Universidade do Minho
Escola de Engenharia

Ana Rita da Silva Reis Dourado

Impact of the ablation of hippocampal astrocytes in neurophysiology and behavior of rodents

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Impact of the ablation of hippocampal astrocytes in neurophysiology and behavior of rodents

Master Dissertation
Master in Biotechnology

Work carried out under the guidance of:
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October 2023

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

As células da Glia são células de suporte ao Sistema Nervoso Central, das quais fazem parte os astrócitos. Estes estão envolvidos em processos emocionais e cognitivos, podendo ser encontrados em várias partes do cérebro, incluindo o hipocampo, onde se encontra o Giro Dentado (DG). Esta é uma das áreas do cérebro onde se observa a formação de novos neurónios (neurogénese) e astrócitos (astrogliogénese), sendo uma estrutura base no controlo emocional. Apesar desta importante característica, os astrócitos são também conhecidos por muitas outras funções, como por exemplo armazenamento de energia, homeostase da água e proteção contra o stress oxidativo. Ademais, alterações no número e morfologia dos astrócitos tem sido descrita em várias patologias, levantando questões relativas à função dos astrócitos nas mesmas. No entanto, uma vez que os neurónios e os astrócitos estão tão intimamente ligados, o estudo da função individual de cada torna-se num desafio.

Dessa forma, para a realização deste trabalho recorremos à utilização de uma ferramenta viral que irá atuar sobre os astrócitos maduros do hipocampo de um modelo animal de roedor, marcando-os com GFP ou eliminando-os. Pretendemos assim avaliar os efeitos da ablação dos astrócitos no comportamento animal e na fisiologia cerebral, contribuindo assim para o aumento do conhecimento da função astrocítica.

Neste trabalho conseguimos eliminar significativamente mais astrócitos do que neurónios, o que resultou em diferenças comportamentais no nosso modelo de roedor, nomeadamente ao nível de self-care e behavioral despair. Novas otimizações deverão ser realizadas futuramente, de forma a caracterizar completamente a eficácia da nossa ferramenta, bem como para a melhorar, de forma a não só aumentar o conhecimento já existente relativamente aos astrócitos maduros, mas também para chegarmos ao nosso objetivo inicial de criar uma ferramenta viral capaz de eliminar especificamente astrócitos maduros hipocampais.

Palavras-Chave: Astrócitos; Giro Dentado; Hipocampo

Abstract

Glial cells are support cells for the Central Nervous System, of which astrocytes are a part. These are involved in emotional and cognitive processes and can be found in various parts of the brain, including the hippocampus, where the Dentate Gyrus (DG) is located. This is one of the areas of the brain where the formation of new neurons (neurogenesis) and astrocytes (astrogliogenesis) is observed, being a basic structure in emotional control. Despite this important characteristic, astrocytes are also known for many other functions, such as energy storage, water homeostasis and protection against oxidative stress. Furthermore, changes in the number and morphology of astrocytes have been described in several pathologies, raising questions regarding the function of astrocytes in such disorders. However, because neurons and astrocytes are so closely linked, studying their individual function becomes a challenge.

Therefore, to carry out this work we resorted to using a viral tool that will act on mature astrocytes from the hippocampus of a rodent animal model, marking them with GFP or ablating them. We thus intend to evaluate the effects of astrocyte ablation on animal behavior and brain physiology, thus contributing to increased knowledge of astrocytic function.

In this work we managed to eliminate significantly more astrocytes than neurons, which resulted in behavioral impairments in our rodent model, particularly in terms of self-care and behavioral despair. New optimizations should be carried out in the future, in order to fully characterize the effectiveness of our tool, as well as to improve it, in order to not only increase the existing knowledge regarding mature astrocytes, but also to reach our initial objective of creating a viral tool capable of specifically eliminating mature hippocampal astrocytes.

Key-Words: Astrocytes; Dentate Gyrus; Hippocampus

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Lista de Abreviaturas e Unidades

AAV	Adeno Associated Virus
ANPs	Amplifying Neural Progenitors
A-P	Anterior-Posterior
APC	Astrocytes Progenitor Cells
BrdU	Bromodeoxiuridine
CA	Cornu Ammonis
cm	Centimeter
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate Gyrus
DGAV	Direção Geral de Alimentação e Veterinária
DGC	Dentate Granule Cell
DLB	Dark/Light Box
dTK	Delta Thymidine Kinase
D-V	Dorso-Ventral
EtBr	Ethidium Bromide
EPM	Elevated Pus Maze
GCL	Granular Cell Layer
GCV	Ganciclovir
GFAP	Green Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GLAST	Glutamate Aspartate Transporter
h	Hour
H	Hilus
HCl	Hydrochloric Acid
HMGN	High Mobility Group Nucleosome-binding
ICVS	Life and Health Sciences Research Institute
i.p.	Intraperitoneal
INP	Intermediate Neuronal Progenitors
kg	Kilogram

M	Molar
MDD	Major Depressive Desorder
mg	Milligram
min	Minute
mL	Millilitre
M-L	Medial-Lateral
ML	Molecular Layer
NFIA	Nuclear Factor I A
NGS	Normal Goat Serum
nL	Nanolitre
NPC	Neural Progenitor Cell
NOR	Novel Object Recognition
OPC	Oligodendrocyte Progenitor Cells
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline-Triton
PNS	Peripheral Nervous System
PTSD	Post-Traumatic Stress Disorder
QNPs	Quiescent Neuronal Progenitors
RGL	Radial Glia Like cell
S100β	S100 calcium-binding protein β
SEM	Standard Error of the Mean
SGZ	Subgranular Zone
SPT	Sucrose Preference Test
ST	Splash Test
STAT	Signal Transducer and Activator of Transcription
SVZ	Subventricular Zone
TST	Tail Suspension Test
uCMS	Unpredictable Chronic Mild Stress
WT	Wild-Type
µg	Microgram
µL	Microliter

µm	Micrometre
%	Percentage
°C	Degrees Celsius

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1. Introduction

1.1 The brain and the Central Nervous System

Through the decades, research aimed to solve the big mysteries of the brain, from its constitution to its function and importance. However, some of the raised questions have no definitive answer, therefore there is still a lot to be studied and to be found in this field. One paradigmatic example of these unanswered questions is regarding the brain's development process or even its plasticity in pathological processes. Researchers have also leaned over the proliferation of brain cells like neurons, and are trying to assess the fascinating process of neurogenesis in the adult brain and its possible roles.

Like the brain, the Nervous System has raised a lot of important questions to researchers, and a lot is already known, such as its function. This system is composed of the Central Nervous System (CNS) and the Peripheral Nervous System (PNS), but as the main focus of this work is the brain, understanding the CNS becomes essential. The CNS function has been studied for many years and nowadays we know it is responsible for our movement – by controlling the voluntary movements of our muscles, allowing us to walk – our ability to feel things by touch – by receiving the signals sent by the surface of our skin – and other functions like the homeostatic control of our organism (Haschek et al., 2010).

Studies of the development of the CNS and more specifically of the brain have allowed us to understand their constitution. Two types of cells should be highlighted as the main components of the brain: the neurons and glial cells (He & Sun, 2007; Jessen, 2004). In fact, the current knowledge of the types of cells forming the brain tissues, their differentiation and their birth during development is an important subject under study namely because of their functional relevance, which will be discussed in the next sections.

1.2 Neurons and Neurogenesis

As mentioned, the understanding of the brain needs further comprehension of its cells. Neurons are the electrical cells responsible for the brain's information mechanisms such as information processing and storing (Jessen, 2004; Kimelberg & Norenberg, 1989; Holt et al., 2019). The transmission of that stored information and the control of cognitive functions like learning and memory are also managed by neurons (Holt et al., 2019; Tsutsui et al., 2000; Kandel et al., 2013). Another interesting characteristic of these cells is their ability to communicate through synapses by releasing neurotransmitters into the synaptic cleft (Lin & Iacovittin, 2015). Interestingly, learning leads to changes in the neurons at the synapse level, altering their number and effectiveness, which confers plastic characteristics to neurons (Kandel et al., 2013).

One of the main questions raised by neuroscientists is the possibility of generating cells, like neurons, in the brain. Lin & Iacovittin (2015) characterised neurons as quiescent cells and explained that most of the neurons cannot be substituted by a new one. In fact, neurogenesis, may – and is – a possible process, although not all neurons can be regenerated. In fact, even though the majority of the cells die during the initial hours post-mitosis, namely during the network integration stage of the neurogenesis process, some of the new neurons end up being integrated into the existing neuronal circuits (Jiang et al., 2023).

For many years, the study of neurogenesis was mainly focused on the embryonic stages in the neocortex (Jiménez et al., 2019). Although scientists believed for a long time that the adult brain did not possess the capacity to generate new cells, nowadays, we know that there are stem cells with the capacity of self-renewal in the adult brain, even though in specific microenvironmental niches (Schneider et al., 2019). Therefore, the study of adult neurogenesis was neglected for many years and its study just stood out some decades ago.

In fact, the beginning of the adult neurogenesis study takes us back to the last century, when Altman and Das observed, for the first time, newborn cells in the rat brain (Altman & Das, 1965). Thanks to their work, we now know that neurogenesis is possible, but also how, when, and where new neurons are formed. This interesting process is found through many stages of life and in specific regions of the adult brain, namely subgranular zone (SGZ) of the hippocampal Dentate Gyrus (DG) and the subependymal zone (Parpura et al., 2001; Deng et al., 2010; Petrik et al., 2012), which compose the consensual cytogenic niches where Neural Stem Cells (NSCs) are found (Yeh et al., 2022). However, some other researchers recently reported neurogenesis in other brain regions, such as the hypothalamus and the striatum (Ernst et al., 2014; Xu et al., 2005). Despite all these facts, only in the 90's neurogenesis was observed in the hippocampal DG of the human *post-mortem* tissues (Eriksson et al., 1998).

Neurogenesis **(Figure 1)** passes through many stages namely proliferation, differentiation, migration, and synaptic integration (Toda et al., 2019; Li et al., 2009).

Briefly, specifically in the hippocampal DG, these events consist on the differentiation of a Neural Progenitor Cell (NPC) to a Dentate Granule Cell (DGC) that will migrate to the granular cell layer of the DG to elongate their cellular processes and maturate (Deng et al., 2010). More specifically, neurogenesis starts in the SGZ, with the activation of Radial Glia-like Cells (RGL) (type 1 cells), representing a pool of pluripotent stem cells (Yeh et al., 2022, Alvarez-Buylla & Kriegstein, 2013). Once activated, RGL will produce Intermediate Neuronal Progenitor (INP) (type 2 cells), which will differ from the first ones for not having gap junctions nor radial polarity (Alvarez-Buylla & Kriegstein, 2013), and will differentiate into

neuroblasts (type 3 cells) (Gonçalves et al., 2016; Toda et al., 2019). Finally, the neuroblasts will originate mature neurons that upon migration to the DG, will develop their cellular processes, axons, and other important neuronal structures (Deng et al., 2010). At this stage, the cellular processes will be able to interconnect with other cells, initiating the integration of the new cells in the local neuronal networks, such as the memory networks (Deng et al., 2010; Deng et al., 2011). Therefore, neurogenesis may be defined as the process in which new functional neurons are integrated into the brain's neuronal circuits (Parpura et al., 2001; Toda et al., 2019; Kitchigina et al., 2022).

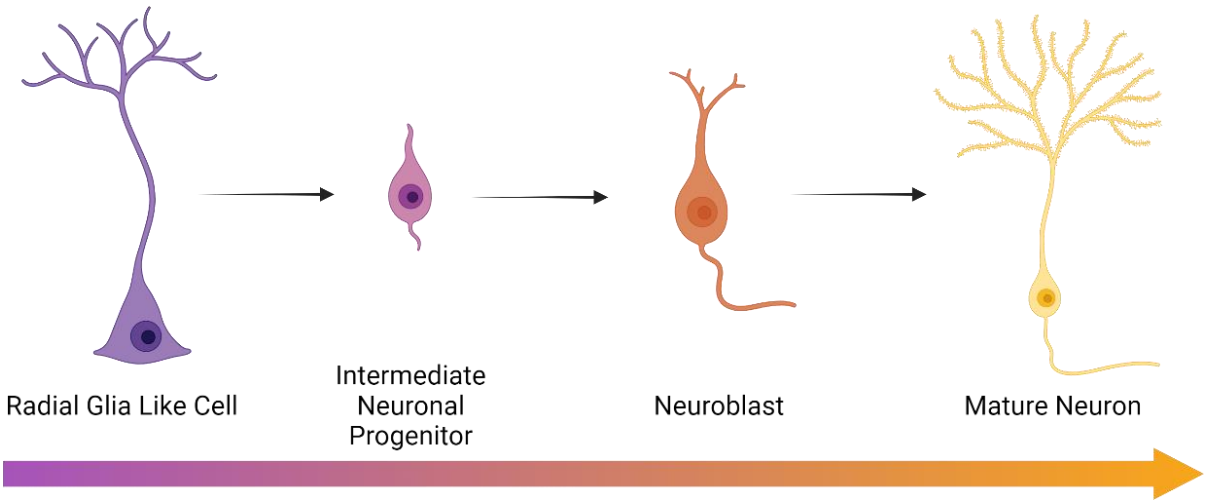


Figure 1 – Neurogenesis process

An activated Radial Glia Like cell will produce an Intermediate Neuronal Progenitor that will differentiate in a Neuroblast to finally originate a mature neuron. Figure created by Biorender.com

This process of neurogenesis is known to be relevant for many functions, including hippocampal-dependent learning (Winocur, et al., 2006), antidepressant drug-positive response (Santarelli, et al., 2003) and even brain homeostasis and memory (Park et al., 2022). Curiously, even the application of Lavender Essential Oil was recently described to allow an improvement in both neurogenesis and depression-like behavior (Isabel et al., 2019) and, interestingly, the injection of the fungi *Hericium erinaceus* extract had also led to an increase in neurogenesis (Chong et al., 2021). However, neurogenesis can also be influenced by many other factors like environmental enrichment (Kempermann et al., 2002), exercise (van Praag et al., 2005), growth factors (Schänzer et al., 2004) and even hormones (Gheorghe et al., 2018), which includes the female estrus cycle, namely in the proestrus phase, in which we can observe an increase in neurogenesis (Tanapat et al., 1999). Moreover, recent studies have shown other relevant features regarding the neurogenesis process, as for example, researchers have leaned over relevant diseases and reported the influence of cytogenesis in epilepsy (Cho et al., 2015), anxiety (Revest et al., 2009) and stress (Czéh et al., 2002; Alonso et al., 2004). In fact, Chronic Stress induced depression decreases hippocampal cytogenesis in mice, while an increase in neurogenesis leads to a

reduction in depressive-like behavior (Planchez et al., 2021). The deficit of newborn cells was further considered to be related to the motor and cognitive impairments of some diseases like Parkinson's and Alzheimer's diseases (Abdipranoto et al., 2008). Aging, for example, is also an important factor in cognitive processes and neurodegenerative disorders (Toda et al., 2019), leading to a decrease in adult cytogenesis (Aizawa et al., 2009).

Despite these findings, there is opposite evidence (Mineur et al., 2007; Revest et al., 2009) concerning the impact of cell cytogenesis on behavior in animal models of disease. For example, the ablation of adult-born neurons was reported to not influence anxiety-like behavior or spatial learning in mice (Meshi et al., 2006; Groves et al., 2013), while others concluded that hippocampal neurogenesis is an important process in stress response (Tsai et al., 2015) and that the reduction of adult-born cells induced anxiety-like behavior (Yun et al., 2016). Although some recent studies using GFAP-tk transgenic rats had reported changes in anxiety and anhedonic behaviors after hippocampal cytogenesis abrogation (Mateus-Pinheiro et al., 2021), studies with X-ray irradiation indicate a clear necessity for hippocampal new-born cells for contextual discrimination, namely in the dorsal hippocampus (Wu & Hen, 2014). As such, more studies are needed to elucidate and clarify the specific role of neuro and astrogliogenesis in pathology and behavior (Lucassen et al., 2016), since it is still unclear whether the changes in the number of these adult-born cells in the brain are a cause or consequence of certain pathologies.

On the other hand, other studies focused on cytogenesis's importance for brain adaptability and plasticity (Kempermann et al., 2018), including hippocampus-dependent learning (Pittenger & Duman, 2008) suggested an essential role in hippocampal cognitive plasticity (Leuner & Gould, 2010), but also emotional impairment recovery (Mateus-Pinheiro et al., 2013). In fact, during perinatal periods, neurogenesis is considered one of the main forms of plasticity in the human brain that allows adaptation to the environment during postnatal periods (Toda & Gage, 2018, Kitchigina et al., 2022). Newborn neurons play a crucial role in hippocampal plasticity, by improving memory processing (König et al., 2016) and can contribute to cognitive flexibility by controlling the strategies we use to complete a new task or whether that ability is just a consequence of improved spatial memory (Gonçalves et al., 2016). Besides, adult-born neurons may also contribute to synaptic plasticity (Ge et al., 2007; Marín-Burgin et al., 2012), since the learning and memory processes are linked to the synaptic circuit, suggesting that neurogenesis in the hippocampal DG may be a key functional feature of the hippocampus plasticity, for example, the regulation of the integration of adult-born neurons in the neuronal circuits during neurogenesis is important for hippocampal structural plasticity (Toda & Gage, 2018). Besides, the

regulation of pattern integration leads to the association of temporally separated events and, eventually, to encode new memories (Aimone et al., 2009; Becker & Wojtowicz, 2007; Ragel et al., 2014).

To study neurons and, of course, neurogenesis, multiple markers can be used. For example, markers such as Nestin and Glial Fibrilary Acidic Protein (GFAP) are expressed by NSC with self-renewal characteristics (Lam et al., 2009), whereas GLAST (Glutamate Aspartate Transporter) is expressed during neuronal migration by Radial Glial Cells (Shibata et al., 1997). For instance, NeuN is known to be expressed in mature newborn neurons (Mullen et al., 1992) and Doublecortin (DCX) is recognised to be expressed by immature neurons (Parent, 2007). These markers are very important to assess the different phases of the neurogenesis process. Besides, the regulation of gene expression by Transcription Factors (TF) has a pivotal role in RNA transcription to DNA, since it leads to the expression of functional proteins (Latchman, 1993). Many TFs are involved in neurogenesis, as for example, Pax6 which is expressed by radial glial cells and NSC (Englund et al., 2005) and is co-expressed by Type 1-cell markers such as GFAP (Nacher et al., 2005). The same happens with the TF Sox2 (Ferri et al., 2004) which is expressed by most of the dividing cells in the SGZ (Lugert et al., 2010). In turn, CREB which is involved in neuronal survival (Nakagawa et al., 2002), is mostly expressed by immature neurons and co-localizes with DCX (Jagasia et al., 2009). All of these factors are, therefore, important for neurogenesis occurrence and ultimately for its assessment.

To sum up, despite the initial thought regarding the formation of new neurons in the adult brain, the discovery of the neurogenesis process overcame the belief that new cells couldn't be generated in the brain. Even so, the existence of adult neurogenesis in humans is a process that is still under discussion. Some authors reported that neurogenesis persists throughout life in the human brain (Spalding et al., 2013), meanwhile, other researchers questioned the persistence of adult neurogenesis after the first years of life (Sorrells et al., 2018). However, in other species, for example, fishes (Oliveira-Pasilio et al., 2017), birds (Barkan et al, 2017), other mammalian species (Taylor et al., 2016; Oosthuizen, 2017) and even in invertebrates (Benton et al., 2022) it seems to be clear that neurogenesis does exist. More recently the studies on brain cytogenesis proceeded by approaching other brain cells, namely glial cells, increasing the knowledge in this field.

1.3. Glial Cells

Glial cells were originally described by the German Virchow as the glue cells of the CNS since the spaces between the nerve elements were filled by them (Messam et al., 2002). These cells can be divided into three major groups of cells: astrocytes, oligodendrocytes, and microglia (He & Sun, 2007), however,

some authors also consider a fourth group: the glial progenitors NG2-glia (Jäkel & Dimou, 2017). Glial cells were discovered more than two centuries ago (Jäkel & Dimou, 2017) and, despite being different from neurons, glial cells and neurons may come from common lineages that undergo a switch during development (Jones et al., 1995). In fact, (Bond et al., 2020) reports recognize that neurogenesis and gliogenesis are sequential over time during embryonic development, describing that the astrogliogenesis process starts in late embryonic development until early postnatal stages. This process will be further discussed in the next section.

The definition of glial cells' role is a challenging process, however, now it is known that they are involved in almost every neurological process (Ransom et al., 2003). These cells were first recognized to have an essential role in giving support to neurons and, therefore, to the nervous system (He & Sun, 2007; Snyder et al., 2018), but the advances in this field allowed us to understand that they have additional roles in the CNS besides the originally discovered. In fact, glial cells constitute the majority of the human brain cells and, apart from their supportive function, they also play important roles in pathophysiology (Benedetto et al., 2013), regulate the dopamine levels in the brain (He & Sun, 2007), but they can also avoid toxic substances to pass the blood-brain barrier (Montgomery, 1994; Jessen, 2004). Besides, other studies have been suggesting a new important role of these cells at the synapse level (Allen & Barres, 2005), but also in the neurogenesis process, namely during axonal growth and neuronal migration, but also as progenitor cells (Montgomery, 1994; Jessen, 2004; Alvarez-Buylla & Kriegstein, 2013).

Specifically, microglia are immunocompetent cells that are activated upon brain injury (Jäkel & Dimou, 2017), important for protection, function and normal maturation of the CNS (van Rossum & Hanisch, 2004). Oligodendrocytes are responsible for myelination and, therefore, are also important for neuronal transmission conservation (Goldman & Kuypers, 2015). Astrocytes, on the other hand, were considered nothing else but supportive cells for many years (Schneider et al.,2022), however, as new evidence has been collected, the astrocytes' importance is unquestionable. This thesis will mainly focus on astrocytes, their function, and their importance, which will be further assessed in the next section.

1.3.1. Astrocytes and Astrogliogenesis

Astrocytes are star-shaped cells (Bernardinelli et al., 2014) and are the most frequently found cells in the white and grey matter of the brain (Kimelberg & Norenberg, 1989; Molofsky et al., 2013; Snyder et al., 2018), being highly organized and curiously arranged in a non-overlapping manner in the CNS environment (Haim & Rowitch, 2017, Sofroniew & Vinters, 2010, Volterra & Meldolesi, 2005). These

cells vary according to their morphology, being classified as plasmic, fibrous or specialized astrocytes (Shao & McCarthy, 1994), in which the protoplasmic astrocytes are considered the most abundant cell type in the grey matter with a spongiform morphology (Bushong et al., 2004). These cells are highly ramified, with their processes extending from the soma to the neighbour astrocyte (Bushong et al., 2004).

There are several functions of astrocytes that are currently known, such as energy storage, mitochondrial biogenesis, water homeostasis and protection against oxidative stress (Martins-Macedo et al., 2021a). Astrocytes are involved in neurons and blood vessel linkage (Li et al., 2019; Parpura et al., 2001), allowing their metabolic and structural support, but also in CNS blood flow control (Kimelberg & Nedergaard, 2010). Although these cells are found in the brain, they are not capable of conducting electrical signals like neurons (Nag, 2011, Kuffler et al., 1996, Volterra & Meldolesi, 2005), however, they can be excited through internal or external signals that allow them to communicate with neighbouring cells (Volterra & Meldolesi, 2005, Bezzi & Volterra, 2002). This way, astrocytes can be excited in a neuron-dependent manner or spontaneously and then act as a link between other cells or blood vessels (Volterra & Meldolesi, 2005, Fellin et al., 2004, Hirase et al., 2004). Besides, their characteristic processes – about five to eight highly ramified processes (Nag, 2011; Bushong et al., 2002) – together with the ion channels and the gap junctions, they can communicate with multiple structures like blood vessels – curiously, almost all astrocytes can form end-feet processes with at least one blood vessel (Bushong et al., 2002, Koehler, et al., 2006) – and other astrocytes. Therefore, astrocytes communicate with the extracellular environment and the surrounding neuronal networks to fulfil their functions (Kuffler et al., 1996), for example, by regulating the brain blood flow during Ca^{2+} wave, leading to vascular constrictions (Mulligan & MacVicar, 2004).

Other remarkable functions of astrocytes have been reported over the years, for example, these cells are known to play a crucial role in brain injury mechanisms (Chen & Swanson, 2003), including neuronal survival (Parpura et al., 2001; Hart & Karimi-Abdolrezaee, 2021). In fact, during the glycolytic and respiratory blockade, astrocytes are capable of creating a protection mechanism by maintaining a gradient of Na^{+} for a while (Rose et al., 1998), which indicates an incredible capacity for resilience during harsh events. After all, (Angelova et al., 2015) describes astrocytes as O_2 sensors specialized to detect physiologic alterations in brain oxygenation, by stimulating respiratory neuron networks and responding accordingly to ensure appropriate blood oxygenation. These cells are also responsible for many other functions, namely metabolic, neural and vascular homeostasis (He & Sun, 2007; Popovich et al., 2009), blood-brain barrier maintenance (Popovich et al., 2009; Leis et al., 2005), synaptic connectivity, formation, and control (Verhratsky, et al., 2015; Allen & Barres, 2005; Kriegstein & Buylla, 2009),

regulation of neuronal networks (Yu et al., 2020), information (Jessen, 2004), learning and memory functions (Verkhratsky, et al., 2015), daylight rhythm regulation (Tso et al., 2017) and enduring the extracellular milieu composition by giving mechanical integrity to the brain tissue (Bushong et al., 2004). Curiously, the glial cells increasing complexity during evolution is believed to have conceded to humans' incredible capacities namely regarding cognition (Molofsky et al., 2013).

The communication provided by the astrocytes processes is a pivotal way to control synapse activity (Koehler, et al., 2006). The astrocytic processes are known to contact with thousands of synapses (Bushong et al., 2002), forming the tripartite synapse along with the presynaptic and postsynaptic structures (Araque et al., 1999). This communication between astrocytes and synapses is moderated by neurotransmitter receptors, transporters and cell-adhesion molecules of the astrocytic processes (Chung et al., 2015). At this level, the clearance of neurotransmitters may be one of the primary functions of astrocytes (Chung et al., 2015). During development, synapse formation overlaps with astrocytic differentiation and maturation, which suggests that astrocytes are a key player in synapse formation (Chung et al., 2015). Unfortunately, this was a hard subject to study for many years, since astrocytes are crucial for neuronal survival (Banker, 1980), however, thanks to further developments over the years, and the more recent usage of purified cultures of retinal ganglion cell neurons that aimed to overcome the purification challenges in these cell culture studies (Meyer-Franke et al., 1995), now we know that astrocytes can control the excitatory synapses by increasing the number of synaptic structures, improve postsynaptic activity and intensify the presynaptic function (Chung et al., 2015). Astrocytes are also known to directly control synapse elimination through their phagocytic activity, for example, by phagocytosing synapses through MEGF10 and MERTK pathways (Chung et al., 2013).

Astrocytes are also important in neuronal glutamate production by catalysing its production (Messam et al., 2002; Hertz & Zielke, 2004). They play a crucial role in the pump Na^+/K^+ homeostasis by taking up the excess of K^+ after neuronal depolarization and redistributing that excess through the gap junctions to other parts of the brain (Messam et al., 2002; Popovich et al., 2009; Leis et al., 2005; Kuffler et al., 1996). These cells also have an incredible way of modulating the synaptic function by participating in glutamate synthesis and removal (Tekki-Kessaris, 2001). Additionally, through the release of chemokines that activate other cell receptors, astrocytes can coordinate oligodendrocytes' position during development (Tsai et al., 2002) and attract microglia during inflammatory reactions (Marella & Charby, 2004). Researchers also reported an interesting increase in lactate during neuronal activation (Hu & Wilson, 1997), however, since the glucose metabolism is not balanced (Madsen et al., 1999), other researchers hypothesise that aerobic glycolysis occurs in astrocytes when exposed to glutamate, which

will increase the production of lactate and trigger the glucose utilization (Pellerin & Magistretti, 1994). This way, astrocytes seem to have an important role in this alternative neuronal energy substrate for neuronal activity fluctuations (Koehler, et al., 2006).

Moreover, the Radial Glia may be responsible for giving rise to new neurons and other glial cells and, therefore, they do play an important role in neurogenesis (Kriegstein & Bullya, 2009; Kanski et al., 2014), as such, they can be considered one of the main responsible for brain plasticity (Pittenger & Duman, 2008; Deng et al., 2010; König et al., 2016). During late embryonic development, around (E17) (Vallejo, 2009), neurogenesis is suspended and gliogenesis begins, although there is evidence of overlapping between these two processes in the DG (Bond et al., 2020). At this phase, progenitor cells pass through a stage of symmetrical divisions in which the RGL will arise, these cells will then produce postmitotic neurons and finally undergo a switch to produce either Astrocytes Progenitor Cells (APCs) or Oligodendrocyte Progenitor Cells (OPCs) (Zheng et al., 2021). Finally, astrocytes will be continuously produced until around the first post-natal week and their peak depends on the region where astrogliogenesis is occurring, for example, while in the hillus, astrocytes are predominantly formed at the beginning of the first post-natal week, the peak in the molecular layer of the DG occurs at the end of that same week (Bond et al., 2020).

Just like the neurons, new astrocytes can be born in the brain, in a process called astrogliogenesis (Parpura et al., 2001; Toda et al., 2019) **(Figure 2)**. This, although being a process still not fully understood in the adult brain, it can be found in many areas of the brain, including the hippocampal cytogenic niches subependymal zone and the Hippocampal DG (Luskin & McDermott, 1994; Holmin, et al., 1997, Yeh et al., 2022), but also in other brain regions, as for example, the Neocortex (Huang et al., 2016) and the Cerebellum (Carrato et al., 2018). In the cortex, astrocytes are primarily originated from stem cells in the ventricular zone that migrate to their final region, passing transiently through the SVZ (Messam et al., 2002). In the adult hippocampal DG, this process starts, with a multipotent stem cell, the RGL, that will give rise to a progenitor cell which will then differentiate into an astrocyte (Molofsky et al., 2013, Mori et al., 2005). According to Encinas et al., (2011), this process needs to take into account two types of cells: the Quiescent Neuronal Progenitors (QNPs) and the Amplifying Neural Progenitors (ANPs), which are found in the SGZ of the hippocampus and are the protagonist cells of the astrogliogenesis process. ANPs have a higher level of proliferation than QNPs (Encinas et al., 2011), which may lead to different roles in cytogenesis, namely because they can differentiate in new mature neurons by temporarily becoming neuroblasts in a seven-day process. Differently, the QNPs stay unmodified for those seven days, and only on the tenth day does their number decrease (Encinas et al.,

2011). In consequence, Encinas and his colleagues believe that the QNPs are newborn astrocyte progenitors, once the number of astrocytes at the end of the process equals the number of QNPs at the beginning. Summing up, over the differentiation stage of cytogenesis, some of the cells differentiate into neuroblasts and give rise to newborn neurons – neurogenesis –, while the other cells will differentiate in QNPs and give rise to newborn astrocytes – astrogliogenesis.

Regarding the post-natal astrocytic formation, (Schneider et al., 2022) discovered that astrogliogenesis mediated by radial NSCs mostly occurs at the second post-natal week – around the post-natal days 10 (Toda & Gage, 2018) and P14, when it reaches a plateau, and proceeds until P21. They also found that the different DG layers are composed of different types of astrocytes and that astrogliogenesis occurs in a pattern that starts from the Molecular Layer to the Granular Zone. As such, although the described timings do not completely follow those noted by Bond et al., 2020, both studies found an overlap between astrogliogenesis and neurogenesis (Bond et al., 2020; Schneider et al., 2022). Schneider et al. (2022) also concluded that the majority of new astrocytes formed in the DG during adulthood were formed by the locally divided astrocytes.

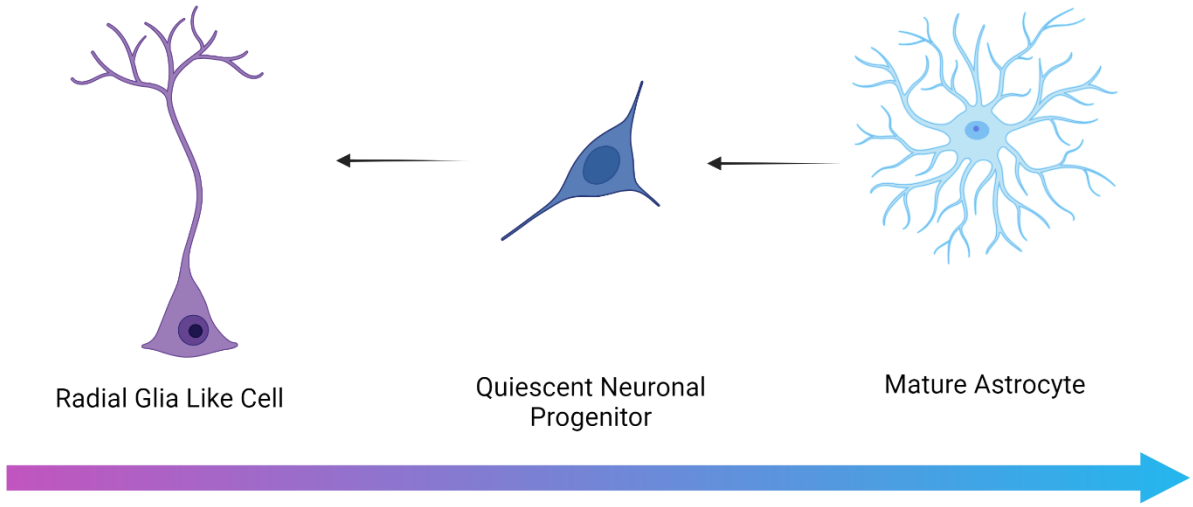


Figure 2 - Astrogliogenesis process

An activated Radial Glia Like cell will produce a Quiescent Neuronal Progenitor that will differentiate into a mature astrocyte. Figure created by Biorender.com

The different markers expressed by astrocytes allow us to distinguish these star-shaped cells from others, however, it is important to note that the specific definition of a mature astrocyte has been subject of debate. For that reason, (Kimelberg, 2010) reunited the most settled properties, mostly agreed by other authors (Parpura & Haydon, 2009; Barres, 2003; Barres, 2008), that describe what a mature astrocyte is. Those properties include some, but not only, like the incapacity of these cells for being excitable, the existence of intermediate filaments that can express GFAP and the usage of gap junctions

as a brink between astrocytes. Some studies even reported that due to a misstep during migration, mature astrocytes effectively extend their end feet to blood vessels, helping the glial scar formation (Smith & Miller, 1991).

To study astrocytes, it has become essential the knowledge regarding the specific markers expressed by the cells that are involved in each stage of the cytogenic process. Starting with KI67 and MCM2, which are cell cycle markers (Schneider et al., 2022) and 5'-bromo2'-deoxyuridine (BrdU) which can incorporate into the DNA molecule during cell division and therefore, are important to assess the new cells generated in the brain by immunostaining (Bond et al., 2020). Other similar thymidine analogues such as IdU and CldU can also be used for the same purpose (Veja & Peterson, 2005). Nestin, for instance, is presented both in RGLs and NSC (Petrova, 2012), while Sox2 is widely expressed by adult neural stem cells, being essential for cell proliferation (Ferri et al., 2004) and actively inhibits neuronal differentiation (Wegner & Stolt, 2005). Furthermore, we have the two most common astrocyte markers used to study the cellular fate and differentiation of astrocytes (Namihira & Nakashima, 2011): S100β is a marker secreted by astrocytes (van Eldik & Zimmer, 1987) and helps them to proliferate (Selinfreund et al., 1991); and GFAP which only marks astrocyte's bigger projections (Nag, 2011) and is initially expressed by type 1 cells and stops being expressed after their differentiation to type 2 cells (Deng et al., 2011) even though it is also expressed by mature astrocyte's filaments (Barres, 2003; Kimelberg, 2010; Eng et al., 1992). Other markers such as ALDH1L and ACSBG1 can also be used to mark astrocytes (Schneider et al., 2022; Cahoy et al., 2008). As such, the expression of these markers is important to evaluate whether the marked cells are progenitor cells or adult cells (Seki, 2011).

Besides the cellular markers above mentioned, TFs like the Signal Transducer and Activator of Transcription (STAT), Sox9, Nuclear Factor I A (NFIA) and High Mobility Group Nucleosome-binding (HMGN) are important elements in gene expression for astrogliogenesis induction and astrocyte differentiation (Bonni et al., 1997; Kang et al., 2012; Nagao et al., 2014). TFs like the ones mentioned are responsible for regulating astrocyte genes by interacting with epigenetic mechanisms during development (Tirari et al., 2018). Therefore, they are key players in the formation of new astrocytes, allowing precursor cells to follow their specific fates and differentiate into mature astrocytes.

1.4. The Hippocampus and the Dentate Gyrus

In this work, we will be focusing on the hippocampus, namely the DG, in which region we can find both astrogliogenesis and neurogenesis, as mentioned above (Deng et al., 2010; Eriksson et al., 1998).

The DG is the most central part of the cerebral cortex (Schultz & Engelhardt, 2014) and is divided into three layers: the molecular layer, which has few cells (Treves et al., 2008); the granule cell layer, composed of small granule cells that constitute the majority of the cells of the DG and generally, there are only few glial cells between them (Treves et al., 2008; Amaral et al., 2007); and the hilus, which is the deepest of the three layers mainly composed by mossy fibres that form synapses between this layer and the subjacent areas of the DG (Treves et al., 2008; Amaral et al., 2007).

Regarding the known functions of the hippocampus, Eichenbaum et al. (1992) stated that the hippocampus is responsible for our memory (Toda et al., 2019; Gonçalves et al., 2016; Serafini, 2012; Eichenbaum et al., 1992). However, Eichenbaum et al. (1992) also concluded that the hippocampus is just one part of a major system composed of other important structures like the DG. This small structure helps the hippocampus in its major function by encoding sensory inputs and spatial information and being involved in spatial pattern separation (Kesner, 2013; Dayer et al., 2003). Thus, the DG is an important structure to separate spatial events by using the sensory information it receives to make sensory markers separate spatial locations, allowing us for example to perceive two different locations. Pattern separation is affected by DG dysfunction (Kesner, 2007). In turn, Seress (2007) considers that the DG function is complex and admits that DG lesions can lead to a severe functional lesion of the hippocampus itself. The DG was also described to be relevant for cognitive deficits observed in some diseases like Alzheimer's disease (Dias et al., 2016) and depression (Bramham, 2007). Although the DG is a relevant structure for learning and memory, it was also shown to be important for emotional behavior, being a key structure for brain plasticity (Aniol et al., 2022).

To sum up, the hippocampus, and specifically, the DG is a crucial brain structure to study the mechanisms of cognitive and emotional behavior control. As such, it is important to understand the impact of cellular dysfunction in the DG, in the healthy brain but also in the context of pathologies.

1.5. Impact of Astrocytes on Behavioral Performance

One of the most important aspects of this thesis regards animal behavior since is one of the major forms to assess astrocytes' function after their ablation. Emotional, sensorial and motor aspects of behavior can be assessed through multiple tests during the experimental course, but also other cognitive aspects such as learning and memory (Oliveira et al., 2015). For example, as stated in the previous sections, astrocytes have important roles in cognition features such as memory and learning (Verkhratsky, et al., 2015), therefore, it seems natural to admit that dysfunctional astrocytes may alter those functions. To assess those alterations, many behavioral tests can be applied to animal models. For example, it has

been reported that D-serine has an important role in memory enhancement (Zhang et al., 2008), meanwhile, astrocytic ablation can reduce extracellular D-serine in the hippocampus, resulting in damaged object recognition memory (Robin et al., 2018). On the other hand, astrocyte impairments can lead to some emotional state alterations, such as anhedonic behavior (Banasr & Duman, 2008). Reports also show that obstruction of the glutamate uptake by astrocytes and astrocyte gap junction damage can lead to anhedonic and anxiety-like behaviors (Banasr & Duman, 2008; John et al., 2012; Sun et al., 2012). Besides, studies revealed that astrocytic activation increases ketamine efficacy and, therefore, its anti-depressant effects, decreasing anxiety-like behavior in mice (Wang et al., 2018). Animals can act in an instinctive or learned form to certain tasks or survival threats (Medlej et al., 2019) that can be altered during astrocytic manipulations.

Many behavioral tests can be performed to study the above-mentioned behavioral changes. Features such as exploratory and anxiety-like behaviors, animal preference and even learning can be assessed during behavioral testing (Medlej et al., 2019). Preference tests are commonly used and consist of allowing the animal to arbitrarily choose between two conditions (Gonyou, 1994), however, the subjectivity of the animal's emotional features is an obstacle to precisely evaluating these states (Medlej et al., 2019). As emotions can lead to learned responses, it is hard to evaluate emotion and cognition separately, which can be a relevant clinical advantage (Medlej et al., 2019). For example, this thesis will be assessing behavioral tests in rodents such as Novel Object Recognition, which measures the animal preference for a new object to study recognition memory (Halassa et al., 2009). In turn, the anxiety-like behaviors can be assessed with Elevated Plus Maze and Dark-Light Box tests (Aida et al., 2015; Medlej et al., 2019), as for anhedonia Sucrose Preference Test seems to be one of the most used tests (Markov, 2022). Of course, these are not the only available tests to follow the behavioral changes while studying astrocytes, other tests such as Novelty-Suppressed Feeding to study anxiety-like behaviors (Samuels & Hen, 2011) or Contextual Fear Conditioning for memory assessments (Rudy et al., 1004) can also be used.

To sum up, astrocytes are important players for animal behavior which gives them some clinical potential to treat some disorders (Cahoy et al., 2008), as it will be further assessed in the next section. Thanks to new advances in astrocytic functional studies, the comprehension of both their role in the brain and their importance for behavioral control (Cahoy et al., 2008) is now possible. Still, many studies are needed to further understand astrocytes and their role in the modulation of more complex behavioral features.

1.6. Astrocytes Involvement in Pathology

Many may describe pathology as an imbalance in CNS homeostasis (Verkhratsky et al., 2015; Pekny et al., 2016). Therefore, if we bear in mind that astrocytes are a key regulator of CNS homeostasis, it is plausible to envisage that a deficiency of astrocytes in the brain may lead to CNS disorders. According to Zhao and colleagues (2022), astrocytes functional impairments in pathologies such as depression, undergo changes at the level of synaptic plasticity, neuroimmune state and neuronal transmission. Besides, some scientific articles have been reviewing and reporting hippocampal volume loss and astrocytic degeneration in some diseases like Major Depressive Disorder (Zhu et al., 2010; Banasr & Duman, 2008; Peng et al., 2015). In recent years, the relevance of studying mental diseases such as depression has been increasing because of its personal and public consequences (Burcusa & Iacono, 2007) and its relevance as a factor for stroke (Pan et al., 2011).

Specifically tackling the relevance of astrocytes, they seem to be altered in some pathologies (Volterra & Meldolesi, 2005). Besides, astrocytes are known to have neuroprotective functions regarding some pathological conditions (Sidoryk-Wegrzynowicz et al., 2011), however, multiple studies have described a clear reduction in the number and density of astrocytes in *post-mortem* tissues of mood disorder patients, for example, in Major Depressive Disorder (MDD) (Cotter et al., 2002; Gittins & Harrison, 2011; Rajkowska et al., 1999) and a clear decrease in S100β and GFAP expression in the hippocampus (Gos et al., 2013; Cobb et al., 2016). The use of antidepressants can, however, reverse these changes, for example, by using fluoxetine (Rong et al., 2010). Some studies also reveal an alteration of the astrocyte's nucleus size in some brain regions of MDD patients (Rajkowska et al., 1999), but also, in their branching, in both human and animal studies (Saur et al., 2015; Torres-Platas et al., 2011). In fact, a previous study reported a decrease in the number of cells expressing GFAP after Unpredictable Chronic Mild Stress (uCMS) protocol (Machado-Santos et al., 2021), but also reported a slight increase in GFAP-expressing cells in animals previously treated with fluoxetine or imipramine (Machado-Santos et al., 2021). The same study also reported an increase in $S100\beta^{+}$ cells after subsequent exposure to uCMS protocol, which they speculated to be promoting astrogliosis (Machado-Santos et al., 2021), curiously, there were no changes in S100β expressing cells in the first stress exposure, that occurred after fluoxetine or imipramine treatment (Machado-Santos et al., 2021). Previous studies suggested that the S100β increase may lead to pro-inflammatory cytokines release and stress exposure can induce neuroinflammatory effects in glial cells (Dowlati et al., 2010; Yuekui et al., 2001).

Nevertheless, studies assessing MDD subjects reported glial cell density increase in some brain regions such as in the pyramidal cell layer of the Cornu Ammonis (CA) and the DG (Stockmeier et al.,

2004), but the opposite was also described by a different study (Cobb et al., 2013). Studies reporting a reduction in astrocyte length, branching and volume may justify hippocampal volume loss in some diseases such as MDD (Czéh et al., 2006; Rajkowska et al., 1999; Saur et al., 2015; Torres-Platas et al., 2011), besides, changes in cognition are connected with alterations in astrocyte number and morphology that can induce other functional and physiological modifications (Lima et al., 2014), in fact, their physiological and anatomical properties play a crucial role in information processing and organizational levels in the brain (Martins-Macedo et al., 2021b). Furthermore, emotional behavior changes, that are profoundly connected to the hippocampus, are also known to be altered in depression (Harrison, 2002). These findings urge the necessity to proceed with the study of astrocyte importance to further understand neurological conditions and assess new treatment approaches.

1.7. Astrocytes Impact on Plasticity

Brain plasticity is the ability of the brain to respond to injury and stimuli by changing its structure or its function (Su & Xu, 2020). For instance, astrocytes respond to CNS insults in a process called astrogliosis (Sofroniew, 2014; Pekny et al., 2007), in which they change both their gene expression and morphology (Araújo et al., 2022). In fact, they can avoid damage extension by creating the glial scar through increased axonal regeneration (Zamanian et al., 2012). Furthermore, reactive astrocytes are being related to phenotypical changes at the level of altered synapses phagocytosis, gain of neurotoxic function and myelin debris (Liddelow et al., 2017). Besides, they can secrete some proteins at the synaptic level, which implies their important function in homeostatic plasticity modulation (Shao & McCarthy, 1994). For example, the activation of the G-protein coupled receptors can promote the release of gliotransmitters, such as ATP and glutamate, in consequence of an increase of the Ca^{2+} intracellular levels (Araque et al., 2014; Perea & Araque, 2005; Bezzi & Volterra, 2001). Besides, although multiple gliotransmitters can be released by astrocytes, one single gliotransmitter can target different pathways and synchronize neuron and synapse networks (Araque et al., 2014). Regarding the challenges provoked by the distance between astrocytes and the synaptic cleft, evidence suggests that astrocytes do not need high concentrations of neurotransmitters to communicate with distant synapses (Rusakov & Kullmann, 1998; Araque et al., 2014).

Interestingly, they can also change their morphology depending on their environment, for example, during neuronal migration throughout early developmental stages, or even during brain injury (Shao & McCarthy, 1994). Moreover, astrocytes can respond to some physiological alterations, by wrapping their processes around synapses and, therefore, are able to alter the synaptic contact during

development (Allen & Eroglu, 2017; Theodosis et al., 2008). As stated earlier, changes in astrocyte morphology were observed in some diseases (Rajkowska et al., 1999), however, some of those alterations can be reverted by using antidepressants (Iwata et al., 2011). Besides, astrocytes are responsible for experience-dependent plasticity regulation (Ribot et al., 2021).

Astrocytic cells are indeed important in plasticity that once engrafted in the mouse brain, human glial cells can differentiate into astrocytes and lead to different functions (Han et al., 2013). This fact made some authors hypothesise that astrocytes have a higher level of plasticity when compared to neurons which helps them, depending on the context, to get to the final phenotype, even after differentiation and maturation (Villarreal & Vogel, 2021). Moreover, astrocytes contribute to brain plasticity in two other forms: primarily by helping the cytogenic niche cells in controlling the NSCs and secondarily by helping the NSCs differentiate into both neurons and glial cells (Schneider et al., 2019).

Interestingly, Bielefeld and colleagues (2023) showed recently that traumatic Brain Injury modifies the anatomic location of some cell populations derived from NSC in the DG and promotes neurogenesis by inhibiting astrogliogenesis, showing the high inter-relationship between both processes. Besides, there were reported changes in astrocyte morphology, proliferation and differentiation in the CNS during injury, at the lesion proximities, whereas during astrogliosis there is an increase in astrocytic proliferation, hypertrophy of their cellular processes and cell body and an improvement of GFAP synthesis in the intermediate filaments (Eng et al., 1992).

1.8. Challenges in the study of astrocytes and astrogliogenesis

At this point, we revised the state of the art about adult brain astrogliogenesis and mature astrocytes function, however, as mentioned above, there is still a lot to be studied. Therefore, it is important to understand what are the major obstacles to studying specifically astrocytes and how our team aims to overcome them. There are several ways to study cells in the brain.

Regarding the *in vivo* study of astrocytes, the fine interactions between these and other glial cells are the biggest obstacle to successfully manipulating astrocytes individually, once most of the proteins are expressed by many types of cells (Liddelow & Barres, 2017). These methods could, therefore, be substituted by *in vitro* approaches, like cell culture systems, however, these are not ideal, since they can be easily contaminated and depending on the media used, the morphology of the cells can be irreversibly changed (McCarthy & de Vellis, 1980; Foo et al., 2011). Besides, the *in vitro* models do not fully replicate the exact astrocytic features of an *in vivo* astrocyte (Liddelow & Barres, 2017), which leads to the necessity to improve *in vivo* approaches, even though relevant advances were made to avoid cell culture difficulties.

One of the major methods used to study adult cytogenesis *in vivo* is inducing the ablation of cells to assess the effects of that ablation in the brain, either to explore the effects in physiology, pathology or behavior (Deng et al., 2010). In fact, at the beginning of the study of glial cells' functions, scientists were also using non-specific methods to ablate these cells, and more recently, they started to couple "suicide" genes with specific promotors to specifically target the cells of interest (Jäkel & Dimou, 2017; Cahoy et al., 2008). For example, (Takada & Hattori, 1986) injected stereotaxically alpha-Aminoadipic Acid as a gliotoxin for astrocytes, while (Chiang et al., 1993) irradiated astrocytes to study its response to brain injury. In addition, toxic substances like ethidium bromide (EtBr) were also used to study white matter glial cell impairment (Blakemore, 1982). As these methods are not specific for astrocytic ablation (Jäkel & Dimou, 2017), they are unsuitable approaches to effectively study astrocytes and therefore, there was an increasing need to create better tools.

Besides, researchers have been exploring retroviruses' usage to ablate and mark adult-born cells, namely to assess the morphology of newborn cells (van Praag et al., 2002). Therefore, the usage of retroviruses to ablate cells in the brain may be an appellative way to effectively study astrocyte's function and morphology, without manipulating neurons at the same time, for example, by using a specific promoter that will only target astrocytes. As it will be further described, in this work, we will use a virus to specifically ablate astrocytes in the adult brain, by using an S100β promotor that may will only target astrocytes.

On the same line of thought, the same principles can be applied to study the formation of new cells in the brain. As described, neurogenesis and astrogliogenesis are not the same processes, although some of their phases may coincide. This is where the study of astrogliogenesis turns out to be demanding, as there are no reliable methods to study specifically astrogliogenesis without interfering with neurons at the same time (Molofsky & Deneen, 2015). However, it does not mean that we cannot use similar techniques and adapt them to effectively study the formation of new astrocytes in the brain.

2. Aims of this work

In this work, we used an Adeno Associated Virus (AAV) with an S100β promoter that will specifically target the existing hippocampal astrocytes in the brain of adult mice. We developed two viruses: one for astrocyte labelling with Green Fluorescent Protein (GFP) and another for astrocyte ablation using the delta Thymidine-Kinase (dTK) cassette. Finally, to successfully complete this work, two main goals were set up:

- Assess the performance of these viruses in the hippocampal DG and the impact of ablating mature hippocampal astrocytes on the niche existing cells, using immunofluorescence technique.
- Test the influence of the ablation of hippocampal DG astrocytes on the normal behavior of rodents, namely regarding emotional and cognitive behavior.

3. Materials and Methods

All animal's procedures used in this work are depicted in **Figure 3**.

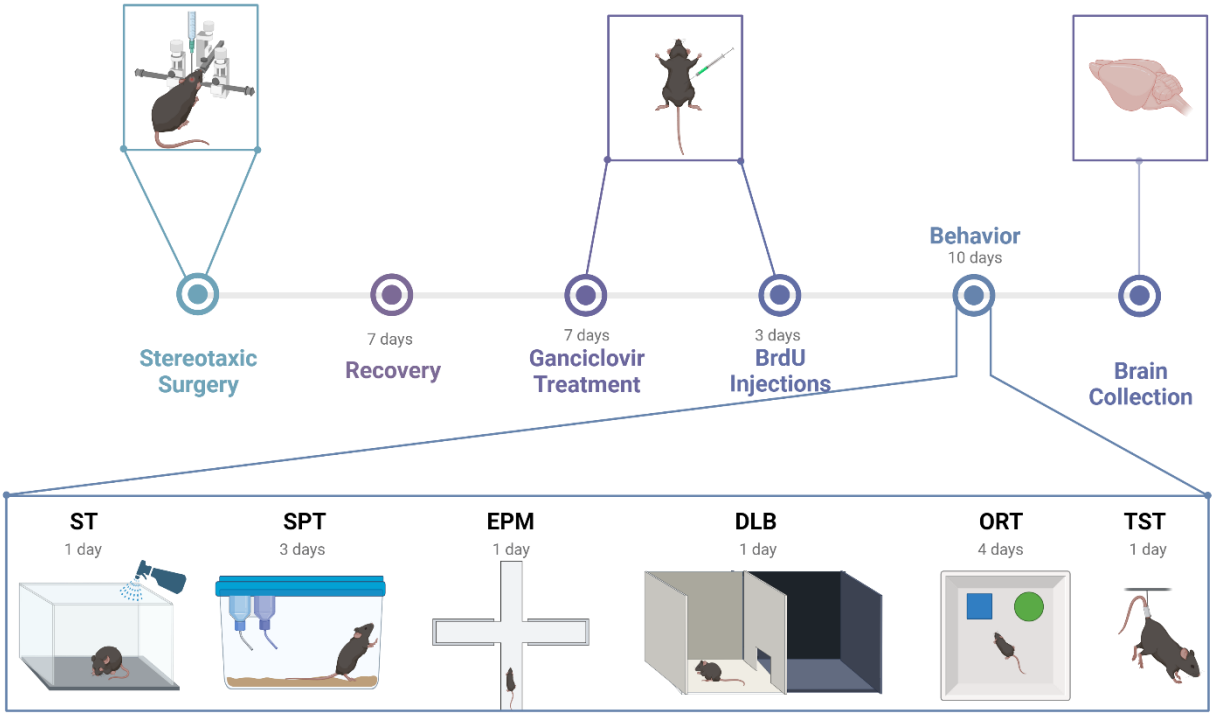


Figure 3 - Experimental timeline for animal procedures

After stereotaxic injection, animals were allowed to recover from surgery for 7 days, followed by Ganciclovir treatment (7 days) and BrdU injections (3 days). Brain collection followed behavior assessment. Created by biorender.com

3.1. Animals

8-26 weeks-old C57BL/6J Wild-Type (WT) and Nestin-Cre transgenic mice (bred at the Life and Health Sciences Research Institute) were used in this work to study the impact of mature hippocampal astrocytes on brain physiology and behavior. Animals were kept under standard experimental conditions at the School of Medicine of the University of Minho animal house ((22 ± 1) °C, 55 % relative humidity, 12 h light/dark cycle (lights from 8 A.M. to 8 P.M.)) with access to food and water *ad libitum*. Experimental procedures were performed by the European Regulation (European Union Directive 2010/63/EU) and approved by the Portuguese National Authority for Animal Experimentation, *Direção Geral de Alimentação e Veterinária* (DGAV 014085). All efforts were made to minimize both the number of animals and their suffering during experimental procedures. Weights were recorded as often as possible, to safeguard the welfare of the animals.

3.2. Virus Preparation

To study the relevance of mature hippocampal astrocytes on the rodent brain, two vectors were developed with the help of commercial Adeno Associated Virus (AAV): AAV-dTK (deltaTK suicide gene)

and AAV-GFP (Vector Builder) **(Figure 4)**, in which a minimal promoter of S100β (already validated by the team) was cloned. Thus, the AAV-S100β-GFP virus will be used to label the hippocampal astrocytes, while the AAV-S100β-dTK virus will be used to ablate the hippocampal astrocytes upon Ganciclovir (GCV)

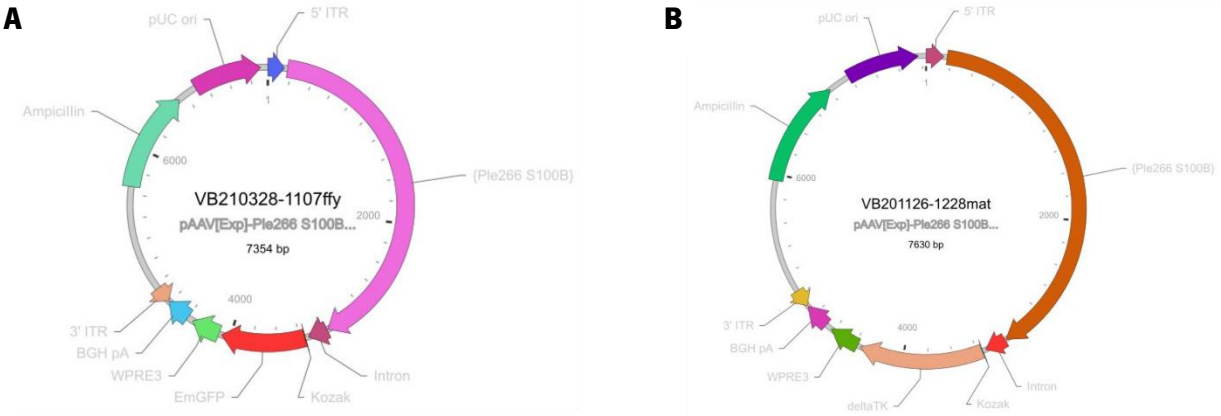


Figure 4 - Viral vectors representation

(A) represents the virus used in the control group (AAV-GFP); (B) represents the virus injected in the experimental group (AAV-dTK). Addapted from en.vectorbuilder.com

3.3. Stereotaxic Injection

Mice were deeply anaesthetized with gas anaesthesia (sevoflurane 2.5 %; 800 mL/min) and, after confirming the absence of responsiveness to the toe pinch-response method, animals were placed in the stereotaxic frame to adjust their noses in the nose clamp. When correctly positioned, the scalp was opened with a scalpel until the skull surface was visible. The viruses above mentioned were then injected (500 nL at a rate of 200 nL/min) with a needle (model 701 RN SYR, ref: 7635-01, Hamilton) attached to an automatic injector in the hippocampal DG, using the following coordinates: Medio-Lateral (M-L): -1.2 or 1.2 (taking into account that the injection was performed in both brain hemispheres), Anterior-Posterior (A-P): -2, Dorso-Ventral (D-V): -1.6. The syringe was left in place to allow viral diffusion for 5 minutes. Once the procedure was over, animals were allowed to fully recover by themselves, and Buprenorphine (50 µg/kg, Bupaq®, Richter Pharma) was administered intraperitoneally (i.p). The stereotaxic injection was previously optimized, including its volumes and rates of injection. Sutures were regularly monitored and reapplied if necessary. If a suture opened during the procedures, Bacitracin was applied as a prevention measure for possible bacterial infections, before a new suture was applied.

3.4. Ganciclovir and BrdU Preparation

Ganciclovir solution was prepared in a base solution of hydroxyethylcellulose (0.5 % in ultrapure water), at a concentration of 20 mg/kg and a dose of 2 mg/mL. Injections (i.p.) were performed for 7 days.

BrdU was prepared at a concentration of 50 mg/kg and a dose of 5 mg/mL, diluted in Saline (0.9 %). BrdU was administered (i.p.) for 3 days, starting with the day after the last Ganciclovir injection.

Both injections were performed taking into account the individual weight of each animal.

3.5. Behavioral Tests

3.5.1. Splash Test (ST)

To assess well-being behavior ST was performed. Some hours after the last GCV injection animals were separated into empty cages and their coat was sprayed with a sucrose 10 % solution. Animals were allowed to groom their coats for 5 minutes. After the trial was over, animals returned to their home cages. This test was only performed in the last set of animals.

3.5.2. Sucrose Preference Test (SPT)

To study anhedonic-like behavior we performed the SPT. Before the first Ganciclovir (GCV) administration, a first baseline for the SPT was performed to allow habituation of the mice to the sucrose 2 % solution and the test was performed after GCV and BrdU injections. Thus, mice were food and water deprived for 12 h before the beginning of the trial. Mice were then individually housed, and each cage presented one bottle of water and one of sucrose 2 % solution, and some food pellets in the bedding. All animals were weight before the baseline was performed. The test was initiated at the beginning of the animal's active period (around 8 P.M.) and the light cycles were respected during the 48 h of the test. Initially, the bottle of water was placed on the right side of the grade (as the animals are used to that arrangement) and the bottle of sucrose solution was placed on the left side of the grade. After a 24 h period, the position of the bottles was switched (bottle of water on the left side and sucrose solution bottle on the right side). Once the trial was over, the animals returned to their home cages and they were given access to food and water *ad libitum*. Both bottles were weighed before and after the trial and the sucrose preference was measured.

3.5.3. Elevated Plus Maze (EPM)

The EPM was used to study anxiety-like behavior in rodents. The apparatus consists of a plus-cross-shaped maze with two opposite open arms (50.8 cm x 10.2 cm) and two opposite closed arms (50.8 cm x 10.2 cm x 40.6 cm) linked by a central area, elevated 72.4 cm above the floor. The animals were placed in the maze in the central area and left to explore the arena for 5 min. Behavior was measured using an infrared system connected to the computer with specific software (Ethovision XT 13.0 software).

The apparatus was cleaned with ethanol 10 % between animals. Time spent in the open arms was calculated as a percentage considering each trial's full time. The same protocol was used in rats and in mice.

3.5.4. Dark/Light Box (DLB)

The Dark/Light test was also used to measure anxiety-like behavior in rodents. The test arena consists of a square-shaped polypropylene transparent box that is divided by a smaller black box of the same material. In this black chamber, one of the walls has a small opening connecting both chambers of the apparatus. The light chamber has a light source illuminating it (around 600 lux). Mice were placed in the light chamber, facing the opening between the two chambers, and allowed to freely explore both for 10 min. The latency to enter both chambers, the number of transitions, the time spent in each chamber and the ratio of time in light/dark chamber were measured. The transition of the four paws of the animal was considered as the transition between chambers.

3.5.5. Object Recognition Test (ORT)

ORT was used to measure recognition memory. Animals were habituated to the testing box for 20 min on Day 0. On day 1, animals were positioned in the same box with two indistinguishable objects in the middle of the arena and allowed to explore for 10 min (sample phase). After one hour, animals returned to the same box, with the same objects, one of them in a different position (displacement/recognition memory assessment) and were allowed to explore for 10 min. On the following day (day 2), animals returned to the arena, where one of the objects had been replaced by a different object (long-term memory assessment) and were allowed to explore for 10 min. On day 3, animals returned to the arena with two new objects, indistinguishable from each other and were allowed to freely explore for 10 min. After one hour, animals returned to the same box, with one object replaced by a novel one and were allowed to explore for 10 min (short-term memory assessment). The arena was cleaned with ethanol 10 % between each animal. Objects were as different as possible whenever they needed to be different, including shape, texture, format and height. The discrimination index and the preference for the new object were then measured. The test was performed under dim light (around 120-130 lux).

3.5.6. Tail Suspension Test (TST)

The TST was used to measure depressive-like behavior in rodents. Mice were suspended by the tail to the edge of a trolley (using adhesive tape) for 6 min. Trials were recorded and immobility time was measured. Immobility was considered as the time that animals were not moving their back paws.

3.6. Oestrus Cycle Control

The female Oestrus Cycle was monitored immediately after the Behavioral Tests days through vaginal cytology. Vaginal smears were collected from the female mice using inoculating loops gently rubbed against the vaginal wall. To help, the vaginal opening was wetted with 0.9 % saline solution. Cells were then transferred to a glass slide and let dry. Slides were post-fixed with alcohol 90 %. The analysis was performed using the bench Leica brightfield microscope.

3.7. Perfusion

Terminal anaesthesia was applied to animals by pentobarbital over-dose (intraperitoneal (i.p.); Medetomidine-ketamine mixture; 0.5 mL per mice; Eutasil; 0.9 mL per rat). Then, to clean blood from the systemic circulation, animals were transcardially perfused with 0.9 % saline solution and, to fix and preserve the cells for histology study, they were transcardially buffered with 4 % Paraformaldehyde (in Phosphate Buffered Saline 1x (PBS 1x)) (PFA). Brains were removed, post-fixed in 4 % PFA and stored in falcon tubes at 4 °C. The next day, PFA was replaced by 30 % sucrose + 0.02 % azide (PBS 1x) solution, to protect the brain cells by changing the water molecules from the tissue and avoid fungi contamination. Brains were stored at 4 °C until they could be cut.

3.8. Vibratome

After storage, the cerebellum was cut out of the brain and agarose 3 % was carefully heated in the microwave until it was translucid and free of bubbles. Agarose was carefully poured into a twenty-four-well plate to cover the bottom and the brain sample was placed in it (ventral side facing bottom) fully covered with agarose. After solidifying, the sample was removed from the plate and the agarose excess was carefully removed. The sample was then glued to a base and, once the glue was dried, the base was placed in the vibratome. After putting the cutting blade, the bath was filled with PBS 1x until it covered the cutting blade. Finally, the limits for an automated cut were established and the sample was continuously cut at 40 μm. The slices with the hippocampus were collected to a twenty-four-well plate and covered with PBS 1x + azide 0.02 %. Brain slices were stored at 4 °C.

3.9. Immunohistochemistry

Brain slices with ventral and dorsal hippocampus were selected, agarose residues were carefully removed and slices were placed in a twenty-four-well plate. Permeabilization was done under agitation at room temperature for 10 min with 0.5 % Triton in PBS (PBS-T 0.5%). Primary antibodies were prepared with PBS-T 0.5 % and 4 % normal goat serum (NGS). Brain slices were incubated overnight with the primary antibodies at 4 °C and continuous agitation. Slices were stained with anti-S100β (rabbit polyclonal, 1:200, Dako, Glostrup, Denmark), anti-NeuN (rabbit monoclonal, 1:200, Cell Signaling Technologies), anti-NeuN (mouse monoclonal, 1:200, Chemicon), anti-GFAP (rabbit polyclonal, 1:200, Dako, Glostrup, Denmark), anti-GFAP (mouse monoclonal, 1:200, Epredia), anti-BrdU (mouse monoclonal, 1:200, Palex) and anti-BrdU (rat monoclonal, 1:200, Abcam). For the immunohistochemistry using BrdU antibodies, there was an additional step after permeabilization, where slices were incubated for 30 min in HCl 1 M followed by 3 washes with PBS-T 0.5 % for 10 min each to wash excess residues of HCl.

After washing the slices 3 times for 10 min in PBS 1x, the secondary antibodies were prepared in PBS 1x (Alexa-Fluor® 594 goat anti-rabbit, 1:1000, #A11037; Alexa-Fluor® 594 goat anti-mouse, 1:1000, #A11020; Alexa-Fluor® 488 goat anti-rabbit, 1:1000, #A11034; Alexa-Fluor® 488 goat anti-mouse, 1:1000, #A11001; Alexa-Fluor® 647 donkey anti-mouse, 1:1000, #31571) and the slices were incubated for 2 h at room temperature, under agitation. Brain slices were then washed 3 times for 10 min with PBS 1x and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1:1000) at room temperature for 10 min. The slices were washed 3 times for 10 min with PBS 1x and finally mounted. The excess of PBS 1x was removed with absorbent paper and the slices were let dry. Drops of PermaFluor Aqueous Mounting Medium were carefully placed over the slices and the coverslips were immobilized at room temperature until completely dry.

Imaging was obtained by fluorescence microscopy (Olympus IX81; Olympus) and by confocal microscopy (Olympus FluoViewTM FV1000).

3.10. Data and Statistical Analyses

All statistical analyses were performed using Graph Pad Prism version 8. Data were described as mean ± standard error of the mean (SEM) and graphically represented. Normal distribution between groups was assessed with Shapiro-Wilk test. Welch t-test was performed only when normality was achieved and Mann-Whitney test was used when normality was not present. Two-way ANOVA was performed to assess repeated measurements. Statistical significance was assumed when $p<0.05$.

Behavioral data was analysed using EthoVision (Ethovision, Noldus Information Technologies, Netherlands) and Kinoscope programs.

4. Results

4.1. Virus specificity assessment

To evaluate viral specificity, we used immunofluorescence staining in the control group injected with the GFP virus. We expected that animals injected with the GFP virus express that protein in hippocampal cells expressing S100β **(Figure 5A, B and C)** while in the dTK group we expected to see some reduction in the S100β expressing cells **(Figure 5B)**. As stated in previous sections, S100β is a commonly used astrocytic marker and, therefore, we performed immunofluorescence staining with S100β primary antibody in control animals to confirm co-localization between GFP^+ cells and $S100\beta^+$ cells. Separately, we also performed immunofluorescence staining in the control group with NeuN antibody to confirm that GFP^+ are only co-localizing with hippocampal mature astrocytes and not neurons **(Figure 5D, E and F)**.

At the beginning of this project, animals were injected in only one side of the brain to assess the injection location. Although by that time the injection seemed to be in the right place, we noticed that in more recent sets there were some displacements regarding the control injection **(Figure 6A)**, which we were not able to identify in the experimental group because there was no marker that could help to identify the place of the injection **(Figure 6B)**. For that reason, on the latest set, we used DiI dye that stained red to evaluate the needle trajectory during the surgery **(Figure 7)**. Still, we watched some animals in which the local of the injection was completely outside the hippocampal DG. Regarding the unilateral injection, we carefully observed the collected hippocampal DGs and we noticed that after an 18-day period of GCV injection, there seemed to be a recovery of the ablated astrocytes.

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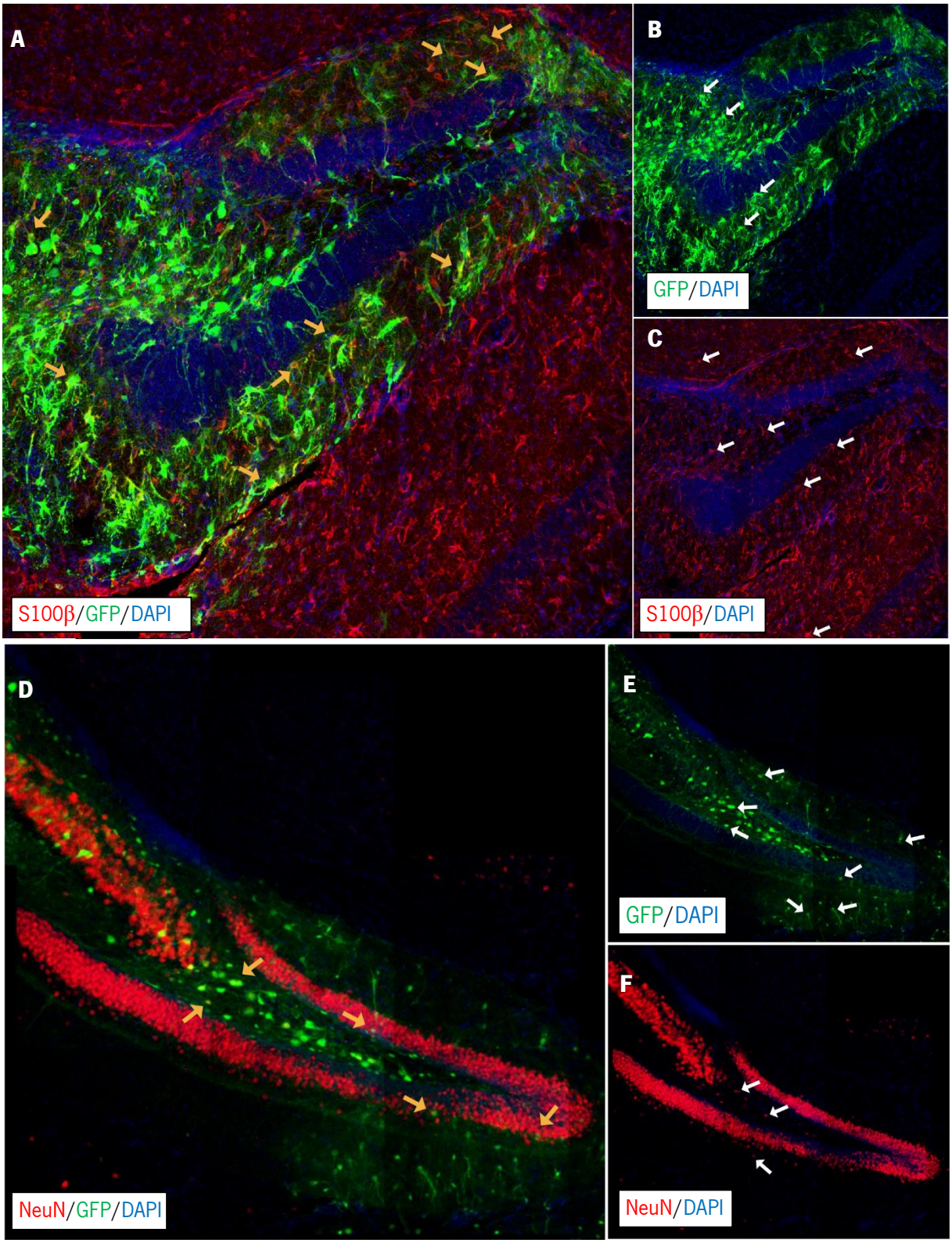


Figure 5 - NeuN and S100β staining's

Representative figure of immunofluorescence staining in a control animal for S100β (A to C) and NeuN (D to F). S100β staining (A to C): GFP (green), S100β (red), DAPI (blue). NeuN staining (D to F): GFP (green), NeuN (red), DAPI (blue). Yellow arrows indicate co-localizations between $S100\beta^{+}/GFP^{+}$ (A) or $NeuN^{+}/GFP^{+}$ cells (D). White arrows indicate $S100\beta^{+}$ cells (C) $NeuN^{+}$ cells (F) or GFP^{+} cells (E and B).

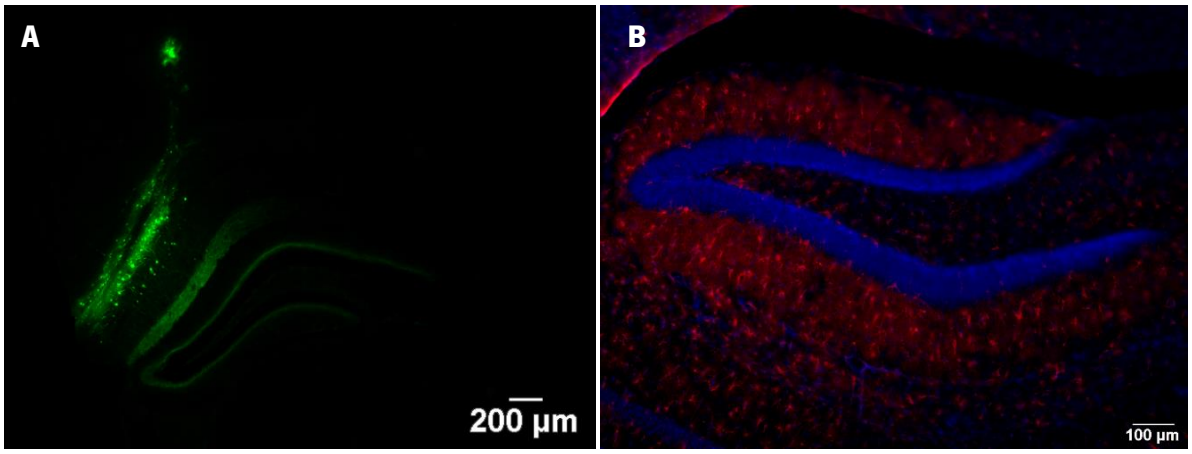


Figure 6 - GFP and S100β staining's in the control and experimental groups

(A) is a representative figure of GFP fluorescence in a control animal in which the injection site was not the correct one. Although the coordinates were the same in all the animals, some of them present the GFP completely outside the DG. Scale bar represents 200 µm (B) is a representative figure of a brain slice of a dTK animal after GCV 7-day treatment stained with S100β. Scale bar represents 100 µm.

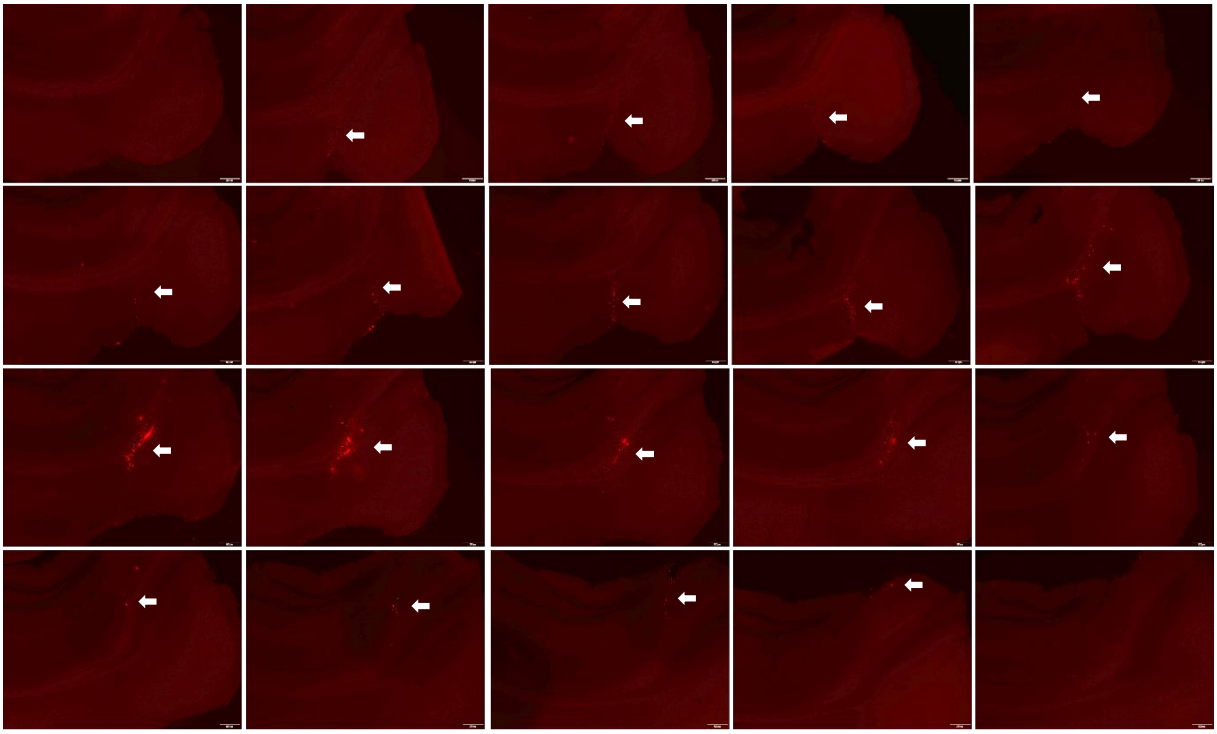


Figure 7 - Representative figure of DiI dye fluorescence in a dTK animal

The tip of the needle used was stained with the DiI dye, therefore, the DiI also represents the trajectory of the needle inside the animal brain. The white arrow points to the DiI positioning through 20 slices of brain from the same animal.

Regarding the specificity of the virus injected, the percentage of GFP⁺/S100β⁺ and GFP⁺/NeuN⁺ were taken in consideration in three Dentate Gyrus layers: Molecular Layer (ML), Hilus (H) and Granule Cell Layer (GCL) **(Figure 8)**. Although in the total DG we observed more S100β⁺ co-localizing with GFP⁺ cells **(Figure 8A)**, that difference was not enough to be statistically significant, even though there is a clear tendency (p = 0.0685). However, when we analyse each substructure individually, we could observe that in the ML **(Figure 8B)** there was a significant difference in the S100β⁺/GFP⁺ cells when compared with the NeuN⁺/GFP⁺ cells (p = 0.0050). The opposite was clearly observed in the GCL **(Figure 8C)**, in which there were significantly more NeuN⁺/GFP⁺ cells compared to S100β⁺/GFP⁺ cells (p = 0.0354). Inside the Hilus, however, we did not observe any differences regarding the percentage of S100β⁺/GFP⁺ and NeuN⁺/GFP⁺ **(Figure 8D)**.

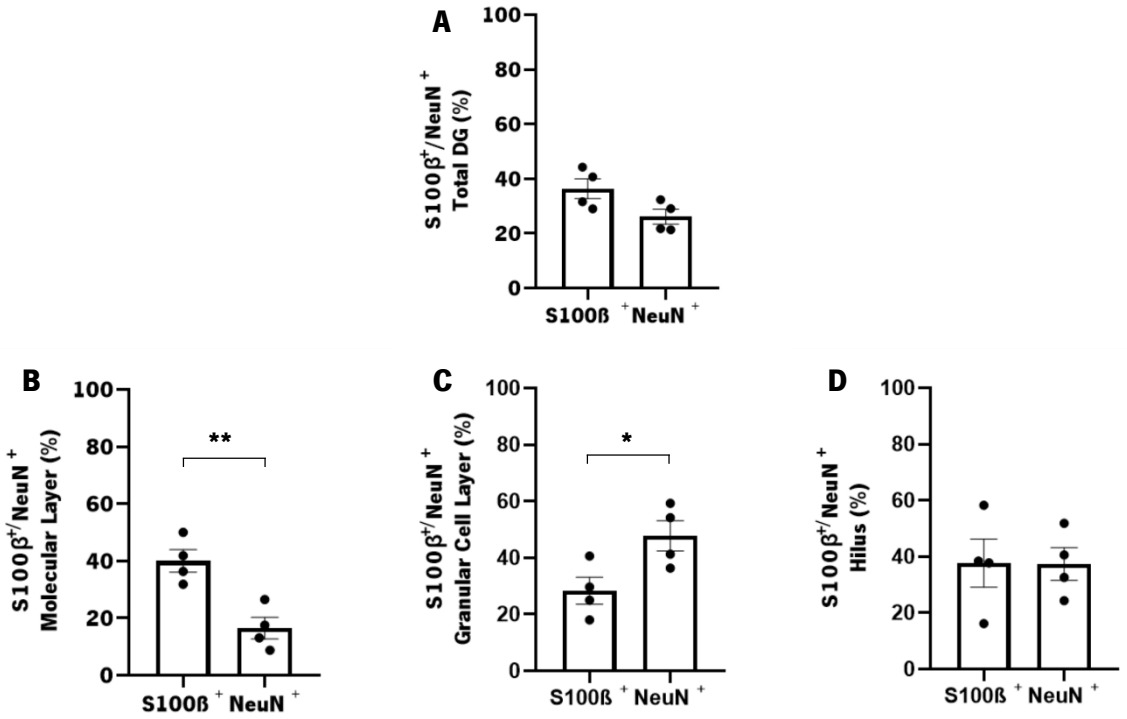


Figure 8 - Percentage of S100β⁺/GFP⁺ cells and NeuN⁺/GFP+ cells

Total of cells co-localizing simultaneously with S100β and GFP or with NeuN and GFP in the total DG (A), Molecular Layer (B), Granular Cell Layer (C) and Hilus (D). n= 4 for S100β and n=4 for NeuN. Data represented as mean ± SEM. *=p<0.05; **=p<0.01

4.2. Impact of mature astrocytes viral ablation on animal behavior

Three different animal sets were considered for the behavioral assessments. Set 0 aimed to evaluate possible differences on behavior after 7 or 18 days of GCV treatment upon astrocytic recovery suspicion and optimize some behavioral tests to be performed in future sets. In this set, we used 3 males and 3 females, in which the female group was injected with AAV-dTK virus and the male group was injected with AAV-GFP virus. Of those, two animals of each group were submitted to 7 days of Ganviclovir treatment, while the third animal was submitted to an 18-day treatment. As Set 1 was only composed of female mice, Set 2 aimed to proceed with female assessments and start male mice assessment. Both Set 1 and Set 2 results were conjugated on behavioral assessments in which the performed protocol was the same. The oestrus cycle influence in the behavioral results are represented in Annex A.

4.2.1. Astrocytes ablation influence in animal welfare and self-care

Animal welfare was monitored during the experimentation course. Animal weight was monitored every week from the surgery day (Week 0) until the sacrifice day (Week 3), completing an overall 3-week period **(Figure 9)**. Overall, all the animals did not present high variances in their weights, having recovered well from the surgery. There were no differences observed between the two groups weights in either males of females.

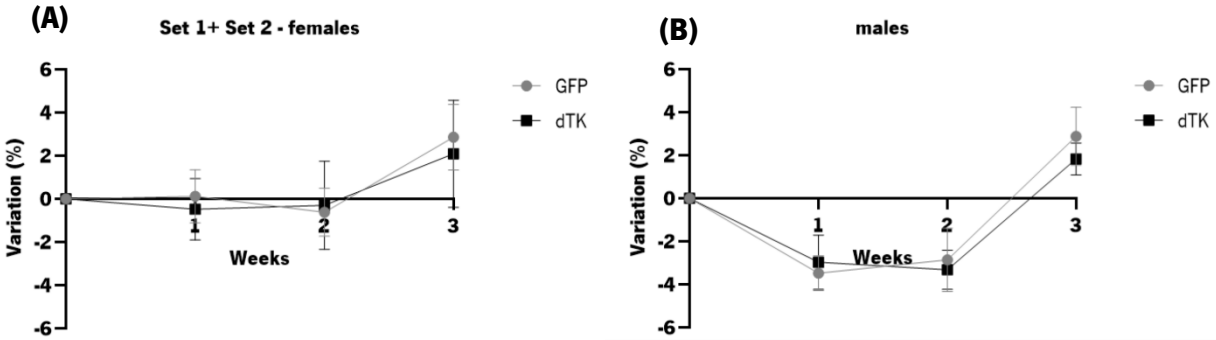


Figure 9 - Weight monitoring through the procedures

Weight variation from week 0 (surgery day) until sacrifice day (week 3) (represented in percentage). Values are represented as the mean of the weight of each animal per week compared to week 0. Both Set 1 and Set 2 females (A) weights were joined, males' weights are depicted on B. n= 15 (GFP females), 9 (dTK females), 6 (GFP males), 8 (dTK males).

The Splash Test was performed on Set 2 to assess animal self-care differences between groups and analyse sex differences. Males and females' grooming time was shown to be statistically significantly different between groups, where GFP animals spent more time grooming after splashing a sucrose

solution 10 % on their coats, compared to dTK animals (p= 0.0252 (females)/p= 0.0425 (males); **Figure 10A and B**).

When assessing the latency for grooming **(Figure 10C and D)**, not only the female experimental group presents a higher latency comparing to control group, but also, we see the opposite regarding the male groups. Even so, there is no statistically significant differences between groups.

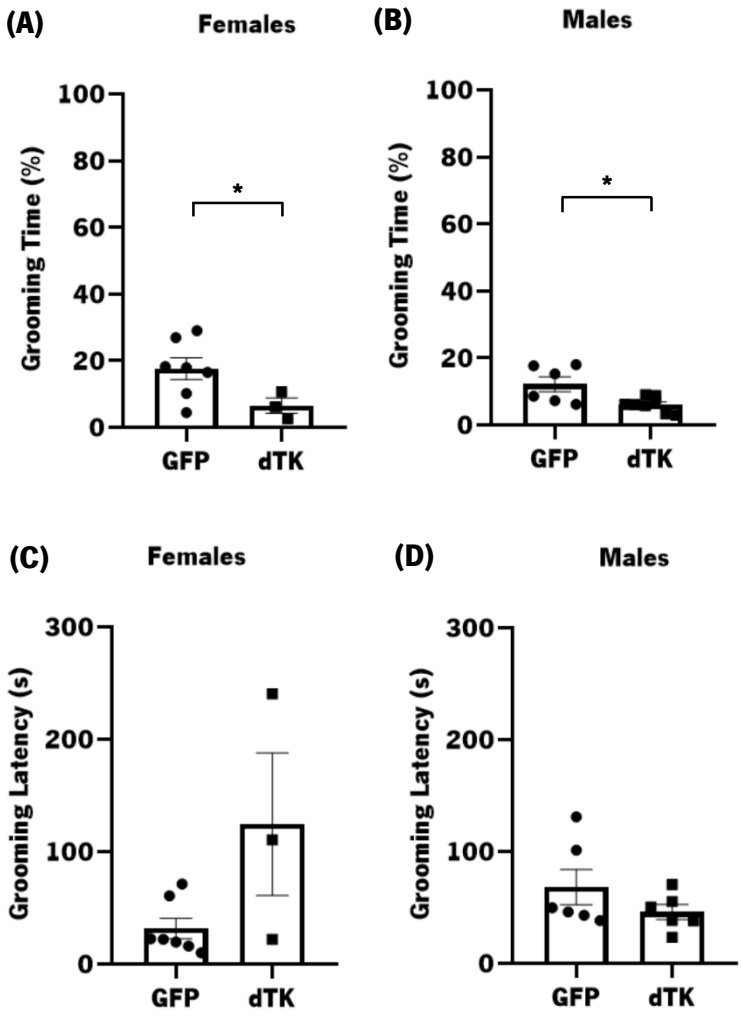


Figure 10 - Evaluation of animal self-care behavior through splash-test

Splash-test was only performed on Set 2. Grooming time (represented as percentage) (A and B) and Grooming latency (represented in seconds) (C and D) measures used to assess self-care behavioral alterations upon viral injection. *=p<0.05

4.2.2. Astrocytes ablation influence in anhedonic-like behavior

To assess anhedonic-like behavior the SPT test was performed in all mice. However, there was a need for altering the protocol, in which Set 2 was not submitted to food nor water deprivation in the previous 12 hours as described above. Taking into account the aims mentioned for Set 0 **(Figure 11A and B)**, the animals were not submitted to the baseline test. As shown by **Figure 11A**, females with 7

days treatment of GCV had a major preference for sucrose, while in the males we observed the opposite **(Figure 11B)**. However, and as the number of animals was too small, we were not able to conduct statistical analysis and, therefore, we could not assess possible significances between groups, what urged the need to develop a new female set only with 7 days treatment of GCV to confirm these results.

Considering sucrose preference in both sets baseline **(Figure 11C and E)**, curiously, only females on set 2 got a significant difference between both control and test groups (p= 0.0478) **(Figure 11D)**. However, regarding the SPT test **(Figure 11F and H)**, neither sets had significant differences even though it is clear that all animals preferred consuming sucrose over water **(Figure 11F and H)**.

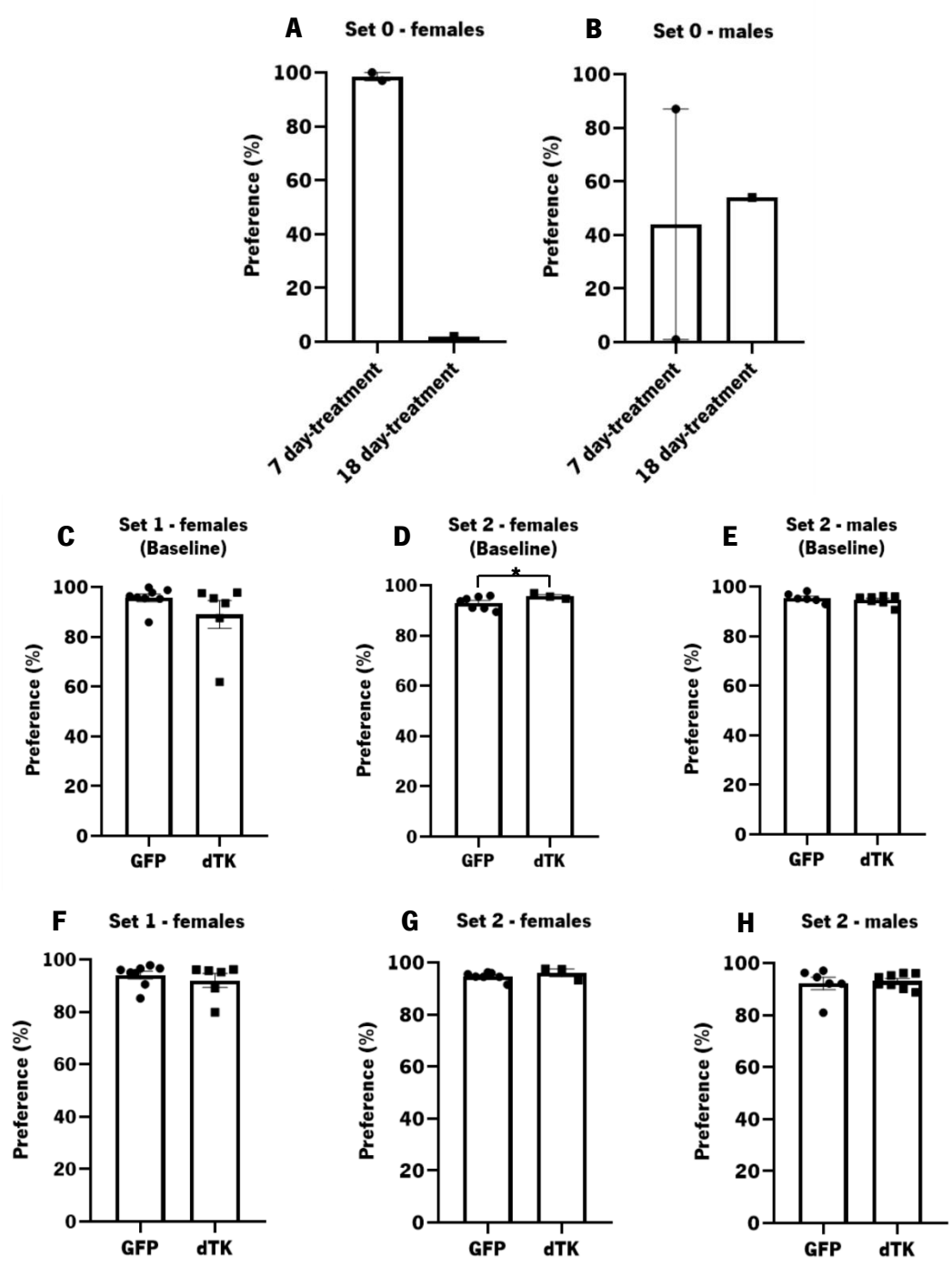


Figure 11 - Evaluation of anhedonic-like behavior through the SPT test

Sucrose consumption (represented as percentage) was used to assess anhedonic behavioral alterations upon viral injection. Set 0 (A and B) aimed to assess possible differences upon 7 or 18-days of GCV injections for viral activation. Baseline trial was only performed on Set 1 and Set 2 (C, D and F). SPT test preference is displayed for Set 1 (F) and Set 2 (G and H). n = 3 (GFP) and 3 (dTK) on Set 0; n = 8 (GFP) and 6 (dTK) on Set 1; n = 7 (GFP) and 3 (dTK) on Set 2. *=p<0.05

4.2.3. Astrocytes ablation influence on anxiety-like behavior

Two behavioral tests were performed to assess possible alterations in anxiety-like behaviors upon viral injection. The first one, that was performed in all sets, is the EPM **(Figure 12)**. The second, that was only performed on Set 1 and 2, is the Dark-Light Box **(Figure 13)**.

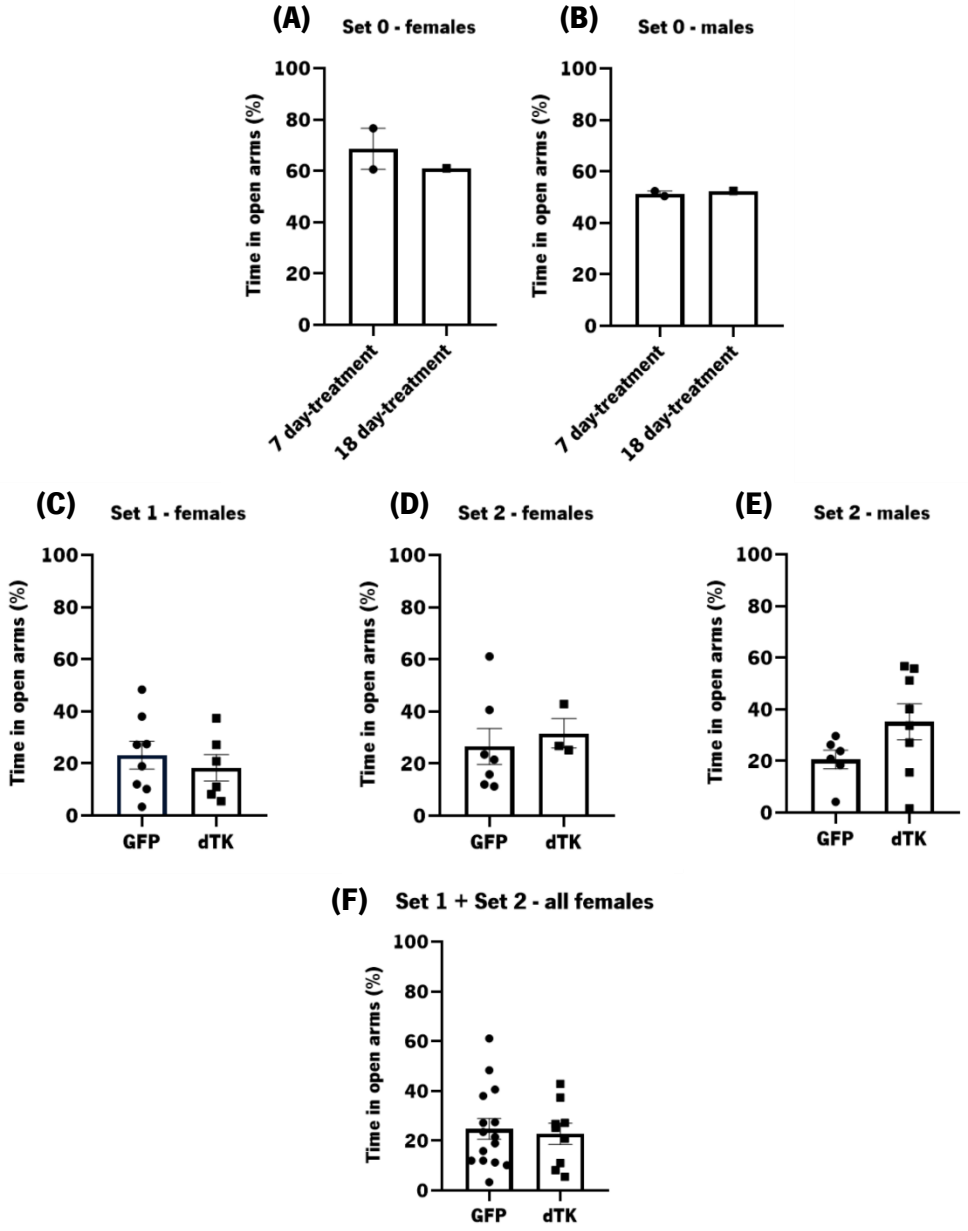


Figure 12 - Evaluation of anxiety-like behavior through the EPM test

Time spent in open arms (represented as percentage) used to assess anxiety-like behavioral alterations upon viral injection. Set 0 (A and B) was used to assess possible behavioral differences after 7 and 18-days of GCV treatment. Set 1 females are depicted on C, Set 2 is depicted on D and E. As the protocol was the same between Set 1 and Set 2, the percentage of all females is depicted in the same graphic (F). n = 3 (GFP) and 3 (dTK) on Set 0; n = 8 (GFP) and 6 (dTK) on Set 1; n = 7 (GFP) and 3 (dTK) on Set 2.

Regarding the EPM test, **Figure 12A**, shows that females treated with GCV for a 7 day-period spent more time in open arms that the animal treated for 18 days, while in the males the time spent in open arms is approximately similar, however, the number of animals was again too small for statistical comparisons. Regarding set 1, the control group spent more time in open arms comparing to the experimental group, however, we did not see that tendency in the set 2 of females. Since the protocol performed between set 1 and set 2 was the same, we were able to join both sets **(Figure 12F)**, and no significant differences between groups were observed. Regarding the males, the experimental group spent more time in the open arms in comparison to the control group, which is the opposite observed in the females. Still, there was no significant difference between groups (p= 0.0918), even though the results show a clear tendency.

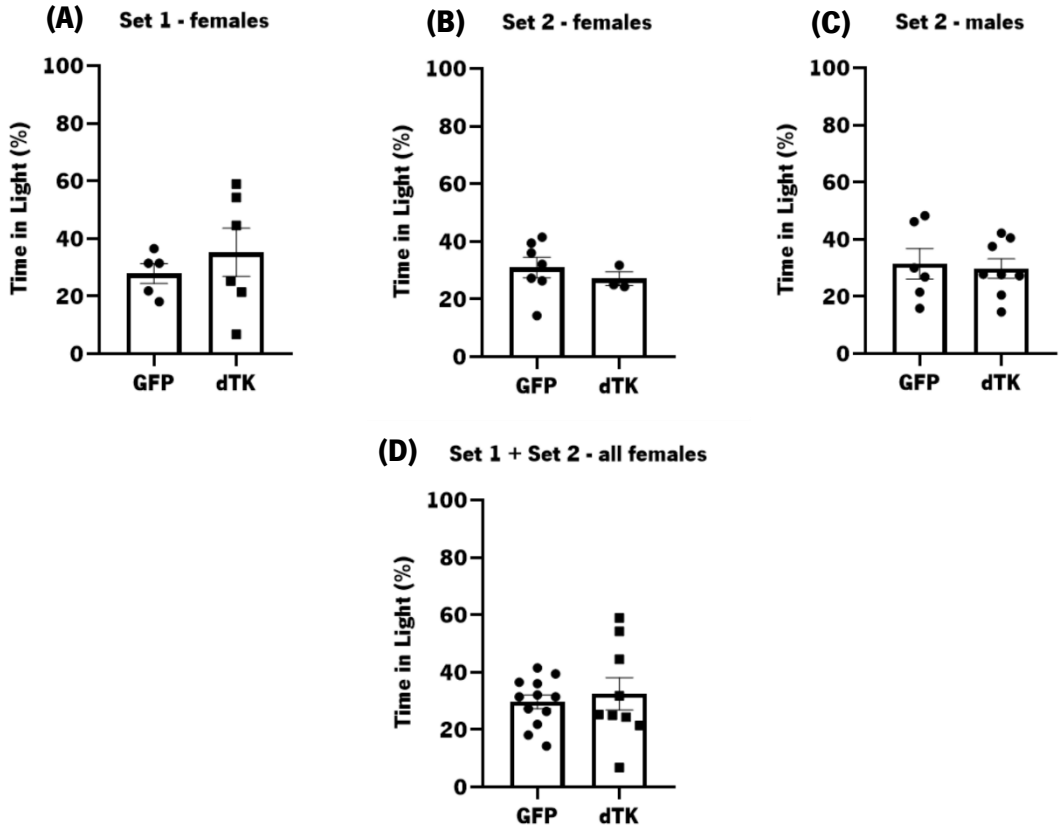


Figure 13 - Evaluation of anxiety-like behavior through the Dark/Light box test

Time spent in light chamber (represented as percentage) used to assess anxiety-like behavioral alterations upon viral injection. This test was only performed on Set 1 (A) and Set 2 (B and C). As the protocol was the same in both sets, we were able to join all female percentages in the same graphic (D). n = 8 (GFP) and 6 (dTK) on Set 1; n = 7 (GFP) and 3 (dTK) on Set 2.

Concerning the Dark/Light box test, we observed that the experimental group of Set 1 spent more time in the light, when compared to the control group **(Figure 13A)**, while on Set 2, both in males and in females, the control group spent similar time in light when compared to the experimental group **(Figure 13B and C)**. When combined Set 1 and Set 2 results, the experimental group shows similar time spent in the light chamber, compared to the control group.

4.2.4. Astrocytes ablation influence on memory and cognition

The influence of astrocytic ablation on memory and cognition was evaluated through the ORT in all sets. However, as the objects used were different in every set, we are not able to join the information and, therefore, the results must be considered individually **(Figure 14)**. Regarding Set0, as it was a small Set used to evaluate and optimize the behavioral protocols, only two tasks were performed, following the sequence bellow:

-Task 1 – Long-term memory assessment

-Task 2 – Displacement assessment

On Set 1, animals were submitted to the following sequence:

-Task 1 – Displacement assessment

-Task 2 – Long-term memory assessment

-Task 3 – Short-term memory assessment

However, as a different set (used for another part of the project) showed promising results when the chronology of the tasks was changed (data not shown), we decided to use the same protocol on Set2, with the following sequence:

-Task 1 – Short-term memory assessment

-Task 2 – Displacement assessment

-Task 3 – Long-term memory assessment

The changing of the position of the object in the displacement assessment was different in the three sets (as shown in the Annex B). This alteration was made in hope to achieve better results in the displacement task.

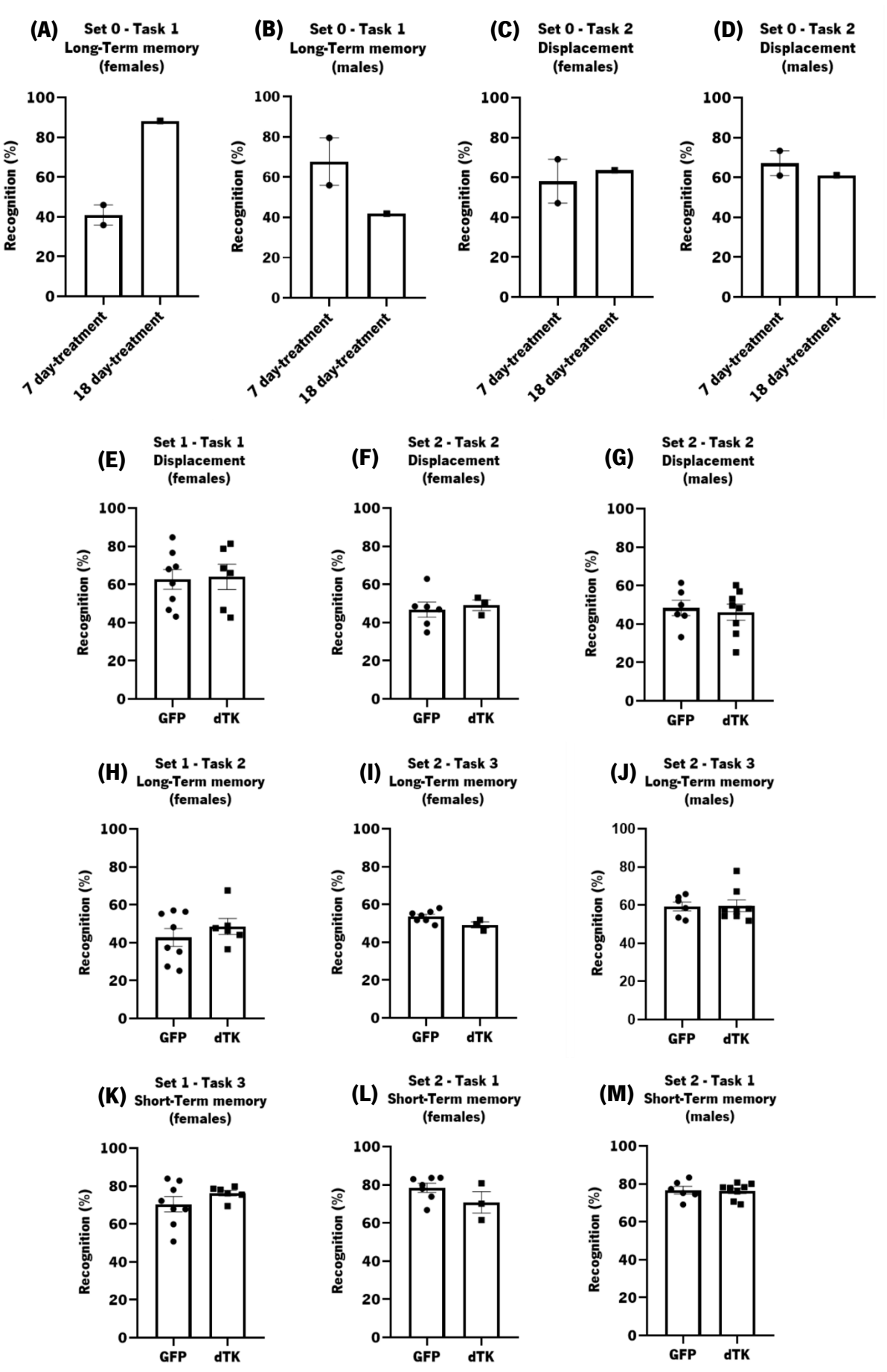

(A)
Set 0 - Task 1
Long-Term memory
(females)
(B)
Set 0 - Task 1
Long-Term memory
(males)
(C)
Set 0 - Task 2
Displacement
(females)
(D)
Set 0 - Task 2
Displacement
(males)
Recognition (%)
100
80
60
40
20
0
7 day-treatment
18 day-treatment
(E)
Set 1 - Task 1
Displacement
(females)
(F)
Set 2 - Task 2
Displacement
(females)
(G)
Set 2 - Task 2
Displacement
(males)
GFP
dTK
(H)
Set 1 - Task 2
Long-Term memory
(females)
(I)
Set 2 - Task 3
Long-Term memory
(females)
(J)
Set 2 - Task 3
Long-Term memory
(males)
(K)
Set 1 - Task 3
Short-Term memory
(females)
(L)
Set 2 - Task 1
Short-Term memory
(females)
(M)
Set 2 - Task 1
Short-Term memory
(males)

Figure 14 - Evaluation of cognitive behavior through the NOR test

Object recognition (represented as percentage) used to assess cognitive behavioral alterations upon viral injection, according to task. Set 0 (A-D) aimed to assess possible alterations on animal behavior after 7 or 18-days of GCV injections for viral activation. Only Long-term memory and Displacement were analysed in this Set. Set 1 (E, H and K) evaluated the same tasks and additionally analysed the Short-Term memory of the animals. Set 2 (F, G, I, J, L and M) aimed to test a new protocol in which the order of the tasks was changed. n = 3 (GFP) and 3 (dTK) on Set 0; n = 8 (GFP) and 6 (dTK) on Set 1; n = 7 (GFP) and 3 (dTK) on Set 2.

4.2.4.1. Memory and Cognition assessment: Set0

As shown by **Figure 14A-D**, males had better recognition percentages both regarding the new object and the new object position after a 7-day treatment, while females got the opposite results in both tasks. Again, as the number of animals was small, there was an urge to increase the number of animals to be possible to take some significant conclusions.

4.2.4.2. Memory and Cognition assessment: Displacement

4.2.4.2.1. Set 1

Regarding the displacement assessment, **Figure 14E** shows that, both the control and the experimental group recognized the change in the object position inside the box, showing similar recognition percentages.

4.2.4.2.2. Set 2

In the displacement assessment, **Figure 14F and G** shows that both the female and the male groups of Set 2 presented recognition percentages below 50 %, meaning that the animals of both groups were not able to recognize the new position of the object in the arena.

4.2.4.3. Memory and Cognition assessment: Long-Term memory

4.2.4.3.1. Set 1

As to the second task performed by Set 1 animals, **Figure 14H** shows that the experimental group had slightly higher values than those of the control group. However, as the percentage of recognition was below 50 % even to the control animals, it means that the animals were notable to recognize the new object, thus not allowing to drive any significant conclusion from this task.

4.2.4.3.2 Set 2

Regarding the long-term memory assessment of Set2, **Figure 14I** shows that males did not recognize the new object after a period of 24h, while **Figure 14J** shows that males recognized the new object. Even so, we can see that the females experimental group has lower values, comparing to the control group, while both male groups have similar values of recognition. Although the difference seems considerable, it is not significant (p= 0.0863).

4.2.4.4. Memory and Cognition assessment: Short-term memory

4.2.4.4.1. Set 1

Regarding the short-term memory assessment, **Figure 14K** shows that both groups not only recognized the new object, but also the dTK group presented slightly higher recognition percentages, compared to the control group.

4.2.4.4.2. Set 2

On the Short-term memory assessment **(Figure 14L and M)**, both males and females recognized that there was a different object inside the box arena after a short delay between the habituation and the replacement of the new object. However, the female experimental group presented lower values, when compared to the control group, although it is not statistically significant.

4.2.4.5. Memory and Cognition assessment: final remarks

Overall, the changes in the protocol did not seem to be enough to successfully achieve results. In the Displacement assessment, the animals seemed to recognize the change in the position only on Set1, however, in the Long-term assessment, both males and females on Set 2 seem to have a higher recognition for the new objects. Either way, none of the differences between groups in both sets were statistically significant. Regarding Short-term memory analysis, although we had better results in what concerns the recognition percentage, neither set seem to have a significant difference among groups in this test.

4.2.5. Hippocampal astrocytes ablation influence on behavioral despair

The TST was performed in order to assess the animal's behavioral despair **(Figure 15)**. Results showed that dTK animals of the set 1 and of the set 2 of females presented similar immobilization time in the TST when comparing to the control animals **(Figure 15A and B)**. However, on Set 2, dTK male animals **(Figure 15C)** presented a significantly higher immobility time when compared to the males

control group (p= 0.0327). There was no significant difference between both female groups on immobility time, neither when combining both sets. As the protocol for all sets was the same, data was combined.

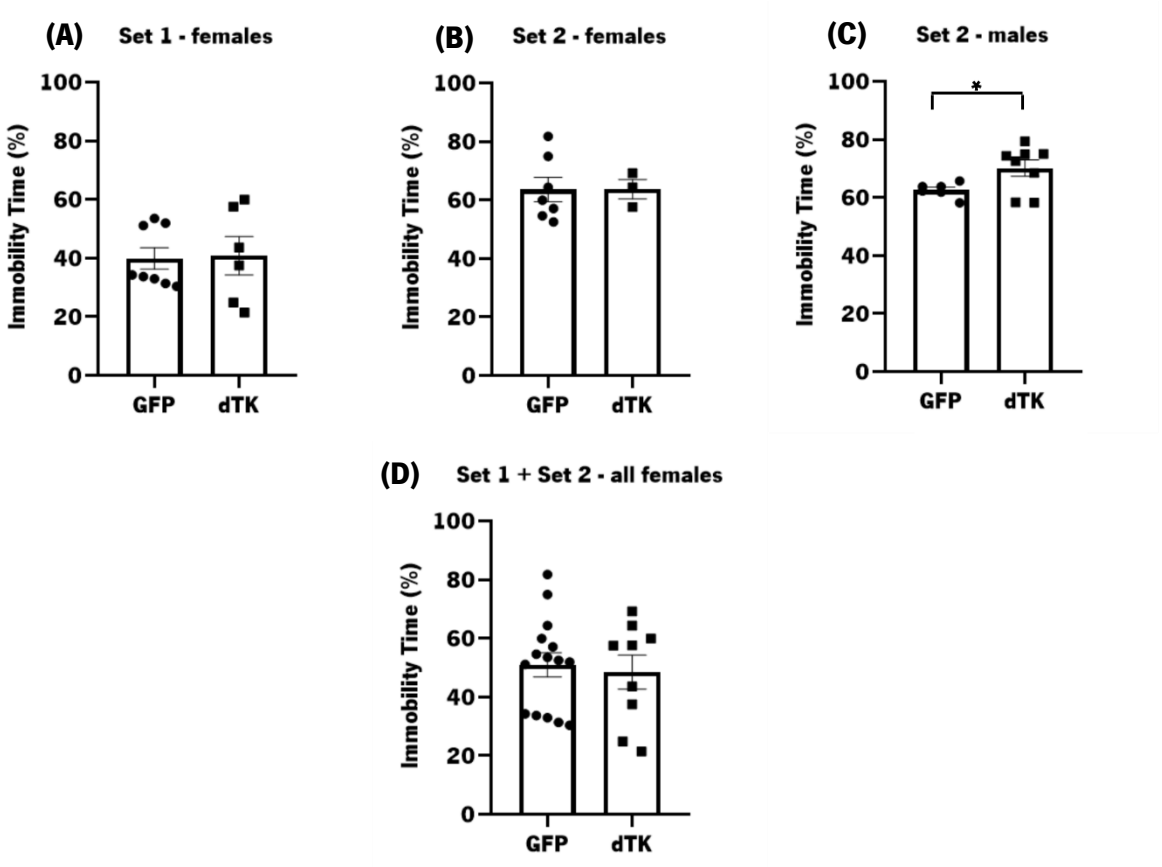


Figure 15 - Evaluation of behavioral despair through the TST test

Immobility time (represented as percentage) used to assess behavioral despair alterations upon viral injection. Neither female groups from Set 1 (A) nor from Set 2 (B) presented any significant differences. Males from Set 2, however, got significant differences in immobility time (C). As the protocol followed in both sets was the same, all female's percentages were put together in one graph (D). *=p<0.05

5. Discussion

5.1. On the virus specificity

At the beginning of the viral assessments, we began to study the specificity of our viruses only in one side of the brain, specifically in the left hippocampus. In the beginning of our experiments, the protocol we were following implied an 18-day treatment with GCV for viral infection. However, as we already know, astrocytes take a 15-day period to self-regenerate (Patrício et al., 2015). In fact, when we performed immunohistochemical analyses, we didn't observe much astrocytic reduction, which led us to define two other approaches: reduce the GCV treatment for 7 days, and additionally evaluate cell death with staining for caspase-3.

The GCV treatment is used as an anti-viral drug that will be phosphorylated by the dTK cells unto a monophosphate analog that will then be converted by the cellular enzymes enabling its toxicity by incorporating the converted GCV into the DNA strands (Alastair & Wook, 2014; Cheng et al., 1983). Therefore, we hoped that the reduction on the number of days of the GCV treatment would be enough to promote cell death of S100β$^+$ cells and observe its behavioral impact. For that reason, we decided to firstly change the protocol and evaluate whether or not we saw differences regarding the behavior when animals were injected for 7 or 18 days, which will be discussed in the next section. For the caspase-3 assessment, we were not able to fully assess the cell death due to setbacks regarding the staining procedure. Therefore, one of the future steps to be taken is to fully optimize the immunohistochemistry protocol to reduce those setbacks. Either way, although the number of animals used in both treatments was extremely small, we decided to go through the new protocol of 7 days of GCV treatment with more animals to fully assess the results.

Furthermore, we observed some misplacement in the viral location in some animals that could be provoked by a wrong animal' position in the stereotaxic apparatus or due to the sensibility of the needle and the interconnection between every hippocampal substructure which can lead to a misdirection of the tip of the needle that will end up spreading the virus above the DG and alongside the hippocampal structure, instead of penetrating the DG structure and infect its substructures. Besides, it is also possible that the slides chosen in the immunohistochemical assessment were extremely dorsal or extremely ventral and, as such, there is a possibility that we are not exactly observing the correct place of the injection for not being observing the right hippocampal slices. Therefore, as we may not have the virus correctly injected in the DG of some animals, we need to look carefully to the results of the behavioral assessment. This can only be corrected by more careful verification of the virus location in each animal, which it was not possible to perform so far due to lack of time, but it is planned to be done in the future.

As such, further immunohistochemistry analysis should be taking into consideration a previous selection of the slices that have the injection in the right place, once in the control group we can assess the local of the injection by using the natural fluorescence of the GFP. For the experimental group, the usage of the DiI dye seems to be helping on the assessment of the local of the injection by its autofluorescence. Moreover, we also need to evaluate the astrocytic reduction in the hippocampal DG of our experimental group through immunostaining procedure. In what concerns the BrdU treatment, we were also not able to fully evaluate the number of $BrdU^+$ cells, due to problems with the staining. Therefore, further staining's are needed as well as to optimize the protocol for immunostaining.

On the viral specificity for astrocytes, previous studies showed that S100β – which is the promotor that we used for our virus – is not only expressed by astrocytes, but also some other cell populations in a spatiotemporal manner, for example, in the forebrain (Vives et al., 2003), even though it is highly specific for astrocytes in the hippocampus (Ogata & Kosaka, 2002). In fact, not all the GFP^+ cells that we observed showed a clear astrocytic morphology and some co-localization was observed between those cells and the neuronal marker NeuN, which suggests that our virus is not totally specific for astrocytes. Therefore, on a future perspective to further assess the virus specificity and the types of cells that it affects, more quantification of double immunostaining's will be needed to evaluate how many cells are simultaneously GFP^+ and $NeuN^+$ and/or how many cells are simultaneously GFP^+, $S100\beta^+$ and/or $GFAP^+$. That way, we will be able to assess the type of cells we are affecting and their proportion.

In fact, as observed, the overall number of $S100\beta^+$ that is co-localizing with GFP^+ cells in the DG is relatively small (around 36%) but the percentage of $NeuN^+$ cells that are co-localizing with the GFP^+ cells is also low (around 26%). The fact that our virus is working under a S100β promotor should be leading to higher levels of co-localization in the S100β stainings, meaning that the GFP^+ cells that are not co-localizing neither with NeuN nor S100β may be, in fact, other types of cells. Therefore, there is some possibility that the promotor is allowing our virus to infect other types of cells such as oligodendroglial precursors (Vives et al., 2003), which will also need to be assessed through the usage of other cell markers, such as Olig2. The usage of other neuronal markers such as DCX may also be interesting to ensure the lack of influence of our virus upon other neuronal populations (Rao & Shetty, 2004), such as the GFAP assessments, since it had been described a decrease of the GFAP marker in various areas of the brain in some animal models of depression (Machado-Santos et al., 2021, Gosselin et al., 2009) and its analysis may be interesting, taking into account the results observed in the next section.

In a deeper analysis, when observing the amount of $S100\beta^+$ cells co-localizing with the GFP^+ cells, we were able to observe a statistical difference in the ML. In fact, we observed more $S100\beta^+$ cells co-

localizing with GFP+ cells than NeuN+ cells co-localizing with GFP+ cells. However, the opposite was observed in the GCL. As depicted in Figure 4, NeuN stain is condensed in the GCL, which makes it difficult to observe a GFP+ cell that is not NeuN+ cell. We can also observe, in the same figure, that there is not much staining for S100β in that layer, which can be the reason why we observe much more GFP+ cells co-localizing with NeuN in the GCL. Furthermore, not all GFP+ cells with astrocyte morphology co-localized with S100β, which does not mean that these cells are not, in fact, astrocytes. Therefore, it will be necessary to explore other astrocytic markers such as GFAP, in order to better confirm the total number of GFP+ cells that are astrocytes.

We also observed a high variance on the overall number of cells counted even in the same animal, namely on the GFP+ cells. As said above, we cut brain slices at a size of 40 μm, which implies that the number of cells in one slice can be different in any other slice, since we may be cutting in the spaces in between the cells. For that reason, we counted the GFP+ cells that were simultaneously S100β+ and normalized that number for the total amount of GFP+ in order to reduce that variance and have more reliable results. Besides, as the slices from the same animal were used for more than one staining, the slices of interest are rapidly exhausted and sometimes the staining procedure does not go as planned. Of course that's up to the user to be careful with the staining's, however, there is always the need to use as much sample in the most optimize way as possible. For example, by using double staining's in the same animal slices, so that way we can assess more than one thing at the same time, while we are increasing our information with the same amount of sample. For instance, although the number of animals and the number of hippocampus used for is work is relatively small (4 animals and 3 to 4 hippocampus per animal), we still got future opportunities to increase this number and have a more accurate number of cells to completely assess the virus specificity.

The difficulty in study astrocytes is clear and the lack of reliability in the available methods turns it all much harder. After all, some astrocyte populations may not have been affected by our virus, which could lead to different expressed characteristics that would otherwise be visible if we were able to target all astrocyte populations (Karpf et al., 2022). As previously described, the number of hippocampal astrocytes can be correlated with the expression of depressive-like behaviors (Machado-Santos et al., 2021), therefore, it became imperative to evaluate possible changes in animal behavior caused by our virus. Either ways, the targeting of mature astrocytes is not totally impossible, since it has been described that markers such as GLAST is only expressed in astroglia (Karpf et al., 2022), turning this into a great opportunity to more specifically target hippocampal mature astrocytes. However, it is mostly expressed in areas such as the ML, but not the Hilus, therefore, there is a need to combine this marker with others

such as ATP1B2, since it is also a specific marker for mature astrocytes, but also it is present in the Hilus (Karpf et al., 2022).

To sum up, our results on the viral specificity show that although not being totally specific for S100β$^+$ cells, we were able to develop a viral tool capable of abolishing hippocampal astrocytes, which allows us to add knowledge to what is already known about these cells. Future developments of the project will further help to understand other aspects of mature hippocampal astrocytes so that definitive conclusions can be drawn regarding the function of these cells.

5.2. On the behavioral analysis

Although in previous sections we observed that our virus is not only affecting astrocytes, we cannot fully conclude that our behavioral results are not relevant. On the contrary, as we continue to affect some astrocytes, they can still contribute to increasing the understanding of these cells and help, in future stages, to improve our approaches to better study and understand the importance of hippocampal astrocytes in animal behavior and brain physiology.

We started to approach behavioral changes with one simple set in which we assessed possible differences between the behavioral performance after 7 and 18-days of GCV treatment in both male and female mice. However, due to the reduced number of animals used, we were not able to analyse possible statistical differences and, therefore, we decided to perform one bigger set of female mice to test the 7-day protocol. Although there were no significant conclusions, the results were promising and, therefore, we developed a new set with both males and females to be able to assess not only possible sex differences, but also to assess possible behavioral changes on male mice that were not shown by the reduced number of animals used on Set 0.

In what regards the animal welfare during the experimental procedures, all the animals recovered well from the surgery day and no major weight differences were noticed. The differences in body weight between groups were not significant, which not only assures their welfare, but also shows that the GCV injections are not significantly affecting their weights. The viral injection and cellular ablation also does not seem to have a significant impact on their weight.

Concerning the behavioral analysis of the most recent sets (set 1 and 2), our results show that the experimental group has lower grooming time than the control group both in males and females, indicating a clear decrease in self-care behavior in these animals. Interestingly, dTK males presented higher immobility time in the TST test, indicating higher levels of behavioral despair than in the control group, which was not observed in the female group. Surprisingly, we also observed that females on Set

2 had higher preference for sucrose over water consumption in the SPT test, in the baseline (before the GCV treatment). However, the difference in the sucrose preference between the two groups is very small and we do not consider the result biologically significative.

Although these results are outside of expectations, they can lead to some misinterpretation of the following results such as the possibility of the virus decreasing anhedonic-like behaviors in females. Moreover, as the number of animals in each group, namely the female experimental group on Set 2, is so small, the significant result in the baseline may be due to that low number, as all the other baselines, which have more animals, did not have similar differences and the test itself presented no differences between groups. Notably, all animals from both Sets 1 and 2 presented high sucrose preference values, indicating that the experimental virus did not affect their behavior at the hedonic level.

Regarding the Splash test, it is used to evaluate self-care and motivational behaviors in animals (Isingrini et al., 2010) which is assessed by measuring the grooming time. For instance, animals use grooming to maintain their hygiene and is considered to have an anxiolytic role (Kompagne et al., 2008). Besides, decreases in grooming time can be reversed by using drugs such as pramipexole which has been reported as having antidepressant-like effects (Lieberknecht et al., 2017). Our results show a clear and significant decrease in grooming time by the experimental group in both males and females. Interestingly, in studies using UCMS in which astrocyte morphology alterations and astrocytic network disruptions are reported, the authors correlated those changes with emotional alterations such as reduction in grooming time that were translated as an increase of depressive and anxiety-like behaviors (Aten et al., 2023). Therefore, as the decrease in grooming frequency has been associated to animals with increased stressed levels, these results suggest that our experimental group may be experiencing higher stress levels than the control group. For instance, these results indicate a loss of motivation identical to human apathy (Humo et al., 2020) that is translated into neglect self-care behaviors such as grooming. In fact, pathological disorders such as depression are characterized by anhedonic and motivational impairments (Amiri et al., 2016), however, in this study, we observed motivational changes after viral infection, but not anhedonic alterations. Besides, the low grooming time values, however, can be due to the novelty of the cage, since they were isolated for 5 minutes in new cages. In fact, it was already shown that comfortable environments such as their home cage can lead to an increase in grooming time (Reis-Silva et al., 2019).

On the EPM and DLB tests, used to evaluate anxiety-like behavior, there were no statistically significant differences. We could observe some tendency in the male group from Set 2 ($p = 0.0918$), where the experimental group spent more time in the open arms compared to the control group, which

indicates a promising outcome in further sets. Besides, regarding the females, in Set 1, if the EPM showed that the control group spent more time in the open arms, the opposite was verified in Set 2. However, on the DLB test, the females in the Set 1 experimental group spent more time in the light compared to the control group, but the opposite was seen in Set 2. When putting together all the females from Set 1 and Set 2, since the protocol followed was exactly the same, we could not see any significant difference between the groups. In summary, we can only conclude that there were no differences provoked by the viral injection and, therefore, animals did not show any anxiety-like behavior alterations caused by the virus.

Recent studies on hippocampal astrocytes revealed their high influence on anxiety-like behaviors and associated elevated anxious-like behaviors with psychiatric disorders such as MDD and Post-Traumatic Stress Disorder (PTSD) (Cho et al., 2022). The fear of open and elevated spaces has also been associated with the animal's higher presence in the closed arms (Komada et al., 2008). For instance, the speculation of opposite observations between EPM and DLB tests doesn't seem to be uncommon in the literature. For example, Nakajima and colleagues (2008) concluded in their study that their animals showed a higher anxiety-like behavior expression in the EPM test, namely in the number of entries in the arms, but not in the Dark/Light test. In fact, both tests measure anxiety-like behaviors, however, they assess different aspects of anxiety, for example, while the EPM assess anxiety-like behavior associated with the open unprotected space, the DLB test analyses the anxiety provoked by a brighter space in comparison with a darker compartment and their will to explore the lighter space (Bourin & Hascoët, 2003; Pellow & File, 1986). Therefore, a lower time spent within open arms and an increase in the time spent within the dark side chamber can be considered as higher anxiety-like behaviors and seems to be the major interpretation of both tests. Nevertheless, it seems contradictory to say that an animal that spends more time in the dark presents more anxiety-like behavior, when the natural behavior of nocturnal animals such as mice is precisely to spend more time in closed and dark areas (Bourin & Hascoët, 2003). Once again, future sets will be needed to fully confirm our virus's lack of influences on anxiety-like behaviors.

As astrocytes are known to use synaptic modulation for cognitive information processing (Dallérac & Rouach, 2016), we envisioned that our virus could lead to some cognitive impairments in the experimental group. As the changes in the protocol do not allow us to combine data from the different sets, we can only discuss those changes and future directives to take into consideration. Regarding the Displacement task, we showed that, although without differences, the object positioning performed on Set 1 seems to be the one with more promising results, while the objects used both in the Long-term and

the Short-term assessments appear to have similar performances. However, we cannot draw any kind of conclusions about the objects used nor their positioning, since the objects are not fully validated and we did not assess possible preferences for one side of the box or object. In fact, when first exposed, animals should not demonstrate any kind of preference for either object, since they are both similar in size, shape and texture and they are both considered novel, however, if the animals show any preference, the objects should not be used, since it will interfere in the test itself (Denninger et al., 2019). Furthermore, it is considered that an animal recognises the change in the object's position if it spends more than 50% of the exploration time exploring the dislocated object (Denninger et al., 2019). On the same line of thought, if there is no preference towards one of the novel objects used in the Long-term and Short-term memory assessments, the investigation ratio should be around 50%, meaning that higher ranks towards the novel object lead to the conclusion that the animals recognized the new object (Akkerman et al., 2012). In fact, Heyser & Chemero (2012) concluded that animals not only preferred, but also showed higher preference indexes when presented with climbable objects but also demonstrated a faster habituation to objects that the animals could only touch. Therefore, we can exemplify that a climbable object should not be used as a novel object after a non-climbable object is used as a familiar object since animals can show some innate preference for the novel object and explore it, not because they recognise the new object, but because they prefer the climbable object. The full validation of the objects used in this test is, therefore crucial in future sets to fully understand the possible influences of our virus on cognitive behaviors. Besides, and as this is the only cognitive test we are assessing, to fully understand possible cognitive implications caused by our virus, future sets will be necessary, as well as to perform another cognitive-behavioral tests, such as Contextual Fear Conditioning. In fact, studies revealed that astrocytes use the interleukin-1 signalling, to support hippocampal-dependent memory that can be assessed by, for example, Contextual Fear Conditioning (Menachem-Zidon et al., 2011). Therefore, using other cognitive tests can be useful to evaluate cognitive implications upon the injection of our virus.

In what concerns the TST, studies have been reporting the increase in immobility time as the unwillingness or inability of the animal to keep the escape-directed behavior (Cryan et al., 2005), although it was initially described that immobility was simply the result of a state of hopelessness which could be treated by drugs used to ameliorate depression symptoms in humans (Porsolt et al., 1978). Taking that into consideration, and as we show that our male experimental group spent significantly more time immobile in this test when compared to the control group, we can conclude that our experimental group was experiencing a bigger hopelessness state by the time the test was conducted. These results not only raise the expectation of the astrocytic importance in behavioral despair but also allowed us to hope that

the virus could still be active on the last day of behavior and that a 7-day GCV treatment could be enough to not only activate the virus but also to keep it active during the behavioral period under analysis. On a deeper line of thought, as we did not observe any differences in the female groups, our results suggest that there is some kind of sex-dependent difference between males and females in regard behavioral despair. However, in recent studies assessing depressive-like bahaviors in WT animals, the authors did not observe any sex differences on their behavioral tests, nor on the TST test (Eltokhi et al., 2021). However, in another study with a different animal model showing a reduction of hippocampal astrocytes after the activation of glucocorticoid receptors, the authors showed an increase in behavioral despair (Lou et al., 2018), while in a different study using astrocyte atrophy, the behavioral despair observed in mice injected with corticosterone was reversed by fluoxetine treatment (Zhao et al., 2018). Furthermore, when astrocytes were inhibited in a third study using male mice, the anti-depressant effects caused by ketamine were reduced, translating in an increase in the immobility time in the TST and Forced Swimming Test (Wang et al., 2018). Therefore, the increase in immobility time and consequent translation into behavioral despair is not new in astrocytic modulation of animal models such as rats and mice.

Furthermore, we cannot forget that vaginal smears were collected on test days – but not habituation or baseline days – which has been considered by some authors a cause of stress in female mice when continually done (Varol et al., 2022). Therefore, the continuous collection of vaginal smears during the nine days of trials may lead the females of both groups to feel more stressed during the tests, inhibiting the presence of some differences between groups during the behavioral assessments.

On a quick note, the fact that we are using GCV treatment in both experimental and control groups, it may raise some issues. In truth, the AAV-GFP virus does not need GCV to be activated. For the GCV known toxicity properties as a cytotoxic agent, the hematological effects (Al-Badr & Ajarim, 2018; Gwee et al., 2014), of course the results on the control group behavior can be affected by the GCV injection itself. In further analysis it will be necessary to control this aspect through saline injections to ensure that there is no interference from the AAV-GFP virus in other aspects other than cell infection. However, this is just the very beginning of the study on our virus and, therefore, the first approach was to eliminate variables between the control and the experimental group by, for example, maintain the GCV injections in both groups.

In conclusion, the injection of our virus allowed us to observe some important behavioral changes, namely in terms of self-care behavior in males and females, which resulted in a decrease in grooming time, as well as an increase in behavioral despair. in males, resulting in a decrease in immobile time. These results thus become the beginning of a deeper and more targeted analysis of the impact that our

viral tool has on animal behavior and, possibly, will allow us to advance the study and add information to the current knowledge regarding astrocytes and their multiple functions.

6. Concluding Remarks

In this work we committed to testing a new viral tool that aimed to eliminate existing mature astrocytes from the hippocampus of a rodent animal model. Immunohistochemical procedures demonstrated that this virus eliminates significantly more astrocytes in the molecular layer than neurons, although the opposite happened in the granular cell layer. These results lead to future analysis taking into account the possible ablation of neurons and other types of cells in addition to astrocytes.

With regard to behavioral results, our viral tool administered into the hippocampus of mice demonstrated to be effective in reducing grooming time in both males and females, but also demonstrated to have an influence on the immobility time of males in the TST, increasing it. Although we did not observe other behavioral changes, the tendencies observed in the anxiety tests appear to be promising, and there is a need to, in the future, increase the number of animals in order to draw definitive conclusions regarding the impact of our virus on the animals' anxious-like behavior.

In conclusion, new tests should be applied in the near future in order to fully characterize the effectiveness of our viral tool, as well as its effects on both astrocytes and other types of hippocampal cells. Additional behavioral tests should also be applied, particularly cognitive tests, in order to confirm that our virus does not have any influence at this level, as suggested by our ORT results. Continuing to improve this tool is essential not only to add knowledge to what already exists, but also to reach our initial objective of creating a tool that is capable of exclusively and specifically targeting mature hippocampal astrocytes.

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Annexes

Annex A – Oestrus cycle influence on animal behavior

To assess possible differences between the females during the behavioral trials we collected vaginal smears daily. Although the considerable number of females used, the number of animals in the proestrus phase was extremely small (only 1 animal per group or per day) and we did not observe that phase every day. Therefore, we compared the animals in the non-proestrus phase with the ones in proestrus phases and considered comparisons between the experimental and the control groups when possible. As observed in **Figure 16**, only in the Dark/Light Box of the set 2 we observed one control group female and one experimental group female in the proestrus phase **(Figure 16G)**, while in all other behavioral test we observed that only one control female or one experimental female was in that phase. In addition, as the number of animals in the proestrus phase was extremely small, unfortunately we were not able to assess possible statistical differences and, as such, it is sensible to draw any type of definitive conclusions out of these results, not even if the GCV treatment is somehow influencing the oestrus cycle. As stated in previous sections, the oestrus cycle can influence behavior and we observed the same phase in the cycle for multiple days during the behavioral assessment, indicating that the females cycle may eventually have stopped. Either way, only a deeper analysis in which the cytological smears are collected at the same hour every day can confirm that. However, as previously mentioned, we should be aware that the continuous collection of vaginal smears can elevate the female stress levels, which can influence possible behavioral outcomes. For that reason, to study those issues individually can be an interesting way to assess possible influences of the GCV treatment on the oestrus cycle.

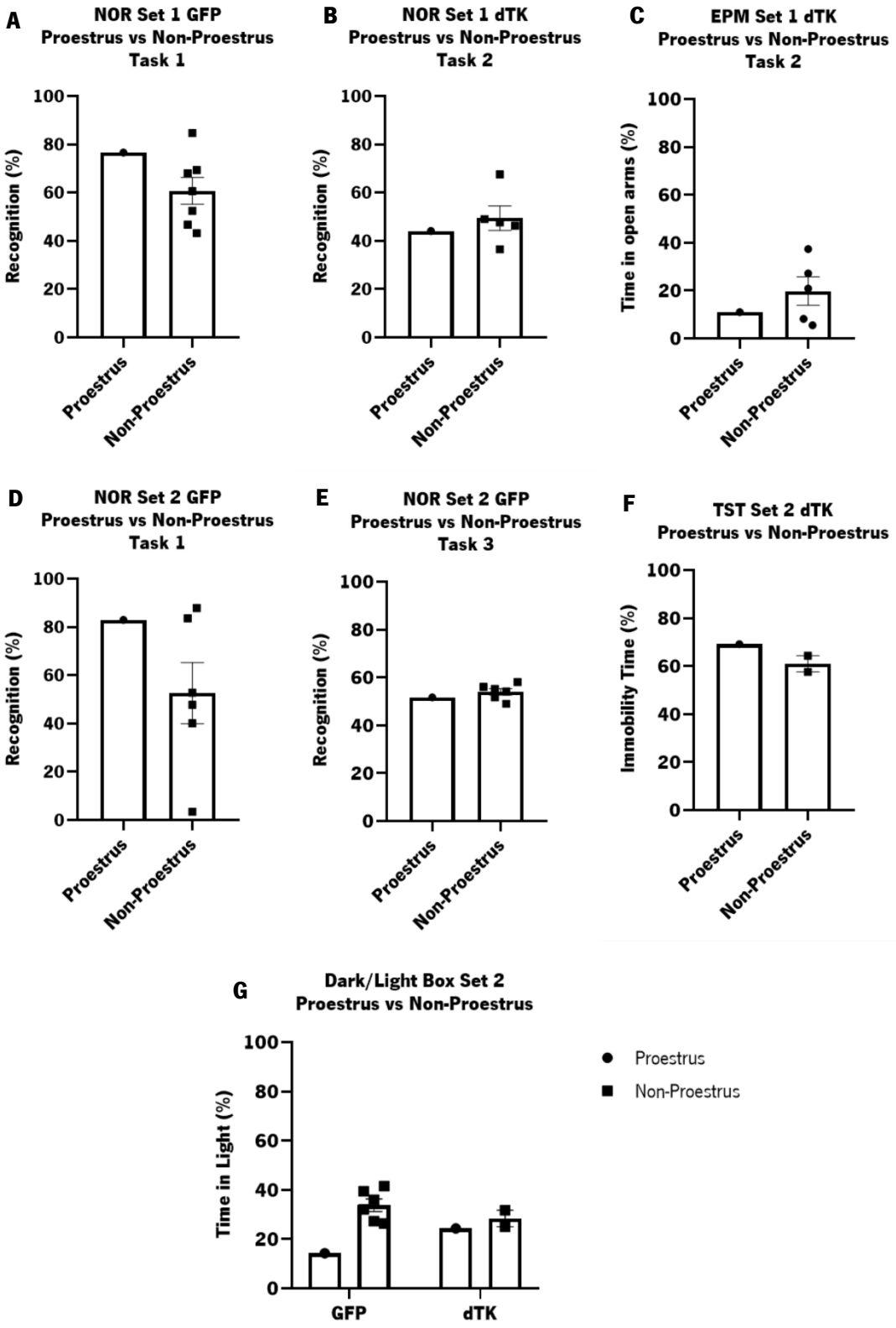


Figure 16 – Oestrus cycle influence on behavior

Vaginal smears were collected during the behavioral assessments. Set 1 (A, B and C) had only one female GFP or one female dTK in Proestrus phase during the behavioral trials. Set 2 (D, E, F and G) had one GFP or one dTK female in the Proestrus phase, except for the Dark/Light box test (G), where there were 1 GFP and 1 dTK female in the Proestrus phase. n= 8 (GFP) and 6 (dTK) for Set 1; n= 7 (GFP) and 3 (dTK) for Set 2.

Annex B –Object Recognition Test through the Sets

As described, multiple alteration on the ORT were performed through the 3 sets. For a better observation of these changes, the objects used and subsequent manipulations are represented in the **Figure 17**.

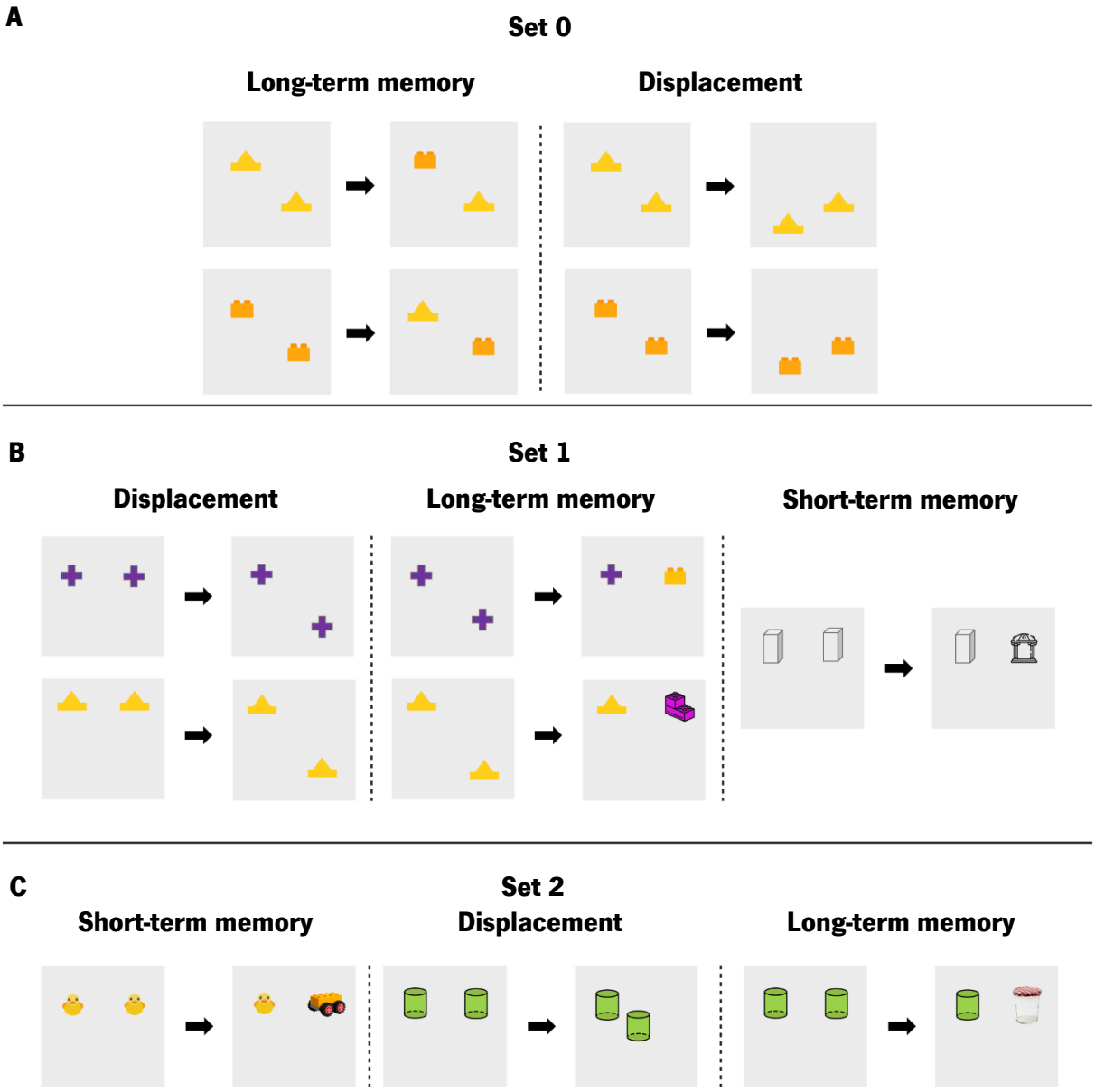


Figure 17 – Objects used on Object Recognition Test assessment

Different protocols were applied in the three sets. Set 0 (A) only assessed Long-term memory and displacement. Set 1 (B) and Set 2 (C) assessed both cognition tests from Set 0 plus Short-term memory.