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Tim Hendrik Boy

THE EFFECTS OF LONG-TERM DIABETES MELLITUS ON ADULT NEUROGENESIS IN THE SUBVENTRICULAR ZONE OF THE RAT BRAIN

Diploma Thesis

Academic Year:
2018/2019

Mentor:
Assist. Prof. Natalija Filipović, DVM, PhD

Split, December 2018

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1.INTRODUCTION

1.1. Adult neurogenesis

For long periods of time it was believed that mammals were born with a fixed number of neurons. This theory was consistent with the clinical picture of minimal recovery after neuronal injuries such as stroke and brain trauma as well as the decreased neuroplasticity observed with age (1). Adult neurogenesis describes the ability of non-embryonic neural cells to divide and differentiate into specific neurons. Along with other processes, such as the axonal regeneration, collateralization, reactive synaptogenesis, long term potentiation and activation of silent synapses, it contributes to neuroplasticity (1, 2).

The use of modern technology has enabled to proof adult neurogenesis in various brain tissues of several species such as birds, fish, reptiles and mammals including humans (3-5). However, an article published in Nature in 2018 didn't find any sign of neurogenesis in human dentate gyrus tissues after childhood (6). This has once more put the field of adult neurogenesis in the spotlight of scientific discussion. In the following paragraphs we are trying to delineate the basic methods of researching adult neurogenesis, what is currently known and future perspectives on adult neurogenesis to give some understanding on the scientific dispute caused by this article.

1.1.1. Basic methods of researching adult neurogenesis

Adult neurogenesis is mostly researched using immunohistochemistry methods or DNA labeling. Early research relied on injection of radioactively labeled thymidine, which integrates into the DNA of dividing cells. By using radiographic microscopy, it was then possible to identify the neurons which had been born after the injection (7).

Immunohistochemistry techniques enable the visualization of molecules of interest in the tissue sections. Its' advantage is that several different targets could be marked with different fluorescent colors all on the same specimen. This allows for analyzing neurogenesis in specific cell lines or the effects of specific molecules and receptors on neurogenesis by employing markers targeted at them and observing co-localization of these markers. The most important markers will be briefly discussed below. These methods are supplemented by genetic methods such as genetic marking of particular cell populations using Cre-Lox recombination or viral vectors to fluorescently mark cells expressing genes of interest (8).

Knockout animals and viral-mediated gene transfer are used to determine the effect of gene expression in neurogenesis on a molecular to behavioral spectrum (8). The use of pharmacologic and pharmacogenetic inhibitors of neurogenesis as well as brain irradiation have a similar application (9).

In the recent years the use of computational models has found its application into this area of study. Using modern high-powered computers, neuroanatomic and neurophysiologic knowledge it is now possible to build software simulating complex brain functions and to test hypothesis about them (10).

1.1.2. Common markers used in adult neurogenesis research

DAPI or 4′,6-diamidino-2-phenylindole strongly binds to adenine-thymine rich regions of DNA and absorbs blue light of 347 nm wavelength. It emits light of 453 to 460 nm wavelength making DNA and therefore nuclei visible under fluorescent microscopes (11). This allows for easy recognition of cells and enables to colocalize other markers to them.

Ki-67 is a protein expressed exclusively in the nucleus of dividing cells. Beside its role in basic research it has found application in cancer prognostics. The Ki-67 labeling index is used to find patients with highly proliferating carcinomas of breast, brain, prostate, nephroblastoma and neuroendocrine tumors benefiting from adjuvant therapy (12).

Bromodeoxyuridine (BrdU) is a thymidine analog that incorporates into the DNA of dividing cells. It is one of the earliest and most common markers used in adult neurogenesis research. BrdU is often considered the gold standard of measuring adult neurogenesis (7).

Nestin is a marker of neuronal stem cells, neural crest progenitors and it is also expressed in stem cells of the hair follicle which can differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes in vitro. It has also been used to identify endothelial cells and neovasculogenesis in colorectal cancers (13, 14).

Glial fibrillary acidic protein (GFAP) is expressed in several CNS cells including astrocytes and ependymal cells. It is an intermediate filament in mitosis and plays roles in myelination, cell-cell communication and the functioning of the blood brain barrier (15).

Sox2 is a transcription factor that plays a key role in maintaining pluripotency of embryonic and neural stem cells. Together with other key factors such as Oct4 it binds DNA to activate transcription of pluripotency circuitry proteins. Neural cells expressing Sox2 can divide and differentiate into mature neural cell types (16).

Doublecortin (DCX) is a protein expressed in immature neurons and neuronal precursors. It is associated with microtubule formation and downregulated in maturity. Mutations of the DCX gene have been associated with lissencephaly and epilepsy due to abnormal migration of developing neurons (17).

1.1.3. Known locations of adult neurogenesis

In the human adult brain neurogenesis has been found in the subgranular zone (SGZ) of the hippocampal dentate gyrus and in the subventricular zone (SVZ) of the ventral telencephalon (10). There is controversy about the persistence of neurogenesis in other areas of the adult mammalian brain.

1.1.3.1.Hippocampus

The hippocampus is a structure of the limbic system located under the cerebral cortex and medial temporal lobe. It can be divided into the hippocampus proper and the dentate gyrus.

Defects in the hippocampus are known to cause problems with learning and memory formation. Several human patients with lesions confined to the hippocampus showed profound anterograde amnesia in declarative memory formation, all whilst intelligence and retrograde memory was unaffected (18). These unfortunate patients were unable to form new memories. Rats with damaged hippocampi that were tested in the water maze, were impaired in learning the position of the hidden platform by orienting on the landmarks in the surrounding (19). This implicates that orienting is dependent on the same structures responsible for declarative memory formation. This correlates with a study of taxi drivers in London, who had increased posterior hippocampal volume compared to age-matched controls. Interestingly the increase was proportional to the months of job experience as a cab driver (20).

Monkeys with damaged hippocampus and parahippocampal gyrus had severe difficulty with remembering newly learned information but performed on par with controls (21). A Study using fMRI perfusion scans showed increased perfusion in the bilateral anterior hippocampus, the left middle temporal gyrus, bilateral lingual gyrus and the anterior cingulate after a face-name memorization task. Correct retrieval of items during the recall test were associated with increased prefusion in the right anterior hippocampus. This was especially true for recently learned items in comparison to items already recalled in a previous recall test (22).

When viewed together this information supports the theory that hippocampal structures play a key role in consolidating memories from short to long-term and in the formation of "mental maps" for orientation.

1.1.3.2.Subventricular zone

The subventricular zone (SVZ) is a thin lining surrounding the lateral ventricles of the brain. In nonhuman mammals, progenitors derived from the SVZ migrate via the rostral migratory stream (RMS) into the olfactory bulb (23). Stem cells from the SVZ also migrate into the neurodegenerating cortex after NDMA induced injury, where they differentiate into astrocytes and to a lesser extent into tbr1+ cortical glutamatergic neurons and calretinin+ interneurons. However, these neurons integrate into the existing circuitry only to a minor degree (24). It is likely that in humans the neuroblasts of the SVZ migrate into the striatum instead of the olfactory bulb (25).

1.1.4. Possible Functions of adult neurogenesis

The functions of adult neurogenesis are still under investigation. It is known that the progenitors in both zones become granule interneurons and integrate into existing circuitry. As stated above the presumed functions of the hippocampus are declarative memory and spatial memory. Several studies have been conducted on the effects of inhibiting adult neurogenesis in the hippocampus on learning and memory.

In one study neurogenesis in rats was blocked with the DNA-methylating agent methylazoxymethanol (MAM). While under the effect of the drug the rats had impaired learning of trace eye-blink conditioning. Three weeks after the treatment, when neurogenesis returned to normal levels, they were unimpaired in trace eye-blink conditioning (26). This example demonstrates the importance of young neurons in hippocampal learning processes. In a follow up study using the same MAM protocol affected mice showed reduced acquired fear on a fear conditioning paradigm but there were no impairments in performance in tests of contextual fear conditioning and the Morris water maze (27).

It has also been shown that predominantly neurons born one week before the onset of training will survive. Neurons born shortly before and in the early phase of training, when performance improves rapidly, selectively undergo apoptosis during the late phase of the Morris water maze (MWM) training, during which performance improvements slow down. This selective apoptosis is important for learning. Blocking this apoptosis prevents the enhanced survival of the neurons that were born one week prior to the MWM training and impairs performance in the MWM (13). This means that some degree of neuronal maturity is required for neurons to be relevant for the learning process, whereas neurons that are too immature obstruct the learning process and need to die for optimal learning.

This process contributes to the neuroplasticity of the dentate gyrus. Using computational models several theories regarding how these processes contribute to memory formation have been developed.

In replacement models, old neurons are simply replaced by newer neurons with naïve synapses. This allows for great learning capacity with the downside of loss of older neuronal circuits and therefore loss of old memories. This however doesn't necessarily lead to forgetting as cell death could be targeted to unused circuits and memory could be transferred to different parts of the brain. This is the case in Becker's replacement model. Here the role of the dentate gyrus is encoding while storage and recall depend on other hippocampal areas (10).

Addition models presume that existing adult neurons are not removed from the dentate gyrus. This allows mature neurons to stay specific for the same memories over long periods of time. In this model subsets of mature neurons could be used to encode new information in familiar contexts. It could also prevent instability in the network because old neurons are not affected by new information entering the network. Here the function of apoptosis is explained in that young neurons are physiologically less selective in what activates them and become more selective as they age, so that some sort of pruning is required for the selection of appropriate circuitry (10).

Both types of models provide valid methods of pattern separation. Pattern separation is a task presumed to be dependent on the dentate gyrus. This is based on several facts. The dentate gyrus has 5 to 10 times more neurons than its primary input the entorhinal cortex, many of those are interneurons (28, 29). This seems ideal regarding the computational needs for pattern separation. *In vivo* dentate neurons are rarely active during behavior (30). Also, their mossy fibers are capable of depolarizing pyramidal neurons in the CA3 area of the hippocampus, suggesting that they could drive memory encoding (31, 32). Pattern separation describes the ability of a network to discriminate between two input signals by providing two output signals that are less similar to each other than the inputs. In short, it is the computational mechanism behind differentiating one information from another and preventing interference between memories, that is making sure only the appropriate memory circuits are activated (10).

Behavioral studies show significant inconsistency of results. A review comments that different knockdown methods didn't produce the same results and many report deficits in long-term but not in short-term memory regarding navigation in the MWM. New neurons may help stabilizing old memories when new information is being encoded. The review continues that these inconsistencies could be attributed different rat strains, the ages of the rats, the interval between ablation and testing and the effectivity and side effects of ablation procedures. Some tests of hippocampus dependent learning might not be dependent on the dentate gyrus. It even cites a study in which mice who had neurogenesis ablated performed better than controls in a spatial working memory task with high interference. The lack of neurogenesis might reduce intra- and inter-trial interference with the tasks due to more distinctive encoding.

The conclusion was that despite inconsistencies studies suggest that neurogenesis in the dentate gyrus contributes to learning and memory (10).

1.1.5. The neurogenic niche

We have already discussed where adult neurogenesis happens, what its possible functions are and some of the possible neurological mechanisms of it. However, in order to understand the possible benefits of this research for clinical medicine, we first need to discuss some molecular and cellular mechanisms behind it, as they represent possible disease mechanisms and targets for therapy. Why does neurogenesis happen only in the dentate gyrus and the subventricular zone? One of the possible reasons is the microenvironment. The neurogenic niche has many factors that enable and regulate adult neurogenesis. The next paragraphs discuss some transcription factors, signaling molecules, neurotransmitters and supportive cells of the neurogenic niche.

1.1.5.1. Notch signaling

Notch1 is a fate signal integrator in stem cells which binds to its receptor on the cell membrane and activates downstream signaling involving gamma secretase, Notch intracellular domain (NICD), mastermind-1 (mam1), the transcriptional cofactor recombining binding protein suppressor of hairless (RBP-J) and transcribes genes including Hes1, Hes3 and Hes5. This maintains a stable pool of stem cells by preventing terminal differentiation of stem cells (33). Loss of Notch signaling from stem cells and their progeny in the SGZ shifted the cells from stem-like (GFAP+) to neuronal (DCX+) with stunted dendritic arbors and fewer varicosities, decreasing the progenitor pool. Overactivation of Notch signaling in NICD transgenic mice led to persistent GFAP+ stem cells, fewer DCX+ neurons with enlarged arbors and increased varicosities, increasing the progenitor pool (34). There seems to be differences in Notch signaling in the SVZ compared to the SGZ. In the absence of the essential Notch transcription factor RBP-J ependymal cells of the SVZ began generating granule and periglomerular neurons that migrated to the olfactory bulb, demonstrating their multipotency (35).

1.1.5.2. Cyclin dependent kinase 5

Cdk5 is involved in cell maturation and migration and phosphorylates molecules regulating neurite growth and synaptogenesis in post-mitotic neurons (35, 36). In a retroviral Cdk5-knockdown study targeting dividing cells in the anterior SGZ neuroblasts had ectopically projecting dendrites, abnormal migration and dendritic spine formation with minimal effect on survival of immature neurons (37). In contrast to that a study ablating Cdk5 in stem cells using a Nestin-CreERT2 system throughout the entire SGZ led to decreased accumulation of immature neurons, but no change in total proliferation (38). Both studies indicate that Cdk5 has only minimal influence on proliferation or cell-cycle progression in adult neurogenesis (8).

1.1.5.3. Neurotrophin Receptors

TrkB and p75 are membrane bound receptors for the neurotrophins brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) respectively. They are both expressed in dividing SVZ cells. Dividing SGZ cells express p75, but TrkB expression drops as the stem cells start proliferating and is upregulated later as the progeny mature (39, 40). Knocking out TrkB from stem cells and their progeny in the SGZ blocked antidepressant mediated increase in neurogenesis and its behavioral effects on rodent tests of anxiety and depression (41, 42). Another study ablating TrkB signaling showed abnormal dendritic morphology, ability to develop long-term potentiation and decreased neuronal survival. The ablation of TrkB in adult cells led to marked increases in two rodent tests for anxiety (44). The role of TrkB signaling in the SVZ is less certain, although these receptors are expressed, BDNF did not stimulate neurogenesis (, 45).

1.1.5.4. Wnt and BMP signaling

Bone morphogenic protein (BMP) and Wingless/Integrated (Wnt) are key regulators of neurogenesis in embryonic development. Wnt signaling is necessary for the upregulation of neurogenesis by lithium (46). Blocking it causes impairments in spatial and object recognition memory in rats. Rats with higher percentage of knockdown performed poorly in remembering the location of the platform in a MWM task at 2, 4 and 8 weeks after training while not being impaired in learning a new MWM task. For the object recognition tasks, the high-knockdown animals showed lack of remembrance 3 hours after first exposure (47). Smad4 a downstream target of BMP signaling, is exclusively expressed in SVZ progenitors. Its deletion in stem cells via the GLAST-CreERT2 system shifts cell progeny to oligodendrocyte precursors. However, it did not have any effect on SVZ neurogenesis (48). This is consistent with what Ortega *et al.* showed in 2013, that neural stem cells of oligodendrogliogenic and neurogenic lineage respond differently to Wnt and BMP signaling (49).

1.1.5.5. DISC1

The Gene "disrupted in schizophrenia 1" is an intracellular signaling molecule. DISC1 mutations have been associated with an increased risk to develop schizophrenia (50). Its knockdown enhanced neurogenesis in the dentate gyrus, affecting stem cells and immature neurons. The adult neurons showed longer dendrites, ectopic dendrites, more dendritic crossings, enhanced spine formation, synaptogenesis, increased excitability, ectopic cell bodies and reduced neuron maturation (51). In another study knockdown of DISC1 immature neurons showed decreased SGZ proliferation at 5 weeks without increased cell death. The animals from the same experiment showed behavioral abnormalities such as hyperactivity in an open field test, novelty-induced hyperlocomotion and decreased latency to immobility in a forced swim test. The application of a Glycogen synthase kinase 3 beta (GSK3β) inhibitor prevented this decrease and behavioral abnormalities (52).

1.1.5.6. NMDA receptors

It is known that N-Methyl-D-aspartate (NMDA) glutamate receptors can potentiate synapses and cause long-term changes in neuronal circuitry. Immunohistochemical studies have found glutamate receptors in astrocytes and neuroblasts of the SVZ (53). Knocking down the NR1 subunit of NMDA receptors via retrovirus decreases maturing neurons in the SGZ and increases cell death at 3 weeks (54). Activation of NMDA receptors inhibited cell proliferation, while blockade had the opposite effect (55).

1.1.5.7. GABA Receptors

$GABA_A$ receptors are involved in regulating the proliferation, migration and maturation in juvenile SGZ and SVZ neurons. Contrary to their hyperpolarizing inhibitory functions in the mature neuron, $GABA_A$ receptors are excitatory in immature neurons until the KCC2 chloride transporter is expressed. This excitation promotes maturation of neural stem cells (56). Retroviral knockdown of the NKCC1 chloride transporter results in cell hyperpolarization in response to GABA stimulation. This leads to reduced formation of GABAergic and glutamatergic synapses with deficits in neuronal maturation, as witnessed by decreased dendritic length, branch number and number of synaptic crossings (57).

1.1.5.8. Serotonin

Serotonin synthesis inhibition leads to reduced cell proliferation and decrease in immature neurons in the hippocampus. Many antidepressants that are believed to act via their serotonergic activity and ability upregulate neurogenesis.

In the dentate gyrus activation of 5-HT1A receptors increases proliferation and increases the number BrdU positive neurons (58). Serotonin depleted rats regain normal proliferation after 5-HT1B receptor activation, indicating a permissive function of these receptors (58). The 5-HT2A/2C ketanserine produces a large decrease in proliferation and 5-HT2C agonism did not have any effect after either acute or chronic administration (58).

In the SVZ 5-HT1A receptor stimulation increased proliferation by 53% (58). In contrast 5-HT1B activation lead to a 26% decrease in BrdU positive cells (58). Agonists of 5-HT2A/2C receptors increased proliferation by 24% but antagonism had no effect (58).

Olfactory bulb neurons increased in neurogenesis by 48% several weeks after a single dose of 5-HT1A agonists, the same effect was observed in chronic treatment (58). 5-HT2C agonists persistently increased number of BrdU positive cells both in acute and chronic treatment (58). In the olfactory bulb there seems to be no effect on cell line fate from serotoninergic stimulation (58).

1.1.5.9. Microglia

Microglia are the phagocytes of the brain and mount the brains innate immune response. They are capable of secreting proinflammatory cytokines and phagocytosing dying cells (59). As mentioned above, in the SGZ most immature neurons fail to be integrated into functional circuitry and most of them undergo apoptosis during their first 4 days of life. These cells are rapidly approached and phagocytosed by "unchallenged" microglia cells, in the absence of an inflammatory response or pathogens (60). This phagocytosis declines in parallel with the decline in adult neurogenesis induced by aging and is not affected by local inflammation as tested by peripheral infusion of gram-negative endotoxin lipopolysaccharide (LPS) (60).

1.1.5.10. Astrocytes

Astrocytes are macroglial cells derived from ectoderm. They can be classified in neurogenic and non-neurogenic. Neurogenic astrocytes are neuronal stem cells (NSCs) generating the new neurons of adult neurogenesis. Non-neurogenic astrocytes are part of the supporting structure necessary for adult neurogenesis as contact of astrocytes with SVZ and SGZ precursors supports neuronal proliferation (61, 62). Non-neurogenic astrocytes secrete several morphogens such as Sonic Hedgehog, which stimulates progenitors to reenter the cell cycle and the BMP, Wnt and Notch ligands like Wnt7a and Dlk1 (63, 64). They secrete proinflammatory cytokines such as IL-1β and IL-6 promoting neuronal differentiation. Astrocytes can also inhibit differentiation by secreting insulin-like growth factor binding protein 6 (IGFBP6) (65).

In the RMS neural stem cell progenitors travel along an astrocytic chain from the SVZ to the olfactory bulb (66). The astrocytes that form these chains have an elongated morphology forming along with blood vessels a guiding scaffold for the neuroblasts. The astrocytes form a glial tube to ensheath the neuroblasts in the RMS. The inhibition of GABA signaling in these astrocytes reduces migration and GABAergic stimulation of astrocytes by neuroblast leads to TrkB receptor insertion, this traps extracellular BDNF and stops migration (67, 68).

They take part in the early apoptosis in the olfactory bulb, releasing transforming growth factor-β2 (TGF-β2), which is proapoptotic to periglomerular cells (69). And the presence of connective tissue growth factor (CTGF) enhances this activity (69). They influence blood flow through calcium signaling and can channel glucose in a glutamate dependent fashion from the blood vessels to distant neurons via gap junctions (70, 71).

1.1.6 Linking adult neurogenesis to pathology

Impairments of cognitive function, memory and other mental processes have been observed in many somatic and psychiatric diseases such as diabetes, *Systemic lupus erythematosus* (SLE), anxiety disorders, depression and schizophrenia. Discovering the complex regulation of adult neurogenesis has enabled the creation of plausible theories and research on possible causes of such impairments. Two of the possible causes common to many of those diseases are chronic inflammation and stress.

1.1.6.1. Inflammation

As mentioned above immune cells play a role in supporting adult neurogenesis. Peripheral proinflammatory cytokines can disrupt the blood brain barrier leading to astrocyte activation and local CNS inflammation. Although neurogenesis can be unaffected by the activation of microglia, most of the time, it suppresses hippocampal neurogenesis (72). The administration of LPS disrupts neural progenitor proliferation, decreases differentiation into neurons and reduces survival of neuroblasts. In rodents LPS administration as shown to disrupt spatial memory, cause depression-like behavior and anxiety (73). *In vitro* studies showed that IL-6 and p21, an inhibitor of Cdk, arrests proliferation of neural progenitors while permitting astroglial proliferation (74).

1.1.6.2. Stress

Inflammation also activates the Hypothalamus-Pituitary-Adrenal (HPA)axis causing the release of ACTH and cortisol (75). Steroids released in stress are capable of influencing proliferation, differentiation and survival of developing neurons (76). Stress affects neurogenesis trough many different pathways, in the following text a few of them will be discussed briefly.

While low levels of glucocorticoids increase proliferation of progenitors and decrease neurogenesis via mineralocorticoid receptors, high levels of glucocorticoids suppress neural progenitor proliferation and neurogenesis via glucocorticoid receptors (77). Adrenalectomy combined with corticosterone replacement therapy desensitizes rats to fox odor induced decreases in neurogenesis (78). *Ergo* disrupting the HPA axis (and therefore disrupting the stress response) enhances neurogenesis and administration of corticosterone reduces neurogenesis.

Chronic stress also reduces the expression of VEGF and VEGF2-Receptor in the hippocampus, decreases neurogenesis more in vicinity of blood vessels and these regions take longer to recuperate from stress (79). Both, IL-1β administration and acute stress impair hippocampal proliferation and blockade of IL-1β signaling blocks the effects of stress on neurogenesis (80). BDNF expression and cAMP response element-binding protein (CREB) phosphorylation is downregulated in the dentate gyrus by stress (81). Exogenous glucocorticoids suppress hippocampal BDNF mRNA and translation into protein (82).

Early separation of rat pups from their mothers and administration of inescapable electroshocks caused decreased neurogenesis lasting well into adulthood, even in the presence of normal glucocorticoid levels (83, 84). These experiments demonstrate that psychologic trauma can have long-term effects on neurogenesis.

As elaborated stress has many ways of affecting neurogenesis, however, stress also affects the brain on many other levels and suppressing neurogenesis itself leads to activation of the HPA axis (85). Rats with disrupted neurogenesis take longer to recover cortisol levels to baseline after moderate stress tests, are less responsive to HPA suppression with dexamethasone and showed decreased coping capability in the forced swim test, novelty induced food avoidance test and showed signs of anhedonia. This means that the hippocampus itself contributes to the control of stress (86).

Much remains to be discovered about the relationship of stress and adult neurogenesis and further studies will have to elaborate on the mechanisms underlying the observed neurocognitive decline in stress.

1.1.6.3. Ageing

As mentioned in the introduction, the effects of ageing on adult neurogenesis are controversial. There is conflicting data on its persistence in adulthood in humans and other species. Most studies have been conducted on rodents and it is generally agreed upon that adult neurogenesis persists in them. In the following we are presenting two opposing studies on human adult neurogenesis, before going into more intricate fields of studying adult neurogenesis in the context of ageing.

In March 2018 Sorrels *et al.* published the following findings in Nature. They conducted an immunohistochemical study on human and monkey hippocampi. They found that in the 59 post-mortem and live-samples from epilepsy-surgeries there was a drastic decline in neurons marking for several neurogenic markers with ageing. For example, they found a drop from 1,618 ± 780 (mean ± SD) at birth to 292.9 ± 142.8, 12.4 ± 5.3 and 2.4 ± 0.74 $DCX^+PSA\text{-}NCAM^+$ cells/mm^2 at 1, 7 and 13 years of age respectively. They also reported that these neurons had a progressively mature morphology and that there was no continuous layer that would resemble the SGZ of rodents in either adult humans or monkeys (6). The publication is in support of other publications that found similar results however, the limitations include that all samples were taken from patients suffering from various diseases.

This was in turn addressed by Boldrini *et al.* in a Cell Stem Cell article from April 2018. Their publication is making a case for the persistence of neurogenesis in healthy humans with no reported impairments, good global functioning and low recent (last 3 months) life event related stress. They found similar numbers of intermediate progenitors and immature neurons, glia and mature granule neurons in the dentate gyrus across a range of 14 to 79 years for both sexes. However, they reported that the pool of quiescent neural progenitor cells declined with ageing in the anterior-mid dentate gyrus. They suggested that the decline in DCX^{-} $PSA\text{-}NCAM^{+}$ cells of intermediate neural progenitor and immature granule neuron morphology might indicate a decrease in neuroplasticity in the anterior dentate gyrus. This might include blunted migration, dendrite sprouting, long-term potentiation and activity-dependent plasticity. Further they found a correlating decline in angiogenesis as measured by length, area and number of bifurcations of capillaries per mm^3 using Nestin as marker of neovasculogenesis (1).

It is not yet clear to what extent adult neurogenesis persists in humans. Further studies will have to unveil more information and take into consideration the molecular factors affecting neurogenesis such as inflammation and stress.

Interestingly ageing itself seems to be causing dysregulation of the immune system responsible for chronic low-grade inflammation and microglia produce more IL-6, TNF-α and IL-1β in response to inflammatory stimuli such as LPS in aged human subjects (87, 88). Increased levels of IL-6 have been linked to aging-related cognitive decline (89).

1.1.6.4. Obesity

Overnutrition is not only one of the most common preventable cause of diseases such as diabetes, ischemic heart disease, certain cancers and elevated blood pressure, it has also been found to increase the risk of dementia (90). Obesity is more than just an excess of calories and lack of physical activity or failure to control unhealthy feeding habits. A recent study has uncovered neuroinflammation as important component of the pathology (91).

Peripheral tissues, the blood system and the hypothalamus respond to obesity with a chronic inflammatory reaction through the inhibitor of nuclear factor kappa-B kinase subunit beta/Nuclear Transcription factor kappa-B pathway (IKKβ/NF-κB) . This affects hypothalamic neurogenesis and functions such as feeding regulation, energy management and metabolic control (91). Overeating, glucose intolerance, insulin resistance and impaired insulin secretion can be caused by neuroinflammation (91). Blocking the IKKβ/NF-κB pathway protects from obesity and systemic glucose and insulin disorders in high-fat diet (HFD) feeding (92).

Dietary and leptin deficiency-induced obesity are both associated with reduction of neurogenesis (93). Excessive secretion of microglial cytokines such as TNFα and IL-1β from the IKKβ/NF-κB pathway causes neurodegeneration via chronic inflammation while microglia-specific IKKβ knockout prevents upregulation of apoptosis and Notch mediated differentiation arrests (92). Chronic overnutrition with HDF leads to reduction of proopiomelanocortin (POMC)positive neurons, involved in energy balance and glucose homoestasis, and increases in apoptosis of mature and newborn neurons (91, 94). Some of these effects can be reversed by caloric restriction (93). However hypothalamic neurodegeneration itself is a cause of adult onset obesity and prediabetic changes since mice having genetically impaired neurogenesis in the mediobasal hypothalamus develop metabolic disorders (91).

Obese animals have shown impaired hippocampus dependent learning, spatial memory, long-term memory and contextual fear conditioning (95). This study also reports depression-like behavior, especially under stress conditions or LPS induced inflammation. In the human obesity has also been associated with several types of cognitive impairment (96).

1.1.6.5. Diabetes Melitus

Human diabetes has been associated with hippocampal atrophy, increased risk of dementia, cognitive impairment and depression (97). *Diabetes mellitus* is a disease of glucose metabolism and results from the lack or reduced effectiveness of endogenous insulin. Hyperglycemia is the characteristic hall mark of *diabetes mellitus* and causes microvascular problems such as retinopathy, neuropathy and nephropathy. Macrovascular complications of diabetes include stroke, renovascular disease, limb ischemia and heart disease (98). However, there is more to how diabetes affects the brain.

On a neurological level decreases in hippocampal synaptic plasticity, neurotoxicity and changes in glutamate neurotransmission have been observed. In animal models of diabetes, reduced neurogenesis is associated with learning and memory deficits and depression-like behavior. Both the reduced hippocampal neurogenesis and the behavioral manifestations can be reversed by insulin treatment (97).

Type 1 diabetes is caused by B-lymphocyte-mediated destruction of pancreatic beta-cells. It is usually of adolescent onset and there is absolute insulin deficiency. Patients are dependent on insulin therapy and are prone to wasting and ketoacidosis (98). Streptozotocin induced type 1 diabetes mice have reduced proliferation and survival of neurons in the hippocampus. These changes can be prevented by the administration of estrogen or fluoxetine (99, 100).

Type 2 diabetes has reached epidemic levels of prevalence in the western world. This is due to the sedentary lifestyle and high consumption of high caloric density foods containing refined carbohydrates. Key features are insulin resistance, obesity and lack of exercise (98). Knockout mice lacking leptin receptors have deficits in Long-term-potentiation (LTP) in the dentate gyrus and show impaired learning in the MWM, decreased hippocampal cell proliferation and survival due to increases in glucocorticoid levels (101). As mentioned in the section about stress IL-1β signaling is essential for the detrimental effects of glucocorticoids on adult neurogenesis.

The changes in type1 and type 2 diabetes are associated with increased levels of circulating corticosterone and maintaining physiological levels of corticosterone prevents these changes (101). Indomethacin blocks decreases in neurogenesis in the streptozotocin model (97).

It is possible that the sustained inflammatory and stress response in both types of diabetes activates the microglia, reducing neurogenesis and thereby causes the neuropsychological symptoms that have been observed in diabetes patients.

1.1.6.6. Anxiety

As mentioned above many studies on adult neurogenesis found increased anxiety-like behavior as a result of impaired neurogenesis in animals and many inflammatory diseases in humans have been associated with increased risk of anxiety. A study found that higher levels of anxiety were correlated with higher levels of IL-6, which can negatively affect adult neurogenesis (102). While there is no research directly linking decreased adult neurogenesis in humans to anxiety, patients suffering from post-traumatic stress disorder (PTSD) and social anxiety have reduced hippocampal volume compared to healthy controls and are more likely to develop dementia (103, 104,105). This could be related to problems in pattern separation and the tendency to misinterpret neutral stimuli as threatening in anxiety disorders (106). Interestingly anxiety has also been linked to diabetes (107).

1.1.6.7. Depression

Anxiety and depression are often said to go hand in hand and many of the findings we presented about anxiety are equally found in depression. Depression-like behavior was found in animal studies where neurogenesis was impaired and most treatments for depression act by increasing neurogenesis (108). Fluoxetine, one of the most commonly prescribed antidepressant drugs, at least partially depends on intact neurogenesis for its effects (109). IL-1β, IL-6 and TNFα are reliably elevated in depressed subjects and dysregulation of the HPA axis is one of the most reliable predictors of depression (110, 111). A longitudinal study measuring mRNA for NF-κB, its inhibitor I-κB, IL-6 and c reactive protein (CRP) in adolescent women at risk of developing depression found that upregulated inflammatory gene expression was positively correlated to noxious social events such as targeted rejection, for example being fired from a job or being bullied (112). As mentioned above, all of these are capable to negatively affect neurogenesis, representing possible contributors to the development of depression.

1.1.7 Possible Influences on adult neurogenesis

The notion that adult neurogenesis is impaired in many diseases and potentially causative of many of their symptoms and complications gives hope to treat these conditions with therapies targeting adult neurogenesis. In the following section we will elaborate some of these potential therapies.

1.1.7.1. Environmental enrichment

Environmental enrichment (EE) describes a positive change to the standard housing of laboratory animals. This means larger cages, more social contact, more toys and more opportunity for exploratory activity (7). Long-term EE has been found to increase survival of new born neurons up to fivefold (113). These animals demonstrated improved learning, locomotion and brain aging. Further EE has been shown to reverse consequences of prenatal stress in rats on play behavior and HPA dysregulation (114). Rats living in EE used social play as a coping strategy in novel situations while controls did not. This might have important implications for survival in social groups and may explain the importance of an active lifestyle for long term survival and health.

1.1.7.2. Exercise

Exercise has long been known to be beneficial to physical and mental health. There is strong evidence that physical activity can help with symptoms of depression, substance abuse disorders and anxiety (115). Further, it may help with self-image, cognitive function and social skills and improves quality of life (115, 116). Regular exercise not only causes the release of growth factors like BDNF, that can stimulate neurogenesis, and anti-inflammatory molecules, it also reduces body fat and decreases the risk of diabetes both of which contribute to chronic inflammation (117). Regular exercise in mice led to increased neurogenesis in the dentate gyrus, improved learning and long-term potentiation (118). It is likely that some of the benefits of exercise are mediated via its effects on adult neurogenesis.

1.1.7.3. Pharmacologic agents

Recently, there have been first studies into pharmacological approaches to improve adult neurogenesis. Antidepressant effects of Fluoxetine depend, at least in part, on functional neurogenesis and mice who had neurogenesis ablated by brain irradiation did not respond to the tricyclic antidepressants imipramine and desipramine (119).

Psychedelic substances such as psilocybin produced large and long-lasting antidepressant effects in clinical trials and, in contrast to conventional antidepressants work, without blunting emotional responses of the amygdala (120, 121). Another study reported that mice, receiving low doses of psilocybin after a fear conditioning task, showed increased extinction of that fear, implicating possible uses for anxiety disorders such as post-traumatic stress disorder (PTSD), related these to its effects on neurogenesis (122). A single dose of the natural psychedelic drug dimethyltryptamine (DMT) increased neurogenesis in the dentate gyrus of mice (123). It promoted granule cell differentiation, dendritic complexity and changed the electrophysiologic properties of these cells. The study concludes that these changes may be responsible for the known antidepressant properties of psychedelic drugs. Another study suggests that 5-HT2A receptor agonism of psychedelic drugs has potent anti-inflammatory effects even at doses too small to produce behavioral effects (124).

Another drug affecting neurogenesis is cannabidiol (CBD) the non-psychoactive brother of tetrahydrocannabinol (THC) of the cannabis sativa plant. CBD decreases anxiety in patients with social anxiety disorder (125). CB1 receptor agonism stimulates neurogenesis without affecting cell fate and has antidepressant and anxiolytic properties in rats, which depend on functional neurogenesis (126). CBD also demonstrated antipsychotic properties in a clinical trial on acute schizophrenia, an effect which might be related to increase of the endogenous cannabinoid receptor antagonist anandamide (127).

So far clinical trials on pharmacologic agents affecting neurogenesis are rare and further studies will have to improve our knowledge on the effects and safety of these agents. Nevertheless, these agents are showing promising results for a variety of severe disorders such a schizophrenia and treatment resistant depression, related to the impaired neurogenesis.

1.2 DSTYK

Dual serine/threonine and tyrosine kinase (DSTYK) also known as dusty protein kinase and formerly known as receptor interacting protein 5 or rip kinase 5 (RIP5) is a dual serine-threonine and tyrosine protein kinase involved in the regulation of caspase dependent and caspase independent cell death (128). It was found in all vertebrates, in early metazoans and echinoderm but is absent in unicellular organisms (129). Its C-terminal kinase domain is mostly homologous with kinase domains of RIP family members, while its N-terminus has no detectable homology with known proteins (128).

Research of this gene is relatively novel but its wide expression in human tissues and role in embryogenesis make it an interesting candidate for a wide array of research fields including nephrology and neurology. So far not much is known about its contribution to the function and development of life, but it has already been connected to in congenital abnormalities of kidneys and urinary tract (CAKUT) syndromes and spastic paraparesis in humans and possibly to learning and memory in mice (130-131).

1.2.1 Tissue expression (RNA)

One study found that DSTYK is expressed at variable levels in brain, heart, kidney, lung, muscle and testis and certain cancer cell lines such as erytholeukemic K562 and colon SW480 cancer cell lines, but it is also reduced in some tumors of breast, ovary, lung and pancreas (129). Another study reported that RIP5 mRNA was weakly expressed in several tissues, including heart, brain, placenta, skeletal muscle, kidney, pancreas, and testis, but was undetectable in lung, liver, spleen, thymus, prostate, ovary, small intestine, colon, and leukocytes (128). It is expressed in mesenchymal-derived cells of all major organs (130). In the mouse embryo fluorescent in situ hybridization analysis (FISH) showed expression in lung, muscle, skin, whisker, gut, heart, kidney, ovary and testis (129). DSTYK was also highly expressed in brain tissues including cerebellum, olfactory, hippocampus and cerebral cortex (129). In the developing mouse kidney, DSTYK is expressed at low levels in the nephrogenic zone but is more highly expressed in maturing tubular epithelia, with the most prominent expression in the medulla and the papilla. In postnatal mouse and human pediatric kidneys, DSTYK is detected in the basolateral and apical membranes of all tubular epithelia (130). It was also detected in all layers of transitional ureteric epithelium and in the ureteric smooth-muscle cells (130).

At protein level dusty expression has been demonstrated in mouse brain, heart, skeletal muscle, kidney and lung (132).

1.2.2 Role in the pathogenesis of congenital abnormalities of kidneys and urinary tract

DSTYK has also been studied for its role CAKUT syndromes. So far, an autosomal dominant splice-site mutation, resulting in aberrant splicing of mRNA and other mutations in DSTYK have been found to cause CAKUT.

In a knockout zebrafish model defects in multiple organs (growth retardation, as evidenced by small fins, abnormal morphogenesis of the tail, and loss of heartbeat with cloacal malformations that correspond to lower genitourinary defects in mammals and defects in jaw development, as well as specific loss of the median fin fold) were found, supposedly due to fibroblast growth factor (FGF) signaling loss and inhibition of FGF stimulated phosphorylation of extracellular-signal-regulated kinase (ERK) (130). Joint immunostaining confirmed that DSTYK colocalizes with both fibroblast growth factor receptor (FGFR) 1 and 2 in the ureteric bud and comma-shaped bodies and with FGFR2 in distal tubular cells in the adult renal medulla and papilla (130). siRNA knockdown of DSTYK in the human embryonic kidney-cell line 293T, which resulted in a reduction of up to 80% in transcript levels and a pronounced reduction of DSTYK protein levels within the first 96 hours after transfection prevented ERK phosphorylation stimulated by FGF (130).

Research identified a nonsense mutation in a patient with ureteropelvic junction obstruction and early-onset ataxia, and a splice-site mutation in two siblings affected by ureteropelvic junction obstruction. Sequence analysis in an independent cohort of 311 patients with congenital abnormalities of the kidney and urinary tract identified five DSTYK mutations in 7 patients (2.3% of this cohort) (130).

1.2.3 Role in Spastic paraparesis SPG23

Spastic paraparesis type SPG23 is a progressive childhood onset hereditary disorder of spastic paralysis of the lower limbs, associated with peripheral neuropathy, vitiligo-like skin depigmentation and premature graying of hair. Only 6 cases (2 Arab Israeli, 2 Jordanian, 1 North European and 1 Indian family) have been described. In one of those Jordanian and in the 2 Israeli cases analysis revealed a deletion in DSTYK removing exons 12 and 3 (131). In affected skin of these individuals a focal loss of melanocytes with swollen mitochondria and cytoplasmic vacuoles in remaining melanocytes was found. These changes were also found in fibroblasts and keratinocytes, indicating increased susceptibility to stress and cell death (131). siRNA knockout mice fibroblasts and cells from affected individuals exhibited increased cell death and apoptotic markers after experimental UV exposure. These cells also showed decreased phosphorylated ERK activity in response to FGF stimulation (131).

1.2.4 Memory and learning in DSTYK knockout mice

In a knockout mice model ablating the C-terminal from exon 4 to 12 of DSTYK including the kinase domain, impairment of learning and memory has been demonstrated using the Morris water maze test. The knockouts were fertile and showed no significant morphological defects, balance or motor deficits when compared to wildtype mice (132). The DSTYK mRNA level of DSTYK+/- mouse were reduced to about 60% of DSTYK+/+ mouse and no DSTYK mRNA was detected in DSTYK-/- mice. Western blot showed lack of the 119kDa protein band in brain tissue confirming successful knockout (132).

At the beginning of MWM training there was no significant difference between the groups, but in trial 3-4 of visible platform water maze test, DSTYK-/- mice needed significantly more time to swim to the target platform than DSTYK+/- mice, there was no significant difference in the average swimming speed. In the probe trial of hidden platform water maze test, the latency to target the platform of DSTYK-/- mice was significantly higher than that of DSTYK+/- mice, other parameters such as number of target platform crossing, time spent in target quadrant and relative total distance moved showed no significant differences. The performance of the knockout mice indicates impairment in spatial memory and learning (123).

2. OBJECTIVES

AIMS:

1. Determine the influence of aging on the rate of adult neurogenesis in subventricular zone of rats.
2. Determine the influence of *diabetes mellitus* on the adult neurogenesis in subventricular zone of rats over a long-term period.
3. Determine the possible role of DSTYK protein in the adult neurogenesis in subventricular zone of rats.

HYPOTHESIS:

1. Ageing is causing decline in number of doublecortin immunoreactive neuroblasts within the the subventricular zone.
2. Long-term diabetes is causing decline in number of doublecortin immunoreactive neuroblasts within the the subventricular zone.
3. DSTYK protein is expressed in neural progenitors in adult neurogenesis-active areas of rat brain and could have role in adult neurogenesis.

3. METHODS

3.1 Ethics

The Ethics Committee of the University of Split School of Medicine approved the experimental design according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

3.2 Experimental animals

For the experiment 35 male Sprague-Dawley rats, weighting from 160-200 g were obtained from the University of Split Animal Facility. The rats were all raised under the same controlled conditions. The temperature was set to 22 ± 1°C and the light was scheduled to 12 hours light and 12 hours darkness.

3.3 Groups

The rats were randomly divided into experimental animals and controls. Each of these groups was further subdivided into groups to be sacrificed 2 weeks, 6 months and 1 year after induction of the DM (Figure 1).

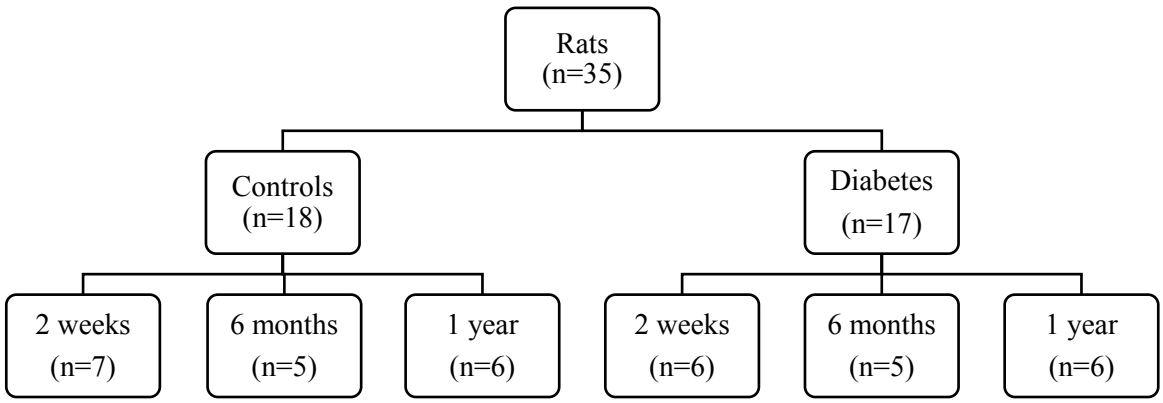


Figure 1. Division of Animals into groups.

3.4 Induction of diabetes mellitus

Type 1 *diabetes mellitus* was induced with streptozotocin (STZ) at a dose of 55 mg/kg. The medication was given as intraperitoneal injection in a citrate buffer (pH 4.5) after overnight fasting. The rats were fed *ad libitum* with laboratory chow containing 27% proteins, 9% fat and 64% carbohydrates (4RF21 GLP, Mucedola, Settimo Milanese, Italy). The control animals were injected with citrate buffer only. In order to prevent ketoacidosis 1 U of long-acting insulin (Lantus Solostar; Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany) was injected once a week to diabetic rats.

3.5 Validation of diabetes mellitus

We confirmed the development of diabetes in the rats by measuring of plasma glucose. Our criteria for diabetes were plasma glucose levels above 16.5mmol/L on day 4 after streptozotocin injection. Blood samples were taken from the tail vein and glucose level was measured using One Touch Vita instruments (LifeScan, High Wycome, UK). Weight measurement was conducted using a scale.

3.6 Tissue collection and Immunohistochemistry

Anesthesia was induced with isoflurane (Forane, Abott Laboratories, Queenborough, UK). The animals were sacrificed by perfusion through the ascending aorta via the left ventricle. Tissues were harvested, dehydrated in ethanol and embedded into paraffin blocks. Then slides were cut into sections of 5 μm using a microtome (LEICA RM 2155, Leica Microsystem Nussloch GmbH, Nussloch, Germany). Sections corresponding to 2 mm anterior from bregma (bregma +2mm) were chosen. According to the Paxinos and Watson stereotaxic atlas (133) the tissues were deparaffinized and rehydrated using alcohol and water, briefly rinsed in PBS and microwaved in a citrate buffer (pH 6.0) for 12 minutes at 95°C.

After cooling the slides were incubated overnight with mouse monoclonal Anti-Doublecortin Antibodies (Doublecortin (E-5): sc-390645, diluted at 1:50) and/or goat polyclonal anti-DSTYK (RIP5 (N-16): sc-162109, diluted at 1:100) (both Santa Cruz Inc., Santa Cruz, CA, USA), at room temperature in a humidified chamber. After washing in PBS, sections were incubated in secondary antibodies (Donkey Anti-Mouse IgG H&L, Alexa Fluor® 488, Donkey Anti-Goat IgG H&L, Alexa Fluor® 488, ab150129; both Abcam plc. 330 Cambridge, CB4 OFL, UK; and/or donkey Anti-Mouse IgG-Rhodamine, 715-295-151, Jackson Immuno Research Laboratories, Inc., Baltimore, PA, USA) for 1,5 hours. All the secondary antibodies were diluted at 1:400. After washing in PBS nuclei were stained with DAPI. Slides were air-dried and cover slipped (Immu-Mount, Shandon, Pittsburgh, PA, USA).

The finished slides were digitalized using a Microscope (BX61, Olympus, Tokyo, Japan) with cooled digital camera (DP71, Olympus, Tokyo, Japan) at 40x magnification using the Cell A Imaging Software for Life Sciences Microscopy (Olympus, Tokyo, Japan).

3.7 Image processing and analysis

In order to analyze the microscopic images and create data images of the same microscopy fields using different light filters had to be combined into one fusion image. We used ImageJ (National Institutes of Health, Bethesda, MD, USA) to isolate the green fluorescence of the antibodies in the image by deleting the red color channel via the split color- and Image calculator subtract function. In the next step a threshold was applied to the green signal and we pseudo-colored it into red. Analysis of threshold percentage area (for both, thick and thin parts of the SVZ) was made by using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Initially, area of interest was isolated by manually outlining and erasing the surrounding tissue area from the picture, after that selection by color function was used to select area of red and black, in pixels. For thick zone total area of interest was calculated by summing the area of black and red and percentage of red area in total area of interest was calculated.

For the thin area, figures of nuclear DAPI staining a threshold was applied by using ImageJ, and area of nuclei (in pixels) was measured on afterwards by using selection by color function in the Adobe Photoshop. Ratio of red area (doublecortin) per area of nuclei was calculated. Ratio per nuclei was selected as appropriate for thin part of the SVZ because this part contains clearly distinguishable cells, they do not form compacted groups but are rather dispersed. This part also contains many cells that were not doublecortin immunoreactive. In opposite, thick part of the SVZ contains a mass of densely packed cells that are not clearly distinguishable from each other and are mostly doublecortin immunoreactive. That was the reason why we used percentage area as the arbitrary unit for doublecortin immunoreactivity in this part.

For possible co-localization of DSTYK and doublecortin we inspected sections of the brain from all of the instigated animals, by using Olympus BX61 Microscope, at 40x magnification. Since no sign of co-localization was observed in any of the sections, only representative images were captured.

Figures were prepared for presentation by using GNU Image Manipulation Program GIMP 2.8.22 (GIMP Development Team, https://www.gimp.org). The contrast of the image was adjusted, and the background of the image deleted by converting the color black into alpha channel. In similar fashion we improved the contrast and removed the background of the blue DAPI image of the nuclei. The last step was to fuse these layers with the original green image, which in some cases was tinted yellow using the colorize command or the antibody layer was duplicated to improve visibility of the antibodies.

For preparation of DSTYK figures Adobe Photoshop was used. Nuclei were pseudo-colored into magenta, in order to improve the contrast to the green DSTYK immunoreactivity and magenta and green figures were then merged. For co-localization of DSTYK with doublecortin, red (doublecortin), green (DSTYK) and blue (nuclei) figures were merged, after being slightly contrasted.

3.8 Statistical Analysis

We used Shapiro-Wilk testing for normality distribution. Normal Distribution was confirmed for all tested parameters. All data were expressed as mean with 95% Confidence Interval).

Differences were tested with Mann-Whitney test and P-values <0.05 were considered statistically significant. We used MedCalc Statistical Software version 18.5 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018) for all statistical analysis).

For intergroup comparisons Kruskal-Wallis tests were performed in GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

4. RESULTS

4.1 Changes in expression of doublecortin in the thick part of the SVZ

Regarding the thick part of the SVZ we measured the percentage of Doublecortin (DCX) staining surface compared to the total surface visible (Figure 3). There was no significant difference between the control group and the diabetic group at 2 weeks, 6 months or 1 year (Figure 2). However, there was a statistically significant difference in the intragroup comparison of the control group when comparing the subsets of 2 weeks versus 6 months ($P<0.01$). There were no statistically significant differences in the other intragroup comparisons (Figure 2).

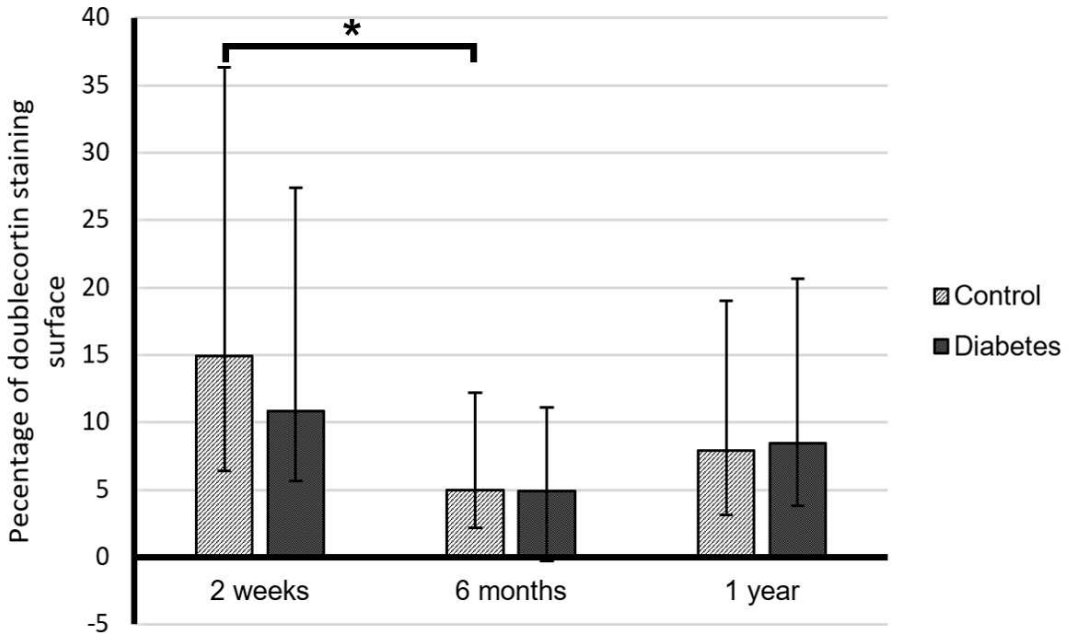


Figure 2. Changes in adult neurogenesis in the thick part of the SVZ of diabetic rats. Data are expressed as mean with 95% confidence interval.
*intragroup comparison ($P<0.01$)

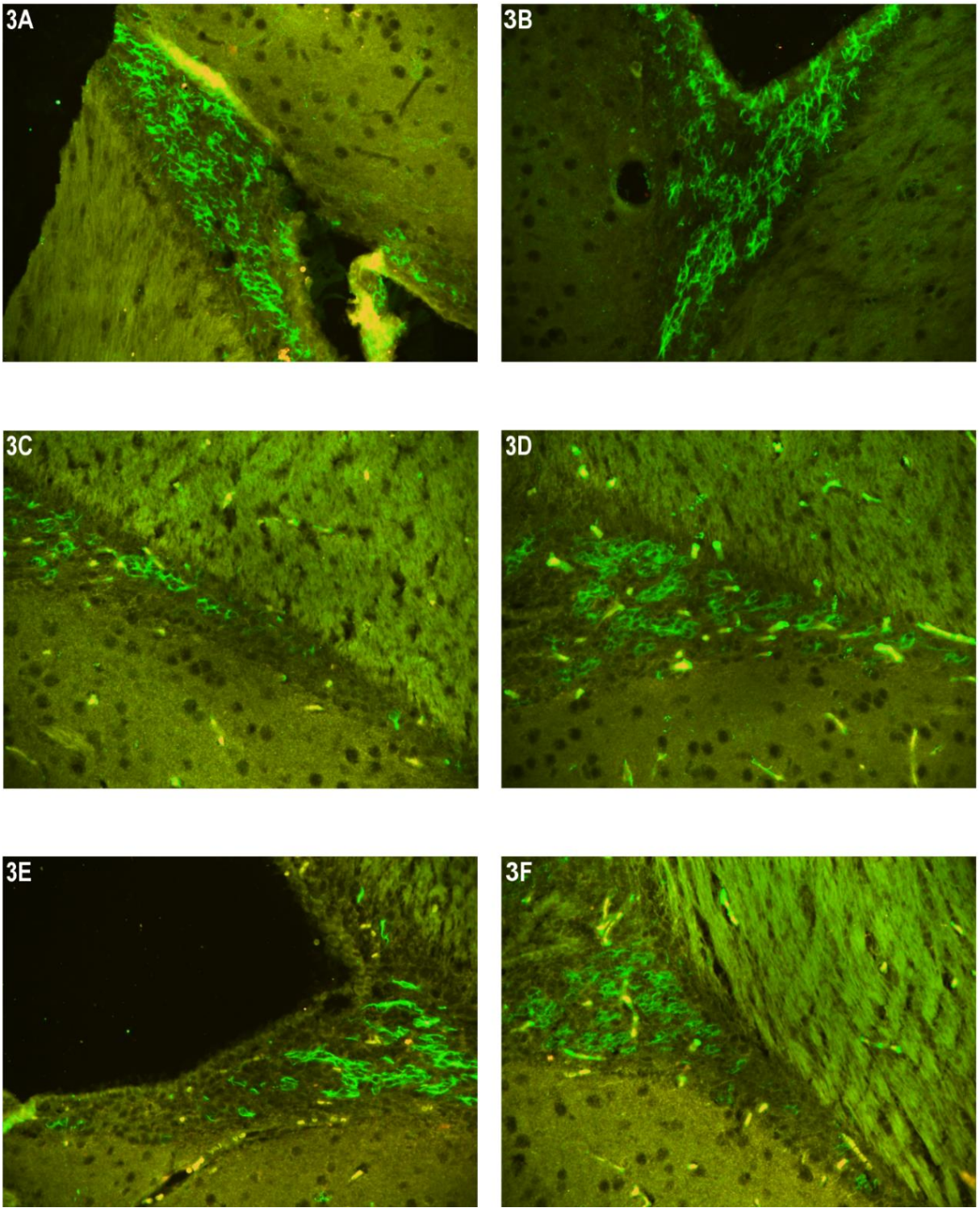


Figure 3. Doublecortin immunoreactive neuroblasts in the subventricular zone of rats. Representative images of doublecortin staining (green) at 2 weeks in controls (3A) and diabetic rats (3B), 6 months in controls (3C) and diabetic rats (3D) and at 1 year in controls (3E) and diabetic rats (3F) respectively. Samples were visualized with fluorescent microscopy using 40x magnification.

4.2 Changes in neurogenesis in the thin part of the SVZ

For the thin part of the subventricular zone we measured the ratios of doublecortin (DCX) staining surface to total neuronal nuclear surface (Figure 5). At two weeks and at 6 months the differences between the controls and the diabetic rats were not statistically significant. At 1 year there was a significant difference (P = 0.025) between the controls and diseased animals (Figure 4). There were statistically significant differences (P=0.045) comparing neurogenesis at 6 months and 1 year within the within the control group, but there were no statistically significant differences in the other intragroup comparisons.

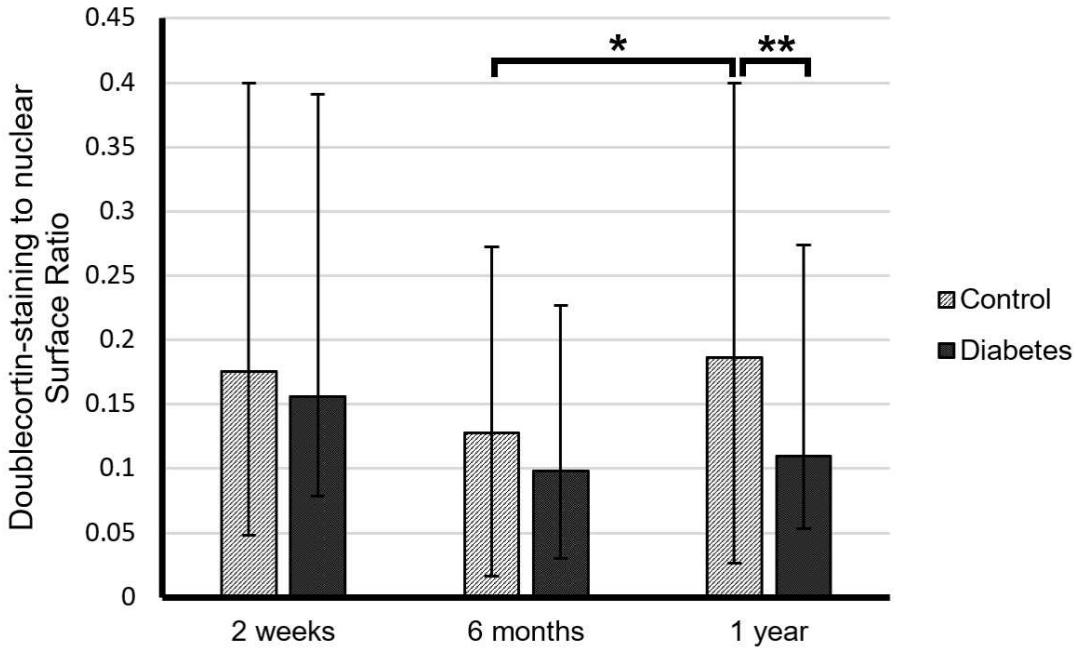


Figure 4. Changes in adult neurogenesis in the thick part of the SVZ of controls and diabetic rats. Data are expressed as mean with 95% confidence interval.

*intragroup comparison (P=0.045)

**comparison at 1 year (P=0.025)

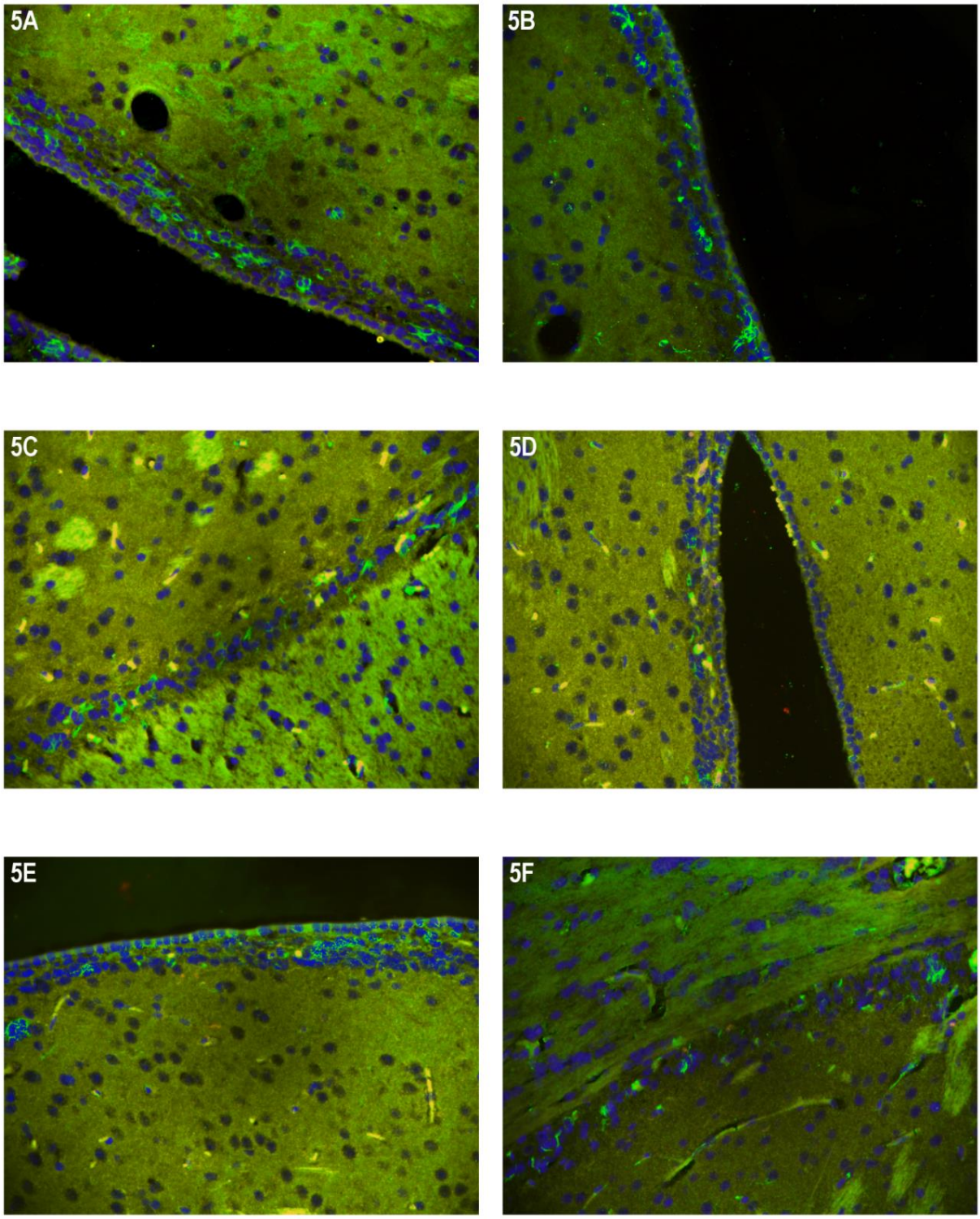


Figure 5. Doublecortin immunoreactive neuroblasts in the subventricular zone of rats. Staining of nuclei (blue) and doublecortin (green) in controls and diabetic rats at 2 weeks (5A, 5B), 6 months (5C, 5D) and 1 year (5E, 5F) respectively. Samples were visualized with fluorescent microscopy using 40x magnification.

4.3 DSTYK protein expression in the rat brain

DSTYK immunoreactivity was observed in different parts of the rat brain (Figure 6). In most of the locations, DSTYK immunoreactivity was restricted to a wall of the brain blood vessels (Figure 6A and B). Besides that, DSTYK expression was also found in cells of the pia mater (Figure 6C), as well as in glia of the white matter in different parts of the brain and spinal cord (Figure 6 D-G). In addition, we found expression of DSTYK on a regular base in ependymal cells of the central canal of the spinal cord (Figure 6G) and lateral ventricles of the brain (Figure 7).

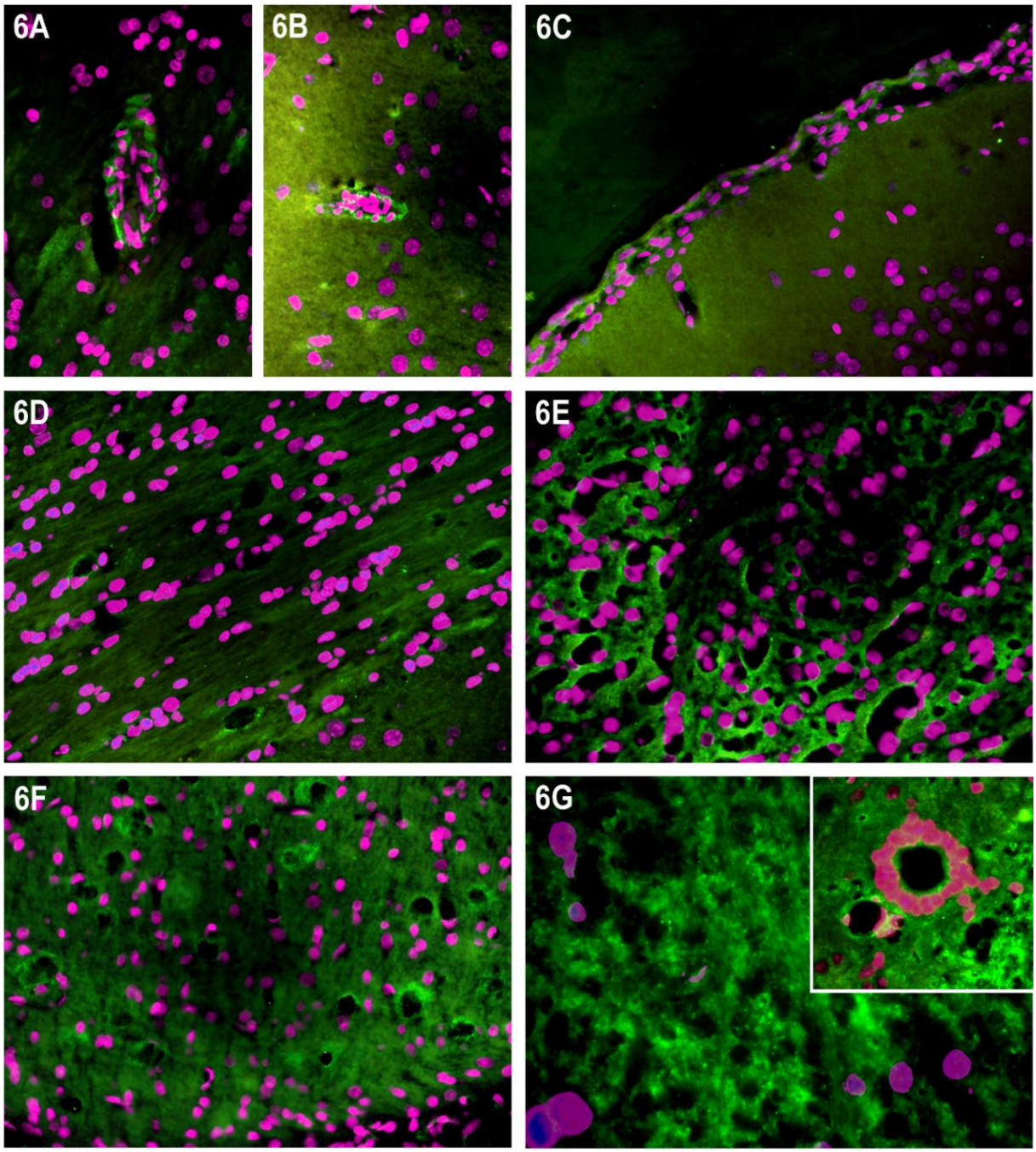


Figure 6. DSTYK immunoreactivity in rat central nervous system.

Staining of nuclei (magenta) and DSTYK (green) using 40x magnification in rat brain (6A-F) and 100x magnification in spinal cord (6G). Staining in blood vessels of the brain (6A and B). DSTYK immunoreactivity in pia mater (6C). DSTYK immunoreactivity in white matter of corpus callosum (6D), anterior commissure (6E), olfactory tubercle (6F) and white matter of the spinal cord (6G). DSTYK was also present in ependymal cells of the central canal of the spinal cord, as well as the blood vessels in the spinal cord (inset in 6G).

4.4 DSTYK expression in the neurogenic niche of the lateral ventricle in the rat brain

DSTYK immunoreactivity found in the lateral ventricle wall was restricted on one layer of ependymal cells lining the wall (Figure 7). We did not find any signs of co-localization of the DSTYK with DCX, a marker for the neuroblasts in thin (Figure 7A and B) or thick (Figure 7C and D) of the subventricular zone, in any of studied groups of animals.

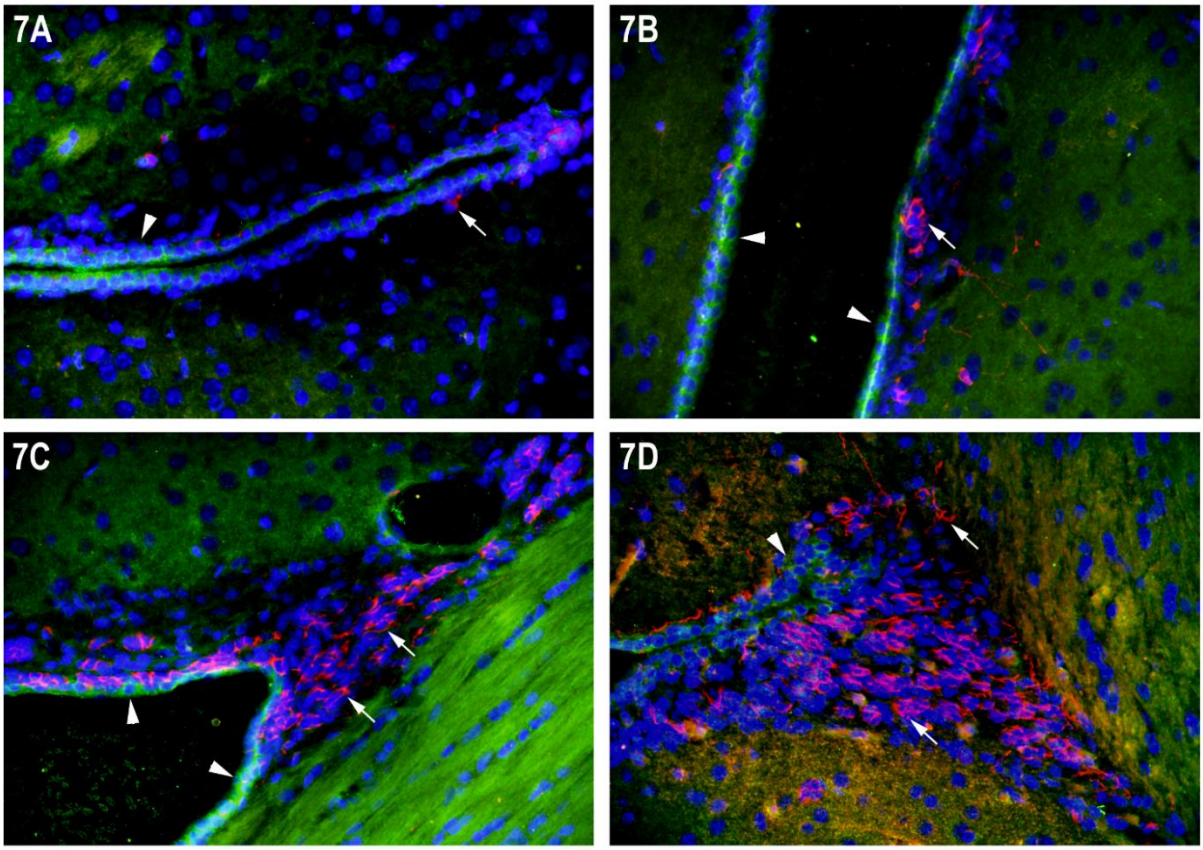


Figure 7. DSTYK immunoreactivity in the neurogenic area of the lateral ventricle in rat. Staining of nuclei (blue), DSTYK (green; arrowheads) and doublecortin (DCX, red; arrow), a marker for neuroblasts in the rat brain. (7A and B) thin area of the subventricular zone (SVZ); (7C and D) - thick part of the SVZ. Samples were visualized with fluorescent microscopy using 40x magnification. DSTYK was immunoreactive in the layer of ependymal cells of the lateral ventricle, while DCX-antibody stained neuroblasts of the SVZ. No co-localization of DSTYK with DCX was found in brain.

5. DISCUSSION

In this study, we used long-term rat model of DM1, induced by streptozotocin injection, in order to explore whether the DM1 is causing damage of adult neurogenesis in SVZ of the rat brain. We were using an immunohistochemistry for DCX, a marker specific for the neuroblasts, cells that are already destined to a neuronal fate (17). We measured expression of the DCX in two parts of the SVZ, a "thin" layer of cells in the subependymal layer, and the "thick" part of the subependymal layer of ventricular angle, the location of intense neuroblast collection in the cells are not in contact with the ependymal layer. For the thin part of the subventricular zone we measured the ratios of DCX staining surface to total neuronal nuclear surface. We found a significant decrease in DCX expression in this part 1 year after DM1 induction. On the other hand, there were no significant differences between the controls and diabetic animals 2 weeks or 6 months after the DM1 induction. In thick part we did not find significant changes in DCX expression between control and diabetic animals in any of the investigated periods. These data support our hypothesis that DM1 is causing decrease in adult neurogenesis in SVZ.

However, only a 1 year-long DM1 model was long enough to provoke visible changes in DCX expression. The meaning of this result for the future studies is that usually used 2 week and 2 months short term DM1 models are not appropriate to study the influence of DM1 on adult neurogenesis, at least in SVZ.

In support to our conclusions, it was found that chronic, but not the acute hyperglycemia decreased the cell proliferation in all parts of the brain in zebrafish (134). Nevertheless, in most of the short-term DM1 models, an increase in proliferation in hippocampus and/or SVZ was observed, measured mostly by BrdU incorporation (135-137). However, it is not necessary that all of these cells develop to neuroblasts/neurons. Tirassa et al. found increase in Ki67 proliferation marker but decrease in BrdU incorporation and number of doublecortin immunoreactive cells in SVZ of rats 66 days after induction of DM1 (138). In support of our findings, Lang *et al* observed that 6 weeks old Goto Kazaki rats (a model for DM2) did not have any difference in number or survival of neuronal progenitors in the SVZ or hippocampus (139). However, at 16 weeks of age, while proliferation of neuronal progenitor cells increased, their survival actually decreased.

There are quite clear differences in the influence on the adult neurogenesis between DM1 and DM2. Bachor *et al* found that DM2 caused marked drop in markers of neurogenesis, including doublecortin, in SVZ of the mice already 7 days after induction, while DM1 did not cause any change at the same time (140). Most of the animal studies agree that DM2 causes decrease in proliferation, as well as the number of doublecortin immunoreactive neuroblasts in hippocampus, while data for the SVZ are scarce (141-143).

Concerning the influence of age on the neurogenesis in the thick part of SVZ in control animals, a significant decrease in DCX expression was found 6 months after the beginning of the experiment (in age of 8 months). This is in agreement with what we expected, as well as data from the literature that observed decrease in number of doublecortin immunoreactive cells in dorsal and ventral hippocampus of ageing mice (144). On the other hand, in the thin part, DCX expression was slightly but insignificantly decreased at 6 months, but then surprisingly increased 12 months after the begging of the experiment (in age of 14 months). These data only partially support hypothesis that age negatively affects neurogenesis in the SVZ, confirming it only for the thick part of the subependymal layer of the SVZ. However, in both parts we found an increase in DCX expression, that points to a biphasic pattern of neurogenesis in SVZ of rat, that increases at the age of 14 months, after initial transient decrease (in age of 8 months).

In addition, we also explored a hypothesis about the influence of the novel molecule DSTYK on neurogenesis in SVZ. Namely it was found that DSTYK knock-out diminishes cognitive abilities in mice (132), which could also point to its possible role in neurogenesis in hippocampus. We wanted to see if it has any connection with the neurogenesis in SVZ. We managed to see that DSTYK is strongly expressed in ependymal cells of the SVZ and the central canal of the spinal cord, that are known as progenitors capable of adult neurogenesis. However, we did not find any signs of co-localization of the DSTYK with DCX in any of studied groups of animals. These findings point to the probable role of DSTYK in initial steps of the adult neurogenesis. On the other hand, a lack of co-localization with DCX indicates that DSTYK is probably not needed in later steps of neuronal differentiation during the adult neurogenesis, once when they are already destined to the neuronal fate.

Regarding to the expression of the DSTYK in rat brain, in general DSTYK immunoreactivity was mostly restricted to a wall of the brain blood vessels. It was also found in cells of the *pia mater*, as well as in glia of the white matter in different parts of the brain and spinal cord. Taking into consideration the lack of data about the DSTYK expression in general, in the brain in particular, as well as fact that most of the studies were used Western blot of PCR techniques, that do not give any information on cellular distribution of DSTYK in particular tissues, we believe that our findings will help to enlighten the possible function of this novel molecule in central nervous system.

6. CONCLUSION

1. Long term experimental DM1 is decreasing neurogenesis in thin part of the SVZ in rat brain.

2. Short term experimental DM1 is not influencing neurogenesis in the SVZ of the rat brain. Hence, short term studies are not relevant in conclusions for the influence of DM1 on neurogenesis in SVZ of rat.

3. Ageing has an influence on neurogenesis in thick part of the SVZ of the rat brain and the pattern of changes is bi-phasic, with initial transient decrease and increase at the age of 14 months.

4. DSTYK is expressed in rat brain in neurogenic areas of the SVZ, hence could have role in initial steps of the adult neurogenesis.

5. DSTYK in SVZ is not expressed in neuroblasts, hence it probably does not have role in their differentiation.

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8. SUMMARY

Objectives: Diabetes mellitus has been associated with increased risk of cognitive decline, dementia, depression and anxiety. The aims of this study were to determine the influence of aging and long-term type 1 diabetes mellitus (DM1) on the rate of adult neurogenesis in subventricular zone of rats, as well as to determine the possible role of DSTYK protein in the adult neurogenesis in subventricular zone of rats as a possible disease mechanism.

Material and Methods: Thirty-five male Sprague-Dawley rats randomly divided into two groups: control or diabetic. DM1 was induced by intraperitoneal injection of streptozotocin (55 mg/kg). DM1 was confirmed by measuring of plasma glucose 4 days after induction. Rats were sacrificed 2 weeks, 6 months and 1 year after induction of the DM1. Tissues of brain were processed for the immunohistochemistry for doublecortin (DCX) alone, or in combination with DSTYK.

Results: For the thick part of the subependymal layer of the SVZ, the percentage of DCX area in the sections from a control group significantly decreased 6 months in comparison to the 2 weeks after the beginning of the experiment ($P<0.01$). For the thin part of the SVZ we measured the ratios of DCX staining surface to total nuclear surface. At two weeks and at 6 months the differences between the controls and the diabetic rats were not statistically significant. At 1 year there was a significant decrease ($P = 0.025$) between the controls and diseased animals. We also observed significant increase ($P=0.045$) comparing neurogenesis at 6 months and 1 year within the within the control group.

DSTYK immunoreactivity was restricted to a wall of the brain blood vessels, the cells of the pia mater, as well as in glia of the white matter in different parts of the brain and spinal cord. In addition, we found expression of DSTYK on a regular base in ependymal cells of the central canal of the spinal cord and lateral ventricles of the brain. We did not find any signs of co-localization of the DSTYK with DCX, a marker for the neuroblasts in any of studied groups of animals.

Conclusion: We concluded that long term experimental DM1 is decreasing neurogenesis in thin part of the SVZ in rat brain, while the short term DM1 did not have influence. Hence, short term studies are not relevant in conclusions for the influence of DM1 on neurogenesis in SVZ of rat. Ageing has an influence on neurogenesis in thick part of the SVZ of the rat brain and pattern of changes is bi-phasic, with initial transient decrease and increase in age of 14 months. DSTYK is expressed in rat brain in neurogenic areas of the SVZ, hence could have role in initial steps of the adult neurogenesis. Since DSTYK is not expressed in neuroblasts, it probably does not have role in their differentiation.

9. CROATIAN SUMMARY

Naslov: UTJECAJ DUGOTRAJNE ŠEĆERNE BOLESTI NA NEUROGENEZU U SUBVENTRIKULARNOJ ZONI MOZGA ODRASLIH ŠTAKORA

Ciljevi: Šećerna bolest povezana je s povećanim rizikom slabljenja kognitivnih sposobnosti, demencija, depresije i anksioznosti. Ciljevi ovog istraživanja bili su odrediti utjecaj starenja i šećerne bolesti tipa 1 (DM1) na neurogenezu u subventrikularnoj zoni (SVZ) mozga štakora, kao i odrediti moguću ulogu DSTYK u adultnoj neurogenezi u subventrikularnoj zoni, kao moguće mehanizme u nastanku ove bolesti.

Materiali i methode: Trideset pet štakora muškog spola soja Sprague-Dawley podijeljeni su nasumično u dvije skupine: kontrolne i dijabetične životinje. DM1 je izazvan intraperitonealnom aplikacijom streptozotocina (55 mg/kg). DM1 potvrđen je mjerenjem glukoze u plazmi 4 dana nakon indukcije. Štakori su žrtvovani 2 tjedna, 6 mjeseci ili 1 godinu nakon indukcije DM1. Tkiva mozga pripremljena su te obojena imunohistokemijski na doublecortin (DCX) samostalno, ili u kombinaciji s DSTYK.

Rezultati: U debljem području subependimskog sloja SVZ, postotak površine pod DCX u rezovima mozga kontrolnih štakora značajno se smanjio 6 mjeseci, u usporedbi s 2 tjedna nakon početka pokusa ($P<0,01$). U tanjem dijelu subependimskog sloja SVZ mjerili smo omjer površine pod DCX prema ukupnoj površini jezgara. Dva tjedna i 6 mjeseci nakon početka pokusa nisu pornađene značajne razlike između kontrolnih i dijabetičnih štakora. Dvanaest mjeseci nakon početka pokusa uočen je značajni izražaja DCX u dijabetičnih, u usporedbi s kontrolnim štakorima ($P = 0,025$). Također je uočen značajni porast izražaja DCX ($P=0,045$) 12 mjeseci nakon početka pokusa u usporedbi s 6 mjeseci, unutar kontrolne skupine životinja.

Imunoreaktivnost na DSTYK bila je ograničena na stjenke krvnih žila mozga, stanice meke moždane ovojnice (pia mater), kao i na glija stanice u bijeloj tvari različitih dijelova mozga i kralježnične moždine. Nadalje, redovito je pronađen izražaj DSTYK u ependimskim stanicama središnjeg kanala kralježnične moždine i lateralnih komora mozga. Nisu pronađeni znakovi ko-lokalizacije DSTYK s DCX, biljegom neuroblasta, niti u jednoj od istraživanih skupina.

Zaključci: Dugotrajni DM1 smanjuje neurogenezu u tanjem dijelu SVZ mozga štakora, dok kratkotrajni DM1 nije imao nikakvog utjecaja. Stoga se zaključuje kako kratkotrajne studije nisu u potpunosti relevantne za zaključke o utjecaju DM1 na neurogenezu u SVZ štakora. Nadalje, starenje utječe na neurogenezu u debljem dijelu SVZ a uzorak promjena izazvanih starenjem je bifazičan, s početnim prolaznim smanjenjem te porastom u dobi od 14 mjeseci. DSTYK je izražen u neurogenim područjima SVZ štakorskog mozga, te bi stoga mogao imati ulogu u početnim stadijima adultne neurogeneze. Budući da nije izražen u neuroblastima, vjerojatno je da DSTYK nema ulogu u njihovoj diferencijaciji u neurone.

10. CIRCUM VITAE

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2016-2018	Membership of NeuroMefst: Psychiatry Subsection, School of Medicine Split
Nov. 2017	Participation at the 1st Croatian Congress on psychological trauma
Oct. 2011	Nursing Internship Bezirkskrankenhaus Niederbayern, Landshut
2011-2012	Volunteering as Trainer for Parkour at ETSV09, Landshut
2007 & 2008	Participation at Bundeswettbewerb für politische Bildung
2005	1st Place at local level at 35th international Youth-Competition “Youth Creative”

Presentations:

Early History of Psychiatry in Europe

Mindfulness and Selflove

Rehabilitation, Well-being and the Mind/ Tools of Emotion Regulation and Well-being

Mindfulness in Therapy and Everyday Life