Studies on neurogenesis in the adult human brain

Av: Annika Andersson
Handledare: Olof Bendel
Abstract

Many studies on neurogenesis in adult dentate gyrus (DG) have been performed on rodents and other mammalian species, but only a few on adult human DG. This study is focusing on neurogenesis in adult human DG. To characterize the birth of cells in DG, the expression of the cell proliferation marker Ki67 was examined using immunohistochemistry. Ki67-positive labelling was indeed observed in the granular cell layer and the molecular layer of dentate gyrus and in the hilus of hippocampus, as well as in the subgranular zone (SGZ). The Ki67 positive nuclei could be divided into three groups, based on their morphology and position, suggesting that one of the groups represents neuronal precursors. Fewer Ki67 positive cells were seen in aged subjects and in subjects with an alcohol abuse. When comparing the Ki67 positive cells and the amount of blood vessels as determined by anti factor VIII, no systematic pattern could be discerned. To identify possible stem/progenitor cells in DG a co-labelling with nestin and glial fibrillary acid protein was carried out. Co-labelling was found in the SGZ, but most of the filaments were positive for just one of the two antibodies. Antibodies to detect immature/mature neurons were also used to investigate adult human neurogenesis in DG. The immature marker βIII-tubulin showed a weak expression. The other two immature markers (PSA-NCAM and DCX) used did not work, probably since they were not cross-reacting against human tissue. In summary, this study shows that new cells are continuously formed in the adult human hippocampus, but at a slower pace compared to the rat, and that some of these new cells may represent neuronal precursors.
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### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>BB</td>
<td>Blocking buffer</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CA 1-3</td>
<td>Cornu Ammonis 1-3</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3´-diaminobenzidine</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex, forms the main input to the hippocampus.</td>
</tr>
<tr>
<td>GCL</td>
<td>Granule cell layer</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>KI</td>
<td>Karolinska Institutet</td>
</tr>
<tr>
<td>ML</td>
<td>Molecular layer</td>
</tr>
<tr>
<td>MAOI</td>
<td>Monoamine oxidase inhibitor</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NPCs</td>
<td>Neural progenitor cells</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PMI</td>
<td>Post-mortem interval, time between death and autopsy.</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Polysialic acid-neural cell adhesion molecule</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
</tbody>
</table>

**Keywords:** Neurogenesis, subgranular zone, adult human DG, Ki67.
1 Introduction

For many years scientists have believed that no neurogenesis occurs in the adult mammalian brain, but rather that neurogenesis is restricted to development. One of the first support for the opposite, i.e. that neurogenesis does occur in the adult mammalian brain was reported by Altman and Das (Altman et al., 1965), who described formation of new cells in the hippocampal formation. The hippocampal formation is a structure located in temporal lobe of the brain (Parent, 1996.), which plays an important role in several cognitive functions, including learning and memory (Morris, 2007).

The principal neurons within this structure include the pyramidal neurons of the hippocampus proper (CA1 to CA3) and granule cells of the dentate gyrus (DG). Within the DG, neuronal density is particularly high in the granular cell layer (GCL) (Figure 1A). Based on animal research it is known that, new granule cells are constantly generated in the subgranular zone (SGZ) (figure 1B) (Bohlen et al., 2007). The molecular layer (ML) is located on the opposite side of the GCL.

![Figure 1. A coronal section of a human DG labelled with Hoechst (A-B). The densely packed granular cells build up the winding band called the granular cell layer (A). Figure 1B is a magnification of the marked area in Figure 1A. This close-up view of DG (B) shows the molecular layer (ML), the granular cell layer (GCL) and the sub granular zone (SGZ). Scale bar = 1 mm (A) and 50 µm (B).](image)

In adult rodents, neural stem cells (NSCs) divide slowly, but their progeny (neural progenitor cells (NPCs)) divides rapidly, and this generates a pool of immature granule cells (Figure 2). Following proliferation, the newly formed cells start to migrate into the GCL, where their dendrites and axons develop (Bohlen et al., 2007; Christie et al., 2006). The mature granule neurons establish their synaptic contacts, receiving their projections from the entorhinal cortex (EC) and sending their projections to the CA3 and hilus regions. If a newly formed neuron fails to tie contact with CA3 or other neurons the newly formed neuron will die.
(Bohlen et al., 2007; Christie et al., 2006). It has also been proposed that integration of new neurons into functional circuits is necessary for learning and memory processes (Kempermann et al., 1997; Mirescu et al., 2006)

**Figure 2.** A schematic representation of DG. Neurogenesis in DG can be divided into three major steps; proliferation, migration and differentiation. During proliferation (1-2), neuronal stem cells (NSCs; blue) give rise to mitotically active neuronal progenitor cells (NPCs; yellow). Subsequently, the newly formed granule cells (orange) start to migrate into the GCL (3). During differentiation (3-4), dendrites and axons emerge from the granule cells and their axons project to CA3 neurons or hilar neurons (not shown).

Adult neurogenesis in the DG is positively affected by enriched environments, exercise and hippocampus-dependent learning tasks (Kempermann et al., 1997; Mirescu et al., 2006). At the cellular level, a variety of signaling molecules play important roles in this response (Lee et al., 2009). Some of the signaling molecules will bind to their receptors and activate downstream signalling pathways. These pathways will ultimately regulate genes responsible for proliferation, differentiation, and/or migration of cells. Adult neurogenesis is promoted by several neurotrophins, including brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3). Notably, BDNF expression in the hippocampus is up-regulated by a variety of antidepressant drugs, including selective serotonin reuptake inhibitors (SSRI), and monoamine oxidase inhibitors (MAOI) (Huang et al., 2008). Following SSRI- treatment, patients with major depressive disorder were reported to show more NPCs and proliferating cells in the SGZ (Boldrini et al. 2009), possibly caused by an increased expression of BDNF or other signaling molecules. Some of the signaling molecules that promote adult neurogenesis also stimulate the formation and growth of new blood vessels from pre-existing vessels, i.e. angiogenesis. In particular, vascular endothelial growth factor (VEGF) is a potent modulator of angiogenesis but also neurogenesis (Lee et al., 2009; Mudò et al., 2009). VEGF is also up-regulated by antidepressants, and is essential in the mechanisms that promote neurogenesis in adult mice following exercise and environmental enrichment (Cao et al., 2004; Fabel et al., 2003). Moreover, it has been shown that adult
neurogenesis occurs within an angiogenetic niche, suggesting that these processes might not only represent parallel phenomena, but may be functionally linked (Palmer et al., 2000).

Adult neurogenesis is negatively affected by stress and corticoids (Cameron et al., 1994; Gould et al., 1997), the same effect have endogenous glucocorticoids on angiogenesis (Small et al., 2005). It should be noted that aging has been associated with increased levels of cortisol (Lupien et al., 1994) and reduced neurogenesis (Cameron et al., 1999; Kuhn et al., 1996). Taken together, a variety of growth factors and hormones appear to act in concert to regulate adult neurogenesis and that angiogenesis may play an important role in the formation of new nerve cells in the brain.

1.1 Neurogenesis in the adult human dentate gyrus.

Neurogenesis in adult mammalian DG has been investigated for many years, but few studies have been conducted on adult neurogenesis in human DG. The knowledge regarding neurogenesis in DG is mainly based on studies done in animals, mostly rats and mice. Today we do not know if there are species-dependent differences in adult neurogenesis. In fact, the volume of a human hippocampus is approximately 100 times greater than the volume of a rat hippocampus (Morris et al., 2007), but given the difference in complexity of the brain across species, it is difficult to predict the cell turnover in human hippocampus from animal data. For ethical and practical reasons, many studies performed in rodents cannot be repeated in humans. However, in 1998 Peter S. Eriksson’s group in Gothenburg designed a study on post-mortem brains from cancer patients who had received bromodeoxyuridine (BrdU) for diagnostic purposes (Eriksson et al., 1998). BrdU is an analog to thymidine, which integrates into the DNA during the S phase in dividing cells (Taupin, 2007). By using triple immunohistochemistry labelling for BrdU and other cell-specific markers, these authors provided the first line of evidence of neurogenesis in the adult human DG (Eriksson et al., 1998). Only a limited number of studies on neurogenesis in the adult human brain has since been performed, whereas there has been an immense increase in reports regarding experimental studies on rodents.
1.2 Specific markers involved in neurogenesis

1.2.1 Neural stem cells

By using non-neuronal cells markers information about neurogenesis can also be obtained. Glial Fibrillary Acidic Protein (GFAP) is an intermediate filament. This protein is routinely used by pathologist as a marker for astrocytes, but a large amount of newborn cells in SGZ also express GFAP (Bohlen et al., 2007). Nestin is a neural-specific intermediate filament protein expressed in CNS stem cells (Bohlen et al., 2007; Frederiksen et al., 1998; Lendahl et al., 1990). It has been show that NSCs express both nestin and GFAP at the same time (Encinas et al., 2008).

1.2.2 Cell proliferation

Ki67 and Proliferating Cell Nuclear Antigen (PCNA) are two markers that can be used to detect cell proliferation. Ki67 is a nuclear protein that is expressed in phase G1, S, G2 and M in the cell cycle, but its function is not known today (Kee et al., 2002). PCNA is a nuclear protein which is synthesized in the G1 and S phases of the cell cycle (Raucci et al., 2006).

1.2.3 Immature neurons

In order to identify immature neurons, several markers can be used; hence their expression of certain proteins such as PSA-NCAM, βIII-tubulin and DCX can be examined. These proteins are almost exclusively active in immature neurons and when the neurons mature the expression of these proteins is down-regulated (Bohlen et al., 2007). Polysialic acid (PSA)-neural cell adhesion molecule (NCAM) is expressed by migrating neuroblasts and early post-mitotic neurons. PSA is a long, linear homopolymer which is attached to NCAM immature nerve cells, but is lost when the cell matures (Bohlen et al., 2007; Gascon et al., 2008). Doublecortin (DCX) is a protein that promotes microtubule polymerization, which is expressed in migrating neuroblasts and young neurons (Bohlen et al., 2007). βIII-tubulin is a unique microtubular subunit, which is expressed almost exclusively in young neurons (Braun et al., 2002).
1.2.4 Mature neurons

NeuN protein is a soluble nuclear protein which is present in the neuronal cytoplasm of post-mitotic neurons (Bohlen et al., 2007). NeuN is a neuron-specific protein that is present in most CNS and PNS neuronal cell types. Unfortunately, this very specific marker is not expressed by neuronal precursors, making it difficult to separate these from other immature cells.

1.3 Aim of the study

This study was designed to address questions regarding the adult human DG:
1. Where are new cells formed in relation to the GCL?
2. Are new neurons formed in DG?
3. Do the markers: PSA-NCAM, βIII-tubulin and DCX, for immature neurons work on post-mortem human tissue?
4. Does cell renewal in DG decrease with age?
5. Is cell renewal in DG reduced in alcoholics?
2 Material and Methods

2.1 Brain samples and preparation.

Human hippocampal tissues were identified and sampled during forensic autopsy (at the Department of Forensic Medicine at KI in Solna) and frozen in iso-pentane/dry ice at -35°C. Coronal sections of hippocampus were cut in 20 µm. Sections were thaw-mounted onto slides (Superfrost Plus, Menzel-Gläser, Germany), air-dried for 1-2 hours at room temperature (RT) and then stored at -20°C until preparation for immunohistochemistry. Table 1 show the cases included in this study.

Table 1. Information about the cases.

<table>
<thead>
<tr>
<th>Cases</th>
<th>Sex</th>
<th>Age</th>
<th>Abuse</th>
<th>Depression</th>
<th>Suicide</th>
<th>PMI (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>19</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Y</td>
<td>&gt; 48</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>28</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>28</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>37</td>
<td>Alcohol</td>
<td>Y</td>
<td>Y</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>51</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Y</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>57</td>
<td>Alcohol</td>
<td>Y</td>
<td>Y</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>59</td>
<td>Alcohol</td>
<td>N</td>
<td>N</td>
<td>&gt; 48</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>73</td>
<td>Alcohol</td>
<td>Y</td>
<td>Y</td>
<td>23</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>89</td>
<td>N</td>
<td>n.d.</td>
<td>n.d.</td>
<td>35.5</td>
</tr>
</tbody>
</table>

Y = Yes, N = No, n.d. = not determined.

2.2 Immunohistochemistry

All sections that were used in this study were air-dried and rehydrated in phosphate buffered saline (PBS) comprising (in mM) NaCl 137, KCl 2.7, KH2PO4 15, HNa2PO4 13.6, pH 7.7, and then fixed in 4% paraform-aldehyde in PBS for 30 min before the preparation of the fluorescence immunohistochemistry and ABC method, these methods were performed separately.

For fluorescence immunohistochemistry, the sections were blocked for 1 hour (4°C) with blocking buffer (BB), containing: 1% BSA, 0.3% Triton-X and 0.01% NaN3 in PBS. The blocking was gently removed by tilting the sections. Primary antibodies (see Table 2) were diluted in BB and then added to the sections, and incubated at 4°C overnight. No primary antibodies were applied on negative controls, which were processed on adjacent sections, including one section per subject and staining. Following the incubation with primary
antibodies, the blocking step was repeated, and sections were incubated with secondary antibodies, which were diluted in BB. Sections were thoroughly washed for 3x10 min in PBS between steps, except after BB steps. Finally, the sections were mounted in cytomatic (DAKO Cytomation, DK).

For ABC method, the sections were incubated for 30 min in 0.3% H₂O₂ in PBS, to reduce endogenous peroxidise activity. Subsequently, the sections were incubated for 1 hour at 4°C in BB and avidin, which was diluted according to the manufacturer’s instructions (Avidin/Biotin Blocking kit, Vector Laboratories, CA, USA). Primary antibodies: TU20 (1:1000; Millipore, CA, USA) or nestin (1:1000; Millipore, CA, USA), were diluted in a solution containing BB and biotin (Vector Laboratories). The sections were then incubated with the diluted antibodies overnight at 4°C. No primary antibodies were applied on negative controls, which were processed on adjacent sections, including one section per subject and staining. After the overnight incubation, the sections were incubated for 1 hour at RT with the secondary antibody (α-mouse IgG Vectastain® Elite® ABC kit, Vector Laboratories), which was diluted (1:250) in PBS. The sections were then incubated with avidin-biotinylated enzyme complex (ABC solution) for 1 hour. The signal was visualized using 3, 3’- diaminobenzidine (DAB), and H₂O₂ (DAB substrate kit for peroxidise, Vector Laboratories). Sections were thoroughly washed for 3x10 min in PBS between steps. Finally, the sections were dehydrated in graded series of ethanol, immersed in xylene, mounted in DPX.

2.3 Histological analysis

Sections were analyzed using a Nikon Microscope (ECLIPSE E400) equipped with a Nikon camera unit (Nikon Digital Sight: DS-2Mv, DS-U2), fluorescent filters (UV, rhodamine and FITC), power supply for mercury lamp (C-SHG1) and magnification (TV-lens 0.55x, 10x ocular, 2-60x objectives). Each section was photographed using the NIS-Elements BR 2.30 program (Nikon Corporation, Japan). The images were then edited in Adobe Photoshop CS 8.0 (Adobe Systems Incorporated, USA). Quantification of the number of dividing cells (Ki67-positive nuclei) was performed in sections including the ML, GCL and SGZ. The boundaries of DG were defined at 400x magnification, and included areas 0.3 mm on each side of GCL. Ki67-positive nuclei on five sections per case were counted and the mean values were calculated. For each case the mean of the Ki67-positive nuclei result was divided with the length of DG, this value is identified as the Ki67-index. The length was measured using a
2x objective and documented with NIS- Elements BR 2.30. To identify DG, Hoechst 33342 ([0.2 µl/ml BB], Invitrogen Molecular Probes, UK) was used, see Figure 1. As a measure of the vascular density, vWF-positive vessels exceeding 100 µm were counted, and only those that also were in contact with GCL. A grid eyepiece was used to determine the length of the vessels. Measurements of long blood vessels were taken three sections per case and the mean values were calculated. Like the quantification of the number of dividing cells (Ki67-index) and the mean value of the long blood vessels, was then divided by the representative length of DG for each case, this value is identified as the blood vessel-index. The graphs were done in OriginPro 8.0. The Ki67-index and blood vessel-index data are present in scatter plots, expressed as means ± S.E.M (standard error of the mean).

Table 2. Primary and secondary antibodies

<table>
<thead>
<tr>
<th>Marker/Antibody</th>
<th>Type/Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td>Polyclonal / rabbit</td>
<td>1:250</td>
<td>Millipore, CA, USA</td>
</tr>
<tr>
<td>PCNA</td>
<td>Monoclonal / mouse</td>
<td>1:100- 1:800</td>
<td>Millipore</td>
</tr>
<tr>
<td><strong>Immature neurons</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Monoclonal / mouse</td>
<td>1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>TU20</td>
<td>Monoclonal / mouse</td>
<td>1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>DCX</td>
<td>Polyclonal / goat</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td><strong>Mature neurons</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NeuN</td>
<td>Monoclonal / mouse</td>
<td>1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td><strong>Non-neuronal cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFAP</td>
<td>Monoclonal / mouse</td>
<td>1:1000</td>
<td>Serotec, UK</td>
</tr>
<tr>
<td>GFAP</td>
<td>Polyclonal / rabbit</td>
<td>1:1000</td>
<td>Dako Cytomation, DK</td>
</tr>
<tr>
<td>Nestin(^1)</td>
<td>Monoclonal / mouse</td>
<td>1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>Nestin(^2)</td>
<td>Monoclonal / mouse</td>
<td>1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>vWF</td>
<td>Monoclonal / mouse</td>
<td>1:2000</td>
<td>Dako Cytomation</td>
</tr>
<tr>
<td><strong>Secondary Antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α -rabbit Alexa Fluor 555</td>
<td></td>
<td>1:500</td>
<td>Invitrogen Molecular Probes, UK</td>
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<tr>
<td>α -mouse Alexa Fluor 488</td>
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<td>1:500</td>
<td>Invitrogen Molecular Probes</td>
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<td>α -goat Alexa Fluor 488</td>
<td></td>
<td>1:500</td>
<td>Invitrogen Molecular Probes</td>
</tr>
<tr>
<td>Biotinylated α -mouse</td>
<td></td>
<td>1:250</td>
<td>Vector Laboratories, CA</td>
</tr>
</tbody>
</table>

\(^1\) Human specific, \(^2\) Rat specific.3 Results
3 Results

3.1 Cell proliferation and blood vessels

By double labelling with Ki67 and Hoechst, the morphology of the Ki67-positive nuclei in DG sections could be examined. Ki67-positive nuclei could be divided into three categories. The first category of nuclei was big and rounded (Figure 3A) where the majority were found as singles, both in the SGZ and in the ML. The second category of nuclei were small and rounded (Figure 3B). Many were found in pairs in the DG, suggesting symmetric cell division. These nuclei were smaller in size compared to the first category of nuclei. Moreover, a third category of Ki67-positive nuclei had an elongated shape (Figure 3C). We also observed Hoechst-positive nuclei with the same morphology categories as the Ki67-positive nuclei (Figure 3D). The other cell proliferation marker PCNA, which marks the G1 and S phases of the cell cycle (Raucci et al., 2006), was also tested, but did not work on these post-mortem human sections.

Figure 3. Photomicrographs showing Ki67-positive nuclei (A-C) and Hoechst-labelling (D) in the adult human DG. Arrowheads indicate each of the three categories Ki67-positive nuclei, including a large rounded nucleus (A), one pair small rounded nuclei (B), and a single elongated nucleus (C). The characteristics of these three different categories of nuclei were also seen with Hoechst labelling (D). Insets show the characteristics of these nuclei at higher magnification (A-D). Ki67-negative nuclei are visible in grey (A-C). Broken lines show the boundary between the GCL and SGZ (A-D). Scale bar = 50 µm
To analyse for individual differences in cell proliferation the Ki67-index was calculated, mean values (calculated for five sections per case) of Ki67-positive nuclei were divided by the length of the DG on each section. Ki67-index was highest in two of the youngest individuals, and quite low among the elderly (Figure 4). Notably, the Ki67-indexs for the alcoholics (asterisk in Figure 4) were all below the trend-line.

![Figure 4. Ki67-index in human DG vs. ages. Note that the older subjects have lower Ki67-index. The asterisks mark subjects with alcoholic abuse. These subjects had Ki67-index that were under the trend line. Bars show standard error of the mean.](image)

In adult mice, it has been shown that neurogenesis occurs within a vascular niche (Palmer et al., 2000). Newly formed cells were in this study often associated with cerebral blood vessels, Ki67-positive nuclei co-localized with both nestin-positive vessels (Figure 5A) and von Willebrant factor (vWF) (Figure 5C).
Cell proliferation was shown to be negatively affected by aging (Cameron, et al., 1999; Klempin, et al., 2007; Kuhn, et al., 1996). To see if long blood vessels (≥100 µm) in the GCL are affected the same as Ki67-positive nuclei the long blood vessel in the GCL were counted. The mean for three sections was used as a measure of the vascular density in DG for each individual. To get comparable data, the mean value for each subject was divided by an estimated length of the DG. These results were compared with the age of the individuals (Figure 6). Relatively few long blood vessels were observed in one of the youngest subjects, but there was little variation among the others.
Figure 6. Blood vessel-index in human GCL vs. ages. Each value represents the mean of the number of long vessels, divided by the length of DG on three coronal sections. Bars show standard error of the mean.

To see whether the degree of cell proliferation was associated with the degree of vasculaturation, blood vessels-indexes were plotted against Ki67-indexes (Figure 7). There was no obvious correlation between markers for cell proliferation and vascular density in our experiment; except for one of the subjects which had both high proliferation and vascular score. This subject was one of the youngest in the study. Interestingly, there was no correlation between blood vessels-indexes and Ki67-indexes and the age of the individual.
3.2 Nestin and GFAP

The intermediate filament protein nestin is a protein, which is expressed in CNS stem cells (Lendahl et al. 1990). It has since its discovery been used as a marker for NSCs and NPCs. In this study, nestin-positive cells were found mostly in the SGZ (Figure 8A-8B (arrow)). No quantification of the nestin positive cells was performed since the labelling often was restricted to filaments outside the cell body, implying that several adjacent dendrites or axons either could represent one cell, or two or more cells. However, NSCs have a unique molecular signature, and have been shown to express both nestin and GFAP (Encinas et al., 2008). GFAP is an intermediate filament, and is present in both astrocytes and newborn cells in SGZ (Bohlen et al., 2007). To improve the identification of NSCs, sections were double-labelled for GFAP and nestin (Figure 8C-8D). Only a few of the intermediate filaments were double positive for nestin and GFAP (Figure 8C-8D).
Figure 8. NSCs and NPCs in the adult human SGZ. At low magnification, GCL is shown by Hoechst labelling (A). The area within the box in (A) is displayed at higher magnification in (B-D) Nestin-positive cells with different morphologies were present in the SGZ (B); the arrows point at a cell with nestin-positive filaments and the arrowhead points at a nestin-positive blood vessel. Broken lines show the boundary between the GCL and SGZ, Hoechst positive nuclei are shown in grey. Some filaments were positive for both GFAP (C) and nestin (D), as shown by arrows. However, the majority of filaments were only GFAP-positive (arrowhead, C). Scale bar = 1 mm (A), 50 µm (B) and 17 µm (C and D)

3.4 Immature neurons

There are a number of markers which could be used to identify immature neurons (Bohlen et al., 2007; Braun et al., 2002; Gascon et al., 2008). We used βIII-tubulin, detected by both florescence and ABC immunohistochemistry methods. It was a weak expression of βIII-tubulin in all the subjects, the majority of the filaments were observed in the GCL (Figure 9A). When using the ABC method the expression of βIII-tubulin was seen both in cells bodies and in filaments (Figure 9B) in the SGZ and hilus. The other two immature neuron markers that were tested, DCX and PSA-NCAM, did not work on these post-mortem human samples.
3.3 Mature neurons

The most widely used marker for mature neurons is NeuN, which typically strongly stains their nuclei. In this study NeuN were used together with Hoechst (Figure 10A and 10B) to mark mature neurons. In agreement with previous reports (Kee, et al., 2002) no co-localization of Ki67 and NeuN could be detected in any of the sections examined.
4 Discussion

4.1 Cell proliferation and blood vessels

Neurogenesis in the adult DG is a process involving many stages. It can broadly be defined as the cell proliferation, migration, differentiation and ultimately the maturation of granule cells. By using a battery of antibodies, this study aimed to characterize this phenomenon in the adult human DG. Cell proliferation is a prerequisite for adult neurogenesis, and Ki67 protein is exclusively expressed in the interphase and M phase of the cell cycle. In this study, three categories of Ki67-positive nuclei were observed in the adult human DG (Figure 3A-C). The first category of Ki67-positive nuclei consisted of nuclei with size and shape similar to the nuclei of granule cells (Figure 3A). This suggests that these nuclei may be destined to become granule cells. The second group of Ki67-positive nuclei may represent dividing small glia cells (Figure 3B), but since they often occurred in pairs, alternatively they represent newly formed daughter cells of category one (i.e. in cytokinesis phase). The third category of Ki67-positive nuclei had an elongated shape. These nuclei were typically located close to blood vessels and may represent endothelial cells.

In the adult human DG, we observed relatively few Ki67-positive nuclei. This agrees with earlier Ki67 data from postmortem studies (Boekhoorn et al., 2006; Reif et al., 2006). Recently, it was reported that age has no effect on cell proliferation in the adult human DG (Boldrini et al. 2009). In contrast these observations are not in agreement with animal studies which show that age was an effect on cell proliferation (Cameron, et al., 1999; Klempin, et al., 2007; Kuhn, et al., 1996). In our study the older subjects generally had lower Ki67-indexes then the younger; an age trend can be suspected. There may be different reasons for such a decline, but the fact that older people generally become less active, both mentally and physically, is probably important. Animal studies have clearly established that both exercise and living in an enriched environment have positive effects on cell proliferation/neurogenesis in adult DG (Gascon, et sl., 2008; Kempermann, et al., 1997; Mirescu, et al., 2006).

In this study, Ki67-indexs were particularly low in subjects that had a history of alcohol abuse, regardless of their age. Likewise, adult rats were receiving alcohol to drink for five weeks had fewer BrdU positive cells in the SGZ than the control group that was given water (Herrera, et al., 2003). However, the low number of subjects in this study does not allow for a firm conclusion regarding the influence of alcohol exposure and rate of cell proliferation in
DG. In order to more closely address this issue, not only more cases, but also a thorough exploration of other factors, such as depression and physical activity etc, would be necessary.

In adult mice SGZ, it has been shown that NPCs proliferate within an angiogenic niche and that more than 70% of the newborn cells express the neuronal stem cell marker nestin (Palmer et al., 2000). Nestin has also been reported to be expressed in mature capillary endothelium (Palmer, et al., 2000). To see whether similar mechanisms occur in human cerebral blood vessels, sections were double stained for Ki67 and either nestin or vWF, which is found in blood vessels. Co-localized between Ki67 and both nestin-positive vessels and vWF were found (figure 5A-D). vWF was also used when long blood vessel (≥100 µm) in the CGL were counted and compared with age of the subjects. There was little variation between the subjects (Figure 6), except for one subject. This subject was one of the youngest (28 years old female) and had one of the lowest values of blood vessel-index, the subject also had a low Ki67-index value (Figure 7). However, no correlation between blood vessel-index, Ki67-index value and the age of the individual were found among the cases.

4.2 Nestin and GFAP

Neurogenesis has for many years been studied in animal’s models. Neurogenesis starts with an asymmetric cell division of a NSC. The NSC will give rise to NPCs which will generate a pool of immature granule cells (Bohlen et al., 2007; Christie et al., 2006; Encinas JM, et al., 2008). Neural stem cells (NSCs) are defined as self-renewing, multipotent cells that have a long life span. Compared to neural progenitors cells (NPCs) which have less self-renewal ability, and can be both multi- and uni-potential in terms of the types of mature cells which can be form (Encinas JM, et al., 2008). To identify NSCs and NPCs in adult neurogenesis, we used the expression of the intermediate filament protein nestin as a marker (Figure 8B) (Encinas JM, et al., 2008; Lendahl U, et al., 1990). If a cell express both nestin and GFAP it might be a neural stem cells, while neural progenitors cell will only express nestin (Encinas JM, et al., 2006). In this study, filaments that only expressed one of the two proteins were found. Co-expression of nestin and GFAP was found in the SGZ (Figure 8C) which indicates that NSCs are found in the adult human DG.
4.3 Immature neurons

For the identification of immature neurons, the immunoreactivity against βIII-tubulin, DCX and PSA-NCAM was studied. βIII-tubulin which is a component of the cytoskeleton and marks newborn neurons in the GCL (Braun, et al., 2002), showed a weak labelling with the florescence method (Figure 9A). Expression of βIII-tubulin, detected with ABC method, was found in the GCL and SGZ (Figure 9B) but the staining pattern was difficult to analyze. DCX is present in migrating neuroblasts and early post-mitotic neurons (Bohlen, et al., 2006). The α-DCX antibody was incubated either overnight or for 48 hours, but neither procedure produced any specific labelling on these post-mortem human samples. The PSA-NCAM antibody worked very well on rat brain sections (see appendix 1), but did not produce any labelling on the post-mortem sections from human DG.

When working with human material it is important to consider that more than one factor which can have an effect on the outcome of the result, one of the most problematic factors is the PMI (post-mortem interval). Which is the time interval between the time of death to the time of collection and preparation at an autopsy (Chandana, et al., 2009; Lewis, et al., 2002)? In this study the PMI varies between 21 to 64 h. A long PMI can result in a degradation or changes in the conformation of certain proteins so that the epitopes may be hidden or destroyed, and thus hampering the binding of the antibodies to the proteins (Lewis, et al., 2002). Post-mortem changes may affect some proteins more than others, which could explain the good labelling for NeuN and GFAP, somewhat weaker signal for βIII-tubulin, and the lack of labelling for PSA-NCAM, DCX and PCNA antibodies.

4.4 Conclusions

This study shows that cell proliferation (Ki67-positive nuclei) occurs in the human DG, which seems to decrease with age. Cell proliferations in the adult human DG don’t have to be neurogenesis, but it is a requirement for neurogenesis. Subjects with a history of alcohol abuse displayed low numbers of Ki67-positive nuclei despite low age, suggesting that heavy alcohol consumption may reduce cell proliferation (neurogenesis). By using immunohistochemistry labelling cells and filaments that expressed nestin was found, some filaments were also expressing GFAP. This indicates that NSCs and NPCs might exist in the adult human DG. Over all more studies has to be done on adult human DG to learn more about neurogenesis in human DG.
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6 References


Reif A, et al. (2006) "Neural stem cell proliferation is decreased in schizophrenia, but not in depression." Mol Psychiatry. 11(5):514-22.


Appendix 1

To evaluate the specificities of the antibodies, a rat DG (Figure 11A) was used as a control. The majority of the antibodies were used in this study produced labelling both in rat and human, see table 3. However, the antibody raised against PSA-NCAM, which is a protein expressed by migrating neuroblasts and early post-mitotic neurons (Bohlen et al., 2007; Gascon et al., 2008), only produced labelling in the rat DG (Figure 11B). In the SGZ this marker co-localised with Ki67-positive nuclei (red).

Table 3; Summary of labelling in DG.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Human DG</th>
<th>Rat DG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>Labelling</td>
<td>Labelling</td>
</tr>
<tr>
<td>PCNA</td>
<td>No labelling</td>
<td>No labelling</td>
</tr>
<tr>
<td>GFAP</td>
<td>Labelling</td>
<td>Labelling</td>
</tr>
<tr>
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<td>Labelling</td>
<td>-</td>
</tr>
<tr>
<td>Nestin 2</td>
<td>-</td>
<td>Labelling</td>
</tr>
<tr>
<td>PSA-NCAM</td>
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<td>Labelling</td>
</tr>
<tr>
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<td>Labelling</td>
<td>No labelling</td>
</tr>
<tr>
<td>DCX</td>
<td>No labelling</td>
<td>No labelling</td>
</tr>
<tr>
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<td>Labelling</td>
<td>Labelling</td>
</tr>
<tr>
<td>vWF 1</td>
<td>Labelling</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Human specific, 2 Rat specific

Figure 11. Neurogenesis in the adult rat DG. Photomicrographs show a rat hippocampal formation labelled with Hoechst (A). The square-marked area indicating where figure 11B was taken. PSA-NCAM and Ki67 could both be detected in the GCL/SGZ (B). Hoechst labelled cells (grey) were also be observed. Scale bar = 1 mm (A) and 50 µm (B).