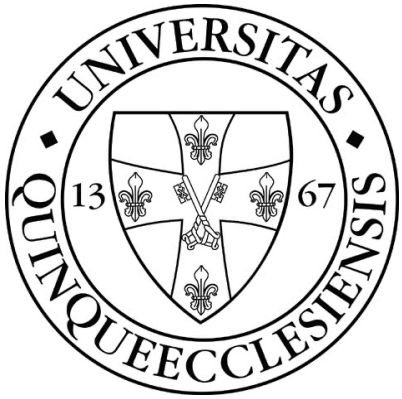
Investigation of adult hippocampal neurogenesis in mouse models of chronic stress and peripheral inflammation

PhD thesis



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1. Introduction

1. Adult hippocampal neurogenesis and its investigation

The development of the central nervous system is a complex dynamic process, precisely controlled in time and space, involving a precisely coordinated series of genetic, environmental, biochemical and physical factors from the early embryonic stage to postnatal life (Greig et al, 2013), At the same time, a small subset of stem cells persist throughout life and provide plasticity for neural function, repair and alteration (Gage and Temple, 2013). Embryonic and early postnatal neural stem cells (NSCs) generate neurons and glial cells and then proliferative and multipotent NSCs transform into fully differentiated neurons or glial cells. In the mammalian brain, neurons are generated from early embryonic development to the early postnatal stage, and only a few neurogenic zones remain active in adulthood (Urbán and Guillemot, 2014). In the adult brain, a very small number of stem cells persist in certain brain structures and contribute to the continued formation of certain neurons (and glial cells). This process is called adult neurogenesis. Neuronal stem cells are the origin of both embryonic and adult neurogenesis (Engler et al., 2018), although some theories suggest that in the adult brain there are no longer true stem cells, only neuronal progenitor cells. One of the landmark events in neuroscience research over the past 25 years has been the establishment of the term neural stem cell as a lifelong source of neurons and glia, a concept that has shattered the dogma that the nervous system lacks regenerative power.

Adult neurogenesis has emerged as one of the most researched areas of neuroscience in recent decades (Yun et al., 2016). Adult neurogenesis in humans is thought to be very rare and inversely proportional to ageing, so that the rate of new neuronal cell formation decreases significantly with age (Isaev et al.,2018). Progenitor cells divide and differentiate to form new neurons that are incorporated into the neuronal network. Only 40-50% of them can integrate, the rest of the neurons die (Biebl et al., 2000). In the adult, mature brain, new neurons are formed in the hippocampal gyrus dentatus and in the subventricular zone of the lateral ventricles. Neonatal neurons from the subgranular zone migrate to the granular cell layer of the gyrus dentatus, while new neurons from the subventricular zone migrate to the olfactory bulb. From a group of neural stem cells, new neurons develop in an unique and specialised microenvironment known as the "neurogenic niche" (Bátiz et al., 2016).

2. Arthritis and its effects

Arthritis is an inflammatory reaction of the joints, either acute or chronic, which is characterised by swelling, redness, warmth, pain and loss of function of the affected joint. Inflammation is caused by inflammation of the synovial membrane that lines the inside of the joint capsule, which causes synovial fluid to build up, causing swelling and pain due to tightness. In a chronic condition, there is usually significant limitation of movement, pain, joint stiffness and deformity (Harth and Nielson, 2019, Tang, 2019).

Rheumatoid arthritis, a systemic chronic inflammatory disease of unclear etiology, manifests as progressive and destructive polyarthritis and is one of the major health problems worldwide due to its high prevalence and ineffective therapeutic options. It is characterised by chronic pain and joint damage, usually progressing from the distal to the more proximal joints (Kourilovitch et al., The annual prevalence of rheumatoid arthritis is approximately 40 per 100 000 people worldwide, with women affected at a rate two to three times higher than men (Ospelt et al, 2017). For patients with arthritis, pain is the most common complaint (Horváth et al, 2016). Consequently, inflammatory joint disorders can severely impair quality of life. The early stage is characterised by oedema and tenderness around the affected joints, which may later be accompanied by progressive, irreversible degeneration and bone remodelling (Botz et at., 2014).

In the pathogenesis of rheumatoid arthritis, as in many other diseases, the interaction of genetic and environmental factors results in a cascade of immune responses. The main histocompatibility complex genes of the HLA have been identified as the genetic factors responsible for disease susceptibility, and other cytokine promoters and T-cell signalling genes are also involved in pathogenesis (Gregersen et al., 1987). The joints of the hand and foot are most affected. In a healthy state, the synovial membrane, a special connective tissue consisting of one or two layers of cells, produces synovial fluid that helps the joints to move and reduces friction on cartilage surfaces. During rheumatoid arthritis, this membrane thickens and inflammation leads to the production of T and B cells, fibroblasts, monocytes and macrophages (Strand et al., 2007). Since autoimmunity plays an important role in the pathogenesis of the disease, the antibodies produced activate the complement system and the production of inflammatory cytokines (TNFα, IL-1, IL-6). These cytokines induce the accumulation of matrix metalloproteases (MMPs) which lead to cartilage loss and free bone resorption, and thus bone erosion (Dolati et al., 2016, Harre et al., 2012).

Complete Freund's adjuvant (CFA) is a paraffin-embedded suspension of antigens containing killed Mycobacterium tuberculosis that attract macrophages and other antibodies to the injection site, thus enhancing the immune response. The administration of CFA induces prolonged nociception, which is associated with glia activation, production of pro-inflammatory mediators and pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in peripheral tissue, and induces thermal and mechanical hyperalgesia (Zucoloto et al, 2019). CFA-induced arthritis is a well-established inflammatory animal model used to test a number of immunomodulatory and anti-inflammatory drugs today. By administering the suspension intraplantar and into the tailbone, destructive monoarthritis can be induced in mice by inflammatory mechanisms similar to rheumatoid arthritis, where inflammatory mechanisms can be studied in both acute and chronic conditions (Billiau al., 2001). In the present study, we used this animal model because we wanted to investigate the effects of peripheral inflammation and, among other things, to demonstrate the existence of inflammation in vivo. Moreover, it is a common disease that may be associated with depression.

3. Cannabis and the endocannabinoid system

Marijuana is one of the most widely used illicit stimulants and is regularly consumed in some form by around 2.5% of the world's population (UNODC, 2017), 2017; EMCDDA, 2017; Webster, 2018) leading to increased consumption, while the long-term health effects are not yet fully understood (Volkow et al., 2014, 2016; Levine et al., 2017). It is therefore important to examine the consequences of long-term use of marijuana smoke. Cannabidiol (CBD), a major constituent of Cannabis sativa and a participant in the endocannabinoid system, is increasingly being considered as a potential therapeutic option for the treatment of anxiety (Blessing et al., 2015; Lee et al., 2017; Patel et al., 2017) as several studies suggest that promoting cannabinoid signalling may prevent stress-induced behavioural changes (Campos et al, 2013; Scarante et al., 2017; Fogaça et al., 2018). The anti-anxiety efficacy of marijuana is still a controversial issue as several research findings suggest that one of the five side effects induced by marijuana is anxiety itself (Turna et al., 2017). The relationship between the improvement or even impairment of cognitive function due to cannabis use is also a controversial topic among researchers. Several clinical and preclinical studies suggest that there may be a strong association between marijuana exposure and reduced cognitive abilities (Broyd et al., 2016; Curran et al., 2016; Volkow et al., 2016). Marijuana use may not only have adverse effects on cognitive function, but may also trigger macrostructural lesions of the brain. It can also alter the morphology of the grey matter, the integrity of the white matter tract. Furthermore, it can result in abnormal brain functions such as increased brain activation or induce abnormal neurovascular functioning (Jacobus et al., 2012). In contrast, some research suggests that cannabis use has positive effects on various cognitive and executive functions (Osborne et al.,

2017; Gruber et al., 2018; Tervo-Clemmens et al., 2017). Furthermore, the effects of regular inhalation of marijuana smoke on the airways and the increased risk of developing lung cancer are debated (Gates et al., 2014; Martinasek et al., 2016; Chatkin et al., 2017; Stone et al., 2018). There are also conflicting research findings on the effects of cannabis use on body weight. It is already known that marijuana consumption stimulates appetite and promotes weight gain in patients with human immunodeficiency virus (HIV) or cancer. In contrast, extensive epidemiological studies in the general population have shown that marijuana users tend to have lower body mass indexes (Sansone, 2014).

The endocannabinoid system plays a central role in the development of a healthy developing nervous system and modulates the activity and function of neural networks in adulthood. The endocannabinoid system is composed of endogenous cannabinoids, i.e. endocannabinoids, cannabinoid receptors, and proteins that transport, synthesize, and degrade endocannabinoids, and the endocannabinoid system influences many other signaling pathways (Lu and Mackie, 2020). Cannabinoids include both endocannabinoids and synthetic cannabinoids, which are active constituents of the Cannabis sativa plant. The neuronal cannabinoid system is a complex series of receptor-ligand and receptor-receptor interactions involving diverse signaling pathways, such as growth factor receptor and G-protein receptor signaling, that regulate a wide range of physiological processes, including adult neurogenesis. Cannabinoids exert their effects through the activation of G-protein coupled type 1 and type 2 cannabinoid receptors (CB1 and CB2 receptors, respectively), which are localized in the central nervous system on astrocytes, microglia and neurons (Prenderville et al., 2015). Cannabinoid receptors are also highly expressed in the hippocampus. Research has shown that various cannabinoid ligands regulate both cell genesis and neurogenesis in the mammalian brain (Campos et al., 2013).

4. Effects of stress on the central nervous system

In today's fast-paced world, it is almost accepted that we are exposed to stress on a daily basis. Our body experiences unpleasant, threatening or dangerous events, whether external or internal, as stress (McEwen et al., 1998). In the short term, stress causes an acute increase in both glucocorticoids and catecholamines, which help to prepare the whole body to cope with the stressful event and help to transform stressful events into memories, thereby providing a protective function (McGaugh et al., 2000, Roozendaal et al., The hippocampus is involved in episodic, declarative, contextual and spatial learning and memory, and also plays a role in the regulation of autonomic and autonomic functions such as ACTH secretion (Eichenbaum et al., 1992, Sapolsky et al., 1992) and therefore, in the long term, stress hormones, and glucocorticoids in particular, contribute to impaired cognitive function and damage to brain structures such as the hippocampus (McEwen et al., 1995, Sapolsky et al., 1992). Among other things, neurons in the hippocampus express adrenal steroid receptors. Two types of adrenal

hormone receptors are distinguished: type I (mineralocorticoid) and type II (glucocorticoid), which are also found in the hippocampus and these receptors mediate various effects on neuronal excitability, neurochemical and structural plasticity changes (DeKloet et al., 1998). Hormone-mediated effects not only affect gene expression, but also the rearrangement of pyramidal cell dendrites in the Ammon's horn (Kerr et al., 1992, McEwen et al., 2000). In acute stress, the brain tries to adapt to changing conditions. In chronic stress, plasticity is compromised and the brain becomes less able to adapt. During prolonged stress, local neurotransmitters and systemic hormones interact to induce structural and functional changes in the brain, including inhibition of hippocampal neurogenesis (McEwen et al., In rats exposed to stress, morphological changes such as dendritic shrinkage of neurons in the dendritic tree of the gyrus dentatus, CA1 and CA3 regions, and a reduction in the number of spines in the dendritic tree, which also leads to a reduction in the number of synapses, so that the conduction of stimuli is no longer as efficient as in the intact state (Joëls et al., 2007). Hippocampal adult neurogenesis is a unique form of neuroplasticity and has received considerable attention over the last twenty years. These newly born neurons in adulthood play a fundamental role in normal cognitive function and learning (Denny et al., 2014; Anacker and Hen, 2017). Stress strongly inhibits the proliferation of precursor cells and also inhibits the survival of newly generated neurons (Gould et al., 1997; Czéh et al., 2001, 2002; Cameron and Schoenfeld, 2018) and from these research findings we can speculate that stress may play a role in the development of various psychiatric disorders such as depression, anxiety or schizophrenia by inhibiting neurogenesis (Santarelli et al., 2003; Snyder et al., 2011; Surget et al., 2011; Kim et al., 2012; Schoenfeld and Cameron, 2015).

2. Objectives

1. effects of acute and chronic peripheral inflammation on adult hippocampal neurogenesis

The main objective of our study was to investigate how peripheral inflammation affects the central nervous system, as experimental results on this issue are highly controversial in the literature. In addition, we aim to explore the effects of acute and chronic arthritis on the central nervous system of mice and adult neurogenesis in the hippocampus. Furthermore, our research aims to prove our hypothesis that peripheral inflammation causes inflammatory cytokines released in the periphery to cross the blood-brain barrier or to be expressed in the brain.

Hypotheses

1. peripheral arthritis negatively affects the central nervous system, inhibiting neurogenesis and inducing a concomitant neuroinflammatory response in the hippocampus.
2. peripheral inflammation will also lead to an increase in cytokines in the central nervous system.

2. Effects of chronic stress and marijuana smoke on behavior and adult hippocampal neurogenesis in experimental mice

The part of the young adult population that regularly uses marijuana is thought to use marijuana to relieve stress, so the main objective of our research was to mimic real-life conditions and therefore experimental mice were stressed daily for several weeks and at the end of the stress exposure the animals inhaled marijuana smoke. We investigated the effect of chronic stress and/or chronic marijuana smoke exposure on adult neurogenesis. In addition, we also examined the effects of marijuana smoke exposure on the animals' vital functions (weight gain) and health status (respiratory function tests) and the effects on the animals' behaviour, partly by cognitive behavioural tests and partly by tests measuring the animals' anxiety levels.

Hypotheses

1. marijuana smoke has a stress-relieving effect. 2. mice inhaling marijuana smoke have impaired respiratory function. 3. Weight gain is reduced in animals exposed to chronic stress, and weight gain is increased in animals exposed to marijuana compared to control mice. 4. Chronic exposure to marijuana smoke stimulates neurogenesis and chronic stress inhibits it. 5. Cognitive function is altered - impaired or enhanced - by exposure to marijuana smoke.

3. Experimental models, test methods

1. Animals used

1.1 Mice used in the arthritis animal model

Laboratory mice (Mus musculus) from 82 young adult (8-12 weeks old) male strain C57BL6/J were used. We had ethical approval for the experiment with the licence number BA02/2000-28/2020.

The experimental animals were subjected to acute (7 days) and chronic (21 days) treatment, both groups had control and CFA treated animals, so they were divided into 4 different groups according to the rules of randomization: 1) acute control group, 2) acute CFA group, 3) chronic control group, 4) chronic CFA group.

1.2 Mice used during chronic stress and marijuana exposure

Laboratory mice (Mus musculus) from 36 adult male NMRI strains were used in this study. The experiment was performed under an animal health permit with the license number BA02/2000-35/2016.

The mice were divided into 4 different groups according to the randomization rules: 1) control group, 2) stress group, 3) cannabis group, 4) stress+cannabis group.

2. Experimental models

2.1 Complete Freund's adjuvant (CFA)-induced arthritis model

CFA model is a prevalent chronic arthritis model. Complete Freund's adjuvant contains 1 mg/ml of heat-killed Mycobacterium tuberculosis in a paraffin oil suspension (Sigma Aldrich, St. Louis, MO, USA), of which 20 µl can be administered to mice by subcutaneous injection into the right hindfoot and hindlimb of the hindfoot to induce destructive monoarthritis with inflammatory mechanisms similar to rheumatoid arthritis.

2.2 Animal model of marijuana smoke exposure

For marijuana exposure, to mimic a real-life situation, a whole-body marijuana smoke exposure model was chosen. Exposure occurred on weekdays, 2x30 min per day, for 2 months. During one exposure, 2 strands of 0.8 g of marijuana per cigarette were burnt simultaneously over 10 min (with a puffing time of 2 s, at a puffing rate of 1/min/cigarette), and the smoke was inhaled for 30 min.

2.3. Chronic stress model

Mice were immobilized for 6 hours daily between 8:00 am and 2:00 pm. During the

immobilization stress, the mice were deprived of food and water, and were unable to move and clean themselves. Control mice were not exposed to any stress except for daily handling.

3. In vivo measurements

3.1 In Vivo Techniques to Confirm Peripheral Inflammation

3.1.1 Mechanical hypersensitivity measurement (DPA)

In the hind limb, mechanical hypersensitivity was determined using a dynamic plantar esophageometer (DPA, Ugo Basile Dynamic Plantar 37400, Comerio, Italy). Mechanonociceptive thresholds were expressed in grams (g).

3.1.2 Measurement of the latency time of the suppressive reaction (Hot Plate)

The latency of the avoidance reaction was determined using a hot plate (IITC Life Science Woodland Hills, CA, USA) at a constant temperature (50°C). The time corresponding to a thermonociceptive threshold was read from the display of the device.

3.1.3 Determination of leg volume

The leg volume was measured using a plethysmometer (Ugo Basile Plethysmometer 7140, Comerio, Italy). The hind leg of the animal was immersed in a cylinder of fluid for this instrument up to a predetermined mark. The instrument sensed the fluid volume displacement.

3.1.4 Determination of spontaneous weight distribution

The degree of spontaneous weight bearing on the hind legs was determined using the BioSeb inertia test (Vitrolles, France).

3.1.5 In vivo optical imaging

Neutrophil myeloperoxidase (MPO) activity was assessed by in vivo bioluminescence imaging (IVIS Lumina II, PerkinElmer, Waltham, USA) using luminol (5-amino-2,3-dihydro-1,4-phalazineindione) and i.p. lucigenin (bis-N-methyl acridinium nitrate) bioluminescent contrast agent to detect macrophage NADPH oxidase activity. Data were analysed using Living Image® software (Perkin-Elmer, Waltham, USA) and values from ROIs are plotted on graphs.

3.2 Respiratory function test

Respiratory function testing was performed using a whole-body plethysmograph (PLY 3211, Buxco Europe Ltd., Winchester, UK) in awake, spontaneously breathing animals as an intact measurement at the beginning of the two-month treatment period and during the experiment at the end of the first and second month. All measurements were performed in a self-controlled manner in all animals.

3.3 Behavioural studies

3.3.1. Y-maze test

Mice were subjected to behavioural tests at weekends when the animals were not acutely exposed to marijuana or stress. The Y-maze test is used to assess the short-term memory of mice.

3.3.2 Open Field test (OF)

The test is used to assess the anxiety level of mice and to measure locomotor activity. Behaviour was tested in a square box in a new environment.

3.3.3 Novel Object Recognition Test (NOR)

The novel object recognition test, which assesses cognitive function, is used in particular to assess recognition memory.

4. Post mortem histopathology methods

4.1 Tissue preparation for immunohistochemistry

Animals were injected intraperitoneally with BrdU (200 mg/kg, ip) on days 3 and 4 before perfusion. BrdU is a thymidine analogue compound, so it is incorporated into DNA and newly formed neurons can be detected. On the day of sacrifice, mice were overmedicated by intraperitoneal administration of ketamine-xylazine solution (0.1ml solution/gram body weight). When the mice's nociceptive reflexes ceased, transcardiac perfusion was initiated.

4.2 BrdU immunohistochemistry

Immunohistochemical labeling was performed using anti-BrdU antibody (DAKO, clone Bu20a, catalogue number M074401) at a dilution of 1:5000. This immunohistochemical procedure was performed using the avidin-biotin/diaminobenzidine visualization method, thus biotinylated anti-mouse IgG was used as a secondary antibody.

4.3 Doublecortin immunohistochemistry

Immunohistochemical labeling is used to visualize immature neurons in the dentate gyrus. Goat anti-DCX antibody was used at a dilution of 1:3000. This immunohistochemical procedure was performed using the avidin-biotin/diaminobenzidine visualization method, thus using biotinylated anti-goat IgG as secondary antibody.

4.4 Iba-1 immunohistochemistry

Iba-1 immunohistochemical labeling was used to visualize microglia on brain slices. The method is the same as described above. The primary antibody was polyclonal rabbit anti-Iba1 antibody. The secondary antibody was anti-rabbit antibody.

4.5. CD68 immunohistochemistry

CD68 immunohistochemical labeling is used to visualize activated microglia and macrophages in the hippocampus. The steps of the method are almost identical to those described above, the only difference being the primary antibody used. Rabbit monoclonal anti-CD68 antibody was used for immunostaining.

4.6 Determination of the immunopositivity level

After randomization and encryption, cell counting was performed using the Neurolucida (version 7) reconstruction system (Microbrightfield, Colchester, VT, USA), coupled with a Nikon Eclipse light microscope to examine cells under 20x magnification. For cell quantification, a modified stereological protocol was used

5. Other measurements

5.1. Blood count analysis

At slaughter, blood was collected from the animals by cardiac puncture and stored in a 250µl K2EDTA tube (Becton Dickinson, Hungary) used for infants. Blood analyses were determined using an automated small animal haematology analyser (Sysmex XN-V). The following parameters were measured: white blood cell count, red blood cell count, platelet count, haemoglobin concentration, neutrophil granulocyte, basophil granulocyte, eosinophil granulocyte, lymphocyte, and monocyte count and percentage distribution.

5.1.2 Measurement of inflammatory cytokine concentrations

At slaughter, tissue samples were collected to measure the concentration of inflammatory cytokines in the ankle joint and hippocampus. A Luminex multiplex immunoassay was performed to determine the concentration of 8 characteristic inflammatory cytokines using a Milliplex mouse cytokine/chemokine magnetic bead panel custom designed for this study. The cytokines and chemokines tested were IL-1a, IL-4, IL-6, IL-10, KC, MIP-2, TNF-a, IL-16.

5.1.3 Determination of urinary marijuana content

Urine analysis was performed at the Institute of Forensic Medicine, University of Pécs. Concentrations of delta-9-THC (tetrahydrocannabinol), cannabidol cannabinol and 11-nor-9-carboxy-THC were determined by fluorimetric polarized immunoassay.

5.1.4 Determination of the constituents of marijuana smoke

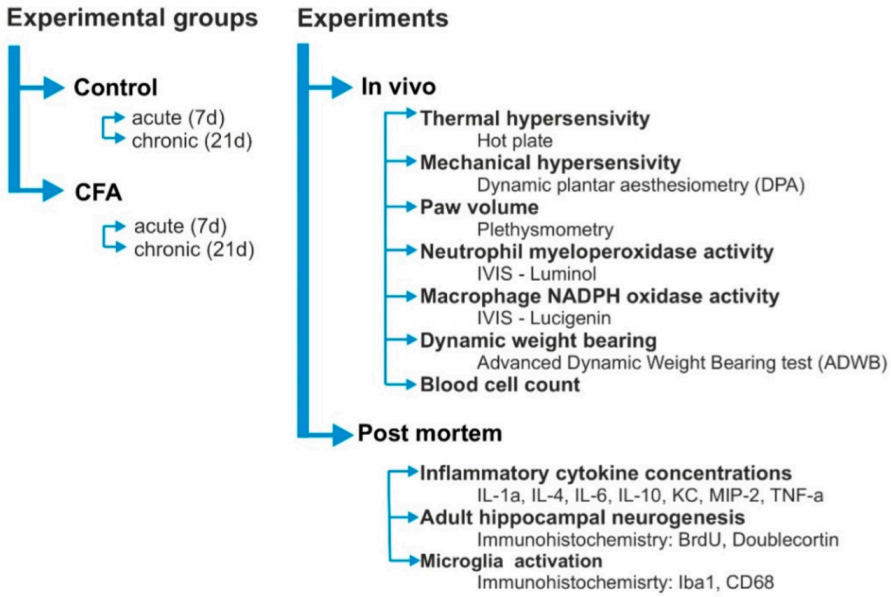
High Performance Liquid Chromatography (HPLC) was used to determine the components of marijuana smoke.

6. Statistical analyses

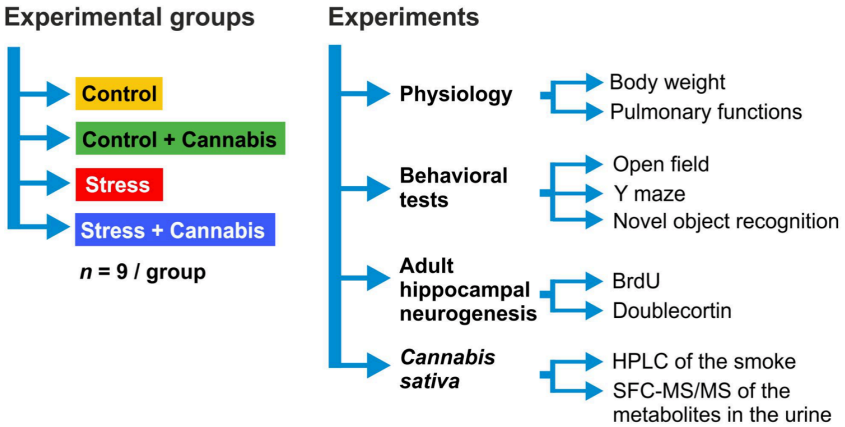
Results are expressed as mean ± SEM. Data analysis was performed using GraphPad Prism version 7. Behavioural data were analysed using two-way repeated measures analysis of variance (ANOVA) or two-way ANOVA (time × CFA treatment) followed by Sidak's multiple comparison post hoc test. In vivo imaging data, blood cell counts, cytokine concentrations and cell counts in the hippocampal gyrus dentatus were analyzed by two-way ANOVA (time × CFA treatment) followed by Tukey's post hoc test. Data on body weight and lung function tests were analysed with three-way ANOVA (time × stress × cannabis treatment) followed by Tukey's multiple comparison post hoc tests. Other data were analysed with two-way ANOVA (stress × cannabis treatment) and Tukey's post hoc test. The significance level was set at $p < 0.05$.

7. Experimental design and timing

1. Effect of acute and chronic peripheral inflammation on adult hippocampal neurogenesis



2. Effects of chronic stress and marijuana smoke on the behaviour and adult neurogenesis in the hippocampus of experimental micey



3.) Results

1. Effect of acute and chronic peripheral inflammation on adult hippocampal neurogenesis

1.1. Behavioural and pain tests

CFA treatment induced edema, thermal and mechanical hyperalgesia, and upset weight distribution in the hind paws. In CFA-injected mice, the mechanonociceptive threshold was reduced in acute and chronic inflammation conditions as detected by the dynamic plantar esotesiometer. Mice with arthritis in acute and chronic inflammatory states showed increased thermal sensitivity to warm heat stimuli, as assessed by the hot plate test. Animals treated with CFA developed persistent inflammatory edema in both acute and chronic conditions. The body weight distribution of the mice was also altered. The animals placed less weight on their inflamed right leg in the acute phase.

1.2 In vivo imaging

CFA treatment induced local inflammation. The inflammation-related increase in reactive oxygen species production was detected by bioluminescence imaging. These results also confirm that CFA-injected mice suffered from arthritis.

1.3. Blood count analysis

Changes in blood cell count are a characteristic symptom of systemic inflammation. In our experiment, the platelet count in the chronic group was reduced in CFA-treated animals compared to the acute phase. Increased neutrophil granulocyte ratio is seen in CFA treated animals in the acute phase and increased monocyte ratio is seen in CFA treated animals in the chronic phase. The percentage of eosinophilic and basophilic granulocytes is significantly decreased in CFA-treated mice.

1.4. Immunohistochemical results

1.4.1 BrdU immunohistochemistry results

Cell proliferation was assessed by anti-BrdU immunohistochemistry. Acute inflammation did not affect the number of BrdU-labelled cells, but chronic inflammation resulted in a significantly lower number of neonatal cells.

1.4.2 Results of DCX immunohistochemistry

The number of immature neurons was assessed by anti-doublecortin immunohistochemistry. Chronic arthritis reduced the number of DCX+ cells in the hippocampus in CFA-treated

animals. This reduction in DCX+ cell number in the chronic CFA group demonstrates the inhibitory effect of chronic arthritis on adult hippocampal neurogenesis.

1.4.3 Results of Iba1 immunohistochemistry

Microglia may be the primary determinants of the regulation of hippocampal neurogenesis, and we quantified the number of Iba1+ microglia in the dentate gyrus. We did not find any treatment-induced effects on Iba1+ microglia cell numbers. Thus, we conclude that arthritis did not affect the number of Iba1-immunopositive microglia in the gyrus dentatus. Based on semiquantitative morphological analysis of Iba1+ cells, no significant differences in their activation were observed between the groups studied.

1.4.4 Results of CD68 immunohistochemistry

CD68 is a glycoprotein located mainly in the endosomal/lysosomal compartment and is highly expressed in macrophages and other mononuclear phagocytes, and is therefore typically used as a cytochemical marker to visualise monocytes/macrophages in inflamed tissues. In contrast to the Iba1 data, there was a significant increase in CD68+ cell density in the dentate gyrus

1.5 Levels of inflammatory cytokines in the hind paws and hippocampus

In the hind paws, we see an increase in total protein concentration induced by inflammation. In the hindlimbs, acute CFA treatment increased cytokine concentrations of TNF-α, IL-4, IL-6, KC and MIP-2, whereas TNF-α and KC were elevated during the chronic phase. In some cases, the measured cytokine levels were either unchanged in CFA-treated hind limbs (IL-1α, IL-10) or decreased (IL-1α). In the hippocampus, cytokine levels were not altered by CFA treatment.

2. Effects of chronic stress and marijuana smoke on behaviour and adult hippocampal neurogenesis in experimental mice

2.1 Analysis of marijuana smoke and mouse urine

In our study, we determined the 9-THC, CBD and CBN content of marijuana smoke using HPLC. Furthermore, urine samples were collected from mice inhaling marijuana smoke at weeks 6 and 7. Urinary concentrations of 9-THC, 11-nor-9-carboxy-THC, CBD and CBN were

	delta-9-THC ng/ml	cannabidiol ng/ml	cannabinol ng/ml	11-nor-9-carboxy-THC ng/ml
Marijuana smoke (ng/ml)*	427000± 2000	35800± 1100	258000± 8000	-
Urine sample (ng/ml)	65,61 ± 22,77	2,44 ±0,56	41,92 ± 17,62	1,81 ± 0,16
* Quantitative data for compounds are from 1.6g of burnt marijuana smoke. Data are expressed as mean ± SEM.				

Table 1: Chemical analysis of marijuana smoke and urine samples

2.2. Body weight change in response to marijuana and stress treatment

Stress and cannabis exposure reduced the body weight gain of the animals. The results suggest that cannabis treatment inhibited the animals' weight gain and that the inhibition of stress-induced weight gain by inhalation of cannabis smoke was somewhat attenuated in the animals.

2.3. Respiratory function study

Stress impaired lung function and normal lung development, whereas marijuana smoke did not affect lung function. Chronic stress also negatively affected the development of several lung parameters, such as frequency, number of breaths per minute, peak inspiratory flow, expiratory time, relaxation time and respiratory volume.

2.4 Behavioural tests

2.4.1. Cognitive test - Y maze test / New item recognition test

Our results show that neither stress nor marijuana exposure affects the cognitive functions of the mice.

2.4.2. Open field test

This test is used to assess the locomotor activity and anxiety levels of the animals. Anxiety-related spontaneous locomotor activity and self-care, i.e. time spent washing, were measured in the animals. Stressed mice spent less time in the middle of the available area. Animals that were anxious tended to spend more time in the corner. The results of the movement speed measurements show that the stressed mice showed reduced interest in the new environment and were slower. Animals exposed to marijuana smoke tended to spend more time in the middle of the area. Both the stressed and cannabis-treated mice spent significantly more time washing themselves.,. In addition, the stressed and/or cannabis-treated mice started self-grooming much earlier than the control mice.

2.5. Immunohistochemical results

2.5.1 BrdU immunohistochemistry results

Cell proliferation in gyrus dentatus was visualized using the exogenous proliferation marker BrdU. We could not detect inhibition of cell proliferation by statistical analyses, but at trend level it can be seen that stress and cannabis treatment resulted in a decrease in the number of BrdU+ cells in the gyrus dentatus compared to the control group.

2.5.2 Results of DCX-immunohistochemistry

Inhalation of cannabis smoke affected the number, morphology and migration of immature neurons. Stress had no effect, but cannabis treatment significantly reduced the number of

DCX+ neurons in the gyrus dentatus. The incidence of abnormal-looking DCX+ cells was significantly higher in cannabis-treated animals. In control mice, the majority (more than 90%) of immature neurons were located in the germinative subgranular zone (sgz). A small percentage of cells (<5%) migrated to the granule cell layer (gcl) (Figure 29), and some cells were also found in the hilus. Compared to the data from control animals, the migration rate was significantly altered by marijuana exposure. Long-term exposure to cannabis altered the morphology of DCX+ neurons. DCX+ cells in the gyrus dentatus of cannabis-treated mice often showed an unusual, abnormal appearance. For example, cannabis treatment significantly altered the dendrites of neurons and many cells lost their dendrites, and bipolar DCX+ neurons or neurons with basal dendrites were observed.

2.5.2. Results of DCX immunohistochemistry

Inhalation of cannabis smoke affected the number, morphology and migration of immature neurons. Stress had no effect, but cannabis treatment significantly reduced the number of DCX+ neurons in the gyrus dentatus. The incidence of abnormal-looking DCX+ cells was significantly higher in cannabis-treated animals. In control mice, the majority (more than 90%) of immature neurons were located in the germinative subgranular zone (sgz). A small percentage of cells (<5%) migrated to the granule cell layer (gcl) (Figure 29), and some cells were also found in the hilus. Compared to the data from control animals, the migration rate was significantly altered by marijuana exposure. Long-term exposure to cannabis altered the morphology of DCX+ neurons. DCX+ cells in the gyrus dentatus of cannabis-treated mice often showed an unusual, abnormal appearance. For example, cannabis treatment significantly altered the dendrites of neurons and many cells lost their dendrites, and bipolar DCX+ neurons or neurons with basal dendrites were observed.

4) Summary of our results

1. Effect of acute and chronic peripheral inflammation on adult hippocampal neurogenesis

We hypothesized that peripheral arthritis would increase the levels of a cytokines in both joints and hippocampus, but we could not confirm the latter hypothesis based on our research results. Total protein concentrations were elevated in inflamed joints and a similar trend was observed in the hippocampus of CFA-treated animals, especially in the acute phase. We conclude that the increase in total protein concentration is due to increased production of inflammatory mediators. Furthermore, our hypothesis that peripheral inflammation reduces neurogenesis in the gyrus dentatus and affects CD68+ macrophage/activated microglia density was fulfilled, and our data suggest that acute peripheral inflammation triggers a cascade of molecular and cellular changes that eventually lead to a reduction in adult hippocampal neurogenesis, which was only detectable in the chronic inflammatory phase.

2. Effects of chronic stress and marijuana smoke on the behaviour and adult hippocampal neurogenesis in experimental mice

In conclusion, our results do not fully support any of our hypotheses. Marijuana smoke does not have a stress-relieving effect as assessed by behavioural tests, and we observed abnormal washing behaviour in this group. Furthermore, the smoke inhaled mice did not show impaired respiratory function, but the stressed animals did, probably because their lungs did not develop as they normally would have during the time in the tube. Contrary to what was expected, the weight gain of each group was lagging behind that of the control mice. Furthermore, we could not prove that chronic marijuana smoke exposure stimulated neurogenesis in the hippocampus, on the contrary, marijuana smoke reduced neurogenesis as did stress. Inhalation of marijuana smoke altered the dendrites of neurons and many cells lost their dendrites, with many neurons migrating to unusual areas. All of this can affect normal conduction and predispose to pathological neurological conditions.

5. Conclusions, discussion

Adult hippocampal neurogenesis is an intensively researched topic among researchers, but its significance is still a controversial issue. It is important to emphasise that most of the evidence in the literature comes from experimental animals (mice and rats) and much less evidence comes from primates or human samples. The two most important questions that remain to be clarified are: to what extent do the biological processes observed in experimental animals reflect the functioning of the human brain? What is the amount of new neurons formed in the adult human hippocampus and what exactly are the physiological processes they are involved in (Kempermann et al., 2018; Lucassen et al., 2020)?

The main problem is that this cellular process can best be investigated only by post-mortem histological methods. From the literature, we know that this process occurs in the brains of most animal species studied so far, including several primate species (Augusto-Oliveira et al., 2019). Several studies support that adult hippocampal neurogenesis in primates and rodents is similar, but significant differences have also been shown (Miller et al., 2013; Fasemore et al., 2018; Augusto-Oliveira et al., The maturation of newborn cells is slower in primates than in rodents, as the period of maximum cellular excitability can be significantly prolonged in organisms that live for years or decades (Kohler et al., 2011; Yuan et al., 2014). Despite methodological difficulties, there is also evidence that adult hippocampal neurgenesis also occurs in the human brain. In 1998, the first research on whether adult neurogenesis can occur in humans was published. The authors showed that BrdU is incorporated into certain cells of the adult human gyrus dentatus. A few months after administration of the thymidine analogue, BrdU+ cells were detectable alongside several neuronal markers, demonstrating the presence of a limited population of adult-derived granule cells in humans (Eriksson et al., 1998). And a study published in 2013 used another innovative approach to detect and quantify the number of newly generated cells in the human brain. In this, researchers confirmed the existence of newly formed neurons in the adult human brain by examining radioactive carbon isotopes (Spalding et al., 2013). These two studies took advantage of the greater postmortem stability of nucleic acids compared to proteins and paved the way for future advances in the field. In the following years, other groups, mainly using techniques based on immunohistochemistry, have contributed to the evidence supporting the occurrence of adult hippocampal neurogenesis in humans (Boldrini et al., 2009; Crews et al., 2010; Knoth et al., 2010; Ernst et al., 2014, Moreno-Jiménez et al., 2021). Attempts have also been made to visualise this specific cellular process using in vivo magnetic resonance imaging studies, but these attempts have not yet yielded any breakthrough success (Ho et al., 2013).

In summary, the research presented here focuses on everyday diseases and health problems that are common. Approximately 2.5% of the world's population consumes marijuana (UNODC, 2017), 0.5-1% suffer from rheumatoid arthritis (Gibofsky et al., 2012) and 40% of the population has been exposed to a source of stress and approximately 30% have felt physical pain or sadness in the days prior to the survey (Gallup Global Emotions Report, 2022). All of these factors negatively impact adult hippocampal neurogenesis, which can lead to the development of additional health problems. Neurogenesis can contribute to stress management and regulation of stress hormones, but reduced neurogenesis can lead to a reduced ability to cope with stress and an increase in emotional reactivity. The formation of new neurons in the hippocampus may also be associated with mood regulation, so reduced neurogenesis may increase the risk of depression, anxiety and other mood disorders (Santarelli et al., 2003; Snyder et al., 2011; Surget et al., 2011; Kim et al., 2012; Schoenfeld and Cameron, 2015). Chronic systemic inflammation may contribute to the development or exacerbation of mental disorders, depression and neurodegeneration, and may also reduce neurogenesis (Dantzer et al., 2008; Perry et al., 2010; Berk et al., 2013). Neurogenesis may play a role in the regenerative capacity of the brain and may contribute to reducing the risk of neurodegenerative diseases. Reduced neurogenesis may slow recovery processes after brain injury, such as stroke or rehabilitation after head trauma, and increase the risk of developing neurodegenerative diseases such as Alzheimer's and Parkinson's (Hanspal and Gillotin, 2022; Disouky and Lazarov, 2021; Marchetti et al., 2020; Tang et al., 2022).

The endocannabinoid system and endocannabinoids play an important role in brain development, regulating cell proliferation, migration, differentiation and survival (Harkany et al., 2007). Marijuana use may inhibit the production and integration of new neurons in the hippocampus (Rusznák et al., 2018). Long-term marijuana use may cause perceptual learning difficulties and memory impairment. Decreased brain plasticity and inhibition of new neuronal production may also have negative effects on learning, memory and cognitive functions (Denny et al., 2014; Anacker and Hen, 2017), thus, decreased neurogenesis may lead to deterioration of cognitive functions, such as reduced learning abilities or memory problems, or a decrease in flexibility of thinking (Gillotin et al., 2021).

All this suggests that adult hippocampal neurogenesis is thought to play an important role in the healthy functioning of the brain and that any disruption of this physiological process could lead to the development of various pathologies.

List of abbreviations

2-AG: 2-arachidonoylglycerol
5-HT: 5-hydroxytryptamine/serotonin
ABC: avidin-biotin complex
ACTH: adrenocorticotropic hormone
AEA: anandamide / N-arachidonoyl ethanolamine
AIDS: acquired immunodeficiency syndrome
ANOVA: analysis of variance
BrdU: 5-bromo-2'-deoxyuridine
CaCl2: calcium chloride
CA1: cornu ammonis 1 region of the hippocampal formation
CA3: cornu ammonis 3 region of the hippocampal formation
CBC: cannabichromene
CBD: cannabidiol
CBDV: cannabidivarin
CBG: cannabigerol
CBN: cannabinol / cannabinol
CD68: cluster of differentiation 68
CFA: complete Freund's adjuvant
DA: dopamine
DAB: 3,3'-diaminobenzidine
DCX: doublecortin
DG: dentate gyrus / gyrus dentatus
DNS: deoxyribonucleic acid
DPA: dynamic plantar aesthesiometer
DSS: dextran sulphate sodium
EDTA: ethylene-diamine tetra-acetic acid
FAAH: fatty acid amide hydrolase
GABA: gamma-aminobutyric acid
GCL: granular cell layer
GPR6: G protein-coupled receptor 6
HIV: human immunodeficiency virus
HLA: human leukocyte antigen
HPLC: high performance liquid chromatography
Iba1: ionized calcium-binding adapter molecule 1
IgG: immunoglobulin G
IL: interleukin
i.p.: intraperitoneal
i.pl.: intraplantar
i.v.: intravenous
K2EDTA: dipotassium ethylene diamine tetraacetic acid
KC: keratinocyte chemoattractant
LPS: lipopolysaccharide
LV: lateral ventricles
MDMA: 3,4-methylenedioxy methamphetamine (or ecstasy)
MIP-2: macrophage inflammatory protein-2
MPO: myeloperoxidase
MS: mass spectrometry
NADPH: kikotinamide adenine dinucleotide phosphate
NaOH: sodium hydroxide
NGS: normal goat serum
NSCs: neural stem cells
OB: olfactory bulb
OF: open field test
PBS: phosphate buffered saline
PFA: paraformaldehyde
PCNA: proliferating cell nuclear antigen
s.c.: subcutaneous/subcutaneous
S.E.M.: standard error of mean
SVZ: subventricular zone
THC: Δ9-tetrahydrocannabinol
THCV: tetrahydrocannabivarin
TNF-α: tumour necrosis factor alpha
TRIS: tris-(hydroxymethyl)amino-methane
TRP: transient receptor potential

Publication list

Original publications on which the thesis is based

Kitti Rusznák, Csekő Kata, Varga Zsófia, Csabai Dávid, Bóna Ágnes, Mayer Mátyás, Kozma Zsolt, Helyes Zsuzsanna, Czéh Boldizsár
Chronic marijuana smoke exposure does not influence the impact of long-term stress on physiology, cognition, emotion and adult hippocampal neurogenesis.
Frontiers in Pharmacology, 23 July 2018 | https://doi.org/10.3389/fphar.2018.00786

(Q1, IF: 3.8) Shared first author

Kitti Rusznák, István Horváth Ádám Horváth, Kinga Pohli-Tóth, Anett Futácsi, Ágnes Kemény, Gabriella Kiss, Zsuzsanna Helyes, Boldizsár Czéh
Experimental Arthritis Inhibits Adult Hippocampal Neurogenesis in Mice. Cells 2022, 11, 79., https://doi.org/10.3390/cells11050791 (Q1, IF: 7,666)

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Additional publications
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Participation in scientific conferences, poster presentations

Poster presentations at international conferences

Kitti Rusznák, István Horváth Ádám István, Kinga Pohli-Tóth, Anett Futácsi, Ágnes Kemény, Gabriella Kiss, Zsuzsanna Helyes, Boldizsár Czéh
Experimental arthritis inhibits adult hippocampal neurogenesis in mice
FENS (Federation of Neuroscience Societies) Paris, France, 9-13 July 2022

Rusznák Kitti, Csekő Kata, Varga Zsófia, Csabai Dávid, Bóna Ágnes, Mayer Mátyás, Kozma Zsolt, Helyes Zsuzsanna, Czéh Boldizsár
Long-Term Stress and Concomitant Marijuana Smoke Exposure Affect Physiology, Behavior and Adult Hippocampal Neurogenesis. EBBS (European Brain and Behaviour Society) 2019, Prague, Czech Republic, 2019.09.21-24.

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Long-Term Stress and Concomitant Marijuana Smoke Exposure Affect Phys- iology, Behavior and

Adult Hippocampal Neurogenesis,
IDK-Interdisciplinary PhD Conference, Pécs, Hungary 24-25 May 2019.

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The effect of chronic stress and concomitant cannabis exposure on behaviour and adult hippocampal neurogenesis in mice,
ECNP (European College of Neuropsychopharmacology) Workshop for Early Career Scientists in Europe, Nice, France, 2019, March 7 - 10.

Rusznák Kitti, Csekő Kata, Varga Zsófia, Csabai Dávid, Helyes Zsuzsanna, Czéh Boldizsár
The Effect of Chronic Stress and Concomitant Cannabis Exposure on Behavior and Adult Hippocampal Neurogenesis in Mice,
FENS (Federation of Neuroscience Societies) Regional Meeting, Pécs, Hungary 2017 September 20-23.

Poster presentations at Hungarian conferences

Rusznák Kitti, Csekő Kata, Varga Zsófia, Csabai Dávid, Bóna Ágnes, Mayer Mátyás, Kozma Zsolt, Helyes Zsuzsanna, Czéh Boldizsár
The effects of long-term cannabis smoke exposure in mice,
59th General Meeting of the Hungarian Laboratory Diagnostics Society Pécs, 30 August - 1 September 2018.

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