progression through developmental stages in the dentate gyrus at three different time points (see next chapter).

Experiment II: Serotonin action in the adult hippocampus

Ten to fourteen animals per experiment were randomly distributed into two groups. One group received the drug, whereas saline was injected to the control group. Six experiments with different pharmacological treatments have been conducted. Each experiment was developed for two different time points (see chapter 3.3.4 “Experimental design”).

3.2 BrdU administration

The thymidine analogon BrdU (5-Bromo-2’-deoxyuridine; SIGMA, Germany) is added during the S-phase into the DNA cell cycle, and can be detect by immunohistochemistry.

BrdU can be verified for two hours, which assist the conclusion of the detection of dividing cells just after exogenous BrdU amplification (Nowakowski et al. 1989; Kuhn et al. 1996). BrdU was dissolved in 0.9% NaCl, filtered (10 mg BrdU/ml), and administered intraperitoneally at a concentration of 50mg/kg body weight once daily (Cameron and McKay 2001). For determination of proliferating cells in nestin-GFP expressing mice, animals were killed 3 hours or 1 week after the BrdU-injection. Proliferative activity in the dentate gyrus of DCX-EGFP mice has been investigated 24 hours or 3 days after a single BrdU injection. One group of animals received a seven-day series of BrdU and was killed at 4 weeks after the first BrdU injection.

The BrdU administration time schedule for "Experiment II: Serotonin action in the adult hippocampus" is shown in chapter 3.3.4.

3.3 Drug treatments and experimental design

The antidepressant fluoxetine and pharmacological specific agonists and antagonists were used to dissect the interplay of various serotonin receptor subtypes in regulation of adult hippocampal neurogenesis. All components were administered intraperitoneally (i.p.).

3.3.1 Fluoxetine

Fluoxetine (Flx) is a widely used drug in the treatment of depression. Flx enhances serotonergic transmission by serotonin reuptake inhibition in the central nervous system. The experimental groups of mice for either determination of proliferating cells or survival of newly generated cells received fluoxetine (10 mg/ kg, dissolved in saline solution, 0.1 ml/20 g), whereas the control groups received an equivalent volume of 0.9% saline (NaCl).

3.3.2 5-HT1a receptor agonist and antagonist

All drugs used for pharmacological manipulation of receptors were purchased from TOCRIS and first dissolved in double distilled water as stock solutions. For the *in vivo*

experiment, the standard selective 5-HT1a agonist 8-OH DPAT (8-hydroxy-2-dipropylaminotetralin hydrobromide) and WAY100135 (S-N-tert-Butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide dihydrochloride), a potent selective 5-HT1a receptor antagonist with partially agonistic affinity, were dissolved in 0.9% saline. Dose calculations were based on (Rodgers and Cole 1994), and (Yoshitake and Kehr 2004). One group of mice received either 8-OH DPAT (1 mg/kg dissolved in saline, 0.1 ml/20 g) or WAY100135 (10 mg/kg dissolved in saline, 0.1 ml/20 g), and the control group received an equivalent volume of saline.

3.3.3 5-HT2 and 5-HT2c receptor agonists and antagonists

Cinanserin hydrochloride (N-(2-((3-(Dimethylamino)propyl)thio)phenyl)-3-phenyl-2-propenamide hydrochloride) as a widely used drug blocks the entire 5-HT2 receptor family. One group of mice received 2 mg/kg body weight of Cinanserin dissolved in saline. Another group of animals was treated with α-Methyl-5-hydroxytryptamine-maleate, which is used as an entire 5-HT2 receptor family agonist with high affinity in the following concentration: 0.5mg/ kg body weight.

The potent and selective 5-HT2c receptor agonist WAY161503 (8.9-dicloro-2.3.4.4a-tetrahydro-1H-pyrazino(1.2-a)quinoxalin-5(6H)-one hydrochloride) has partially 5-HT2a agonistic activity and is used as an antidepressant following systemic administration *in vivo*. Dose calculations were based on (Welmaker et al. 2000) and (Cryan and Lucki 2000). One group of mice received 10 mg/kg per body weight of WAY161503 dissolved saline; the control group received an equivalent volume of saline.

Table 4. Treatment concentrations and mechanisms of the antidepressant fluoxetine and of serotonin receptor agonists and antagonist used for the experiments.

Name of the drug	Mechanism of action	Treatment concentration per body weight
Fluoxetine	antidepressant drug, acts on SERT, serotonin reuptake inhibition	10 mg/ kg
8-OH DPAT	selective 5-HT1a agonist	1 mg/ kg
WAY100135	5-HT1a receptor antagonist (high antagonistic activity, partially agonistic action)	3 mg/ kg
Cinanserin	5-HT2 receptor family antagonist	2 mg/ kg
α-Methyl-5-HT-maleate	5-HT2 receptor family agonist	0.5 mg/ kg
WAY161503	selective 5-HT2c receptor agonist (partially 5-HT2a agonistic activity)	10 mg/ kg

3.3.4 Experimental design

The effect of serotonin on different stages of neuronal development in the adult hippocampal dentate gyrus has been investigated. The experimental design for the fluoxetine (Flx) experiment was split into three parts (Fig. 9 A). The first setting analyses the amount of dividing precursor cells after one day of BrdU injection to investigate an acute effect of the drugs on cell proliferation. The mice were first injected with fluoxetine or 0.9% saline and 24 hours later with BrdU. The animals were then killed after 2 hours. The second part focused on cell differentiation and survival of newly born neurons by a daily drug administration for a period of 21 days. BrdU was given once before initiation of fluoxetine or NaCl. The third part was needed to investigate the effect on precursor cell survival by a single injection of BrdU followed by one injection of fluoxetine 24 hours later, and followed by 20 days of 0.9% saline.

The investigation of the role of serotonin receptor subtypes in adult neurogenesis contained two parts per experiment (Fig. 9 B). In the first part, the effect on cell proliferation has been examined. One group of mice was first injected with selective receptor agonists or antagonists or 0.9% saline. It takes 2 hours to let the drugs take effect. One single BrdU injection followed. Mice were then killed after 24 hours. In the second group of animals BrdU

was given 8 hours before initiation of receptor stimulation or blockade (to let the marker be incorporated by proliferating cells). Cell differentiation and survival was analyzed in a paradigm with one daily agonist or antagonist injection over 7 days.

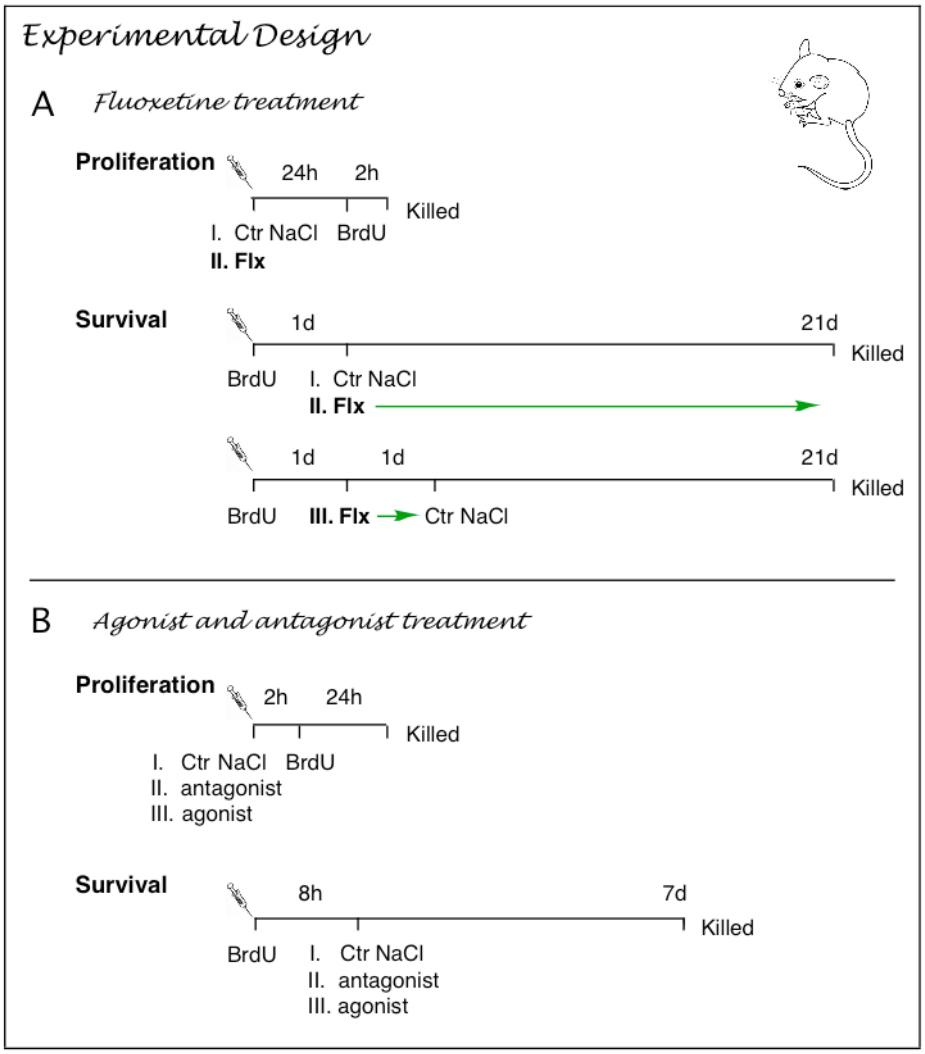


Fig. 9. Experimental design. A. Time course of fluoxetine (Flx) treatment for proliferation and survival experiments. Mice were injected either with NaCl (I, Ctr-group) or Flx (II) once followed by one single injection of BrdU 24 hours later. Animals were killed 2 hours after BrdU injection. The survival paradigm contains two parts: Mice were injected with BrdU 1 day before initiation of either NaCl (I) or Flx (II, long green arrow) for a period of 21 days; a third group received BrdU followed by one single injection of Flx 1 day later (III, short green arrow) followed by NaCl once daily over 20 days. **B.** Time course of serotonin receptor agonist and antagonist treatments for proliferation and survival experiments. Mice were injected once with NaCl (I), the antagonist (II) or agonist (III) followed by one single injection of BrdU after 2 hours, and they were killed 24 hours later. For cell survival animals were injected with BrdU 8 hours before initiation of NaCl, the antagonist or agonist for a period of 7 days.

3.4 Tissue preparation

Animals were deeply anesthetized with ketamine (0,15 ml/20 g body weight) and perfused transcardially with 0.9% NaCl to rinse the blood out of the brain followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (approximately 50 ml per animal) to fix the brain tissue. The brains were dissected from the skulls, postfixed in 4% PFA at 4°C overnight, and transferred into 30% sucrose to eliminate the water for a save cold storage.

Brains were cut on a dry-ice-cooled copper block with a sliding microtome (Leica, Bensheim, Germany) into 40 µm thick coronal sections and stored at –20°C in a cryoprotectant solution containing 25% ethylene glycol, 25% glycerin, and 0.05M phosphate buffer.

3.5 Immunohistochemistry

As a pretreatment, free-floating slices were rinsed in Tris-buffered saline (TBS) for 5 minutes each.

For BrdU staining, DNA was denatured in 2N HCl for 30 minutes at 37°C. Sections were then rinsed in 0.1 M borate buffer and washed in TBS. Primary and secondary antibodies were diluted in TBS containing 0.1% TritonX-100, and 3% donkey serum (TBS+). The detergent TritonX-100 breaks the lipid membrane, whereas donkey serum saturates unspecific bindings of the antibodies.

For immunohistochemistry followed the peroxidase method with biotinylated secondary antibodies, sections were pretreated with 0.6% H_2O_2 and incubated with the secondary antibody for 2 hours at room temperature. ABC reagent (Vectastain Elite, Vector Laboratories) was applied for 1 hour at a concentration of 9 µl/ml. Diaminobenzidine (DAB, Sigma, Germany) was used as a chromogen at the concentration of 0.25 mg/ml in TBS with 0.01% H_2O_2 and 0.04% NiCl. The primary antibody was applied in the following concentration: anti-BrdU (rat, 1:500, Harlan Seralab). The following Biotin-SP conjugated secondary antibodies were used: anti-rat, anti-goat and anti-mouse (donkey, all 1:250, Jackson Immuno; distributor: Dianova).

3.6 Immunofluorescence

For immunofluorescence, one-in-twelve series of brain sections were triple-labeled. Sections were incubated with the primary antibody for 48 hours at 4°C, washed with TBS and TBS+ and incubated with the secondary antibodies in TBS+ for 4 hours at room temperature protected from light. Fluorescent sections were then washed with TBS and coverslipped in polyvinyl alcohol with diazabicyclooctane (DABCO) as anti-fading agent.

The primary antibodies were applied in the following concentrations (Table 5): anti-BrdU (rat, 1:500), anti-DCX (goat, 1:200), anti-GFAP (glial fibrillary acidic protein, guinea pig, 1:1000), anti-NeuN (neuronal nuclear marker, mouse, 1:100), anti-S100β (rabbit, 1:2500), anti-S100β (mouse, 1:1000), anti-GFP (green fluorescent protein, rabbit, 1: 400), anti-GFP (goat, 1:1000), anti-GFP (mouse, 1:400), anti-Calretinin (goat, 1:250), anti-NG2 (rabbit, 1:200), anti-Parvalbumin (mouse, 1:500), anti-BLBP (brain lipid-binding protein, rabbit, 1:2000), anti-Sox2 (rabbit, 1:1500), anti-Ki67 (rabbit, 1:500), anti-Glut1 (blood vessel capillary marker, goat, 1:100).

The antibodies for serotonin and the receptors were used under the following conditions: anti-Serotonin (rabbit, 1:5000, Sigma), anti-Serotonin Receptor 1a (guinea pig, 1:500, Chemicon), anti-Serotonin Receptor 2a (rabbit, 1:200, Sigma, Abcam), anti-Serotonin Receptor 2c (rabbit, 1:250, Chemicon).

For immunofluorescence, the antigen-primary antibody-complexes were marked with a secondary antibody, which was coupled on a specific fluorescent molecule and enhance the signal. The following secondary antibodies were used: anti-goat FITC, anti-rabbit FITC, anti-mouse FITC, anti-rat Rhodamine RedX, anti-goat Cy5, anti-rabbit Cy5, anti-mouse Cy5 and anti-guinea pig Cy5 (donkey, 1:125, Jackson Immuno; distributor: Dianova).

Table 5. Primary antibodies for immunofluorescence. Alphabetically listed in according to description, company and dilution.

Antigen	Description	Dilution	Company
BLBP	Rabbit,	1:2000	Gift from Nathaniel Heintz, The Rockefeller University, NYC
BrdU	Rat, IgG	1:500	Harlan Seralab, Biolzol
Calretinin	Goat, polyclonal	1:250	Swant
Doublecortin (C-18)	Goat, polyclonal	1:250	Santa Cruz Biotechnologies
GFAP	Guinea pig, polyclonal	1:1000	Advanced ImmunoChemical
GFP	Goat Rabbit, IgG Mouse, IgG	1:1000 1:400 1:400	Acris Antibodies (DPC Biermann)
Glut1	Goat, polyclonal	1: 100	Chemicon
Ki67	Rabbit, polyclonal	1:500	Novocastra (NCl-Ki67)
NeuN	Mouse, IgG	1:100	Chemicon
NG2	Rabbit, polyclonal	1:200	Swant
Parvalbumin	Mouse, monoclonal	1:500	Swant
S100β	Rabbit, polyclonal Mouse, monoclonal	1:2500 1:1000	Swant SIGMA
Serotonin, 5-HT	Rabbit, polyclonal	1:5000	SIGMA
5-HT1a	Guinea pig, polyclonal	1:500	Chemicon
5-HT2a	Rabbit, polyclonal	1:250	SIGMA
5-HT2c	Rabbit, polyclonal	1:250	Chemicon
Sox2	Rabbit, polyclonal	1:1500	Chemicon

3.7 Quantification and imaging

One-in-six series of the coronal sections of each brain were stained with BrdU followed the peroxidase method, and immunoreactive cells were counted throughout the rostrocaudal

extent of the dentate gyrus with a light-optical microscope (Leica DME, Fig. 10 A). Results were multiplied by six to obtain the total number of BrdU-positive cells per granule cell layer.

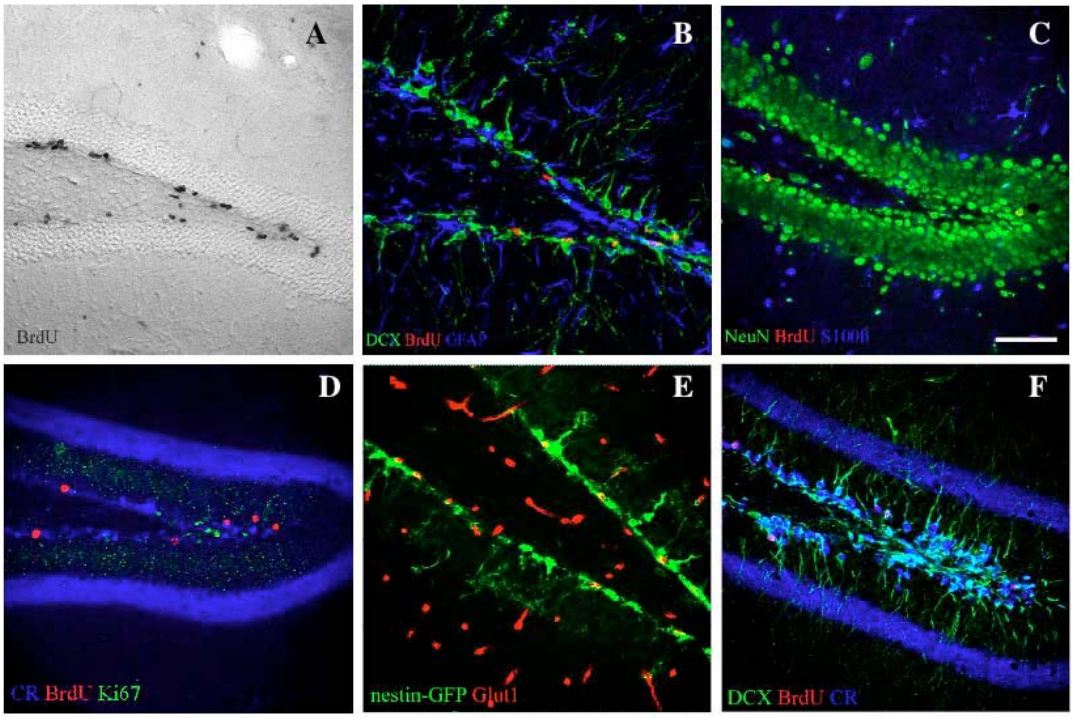


Fig. 10. Examples for immunohistochemistry and immunofluorescent stainings. A. BrdU staining in the adult mouse dentate gyrus followed the peroxidase method; BrdU is added during the S-phase into the DNA cell cycle; proliferating cells form cluster in the subgranular zone. **B and C**. Triple-labeling of newly generated cells in the adult dentate gyrus; slices were typically stained with DCX/BrdU/GFAP to investigate the fate choice of proliferating cells one day after they were born (B), and with NeuN/BrdU/S100β to examine the distribution of surviving cells due to their phenotype. **D-F.** Additional stainings were made to investigate transient Calretinin (CR) expression (D, F), and co-labeling with endothelial cells (Glut1, E). Scale bar 150 µm

One-in-twelve series of sections were triple-labeled. For phenotypic analysis of BrdU-labeled cells, 100 randomly selected cells per animal were analyzed for co-staining with glial or neuronal markers, using a Leica TCS SP2 confocal microscope. The confocal fluorescence microscopy allows the determination and construction of thin (1 µm) optical slices. BrdU/DCX/GFAP staining was used to estimate the phenotype of proliferating cells after one day of drug treatment (proliferation experiment). The phenotype of newly generated cells after three days, seven days or 4 weeks (differentiation or survival experiment) was determined with BrdU/NeuN/S100β co-labeling (Fig. 10 B, C). For investigations of

specified developmental steps within neurogenesis additional stainings were made, e.g. CR/BrdU/Ki67 and DCX/BrdU/CR (Fig. 10 D, F). All images were taken in sequential scanning mode and processed in Adobe Photoshop 7.0 for Macintosh, where input levels were adjusted to the given distribution.

4 Results

4.1 Transgenic GFP-expressing mice represent a powerful tool to visualize specific cell types

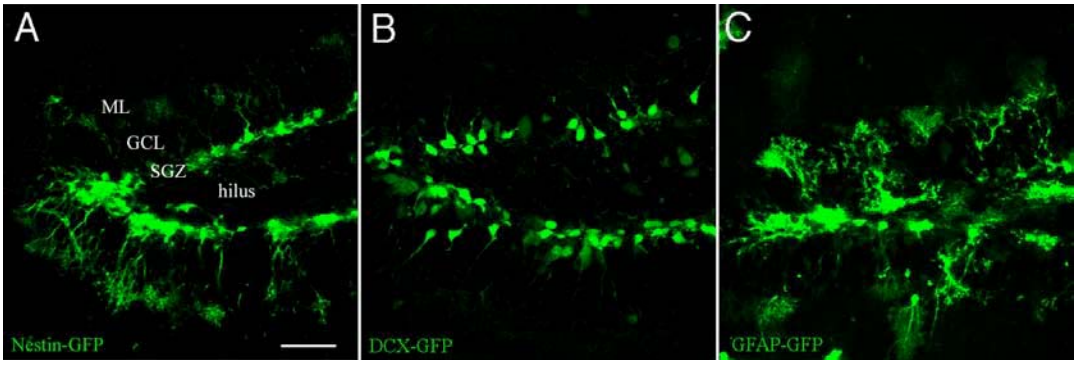


Fig. 11. The dentate gyrus of three different transgenic GFP-expressing mice strains. A. Nestin-GFP-expressing putative stem or progenitor cells reside in the subgranular zone. One subtype is characterized by a long process, which reaches the molecular layer, Scale bar 120 µm. **B.** DCX-EGFP-expressing cells in the subgranular zone represent early steps of neuronal development. Furthermore, weak GFP expression was found in hilar interneurons. **C.** GFAP-GFP-expressing astrocytic-like type-1 cells show radial morphology and vascular end feet. Their processes reach the molecular layer. GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.

In the first part of my study, I characterized type-2 and type-3 cell populations in adult hippocampal neurogenesis using reporter gene mice expressing GFP under the promotor for nestin, GFAP or DCX (Fig. 11 A-C). Nestin expressing putative stem or progenitor cells form cluster along the subgranular zone (Fig. 11 A). One subtype shares features with mature astrocytes such as a characteristic long process (type-1). Furthermore, we have previously described a second subtype as type-2a glial cells, whereas a third subtype mirrors morphological and electrophysiological properties of early neuronal development (type-2b). GFAP-GFP mice were used to characterize an astrocytic-like type 1 cell with low proliferative activity, a typical morphology, e.g. vascular end feet and processes reaching the molecular layer (Fig. 11 C). Whereas type-2b cells in nestin-GFP mice co-express markers of

the neuronal lineage like DCX, in GFAP-GFP mice expression of GFP and DCX was mutually exclusive. Early steps of neuronal development are characterized by DCX expression. In the DCX-EGFP transgenic mice, strong EGFP expression was observed in the nucleus and soma of granule cells and some hilar interneurons, whereas dendritic trees entering the molecular layer showed a weak EGFP-expression (Fig. 11 B).

4.2 Type-2 cells represent an intermediate precursor cell stage

In the following chapter, various markers expressed in and associated with early developmental stages will be characterized in more detail.

The transcription factor gene Sox2 is expressed in proliferating CNS precursor cells and downregulated during their final cell cycle thus inhibits terminal neuronal differentiation. This study at first characterized the expression of Sox2 in type-1, type-2 and type-3 cells of the adult subgranular zone (Fig. 12). The expression of Sox2 during different developmental stages was estimated by characterizing co-expression with nestin-GFP or DCX. Glia-like stem cells with radial morphology (type-1) were also characterized in the transgenic GFAP-GFP mice. Of 100 Sox2-positive cells in the dentate gyrus, 23.4% were type-1 cells with radial morphology and 30.9% were type-2a cells, whereas only 2.7% were type-2b cells, and 1.3% type-3 cells (Fig. 14 A), defined by co-expression of DCX (Fig. 12 A). The results implied that more than 40% of all Sox2-positive cells did not show a co-expression with either nestin-GFP or DCX. Inversely, 94.1% of all nestin-GFP-positive type-1 cells expressed Sox2, 82.0% of type-2a cells, and 25.5% of type-2b cells (Fig. 12 B, 14 B). However, only 6.5% of all DCX-expressing cells showed an overlap with Sox2 and approximately half of these cells were nestin-GFP-positive type-2b cells (Fig. 14 C). These results demonstrate that putative precursor cells in the subgranular zone of the adult hippocampus express stem cell-marker Sox2, whereas type-3 cells displaying early neuronal features mostly lack Sox2. The down-regulation of Sox2 thus occurs at the transition level between type-2a and type-2b cells, in coincidence with the increase in DCX expression. The remainder of Sox2-expressing cells was largely co-labeled with the astrocytic marker S100β. Some of these S100β-positive cells resided in the subgranular zone; however, most were found in the molecular layer of the adult dentate gyrus (Fig. 12 C, C′).

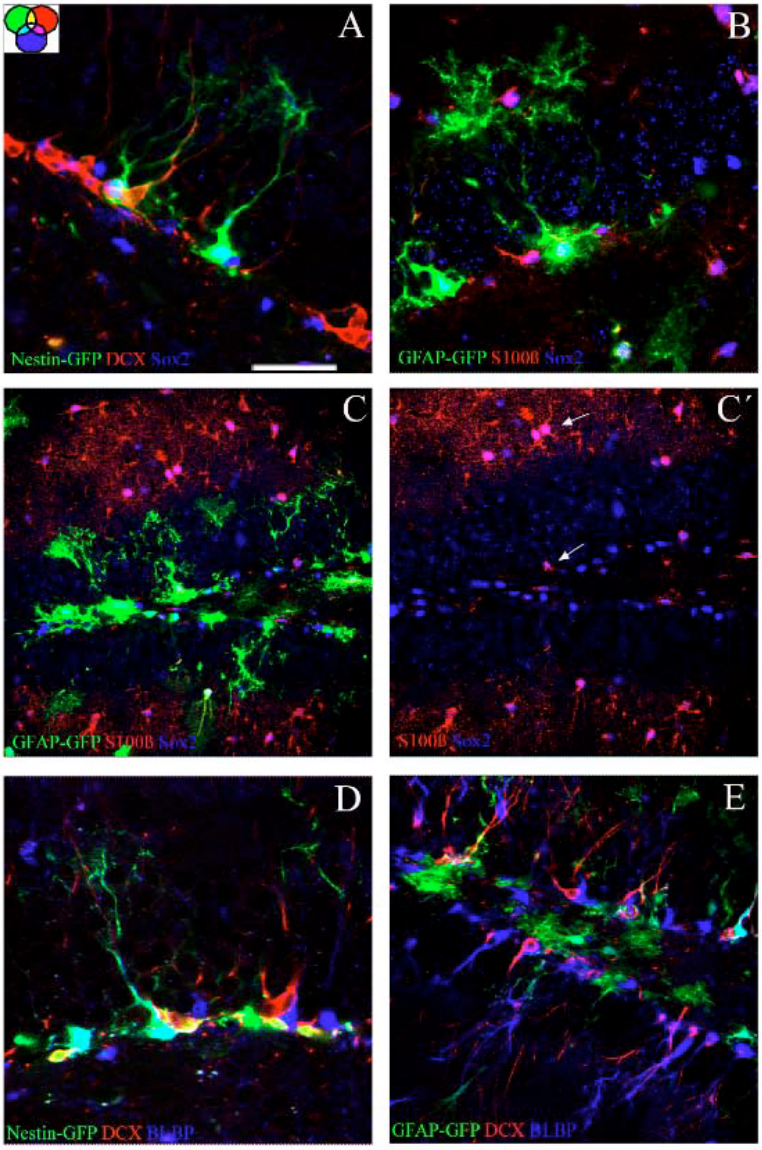


Fig. 12. Precursor cells and lineage marker expression in the adult dentate gyrus. A. Nestin-GFP (green), doublecortin (DCX, red) and Sox2 (blue) show varying degrees of overlap in the subgranular zone, Scale bar 40 µm. **B-C´.** All radial glia-like type-1 cells are Sox2-positive (GFAP-GFP in green, S100β in red, Sox2 in blue). **C/C´.** Essentially all S100β-positive astrocytes in the dentate gyrus are Sox2-positive (arrows in C´, Scale bar 100 µm). **D-E.** The radial glia maker BLBP identifies both type-1 cells with their radial morphology and type-2 cells, which lack the radial process (type-2 cells appearing yellow in D; nestin-GFP is in green, DCX in red, BLBP in blue). Analysis of the GFAP-GFP and nestin-GFP transgenic mice revealed that radial glia-like cells are BLBP-positive. GFAP-GFP and DCX are mutually exclusive (E, GFAP-GFP in green, DCX in red, BLBP in blue). *Published in (Steiner et al. 2006)*

The brain lipid binding protein BLBP is transiently expressed in radial glia during development and actively blocks differentiation, presumably downstream of Notch signaling

(Feng et al. 1994; Anthony et al. 2005). In the adult dentate gyrus, almost all type-1 cells express BLBP supporting the radial-glia like nature of type-1 cells (Steiner et al. 2006). Furthermore, type-2 cells showed BLBP in co-expression with nestin-GFP and DCX (Fig. 12 D-E). Systemic application of BrdU to study the proliferative activity of radial glia-like cells revealed that type-1 cells, which are co-labeled with nestin-GFP and BLBP represented a cell population with low proliferative activity (less than 5%). The majority of BrdU+/nestin-GFP+/BLBP-positive cells 24 hours after a single injection of BrdU are type-2 cells. Furthermore, 50% of the proliferating BLBP population (12% of all BrdU-positive cells) is comprised of nestin-GFP-negative cells without radial glia-like shape (Fig. 14 D). These results suggested that nestin-GFP expression might indicate an actively dividing cell state (activated precursor cells).

Radial glia-like stem cells (type-1) give rise to highly proliferative type-2 cells, which still express BLBP and Sox2. Like Sox2, BLBP is a maintenance gene that preserves the precursor cell pool. On the stage of transiently amplifying progenitor cells in the dentate gyrus type-2 cells represent an intermediate precursor cell stage. We described an overlap in the expression of markers associated with early progenitor cells (nestin-GFP, Sox2 and BLBP), and with the neuronal lineage (DCX), which needs to be further distinguished in a pre-neuronal stage (type-2a), a transitional stage (type-2b) and an early neuronal stage (type-3), all associated with cell proliferation (Fig. 13).

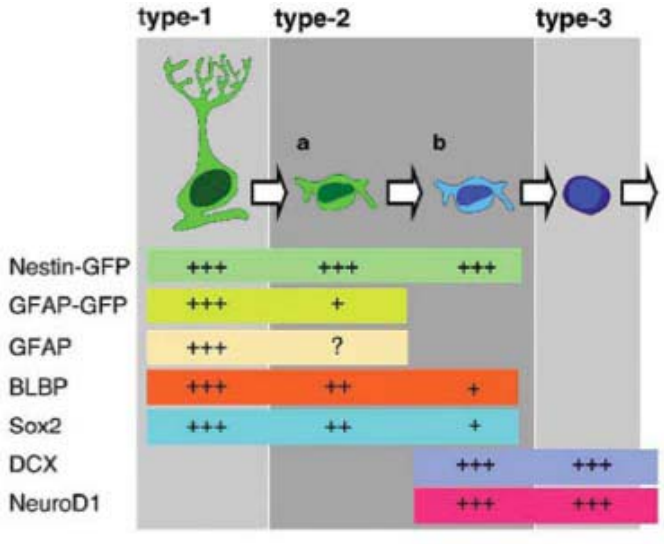


Fig. 13. Scheme of marker expression within developmental steps of adult neurogenesis. This is a qualitative summary of the findings, high lightening the transition from type-2a to type-2b cells in the dentate gyrus. *Published in (Steiner et al. 2006)*

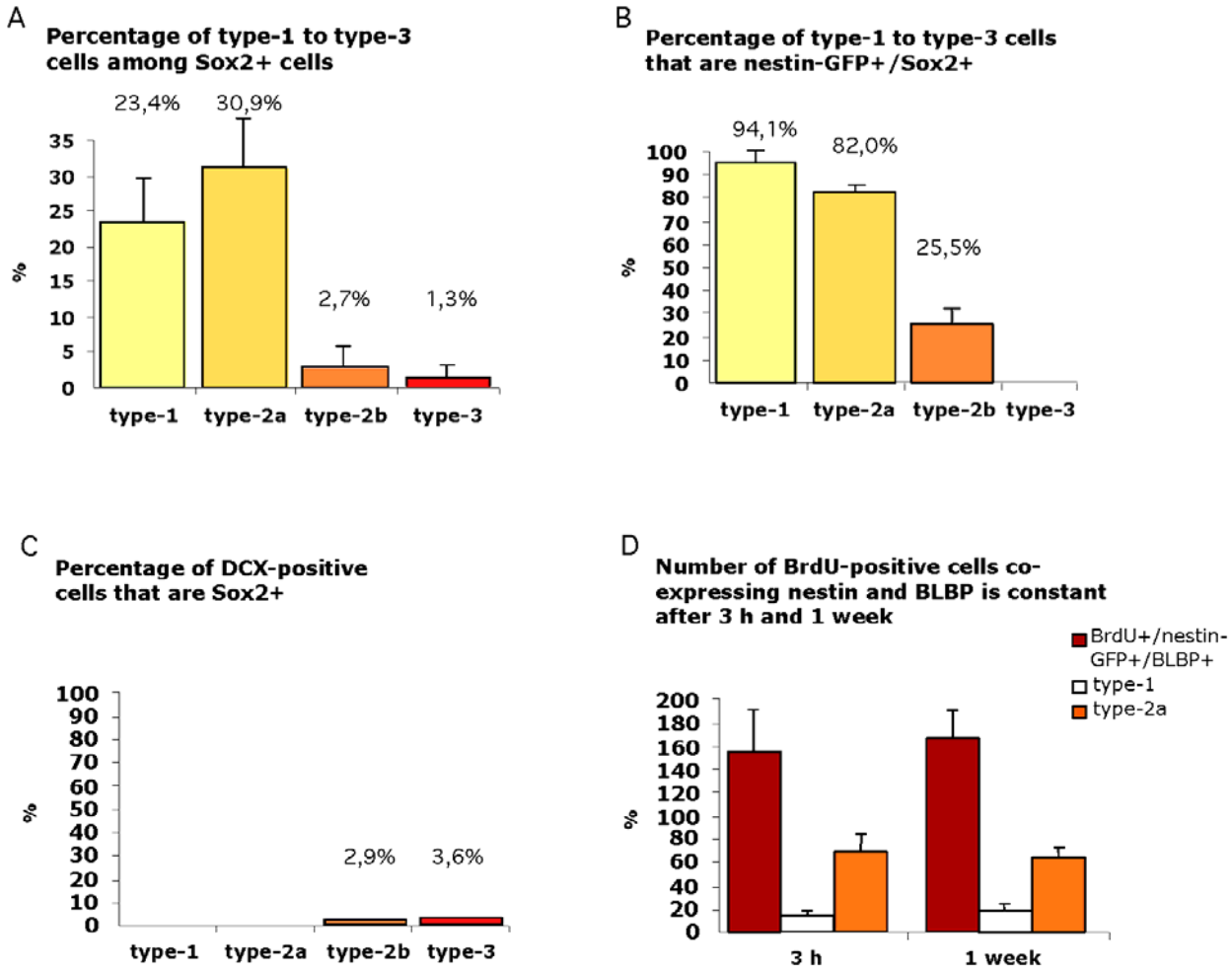


Fig 14. Quantitative results for Sox2 and BLBP expression in the adult dentate gyrus. A. The graph shows the distribution of 100 Sox2-positive cells into the different categories of precursor cells *in vivo*. 41% of Sox2-positive cells do not belong to the precursor cells. More than the half of these cells is S100β-positive astrocytes (not shown here). **B.** Here, the precursor cells as defined by nestin-GFP expression are taken as the basis of the analysis; essentially all type-1 cells are Sox2-positive and 4/5 of type-2 cells. One quarter of DCX-positive type-2b cells is Sox2-positive. Because type-3 cells are by definition nestin-GFP-negative they are not listed here. **C.** Here, DCX-expressing cells are taken as the basis of the analysis; only a very small percentage of DCX-positive cells co-express Sox2. **D.** The actively dividing precursor cell population represents only a small subset of all BrdU+/BLBP+/nestin+ cells in the subgranular zone. The size does not change between 3 h and 1 week after BrdU, which is consistent with the idea that these cells constitute an asymmetrically dividing group. *Published in (Steiner, Klempin et al. 2006)*

4.3 Doublecortin expression as an indicator for adult hippocampal neurogenesis

Type-2b and 3 cells mirror a marker progression during earliest neuronal development, which can be influenced by different intrinsic factors and environmental stimuli. When adult hippocampal neurogenesis is stimulated, the most significant expansion occurs in the population of transiently amplifying progenitor cells that express DCX or PSA-NCAM (Type-2b, and 3). In the adult mouse brain strong DCX-EGFP expression was observed in the subgranular zone of the dentate gyrus, in some migrating cells of the inner granule cell layer, and in layer II and III of the non-neurogenic piriform cortex. Furthermore, EGFP is expressed in the subventricular zone and corpus callosum, whereas a fainter signal was observed in striatum. In the adult dentate gyrus EGFP is expressed in the nucleus and soma of cells located in the subgranular zone, the inner granule cell layer, and also in hilar interneurons. As compared to direct staining against DCX, transgenic DCX-EGFP mice displayed only weak EGFP-expression in dendritic trees arborizing in the molecular layer (Fig. 15). Analysis of co-labeling of DCX protein showed an overlap of 90% in the subgranular zone.

Characterizations of developmental stages of DCX-EGFP-positive cells in the adult dentate gyrus according to a previously described classification (Kempermann et al. 2004a) confirmed that DCX is expressed transiently during type-2b and type-3 cell stages of neuronal development as well as in immature postmitotic neurons, yet is absent from mature granule cells and glia-like type-1 stem cells. We described previously that approximately 20% of the actively dividing precursor cell population in the dentate gyrus (type 2b cells), and a large number (70%) of type-3 cells and more mature neurons express DCX in concert with Calretinin (Brown et al. 2003b; Plumpe et al. 2006). In the transgenic mice, 72±6 cells out of 200 DCX-EGFP cells analyzed display a co-labeling with early postmitotic marker Calretinin (36%; Fig. 16 A, 17 D), conversely 70% of Calretinin-positive cells also express EGFP (140±6 cells out of 200 CR+ cells). Approximately 14% of DCX-EGFP cells co-expressed neuronal marker NeuN (Fig. 16 A, 17 C; 28±3 out of 200 cells). The morphology suggests these cells as immature neurons with short and thin processes. In the hippocampus, transgenic DCX-EGFP expression, thus seem to visualize primarily the actively dividing precursor cell stages of type-2a and type-2b cells with less overlap with CR and NeuN.

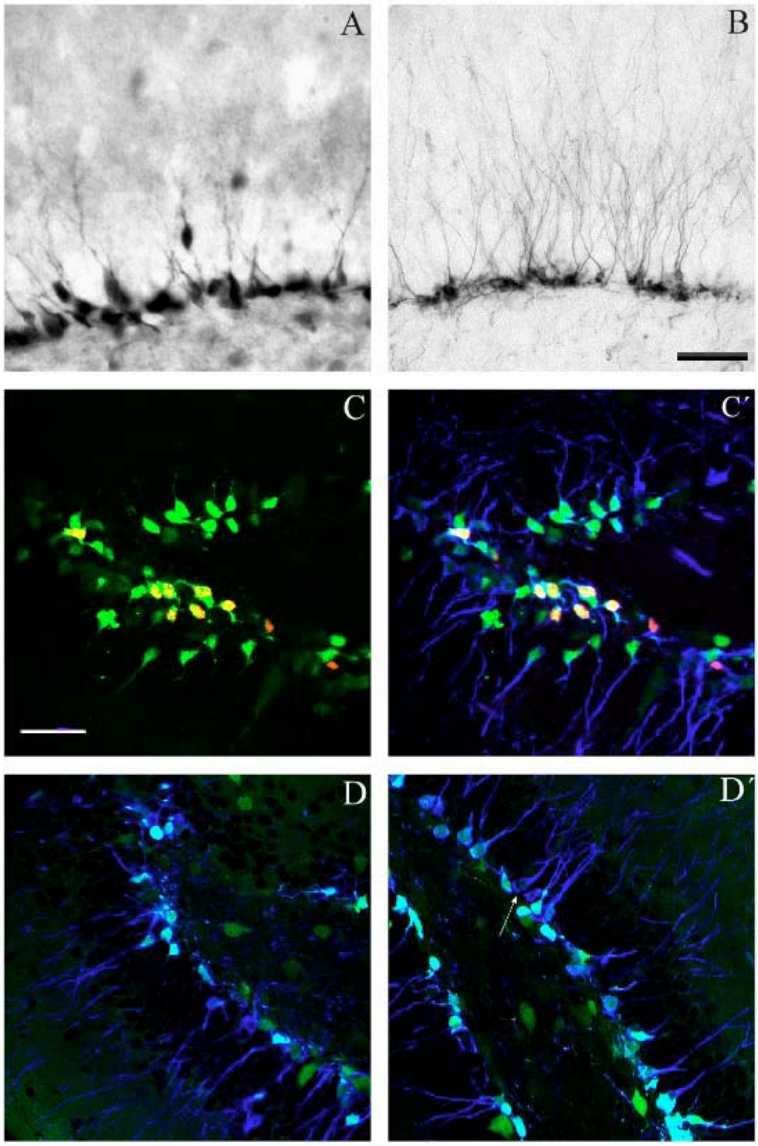


Fig. 15. Features of the DCX-EGFP transgenic mouse in comparison with DCX-antibody staining in the adult dentate gyrus. DCX-EGFP (green) and DCX-protein (blue) show approximately 90% overlap in the subgranular zone (C-D, Scale bar 80 µm). **A, B.** Whereas EGFP-expression in the transgenic mice is restricted to the soma and proximal processes (A), DCX-protein expression (B) can be traced up to the molecular layer revealed by immunohistochemistry, Scale bar 40 µm. **C, C´.** Roughly 80% of DCX-EGFP cells in the subgranular zone incorporate BrdU (in red) and almost all of them are horizontal type-2 cells. **D, D´.** There is weak DCX-EGFP staining in the hilus. Some of the vertical DCX-positive cells with longer processes (postmitotic cell stage, dendrites are branching into the molecular layer) show no overlap with DCX-EGFP (D´, arrow).

10% of the green fluorescent hilar interneurons of DCX-EGFP mice also showed an overlap with parvalbumin (Fig. 16 A, 17 F), a marker for basket cells (12±2 cells out of 110). Both DCX and DCX-EGFP were not expressed in multipotent neural stem cells or glia cells,

and constitute a population distinct from GFAP-expressing cells (type-1; Fig. 16 A, 17 B) (Nacher et al. 2001; van Praag et al. 2002; Brown et al. 2003b). Further phenotypic analysis of DCX-EGFP cells also revealed no overlap with the astrocytic marker S100β (Fig. 16 A, 17 A). Some of the fainter EGFP-positive cells in the hilus and subgranular zone showed a weak overlap with the proteoglycan NG2 (Fig. 17 E).

The proliferative activity of DCX-EGFP-expressing cells and the progression trough developmental stages in the dentate gyrus was estimate using S-phase marker BrdU (Fig. 16 B). After 1 day of a single BrdU injection roughly 73% and after 3 days 80% of the DCX-EGFP-expressing cell population in the subgranular zone had incorporated BrdU. Most of these cells could be classified as horizontal type-2 cells. 3 weeks after a seven-day series of BrdU, 85% of DCX-EGFP-expressing cells were dividing. Furthermore, there was weak overlap of approximately 10% of BrdU+/DCX-EGFP-positive cells with Calretinin but not NeuN as postmitotic markers (Fig. 16 B). The transgenic DCX-EGFP mice showed weak EGFP expression in processes and no BrdU-staining in migrating cells either after 1 day or 4 weeks after BrdU-labeling. This indicates that the DCX promotor is downregulated in non-dividing cells of categories E (when one strong apical dendrite is branching into the molecular layer) and F (when delicate dendritic trees branching into the granule cell layer) of DCX-protein-immunoreactive cells (postmitotic stage) (Plumpe et al. 2006).

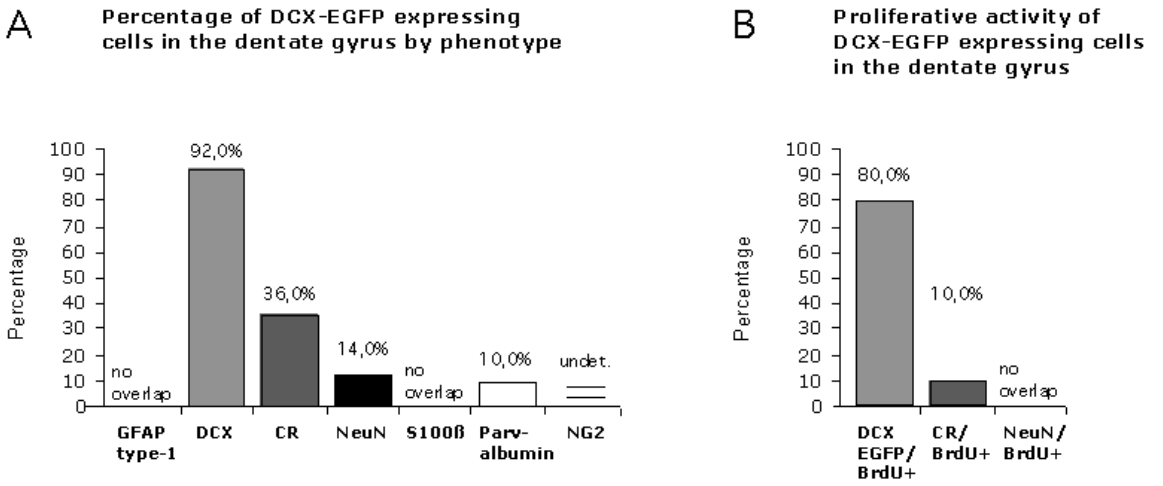


Fig. 16. DCX-EGFP-expressing cells in the adult dentate gyrus. A. Distribution of DCX-EGFP-expressing cells by phenotype. Co-labeling of DCX-EGFP with DCX-protein revealed 92% overlap. EGFP signaling goes down with neuronal marker expression: DCX-EGFP/CR 36%, DCX-EGFP/NeuN 14%. DCX-EGFP is not expressed in glia-like stem cells (type-1, GFAP expression), and not in mature astrocytes (S100β). Some of the fainter signal was observed in basket cells (10% overlap with Parvalbumin) and oligodendrocytes (NG2, undetermined). **B.** Approximately 80% of DCX-EGFP-positive cells were dividing 3 days after BrdU, a weak co-labeling was observed with Calretinin (10%), but not NeuN as postmitotic marker.

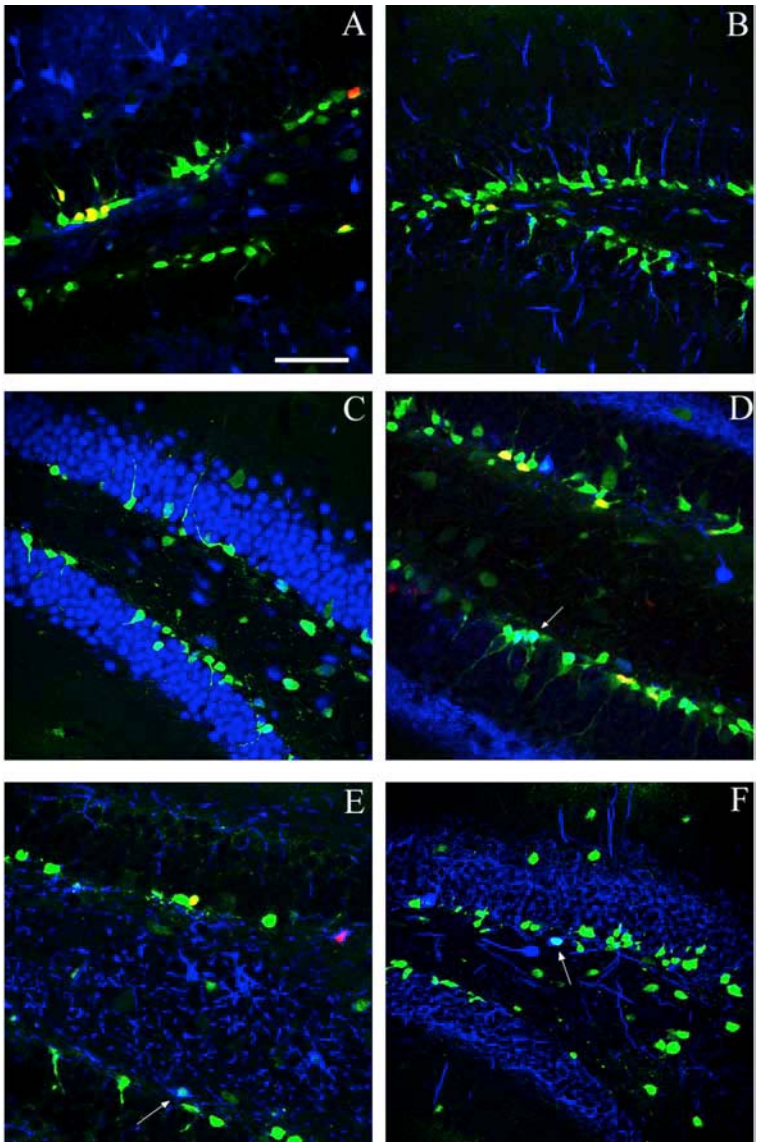


Fig. 17. Confocal microscopy of DCX-EGFP-expressing cells in overlay with precursor cell marker and lineage marker expression in the adult dentate gyrus. A, B. DCX-EGFP cells show no overlap with the astrocytic marker S100β (A, blue, Scale bar 150 µm), and the stem cell/glia marker GFAP (B, blue). **C-E.** Some of the DCX-EGFP-expressing cells overlap with the neuronal lineage-determined cell marker Calretinin (D, arrow, BrdU in red), NeuN (C, blue), and the oligodendrocytic marker NG2 (E, blue, arrow). **F.** Some Parvalbumin-expressing basket cells in the hilus show an overlap with DCX-EGFP (F, blue, arrow).

Beside the hippocampus, strong DCX-EGFP expression occurs in the non-neurogenic piriform cortex. So far, based on morphological criteria and precise location, DCX-expressing cells in the piriform cortex have been grouped into the following categories: neurogliaform

cells, pyramidal-semilunar neurons and large interneuron-like neurons (Haberly 1983; Nacher et al. 2001). In tissue sections of transgenic mice DCX-EGFP expression was observed in all of these three cell types. Immunoreactivity revealed strong EGFP expression in neurogliaform cells, which form characteristic clusters in layer II (Fig. 18 A). Approximately 64% of these DCX-EGFP-expressing neurogliaform also revealed DCX-immunoreactivity. These clusters of DCX-EGFP neurogliaform cells are often found close to DCX-positive cells, which where stained with the protein (Fig. 18 B). Postmitotic neuronal markers CR and NeuN are rarely expressed in neurogliaform cells (30% were CR-positive, 25% were NeuN-positive cells (Fig. 18 C-D). EGFP signal intensity decreases with postmitotic neuronal marker expression.

Semilunar-pyramidal neurons displayed only weak EGFP fluorescence intensity. These cells are characterized by two apical processes extending into layer I (Fig. 18 A). The piriform cortex receives afferent fibers from the olfactory bulb and other cortical regions (association fibers) where they predominantly synapse with semilunar-pyramidal cell spines in layer I (Haberly and Feig 1983). Detailed phenotypic analysis of semilunar-pyramidal DCX-EGFP cells revealed a high percentage (60%) of NeuN co-expression (Fig. 18 D). Only one third of semilunar-pyramidal DCX-EGFP cells displayed co-labeling with DCX (Fig. 18 B), and 20% were CR-positive (Fig. 18 C). The third population of DCX-EGFP cells composed of large interneuron-like cells was mainly located in layer III and showed weak EGFP expression. No overlap with the DCX-protein was observed. However, all DCX-EGFP-positive large interneuron-like cells expressed NeuN (Fig. 18 D).

None of these cells of the different cell types in the piriform cortex was BrdU-positive either after 1 day, 3 days or 3 weeks postinjection. (Fig. 18 E). In contrast, proliferating cells in the neurogenic region dentate gyrus become mature neurons within 3 weeks (van Praag et al. 2002; Plumpe et al. 2006) and migrate into the inner granule cell layer. BrdU-positive cells are found in the piriform cortex detected in some S100β-positive cells in layer II (Fig. 18 F).

In summary, in the adult brain, DCX expression is retained in areas of continuous neurogenesis, and is also observed in non-neurogenic regions, such as the piriform cortex. DCX-expressing cells do not constitute a homogeneous population. Whereas DCX is expressed in proliferating and migrating precursor cells in the adult hippocampal dentate gyrus, DCX expression in the piriform cortex can be detected in three cell types with distinct morphology.

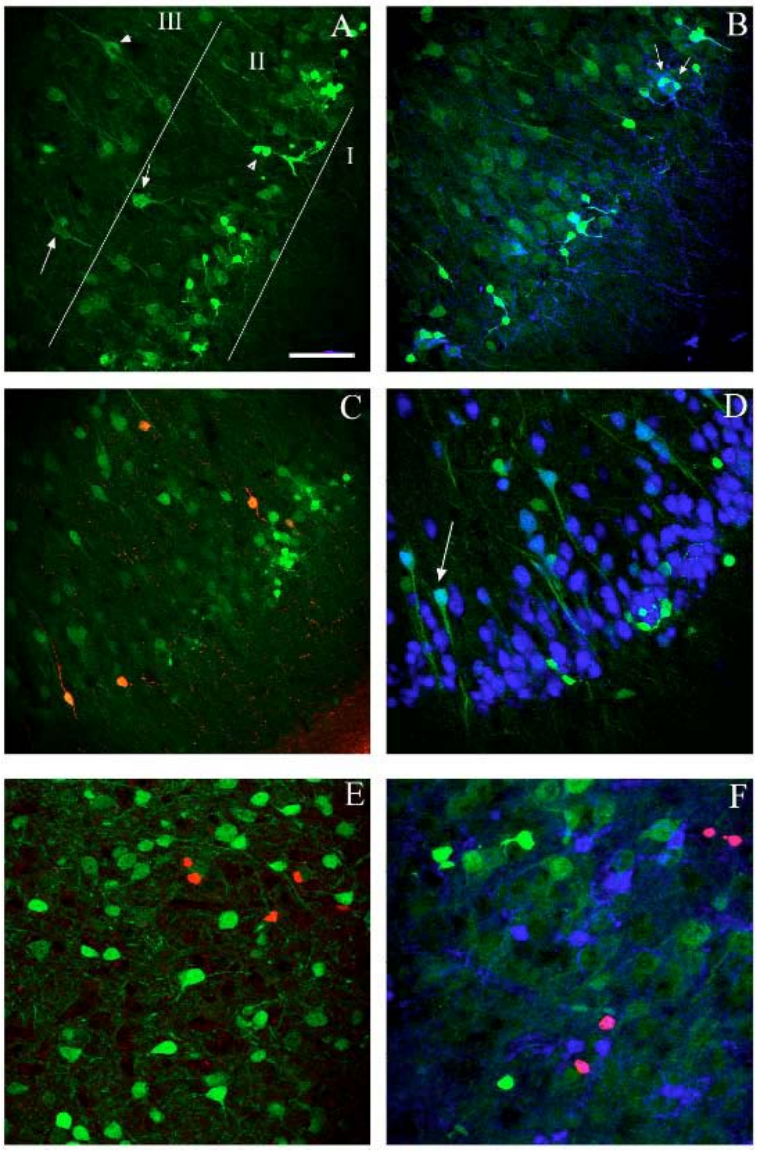


Fig. 18. Features of the transgenic DCX-EGFP mouse in the piriform cortex layer II and III. A-F. DCX-EGFP (green) expression in the adult piriform cortex approximately at Bregma –0.82/ 2.98. DCX-EGFP-expressing cells with round somata, short processes and irregular trajections form cluster in layer II (neurogliaform, arrowhead in A), and show approximately 64% overlap with the DCX-protein or build cluster surrounding DCX-positive cells (blue, arrows in B). Two or three apical processes towards layer I characterize semilunar-pyramidal neurons in layer II and III (short arrows in A), and show one third overlap with the DCX-protein. Large interneuron-like cells in layer III show no overlap with DCX. **C.** Calretinin is expressed in some of the neurogliaform cell population as well as in semilunar-pyramidal neurons in layer II and III. **D.** Large interneuron-like cells are always co-labeled with NeuN, approximately 60% of pyramidal-semilunar neurons and 25% of neurogliaform overlap with NeuN. **E-F.** None of these cells was BrdU-positive (E); BrdU-positive cells are found in the piriform cortex detected in some S100_-positive cells in layer II (F); Scale bar 60 µm (A-D), 20 µm (E and F).

Serotonin action in the adult hippocampus

4.4 The adult dentate gyrus receives serotonergic input

The hippocampal dentate gyrus is a major efferent target of the raphe nuclei. During embryonic development the hippocampus receives dense serotonergic fiber afferents suggesting a role for regulation of hippocampal development by serotonin. Immunohistochemistry in coronal brain sections of adult wild-type mice showed the presence of serotonergic fibers, which terminate in the molecular layer and the hilus of the dentate gyrus, and they are in proximity to the subgranular zone (Fig. 19). Immunohistochemistry with a selective 5-HT2a receptor antibody shows a dense staining in the hilus (Fig. 19 B, 20 C) whereas staining for 5-HT2c receptors marks the granule cell layer of the dentate gyrus (Fig. 20 D). The staining might indicate two different serotonergic fiber projections into the hippocampus originating from different raphe nuclei as described in the *introduction*: serotonergic fibers from the dorsal raphe nuclei (DRN) enter the entorhinal cortex and terminate in the molecular layer (De Foubert et al.) of the dentate gyrus, whereas those from the median raphe nuclei (MRN) project mainly to the hilus.

Furthermore, immunohistochemistry revealed that 5-HT1a receptors are expressed in neurons both in hilus and granule cell layer, and also in the subgranular zone of the dentate gyrus (Fig. 19 A). The images show a 5-HT1a receptor co-labeling with DCX-EGFP in the subgranular zone of the adult hippocampus (Fig. 20 B). In cooperation with Harish Babu I was further able to show that proliferating precursor cells in stem cell culture also display intense 5-HT1a receptor expression (Fig. 20 A).

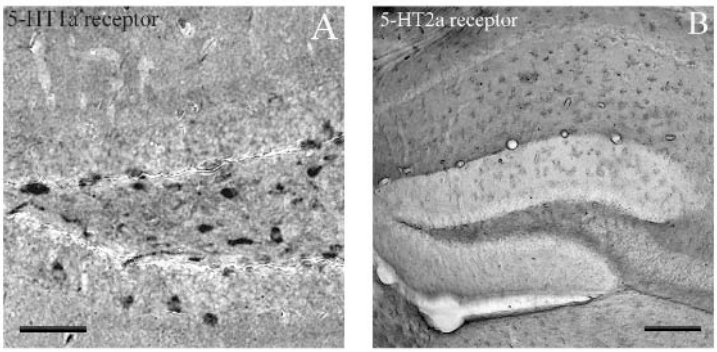


Fig. 19. Serotonin receptor staining in the adult dentate gyrus. A. The 5-HT1a receptor is expressed in neurons both in the hilus and granule cell layer, and also in the subgranular zone. In contrast, 5-HT2a receptor staining (**B**) shows a dense fiber staining in the hilus; Scale bar in A 80 µm, in B 200 µm.

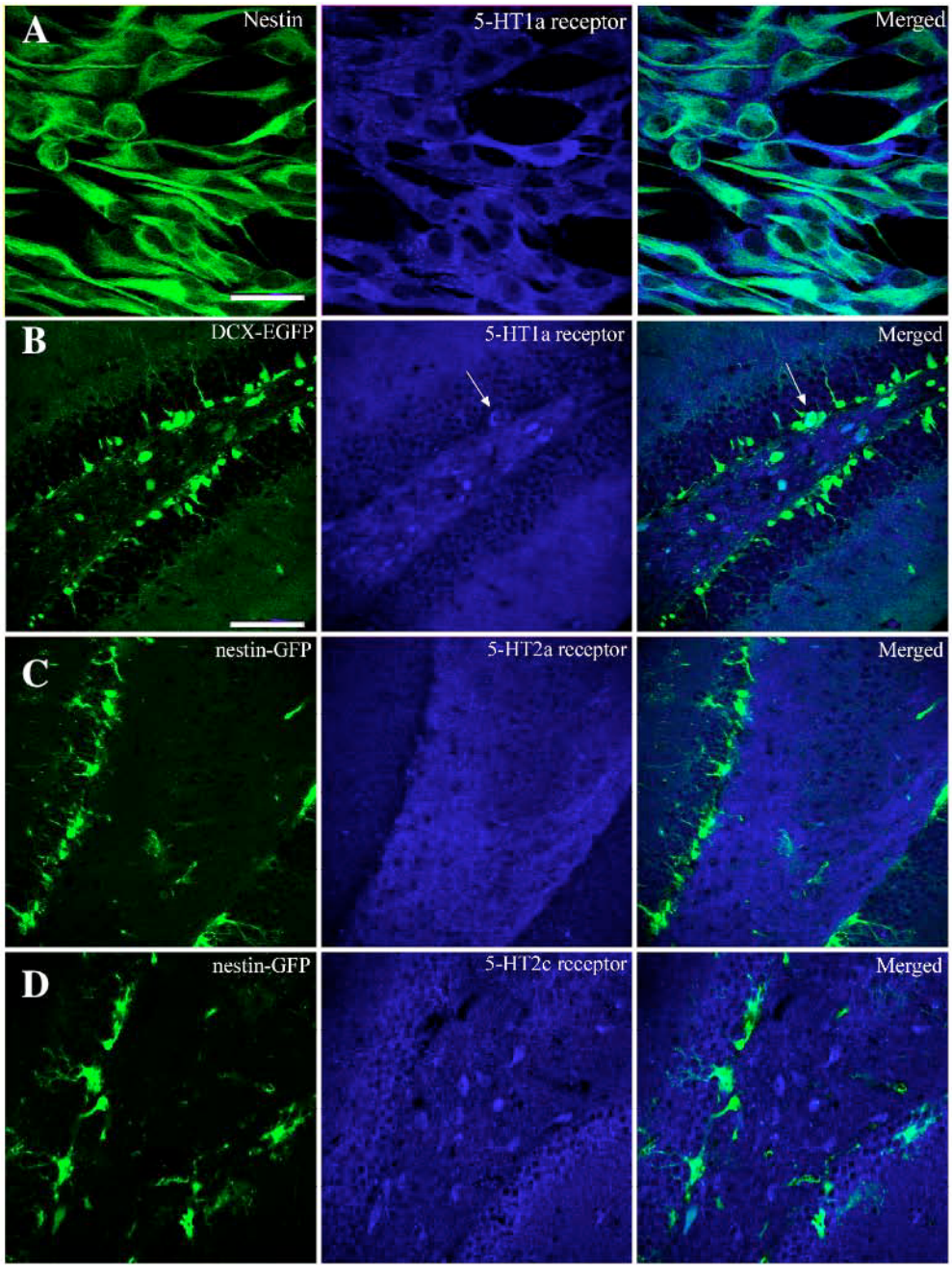


Fig. 20. Confocal microscopy of serotonin receptor expression. A. Precursor cells in culture (Nestin, green) show an intense 5-HT1a receptor expression, Scale bar 10 µm. **B-D.** Serotonin receptor expression *in vivo* in the adult dentate gyrus. 5-HT1a receptor (B, blue) is expressed in neurons both in the hilus and granule cell layer and shows an overlap with DCX-EGFP-expressing cells (B, green, arrow, Scale bar 150 µm). **C.** 5-HT2a receptor expression in the adult mouse dentate gyrus revealed a dense fiber staining in the hilus, whereas 5-HT2c receptors are expressed in the granule cell layer (D, blue).

4.5 Fluoxetine has no effect on cell proliferation but increases survival of BrdU-positive cells

Fluoxetine promotes serotonergic neurotransmission by reuptake inhibition of serotonin into presynaptic neurons (Lesch et al. 1993; Emslie et al. 2002). Fluoxetine also exerts an inducing effect on adult hippocampal neurogenesis (Malberg and Duman 2003; Santarelli et al. 2003; Czeh et al. 2007). Here, serotonergic effects on adult hippocampal neurogenesis were dissected in more detail. In the first experimental paradigm the influence of the antidepressant fluoxetine on precursor cell proliferation in the subgranular zone was investigated. Animals received a single injection of the drug followed by one injection of BrdU 24 hours later, and were then killed after another 2 hours. The results show no acute effect of fluoxetine on cell proliferation (Fig. 21 A). After 1 day, immunohistochemistry revealed that 749±79 cells in the drug treated group were BrdU-positive versus 760±95 for the control group (ANOVA, F (1.8)=0.011; p=0.9180). In the second experimental paradigm the effect on precursor cell survival was investigated by a single injection of BrdU followed by only one injection of fluoxetine 24 hours later, followed by NaCl for 20 days. Here as well no difference in the number of BrdU-positive cells was observed (Fig. 21 B; 178±90 cells in the experimental group vs. 185±114 cells in the Ctr-group (ANOVA, F (1.10)=0.014; p=0.9086).

In the third experiment the influence of fluoxetine on precursor cell survival was investigated by giving BrdU 1 day before initiation of fluoxetine treatment for a period of 21 days. The number of BrdU-positive cells was significantly increased by 42 % after 21 days of fluoxetine administration relative to the control group (Fig. 21 B). BrdU-immunohistochemistry revealed that 322±94 cells in the drug treated group had incorporated BrdU versus 185±114 cells in control (ANOVA, F (1.10)=5.122; p=0.0471). Fluoxetine needs to be administrated for more than one day to influence neurogenesis; that is in line with previous investigations by Malberg and Santarelli (Malberg et al. 2000; Santarelli et al. 2003). In contrast to our findings, they described that treatment effects can only be seen after a time interval and that in fact, suspending the treatment is required for the action of antidepressant. Here, however, the data present that already one day after a chronic fluoxetine treatment for 21 days the drug has a survival effect on newly generated neurons. Compelling evidence shows that a treatment suspending is not required.

Differentiation profile

Next, the phenotype of BrdU-positive cells in the subgranular zone of the dentate gyrus after chronic administration of fluoxetine was determined by immunofluorescence. Sections from the third experimental paradigm (precursor cell survival) were stained with the neuronal marker NeuN and the astroglial marker S100β. Confocal analysis indicated that fluoxetine treatment for 21 days did not effect precursor cell differentiation into either neurons or glia cells (Fig. 21 C). BrdU/NeuN co-labeling based on an analysis of 100 cells randomly selected BrdU-positive cells revealed (in %) 81.8%±8.1% in Ctr-animals vs. 84%±10% in the Flx-group (ANOVA, F (1.10)=0.088; p=0.7728); and for BrdU/S100β co-expression (%): 5.4%±3% versus 3.1%±1.6% of these cells (ANOVA, F (1.10)=0.477; p=0.5056). In addition, calculations based on the absolute number of new neurons per dentate gyrus (BrdU+/NeuN-positive cells) showed a slightly increase in the Flx-treated animal group: 147±97 vs. 279±110 cells (ANOVA, F (1.10)=4.784; p=0.0536), whereas no change in the number of BrdU+/S100β-positive new astrocytes was observed: 10±5 vs. 8±4 cells (ANOVA, F (1.10)=0.086; p=0.7748). Thus, the primary result of chronic fluoxetine administration over 21 days was a net increase in the number of newly generated neurons.

Immunohistochemistry for BrdU and the calcium binding protein Calretinin, which is expressed in immature postmitotic granule cells revealed a significant decrease in the percentage of BrdU+/CR-positive cells in all BrdU-positive cells in the fluoxetine treated group (Fig. 21 D). Surprisingly, significantly fewer newly generated cells expressed Calretinin after 21 days of chronic antidepressant administration: Flx-group 32.6%±4.8% versus Ctr-group 49.0%±8.3%, ANOVA, F (1.10)=17.523, p=0.0019). However, the absolute number of BrdU/CR-expressing cells (Fig. 21 D´) is not significantly reduced in the Flx-group due to the increased total number of BrdU-positive cells in the dentate gyrus: 106±15 cells vs. Ctr-group 87±20 cells (ANOVA, F (1.10)=0.534; p=0.4819). There is a similar effect on the population of BrdU-positive cells chronic that do not show co-expression with the other markers investigated: the percentage of BrdU-positive cells in the Flx-treated group was significantly increased: 66.5%±4.7% versus Ctr-group 48.2%±5% (ANOVA, F (1.10)=43.151; p<0.0001). I also calculated the absolute number of BrdU-positive cells in the dentate gyrus that fall into this category: Flx-group 214±62 vs. Ctr-group 90±60 cells (ANOVA, F (1.10)=12.341; p=0.0056). These results might suggest that Flx treatment might accelerate neuronal development through the Calretinin stage.

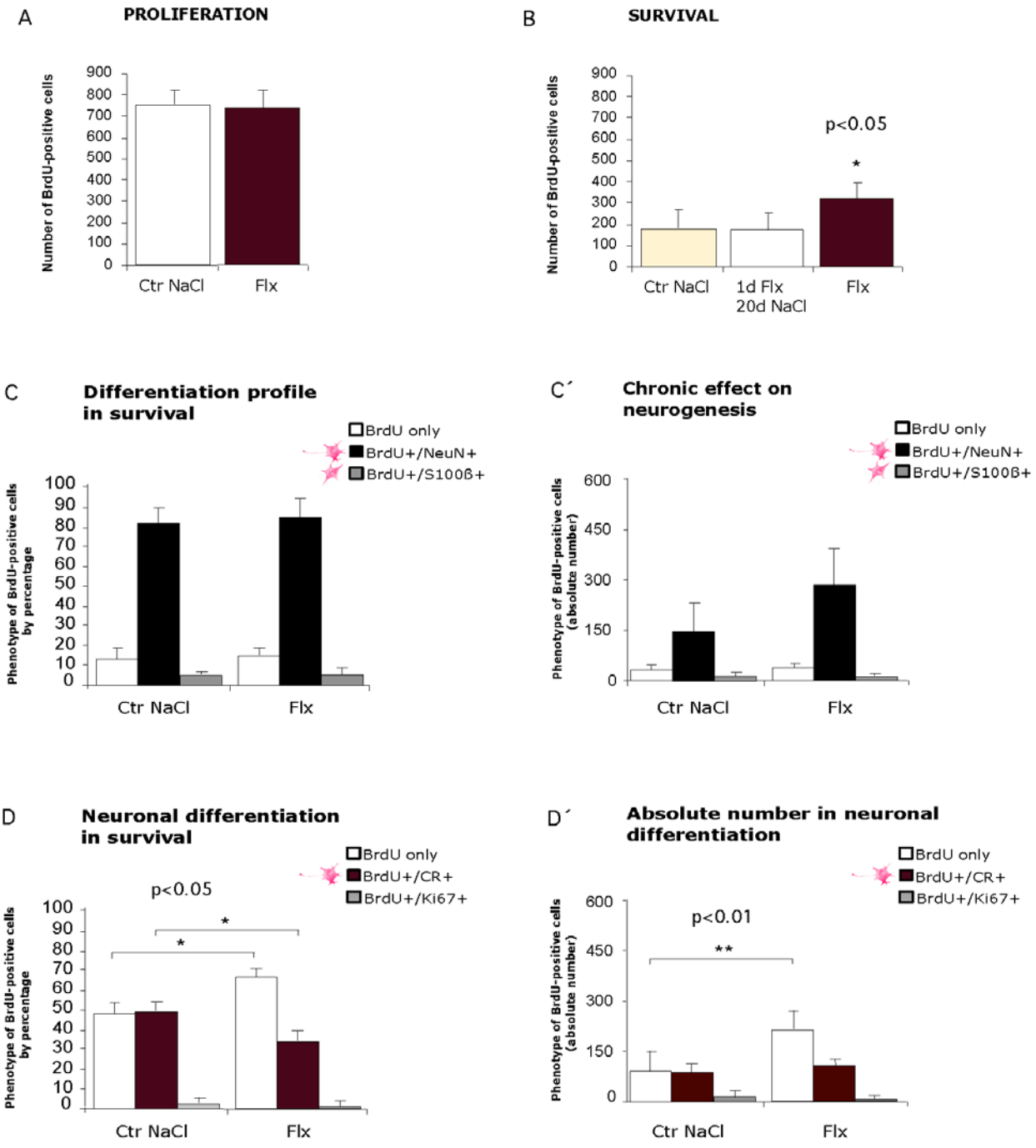


Fig. 21. Effects of acute and chronic fluoxetine (Flx) treatment on precursor cells proliferation and differentiation in the subgranular zone. A. As compared to control (Ctr NaCl), no changes in the number of proliferating cells are observed after 1 day of fluoxetine treatment. **B.** The number of BrdU-positive cells was significantly increased after one single injection of BrdU followed by 21 days of chronic fluoxetine administration, whereas only one single injection of Flx followed by 20 days of saline had no effect on neurogenesis. Data are presented as numbers of BrdU-positive cells per dentate gyrus, means±SD. **C, C´.** Chronic fluoxetine administration for 21 days increases neurogenesis indicated by a higher number of surviving neurons. **D.** Surprisingly, significant fewer newly generated cells expressed Calretinin after 21 days of Flx administration, which let rised the population of BrdU-only cells in a corresponding effect (D, D´). Data present the phenotype of BrdU-positive cells per dentate gyrus by percentage and absolute number, means±SD.

The next chapter shows the results of serotonin receptor stimulation in adult hippocampal neurogenesis. Pharmacological specific agonists and antagonists for the 5-HT1a and 5-HT2 receptors were used to dissect the interplay of these various receptors in the regulation of adult hippocampal neurogenesis.

4.6 Serotonin differentially influences adult hippocampal neurogenesis via various receptor subtypes

4.6.1 Chronic 5-HT1a receptor blockade decreases the survival of BrdU-labeled cells

The actions of fluoxetine are ascribed to increased serotonin levels in the synaptic cleft by reuptake inhibition (Lesch et al. 1993). The effects of serotonin are subserved through various membrane-bound receptors. The 5-HT1a receptor subtype is a major mediator of serotonin action in the hippocampus.

5-HT1a receptor agonist treatment

8-OH DPAT is a specific 5-HT1a receptor subtype agonist. Proliferating cells in the 8-OH DPAT treated group formed more clusters in the subgranular zone as compared to control animals (Fig. 22).

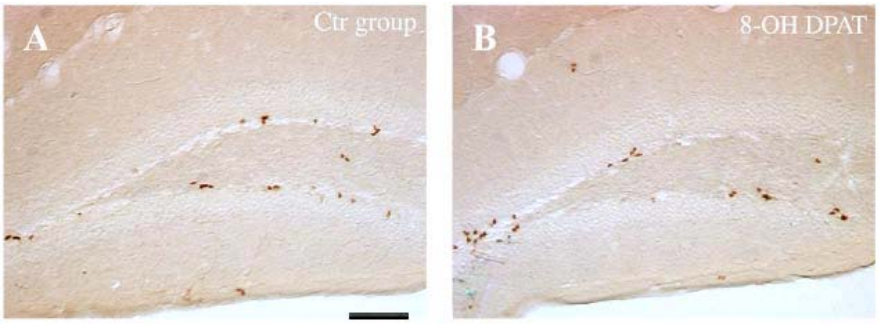


Fig. 22. BrdU-labeled cells in the subgranular zone shown by peroxidase immunostaining. As compared with the Ctr-group (A), an increase in the number of BrdU-positive cells was observed after one day treatment with the 5-HT1a receptor agonist 8-OH DPAT (B). Proliferating cells in the agonist treated group form clusters of more than 2 cells; Scale bar 120 µm.

Acute activation of 5-HT1a receptors by a single injection of the agonist followed by BrdU 2 hours later significantly increased precursor cell proliferation after one day by more than 50% (Fig. 23 A). As compared to control animals the total number of BrdU-positive cells per dentate gyrus (1052±106) was markedly increased to 1627±279 cells in agonist treated animals (ANOVA, F (1.7)=14.925; p=0.0062).

In the chronic setting, where BrdU was injected 8 hours before initiation of 8-OH DPAT treatment for a period of 7 days, BrdU-positive cells were primarily located in the granular cell layer, whereas some cells had already migrated into the mid granular cell layer. After one week about 65% of the proliferating cell population survived in the adult dentate gyrus, which is in line with previously reported numbers (Gould 1999; Steiner et al. 2004). Chronic 5-HT1a agonist administrations over 7 days showed no difference in the number of dividing cells within the two groups (Fig. 23 B). BrdU-immunohistochemistry revealed 833±208 cells in the drug treated group versus 679±122 cells for the Ctr-group (ANOVA, F (1.9)=2.127; p=0.1787).

Further phenotypic analysis using confocal microscopy indicated that 5-HT1a receptor agonist treatment caused no effect on the differentiation of BrdU-positive cells into neurons or glia as assessed by co-expression of DCX (68.4%±7.4% versus Ctr-group 72.5%±7.6%) or GFAP (2.9%±3.1% versus Ctr-group 4.2%±4.0%), NeuN (83.7%±5.2% versus Ctr-group 83.9%±4.9%) or S100β (1.7%±1.1% versus Ctr-group 2.0%±1.0%) (Fig. 23 C-D). However, in addition, calculations based on the absolute BrdU number showed a significant increase of BrdU+/DCX-positive cells due to the increased total number of proliferating cells (Fig. 23 E; 1104±172 vs. Ctr-group 763±116 cells, ANOVA, F (1.7)=11.346; p=0.0119). This result suggests a net increase in proliferating type-2b and type-3 cells after acute 5-HT1a receptor stimulation.

5-HT1a receptor antagonist treatment

In contrast, acute 5-HT1a receptor blockade using WAY100135 caused no change in cell proliferation, but decreased the survival of BrdU-labeled cells (Fig. 23 A, B). The number of BrdU-positive cells in WAY100135-injected animals was similar compared to control animals after one day (1207±120 versus 1052±106, ANOVA, F (1.7)=4.665; p =0.0676; a single BrdU-injection was given 2 hours after one injection of the antagonist; animals were killed 24 hours later). The number of BrdU-labeled cells was significantly decreased to

480±55 cells (Ctr: 679±122) in animals that received the 5-HT1a receptor antagonist for 7 days (ANOVA, F (1.11)=16.544; p=0.0019; a single BrdU-injection was given 8 hours before initiation of WAY100135 for a period of 7 days).

To examine whether this affected only the total number of surviving cells or also caused a shift in the balance of neurons or glial cells were generated the brain slices after 7 days of treatment were triple-labeled with BrdU, the neuronal marker NeuN, and the astroglial marker S100β. Confocal analysis was used to determine the phenotype of newly generated cells. 83.9%±4.9% of BrdU-positive cells in the Ctr-group were co-labeled with NeuN after 7 days as compared to WAY100135-treated animals (86.6%±5.5%; ANOVA, F (1.10)=0.730; p=0.4129). However, calculations based on the absolute number revealed a significant higher amount of BrdU+/NeuN-positive cells for the Ctr-group: 572±124 vs. 404±51 cells (ANOVA, F (1.10)=10.757; p=0.0083), which can be again explained by the higher total number of surviving cells in Ctr-animals. No significant changes either in the percentage nor in the absolute number of BrdU+/S100β-positive cells were observed (1.5%±1.5% vs. Ctr-group 2.0%±1.0%, absolute number 7±7.2 vs. 15±10 cells). As well as for the stimulation experiment with 8-OH DPAT, 5-HT1a receptor blockade caused a net shift in the number of cells produced, in fact less neurogenesis occurs.

Taken together, while acute receptor stimulation with 5-HT1a receptor agonist 8-OH DPAT resulted in an increase of the population of proliferating precursor cells, and chronic treatment showed no differences, acute pharmacological blockade of 5-HT1a receptors with WAY100135 showed no significant change, whereas chronic administration of the antagonist significantly reduced the survival of BrdU-positive cells in the dentate gyrus after 7 days.

To summarize these data so far, chronic fluoxetine administration for a period of 21 days increased adult hippocampal neurogenesis. Acute 5HT1a receptor stimulation also increased the population of proliferating precursor cells whereas blocking of 5-HT1a receptors caused significantly reduced survival of BrdU-positive cells in the dentate gyrus after 7 days of antagonist treatment. The time-latency required for antidepressant action on the precursor cells might thus be either a result of presynaptic (serotonergic neurons in the raphe nuclei) versus postsynaptic (hilar interneurons) effects (feedback inhibition), or due to the time course of cell differentiation associated with different receptor expression in the dentate gyrus.

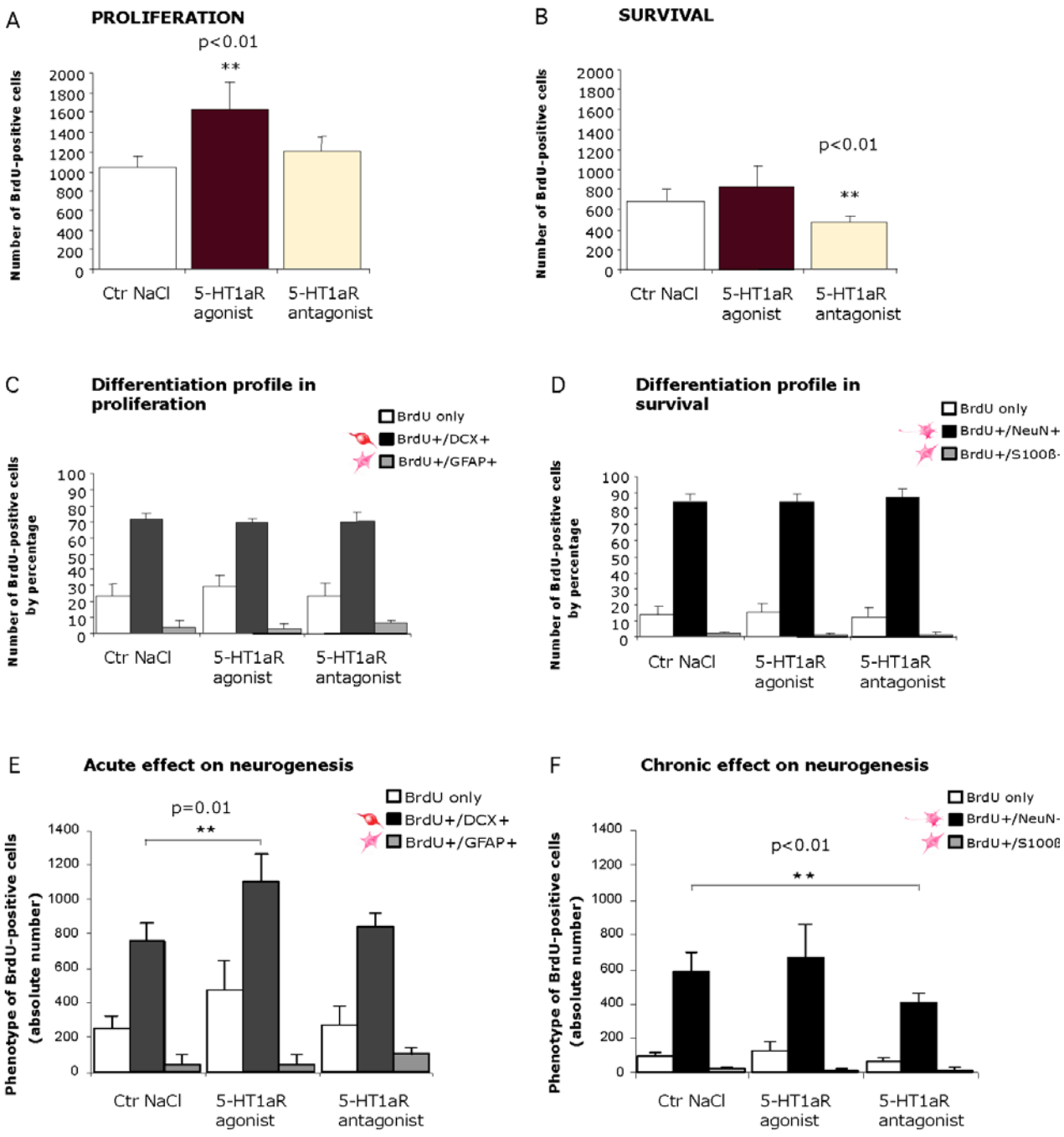


Fig. 23. Bar graphs showing the effect of 5-HT1a agonist and antagonist treatment on cell proliferation and differentiation in the adult dentate gyrus. **A.** Acute treatment with the 5-HT1a agonist 8-OH DPAT produced a significant increase in cell proliferation *in vivo* after one day by more than 50% whereas chronic stimulation for a period of 7 days showed no significant difference. **B.** Acute 5-HT1a receptor blockade with WAY100135 caused no change in cell proliferation after one day but decreased the survival of BrdU-positive cells after 7 days of administration; data are presented as numbers of BrdU-positive cells per dentate gyrus, means±SD. **C-F.** Confocal analysis of the differentiation profile revealed a net increase in the number of BrdU/DCX-expressing cells after acute stimulation, whereas chronic 5-HT1a receptor blockade over 7 days caused less neurogenesis. Data present the phenotype of BrdU-positive cells per dentate gyrus by percentage and absolute numbers, means±SD.

4.6.2 The 5-HT2 receptor family regulates adult neurogenesis in the opposite way

The second important receptor complex in the brain is the 5-HT2 receptor family. Cinanserin as a widely used drug blocks the entire 5-HT2 receptor family, whereas the agonist α-Methyl-5-HT-maneate activates it. For further experiments, the selective 5-HT2c receptor subtype agonist WAY161503 was used.

5-HT2 receptor antagonist treatment

Surprisingly, acute inactivation of 5-HT2 receptors with one single injection of Cinanserin resulted in significantly more BrdU-positive cells in the adult dentate gyrus as compared to the control group. This is in contrast to the results for 5-HT1a receptors, where agonist activation significantly increased the proliferation of BrdU-positive cells. The number of proliferating cells after one day of a single 5-HT2 receptor antagonist injection followed by BrdU 2 hours later (1043±147) was significantly increased by roughly 48% (1593±319 cells, Fig. 24 A; ANOVA, F (1.13)=15.367; p=0.0018). There was no difference in the number of BrdU-positive cells in the survival paradigm where BrdU was given 8 hours before initiation of the 5-HT2 receptor antagonist for a period of 7 days (Fig. 24 B; 975±214 versus Ctr-group 973±248; ANOVA, F (1.16)=3.757E-4; p=0.9848).

Differentiation profile after 5-HT2 receptor antagonist treatment

Further phenotypic analysis indicated that 7 days of Cinanserin treatment caused no effect (by percentage) on the differentiation of BrdU-positive cells into neurons or glia as assessed by co-expression of NeuN (Fig. 24 D; 76.9%±5.6% vs. Ctr-group 82.8%±9.9%, ANOVA, F (1.7)=2.382; p=0.1666) or S100β (10%±4.9% vs. Ctr-group 4.3%±3%, ANOVA, F (1.7)=4.575; p=0.0698). However, in addition, calculations based on the absolute BrdU number showed a significant decrease of BrdU+/S100β-positive cells due to the decreased total number of BrdU-labeled cells (Fig. 24 F, ANOVA, F (1.6)=6.626; p=0.0421).

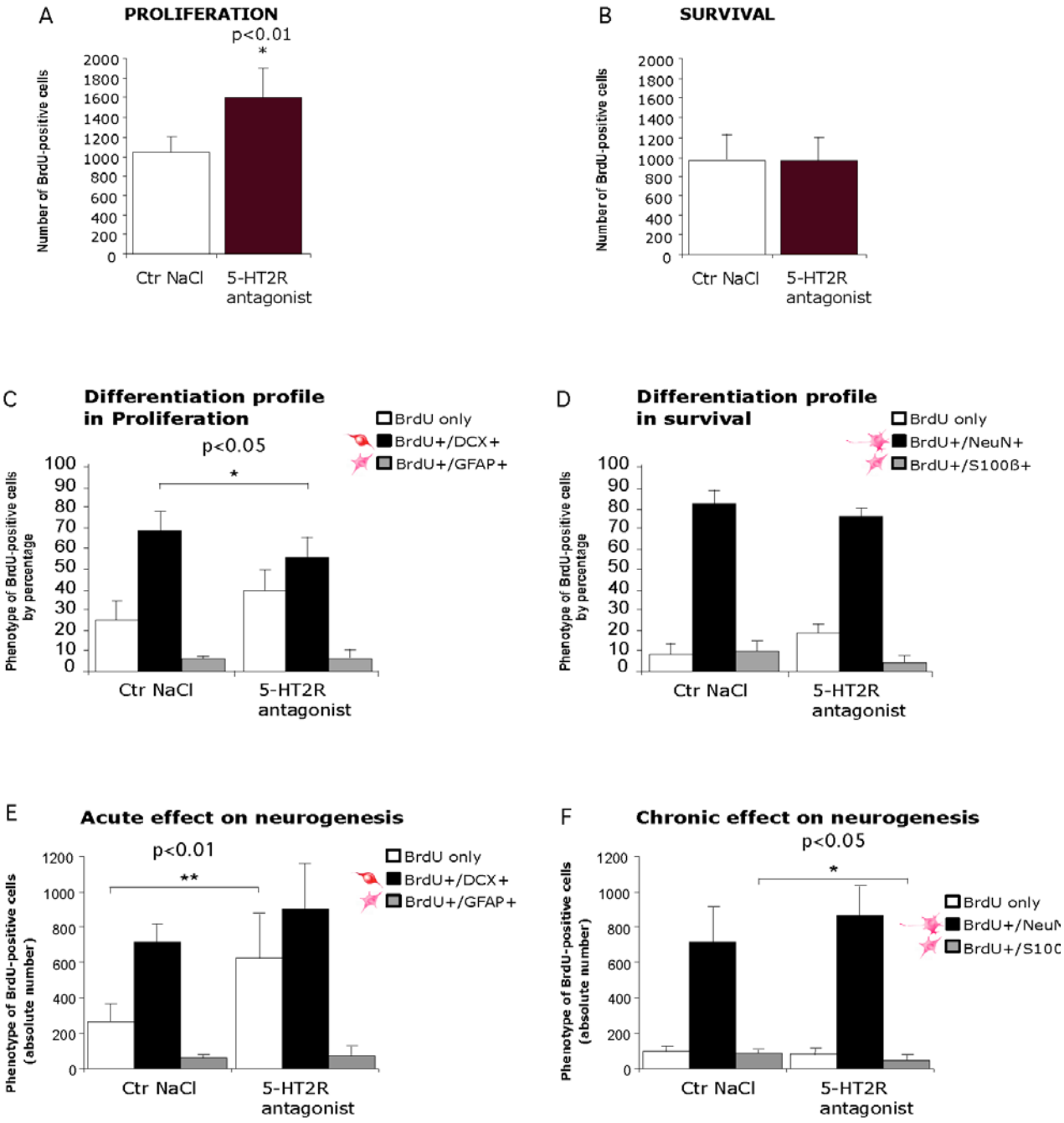


Fig. 24. Effects of 5-HT2 receptor blockade on precursor cell proliferation and differentiation in the adult dentate gyrus. A-B. As compared to control, a surprisingly large increase by 48% in the number of proliferating cells in Cinanserin treated mice after 1 day of BrdU was observed. Chronic blockade for a period of 7 days caused no significant difference. Data are presented as numbers of BrdU-positive cells per dentate gyrus, means±SD. **C-F.** Confocal analysis revealed no differences in the differentiation profile by co-expression of BrdU/NeuN (D, F), but a net decrease in the number of surviving astrocytes (F). Surprisingly, the number of BrdU-positive cells co-expressing DCX was decreased in the drug treated group after one single BrdU injection followed by 7 days of Cinanserin, which let raise significantly the population of BrdU-only cells. Data present the phenotype of BrdU-positive cells per dentate gyrus by percentage and absolute numbers, means±SD.

Further phenotypic analysis using confocal microscopy indicated that acute 5-HT2 receptor blockade for one day with one single injection of Cinanserin had stimulatory effects on subpopulations of precursor cells in the dentate gyrus. Surprisingly, the number of BrdU-positive cells (by percentage) co-expressing DCX was significantly decreased in the experimental group from 68.5%±8.9% (Ctr-group) to 56.1%±9.4% (Fig. 24 C; ANOVA, F (1.12)=6.065; p=0.0299). However, calculations based on the absolute number, revealed a little increase in the population of BrdU+/DCX-positive cells due to the significant higher total number of proliferating cells in the Cinanserin-treated group (900±256 cells compared to 715±100 cells; Fig. 24 E). The shift in the BrdU/DCX-population by percentage was reflected in the size of the BrdU-only cell population: 38.6%±12% vs. Ctr-group 25.4%±10%, ANOVA F(1.12)=4.181, p=0.0634. In addition, the absolute number of BrdU-only cells indicated significantly more BrdU-positive cells for the experimental group: 626±245 vs. Ctr-group 267±115 cells, ANOVA F (1.12)=10.923; p=0.0063; Fig. 24 E. The number of proliferating astrocytes after one day of a single injection of Cinanserin and BrdU showed no differences between the groups and thus does not compensate the smaller number of BrdU/DCX-expressing cells (BrdU/GFAP, ANOVA, F (1.12)=0.226; p=0.6427).

In summary, acute inactivation of 5-HT2 receptors increased the number of proliferating precursor cells, but slowed neuronal lineage commitment.

In the line with Palmer and Fabel who mentioned a correlation of precursor cell and endothelial cell proliferation (Palmer 2002; Fabel et al. 2003), in the following step the endothelial marker Glut1 was used to estimate the number of proliferating endothelial cells in relation to the drug treatment. Surprisingly, significant fewer endothelial cells were proliferating in the antagonist treated animal group (Fig. 25). In fact, blockade of 5-HT2 receptors revealed an acute effect on precursor cells and endothelial cells. The significant lower number of proliferating Glut1-positive cells (4.9%±2.7%) for the experimental group vs. 11.8%±3.8% in the Ctr-group (ANOVA, F (1.12)=11.151; p=0.0102) might correlate with the decreased number of DCX-positive cells and vice versa (Fig. 25 A).

Surprisingly, an increase in the BrdU/DCX-population by roughly 40% up to 90.2% after 7 days of drug treatment was observed (Fig. 25 B). However, the number of proliferating endothelial cells remained relatively constant in drug treated animals, but significantly decreased by approximately 80% to 2.4% in control animals. The survival of Glut1-positive

cells after 7 days of Cinanserin treatment might lead to an increased survival of neurons. The results suggest a yet unidentified role of serotonin regulation of endothelial cell biology that impinges on precursor cell behavior.

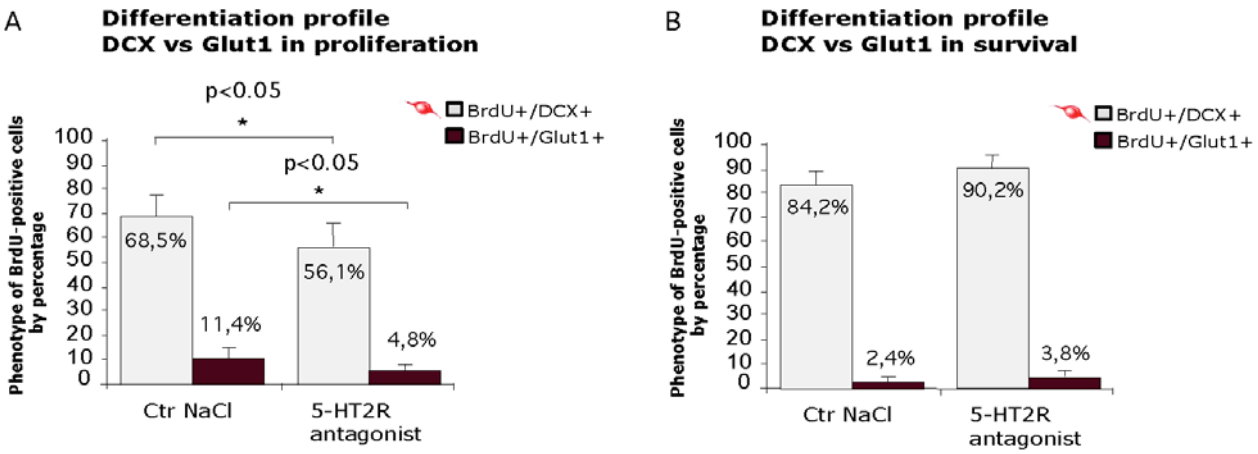


Fig. 25. Acute 5-HT2 receptor blockade caused an effect on precursor cells and endothelial cells in the dentate gyrus. A. Significantly fewer BrdU/DCX-expressing cells as well as endothelial cells were proliferating after one day of Cinanserin treatment. **B.** After seven days the population of BrdU+/DCX-positive cells in the drug treated group increased by roughly 40%, simultaneously the number of proliferating BrdU/Glut1-expressing cells remained constant. Data present the phenotype of BrdU-positive cells per dentate gyrus by percentage, means±SD.

5-HT2 receptor agonist administration

In contrast, acute as well as chronic activation of the entire 5-HT2 receptor family significantly decreased the number of BrdU-positive cells in the subgranular zone (Fig. 26 A-B). Animals treated once with the 5-HT2 receptor agonist α-Methyl-5-HT-maneate followed by BrdU 2 hours later revealed roughly 47% less proliferating cells after one day of BrdU (542±165 versus Ctr-group 1017±250 cells, ANOVA, F (1.10)=14.982; p=0.0031). Chronic agonist administration (after one single BrdU injection) for a period of 7 days showed the same proportion, almost only the half of BrdU-positive cells survived in the drug treated animal group (Ctr-group 911±114 to 488±170 cells (ANOVA, F (1.8)=21.211 p=0.0017).

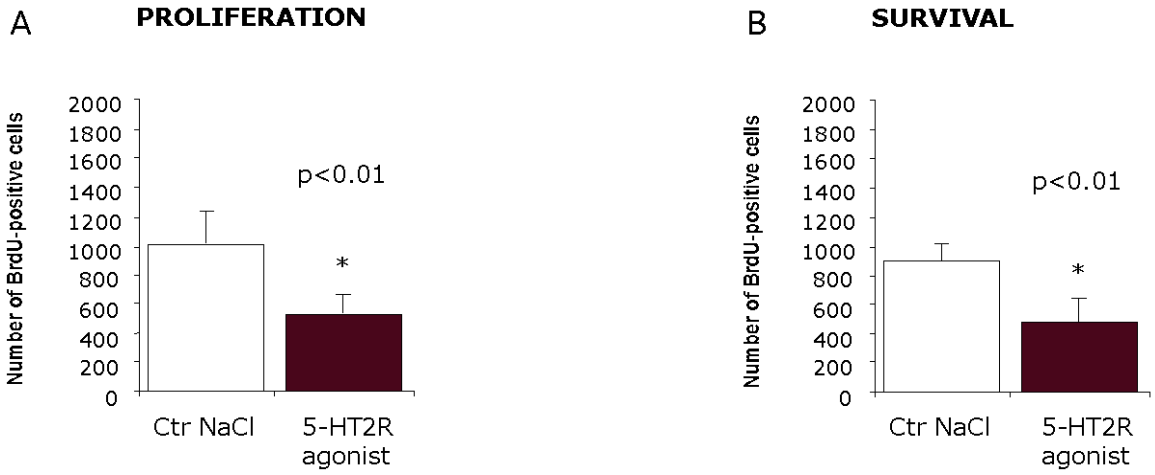


Fig. 26. Effects of acute and chronic 5-HT2 receptor stimulation on proliferating precursor cells in the adult dentate gyrus. A. In contrast to 5-HT2 receptor blockade, acute stimulation with α-Methyl-5-HT-maleate for one day and chronic stimulation over 7 days (B) significantly decreases the number of BrdU-positive cells in the subgranular zone by roughly 47%. Data are presented as numbers of BrdU-positive cells per dentate gyrus, means±SD.

Differentiation profile after 5-HT2 receptor agonist administration

Further phenotypic analysis using confocal microscopy indicated that acute stimulation of 5-HT2 receptors caused an effect on a subpopulation of precursor cells in the adult dentate gyrus. Calculations based on the percentage showed a significant increase in the number of type-2b cells (Fig. 27 A). The population of these proliferating transiently amplifying progenitor cells that co-expresses nestin-GFP and DCX raised from 18.3%±5% (Ctr-group) to 27.3%±8.4% in the agonist treated animal group (ANOVA, F (1.10)=5.161; p=0.0464). The percentage of BrdU+/DCX-positive cells (type-3 and early postmitotic immature neurons) was pretty evenly between the groups (Ctr-group 37.0%±8% vs. 39.5%±8,4%) whereas the number of BrdU/nestin-GFP-positive cells was slightly decreased from 20.6%±7.2% in control to 14.8%±7.5% (Fig. 27 A). However, in addition, calculations based on the absolute number showed no differences in the population of type-3 cells and early postmitotic neurons (Ctr-group 183±55 vs. 142±51 cells), but a significant decrease in the number of BrdU+/nestin-GFP-positive cells as well as BrdU+/nestin-GFP+/DCX-positive cells in drug-treated animals due to less proliferating cells in total (Fig. 27 C; BrdU/DCX: Ctr-group 377±123 vs. 213±81 cells, ANOVA, F (1.10)=7.484; p=0.0210; BrdU/nestin-GFP Ctr-group 209±87 vs. 88±55 cells, ANOVA, F(1.10)=8.274; p=0.0165).

Further analysis after 7 days revealed a slightly decrease in the number of newly generated neurons co-expressing BrdU/NeuN in agonist-treated animals (Fig. 27 B; Ctr-group 86.2%±7.3% vs. 80.9%±4.5%); no difference for BrdU/S100β-co-expression was observed (Ctr-group 2.4%±2% vs. 2.5%±2.9%). Calculations based on the absolute number showed a significant decrease of BrdU/NeuN-positive cells in the 5-HT2 receptor agonist-treated group as compared to the Ctr-group (from 787±126 to 371±145 cells in control) due to a smaller total number of proliferating cells (Fig. 27 D; ANOVA, F (1.7)=21.167; p=0.0025).

Taken together, acute as well as chronic 5-HT2 receptor activation influences adult neurogenesis via an effect on type-2b progenitor cells.

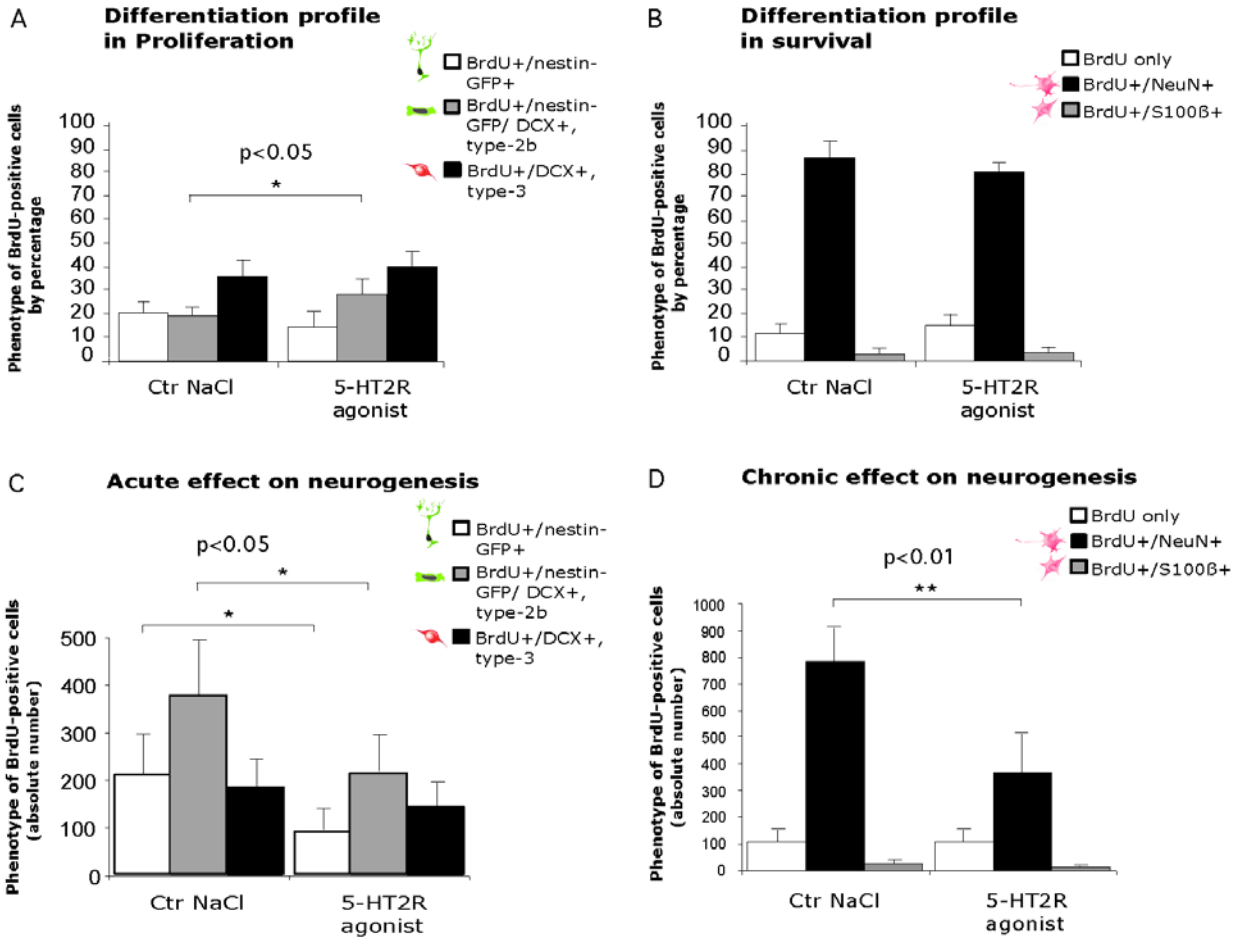


Fig. 27. Bar graphs showing the result of confocal analyzing of precursor cell differentiation after acute and chronic stimulation of 5-HT2 receptors. A-B. One day after a single injection of α-Methyl-5-HT-maleate and BrdU the population of type-2b cells increases (A), whereas no differences in the differentiation profile were observed after 7 days of drug treatment co-expressing BrdU/NeuN or BrdU/S100β (B) **C-D.** Calculations based on the absolute number show a significant effect on BrdU/nestin-GFP-expressing cells and type-2b cells (C), as well as a net decrease in the number of newly generated neurons (D). Data present the phenotype of BrdU-positive cells per dentate gyrus by percentage and absolute numbers, means±SD.

5-HT2c receptor subtype stimulation

For further experiments, the selective 5-HT2c receptor agonist WAY161503 was used to get more and detailed information about the 5-HT2 receptor family action. Surprisingly, acute activation of 5-HT2c receptors by a single injection of the agonist followed by BrdU 2 hours later significantly decreased the number of BrdU-positive cells in the adult dentate gyrus after one day, but not as much as in the previous experiment (Fig. 28 A). The amount of proliferating cells declined from 1129±169 (Ctr-group) to 864±161 cells in the experimental group (ANOVA, F (1.7)=5.715; p=0.0481).

Agonist treatment after one single BrdU injection for a period of 7 days showed no significant difference between the groups (Fig. 28 B). In control animals the total number of BrdU-positive cells per dentate gyrus was 911±114, decreased to 813±314 cells in agonist treated animals (ANOVA, F (1.7)=0.433), p=0.5314). But here, statistical analysis revealed a high variance.

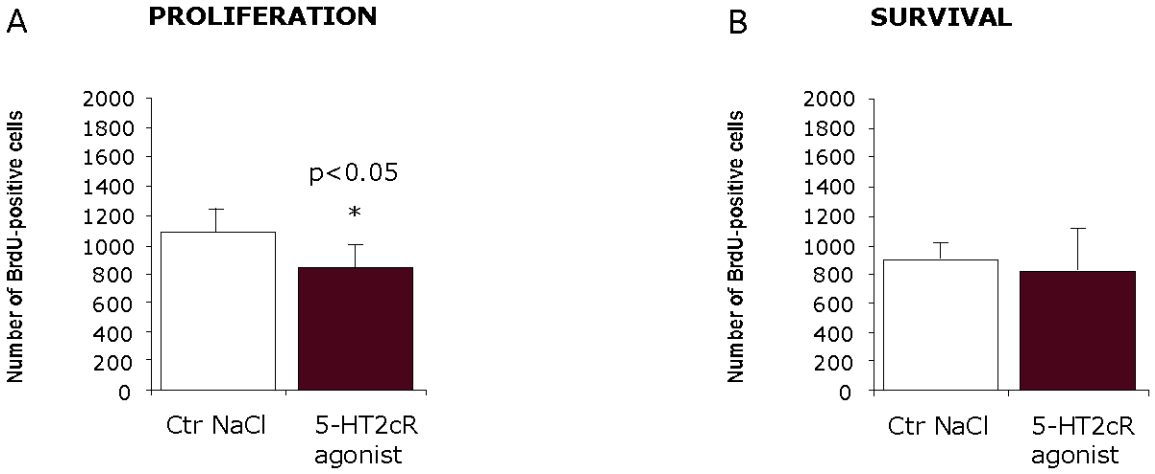


Fig. 28. Absolute number of BrdU-positive cells in the dentate gyrus after acute and chronic stimulation of 5-HT2c receptor subtypes. A. Acute activation by a single injection of WAY161503 and BrdU significantly decreases the number of proliferating cells after one day. **B.** In contrast to the action of α-Methyl-5-HT-maleate on precursor cells, a seven-day treatment with the selective 5-HT2c receptor agonist does not effect the survival of newly born cells. Data are presented as numbers of BrdU-positive cells per dentate gyrus, means±SD.

Differentiation profile after 5-HT2c receptor agonist treatment

Interestingly, further phenotypic analysis after acute activation of 5-HT2c receptor subtypes also showed an effect on the DCX cell population. In the experimental group the number of type-3 cells (BrdU+/DCX+/nestin-GFP-negative) and early postmitotic immature neurons significantly increased from 29.7%±3% in the Ctr-group to 51.6%±9% (Fig. 29 A; ANOVA, F (1.6)=19.054; p=0.0047). Simultaneously, the amount of proliferating nestin-GFP-positive cells (type-1, type-2a) is decreased by more than 40% from 29.2%±1.4 to 16.5%±9.7% in the experimental group (ANOVA, F (1.6)=6.676, p=0.0416). In addition, calculations based on the absolute number (Fig. 29 C) indicated even less BrdU+/nestin-GFP-positive cells (328±42 to 133±74 cells, ANOVA, F (1.6)=20.770, p=0.0039). The absolute number of type-3 cells and early postmitotic immature neurons instead revealed no difference between the groups (Ctr-group 336±75 vs. 435±129 cells). Both, calculations by percentage as well as based on the absolute number of triple-labeled cells (type-2b) showed no differences (Ctr-group 16.5%±5% or 180±76 cells vs. 17.0%±3.4% or 135±33).

In summary, acute stimulation of 5-HT2c receptor subtypes effected more advanced stages of neurogenesis in comparison with the activation of 5-HT1a receptors and the entire 5-HT2 receptor family. Here, in addition, the effect of stimulation of the 5-HT2a receptor subtype needs to be investigated.

After 7 days of chronic treatment with WAY161503 (BrdU was given once before drug initiation) no differences in the phenotypes of BrdU-positive cells were observed between the groups co-expressing BrdU/NeuN (Fig. 29 B, D; Ctr-group 86.2%±7.3% vs. 82.2%±1.5%, absolute number 787±126 vs. 668±174 cells) and BrdU/S100β (Ctr-group 2.4%±2.0% vs. 2.9%±2.8%, Fig. 29 B).

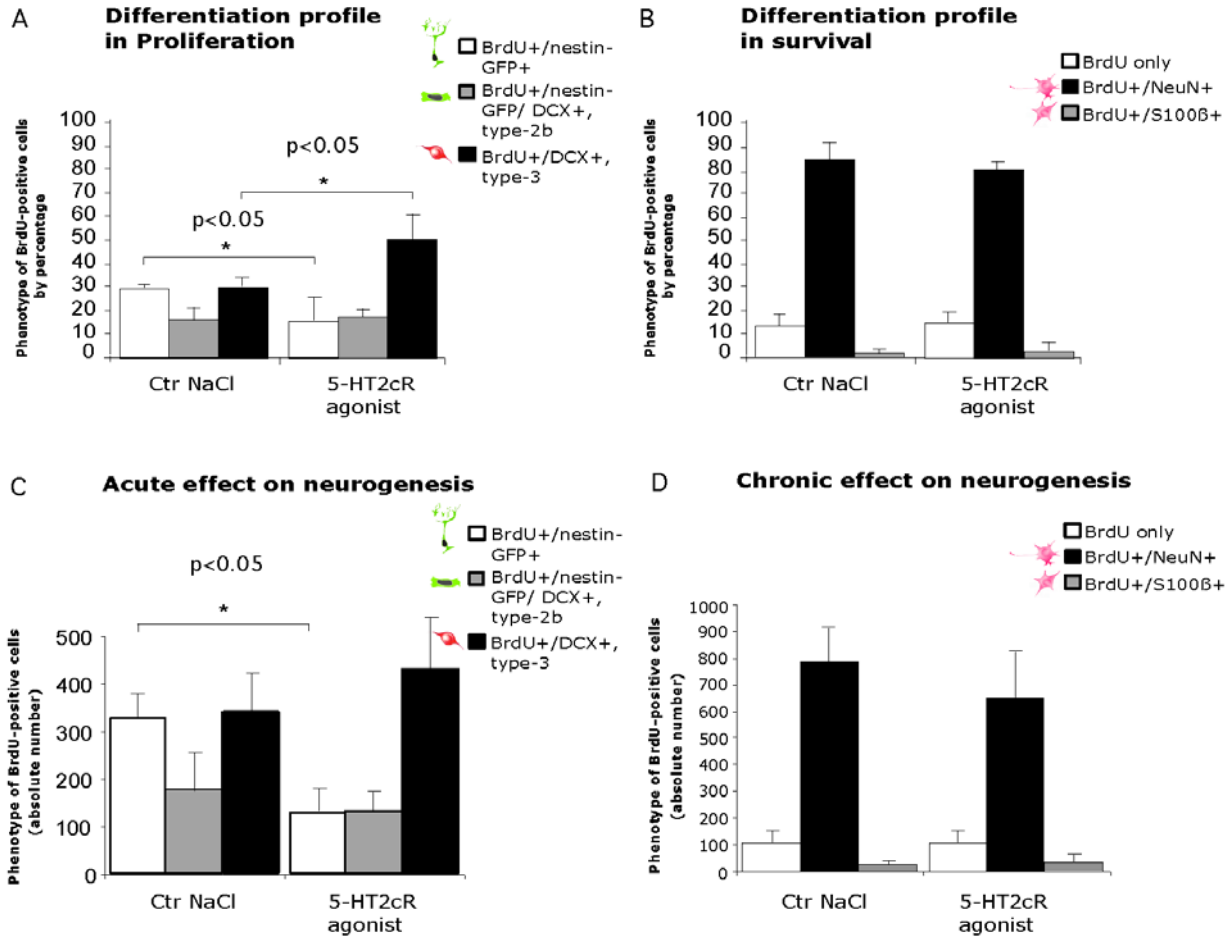


Fig. 29. Bar graphs showing the result of confocal analyzing of precursor cell differentiation after acute and chronic stimulation of 5-HT2c receptor subtypes. A, C. One day after a single injection of WAY161503 and BrdU the population of type-3 cells and early postmitotic immature neurons increases, simultaneously the population of BrdU/nestin-GFP-expressing cells gets smaller. **B, D.** No differences in the differentiation profile of precursor cells were observed after BrdU and 7 days of drug administration co-expressing BrdU/NeuN or BrdU/S100β. Data present the phenotype of BrdU-positive cells per dentate gyrus by percentage and absolute numbers, means±SD.

4.6.3 5-HT2 receptor and 5-HT2c receptor subtype stimulation affects early postmitotic immature neurons

Furthermore, confocal analysis of both chronic stimulation of the entire 5-HT2 receptor family as well as of 5-HT2c receptor subtypes for a period of 7 days showed an effect on type-3 cells and early postmitotic immature neurons in the adult dentate gyrus. Co-labeling of BrdU/DCX with Calretinin revealed a significant increase in the number of these cells after treatment with α-Methyl-5-HT-maleate, the 5-HT2 receptor agonist (Fig. 30 A; 72.1%±7.6%

versus Ctr-group 62.5%±3.0%; ANOVA, F (1.7)=5.493; p=0.0516). No changes were observed in the population of BrdU+/CR-positive cells did not express the transient marker DCX (ANOVA, F (1.7)=2.531; p=0.1557) as well as in the population of BrdU/DCX expressing cells (ANOVA, F (1.7)=1.314; p=0.2893). In contrast, calculations based on the absolute number indicated that 5-HT2 receptor stimulation for a period of 7 days caused a significant decrease in the population of triple-labeled cells (Fig. 30 A′) due to the almost half total number of BrdU-positive cells (564±79 vs. 363±117, ANOVA, F (1.6)=8.098; p=0.0293), and similarly for BrdU+/DCX-positive type-3 cells (73±28 vs. 27±28, ANOVA, F (1.7)=5.987; p=0.0443) and BrdU/CR (198±50 vs. 53±24, ANOVA, F (1.6)=28.162; p=0.0018).

Further phenotypic analysis after stimulation of 5-HT2c receptor subtypes for a period of 7 days revealed similar effects (Fig. 30 B, B′): Co-labeling with BrdU/DCX/CR showed an increase from 62.5%±3.0% in the Ctr-group to 79.9%±10.4% in the experimental group (ANOVA, F (1.6)=10.281; p=0.0185). Due to the increased number of triple-labeled cells, the population of BrdU/DCX co-expressing type-3 cells was smaller in the 5-HT2c receptor subtype agonist treated group (by percentage): Ctr-group 7.9%±2.3% vs. 1.6%±1.9%, ANOVA, F (1.6)=18.210; p=0.0053). In addition, calculations based on the absolute number indicated similar effects (Fig. 30 B′): The population of surviving BrdU/DCX-expressing type-3 cells showed a significant smaller cell number in the agonist treated animals (73±28 vs. 10±12 cells, ANOVA, F (1.6)=16.773; p=0.0064). No differences have been found for the absolute numbers of triple-labeled cells as well as BrdU+/CR-positive cells.

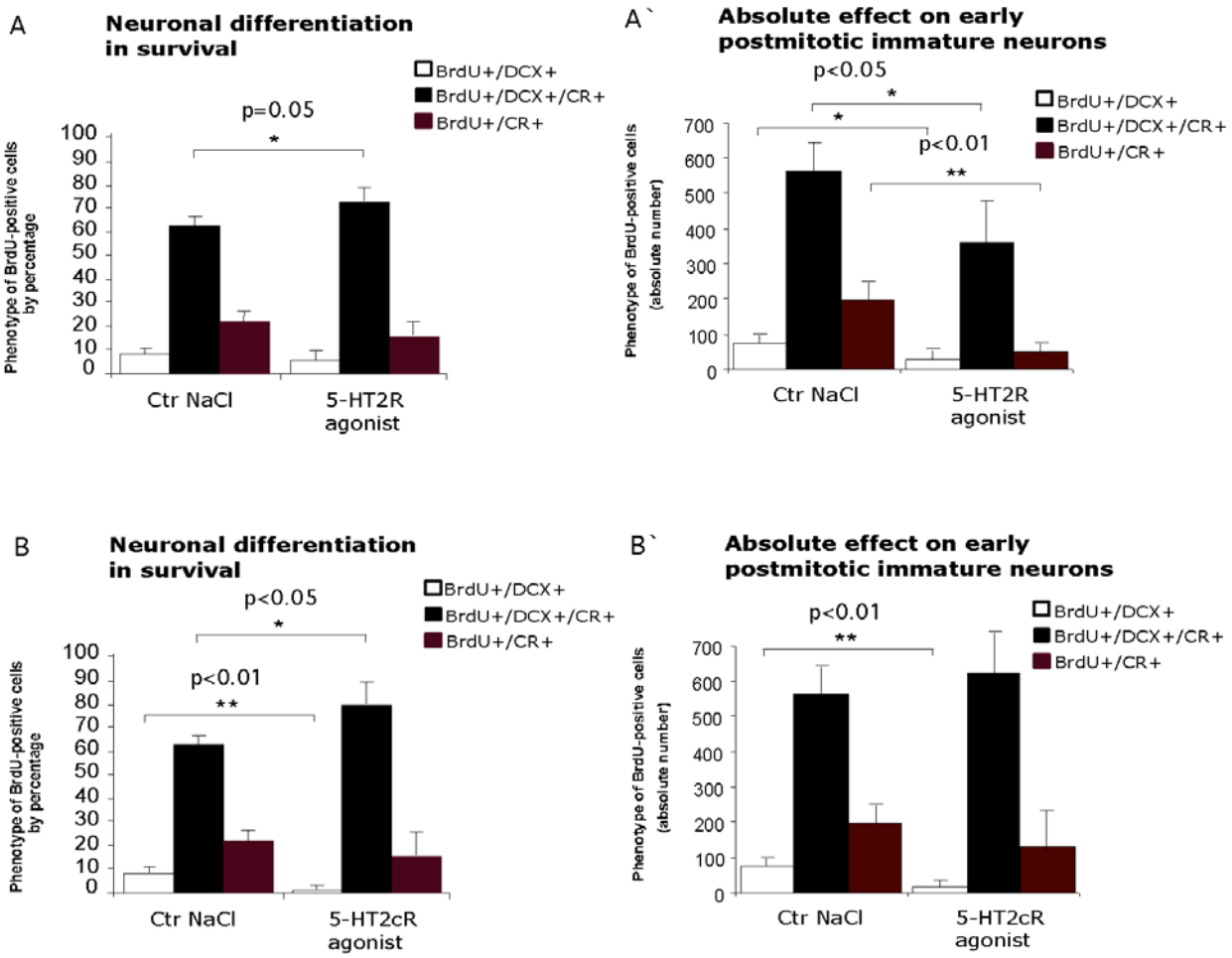


Fig. 30. Chronic 5-HT2 receptor and 5-HT2c receptor subtype stimulation effects the population of early postmitotic immature neurons in the dentate gyrus. A-B. The population of triple-labeled BrdU/DCX/CR cells is significant higher in the hippocampus after 7 days of treatment with the 5-HT2 receptor agonist α-Methyl-5-HT-maleate or the 5-HT2c receptor subtype agonist WAY161503. Simultaneously the percentage of BrdU+/DCX-positive cells goes down (B). **A´.** Calculations based on the absolute number of BrdU+/DCX+/CR-positive and CR-negative cells as well as BrdU/CR-expressing cells indicate a significant decrease when animals were exposed to 5-HT2 receptor agonist treatments for 7 days. **B´.** 5-HT2c agonist treated animals show a decrease in the number of BrdU/DCX-expressing type-3 cells. Data present the phenotype of BrdU-positive cells per dentate gyrus by percentage and absolute numbers, means±SD.

Table 6. Summary of the effects of fluoxetine and 5-HT receptor agonists and antagonists on cell proliferation and differentiation in the SGZ of the adult dentate gyrus (↑↓ increase/decrease in the total number of BrdU-positive cells, ⇑/⇓ increase/decrease in the marker expression of BrdU-positive cells, - no significant difference); CR, Calretinin; Ctr., control group; Exp., experimental group; DCX, doublecortin; Glut1, endothelial marker

Treatment	***Effect on cell proliferation*** *absolute number of BrdU+ cells (Ctr. vs. Exp.)*	***Differentiation profile in proliferation***	***Effect on cell survival*** *absolute number of BrdU+ cells (Ctr. vs. Exp.)*	***Differentiation profile in survival***
Fluoxetine	—	—	↑ *P=0,0471* *185±114 vs. 322±94*	⇑ *in the number of BrdU+/NeuN+ cells* ⇓ *in the number of BrdU+/CR+ cells*
5HT1a *8-OHDPAT (agonist)*	↑ *P=0,0062* *1052±106 vs. 1627±279*	*net* ⇑ *in BrdU+/DCX+ cells*	—	—
WAY100135 (antagonist)	—	—	↓ *P=0,0019* *679±122 vs. 480±55*	⇓ *in the number of BrdU+/NeuN+ cells*
5-HT2 *α-Methyl (agonist)*	↓ *P=0,0031* *1017±250 vs. 542±165*	⇑ *in the number of type-2b cells*	↓ *P=0,0017* *911±114 vs. 488±170*	⇓ *in the number of BrdU+/NeuN+ cells* ⇑ *in the number of BrdU+/DCX+/CR+ cells*
Cinanserin (antagonist)	↑ *P=0,0018* *1043±147 vs. 1593±319*	⇓ *in the number of BrdU+/DCX+ cells*	—	⇑ *Glut1⇒* ⇑ *DCX*
5-HT2c *WAY161503 (agonist)*	↓ *P=0,0481* *1129±169 vs. 864±161*	⇑ *in the number of type-3 cells & early postmitotic immature neurons*	—	⇓ *in the number of type-3 cells,* ⇑ *in the number of BrdU+/DCX+/CR+ cells*

5 Discussion

Adult hippocampal neurogenesis is a prominent aspect of neural stem cell development. The addition of newly generated neurons into a preexisting circuitry during adulthood constitutes an adaptation to internal and external changes, thus might compensate structural and functional cell loss. Thereby, the stages of cell differentiation, maturation and integration following cell proliferation are of utmost functionally relevance. Serotonin, as an important neurotransmitter plays a crucial role in the regulation of neurogenesis in the adult dentate gyrus (Vaidya et al. 1997; Brezun and Daszuta 1999, 2000; Banasr et al. 2004). Furthermore, it is an essential molecule in neuro-psychiatry (Malberg et al. 2000; Czeh et al. 2001; Holmes et al. 2002; Santarelli et al. 2003) and learning and memory (Wolff et al. 2003; Sanberg et al. 2006). In a hypothesis-driven approach I here investigated the effects of serotonin on cellular development of various stages of hippocampal neurogenesis by pharmacologically modulating specific receptor subtype activity. The present findings demonstrate that serotonin influences cell proliferation as well as differentiation in the subgranular zone. These results suggest differential roles of 5-HT1a and 5-HT2c receptor subtypes in affecting sequential steps in adult hippocampal neurogenesis.

5.1 5-HT1a and 5-HT2 receptors mediate an opposite effect on precursor cell proliferation and differentiation in the adult dentate gyrus

Since serotonin provides a necessary signal contributing to adult neurogenesis, serotonin receptors might possibly affect this diverse process. The present study demonstrates that in adult mice, serotonin receptors are differentially expressed in the hippocampal dentate gyrus suggesting that divergent serotonin receptors may have unique and specific function within the dentate gyrus.

In the present study, immunohistochemistry revealed that 5-HT1a receptor are expressed in neurons both in the hilus and subgranular zone. Here, the highly selective 5-HT1a receptor

agonist 8-OH DPAT and the antagonist WAY100135 were used to stimulate or inhibit 5-HT1a receptor activity. They are acting at both somatodendritic and postsynaptic 5-HT1a receptors (Fletcher 1993; Routledge et al. 1993). WAY100135 has also been identified to have partially agonistic affinity which is dose-dependent (Fletcher et al. 1993; Assie and Koek 1996). Acute treatment (animals were killed 24 hours later) with 8-OH DPAT produced a significant increase in cell proliferation *in vivo* by more than 50% whereas chronic stimulation showed no significant difference. In contrast, acute 5-HT1a receptor blockade caused no change in cell proliferation but decreased the survival of BrdU-positive cells after chronic treatment for 7 days. The balance between activation and inhibition is consistent with the literature (Radley and Jacobs 2002; Blier and Ward 2003; Banasr et al. 2004).

Presynaptic 5-HT1a autoreceptors are present on serotonergic neurons in the raphe nuclei and provide a mechanism for feedback inhibition of the serotonin system (Blier 2003). It has been shown, that activation of these receptors leads to a cell hyperpolarization and thus decreased firing rate. Less firing rate of presynaptic receptors results in a suppression of 5-HT release of serotonergic raphe neurons (Jolas et al. 1995) and a decreased firing rate of postsynaptic cells. Both the literature and this study indicate that serotonergic projections from the median raphe nucleus terminate on hilar inhibitory interneurons. If 5-HT1a receptors exert an indirect effect on precursor cells through hilar interneurons, a decreased firing rate leads to less inhibitory action on precursor cells (Fig. 31 A). Systemic application of 8-OH DPAT to the dentate gyrus reduced somatic GABAergic inhibition (Sanberg et al. 2006). But, the present study also shows 5-HT1a receptor expressions on precursor cells in the subgranular zone suggesting a direct effect. Here, the enhanced number of BrdU- positive cells after one day of acute administration of the selective agonist 8-OH DPAT could be explained by less inhibitory activity of hilar interneurons on precursor cells. No effect was seen in the survival paradigm after 7 days of 8-OH DPAT treatment But this observation might be a dose-dependent effect.

In the present study, treatment with WAY100135 showed no acute effect on cell proliferation but revealed less survival of BrdU-positive cells after chronic treatment for seven days. This indicates that 5-HT1a receptors are involved in the survival of newly born cells post proliferation. This is interesting as 5-HT1a receptors are present even in proliferating cell in the adult dentate gyrus. The suppression of the survival of newly

generated cells might be a postsynaptic effect mediated through 5-HT1a receptors. Chronic blocking of 5-HT1a receptors could disinhibit neuronal circuitry in the dentate gyrus.

In contrast to the literature, I used a modified design for the experiments in the present study. Here, for both the proliferation and survival paradigm the animals received only one single BrdU-injection. Furthermore, the survival of BrdU-positive cells right after chronic drug treatment for 7 days was estimated.

5-HT2 receptors are expressed on GABAergic neurons in the brain (Hoyer et al. 1986). Since the dentate gyrus is a major efferent target of the raphe nuclei, immunohistochemistry in the present study showed a dense presence of serotonergic fibers in the hilus stained with a 5-HT2c receptor antibody whereas 5-HT2a receptors are stained in the granule cell layer. Both acute and chronic activation or inhibition of 5-HT2 receptors with the antagonist Cinanserin and α-Methyl-5-HT-maleate or WAY161503 as agonists have been investigated. Surprisingly, the present results demonstrate that acute 5-HT2 receptor blockade significantly increases the number of BrdU-positive cells in the dentate gyrus by roughly 48%. In contrast, both acute (24 hours) and chronic (for 7 days) stimulation of 5-HT2 receptors with the agonist α-Methyl-5-HT-maleate produces significantly less BrdU-positive cells. A differentiated analysis revealed that the acute effect is mediated via 5-HT2c receptor subtypes. Recent publications indicate that WAY161503 inhibits the firing rate of serotonergic dorsal raphe neurons by a mechanism that involves 5-HT2c receptor-mediated activation of GABAergic neurons (Boothman et al. 2006) through increasing cellular levels of IP3 and DAG. Serotonergic raphe afferents also establish synaptic contacts with hilar interneurons thus are able to modulate dendritic inhibition of principle neurons in the dentate gyrus. Activation of 5-HT2 receptors on GABAergic interneurons by selective agonists is believed to result in an increase in spontaneous GABA release. The release in turn leads to an inhibitory control of precursor cell proliferation and differentiation in the subgranular zone. Taken together, even though immunohistochemistry revealed a 5-HT1a receptor staining on precursor cells in the dentate gyrus, serotonergic input from raphe nuclei might also exerted a general control over hippocampal neurogenesis via modulation of local inhibitory neurons.

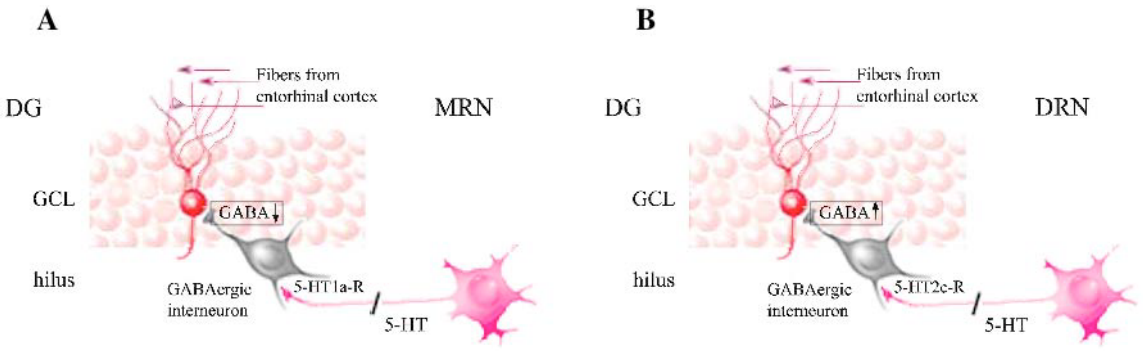


Fig. 31. Serotonergic afferences from A. median raphe nucleus (MRN) and B. dorsal raphe nucleus (DRN) establish synaptic contacts with GABAergic interneurons. A. Serotonergic projections terminate on hilar interneurons, which express 5-HT1a receptors. 5-HT1a receptor stimulation decreases the firing rate of interneurons, which exert a less inhibitory effect on precursor cells in the granule cell layer (GCL). **B.** Shows 5-HT2c receptor-mediated activation of GABAergic neurons that modulate dendritic inhibition of principle neurons in the dentate gyrus (DG). *Modified from (Lie et al. 2004)*

Furthermore, the present findings demonstrate that the effect of serotonin receptor modulation is seen on sequential steps in adult neurogenesis. Here, transgenic nestin-GFP mice and triple immunohistochemistry were used to characterize the different stages of neuronal development. DCX and Calretinin as selective cell markers are committed to the neuronal lineage in the adult brain. DCX expression has been connected with neuronal migration and dendritic outgrowth, whereas Calretinin characterizes an early postmitotic step in neuronal differentiation. The present data shows that the phase of neuronal survival is influenced by 5-HT1a receptors. Whereas acute stimulation of this receptor subtype produces a net increased in the BrdU/DCX-expressing cells population, blocking decreases the absolute number of BrdU+/NeuN-positive cells. The result is an indirect effect on the survival of newly generated neurons. Furthermore, the findings represent that the phase of neuronal differentiation during DCX expression is regulated by 5-HT2 receptors. Acute agonist treatment results in an increase of the type-2b cell population compare to the control group, which leads to an increase in the number of BrdU/DCX/CR-expressing cells after 7 days of daily administration. No differences were observed in the number of BrdU+/NeuN-positive cells, which indicate that 5-HT2 receptors affect early postmitotic immature neurons, characterized by transient Calretinin expression. The transgenic DCX-EGFP mouse could be

used to analyze differences in serotonin receptor function on specifically type-2 cells in adult neurogenesis.

Endothelial cells are an important cell type constituting a major component of the neurogenic niche. Cell division in the subgranular zone of the adult dentate gyrus occurs in clusters and in close proximity to blood vessels. Precursor cell proliferation is paralleled and can be induced by proliferation of endothelial cells (Palmer et al. 2000; Fabel et al. 2003; Cao et al. 2004). Interestingly, the results indicate a role of endothelial cells in serotonin-mediated effects. The present findings demonstrate a correlation in the number of BrdU/DCX-expressing cells with the amount of proliferating endothelial cells in the subgranular zone after acute and chronic blocking of 5-HT2 receptors. Acute receptor blocking with Cinanserin produced significant less BrdU+/DCX-positive cells as well as BrdU/Glut1-expressing endothelial cells in these animals. Remarkably, after chronic treatment over 7 days the same number of Glut-1-expressing cells survived leading to a 40% increase in the number of new neurons (BrdU/DCX-expressing cell population). The results suggest an as yet unidentified role of serotonin regulation of endothelial cell biology that impinges on precursor cell behavior.

5.2 5-HT1a and 5-HT2 receptors are critical components in the mechanism of fluoxetine action

Serotonin has an overall positively regulating effect on adult hippocampal neurogenesis. Animals injected with agents neurotoxic to serotonergic input showed reduced neural production in the adult hippocampus. Fetal serotonergic neurons grafted to the rat dentate gyrus restored this loss of neurons (Brezun and Daszuta 1999, 2000). Regulation of neurogenesis by serotonin could occur at several stages of differentiation, including proliferation, differentiation, and survival. Furthermore, preclinical studies suggest that enhanced serotonin transmission via 5-HT1a receptors is particularly important in antidepressant response. Here, the analysis of the surviving BrdU-positive cells in the dentate gyrus demonstrates that chronic fluoxetine treatment for a period of 21 days significantly increased the amount of newly generated cells relative to control. This is in agreement with several reports that used rats and have seen similar effects (Malberg et al. 2000; Santarelli et al. 2003). Administration of the antidepressant for 1 day did not affect the number of BrdU-

positive cells in the dentate gyrus either when the animals were killed one day nor 20 days later. The present study indicates that continuous antidepressant treatment is necessary to let the drug takes effect; fluoxetine needed to be administered over a chronic period of time to observe an effect on neurogenesis. Thus, antidepressant treatment in these paradigms had no effect on cell proliferation. Malberg in contrast, revealed a proliferating not a survival effect after chronic antidepressant treatment (Malberg et al. 2000). They suggest that antidepressants do not influence cell survival independently from increased cell proliferation. This discrepancy may be a species related effect. In this study adult mice were used in contrast to Malberg who used rats to study the effects of fluoxetine on adult neurogenesis.

Surprisingly, systemic antidepressant administration leads to a reduced number of BrdU/CR-expressing cells in the dentate gyrus. The result might suggest that fluoxetine enhances the speed of neuronal differentiation during maturation (early expression of NeuN), indicated by a loss of transient Calretinin expression. Recently the group of Encinas et al. (Encinas et al. 2006) has published that fluoxetine targets amplifying neural progenitor cells. Here, the present study has focused in more detail on the individual neuronal developmental steps. Although the independent role of fluoxetine in regulating Calretinin was not studied in this work, the data might suggest that fluoxetine treatment might accelerate neuronal development through the Calretinin stage.

A serotonin-induced increase of adult neurogenesis might promote recovery from depression. The time delay in the onset of antidepressant action is a function of the drug rather than the disease. The present findings might indicate a balance of fluoxetine action due to different receptors. Acute stimulation of 5-HT1a receptors with the direct agonist 8-OH DPAT increases precursor cell proliferation in the dentate gyrus whereas acute stimulation with the selective 5-HT2c receptor agonist WAY161503 significantly decreases the number of BrdU-positive cell. Here, one can conclude that fluoxetine indirectly activates the 5-HT1a receptors via increasing serotonin levels, and suggests a negative regulation of neurogenesis through 5-HT2 receptors. Taken together, the latency in the onset of fluoxetine action can be explained by the interaction of 5-HT1a and 5-HT2c receptor activities. Fig. 32 A shows that acute stimulation of both receptors result in a balanced serotonin level.

In a long-term setting over a period of 21 days, continuous administration of fluoxetine significantly increased the survival of newly generated cells (Fig. 32 B). Chronic stimulation of 5-HT1a receptors for a period of 7 days showed no significant changes, but a decreased

number of BrdU-positive cells by blocking. In contrast, 5-HT2 receptor agonist treatment significantly decreased the number of BrdU-positive cells whereas chronic inhibition revealed no changes relative to control (Fig. 32 B). Also chronic stimulation or inhibition of 5-HT1a and 5-HT2 receptors for 7 days revealed a balanced regulation of serotonin levels via a opposed action of these receptors.

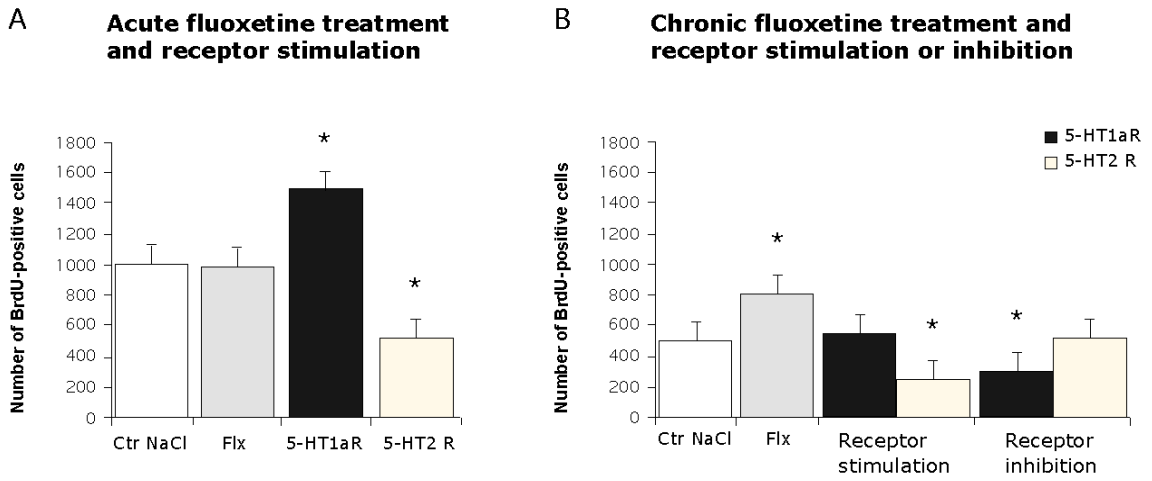


Fig. 32. Serotonergic input exert a balanced regulation of hippocampal neurogenesis via 5-HT1a and 5-HT2 receptors. A. Acute fluoxetine treatment does not effect the number of BrdU-positive cells in the adult dentate gyrus after one day. Based on 1000 proliferating cells in control (NaCl), acute stimulation of 5-HT1a receptors significantly increased the number of proliferating cells by 50% whereas acute blockade of 5-HT2 receptors halved the cell number. Taken together, acute stimulation of both receptors result in balanced serotonin level. **B.** Chronic antidepressant treatment over 21 days significantly increased the number of BrdU-positive cells (500 BrdU-positive cells in control are taken as basis). The number of newly generated cells is significantly decreased after 7 days of stimulation with the 5-HT2 receptor agonist α-Methyl-5-HT-maleate as well as by 5-HT1a receptor inhibition with WAY100135. Taken together, both receptors balance the level of serotonin in the dentate gyrus. Data are presented as numbers of BrdU-positive cells per dentate gyrus, means±SD.

Additionally, the effect of stimulation and blocking of two different receptors at the same time in one experiment could be investigated. The 5-HT1a receptor agonist 8-OH DPAT and the 5-HT2 receptor antagonist Cinanserin can produce several changes in serotonin system that exert antidepressant action. An antidepressant in the form of 8-OH DPAT and Cinanserin together could have the effect on both an increase in the number of proliferating cells in the dentate gyrus and an enhanced neuronal survival after long-time administration. Since long-

term stimulation with fluoxetine increases the survival of newly generated cells in the dentate gyrus, the effect might be mediate via both receptors. On the other hand, the time latency in action of antidepressant drugs can be explained by a time neurons need to recover their normal firing rate after a desensitization of raphe autoreceptors. Blier et al. already described a postsynaptic sensitization of 5-HT1a receptors in the adult hippocampus (Blier 2003).

Since enhanced serotonin neurotransmission underlies antidepressant response thus the neurotransmitter and its regulation of neurogenesis seem to be necessary for antidepressant treatment. The literature already suggests conditions that lower serotonin levels are associated with depression; slightly elevated serotonin levels due to different receptor function, e.g. alteration in postsynaptic 5-HT1a and 5-HT2 receptors in the dentate gyrus, tend to elevate mood and prevent depression.

5.3 Possible role of serotonin in brain plasticity, e.g. learning and memory

The ability to learn or unlearn, that is to remember or to forget an experience, may be dependent upon the rheostat function of neurochemicals like serotonin that fix the gain of neurogenesis. The hippocampus has long been associated with learning and memory processes, and as part of the limbic system with the modulation of emotional responses. This study demonstrates that the regulation of serotonin receptor subtypes assist the generation and survival of newly born neurons in the adult hippocampus. In fact, adult neurogenesis appears to be mediated by a balanced activation of 5-HT1a and 5-HT2 receptors. Newborn neurons get integrated into the circuitry thus might contribute to cognitive function mediated by the hippocampus.

Several studies suggest a role for serotonin in modulating long-term potentiation (Sakai and Tanaka 1993; Tecott et al. 1995b; Sanberg et al. 2006). They confirm that activation of 5-HT1a receptors generally has an inhibitory effect on LTP in the perforant path projection to the dentate gyrus. The perforant path constitutes the primary projection system relaying information from the neocortex to the hippocampal formation. Sanberg et al. suggest that 5-HT1a receptors contribute to LTP induction via inhibition of GABAergic interneurons. Systemic administration of the 5-HT1a agonist 8-OH DPAT to the median raphe nuclei activates 5-HT1a autoreceptors thus blocks LTP due to reduced serotonin levels (Sanberg et

al. 2006). In contrast, the antagonist WAY100635 also blocks LTP via the blockade of postsynaptic receptors in the dentate gyrus. The study attributes serotonin receptors in the hippocampus a functional role not only in depression but also in memory processes.

Although serotonin is an important neurotransmitter and neuromodulator, BDNF or VEGF (Warner-Schmidt and Duman 2007) are essential for neurogenic action in the hippocampus by mediating serotonin effects. The neurotrophic factor BDNF is known to play a role in neuronal survival and synaptic plasticity underlying the consolidation of certain forms of memory (Heldt et al. 2007). Additionally, studies indicated a role for neurotrophic function in the hippocampus to protect from anxiety and depression. They show that cognitive deficits in depression may be related to decreased hippocampal BDNF, which is reversed by antidepressant drugs that increase the expression of BDNF in the brain. Interestingly, 5-HT2 receptor subtypes stimulation caused a downregulation of BDNF mRNA expression in the dentate gyrus, which suggest a role of BDNF in mediating serotonin effects (Vaidya et al. 1997).

The hippocampus is involved in both long-term memory formation and cognitive processing. Intrinsic GABAergic interneurons, and neurotrophic factors including BDNF are present in the hippocampus. Furthermore, this brain region receives extrinsic inputs of various neuromodulatory neurotransmitters such as fiber projections that are serotonergic, dopaminergic, cholinergic, and noradrenergic. All these agents are participate in synchronizing hippocampal neuron firing patterns by various mechanisms and constitute a functional role to optimize hippocampal function. The integration of newly born neurons of the adult dentate gyrus into a preexisting circuitry plays an important role in cognition, and may compensate in situations of structural and functional loss.

The hypothesis presented here purports that the interaction of adult hippocampal neurogenesis and serotonergic signaling contribute to memory formation and might promote recovery from major depression. In this context, the present study described in more details the functional role of serotonergic receptor control on adult hippocampal neurogenesis. Here, compelling evidence suggest that acute and long-term modulation of serotonergic neurotransmission via 5-HT1a and 5-HT2c receptor subtypes mediate opposing effects on proliferation, differentiation and survival of newly generated neurons. The current knowledge of serotonin receptor function and its control of precursor cell development also contribute to

understand neural mechanisms underlying the cellular responses of antidepressant action. It furthermore underscores the importance of research on basic principles that afterwards will be translated into clinical relevance. Further studies are required to clarify aspects of interactions with other serotonin receptors, especially the 5-HT3 receptor family, and in association with pharmacological action of antidepressants.

In summary, I here have challenged and manipulated the serotonin system in order to test whether 5-HT1a or 5-HT2c receptor subtypes directly or indirectly modulate neuronal differentiation and survival. I have done so with caution because the dependent variable, aspects of adult neurogenesis, is locally and systemically influenced by many biological processes, which cannot be controlled or even monitored. Hence, these experiments do not verify my hypothesis as 'true', but they also do not vitiate the arguments I have put forth. As it stands, my hypothesis is conceptionally correct, at least until the never-ending pursuit of falsification proceeds by another.

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Appendix

Solutions for the tissue preparation step

0.9% Sodiumchloride	*50 ml per 20 g animal*
Paraformaldehyde (PFA), 4%	*50 ml per 20 g animal*
	500 ml dd H_2O at 65°
	500 ml 0.2 M PO_4 buffer (on Ice)
	40 g PFA / NaOH to adjust the pH
0.2 M Phosphate Buffer	6.35 g NaH_2PO_4 x 1 H_2O
	27.46 g Na_2HPO_4 x 2 H_2O
	fill up to 1 l H_2O
Saccharose 30%	150 g Saccharose
	400 ml 0.1M PO_4

Buffers and solutions for immunochemistry

0.1 M Phosphate Buffer	3.18 g NaH_2PO_4 x 1 H_2O
	13.73 g Na_2HPO_4 x 2 H_2O
	fill up to 1 l with dd H_2O
10x Tris buffer solution den 12. Oktober 2007	132.20 g Trizma HCL
	19.40 g Trizma Base
	90 g NaCl
	fill up to 1 l with dd H_2O

0.1M Borate Buffer (pH 8,5)	3.08 g Boric Acid 450 ml dd H_2O 5N NaOH to pH 8,5 fill up to 500 ml with dd H_2O
Cryoprotection solution (CPS)	250 ml Glycerol (25%) 250 ml Ethylenglycol (25%) 500 ml 0.1 M PO_4
Diamino-benzidin (DAB)	Stock Concentration: 20 mg/ml 10 mg DAB tablet (in 40 ml TBS)
Nickelchloride 8%(NiCl)	8 g Nickelchloride (Hexahydrate) Fill up to 100ml with dd H_2O

Selbständigkeitserklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst und nur die angegebene Literatur und erlaubte Hilfsmittel verwendet zu haben.

Berlin, den 12. Oktober 2007

Friederike Claudia Klempin

Publications

Friederike Klempin, Golo Kronenberg, Giselle Cheung, Helmut Kettenmann, and Gerd Kempermann (2008). Investigation into the properties of doublecortin-expressing cells in the adult mouse piriform cortex as compared to the dentate gyrus. *Manuscript in preparation*

Gerardo Ramírez-Rodríguez, **Friederike Klempin**, Harish Babu, Gloria Benítez-king, Gerd Kempermann (2008). Melatonin modulates cell survival of newly formed cells in the adult hippocampus. *Submission to Biological Psychiatry*

Friederike Klempin, Harish Babu, Klaus Fabel, and Gerd Kempermann (2008). Differential acute and chronic effects of serotonin receptor activation and blockade on sequential stages of adult hippocampal neurogenesis. *Submission to Molecular Psychiatry*

Dan Ehninger, LiPing Wang, **Friederike Klempin**, Benedikt Römer, Helmut Kettenmann, and Gerd Kempermann (2008). Activity-dependent plasticity of NG2 cells in the adult murine amygdala. *Manuscript in preparation*

Barbara Steiner, Andreas Kupsch, Eberhard Siebert, Kai Hosmann, Friederike Klempin, Rudolf Morgenstern, Christine Winter (2007). Unilateral lesion of the subthalamic nucleus transiently provokes bilateral subacute glial cell proliferation in the adult rat substantia nigra. *Neurosci Lett. Nov 06*

Friederike Klempin & Gerd Kempermann (2007). Adult hippocampal neurogenesis and aging, *Review. Eur Arch Psychiatry Clin Neurosci. April 1*

Tobias Plümpe, Dan Ehninger, Barbara Steiner, **Friederike Klempin**, Sebastian Jessberger Moritz Brandt, Benedikt Römer, Gerardo Ramirez Rodriguez, Golo Kronenberg, and Gerd Kempermann (2006). Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation. *BMC Neurosci 7:77*

Barbara Steiner*, **Friederike Klempin***, Liping Wang, Monika Kott, Helmut Kettenmann, and Gerd Kempermann (2006). Type-2 cells as link between glial and neuronal lineage in adult hippocampal neurogenesis. *Glia 54(8)*

Curriculum vitae

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Academic position: Since January 2008 Research Associate in Daniel A. Peterson's Lab at the Center for Stem Cell and Regenerative Medicine at Rosalind Franklin University of Medicine and Science, North-Chicago, IL, USA

Academic profile/Education:

2004 – 2007 PhD student at the Max Delbrueck Center for Molecular Medicine, Neuronal Stem Cells Group of Prof. Gerd Kempermann, Berlin, Germany, German degree of Doctor rerum naturalium (Dr. rer. nat.), subject of the thesis: Adult Brain Plasticity: Serotonin receptor subtypes mediate opposing effects on adult hippocampal neurogenesis
Since 2006 Member of the German Neuroscientific Society (NWG)
Since 2008 Member of the Society for Neuroscioence (SfN)

1997 – 2003	Study of biology with diploma at the Humboldt University of Berlin
1991 – 1996	German secondary school with degree
1983 – 1991	German primary school

Practical courses:

2000	Work experience in biophysics at the IBPC – Institute de Biologie Physico-Chimique, Paris, France
2008	Practical Training Course in Confocal Microscopy and Stereology, CSCRM, Dr. Daniel A. Peterson, Rosalind Franklin University of Medicine and Science, Chicago, USA

Scientific award:

Prize at the ROUTE28 workshop, September 2006

Spoken languages:	Native language	German
	Foreign language	English
	Beginner to moderate	Russian, Spanish, French

Meetings, abstracts, poster and talks:

2004	In April, I attended the BNF - Berlin Neuroscience Forum in Liebenwalde, Germany In October, I attended for the SfN, San Diego, USA
2005	In October, I participated in the Neuro-Retreat, Doellnsee, Germany, with oral presentation: Serotonin receptor stimulation differentially regulates adult hippocampal neurogenesis.

2006 Oral presentation at the meeting in Gothenburgborg, Sweden: Serotonergic regulation of the adult hippocampal neurogenesis via the 5-HT1a receptor.

In September, I attended the ROUTE28, Frauenchiemsee, Germany; Poster presentation at the International Stem Cell Meeting in Dresden, Germany: Friederike Klempin, Harish Babu, and Gerd Kempermann. Serotonin receptor stimulation differentially regulates adult hippocampal neurogenesis.

In October, poster presentation at the annual SfN meeting in Atlanta, USA: Friederike Klempin, Harish Babu, and Gerd Kempermann. Serotonergic regulation of the adult hippocampal neurogenesis via the 5-HT1a receptor.

2007 In Mai, oral presentation in Daniel Peterson's Lab, Rosalind Franklin University for Medicine and Science, Chicago, USA: Serotonergic regulation of the adult hippocampal neurogenesis via the 5-HT1a and 2c receptors.

In June, poster presentation at the CRTD (Center for Regenerative Therapy) summer conference, Dresden, Germany: Friederike Klempin, Harish Babu, and Gerd Kempermann. Serotonergic regulation of the adult hippocampal neurogenesis via the 5-HT1a and 2c receptors.

2008 May, Abstract submission for SfN in November 2008, WA D.C., USA, Friederike Klempin & Daniel A. Peterson. Providing a Noggin-containing niche following neural stem cell grafting as strategy for structural repair of the injured entorhinal cortex.

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