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KELLI SOMELAR-DURACZ

The molecular and cellular mechanisms of brain plasticity impairing factors

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The molecular and cellular mechanisms of brain plasticity impairing factors

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Department of Pharmacology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

I **Somelar, K**., Jürgenson, M., Jaako, K., Anier, K., Aonurm-Helm, A., Zvejniece, L., & Zharkovsky, A. (2021). Development of depression-like behavior and altered hippocampal neurogenesis in a mouse model of chronic neuropathic pain. *Brain Research*, *1758*, 147329.

II **Somelar-Duracz, K.**, Jürgenson, M., Viil, J., Zharkovsky, A., & Jaako, K. (2024). Unpredictable chronic mild stress does not exacerbate memory impairment or altered neuronal and glial plasticity in the hippocampus of middle-aged vitamin D deficient mice. *European Journal of Neuroscience*, 59, 1696–1722.

III Anier, K.*, **Somelar, K.***, Jaako, K., Alttoa, M., Sikk, K., Kokassaar, R., Kisand, K., & Kalda, A. (2022). Psychostimulant-induced aberrant DNA methylation in an in vitro model of human peripheral blood mononuclear cells. *Clinical Epigenetics*, *14*(1), 89.

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I The author participated in the behavioral experiments, immunohistochemistry, microscopy, figure preparation, data and image analysis, conducted confocal microscopy experiments, wrote the original draft of the manuscript, participated in manuscript revisions and handled the correspondence.

II The author participated in the study design, behavioral experiments, data and image analysis, conducted immunohistochemistry, microscopy, prepared the figures, wrote the original manuscript draft and participated in manuscript revisions.

III The author participated in the *in vitro* cell culturing, ELISA experiments, and data analysis, carried out the flow cytometry experiments, participated in figure preparation, co-wrote the original manuscript draft, participated in manuscript revisions and handled the correspondence.

ABBREVIATIONS

AID	activation-induced cytidine deaminase
AMP	amphetamine
ANOVA	analysis of variance
APOBEC	apolipoprotein B mRNA-editing enzyme complex
ATP	adenosine triphosphate
ATP2B4	ATPase Plasma Membrane Ca^{2+}
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BER	base excision repair
BrdU	bromodeoxyuridine
CA	cornu ammonis
CalB	calbindin
CNS	central nervous system
COCA	cocaine
CREB	cyclic adenosine monophosphate response element-binding protein
CTRL	control
Cuff	cuffed
CX3CL1	C-X3-C motif chemokine ligand 1
DAC	decitabine
DAPI	4′, 6-diamidino-2-phenylindole
DAT	dopamine transporter
DG	dentate gyrus
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
FBS	fetal bovine serum
GABA	γ-aminobutyric acid
GCL	granule cell layer
GFAP	glial fibrillary acidic protein
HE	hematoxylin-eosin
HPA	hypothalamic-pituitary-adrenocortical
IBA1	ionized calcium-binding adapter molecule 1
IGF-1	insulin-like growth factor 1
IHC	immunohistochemistry
IL	interleukin
LPS	lipopolysaccharide
LTD	long-term depression
LTM	long-term memory
LTP	long-term potentiation
mCSF	macrophage colony-stimulating factor
NAc	nucleus accumbens
NeuN	neuronal nuclear antigen

NGS	next generation sequencing
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NMDA	N-methyl-D-aspartate
NSFT	novelty suppressed feeding test
OFT	open field test
ORT	object recognition test
PBS	phosphate buffered saline
POD	postoperative day
PROX1	Prospero homeobox protein 1
PSA-NCAM	polysialylated-neural cell adhesion molecule
RNA	ribonucleic acid
RT-qPCR	real-time quantitative polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenyl methionine
SD	standard deviation
SNC	sciatic nerve cuffing
SOX2	sex-determining region Y box2
STM	short-term memory
TDG	thymine DNA glycosylase
TET	ten-eleven translocation
TETs	ten-eleven translocation enzymes
TH	tyrosine hydroxylase
TNF	tumor necrosis factor
TST	tail suspension test
UCMS	unpredictable chronic mild stress
VDD	vitamin D deficiency/vitamin D deficient
VDR	vitamin D receptor
VMAT -2	vesicular monoamine transporter 2
VTA	ventral tegmental area

1. INTRODUCTION

Brain plasticity is the innate ability of the brain to adapt and change its structure and function in response to internal and external stimuli (Ganguly & Poo, 2013; Voss *et al.*, 2017; Mateos-Aparicio & Rodríguez-Moreno, 2019). It is essential for development, learning, and memory, as well as for recovery after injury (Murphy & Corbett, 2009; Ganguly & Poo, 2013). The brain is highly plastic during early developmental periods, when widespread structural alterations occur, such as neurogenesis, neuronal migration, and establishment of functional connections (Nayak *et al.*, 2022; Marzola *et al.*, 2023). In adulthood, however, brain plasticity is mainly confined to changes in the number of synaptic connections and their strength (La Rosa *et al.*, 2020), with the remarkable exception of adult hippocampal neurogenesis, which represents a form of structural plasticity (Eriksson *et al.*, 1998; Spalding *et al.*, 2013; Boldrini *et al.*, 2018). Hippocampal neurogenesis introduces plasticity into local networks and contributes to learning, memory encoding, and mood regulation (Anacker & Hen, 2017; Toda & Gage, 2018; Kempermann, 2022).

The hippocampus is also a region vulnerable to stress, aging, and neuroinflammation, which may cause dysfunctional hippocampal plasticity, lead to cognitive deficits, and contribute to the development of depression. Indeed, depression is characterized by neuroplastic alterations in the hippocampus, such as decreased neurogenesis, atrophy, and impaired synaptic plasticity (Bartsch & Wulff, 2015; Toda & Gage, 2018; Price & Duman, 2020; Wu & Zhang, 2023). Chronic neuropathic pain is often co-morbid with depression (Radat *et al.*, 2013; Shaygan *et al.*, 2013; Cohen & Mao, 2014), and chronic stress is a known risk factor for depression (Bekhbat & Neigh, 2018). Vitamin D deficiency has also been associated with a higher prevalence of depression and memory impairments, however the causal link has not been established (Meehan & Penckofer, 2014; Ceolin *et al.*, 2022). Moreover, it is not known, if vitamin D deficiency could exacerbate the negative behavioral effects of chronic stress. The exact mechanisms of how these causal factors, alone or in combination, may lead to dysfunctional hippocampal plasticity and resultant depression, anxiety, and cognitive impairments are unclear.

Impaired neuronal plasticity also occurs in addiction, when the repeated use of narcotics triggers neuroplastic changes in the brain's mesolimbic reward system. These changes may then result in addiction-related behaviors long after the drug use has ceased (Kauer & Malenka, 2007; Nyberg, 2014; Volkow & Morales, 2015; Nestler & Lüscher, 2019). Previous preclinical studies suggest a role for epigenetic alterations, such as aberrant DNA methylation, in mediating addiction-related behaviors by regulating gene expression networks (Anier *et al.*, 2010; LaPlant *et al.*, 2010; Nestler & Lüscher, 2019, 2019; Urb *et al.*, 2020), however the role of epigenetic alterations in the development of addiction in humans needs clarification.

The current thesis aims to elucidate the molecular and cellular mechanisms of the brain plasticity impairing factors, *chronic neuropathic pain, vitamin D deficiency* and its combination with *chronic stress*, as well as *psychostimulants cocaine and amphetamine*, in relevant mouse and human cellular models with a focus on hippocampal neurogenesis, neuroinflammation, and epigenetic DNA modifiers. In addition, we aim to clarify the effects of chronic neuropathic pain, vitamin D deficiency, and chronic stress on depressive-like behaviors, impairments in memory and their correlates with hippocampal plasticity.

2. LITERATURE REVIEW

2.1. Brain plasticity

Brain plasticity is an umbrella term used to describe the innate ability of the brain to adapt and change its structure, connections, and functions throughout life and in response to experience (Voss *et al.*, 2017; Mateos-Aparicio & Rodríguez-Moreno, 2019). The broad term covers structural alterations of neurons and neural networks, such as neurogenesis, axonal sprouting, alterations in dendritic branching, dendritic spine remodeling, and creation or removal of synapses, as well as functional alterations, such as activity-dependent changes in synaptic transmission efficacy and strength (Ganguly & Poo, 2013; La Rosa *et al.*, 2020). Different forms of brain plasticity are essential in the developmental period, for learning and memory and for regaining lost functions and adaption after injury, such as stroke or traumatic brain injury (Murphy & Corbett, 2009; Ganguly & Poo, 2013; Toda & Gage, 2018; Marzola *et al.*, 2023).

During early development, such as the fetal, perinatal, and early childhood periods, the brain exhibits a high degree of structural and functional plasticity while undergoing rapid and extensive growth. Structural plasticity is dominant in the fetal brain, which is characterized by the widespread generation and migration of new neurons, neurite outgrowth, and the establishment of functional connections between the newly generated neurons to form neural circuits (Nayak *et al.*, 2022; Marzola *et al.*, 2023). Initially, new neurons and synapses are generated in surplus, peaking at late embryonic stages, and by early adolescence, synaptic pruning decreases the number of synapses to the adult level, closing the period of elevated neuroplasticity (Tau & Peterson, 2010; Milbocker *et al.*, 2021). Exceptional brain plasticity during perinatal and early childhood periods increases sensitivity to environmental input and promotes the formation of neural connections. During later life, brain plasticity is more context-dependent and a tightly regulated process (Marzola *et al.*, 2023).

In adulthood, brain plasticity is mainly confined to changes in the number of synaptic connections and their strength (La Rosa *et al.*, 2020). The activity-dependent change in neuronal connection strength and synaptic transmission efficacy at pre-existing synapses is termed synaptic plasticity. Synaptic plasticity is considered an important underlying mechanism for learning and memory (Magee & Grienberger, 2020). The roots of the premise that synaptic plasticity contributes to memory formation date back to the late 19th century when the Spanish neuroscientist and Nobel laureate Ramon y Cajal hypothesized that information storage in the brain results from alterations in the strength of synaptic connections between neurons (Ramon y Cajal, 1894; Kauer & Malenka, 2007). The idea was further advanced in 1949 by Donald Hebb, who proposed that memories are formed in the brain by strengthening of synapses between neurons whose activity is correlated (Hebb, 1949), a process which Carla Shatz has described with a phrase “Cells that fire together, wire together” (Shatz, 1992). Following studies have demonstrated that through synaptic plasticity, experiences such as learning,

stressful events, or the use of psychoactive substances can modify the activity and organization of neural circuits (Kauer & Malenka, 2007; Citri & Malenka, 2008). Synaptic plasticity is also involved in the developmental fine-tuning of brain neural circuits and reorganization after damage (Stampanoni Bassi *et al.*, 2019). Examples of activity-dependent synaptic plasticity are the long-term potentiation (LTP) of glutamatergic excitatory synapses, which enhances the efficiency of synaptic transmission, and long-term depression (LTD), which reduces the efficiency of synaptic transmission and reduces responsiveness to behaviorally irrelevant or unused stimuli (Bliss & Lomo, 1973; Allen *et al.*, 2003; Citri & Malenka, 2008; Collingridge *et al.*, 2010).

Structural brain plasticity decreases in mammals in adulthood, with the remarkable exception of neurogenic regions of the brain that demonstrate high levels of structural plasticity throughout life. Adult neurogenesis is evolutionarily conserved across different classes of animals. In sub-mammalian vertebrates like fish, amphibians, and reptiles (Kaslin *et al.*, 2008), new neurons are produced during their whole lifespan; the capacity diminishes in birds (Goldman & Nottebohm, 1983; Alvarez-Buylla *et al.*, 1990) and even more in mammals. In rodents, two main stem cell niches have been identified: the subgranular zone in the dentate gyrus (DG) of the hippocampus and the subventricular zone lining the lateral ventricles, which gives rise to neuroblasts that migrate to the olfactory bulb via rostral migratory stream where they differentiate into neurons (Altman, 1962, 1963, 1969; Corotto *et al.*, 1993; Kuhn *et al.*, 1996; Kempermann *et al.*, 1997; Toda & Gage, 2018; La Rosa *et al.*, 2020; Denoth-Lippuner & Jessberger, 2021). Additionally, neurogenesis to a smaller extent has been reported in the brainstem, hypothalamus, neocortex, striatum, amygdala, and substantia nigra of rodents and other mammals (Denoth-Lippuner & Jessberger, 2021).

In humans, widespread neurogenesis takes place in the hippocampus (Eriksson *et al.*, 1998; Knoth *et al.*, 2010; Spalding *et al.*, 2013; Boldrini *et al.*, 2018), in contrast to only miniscule neurogenesis in the olfactory bulbs (Bergmann *et al.*, 2012) and in the neocortex (Bhardwaj *et al.*, 2006). Adult hippocampal neurogenesis in mammals has gained attention due to its involvement in hippocampal functions, such as memory encoding and mood regulation. Moreover, hippocampal neurogenesis is highly regulated by environmental factors and physiological states, such as aging and chronic stress, which decrease neurogenesis, or environmental enrichment, physical activity, and antidepressant use during depression, which can increase neurogenesis. As some of the symptoms of mood disorders and cognitive decline can be partially attributed to dysregulation of hippocampal neurogenesis (Kempermann *et al.*, 2015; Toda & Gage, 2018), regulating neurogenesis may represent an opportunity for enhancement of brain plasticity, and improvement of memory functions and mood.

2.2. Hippocampal brain plasticity in health

2.2.1. Hippocampal anatomy and functions

The name of the hippocampus brain structure was inspired by its gross dissection resemblance to the seahorse (genus *Hippocampus*). In humans, the hippocampus is buried deep within each of the medial temporal lobes; in rodents, the hippocampus is a relatively big C-shaped structure beneath the neocortex in the caudal part of the brain in each hemisphere. The hippocampal formation (referred to as the hippocampus) consists of three distinct parts: dentate gyrus, hippocampus proper, which is divided into cornu ammonis subregions (CA1, CA2, and CA3), and subiculum (Figure 1) (van Strien *et al.*, 2009; Knierim, 2015). The curved cornu ammonis surrounds a V-shaped DG (Toni & Schinder, 2016), which is organized in three layers. The first, deep layer, is called the hilus and comprises a mixture of afferent and efferent fibers, γ-aminobutyric acid (GABA) interneurons, and glutamatergic mossy cells. Superficial to the polymorph layer is the granule cell layer, which is mainly composed of the bodies of glutamatergic granule neurons. The most superficial layer in the DG is called the molecular layer, and it is occupied primarily by dendrites of the granule cells and axons from the entorhinal cortex and other regions, as well as by pyramidal basket cells (Amaral *et al.*, 2007; van Strien *et al.*, 2009).

The hippocampus receives input from nearly all neocortical association areas through perirhinal, parahippocampal cortices, and finally via the entorhinal cortex (van Strien *et al.*, 2009; Bartsch & Wulff, 2015). The classic hippocampal anatomical connectivity is described as a "tri-synaptic loop," where the entorhinal cortex provides the primary cortical input to the hippocampus via the performant path to the granule cells in the DG region (1st synapse). The DG projects to the CA3 region via the mossy fiber pathway (2nd synapse), and the CA3 projects to the CA1 region then via the Schaffer collateral pathway (3rd synapse). The loop is completed by CA1 projections back to the entorhinal cortex. The entorhinal cortex also projects directly to the CA3 and CA1 regions, and the CA3 provides a feedback projection to the DG via the excitatory mossy cells of the dentate hilus (Knierim, 2015). In addition, the DG receives inputs from local GABAergic interneurons (mainly located in hilus) and from other brain areas, such as cholinergic and GABAergic input from the septal nuclei, serotonergic inputs from raphe nuclei, noradrenergic inputs from the nucleus locus coeruleus and dopaminergic inputs from the ventral tegmental area (VTA) (Toni & Schinder, 2015; Toda & Gage, 2018).

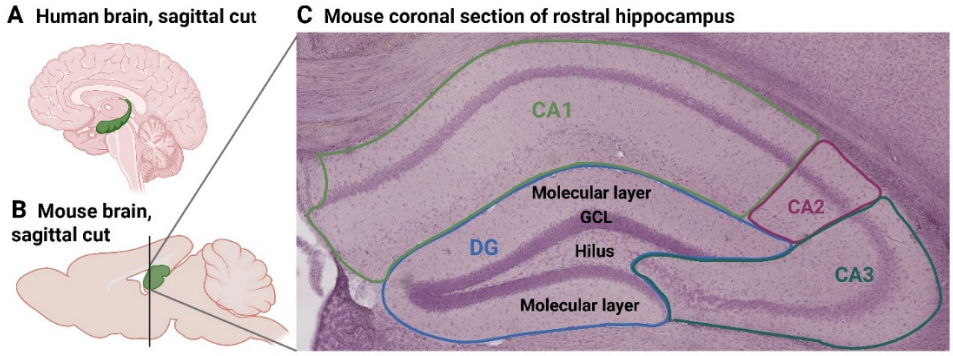

Figure 1. Location of the hippocampus in human and mouse brain and hippocampal subfields in mouse brain. The hippocampus (green) is located (A) deep in the temporal lobes of the human brain and (B) beneath the neocortex in the caudal part of the mouse brain. (C) Hippocampal subfields – dentate gyrus (DG), including molecular layer, granule cell layer (GCL) and hilus, and cornu ammonis 1-3 (CA1-3), are depicted on a mouse coronal section stained with hematoxylin and eosin. Hippocampal subfields were drawn relying on the annotations in the Allen Mouse Brain Atlas (Allen Institute, 2024). This image was created by using Biorender.

The hippocampus is part of the limbic system, a collection of connected cortical and subcortical structures that process sensory input to determine through motivation and memory the emotional, cognitive, autonomic, and motor responses needed for self-preservation and survival (McLachlan, 2009; Catani *et al.*, 2013). The critical functions of the hippocampus are memory processing and spatial navigation, as well as stress and mood regulation (Anand & Dhikav, 2012; Bartsch & Wulff, 2015).

In 1957, Scoville and Milner (1957) described profound deficits in episodic memory in an epilepsy patient H.M, who had double resection of the hippocampi, adjacent temporal and entorhinal cortex, and amygdalae (Corkin *et al.*, 1997). While H.M.'s intellectual abilities and memories previous to the surgery were unaffected, the ability to form new memories related to everyday events and experiences was severely impaired (Squire, 2009). A large number of following studies suggest that the hippocampus is central in encoding, consolidation, and retrieval of episodic memory, which depends on an extensive network of regions, including neocortical association regions, subcortical nuclei, the medial temporal lobe, and parahippocampal areas (Bartsch & Wulff, 2015). Earlier studies have also indicated that the hippocampus has a time-limited function in memory (Zola-Morgan & Squire, 1990; Kim & Fanselow, 1992), since memories that are initially formed in the hippocampus transfer to the medial prefrontal cortex over weeks or months for long-term storage (Nadel & Moscovitch, 1997; Bontempi *et al.*, 1999; Frankland *et al.*, 2004; Toader *et al.*, 2023). Therefore, the hippocampus is involved in memory consolidation, a process during which short-term memory is converted into long-term memory (Wittenberg & Tsien, 2002).

The hippocampus is well known to be involved in the episodic encoding of events by providing spatial and temporal contexts for specific events based on recordings of the hippocampal neurons in preclinical rodent studies (Manns & Eichenbaum, 2009; McKenzie *et al.*, 2013, 2014) and functional magnetic resonance imaging studies in humans (Brown *et al.*, 2014; Hsieh *et al.*, 2014; Nielson *et al.*, 2015). The hippocampal "place cells" in rodents activate as animals place themselves in specific locations in an environment and have been proposed as critical elements of an internal cognitive map (O'Keefe & Dostrovsky, 1971). The place cells are needed for encoding objects and events at specific places, which is associated with forming and retrieving episodic-like memories. In addition to place cells, ensembles of time cells in the hippocampus fire at sequential moments and map the temporal organization of experiences. Time cells are proposed to play an essential role in the memory for ordering events and everyday experiences (Preston & Eichenbaum, 2013).

Behavioral, anatomical, and gene expression studies support a functional separation of rodents' dorsal and ventral hippocampal compartments, corresponding to posterior and anterior compartments in primates (Fanselow & Dong, 2010). While the dorsal hippocampus performs primarily cognitive functions (Moser *et al.*, 1993), as described earlier, the ventral hippocampus relates to emotion, affect, and stress (Fanselow & Dong, 2010). Lesions studies in the ventral hippocampus alter emotional behavior, social interactions (Deacon *et al.*, 2002; McHugh *et al.*, 2004), stress resilience (Henke, 1990; Levone *et al.*, 2015), and optogenetic activation of the ventral DG granule cells reduces the innate anxiety-like behavior in rodents (Kheirbek *et al.*, 2013). The ventral hippocampus shares reciprocal connections with the basolateral amygdala, and these regions and their bidirectional connections are implicated in fear processing, social interaction, and encoding of emotional memories (Richardson *et al.*, 2004; Felix-Ortiz & Tye, 2014; Anacker & Hen, 2017). The ventral hippocampus is also a significant regulator of the hypothalamic-pituitary-adrenocortical (HPA) axis (Fanselow & Dong, 2010; Anacker & Hen, 2017). While the hippocampus and the prefrontal cortex are predominantly inhibitory to HPA axis stimulation and cortisol/corticosterone secretion, the amygdala is proposed to activate glucocorticoid secretion (Herman *et al.*, 2005).

2.2.2. Adult hippocampal neurogenesis

2.2.2.1. Notes on early and recent studies of hippocampal neurogenesis

The belief that neurogenesis is restricted to the early stages of embryogenesis in higher vertebrates was first challenged in the 1960s when Joseph Altman and Gopal Das conducted a series of studies in rodents and cats reporting observations of neurogenesis. Altman and Das used radiolabeled [3-H-]-thymidine and light microscopy to detect newly generated cells in the forebrain, including in the DG of the hippocampus (Altman, 1962, 1963; Altman & Das, 1965, 1967). Although

initial claims were published in prestigious journals at the time and more data emerged substantiating the claim for adult neurogenesis (Kaplan & Hinds, 1977; Bayer, 1982; Bayer *et al.*, 1982; Kaplan & Bell, 1984), these early findings were not universally accepted and instead ignored (Gross, 2000; Bayer, 2016). The debate about the presence of adulthood neurogenesis in mammals continued for decades since the first reports were published (Rakic, 1985; Eckenhoff & Rakic, 1988) until more advanced methods emerged in the 1990s when thymidine analog bromodeoxyuridine (BrdU) was introduced in neurogenesis studies (Kuhn *et al.*, 1996). BrdU is incorporated into the DNA of proliferating cells during the DNA synthesis phase (S-phase). Combining BrdU immunohistochemical labeling with the immunohistochemical detection of a neuron-specific antigen and confocal microscopy weeks after BrdU incorporation into proliferating cells allows the identification of newly generated cell types. Specifying the fate of newly generated cells is necessary for neurogenesis studies, as not all newly generated cells in the brain differentiate into neurons (Kuhn *et al.*, 2016).

Using BrdU in combination with the detection of neuron-specific antigens, adult hippocampal neurogenesis was confirmed in rodents (Kuhn *et al.*, 1996), macaque monkeys (Kornack & Rakic, 1999) and in humans (Eriksson *et al.*, 1998). Although BrdU is not suitable for conducting interventional clinical studies investigating neurogenesis in humans due to toxicity and the need for accessing post-mortem brain tissue, the seminal study by Eriksson and colleagues (1998) used post-mortem brain tissue from cancer patients who had been administered BrdU for tumor progression tracking purposes. More support for neurogenesis in adult humans was provided by a study using nuclear-bomb-test-derived ^{14}C in genomic DNA, which estimated that about 700 new neurons are added in each hippocampus per day and one-third of hippocampal neurons are subject to change during their lifetime (Spalding *et al.*, 2013). Although neurogenesis has been shown to decline with age, studies using immunohistochemical methods have found proliferating cells and neural progenitor cells/neuroblasts/young neurons throughout the life span in humans up to 100 years of age (Knoth *et al.*, 2010; Boldrini *et al.*, 2018; Moreno-Jiménez *et al.*, 2019). Numerous publications investigating the regulation and function of adult hippocampal neurogenesis in mammals followed (Kuhn *et al.*, 1996; Kempermann *et al.*, 1997; Brezun & Daszuta, 1999; Gould *et al.*, 1999; Gould & Tanapat, 1999; van Praag *et al.*, 1999) and the dogma of static adult brain without neurogenesis was overturned.

Despite the above-described evidence, the presence of human adult neurogenesis has been recently challenged. Some reports using post-mortem brain samples have suggested that neurogenesis in the human hippocampus is present only in childhood and ceases to exist or reduces to almost undetectable levels in adulthood (Dennis *et al.*, 2014; Cipriani *et al.*, 2018; Sorrells *et al.*, 2018), contradicting other studies demonstrating adult hippocampal neurogenesis using similar methodology (Boldrini *et al.*, 2018; Moreno-Jiménez *et al.*, 2019). Since assessing neurogenesis in humans has been limited by using postmortem tissue, the majority of knowledge about the roles and regulation of neurogenesis in health and disease states is based on studies using rodent models, which allow tracking

new newly generated neurons and introduction of various manipulations (Kuhn *et al.*, 2016; Toda *et al.*, 2019). New methods enabling the live-tracking of neurogenesis could shed more light on the quantity, regulation, and time course of neurogenesis in humans (Toda *et al.*, 2019).

2.2.2.2. Stages of neurogenesis and survival of newly generated cells in the DG of the hippocampus

The stages and markers for hippocampal neurogenesis in adulthood are presented in Figure 2. Adult newborn neurons in the hippocampus originate from **radial glia-like stem cells (type 1)** located in the subgranular zone, a narrow area between the granule cell layer and the hilus. Radial glia-like stem cells resemble astroglia in their morphology and express astroglial marker glial fibrillary acidic protein (GFAP), in addition to stem cell markers sex-determining region Y box2 (SOX2) and nestin (Eckenhoff & Rakic, 1984; Seri *et al.*, 2001; Moss & Toni, 2013; Kempermann *et al.*, 2015; Denoth-Lippuner & Jessberger, 2021). These stem cells are in a quiescent, non-proliferating state, sustained by local tonic GABAergic input from parvalbumin-expressing interneurons (basket cells). Parvalbumin interneurons can suppress neurogenesis during periods of high network activity and induce neurogenesis during low network activity (Song *et al.*, 2012; Moss & Toni, 2013). When activated, radial glia-like stem cells give rise to transiently amplifying **neural progenitors (type 2)**, which have high proliferative capacity and lack the characteristic morphology of radial glial cells. In addition to neurons, radial glia-like cells produce a small number of astrocytes (Pilz *et al.*, 2018). If a cell fate decision is made, it is made at the stage of type 2 cells. **Type 2a** cells express astroglial cell markers GFAP and, SOX2 and nestin. Type 2a cells progress into **type 2b** cells, which express immature neuronal markers polysialylated-neural cell adhesion molecule (PSA-NCAM) and doublecortin, neuronal markers NeuroD1 and Prospero homeobox protein 1 (PROX1), as well as nestin. Neuronal progenitor cells give rise to **proliferating neuroblasts (type 3**) that have low proliferative activity and do not express stem cell marker nestin but express aforementioned (immature) neuronal markers (Kempermann *et al.*, 2015). During the first week of development in rodents, young neuroblasts migrate first tangentially away from their mother progenitor cells along blood vessels and then radially for a short distance toward the inner granule cell layer (GCL) (Kempermann *et al.*, 2003; Espósito *et al.*, 2005; Sun *et al.*, 2015). The expression of PSA-NCAM has been associated with increased migration potential, as PSA is a large molecule that exhibits steric effects on the cell surface and destabilizes contacts between cells (Brusés & Rutishauser, 2001; Aonurm-Helm *et al.*, 2016).

Neuroblasts differentiate into **immature neurons**, which exit the cell cycle and start to express post-mitotic neuronal marker neuronal nuclear antigen (NeuN) and the transient marker calretinin (Brandt, *et al.*, 2003). These immature neurons undergo neurite growth; their axons extend through the hilus to the target area CA3, and their dendrites extend through the GCL (Toda & Gage, 2018).

During the early phase of newborn neuronal maturation, immature neurons receive mainly ambient, extrasynaptic GABAergic input from local interneurons. GABAergic input is initially excitatory and causes depolarization of newborn neurons, due to high cytoplasmic Cl- content, before it switches to inhibition and hyperpolarization. This ambient GABAergic excitation promotes dendritic maturation and synaptic integration of immature neurons (Tozuka *et al.*, 2005; Ge *et al.*, 2006; Toni & Schinder, 2015; Denoth-Lippuner & Jessberger, 2021). Approximately 12–14 days after cell birth, the first dendritic synapses are formed on immature neurons. Newborn cells will initially receive synaptic GABAergic input from local interneurons in the molecular layer shortly before they receive glutamatergic synaptic inputs from long-range projection neurons from the entorhinal cortex (Espósito *et al.*, 2005; Wadiche *et al.*, 2005; Ge *et al.*, 2006; Markwardt *et al.*, 2009). When newborn granule cells form first dendritic synapses, they also form axonal synaptic connections with pyramidal cells in the CA3 area and hilar cells and release glutamate as their primary neurotransmitter (Toni *et al.*, 2008).

Newborn neurons become fully structurally integrated into the tri-synaptic circuit within four weeks (Kempermann *et al.*, 2015). At this time, they switch their calcium-binding protein from calretinin to calbindin (Brandt, *et al.*, 2003) and stop expressing doublecortin and PSA-NCAM (von Bohlen und Halbach, 2007). Dendritic remodeling continues, however (Gonçalves, Bloyd, *et al.*, 2016) and 4–6 week-old newborn neurons are distinguishable from their older counterparts by differential electrophysiological properties. More specifically, **4–6-week-old newborn neurons are highly excitable** and have a low threshold for plasticity-inducing potentiation, such as LTP, in comparison to mature granule cells (Wang *et al.*, 2000; Schmidt-Hieber *et al.*, 2004; Ge *et al.*, 2007). Weak afferent activity is sufficient to activate newborn cells with relatively low input specificity at this time. After six weeks of age, the threshold for activation increases, and their input responses become more specific. From about 7–8 weeks of age, the electrophysiological properties and morphological measures, such as dendritic complexity and spine density of adult-born neurons, become similar to other mature granule cells (Denoth-Lippuner & Jessberger, 2021).

Only a part of newly generated neurons become fully integrated into the networks and survive, while most die within the first three weeks of their birth (Denoth-Lippuner & Jessberger, 2021). Two specific phases of cell survival and elimination can be distinguished, during which approximately 60% of cells undergo cell death. The first elimination phase occurs within 1–4 days after progenitor cell division due to apoptosis (Sierra *et al.*, 2010; Pilz *et al.*, 2018), and apoptotic cells are eliminated via microglial phagocytosis (Sierra *et al.*, 2010). Apoptosis at this stage depends on BAX, a protein that mediates mitochondria-dependent programmed cell death (Sun *et al.*, 2004). The second elimination takes place approximately 13–18 days after the birth of new neurons and coincides with the start of their synaptic integration (Pilz *et al.*, 2018). The successful survival at this maturational stage is dependent on the glutamatergic input

via N-methyl-D-aspartate (NMDA) receptors (Tashiro *et al.*, 2006) and brain-derived neurotrophic factor (BDNF) signaling (Sairanen *et al.*, 2007).

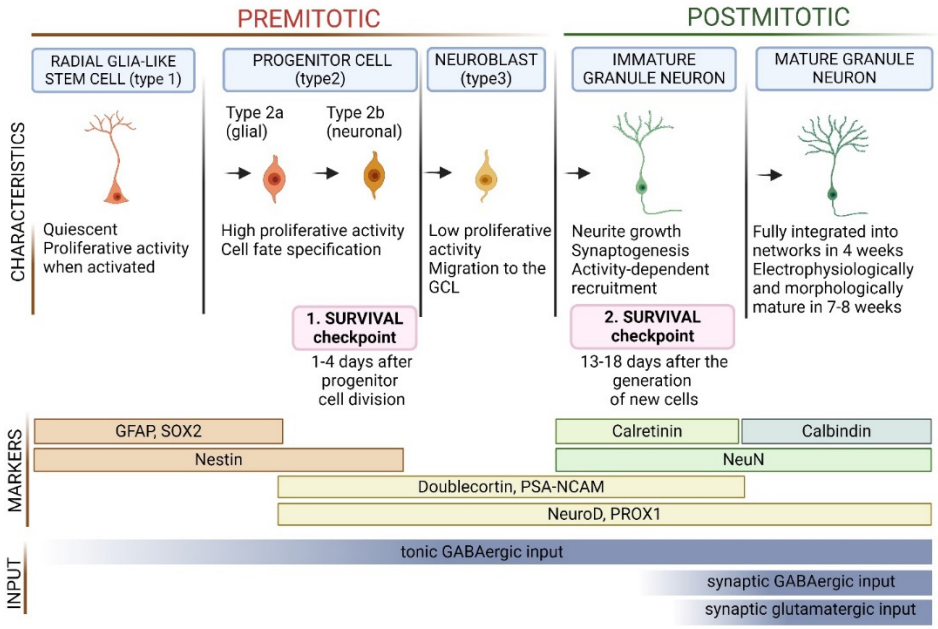

Figure 2. Stages and markers for hippocampal neurogenesis in the adulthood. New neurons in the dentate gyrus of the hippocampus originate from radial glia-like stem cells, which are typically in a quiescent state and proliferate when activated. Radial glial cells (type 1 cells) exhibit stem cell markers sex-determining region Y box2 (SOX2), nestin, and astroglial marker glial fibrillary acidic protein (GFAP). Radial glial cells give rise to neural progenitor cells exhibiting high proliferative activity. During this stage, cell fate specification may take place, as type 2a exhibit glial properties and type 2b cells start to show markers for immature neurons, such as doublecortin, polysialylated neural cell adhesion molecule (PSA-NCAM) as well as neuronal markers NeuroD and Prospero homeobox protein 1 (PROX1). The first survival checkpoint of newly generated neurons occurs 1–4 days after progenitor cell division. Neural progenitor cells give rise to neuroblasts (type 3) with low proliferative activity, which migrate from their original location from the subgranular zone to the inner layers of the granule cell layer (GCL); these cells do not express anymore nestin. Neuroblasts differentiate into postmitotic immature granule neurons, which start to express calretinin and neuronal nuclear antigen (NeuN). This stage is characterized by neurite growth, synaptogenesis, and activity dependent-recruitment to neural networks, as the cells begin to receive synaptic GABAergic input shortly before they receive glutamatergic input, in addition to extrasynaptic tonic GABAergic input. The second survival checkpoint occurs in immature granule neurons approximately 13–18 days after the generation of new cells. Four weeks after the generation of new neurons, mature granule neurons will become fully integrated into networks and switch from calretinin to calbindin expression. 7–8-week-old granule neurons become electrophysiologically and morphologically equivalent to their older counterparts. The image was created with Biorender.

2.2.2.3. Functional significance of adult neurogenesis

Young adult-born hippocampal neurons exhibit a time-limited critical period of increased synaptic plasticity and lower activation thresholds in comparison to mature granule cells, which allows them to make unique contributions to hippocampus-dependent memory functions, behaviors, and information processing. Electrophysiological studies and computational models suggest that young neurons may **encode contextual and temporal information** during their critical period of hyperexcitability (Anacker & Hen, 2017) and be preferentially activated during learning (Kee *et al.*, 2007). Data from rodent studies show that neurogenesis indeed is needed for performance in hippocampus-dependent learning tasks since reducing or blocking hippocampal neurogenesis in rodents impairs fear conditioning (Saxe *et al.*, 2006), long-term spatial memory formation, and spatial learning (Snyder *et al.*, 2005). Another important hippocampal function that newborn neurons contribute to during their critical period is behavioral **pattern separation**, which allows distinguishing similar contexts and environments and object location pairing. The role of hippocampal neurogenesis in pattern separation is evidenced by studies demonstrating impaired or improved ability to discriminate highly similar contexts when neurogenesis is ablated or enhanced, respectively (Clelland *et al.*, 2009; Sahay *et al.*, 2011; França *et al.*, 2017; Toda & Gage, 2018; Denoth-Lippuner & Jessberger, 2021; Kempermann, 2022; Surget & Belzung, 2022). In addition to pattern separation, neurogenesis has been implicated in promoting **relearning** and clearance of previously established memories (Toda & Gage, 2018). The continuous addition of new neurons remodels hippocampal circuits. It is proposed to lead to forgetting of established memories, evidenced by rodent studies where inducing adult neurogenesis after the formation of a new memory mitigated forgetting (Akers *et al.*, 2014). Conversely, decreasing neurogenesis stabilizes existing memories (Epp *et al.*, 2016). Finally, adult hippocampal neurogenesis correlates with **novelty seeking** in rodents (Lemaire *et al.*, 1999; Weeden *et al.*, 2019; Kempermann, 2022), and newborn neurons are involved in biasing the attention toward novelty seeking (Weeden *et al.*, 2019).

2.2.2.4. Regulation of adult neurogenesis in the hippocampus

The regulation of adult neurogenesis can occur at various stages of neurogenesis, such as proliferation, neuronal differentiation, and cell survival. Neurogenesis can be regulated by environmental factors, emotional/physiological states (Figure 3), molecules in the local microenvironment, and cell-intrinsic mechanisms. Environmental factors and emotional and physiological states can alter the local microenvironment, more specifically the extracellular growth factors, such as the BDNF and the insulin-like growth factor 1 (IGF-1), neurotransmitters, such as serotonin, dopamine, and GABA, adhesion molecules, hormones and cytokines in the brain neurogeneic regions (Gonçalves, Schafer, *et al.*, 2016; Toda & Gage, 2018; Toda *et al.*, 2019; Abbott & Nigussie, 2020; Araki *et al.*, 2021). Alterations

of these molecules in local the microenvironment can then induce changes in cell-intrinsic signaling pathways, transcription factors, and epigenetic mechanisms, which manifest in altered neurogenesis (Toda & Gage, 2018; Niklison-Chirou *et al.*, 2020).

Emotional and physiological states can affect hippocampal neural plasticity either positively or negatively. Rodent studies have demonstrated that **voluntary running** increases the proliferation of neural stem or progenitor cells (van Praag *et al.*, 1999; Olson *et al.*, 2006) and that **environmental enrichment** improves the survival of newly generated cells through increased BDNF-mediated integration of immature neurons (Kempermann *et al.*, 1997; Olson *et al.*, 2006; Rossi *et al.*, 2006). Interestingly, hippocampus-dependent associative learning tasks, such as the Morris water maze, can also enhance neurogenesis by improving the survival of newly generated neurons (Gould *et al.*, 1999; Leuner *et al.*, 2004).

Although hippocampal neurogenesis persists throughout life, it decreases significantly during **aging**. Age-related decline in neurogenesis is mainly attributable to reduced proliferation of neural progenitor cells (Kuhn *et al.*, 1996) and reaches a lower threshold in mice already before middle age, around nine months of age (Ben Abdallah *et al.*, 2010; Yang *et al.*, 2015). Another factor that regulates neurogenesis negatively is **chronic stress** and corticosterone, the principal stress hormone in rodents. Chronic stress has been shown to impair neurogenesis at different stages, such as proliferation, differentiation, and long-term survival (Malberg & Duman, 2003; Jayatissa *et al.*, 2006; Mineur *et al.*, 2007; Levone *et al.*, 2015). Moreover, early life stress, such as maternal separation, can have long-term effects on neurogenesis and reduce cell proliferation and immature neuron production in the DG in adulthood (Mirescu *et al.*, 2004) and middle-age period (Suri *et al.*, 2013), possibly via epigenetic mechanisms (McEwen *et al.*, 2016).

Drugs of abuse can modulate neurogenesis. Most addictive drugs decrease the proliferation or survival rate of neuronal progenitors, resulting in global deficits in hippocampal plasticity, but not always (Kang *et al.*, 2016). Most often, repeated administration of cocaine reduces cellular proliferation and neurogenesis immediately or soon after the cocaine administration protocol ends (Yamaguchi *et al.*, 2004; Andersen *et al.*, 2007; Castilla-Ortega *et al.*, 2016). However, cocaine-induced reduction in neurogenesis appears temporary, and hippocampal neurogenesis normalizes or even increases during the withdrawal period following drug administration (Maćkowiak *et al.*, 2005; Noonan *et al.*, 2008; Castilla-Ortega *et al.*, 2016). Similarly, chronic opioid use has been shown to reduce cellular proliferation in the rat DG (Eisch *et al.*, 2000). Adolescents might be more vulnerable to substance abuse-related abnormalities in hippocampal neurogenesis, as repeated exposure to alcohol in adolescent rodents induced long-lasting reduction in hippocampal neurogenesis, while alcohol use in adulthood did not result in reduced neurogenesis (Spear, 2018). While generally, alcohol consumption is negatively correlated with hippocampal neurogenesis, the effects of alcohol on neurogenesis can vary with dosage intake pattern and the duration of exposure (Kang *et al.*, 2016). Interestingly, a single

high dose of alcohol in the early postnatal period has been shown to induce aberrant cellular proliferation in the DG (Zharkovsky *et al.*, 2003).

Brain diseases, such as **depression** (Boldrini *et al.*, 2009) and **Alzheimer's disease** (Moreno-Jiménez *et al.*, 2019; Berger *et al.*, 2020) are associated with decreased adult hippocampal neurogenesis. In contrast, **epileptic seizures** can induce aberrant neurogenesis, characterized by a dramatic increase in cell proliferation and net neurogenesis, abnormal dendritic growth, and dysfunctional integration of newly generated neurons (Jessberger & Parent, 2015).

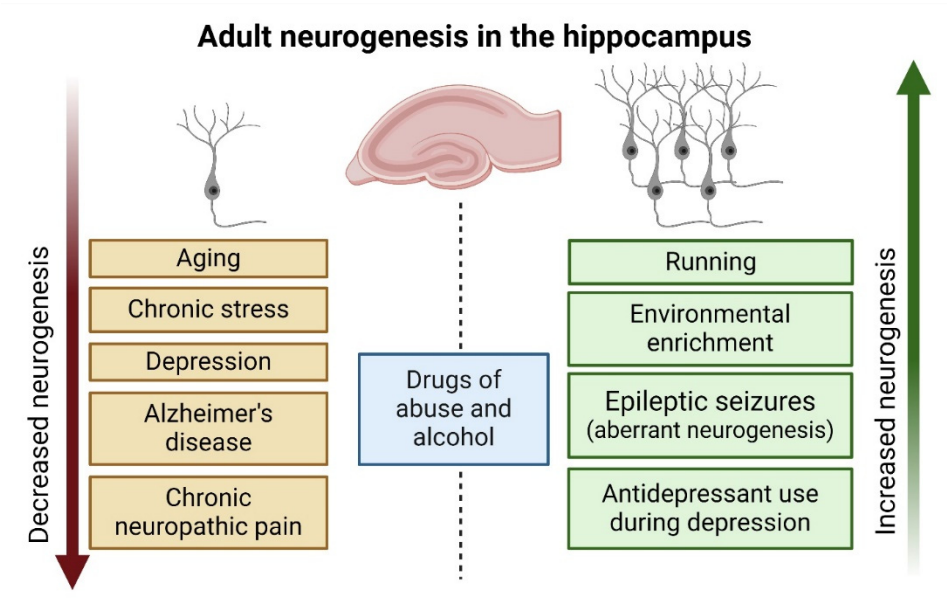

Figure 3. The effect of emotional, psychological/disease states and environmental factors on adult neurogenesis in the dentate gyrus of the hippocampus. The image was created with Biorender.

2.3. Dysfunctional brain plasticity in depression and cognitive decline

2.3.1. Depression, anxious distress, cognitive impairment, and impaired hippocampal plasticity

Depressive and anxiety disorders are major contributors to disease burden and are among the two most disabling mental disorders worldwide. The prevalence of major depressive disorder, referred to as depression, is estimated to be 3.8% of the population, including 5% of adults, 6% among women, 4% among men, and 5.7% in adults older than 60 years (Santomauro *et al.*, 2021; GBD 2019 Mental Disorders Collaborators, 2022; World Health Organization, 2023), demonstrating a higher prevalence of depression in women than in men, and in

older adults than in general population (Bromet *et al.*, 2011; Hasin *et al.*, 2018; Abdoli *et al.*, 2022). Depression is a mental disorder characterized by negative affective biases, meaning that depressed individuals preferentially focus on and remember negative social and affective information while they disregard positive information (Gotlib & Joormann, 2010). The primary symptom of major depressive disorder is depressed mood or loss of interest or pleasure, present for two weeks or more. Additional symptoms include non-somatic symptoms, such as a sense of worthlessness or excessive guilt, impaired ability to think, recurrent thoughts of dying or suicidal idealization, and somatic symptoms, such as fatigue, sleep disturbances, weight change, and psychomotor changes (American Psychiatric Association, 2013; Uher *et al.*, 2014). Major depressive disorder can be further specified into subsequent subtypes, one of which is associated with anxious distress. Most (74.6%) major depressive disorder episodes are associated with anxious distress, described by feeling tensed, unusual restlessness, trouble concentrating due to excessive worry, fear of something awful happening, and fear of losing control (American Psychiatric Association, 2013; Hasin *et al.*, 2018). Moreover, a strong link and high comorbidity between depression and anxiety disorder exist, as estimations suggest that 90% of patients with anxiety disorder have depression (Tiller, 2013). One of the secondary symptoms of depression is cognitive impairment, described by the inability to learn, remember, think, concentrate, and make decisions (American Psychiatric Association, 2013; Uher *et al.*, 2014). Memory impairment, especially in the elderly population, is common in depressive patients (James *et al.*, 2021).

In animal models of depression, depressive-like behaviors often co-occur with increased anxiety behaviors and impairments in memory (La Porta *et al.*, 2016; Mohamed *et al.*, 2020; Xu *et al.*, 2022), suggesting a common underlying neuropathological basis. In basic neuroscience research, depressive-like behaviors and chronic stress have been associated with structural changes, such as neuronal atrophy, due to synaptic loss and decreased neuronal size in the hippocampus and medial prefrontal cortex (Campbell *et al.*, 2004; Duman, 2009; Malykhin *et al.*, 2010; McEwen & Morrison, 2013; Price & Duman, 2020). Findings from rodents and depression patients suggest that atrophy of the dendrites in the CA1 and CA3 and reduced neurogenesis in the DG of the hippocampus may underlie the observed decrease in hippocampal volumes (Duman, 2004; Dranovsky & Hen, 2006; Huang *et al.*, 2013; Samuels *et al.*, 2015). Some depression-related hippocampal plasticity alterations, such as reduced neurogenesis, synaptic plasticity, and atrophy, are also associated with age-related cognitive decline (Bettio *et al.*, 2017).

Depression has also been characterized by decreased levels of monoamines, particularly serotonin (Tartt *et al.*, 2022; Moncrieff *et al.*, 2023), and reduced levels of neurotrophins, such as the BDNF (Neto *et al.*, 2011) in the brain. BDNF signaling is a critical mediator of activity-dependent synaptic plasticity (Castrén & Monteggia, 2021) and is involved in neurogenesis by promoting maturation and long-term survival of adult-born neurons (Sairanen *et al.*, 2007; Bergami *et al.*, 2008; Wang *et al.*, 2015). Therefore, a decrease in BDNF levels could impair

synaptic plasticity and neurogenesis. Preclinical studies have demonstrated impaired neurogenesis in rodent models of depression involving a stressful manipulation (Malberg & Duman, 2003; Van Bokhoven *et al.*, 2011; de Andrade *et al.*, 2013; Du Preez *et al.*, 2021), olfactory bulbectomy (Jaako-Movits & Zharkovsky, 2005; Song & Leonard, 2005; Jaako-Movits *et al.*, 2006) and chronic neuropathic pain (Terada *et al.*, 2008; Mutso *et al.*, 2012; Tyrtyshnaia *et al.*, 2017, 2019). The hypothesis that reduced production of new neurons might contribute causally to the development of depressive behaviors is supported by studies investigating the effects of typical slow-acting antidepressants (Schoenfeld & Cameron, 2015). Slow-acting antidepressants can restore hippocampal neurogenesis by increasing the proliferation of neural stem or progenitor cells (Malberg *et al.*, 2000; Czéh *et al.*, 2001; Jaako-Movits *et al.*, 2006; Surget *et al.*, 2008) and the differentiation, maturation, and integration of new neurons into the DG parallels the timing of antidepressant effect onset in rodents (Schoenfeld & Cameron, 2015). Moreover, the efficacy of some antidepressants is dependent on their neurogenesis-restoring effects in rodent models of depression (Santarelli *et al.*, 2003; Surget *et al.*, 2008). In addition to antidepressants, electroconvulsive shock treatment, a clinically effective method of treating depression, increases neurogenesis in adult rodents (Madsen *et al.*, 2000; Scott *et al.*, 2000). Chronic fluoxetine treatment restores stress-induced reduction of neurogenesis, and electroconvulsive shock treatment improves neurogenesis also in non-human primates (Perera *et al.*, 2007, 2011). Due to technical limitations, less is known about the role of human hippocampal neurogenesis in depression. However, a study using post-mortem human brain tissue has demonstrated fewer neural progenitor cells in the hippocampus of depressed patients. Moreover, treatment with selective serotonin reuptake inhibitors and tricyclic antidepressants was associated with an increased number of neural progenitor cells in depressed patients (Boldrini *et al.*, 2009).

2.3.2. The role of neuroinflammation in impaired brain plasticity, depression, and cognitive impairment

Depression is more prevalent in patients with inflammation-associated diseases, such as type 2 diabetes, rheumatoid arthritis, and cardiovascular disease (Dantzer *et al.*, 2008; Steffen *et al.*, 2020), suggesting a link between depression and peripheral inflammation. Numerous studies have also demonstrated a correlation between depression and increased levels of pro-inflammatory cytokines. More specifically, heightened levels of interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) have been found in the cerebrospinal fluid, peripheral blood, and brain parenchyma of depression patients (Maes *et al.*, 1995, 1997; Dowlati *et al.*, 2010; Enache *et al.*, 2019; Himmerich *et al.*, 2019). A number of studies associate interferon-α, a pro-inflammatory cytokine treatment, with an increased rate of depression (Renault *et al.*, 1987; Miyaoka *et al.*, 1999; Capuron *et al.*, 2002), indicating that inflammation could be a risk factor for depression. Indeed, pre-

clinical studies have shown that immune challenge, such as systemic administration of the bacterial endotoxin lipopolysaccharide (LPS), causes depressive-like "sickness behavior" and microglial activation (Biesmans *et al.*, 2013; Hoogland *et al.*, 2015).

Peripheral inflammation can induce neuroinflammation via pro-inflammatory mediators that disrupt blood-brain barrier (BBB), cytokines that cross the BBB to a limited extent (Gutierrez *et al.*, 1993; Banks, 2005; Yarlagadda *et al.*, 2009), activation of endothelial cells (Liu *et al.*, 2019), or by autonomic nerves via the organ-brain axis (Dantzer *et al.*, 1998; Sun *et al.*, 2022). The term neuroinflammation has been used to describe various pathological conditions, ranging from alterations in the glial cell morphology to the invasion and damaging effects of blood-borne leukocytes (Becher *et al.*, 2017). Most often, neuroinflammation in the central nervous system (CNS) results from local tissue response triggered by infection, injury, abnormal protein aggregates, and severe or chronic stress, and neuroinflammation can also be related to the normal aging process. The local tissue response is mediated mainly by the brain-resident macrophages, i.e., microglial cells, but also astrocytes, oligodendrocytes, and endothelial cells. Microglial cells are known to exhibit both beneficial and neuroprotective effects, such as providing trophic support to neurons and synaptic pruning, as well as detrimental and neurotoxic effects, depending on conditions. Upon pro-inflammatory activation, microglia can change their morphology from ramified to amoeboid shape, secrete pro-inflammatory cytokines, prostaglandins, and reactive oxygen species (Yirmiya & Goshen, 2011; Becher *et al.*, 2017; Cornell *et al.*, 2021; Troubat, Barone, *et al.*, 2021).

Pro-inflammatory mediators in the CNS can lead to synaptic plasticity impairments and decreased neurogenesis, which have been associated with learning, memory deficits, and depressive-like behavior in rodents (Yirmiya & Goshen, 2011; Troubat, Barone, *et al.*, 2021; Wu & Zhang, 2023). Microglial activation upon LPS administration has been shown to reduce neurogenesis, while pharmacological inhibition of microglial activation using minocycline restored neurogenesis (Ekdahl *et al.*, 2003). Inhibition of microglial activation by minocycline also has alleviated depressive-like symptoms and restored decreased hippocampal neurogenesis in a chronic-mild stress rodent model (Bassett *et al.*, 2021). Several preclinical studies have indicated a direct role for IL-1β, a pro-inflammatory cytokine secreted by microglia and astrocytes, in reducing neurogenesis, mediating depressive-like behavior (Goshen *et al.*, 2008; Koo & Duman, 2008; Mendiola & Cardona, 2018), and causing memory impairments by disrupting synaptic plasticity (Barrientos *et al.*, 2002; Cunningham & Sanderson, 2008).

Despite the described evidence suggesting a causal role for microglial activation and neuroinflammation in depressive-like behavior in animal models, anti-inflammatory medications have not proven efficacy for the treatment of depression, and the direct causal role of neuroinflammation in depression is not clear (Hodes *et al.*, 2015; Troubat, Barone, *et al.*, 2021; Hassamal, 2023). Similarly, daily treatment with non-steroidal anti-inflammatory drugs has shown no benefit for dementia prevention (Jordan *et al.*, 2020).

2.3.3. Role of selected causal factors in dysfunctional brain plasticity

2.3.3.1. Chronic neuropathic pain

Pain is defined as: "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" by The International Association for the Study of Pain (Raja *et al.*, 2020). While nociception and acute pain are physiological processes of the nervous system, chronic pain, lasting more than 12 weeks, is a maladaptive process. Chronic pain is triggered by pathophysiological factors, such as neural injury, viral infection, inflammation, or exposure to neurotoxins, and is accompanied by aberrant somatosensory processing in the peripheral and central nervous system (Burma *et al.*, 2017; Kuner & Flor, 2017). Neuropathic pain, caused by a lesion or disease of the somatosensory nervous system, is more likely to become chronic than nociceptive pain. Pathological manifestations of neuropathic pain include allodynia, i.e., pain sensation by normally non-noxious stimuli; hyperalgesia, i.e., exaggerated pain response to a noxious stimulus; and spontaneous pain, which occurs in the absence of an overt stimulus (Baron *et al.*, 2010; Jensen *et al.*, 2011; Cohen & Mao, 2014; Burma *et al.*, 2017; Vasic & Schmidt, 2017).

Chronic pain, particularly chronic neuropathic pain, is often co-morbid with depression and anxiety (Radat *et al.*, 2013; Shaygan *et al.*, 2013; Cohen & Mao, 2014). Co-morbidity of depression and chronic pain is related to pain and depression treatment outcomes, as chronic pain patients co-morbid with depression exhibit poorer responses to pain treatment than non-depressed patients (Doan *et al.*, 2015; Sheng *et al.*, 2017). Moreover, depressed patients co-morbid with chronic pain demonstrate fewer benefits from antidepressant use than those without chronic pain (Roughan *et al.*, 2021). Preclinical rodent studies suggest that neuropathic injury can cause anxiety-like and depressive-like behaviors, as well as memory impairments (Narita *et al.*, 2006; Yalcin *et al.*, 2011; Alba-Delgado *et al.*, 2013; da Silva *et al.*, 2020). The underlying mechanisms connecting chronic neuropathic pain and the development of anxiety and depression are, however, not yet fully understood and further studies are needed in order to elucidate the cellular and molecular mechanisms connecting neuropathic pain, anxiety and depression.

Neuropathic pain induces long-term changes in the plasticity along the sensory pathways from the peripheral to the central nervous system. Nociceptors and projection neurons in the dorsal horn of the spinal cord become sensitized and hyperexcitable; the descendent excitatory pathway of pain strengthens, and the descending inhibitory pathway of pain weakens. Underlying these functional events lie molecular plasticity alterations such as the overexpression of voltage-gated ion channels, an increase of pain sensation-producing chemical (algogen) receptors, and elevated synthesis/release of neurotransmitters, such as glutamate, substance P, calcitonin gene-related peptide and adenosine triphosphate (ATP) (Boadas-Vaello *et al.*, 2016; McCarberg & Peppin, 2019). These functional

alterations are accompanied by structural remodeling and reorganization of synapses in spinal dorsal horn neurons, which are causally associated with nociceptive hypersensitivity (Kuner & Flor, 2017).

Alterations in plasticity in the peripheral nervous system and spinal cord due to chronic neuropathic pain can, in turn, lead to changes in the projection pathways to the prefrontal cortex, hippocampus, and amygdala (Kuner & Flor, 2017; Sheng *et al.*, 2017; Mazza *et al.*, 2018). Chronic pain can disrupt brain structural and functional connectivity (Baliki *et al.*, 2008; Mutso *et al.*, 2014; Kuner & Flor, 2017) and cause brain atrophy, as back pain patients have decreased gray matter volume in the hippocampus (Mutso *et al.*, 2012), thalamus and anterior cingulate cortex (Apkarian *et al.*, 2004). Moreover, in chronic neuropathic pain patients, a shift from brain activation patterns from regions involved in nociceptive processing to regions related to emotional processing takes place (Hashmi *et al.*, 2013; Kuner & Flor, 2017). Alterations in neural plasticity in the brain regions related to mood and emotion regulation, such as the hippocampus, amygdala, and anterior cingulate cortex, may be crucial for the development of depression and anxiety (Mutso *et al.*, 2012, 2014; Yalcin *et al.*, 2014; Barthas *et al.*, 2015; Ru *et al.*, 2022).

Previous reports have demonstrated dysfunctional hippocampal plasticity in rodent models of chronic neuropathic pain, characterized by decreased neurogenesis and impaired synaptic plasticity in the hippocampus. However, the disrupted neurogenesis stage has varied depending on specific studies (Duric & McCarson, 2006; Mutso *et al.*, 2012; Dellarole *et al.*, 2014; Romero-Grimaldi *et al.*, 2015) and the disrupted process of hippocampal neurogenesis as well as molecular mechanisms leading to it are not completely clear. In addition to impaired neurogenesis, increasing number of preclinical studies suggests that peripheral nerve injury induces neuroinflammation in the brain, characterized by supraspinal changes in cytokine levels and glial reactivity. Moreover, neuroinflammation in the brain has been shown to contribute to the pathogenesis of depressive-like behavior and co-occurring memory deficits in chronic neuropathic pain rodent models (Vallejo *et al.*, 2010; Dellarole *et al.*, 2014; Gui *et al.*, 2016; Xie *et al.*, 2017). Specifically, peripheral neuropathic pain has been shown to induce overproduction of IL-1β, which has been demonstrated to contribute to the development of depressive-like behavior and memory deficit in a mouse model of chronic neuropathic pain (Gui *et al.*, 2016).

2.3.3.2. Vitamin D deficiency (VDD)

VDD is a worldwide health concern, estimated to affect about 15.7% of the global population, when a strict definition of deficiency, 25(OH)D <30 nmol/L, is used (Cui, Zhang, *et al.*, 2023). Vitamin D is naturally acquired primarily by ultraviolet B-irradiation-induced synthesis in the skin (vitamin D3, i.e., cholecalciferol) and to a lesser extent by food (vitamin D2, i.e., ergocalciferol and D3) (Holick, 2007; Cashman, 2020). Vitamin D is metabolized in a multi-step process to produce the active form. Firstly, vitamin D is hydroxylated in the liver to 25-hydroxyvitamin

D (25-(OH)D), followed by another hydroxylation step in the kidneys, producing the active form 1,25-dihydroxyvitamin, 1,25-$(OH)_2$D, which acts in genomic or non-genomic manner (Lehmann & Meurer, 2010).

While VDD is prevalent in all age groups, pregnant women, individuals with increased skin melanin pigmentation, obese individuals, and those who practice abstinence from direct sun exposure are at a high risk for VDD (Holick, 2017). Another risk group for VDD is older adults due to the impaired ability of their skin to synthesize vitamin D, a decrease in mobility or residential care resulting in less sun exposure (Meehan & Penckofer, 2014), and reduced gastrointestinal absorption of vitamin D (Boucher, 2012). VDD in older adults has been associated with a heightened risk of developing cognitive impairments and depression (Meehan & Penckofer, 2014; Ceolin *et al.*, 2022). It has even been proposed that prolonged VDD may accelerate brain aging and the development of age-related disorders (Berridge, 2017; Terock *et al.*, 2022). In addition to depression and age-related cognitive decline, previous reports have associated VDD with various brain diseases, such as Alzheimer's disease, multiple sclerosis, schizophrenia, attention deficit disorder, and autism spectrum disorder (Koduah *et al.*, 2017; Mayne & Burne, 2019). While systematic reviews and meta-analyses of observational studies have found an association between VDD and cognitive impairment, dementia, and depression (Etgen *et al.*, 2012; Anglin *et al.*, 2013; Sommer *et al.*, 2017), data from randomized controlled trials have failed to present clear evidence supporting the use of vitamin D in depression or for the prevention of cognitive decline/dementia (Dean *et al.*, 2011; Rossom *et al.*, 2012; Annweiler *et al.*, 2013; Gowda *et al.*, 2015). Therefore, more information is needed to clarify if VDD could be the culprit or secondary to co-occurring cognitive impairment and depression.

Molecular mechanisms mediating the possible effects of VDD during cognitive impairment and depression are not yet fully elucidated. The presence of nuclear vitamin D receptor (VDR) mediating the main genomic effects of vitamin D, membrane receptor of vitamin D (Pdia3) mediating fast-acting non-genomic effects suggested to be related to calcium transport, and metabolizing enzymes in the brain suggest its local autocrine and paracrine properties (Nemere *et al.*, 1994, 2004; Eyles *et al.*, 2005; Landel *et al.*, 2018; Liu *et al.*, 2021). The high density of VDR in limbic structures, such as the hippocampus and amygdala, and the cerebellum, cortex, caudate putamen, and hypothalamus, implies possible functions of vitamin D in cognition, motivational behavior, and stress regulation, which are attributed to aforementioned brain regions (Stumpf *et al.*, 1982; Prüfer *et al.*, 1999; Taniura *et al.*, 2006; Wang *et al.*, 2012; Liu *et al.*, 2021). Several physiologically relevant functions in the brain have been attributed to vitamin D, which might become impaired during VDD. Vitamin D has been shown to promote neuronal differentiation and provide trophic support by inducing the production of growth factors and neurotrophins, such as the nerve growth factor, BDNF, and neurotrophin-3 (Neveu *et al.*, 1994; Naveilhan *et al.*, 1996; Orme *et al.*, 2013; Mayne & Burne, 2019; Bayat *et al.*, 2021). Vitamin D also participates in the regulation of calcium homeostasis (Gezen-Ak & Dursun, 2019) and

prevents oxidative damage (Garcion *et al.*, 1997; Mokhtari-Zaer *et al.*, 2020). Vitamin D has immunomodulatory functions (Sassi *et al.*, 2018), and its administration has been shown to be neuroprotective to dopaminergic neurons via attenuating neuroinflammation in a mouse model of neurodegenerative Parkinson's disease (Calvello *et al.*, 2017). *In vitro* studies have found several inflammation-limiting functions of vitamin D, such as decreasing the activation of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome (Rao *et al.*, 2019), reducing the expression of inducible nitric oxide synthase, IL-6 and IL-1β in inflammatory conditions (Dulla *et al.*, 2016) and inducing the production of anti-inflammatory cytokine IL-10 (Boontanrart *et al.*, 2016).

2.3.3.3. Chronic stress

The basal secretion of cortisol and corticosterone, the primary stress hormones in humans and rodents, respectively, is involved in daily cognitive and behavioral functioning and is finely regulated by diurnal (daytime) rhythm (Adam *et al.*, 2017). Cortisol or corticosterone release is stress-specific when triggered outside diurnal dependencies (Dedovic *et al.*, 2009). Although acute stress response may be essential for survival and have positive effects on recovery, long-term or severe physiological or psychological stress, along with dysregulated activity of the HPA axis, is maladaptive and related to CNS disorders, such as depression and anxiety (Sousa *et al.*, 2021). Indeed, chronic stress, defined as stress that persists for several hours per day for weeks or months (Dhabhar, 2009), is a prominent risk factor for the development of depression and anxiety disorders (Bekhbat & Neigh, 2018). Moreover, chronic life stress has been associated with an increased rate of cognitive decline in older adults and a higher incidence of dementia (Wilson *et al.*, 2007; Aggarwal *et al.*, 2014). Stress hormones cortisol and corticosterone can exert their effects in the CNS, as they can cross the BBB and act via binding to mineralocorticoid and glucocorticoid receptors, which mediate the non-genomic and genomic effects, respectively, and are expressed throughout the brain (McEwen *et al.*, 2016; Sousa *et al.*, 2021).

Various rodent models for mood disorders, such as unpredictable chronic mild stress (UCMS) (Willner *et al.*, 1987), repeated social defeat stress (Golden *et al.*, 2011), and learned helplessness (Chourbaji *et al.*, 2005; Song *et al.*, 2006), apply stress to increase systemic corticosterone levels and induce depressive-like and anxiety behaviors. The widely used UCMS model can also cause memory impairments (Song *et al.*, 2006; Mohamed *et al.*, 2020). Behavioral alterations in the UCMS model are accompanied by sustained reduction of neuroprotective agents, such as the BDNF in the hippocampus and the prefrontal cortex (Song *et al.*, 2006; Zhao, Cao, *et al.*, 2019) and impaired neurogenesis in the hippocampus (Mineur *et al.*, 2007; Goshen *et al.*, 2008; Tanti *et al.*, 2012). Moreover, persistent stress induces synaptic impairment, a decrease in the number of synapses, neuronal (dendritic) atrophy, and neuronal loss in the medial prefrontal cortex and the hippocampus (Watanabe *et al.*, 1992; Stein-Behrens *et al.*, 1994; Liu & Aghajanian, 2008; McEwen *et al.*, 2016; Price & Duman, 2020; Wang *et al.*, 2023).

As acute stress induces the release of glutamate into the extracellular environment in the medial prefrontal cortex and the hippocampus, it has been proposed that glutamate-induced excitotoxicity may contribute to observed neuronal atrophy during stress (Lowy *et al.*, 1995; Moghaddam *et al.*, 1997; Duman *et al.*, 2019).

Depending on the duration of stress (acute vs. chronic), the concentration of produced stress hormones, the timing of observation, and the affected body region, stress can have either immunosuppressive or immuno-enhancing effects. Persistent stress exposure can dysregulate immune function and alter the immune responses and cytokine balance from protective to pro-inflammatory immunity (Dhabhar, 2009). Chronic stress can, therefore, induce chronic low-grade inflammation, both systemically and in the CNS (Hassamal, 2023). In rodents, the UCMS induces neuroinflammation in the hippocampus and medial prefrontal cortex, which is evident by morphological alterations of microglial cells and increased gene expression of pro-inflammatory cytokines, such as IL-1β (Farooq *et al.*, 2012; Mohamed *et al.*, 2020; Nazir *et al.*, 2022). Increased microglial activation and release of proinflammatory cytokines due to chronic stress or other conditions have been proposed to contribute to or mediate the development of depressive-like behavior and underlying neuropathology (Goshen *et al.*, 2008; Hodes *et al.*, 2015).

2.4. Dysfunctional brain plasticity in substance use disorder

2.4.1. Drug addiction and neuroplastic alterations

Substance use disorder, synonymous with drug addiction, is a mental disorder characterized by uncontrolled drug use despite its harmful consequences (American Psychiatric Association, 2013). Additionally, addiction entails physical dependence, characterized by withdrawal symptoms when the drug use is abruptly discontinued (Horowitz & Taylor, 2023). Addiction develops after a period of repeated drug use in a subset of vulnerable individuals. Genetic predisposition, young age, concurrent chronic pain and preexisting mental illness may contribute to individual vulnerability while promotive environments, such as environments associated with chronic stress, particularly social stress and early life stress, peer pressure and access to drugs may further increase the risk of developing addiction (Volkow *et al.*, 2019; Tschetter *et al.*, 2022).

Acutely, drugs of abuse induce excessive dopamine signaling in the brain reward system, which exerts their initial reinforcing effects. Repeated use of drugs can trigger neuroplastic changes in the glutamatergic inputs to the striatum and midbrain dopamine neurons, which form the brain reward system. These neurochemical and structural alterations, which persist long after the drug administration has ceased, create long-lasting memories of the drug experience, enhance the brain's reactivity to drug cues, reduce the sensitivity to rewards that are not associated with drug use, weaken self-regulation, and increase the

sensitivity to stressful stimuli (Kauer & Malenka, 2007; Nyberg, 2014; Volkow & Morales, 2015; Nestler & Lüscher, 2019).

The mesolimbic dopaminergic reward circuitry consists of midbrain VTA dopaminergic neurons and neurons in the nucleus accumbens (NAc, a limbic area) in the ventral striatum. The acute activation and release of dopamine from the VTA neurons to NAc produces reward, and chronic activation can cause complex neural adaptions, which result in addiction (Kauer & Malenka, 2007; Nestler & Lüscher, 2019). The hypothesis that excessive dopamine signaling in the brain reward circuitry drives addiction is based on rodent studies demonstrating that drugs of abuse increase synaptic dopamine levels in the NAc in rats (Di Chiara & Imperato, 1988) and that activation of neurons in the VTA evoke synaptic plasticity and reorganization in the NAc, triggering addiction-like compulsive behaviors and relapse (Witten *et al.*, 2011; Koo *et al.*, 2012; Pascoli *et al.*, 2015). In addition to the VTA dopaminergic neurons, NAc receives input from neurons in interconnected areas, such as glutamatergic neurons in the prefrontal cortex, ventral hippocampus, basolateral amygdala, and thalamus, and alterations in these regions are suggested to contribute to the development of addiction (Nestler & Lüscher, 2019). Neuroplastic alterations in these pathways, such as potentiation of the excitatory glutamatergic afferents from the medial prefrontal cortex and ventral hippocampus onto the G protein-coupled dopamine 1 (D1) receptor-expressing medium spiny neurons of the NAc have been causally associated with drug-seeking behavior (Karler *et al.*, 1989; Jeziorski *et al.*, 1994; Kauer & Malenka, 2007). Interestingly, hippocampal neurogenesis has been shown to be negatively correlated with drug-taking and drug seeking behaviors and the use of neurogenesis-enhancing manipulations are associated with reduced drug taking and lowered rates of relapse in animal models (Kang *et al.*, 2016).

The neuroplastic changes induced by repeated drug use, which underlie addiction are initiated and maintained by molecular and cellular adaptions, which are mediated by altered gene transcription. Intracellular signaling cascades due to the activation of D1 receptors on NAc neurons, such as cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase A pathway, have been shown to be involved in inducing addiction-related behaviors. Changes in intracellular signal transduction can trigger longer-term changes via transcription factors, such as the cAMP response element-binding protein (CREB) and ΔFosB, well-studied in addiction rodent models (Self *et al.*, 1998; Koob & Volkow, 2016). CREB and ΔFosB in the NAc control genes that are involved in cell excitability and synaptic function (Nestler *et al.*, 2001; Renthal *et al.*, 2009; Nestler & Lüscher, 2019). ΔFosB has been proposed to help initiate a state of addiction, as repeated cocaine administration in rodents induces expression of ΔFosB and correlates with increased locomotor activation and reward (Hiroi *et al.*, 1997; Kelz *et al.*, 1999; Colby *et al.*, 2003; Nestler, 2008; Koob & Volkow, 2016). Activation of CREB in NAc, on the other hand, mediates negative feedback and has a homeostatic role, as it opposes the behavioral effects of cocaine and opiates (Carlezon *et al.*, 1998; Nestler & Lüscher, 2019). Transcription factors, such as the well-characterized constitutively expressed CREB and the more stable

isoform of ΔFosB, may mediate some addiction-related behaviors and underlying neural plasticity (Nestler & Lüscher, 2019). However, due to their transient nature, they themselves may not sustain the long-term changes in brain structure and function associated with addiction.

2.4.2. Epigenetic alterations

Several lines of evidence from preclinical studies suggest that epigenetic modifications, such as DNA methylation, are involved in drug-induced stable changes at the cellular level in the brain, which underlie addictive behaviors by regulating gene-expression networks (Anier *et al.*, 2010; LaPlant *et al.*, 2010; Nestler & Lüscher, 2019; Urb *et al.*, 2020). However, much less is known about the role of epigenetic alterations in human addiction, which needs clarification. Some epigenetic modifications can be permanent, although not as static as once thought, and, therefore, are an appealing mechanism to study in the context of addiction, where long-term drug-associated memories can persist for years or even a lifetime (Hyman, 2005; Moore *et al.*, 2013; Nestler, 2013; Nestler & Lüscher, 2019).

The term epigenetics was introduced by Conrad Waddington in the early 1940s, when he defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" (Waddington, 1942; Devaskar & Raychaudhuri, 2007). Later, the term "epigenetics" narrowed and has been characterized as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Wu & Morris, 2001; Dupont *et al.*, 2009). Epigenetic alterations in chromatin structure may alter gene expression and be heritable but also reversible (Nestler & Lüscher, 2019). Different epigenetic alterations exist, such as DNA methylation, histone modifications, and those induced by non-coding RNAs, particularly microRNAs (Moore *et al.*, 2013; Yao *et al.*, 2019; Panni *et al.*, 2020). Even the higher-order 3D structure of chromosomes is proposed to modulate gene expression and might contribute to the inheritance of gene expression (Cashman *et al.*, 2016; Dekker & Mirny, 2016).

Epigenetic mechanisms can regulate gene expression by affecting the readability of the DNA double helix. DNA double helix is wrapped around octamers of histone proteins, which together form a nucleosome, the structural unit of chromatin (Cavalli & Heard, 2019; Nestler & Lüscher, 2019). The more tightly DNA is associated with histones, the less permissive it is for gene expression. DNA methylation and demethylation work in cooperation with histone modifications to regulate gene transcription. Various post-translational chemical modifications of histones exist, such as methylation, acetylation, ubiquitination, and phosphorylation, which are added to three specific amino acids on the N-terminal of histone tails. These modifications affect the packaging of DNA strands and increase or decrease the accessibility of DNA to transcription factors (Bannister & Kouzarides, 2011; Moore *et al.*, 2013).

Epigenetic modifications provide a mechanism that allows long-term gene expression changes in response to environmental cues (Jaenisch & Bird, 2003). Responses to environmental stimuli, such as memory formation during learning (Guan *et al.*, 2002; Zovkic, 2021) or increased vulnerability to psychiatric disorders due to early life stress (Xu *et al.*, 2020; Ochi & Dwivedi, 2023) may induce and sustain physiological and pathophysiological processes via epigenetic mechanisms. Repeated consumption of drugs of abuse may be considered as another environmental factor that alters the epigenetic state of neurons and other cell types within the brain reward system and interconnected regions (Hamilton & Nestler, 2019).

2.4.3. DNA methylation

DNA methylation is critical in gene silencing in conjunction with histone modifications (Kim & Costello, 2017) and exhibits key roles in tissue-specific gene expression, X chromosome inactivation, genomic imprinting, and silencing of retroviral elements (Moore *et al.*, 2013). Although DNA methylation was discovered in mammals simultaneously with the discovery of DNA (Avery *et al.*, 1944; McCarty & Avery, 1946), only several decades later, its involvement in gene regulation and cell differentiation was demonstrated (Holliday & Pugh, 1975; Compere & Palmiter, 1981; Riggs, 2008). While DNA methylation in the gene promoter region has most often a repressive effect on gene expression, methylation of the gene body has been associated with the opposite effect (Hellman & Chess, 2007; Ball *et al.*, 2009; Aran *et al.*, 2011; Wang *et al.*, 2022). Most DNA methylation occurs on cytosine molecules preceding a guanine nucleotide on CpG sites. CpG sites are mostly heterogeneously distributed across the genome and heavily methylated, with the exception of CpG islands. CpG islands exhibit a higher CpG density than the rest of the genome, most of them are located in the gene promoter regions and are often unmethylated (Bird *et al.*, 1985; Moore *et al.*, 2013; Wang *et al.*, 2022). About 70% of gene promoters reside in CpG islands (Saxonov *et al.*, 2006). In gene promoter regions, DNA methylation suppresses gene expression due to the direct impairment of transcriptional factor binding or by proteins that bind to methylated cytosine themselves and contain transcriptional repression domains. Examples of methylated cytosine binding proteins include the methyl-CpG-binding domain (MBD) proteins and the zinc-finger proteins (Watt & Molloy, 1988; Blattler & Farnham, 2013; Moore *et al.*, 2013; Kaluscha *et al.*, 2022). CpG shores are located 2 kb from CpG islands and exhibit highly conserved tissue-specific methylation patterns in contrast to CpG islands, which often do not show tissue-specific methylation patterns (Irizarry *et al.*, 2009; Moore *et al.*, 2013).

2.4.4. DNA methyltransferases (DNMTs) in DNA methylation

DNA methylation is carried out by epigenetic DNA editors, a family of DNA methyltransferases (DNMTs), which catalyze the transfer of methyl group from

S-adenosyl methionine (SAM) to the fifth carbon of a cytosine residue and form 5-methylcytosine or 5mC (Moore *et al.*, 2013; Kim & Costello, 2017). SAM is converted to S-adenosylhomocysteine (SAH), which acts as an inhibitor of DNMT activity (Soda, 2018). DNMT1 maintains the original pattern of DNA methylation in a cell lineage. DNMT1 shows a strong preference for hemi-methylated DNA and copies DNA methylation patterns during DNA replication from the maternal DNA strand to the newly synthesized daughter strand (Pradhan *et al.*, 1999; Hermann *et al.*, 2004). DNMT1 has also been shown to be recruited to sites of DNA repair *in vivo* (Mortusewicz *et al.*, 2005). DNMT3A and DNMT3B, highly similar in structure and function, carry out de novo DNA methylation by transferring methyl groups to cytosines in unmodified DNA (Okano *et al.*, 1999). DNMT3L is a related homolog of DNMT3A and DNMT3B that lacks the catalytic domain but can associate with DNMT3A and DNMT3B and enhance their methyltransferase activity (Hata *et al.*, 2002; Suetake *et al.*, 2004; Jia *et al.*, 2007).

DNMT3A and DNMT3B are recruited to target promoters in complex with other epigenetic repressors, including histone deacetylases and histone methyl-transferases (Fuks *et al.*, 2000, 2003; Geiman *et al.*, 2004; Kim & Costello, 2017). Repressive transcription factors induce chromatin remodeling by recruiting a complex including histone 3 lysine 9 methyltransferase and DNMT3A or DNMT3B. It has been proposed that histone modifications such as methylation at histone 3 lysine 9 initiate heterochromatin formation and that subsequent DNA methylation ensures stable silencing of promoters (Smith & Meissner, 2013; Kim & Costello, 2017).

DNMTs exhibit essential roles in embryonic development (Li *et al.*, 1992; Okano *et al.*, 1999), and deleting *Dnmt1* in mice results in embryonic lethality (Li *et al.*, 1992). In the central nervous system, *Dnmt3a* is highly expressed in both embryonic and postnatal CNS tissues, while *Dnmt3b* expression is high in the early embryonic period and decreases already during E11-15 (Feng *et al.*, 2005). Although DNMT1 is essential in maintaining methylation patterns during cell differentiation, *Dnmt1* is also expressed in postmitotic neurons (Goto *et al.*, 1994; Inano *et al.*, 2000).

2.4.5. Ten eleven translocation (TET) enzymes in DNA demethylation

DNA can be demethylated either passively or actively. Passive demethylation can occur during cell division when the DNMT1 is inhibited or dysfunctional and cannot transfer methyl groups to newly synthesized DNA strands (Rougier *et al.*, 1998; Moore *et al.*, 2013). Active demethylation is carried out by several enzymes that oxidate or deaminate 5-mC to a product, which is finally replaced by un-modified cytosine via the base excision repair (BER) pathway, as no enzyme can cleave the covalent bond between carbon in cytosine molecule and methyl group (Moore *et al.*, 2013). Active DNA methylation and demethylation are described in Figure 4.

The demethylation process at the methyl group on 5-mC is mediated by epigenetic DNA editors and the ten-eleven translocation (TET) enzymes TET1, TET2, and TET3. TET dioxygenases are dependent on two co-factors, α-keto-glutarate and Fe^{2+}, which use O_2 to oxidize and generate succinate and CO_2 as by-products (Huang & Rao, 2014; Wu & Zhang, 2017; Lio & Rao, 2019). TET enzymes add a hydroxyl (OH) group to the methyl group on 5-hmC (Tahiliani *et al.*, 2009; Ito *et al.*, 2010), which can then be further oxidized by TET enzymes to form 5 formylcytosine (5-fC) and then 5-carboxylcytosine (5-caC) (Ito *et al.*, 2011). Alternatively, 5hmC can be deaminated by activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC), converting 5hmC to 5-hydroxymethyl-uracil. AID/APOBEC can also deaminate 5-mC or 5-hmC directly to thymine or 5-hydroxymetyluracil, respectively. In all cases, 5-formylcytosine, 5-carboxylcytosine, 5-hydroxymetyluracil or thymine activate the BER pathway, which uses thymine DNA glycosylase (TDG) to cleave off the modified residue of cytosine and replace it with unmodified cytosine (Cortellino *et al.*, 2011; He *et al.*, 2011; Moore *et al.*, 2013). Oxidized 5-mC products (5-hmC, 5-fC, 5-caC) can also be converted to 5-mC following replication-dependent dilution (Figure 4) (Wu & Zhang, 2017).

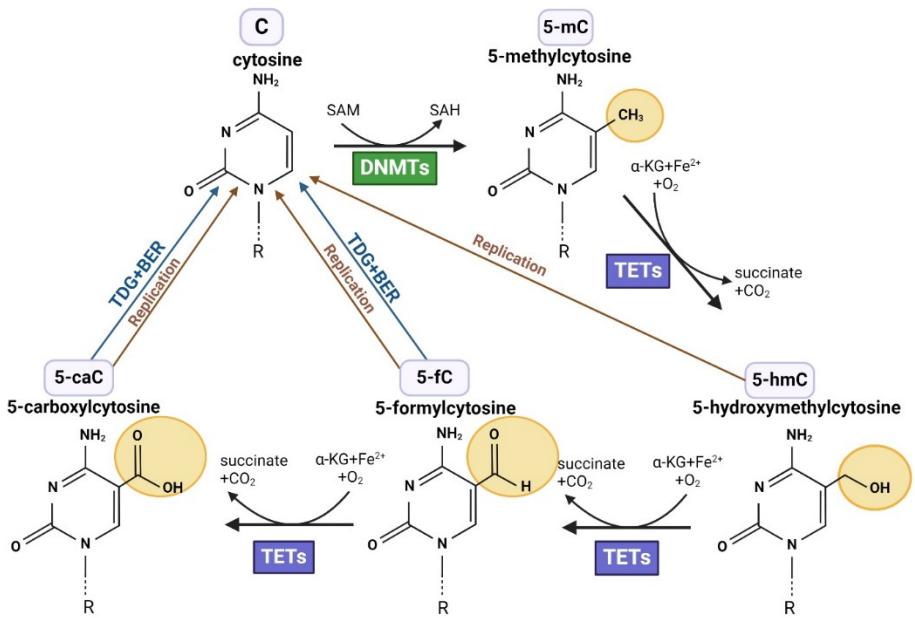

Figure 4. Active DNA methylation and demethylation. DNA methyltransferases (DNMTs) convert unmodified cytosine to 5-methylcytosine (5-mC) by transferring a methyl group from S-adanosyl methionine (SAM) to the carbon-5 position of cytosine, and producing S-adenosylhomocystein (SAH). TET enzymes catalyze the oxidation of 5-mC to 5-hydro-xymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). TET-mediated oxidation uses two co-factors, α-ketoglutarate (α-KG), iron(II) (Fe^{2+}), and O_2,

to produce succinate and CO_2 as co-products. 5-fC and 5-caC can be transformed back to unmodified cytosine (5-C) by thymine DNA glycosylase (TDG), which cleaves off the modified cytosine, coupled with base excision repair (BER), which replaces the modified cytosine with unmodified cytosine. Alternatively, 5-hmC, 5-fC, or 5-caC can be substituted with unmodified cytosine by passive dilution during replication. The AID/APOBEC mediated deamination of 5-mC or 5-hmC and the following replacement of thymine/5-hydroxymethyluracil with unmodified cytosine by the TDG+BER pathway is not depicted here. The image was created with Biorender.

2.4.6. Psychostimulant-induced aberrant DNA methylation in rodent addiction models

The use of psychostimulants is widespread and increasing globally (Lappin & Sara, 2019). The most common illicit psychostimulants are amphetamines (methamphetamine and amphetamine), with an estimated 36 million users globally, cocaine with an estimated 22 million users globally, and "ecstasy"-type drugs, i.e., 3,4-methylenedioxymethamphetamine (MDMA) and its analogs with estimated 20 million users in 2021 (UNDOC, 2023). Approximately two-thirds of illicit psychostimulant users are male, and the average age of use for methamphetamine and cocaine is the mid-30s (Lappin & Sara, 2019). Among cocaine users, about 20% meet the criteria for cocaine use disorder, and among users of amphetamines (including methamphetamine), about 11% meet the criteria for amphetamine-type use disorder in their lifetime (Tardelli *et al.*, 2020; Schwartz *et al.*, 2022). Currently, there is no established pharmacotherapy for cocaine and amphetamine-type use disorder, and psychosocial and behavioral therapies, including cognitive behavioral therapy and contingency management interventions, are the primary treatments for cocaine and amphetamine-type use disorder. Although psychosocial and behavioral therapies are efficient, their effects are not often sustained following their cessation, and they are less effective for severe disorders (Chan *et al.*, 2019; Siefried *et al.*, 2020).

Cocaine is a naturally occurring psychoactive and sympathomimetic tropane alkaloid, which is derived from the leaves of *Erythroxylon coca*. Cocaine acts in the central nervous system by inhibiting presynaptic transporters responsible for the reuptake of serotonin, noradrenaline, and dopamine. The inhibition of presynaptic dopamine transporter (DAT) in the synaptic cleft causes an increase in extracellular dopamine and a euphoric "rush." In addition, cocaine exhibits anesthetic action by blocking the voltage-gated sodium channels (Roque Bravo *et al.*, 2022). Amphetamine, similarly to cocaine, inhibits the presynaptic transporters for serotonin, noradrenaline, and dopamine. In addition, amphetamine has an affinity for vesicular monoamine transporter 2 (VMAT-2), which translocates monoamines from the cytosolic pool into the storage pool. Amphetamine use decreases the translocation of monoamines into the intraneuronal storage vesicles and the release of monoamines to the synaptic cleft resultantly increases (Heal *et al.*, 2013).

Cocaine-induced changes in DNA methylation are proposed to contribute to long-term alterations in brain plasticity, which underlie addiction. In rodent addiction models, acute and repeated use of cocaine has been shown to alter DNA methylation in NAc, and the alterations in DNA methylation have been negatively correlated with altered gene expression (Nestler & Lüscher, 2019). More specifically, repeated administration of cocaine has been associated with increased DNA methylation and activity of DNMTs in NAc (Anier *et al.*, 2010; Massart *et al.*, 2015; Urb *et al.*, 2020).

2.4.7. Pharmacological methods for regulating DNA methylation

Pharmacological inhibition of DNMTs in NAc has suggested a functional role of drug-induced aberrant DNA methylation in mediating addiction-related behaviors in rodent models. More specifically, DNMT inhibition has been shown to abolish cue-induced cocaine seeking (Massart *et al.*, 2015), reduce or delay cocaine-induced behavioral sensitization (Anier *et al.*, 2010; Urb *et al.*, 2020), and potentiate cocaine reward (LaPlant *et al.*, 2010). These studies indicate that inhibiting DNMT activity might represent a viable pharmacological strategy for reversing the addiction-related behavioral effects of repeated psychostimulant use.

Two DNMT inhibitors and hypomethylating agents, decitabine (5-aza-2'-deoxycytidine or DAC) and azacytidine (5-azacytidine), have been approved for clinical use in myelodysplastic syndrome and acute myeloid leukemia. Decitabine and azacytidine are antimetabolites, cytidine analogs incorporated into DNA instead of cytidine during DNA synthesis. Following this, they irreversibly and covalently bind to DNMTs and inhibit DNMT1 at low doses and DNMT3A and DNMT3B at high doses (Agrawal *et al.*, 2018). While decitabine is incorporated into newly synthesized DNA, most azacitidine (80–90%) is incorporated into RNA, and only 10–20% is incorporated into DNA (Stresemann & Lyko, 2008; Agrawal *et al.*, 2018). At high concentrations, azacytidine and decitabine also cause DNA double-strand breaks and cell death. Their anticancer effect is proposed to be related to the reactivation of silenced tumor suppressor genes through CpG demethylation (Gore *et al.*, 2006; Agrawal *et al.*, 2018; Kagan *et al.*, 2023; Laranjeira *et al.*, 2023). In addition to azacytidine and decitabine, other DNMT inhibitors (not in clinical use) have been developed, such as zebularine, a cytidine analog (Ben-Kasus *et al.*, 2005) and a non-nucleoside DNMT inhibitor RG108, which is not incorporated into the DNA and therefore shows less cytotoxicity (Brueckner *et al.*, 2005).

In preclinical rodent studies investigating the CNS and behavior-related effects of DNMT inhibitors, decitabine and azacitidine have been administered either via intracerebroventricular injection (Fonteneau *et al.*, 2017; Qi *et al.*, 2019) directly into the brain area of interest (Sales *et al.*, 2011) or systemically via intraperitoneal injections (Sales *et al.*, 2011). While decitabine has been shown to cross the BBB effectively (Chabot *et al.*, 1983), it is less clear to which extent azacitidine can cross the BBB (McCormack & Warlick, 2010). As the

inhibition of DNMTs in the case of decitabine and azacytidine is dependent on their integration into the DNA during DNA synthesis (Agrawal *et al.*, 2018), it is unclear how well decitabine and azacitidine exert their demethylating effects on post-mitotic neurons. Decitabine and azacytidine could however inhibit DNMTs in proliferating glial cells and proliferating neuronal stem/progenitor cells in neurogenic brain areas.

2.4.8. The effects of psychostimulants on peripheral blood cells and the immune system

Most studies investigating the effects of cocaine and other drugs of abuse on DNA methylation have focused on addiction-specific brain areas, such as the NAc, in rodents (Nestler & Lüscher, 2019). However, obtaining human brain tissue to study drug-related changes in DNA methylation is more complicated. A previous study of our research group identified that repeated cocaine administration induced similar alterations in the mRNA activities and gene expression of epigenetic editors involved in DNA methylation and demethylation in both NAc and the peripheral blood (Anier *et al.*, 2018). More specifically, during the withdrawal period following repeated administration of cocaine, mRNA levels and the enzyme activity of DNMTs increased, and TET enzyme activity decreased similarly in both NAc and peripheral blood cells. Therefore, alterations in the DNMTs and TET enzymes in the peripheral blood may represent to some extent similar alterations in brain regions relevant to addiction, following psychostimulant exposure.

Dopamine is a neurotransmitter in both the CNS and peripheral tissues, and emerging evidence suggests that immune cells themselves can produce dopamine (Pinoli *et al.*, 2017). Dopamine is present in human plasma (Eisenhofer *et al.*, 1997), and peripheral immune cells of lymphoid and myeloid lineage express markers of the dopamine system (Mackie *et al.*, 2018; Gopinath *et al.*, 2020). Peripheral immune cells, such as T-cells and monocytes/monocyte-derived macrophages, express dopamine receptors, DAT, tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis, and VMAT-2 (Mackie *et al.*, 2018). Dopaminergic signaling has been shown to modulate inflammatory response in the brain-resident innate immune cells, microglia in rodents (Vidal & Pacheco, 2020; Pike *et al.*, 2022) as well as in the peripheral innate immune system cells, and human monocyte-derived macrophages (Gaskill *et al.*, 2012; Mackie *et al.*, 2022). Dopaminergic signaling has also been shown to regulate the adaptive immune system (Castellani *et al.*, 2019).

Psychostimulants affect the production and release of cytokines in the brain and the periphery (Bravo *et al.*, 2023). Repeated cocaine use and cocaine abuse have been related to increased serum or plasma levels of proinflammatory IL-6, TNF-α (Narvaez *et al.*, 2013; Moreira *et al.*, 2016; Pianca *et al.*, 2017) and decreased (Moreira *et al.*, 2016) or increased levels of anti-inflammatory IL-10 (Narvaez *et al.*, 2013; Pianca *et al.*, 2017). Postmortem cortical brain tissue of

chronic cocaine-dependent humans demonstrated increased levels of proinflammatory mature IL-1β (Chivero *et al.*, 2021). Rodent studies investigating the effects of repeated administration of cocaine have also detected an increase in IL-1β and TNF-α in the striatum (Lewitus *et al.*, 2016; Chivero *et al.*, 2021) and increased TNF-α, IL-1β, IL-6 levels or gene expression in the hippocampus (Mai *et al.*, 2018; Montesinos *et al.*, 2020). Similarly, repeated methamphetamine use has been associated with increased levels of peripheral and central pro-inflammatory cytokines in rodent models and human users (Bravo *et al.*, 2023). Together, studies mentioned above indicate that repeated psychostimulant use can regulate the immune system in the brain and peripheral blood cells and elicit a pro-inflammatory immune response.

In addition to psychostimulant-induced production of inflammatory cytokines locally in the brain, blood-borne cytokines, such as IL-1β, IL-6, and TNF-α from the periphery can enter the brain to some extent either via saturable transport systems or via simple diffusion in circumventricular organs in areas of the brain, where the BBB is incomplete (Gutierrez *et al.*, 1993; Banks, 2005; Yarlagadda *et al.*, 2009). Chronic psychostimulant use is associated with disruption of the BBB integrity, which can result in increased passage of cytokines into the brain (Kousik *et al.*, 2012; Sajja *et al.*, 2016). Additionally, proinflammatory cytokines can contribute to brain inflammation by activating endothelial cells, as the pro-inflammatory cytokine IL-1β signaling has been shown to mediate microglial activation in the brain parenchyma through IL-1β receptors on the endothelial cells (Liu *et al.*, 2019).

How exactly psychostimulant-induced peripheral and neuroinflammation could contribute to dysfunctional brain plasticity related to substance use disorder needs clarification. However, neuroimmune signaling has been proposed to potentially contribute to the development of maladaptive cocaine-associated memories (Correia *et al.*, 2020), affect glutamate-related plasticity in addiction (Gipson *et al.*, 2021), and regulate the induction and expression of cocaine-induced behaviors (Lewitus *et al.*, 2016).

2.5. Summary of literature review

Brain plasticity is essential for development, learning, adapting to various environmental conditions, and for recovery following injury (Voss *et al.*, 2017; Mateos-Aparicio & Rodríguez-Moreno, 2019). Brain plasticity in adulthood is mainly confined to alterations in synapse strength and number and synaptic plasticity is considered an important underlying mechanism for learning and memory (La Rosa *et al.*, 2020; Magee & Grienberger, 2020). As an exception, a high level of structural neural plasticity remains in the adulthood in the neurogenic regions of the brain, such as the hippocampus, where new neurons are generated through life. Neurogenesis in the hippocampus introduces plasticity into the local networks and contributes to hippocampal functions in episodic

memory and mood regulation (Kempermann *et al.*, 2015; Toda & Gage, 2018, 2018; Denoth-Lippuner & Jessberger, 2021).

Environmental and physiological factors, such as *chronic neuropathic pain, vitamin D deficiency, chronic stress* and *psychostimulants* could impair brain plasticity and result in depression, memory decline and addiction. However, their molecular and cellular mechanisms and behavioral effects are not entirely clear.

Alterations in neuromediators, decrease in trophic factors and neural inflammation can lead to a failure of neuronal plasticity, characterized by synaptic loss, impaired neurogenesis and neuronal atrophy in brain regions related to memory and mood regulation, such as the hippocampus and prefrontal cortex. These dysfunctions in neural plasticity may underlie depression, anxiety, and cognitive impairment (Burke & Barnes, 2006; McEwen *et al.*, 2012; Bannerman *et al.*, 2014; Price & Duman, 2020). However, the exact mechanisms of how different causal factors, such as chronic neuropathic pain and vitamin D deficiency, or a combination of two detrimental factors, such as vitamin D deficiency and chronic stress, could lead to dysfunctional brain plasticity and resultant depression/ anxiety/cognitive impairments, are unclear. Elucidating the cellular and molecular correlates of neuroplasticity-impairing factors could reveal potential crucial processes and targets for prevention and treatment.

Chronic neuropathic pain is often co-morbid with depression (Radat *et al.*, 2013; Shaygan *et al.*, 2013; Cohen & Mao, 2014), and preclinical studies have provided evidence that chronic neuropathic injury and pain can impair hippocampal neurogenesis and induce depressive-like behaviors and memory impairments in rodents. However, the process or stage of neurogenesis which becomes disrupted due to chronic neuropathic pain varies depending on the specific study (Narita *et al.*, 2006; Yalcin *et al.*, 2011; Mutso *et al.*, 2012; Alba-Delgado *et al.*, 2013; Dellarole *et al.*, 2014; da Silva *et al.*, 2020). Therefore, elucidating the impaired stage of neurogenesis following peripheral neuropathic injury and pain and the temporal dynamics of depressive-like behavior development could provide further insight into the pathophysiology of chronic neuropathic pain-induced depressive-like behavior and cognitive impairments. Better understanding the pathophysiology of chronic neuropathic pain-induced depression may help to lead the way to possible new treatment strategies.

Epidemiological data links vitamin D deficiency to depression and memory impairments, particularly in older adults (Meehan & Penckofer, 2014; Ceolin *et al.*, 2022). However, the causal link and underlying mechanisms in the brain remain unclear. Moreover, it is important to understand whether several co-occurring brain plasticity-impairing factors could exacerbate their adverse effects. Considering the known neurobiological functions of vitamin D, such as regulating neurotrophin production and neuroinflammation, and well-known detrimental effects of chronic stress, it can be hypothesized that co-occurrence of vitamin D deficiency and chronic stress could result in exacerbated dysfunctional brain plasticity, depressive-like behaviors, and cognitive impairment.

Finally, addiction represents a form of dysfunctional brain plasticity. Following repeated use of drugs of abuse, long-term alterations in the brain reward

system, such as NAc, and interconnected areas can take place in some vulnerable individuals. These alterations in brain circuits may underlie the strong drug-related memories, even after years of abstinence and compulsive use, regardless of negative consequences (Kauer & Malenka, 2007; Nyberg, 2014; Volkow & Morales, 2015; Nestler & Lüscher, 2019). Epigenetic DNA editors, such as DNMTs and TETs, involved in DNA methylation and demethylation, may mediate and sustain gene expression alterations due to drug use, which then manifest as long-term behavioral changes.

Most of the studies investigating the roles of epigenetic DNA editors in addiction have been conducted in rodent models and focused on alterations in NAc (Anier *et al.*, 2010; LaPlant *et al.*, 2010; Massart *et al.*, 2015; Nestler & Lüscher, 2019; Urb *et al.*, 2020). The extent to which aberrant DNA methylation is involved in the mechanisms of addiction in humans is however not known. A previous study of our research group demonstrated that repeated administration of cocaine-induced similar alterations in epigenetic editor activity and gene expression in the NAc and peripheral blood of mice (Anier *et al.*, 2018). Human peripheral blood mononuclear cells (PBMCs) include immune cells of the lymphoid and myeloid lineage, known to express dopamine system markers (Mackie *et al.*, 2018). As human PBMCs are a more easily obtainable material than human brain tissue and as these cells are possible to culture *in vitro* in laboratory conditions, human PBMCs could pose a putative cellular model for investigating the effects of psychostimulants on epigenetic DNA editors in human cells. Moreover, it is of future interest if alterations in the epigenetic DNA editors DNMTs and TETs in the human PBMCs could reflect alterations in the brain.

3. AIMS OF THE STUDY

The general aim of the study was to elucidate the cellular and molecular mechanisms of brain plasticity-impairing factors (chronic neuropathic pain, vitamin D deficiency, chronic stress, psychostimulants cocaine, and amphetamine) in relevant animal and cellular models.

The specific aims of the study were the following:

1. To specify the temporal dynamics and presence of chronic neuropathic pain-induced anxiety, depressive-like behavior, and memory impairments in mice and to elucidate co-occurring impaired cellular processes of hippocampal neurogenesis and alterations in microglial morphology (Paper I).
2. To clarify whether long-term vitamin D deficiency independently or in combination with chronic stress induces memory impairments and depressive-like behavior in mice and to characterize underlying alterations in neuronal and glial plasticity in the hippocampus (Paper II).
3. To elucidate psychostimulant-induced alterations in epigenetic DNA editors (DNMTs and TETs), DNA methylation, and cytokine profiles by using a human peripheral blood mononuclear cell culture model. To clarify if the DNMT inhibitor decitabine can prevent the effect of cocaine on epigenetic DNA editors (Paper III).

4. MATERIALS AND METHODS

4.1. Experimental design (Papers I–III)

The experimental designs of Paper I, Paper II, and Paper III are described in Figures 5, 6, and 7, respectively.

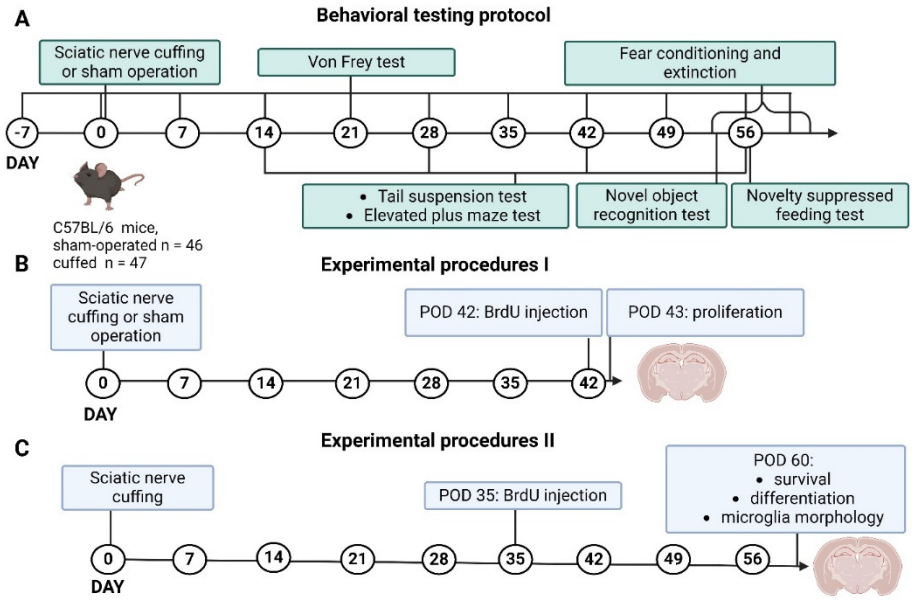

Figure 5. Experimental design for the study presented in paper I investigating the effects of sciatic nerve cuffing-induced chronic neuropathic pain on behavior and hippocampal neurogenesis. A representative graph of (A) behavioral testing protocol and (B, C) experimental procedures is presented. (A) Starting from 7 days (-7) before sciatic nerve cuffing in C57BL/6 mice, Von Frey test was applied weekly, to evaluate mechanical allodynia. Tail suspension test and elevated plus maze tests were carried out biweekly on post-operative days (PODs) 14, 28, 42 and 56 using three different groups of animals in order to avoid habituation effects. One group of animals was used for conducting elevated plus maze test and tail suspension test on POD 14 and 42. A second group of animals was used to conduct elevated plus maze test on PODs 28 and 56, tail suspension test on POD 28 and novel object recognition test on POD 54–55. A third group of animals was used for tail suspension test on POD 56. In addition, in the end of the experiment on POD 56, novelty suppressed feeding test was conducted. A fourth group of animals was used for fear conditioning and extinction test, followed by tone-induced fear memory recall test on PODs 50–59. (B) In order to quantify proliferative activity in the hippocampus, mice were intraperitoneally injected with BrdU on POD 42 and on POD 43 mice were sacrificed. (C) In order to assess the survival and differentiation of newly generated cells, as well as microglial morphology in the hippocampus, mice were intraperitoneally injected with BrdU on POD 35 and sacrificed for immunohistochemistry and histology experiments on POD 60. The image was created with Biorender.

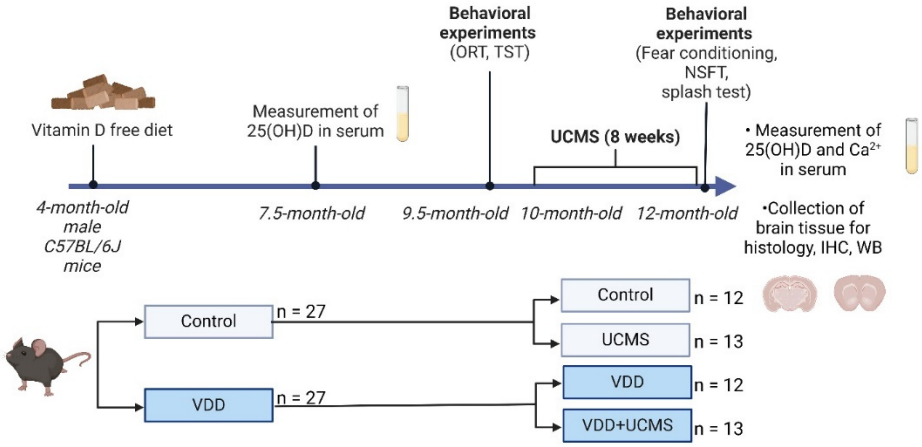

Figure 6. Experimental design for study presented in paper II investigating the effects of vitamin D deficiency and unpredictable chronic mild stress (UCMS) on behavior and hippocampal plasticity. 4-month-old-male C57BL/6J strain mice were divided into two groups: control group had sufficient amount of vitamin D in their diet and vitamin D deficient (VDD) group lacked vitamin D in their diet (n = 27, per group). Two 7.5-month-old mice from each group were euthanized for the measurement of 25(OH)D levels. Novel object recognition test (ORT) and tail suspension test (TST) were carried out to assess memory and depressive-like behaviors in 9.5-month-old middle-aged mice. 10-month-old mice were subjected to unpredictable chronic mild stress (UCMS) for 8 weeks, dividing the 2 groups into further 4 groups (n = 12–13, per group). Following UCMS, behavioral fear conditioning, novelty suppressed feeding test (NSFT) and splash test were conducted, serum was collected for the measurement of 25(OH)D and Ca^{2+}, and brain tissue was dissected and collected for histology, immunohistochemistry (IHC) and western blot (WB) experiments. The image was created with Biorender.

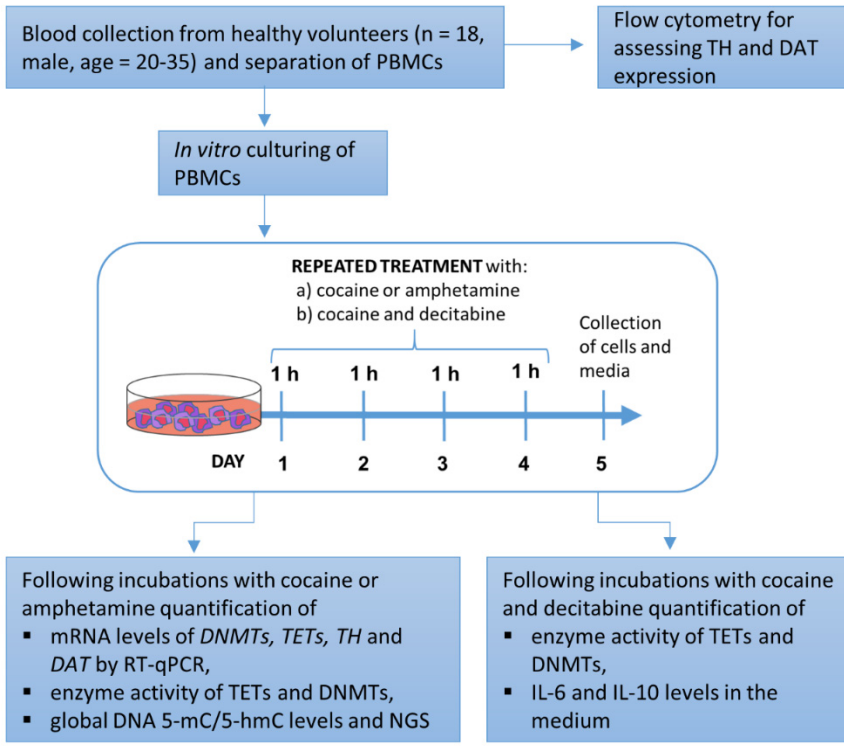

Figure 7. Experimental design for study presented in paper III assessing the effects on psychostimulants and DNMT inhibitor decitabine on epigenetic DNA editors and DNA methylation. Briefly, blood was collected from healthy male volunteers aged 20–35, and peripheral blood mononuclear cells (PBMCs) were separated. A flow cytometry experiment was carried out to assess tyrosine hydroxylase (TH) and dopamine transporter (DAT) expression. In parallel, PBMCs were cultured *in vitro*. Two different experimental designs were applied, one for repeated treatments with a) cocaine or amphetamine and second with b) cocaine and/or decitabine. Cells and media were collected, and downstream experiments were carried out in PBMCs for quantification of mRNA levels of *DNMTs, TETs, TH*, and *DAT* by RT-qPCR, enzymatic activity of ten-eleven translocation enzymes (TETs) or DNA methyltransferases (DNMTs), global DNA 5-methylcytosine (5-mC) or 5-hydroxymethylcytosine (5-hmC) levels and next generation sequencing (NGS). Interleukin 6 and 10 (IL-6 and IL-10) levels were measured in the cell culture medium. After repeated treatment, NGS was carried out.

4.2. Animal models (Papers I-II)

4.2.1. Animals and housing conditions (Papers I-II)

All experiments were undertaken in agreement with the guidelines established in the Principles of Laboratory Animal Care (Directive 2010/63/EU) and the experimental protocols were approved by the Animal Experimentation Committee at the Estonian Ministry of Agriculture (no. 77, 2016 and no 171, 2020). For the

chronic neuropathic pain project presented in paper I, 93 8-weeks-old (at the start of the experiment) male C57/BL/6 mice (from the Laboratory Animal Care Centre, Tartu, Estonia) were used. For the VDD+UCMS project presented in paper II, 54 4-month-old (at the beginning of the experiment) male C57/BL/6J mice (from Envigo), were used. Mice were group-housed, in paper I experiments 5 per cage, in paper II experiments initially 9 per cage, and from the start of UCMS procedure 3–5 per cage. Housing had 12 h light/dark cycle, with water and food available *ad libitum.* All of the behavioral testing was conducted between 9 am and 6 pm in the light phase. Animals were allowed to habituate in the experimental room for at least 30 min before performing the behavioral experiments. The same researcher or researchers throughout the experiment conducted each behavioral experiment and were blinded to the animal groups.

4.2.2. Protocol for sciatic nerve cuffing (SNC, Paper I)

8-week-old mice were randomized into two groups: cuffed (Cuff, n = 47) or sham-operated (Sham, n = 46). Neuropathic pain was induced by sciatic nerve cuffing (SNC) (Yalcin *et al.*, 2014) under isoflurane anesthesia. In cuffed group animals, the common branch of the left sciatic nerve was exposed and a 2 mm long split section of polyethylene tubing (ID = 0.38 mm, ED = 1.09 mm; PE-20, Harvard Apparatus, France) was placed around it. The shaved skin layer was closed using suture. Sham-operated mice underwent the same surgical procedure as described for cuffed animals, except for placing the polyethylene cuff. Previous and following procedures and behavioral experiments for Paper I are described in Figure 5.

4.2.3. Induction of VDD (Paper II)

4-month-old mice were randomized into control (n = 27) or vitamin D deficient (VDD, n = 27) group based on their food consumption. Mice in the control diet group continued to consume food that contained a sufficient amount of vitamin D (Standard Rodent diet with 1500 IU vitamin D3/kg ssniff Spezialdiäten GmbH; E15000-04, Germany), and mice in the VDD group were fed with vitamin D free food (ssniff Spezialdiäten GmbH; E15312-24, Germany) for 8 months in total. Following procedures and behavioral experiments are described in Figure 6.

4.2.4. Assessment of 25(OH) and Ca^{2+} levels via ECLIA method (Paper II)

For the evaluation of 25(OH) and Ca^{2+} levels in the serum, two 7.5-month-old mice and four to five 12-month-old mice from each group were euthanized and their blood was collected via cardiac puncture. Collected blood was allowed to coagulate for 30 min and centrifuged at 2000 × g for 20 min. Next, the serum was collected and stored at −80 °C. 300 µl of serum samples were used to measure 25(OH)D by the electrochemiluminescence method using the biochemistry analyzer COBAS 6000 IT-MW (F. Hoffmann-La Roche Ltd, Switzerland) at the

Tartu University Hospital United Laboratories. The biochemical measurement of calcium concentration in serum was performed by using the Calcium Arsenazo III Colorimetric Method (Giesse Diagnostics, Italy) according to standard operating procedures in the Tartu Health Care College.

4.2.5. Protocol for unpredictable chronic mild stress (UCMS) (Paper II)

10-month-old mice were further randomized for UCMS and the following four groups were formed: 1) control group with sufficient vitamin D in their food (control; n = 12), 2) the VDD group (n = 12), 3) the UCMS group with sufficient vitamin D in their food (UCMS; n = 13) and 4) VDD+UCMS group (n = 13). VDD and VDD+UCMS group continued to be on vitamin D free diet until the end of the experiment. Different stressors presented in Table 1 were applied daily to induce UCMS, and their sequence was randomized to avoid habituation effects. The UCMS protocol was applied for 8 weeks.

Table 1. Applied stressors for UCMS protocol.

Stressor	Duration
Continuous illumination	overnight
Cage tilt at 45°	24 h
Cage shaking	10 min
Physical restraint	2 h
Strobe light	3 h
Wet bedding	16 h
Swimming in 4°C water	3 min
Heat stress applied with a hair dryer	5 min
Food and water deprivation	24 h

4.3. Behavioral experiments (Papers I–II)

4.3.1. Von Frey test for allodynia (Paper I)

Allodynia, a painful response to a stimulus that does not normally provoke pain, was assessed once a week using the Von Frey test. Mice were allowed to acclimate to the plexiglass enclosures (20cm*20cm*15cm), located on top of a wire testing rack for 60 min, on 3 consecutive days before testing and 10-20 minutes before each testing session. Von Frey filaments of increasing bending force (0.0008g; 0.02g; 0.04g; 0.07g; 0.16g, 0.4g; 0.6g; 1.0g; 1.4g; 2.0g) were used to determine the threshold for mechanical pain response. The filaments were repetitively pressed against the lateral area of the left hind paw within the sciatic innervation region, as previously described (Leventhal & Strassle, 2008), and a positive pain reaction was assessed. Positive pain reaction was defined as flinching and/or paw licking induced by the filament or a sudden paw withdrawal.

Mechanical thresholds were tested using the up-down technique (Chaplan *et al.*, 1994) based on stimulus oscillation around the response threshold to determine the median 50% response threshold.

4.3.2. Behavioral tests for assessing depressive-like and anxiety behaviors

4.3.2.1. Tail suspension test (TST, Papers I and II)

The testing apparatus for TST comprised separate compartments for each mouse and a wooden beam. Mice were suspended approximately 1 cm from the tip of their tail to a wooden beam inside a testing apparatus using adhesive tape and video was recorded during the following 6-minute testing period. The total duration of immobility, latency time until the first immobility episode, and the number of immobility episodes were measured based on the video recording. Immobility was defined as a complete lack of movement other than small movement of the forefeet, swinging due to earlier movements, and movements due to respiration.

4.3.2.2. Novelty suppressed feeding test (NSFT, papers I and II)

Twenty-four hours before testing, mice were placed without access to food in a new cage similar to their home cage. The testing apparatus comprised a wooden box with measures 45x45x45cm and was covered with wooden bedding. In the center of the wooden bedding arena, a piece of chow was placed on a highly illuminated (400 lux) white platform. The mouse was placed in one of the arena's corners, facing the center. Latency time until the first eating event was recorded with a stopwatch. Immediately after the mouse started eating or when the 5-minute time limit for testing had passed, the mouse was placed back into the home cage with the piece of chow. In the home cage, latency time until the first eating event and the amount of food consumed were measured.

For the NSFT conducted in Paper II, additional exclusion criteria were applied, since the first animals of every cage (n = 3 per group) exhibited abnormal feeding behavior as they did not start eating in the novel cage and were excluded from the following analysis. As all animals from one cage were tested sequentially and a new piece of chow was placed in the middle of the platform for every cage, observed anomalous behavior could potentially be explained by the previously described phenomenon “social transmission of food preferences,” which entails the rodent pairing food pellet with the smell of carbon dioxide from another rodent and classifying it safe based on familiar smell (Deacon, 2011).

4.3.2.3. Splash test (Paper II)

The splash test was conducted in a room with a red light. Mice were removed from their home cage; viscous 10% sucrose solution was sprayed on their dorsal coat, after which they were placed individually in another cage filled with wooden bedding and time spent grooming, latency until the first grooming event and

number of grooming events were recorded during the following 5 minutes. Time spent grooming was defined as cleaning the fur by licking or scratching. One animal from the control group was excluded from the subsequent data analysis due to a technical error related to spraying sucrose solution during testing.

4.3.2.4. Elevated plus maze test (EPM, Paper I)

The testing apparatus for the EPM test consisted of a plus-shaped apparatus (TSE Systems, Germany) with two open and two closed arms (45cm*10cm*30cm), elevated to a height of 60 cm above the floor. Plus maze was placed in a dimly lit room (8 lux, measured at the center of the maze). The animals were placed in the maze's center and allowed to freely explore the maze for 5 min while an observer recorded the duration and frequency of entries into open and closed arms. All tests were performed at a similar time (between 11 am and 2 pm). The percentage of entries onto open arms from total arm entries was used as a measure of anxiety, and the total number of entries to either of the arms was used as a measure of locomotor activity. On postoperative day (POD) 28, one outlier from the control group and one outlier from the cuffed group were excluded from data analysis, as they spent more than 10 times or more time on open arms compared to the average of their corresponding groups.

4.3.3. Behavioral tests for memory assessment

4.3.3.1. Novel object recognition test (ORT, Papers I and II)

The ORT testing apparatus comprised a brown wooden box (45*45*45cm) with a gray floor. The objects of interest were made of similar materials. However, they had different shapes and were heavy enough to prevent the mice from moving them. To avoid possible effects of anxiety, mice first had a habituation session, in which they were able to explore the box for 5 minutes freely, and during testing, the room was dimly lit (170 lux) to prevent possible anxiety effects. Twenty-four hours after the habituation session, a training session took place with two identical objects (A and A') placed on a diagonal inside the wooden box. Each mouse was placed in the center of the open field, its head positioned away from the objects, and the mouse was left to explore the objects freely for 5 minutes. Three and 24 hours after the training session, short-term memory (STM) and long-term memory (LTM) functions were assessed, respectively, by replacing one of the initial objects with a new one (A' was replaced by object B for STM and by object C for LTM). Similarly to the training session, each animal was left to explore for 5 min. EthoVisionXT video tracking system (version 8, Noldus Information Technologies, Leesburg, VA) was used to video record all trials. Time spent exploring each object was recorded manually with a stopwatch by a trained observer. Sniffing, directing nose towards the object at a distance less than or equal to 2 cm, or touching the object was defined as time spent exploring. Climbing on top of the object was not considered an exploration activity unless the mouse sniffed the object simultaneously. After each trial

session, the box, field, and objects were cleaned with 70% ethanol. One animal was excluded from the long-term memory test, as their overall exploratory activity remained lower than 1 s, which was set as a threshold for exclusion.

4.3.3.2. Contextual fear memory retention and extinction, tone-dependent fear memory recall (Papers I and II)

The testing apparatus for fear memory testing consisted of an experimental chamber made of transparent plastic 22cm*22cm*35cm placed inside a larger noise-attenuating box that exhibited built-in ventilation and a fan providing background noise. The floor of the testing apparatus consisted of stainless steel rods connected to a shock generator (TSE Systems, German). Each mouse was allowed to explore the conditioning chamber freely for 3 minutes immediately before exposure while baseline freezing was measured. Immediately following the pre-exposure session, three conditioned stimuli (85 db, 2800 Hz, 20s tone) and unconditioned stimulus (foot shock at 0.50 mA (Paper II) or 0.70 mA (Paper I), presented during the last 2 s of the conditioned stimulus pairings) were applied with 1 min intervals through the floor, while freezing was simultaneously measured. Twenty-four hours later, fear memory retention was assessed, and contextual fear memory extinction was evaluated on five (Paper II) or eight (Paper I) daily sessions by placing the mice in the conditioning context without applying the shock for 3 min. Freezing time was measured manually with a stopwatch by a trained observer for all sessions and defined as time spent without moving, except for movements due to breathing. Extinction in total freezing time was converted to a percentage $T_{\%Freezing} = T_{freezing} / (100 * T_{context})$, where $T_{freezing}$ is the total amount of freezing (in min), and $T_{context}$ is the amount of time spent in context. Five hours after extinction sessions (Paper II) or 24 hours after the end of the last extinction session (Paper I), a tone-dependent fear memory recall test was performed by exposing the mice to a previously heard tone during the training session in a novel context. Freezing time during the following 3 min was measured. Two animals were excluded from the data analysis due to technical errors during the experiment.

4.4. Injection of BrdU (Papers I–II)

Mice received three (Paper I) or four (Paper II) intraperitoneal (i.p.) injections of thymidine analog, BrdU at 2-hour intervals on the POD 42 for proliferation studies, on POD 35 for survival/differentiation studies (Paper I) and 3 weeks after the start of UCMS (Paper II). Mice were euthanized 24 hours or 25 days after BrdU administration (Paper I) and 6 weeks after BrdU administration (Paper II).

4.5. Tissue collection and processing (Papers I–II)

For immunohistochemistry and histology experiments, animals were deeply anesthetized (100 mg/kg, i.p., Paper I and > 200 mg/kg i.p., Paper II), transcardially perfused with normal saline and then with 4% paraformaldehyde in phosphate-buffered saline (PBS, 0.1M, pH 7.4). The brains were removed, post-fixed in 4% formaldehyde solution for 24–48 hours and cut into 40-µm thick coronal sections on a vibratome (Leica VT1000S, Germany). Sections were stored in a cryoprotectant solution containing 30% glycerol and 30% ethylene glycol in PBS at -20 °C before experiments. For western blot experiments, mice were euthanized by injecting pentobarbital sodium (> 200 mg/kg i.p.), brain tissues were dissected, collected and snap-frozen.

4.6. Hematoxylin-eosin (HE) staining (Papers I–II)

Every sixth section throughout the hippocampal formation (240 µm apart) was used for HE staining. For the staining procedure, brain sections were incubated for 10 min in a 0.1 M TRIS HCl buffer containing 0.025% trypsin and 0.1% $CaCl_2$ and in acidic alcohol (1% HCl in 70% ethanol) solution for 10 s. Then, the sections were stained using HE, washed in PBS and coverslipped in water-based mounting medium (Vector Laboratories, UK) or mounting medium containing glycerol and PBS in 1:9 proportions.

4.7. Volumetric analysis in the dentate gyrus (DG) of the hippocampus (Papers I–II)

An average of 9 sections per animal were analyzed. For the volumetric analysis, HE-stained brain sections were scanned by using Leica SCN400 apparatus (Paper I) or Li-Cor Odyssey®M imager (Paper II). Surface areas of GCL and hilar regions of the DG in the hippocampus were measured using the QuPath software (Bankhead *et al.*, 2017), and volumes of interest were calculated from the surface areas multiplied by the thickness of the sections and the distance between the sections.

4.8. Cell number quantification in the granule cell layer (GCL) of the DG (Papers I–II)

The number of cells in the GCL was quantified using the optical fractionation method (West, 1993). The stereology system used for quantification consisted of a Leica DFC495 microscope, a microcator (Heidenhain, DN 281), and the computer-assisted Stereological Toolbox (VIS – visiopharm Integrator System). Numerical density (Nv) was calculated according to the formula $Nv = \Sigma Q/\Sigma v$

(dis), where ΣQ is the number of cells counted and Σv (dis) is the volume of dissectors (single dissector volume: 10×10×20 μm). The total number of cells in the GCL was calculated based on numerical density (Nv), which was multiplied by the volume of the GCL.

4.9. Immunohistochemistry (IHC) and image analysis (Papers I–II)

Immunohistochemical staining was carried out on free-floating sections. The criteria for selecting sections and processing steps for IHC are provided in Tables 2 and 3. All washing steps were done thrice in phosphate-buffered saline (PBS) solution for 10 minutes. Blocking with either normal goat serum (S-1000, Vector Laboratories) or rabbit serum (S-5000, Vector Laboratories) at 5% concentration at room temperature was conducted before incubations with primary antibodies. Tyrosine hydroxylase (TH), BrdU + calbindin, and BrdU + GFAP immunopositive signals were visualized with fluorochrome-conjugated secondary antibodies. In contrast, BrdU, doublecortin, polysialylated neuronal cell adhesion molecule (PSA-NCAM), caspase-3, and ionized calcium-binding adaptor molecule (IBA1) immunopositive signals were visualized with peroxidase ABC system (Vectastain ABC kit, PK-6100, Vector Laboratories), diaminobenzidine as the chromogen (DAB Peroxidase Substrate, SK-4100, Vector Laboratories) and cleared with xylol before coverslipping. For staining cell nuclei, 0.0001% DAPI (4', 6-diamidino-2-phenylindole) solution (D9542-10MG, Sigma Life Science, USA) in PBS was used in combination with TH, Calbindin + BrdU, GFAP + BrdU IHC. Signal specificity for IHC was verified with negative control samples where the primary antibody was omitted. Image analyses were conducted blinded.

Table 2. IHC protocols using peroxidase ABC and diaminobenzidine visualization system. AB – antibody, HIP- hippocampus, NA – not applicable, RT – room temperature.

Marker for IHC detection	**Selection criteria for brain sections**	**H_2O_2 in PBS**	**HCl / Citrate buffer**	**Blocking buffer (0.5%Tween-20, 0.25% Triton X-100 in PBS)**	**Primary AB in blocking buffer**	**Secondary AB in blocking buffer**
BrdU	Every 6th section from HIP	2% 30 min	HCl 2N 30 min at 37 °C	5% normal rabbit serum 1 h at RT	Rat anti BrdU (1:300, OBT00030, Accurate Chemicals) 24 h at 4°C	Rabbit anti-rat biotinylated (1:400, BA-4000, Vector Laboratories) 30 min at RT
IBA1	Every 6th section (paper I) or 3 sections from the HIP (paper II)	0.6% 30 min	Citrate buffer 30 min 84°C	5% normal rabbit serum 1 h at RT	Goat anti IBA1 (1:750, ab5976, Abcam) 48 h at 4˚C	Rabbit anti-goat biotinylated (1:1000, BA-5000, Vector Laboratories) 30 min at RT
Cleaved caspase-3	Every 10th section from HIP	0.6% 30 min	Citrate buffer 30 min 84°C	5% normal goat serum 1 h at RT	Rabbit anti caspase-3 (1:1000, PA5114687, Invitrogen) 24 h at 4˚C	Goat anti-rabbit biotinylated (1:400, BA-1000, Vector Laboratories) 30 min at RT
PSA-NCAM	Every 12th section from HIP	2% 30 min	NA	5% normal goat serum 1 h at RT	Mouse anti-PSA-NCAM (1:400,MAB5324, Merk Millipore) 48 h at 4˚C	Biotinylated goat anti-mouse (1:500, BA-2020, Vector Laboratories) 1 h RT
Double-cortin	Every 12th section from HIP	2% 30 min	NA	5% normal goat serum 1 h at RT	Rabbit anti-doublecortin (1:500, ab18723, Abcam) 24 h at 4˚C	Biotinylated goat anti-rabbit (1:1000, ref no BA-1000, Vector Laboratories)

Table 3. IHC protocols using secondary fluorescent antibodies for visualization. AB – antibody, HIP – hippocampus, NA – not applicable, RT – room temperature.

Marker for IHC detection	**Selection criteria for brain sections**	**H_2O_2 in PBS**	**HCl / Citrate buffer**	**Blocking buffer (0.5%Tween-20, 0.25% Triton X-100 in PBS)**	**Primary AB in blocking buffer**	**Secondary AB in blocking buffer**
TH	1 section bregma 1.1 mm	NA	NA	5% normal goat serum 1 h at RT	Rabbit anti-TH (1:500, Ab152, Sigma-Aldrich) 24 h at RT	Goat anti-rabbit Alexa594 (1:1000, A11012, Thermo Fisher Scientific) 1 h at RT
BrdU + calbindin	Every 6th section of HIP	NA	HCl 2N 30 min at 37 °C	5% normal goat serum 1 h at RT	Rat anti-BrdU (1:650, OBT00030, Accurate Cheicals) + rabbit anti-Calbindin (1:250, ABN2192, EMD Millipore) 72 h 4 ˚C	Goat anti-rat Alexa-594 (1:800, A11007, Invitrogen) + goat anti-rabbit Alexa-488 (1:800, A11007, Invitrogen) 1 h RT
BrdU + GFAP	Every 12th section from HIP	NA	HCl 2N 30 min at 37 °C	5% normal goat serum 1 h at RT	Rat anti-BrdU (1:650, OBT00030, Accurate Cheicals) + mouse anti-GFAP (1:1000, Z0334, Dako) 24 h RT	Goat anti-rat Alexa-594 (1:800, A11007, Invitrogen) + goat anti-mouse Alexa-488 (1:800, A11001, Invitrogen) 1 h RT

Microscopy and image analysis (Paper I–II)

BrdU. BrdU+ cells were counted in the GCL and hilar areas of the DG using a Leica ICC50 HD microscope equipped with a 40x magnification objective (numerical aperture 1.4). The total number of BrdU+ cells was obtained by multiplying the sum of cell counts from every 6th section (240 μm apart, on average 8–9 sections per animal) by six. Left and right dentate gyri were quantified separately, and estimates were averaged per animal.

BrdU + calbindin (CalB)/ BrdU + GFAP. Immunofluorescent double-labeling was conducted, and fluorescent signals were detected with a confocal microscope (LSM 710 Duo, Carl Zeiss Microscopy GmbH) equipped with an argon laser. 3D images were constructed from a series of scans (12-15) of the DG taken at 2 μm intervals, using 20x objective and 4x digital zoom or 63x oil objective, and GCL and hilar areas were analyzed for colocalization. The sections were analyzed for the co-expression of BrdU with a mature neuronal marker, calbindin, and an astrocyte marker, GFAP. The data were expressed as a percentage of BrdU+ cells found in the DG expressing calbindin or GFAP compared to the average of sham group animals.

Doublecortin and PSA-NCAM. Microphotographs of doublecortin+ and PSA-NCAM+ cells were captured using a Leica ICC50 HD microscope equipped with and 63x magnification objective. The total number of PSA-NCAM+ or doublecortin+ cells in the GCL and hilar areas was obtained by multiplying the sum of cell counts from every 12th section (480 μm apart, in average 4 sections) by 12. Left and right dentate gyri were quantified separately, and estimates were averaged per animal.

Caspase-3. Cleaved caspase-3+ (referred to as caspase-3+) cells were counted in the GCL and hilar areas of the DG using a Leica ICC50 HD microscope equipped with an 40x magnification objective. A total number of caspase-3+ cells was obtained by multiplying the sum of cell counts from every 10th section (on average, 6 sections per animal) by ten. Left and right dentate gyri were quantified separately, and estimates were averaged per animal.

IBA1. In Paper I, IBA1+ cell morphology was assessed and in Paper II, both IBA1+ cell density and morphology were assessed from sections with coordinates Bregma -1.6 (±0.1), 2.4 (±0.1), and 3.3 (±0.1). Microphotographs were acquired using a Leica microscope HCC50 HD; a 10x objective was used for assessing cell density, and a 40x objective was used for assessing morphology. Images were further analyzed using Fiji software (National Institutes of Health). For morphology analysis, the ratio of the area of the cell body and total cell area (with processes) was measured to characterize changes during microglial activation, when the enlargement of the cell body and retraction of the processes occur (Kettenmann *et al.*, 2011). Therefore, an increase in cell body/total cell area ratio could indicate possible activation of microglial cells. Images for morphology analysis were acquired from comparable GCL, hilus, and molecular layer areas of the DG, and a semi-automatic image analysis adapted from Hovens and colleagues (2014) was conducted using Fiji software. Briefly, images were converted to 8-bit grayscale, the area covered by microglial cell bodies and cell processes were

determined by using filters for particle size (30 pixels to infinity) and intensity thresholds, which were manually set for every image based on manually counted numbers of microglial cells. The area of the cell body ratio to the area of the whole cell (%) was calculated based on acquired surface areas. For density analysis, IBA1+ cells were manually counted in the GCL, hilus, and molecular layer, and the surface areas of corresponding regions were measured. Cell density was calculated and expressed as the number of cells per mm^2 surface area.

TH. Brain sections were scanned with Aperio Versa digital pathology scanner (Leica), and images were analyzed using Fiji software. Microphotographs were converted to 8-bit format, the striatum was marked with a polygon tool, and the mean gray value was used as a marker for optical density reflecting TH+ signal intensity.

4.10. Cell culture experiments (Paper III)

4.10.1. Isolation of PBMCs

Fresh whole blood was collected from healthy male volunteers aged 20-35, and blood samples were obtained from Tartu University Hospital Blood Bank. Written informed consent was obtained from study participants, who had to state that they had not used narcotics within the past year. Human PBMCs were isolated by density centrifugation from the anticoagulated whole blood on the day of blood collection or one day later, using Ficoll-Paque PLUS medium (ref no GE17-1440-03, GE Healthcare) according to the manufacturer's instructions. PBMCs were dispersed in fetal bovine serum (FBS) containing 10% DMSO as cryoprotectant, and PBMC concentrated stocks were stored at -150°C until use.

4.10.2. Amphetamine, cocaine, and decitabine treatment

PBMCs were grown at 37°C, in an atmosphere of 95% air, 5% CO_2, and cultured in RPMI-1640 medium (ref no 11875093, Gibco, Thermo Scientific) containing 10% fetal bovine serum (FBS; ref no F0804, Sigma-Aldrich, Merck) on 24-well plates for 24 h before the incubations. For the first set of repeated treatments, PBMCs (donors n = 14–18) from the same donors were divided into three groups (control, amphetamine and cocaine) and incubated with amphetamine (0.3 µg/ml; ref no AMP-96-HC-100, Lipomed) or cocaine (3 µg/ml; ref no COC-156-HC-1000, Lipomed) for 1 hour a day on 4 consecutive days. For the second set of repeated treatments, PBMCs (n = 14) were incubated with a) cocaine (3 µg/ml), b) decitabine (0.3 µM; ref no HY-A0004/CS-0372, MedChemExpress), or c) simultaneously with cocaine (3 µg/ml) and decitabine (0.3 µM) for 1 hour a day on 4 consecutive days. Cocaine and amphetamine were dissolved in cell culture media; decitabine was dissolved first in DMSO, up to a concentration of 20 mM, and then further diluted in cell culture media to 1 mM concentration. For experiments including decitabine treatment, a vehicle treatment was applied to the control group, containing the same volume of DMSO as the decitabine treatment group. The concentrations of cocaine and amphetamine used in the study were

based on forensic medical examinations (Spiehler & Reed, 1985; Jones & Holmgren, 2005). PBMCs were supplemented with fresh medium after every incubation, cells, and medium were collected 24 h after the last treatment. Cell lysates, RNA, DNA, and nuclear extracts were prepared and stored at -80°C.

4.11. Flow cytometry (Paper III)

The flow cytometry protocol was adapted from a method described by Gopinath and colleagues (2020). PBMCs from healthy donors (male, age = 20–35, n = 6) were thawed and suspended in warm RPMI-1640 medium at 37°C (ref no 11875093, Gibco, Thermo Scientific) containing 10% FBS (ref no F0804, Sigma-Aldrich, Merck). Following, naïve PBMCs were stained with cell surface markers for mouse anti-CD3 (1:100, ref no 300454, Biolegend), mouse anti-CD8 (1:200, ref no 563795, BD Biosciences), mouse anti-CD14 (1:500, 301851, Biolegend) and mouse anti-CD16 (1:500, ref no 392406, Biolegend, CA, USA) in flow cytometry buffer containing 2 mM EDTA and 0.5% BSA in PBS, for 30 min at +4°C. Next, the cells were fixed by incubation with IC Fixation Buffer (88-8824-00, eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, Invitrogen) for 45 minutes at room temperature. Cells were then stained for 30 min at room temperature in the dark with primary antibodies: rat anti-dopamine transporter (DAT, 1:100 ref no MAB369, Merck Millipore) and rabbit anti-tyrosine hydroxylase (TH, 1:100, AB152, Merck Millipore), following an incubation with secondary antibodies anti-rat IgG-APC (1:40, ref no 551019, BD Biosciences) and anti-rabbit IgG-B421 (1:40, 565014, BD Biosciences) in permeabilization buffer (eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, Invitrogen, CA, USA) at room temperature in dark. A washing step with 2 ml-s of flow cytometry buffer followed every staining procedure and fixation. Negative controls were used for each donor sample to define TH+ and DAT+-expressing cell populations. Primary antibody staining with anti-TH and anti-DAT antibodies was omitted for negative controls, and only secondary antibodies were used. After the final washing procedure, cells were re-suspended in a flow cytometry buffer and analyzed using LSR Fortessa flow cytometer (BD Biosciences, CA, USA) and BD FACSDiva software (BD Biosciences, CA, USA). Experiments were compensated using single-color compensation controls of SPHERO™ COMPtrol antibody capture beads (CMIgP-70-3K, Spherotech) with aforementioned fluorochrome-conjugated antibodies, except for anti-rabbit and anti-mouse secondary antibodies, which were replaced with APC anti-human CD56 (392406, Biolegend) and BV241 anti-CD127 antibodies (351310, Biolegend). 100 000 events were recorded for every sample. The gating strategy and optical detector configuration are shown in Table 4 and Figure 8.

Table 4. Configuration details of LSR Fortessa optical detectors.

Laser	Detector	LP Mirror	BP Filter	Intended Dye
488-nm blue laser	A	635	710/50	-
	B	505	530/30	FITC
	C		488/10	Side scatter (SSC)
405-nm violet laser	A	670	710/40	-
	B	630	670/30	-
	C	600	610/20	-
	D	535	540/30	-
	E	505	525/50	-
	F	-	440/40	BV421
355-nm UV laser	A	505	530/30	-
	B	-	450/50	BUV395
640-nm red laser	A	750	70/60	-
	B	690	730/45	AF700
	C	-	670/14	APC
561-nm YG laser	A	750	780/60	-
	B	685	710/50	-
	C	635	670/30	-
	D	600	610/20	PE-CF594
	E	-	586/15	-

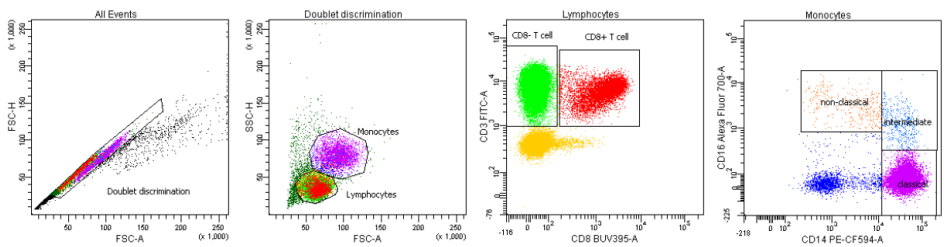

Figure 8. Gating strategy for defining T-lymphocyte and monocyte subsets. Firstly, singlets were selected for further analysis based on forward scatter height (FSC-H) and forward scatter area (FSC-A). Next, lymphocyte and monocyte subsets were chosen based on their side scatter height (SSC-H), describing the size, and forward scatter area (FSC-A), describing granularity. Finally, CD8- and CD8+ T-cell subsets were defined based on their expression of CD8 and CD3 surface markers, and monocytes were divided into non-classical, intermediate, and classical subsets based on the expression of CD16 and CD14.

4.12. Molecular biology experiments (Papers II–III)

4.12.1. Western blot (Paper II)

Hippocampi or striata was lysed in Tris-HCl buffer (Tris-HCl 50mM, pH 7.3, containing protease and phosphatase inhibitors cocktail), incubated on ice for 25 minutes, and centrifuged (13 000 rpm for 20 minutes at 4 °C). Equivalent amounts of proteins were resolved by electrophoresis on 8% or 13% SDS-polyacrylamide gels. Resolved proteins were transferred to Immobilon-FL PVDF Membranes (IPFL00010, Merck Millipore) in 0.1 M Tris-base, pH 8.3, 0.192 M glycine, and 20% (v/v) methanol using an electrophoretic transfer system (Bio-Rad). The membranes were blocked using Odyssey® blocking buffer (ref no 927-50000, Li-Cor Bioscience). After blocking, the membranes were incubated overnight with mouse anti-caspase 1 (1:1000, ref no AG-20B-0042-C100, Adipogen), mouse anti-mCSF (1:1000, ref no ab233387, Abcam) or rabbit anti-TH (1:1000, ref no Ab152, Sigma-Aldrich). Incubations were followed by washing and incubation with goat anti-mouse IR-Dye® 800CW (1:10 000, ref no 926-32210, Li-Cor Biosciences) or goat anti-rabbit IR-Dye® 800 or 680 (1:10 000, ref no 926-32211 or 926-68021, respectively, Li-Cor Biosciences) for 1 hour at room temperature. Immunoreactive bands were detected using the Odyssey Infrared Imaging System (Odyssey CLx®, Li-Cor Bioscience). To normalize immunoreactivity of the proteins, β-actin was measured on the same blot by using the mouse (1:10 000, ref no A2228, Sigma-Aldrich) or rabbit anti-β-actin antibody (1:1000, ref no 926-42210, Li-Cor Biosciences), followed by incubating with goat anti-mouse IR-Dye® 800CW, anti-rabbit IR-Dye® 680LT or 800CW (1:10 000, ref no 926-32210, 926-68021 or 926-32211, respectively, Li-Cor Bioscience). The ratio of proteins of interest to β-actin was calculated and expressed as the mean OD ratio in arbitrary units ± standard deviation (SD).

4.12.2. Real-time quantitative polymerase chain reaction (RT-qPCR) (Paper III)

Total RNA was extracted from the PBMCs by using RNeasy Mini Kit (QIAGEN, Hilden, Germany) as previously described (Anier *et al.*, 2018), and cDNA was synthesized from 375 ng of total RNA using the First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). QPCR was conducted using QuantStudio 12K Flex Real-Time PCR System equipped with QuantStudio 12K Flex Software (Thermo Scientific). The primers (listed in Table 5) were designed using Primer3 with BLAST sequence verification and synthesized by TAG Copenhagen AS (Denmark). Results were normalized to housekeeping gene *B2M* and calculated using the comparative C_T ($2^{-\Delta\Delta C_T}$) method (Schmittgen & Livak, 2008).

Table 5. QPCR primer sequences

Target genes	Primer sequence
DNMT1	Forward: GTTCTTCCTCCTGGAGAATGTT Reverse: GTCTGGGCCACGCCGTACT
DNMT3A	Forward: TATTGATGAGCGCACAAGAGAGC Reverse: GGGTGTTCCAGGGTAACATTGAG
DNMT3B	Forward: GGCAAGTTCTCCGAGGTCTCTG Reverse: TGGTACATGGCTTTTCGATAGGA
TET1	Forward: AATGGAAGCACTGTGGTTTG Reverse: ACATGGAGCTGCTCATCTTG
TET2	Forward: GTGAGATCACTCACCCATCG Reverse: CAGCATCATCAGCATCACAG
TET3	Forward: GAGGAGCGGTATGGAGAGAA Reverse: AGTAGCTTCTCCTCCAGCGT
B2M	Forward: TGCTCGCGCTACTCTCTCT Reverse: TCCATTCTCTGCTGGATGAC
TH	Forward: CGGAAGCTGATTGCAGAGAT Reverse: GGGTAGCATAGAGGCCCTTC
DAT	Forward: CGAGCCTGCTTGCTGATATT Reverse: ATGGCATCCACTTTCCTGTC
DRD1	Forward: AGGGGAATTTGCAGTTCTGT Reverse: AAAAGATGGAGAGGGCCAAT
DRD2	Forward: GCAGACCACCACCAACTACC Reverse: CCACTCACCTACCACCTCCA
DRD3	Forward: CACTGTCTGCTCCATCTCCA Reverse: GAGGATCCTTTTCCGTCTCC
DRD4	Forward: CCTTCTTCGTGGTGCACAT Reverse: AACTCGGCGTTGAAGACAGT
DRD5	Forward: GCCTACCAGAGATGGACCAA Reverse: AAAAGGGAGGGGAGAGCATA
IL10	Forward: GCCTAACATGCTTCGAGATC Reverse: TGATGTCTGGGTCTTGGTTC
ATP2B4	Forward: AACTCTCAGACTGGAATCATC Reverse: ACCTTTCTTCTTTTTCTCCC

4.12.3. DNMT and TET enzymes' activity measurement (Paper III)

Nuclear proteins were extracted from the PBMCs according to the manufacturer's protocol (Nuclear Extraction kit; ab113474; Abcam, Cambridge, UK). The activity of DNMTs was determined using the colorimetric Abcam DNMT activity assay kit (ab113467), and activity of TETs was assessed using the colorimetric Hydroxylase Activity Quantification kit (ab156912) according to the manufacturer's instructions.

4.12.4. Global DNA Methylation/hydroxymethylation assay (Paper III)

Genomic DNA was extracted from the PBMCs using the QIAamp DNA Micro kit (ref no 51304, Qiagen). For the quantification of global DNA methylation and hydroxymethylation, colorimetric Global DNA Methylation Assay (ab233486; Abcam) and colorimetric Global DNA Hydroxymethylation Assay kit (ab233487; Abcam, Cambridge, UK) were used following the manufacturer's instructions. The percentage of methylated DNA (5-mC %) /hydroxymethylated DNA (5-hmC %) in total DNA was quantified according to the manufacturer's protocol and formula.

4.12.5. ELISA assay for quantifying IL-6 and IL-10 concentration in the cell culture media (Paper III)

Supernatants for IL-6 and IL-10 concentration measurements were collected 24 hours after the last treatment with cocaine and/or decitabine for 1 h in 4 consecutive days. The secretion of IL-6 and IL-10 into the cell culture media was determined by using a Human IL-6 high-sensitivity ELISA kit (ab46042; Abcam Cambridge, UK) and a Human IL-10 high-sensitivity ELISA kit (ab46059; Abcam Cambridge, UK) according to the manufacturer's protocol.

4.13. Next Generation Sequencing (NGS, Paper III)

DNA from PBMCs was extracted using a QIAamp DNA Mini kit according to the manufacturer's protocol (ref no, 51304, QIAGEN), and 500 ng DNA was used for input. The NGS library preparation and sequencing were conducted at the Core Facility of the Institute of Genomics at the University of Tartu. Libraries were prepared with Illumina® TruSeq® Methyl Capture EPIC Library Prep kit (Illumina Inc.), which uses bisulfide conversion to detect methylated cytosines, according to manufacturer's instructions and quantified using Illumina-specific KAPA Library Quant Kit (ref no KR0405, Kapa Biosystems). Sequencing was performed on an Illumina NextSeq500 System in paired-end 2 × 100 bp mode. Sequencing data were demultiplexed using the Illumina Local Run Manager Generate FASTQ Analysis Module v2.0.

4.14. Illumina NGS Bioinformatics (Paper III)

Raw data quality control was conducted using FastQC v.0.11.9 (Andrews, 2010). Data was trimmed using Trimmomatic-0.39 (Bolger *et al.*, 2014) in paired-end mode; max 3 low-quality base pairs from both the start and the end of the read were removed. Quality control after trimming was carried out using FastQC v.0.11.9. Next, trimmed and filtered reads were aligned using Bismark-0.19.1 (Krueger & Andrews, 2011) to Human genome version 19 (hg19) in the paired-end mode (Guo *et al.*, 2017). Alignment quality and statistics were obtained using

Picard-tools v.2.0.1 (Broad Institute, 2019) package modules: CollectAlignmentSummaryMetrics, CollectGcBiasMetrics, CollectWgsMetrics, QualityScoreDistribution. The CPG site coverage was calculated using bedtools v2.27.0 (Quinlan & Hall, 2010) and Bismark-0.19.1 Methylation Extractor module was used for methylation extraction and data quality statistics (Krueger & Andrews, 2011). Downstream methylation analyses were conducted with R (3.6.0) package RnBeads 2.8.0 (Assenov *et al.*, 2014; Müller *et al.*, 2019) and Gene Ontology (GO) Enrichment Analysis was conducted using a hypergeometric test addressing the hierarchical structure of the ontology.

4.15. Statistical analysis (Paper III)

In Paper I, statistical comparisons were made using Student's t-test, one-way analysis of variance (ANOVA), repeated measures (RM) one- or two-way ANOVAs followed by Bonferroni post-hoc tests. In Paper II, all datasets were first tested for normal distribution using Shapiro-Wilk's test. If the data was normally distributed and followed Gaussian distribution, statistical comparisons were made using either Student's t-test, two-way ANOVA followed by Tukey's post-tests, or three-way ANOVA followed by Šidak's post-tests. If the data did not follow a normal distribution, the Mann-Whitney or Kruskal-Wallis test, followed by Dunn's post-tests, were used for statistical comparisons. In Paper III, one-way ANOVAs with Bonferroni's post hoc test were applied to make statistical comparisons. GraphPad Prism software was used for all statistical analyses except for the NGS data in Paper III, which was specified in the previous section. P-value ≤ 0.05 was considered statistically significant.

5. RESULTS

5.1. The behavioral effects of neuropathic pain and correlates with impaired neurogenesis (Paper I)

5.1.1. Sciatic nerve cuffing (SNC) caused mechanical allodynia

Mechanical allodynia was tested to validate the neuropathic pain model, as mechanical allodynia is a characteristic feature of SNC (Kremer *et al.*, 2020). No postoperative complications after surgery were observed, and both sham as well as cuffed mice gained weight during the experiment (time: $p < 0.0001$, $F_{(8,162)} = 11.78$, cuffing: $p > 0.0001$; $F_{(1,162)} = 15.42$, interaction: $p = 0.9833$; $F_{(8,162)} = 0.2371$; two-way ANOVA; Figure 9A). The results from the beam test showed no effect of the SNC on motor balance and coordination (data not shown).

Mechanical sensitivity and allodynia were evaluated via the Von Frey test once a week, starting one week before the SNC, to establish a baseline. Ipsilateral allodynia was detected in cuffed mice from the first post-surgery week and sustained until the end of the experiment at week 8 ($p \leq 0.05$, Bonferroni post-test, Figure 9B). Two-way ANOVA revealed a main effect of cuffing ($p < 0.0001$, $F_{(1,162)} = 260.91$), time ($p < 0.0001$, $F_{(8,162)} = 10.21$), and interaction ($p < 0.0001$, $F_{(8,162)} = 6.779$).

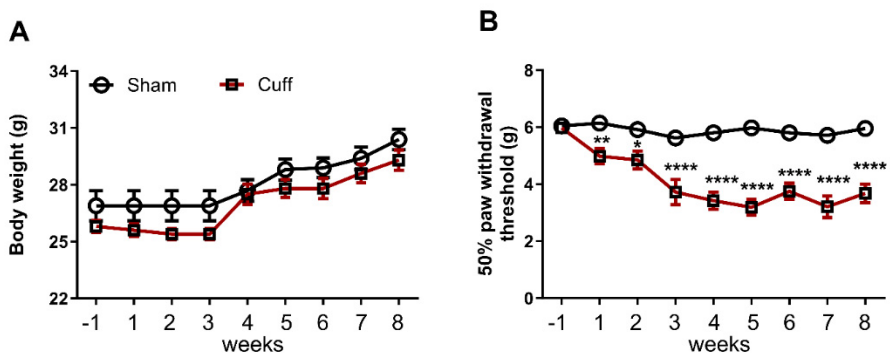

Figure 9. Body weight and mechanical sensitivity before and after the SNC. (A) No changes were detected between the body weight of sham-operated (Sham) and cuffed (Cuff) mice during the testing period. (B) The cuffed mice developed an amplified response to the mechanical stimulus, expressed as a decrease in the 50% paw withdrawal threshold in the Von Frey test. The data are expressed as mean ± SEM, *$p \leq 0.05$, **$p < 0.001$, ****$p < 0.0001$, $n = 10$, per group, two-way ANOVA followed by Bonferroni post-tests.

5.1.2. SNC induced anxiety and depressive-like behavior

EPM and TST were conducted bi-weekly from day 14 following surgery to examine the time course of anxiety and depressive-like behavior development, respectively. Additionally, NSFT was performed on POD 57 to provide additional evidence for the development of anxiety and depressive-like behavior. Cuffed mice demonstrated anxiety-like behavior characterized by a decrease in the percent of open-arm entries on POD 28 in comparison to sham-operated mice ($p = 0.0322$, Bonferroni post-test, Figure 10A), which reached a plateau on POD 28, as percent of open-arm entries did not further decrease on days POD 42 and 56. Two-way ANOVA revealed a main effect of time ($p < 0.0001$, $F_{(3,64)} = 9.581$) and interaction ($p = 0.0078$, $F_{(3,64)} = 4.316$) and a trend towards the main effect of cuffing ($p = 0.0655$, $F_{(1,64)} = 3.512$). Additionally, the cuffed group exhibited decreased total arm entries on PODs 28 ($p = 0.0057$), 42 ($p = 0.0012$), and 56 ($p = 0.0067$, Bonferroni post-tests; Figure 10B), reflecting a decrease in general locomotor activity. Two-way ANOVA revealed the main effect of SNC ($p < 0.0001$, $F_{(1,64)} = 28.2$) and interaction ($p = 0.0256$, $F_{(3,64)} = 3.309$), but not time ($p = 0.6453$, $F_{(3,64)} = 0.5571$) on total arm entries.

Cuffed mice exhibited increased immobility time in TST on PODs 42 ($p < 0.0001$) and 56 ($p = 0004$, Bonferroni post-tests), reflecting depressive-like and despair-behavior (cuffing: $p < 0.0001$, $F_{(1,69)} = 24.84$; interaction: $p = 0.0036$, $F_{(3,69)} = 4.952$; time = 0.0575, $F_{(3,69)} = 2.621$; two-way ANOVA, Figure 10C). NSFT conducted on POD 57 provided additional support for the anxiety and depressive-like behavior inducing effects of 8-week-long chronic neuropathic pain in mice, as the latency to feed was increased in the novel cage ($p = 0.0038$, Student's t-test; Figure 10D), but not in the home cage (data not shown).

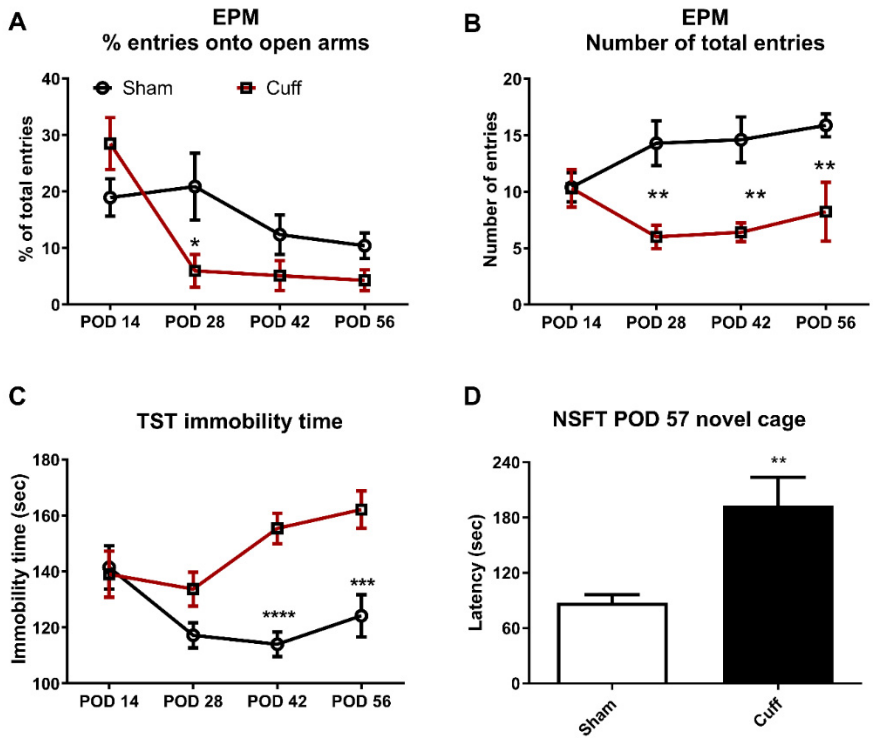

Figure 10. Anxiety and depressive-like behavior after SNC was assessed in an elevated plus maze (EPM) test, tail-suspension test (TST), and novelty-suppressed feeding test (NSFT). (A**)** Cuffed (Cuff) mice exhibited a reduced percentage of entries into open arms in the EPM test in comparison to sham-operated (Sham) mice on POD 28 and (B) fewer total entries into arms compared to sham-operated mice on PODs 28, 42, and 56. (C) Cuffed mice had increased immobility time in TST on PODs 42 and POD 56 (D) and increased latency to feed in NSFT on POD 57 compared to sham-operated mice. The data are expressed as mean ± SEM, *p ≤ 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, (A, B) n = 7–10, per group, (C) n = 8–10, per group, (D) n = 7-8, per group, (A, B, C) two-way ANOVA followed by Bonferroni post-tests and (D) Student's t-test.

5.1.3. SNC impaired memory

Contextual fear memory conditioning and extinction tests were performed on PODs 50-58 to assess whether chronic neuropathic pain can alter the ability of the mice to learn and remember the association between aversive stimulus (foot shock) and environmental cues characterized by fear retention 24 hours after the training, as well as relearning abilities during the following 8 days.

No differences were found in baseline freezing time during habituation or training between the sham-operated and cuffed animals (data not shown). Twenty-four hours after the training session, cuffed mice exhibited a freezing response upon placement in the training chamber, showing long-term retention

of contextual fear memory (p = 0.0378, Student's t-test, Figure 11A). Cuffed mice also exhibited impaired ability to relearn from day 4 to 8 following training, as they spent more time freezing in comparison to the sham-operated group (cuffing: $p < 0.0001$, $F_{(1,144)} = 47.79$; time: $p < 0.0001$, $F_{(7,144)} = 6.470$; interaction: $p < 0.0001$, $F_{(7,144)} = 5.192$; two-way ANOVA). On the 9th day, cuffed mice demonstrated worsened tone-induced fear memory recall compared to the control group (p = 0.0017, Student's t-test; unpublished data). Additionally, cuffed animals demonstrated episodic memory impairments in the ORT, as their preference ratio towards the novel object was lower than the sham group's in both short-term memory (p = 0.0032, Student's t-test, Figure 11B) as well as in long-term memory (p = 0.0419, Student's t-test) tests (unpublished data).

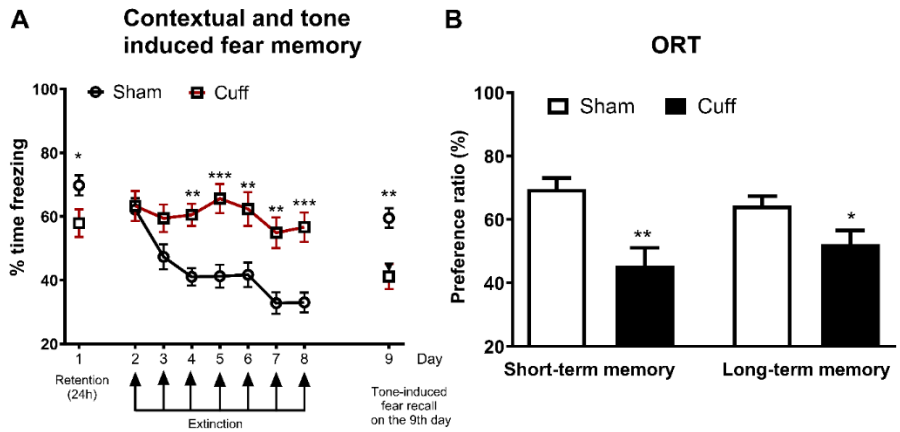

Figure 11. Memory impairment after SNC. (A) Cuffed mice (Cuff) spent less time freezing 24 hours after a training session in the testing chamber describing fear memory retention than sham-operated (Sham) mice. In the fear memory extinction test, cuffed mice spent more time freezing on days 4–8 than sham-operated mice. Cuffed mice spent less time freezing in the tone-induced fear memory recall test on the 9th day than sham-operated mice. (B) In the object recognition test (ORT), cuffed mice exhibited a lower preference ratio towards the novel object in both short-term memory and long-term memory tests. The data are expressed as mean ± SEM, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, (A) n = 10, per group, (B) n = 8–9, per group, (D) n = 7–8, per group, (A) Student's t-test (retention) and two-way ANOVA followed by Bonferroni post-tests (extinction), (B) Student's t-test.

5.1.4. SNC reduced GCL volume and caused cell loss in the GCL of the DG

As the hippocampus is crucial for episodic-like and contextual memory functions (Anacker & Hen, 2017; Toda *et al.*, 2019), we assessed morphological changes in the neurogenic DG. Next, we focused on cellular alterations in the neurogenic DG area of the hippocampus. Based on the hematoxylin-eosin staining, the

volume of the GCL and hilus in the DG and cell number in the GCL were assessed (Figure 12A). In cuffed animals, the volume of hilus was not altered compared to the sham-operated group ($p = 0.2548$, Student's t-test, Figure 12B). However, we observed a 7.9% (SEM ± 3.4%) decrease in the volume of the GCL ($p = 0.0344$, Student's t-test, Figure 12C) in cuffed mice compared to sham-operated mice. Moreover, the cell number in the GCL ($p = 0.0247$, Student's t-test, Figure 12D) was decreased in cuffed animals.

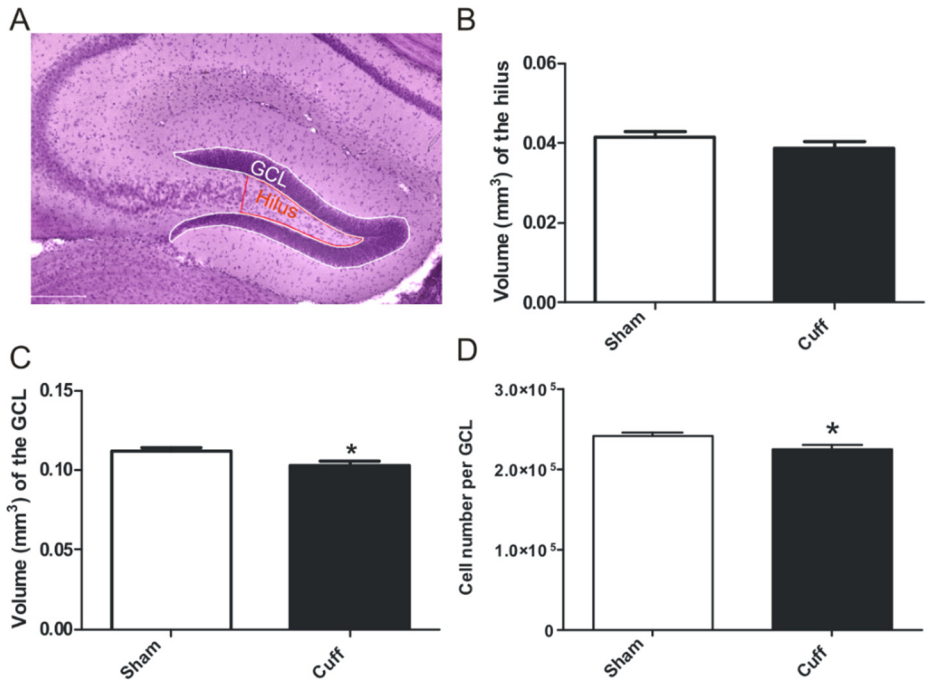

Figure 12. Declined granule cell layer (GCL) volume and reduced cell number in the GCL after SNC. (A) An illustrative image of hematoxylin-eosin staining of the DG, including the GCL and hilus. (B) While the volume of the hilus remained unchanged after SNC, (C) a reduction in the volume of the GCL and (D) cell number in the GCL was observed in cuffed mice (Cuff) in comparison to the sham-operated mice (Sham). The data are expressed as mean ± SEM, *$p \leq 0.05$, $n = 8$, per group, Student's t-test; scale bar = 150 μm.

5.1.5. SNC reduced the long-term survival of newly generated cells in the DG, but did not affect proliferation

Proliferation and long-term survival of newly generated cells were assessed in the GCL and hilus areas of the DG. For assessing proliferation, BrdU+ cells were labeled and counted on brain tissue collected on POD 43, 24 h after BrdU injection. Analysis of BrdU+ proliferating cells revealed no changes in cell proliferation between the groups ($p = 0.2483$, Student's t-test, Figure 13A, B, C).

To determine the long-term survival of newly generated cells, BrdU+ cells were counted on POD 60 (25 days after BrdU administration). A decrease in the long-term survival of newly generated cells was found in cuffed mice compared to sham-operated ($p = 0.0103$, Student's t-test, Figure 13D, E, F).

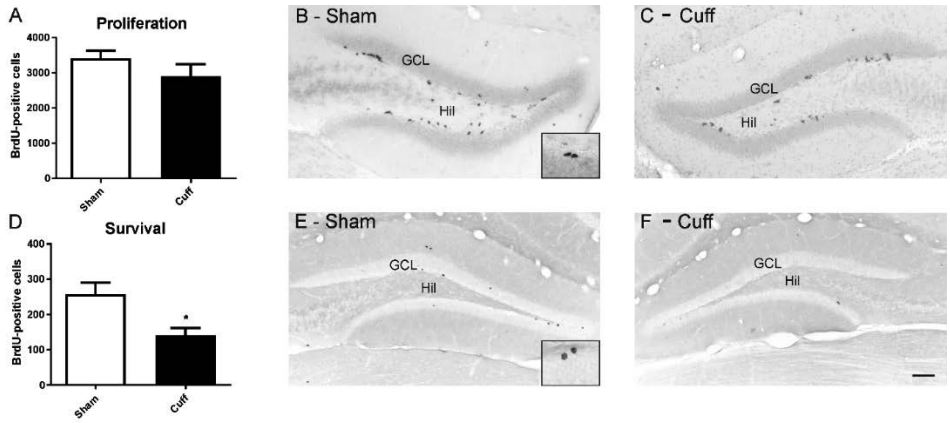

Figure 13. Proliferation and reduced long-term survival of newly generated cells in the DG after SNC. (A, B, C) No differences were observed in the number of proliferating BrdU+ cells in cuffed mice (Cuff) compared to the sham-operated (Sham). (D, E, F) A decline in BrdU+ cells, which survived for 25 days, was found in the cuffed mice compared to sham-operated mice. Illustrative microphotographs of (B, C) proliferating BrdU+ and (E, F) surviving BrdU+ cells taken with 10x objective. Inserts demonstrate BrdU+ cells at a higher magnification, taken with 100x objective. The data are expressed as mean ± SEM, $*p \leq 0.05$, (A) n = 5, per group, (D) n = 8, per group, Student's t-test; scale bar = 150 μm.

5.1.6. SNC did not affect the number of neuroblasts/immature neurons in the DG

To quantify the number of neuroblasts/immature neurons, brain sections were stained with antibodies against doublecortin and PSA-NCAM, and their numbers were quantified in the hilus and GCL regions of the DG. However, no differences were observed in the number of doublecortin+ ($p = 0.8682$, Student's t-test, Figure 14A, B, C) and PSA-NCAM+ cells ($p = 0.3580$, Student's t-test, Figure 14D, E, F) between the groups.

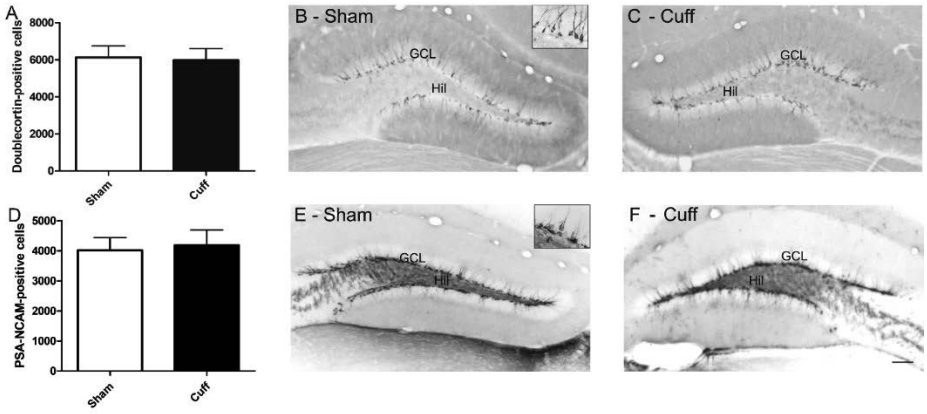

Figure 14. The number of doublecortin+ and PSA-NCAM+ cells in the DG after SNC. No alterations in the number of (A, B, C) doublecortin+ or (D, E, F) PSA-NCAM+ cells in the DG were found after SNC. Representative microphotographs of (B, C) doublecortin+ and (E, F) PSA-NCAM+ cells in the subgranular cell layer in the DG were taken with a 10x objective. Inserts represent (B, C) doublecortin+ and (E, F) PSA-NCAM+ cells at a higher magnification, taken with 100x objective. The data are expressed as mean ± SEM, $p > 0.05$, $n = 5$, per group, Student's t-test; scale bar = 150 μm.

5.1.7. SNC reduced differentiation into mature neurons in the DG

To assess the differentiation profile of newly generated cells, BrdU+ cells that survived long-term (25 days) were co-stained with calbindin, a marker for mature neurons, or with GFAP, a marker for astrocytes/radial glial cells. A significantly lower percentage of BrdU+ cells differentiated into mature neurons in cuffed mice than in sham-operated ($p = 0.0010$, Student's t-test; Figure 15A, B, C). In cuffed mice, 35.8% (SEM ± 5.2%) of total BrdU+ cells co-localized with CalB, while in sham-operated mice, the percentage was 59.9% (SEM ± 2.6%). In parallel, a significantly higher percentage of BrdU+ cells co-expressed GFAP in cuffed mice in comparison to sham-operated ($p = 0.04333$, Student's t-test; Figure 15D, E, F), as the percentage of BrdU+/GFAP+ cells in cuffed mice was 44.1% (SEM ± 7.3%) and in sham-operated mice was 26.2% (SEM ± 2.6%).

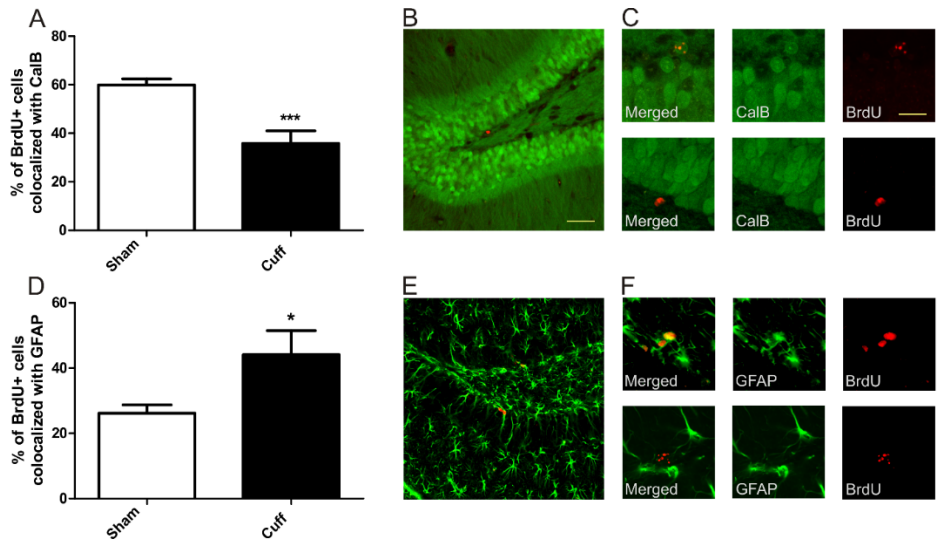

Figure 15. The phenotype of newly generated cells in the GCL and hilus of the DG after SNC. (A, B, C) Cuffed mice (Cuff) demonstrated a significantly lower percentage of BrdU+ cells co-localizing with CalB than sham-operated (Sham) mice on POD 60, 25 days after BrdU injection. (D, E, F) In parallel, cuffed mice demonstrated an increased percentage of BrdU+ cells that co-localized with GFAP compared to sham-operated mice. (B) The illustrative microphotograph demonstrates the CalB (green) signal in the GCL and BrdU (red) signal in the subgranular layer of the DG (20x objective). (C) Representative confocal microphotographs demonstrating BrdU+/CalB+ cells (upper panels) and BrdU+/CalB- cells (lower panels) (63x objective and 2x digital zoom). (E) Illustrative microphotograph demonstrating GFAP (green) signal in the GCL and BrdU red signal (20x objective) in the DG. (F) Representative confocal microphotographs showing BrdU+/GFAP+ cells (upper panels) and BrdU+/GFAP- cells (lower panels) in the DG. The data are expressed as mean ± SEM, (A) *** $p < 0.001$, n=8, per group, (D) *$p \leq 0.05$, n = 5, per group, Student's t-test; scale bar = 20/100 µm.

5.1.8. SNC altered microglial morphology in the DG

To characterize microglial reactivity in the DG of the hippocampus, brain sections were stained with an antibody against IBA1, and image analysis was conducted to characterize the cell-body-to-cell size ratio. A retraction of microglial processes and enlargement of the cell body has been described during microglial activation. Therefore, an increase in the cell body to whole cell size ratio indicates the activation process of microglial cells (Kettenmann *et al.*, 2011; Hovens *et al.*, 2014). Indeed, we observed an increase in the cell body to cell size ratio in the microglial cells of the cuffed mice in comparison to sham-operated ($p = 0.0227$, Student's t-test, Figure 16; unpublished data).

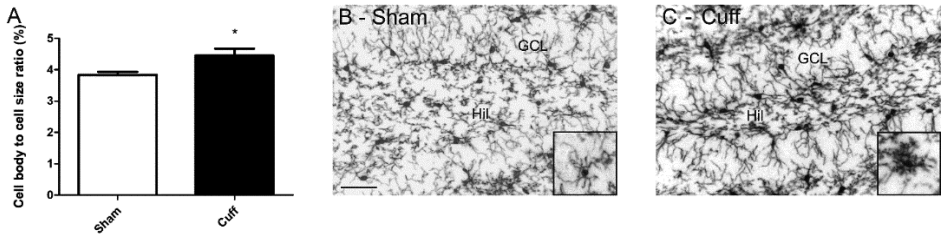

Figure 16. Microglial activation in the DG after SNC. (A) Cuffed (Cuff) mice demonstrated an increased cell body-to-cell size ratio compared to sham-operated (Sham) mice in the DG of the hippocampus. Representative microphotographs of IBA1+ microglial cells in the DG in (B) sham-operated and (C) cuffed mice taken with 40x objective inserts demonstrate IBA1+ cells at a higher magnification. The data are expressed as mean ± SEM, *$p \leq 0.05$, n = 8, per group, Student's t-test; scale bar = 50 μm.

5.2. The behavioral effects of long-term UCMS, VDD, VDD+UCMS and their correlates with brain plasticity (Paper II)

5.2.1. Long-term vitamin D free diet reduced serum 25(OH)D concentration

To confirm that a vitamin D-free diet results in VDD, 25(OH)D levels were measured in the serum of 7.5-month-old mice before the start of UCMS and at the end of the experiment. Indeed, 3.5 months of vitamin D-free diet resulted in significantly lower levels of 25(OH)D in 7.5-month-old VDD mice in comparison to the control ($p > 0.0001$, Tukey's post-test, Figure 17A). Similarly to the earlier time-point, 12-month-old VDD mice demonstrated significantly lower levels of 25(OH)D in their serum than in control mice ($p = 0.0036$, Tukey's post-test). In addition, 12-month-old control mice had significantly lower 25(OH)D levels in comparison to their younger, 7.5-month-old counterparts ($p < 0.0001$, Tukey's post-test). Two-way ANOVA analysis revealed main effect of VDD ($p < 0.0001$, $F_{(1,9)} = 157.2$), main effect of time ($p = 0.0003$, $F_{(1,9)} = 32.07$) and an interaction effect ($p = 0.0003$, $F_{(1,9)} = 37.77$). No effect of UCMS was observed in 12-month-old mice, as UCMS mice exhibited similar levels of 25(OH)D as control mice ($p = 0.0572$, Tukey's post-test; Figure 17B), and UCMS in VDD mice did not further reduce 25(OH)D levels ($p = 0.9997$, Tukey's post-test). Two-way ANOVA revealed main effect of VDD ($p < 0.0001$, $F_{(1,13)} = 300.5$), but not UCMS ($p = 0.0639$, $F_{(1,13)} = 4.131$) or interaction ($p = 0.0794$, $F_{(1,13)} = 3.622$). No effect of VDD or UCMS was found on Ca^{2+} serum levels in 7.5- and 12-month-old mice (data not shown).

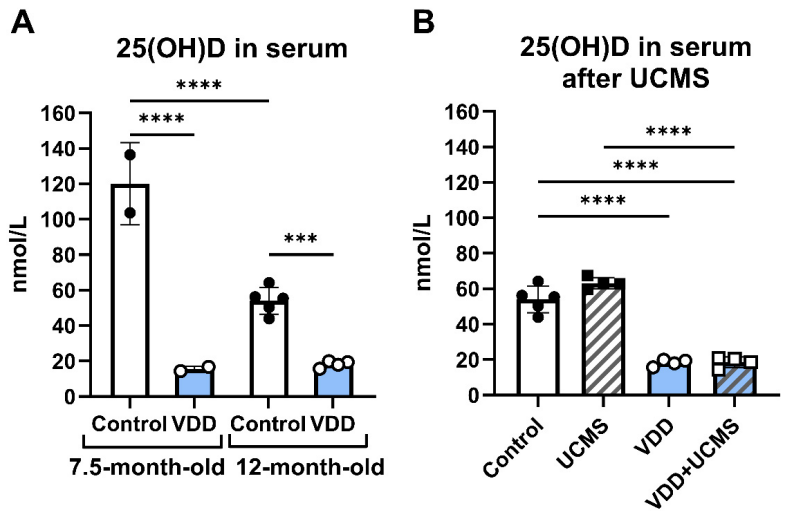

Figure 17. Lowered 25(OH)D levels due to VDD and aging. (A) 7.5-month-old and 12-month-old VDD mice had significantly lower serum levels of 25(OH)D than their age-corresponding control mice. Additionally, 12-month-old control mice had lower 25(OH)D levels than their younger, 7.5-month-old counterparts. (B) While VDD lowered 25(OH)D levels in 12-month-old mice, no effect of UCMS was observed on 25(OH)D levels. The data are expressed as mean ± SD, ***$p < 0.001$, ****$p < 0.0001$, (A) n = 2–5, (B) n = 5, per group, two-way ANOVA followed by Tukey's post-tests.

5.2.2. VDD impaired episodic-like memory, but did not cause depressive-like behavior

To determine the effect of VDD on memory and depressive-like behavior on the baseline, ORT and TST were carried out on 9.5-month-old mice. No differences were detected between groups in locomotor activity (data not shown) during the habituation session in ORT. VDD mice demonstrated a lower preference ratio (%) towards the novel object in comparison to the control mice in both short-term memory ($p < 0.0001$, Mann-Whitney test; Figure 18A) and long-term memory ($p = 0.0007$, Student's t-test; Figure 18B) tests.

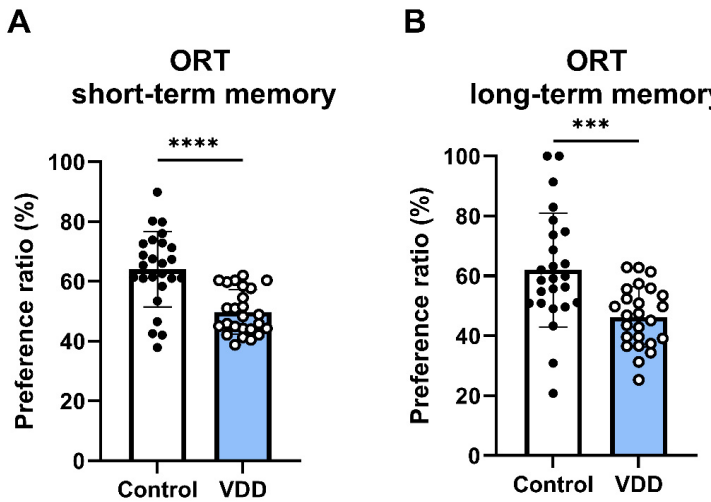

Figure 18. Impaired episodic-like memory in ORT due to VDD. VDD mice demonstrated a lowered preference ratio towards the novel object in both (A) short-term memory and (B) long-term memory tests. The data are expressed as mean ± SD, ***$p < 0.001$, ****$p < 0.0001$, n = 24–25, per group, (A) Mann-Whitney test, (B) Student's t-test.

No effect of VDD was observed on the immobility time reflecting depressive-like and despair-behavior ($p > 0.05$, Student's t-test; Figure 19A). However, VDD mice exhibited decreased latency time to the first immobility episode ($p = 0.0292$, Student's t-test; Figure 19B) and an increased number of immobility episodes ($p = 0.0262$; Mann-Whitney test, Figure 19C).

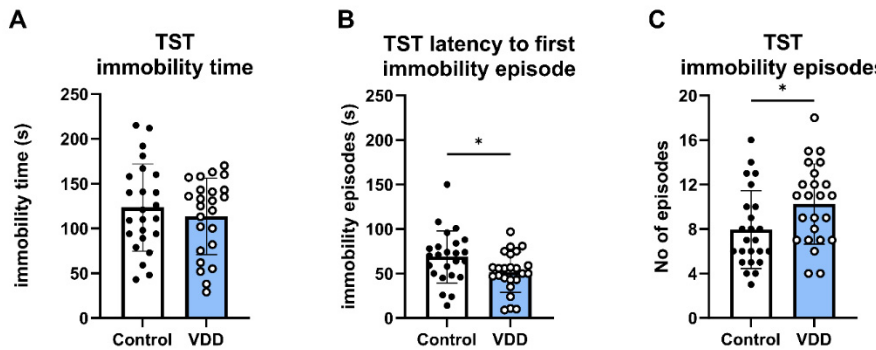

Figure 19. Unaffected depressive-like behavior due to VDD. (A) The immobility time in TST was similar in the control and VDD groups. (B) Latency time to the first immobility episode was shorter in VDD mice, and (C) the number of immobility episodes was increased in VDD mice compared to the control. The data are expressed as mean ± SD, *$p \leq 0.05$, n = 25, per group, (A, B) Student's t-test, (C) Mann-Whitney test.

5.2.3. UCMS and/or VDD impaired contextual fear memory and tone-induced fear memory recall

The contextual fear memory assessment was carried out after 8 weeks of UCMS to assess if VDD independently or in combination with UCMS causes fear memory impairment. When assessing baseline freezing behavior during the training session, which immediately followed the aversive stimulus, no differences between the groups were observed (data not shown). Contextual fear memory retention, which was assessed 24 hours after the training session, was impaired in UCMS, VDD, and VDD+UCMS groups (UCMS vs. control: $p = 0.0348$; VDD vs. control: $p = 0.0164$; VDD+UCMS vs control: $p = 0.0014$, Tukey's post-tests; Figure 20A) as the percent of time spent freezing in the training environment was lower in these groups in comparison to the control. However, UCMS in VDD mice did not further impair contextual fear memory retention (VDD+UCMS vs UCMS: $p = 0.8836$, Tukey's post-test) as the freezing time percentage in these groups was similar. Two-way ANOVA analysis revealed the main effect of UCMS ($p = 0.0158$, $F_{(1,44)} = 6.310$) and VDD ($p = 0.0033$, $F_{(1,44)} = 9.667$) on time spent freezing, but no interaction effect ($p > 0.05$, $F_{(1,44)} = 2.180$).

Next, contextual fear memory extinction was assessed during the following 5 days. When comparing the percent of time spent freezing on 1st day following the training session and the 5th day following the training session, only control mice demonstrated fear memory extinction and relearning abilities, as their percent of time spent freezing decreased from 71.8% (± SD 8.2%) on 1st day to 41.3% (± SD 15.67%) on 5th day ($p < 0.0001$, Šidak's post-test, Figure 20B). Three-way ANOVA revealed the main effect of extinction time ($p = 0.019$, $F_{(1,44)} = 10.95$), extinction time and VDD interaction ($p = 0.0132$, $F_{(1,44)} = 6.676$), and extinction time, VDD and UCMS interaction ($p = 0.0012$, $F_{(1,44)} = 11.99$).

VDD impaired tone-induced fear memory recall, as the percent of time spent freezing was reduced in VDD mice in comparison to control ($p = 0.0002$, Kruskal-Wallis test; control vs VDD: $p = 0.0075$, Dunn's post-test; Figure 20C) and in VDD+UCMS mice in comparison to UCMS ($p = 0.0395$, Dunn's post-test) upon presenting the tone, previously paired with foot-shock. UCMS, however, did not affect tone-induced fear memory recall, as UCMS mice demonstrated a similar percentage of time spent freezing as the control group mice ($p > 0.999$, Dunn's post-test).

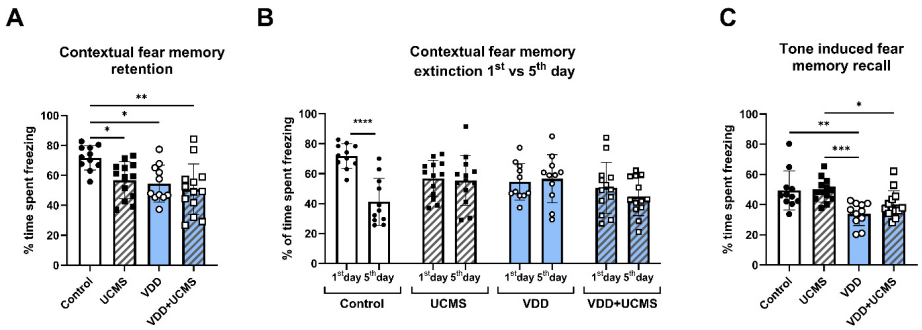

Figure 20. Impaired contextual fear memory and tone-induced fear memory due to VDD and/or UCMS. UCMS, VDD, and VDD+UCMS group mice demonstrated (A) impaired contextual fear memory retention 24 hours after training, reflected by reduced percent (%) time spent freezing. (B) While control mice exhibited contextual fear memory extinction, UCMS, VDD, and VDD+UCMS group mice did not exhibit fear memory extinction on study days 1–5. (C) VDD and VDD+UCMS, but not UCMS mice, demonstrated impaired tone-induced fear recall on study day 5 compared to the control group. The data are expressed as mean ± SD, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n = 11–13, (A) two-way ANOVA, (B) three-way ANOVA or (C) Kruskal-Wallis test followed by (A) Tukey's, (B) Šidak's or (C) Dunn's post-tests.

5.2.4. UCMS and/or VDD induced alterations in weight, depressive-like and self-care behavior

Eight weeks of UCMS caused increased body weight loss in the UCMS and VDD+UCMS group in comparison to the control group (control vs UCMS: $p = 0.0027$; control vs VDD+UCMS: $p = 0.0019$, Tukey's post-tests; Figure 21A). Two-way ANOVA revealed main effect of UCMS ($p < 0.0001$, $F_{(1,46)} = 49.34$), however no effect of VDD ($p = 0.1237$, $F_{(1,46)} = 2.459$) or interaction ($p = 0.0912$, $F_{(1,46)} = 2.976$) was observed. Baseline body weight before the start of UCMS did not differ between the control and VDD groups (data not shown).

To assess anxiety and depressive-like behavior, NSFT was employed (Dulawa & Hen, 2005). We found that UCMS and VDD+UCMS group mice exhibited increased latency time to feed in the novel cage in comparison to the control group ($p = 0.0075$, Kruskal-Wallis test; UCMS vs. control: $p = 0.0424$; VDD+UCMS vs. control: $p = 0.0066$, Dunn's post-tests; Figure 21B). VDD independently, however, did not affect the latency time to feed in the novel cage (VDD vs. control: $p > 0.05$; Dunn's post-test), and no additional effect of VDD in combination with UCMS on latency time to feed was observed, as VDD+ UCMS and UCMS group exhibited similar latency time to feed in the novel cage ($p > 0.999$, Dunn's post-test). In the home cage, no differences between experimental groups were observed in the latency times to feed and the amount of food consumed (data not shown).

To further assess the motivational aspect of depressive-like behavior, self-care assessment by using the splash test was employed. Duration of grooming is a measure of self-care behavior and motivation, recognized as a manifestation of depressive-like behavior (Kalueff & Tuohimaa, 2005; Isingrini *et al.*, 2010). UCMS and VDD groups exhibited significantly decreased total duration of grooming in comparison to the control group (control vs UCMS: $p = 0.0003$; control vs VDD: $p < 0.0001$, Tukey's post-test; Figure 21C). Interestingly, UCMS in VDD mice improved self-care behavior, as VDD+UCMS mice spent more time grooming than VDD mice ($p = 0.0279$, Tukey's post-test). Two-way ANOVA analysis revealed the main effect of UCMS ($p = 0.0219$, $F_{(1,45)} = 5.639$), but not VDD ($p = 0.2444$; $F_{(1,45)} = 1.391$) and interaction ($p < 0.0001$, $F_{(1,45)} = 27.48$) on the total duration of grooming in splash test.

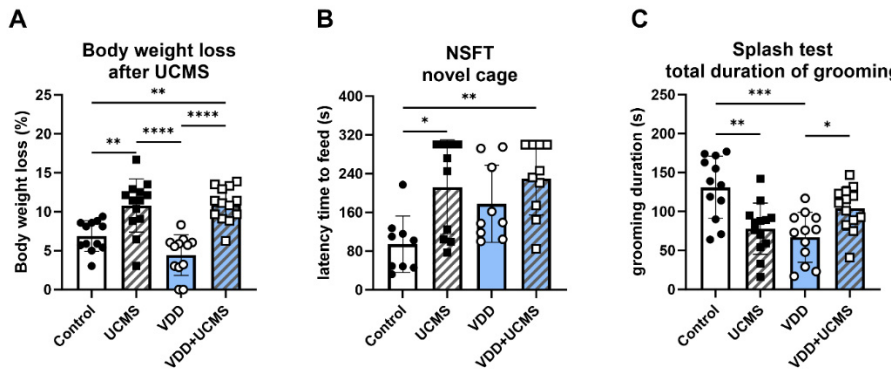

Figure 21. Altered body weight, depressive-, and anxiety behavior and grooming due to VDD and/or UCMS. (A) UCMS and VDD+UCMS mice exhibited increased body weight loss compared to control and VDD group mice, respectively, and (B) increased latency to feed in the novel cage of novelty suppressed feeding test (NSFT) compared to the control group. (C) In the UCMS and VDD groups, total grooming duration was decreased compared to the control group in the splash test. VDD+UCMS mice spent more time grooming than VDD mice. The data are expressed as mean ± SD, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, (A, C) n = 11–13, per group, (B) n = 9–10, per group, (A, C) two-way ANOVA followed by Tukey's post-tests, (B) Kruskal-Wallis test followed by Dunn's post-tests.

5.2.5. UCMS, VDD, and VDD+UCMS reduced the cell number in the GCL of the DG

When estimating the volume of DG subfields, no significant volumetric changes were observed in the hilus region between the groups (data not shown). However, a trend toward a decrease in the volume of neuron-rich GCL was observed in the UCMS, VDD, and VDD+UCMS groups (UCMS vs. control: $p = 0.1370$; VDD vs. control: $p = 0.1063$; VDD+UCMS vs. control: $p = 0.1224$, Tukey's post-tests;

Figure 22A). Two-way ANOVA did not reveal any statistically significant effects on the volume of GCL (VDD: $p = 0.0828$, $F_{(1,25)} = 3.265$; UCMS: $p = 0.1425$, $F_{(1,25)} = 2.293$; interaction: $p = 0.1181$, $F_{(1,25)} = 2.620$).

As a trend towards a decrease in the volume of GCL was observed in UCMS, VDD, and VDD+UCMS groups, the number of cells in the GCL was assessed. A reduced number of cells in the GCL was observed in UCMS, VDD, and VDD+UCMS groups in comparison to the control (UCMS vs. control: $p = 0.0069$; VDD vs. control: $p = 0.0309$; VDD+UCMS vs. control: $p = 0.0029$, Tukey's post-tests, Figure 22B). UCMS in VDD mice did not further reduce the number of cells in the GCL (VDD+UCMS vs VDD: $p = 0.7588$, Tukey's post-test). Two-way ANOVA revealed a main effect of UCMS ($p = 0.0036$, $F_{(1,25)} = 10.36$) and VDD ($p = 0.0209$, $F_{(1,25)} = 6.077$), but not an interaction effect ($p > 0.05$, $F_{(1,25)} = 3.246$).

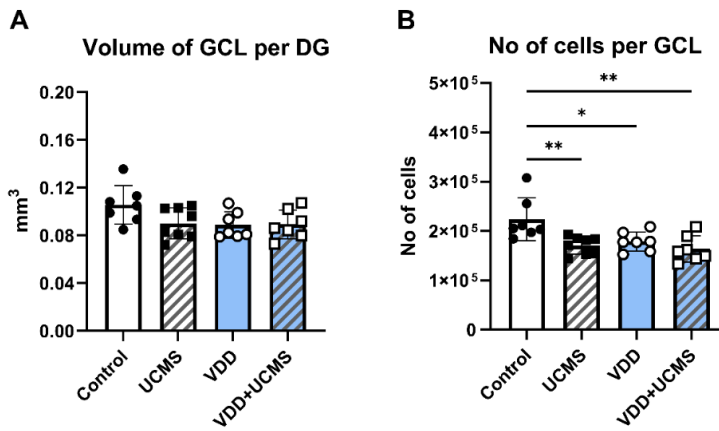

Figure 22. The volume of the GCL and decreased number of cells in the GCL in UCMS, VDD, and VDD+UCMS groups. UCMS, VDD, and VDD+UCMS groups demonstrated (A) a trend towards a decrease in the volume of the GCL in the DG of hippocampal formation and (B) a decrease in the total number of cells in the GCL. The data are expressed as mean ± SD, *$p \leq 0.05$, **$p < 0.01$, $n = 7–8$, per group, two-way ANOVA followed by Tukey's post-tests.

5.2.6. UCMS, VDD, and VDD+UCMS reduced long-term cell survival and VDD increased apoptosis in the DG

To assess if reduced long-term survival of newly generated cells in the DG could contribute to reduced cell number in the GCL, long-term (6 weeks) cell survival was measured via conducting BrdU immunohistochemistry and quantification. UCMS, VDD, and VDD+UCMS groups had a reduced number of BrdU+ cells per DG in comparison to the control group (UCMS vs. control: $p = 0.0084$; VDD vs. control: $p = 0.0052$; VDD+UCMS vs. control: $p = 0.0183$; Tukey's post-test; Figure 23A, B). UCMS in VDD mice, however, failed to further reduce the number of BrdU+ cells in the DG (VDD+UCMS vs VDD $p = 0.9502$, Tukey's

post-test). Two-way ANOVA revealed a main effect of UCMS ($p = 0.0439$, $F_{(1,24)} = 4.523$), VDD ($p = 0.0237$, $F_{(1,24)} = 5.836$) and an interaction effect ($p = 0.0082$, $F_{(1,24)} = 8.297$) on the number of BrdU+ cells in the DG.

To clarify if apoptotic cell death could contribute to the observed reduction in the GCL cell number, quantification of cleaved caspase-3+ cells was carried out. The number of caspase-3+ apoptotic cells was increased in the VDD group in comparison to control group ($p = 0.0058$, Tukey's post-test, Figure 23 C, D). Two-way ANOVA analysis revealed main effect of VDD ($p = 0.0033$, $F_{(1,25)} = 10.57$), no effect for UCMS ($p > 0.05$, $F_{(1,25)} = 0.4301$) and a trend towards an interaction effect ($p = 0.0530$, $F_{(1,25)} = 4.124$).

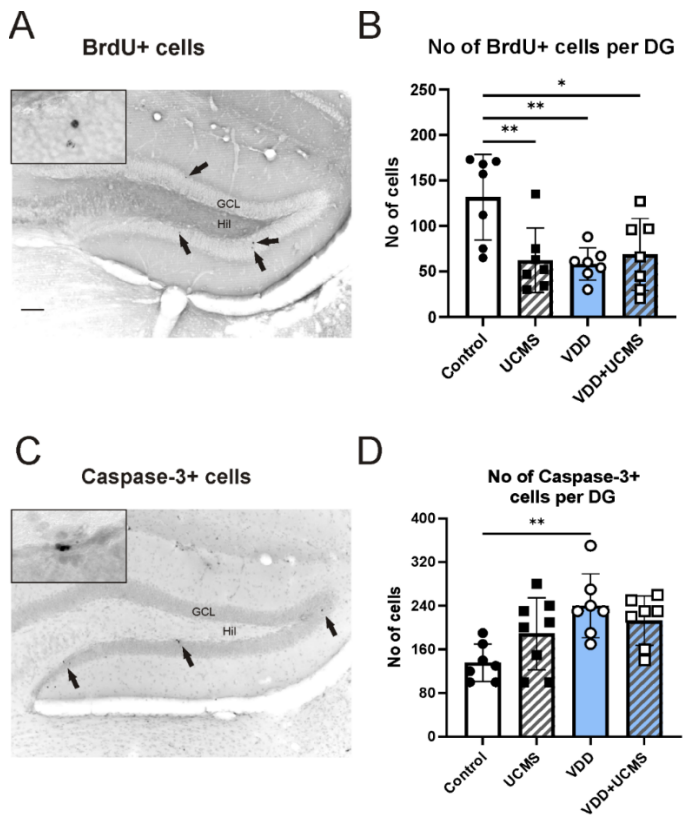

Figure 23. Decreased long-term survival of newly generated BrdU+ cells due to VDD and/or UCMS, increased apoptotic caspase-3+ cells due to VDD in the DG. Illustrative microphotographs displaying (A) BrdU+ and (C) caspase-3+ signal, taken with 10x objective, inserts are taken at higher magnification with 40x objective. (B) Compared to the control group, long-term survival of BrdU+ cells decreased in the DG of UCMS, VDD, and VDD+UCMS groups. (D) VDD increased the number of apoptotic caspase-3+ cells in the DG. The data are expressed as mean ± SD, *$p \leq 0.05$, **$p < 0.01$, n = 7-8, per group, two-way ANOVA followed by Tukey's post-tests; scale bar = 100μm.

5.2.7. UCMS, VDD, and VDD+UCMS altered microglial plasticity and induced signs of neuroinflammation in the hippocampus

5.2.7.1. VDD altered microglial cell density and morphology in the DG

To assess if and how VDD and/or UCMS affect inflammatory status in the DG, we evaluated the density and morphology of microglial cells. We observed that while UCMS increased IBA1+ cell density ($p = 0.0241$, Tukey's post-test; Figure 24A, B), VDD in an opposite manner decreased IBA1+ cell density in the DG in comparison to the control group ($p = 0.0176$, Tukey's post-test). UCMS in VDD mice increased microglial cell density in comparison to VDD mice ($p = 0.0081$, Tukey's post-test) and normalized IBA1+ cell density to the control group level ($p > 0.05$, Tukey's post-test). Two-way ANOVA revealed the main effect of UCMS ($p < 0.0001$, $F_{(1,26)} = 21.75$) and VDD ($p = 0.0002$, $F_{(1,26)} = 18.97$), but no interaction effect was detected ($p > 0.05$, $F_{(1, 26)} = 0.1053$) on the cell density of IBA1+ cells in the DG.

Although UCMS mice exhibited an increased number of IBA1+ cells, no morphology alterations were observed compared to the control group when assessing the cell body-to-cell size ratio ($p = 0.1181$, Tukey's post-test; Figure 24A, B). However, a small increase in the cell body-to-cell size ratio in the DG of VDD group mice in comparison to the control group ($p = 0.0431$, Tukey's post-test), indicating possible increased microglial reactivity, as microglial cells retract their processes and their cell bodies become enlarged during activation (Hovens *et al.*, 2014). Two-way ANOVA revealed an interaction effect ($p = 0.0301$, $F_{(1,26)} = 5.263$), but no main effect of VDD ($p = 0.0944$, $F_{(1,26)} = 3.014$) or UCMS was found ($p = 0.3292$, $F_{(1,26)} = 0.9889$).

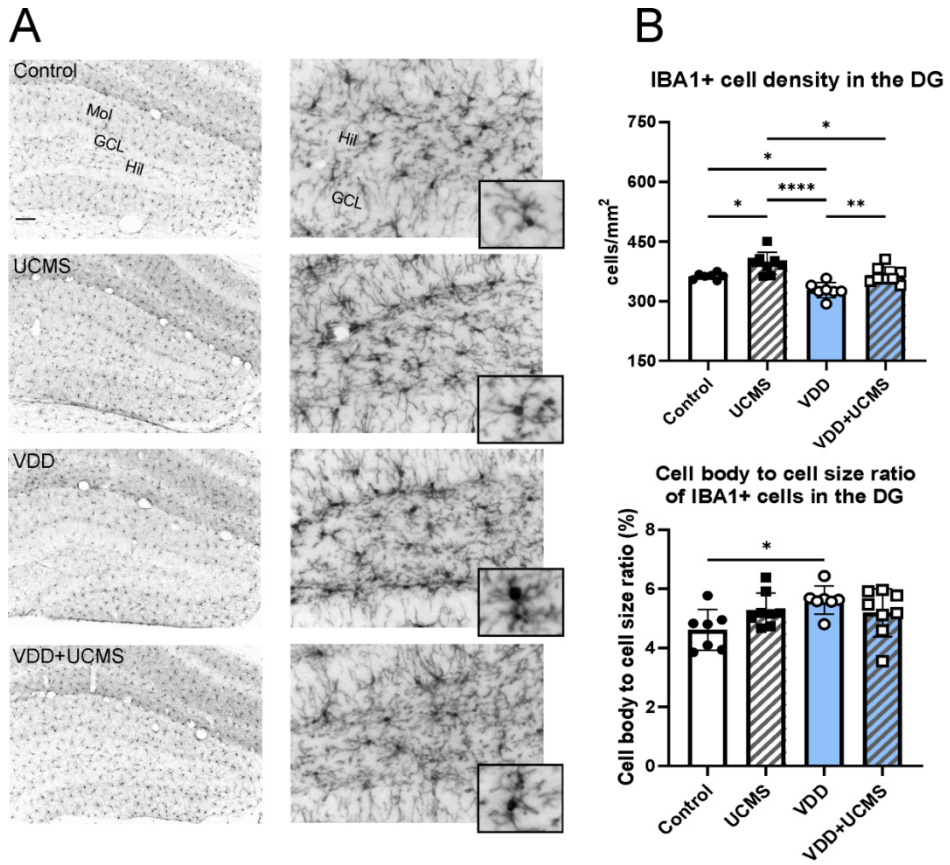

Figure 24. Altered IBA1+ microglial cell density and morphology in the DG due to VDD and/or UCMS. (A) Illustrative microphotographs demonstrating IBA1+ cells in the DG are taken with (A, left panels) 10x and (A, right panels) 40x objective; inserts demonstrate IBA1+ cell morphology at a higher magnification. (B, upper graph) UCMS increased, and VDD decreased the density of IBA1+ cells in the DG compared to the control group. However, UCMS in VDD mice normalized IBA1+ cell density to the control group level. (B, lower graph) VDD increased the cell body-to-cell size ratio in the DG compared to the control group. The data are expressed as mean ± SD, *$p \leq 0.05$, **$p < 0.01$, ****$p < 0.0001$, n = 7–8, per group, two-way ANOVA followed by Tukey's post-tests. GCL – granule cell layer, hil – hilus, mol – molecular layer; scale bar = 100 µm.

5.2.7.2. UCMS, VDD, and UCMS+VDD increased cleaved caspase-1, and UCMS increased macrophage colony-stimulating factor (mCSF) protein levels in the hippocampus

Caspase-1 and mCSF protein levels were measured in the hippocampus to explain possible reasons behind altered microglial cell density. Caspase-1 is an inflammatory caspase that processes cytokines IL-1β and IL-18 and induces pyroptotic, i.e. inflammatory cell death (Miao *et al.*, 2011; Wu *et al.*, 2022). Our results demonstrated no alterations in pro-caspase-1 protein levels (data not shown). However, an increase in cleaved caspase-1 protein levels was observed in the hippocampus of UCMS, VDD, and VDD+UCMS groups (UCMS vs. control $p = 0.0150$; VDD vs. control: $p = 0.042$; VDD+UCMS vs. control $p = 0.012$, Tukey's post-test; Figure 25A). UCMS in VDD mice, however, failed to increase further cleaved caspase-1 protein levels (VDD+UCMS vs VDD: $p > 0.05$, Tukey's post-test). Two-way ANOVA revealed a main effect for UCMS ($p = 0.015$, $F_{(1,16)} = 8.406$) and VDD ($p = 0.047$, $F_{(1,16)} = 4.638$), but no interaction effect was detected ($p = 0.061$, $F_{(1, 16)} = 4.037$).

Since we observed an increase in IBA1+ microglial cells in the DG of UCMS mice, mCSF protein levels were assessed in the hippocampus, as this cytokine has shown to be crucial in the regulation of microglial activation, proliferation, and migration in the CNS (Imai & Kohsaka, 2002; Smith *et al.*, 2013; Pons & Rivest, 2018). MCSF levels in the hippocampus of UCMS group mice increased compared to the control group ($p = 0.0355$, Tukey post-test; Figure 25B). Two-way ANOVA analysis revealed a main effect for UCMS ($p = 0.0338$, $F_{(1,16)} = 5.385$) and interaction ($p = 0.0467$, $F_{(1,16)} = 4,647$), but no main effect of VDD was observed ($p = 0.4864$, $F_{(1,16)} = 0.5076$).

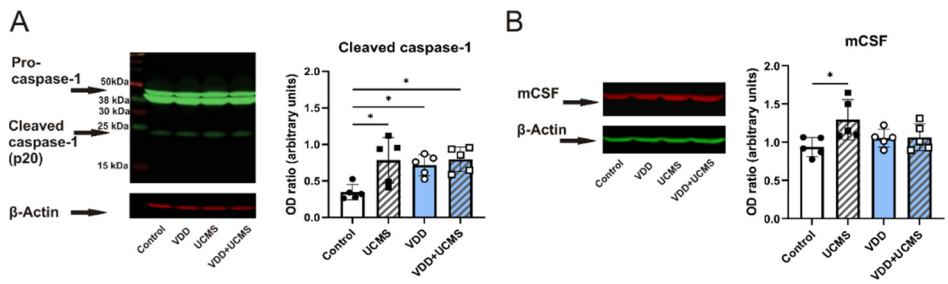

Figure 25. Increased caspase-1 and mCSF protein levels in the hippocampus due to VDD and/or UCMS. (A) Representative image of Western blot membrane demonstrating pro-caspase-1 and cleaved caspase-1 bands in the hippocampus and β-actin bands as internal loading control. UCMS, VDD, and VDD+UCMS increased cleaved caspase-1 levels in the hippocampus compared to the control. (B) The representative image of the Western blot membrane demonstrates mCSF bands in the hippocampus and β-actin bands, which were used as an internal loading control. UCMS increased mCSF protein levels in the hippocampus in comparison to the control. The data are expressed as mean ± SD, *$p \leq 0.05$, $n = 5$, per group, two-way ANOVA followed by Tukey's post-tests.

5.2.8. VDD and UCMS altered TH levels in the striatum

TH catalyzes the rate-limiting step in the biosynthesis of dopamine, and its dysfunction can lead to dopamine deficiency associated with reduced motivation (Daubner *et al.*, 2011; Wise & Jordan, 2021). Therefore, TH-immunopositive signal intensity and protein levels were measured and used as a proxy to characterize dopamine production.

TH+ IHC signal intensity in the striatum was reduced in the UCMS ($p = 0.0052$, Tukey's post-test; Figure 26A, B) and the VDD group mice ($p = 0.0036$, Tukey's post-test) in comparison to the control group mice. However, no differences in TH signal intensity were found between the control and VDD+UCMS group ($p = 0.3319$, Tukey's post-test). Two-way ANOVA revealed an interaction effect ($p = 0.0003$, $F_{(1,23)} = 17.90$), no independent main effects of UCMS ($p = 0.2416$, $F_{(1,23)} = 1.445$) or VDD were detected ($p = 0.2445$, $F_{(1,23)} = 1.427$).

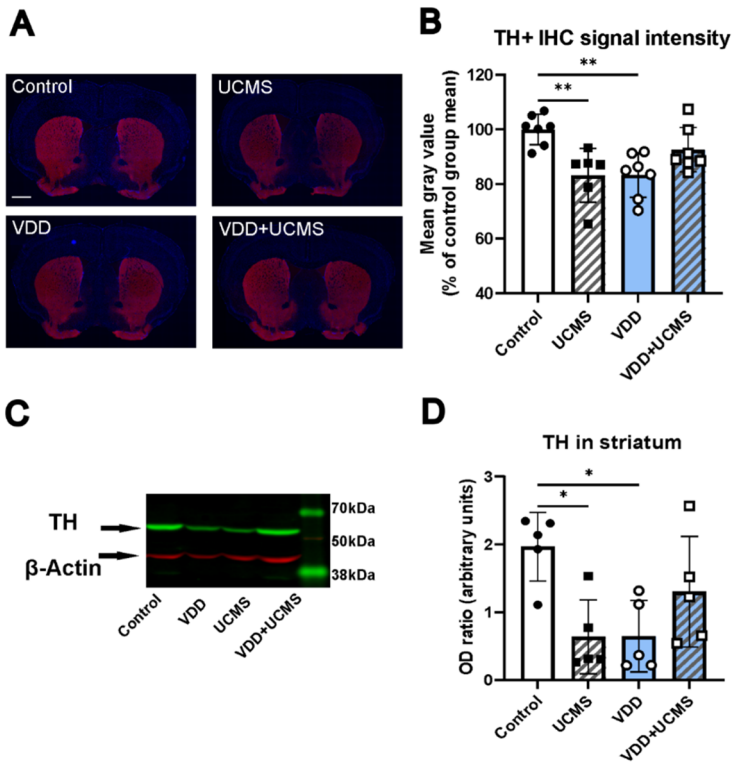

Figure 26. Reduced TH levels in the striatum due to VDD and UCMS. (A) Illustrative microphotographs of TH+ signal (red) and DAPI (violet) in the striatum. (B) UCMS and VDD decreased TH+ signal intensity in the striatum compared to the control group. (C) Representative image of Western blot membrane demonstrating TH bands in the striatum and β-actin bands, which were used as an internal loading control. (D) VDD and UCMS decreased protein levels of TH in the striatum. The data are expressed as mean ± SD, *$p \leq 0.05$, **$p < 0.01$, (B) $n = 6–8$, per group, (D) $n = 5$, per group, two-way ANOVA followed by Tukey's post-tests; scale bar = 1 mm.

TH protein levels in the striatum, assessed by western blot, were decreased in UCMS ($p = 0.0159$, Tukey's post-test; Figure 26C, D) and VDD ($p = 0.0168$, Tukey's post-test) groups compared to the control group. However, no statistically significant differences were found between the VDD+UCMS and control group ($p = 0.3453$, Tukey's post-test). Two-way ANOVA analysis revealed an interaction effect ($p = 0.0022$, $F_{(1,16)} = 13.19$), no independent main effect of UCMS ($p = 0.2340$; $F_{(1,16)} = 1.530$) or VDD ($p = 0.2484$; $F_{(1,16)} = 1.435$) were detected.

5.3. The effects of psychostimulants on epigenetic DNA editors, DNA methylation, and cytokine levels in human peripheral blood mononuclear cells *in vitro* (Paper III)

5.3.1. T-cells and monocytes express TH and DAT

We aimed to investigate the effects of psychostimulants, which acutely increase the extracellular concentration of dopamine, in a human-relevant cell culture model. To confirm the suitability of PBMCs as a cellular model for assessing dopamine-related effects, we conducted flow cytometry and assessed the expression of TH and DAT on subpopulations of T-cells and monocytes of PBMCs.

We found that both T-cells and monocytes expressed TH and DAT, while the percentage of cells co-expressing TH and DAT was significantly higher in monocytes than in T-cells (CD8- T-cells vs. non-classical monocytes: $p < 0.0002$; CD8+ T-cells vs. non-classical monocytes: $p < 0.0001$; CD8- T-cells vs classical/intermediate monocytes: $p < 0.0001$; CD8+ T-cells vs classical/intermediate monocytes: $p < 0.0001$, Bonferroni post-tests); one-way ANOVA revealed main effect of cell type group ($p < 0.0001$, $F_{(4,25)} = 26.69$; Figure 27A, B). A higher percentage of T-cells expressed only TH in comparison to monocytes (CD8- T-cells vs. classical monocytes: $p = 0.0090$, vs. intermediate monocytes: $p = 0.0090$, vs. non-classical monocytes: $p = 0.0293$; CD8+ T-cells vs. classical monocytes: $p = 0.0220$, vs intermediate monocytes = 0.0269, Bonferroni post-tests), one-way ANOVA revealed main effect of cell type group ($p = 0.0006$, $F_{(4,25)} = 7.052$). The percentage of T-cells, which did not express either TH or DAT, was significantly higher than monocytes (CD8- T-cells vs. classical and intermediate monocytes: $p = 0.0006$, vs. non-classical monocytes: $p = 0.0055$; CD8+ T-cells vs classical monocytes: $p = 0.0012$, vs intermediate monocytes: $p = 0.0013$, vs non-classical monocytes $p = 0.0111$, Bonferroni post-tests; $p < 0.0001$, $F_{(4,25)} = 11.82$, one-way ANOVA main effect of cell type group). No differences were detected in the percentage of cells co-expressing TH and DAT, expressing only TH or expressing neither TH or DAT within the monocyte or T-cell subsets ($p > 0.05$, Bonferroni post-tests).

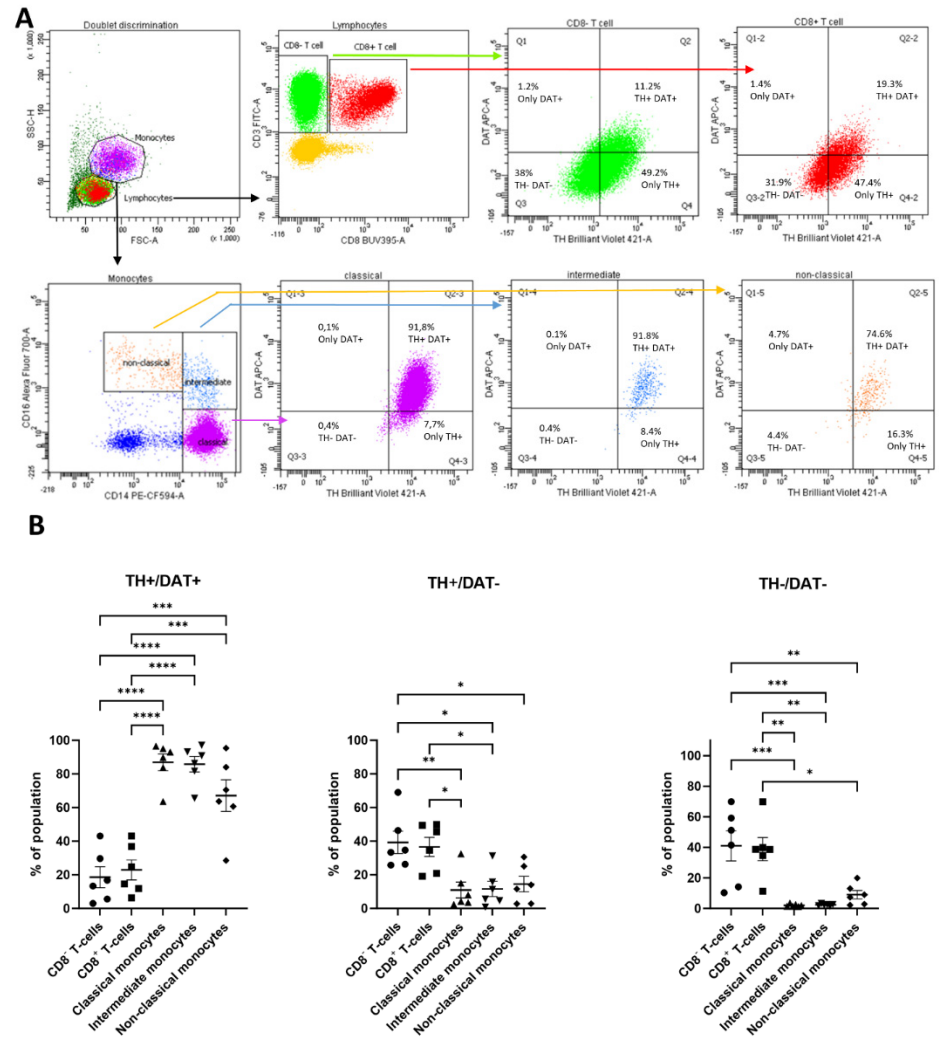

Figure 27. Human T-cells and monocytes express TH and DAT. (A) Representative plots demonstrate the gating strategy for determining lymphocyte and monocyte subsets and the expression of TH and DAT on one naïve PBMC sample. Lymphocytes and monocytes were determined by side-scatter height (SSC-H) and forward-scatter area (FSC-A). T-cells stained with surface markers CD8 and CD3 were divided into CD8+ and CD8- T-cell subsets. Monocytes were split into classical, intermediate, and non-classical monocytes based on the expression of CD16 and CD14. (B) A higher percentage of different monocyte subsets were TH+/DAT+ than T-cell subsets. In addition, a higher percentage of different T-cell subsets were TH+/DAT- than monocyte subsets, and a higher percentage of T-cell subpopulations were TH-/DAT- than monocyte subsets. The line indicates mean and error bars ± SEM, *p ≤ 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n = 6, one-way ANOVA followed by Bonferroni's post-test.

5.3.2. Repeated incubations with psychostimulants induce alterations *in DNMT1, TET1, TET2, DAT* and *TH* mRNA levels

To assess the effects of repeated incubations with cocaine (COCA) or amphetamine (AMP) compared to the control group (CTRL) on the gene expression of enzymes conducting DNA methylation and hydroxymethylation, the mRNA levels of *DNMT*s and *TET*s were quantified via qPCR. In addition, *TH* and *DAT* (*SLC6A3* gene) mRNA levels were measured to investigate if repeated *in vitro* incubations with psychostimulants affect gene expression of dopamine transportation and synthesis-related genes, *DAT*, and *TH* in human PBMCs.

Repeated psychostimulant treatment upregulated mRNA levels of *DNMT1* (AMP vs CTRL: $p = 0.0414$, COCA vs CTRL: $p = 0.0050$, Bonferroni post-tests; one-way ANOVA main effect of group: $p = 0.0044$, $F_{(2,51)} = 6.035$; Figure 28A-C), but no effect was observed on *DNMT3A* and *DNMT3B* mRNA levels in PBMCs ($p > 0.999$, Bonferroni post-tests). In addition, repeated *in vitro* psychostimulant exposure reduced *TET1-3* mRNA levels (*TET1* AMP vs. CTRL: $p < 0.0001$; *TET1* COCA vs. CTRL: $p < 0.0001$; *TET2*: AMP vs. CTRL: $p = 0.0106$; *TET2* COCA vs. CTRL: $p = 0.0191$; *TET3* AMP vs CTRL: $p = 0.0154$; *TET3* COCA vs CTRL: $p = 0.0154$, Bonferroni post-tests; Figure 28D-F). One-way ANOVA analysis revealed main effect of group for *TET1* ($p < 0.0001$, $F_{(2,51)} = 18.93$), *TET2* ($p = 0.0052$, $F_{(2,51)} = 5.831$) and *TET3* ($p = 0.0207$, $F_{(2,51)} = 4.188$).

Furthermore, repeated psychostimulant incubations significantly decreased *DAT* (AMP vs. CTRL: $p = 0.0383$; COCA vs. CTRL: $p = 0.0166$, Bonferroni's post-test; one-way ANOVA main effect of group: $p = 0.0099$; $F_{(2,51)} = 10.36$; Figure 28G-H) and increased *TH* mRNA levels (AMP vs CTRL: $p = 0.0026$; COCA vs CTRL: $p < 0.0001$, Bonferroni post-tests; $p < 0.0001$, $F_{(2,51)} = 11.57$, one-way ANOVA main effect of group). We additionally assessed repeated amphetamine and cocaine treatment effects on the mRNA levels of dopamine receptors (*DRD1-5*), but no statistically significant changes were identified (data not shown). Western blotting did not reveal any significant alterations in DNMT1 and TET1 protein levels in PBMCs after treatments with psychostimulants (data not shown).

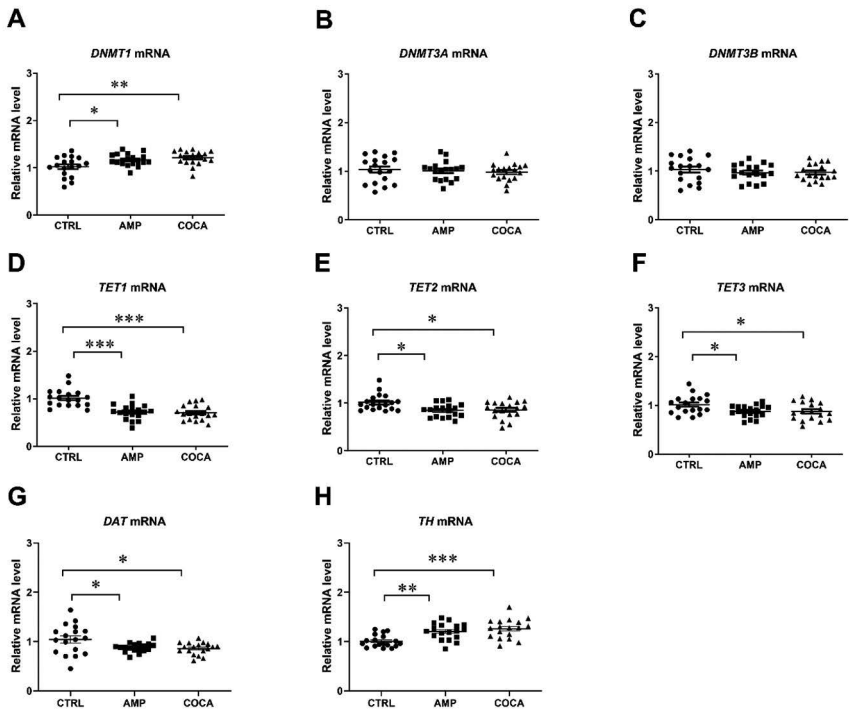

Figure 28. Alterations in the mRNA levels of (A-C) *DNMTs*, (D-F) *TETs*, (G) *DAT*, and (H) *TH* in human PBMCs following repeated amphetamine (AMP) or cocaine (COCA) *in vitro* treatments in comparison to control (CTRL). The line indicates mean and error bars ± SEM, *p ≤ 0.05, **p < 0.01, ***p < 0.001, n = 18, per group, one-way ANOVA followed by Bonferroni's post-tests.

5.3.3. Repeated incubations with psychostimulants reduce TET enzymes activity

To determine if changes in the *DNMT* and *TET* mRNA levels translate into alterations in their enzymatic activity, the total activity of DNMT and TET enzymes was assessed using colorimetric assay kits in the PBMCs after repeated treatments with psychostimulants. No statistically significant alterations in the DNMT activity were observed in response to repeated psychostimulant treatments ($p > 0.05$, one-way ANOVA followed by Bonferroni's post-tests, Figure 29A). However, the repeated psychostimulant treatment decreased the activity of TET enzymes in PBMCs (AMP vs. CTRL: $p = 0.0019$, COCA vs. CTRL: $p < 0.0001$, Bonferroni post-tests; one-way ANOVA main effect of group: $p < 0.0001$, $F_{(2,51)} = 11.87$; Figure 29B).

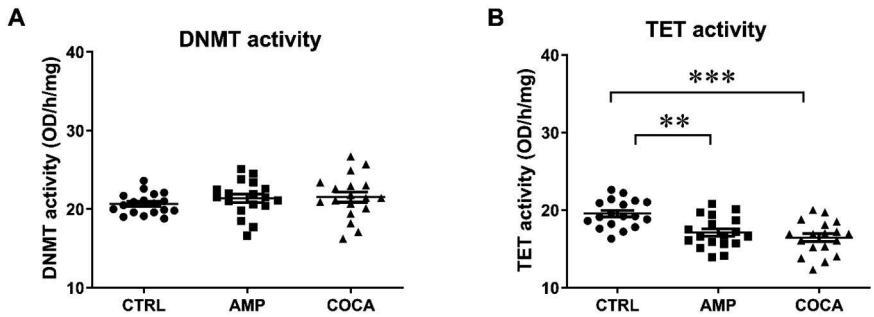

Figure 29. The unaltered activity of (A) DNMTs and (B) reduced activity of TET enzymes in human PBMCs following repeated amphetamine (AMP) or cocaine (COCA) treatment in comparison to control (CTRL). The line indicates mean and error bars ± SEM, **p < 0.01, ***p < 0.001, n = 18, per group, one-way ANOVA followed by Bonferroni's post-tests.

5.3.4. Repeated incubations with psychostimulants reduce DNA hydroxymethylation

To evaluate if repeated treatments with psychostimulants affect global DNA methylation and/or hydroxymethylation profiles, the levels of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) were measured using colorimetric assay kits. While no changes in the percentage of 5-mC of total Cs in the DNA were observed (AMP vs. CTRL: $p > 0.9999$; COCA vs. CTRL: $p = 0.2935$; one-way ANOVA main effect of group: $p = 0.244$, $F_{(2,21)} = 1.509$, Figure 30A), a decrease in the 5-hmC levels due to repeated treatments with amphetamine or cocaine was detected (AMP vs. CTRL: $p = 0.0004$; COCA vs. CTRL: $p = 0.0002$; one-way ANOVA main effect of group: $p < 0.0001$, $F_{(2,21)} = 15.58$, Figure 30B).

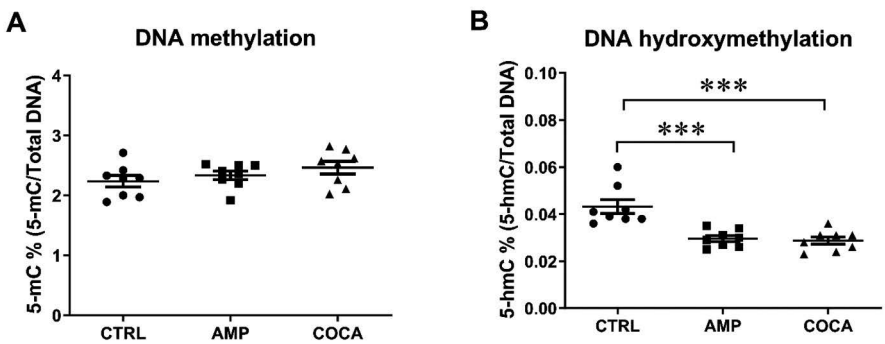

Figure 30. Unaltered (A) global DNA methylation and (B) reduced hydroxymethylation in human PBMCs following repeated amphetamine (AMP) or cocaine (COCA) treatment in comparison to control (CTRL). The line indicates mean and error bars ± SEM, ***p < 0.001, n = 8, per group, one-way ANOVA followed by Bonferroni's post-tests.

5.3.5. Repeated incubations with psychostimulants alter DNA methylation profiles based on next generation sequencing (NGS) results

A targeted methylation sequencing (Methyl-Seq) approach was used to find differentially methylated regions in the DNA, marker genes, and pathways following repeated psychostimulant treatments. NGS analysis demonstrated that after repeated treatments with amphetamine, 76.21 % of CpG sites were hypermethylated, and 23.78 % of CpG sites were hypomethylated compared to the control group. Following repeated treatments with cocaine, 76.47 % of CpG sites were hypermethylated, and 23.52 % of CpG sites were hypomethylated in comparison to the control group. Repeated incubations with amphetamine resulted in hypermethylation of 52.62% of total altered genes and in hypomethylation of 47.38% of total altered genes in comparison to the control group. Following repeated incubations with cocaine, 54.04% of total altered genes were hypermethylated, while 45.96% of total altered genes were hypomethylated. Gene ontology (GO) enrichment analysis revealed that several genes regulating biological processes related to the immune responses, inflammatory and cytokine responses, leukocyte cell-cell-adhesion, locomotion, and the regulation of transcription were hypermethylated following repeated treatments with amphetamine and/or cocaine ($p < 0.001$). Moreover, CNS-related biological processes, such as microglial/glial/radial glial cell guided cell migration, negative regulation of neuroinflammatory response, immune response in brain or nervous system, astrocyte activation, and central nervous system maturation, were hypermethylated following repeated treatments with cocaine ($p < 0.001$). Hypomethylated genes after repeated amphetamine and cocaine treatments were involved in nervous system processes, immune responses, cytokine responses, and receptor signaling pathways ($p < 0.001$), among other biological processes (see supplementary table Anier *et al.*, 2022).

5.3.6. Repeated incubations with psychostimulants alter *IL10* and ATPase Plasma Membrane Ca^{2+} (*ATP2B4*) mRNA levels

IL10 and *ATP2B4* were chosen as marker genes for validation since the *IL10* gene was listed in the top hypermethylated gene list in GO analysis for both amphetamine and cocaine treatments ($p < 0.0001$), and *ATP2B4* was listed in the top hypomethylated gene lists for both amphetamine and cocaine treatments ($p < 0.0001$) in GO analysis. Additionally, previous studies suggest that following repeated psychostimulant use, IL-10 concentration in the serum and plasma of drug users is decreased (Fox *et al.*, 2012; Moreira *et al.*, 2016), and *ATP2B4* gene expression in the human blood (Yarosh *et al.*, 2015) and in the NAc of macaque monkeys (Vallender *et al.*, 2017) is increased. The mRNA levels of *IL10* and *ATP2B4* were measured to clarify if hypermethylation of *IL10* and hypomethylation of *ATP2B4* following repeated treatment with psychostimulants correlate with altered gene expression. Cells from the same donors were used for both NGS and RT-qPCR to measure mRNA levels of *IL10* and *ATP2B4*. While

both amphetamine and cocaine repeated treatment reduced *IL10* mRNA levels in comparison to control (AMP vs. CTRL: $p = 0.0011$; COCA vs. CTRL: $p < 0.0001$, Bonferroni's post-tests; one-way ANOVA main effect of group $p < 0.0001$, $F_{(2,9)} = 64.64$; Figure 31A), cocaine decreased *IL10* mRNA levels to a greater extent (COCA vs AMP: $p = 0.0007$). Similarly, both amphetamine and cocaine treatment increased *ATP2B4* mRNA levels (AMP vs. CTRL: $p < 0.0001$; COCA vs CTRL: $p < 0.0001$, Bonferroni's post-tests; one-way ANOVA main effect of group $p < 0.0001$, $F_{(2,9)} = 145.9$; Figure 31B), while cocaine increased *ATP2B4* mRNA levels to a greater extent (COCA vs AMP: $p = 0.0001$).

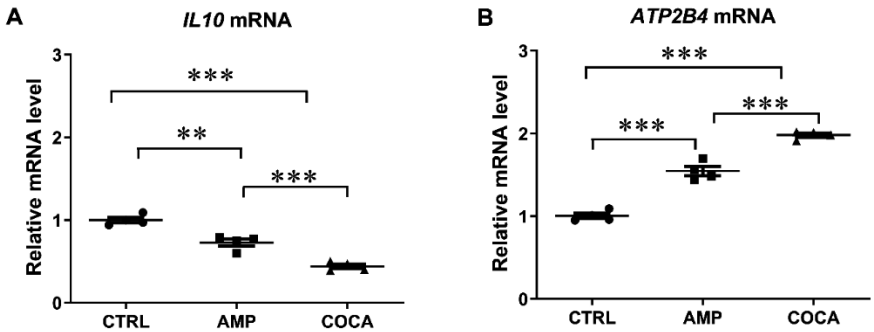

Figure 31. Alterations in the mRNA levels of (A) *IL10* and (B) (*ATP2B4*) in human PBMCs following repeated amphetamine (AMP) and cocaine (COCA) treatment in comparison to control (CTRL). The line indicates mean and error bars ± SEM, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 4$, per group, one-way ANOVA followed by Bonferroni's post-tests.

5.3.7. Repeated incubations with cocaine and decitabine (DAC) normalize the activity of DNMT and TET enzymes to control level

To prevent the effects of cocaine on the activity of DNMT and TET enzymes, we incubated DNMT inhibitor decitabine together with cocaine. A pilot study was conducted where PBMCs were incubated with different doses of decitabine for 1 h on four consecutive days to find an optimal dose. At the end of the pilot experiment, a colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay for cell viability was applied to assess viability (data not shown) and a non-toxic concentration of 0.3 μM decitabine was chosen for subsequent studies. While repeated cocaine treatment significantly increased the activity of DNMTs ($p = 0.0029$, Bonferroni post-test; one-way ANOVA main effect of group: $p < 0.0001$, $F_{(3,52)} = 25.74$; Figure 32A) and repeated decitabine treatment significantly decreased the activity of DNMTs ($p < 0.0001$, Bonferroni's post-test), co-treatment with cocaine and decitabine normalized the activity of DNMTs to a similar level as the control ($p = 0.4311$, Bonferroni's post-test) and reduced the activity of DNMTs in comparison to cocaine alone

($p < 0.0001$, Bonferroni's post-test). When assessing the activity of TET enzymes, a decrease in the activity of TET enzymes following repeated cocaine treatment ($p = 0.0004$, Bonferroni's post-test; one-way ANOVA main effect of group: $p < 0.0001$, $F_{(3,52)} = 5.23$; Figure 32B) and an increase in the activity of TET enzymes following repeated decitabine treatment was observed. Co-treatment with cocaine and decitabine normalized the activity of TET enzymes to a similar level as control ($p > 0.999$, Bonferroni's post-test) and increased activity in comparison to cocaine alone ($p = 0.0034$, Bonferroni's post-test).

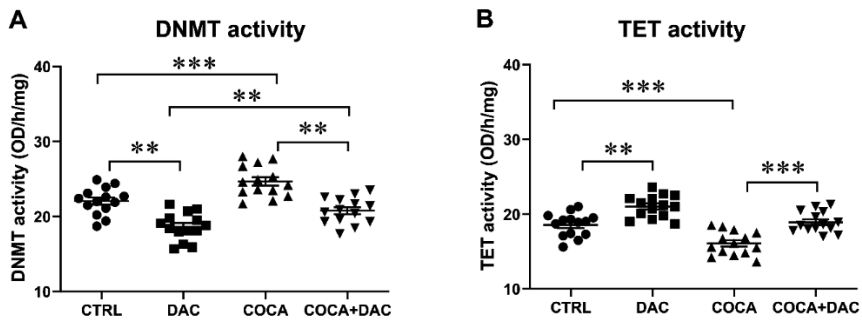

Figure 32. Alterations in the activity of (A) DNMT and (B) TET enzymes following repeated decitabine (DAC), cocaine (COCA), and cocaine+decitabine (COCA+DAC) treatment in comparison to control (CTRL) in human PBMCs. The line indicates mean and error bars ± SEM, $^{**}p < 0.01$; $^{***}p < 0.001$, n = 14, per group, one-way ANOVA followed by Bonferroni's post-tests.

5.3.8. Repeated incubations with cocaine and/or decitabine affect the concentration of IL-6 and IL-10

Next, the concentration of proinflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 in cell culture media was measured to assess if repeated *in vitro* treatments with cocaine and/or decitabine could affect inflammatory status. Repeated cocaine treatment significantly increased IL-6 concentration ($p = 0.0495$, Bonferroni's post-test, one-way ANOVA main effect of group: $p < 0.0001$, $F_{(3,53)} = 11.70$; Figure 33A) while co-treatment with cocaine+decitabine reduced the IL-6 concentration in comparison to cocaine ($p = 0.0014$, Bonferroni's post-test) and to a similar level as control ($p = 0.1298$, Bonferroni's post-test). When assessing IL-10 levels, all treatments resulted in a slight decrease in IL-10 concentrations (DAC vs. CTRL: $p = 0.0499$; COCA vs. CTRL: $p = 0.0065$; COCA+DAC vs. CTRL: $p = 0.0038$, Bonferroni's post-tests; one-way ANOVA main effect of group: $p = 0.0004$, $F_{(3,52)} = 11.72$; Figure 33B), while co-treatment with cocaine and decitabine further lowered the IL-10 concentration in comparison to cocaine alone ($p = 0.0038$, Bonferroni's post-test).

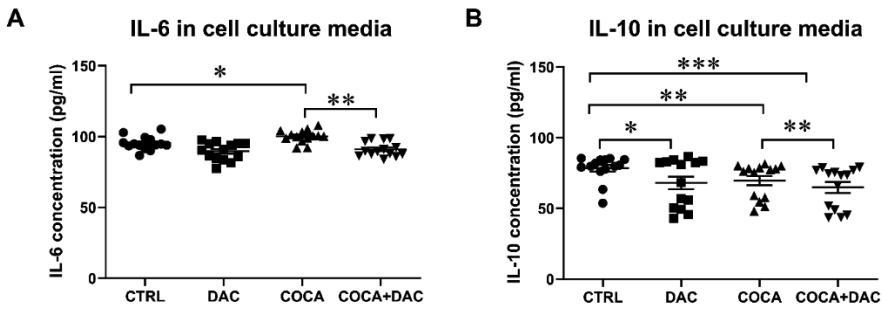

Figure 33. Altered (A) IL-6 and (B) IL-10 concentrations following repeated treatments with decitabine (DAC), cocaine (COCA), and cocaine+decitabine (COCA+DAC) in comparison to control (CTRL) in human PBMC cell culture media. The line indicates mean and error bars ± SEM, *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 14$, per group, one-way ANOVA followed by Bonferroni's post-tests.

6. DISCUSSION

6.1. The effects of sciatic nerve cuffing (SNC), unpredictable chronic mild stress (UCMS), and vitamin D deficiency (VDD) on depressive-like behavior, cognitive impairment, and hippocampal plasticity (Paper I and II)

6.1.1. Establishing the SNC, UCMS and VDD models

We used three different interventions – SNC, UCMS and VDD to investigate their potential negative effects on behavior and underlying cellular and molecular mechanisms. Following the SNC, cuffed mice developed chronic neuropathic pain for eight weeks according to ipsilateral allodynia measurements. Persistent mechanical allodynia due to peripheral nerve injury allowed us to elucidate the time dynamics of comorbid anxiety and depressive-like behavior and underlying alterations in hippocampal neurogenesis due to chronic neuropathic pain.

The UCMS procedure was successful in modeling chronic stress, as it resulted in increased weight loss, altered memory function and mood (described below), and impaired hippocampal plasticity. The effectiveness of a vitamin D-free diet on lowering serum concentration of the circulating form of vitamin D, 25(OH)D, was assessed twice in 7.5-month-old mice and 12-month-old mice. In both cases, 25(OH)D levels were significantly lower than in the control mice. In addition, our results indicate that aging is a risk factor for VDD, as the 12-month-old control mice had significantly lower 25(OH)D levels in comparison to 7.5-month-old control mice, which is in line with the results acquired by Roizen and colleagues (2018), who demonstrated age-related decrease in serum 25(OH)D levels in male C57BL/6 mice. The reason behind a reduction of 25(OH)D serum could be decreased gastrointestinal absorption (Holt & Dominguez, 1981) or inhibited bio-activation due to the lower liver CYP2R1 protein expression, or both, which could contribute to lower circulating levels of 25(OH)D (Roizen *et al.*, 2018).

6.1.2. Alterations in depressive-like and anxiety behaviors due to SNC, UCMS, VDD and VDD+UCMS

The two studies assessing the effects of a) SNC and b) UCMS, VDD, and VDD+UCMS exhibited several similarities in their design, which allows us to make indirect comparisons, although our experiments were not designed to compare the effects of SNC with UCMS or VDD. While the mouse strain used and their sex (male) were the same for both studies, the ages of the mice differed. In the case of SNC, depressive-like and anxiety behaviors were assessed in young adult, 2–4-month-old male mice and the effects of UCMS and VDD on depressive-like and anxiety behaviors were evaluated in middle-aged, 9.5- and 12-month-old mice. A later period was chosen for examining the behavioral

effects of VDD because aging has been considered a risk factor for vitamin D deficiency (Laird *et al.*, 2018; Aspell *et al.*, 2019) and since VDD has been suggested to play a causal role in brain aging (Berridge, 2017). Moreover, hippocampal plasticity has been shown to exhibit vulnerability to deleterious conditions such as aging and chronic stress, leading to cognitive deficits (Bartsch & Wulff, 2015). Therefore, VDD in adulthood and middle age could increase the vulnerability of the hippocampus to chronic stress by contributing to processes related to brain aging. Middle age was particularly interesting because the buildup of age-related pathologies may affect brain functions during this period and result in mild cognitive decline, as reported (Ferreira *et al.*, 2017).

Based on epidemiological studies, chronic (neuropathic) pain (Nicholson & Verma, 2004; Radat *et al.*, 2013; IsHak *et al.*, 2018), chronic stress (Pêgo *et al.*, 2010; Dudek *et al.*, 2021; Yuan *et al.*, 2023) and VDD (Maretzke *et al.*, 2020; Zhang *et al.*, 2021; Musazadeh *et al.*, 2023) have all been proposed as risk-factors for major depressive disorder and anxiety. While chronic neuropathic pain (Kremer *et al.*, 2020) and UCMS mouse models (Antoniuk *et al.*, 2019) have been well-established to induce anxio-depressive phenotype, information is scarce about the effects of VDD in mouse models (Groves *et al.*, 2016).

We observed the development of depressive-like behavior due to SNC and UCMS. VDD did not induce depressive-like behavior. However, it impaired grooming behavior describing self-care.

In traumatic neuropathic pain rodent models, the time factor seems to be critical for the development of depressive-like and anxiety behaviors (Yalcin *et al.*, 2011; Kremer *et al.*, 2020). Several reports have not been able to detect mood and anxiety-related behaviors in rodent models of chronic neuropathic pain during the first weeks (Mutso *et al.*, 2012; Pitzer *et al.*, 2019; Wang *et al.*, 2019) following neuropathic injury, and some have reported spontaneous recovery from pain (Mosconi & Kruger, 1996; Benbouzid *et al.*, 2008), anxiety and depressive-like behaviors (Dellarole *et al.*, 2014) after specific time has passed from the neuropathic injury. The exact time when affective behavior-related changes have been observed depends on the pain model, species, strain, and test used for evaluation or behavior being assessed (Kremer *et al.*, 2020). Our EPM test results showing a decreased percentage of entries into the open arms in cuffed mice compared to the sham mice on POD 28 (week 4) corroborate with other results of EPM test conducted in traumatic neuropathic pain mouse models, showing anxiety-related phenotypes starting from week 3-4 (Benbouzid *et al.*, 2008; Ji *et al.*, 2017; Ferreira-Chamorro *et al.*, 2018). On PODs 42 and 56 (weeks 5 and 7, respectively), the mice in our experiment went though the second EPM test, and no differences were observed in the percentage of entries into the open arms. No differences between the study groups could be attributed to the one-trial tolerance effect in which a decrease in the open arm entries occurs upon repetitive testing (Tucker & McCabe, 2017). However, as we detected an increase in the latency time to feed in the NSFT on POD 57, describing both anxiety and depressive-like behaviors, anxiety could be interpreted to remain until POD 57. Anxiety in cuffed

mice was later followed by the development of depressive-like behavior on POD 42, evident by increased immobility time in TST.

Similarly to the neuropathic pain study, TST and NSFT were used for assessing depressive-like behavior and anxiety in VDD, UCMS, and VDD+UCMS mice. In addition, the open field test was conducted to assess anxiety (Seibenhener & Wooten, 2015) and the splash test to assess grooming and self-care behavior (Kalueff & Tuohimaa, 2005) in VDD, UCMS, and VDD+UCMS mice.

In line with earlier studies, we detected depressive-like and anxiety behavior in UCMS mice (Willner *et al.*, 1996; Pothion *et al.*, 2004; Mineur *et al.*, 2006; Zhu *et al.*, 2014; Alqurashi *et al.*, 2022). Eight weeks of UCMS in middle-aged mice induced depressive-like and anxiety behavior characterized by increased latency time to feed in the NSFT and decreased time spent grooming in the splash test. Our panel of behavioral tests did not, however, detect clear depressive-like or anxiety behavior due to VDD, as VDD failed to increase immobility time in the TST or affect latency time to feed in the NSFT. In contrast to other tests characterizing depressive-like behavior, VDD mice exhibited decreased time spent grooming in the splash test. A discrepancy between different test results might be due to assessing different domains of depressive-like behavior. While increased immobility time in the TST reflects despair-like behavior (Porsolt *et al.*, 2001; Castagné *et al.*, 2011) and NSFT is used for describing both anxiety and depressive-like behaviors (Dulawa & Hen, 2005; Bessa *et al.*, 2009), the splash test reflects self-care and motivational aspects of depressive-like phenotypes and decreased grooming is considered to parallel some symptoms of depression, such as apathy (Willner, 2005; Yalcin *et al.*, 2008; Isingrini *et al.*, 2010; Shiota *et al.*, 2016). Therefore, our results suggest that VDD preferentially disrupts motivational and self-care behavior.

Surprisingly, we did not find any further worsening of depressive-like behavior when VDD and UCMS co-occurred, meaning that previous VDD did not manifest as an additional risk factor in combination with UCMS for the development of depressive-like and anxiety behaviors. In fact, VDD+UCMS mice spent more time grooming than VDD, indicating that UCMS in VDD mice improved self-care.

To conclude, SNC induced first anxiety behavior, followed by depressive-like behavior. Similarly to SNC, UCMS induced depressive-like behavior. VDD primarily reduced self-care but did not cause depressive-like behavior or exacerbate the behavioral effects of UCMS. Interestingly, UCMS in VDD mice improved self-care.

6.1.3. Alterations in cognitive functions due to SNC, UCMS, VDD, VDD+UCMS

Depressed individuals often exhibit disruptions in episodic memory, suggesting a link between depression and cognitive functions (Dillon & Pizzagalli, 2018; James *et al.*, 2021). In line with data from epidemiological studies, chronic neuropathic pain (Hu *et al.*, 2010; Gui *et al.*, 2016; Wang *et al.*, 2019) and chronic

stress (Zhao, Wang, *et al.*, 2019; Mohamed *et al.*, 2020) in rodent models have shown to induce memory impairments, in addition to depressive-like and anxiety behaviors. Similarly, in our studies, chronic neuropathic pain caused short-and long-term episodic-like memory impairments on POD 54–55 in the ORT. Both, chronic neuropathic pain and long-term UCMS caused impairments in the contextual fear memory retention and extinction. While cuffed mice also demonstrated impaired tone-induced fear memory recall, UCMS mice did not, as their freezing time after hearing a tone cue did not differ from control mice. This was expected however, as chronic stress has been reported not have a negative effect on tone-induced fear memory and reports have even demonstrated improvement of tone-induced fear memory due to UCMS (Hatherall *et al.*, 2017; Marks & Kalynchuk, 2017).

In comparison to chronic neuropathic pain and UCMS, the effects of VDD on cognitive functions in preclinical studies are more controversial (Brouwer-Brolsma *et al.*, 2014; Latimer *et al.*, 2014; Bakhtiari-Dovvombaygi *et al.*, 2021; Al-Amin *et al.*, 2022). VDD has been shown to impair hippocampal-dependent spatial learning and memory formation in active and passive avoidance tasks in adult mice (Bakhtiari-Dovvombaygi *et al.*, 2021; Al-Amin *et al.*, 2022). In the context of aging, a vitamin D-enriched diet during adulthood prevented age-related cognitive decline in middle-aged F344 rats (Latimer *et al.*, 2014). However, VDD during the middle age period did not have any effect on cognitive decline and emotional reactivity in 18-month-old C57BL/6 mice (Brouwer-Brolsma *et al.*, 2014). The variation in reported results could be due to different times when VDD was induced and cognitive functions assessed. Inducing VDD when cognitive decline has already started and aging-related pathologies are in the process may not worsen memory functions due to the floor effect. In order to avoid this floor effect, VDD in our experiments was induced already during early adulthood (starting in 4-month-old-mice), and cognition was assessed in middle age. We observed impairments in short-and long-term episodic-like memory in 9.5-month-old VDD mice compared to age-matched control mice with sufficient vitamin D in their diet, evidenced by the decreased preference ratio of the novel object in the ORT. Alternatively, as the ORT relies on the inherent preference of the mice to explore novel objects (Denninger *et al.*, 2018), a reduction in the exploratory activity could describe a loss of interest in novelty and apathy (Cathomas *et al.*, 2015). Apathy and reduced exploratory activity have been described during aging (Traschütz *et al.*, 2018; Jackson *et al.*, 2021), which might be more pronounced in middle-aged VDD mice than young adult VDD mice. However, as the 12-month-old VDD and VDD+UCMS mice also exhibited impaired contextual fear memory retention and extinction and tone-induced fear memory recall; the memory-impairing effects of VDD seem to be global, i.e. present across different paradigms and not reliant only on exploratory activity. Interestingly, UCMS in VDD mice did not further worsen fear memory retention and extinction or tone-induced fear memory recall, as VDD+UCMS mice exhibited similar freezing times to the VDD group.

Impaired ability to recall stressful events, which were present in VDD and VDD+UCMS mice, could affect sensitivity to chronic stress and lead to decreased awareness or recollection of the stressful events. The recollection of aversive situations is required for anticipation of stressful experiences, which implies psychological states, such as apprehension, worry, and anxiety in humans, and is considered to play a role in adapting to stress (McEwen & Gianaros, 2011). Therefore, we hypothesize that hippocampus-dependent episodic memory impairments, which were observed in VDD mice already before the UCMS, could have affected their stress experience via impaired ability to consolidate or recall stressful events. As VDD and VDD+UCMS mice exhibited impaired tone-induced fear memory recall, which is at least partly dependent on the functions of the amygdala (Do Monte *et al.*, 2016), possible dysfunctions in the amygdala, the brain region involved in forming strong memories about emotional, arousing experiences (Roozendaal *et al.*, 2009), could also contribute to altered stress response in VDD+UCMS group mice.

To summarize, when comparing the effects of chronic neuropathic pain, UCMS, and VDD on memory, we observed that the memory impairments induced by chronic neuropathic pain and VDD were more global, as they also caused impaired tone-induced fear memory recall, in contrast to UCMS. Observed differences in contextual fear memory and tone-dependent fear memory functions might reflect impairments in different areas of the brain, depending on the specific brain-plasticity impairing factor. Contextual fear memory extinction depends on hippocampal integrity (Kim & Fanselow, 1992; Phillips & LeDoux, 1992), as hippocampal functions are needed for relearning. Therefore impairments in contextual fear memory extinction could indicate impaired hippocampal functions. Similarly, performance in the ORT has been shown to rely on the activity of several brain regions, such as the prefrontal cortex and the hippocampus, as some studies that induced hippocampal lesions showed declined episodic-like memory in the ORT (Cohen & Stackman, 2015; Denninger *et al.*, 2018). The acquisition of auditory fear memories, however, requires the integration of sensory information in the amygdala and a transfer of emotional memories to cortical structures (e.g., medial prefrontal cortex) via the paraventricular nucleus of the thalamus (Do Monte *et al.*, 2016). Therefore, dysfunction in one or several of the structures mentioned above, in addition to the hippocampus, might occur in cuffed and VDD mice but not in UCMS mice, who did not exhibit any disruptions in tone-induced fear memory.

6.1.4. Alterations in neurogenesis and neuronal plasticity in the hippocampus due to SNC, UCMS, VDD, and VDD+UCMS

Depressive-like behavior and impaired memory may share common pathophysiological mechanisms in the hippocampus, such as impaired hippocampal plasticity due to decreased neurogenesis, reduced cell survival, and neuronal loss (Bartsch & Wulff, 2015).

Volumetric analysis of the GCL of the DG in the hippocampus, where newly generated cells are being integrated, revealed a decrease in GCL volume following chronic neuropathic pain. UCMS, VDD, and VDD+UCMS mice exhibited similar trends toward a decrease in the volume of the GCL. However, no statistically significant changes were observed. The subsequent analysis showed a decrease in the cell number of the GCL in cuffed, UCMS, VDD, and VDD+ UCMS mice. To reveal the reasons behind decreased cell number in the GCL, a thorough analysis of neurogenesis in the DG of the hippocampus was conducted in brain sections from cuffed mice. Interestingly, we did not detect any alterations in the proliferative activity assessed on POD 43 or in the number of PSA-NCAM+ and doublecortin+ cells on POD 60 between the cuffed and sham-operated mice. This indicates that chronic neuropathic pain did neither affect the proliferation of neural stem or progenitor cells, the cell fate specification, nor early survival of neural progenitors/neuroblasts/immature neurons expressing PSA-NCAM and doublecortin at a time point when depressive-like behavior was present. The decrease in the number of GCL could be dependent on long-term (25-days) cell survival, as we observed a reduction in long-term survival of BrdU+ cells in the DG on POD 60. Moreover, co-localization of BrdU+ cells with either CalB, a marker for mature neurons, or GFAP, an astroglial marker, showed that the percentage of BrdU+ cells co-localizing with CalB was significantly lower in cuffed mice than in sham-operated mice and that the percentage of BrdU+ cells co-localizing with GFAP was significantly higher in cuffed mice than in sham-operated mice. The decrease in the percentage of BrdU+/CalB+ cells out of all BrdU+ cells demonstrates a decrease in neurogenesis and could be a result of decreased long-term survival of newly generated neurons. It is likely that newly generated neurons in cuffed mice are being eliminated during the final stages of differentiation. Two elimination phases occur after the division of neural stem or progenitor cells: the first elimination phase occurs around 1–4 days, and the second approximately around 13–18 days after their generation (Pilz *et al.*, 2018). The second phase coincides with the start of synaptic integration of newborn neurons (Denoth-Lippuner & Jessberger, 2021) and is dependent on the newly generated neurons receiving glutamatergic input via NMDA receptors (Tashiro *et al.*, 2006). Therefore, impaired input from glutamatergic neurons through NMDA receptors on newborn granule cells may underlie the decrease in survival of CalB+ cells in cuffed mice. Decreased neurogenesis in cuffed mice may also result from concurrent increased microglial reactivity and neuroinflammation, which could contribute to reduced survival of newly generated neurons. This hypothesis is based on an earlier study, which demonstrated that LPS-induced inflammation did not significantly alter cell proliferation or the number of doublecortin+ cells in the hippocampus but reduced the long-term survival of newly generated neurons (Ekdahl *et al.*, 2003).

It was rather surprising to find that proliferative activity or the number of PSA-NCAM+ and doublecortin+ neural progenitors/neuroblasts/immature neurons was not decreased in cuffed mice compared to sham-operated mice. Several preclinical studies have earlier demonstrated an association between long-term

pain or neuropathic injury and reduced hippocampal neurogenesis or decreased immature hippocampal neuronal cells (Mutso *et al.*, 2012; Dellarole *et al.*, 2014; Romero-Grimaldi *et al.*, 2015; Jiang *et al.*, 2019; Wang *et al.*, 2019). Thus, our data contrast with earlier studies showing a decrease in proliferative activity in the DG. However, the reduction in proliferative activity in these studies has been previously demonstrated at earlier time points, between 1 and 4 weeks after neuropathic injury in different rodent models of neuropathic pain, such as the chronic constriction injury and spared nerve injury (Mutso *et al.*, 2012; Romero-Grimaldi *et al.*, 2015; Tyrtyshnaia *et al.*, 2017; Jiang *et al.*, 2019). The SNC model used in our study has proven to be a reliable model for inducing neuropathic pain and related anxious-depressive behaviors in rodents (Benbouzid *et al.*, 2008; Yalcin *et al.*, 2011) and the use of standardized cuff placed around the sciatic nerve minimizes the variability of the extent of nerve constriction (Mosconi & Kruger, 1996). However, the pain-related behavior caused by the SNC is not as severe as those caused by the widely used chronic constriction injury model, where four ligatures are tied around the sciatic nerve (Austin *et al.*, 2012). Chronic constriction injury has been shown to cause long-lasting (week 2–7 after injury) hyperalgesia (Dellarole *et al.*, 2014), while in the SNC model animals recover from the most severe pain-related behaviors, including hyperalgesia and postural asymmetries earlier, by postoperative week 4 (Mosconi & Kruger, 1996; Benbouzid *et al.*, 2008). Weaker pain induced by the SNC model in our study may be one reason why no changes in the proliferative activity or in the number of neuroblasts/immature neurons in the DG of the hippocampus were detected. It is possible that the proliferative activity is not affected by SNC at all or that similarly to the recovery from hyperalgesia in the SNC model, the proliferative activity in the later stages of neuropathic pain is restored.

Adult-born neurons contribute to hippocampal plasticity due to the distinct electrophysiological properties of 4-6 week old newborn neurons, which are highly excitable and have a low threshold for plasticity-inducing potentiation in comparison to mature granule cells (Wang *et al.*, 2000; Schmidt-Hieber *et al.*, 2004; Ge *et al.*, 2007). These newborn neurons are integrated into functional networks around four weeks of age (Gonçalves, Bloyd, *et al.*, 2016), which is marked by the expression of calbindin. Newborn neurons are shown to contribute to hippocampus-dependent functions, such as episodic-like memory formation, contextual learning and relearning (Saxe *et al.*, 2006) and a decrease in neurogenesis is associated with depressive-like behaviors (Malberg & Duman, 2003; Boldrini *et al.*, 2009; Du Preez *et al.*, 2021). As the proliferating cells and neural progenitor cells/neuroblasts/immature newborn DG cells lack synaptic input and output during the first week (Espósito *et al.*, 2005), they most likely do not make significant contributions to hippocampal plasticity. It is rather those newborn cells that acquire neuronal phenotype and are integrated into networks that contribute to synaptic plasticity, memory (Kee *et al.*, 2007; Deng *et al.*, 2009), and mood regulation (Sun *et al.*, 2023). Therefore, SNC-caused decline in long-term neuronal survival may reduce hippocampal plasticity and contribute to decreased synaptic plasticity, impaired memory and depressive-like behavior.

When investigating the effects of UCMS, VDD, and VDD+UCMS on hippocampal plasticity, we focused on long-term alterations, such as long-term cell survival of the newly generated cells and cell loss in the GCL. Both UCMS and VDD impaired the long-term (6 weeks) cell survival of newly generated cells compared to the control group. The reduction was evident despite the decreased neurogenesis in the hippocampi of middle-aged mice compared to young adult mice (Yang *et al.*, 2015). The detrimental effects of chronic stress on various stages of hippocampal neurogenesis, such as the proliferation, number of neuroblasts/immature neurons, and long-term survival, are well known (Mineur *et al.*, 2007; Levone *et al.*, 2015) and have been linked to cognitive impairment (Raber *et al.*, 2004; Monje & Dietrich, 2012) and depression (Santarelli *et al.*, 2003; Lucassen *et al.*, 2010; Anacker & Hen, 2017). However, it remains unclear if reduced hippocampal neurogenesis is directly responsible for the development of depression (Levone *et al.*, 2015; Schoenfeld & Cameron, 2015), as inhibiting neurogenesis does not always induce depressive-like behavior in rodents and depressive symptoms induced by chronic stress do not always correlate with decreased neurogenesis (Vollmayr *et al.*, 2003; Jayatissa *et al.*, 2010), although the effect of some antidepressants has been shown to rely on increasing neurogenesis (Santarelli *et al.*, 2003). Therefore, a reduction in the survival of newly generated cells, possibly indicating impaired neurogenesis, could contribute to decreased hippocampal plasticity underlying observed cognitive decline and depressive-like behavior, but may not be the sole reason behind observed behavioral alterations.

Similarly to UCMS, VDD impaired the survival of newly generated BrdU+ cells in the DG and reduced the total cell number in the GCL. GCL mostly comprises neurons and is the destination where newly generated neurons from the subgranular zone migrate and integrate (Kuhn *et al.*, 1996; van Praag *et al.*, 2002; Keller *et al.*, 2018). Therefore, a decrease in the number of cells in the GCL indicates possible neuronal loss. A limitation of our study is not determining the phenotype of newly generated cells in the DG. However, as we observed a significant decrease in the survival of newly generated cells in the DG of VDD mice (55% ± 14% SD), and most of the newly generated cells differentiate into neurons in rodents (Steiner *et al.*, 2004; Bekiari *et al.*, 2015), decreased survival of newly generated neurons is likely a significant contributor to the decreased overall survival of newly generated cells. Moreover, in parallel with a lower survival rate of newly generated cells, we detected an increase in the number of apoptotic caspase-3+ cells in the DG of VDD mice. As new neurons are created in surplus and the excess is eliminated via apoptotic mechanisms (Kempermann *et al.*, 2015), increased apoptotic elimination of newly generated cells could result in their reduced long-term survival and might underlie observed cell loss in the GCL of VDD mice.

Our results showing decreased survival of newly generated cells in the DG of VDD mice are in line with an earlier study by Zhu and colleagues (2012) demonstrating a reduction in the survival of newly generated cells and an increase in the number of apoptotic cells in the DG of mice when the bioactive form of vitamin

D was absent. Morello and colleagues (2018) also observed a decrease in the number of doublecortin+ cells and reduced proliferation in the DG of middle-aged VDD mice. In contrast, Groves and colleagues (2016) found no effect of VDD on adult hippocampal neurogenesis in younger adult mice. The different effects of VDD on neurogenesis could be due to various reasons, such as different ages when VDD was induced, and the length of deficiency. Relying on earlier studies, VDD could affect multiple aspects of neurogenesis, such as the proliferation, survival, or differentiation (Zhu *et al.*, 2012; Shirazi *et al.*, 2017; Morello *et al.*, 2018), resulting in a net effect of reduced neuronal survival. The mechanism of how VDD reduces neurogenesis is not fully clear, but earlier studies indicate that VDD could decrease the production of growth factors such as the BDNF, nerve growth factor and IGF-1, which promote neuronal survival (Bakhtiari-Dovvombaygi *et al.*, 2021; Bayat *et al.*, 2021) and modulate microglial phenotypes and neuroinflammation (Cui, Lu, *et al.*, 2023; Mirarchi *et al.*, 2023), which can regulate neurogenesis (Ekdahl *et al.*, 2003; Amanollahi *et al.*, 2023).

When assessing the effects of chronic stress on neurogenesis in VDD mice, we found that in the VDD+UCMS group, the long-term cell survival was impaired to a similar extent to UCMS and VDD in the DG. Therefore, the application of the UCMS procedure in VDD mice did not result in the amplification of the cumulative effects of UCMS and VDD, as VDD+UCMS mice did not show a further decline in long-term cell survival. A ceiling effect could explain the lack of further worsening of the negative effect of UCMS in VDD mice.

To conclude, SNC impaired long-term neuronal survival but not proliferative activity or the number of immature neurons in cuffed mice at a time point when depressive-like, anxiety behaviors and memory impairment had developed. Similarly to SNC, UCMS, VDD, and VDD+UCMS reduced the long-term cell survival of newly generated cells in the DG, and decreased the cell number in the GCL all to a similar extent. The increased number of cleaved caspase-3+ cells in the DG of VDD mice indicates that cells are being removed via apoptotic mechanisms. Reduced survival of newly generated neurons in the DG of the hippocampus could contribute to observed impairments in memory and mood regulation (Ge *et al.*, 2007; Kee *et al.*, 2007; Deng *et al.*, 2009; Sun *et al.*, 2023).

6.1.5. Alterations in glial reactivity in the hippocampus due to SNC, UCMS, VDD, and VDD+UCMS

Increased glial reactivity and neuroinflammation have been shown to lead to impaired hippocampal plasticity (Liu *et al.*, 2017, 2018) and decreased neurogenesis (Ekdahl *et al.*, 2003; Troubat, Leman, *et al.*, 2021). Our results demonstrated signs of increased microglial reactivity based on microglial morphological changes in the DG of the hippocampus due to chronic neuropathic pain and VDD, while UCMS induced proliferation of microglial cells in the DG of the hippocampus. Moreover, UCMS, VDD, and VDD+UCMS caused an increase in the hippocampal protein levels of a key mediator of inflammation, cleaved caspase-1, which activates proinflammatory cytokines IL-1β and IL-18, and is involved

in pyroptotic cell death (Arend *et al.*, 2008; Miao *et al.*, 2011; Denes *et al.*, 2012; Zhou *et al.*, 2023).

Chronic neuropathic pain altered microglial morphology, namely causing an increase in cell body/total cell area ratio in the DG of the hippocampus, which is characteristic of microglial activation (Kettenmann *et al.*, 2011; Hovens *et al.*, 2014). Therefore, an increased cell body/total cell area ratio in the DG of cuffed mice indicates increased microglial reactivity. Our studies corroborate with others, demonstrating brain microglial activation in the hippocampus of chronic neuropathic pain rodent models (Liu *et al.*, 2017; Barcelon *et al.*, 2019; Chen *et al.*, 2023). Moreover, microglial cells seem to contribute or even be responsible for mediating the memory-impairing effects of neuropathic pain, as pharmacological inhibition of microglial activation or ablating microglial cells has prevented peripheral nerve injury-induced hippocampal synaptic alterations and memory impairments (Liu *et al.*, 2017). Similarly, earlier findings have highlighted the role of peripheral and central inflammation in mediating chronic neuropathic pain or injury-induced depressive-like behavior (Gui *et al.*, 2016; Chen *et al.*, 2023) and impaired hippocampal neurogenesis (Dellarole *et al.*, 2014). More specifically, inhibiting pro-inflammatory IL-1β via peri-sciatic administration of IL-1β neutralizing antibody in spared nerve injury model substantially prevented memory deficits, depressive and pain behaviors in rodents (Gui *et al.*, 2016), and following chronic constriction of the sciatic nerve, TNF receptor 1 double knock-out mice did not develop depressive-like behavior or exhibit impairments in hippocampal neurogenesis in contrast to their wild-type counterparts (Dellarole *et al.*, 2014). Therefore, observed alterations in microglial reactivity and potential subsequent alterations in cytokine profiles in cuffed mice could contribute to impaired hippocampal plasticity and concurrent behavioral alterations.

UCMS induced signs of neuroinflammation, evidenced by increased density of IBA1+ cells in the DG of the hippocampus and increased levels of cleaved caspase-1 in the hippocampus. Elevated hippocampal protein levels of mCSF, a secreted growth factor that regulates the proliferation and survival of microglial cells (Imai & Kohsaka, 2002; Smith *et al.*, 2013; Pons & Rivest, 2018), might contribute to the observed increase in microglial cell density. In contrast to UCMS, VDD induced a decrease in microglial density in the DG and an increase in cell body/total cell area ratio indicating microglial reactivity. A reduction in microglial density in VDD mice could be attributed to the augmented elimination process, impaired renewal mechanisms of microglial cells, or both. As we detected increased caspase-1 protein levels in the hippocampus of VDD mice, we hypothesize that an inflammatory form of cell death, termed pyroptosis, contributes to the observed decrease in microglial cell density. In addition to the detrimental effects of caspase-1 mediated pyroptosis and pro-inflammatory cytokine release (Miao *et al.*, 2011), microglial cell depletion in VDD mice could also impair the physiologically relevant functions of microglia, such as providing trophic support to neurons via secretion of insulin-like growth-factor-1 and transforming growth factor β, phagocytosis and removal of apoptotic neurons (Wohleb

et al., 2015; Amanollahi *et al.*, 2023). Therefore, pyroptotic microglial cell death, neuroinflammation, and loss of microglial functions could potentially contribute to observed behavioral phenotype and neuronal damage in VDD mice. Our results corroborate with earlier studies that showed the importance of vitamin D in the regulation of neuroinflammation (Balden *et al.*, 2012; Rao *et al.*, 2019; Alessio *et al.*, 2021; Bellettini-Santos *et al.*, 2023).

When assessing microglial cell density in the DG of VDD+UCMS mice, no density alterations were observed compared to the control group. As long-term VDD decreased and UCMS increased microglial cell density in the DG, the cumulative effect of both factors could result in the normalization of microglial cell density compared to the control group. The normalization of IBA1+ microglial cell densities in VDD mice after UCMS could be due to decreased elimination or reduced pyroptosis, increased replacement/proliferation of microglia, and/or increased monocyte invasion. Even though we did not detect increased mCSF protein levels in VDD+UCMS mice, other regulators of microglial density and phenotype, such as the granulocyte-macrophage colony-stimulating factor, ATP and purinergic signaling, certain chemokines and growth factors, such as C-X3-C Motif Chemokine Ligand 1 or IGF-1 could be involved (Liva *et al.*, 1999; Loane & Kumar, 2016; Labandeira-Garcia *et al.*, 2017; Suresh *et al.*, 2021; Afridi & Suk, 2023). In addition to the density of microglial cells, the phenotype of microglial cells in the VDD+UCMS group in DG might have been altered, as microglia can exhibit a wide range of functional phenotypes in response to changes in their local environment (Ransohoff, 2016). Further studies are needed to elucidate whether observed changes in microglial density and possible alterations in their phenotype could also contribute to behavioral outcomes.

To summarize, signs of increased microglial reactivity and neuroinflammation were observed in cuffed, VDD, UCMS, and VDD+UCMS mice hippocampi. Increased microglial reactivity and neuroinflammation could contribute to impaired hippocampal plasticity (Liu *et al.*, 2017, 2018) and decreased neurogenesis (Ekdahl *et al.*, 2003; Troubat, Leman, *et al.*, 2021).

6.1.6. Alterations in the striatal TH protein levels due to UCMS and VDD

We measured striatal TH protein levels in UCMS, VDD, and VDD+UCMS group mice, since we observed a decrease in grooming behavior describing self-care and motivation in UCMS and VDD mice and because the dopaminergic system is considered fundamental in motivational and reward-associated behaviors (Bromberg-Martin *et al.*, 2010; Daubner *et al.*, 2011). TH is the rate-limiting enzyme in dopamine synthesis, and a decrease in the protein levels of TH indicates decreased dopamine production (Daubner *et al.*, 2011). Indeed, striatal TH protein levels correlated with observed changes in grooming time in the splash test, as both UCMS and VDD decreased striatal TH protein levels and grooming time. However, no differences were detected in TH levels between the VDD+UCMS and control group, which also correlates with splash test results

showing no statistically relevant differences between the VDD+UCMS and control group. Similar levels of TH in the VDD+UCMS and control group could explain improved self-care and motivation in the VDD+UCMS group. The presence of VDR in mesolimbic dopamine neurons and projection targets in the striatum indicates possible functions of vitamin D in regulating the dopamine system (Kesby *et al.*, 2011; Cui *et al.*, 2013; Pertile *et al.*, 2023). Based on previous studies, VDD could affect TH levels either via direct regulation of the *TH* gene expression or loss of neuroprotection of dopamine-producing neurons, or both (Puchacz *et al.*, 1996; Cass *et al.*, 2014; Lima *et al.*, 2018; Magdy *et al.*, 2022). The exact mechanism by which chronic stress could increase TH levels and improve self-care in VDD mice is unclear. UCMS has been shown to locally and region-specifically induce the synthesis of $1,25(OH)_2D$ without affecting the serum 25(OH)D levels. More specifically, UCMS has been shown to enhance the gene expression of VDR and vitamin D activating and catabolizing enzymes in the rat hippocampus (Jiang *et al.*, 2013). However, it is not known whether UCMS could have similar effects in the striatum of VDD mice and induce local production of $1,25(OH)_2D$.

6.1.7. Limitations of assessing behavioral alterations and impaired brain plasticity induced by chronic neuropathic pain, UCMS, VDD, and UCMS+VDD in male mice

It should be noted that a limitation of our studies was investigating the behavioral and brain plasticity-impairing effects of neuropathic pain, UCMS, and VDD only in male mice. Significant differences between the prevalence of depression and chronic pain in male and female populations exist, as women are 1.7 times more likely to experience depression (Albert, 2015), and the majority of patients with chronic pain are women (Breivik *et al.*, 2006; Mogil, 2012). Moreover, preclinical studies have demonstrated significant sex differences in the affective-motivational pain component and treatment response to neuropathic pain (Baggio *et al.*, 2024), in stress regulation (Wellman *et al.*, 2018), vulnerability and resilience to stress (Krispil-Alon *et al.*, 2022). Perimenopause and menopause lead to changes in the HPA axis regulation, which may amplify the adverse effects of stress (Otte *et al.*, 2005; Hodes & Epperson, 2019). Therefore, chronic neuropathic pain, UCMS, and VDD could exhibit differential effects in male and female mice, and future studies could elucidate the differences in male and female mice. While generalizing the results of our studies, it is advisable to remember, that these may not be wholly representative of the female population.

6.1.8. Future perspectives

We demonstrated that decreased long-term neuronal survival is the impaired process of neurogenesis due to SNC. Future studies could further elucidate molecules or cellular processes impairing survival with the aim to prevent or reverse decreased neuronal survival. Moreover, further studies could elucidate if

the reversal of reduced neuronal survival helps to improve memory functions and depressive-like behavior associated with chronic neuropathic pain.

We also observed alterations in microglial morphology in the DG of the hippocampus of cuffed mice. Previous reports have demonstrated that targeting specific inflammatory cytokines can improve pain-related behaviors, impaired memory functions (Gui *et al.*, 2016; Liu *et al.*, 2016; Saffarpour *et al.*, 2021), and depressive-like behavior (Dellarole *et al.*, 2014; Chen *et al.*, 2023) in rodent models of neuropathic pain. Since antidepressant treatment has been shown to be less effective in depression patients co-morbid with chronic pain than those without chronic pain (Roughan *et al.*, 2021), it could be further investigated if combining antidepressant treatment with anti-inflammatory treatment improves depression treatment outcomes, which are comorbid with chronic neuropathic pain.

We found that long-term VDD during adulthood induced memory impairments and reduced self-care in middle-aged mice, but in this study, we did not aim to reverse the behavioral effects of VDD. Further work is needed to clarify if subsequent vitamin D supplementation could reverse the effects of long-term VDD on memory and self-care. Although we expected the effects of long-term VDD to be more pronounced in middle age, our study did not compare different age groups. Therefore, future studies comparing VDD at different life periods could clarify, if the effects of VDD on memory and self-care are age-specific and provide more information about possible critical periods.

In addition, we observed decreased TH protein levels in the striatum of VDD mice. However, the underlying reason behind decreased TH protein levels, indicating possible loss of dopamine, was not investigated and could be further elucidated. Previously, in a mouse model of Parkinson's disease, vitamin D administration alleviated the loss of TH+ neurons, which was associated with decreased pro-inflammatory microglia activation (Calvello *et al.*, 2017). Therefore, long-term VDD could induce neuroinflammation and neuronal loss in the VTA and substantia nigra regions, where dopamine-producing neurons reside, or alter TH gene expression.

6.2. The effects of psychostimulants on aberrant DNA methylation and cytokine profiles in an *in vitro* cell culture model of human PBMCs (Paper III)

6.2.1. Suitability of human PBMC *in vitro* model for investigating the effects of psychostimulants

Altered DNA methylation and changes in epigenetic DNA editors in the brain reward system due to repeated administration of cocaine have been shown to contribute to addiction-related phenotypes in rodents (Anier *et al.*, 2010; Massart *et al.*, 2015; Urb *et al.*, 2020). However, there is little information about how psychostimulants affect DNA methylation and epigenetic DNA editors in

humans. Since no methods are available for assessing epigenetic DNA editors involved in DNA methylation and demethylation in the brains of living humans, we used an alternative cell culture model of human PBMCs. PBMCs can be isolated from whole blood and consist of cells with a single round nucleus; 70–90% of PBMCs are lymphocytes (T-cells, B-cells, and natural killer cells), and the rest are monocytes and dendritic cells (Kleiveland, 2015; Alexovič *et al.*, 2022).

As the primary effect of psychostimulants is transiently increasing the extracellular levels of dopamine via inhibiting dopamine transport into the intracellular space (Heal *et al.*, 2013; Roque Bravo *et al.*, 2022), a model for studying the possible downstream effects of psychostimulants on epigenetic DNA editors and DNA methylation should exhibit primary targets for psychostimulants and a working dopamine system. We characterized the expression of DAT, one of the primary targets for cocaine and amphetamine, and TH, the rate-limiting enzyme of dopamine synthesis, in T-cells and monocytes. T-cells were chosen to characterize the expression of DAT and TH as CD3+ T-cells are the most abundant lymphocyte population in PBMCs, accounting for 70–85% of lymphocytes, and monocytes were chosen for characterizing the expression of DAT and TH to represent myeloid lineage cells (Kleiveland, 2015; Alexovič *et al.*, 2022).

Our flow cytometry and RT-qPCR results confirmed that T-cells and monocytes of the PBMCs express DAT and TH, corroborating with earlier studies (Mackie *et al.*, 2018). Most naïve monocyte subpopulations expressed both DAT and TH, but a significantly lower percentage of naïve T-cells expressed both DAT and TH. Interestingly, a significantly higher percentage of T-cells expressed only TH than monocytes. As dopamine is a precursor for noradrenaline and as noradrenaline has been shown to regulate memory T-cells' functions (Slota *et al.*, 2015), it could be proposed that T-cells expressing only TH, but not DAT, may be involved in noradrenaline synthesis. However, this hypothesis needs further investigation. While the co-expression of DAT and TH has been shown previously on monocytes by Gopinath and colleagues (2020), whose protocol was adapted to our studies, to our knowledge, examination of DAT and TH co-expression in T-cell subsets using flow cytometry has not been previously conducted. Based on DAT and TH expression, our results indicate that most monocytes can synthesize and transport dopamine into the intracellular space. However, TH expression suggests that about half of the T-cells can synthesize dopamine, and DAT expression indicates that a significantly lower percentage of T-cells can transport dopamine into the intracellular space. In addition to DAT and TH, peripheral immune cells, such as T-cells and monocytes or monocyte-derived macrophages, have been shown to express dopamine receptors, and VMAT-2, another primary target of amphetamine, which translocates monoamines, including dopamine, from the cytosolic pool to the storage pool (Heal *et al.*, 2013; Mackie *et al.*, 2018). Together, our and others' results demonstrating the expression of TH and DAT on immune system cells support the suggestion that the dopamine system could contribute to the regulation of the immune system (Feng & Lu, 2021; Li *et al.*, 2022).

Repeated amphetamine and cocaine treatment resulted in decreased *DAT* mRNA levels and increased *TH* mRNA levels in our *in vitro* model of human PBMCs. These results corroborate earlier *in vivo* studies in rodents, demonstrating decreased *Dat* mRNA (Letchworth *et al.*, 1997) and increased *Th* mRNA (Vrana *et al.*, 1993) in the VTA and in substantia nigra, where dopamine-producing neurons reside, following chronic cocaine treatment. Repeated administration of methamphetamine has shown similar effects on *Th* mRNA levels, which increased in the VTA and substantia nigra a day following ten days of self-administration, although no differences or even an increase has been observed in *Dat* mRNA levels in the VTA (Shepard *et al.*, 2006; Hong *et al.*, 2015). The variability in reported effects of methamphetamine on *Dat* mRNA levels in NAc might be due to different treatment protocols (different lengths and concentrations of methamphetamine) and assessment time following the last treatment. Together, our results mimic, to some extent, the effects of *in vivo* administration of psychostimulants on *Th* and *Dat* mRNA levels.

The use of PBMCs as a cell culture model for investigating the effects of repeated exposure to psychostimulants was also supported by our previous study demonstrating similar alterations in the gene expression and enzymatic activity of DNMTs and TETs in NAc and peripheral blood of mice following repeated administration of cocaine (Anier *et al.*, 2018).

6.2.2. The effects of repeated amphetamine and cocaine treatment on epigenetic DNA editors

Cells rely on the enzymatic activities of epigenetic DNA editors, DNMTs, and TETs to maintain genomic methylation homeostasis, and alterations in their activities can lead to aberrant DNA methylation. Epigenetic editors involved in DNA methylation and demethylation are proposed to contribute to the development of addiction by maintaining drug-induced stable changes at the cellular level in the brain (Anier *et al.*, 2010; Feng *et al.*, 2015; Massart *et al.*, 2015; Nestler & Lüscher, 2019; Urb *et al.*, 2020).

Our experimental results demonstrate that DNMT1 was the only affected DNMT enzyme following repeated amphetamine and cocaine exposure in PBMCs. More specifically, we observed an increase in the *DNMT1* mRNA level in PBMCs following repeated cocaine and amphetamine treatment but not in the mRNA levels of *DNMT3A* and *DNMT3B* or the enzymatic activity of DNMT. DNMT3A has been highlighted as the prominent enzyme involved in *de novo* DNA methylation in the brain, as the gene expression of *Dnmt3b* significantly decreases during the embryonic period in the brain (Feng *et al.*, 2005). Our research group's earlier study in mice (Anier *et al.*, 2018) found that mRNA levels of *Dnmt1* and *Dnmt3a* and the activity of DNMTs increased in both NAc and in the peripheral blood cells following repeated cocaine treatment, which comprised of 7 daily administrations of cocaine and a 21-day-long withdrawal period followed by a single administration of cocaine. Although it is not possible to draw direct comparisons between the current *in vitro* study in human PBMCs

and previous mouse studies due to differences in study designs, in both studies, the *DNMT1* mRNA increased after repeated cocaine treatment. *Dnmt3A* mRNA levels were increased only in mouse NAc and peripheral blood but not in human PBMCs. Differences in *DNMT3A* mRNA levels between the two studies might be due to using different materials (whole blood/NAc vs. PBMCs), different cocaine exposure protocols (longer treatment in mice vs. shorter treatment in PBMCs), or it could be a species-specific effect (mouse vs. human).

Amphetamine and cocaine repeated treatments significantly decreased *TET1, TET2,* and *TET3* mRNA levels and TET enzymes activity in comparison to controls. These results indicate that in human PBMCs, repeated treatment with psychostimulants may cause aberrant DNA hydroxymethylation via impairing the activity of TET enzymes, with less contribution to increased activity of DNMTs. A decrease in TET enzyme activity after repeated psychostimulant treatment is in concordance with our research group's earlier study showing a reduction in the activity of TET enzymes in NAc and peripheral blood of mice after repeated cocaine treatment (Anier *et al.*, 2018). Feng and colleagues (2015) also demonstrated a decrease in *Tet1* mRNA, but not in *Tet2–3* mRNA, following repeated cocaine administration in mouse NAc. Together, increased mRNA expression of *DNMT1,* a decrease in *TET1–3* mRNA levels, and TET enzyme activity in human PBMCs corroborate to some extent with earlier mouse studies assessing the effects of repeated cocaine treatment in NAc. However, whether we can extrapolate the activities of DNMT and TET enzymes in human PBMCs to the brain is not known. Previous studies have demonstrated tissue- or cell-specific methylation patterns (Liyanage *et al.*, 2014), and therefore, caution should be taken when extrapolating results from peripheral tissues to the brain. However, we hypothesize that the activity of epigenetic DNA editors varies less between tissues and that the activity of TET enzymes could pose as a putative biomarker for repeated psychostimulant use. Further studies assessing alterations in the activity of TET enzymes in the brain tissue and peripheral blood cells of psychostimulant users could provide more information for the potential value of the activity of TET enzymes as a biomarker for conditions associated with substance use disorder, such as repeated use of psychostimulants and treatment response of substance use disorder.

6.2.3. The effects of repeated amphetamine and cocaine treatment on DNA methylation, hydroxymethylation, and marker genes' expression

To analyze the balance between DNA methylation and hydroxymethylation, we assessed global 5-mC and 5-hmC levels in PBMCs. We observed a decrease in the percentage of 5-hmC of total DNA but no alterations in the percentage of 5-mC of total DNA. Our results suggest that the reduced activity of TET enzymes due to repeated cocaine and amphetamine treatment leads to reduced 5-mC conversion to 5-hmC, decreasing the 5-hmC levels, which may result in promoter hypermethylation and transcriptional repression, respectively. An increase in 5-

hmC levels due to repeated psychostimulant treatment in PBMCs corroborates with our research groups' earlier study demonstrating increased 5-hmC in the NAc of mice following repeated cocaine treatment (Anier *et al.*, 2018). However, Feng and colleagues (2015) did not detect a decrease in overall 5-hmC levels after repeated cocaine administration in the NAc of mice and demonstrated that *TET1* downregulation was associated with increased enrichment of 5-hmC at the putative enhancers and coding (gene body) regions of genes, which have important roles in drug addiction. Moreover, site-specific enrichment of 5-hmC was correlated with increased expression of these genes and with their alternative splicing. Therefore, a decrease in the expression of TETs may result in differential global and site-specific DNA hydroxymethylation, which may contribute to gene expression alterations related to addiction phenotypes.

We conducted genome-wide targeted methylation sequencing and gene ontology enrichment analysis to gain insight into the effects of repeated cocaine and amphetamine treatment on genome-wide DNA methylation profiles and the associated biological functions of differentially methylated genes in PBMCs. When analyzing alterations in DNA methylation at the gene level, we observed that both amphetamine and cocaine exposure caused relatively equal hyper-and hypomethylation. When analyzing all CpG sites, hypermethylation due to repeated cocaine and amphetamine treatment predominated. However, a limitation of the targeted methylation sequencing used in our study, which implemented bisulfite conversion, is the inability to differentiate between 5-mC and less common 5-hmC (Mehrmohamadi *et al.*, 2021).

Our gene ontology analysis revealed that after repeated psychostimulant treatment, both the genes involved in biological processes related to immune and cytokine responses were hyper- and hypomethylated. Repeated cocaine treatment also found hypermethylated genes involved with biological processes related to the CNS and neuroinflammation, such as microglial/glial/radial glial cell guided cell migration, negative regulation of neuroinflammatory response, immune response in brain or nervous system and central nervous system maturation. These results suggest that repeated cocaine administration could potentially affect brain plasticity via hypermethylation of genes related to the regulation of glial functions and neuroinflammation.

Next, we chose two marker genes, *IL10* and *ATP2B4*, for further analysis via RT-qPCR as the *IL10* gene was hypermethylated and the *ATP2B4* gene hypomethylated in PBMCs after repeated exposure to both cocaine and amphetamine, based on the NGS and bioinformatics analysis. The psychostimulant treatment significantly hypermethylated *IL10*, a gene encoding for an anti-inflammatory cytokine, and the hypomethylated *ATP2B4* gene, encoding for ATPase plasma membrane Ca^{2+} transporting 4 protein (ATP2B4), involved in calcium homeostasis. These alterations in gene methylation correlated with decreased *IL10* mRNA levels and increased *ATP2B4* mRNA levels, indicating that their gene expression was regulated via psychostimulant-induced changes in DNA methylation. In humans, the cytokine IL-10 is mainly produced by lymphocytes, monocytes, macrophages, and dendritic cells (Iyer & Cheng, 2012), and in the CNS,

IL-10 can be produced by microglia and astrocytes (Williams *et al.*, 1996; Ledeboer *et al.*, 2002). IL-10 regulates various immune cells to limit and stop the inflammatory response and thus plays an important role in autoimmune diseases, inflammatory diseases, and cancer (Zheng *et al.*, 2020). Interestingly, decreased IL-10 has been reported in the serum of subjects who reported cocaine use (Moreira *et al.*, 2016) and in the plasma of cocaine dependent individuals (Fox *et al.*, 2012), which is in line with our results showing decreased mRNA levels of *IL10. ATP2B4* is highly expressed in numerous tissues and cell types, including the brain, heart, and spermatozoa (Lopreiato *et al.*, 2014). Our results showing increased mRNA levels of *ATP2B4* due to repeated psychostimulant treatment corroborates with studies showing differential expression of *ATP2B4* in human blood due to repeated d-amphetamine administration (Yarosh *et al.*, 2015) and in the NAc of rhesus macaques due to repeated self-administration of cocaine (Vallender *et al.*, 2017). However, the potential role of ATP2B4 in mediating the effects of psychostimulants is not known. Together, previously described alterations in IL-10 levels and in *ATP2B4* gene expression, indicate that repeated *in vitro* psychostimulant treatment of PBMCs produced similar effects to *in vivo* administration of psychostimulants.

6.2.4. The effects of cocaine and decitabine on the activity of epigenetic DNA editors in human PBMCs

Our results demonstrated that repeated treatment with psychostimulants cocaine and amphetamine significantly reduced the activity of TET enzymes. Therefore, our next aim was to investigate whether this effect of cocaine could be pharmacologically inhibited. We chose the DNMT inhibitor decitabine (Gore *et al.*, 2006) for our subsequent studies, because a recent study demonstrated that decitabine caused an increase in 5-hmC levels in human leukemia cells, indicating possible TET-activating effects (Chowdhury *et al.*, 2015) and since no selective TET activator is known. Moreover, DNMT inhibition in animal models has been shown to reverse some of the behavioral effects of psychostimulants (Anier *et al.*, 2010; Massart *et al.*, 2015; Urb *et al.*, 2020). Interestingly, in addition to the decrease in DNMT activity due to decitabine treatment, we found that repeated decitabine incubations significantly increased the basal activity of TET enzymes and normalized the activity of TET enzymes following repeated cocaine treatment in human PBMCs. As the primary mechanism of action of decitabine is inhibiting the activity of DNMTs, the exact molecular mechanism by which DAC increases TET enzyme activity is not fully elucidated. However, Chowdhury and colleagues (2015) have suggested that the increased abundance of 5-hmC after decitabine treatment may be due to increased recruitment of TET at hemimethylated dyads. Taken together, our results are consistent with previous studies demonstrating that decitabine decreases the activity of DNMTs and increases the activity of TET enzymes in mitotic cells, and treatment with decitabine was able to reverse the effects of repeated cocaine treatment on the activities of epigenetic DNA editors.

6.2.5. The effects of cocaine and decitabine on IL-6 and IL-10 concentration in cell culture media

Previous studies have indicated that, in general, repeated psychostimulant exposure results in increased levels of pro-inflammatory cytokines and decreased levels of inflammatory cytokines (Narvaez *et al.*, 2013; Moreira *et al.*, 2016; Bravo *et al.*, 2023). Moreover, it has been suggested that immune responses may mediate some of the behavioral effects of psychostimulants (Lewitus *et al.*, 2016; Correia *et al.*, 2020). Therefore, to further explore the relationship between repeated cocaine exposure and immune response in a human PBMC *in vitro* cell culture model and the potential of DNMT inhibition to reverse these effects, we assessed how repeated treatment with cocaine and decitabine influences cytokine concentrations. Here, we observed that repeated cocaine treatment slightly increased the proinflammatory IL-6 concentration and slightly decreased the anti-inflammatory IL-10 concentration in cell culture media, which was in line with observed *IL10* gene hypermethylation and reduced mRNA expression. Our results corroborate previous studies demonstrating increased plasma pro-inflammatory molecules, such as IL-6 and TNF-α, and decreased anti-inflammatory molecules, such as IL-10, in cocaine users compared to healthy controls (Bravo *et al.*, 2023). Epigenetic mechanisms have been shown to contribute to regulating *IL6* and *IL10* gene expression. Tang and colleagues (2011) showed that the up-regulation of *IL6* was modulated by its promoter demethylation in human lung epithelial cells and that the administration of decitabine enhanced *IL6* promoter activity in a dose-dependent manner. Accumulating data suggests that epigenetic modifications also play a role in the regulation of *IL10* (Alipour *et al.*, 2018; Zheng *et al.*, 2020) and that hypomethylation of the *IL10* promoter leads to higher *IL10* expression in the PBMCs (Larsson *et al.*, 2012). This is in line with our results, indicating that DNA methylation may be a key regulatory mechanism for decreased *IL10* expression following repeated cocaine exposure.

Cocaine may affect brain plasticity via indirect modulation of the peripheral immune system and production of cytokines, which then may cross the BBB (Salvador *et al.*, 2021; Bravo *et al.*, 2023). In addition, monocytes, whose function peripherally has been altered by psychostimulant exposure, may penetrate the brain and affect neural plasticity. Although the BBB prevents the penetration of blood-borne immune cells into the brain, previous findings suggest that psychostimulants may induce dysfunction of the BBB by inducing neuroinflammatory pathways, increasing enzyme activation related to BBB remodeling and alterations in tight junction protein expression (Kousik *et al.*, 2012). A study by Niu and colleagues (2019) demonstrated the penetration of monocytes into the brains of cocaine users by examining their post-mortem brain tissue.

While cocaine treatment increased IL-6 concentration in cell culture media, decitabine and cocaine co-treatment normalized the concentration of IL-6 to a similar level to the control. Decitabine treatment significantly reduced IL-10 basal concentrations and further reduced cocaine-induced decreases in IL-10 concentration in the cell culture media. In this regard, decitabine could not reverse

the effects of repeated cocaine treatment. The mechanism underlying reduced IL-10 concentration in human PBMCs due to decitabine treatment was not clarified, but decitabine may contribute to reduced cytokine levels via a possible reduction in cell proliferation.

6.2.6. Limitations of investigating the effects of repeated psychostimulant and decitabine treatment in an *in vitro* model of PBMC and future perspectives.

We demonstrated that decitabine was able to inhibit the effects of cocaine on the activities of DNMT and TET enzymes. However, even though decitabine is a medicine approved by regulatory authorities, it is a cytotoxic chemotherapy drug with severe side effects (Gore *et al.*, 2006). Current *in vitro* study is insufficient for making conclusions about the therapeutic potential in a more complicated system at the organism level. However, our study shows proof that DNMT inhibition reverses the cocaine-induced changes in the activity of DNMT and TET enzymes and modulates the immune response in the PBMCs. As decitabine is a DNMT inhibitor, which is incorporated into DNA strands upon replication (Agrawal *et al.*, 2018), it is plausible that it does not have a significant effect on post-mitotic neurons. However, it could affect neural stem or progenitor cells and pre-mitotic glial cells. Alternative, non-toxic DNMT inhibitors, which are not incorporated into the DNA, could exhibit more therapeutic potential in reversing the psychostimulant-induced epigenetic effects.

Our study did not assess the effects of cocaine, amphetamine, and decitabine *in vivo* or in central nervous system regions directly involved in addiction. Further studies are needed to clarify if cocaine and amphetamine could exert similar effects on epigenetic DNA editors and DNA methylation *in vivo* in human blood and human brains, e.g., by assessing the post-mortem tissue of people with an addiction. Alternative models using human-induced pluripotent stem cell-derived dopaminergic neurons or glial cells could provide further information about the effects of psychostimulants on epigenetic DNA editors and DNA methylation in nervous system cells.

7. CONCLUSIONS

1. Chronic neuropathic pain caused anxiety, followed by the development of depressive-like behavior, which was accompanied by impaired episodic-like memory and disrupted contextual fear memory in adult male mice. The long-term alterations in hippocampal plasticity, which co-occurred with behavioral changes, were a reduction in newly generated mature neurons and a decrease in the long-term survival of newly generated cells rather than reduced proliferation or decreased number of immature neurons. In addition, microglial cells exhibited morphological alterations indicating increased reactivity in the DG of the hippocampus. Our results suggest that newly generated neurons are not eliminated in earlier survival stage, right after proliferation, but at the later critical time-point for survival.

   To summarize, chronic neuropathic pain caused anxiety, depressive-like behavior, and memory impairments, which co-occurred with impaired neurogenesis and signs of increased microglial reactivity, possibly contributing to observed alterations in behavior.
2. Long-term VDD induced episodic-like and contextual fear memory impairment, and a reduction in self-care in middle-aged male mice. VDD did not amplify the adverse behavioral effects of chronic stress, which were depressive-like behavior, decreased self-care, and disrupted episodic-like and contextual fear memory. In fact, self-care even improved in response to chronic stress in VDD mice. We hypothesize that preceding cognitive impairments induced by VDD may have dampened some of the adverse effects of chronic stress. In parallel with the behavioral changes, both VDD and UCMS reduced the long-term survival of newly generated cells in the hippocampus and cell loss of the GCL; VDD also induced increased apoptotic cell death in the hippocampus. In addition, signs of neuroinflammation were observed in the hippocampus of VDD mice, namely morphological alterations of microglial cells indicating increased reactivity, microglial cell loss, and increased protein levels of pro-inflammatory cleaved caspase-1. Signs of decreased neurogenesis, increased cell death, and neuroinflammation in the hippocampi of VDD mice may contribute to observed memory impairments. Reduced TH levels in the striatum of VDD and UCMS mice, but not in VDD+UCMS mice, may contribute to reduced self-care behavior related to reduced motivation.

   To summarize, our results demonstrate that maintaining adequate vitamin D levels throughout adulthood and middle age is necessary to preserve cognitive functions and self-care in middle-aged male mice. However, based on the obtained results, long-term VDD does not cause depressive-like behavior on its own or contribute to decreased stress resilience in the middle-age period. Manifestations of neuroinflammation could contribute to reducing the long-term survival of newly generated cells in VDD mice. Signs of decreased neurogenesis, neuroinflammation, and cell loss in the hippocampus may

contribute to observed memory decline, while reduced TH levels may underlie the observed reduction in self-care and motivation.

3. Repeated treatments with cocaine or amphetamine increased *DNMT1* mRNA levels, reduced mRNA levels of *TET1–3* and the activity of TET enzymes, and reduced global DNA hydroxymethylation in an *in vitro* model of human PBMCs. Data from our previous study in mice suggest that alterations in the activities of epigenetic DNA editors after cocaine treatment are similar in the CNS and peripheral blood cells, but it is unknown whether it is possible to extrapolate the activity of epigenetic DNA editors in human PBMCs to the CNS. Further investigation is needed to clarify whether similar alterations in *TET* mRNA and activity of TET enzymes take place in the CNS and if altered TET activity could contribute to the development of addiction.

   Our results indicate that cocaine induces alterations in cytokine concentrations in cell culture media of PBMCs and that psychostimulant use may regulate *IL10* gene expression via altered DNA methylation. Finally, our results suggest that DNMT inhibition by decitabine inhibits the effects of cocaine on the activity of TET and DNMT enyzmes and modulates the immune response in PBMCs. Further investigation is needed to clarify if DNMT inhibition may pose therapeutic potential.

8. SUMMARY IN ESTONIAN

Aju plastilisust kahjustavate tegurite molekulaarsed ja rakulised mehhanismid

Aju plastilisus on aju kaasasündinud võime muuta oma struktuuri ja funktsioone, et kohaneda muutuvate sise- ja väliskeskkonna tingimustega. Aju plastilisus on vajalik arenguperioodidel, õppimisel ja mälu protsessides ning funktsioonide taastamiseks pärast kahjustust (Murphy & Corbett, 2009; Ganguly & Poo, 2013; Voss *et al.*, 2017; Mateos-Aparicio & Rodríguez-Moreno, 2019). Täiskasvanuea neurogenees hipokampuses on unikaalne aju plastilisuse vorm, mis panustab mälu funktsioonidesse ja meeleolu regulatsiooni ning vähenenud hipokampuse neurogeneesi seostatakse mäluhäirete ja depressiooniga (Eriksson *et al.*, 1998; Spalding *et al.*, 2013; Boldrini *et al.*, 2018; Toda & Gage, 2018; Kempermann, 2022). Uuringud näriliste mudelitel on näidanud, et vähenenud neurogenees hipokampuses võib olla üheks hipokampuse atroofia põhjuseks (Santarelli *et al.*, 2003; Surget *et al.*, 2008). Hipokampuse plastilisusele võib kahjustavalt mõjuda ka mikrogliia rakkude suurenenud reaktiivsus ja lokaalne neuropõletik, mis võivad viia neurogeneesi ja sünaptilise plastilisuse languseni (Ekdahl *et al.*, 2003; Yirmiya & Goshen, 2011; Becher *et al.*, 2017; Cornell *et al.*, 2021; Troubat, Barone, *et al.*, 2021).

Patoloogiad ja terviseseisundid, nagu krooniline neuropaatiline valu ja krooniline stress võivad põhjustada düsfunktsionaalset hipokampuse plastilisust, mida seostatakse mäluhäirete ja depressiooniga (Mineur *et al.*, 2006; Song *et al.*, 2006; Mutso *et al.*, 2012; Dellarole *et al.*, 2014; Gui *et al.*, 2016; Toda *et al.*, 2019). Epidemioloogilised vaatlusuuringud on leidnud seose ka D-vitamiini defitsiidi, mäluhäirete ja depressiooni vahel, kuid põhjuslik seos ei ole selge (Meehan & Penckofer, 2014; Ceolin *et al.*, 2022). Aju plastilisuse häired esinevad ka sõltuvuse korral, kui korduv uimastite kasutamine põhjustab hipokampuse neurogeneesi häireid ja neuroplastilisi muutusi glutamatergilises innervervatsioonis juttkehale ning dopaminergilistele neuronitele keskajus (Yamaguchi *et al.*, 2004; Witten *et al.*, 2011; Koo *et al.*, 2012; Pascoli *et al.*, 2015; Nestler & Lüscher, 2019). Varasemad loomuuringud on viidanud, et psühhostimulantidest tingitud häirunud DNA metülatsioon võib vahendada geeniekspressiooni muutusi, mis omavad olulist rolli sõltuvuskäitumises (Anier *et al.*, 2010; LaPlant *et al.*, 2010; Nestler & Lüscher, 2019; Urb *et al.*, 2020). Meie töögrupi eelnev uuring näitas, et pärast kokaiini manustamist hiirtele toimusid sarnased muutused epigeneetilistes DNA modifitseerijates – DNA metüültransferaasides (DNMTdes) ja *ten-eleven* translokatsiooni (TET) ensüümides, nii sõltuvusega seotud juttkeha piirkonnas naalduvas tuumas kui ka perifeerses veres (Anier *et al.*, 2018). Aberrantse DNA metülatsiooni ja epigeneetiliste DNA modifitseerijate roll sõltuvuse tekkes inimestel vajab lisauuringuid.

Töö eesmärgid

Käesoleva doktoritöö eesmärk oli selgitada aju plastilisust kahjustavate faktorite (krooniline neuropaatiline valu, D-vitamiini defitsiit, krooniline stress ja psühhostimulandid amfetamiin ja kokaiin) rakulisi ja molekulaarseid mehhanisme asjakohastes hiiremudelites ja *in vitro*. Täpsemad töö eesmärgid olid järgmised.

1. Täpsustada kroonilise neuropaatilise valu hiiremudelis depressioonilaadse käitumise, ärevuse ja mäluhäirete tekke ajadünaamikat. Samuti soovisime selgitada käitumuslike muutustega kaasnevaid häireid hipokampuse neurogeneesis ning mikrogliia rakkude morfoloogias.
2. Selgitada, kas pikaajaline D-vitamiini defitsiit (DVD) põhjustab iseseisvalt või kombinatsioonis kroonilise stressiga mäluhäireid ja depressioonilaadset käitumist hiirtel. Samuti oli eesmärgiks iseloomustada käitumuslike muutustega kaasuvaid häireid närvi- ja gliiarakkude plastilisuses hipokampuse piirkonnas.
3. Selgitada psühhostimulantidest tingitud muutusi epigeneetilises DNA modifitseerijates, DNA metülatsioonis ja tsütokiinide profiilis, kasutades inimese perifeerse vere mononukleaarsete rakkude (PBMC) kultuuri. Täpsustada, kas DNA metüültransferaasi (DNMT) inhibiitor detsitabiin võib takistada kokaiini toimeid DNA modifitseerijatele.

Töö tulemused ja järeldused

1. Leidsime kroonilise neuropaatilise valu hiiremudelis, et esmalt tekkinud ärevuskäitumisele järgnes depressioonilaadse käitumise teke, millega kaasnesid lühi- ja pikaajalise mälu häired. Samaaegselt käitumuslike häiretega vähenes uute küpsete neuronite hulk ja uute tekkinud rakkude elulemus, kuid mitte proliferatiivne jagunemine ega ebaküpsete neuronite arv. Samuti oli hipokampuse hammaskäärus muutunud mikrogliiarakkude morfoloogia, mis viitas suurenenud mikrogliia reaktiivsusele. Kokkuvõttes põhjustas krooniline neuropaatiline valu ärevust, depressioonilaadset käitumist ja mäluhäireid, millega kaasnesid neurogeneesi langus ja mikrogliia morfoloogia muutused hipokampuses. Need struktuursed muutused hipokampuses võivad panustada neuropaatilise valu foonil kujunenud käitumuslikesse muutustesse.
2. Kasutades hiiremudelit näitasime, et pikaajaline DVD põhjustas lühi- ja pikaajalise mälu häired ning vähenenud enesehoolituskäitumist, kuid mitte depressioonilaadset käitumist. DVD foonil ei süvenenud kroonilise ettearvamatu stressi negatiivsed efektid käitumisele, milleks olid depressioonilaadne käitumine, enesehoolitsuse langus ja mäluhäired. Enesehoolitsuskäitumine isegi paranes neil DVD hiirtel, kes kogesid pikaajalist stressi. Pole välistatud, et eelnevad ulatuslikud kognitiivsed häired DVD hiirtel võisid takistada kroonilise stressi negatiivseid toimeid käitumisele. Samaaegselt DVD ja kroonilise stressi tulemusena kujunenud käitumuslike muutustega leidsime vähenenud uute tekkinud rakkude elulemuse hipokampuses. Samuti tuvastasime, et DVD suurendas apoptootiliste rakkude hulka hipokampuses ning esinesid neuropõletiku ilmingud,

milleks olid muutused mikrogliia rakkude morfoloogias, vähenenud mikrogliia rakkude arv ja põletikku soodustava aktiveeritud kaspaas-1 valgutaseme tõus. Vähenenud enesehoolitsus- ja motivatsioonikäitumine võivad olla põhjustatud dopamiini sünteesis kriitilise tähtsusega ensüümi, türosiinhüdroksülaasi (TH) taseme vähenemisest. TH tase oli alanenud DVD hiirtel, kuid mitte stressi kogenud DVD hiirtel, kellel muutusi enesehoolitsuses ei täheldatud.

Kokkuvõttes näitavad meie tulemused, et D-vitamiini piisav hulk organismis täiskasvanueas on vajalik kognitiivsete võimete säilitamiseks keskeas. Küll aga ei näidanud meie töö tulemused, et pikaajaline DVD põhjustaks depressioonilaadset käitumist või suurendaks haavatavust stressi suhtes. Häirunud neurogeneesi ja neuropõletiku ilmingud ning rakkude kadu DVD hiirte hipokampuses võivad olla seotud mäluhäirete tekkega.

3. Kasutades *in vitro* inimese perifeerse vere rakkude mudelit, leidsime, et korduv kokaiini ja amfetamiini töötlus suurendas *DNMT1* mRNA taset, vähendasid *TET1–3* mRNA tasemeid, TET ensüümide aktiivsust ja globaalset DNA hüdroksümetülatsiooni. Korduv inkubatsioon kokaiiniga tõstis põletikku soodustava tsütokiini, interleukiin (IL)-6 taset ning vähendas põletikku pärssiva IL-10 taset rakusöötmes. *IL10* geeni hüpermetülatsioon ning vähenenud *IL10* mRNA tase viitab, et IL-10 kontsentratsiooni langus kokaiini toimel võib olla vahendatud epigeneetiliste mehhanismide läbi. DNMT inhibiitor detsitabiin pärssis kokaiini toimet DNMT ja TET ensüümide aktiivsusele.

   Meie tulemused näitavad, et psühhostimulandid kokaiin ja amfetamiin põhjustasid muutusi epigeneetilistes DNA modifitseerijates, TET ensüümides ning et DNMT inhibeerimine takistab kokaiini toimet epigeneetilistele DNA modifitseerijatele. Edasised uuringud on vajalikud, et selgitada, kas TET ensüümide aktiivsuse langus psühhostimulantide toimel inimese perifeerse vere rakkudel *in vitro* sarnaneb muutustele psühhostimulante tarvitavate inimeste veres ja ajus. Selgitamist vajab ka epigeneetiliste DNA modifitseerijate ja aberrantse DNA metülatsiooni roll sõltuvuse tekkes inimestel ning DNMT inhibeerimise terapeutiline potentsiaal.

Käesolev doktoritöö näitab, et aju plastilisust mõjutavad tegurid (krooniline neuropaatiline valu, DVD ja krooniline stress) põhjustavad häireid hipokampuse neuroplastilisuses ja mikrogliia rakkude morfoloogias, mis võivad olla käitumuslike häirete aluseks hiirtel. Samuti näitavad meie tulemused, et psühhostimulandid põhjustavad inimese perifeerse vere rakkude mudelis muutusi tsütokiinide profiilis ja epigeneetiliste DNA modifitseerijate, TET ensüümide aktiivsuses, mida on võimalik inhibeerida DNMT inhibiitori detsitabiiniga. Meie leiud neis molekulaarsetes ja rakulistes alusmehhanismides võivad aidata tulevikus välja töötada uusi ennetus- ja ravistrateegiaid.

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PUBLICATIONS

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4. Chithanathan, K., **Somelar, K.**, Jürgenson, M., Žarkovskaja, T., Periyasamy, K., Yan, L., Magilnick, N., Boldin, M. P., Rebane, A., Tian, L., & Zharkovsky, A. (2022). Enhanced Cognition and Neurogenesis in miR-146b Deficient Mice. *Cells*, *11*(13), 2002. https://doi.org/10.3390/cells11132002
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