Università di Foggia
Medicina Clinica e Sperimentale

PhD thesis
Basic and Clinical Neuroscience
XXXVIII cycle

Ketone bodies as modulators of neuronal function: from cellular mechanisms in human hippocampal neurons *in vitro* to cognitive outcomes *in vivo*

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Academic year 2024/2025

Abstract

This work investigates the impact of ketone metabolism on neuronal development, mitochondrial function and cognitive performance by integrating mechanistic analyses in a human stem cell derived neuronal model with a dietary intervention study in healthy adults. First, we established a simplified, scalable and cost-effective protocol for generating hippocampal neurons from human induced pluripotent stem cells (hiPSCs), preserving an active neurogenic niche composed of both immature and mature neuronal populations. Using this platform, we examined the effects of β-hydroxybutyrate (BOHB) on neuronal differentiation, dendritic maturation and bioenergetics. BOHB treatment did not modify neuronal yield or lineage specification, but significantly increased MAP2 expression, indicating enhanced dendritic arborization. Real-time PCR analyses showed upregulation of mitochondrial biogenesis regulators (PGC-1α, TFAM) and mitochondrial markers (MT-ND1, CS), while respirometric profiling revealed elevated basal respiration, increased maximal OCR and higher spare respiratory capacity. These findings demonstrate that BOHB promotes coordinated structural and metabolic maturation of hippocampal neurons by stimulating mitochondrial biogenesis and expanding mitochondrial capacity to support dendritic development.

In parallel, we conducted an exploratory isocaloric ketogenic diet intervention in healthy young students to evaluate whether systemic ketosis modulates cognitive performance. After three weeks of dietary ketosis, participants exhibited faster completion times in visuomotor and processing-speed tasks (DST, TMT, Stroop) but showed decreased accuracy on a high-load working memory test (Aospan). These results suggest that ketosis may differentially influence cognitive domains, enhancing speeded processing while impairing complex working memory performance, potentially through stress-related mechanisms.

Collectively, this work provides mechanistic evidence that ketone bodies enhance neuronal plasticity by boosting mitochondrial biogenesis and

dendritic maturation and offers preliminary *in vivo* data indicating that ketogenic diet may exert specific effects on cognition. The combination of hiPSC-derived hippocampal modelling and controlled nutritional interventions highlights a translational framework for understanding how metabolism shapes neuronal function and cognitive outcomes and sets the stage for future investigations into metabolic modulation of brain plasticity, learning and neuronal resilience.

Literature Review

1.1 Central nervous system and neurogenesis

The nervous system is divided into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is composed of the brain and spinal cord, while the PNS includes all other neural structures. The CNS is responsible for receiving sensory input, processing that information and generating appropriate responses.

The brain is the core of CNS and controls sensation, movement, emotional responses, communication, thought and memory. It is protected by the skull, the meninges and cerebrospinal fluid. Additionally, the blood-brain barrier shields it from potentially harmful substances circulating in the bloodstream. The spinal cord, located within the vertebral column, is another essential component of the CNS. It transmits motor signals from the brain to the rest of the body and carries sensory information from sensory receptors back to the brain. Like the brain, the spinal cord is protected by bone, meninges and cerebrospinal fluid.

The brain is divided into two hemispheres, the left and the right which remain in constant communication but specialize in different functions, a concept known as brain lateralization. The left hemisphere is primarily associated with language, logical reasoning and mathematical skills, while the right hemisphere is more involved in creativity, artistic and musical abilities and intuitive thinking.[1]

To better understand brain's organization, its stuctures can be listed as follows:

Cerebral Cortex: the outer gray-matter layer of the brain responsible for higher executive functions. It is divided into four lobes: frontal, parietal, occipital and temporal.

Frontal Lobe: controls voluntary movement, problem-solving, attention, memory and language. Contains the motor cortex and Broca's area, which regulates speech production.

Parietal Lobe: processes sensory information and contains the somatosensory cortex. It interprets touch, pressure and body position based on past experiences.

Occipital Lobe: the visual center of the brain. Contains the visual cortex, which interprets information from the retina and enables recognition of visual stimuli.

Temporal Lobe: processes auditory input through the auditory cortex and stores sound-related memories. Contains Wernicke's area, responsible for language comprehension.

Basal Nuclei (Basal Ganglia): group of deep gray-matter structures including the caudate nucleus, putamen and globus pallidus, responsible for motor control, movement regulation and coordination.

Thalamus: acts as the brain's relay station, directing sensory information to appropriate cortical areas. Also regulates consciousness and sleep-wake cycles.

Hypothalamus: maintains homeostasis and links the nervous system to the endocrine system. Regulates heart rate, blood pressure, hunger, thirst, temperature and hormone release.

Pons: part of the brainstem that connects the medulla, cerebellum, and thalamus. It contains tracts that relay motor information between brain regions.

Medulla Oblongata: the lowest part of the brainstem, controls vital autonomic functions like respiration, blood pressure, heart rate and reflexes such as swallowing and coughing.

Cerebellum: coordinates voluntary movements, balance and posture. It communicates with other brain regions through cerebellar peduncles and uses stored information to refine motor activity.

Limbic System: a group of structures, including the **hippocampus**, amygdala, hypothalamus, nucleus accumbens responsible for emotion, memory, motivation and olfaction.

Reticular Formation: network of neurons extending through the brainstem that regulates alertness and consciousness via the reticular activating system (RAS), which filters sensory input.

Spinal Cord: a two-way communication pathway between the brain and body. Divided into cervical, thoracic, lumbar, and sacral regions with 31 pairs of spinal nerves. Contains central gray matter and peripheral white matter.

Ascending Pathways: sensory pathways that carry information from the body to the brain using first-, second-, and third-order neurons. Transmit sensations like touch, pain, temperature and proprioception.

Descending Pathways: motor tracts that send signals from the brain to lower motor neurons, producing voluntary and involuntary movements.[1]

These structures are composed of several different cytotypes that work together to support normal neural function. Among them, **neurons** remain the central players, since they are the cells responsible for transmitting and integrating electrical and chemical signals. Their ability to form highly organized and dynamic networks is what ultimately allows the brain and spinal cord to carry out processes such as sensory interpretation, motor control, memory formation and higher-order cognition. Based on their function we can distinguish 3 main neuron types:

- **Sensory neurons** transmit sensory information from organs, such as the eyes and ears, to the brain;
- **Motor neurons** send information from brain cells to muscles throughout the body. Motor neurons are also in charge of voluntary muscle movements like speaking;
- **Interneurons** are all neurons that don't fit into sensory or motor categories.

Glial cells, although often discussed as supporting elements, are essential for maintaining the environment neurons rely on, providing metabolic support, regulating synapses, contributing to immune defence and ensuring proper insulation of axons.

- **Oligodendrocytes** wrap around the axon of neurons and make-up a myelin sheath to act as insulation. This protective insulation of the axon allows for high speed transmission of signals;
- **Microglial cells** are constantly surveying different areas throughout the brain for signs of distress, disease and infection. When there is a danger there is a swarming of microglia in the target area where they work together to clear out any debris and buildups, they then go back to a resting state of constant surveying;
- **Astrocytes** are star-shaped cells that surround neurons and they are the most abundant of all glial cells. The overall function of astrocytes is to keep the brain in homeostasis in many ways: including glutamate, ion (i.e., Ca2+, K+) and water homeostasis, defence against oxidative/nitrosative stress, energy storage, mitochondria biogenesis, scar formation, tissue repair via angiogenesis and neurogenesis and synapse modulation.

Still, the functional output of each nervous system structure is fundamentally driven by neuronal activity.

Contrary to the long-held belief that the adult brain is a fixed and unchanging organ, Altman's pioneering studies[2] decades ago provided the first anatomical evidence for the presence of newly generated dentate granule cells in the postnatal rat hippocampus, this advancement, along with evidence showing continuous neurogenesis throughout life in nearly all mammals investigated, including humans[3], helped support the hypothesis that adult neurogenesis is indeed present. Further research have demonstrated that the mature brain retains neurogenic potential throughout life.

Neurogenesis refers to the formation of new neurons from neural stem or progenitor cells, it plays a crucial role in brain development, functions and adaptability.

Temporally, we can distinguish between two types of neurogenesis:

- **Developmental Neurogenesis**

This occurs during embryonic and early postnatal development. During this period billions of neurons are generated and organized into the complex architecture of the brain and spinal cord. Developmental neurogenesis lays the foundations for the entire nervous system.

- **Adult Neurogenesis (AHN)**

In the adult brain, under normal physiological conditions, neurogenesis is largely restricted to two defined neurogenic regions, known as **neurogenic niches**: the first is the **subgranular zone (SGZ)** of the dentate gyrus in the hippocampus, where new granule neurons are produced and the second one is the **subventricular zone (SVZ)** lining the lateral ventricles, where newly generated neurons migrate along the rostral migratory stream (RMS) to the olfactory bulb, where they ultimately differentiate into interneurons[4]. Although most research on adult neurogenesis focuses on the two major niches in DG and SVZ, many studies have reported that it also occurs in other brain areas including the hypothalamus, amygdala and striatum. However, the stability and functional implication of these ‘’non-classical” neurogenic niches are controversial[5].

Developmental neurogenesis and adult neurogenesis differ both functionally and temporally. Developmental neurogenesis drives the formation of the central nervous system during early embryonic and postnatal stages, whereas adult neurogenesis supports the generation of new neurons within distinct neurogenic niches throughout the lifespan.

Focusing only on adult neurogenesis, this process comprises a series of tightly regulated steps, consisting of proliferation of neuronal stem/precursor cells (NPCs), migration of neuroblasts, apoptosis/survival during critical periods of development, differentiation into mature neurons, and finally, functional integration into preexisting neuronal circuitries[5].

Although the two neurogenic niches in the adult brain share fundamental features, they exhibit distinct characteristics. In the SGZ, radial glia-like neural progenitor cells (type 1) proliferate and give rise to intermediate progenitors (type 2a), which subsequently generate doublecortin (DCX)-positive neuroblasts (type 2b). These neuroblasts migrate a short distance to their final position within the dentate gyrus (DG), where they develop into immature neurons (type 3) **Figure 1**. Only a portion of these cells survives and differentiates into granule neurons expressing the mature neuronal marker NeuN and Map2[5]. These newly generated granule cells extend their dendrites into the molecular layer and project axons through the hilus toward the CA3 region, where they integrate into the hippocampal network.

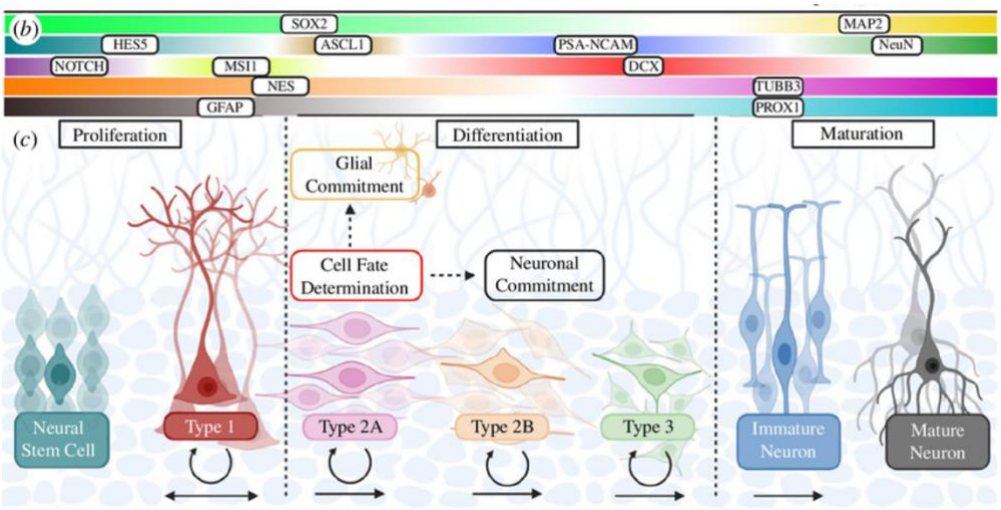


Figure 1 The diagram outlines the sequential phases of AHN, elucidating the progression from quiescent neural stem cells to the generation of diverse neural types, including Type 1 neural stem cells, Type 2A and 2B neural precursors, Type 3 neuroblasts, and their maturation into immature and eventually mature neurons. Circular arrows signify the proliferative potential at each stage, while straight arrows illustrate the directional transitions, with the capability of Type 1 cells to revert to quiescence. Additionally, the figure highlights the pivotal decision point where Type 2A precursor cells commit to either a neuronal or glial lineage. Image courtesy of Gyimesi M. et al, 2024[6].

In the SVZ instead, the activated radial glia-like progenitor cells (type B), located along the lateral ventricular wall, proliferate to form transient

amplifying progenitors (type C), which in turn generate neuroblasts (type A). These neuroblasts migrate in chains over a considerably longer distance along the rostral migratory stream (RMS), supported by astrocytic scaffolding and guided by neurotrophic factors and cytokines, until they reach the olfactory bulb (OB) **Figure 2**.Upon arrival, they dissociate from the chains and differentiate into interneurons that integrate into the granule cell layer (GCL) and the glomerular layer (GL)[5].

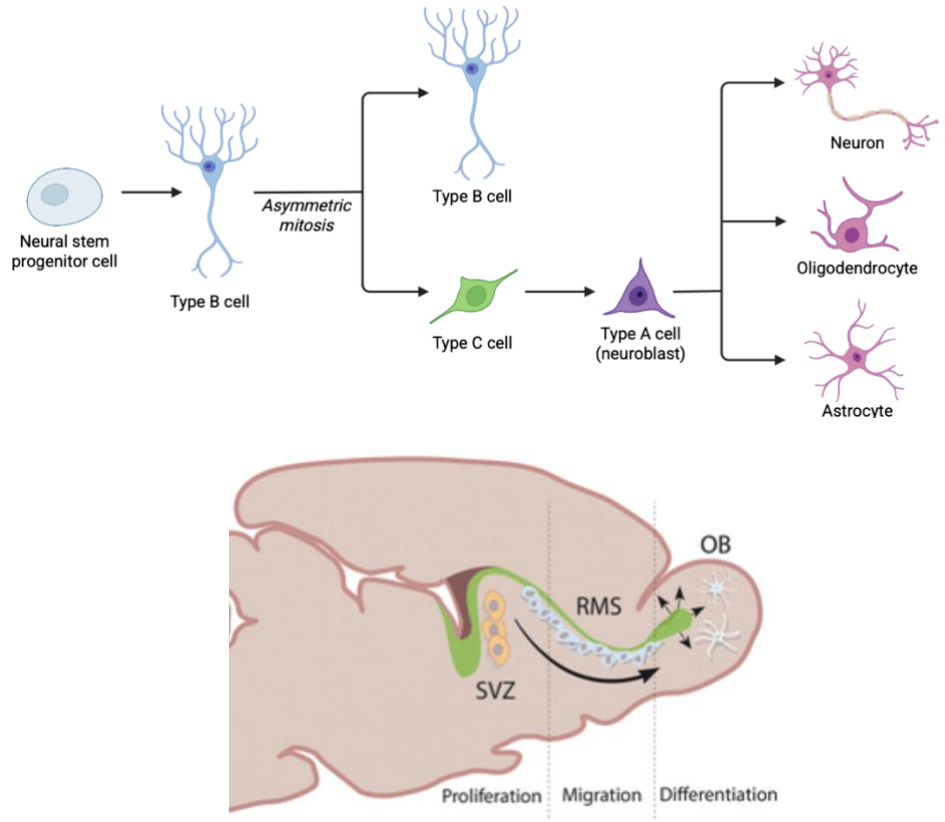


Figure 2 The quiescent NSC (Neural Stem Cells) in the adult SVZ are known as type B cells or radial glial-like cells. These cells give rise to proliferating neural progenitors, called type C cells or transient amplifying cells, which generate neuroblasts or type A cells. Adult subventricular neurogenesis is a multiple-step process, occurring in three different subregions: the ventricular-subventricular zone (V-SVZ), the rostral migratory stream (RMS), and the olfactory bulb (OB). As the cells migrate towards the OB, they undergo terminal differentiation. Images created with Biorender.

The presence of quiescent neural stem cells (NSCs) questions their purpose, what regulates their activation and differentiation, and whether there is a

connection between NSCs and the natural cognitive decline observed during human ageing.

Are these new neurons merely replacing dying mature neurons, or are they making unique contributions to specific brain functions that could not be achieved by existing mature neurons?

1.2 Importance of hippocampal neurogenesis

New neurons generated in the subgranular zone (SGZ) of the dentate gyrus (DG) play a crucial role in learning and memory and disruptions in hippocampal neurogenesis have been associated with epileptic seizures Alzheimer's disease and the cognitive impairments observed in depression and schizophrenia (SCZD)[7]. However, investigating the early stages of these central nervous system (CNS) disorders in humans remains challenging and it is unclear to what extent findings from rodent models can be reliably translated to human biology.

The advent of stem cell biology has created new opportunities for neuroscience research and for developing treatments for neurological disorders. While it is now well established that human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), can differentiate into functional neurons, a major current challenge is designing differentiation methods that generate neuron subtypes relevant to specific diseases.

Adult hippocampal neurogenesis (AHN) contributes to maintaining and enhancing hippocampal neural plasticity throughout aging. It plays an essential role in several hippocampus-dependent processes, including memory, cognitive ageing as well as mood regulation[8]. Specifically, AHN is involved in pattern separation that is the brain's process of transforming similar inputs into distinct, non-overlapping neural representations to prevent memory interference, spatial memory, the forgetting of previously formed memories, behaviours related to depression and anxiety[8]. The continuous production of new neurons is supported by the neurogenic niche located along the inner border of the dentate gyrus (DG), adjacent to the hilus. This specialized microenvironment contains a dense vascular

network, astrocytes, microglia and immature neurons that all together, through complex signalling interactions, tightly regulate AHN in mammals.

A central role of AHN is, as said, the regulation of learning and memory[9]. Newly generated granule cells in the dentate gyrus (DG) show increased excitability and synaptic plasticity during a critical maturation window, making them particularly receptive to activity-dependent circuit integration[10]. This property supports pattern separation, a computational process that facilitates discrimination between similar sensory inputs and reduces memory interference[11].

AHN also plays a critical role in mood regulation and stress responses. Chronic stress, mediated via elevated glucocorticoid signalling, suppresses progenitor proliferation and neuronal survival in the SGZ[12]. Reduced neurogenesis is strongly associated with depressive- and anxiety-like behavioural phenotypes in rodent models and importantly, chronic antidepressant treatments increase adult hippocampal neurogenesis, suggesting a potential mechanistic link between neurogenesis and affective recovery. The functional significance of this effect was demonstrated by Santarelli et al., who showed that disrupting antidepressant-induced neurogenesis, using either genetic approaches or localized X-irradiation of the hippocampal region, abolished the behavioural effects of antidepressants[13]. For example, serotonin 1A receptor-null mice were insensitive to both neurogenic and behavioural actions of the SSRI fluoxetine (Fluoxetine is a type of antidepressant known as a selective serotonin reuptake inhibitor (SSRI)), indicating that stimulation of hippocampal neurogenesis is required for antidepressant efficacy.

Neurogenesis is tightly coupled with aging and neurological disease but the extent to which neurogenesis occurs in humans is highly debated and quantitative studies are scarce. Boldrini et al., 2018 analysed the role of neurogenesis thought life using postmortem assessments on hippocampuses from 28 women and men, 14 to 79 years of age that had no neuropsychiatric disease or treatment. They characterized and quantified

angiogenesis, volume and cells at different maturational stages in the DG neurogenic niche, using unbiased stereological methods. They found persistent adult neurogenesis in humans into the eighth decade of life, despite declines in quiescent stem cell pools, angiogenesis and neuroplasticity. Over a 65-year age span, proliferating neural progenitors, immature and mature granule neurons, glia and dentate gyrus volume were unchanged[14]. What we can conclude is that physiological aging targets AHN and the integrity of the hippocampal neurogenic niche. Decreased angiogenesis and capillary density in aged brains correlates with reduced neuroplasticity so an exhausted quiescent progenitor pool together with less vascularization of the neurogenic niche resulting in less neuroplasticity, may explain some age related cognitive/emotional changes.

Impaired or dysregulated neurogenesis is also implicated in neurodegenerative and neurological disease such as schizophrenia, post-traumatic stress disorder and epilepsy, where disturbances to contextual memory processing and stress adaptability are common[9].

In Alzheimer's disease, reductions in AHN correlate with memory decline and hippocampal circuit dysfunction[15]. GABAergic signalling is enhanced in immature DGCs in a human Amyloid Precursor Protein (APP) transgenic mouse line, which consequently impairs morphological and functional maturation of adult-born DGCs[16]. Since GABAergic signalling from the local neural network appears to be crucial for the regulation of adult hippocampal neurogenesis, defective GABAergic signalling could be one of the mediators of AD pathology. Importantly, knock-out of apoE and knock-in of human apoE4, one of the major genetic risk factors for AD, impairs GABAeric signaling onto immature adult-born DGCs and reduce neurogenesis while increasing gliogenesis[16].

1.3 Regulation of hippocampal neurogenesis

Hippocampal neurogenesis is regulated by a complex interplay of intrinsic and extrinsic factors, including physical activity, stress, learning and

hormones, which influence the birth, survival and maturation of new neurons. Environmental enrichment and exercise stimulate neurogenesis, while stress, aging and certain diseases inhibit it. Molecular regulators like neurotrophins (e.g., BDNF), epigenetic factors and neural circuit activity also play a crucial role in fine-tuning this process.

The molecular regulation of neurogenesis has been dissected in greatest detail during embryonic development. Consequently, we now have a comprehensive framework describing the transcriptional programs and extracellular pathways that orchestrate the behavior of embryonic neural progenitor populations. While many of these regulatory axes such as Notch, Wnt/β-catenin, BMP and Shh signaling are also present in the adult neurogenic niches, their functional outcomes diverge in important ways.[17] A major distinction lies in the physiological state of adult neural stem cells (NSCs), which predominantly remain in a quiescent or slow cycling condition under baseline circumstances. This quiescence imposes additional layers of control over activation, self-renewal and lineage progression, producing regulatory dynamics that have no direct equivalent during embryogenesis, when precursor cells are highly proliferative.[17]

Brain-derived neurotrophic factor (BDNF)

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophins (NT) family, proteins that are essential for supporting central nervous system functions. Although neurotrophins are produced primarily within the CNS, they are also synthesized in various non-neuronal peripheral cells, including T and B lymphocytes, monocytes, vascular endothelial cells and both smooth and skeletal muscle cells[18]. BDNF expression has been identified in numerous brain regions, such as the hippocampus, frontal cortex, midbrain, amygdala, hypothalamus, striatum, pons and medulla oblongata. It plays a critical role in nervous system development by regulating cell differentiation, neuronal growth and survival, neurogenesis, synaptogenesis and synaptic plasticity. Evidence suggests that impaired synaptic plasticity, linked to reduced availability of BDNF and other neurotrophic factors, may contribute to the development of neurodegenerative and neuropsychiatric disorders[18]. Therefore,

developing strategies to enhance BDNF levels is considered an important objective in both prevention and treatment of neurological diseases.

BDNF in adult hippocampal neurogenesis: in DG, neural progenitor cells (NPCs) constantly differentiate into excitatory granule neurons which migrate to the granule cell layer and are integrated into hippocampal neural circuits[19]. An in vitro study using neutrospheres culture of NPCs isolated from adult rat hippocampus demonstrated that BDNF promoted cell proliferation, neural differentiation and cell survival[20]. It has also been reported that the neurogenesis-promoting effect of BDNF in organotypic mice hippocampal slice culture was inhibited by pre-incubation with a Tropomyosin receptor kinase B TrkB antagonist (TrkB is BDNF receptor), indicating important contribution of the BDNF/TrkB system[21]. Intra-hippocampal infusion of BDNF in adult rats increased the number of newborn neurons and caused appearance of ectopic granule cells, suggesting that BDNF influences not only neurogenesis but also migration of newborn neurons. To reveal a contribution of endogenous BDNF, several lines of genetically modified mice targeting BDNF or TrkB were generated. BDNF heterozygous mice ($BDNF^{+/-}$) showed a decreased number of proliferative cells and newborn neurons in DG[22]. Genetic ablation of TrkB in NPCs (but not neurons) caused a reduction of proliferative hippocampal NPCs and newly generated granule neurons[23]. Wang et al. showed that newborn granule neuron-specific BDNF deletion results in repression of dendrite growth and synaptogenesis to a similar extent of brain-specific BDNF conditional knockout mice[24]. Interestingly, they also found that newborn neuron-targeted BDNF gene transfer in brain-specific BDNF knockout mice recovered the deficit in dendrite and synapse in these neurons, demonstrating cell-autonomous actions of BDNF[24].

WNT pathway

The Wnt/β-catenin pathway comprises a family of 19 Wnt glycosylated proteins that are secreted and act as signalling molecules in an autocrine or paracrine manner. They play critical roles in embryonic development, cell renewal, cell proliferation, cell fate determination and adult tissue homeostasis[25].

Wnt ligands are involved into different signalling cascades known as canonical and non-canonical pathways. The canonical Wnt pathway, more widely studied, acts by inhibiting GSK3β, which leads to the increase of unphosphorylated β-catenin levels in the cytosol, allowing its translocation to the nucleus and its subsequent interaction with transcription factors such as the LEF/TCF family. Other two non-canonical Wnt pathways have been described: the Wnt/PCP also known as the Wnt/JNK pathway, and Wnt/Ca^{2+} pathways. The first one, participates in the modulation of gene expression, cytoskeletal organization and cell motility, while the Wnt/Ca^{2+} pathway activates the phospholipase-C (PLC), which in turns produce diacylglycerol (DAG) and inositol triphosphate (IP3). Wnt pathway activation and signal transduction is further complicated by endogenous antagonist such as Dickkopf proteins (DKK) and the soluble Frizzled-related proteins (SFRP) which can prevent ligands to bind their receptors.

In the central nervous system, Wnt signalling pathways play pivotal roles during development, controlling cell division, differentiation, polarity, migration and synaptogenesis[26]. Wnt pathway is active in the subgranular zone (SGZ) of the hippocampus, where it controls different stages of adult neurogenesis[25]. Particularly, Wnt3 ligand stimulates neuroblast proliferation and differentiation, as shown by *in vitro* and *in vivo* experiments. Interestingly, the treatment with the GSK3β inhibitor ,lithium, which then results in the activation of the pathway, increases the number of new cells generated in the adult rodent hippocampus[25] as well as the hippocampal volume in patients with the diagnosis of with bipolar disorder[27]. Wnt ligands released by hippocampal astrocytes, such as Wnt-3, activate the canonical Wnt pathway in adult hippocampal progenitor cells, thereby promoting their proliferation. Through the Wnt/β-catenin cascade, these signals also drive the differentiation of progenitors toward a neuronal fate; for example, Wnt-dependent activation of transcriptional targets, such as NeuroD1, plays a crucial role in generating new neurons[28]. Beyond proliferation and differentiation, Wnt signaling is also essential for the proper integration and maturation of newborn neurons within the

hippocampal circuitry. Importantly, while early activation of Wnt/β-catenin supports neuronal development, excessive or prolonged activation can negatively impact later maturation steps, such as dendritic refinement[28].

Wnt5a was also identified as an endogenous niche factor that regulates hippocampal neurogenesis. Arredondo et al., 2020 determined that reducing the levels of Wnt5a in the dentate gyrus of adult mice decreased the generation of new neurons[29]. Lentivirus-mediated knockdown of Wnt5a reduced the differentiation of neuronal committed progenitor cells, which remained as non-proliferative intermediate Sox2-expressing progenitors that failed to continue with the neuronal differentiation program. In addition, impaired dendritic arborization of newborn neurons was observed when knocking down Wnt5a. A similar effect was observed when Wnt5a was reduced in cultured adult hippocampal progenitors, in which neuronal differentiation and morphological development of the derived neurons were reduced, while treatment with Wnt5a had the opposite effect[29]. In agreement, chronic infusion of Wnt5a and Wnt7a ligand into the adult rat hippocampus increased the rate of newly generated neurons, furthermore only Wnt5a exerted additional effects by promoting neurite growth and neurite orientation in the dentate gyrus of adult rats[25], additionally the chronic exposure to Dkk-1 that led to the inhibition of Wnt signalling (antagonist) also generated aberrant location of growing neurites[25]. These findings underline the importance of a finely balanced Wnt signalling environment that support neuronal development and their structural integration within the hippocampal circuitry.

Glutamate is a well-established regulator of adult hippocampal neurogenesis[30], yet only recently have studies begun to uncover cell-intrinsic roles for specific glutamate receptor subtypes. The NMDA glutamate receptor (NMDAR) acts as a key integrator of synaptic activity and cellular signals that drive synaptic potentiation and long-term circuit remodelling. Tashiro et al. investigated the involvement of NMDAR signalling in adult SGZ neurogenesis using retroviral knockdown of the NR1

subunit in newborn granule neurons[31]. They found that maturing neurons lacking functional NMDARs exhibited reduced density in the SGZ at three weeks post-mitosis, accompanied by increased cell death. These findings suggest the presence of a critical period in which NMDAR-mediated synaptic activity is required for the survival and successful integration of newborn neurons. However, many questions remain unresolved. Pharmacological and biochemical studies *in vivo* and *in vitro*[31] also implicate NMDAR signalling in earlier stages of neurogenesis, including progenitor proliferation and cell-fate specification, raising the possibility that NMDARs may serve as cell-intrinsic regulators beyond the survival and maturation phase.

In addition, other glutamate receptor classes, including kainate, AMPA and metabotropic mGluR receptors, have been proposed as intrinsic modulators of SGZ neurogenesis[30], highlighting the need for further investigation using modern inducible and cell-type-specific approaches. Neurotransmitter regulation of AHN is not restricted to glutamatergic signaling: local GABA, the primary inhibitory transmitter in the brain, suppresses proliferation yet promotes differentiation and survival, whereas modulatory transmitters associated with psychiatric function appear to act at distinct neurogenic stages[30] for example, serotonin promotes proliferation and acetylcholine is required for proper maturation and survival. Together, these findings illustrate a complex interplay between excitatory, inhibitory and modulatory neurotransmitter systems in orchestrating adult neurogenesis.

A notable and intriguing characteristic of adult hippocampal neurogenesis is its strong regulation by environmental conditions and an individual's emotional, exercise, dietary interventions and physiological state. Potentially, adult born dentate granule cells (DGCs) may be generated on demand in response to external cues, contributing to plasticity within neurogenesis-dependent remodelling of hippocampal circuitry. For example, exposure to an enriched environment, featuring increased space, novel objects and running wheels, has been shown to markedly enhance the number of adult-born neurons, expand the granule cell layer, resulting in

approximately a 15% increase in granule cell neurons within the dentate gyrus, ultimately leading to improved spatial learning performance in rodents[32].

The fact that some of these modulatory signals, such as environmental conditions and an individual's emotional or physiological state, originate externally underscores the possibility that adult neurogenesis may be, at least in part, inducible and potentially amenable to therapeutic manipulation. Moreover, the sensitivity of adult hippocampal neurogenesis to such factors suggests that it is not only targetable in pathological contexts but can also be deliberately enhanced under non-pathological conditions, for example to promote improved or more refined cognitive functions.

A great deal of work has been devoted to dissecting the role of cytokines in the direct and indirect regulation of neurogenesis NPC express receptors for **pro-inflammatory cytokines**[33]. Despite some controversies and discrepancies arising from the use of different experimental models, most *in vitro* experiments have shown that pro-inflammatory cytokines suppress NPC proliferation.

For example, it has been showed in *in vitro* experiments that interleukin (IL)-6 markedly induces expression of p21 in hippocampus-derived murine NPC, which in turn, arrests proliferation of progenitors of neuronal lineage, while astroglial cells continued to proliferate[34]. This could be one of the mechanisms underlying cytokine-induced decreases expression of the early neuronal marker doublecortin (DCX), suggesting preferential differentiation into astrocytes rather than into neurons. High levels of cytokines induce apoptosis of newborn neurons and trigger oxidative stress that directly damages developing neurons. As an example of an indirect mechanism, pro-inflammatory cytokines released in the periphery activate the HPA axis (Hypothalamic–pituitary–adrenal axis is a major neuroendocrine system that controls stress, immune response and many other physiological functions) and high levels of glucocorticoids strongly suppress NPC proliferation[12]. In contrast to experiments with single cytokine exposure, multiple cytokines, chemokines, prostaglandins and

other inflammatory molecules and hormones act cooperatively during acute or chronic peripheral inflammation. Peripheral administration of lipopolysaccharide (LPS), an experimental method commonly used to stimulate the innate inflammatory response, promotes release of the pro-inflammatory cytokines from peripheral immune cells that serve as critical mediators of the communication between the periphery and the CNS. Acute administration of LPS disrupts NPC proliferation, decreases differentiation into neurons, and reduces survival of neuroblasts[34]. Experiments with intraventricular LPS injections[35] and *in vitro* studies[35] show that, during an innate immune response, cytokines and activated microglia are, to a large extent, responsible for the decrease in neurogenesis.

The mammalian hippocampus is densely packed with stress hormone receptors and is the key brain region involved in both sensing and regulating the response to stress. Chronic and uncontrollable stress is a major risk factor for a wide range of neuropsychiatric disorders, including anxiety and depression[36]. Stress and clinical antidepressants have been identified as major regulators of adult hippocampal neurogenesis, with stress generally decreasing and antidepressants increasing the number of newborn neurons. Gould et al. demonstrated that exposure to stress reduces cell proliferation in the adult hippocampus, an effect thought to be mediated by elevated levels of the stress hormone glucocorticoid. Supporting this idea, animals that underwent adrenalectomy exhibited increased cell proliferation, whereas administration of corticosterone, the primary glucocorticoid in rodents, led to a decrease in hippocampal cell proliferation[12].

1.4Metabolic regulation of adult neurogenesis

The developing brain follows a well-organised differentiation protocol during embryogenesis and it is considered to predominantly metabolically rely on glycolysis[37]. During adolescence, the developing brain of both rats and humans has been shown to have a metabolic shift from fatty acid oxidation to glucose-based metabolism[37], by adulthood the brain consumes approximately 25% of the body's glucose intake, despite representing only about 2% of total body weight. Simultaneously, roughly 20% of inhaled

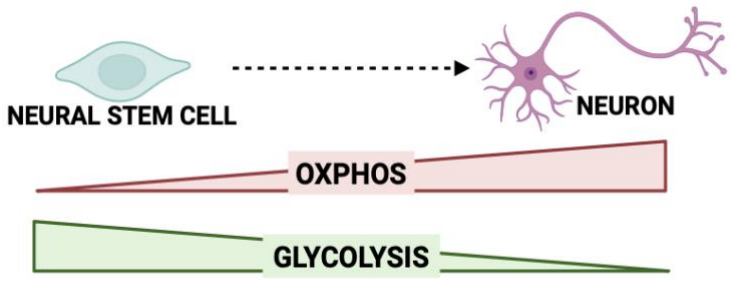
oxygen is utilized by the brain, reflecting a substantial bioenergetic demand that is largely met through oxidative phosphorylation (OXPHOS)[38].

Glucose metabolism in the brain involves glycolysis in astrocytes, producing pyruvate that is often converted to lactate and then supplied to neighbouring neurons. Neurons can reconvert lactate to pyruvate, which is imported into mitochondria and transformed into acetyl-CoA via the pyruvate dehydrogenase complex, entering the tricarboxylic acid (TCA) cycle. Within the TCA cycle, citrate may either serve as a precursor for cytosolic acetyl-CoA or continue through the cycle to generate α-ketoglutarate, with reducing equivalents NADH and $FADH_2$ feeding into the electron transport chain to drive oxidative phosphorylation (OXPHOS). While stem cells were traditionally considered glycolytic due to their high biosynthetic needs and residence in hypoxic niches, it is now clear that they are fully capable of mitochondrial respiration[39].

This shift in metabolic pathway utilization demonstrates that the maturation and differentiation of neural stem cells into fully integrated neurons within existing circuits requires extensive metabolic reprogramming. Mitochondria play a pivotal role not only by providing ATP but also by regulating redox balance, signalling cascades and epigenetic states[40] and they are increasingly recognized as a central regulatory hub in determining neural stem cell fate and orchestrating neurodevelopment.

This means that cells will require different metabolic profiles during a variety of stages and understanding these requirements might allow us to improve their cellular function.

The transition from neural stem cells to neurons is accompanied by increased oxidative phosphorylation and mitochondrial biogenesis , as well



as the downregulation of glycolysis and fatty acid oxidation pathway[41] (**Figure 3**).

Figure 3 Schematic representation of neuronal differentiation. During neurogenesis, neural stem cells (NSCs) differentiate, following a series of steps, into mature neurons. This developmental progression is accompanied by major metabolic transitions, including increased mitochondrial biogenesis and a shift from glycolytic metabolism to aerobic energy production, with a greater dependence on oxidative phosphorylation (OXPHOS) for ATP generation.

During the progressive differentiation from neural stem cells to neurons is observed a strong reduction in glycolysis-related proteins, such as hexokinase 2 (HK2) that phosphorylate glucose to produce glucose 6-phosphate, the isoform A of lactate dehydrogenase (LDHA) which catalyses the reduction of pyruvate to lactate and also an upregulation of OXPHOS-related genes has been observed[42]. Downregulation of HK2 and LDHA is required for neurodifferentiation and overexpression of either HK2 or LDHA in NPCs (neural progenitor cells) blocks neurodifferentiation and promotes astrocyte formation, thus guiding differentiation away from a neuronal phenotype towards a glial profile[42].

Additionally, the TP53-inducible glycolysis and apoptosis regulator (TIGAR) was shown to increase during brain development and neuronal development[43]. TIGAR is an important endogenous inhibitor of glycolysis, it has similar function of fructose-2,6-bisphosphatase that being part of PFK2 (phosphofructokinase 2) regulates the levels of fructose-2,6-bisphosphate, which is the most potent activator of glycolysis, therefore TIGAR acts as an inhibitor of glycolysis. Silencing of TIGAR in NSCs resulted in reduced neuronal differentiation and decreased expression of neuronal markers, such as β-III tubulin, microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP), Ngn1 and NeuroD1[43]. Moreover, TIGAR expression was accompanied by decreased lactate production and increased expression of the neuron-specific lactate dehydrogenase B (LDHB), thus, shifting towards pyruvate production and mitochondrial metabolism[43]. Neural stem cells (NSCs) in the subgranular and subventricular zones are predominantly glycolytic[38] and while

glycolysis is necessary for preserving stem cell identity, fatty acid oxidation can also serve as a fuel source, particularly during NSC activation, which depends on a functional electron transport chain and OXPHOS[39].

Increasing experimental evidence directly links mitochondrial functionality with the success of adult neurogenesis.

Several studies using conditional gene deletion models have demonstrated that impairing mitochondrial respiratory capacity compromises NSC function in the adult mouse. Deletion of the mitochondrial transcription factor A (TFAM), which regulates the expression of mtDNA-encoded ETC components, results in a marked reduction in the proliferation and survival of intermediate progenitor cells (IPCs)[44]. Restoration of ETC complex expression and mitochondrial membrane potential in aged mice rescues IPC proliferation and restores hippocampal neurogenesis[44].

Likewise, deletion of subunits of the mitochondrial α-ketoglutarate dehydrogenase complex (KGDHC) leads to a reduction in neuroblasts and impaired proliferation in the SGZ. Loss of the mitochondrial oxidoreductase apoptosis-inducing factor (AIF) in NSCs disrupts self-renewal, proliferation and differentiation from embryogenesis into adulthood, ultimately causing locomotor and cognitive deficits[44 45]. In addition to respiratory capacity, mitochondrial dynamics also contribute to NSC fate decisions: deletion of the fusion proteins MFN1 and/or MFN2 compromises NSC self-renewal in the adult hippocampus and leads to deficits in spatial learning[46]. Together, these findings demonstrate that intact mitochondrial function is essential for NSC proliferation, differentiation and self-renewal and is consequently critical for sustaining adult neurogenesis.

Age-associated accumulation of mitochondrial DNA (mtDNA) mutations, considered a major driver of ageing, further highlights the importance of mitochondrial integrity for NSC homeostasis. mtDNA mutations elevate mitochondrial ROS production, which can impair the self-renewal capacity of stem cells. In the polymerase gamma (Polg) mutator mouse model of accelerated aging, which lacks mitochondrial DNA (mtDNA) proofreading capability and consequently exhibits progressive accumulation of mtDNA mutations, researchers observed a reduction in quiescent neural stem cells

(NSCs) in the adult subventricular zone (SVZ) along with impaired self-renewal capacity. Notably, both deficits were reversed following antioxidant treatment[47].

Reactive oxygen species (ROS) are increasingly recognized as critical signalling molecules that dynamically regulate transcriptional mechanisms underlying NSC fate specification.

Proliferating intermediate progenitors in the adult SGZ produce transient bursts of ROS that activate expression of oxidation-responsive genes and rapidly induce antioxidant and uncoupling proteins to counterbalance oxidative stress[46]. However, excessive ROS levels can disrupt adult neurogenesis. Deletion of the extracellular antioxidant enzyme superoxide dismutase 3 (SOD3) leads to reduced neurogenesis in the SGZ and impaired cognitive function. Elevated ROS can also shift NSC fate towards astroglial differentiation at the expense of neuronal production[48]. Mice deficient in cytoplasmic SOD1 or mitochondrial SOD2 exhibit reduced adult hippocampal neurogenesis and increased astrocyte generation. Moreover, deletion of the circadian regulator Bmal1 results in elevated ROS and increased SIRT1 expression, correlating with reduced hippocampal neurogenesis and preferential astrocytic differentiation[46].

Collectively, these observations underscore that the metabolic transition, from glycolysis toward oxidative phosphorylation, together with mitochondrial integrity and tightly controlled ROS signalling, are fundamental determinants of neural stem cell homeostasis and lineage commitment. Proper coordination of these bioenergetic and redox mechanisms is essential to sustain successful adult neurogenesis and preserve neurogenic potential, particularly in the context of aging and susceptibility to neurodegenerative processes.

1.5 Brain metabolism and the functional role of ketone bodies

Although the human brain accounts for only ~2% of total body mass, it consumes approximately 20% of the body's resting energy expenditure. This

high energetic demand is primarily sustained by glucose oxidation, which provides ATP required to support synaptic transmission and the maintenance of ionic gradients. In addition, fundamental neuronal activities, including cytoskeletal remodelling, phospholipid synthesis and axonal transport also rely heavily on ATP availability. Because the brain possesses minimal glycogen reserves, a continuous and adequate supply of metabolic substrates is essential to maintain neuronal and glial function[49].

The energy metabolism of the brain is complex, where the usage of the glycolysis and mitochondrial respiration is distributed differently across different cell types and different parts of the brain. Glucose, as said, is the predominant energy substrate for the adult brain but alternative fuels, including ketone bodies (KBs) and lactate, can significantly support cerebral metabolism under particular physiological or pathological conditions. Both ketone bodies and lactate can cross the blood brain barrier (BBB) via monocarboxylate transporters (MCTs), enabling their utilization by neural cells[49]. Under human physiological condition plasma KBs concentration fluctuate between 0,05-0,1 mM, increased to ~1 mM after prolonged exercise or 24 h of fasting and to 6–8 mM during a prolonged fast without giving rise to clinically hazardous acidosis. In diabetic ketoacidosis, plasma concentration of KBs can exceed 20 mM[50].

During states of ketosis such as prolonged fasting, high-fat/low-carbohydrate ketogenic diets, uncontrolled diabetes or severe caloric restriction, systemic KB levels increase substantially and the brain progressively upregulates their uptake and oxidation in proportion to their availability[49]. β-hydroxybutyrate (βHB) and acetoacetate (AcAc) can replace glucose in the brain, whereas acetone, produced by spontaneous decarboxylation of AcAc, is rapidly eliminated through urine and lungs.

Ketone bodies are primarily synthesized in hepatocytes, though lower levels of production also occur in kidneys and astrocytes. Ketogenesis requires high intracellular levels of acetyl-CoA derived predominantly from fatty acid β-oxidation. When acetyl-CoA exceeds the capacity of the tricarboxylic acid

(TCA) cycle, the excess amount of acetyl-CoA are redirected to ketone body formation[49] in a process called ketogenesis. The first catalytic step involves the condensation of two acetyl-coenzyme A (acetyl-CoA) molecules into acetoacetyl-coenzyme A (acetoacetyl-CoA) catalyzed by the enzyme acetyl-CoA acetyltransferase, an enzyme present both in the cytosol and mitochondria. The mitochondrial isoform serves in ketone body synthesis, while the cytosolic enzyme is involved in cholesterol synthesis. Acetoacetyl-CoA is subsequently converted into 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) through the action of 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMG-CoA synthase 2, HMGCS2), the rate-limiting enzyme of ketogenesis, whose transcription is regulated by insulin and glucagon via the forkhead box protein A2 (FOXA2). 3-Hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase) then cleaves HMG-CoA to yield acetoacetate. Acetoacetate may either spontaneously decarboxylate to form acetone, a minor ketone body, or be reduced to beta-hydroxybutyrate (β-hydroxybutyrate, BHB), the most abundant circulating ketone body, through the action of beta-hydroxybutyrate dehydrogenase (BDH) in a nicotinamide adenine dinucleotide (NAD^+/NADH) dependent reaction. Because the liver lacks the enzymes required for ketolysis, it exports ketone bodies (KBs) into the bloodstream for utilization by extrahepatic tissues[49].

Once released into circulation, KBs cross the BBB primarily through MCT1, MCT2 and MCT4. MCT1 is expressed in endothelial cells and astrocytes, whereas neurons predominantly express MCT2. During early postnatal development, lactate and ketone bodies are abundant energy substrates, supported by high transporter expression and their presence in maternal milk, suggesting an important metabolic role in neonatal brain development[51] and so in neurogenesis.

Beyond acting as alternative metabolic substrates, KBs exert multiple and diverse effects. BHB through β-hydroxybutyrylation of histones and other proteins can regulate gene expression. One major effect is the upregulation of the transcription of detoxifying genes including catalase, mitochondrial

superoxide dismutase (mn-SOD) and metallothionein 2 in order to counteract oxidative stress[52].

A further mechanism explaining the protective activity of the ketogenic diets against oxidative stress is the intracellular modulation of the NAD+/NADH ratio. KB metabolism increases the NAD^+/NADH ratio, favouring the activation of NAD^+-dependent enzymes such as sirtuins (SIRTs), a class III histone deacetylase (Class III HDACS). NAD^+ is a co-substrate of SIRTs which have been involved in metabolic diseases and aging. SIRTs are important regulators of autophagy and mitophagy through the deacetylation of FOXO1 and FOXO3a transcription factors[53]. SIRT1 deacetylates PGC-1α, a key regulator of mitochondrial biogenesis, promoting mitophagy of damaged mitochondria and the renewal of the mitochondrial population. This regulatory pathway also involves activation of Nrf2 and induction of PPARGC-1γ (PGC-1α), ultimately controlling TFAM transcription and mtDNA replication.

The ketogenic diet (KD) stimulates the cellular endogenous antioxidant system with the activation of nuclear factor erythroid-derived 2 (NF-E2)-related factor 2 (Nrf2), the major inducer of detoxification genes[52]. Nrf2 is sequestered and inhibited in the cytoplasm by kelch-like ECH associated protein 1 (Keap1) interacting with Neh2 domains of Nrf2. Keap 1 is a substrate adaptor for different ubiquitin ligase systems, Cullin 3 (Cul3) RING-box 1 (RBX1) E3 ubiquitin ligase complex being the most studied. They constantly ubiquitinate Nrf2 which is then degraded by the proteasome. Nrf2-Keap 1 can be considered a sensor of cellular redox status since Keap 1 is a cysteine-rich protein. When distinct cysteine residues (Cys273 and Cys288) are oxidized, a conformational change takes place in Keap1 and so Nrf2 is able to translocate to the nucleus[50]. Once translocated into the nucleus, nuclear factor erythroid 2–related factor 2 (Nrf2) forms a heterodimer with small Maf transcription factors and binds to the Antioxidant Response Element (ARE), an enhancer sequence located in the promoter region of several cytoprotective genes. The ARE regulates the

expression of key antioxidant proteins containing thiol (-SH) groups, such as glutathione (GSH), thioredoxin (TXN) and peroxiredoxin (PRDX), as well as detoxifying enzymes including superoxide dismutase (SOD), catalase (CAT), heme oxygenase-1 (HO-1) and glutamate–cysteine ligase (GCL)[50]. Through this pathway, Nrf2 promotes the transcription of enzymes such as glutathione reductase, thioredoxin and peroxiredoxin, which are essential for regenerating and maintaining endogenous antioxidants in their active form.

Ketones increase the efficiency of electron transport chain (ETC) through the expression of uncoupling proteins (UCP). UCP reduces the mitochondrial membrane potential and diminishes the production of ROS and reactive oxygen nitrogen species (RONS)[52]. UCP4 and UCP5 are increased in brains of ketone ester-supplemented rats[52]. The KD inductive effect on UCP2, 4 and 5 expression could be a consequence of SIRT1 activation. These proteins reduce mitochondrial membrane potential and ROS production, while secondarily stimulating mitochondrial biogenesis to compensate for reduced ATP yield per mitochondrion.

Collectively, these findings demonstrate that ketone bodies are not simply alternative fuels but pleiotropic signalling molecules capable of modulating oxidative stress, mitochondrial dynamics and cellular homeostasis.

1.6 Therapeutic potential and neuroprotective effects of ketone bodies in neurodegeneration and cognitive function

As discussed in the previous section (1.5), multiple pathways mediate the positive effects of ketone bodies. Recently, there has been growing interest in the clinical use of ketone bodies, both for the treatment of neurological disorders and for enhancing physiological cognitive function. While exogenous administration or infusion of ketone bodies is possible, dietary approaches that stimulate endogenous ketone production are increasingly

favoured due to their practicality and ease of management. Various dietary interventions, including low-carbohydrate (typically ≤50 g/day), high-fat regimens and fasting protocols, are characterized by their capacity to markedly reduce blood glucose levels while promoting ketogenesis. The ketogenic diet (KD) is a high-fat, adequate-protein, low-carbohydrate regimen that induces nutritional ketosis. Several variants KD that show similar efficacy have been developed to date, this offers flexibility to increase compliance with the regimens. There are four major types of the KD with proven efficacy: the classic long-chain triglyceride (LCT) KD, medium-chain triglyceride (MCT) KD, modified Atkins diet (MAD) and low glycaemic index treatment. While ketogenic diet (KD) has demonstrated strong efficacy in the treatment of refractory epilepsy especially in children, as supported by robust and extensive clinical evidence[54], its broader application in the management of other neurological disorders remains in the exploratory phase.

Alzheimer's disease (AD) affects ~50 million people worldwide and is characterized by cognitive impairment that is associated with a progressive decline in memory. AD induces changes in amyloid precursor protein cleavage and production of the amyloid precursor protein fragment beta-amyloid (Aβ) along with hyperphosphorylated tau protein aggregation. Patients with AD have mitochondrial dysfunction and metabolic changes, such as impaired glucose utilization in the brain. Kashiwaya et al. treated cultured rat hippocampal cells with Aβ, β-HB(β-hydroxybutyrate), or both Aβ plus β-HB. Treatment with Aβ alone resulted in reduced neurite numbers and length compared to controls, and the additional treatment with β-HB reversed Aβ toxicity[55], suggesting that β-HB could potentially play a neuroprotective role against Aβ toxicity. In addition, the development of AD is associated with hypometabolism, mitochondrial dysfunction, oxidative stress and inflammation. Impaired glucose uptake and utilization in regional brain energy-substrate hypometabolism may be one of the earliest hallmarks of AD and suggests a potential avenue for compensating the brain energy deficit in AD dementia with ketones. Both

β-HB and acetoacetate bypass glycolysis to produce acetyl-CoA, which can then be channelled into the Krebs cycle and can thus increase energy availability in the brain. In AD, brain ketone uptake is unimpaired, which makes KBs a viable alternative energy source. Studies in Alzheimer's rodent models have found that ketogenic diet and/or exogenous treatment with ketone supplements attenuated cognitive/behavioural impairments, neuroinflammation, oxidative stress and Aβ accumulation and enhanced mitochondrial function and synaptic plasticity[56].

Several studies have demonstrated that ketogenic interventions enhance neuronal function through multiple converging mechanisms. Elevated circulating ketone levels improve mitochondrial efficiency and expand cellular bioenergetic reserves, while reducing oxidative stress via increased expression of antioxidant proteins. Ketogenic diets also modulate neurotransmitter balance and upregulate brain-derived neurotrophic factor (BDNF), contributing to reduced neuroinflammation and stabilization of neuronal membrane potential. Together, these effects highlight the potential of ketosis as a metabolic strategy to support brain resilience and function in neurological disorders.

The ketogenic diet has also been shown to have a strong influence on the gut–brain axis. KD changes the composition of the gut microbiome, promoting the growth of anti-inflammatory microbial species. These changes may modulate central nervous system inflammation via microbial metabolites, such as short-chain fatty acids and neurotransmitter precursors, thereby contributing to improved cognitive and motor outcomes in neurodegenerative conditions. Short-chain fatty acids (SCFAs), such as acetate, butyrate and propionate, produced via microbial fermentation of dietary fibers, influence blood–brain barrier transport, neuroinflammation, glial activity and neurotrophin expression, including BDNF[57]. Dysbiosis, characterized by an imbalance between Firmicutes and Bacteroidetes, has been associated with chronic inflammation and metabolic dysfunction. Ketogenic diets have been shown to reshape the gut microbiota, increasing

Lactobacillus species capable of producing SCFAs while reducing pro-inflammatory Firmicutes. Clinical studies in drug-resistant epilepsy have reported significant increases in Bacteroides and decreases in Firmicutes following KD, suggesting a potential link between ketone body metabolism, gut microbiota modulation and neurological cognitive improvement[58].

Taken together, these findings highlight ketone bodies as powerful regulators of neuronal homeostasis with potential therapeutic applications in neurodegenerative disorders. However, despite extensive evidence describing their metabolic and signalling functions, little is currently known about how ketone bodies influence adult neurogenesis. Understanding this relationship is essential to determine whether ketone-based interventions can promote cognitive resilience and neural repair. This thesis aims to build the foundation to address this gap.

Research Objectives

Despite widespread interest in using human induced pluripotent stem cells (hiPSCs) in the study of central nervous system (CNS) and in neurological disease modeling there are not standardized protocols for hippocampal neurons differentiation from stem cells, challenging scientists in the study of hippocampal physiological and pathological mechanisms.
Most studies investigating the hippocampus and hippocampal neurogenesis have primarily relied on animal models, especially mice. Phylogenetic differences between humans and rodents make necessary the assessment of the different stages of neuronal maturation in the human DG. For example, striatal neurogenesis is found only in humans, while olfactory bulb neurogenesis is absent in humans but present in other mammals[14]. Although these models have contributed significantly to our understanding of hippocampal functions, their relevance to human biology is often limited by fundamental differences between species at genetic, cellular, systemic and functional levels. These interspecies variations sometimes result in findings that do not fully translate to humans, posing challenges for the

applicability of the research. Moreover, the use of animal models involves ethical concerns, as experiments must comply with strict animal welfare regulations designed to reduce suffering. Conducting research with such models also demands highly trained personnel who are skilled in handling and experimental techniques specific to these animals. In addition, the maintenance of animal colonies requires substantial financial resources, including costs related to housing, care and specialized facilities. Considering these factors, there is a clear need for alternative methods that can complement or eventually reduce the dependence on animal models in the study of central nervous system, especially in hippocampal research.

The advantage of using hiPSC is to gain information on each phase of differentiation by controlling it and allows to capture the complexity of the process of neurogenesis. Some attempts to create a protocol of differentiation were done previously[7,59], with remarkable results in differentiating hiPSC into hippocampal neurons.

This research aims to address the following key questions: (1) Can β-hydroxybutyrate enhance the differentiation efficiency and functional maturation of hiPSC-derived hippocampal neurons? (2) How does β-hydroxybutyrate modulate cellular metabolism and gene/protein expression during neurogenesis?

The novelty of this approach lies in its focus on metabolic modulation to influence neurogenesis, which may open new avenues for non-invasive therapeutic strategies. While this study focuses on a single *in vitro* model, future work will be needed to implement other *in vivo* research systems/models to confirm these findings.

The primary expected outcomes of this project include the development of a more standardized, accessible and reproducible protocol for the *in vitro* differentiation of human hippocampal neurons, alongside a comprehensive analysis of the cellular biochemistry involved in neuronal differentiation and a detailed metabolic characterization of these neurons. Additionally, we aimed to investigate whether the application of ketone bodies could enhance neurogenesis and improve mitochondrial function. Altogether, this research was designed to determine if ketone bodies can effectively modulate the

neurogenesis process *in vitro* and if, such approach, could potentially be applied to treat various pathologies affecting the hippocampus, offer a non-invasive strategy to improve cognitive deficits in patients or cognitive decline in aging, or even enhance cognitive performance in healthy individuals.

In addition to the cellular and molecular objectives, this project incorporated an *in vivo* exploratory study to assess the potential cognitive impact of ketone metabolism in humans. Given the emerging evidence that ketone bodies can influence neuronal energetics, synaptic function and plasticity, we aimed to determine whether sustained, diet-induced ketosis could modulate specific cognitive domains in healthy adults.

Thus, an additional goal of this research was:
(3) To evaluate whether a controlled ketogenic diet intervention can alter cognitive performance *in vivo*. This translational component provides a bridge between mechanistic insights obtained from human stem cell derived neurons and potential functional outcomes in living humans, ultimately strengthening the relevance of metabolic interventions as tools for modulating hippocampal function and cognition.

Materials and Methods

2.1 Experimental model

2.1.1 Derivation of NPCs from iPSCs

The human induced pluripotent stem cells (hiPSCs) used in this study were generated from dermal fibroblasts obtained from a healthy control donor. These cells were pre-established and accessible within the laboratory prior to the beginning of the project.

Under specific conditions, hiPSCs can be directed to differentiate into neural progenitor cells (NPCs), which are characterized by their capacity to expand and generate the major differentiated cell types of the central nervous system (CNS). NPCs were obtained using STEMdiff™ SMADi

Neural Induction Kit, NPCs following an embryoid body (EB) protocol (StemCell Technologies, Vancouver, Canada, cat. #08581).

This kit is a serum-free medium kit for highly efficient SMAD inhibition-mediated neural induction of human ES and iPS cells, the resulting cultures are enriched for central nervous system (CNS)-type NPCs, which express SOX1 and Nestin.

2.1.2 Differentiation of NPCs in Hippocampal neurons

To obtain mature neurons, NPCs were plated on a double coating of polyornitine (15 ug/ml) and laminin (10 ug/ml) in DMEM/F12 medium supplemented with N2 (17502048 Thermo Fisher Scientific, Waltham, Massachusetts, USA), B27 (17504044 Thermo Fisher Scientific, Waltham, Massachusetts, USA, ascorbic acid (200 nM), cyclic AMP 500 µg/ml (Sigma A9501), laminin 1 µg/ml (Sigma L2020), BDNF 20 ng/ml (78005 Stem Cell), Wnt3a 20 ng/ml (Sigma H17001), 1% fetal bovine serum and thymidylate synthase inhibitor FudR 10 µM (Sigma F0503), for 3 weeks. Wnt3a and FudR were removed after 3 weeks and the differentiation continued for other 2 weeks (5 weeks total).

Cells were differentiated under two different culturing conditions that differ for the concentrations of glucose 17,5 mM and 5 mM, the presence of β-hydroxybutyrate (BOHB) 5 mM was tested from the start of differentiation maintaining a control without ketone bodies. iPSC-derived hippocampal neurons express mature neuronal markers: NeuN, Map2 and Prox1.

2.2 Immunocitochemistry

For immunofluorescence staining, cells were fixated and stained right after the Seahorse XFe 96 analyses, after the overall 5 weeks of neuronal differentiation. Fixation was performed with 4% paraformaldehyde for 20 min at room temperature, after it was removed and wells were washed twice with PBS. Then, the samples were permeabilized with 0.1% Triton X-100 in PBS for 20 minutes at room temperature. Non-specific binding sites were blocked using a solution containing PBS+ 5% FBS + 0,1% Triton X-100 + 0,02% NaN3 + 3% BSA 0.25%.

For the immunocytochemical analysis of iNPCs, the STEMdiff™ Human Neural Progenitor Antibody Panel (#69001; STEMCELL Technologies Inc., Vancouver, BC, Canada) was used, including antibodies against Nestin, PAX6, OCT4 and SOX1. The working dilutions were as follows: Nestin 1:1000, PAX6 1:200, OCT4 1:500 and SOX1 1:500. Primary antibodies were incubated overnight at 4°C. After washing with PBS, the following secondary antibody were incubated, also for 1 h: Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 567 (Life Technologies, Carlsbad, CA, USA).
After washing again with PBS, nuclei were stained with DAPI using a final concentration of 1ug/ml D1306 Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescent signals were detected using Cytation™ 5 Cell Imaging Multi-Mode Reader (Agilent Technologies, formerly BioTek Instruments, Winooski, VT, USA) and images were processed with ImageJ. Pictures and data were collected from 4 independent experiments. The image analysis was performed using the ImageJ software package.

2.2 Characterization of active neurogenesis and neural cell types

Neural progenitor markers, SRY-box transcription factor 1 (SOX1), PAX6 and Nestin were used to characterize NPCs. Immunostainings showed that the cultures expressed both proteins. After 5 weeks of further neuronal differentiation of NPCs, MAP2, NeuN and PROX1 immunostaining was used to characterize the neuronal culture. MAP2 and NeuN are general neuronal marker while PROX1 is specific and essential for the survival and commitment of the dentate gyrus (DG) neuronal progenitors and their progeny[60] [61]. At the end of the differentiation, the cells exhibited PROX1 and MAP2 positivity, demonstrating the efficiency of the protocol. DCX has been used as a marker used for the identification of newborn neurons in the adult human dentate gyrus, index of active neurogenesis[14].
Ab I° anti MAP-2 rabbit Chemicon #AB5622 1:1000, anti PROX1 rabbit Cell Signaling #14963s, anti DCX mouse Invitrogen #ma517066, anti Neun #A19086. Nuclei were stained with DAPI using a final concentration of

1ug/ml. Fluorescent signals were detected using Cytation™ 5 Cell Imaging Multi-Mode Reader (Agilent Technologies, formerly BioTek Instruments, Winooski, VT, USA) and images were processed with ImageJ.
Pictures and data were collected from 4 independent experiments. The image analysis was performed using the ImageJ software package.

2.3 Metabolic flux analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) and ATP production were measured in adherent cells using an XF96 Extracellular Flux Analyzer using MitoStress test (Seahorse Bioscience, Billerica, MA, USA). For OCR analysis, after replacing the growth medium with 180 µL of bicarbonate-free DMEM supplemented with 17.5 mM glucose for the High glucose condition and 5 mM glucose for the Low glucose condition, 2 mM L-glutamine, 1 mM sodium pyruvate and 5mM Hepes (pre-warmed to 37 °C pH 7.4), cells were preincubated for 45 minutes before beginning the measurement procedure. After measuring basal respiration, oligomycin (1 µM), FCCP (1 µM) and rotenone + antimycin A (1 µM + 1 µM) were sequentially injected into each well to assess ATP-linked respiration, maximal respiration and non-mitochondrial oxygen consumption, respectively.
For ECAR analysis, the Mito Stress Test was followed by the injection of 2-deoxy-D-glucose (2-DG) to assess glycolytic flux, including basal glycolysis, glycolytic capacity and glycolytic reserve.
Glycolytic and oxidative ATP rates production were calculated using extracellular flux measurements following a well-established protocol[62].
OCR, ECAR and ATP values were normalized post experiment to cell count (cell number), as determined by DAPI nuclear staining.

2.4 Gene expression analysis (RT-PCR)

Total RNA was isolated using TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and subsequently quantified using Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

One microgram of RNA was first treated with DNAse (Thermo Fisher Scientific, Waltham, MA, USA) and then reverse-transcribed to generate cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA).
Semi-quantitative determination of mRNA levels was performed using real-time qRT-PCR with the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, California, USA) and primers for the following genes: MAP2 (Bio-Rad, qhsaCID0017831), TBP (Bio-Rad, qhsaCID0007122), MT-ND1 (Bio-Rad, qhsaCED0021828), CS (Quiagen QT00068138) TFAM, (Quiagen QT00012782) PGC1α (Quiagen QT00095578).

qRT-PCR reactions were performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Relative mRNA expression levels were calculated using the ΔΔCt method, with normalization for each sample performed using TATA- Binding Protein (TBP) as internal controls. The Ct (threshold cycle) is defined as the cycle number at which fluorescence of the amplified target exceeds background fluorescence.
ΔΔCt = Ct of sample of interest – Ct of reference sample
ΔΔCt describes the difference between the average ΔCt of the gene of interest under a given condition relative to the reference condition. The reference sample is also referred to as the calibrator. Relative Quantification (RQ) = 2^–ΔΔCt.

2.5 Statistical analysis

Data analysis was performed on GraphPad Prism 9, using t-test, one-way ANOVA or two-way ANOVA. All data are expressed as mean ± standard error of the mean (SEM); a p-value < 0.05 was considered statistically significant.

2.6 Explorative study on the effects of three weeks of ketogenic diet on cognitive functions in undergraduate students

All research activities were conducted in close collaboration between researchers from medical and psychological background respectively from the Department of Clinical and Experimental Medicine and the Department of Humanistic Studies, Letters, Cultural Heritage, and Educational Sciences, University of Foggia, Italy. This study procedures were approved by the Ethics Committee of the University of Foggia [Foggia, 10/10/2022, Prot.007/CEpsi] and met the ethical standards of the 1964 Declaration of Helsinki (World Health Organization, 2013). The recruitment period for this study started on April 17, 2023, and ended on July 13, 2023.

2.6.1 Experimental layout

The study followed a repeated measures design involving cognitive assessments at two time points (T0 or baseline, T1 or follow-up) at the beginning and end of three weeks of an isocaloric ketogenic diet. Each participant who showed interest in the research underwent a screening phase through a medical check-up and an online survey. The medical check-up excluded risk conditions for the participants undergoing the diet. The online survey aimed to investigate the psychological chronotype, sleep quality, eating habits, sporting activities and academic performance. The participants who signed the written informed consent underwent a medical examination to verify the absence of risks in embarking on the diet and completed the online questionnaires and the cognitive assessment (T0). After three weeks of the ketogenic diet, the participants underwent the cognitive assessment again (T1). A team of experts provided customized meal plans based on individual preferences, daily calories by macronutrients and other educational resources for pairing alternative foods.

2.6.2 Participants

Thirty undergraduate volunteer students, who had been free from chronic diseases and psychiatric disorders for at least six months before the study, initially participated in the online screening survey. Six participants were excluded due to missing or incomplete information, five due to sleep quality problems, three due to high total anxiety levels and three due to a decided serotonin or morning chronotype. Additionally, three participants did not show up for the initial cognitive assessment. A total of ten participants took part in the cognitive assessment at T0 before starting the ketogenic diet. Two participants did not attend the second cognitive assessment after three weeks of the ketogenic diet. Thus, the final sample consisted of eight participants (6 women and 2 men). None of the participants reported any side effects related to the dietary treatment.

2.6.3 Dietary intervention

Participants followed an isocaloric ketogenic diet in which 70–75% of daily caloric intake was provided by lipids, while carbohydrate intake was restricted to 5–10%. Protein content was individually adjusted to preserve muscle mass. Dietary adherence was monitored weekly in the morning under fasting conditions, since ketone levels fluctuate throughout the day depending on food intake and physical activity. Ketosis was assessed using Bayer Ketostix (Bayer Leverkusen, Germany), and nutritional ketosis was defined according to the manufacturer guidelines.

2.6.4 Screening Survey

A preliminary screening survey was administered using an online platform (SurveyMonkey Inc.) to collect information regarding participants' nutritional habits (including alcohol, sugary beverage, caffeine and supplement consumption), physical activity and academic performance over the preceding year. Participants also completed the Morningness–Eveningness Questionnaire (MEQ-SA) to determine chronotype, the Global Sleep Assessment Questionnaire (GSAQ) to assess sleep quality, and the State–Trait Inventory of Cognitive and Somatic Anxiety (STICSA) to

evaluate anxiety levels. To ensure reliability of the cognitive performance data, individuals were excluded if they reported poor sleep quality (GSAQ total score > 30), extreme morning or evening chronotypes (MEQ score < 31 or > 69) or elevated anxiety levels (STICSA total score > 42).

2.6.5 Assessment of cognitive functions

A neuropsychological test battery was administered to assess participants' cognitive functions (**Table 1)** at the beginning (T0) and at the end (T1) of the ketogenic diet. The second testing session occurred three weeks after the first, at the same time of day for each participant. All assessments were conducted individually by the same experimenters on weekdays between 9:00 a.m. and 5:00 p.m., and each session lasted approximately one hour. All tasks were administered in paper-and-pencil format, except for the Automated Operation Span Task, which was delivered using E-Prime software (version 3.0; 2016). To reduce practice effects, parallel versions of the cognitive tests were used at T0 and T1. In addition, prior to cognitive testing at both time points, participants completed the State-Trait Inventory of Cognitive and Somatic Anxiety (STICSA).

Task	**Cognitive Domain Functions**
Digit Span - **DS**	Short-term verbal memory
Rey Auditory Learning Test - **RALT**	Long-term verbal memory
Digit Symbol Test – **DST**	Attention
Attentional Matrices – **AM**	Attention
Trail Making Test - **TMT**	Set-shifting
Stroop Test - **StroopT**	Cognitive inhibition
Automated Operation Span Task - **Aospan**	Working memory

Table 1. Cognitive Assessment

2.6.6 Statistical analysis

Bayesian Wilcoxon signed-rank test was used to analyze the data. The Bayes factor (BF), specifically BF_{10}, evaluates how much the observed data supports the alternative hypothesis (H1) over the null hypothesis (H0).

Larger values of BF_{10} indicate stronger support for H1. Bayes factors range from 0 to ∞, and a BF_{10} value > 3 suggests moderate support in favor of the alternative hypothesis, while a BF_{10} value > 10 indicates strong support in favor of the alternative hypothesis. Analyses were conducted on raw data with JASP software (version 0.19; JASP Team, 2024).

Results

3.1 Generation of hippocampal granule neurons from hiPSCs using NPCs

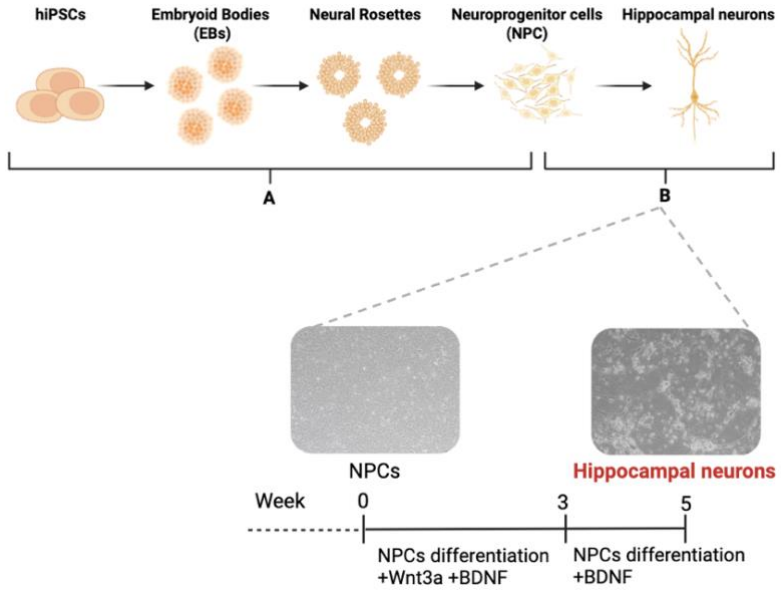


Figura 4 Schematic representation of the differentiation protocol. It consists of two parts: Part A, which generates NPCs from hiPSCs, and Part B, which enables the differentiation of hippocampal neurons.

We successfully generated hippocampal granule neurons from human induced pluripotent stem cells (hiPSCs) using a modified differentiation protocol. This new protocol builds upon the framework established by Yu et al.[7], but introduces key modifications, particularly in the initial differentiation phase and the culture conditions. The modified protocol significantly reduces costs, shortens the differentiation timeline and improves reproducibility, as it utilizes a commercially available kit for the differentiation of NPCs.

Part A: Differentiation of hiPSCs into neural progenitor cells (NPCs).

In the first stage, hiPSCs previously established and characterized in our laboratory, were differentiated into neural progenitor cells (NPCs) using a commercial differentiation kit from StemCell Technologies. The NPCs were characterized by the expression of typical CNS markers, including SOX1**,** Nestin and PAX6, as assessed by immunocytochemistry (**Figure 5**). The cells exhibited the expected localization patterns, with SOX1 and PAX6 localized in the nucleus, and Nestin displaying a filamentous cytoplasmic staining. Importantly, OCT4, a marker of pluripotency, was largely absent in NPCs, indicating successful exit from the pluripotent state. These results confirm that the differentiation protocol efficiently generates NPCs from hiPSCs and that the culture is enriched for CNS-type NPCs capable of further neuronal differentiation.

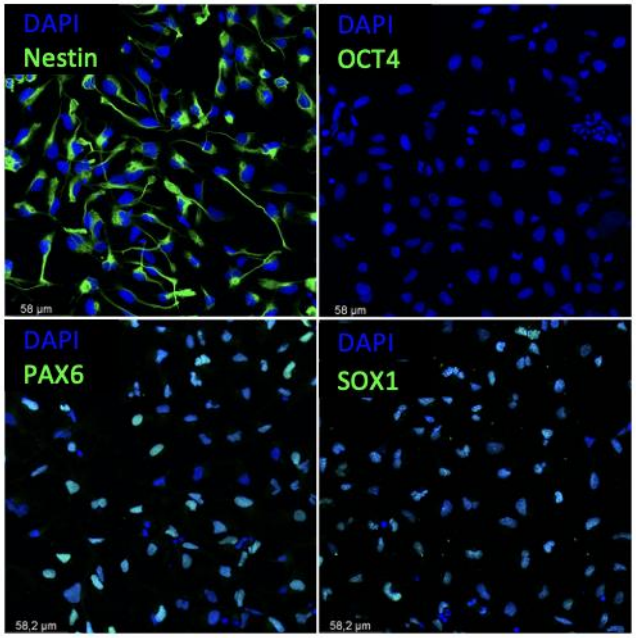


Figure 5 Immunocytochemical characterization of hiPSC-derived NPCs. Confocal merged images show cultures enriched for central nervous system (CNS)-type NPCs, which express SOX1, Nestin and PAX6. OCT4 serves as negative control to assess the loss of pluripotency typical of hiPSC. Scale bar 58,2 µm.

Part B: Differentiation of NPCs into Hippocampal Granule Neurons

In the second stage, NPCs were seeded onto polyornithine-laminin coated plastic supports, replacing the monolayer of astrocytes used in the original protocol by Yu et al, this modification eliminated the need to use human hippocampal astrocyte. NPCs were cultured for a total of 5 weeks under two distinct conditions:

1. Weeks 1–3: The cultures were maintained in presence of Wnt3a and BDNF to promote neuronal differentiation; NPCs were cultured with differentiation media containing Wnt3a. DG primordia lie next to the cortical hem and presumably are exposed to high levels of Wnt3a[59]. 5-fluoro-2′-deoxyuridine (FUdR) was also maintained in the culture medium to control the proliferation of dividing non-neuronal cells, preventing overgrowth that could confound morphological and metabolic analyses.
2. Weeks 4–5: Wnt3a was removed from the medium, BDNF alone was left to support neuronal maturation.

During this period, the NPCs underwent significant morphological changes, including the development of dendritic processes and synaptic structures. Immunocytochemical analysis confirmed the expression of **MAP2** (a dendritic marker), **NeuN** (a neuronal marker, located in the nucleus of post-mitotic neurons) and **PROX1** (a nuclear marker specific for hippocampal granule neurons) **figure 6A**, indicating successful differentiation into hippocampal granule-like neurons.

Challenges encountered: the development of this protocol presented several challenges, particularly when optimizing the differentiation process and ensuring consistency across experiments. The 5-week duration of Part B required careful monitoring and fine tuning of the culture conditions. Finding a good cellular support and proper coating, maintaining neuronal maturation over an extended period and adjusting growth factor concentrations were the primary hurdles encountered

throughout the project. Despite these difficulties, the protocol was successfully optimized and the final method allowed for the reproducible generation of hippocampal granule neurons from hiPSCs.

3.2 Effects of glucose concentration and β-hydroxybutirrate on hippocampal neurons differentiation

Neuronal differentiation was performed under varying glucose concentrations to assess the impact of metabolic substrate availability. The standard high-glucose condition **HG** (17.5 mM) was compared to a low-glucose condition **LG** (5 mM). Further experiments with severely restricted glucose levels (2.5 mM and 1 mM) revealed rapid cell death, indicating that a minimal glucose supply is essential for NPC/neuronal survival. Under low-glucose conditions (5 mM), neuronal differentiation proceeded efficiently. Immunocytochemistry for **MAP2** and **NeuN** (**Figure 6B**) showed no significant differences in expression levels compared to the high-glucose condition, indicating preserved neuronal maturation. Real-time PCR analysis of MAP2 transcript level confirmed these results. Similarly, expression of **Prox1** measured with immunocytochemistry, a marker of hippocampal granule cell identity, did not differ between high- and low-glucose conditions. These findings demonstrate that neuronal differentiation and hippocampal identity are maintained under reduced glucose availability. The differentiation model also maintained populations of immature neurons, as indicated by **DCX** expression, alongside mature neurons (MAP2 and NeuN), suggesting the presence of a proliferative stem and progenitor compartment and ongoing neurogenic activity. When **β-hydroxybutyrate** (5 mM) was added to the culture medium under both high- and low-glucose conditions, DCX, Prox1 and NeuN expression remained unchanged, indicating that neurogenesis, neuronal lineage specification, and overall production of post-mitotic neurons were not affected (**Figure 7**). Attempts to supplement cultures with acetoacetate

alone or combined with β-hydroxybutyrate resulted in marked cytotoxicity. Notably, MAP2 expression was significantly increased in ketone-treated cultures under both glucose conditions, indicating enhanced dendritic complexity and arborisation (**Figure 7D**).

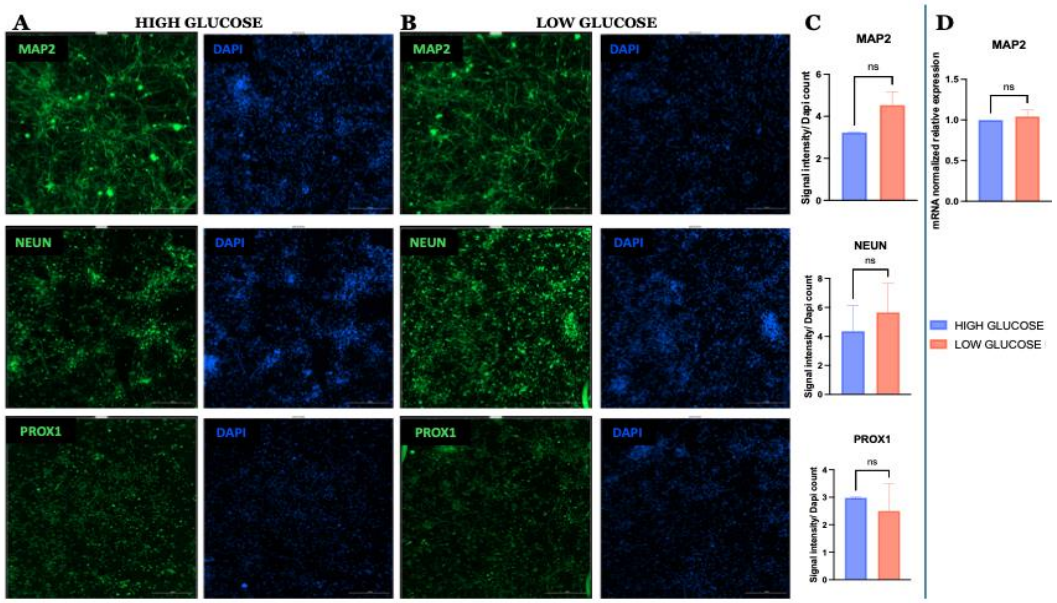
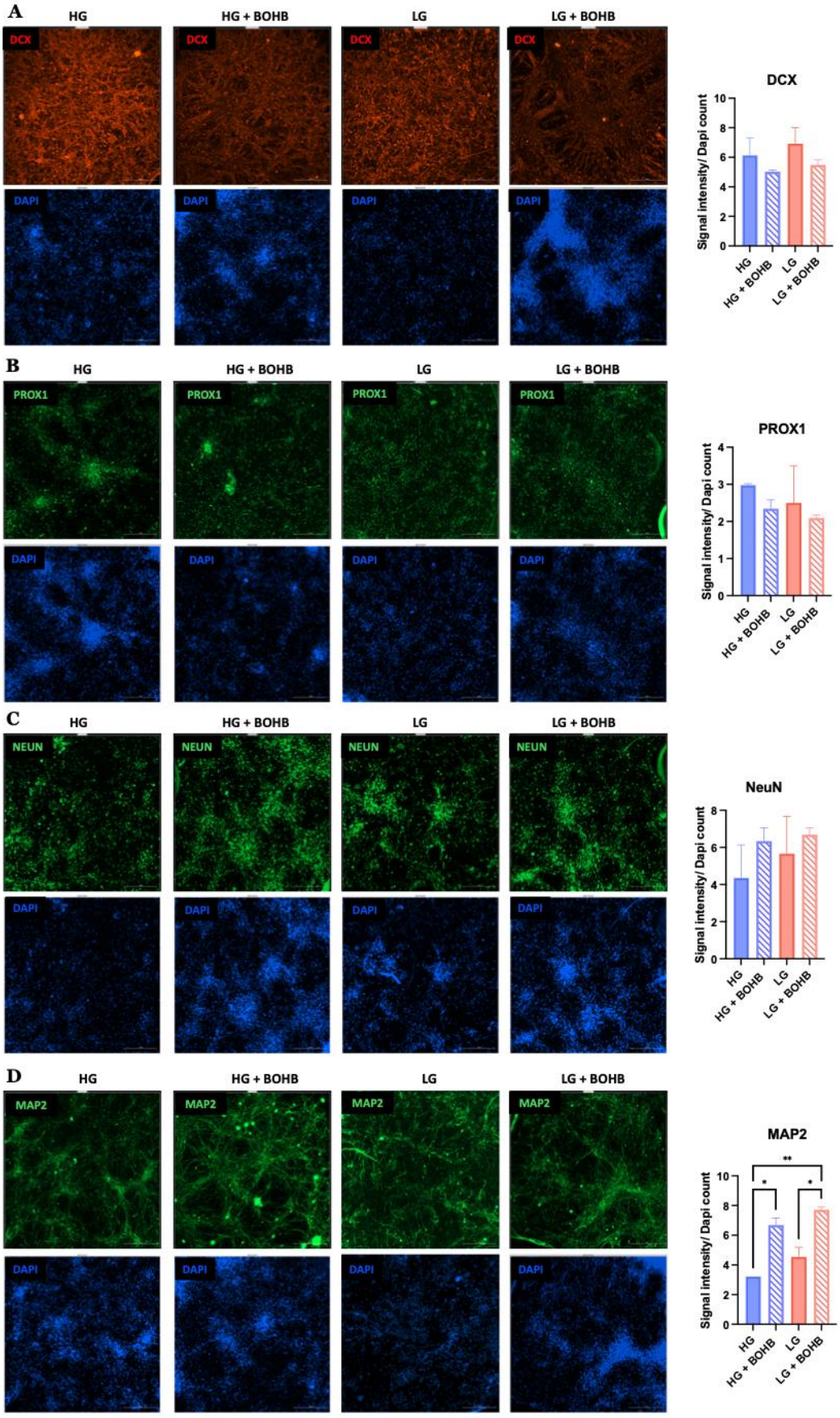


Figura 6 (A) Representative images of immunocytochemisty showing the presence of MAP2, NeuN and PROX1 and the correspective DAPI acquisition of NPCs-derived hippocampal neurons differentiated in **high glucose** (HG) condition 17.5 mM. Scale bar, 300 µm. **(B)** Representative images of immunocytochemisty showing the presence of MAP2, NeuN and PROX1 and the correspective DAPI acquisition of NPCs-derived hippocampal neurons differentiated in **low glucose** (LG) condition 5 mM. Scale bar, 300 µm. **(C)** Histograms of the quantification of MAP2, NeuN and PROX1 signal intensity normalized on DAPI count (cell number) in both HG and LG condition. n = 4 biological replicates; Statistical analysis was conducted using a t-test, all data are presented as mean ± SEM. **(D)** Bar graph representing relative mRNA expression of *MAP2* (normalized to TATA-binding protein (TBP); Statistical analysis was conducted using t-test, all data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.


A
HG
HG + BOHB
LG
LG + BOHB
DCX
DAPI
DCX
Signal intensity/ Dapi count
HG
HG + BOHB
LG
LG + BOHB
B
PROX1
PROX1
Signal intensity/ Dapi count
C
NEUN
NeuN
D
MAP2
MAP2
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*

Figure 7 Representative images of immunocytochemisty showing the presence of DCX **(A)**, PROX1 **(B)**, NeuN **(C)** MAP2 **(D)** and the correspective DAPI acquisition of NPCs-derived hippocampal neurons differentiated in high glucose **(HG),** high glucose supplemented with β-Hydroxybutyrate **(HG + BOHB),** low glucose **(LG)** and low glucose supplemented with β-Hydroxybutyrate **(LG + BOHB).** Scale bar, 300 μm. For each protein, there is a histogram of the signal quantification intensity normalized on DAPI count (cell number). N = 4 biological replicates; Correspondent statistical analysis was conducted using ordinary one-way ANOVA followed by Bonferroni's multiple comparison test, all data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

3.3 Hippocampal neurons metabolic profile

To characterize the metabolic phenotype of differentiated hippocampal neurons, mitochondrial respiration and glycolytic function were evaluated using the Seahorse XFe96 Flux Analyzer. Oxygen consumption rate (OCR) was assessed with the Mito Stress Test, and extracellular acidification rate (ECAR) was evaluated with the Glycolysis Stress Test.

The Agilent Seahorse XF Cell Mito Stress Test allows real-time measurement of mitochondrial activity through dynamic OCR analysis. Overall, BOHB treatment resulted in an increase in overall mitochondrial respiration under both HG and LG conditions (**Figure 8A–B**). Although basal respiration was higher in BOHB-treated neurons, this increase did not reach statistical significance when compared with the corresponding control conditions (HG and LG). To further characterize mitochondrial function, OCR was measured following the injection of oligomycin, an ATP synthase (complex V) inhibitor. Under these conditions, BOHB-treated samples exhibited higher OCR values compared with their respective controls in both HG and LG conditions. ATP-linked respiration was subsequently calculated based on basal and post-oligomycin OCR values. BOHB-supplemented neurons (HG+BOHB and LG+BOHB) showed a statistically significant increase in ATP-linked respiration (**Figure 8A–B**), indicating enhanced mitochondrial ATP production.

BOHB also significantly increased maximal respiration following FCCP exposure under both HG and LG conditions, demonstrating a marked

enhancement in maximal mitochondrial respiration (**Figure 8A-B**). The consistency of this effect across glucose conditions indicates that it is BOHB-dependent, suggesting improved mitochondrial adaptability and performance. Representative OCR traces (**Figure 8C-D**) and corresponding bar plots consistently show enhanced respiration throughout the assay in BOHB-treated neurons compared with controls across both HG and LG conditions.

Extracellular Acidification Rate (ECAR)

The Glycolysis Stress Test measures extracellular acidification rate (ECAR) as an indicator of glycolytic activity. Glycolysis, the conversion of glucose to pyruvate, produces ATP, NADH and protons.

In our experiments, BOHB markedly enhanced glycolytic capacity (**Figure 8E-F**). in both HG and LG conditions. A similar trend toward higher glycolytic reserve and basal glycolysis was observed in BOHB-treated neurons, although these differences did not reach statistical significance. Representative ECAR traces illustrate elevated acidification responses in HG and LG +BOHB samples (**Figure 8G-H**).

ATP Production Rate

Total ATP Production Rate is the sum of the ATP Production Rate from glycolysis and mitochondrial oxidative phosphorylation. Using calculations previously validated for the Seahorse XF Glycolytic Rate Assay and based on known reaction stoichiometry, ECAR data can be converted to glycolytic ATP Production Rate. During oxidative phosphorylation, OCR that drives mitochondrial ATP synthesis can be calculated by addition of oligomycin. To convert ATP-coupled OCR into mitochondrial ATP Production Rate, we must know the stoichiometry of ATP phosphorylated per atoms of oxygen reduced. This value is known as the P/O ratio. An average P/O value of 2.75 was validated that accurately represents cell experimental conditions where cells oxidize a mixture of available fuels.

ATP production rate analysis revealed that total ATP (mitochondrial+ glycolytic ATP) was higher in BOHB-treated samples compared with their

respective controls **(Figure 8 I)**, these findings are consistent with mitochondrial respiration and glycolytic acidification data. However, this increase reached statistical significance only in the HG condition. Notably, the rise in total ATP was not driven by glycolytic ATP generation but was instead attributable to a selective increase in mitochondrial derived ATP, although statistically significant only in HG samples.

The observed increase in mitochondrial ATP in BOHB-treated high-glucose samples is consistent with elevated ATP-linked respiration measured via OCR, indicating enhanced mitochondrial ATP synthesis capacity **(Figure 8 I).**

3.4 Effects of β-hydroxybutirrate on mitochondrial biogenesis and content related gene expression

To further investigate the molecular mechanisms underlying BOHB-induced metabolic changes, we analysed the expression of a set of genes associated with mitochondrial biogenesis and mitochondrial content/function.

PGC1α: A master transcriptional co-activator of mitochondrial biogenesis, PGC1α activates downstream transcription factors such as NRF1/NRF2, which promote the expression of mitochondrial proteins and the generation of new mitochondria.

TFAM: A direct target of PGC1α, TFAM regulates mitochondrial DNA (mtDNA) transcription and replication, facilitating mitochondrial genome maintenance and protein synthesis.

MT-ND1: A mitochondrial-encoded subunit of complex I, MT-ND1 expression reflects mitochondrial gene transcription and can indicate increased mitochondrial content or enhanced respiratory capacity.

Citrate Synthase (CS): A key enzyme of the tricarboxylic acid (TCA) cycle, CS is commonly used as a marker of mitochondrial mass.

- PGC1α was significantly upregulated in BOHB-treated neurons in both HG and LG conditions, suggesting BOHB-induced robust activation of the mitochondrial biogenesis program **(Figure 8 L).**
- TFAM expression increased in both HG and LG neurons upon BOHB treatment, but the upregulation reached statistical significance only in HG + BOHB, suggesting stimulation of mtDNA transcription and replication with potential modulation by glucose availability **(Figure 8 L)**.
- MT-ND1 was increased in both HG + BOHB and LG + BOHB samples, consistent with enhanced mitochondrial gene expression and likely an expansion or reinforcement of the mitochondrial compartment **(Figure 8 L)**.
- Citrate Synthase (CS) was elevated in the presence of BOHB under both HG and LG conditions, indicating increased mitochondrial content and/or metabolic capacity **(Figure 8 L)**.

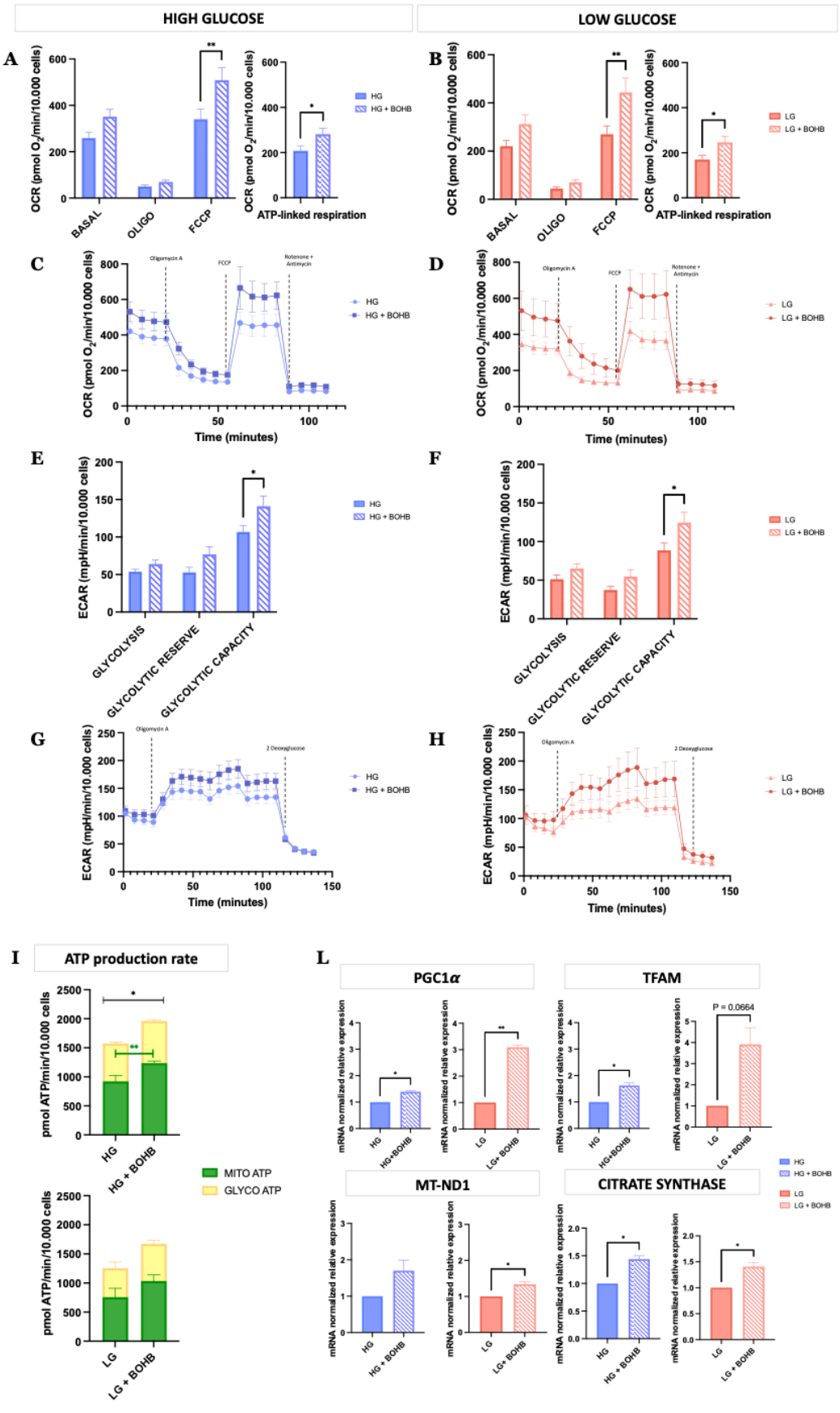
HIGH GLUCOSE
LOW GLUCOSE
A
B
C
D
E
F
G
H
I
L
OCR (pmol O2/min/10.000 cells)
BASAL
OLIGO
FCCP
ATP-linked respiration
HG
HG + BOHB
LG
LG + BOHB
Time (minutes)
Oligomycin A
FCCP
Rotenone + Antimycin
ECAR (mpH/min/10.000 cells)
GLYCOLYSIS
GLYCOLYTIC RESERVE
GLYCOLYTIC CAPACITY
2 Deoxyglucose
ATP production rate
pmol ATP/min/10.000 cells
MITO ATP
GLYCO ATP
PGC1α
TFAM
MT-ND1
CITRATE SYNTHASE
mRNA normalized relative expression
P = 0.0664

Figure 8 histograms in **(A)** and **(B)** show OCR HG (high glucose) and LG (low glucose) values respectively normalized to 10.000 cells. Basal is the resting OCR; Oligo is the OCR measured after the addition of the ATP synthase inhibitor oligomycin; FCCP is the OCR measured after the addition of the uncoupler FCCP eliciting the maximal respiratory capacity. The shown OCRs were corrected for the residual OCR measured after the addition of the CI inhibitor Rotenone and CIII inhibitor Antimycin. N = 4 biological replicates; Correspondent statistical analysis was conducted using ordinary two-way ANOVA, followed by Bonferroni's multiple comparison test, all data are presented as mean ± SEM; *p < 0.05; **p < 0.005; ***p < 0.0005; ATP-linked respiration is also shown in the respective panels. Data are presented as mean ± SEM. Statistical significance for ATP-linked respiration was assessed using an unpaired Student's t-test. Differences were considered statistically significant when *p < 0.05. **(C)** and **(D)** show representative OCR traces. **(E)** and **(F)** histograms of ECAR values in HG and LG condition with or without BOHB. EGlyc (Glycolysis), resting ECAR; Reserve, difference between ECAR measured in the presence of oligomycin and under resting conditions; Capacity, ECAR measured after the addition of oligomycin and refers to the maximal glycolytic activity with the OxPhos inhibited. The shown ECARs were corrected for the 2-DG-insensitive ECAR. Correspondent statistical analysis was conducted using ordinary two-way ANOVA, followed by Bonferroni's multiple comparison test, all data are presented as mean ± SEM; *p < 0.05; **p < 0.005; ***p < 0.0005; **(G)** and **(H)** show representative ECAR traces. **(I)** Bar graph showing relative ATP levels generated through mitochondrial (green) and glycolytic (yellow) pathways under the four experimental conditions. Data are presented as mean ± SEM. Statistical significance was assessed using ordinary two-way ANOVA followed by Bonferroni's multiple comparison test *p < 0.05; **p < 0.005; ***p < 0.0005; **(L)** histograms showing the relative mRNA expression of ***PGC1α, TFAM, MT-ND1 and CS*** (normalized to TATA-binding protein (TBP); Statistical analysis was conducted using a t-test, *a*ll data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

3.5 Analysis of cognitive outcomes following a ketogenic dietary intervention

Thirty undergraduate students (23 women and 7 men; mean age = 23.7, standard deviation = ± 6.3) completed an online screening survey. Out of these, 8 participants (6 women and 2 men; mean age = 22.9; standard deviation = ± 6.9) completed all phases of the research. This included two

cognitive assessments (T0 and T1) conducted immediately before and after three weeks of an isocaloric ketogenic diet. Participants had similar sleep patterns (MEQ score between 31 and 69), low anxiety levels (STICSA score < 42) and good sleep quality (GSAQ score < 30) (**Table 2**).

	Age	MEQ	GSAQ	STICSA(t)
Mean	22.9	55.25	22.13	33
SD	6.9	8.6	3.27	5.6

Table 2. Descriptive Statistics (N=8).

MEQ= Morningness-Eveningness Questionnaire; GSAQ= Global Sleep Assessment Questionnaire; STICSA= State-Trait Inventory of Cognitive and Somatic Anxiety.

None of the participants engaged in intensive sports activity (< 3 hours a week) or took any medications or supplements. Additionally, participants had similar dietary habits (consuming < 3 sugary and alcoholic drinks per week and < 3 coffees per day) and had passed at least four exams in the last academic year.

As shown in **Table 3**, participants at T1 performed faster in the DST (number of items reported correctly; BF_{10} = 25.303), the TMT (part A, BF_{10} = 3.931), the StroopT (part B, BF_{10} = 7.115; part C, BF_{10} = 3.184) and in solving the mathematical expressions of the Aospan task (BF_{10} = 45.075), compared to T0. However, accuracy performance was lower in span absolute score (the sum of all correctly recalled set sizes, BF_{10} = 4.080) and in solving mathematical expressions of the Aospan task (BF_{10} = 6.512) at T1 compared to T0 (**Table 3**). Regarding hippocampal function, the RALT scores remained stable, showing no substantial evidence of change between T0 and T1 (**Table 3**). Finally, state anxiety measured with the STICSA did not differ between T0 and T1 (**Table 4**). To confirm the results, the same analyses were recomputed by changing the Bayes Factor (BF_{01}; H0 over H1) and formulating alternative hypotheses (i.e., T0 > T1 and T0 < T1). In summary, these analyses confirmed the earlier reported results.

	T0		T1		95% CI		BF_{10}
	M	SD	M	SD	Lower	Upper	
DS	6.75	1.58	6.125	1.12	-0.370	1.203	0.621
RALT(i)	47.75	9.16	48.25	10.57	-0.582	0.658	0.347
RALT(d)	11.12	1.88	10.12	2.69	-0.161	1.375	1.160
DST	73.75	11.71	79	12.11	-5.643	-0.371	25.303
AM1(i)	9.75	0.46	9.75	0.46	-0.912	0.945	0.490
AM2(i)	18.87	1.12	18.62	1.59	-0.582	0.699	0.346
AM3(i)	26.62	3.33	27.62	1.40	-0.886	0.386	0.454
AM1(t)	23.68	10.40	22.29	6.86	-0.551	0.670	0.342
AM2(t)	32.31	10.82	29.96	7.97	-0.340	1.004	0.566
AM3(t)	40.36	6.24	37.55	8.31	-0.247	1.116	0.733
TMT(A)	31.17	7.99	25.98	4.22	0.067	1.896	3.931
TMT(B)	52.20	15.39	49.57	16.59	-0.417	0.845	0.457
StroopT(A)	12.75	2.21	11.61	1.55	-0.079	1.388	1.522
StroopT(B)	14.96	1.75	13.75	1.2	0.155	2.052	7.115
StroopT(C)	24.52	6.09	21.44	4.37	0.028	1.627	3.184
Aospan(A)	27.75	19.45	23.5	17.25	0.067	1.862	4.080
Aospan(B)	5.62	3.11	8.37	3.66	-1.973	-0.129	6.512
Aospan(C)	2680.32	1065.97	2121.78	843.05	0.464	3.653	45.075

Table 3. Bayesian Wilcoxon signed-rank results.

DS= Digit Span; RALT= Ray Auditory Learning Test, (i) immediate, (d) deferred; DST= Digit Symbol Test; AM= Attentional Matrices, (i) item, (t) time; TMT= Trial Making Test, (A) part A, (B) part B; StroopT= Stroop Test, (A) words (B) colored squares, (C) color words in incongruent colors; Aospan= Automated Operation Span Task, (A) Span Absolute Score, (B) Math Errors, (C) Math time.

	T0		T1		95% CI		BF_{10}
	M	SD	M	SD	Lower	Upper	
STICSA	32.37	6.18	30.5	5.15	-0.283	1.082	0.646

Table 4. Anxiety state scores (STICSA).

Discussion and conclusion

The present study established a simplified and reproducible protocol for generating human hippocampal neurons from induced pluripotent stem cells (hiPSCs) and used this platform to investigate the metabolic effects of β-hydroxybutyrate (BOHB) on neuronal differentiation, structural maturation and metabolic function. Overall, the results provide new insights into how ketone metabolism influences neuronal development and bioenergetics and highlight the value of hiPSC-derived hippocampal neurons as a translationally relevant model.

Establishment of a robust and accessible hippocampal neuron differentiation model

A major accomplishment of this work was the development of a cost-effective and scalable protocol for deriving hippocampal neurons exhibiting both immature and mature phenotypes. The ability to efficiently generate hippocampal-like neurons *in vitro* offers substantial advantages over traditional animal-based models, which are constrained by interspecies differences, high maintenance costs and the need for specialized facilities. hiPSC-based systems overcome these limitations by providing human-relevant physiological context, enabling controlled and reproducible experiments. The preservation of an active neurogenic niche, evidenced by the coexistence of DCX and mature neuronal markers (MAP2/NeuN) further strengthens the utility of this model for studying neurogenesis, neuronal maturation and disease processes that specifically affect hippocampal circuitry.

What we first found was that maintaining cells in low-glucose (5 mM) conditions did not impair differentiation, neuronal viability or hippocampal identity when compared to the standard high-glucose condition (17.5 mM). This demonstrates that those two glucose concentrations are compatible with efficient neurogenesis, so this establishes a metabolic baseline suitable for downstream supplementation studies, including ketone treatment.

Effects of BOHB on neuronal differentiation and structural maturation

BOHB did not alter neuronal lineage specification or neuronal yield, as indicated by the stability of neuronal markers such as NeuN. However, a significant increase in MAP2 expression was observed in BOHB-treated cells. Because NeuN remained unchanged, the increase in MAP2 cannot be attributed to greater neurogenesis but instead reflects enhanced dendritic complexity or arborization. This interpretation is consistent with literature demonstrating that upregulation of regulatory pathways for mitochondrial

biogenesis is linked to increased dendritic spine formation, dendritic complexity and synaptic maturation. In particular, PGC-1α overexpression in cultured hippocampal neurons increases dendritic spine density and enhances molecular maturation of synapses; conversely, knockdown of PGC-1α inhibits spinogenesis and synaptogenesis[63].

Linking mitochondrial biogenesis, mitochondrial distribution and dendritic maturation

A coherent interpretation of our findings emerges when considering the well-established coupling between neuronal structural complexity and mitochondrial demand. Pacelli et al. demonstrated, in dopaminergic neurons, that increased axonal and dendritic arborization, together with a higher number of synaptic terminals, substantially elevates energetic requirements due to the costs of action-potential conduction and activity-dependent vesicle cycling[64]. In their model, neurons with more elaborate morphologies exhibit a stronger dependence on mitochondrial biogenesis pathways, with PGC-1α acting as a central regulator to scale mitochondrial content and distribution to meet compartment specific energetic needs. This conceptual framework closely parallels our observations in BOHB-treated hippocampal neurons. The increased MAP2 signal detected in our cultures is indicative of enhanced dendritic maturation, while the elevated expression of PGC-1α, TFAM, MT-ND1 and CS supports the conclusion that BOHB stimulates mitochondrial biogenesis and increases mitochondrial mass. Because mitochondrial supply must expand proportionally with neurite outgrowth, the concurrent enhancement of dendritic complexity and mitochondrial gene expression strongly suggests that ketone supplementation promotes neuronal structural development by boosting mitochondrial capacity. Together with the ATP production rate data, these findings indicate that BOHB not only expands mitochondrial content but also enhances the rate at which mitochondria can supply ATP to energetically demanding compartments, such as dendrites and synapses.

This interpretation is further reinforced by our bioenergetic data. BOHB supplementation increased basal, oligomycin induced-OCR as well as FCCP-stimulated respiration. The elevation in FCCP-stimulated OCR particulary, indicates that BOHB-supplemented neurons have a greater capacity to meet increased energetic demands or respond to metabolic stress. This effect, previously observed only in primary cortical neuron cultures[65], reflects a stronger and more sustained maximal uncoupled respiration, demonstrating that BOHB enables neurons to respond more efficiently to energetic challenges.

These functional enhancements are consistent with the notion that neurons undergoing dendritic expansion require a larger, more adaptable mitochondrial pool to sustain elevated energetic and biosynthetic requests. Taken together the convergence of our structural, transcriptional and metabolic data aligns with and extends the model proposed by other groups, supporting the idea that neuronal morphological maturation and mitochondrial biogenesis are deeply interconnected processes[63,64] and that metabolic interventions, such as BOHB supplementation, can bias this relationship toward increased neuronal complexity and functional resilience.

Importantly, mitochondrial biogenesis and dendritic maturation are not independent events but are mechanistically intertwined. Neurons with their highly compartmentalized architecture comprising soma, dendrites, axons and synapses depend on both the generation of new mitochondria and their precise distribution across subcellular compartments to meet local ATP demands, regulate calcium buffering and enable essential biosynthetic processes. Indeed, in primary cultured hippocampal neurons, PGC-1α overexpression has been shown to increase the number and density of mitochondria within dendrites (and axons), indicating that mitochondrial biogenesis directly enhances mitochondrial occupancy of neuronal processes. The same manipulation also promotes dendritic spine formation and synaptic molecular differentiation, leading to increased

expression of postsynaptic markers (e.g., PSD95), presynaptic proteins (e.g., Synapsin I) and improved vesicle recycling. Conversely, PGC-1α knockdown reduces mitochondrial density in dendrites, decreases ATP availability and sharply lowers spine density and synaptic marker expression[63].

Together, these observations reinforce the principle that mitochondrial biogenesis is functionally coupled to neuronal maturation: the development and maintenance of dendritic arborization, spines and synapses requires a robust, appropriately distributed mitochondrial pool. In this context, the increased dendritic MAP2 signal observed in our BOHB-treated hiPSC-derived hippocampal neurons is consistent with a scenario in which BOHB stimulates mitochondrial biogenesis and promotes increased mitochondrial mass and distribution, particularly within dendritic compartments, thereby supporting structural maturation and potentially enhancing synaptic connectivity.

In this model, BOHB promotes a coordinated enhancement of neuronal structural and mitochondrial metabolism. While differentiation and lineage specification remain unaffected, dendritic architecture and mitochondrial function are significantly improved. This dual effect is particularly relevant for hippocampal neurons, which rely heavily on oxidative phosphorylation and require extensive dendritic arborization to form complex synaptic networks. Such improvements may underlie cognitive benefits associated with metabolic interventions (e.g., ketogenic diets or ketone supplementation) reported in various preclinical and clinical studies. Moreover, the hiPSC-derived hippocampal system used here provides a powerful platform for dissecting human-specific metabolic effects on neuronal development, dendritic maturation, mitochondrial biogenesis and synaptic plasticity.

This work demonstrates that BOHB enhances dendritic maturation and mitochondrial biogenesis in human hippocampal neurons while preserving normal neurogenesis. These findings provide mechanistic insight into how

ketone bodies support neuronal structure and function and highlight the potential of metabolic interventions to modulate neuronal plasticity. The study also validates a robust hiPSC-based model that can facilitate future investigations into metabolic regulation of hippocampal physiology and pathology.

Ketogenic dietary intervention on cognitive performance of undergrade students

This study aimed to explore the cognitive effects of an isocaloric ketogenic diet on a group of healthy young adults. The results showed that participants performed the DST, the TMT, the StroopT and solved mathematical operations in the Aospan task faster at T1 than at T0. However, they performed the Aospan task less accurately. Indeed, participants had a lower absolute span score and solved fewer mathematical expressions correctly at T1 than at T0. Furthermore, participants' anxiety levels did not differ between baseline and follow-up.

The findings of this exploratory study indicate that ketosis may have differential effects on various components of cognitive functioning. Specifically, we observed a beneficial impact of ketosis on visual–motor cognition and processing speed, evidenced by reduced completion times in certain cognitive tests at T1 compared to T0. These outcomes are partially consistent with previous research[66,67], which also reported enhanced speed in attention and memory tasks following a low carbohydrate diet. Several mechanisms have been proposed to explain cognitive improvements associated with ketogenic dietary regimens. For instance, ketone bodies have been shown to enhance the expression of Brain-Derived Neurotrophic Factor (BDNF)[68], an essential regulator of synaptic long-term potentiation (LTP), a process closely linked to improved cognitive abilities in adulthood[69].

In contrast, the diet worsened participants' accuracy on the Aospan task, suggesting a deterioration of higher cognitive functions in our sample. An increase in physiological stress following a ketogenic diet has previously been reported and we think that stress-related hormonal responses may

play a key role in this context, as acute corticosteroid administration has been shown to selectively impair performance on working memory tasks, while leaving declarative memory and low working memory load vigilance tasks relatively unaffected[70]. In line with these findings, participants in our study showed reduced accuracy on the Aospan task, suggesting that the ketogenic diet may have negatively affected higher order cognitive functions requiring substantial working memory capacity.

Finally, our results are in contrast with those of Shaw et al.[71], who reported no changes in cognitive functions after the ketogenic diet in a small sample of military personnel. In this case, the results of the studies likely differ due to the use of different cognitive tasks. The Aospan task used in the present study requires reporting the correct sequence of letter presentation and solving mathematical expressions simultaneously. The high commitment of cognitive resources required by the task might make it a more sensitive tool for studying cognitive functions in experimental settings with healthy participants. Instead, in the study by Shaw et al.[71], the working memory task used was a standard one-back, which requires remembering the last number presented and reporting whether it is the same as the current one.

Given the exploratory nature of this study, no formal a priori power analysis was conducted to determine the required sample size. The sample size of 8 participants should therefore be considered a limitation of the present work. The effect sizes observed in this study may serve as a basis for formal power calculations in future.

However, by limiting our participants to healthy, normal-weight individuals free from chronic conditions, obesity and a non-aged sample, we accounted for several important confounding factors in existing studies. Furthermore, we controlled for important psychological and nutritional aspects to exclude further confounding effects on cognitive functions. A second limitation of the present study is the lack of an isocaloric control group with a different macronutrient distribution. This constitutes a potential confounder, making it difficult to attribute the observed effects exclusively to ketosis. Future studies should therefore employ randomized, isocaloric and

preferably crossover designs to disentangle the specific contributions of macronutrient composition and total energy intake.

Additionally, although participants who completed the dietary treatment did not report any side effects, we observed a high dropout rate among participants. This pattern is consistent with previous reports of adherence challenges in ketogenic diet interventions[72]. It is therefore possible that participants who did not attend the second cognitive assessment experienced difficulties in adhering to the dietary plan.

Furthermore, inter-individual variability in ketone metabolism is likely to have introduced an additional layer of complexity in the interpretation of the results. Circulating BHB levels in the general population are influenced by a complex interplay of metabolic, hormonal, and lifestyle factors, including sex, hormonal status and age[73]. Even under controlled dietary conditions, individuals following the same ketogenic protocol may achieve substantially different degrees of ketosis. In the present study, dietary ketosis was monitored weekly using urinary ketone strips, which confirmed adherence to the dietary protocol. However, urinary measurements provide only a semi-quantitative estimate and do not fully capture inter-individual differences in circulating BHB levels. This variability may have contributed to the heterogeneity in cognitive outcomes observed in the present sample and further limits the generalizability of the findings. Related to this, an additional source of inter-individual variability in the present study is the lack of information on participants' menstrual cycle phase. Given that the sample was predominantly composed of women (75%), hormonal fluctuations across the menstrual cycle may have introduced variability in cognitive performance between the two assessment time points. Although the effects of menstrual cycle phases on cognitive function reported in the literature are mixed, and a recent meta-analysis did not observe systematic and robust evidence of significant changes in cognitive performance due to menstrual cycle phases[74], this factor cannot be excluded as a potential confound. Future studies should therefore measure plasma BHB levels as a continuous variable and include it as a covariate in statistical analyses, and

should measure and control for menstrual cycle phase, ideally with hormonal confirmation.

Based on our findings, future studies should investigate different components of cognitive functions. In addition to the dissociation between visual motor and cognitive components, both explicit and implicit aspects of cognitive functions should be investigated. Evidence of a dissociation between performance on implicit/explicit tasks about chronotype is reported in the literature, as well as an important relationship between ketogenic diet, chronobiology and cognitive functions[75]. Furthermore, the role of stress in participants in dietary studies should be further investigated through self-reported and physiological measures concerning the diet. Stress can affect cognitive functions, and increases in physiological stress have been reported during a ketogenic diet[71].

Our findings indicate that the ketogenic diet influenced cognitive functioning by enhancing visuomotor performance and processing speed, while simultaneously reducing accuracy in a complex working memory task. This latter result is particularly relevant, as strong working memory capacity is associated with improved attentional control and plays a crucial role in learning processes. Therefore, the observed decline in working memory performance may carry meaningful implications for both educational and research environments.

Bridging cellular mechanisms and cognitive outcomes: a translational perspective

The present thesis advances our understanding of how ketone body metabolism shapes neuronal function and cognitive performance across two complementary levels of analysis. At the cellular level, we demonstrate that BOHB directly promotes dendritic maturation and mitochondrial biogenesis in human hiPSC-derived hippocampal neurons, establishing a mechanistic link between ketone availability, mitochondrial expansion and structural neuronal maturation.

This mitochondrial functional enhancement provides the energetic substrate that could potentially sustain the dendritic complexity. However,

while our *in vitro* model, we see a clear bioenergetic gain, our exploratory clinical study reveals a more nuanced picture. Diet-induced ketosis in healthy young adults produced domain-specific effects primarily targeting visuomotor processing speed and executive functions (as seen in TMT and Stroop tasks), rather than purely hippocampal-dependent memory (RALT). The lack of significant improvement in RALT scores suggests that while the 'metabolic machinery' is optimized, this may not immediately translate into enhanced long-term memory consolidation in a healthy cohort, possibly due to a ceiling effect or the need for a longer intervention period.

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