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INVESTIGATING CELL TURNOVER IN THE HEALTHY AND DISEASED ADULT HUMAN BRAIN

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Investigating cell turnover in the healthy and diseased adult human brain

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

For decades it was thought that cells that lost in the human central nervous system because of ageing or disease – different from other cell tissues – cannot be replaced and that in humans all neurons are generated during prenatal development. However, over the last 20 years, it became obvious that there is a certain level of adult neurogenesis in most mammals that mainly occurs in the dentate gyrus and the subventricular zone. Whether or not findings from animal studies also hold true in humans was difficult to study as direct evidence – as obtained in animals from genomic labeling using for instance nucleosides like BrdU – was not feasible in humans because of ethical considerations. The establishment of the so-called radiocarbon technique, a method taking advantage of the above-ground nuclear bomb tests during the Cold War to retrospectively birth date cells by determination of the $^{12}C/^{14}C$ ratio in genomic DNA – allowed to investigate the age and the turnover dynamics of cells in various human tissues. Applying this technique we here (i) studied whether there is adult neurogenesis in the healthy human brain, specifically within the hippocampus, (ii) studied whether there is adult neurogenesis in the diseased human brain, specifically in response to cortical stroke, and (iii) investigated the age and growth dynamics of brain tumors, specifically benign meningiomas. In essence we demonstrate (i) that there is a lifelong adult neurogenesis within the human hippocampus and provide an integrated model of hippocampal cell turnover dynamics, (ii) that there is no significant induction of cortical neurogenesis following ischemic cortical stroke in humans, and (iii) that the age of benign meningiomas is significantly older than that of more malignant brain tumors. The clinical implications of these findings are discussed and research projects for future studies identified.

LIST OF SCIENTIFIC PAPERS

I. Spalding KL, Bergmann O, Alkass K, Bernard S, Salehpour M, **Huttner HB**, Boström E, Westerlund I, Vial C, Buchholz BA, Possnert G, Mash DC, Druid H, Frisén J.
Dynamics of hippocampal neurogenesis in adult humans. Cell. 2013; 153:1219-27.

II. **Huttner HB***, Bergmann O*, Salehpour M, Rácz A, Tatarishvili J, Lindgren E, Csonka T, Csiba L, Hortobágyi T, Méhes G, Englund E, Solnestam BW, Zdunek S, Scharenberg C, Ström L, Ståhl P, Sigurgeirsson B, Dahl A, Schwab S, Possnert G, Bernard S, Kokaia Z, Lindvall O, Lundeberg J, Frisén J.
The age and genomic integrity of neurons after cortical stroke in humans.
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III. **Hagen B. Huttner***, Olaf Bergmann*, Mehran Salehpour, Raouf El Cheikhs, Makoto Nakamura, Angelo Tortora, Roland Coras, Elisabet Englund, Ilker Y. Eyüpoglu, Joji B. Kuramatsu, Stephan P. Kloska, Iris Kaschka, Arnd Doerfler, Stefan Schwab, Göran Possnert, Samuel Bernard and Jonas Frisén.
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CONTENTS

LIST OF ABBREVIATIONS

APE-1	apurinic/apyrimidinic endonuclease 1
BrdU	5-bromo-2-deoxyuridine
^{12}C and ^{14}C	Carbon isotope 12 and radioactive isotope carbon-14
CNS	Central Nervous System
DCX	doublecortin
EdU	5-ethynyl-2'-deoxyuridine
GABA	gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
gammaH2AX	H2A histone family, member X
Ki-67	Protein - cellular marker for proliferation
Mem2	Fibulin-1 monoclonal antibody
^{15}N	Stable isotope of nitrogene
NeuN	Neuronal specific nuclear protein
OB	Olfactory Bulb
Prox1	Prospero homeobox protein 1
PSA-NCAM	Polysialylated-neural cell adhesion molecule
RMS	Rostral Migratory Stream
S-Phase	Synthesizing phase of the cell cycle
SGZ	Subgranular Zone
SVZ	Subventricular Zone
WHO	World Health Organization

1 INTRODUCTION

1.1. Cell turnover in the adult human brain

An emerging field at the interface of basic science and clinical research is regenerative medicine (Okano, 2010; Steiner et al., 2006). Although not mandatory for functional recovery, the generation of new cells is linked to increased regenerative capacity of various tissues (Farkas and Huttner, 2008; Koch et al., 2009; Merkle and Alvarez-Buylla, 2006; Okano, 2010). Hence, many investigations aim at understanding the characteristics and behavior of stem and progenitor cells to study mechanisms and techniques to regenerate cells and tissues that may have been lost as a consequence of disease (Barnabe-Heider and Frisen, 2008; Dietrich and Kempermann, 2006; Emsley et al., 2005; Johansson et al., 2010; Kriegstein and Alvarez-Buylla, 2009; Ninkovic and Gotz, 2007; Okano and Sawamoto, 2008; Scheffler et al., 2006; Steiner et al., 2006).

1.1.1. Adult neural stem and progenitor cells

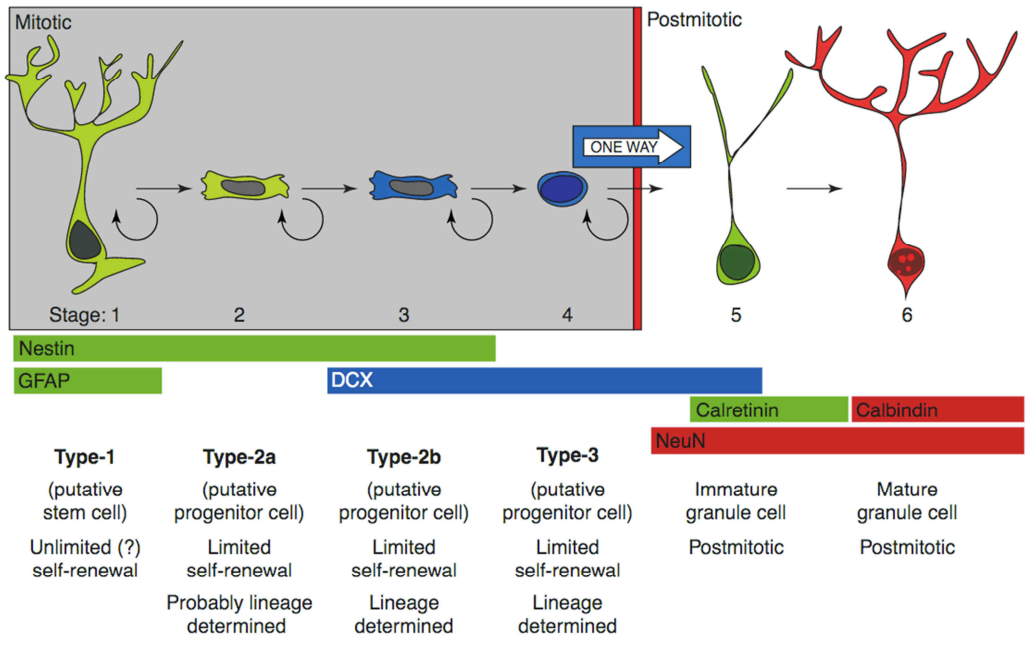
The beginning of human life is the fertilized oocyte (Kempermann, 2011). From this totipotent stage a blastocyst is formed and embryonic stem cells – being pluripotent – emerge (Kempermann, 2011; Kriegstein and Alvarez-Buylla, 2009). Upon further maturation somatic stem cells originate, categorized within the endo-, meso-, and ectoderm (Kriegstein and Alvarez-Buylla, 2009). Focussing on the development of the human central nervous system (CNS), the neuroectoderm contains neuroepithelial cells, which are the primary neural stem cells (Farkas and Huttner, 2008; Kempermann, 2011; Koch et al., 2009). Characteristics of many somatic stem cells are self-renewal and multipotency (Goritz and Frisen, 2012; Kempermann, 2011). There are also progenitor cells, which in the developing CNS comprise some radial glia and the intermediate progenitor cells, and which usually are more proliferative and do not show unlimited self-renewing capacity (Götz and Barde, 2005; Kempermann, 2011).

In the context of investigating cell turnover in the human CNS, neural stem and progenitor cells have been intensely studied and have been characterized in both the embryonic and adult CNS (Kriegstein and Alvarez-Buylla, 2009; Merkle and Alvarez-Buylla, 2006; Okano and Sawamoto, 2008). On the one hand, in order to explore the development of the

mammalian CNS, there have been extensive analyses of embryonic neural stem cells and progenitor cells (Farkas and Huttner, 2008; Jakovcevski and Zecevic, 2005; Okano and Sawamoto, 2008). On the other hand, insights into postnatal processes in the mammalian CNS have been obtained from the discovery and subsequent characterization of adult neurogenesis (see 1.2.) (Dietrich and Kempermann, 2006; Falk and Frisen, 2005; Zhao et al., 2008). The adult human brain contains a variety of neural progenitor cells, i.e. neuronal and glial progenitors, as well as certain radial glial cells and astrocytes that serve as neural stem cells (Kempermann, 2011; Kriegstein and Alvarez-Buylla, 2009). Adult neurogenesis involves the divisions of adult neural stem cells, resulting in (i) self-renewal and (ii) the generation of downstream neural progenitor cells, which then undergo amplification and give rise to post-mitotic neurons (Kempermann, 2011; Kriegstein and Alvarez-Buylla, 2009).

A basic model describing the process from a neural stem cell to a post-mitotic neuron is shown in Figure 1; a neural stem cell gives rise to neural progenitor cells that in turn generate immature neurons which then mature to post-mitotic neurons (Kempermann, 2011).

Figure 1. Basic model of adult neurogenesis in the hippocampus.



Proposed basic model of cell types in adult hippocampal neurogenesis based on morphology and molecular markers. The putative stem cell exhibits radial glia and astrocytic properties and progresses over three stages of putative transiently amplifying progenitor cells which increase their neuronal lineage determination, followed by the post-mitotic stage. Reprinted from Kempermann et al. (Kempermann et al., 2004a) with permission from Elsevier.

1.1.2. Cancer stem cells in brain tumors

Besides stem cells that are present to maintain the function of our organs, in malignant diseases the concept of cancer stem cells has been proposed (Lathia et al., 2015; Singh et al., 2004; Venere et al., 2011). Essentially all tumors, and specifically malignant hematopoetic diseases, arise from cancer stem cells that show similar characteristics as somatic stem cells, such as self-renewal and differentiation into various tissue types (Singh et al., 2004; Venere et al., 2011). Cancer stem cells have been implicated in brain tumors, which coined the term brain tumor stem cells (Schonberg et al., 2014; Zhang et al., 2006a). The two most frequently occurring brain tumors are gliomas and meningiomas (Riemenschneider et al., 2006; Whittle et al., 2004; Wiemels et al., 2010), and tumor generation and resistance to treatment of most malignant variants of glioma and meningiomas have been linked to these stem cells (Bao et al., 2006).

1.2. Neurogenesis in the adult mammalian brain

It has long been thought that the neurons in the adult mammalian CNS are all those that were generated during development (Rakic, 1985, 2002, 2006). However, during the past two decades there has been an enormous progress both in animal and human research establishing that new neurons are continuously generated in at least two regions of the adult brain (Bergmann and Frisen, 2013; Bergmann et al., 2015; Curtis et al., 2011; Eriksson et al., 1998; Ernst and Frisen, 2015; Falk and Frisen, 2005; Goritz and Frisen, 2012; Kempermann et al., 2004a; Lie et al., 2004; Zhao et al., 2008). Thus, adult neurogenesis occurs on the one hand in the dendate gyrus of the hippocampus and on the other hand in the subependymal zone of the lateral ventricle (Bergmann et al., 2015; Goritz and Frisen, 2012; Kempermann, 2011).

1.2.1. Methodological approaches

Experimental approaches to study adult neurogenesis in the mammalian brain mainly relied on immunohistochemical analyses, i.e. on investigating molecular markers of cell proliferation, and using nucleosides to label newly synthesized DNA (Bergmann et al., 2015; Eriksson et al., 1998; Taupin, 2007; Wang et al., 2011). In both rodents and humans the analysis of molecular markers provides insights into cell proliferation and allows labelling of stem and progenitor cells at a given time-point. However, several shortcomings limit the

interpretation of results (Eriksson et al., 1998; Taupin, 2007). First, the widely used marker DCX has been found in cells that differentiated into glial cells (Kempermann, 2011; Klempin et al., 2011; Seidenfaden et al., 2006). Second, the analysis of molecular markers that label neuronal precursor cells or neuroblasts (defined as cells committed to the neuronal lineage but lacking mature neuronal characteristics) does not provide insight into whether or not these cells become mature neurons which survive long-term and are of functional significance (Kempermann et al., 2004b; Steiner et al., 2006; Zhao et al., 2008). Furthermore, the potential number of neurons that these cells generate, and into which specific regions neurons migrate during maturation, cannot be answered, thus making this approach difficult for drawing definite conclusions on neurogenesis (Bergmann et al., 2015; Ming and Song, 2011).

The second approach involves an analysis of genomic DNA, usually taking advantage of nucleosides such as tritiated thymidine or BrdU/EdU and their stable incorporation into the DNA when it is duplicated during S-phase (Dolbeare, 1996). In addition, other strategies employ labelling with retroviruses or transgenic approaches (Feliciano and Bordey, 2013). The basic idea is to introduce a stable mark in cells that is specific for DNA synthesis (e.g. incorporation of BrdU) and then explore whether such mark is present in mature neurons, as this would indicate true neurogenesis (Jin et al., 2001; Parent et al., 2002; Taupin, 2007). Although this approach is more conclusive in demonstrating the occurrence of neurogenesis, there are also some drawbacks. For instance, a BrdU pulse may underestimate the number of newborn cells as only cells in the S-phase of the cell cycle may have been labeled (Eriksson et al., 1998; Magavi et al., 2000). In this regard false-negative and also false-positive findings (over- and underdosing of BrdU) on neurogenesis have been published (Breunig et al., 2007; Karpowicz et al., 2005; Kunz and Kohalmi, 1991). Furthermore, BrdU incorporation into DNA may also occur in non-proliferating cells as a result of DNA repair (Burns et al., 2007; Feliciano and Bordey, 2013; Taupin, 2007). Finally, although this approach allows prospective labeling of newborn neurons revealing the generation and integration of mature neurons in specific brain regions, it is not possible to retrospectively determine cell turnover in the adult brain.

Most importantly, however, the toxicity of labeled nucleosides limits their use for studies in humans for ethical reasons (Bergmann et al., 2015; Kuhn et al., 1996). There is only one seminal study on 5 cancer patients in whom labeled nucleosides were used (see 1.2.2.) (Eriksson et al., 1998), and it revealed the “dilemma” that one has to wait until the person dies until the brain can be analyzed. Hence, basically all findings regarding adult

neurogenesis in the healthy and diseased human brain (see 1.2.2. and 1.2.3.) were derived from immunohistochemical analyses. Contrary to animal studies in which one can combine the approach of studying molecular markers with the technique of labeling DNA to increase the certainty of the findings obtained (Feliciano and Bordey, 2013; Kokaia and Lindvall, 2012), in humans there is the necessity of novel approaches in addition of immunohistochemistry to study neurogenesis in humans, such as the radiocarbon technique (see 1.3.) (Bergmann et al., 2015; Ernst and Frisen, 2015; Lindvall and Kokaia, 2015).

1.2.2. Findings in the healthy brain of rodents and humans

In the adult mammalian brain there are at least two neurogenic niches in which lifelong adult neurogenesis has been described, that is the subgranular zone (SGZ) of the gyrus dentus of the hippocampus and the subventricular zone of the lateral ventricle (Bergmann et al., 2015; Goritz and Frisen, 2012; Kempermann, 2011).

Hippocampal adult neurogenesis

Adult hippocampal neurogenesis has been intensely studied in rodents (Ekdahl et al., 2009; Fabel and Kempermann, 2008; Kempermann, 2012; Kempermann et al., 2004a; Klempin et al., 2010; Klempin et al., 2011; Lugert et al., 2010; Wolf et al., 2010). The precursor cells of the dentate gyrus are located within the SGZ that provides a neurogenic microenvironment that is often referred to as neurogenic niche (Kempermann, 2011). The SGZ contains astrocytes resembling radial glial cells that represent the stem cells of this area (Kempermann, 2011; Seri et al., 2001), which in sequence give rise to radial glia-like precursor cells, glia-like transient amplifying progenitor cells, neuronally determined transient amplifying progenitor cells, and migratory neuroblasts (Kempermann, 2011; Kempermann et al., 2004a). The phase of post-mitotic maturation – eventually including neuroblasts – is characterized by dendritic maturation, axon elongation and synaptic plasticity (Kempermann, 2011).

In rodents the average adult hippocampal neurogenesis amounts to up to 0.03-0.06% per day in 2 month old mice, and there is an almost 10-fold decline in adult neurogenesis in mice with increasing age between 2 and 9 months (Ben Abdallah et al., 2010; Kempermann et al., 1997a, b). Besides to in vivo imaging studies, in humans neurogenesis is mainly studied by analysis of molecular markers, such as DCX, Ki-67 or NeuN (Attardo et al., 2010; Bergmann et al., 2015; Coras et al., 2010; Kempermann, 2012; Knoth et al., 2010). For instance, Kempermann and coworkers studied the pattern of DCX-positive neuroblasts in the human

dentate gyrus in individuals of all ages and found DCX-positive cells over the entire life, and some DCX-positive cells, notably in young individual, showed features of immature neurons (Bergmann et al., 2015; Knoth et al., 2010). Further markers of proliferation such as Ki-67 and surrogate markers of neurogenesis such as Mem2, Prox1 and calretinin, were expressed during human adulthood (Knoth et al., 2010). In humans there was also a decrease in DCX-NeuN-positive cells with increasing age, though this decrease in neurogenesis is probably not as high as in rodents (Ben Abdallah et al., 2010).

Prior to our study (see 3), the seminal study by Eriksson and colleagues provided the most robust evidence of human hippocampal neurogenesis (Eriksson et al., 1998). Using a single infusion dose of BrdU, administered for diagnostic purposes in patients with oropharyngeal cancer, they were able to demonstrate the presence of BrdU in hippocampal neurons that verified the presence of adult born neurons in the human hippocampus (Bergmann et al., 2015; Eriksson et al., 1998; Falk and Frisen, 2005). This study was of enormous significance as it proved the existence of adult neurogenesis in humans. However, it did not enable any quantitative estimates and was not addressed to clarify the functional significance (Kempermann, 2012; Lucassen et al., 2010; Rakic, 1985).

Neurogenesis in the SVZ, striatum and olfactory bulb

The second neurogenic niche in mammals is the SVZ (Benraiss et al., 2001; Curtis et al., 2007; Ekdahl et al., 2009; Hansen et al., 2010; Komitova and Eriksson, 2004; Reillo et al., 2010; Sanai et al., 2004; Seidenfaden et al., 2006; Wang et al., 2011). In rodents the SVZ is a very tiny, approximately two-cell-body-wide layer below the ependymal cell layer, which is why the SVZ is also referred to as subependymal layer (Kempermann, 2011). The putative progenitor cells are radial astrocytes, which extend their primary cilium into the ventricles with direct contact with the ventricular fluid, which is why the term “periventricular region” may be most appropriate (Beckervordersandforth et al., 2010; Kempermann, 2011; Sanai et al., 2011; Weigmann et al., 1997). Neurogenic progenitor cells of this system, however, also are found within the rostral migratory stream (RMS) and in the olfactory bulb (OB) (see below) (Curtis et al., 2007; Wang et al., 2011).

In the rodent brain there are many neuroblasts migrating from the SVZ to the OB every day (Bergmann et al., 2015; Doetsch et al., 1997; Ming and Song, 2011; Ponti et al., 2006). Within the OB there is a differentiation to interneurons followed by an integration into the existing neuronal circuits, and studies demonstrated that about 40% of these newborn neurons

survive long-term (Winner et al., 2002) and are functionally relevant, participating in olfactory memory (Ming and Song, 2011). Other neuroblasts are also found in the striatum, and low levels of striatal neurogenesis have been reported in rodents but also in monkeys (Bedard et al., 2002a; Bedard et al., 2002b; Dayer et al., 2005; Luzzati et al., 2003; Tonchev et al., 2005). Whether or not these cell originated in the SVZ and eventually migrated towards the striatum is currently under investigation (Magnusson et al., 2014) (and personal communication). Although difficult to study, there might be a decline in OB neurogenesis with mammalian evolution (see below for humans), potentially related to a corresponding concomitant increase of respective striatal neurogenesis. However this remains speculative (Bergmann et al., 2015).

In the human brain the existence of SVZ, RMS and OB adult neurogenesis is much more controversial (Curtis et al., 2011; Curtis et al., 2007; Sanai et al., 2007). There were studies confirming this process also in the human fetal and infant brain; however, the migratory neuroblasts and transiently amplifying cells were not detected in adults, or dividing neuroblasts detected by immunolabeling existed only at very low levels (Bergmann et al., 2015; Sanai et al., 2011). Recently, a human study using the radiocarbon technique (see 1.3.) revealed the absence of significant (<1% over 100 years) OB neurogenesis (Bergmann et al., 2012). Hence the question arose as to what the fate of SVZ progenitors is humans, as there has been reported no significant apoptosis of SVZ neuroblasts (Ernst et al., 2014; Ernst and Frisen, 2015). DCX- and PSA-NCAM-positive neuroblasts have been found in the human striatum next to the SVZ (Ernst et al., 2014); however, it remains speculative whether these were derived from the SVZ (Bergmann et al., 2015). Recently, a human study using the radiocarbon technique (see 1.3.) revealed generation of striatal interneurons at levels of 2.7% per year; however, the origin of these cells as well as their functional significance remain to be established (Ernst et al., 2014).

Neurogenesis in the cerebral cortex

The cerebral cortex harbors the higher cognitive functions, and disease affecting the human cortex is directly linked to severe neurological impairment (Buffo et al., 2008). Hence, in the context of adult neurogenesis, it has been hotly debated whether or not the cerebral cortex has any neurogenic potential (Bhardwaj et al., 2006; Feliciano and Bordey, 2013; Rakic, 2006). Some researchers believe that the dogma that all neurons are generated prenatally at least holds true for the cerebral cortex. In line with this notion, various reports in rodents and humans have failed to provide any immunohistochemical evidence for a significant

occurrence of progenitor cells or newly generated neurons in the neocortex (Ehninger and Kempermann, 2003; Kornack and Rakic, 2001; Tamura et al., 2007). A study based on the radiocarbon approach (see 1.3) demonstrated that there is no significant postnatal neurogenesis (Bhardwaj et al., 2006). This investigation was supported by a retrospective assessment of BrdU and its colabeling with neuronal markers in tissue samples of the Eriksson study of 1998 (Bergmann et al., 2015; Bhardwaj et al., 2006; Eriksson et al., 1998). Based on this combined approach the authors concluded that adult neurogenesis in the human cerebral cortex, if there is any at all, amounts to a maximum of one newborn neuron out of 1000 neurons every five years (Bergmann et al., 2015; Bhardwaj et al., 2006). On the other hand there are also studies reporting on cortical neurogenesis, though at very low levels (Bernier et al., 2002; Dayer et al., 2005; Gould et al., 2001; Luzzati et al., 2003). Studies using tritiated thymidine found neurogenesis rates of 0.011% (Altman, 1969; Kaplan, 1981), and analysis of BrdU/NeuN-co-labeling reported on rates of 0.0026-0.012% in monkeys, or 3 newborn neurons per mm^3 in rats (Dayer et al., 2005). In this regard it needs to be mentioned that long-term neuron survival and the functional significance of potential neurogenesis has not been addressed; occurrence of preferentially small GABAergic interneurons instead of projections neurons may occur only transiently (Gabbott and Bacon, 1996; Gould et al., 2001). In conclusion, it remained controversial if there is any neurogenesis in the mammalian cerebral cortex at all. Furthermore, while the functional significance is questionable in the healthy situation, there may be a mechanism of possible induction of cortical neurogenesis upon neurological disease (Jin et al., 2006).

1.2.3. Findings after cerebral ischemia in rodents and humans

Within established neurogenic niches (hippocampus and SVZ)

Histological changes in response to stroke have been intensely studied in animals and – given the methodological problems in humans – only in a limited way in humans (Kokaia and Lindvall, 2012; Lindvall and Kokaia, 2015). While there are essentially no studies addressing changes in hippocampal adult neurogenesis following stroke in humans, in rodents there have been various analyses demonstrating an increase in the hippocampal neurogenesis rate after stroke (Geibig et al., 2012; Jin et al., 2001; Kernie and Parent, 2010; Zhao et al., 2008; Ziv et al., 2007). These investigations were mainly based on co-labelling with BrdU and NeuN, and reported increased rates of such double-positive cells in the hippocampus ipsilateral to the focal cerebral injury (Geibig et al., 2012). Also within the SVZ, experimental stroke in rodents induced proliferation and neurogenesis (Arvidsson et al., 2002; Jin et al., 2001; Parent

et al., 2002; Zhang et al., 2001). Still of debate is whether this increase in SVZ neurogenesis is transient (Arvidsson et al., 2002; Parent et al., 2002) or persisting (Thored et al., 2006). In humans a similar neurogenic response has been postulated in terms of an increased number of Ki-67-positive cells and periventricular neural progenitors in the SVZ ipsilateral to the ischemic lesion (Macas et al., 2006; Marti-Fabregas et al., 2010).

Within brain regions that are non-neurogenic in healthy conditions (striatum and cortex)

There is a body of evidence that stroke can induce the generation of new neurons in brain regions where – in the healthy physiological setting – there is no adult neurogenesis (see above), specifically the striatum and the cerebral cortex (Jiang et al., 2001; Jin et al., 2003; Jin et al., 2006; Leker et al., 2007; Lindvall and Kokaia, 2015; Tonchev et al., 2005; Ziv et al., 2007). Within the striatum of rodents BrdU-positive neuronal progenitors and mature neurons have been identified by co-labelling BrdU-postive cells with DCX and NeuN (Arvidsson et al., 2002; Parent et al., 2002). Notably, on the one hand, the BrdU-positive cells that co-labeled positive for DCX during the course of the disease expressed NeuN, reflecting neuronal maturation. On the other hand, some of these cells expressed transcription factors that indicated differentiation into projection neurons (Arvidsson et al., 2002). Focussing on the source of these cells, recent evidence showed that neuroblasts originate from both, SVZ astrocytes migrating into the ischemic striatum, and latent neurogenic striatal astrocytes (Magnusson et al., 2014). The latter mechanism is inhibited in the healthy setting by Notch1 signaling; however, the functional relevance of striatal endogenous adult neurogenesis in response to stroke needs to be further established (Magnusson et al., 2014).

Whether or not stroke induces neurogenesis in the cerebral cortex is less studied and also controversial (Kernie and Parent, 2010; Lindvall and Kokaia, 2015). In studies on rodent striatal neurogenesis there had been no evidence of any significant cortical neurogenesis after cortical ischemia (Arvidsson et al., 2002; Parent et al., 2002; Zhang et al., 2001). Contrary, some immunohistochemical studies reported on new neuroblasts and mature neurons in the cerebral cortex after cortical ischemia (Jiang et al., 2001; Jin et al., 2006; Zhang et al., 2006b; Ziv et al., 2007). Furthermore, cortical neurogenesis following stroke may be induced by growth factors (Leker et al., 2007; Taguchi et al., 2004). Addressing to the origin of these neurons, Jin and colleagues described DCX-positive neuroblasts migrating from the SVZ to the periphery of the cortical ischemia (Jin et al., 2003; Osman et al., 2011). In humans, there are also conflicting analyses; however, there is growing evidence from immunohistochemical studies reporting on DCX-positive cells in the vicinity

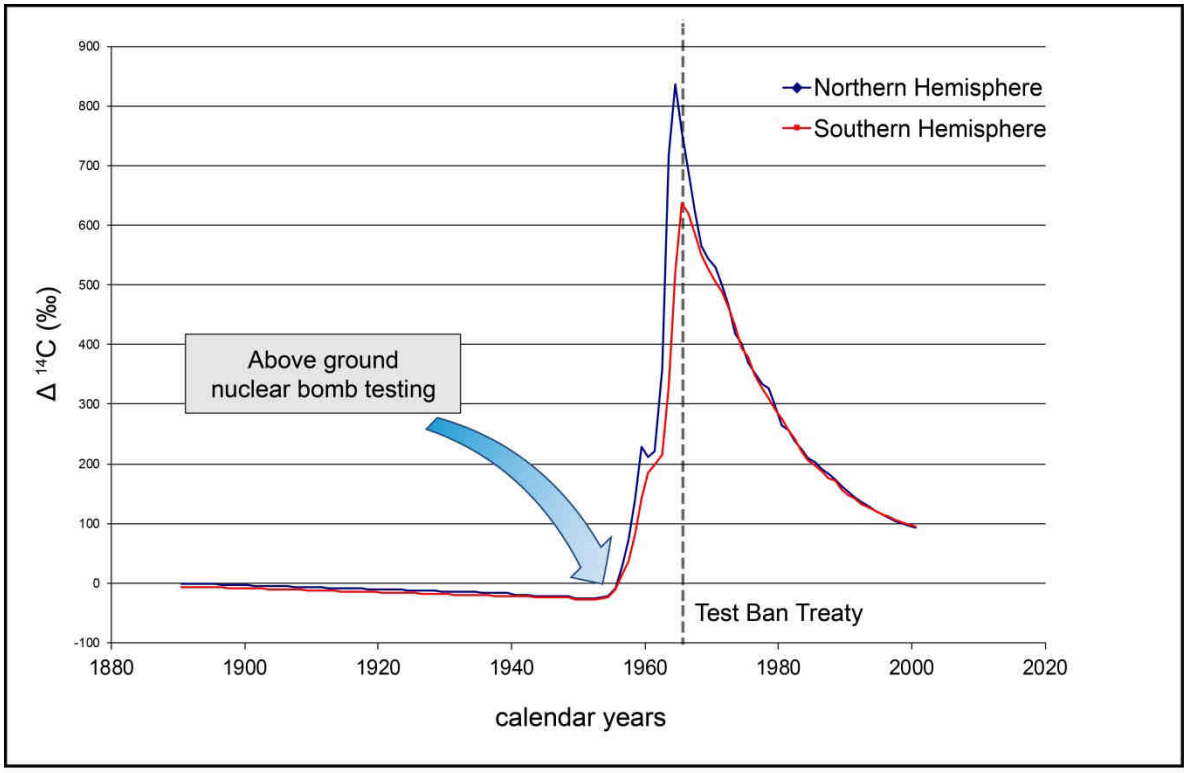
of cortical blood vessels after stroke, as well as cells expressing nestin (Jin et al., 2006; Marlier et al., 2015; Minger et al., 2007; Nakayama et al., 2010). Debate remains whether these cells originated from activation of dormant neural stem cells residing within the cerebral cortex that gave rise to neuroblasts after cortical ischemia (Jiang et al., 2001; Magavi et al., 2000; Yang et al., 2007).

1.3. Radiocarbondating to study cell turnover in the human brain

1.3.1. The technique of ^{14}C-retrospective birth dating of cells

To overcome the difficulties described above in assessing adult neurogenesis in humans, the research group led by Prof. Jonas Frisén at the Karolinska Institute developed a clever method to retrospectively birth date cells in humans (Spalding et al., 2005a). The so-called radiocarbondating – also known from archaeology to date historical finds (Arlotta and Macklis, 2005) – represents a technique which basically analyzes carbon (Spalding et al., 2005a). Carbon usually exists as the isotope ^{12}C (Arlotta and Macklis, 2005); however, within the atmosphere there is also a small amount of the carbon isotope ^{14}C (usually as $^{14}CO_2$) (De Vries, 1958) that naturally occurs in the setting of cosmic radiation colliding with nitrogen nuclei (De Vries, 1958; Stuiver and Polach, 1977). The ratio between both isotopes historically was relatively constant over decades until 1955-63 when ^{14}C levels were drastically increased upon the above-ground nuclear bomb testing during the Cold War (Nydal and Lovseth, 1965; Stuiver and Polach, 1977) (Figure 2). After the test ban treaty in 1963, the ^{14}C levels started to decline again mainly because of diffusion from the atmosphere and binding in the oceans (Harkness, 1972; Libby et al., 1964; Nowakowski, 2006; Nydal and Lovseth, 1965). Importantly, the time resolution of the radiocarbon-technique since the bomb peak in the ^{14}C level and its subsequent decline became very precise as compared to the time prior of the nuclear bomb testings (Harkness, 1972; Spalding et al., 2008; Spalding et al., 2005a; Spalding et al., 2005b; Stuiver and Polach, 1977).

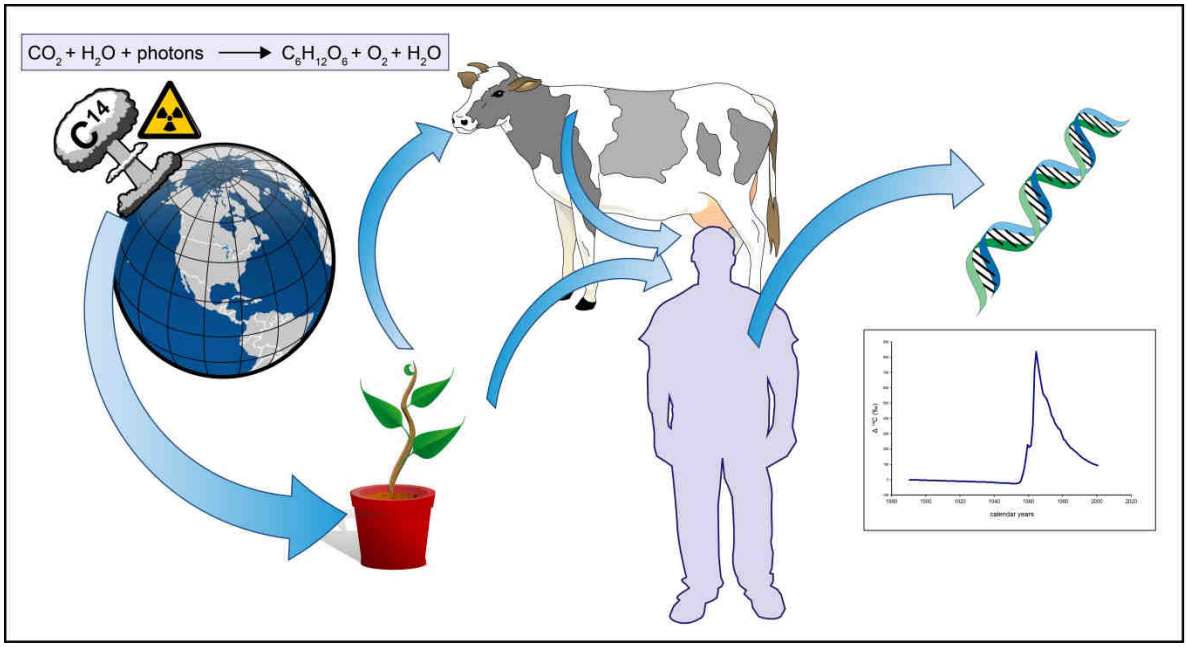
Figure 2. Atmospheric changes of $^{14}C/^{12}C$ ratio caused by nuclear bomb tests.



Ratio of atmospheric carbon isotopes ^{14}C and ^{12}C during the last century (Libby et al., 1964; Nowakowski, 2006; Nydal and Lovseth, 1965; Stuiver and Polach, 1977). Upon the above-ground nuclear bomb tests during the Cold War there was a dramatic increase in the atmospheric levels of ^{14}C which declined since the Test Ban Treaty in 1963. The blue line indicates $^{14}C/^{12}C$ ratio of the Northern hemisphere, the red line of the Southern hemisphere.

The released atmospheric ^{14}C reacts with oxygen and forms CO_2 which enters the food chain upon plant photosynthesis (Bergmann et al., 2009). By eating plants, and animals that live of plants, the ^{14}C concentration in the human body – being at the top of the food pyramid – closely corresponds to that in the atmosphere at any given time (Spalding et al., 2005a). If there is cell turnover, the newly synthesized DNA – which consists of roughly one third of carbon – incorporates exactly this carbon ratio of $^{12}C/^{14}C$ which was present in the atmosphere at the time-point the cell was generated (Spalding et al., 2005a). Hence, as the DNA is stable after a cell has gone through its last cell division, the genomic ^{14}C levels serve as a date mark for when a cell was born and can be used to retrospectively birth date cells in humans (Bergmann et al., 2009; Bhardwaj et al., 2006; Spalding et al., 2005a) (Figure 3).

Figure 3. Concept of ^{14}C retrospective birth dating of cells.



The principle of ^{14}C retrospective birth dating of cells. Nuclear bomb detonations released ^{14}C into the atmosphere which formed $^{14}CO_2$ and entered the food chain by photosynthesis of plants. By eating plants ^{14}C was ingested by animals and humans. Upon generation of a new cell, DNA is synthesized which consists roughly of one third carbon. Almost exactly the $^{14}C/^{12}C$ ratio that was present in the atmosphere in the year a cell was generated was incorporated into the newly synthesized DNA, acting as a stable date mark (Spalding et al., 2005a). By extracting DNA from cell populations of interest (e.g. neurons) and analyzing the $^{14}C/^{12}C$ ratio, the average age of the cell population under investigation can be determined.

The radiocarbon technique requires to first isolate the DNA of the targeted cell population (Spalding et al., 2005a). Human tissue samples may be obtained upon biopsy or – which was the case so far – upon post mortem autopsies (Bergmann et al., 2009; Bhardwaj et al., 2006; Ernst et al., 2014). The methodological approach includes the isolation of cell nuclei in a first step which usually is achieved by sucrose gradient centrifugation (Bergmann et al., 2012; Huttner et al., 2014). Using molecular markers that specifically label the nuclei of the target cell population, these nuclei are isolated by flow cytometry (Bergmann et al., 2009; Ernst et al., 2014). Subsequently, and constituting the most tricky part of the technique, the DNA needs to be isolated: this is performed in clean rooms to minimize contamination with contemporary carbon (Bergmann et al., 2009; Spalding et al., 2013). Finally, the isolated DNA is analyzed by accelerator mass spectrometry to determine the amount of the various

carbon isotopes within the sample (Spalding et al., 2005a). The interpretation of results is schematically explained in Fig. 4.

Fig. 4. Interpretation of radiocarbon technique.

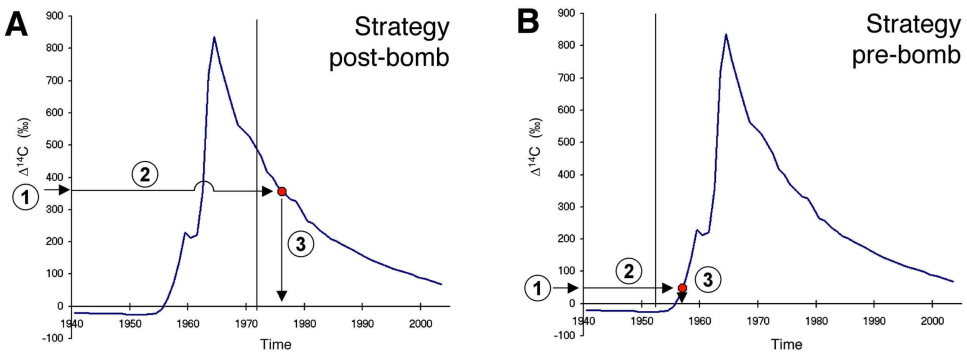


Figure 4: Interpretation of ^{14}C-data obtained; Individual born after (A) and before (B) the bomb peak. The vertical line represents the year of birth of the individuals. First, the ^{14}C concentration in genomic DNA from a given cell population is measured using mass spectrometry. Second, the ^{14}C-value obtained is interpreted in relation to the atmospheric ^{14}C curve (blue) and plotted (red dot) Then the perpendicular on the X-axis reflects the average birth date of the cell population. Reprinted from Spalding et al (Spalding et al., 2005a) with permission from Elsevier.

1.3.2. Implications of radiocarbondating to study cell turnover in the healthy and diseased human brain

It is quite remarkable that today we do not yet understand if neuronal turnover plays a role in disease, and it is not known what regenerative efforts the brain is capable of making following injury. The question whether neural stem cells are activated in the adult brain in response to lesions, notably stroke, and generate functional neurons, is of fundamental importance for regenerative medicine (Barnabe-Heider and Frisen, 2008; Curtis et al., 2011; Minger et al., 2007). Answering this question would be important for identifying diseases where therapies directed at modulating cell replacement would be beneficial (Koch et al., 2009; Lie et al., 2004; Scheffler et al., 2006).

In light of the differences between mouse and human neural stem and progenitor cells that have recently been uncovered (Farkas and Huttner, 2008; Fietz et al., 2010; Goldman, 2003; Hansen et al., 2010; Kriegstein and Alvarez-Buylla, 2009; Merkle and Alvarez-Buylla, 2006;

Reillo et al., 2010), it is unclear to which extent the data from animal models can be extrapolated to humans. Moreover, it is possible that the responsiveness of quiescent neural stem cells to “wake-up” triggers in the adult human brain differs from that in the adult rodent brain (Fancy et al., 2004; Lugert et al., 2010). It is therefore imperative to directly determine the sites, and extent, of adult neurogenesis in the human brain under physiological and pathological conditions (Buffo et al., 2008; Kempermann et al., 2004b; Magavi et al., 2000). The method of ^{14}C-based retrospective birth dating of neurons is at present undisputedly the most valid experimental approach to obtain these data (Arlotta and Macklis, 2005).

Previous findings provided proof-of-principle evidence that the method of retrospective birth dating of cells can be used to determine the age of neurons in specific brain regions, and hence to unequivocally establish the occurrence or absence of human adult neurogenesis. Specifically in stroke, given the different pathophysiological cascades and mechanisms following the various subtypes of stroke, and in light of the differences in clinical significance regarding the location of stroke, it will be important to correlate the ^{14}C data obtained on adult neurogenesis in response to stroke to those on functional recovery to explore the clinical value of possible neurogenesis (Chopp et al., 2009; Jiang et al., 2001). The radiocarbon technique further allows, for the first time, to assess the age of tumor cells and to investigate cell turnover dynamics in brain tumors (Spalding et al., 2005a). As the sensitivity of the ^{14}C-based approach of birth dating cells is increased the longer the time-period between an event (e.g. onset of stroke or tumor growth) and the read-out (tissue assessment upon biopsy or death) dates back, we decided to analyze tumor growth dynamics in meningiomas (Spalding et al., 2005a). This tumor type is thought to grow slowly as compared to more malignant glioma variants (Nakamura et al., 2003; Pompili et al., 1998; Zeidman et al., 2008).

2 AIMS OF THE THESIS

Listed according to publications:

Applying the technique of ^{14}C retrospective birth dating of cells, the aims of this thesis were:

I. to investigate whether or not and to which extent there is adult neurogenesis in the healthy human brain, specifically within the hippocampus;

II. to investigate whether or not and to which extent there is adult neurogenesis in the diseased human brain, specifically in response to cortical stroke;

III. to investigate the age and growth dynamics of brain tumors, specifically benign meningiomas.

3 SUMMARY OF RESULTS

Using the unique opportunity of ^{14}C retrospective birth dating of cells, we have addressed three fundamental research questions. First, the cell turnover in the healthy human brain, specifically adult neurogenesis within the hippocampus. Second, the cell turnover in the diseased human brain, specifically whether or not neurogenesis is induced within the cerebral cortex in response to ischemic stroke. Third, we used the ^{14}C-based birth dating technique to explore the age and growth dynamics of meningiomas.

In the first publication (Spalding et al., 2013), we for the first time demonstrated that adult neurogenesis within the human hippocampus occurs over the entire lifespan and described cell turnover dynamics of this process (Spalding et al., 2013). Prior to our study all information on human hippocampal neurogenesis was based on immunohistochemical analyses, leaving room for uncertainties if the described findings truly reflect neurogenesis in terms of maturation and survival of labeled cells (Knoth et al., 2010; Sanai et al., 2011). Only the landmark study by Eriksson et al. (Eriksson et al., 1998) had provided direct evidence for hippocampal neurogenesis, yet it was based on only few patients and did not address the number of newly generated neurons nor cell turnover dynamics within the human hippocampus. By determining the genomic ^{14}C-concentration in neuronal and non-neuronal DNA, we demonstrated that there is significant hippocampal neurogenesis over the entire human lifespan (Spalding et al., 2013) – in contrast to the previously described absence of neurogenesis in the healthy cerebral cortex and olfactory bulb (Bergmann et al., 2012; Bhardwaj et al., 2006) . We were able to show that hippocampal neurons are exchanged on a population level and that not all neurons are exchanged during life. By mathematical modeling we suggested the existence of a constantly renewing fraction that constitutes approximately 35% of all hippocampal neurons, a number that reflects slightly less neurons than known to exist in the dentate gyrus (Spalding et al., 2013). We calculated that the median turnover rate of neurons within the renewing subpopulation is 1.75% per year during adulthood, which corresponds to ≈700 new neurons per day in each hippocampus or 0.004% of the dentate gyrus neurons per day in the human hippocampus (Spalding et al., 2013). Mathematical modeling also suggested that there is a moderate decline in the amount of neurogenesis with increasing age. The neuronal turnover rates in humans were comparable to previous reports of hippocampal neurogenesis rates in middle-aged mice (Ninkovic and Gotz, 2007; Spalding et al., 2013). We concluded that human adult

hippocampal neurogenesis may be of functional significance and emphasized that further studies in patients with dementia or psychiatric disease may reveal important insights into the hippocampal turnover dynamics in disease (Kempermann, 2011; Santarelli et al., 2003).

In the second publication (Huttner et al., 2014), we investigated whether or not neurogenesis is induced in the human cerebral cortex following ischemic stroke. Prior to our study, knowledge of cortical neurogenesis was mainly based on experimental findings from animal studies, with conflicting results (Arvidsson et al., 2002; Jiang et al., 2001; Jin et al., 2006; Parent et al., 2002; Zhang et al., 2006b; Zhang et al., 2001; Ziv et al., 2007). For humans there were only few immunohistochemical studies that had reported conflicting findings, either suggesting no sustained neurogenesis or claiming signs of adult neurogenesis in the penumbra of cortical infarcts, predominantly in the vicinity of blood vessels (Jin et al., 2006). By measuring the concentration of genomic ^{14}C in cortical neurons after ischemic stroke we demonstrated (i) that the age of neurons surrounding the ischemic infarct core is the same as that of non-affected neurons of the occipital cortex of the healthy contralateral hemisphere of the same individuals (Huttner et al., 2014), and (ii) that this age corresponds to the respective age of the patients included into our study (Huttner et al., 2014). We thus provided evidence that there is no significant cortical neurogenesis in response to stroke and that the neurons surrounding the infarct core are most likely the neurons that were generated during fetal development and that survived the stroke (Huttner et al., 2014). We correlated our findings with immunohistochemical analyses of lipofuscin, an age pigment constituted of protein and lipid clusters that starts accumulating in neurons after 5-10 years and continues to do so during life (Benavides et al., 2002); we did not find any lipofuscin-negative neurons, neither in healthy regions nor within the ischemic lesion of the cerebral cortex, a finding consistent with all neurons being old (Huttner et al., 2014). We further provided evidence that there is cell turnover within the non-neuronal population, most likely reflecting the generation of glial cells in the process of scar formation, as those cells were of younger age than the non-neuronal cells of the control areas, i.e. the non-affected occipital cortex (Huttner et al., 2014). Interestingly, by performing single cell gel electrophoresis assays, we detected a certain level of DNA fragmentation occurring in the early phase after cortical ischemia; in relation to this finding we were able to demonstrate that up to 22% of the non-apoptotic neurons were positive for DNA repair markers, such as APE-1 and gammaH2AX (Huttner et al., 2014; Oezguen et al., 2007; Park et al., 2006). By whole-genome and transcriptome analyses we verified the genomic integrity of neurons present in the chronic phase after cortical stroke. We concluded that there is no relevant neurogenesis – in terms of neuron maturation and

long-term survival – within the human cerebral cortex following ischemic stroke (Huttner et al., 2014).

In the third manuscript (Huttner et al.), we used the ^{14}C-based birth dating technique to explore the age and growth dynamics of meningiomas. Meningiomas are – together with gliomas – the most frequently occurring brain tumors in humans. They arise from arachnoidal cap cells and are usually slowly growing and benign (WHO°1); however, there are also invasive-malignant atypical variants (Perry et al., 2004; Riemenschneider et al., 2006; Whittle et al., 2004). There has been an intensive debate about the age of these tumors; however, there has been no methodological approach to explore the onset of tumor growth, and studies on growth dynamics were mainly based on imaging findings, e.g. serial magnetic resonance imaging (Nakamura et al., 2003; Zeidman et al., 2008). We measured the average ^{14}C-concentrations of all cells within the tumor – obtained as an aliquot upon surgical resection of the tumor – of patients with benign (WHO°1) and atypical (WHO°2) meningiomas, and found the average age of tumor cells of WHO°1 meningiomas to date 2.3 - 9.5 years older than the time-point of surgery, whereas WHO°2 meningiomas dated as old as the year of surgery (this analysis however did not reflect the onset of tumor growth). Based on immunohistochemical analyses and confocal microscopy we obtained various parameters such as Ki-67 indices, tumor cell densities and apoptosis rates, to integrate – together with the ^{14}C-data obtained and the imaging findings of tumor size and growth – our findings into a mathematical model of meningioma growth dynamics. We were able to identify (i) meningiomas that were still residing in the lag phase (i.e. slowly growing but not yet entering the exponential growth curve (Huttner et al.)), (ii) meningiomas that were in the exponential growth, and (iii) meningiomas that had already entered a plateau phase (Huttner et al.). The mathematical modeling allowed us to determine the onset of tumor growth, defined as the generation of the first tumor cell. Benign meningiomas were on average 22.1 years old (ranging from 10.8-31.1 years), whereas atypical WHO° 2 meningiomas originated 1.5 ± 0.1 years prior to surgery (Huttner et al.). Further, we noticed a statistically significant difference in the age of meningioma subtypes, i.e. meningothelial *versus* fibrous meningiomas (meningothelial: 17.8 ± 5.8 years *versus* 26.3 ± 4.0 years in fibrous meningiomas; $p = 0.015$), whereas there was no significant correlation of meningioma age with the age of patients ($R^2 = 0.0275$) or the Ki-67 index ($R^2 = 0.1539$). We concluded that existing models to predict meningiomas growth may complement ^{14}C-data on tumor cells to better estimate growth dynamics (Huttner et al.). The latter may be of specific clinical relevance in patients with a

tumor that is difficult to assess surgically and hence has a high likelihood of incomplete resection (Braunstein and Vick, 1997; Huttner et al.; Pompili et al., 1998).

4 DISCUSSION AND FUTURE PERSPECTIVES

Here we (i) showed that there is continuous adult neurogenesis within the human hippocampus, (ii) demonstrated that there is no significant induction of neurogenesis in the human cerebral cortex in response to stroke, and (iii) established the age and growth dynamics of human meningiomas. These findings have several important implications.

First, the functional significance of human hippocampal cell turnover needs to be further established (Kempermann et al., 2004b; Spalding et al., 2013; Zhao et al., 2008). Hippocampal neurogenesis has been reported to be altered, being either increased or decreased, in experimental models as well as in humans depending on pharmacological medication and disease (Fabel and Kempermann, 2008; Holmes et al., 2004; Knoth et al., 2010; Santarelli et al., 2003). Hence, to elucidate hippocampal cell turnover dynamics in human disease, specific patient groups with clearly defined diagnoses and treatments need to be studied using the radiocarbon-technique (eventually in addition to immunohistochemical analyses) to shed light into the debate of whether or not there is altered neurogenesis in these settings and which contribution such possibly altered neurogenesis may exert on functional outcome (Bergmann et al., 2015; Curtis et al., 2011; Santarelli et al., 2003).

Second, in light of the absence of induction of adult neurogenesis in the human cerebral cortex following ischemic stroke (Huttner et al., 2014), is remains unclear whether this finding also holds true for other brain regions frequently affected by stroke, notably the basal ganglia including the striatum (Lindvall and Kokaia, 2015). These brain structures are topographically closely located next to the neurogenic SVZ niche (Kokaia and Lindvall, 2012). It therefore appears more realistic that newborn neurons may migrate into the penumbra of the ischemic lesion, as shown in rodents (Arvidsson et al., 2002). However, these studies need to be done in humans using methods such as the radiocarbon-technique, as immunohistochemical analyses leave too much room for controversy (Bergmann et al., 2015). The neurogenic potential of these anatomical areas was recently revived by findings of specific striatal neuronal subpopulations showing annual turnover rates >2% (Ernst et al., 2014). In this regard it appears meaningful and valuable to also assess hippocampal cell turnover rates in response to basal ganglia strokes in humans, as increased rates have been proposed in animal studies (Lindvall and Kokaia, 2015).

Third, the determination of tumor onset and growth dynamics of human meningiomas demonstrated that the radiocarbon-technique is feasible also for studying tumor diseases (Huttner et al.). Of clinical relevance would be the investigation of cell turnover dynamics in gliomas (Venere et al., 2011). These tumors usually are more malignant and cross-over from less malignant variants (e.g. WHO°2) to more aggressive and further de-differentiated variants, like glioblastoma (WHO°4). A carbondating-approach of tumor tissue (obtained upon biopsy or tumor resection) may also allow to birth date specific cancer-initiating stem or progenitor cells, which would dramatically increase knowledge of tumor onset and progression in gliomas (Lathia et al., 2015; Venere et al., 2011; Weller et al., 2013). In this regard the recently established ^{15}N-thymidine infusion method may also allow to study human malignant disease in vivo (Alkass et al., 2015; Steinhauser et al., 2012).

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