2022

Constitutive Cell Proliferation and Neurogenesis in the Hypothalamic Sensory Circumventricular Organs of Adult Rats

Suijian Zhou
Integrated Program in Neuroscience
McGill University, Montreal
August 2022

A thesis submitted to McGill University in partial fulfillment of the requirement of the degree of Master of Science.

Table of Contents

Abstract

The organum vasculosum of laminae terminalis (OVLT) and subfornical organs (SFO) are sensory circumventricular organs (CVOs) involved in the regulation of body fluid and electrolyte balance as well as autonomic cardiovascular response. Recent studies showed that the secretory CVO, median eminence (ME), contains progenitor cells and can undergo adult neurogenesis and therefore serves as an adult neural stem cell (NSC) niche. Furthermore, cell proliferation and neurogenesis in the ME are associated with its regulation of energy metabolism. However, whether sensory CVOs such as the OVLT and SFO also serve as adult NSC niches and how cell proliferation in the OVLT and SFO is linked to their physiological functions remain unknown. In this study, we used bromodeoxyuridine (BrdU) and Ki67 to label newborn cells and employed immunohistochemistry to identify proliferating cell types in adult rats. We found that the OVLT and SFO contain highly proliferative cell types capable of constitutive proliferation and represent an additional site of adult neurogenesis. Further, since both the OVLT and SFO are involved in regulating body fluid and electrolyte balance and autonomic cardiovascular function, we investigated the effect of high NaCl diet-induced hypertension on cell proliferation and adult neurogenesis. We observed an increase in cell proliferation and adult neurogenesis in the OVLT and SFO in response to high NaCl diet-induced hypertension. Our results suggest that the OVLT and SFO are adult NSC niches that are responsive to perturbation on fluid balance.

Résumé

L'organum vasculosum des laminae terminalis (OVLT) et les organes sous-forniques (SFO) sont des organes sensoriels circumventriculaires (CVO) impliqués dans la régulation de l'équilibre des fluides corporels et des électrolytes ainsi que dans la réponse cardiovasculaire autonome. Des études récentes ont montré que le CVO sécrétoire, l'éminence médiane (ME), contient des cellules progénitrices et peut subir une neurogenèse adulte et sert donc de niche de cellules souches neurales (NSC) adultes. De plus, la prolifération cellulaire et la neurogenèse dans le ME sont corrélées à sa régulation du métabolisme énergétique. Cependant, la question de savoir si les CVO sensoriels tels que l'OVLT et le SFO servent également de niches NSC adultes et comment la prolifération cellulaire dans l'OVLT et le SFO est liée à leurs fonctions physiologiques reste inconnue. Dans cette étude, nous avons utilisé la bromodésoxyuridine (BrdU) et Ki67 pour étiqueter les cellules nouveau-nées et utilisé l'immunohistochimie pour identifier les types de cellules proliférantes chez les rats adultes. Nous avons constaté que l'OVLT et le SFO subissent une prolifération cellulaire constitutive, contiennent des types de cellules hautement prolifératives et hébergent la neurogenèse adulte. De plus, étant donné que l'OVLT et le SFO sont tous deux impliqués dans la régulation de l'équilibre hydro-électrolytique et de la réponse cardiovasculaire autonome, nous avons étudié l'effet de l'hypertension induite par le NaCl sur la prolifération cellulaire et la neurogenèse adulte. Nous avons observé une augmentation de la prolifération cellulaire et de la neurogenèse adulte dans l'OVLT et le SFO en réponse à l'hypertension induite par le NaCl. Nos résultats montrent que l'OVLT et le SFO sont des niches NSC adultes qui répondent à l'hypertension induite par le NaCl.

Acknowledgement

This project was initially proposed by Dr. Masha Prager-Khoutorsky in her NSERC grant "Understanding the role of tanycytes in the mammalian brain" as Aim #3: Investigating the stem cell potential of OVLT tanycytes. In the summer of 2019, receiving the NSERC Undergraduate Research Award, I joined Dr. Masha Prager-Khoutorsky's lab and started working on this project. By the time I joined the lab, Jeremy Baylou, a graduate student from the Bordeaux International Master of Neurosciences Program, was already working on this project for months. Unfortunately, Jeremy could not complete the experiments in the designated time and left this project at a very preliminary stage. My initial training was mainly given by Graham Lean, Amirah-Iman Hicks, and Dr. Zsuzsanna Barad as well as the McGill Animal Research Facility at McIntyre. Later, I continued working on this project as my undergraduate Physiology Honours research project and my master's thesis project. My master's study was funded by McGill's Integrated Program in Neuroscience, Canada Graduate Scholarships – Master's Program, and Fonds de recherche – Sante – Master's Training. By the time I started writing my dissertation essay, I had published a third author paper in Frontiers Cellular Neuroscience and a second author on Methods Molecular Biology and currently I am at the final stage of preparing my first author paper, summarizing this project. I had also presented a poster at the 14th Canadian Neuroscience Meeting and a poster at the 2021 Society of Neuroscience Annual Meeting. My accomplishments are indispensable to the supervision of Dr. Masha Prager-Khoutorsky and other supporting people that I will mention below.

First, I would give my thanks to my supervisors. I would like to thank Dr. Anastasia Nijnik, who supervised me on my PHGY 396 research project cloning the histone deubiquitinase protein MYSM1 and its mutant forms into plasmids for bacterial expression. Ana opened the

door of basic science research to me when I was an inexperienced U1 Physiology student. I was able to join the compassionate research community and learned how science is done in a laboratory setting. Further, I would like to thank my Physiology Honours and master's Thesis supervisor, Dr. Masha Prager-Khoutorsky. Joining Masha's Lab, I was entrusted with an independent research project and worked from a preliminary stage to the publication stage. I learned a lot about research, collaboration, communication, problem-solving, and critical thinking. Nonetheless, I believe the most valuable quality I have obtained from this journey is that I became truly passionate about research and believe the advancement of scientific knowledge is fundamental to the progress of humanity. I really appreciate Masha's generousness, kindness, and patience which tolerated my sometimes stubborn and blunt personality and allowed me to have the freedom to conduct research driven by my curiosity. I would also like to give my thanks to my advisory committee members, Dr. Jo Anne Stratton and Dr. Maia Kokoeva, who provided valuable feedback and guidance to my project. Additionally, I would like to thank Dr. Arkady Khoutorsky and Dr. Charles Bourque, who collaborate with our lab.

Secondly, I would like to thank my lab mates and friends. Jieyi, Amirah, Banruo, and Ning, thank you for providing me with emotional and technical support on a day-to-day basis. Also, thanks to other lab members from the Prager-Khoutorsky's lab and Khoutorsky's lab: Misha, Safiya, Alberto, Simona, Anna, Graham, Zsuszanna, Weihua, Alba, Nichole, Calvin, Patricia, Mehdi, Kevin, Shannon, and Carolina. These talented people made our working environment warm and amiable. I would especially like to thank Vicky Zhu, my roommate and very close friend, who is always supportive of my dream and considerate of my emotions. Her tidiness made our common home where two human beings, a cat, and a bird live always neat and

pleasant. I would also like to express my gratitude to my friends who enriched my personal life: Virna, Anita, Sally, Inga, Catherine, Xiaohan, Steve, Ryan, Roger, and William.

Importantly, I would like to thank my mom, dad, and sisters Michelle and Cynthia, who have given me unconditional love and financial support throughout my life. Also, thanks to Nemo and Lotti for being my pets, accompanying me with their lovely furs and feathers. Lastly, I would like to thank the following restaurants in Montreal that gave me comfort through the satiety pathways: Chicha Desserts, Zoe Dessert, Panda Hotpot (closed), Opiano, Dolan Uyghur, Pho Nguyen, YEN Cuisine, Cocodak, Le Coq Frit, Mon Ami, Imperial Restaurant, Li Yuan Beijing Duck, Zhonghua Noodles, Mango Mango (closed), Mei Wei Yuan, Shi Li Xiang, Li Ma Ma, Yin Ji Chang Fen, Egina BBQ, Gateau Brise, Presto Tea, Coco, Gazo (closed), Shuyi Tealicious, and Nos Thes.

Contribution of Authors

This study was proposed and designed by Dr. Masha Prager-Khoutorsky. Suijian Zhou performed the experiments, analysed the data, and drafted the thesis. Specifically, Suijian Zhou performed intraperitoneal injections, transcardiac perfusion, and tissue collection, maintained animals during high salt diet protocols, monitored and measured animal behaviors, collected blood samples, measured plasma osmolality, prepared brain tissues, conducted immunohistochemistry experiments, developed and optimized antigen-retrieval protocols and animal treatment, acquired images using confocal microscopy, analyzed data using Zeiss Zen, Microsoft Excel, and Prism Graphpad, reviewed the literature to troubleshoot and optimize protocols, and wrote the draft for the paper in preparation.

Symbols and Abbreviations

ACC	anterior cingulate cortex
ANG II	angiotensin II
AP	area postrema
Arc	arcuate nucleus
BrdU	bromodeoxyuridine
CNS	central nervous system
CSF	cerebral spinal fluid
CVO	circumventricular organ
Dcx	doublecortin
ECF	extracellular fluid
EdU	5-ethynyl-2-deoxyuridine
FGF	fibroblast growth factor
GnRH	gonadotrophin-releasing hormone
HSD	high salt diet
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
ME	median eminence
MnPO	median preoptic nucleus
NFI	nuclear factor I
NG2	neural/glial antigen 2
NPC	neural progenitor cell

NSC	neural stem cell
och	optic chiasma
OLG	oligodendrocyte
OVLT	organum vasculosum of laminae terminalis
PAG	periaqueductal grey
PBN	parabrachial nucleus
PBS	phosphate buffered saline
PFA	paraformaldehyde
PIN	pineal gland
PVA	Anterior nucleus of paraventricular thalamus
PVN	paraventricular nuclei
SEM	standard error of the mean
SFO	subfornical organ
SGZ	subgranular zone
SON	supraoptic nuclei
Sox2	sex-determining region Y – box 2
SVZ	subventricular zone
THAL	thalamus
vBNST	bed nucleus of the stria terminalis
vhc	central hippocampal commissure
VP	vasopressin

1. Introduction and Statement of Problem

For decades, scientists had accepted the no-new-neuron dogma – the idea that there is no new neuron born in the adult brain [1]. It was not until the 1990s that researchers discovered newly formed neurons in the hippocampus of the adult mammalian brain, contradicting the no-new-neurons dogma [1-5]. Following this breakthrough, the subgranular zone (SGZ) of the hippocampal dentate gyrus and subventricular zone (SVZ) on the lateral ventricular wall were characterized as the two major neural stem cell (NSC) niches in the adult mammalian brain that constantly generate new neurons throughout postnatal life [6-8]. Later, adult neurogenesis was discovered in other brain areas, including the hypothalamus [9-11]. In 2005, Kokoeva et al. discovered that adult neurogenesis in the hypothalamus contributes to the regulation of energy balance [12]. Hypothalamic adult neurogenesis was observed adjacent to the median eminence (ME). The ME is one of the circumventricular organs (CVOs), which are specialized brain structures characterized by a high density of fenestrated vasculature located around the third and fourth cerebral ventricles. CVOs can be classified into sensory and secretory CVOs. Sensory CVOs contain sensory neuronal cell bodies capable of sensing blood-borne molecules from the peripheral circulation. Secretory CVOs have secretory neuronal terminals secreting hormones and glycoproteins into the peripheral circulation. The organum vasculosum of laminae terminalis (OVLT) and the subfornical organs (SFO) are sensory CVOs. They contain neuronal cell bodies that can sense the osmolality of extracellular fluid (ECF) and respond to changes in plasma osmolality to regulate body fluid and electrolyte balance. In contrast, the secretory CVOs, such as the ME, have neuronal terminals that secrete peptides and hormones into the peripheral blood

circulation. Accumulating studies showed that the secretory CVO - ME harbors adult NSCs [13-15], but the presence of adult NSCs in the sensory CVOs remains poorly studied. Although it has been shown that cell proliferation (the generation of new cells) and neurogenesis (the generation of new neurons) in the ME and its adjacent basomedial hypothalamus influence body weight control [12-14], whether cell proliferation and neurogenesis in the sensory CVOs are also link to physiological functions of the organism remains enigmatic. Thus, the present study aims to decipher whether the OVLT and SFO harbor adult NSCs and progenitor cells and if cell proliferation correlates to their regulation in body fluid and electrolytes homeostasis.

2. Background

2.1 Circumventricular Organs

2.1.1 General Introduction to Circumventricular Organs

CVOs are brain structures located in the hypothalamus and brain stem around the ventricular system. CVOs are among the most vascularized brain regions and contain fenestrated vasculatures, thus lacking a complete blood-brain barrier [16]. Due to the unique fenestrated vasculature, the CVOs are exposed to blood-borne molecules such as ions, amino acids, monoamine transmitters, and larger peptides and hormones. Neurons in the sensory CVOs sense these blood-borne molecules and induce neuronal responses in the CVOs as well as modulate neuronal activities in other brain regions. Peptide-secreting neurons in the secretory CVOs receive neuronal signals and release peptides to the fenestrated blood vessels which further travel into the blood circulation. The adjacent location of the CVOs to the ventricular system allows CVOs to facilitate communication between the peripheral blood circulation, the brain parenchyma, and the ventricular system. This communication enables the CVOs to regulate various homeostatic functions including but not limited to energy metabolism, reproduction, circadian rhythms, arousal, neuroinflammation, and body fluid balance [16, 17]. The CVOs are also unique due to the presence of specialized ependymal cells called tanycytes. In contrast to classical ependymal cells, tanycytes have their cell bodies lining the ventricular wall and long processes extending into the parenchyma. While blood vessels in the blood-brain barrier containing brain areas are enwrapped by astrocytic endfeet, fenestrated blood vessels in the CVOs are encased by tanycytic endfeet. Cilia in tanycytes also differ from classical ependymal

cells, as ependymal cells are motile multi-ciliated, while tanycytes in the ME are either uni- or bi-ciliated that potentially also play sensory roles [18].

There are three sensory CVOs: the OVLT, SFO, and area postrema (AP) (Fig. 1), which contain neuronal cell bodies that sense blood-borne molecules. Both the OVLT and SFO are centrally located and embedded in the anterior wall of the third ventricle of the hypothalamus. The OVLT is located dorsally to the optic chiasma and the SFO is at the ventral surface of the fornix adjacent to the interventricular foramina. Both the OVLT and SFO are involved in the regulation of body fluid and electrolyte balance as well as autonomic cardiovascular functions [19-21]. The AP is centrally located in the medulla oblongata on the fourth ventricular wall adjacent to the entrance to the central canal [22]. The AP is known as the chemoreceptor trigger zone which senses toxins and other emetic signals in blood to induce vomiting [23].

The secretory CVOs are the ME and pineal gland (Fig. 1), which contain neuronal terminals that secrete peptides to the peripheral circulation. The ME is an integral part of the hypophyseal portal system in the hypothalamus. Therefore, it is involved in the secretions of hypophysiotropic hormones, such as gonadotrophin-releasing hormone (GnRH), corticotrophin-releasing hormone, and growth-releasing hormone. For instance, the GnRH neurons have their neuronal terminals located proximally to the fenestrated blood vessels in the ME. Upon kisspeptin stimulation, GnRH neurons release GnRH into the fenestrated blood vessels, which then reaches the pituitary gland to trigger the release of luteinizing hormone and follicle-stimulating hormone into the peripheral circulation [24]. Importantly, recent studies have shown that the ME plays a prominent role in regulating energy balance. ME contains NG2 glia which are essential to regulating energy metabolism and the ablation of newborn NG2 glia in the ME affects the bodyweight of the animal [13, 25, 26]. The role of ME in energy balance is also

highlighted by its proximity to the arcuate nucleus and dorsomedial hypothalamus, which are involved in regulating energy balance. The pineal gland regulates the sleep-wake cycle by secreting melatonin in a circadian manner to the bloodstream, which is further delivered to other tissues [27].

In addition to sensory and secretory CVOs, a recent review classified the choroid plexus and the subcommisural organ as ependymal CVOs [28]. Both the choroid plexus and subcommisural organ are involved in the production of the cerebral spinal fluid (CSF). However, this classification remains to be further studied, as both brain regions lack central hallmarks of CVOs. For instance, the choroid plexus lacks tanycytes and the subcommissural organ is not highly permeable and does not contain fenestrated blood vessels [29]. The major focus of the present study is the OVLT and SFO – two sensory CVOs involved in the regulation of body fluid and electrolyte balance.

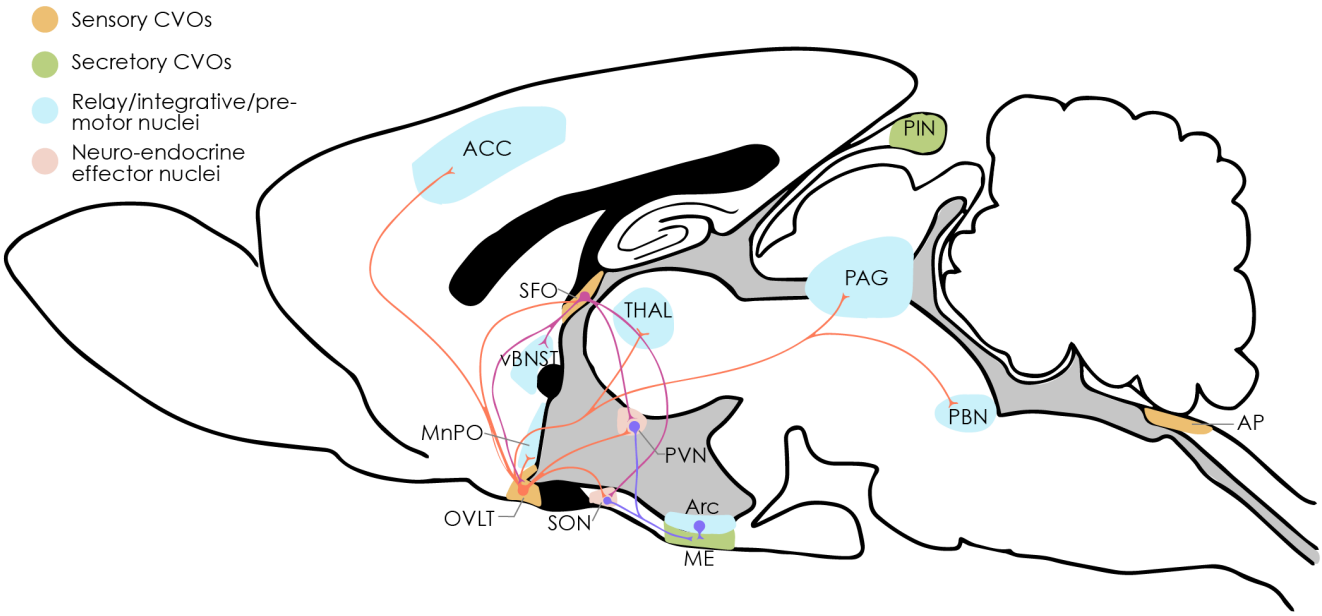

Figure 1. Sagittal illustration of the rat brain depicting the CVOs and relevant structures and nuclei compressed into a single plane. Only structures involved in osmotic regulation and energy balance are shown. Abbreviations: ACC, anterior cingulate cortex; AP, area postrema; Arc, arcuate nucleus; MnPO, median preoptic nucleus; OVLT, organum vasculosum of laminae terminalis; PAG, periaqueductal grey; PBN, parabrachial nucleus; PIN, pineal gland; PVN, paraventricular nucleus; SFO, subfornical organ; SON, supraoptic nucleus; THAL, thalamus, vBNST, bed nucleus of the stria terminalis.

2.1.2 The Organum Vasculosum of Laminae Terminalis

The OVLT is best known as the central ECF osmolality sensor. Neurons in the OVLT can respond to hypertonic NaCl or mannitol solutions and their firing rate is proportional to the ECF osmolality [30-34]. Neurons in the OVLT also express the angiotensin II (ANG II) type 1A receptor (Agtr1a) and therefore respond to angiotensin II (ANG II) stimulations [35-37]. Consistently, various functional studies have demonstrated the central role of the OVLT in sensing ECF osmolality and modifying drinking behavior and autonomic responses. Functional MRI studies have shown that the anterior wall of the third ventricle, where OVLT locates, is activated when humans or other mammals are infused with hypertonic saline [38-40]. Ablation of the OVLT in dogs and sheep impairs the drinking behavior in response to systemic infusion of hypertonic solutions [41, 42]. Furthermore, glutamatergic and GABAergic neurons in the OVLT control drinking behavior in a reciprocal manner, as optogenetic activation of glutamatergic or GABAergic neurons can induce or suppress drinking behavior, respectively [43]. Other studies have shown that optogenetic activation of OVLT Agtr1a-expressing neurons, which are predominately glutamatergic, induces a robust water intake and a rapid and reversible increase in blood pressure [44, 45].

Neurons in the OVLT represent a heterogenous group that express various receptors, including the epithelial Na+ channel, GnRH receptor, leptin receptor [46-48], as well as numerous additional receptors [20]. The expression of these receptors in the OVLT is consistent with its involvement in various homeostatic functions such as body fluid and electrolyte balance, cardiovascular functions, reproduction, circadian rhythms, and thermoregulation [31, 49-53]. OVLT neurons project to multiple brain areas within the hypothalamus, as well as the thalamus

and cortical structures. Neuronal projections to the median preoptic nucleus (MnPO), paraventricular nuclei (PVN), and supraoptic nuclei (SON) are pivotal to the regulation of body fluid balance and autonomic cardiovascular functions. Neurons from the OVLT as well as the SFO project to the MnPO, whose excitation increases water intake and blood pressure [43, 44]. OVLT neurons also project to the PVN and SON which harbor vasopressin- (VP) secreting neurons projecting to the neurohypophysis and regulating diuresis and vascular tone [19]. VP neurons in the PVN and SON receive inputs from the OVLT, MnPO, parabrachial nucleus, and nucleus tractus solitarius, modulating VP release in response to hypotonicity and hypertonicity [54-56]. Additionally, neurons in the OVLT project to the anterior cingulate cortex and thalamus, which, in turn, project to the frontal thirst area, to regulate the emotional and behavioral aspects of drinking [57].

In addition to neurons, the OVLT contains a variety of non-neuronal cell types including tanycytes, pericytes, NG2 glia, oligodendrocytes, endothelial cells, and microglia (Fig. 2), which are discussed in **Section 2.4**. The anatomical structure and cellular composition of the OVLT are comprehensively reviewed in several studies [20, 58, 59]. Nonetheless, the roles of these non-neuronal cell types in regulating body fluid and electrolyte balance remain elusive. One study observed ANG II type 1 receptor – immunoreactive processes in the OVLT, presumably originating from tanycytes, reaching into the retrochiasmatic area, the ventrolateral hypothalamus and the ME [60]. The presence of ANG II receptors in other non-neuronal cell types and their physiological functions remain to be explored.

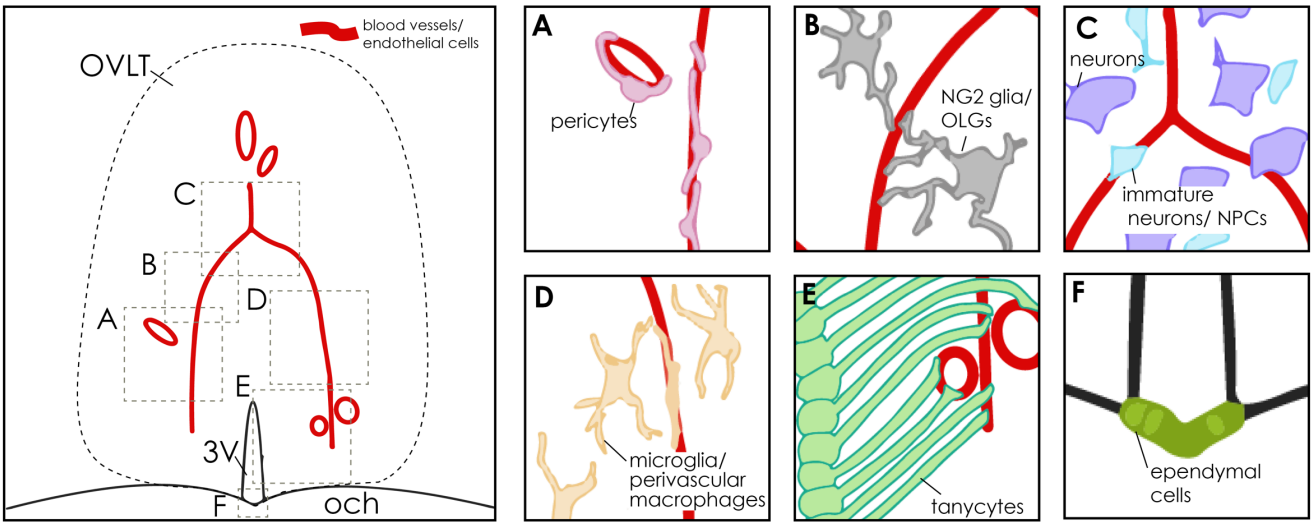

Figure 2. Illustration of the structure and cellular composition of the OVLT in coronal plane. Only cells that are identified in previous studies or the present study in the OVLT are presented. Astrocytes are rarely found in the OVLT, and the blood vessels are enwrapped by tanycytes endfeet instead of astrocytic endfeet. Abbreviations: 3V, third ventricle; NPCs, neural progenitor cells; och, optic chiasma; OLGs, oligodendrocytes; OVLT, organum vasculosum of laminae terminalis.

2.1.3 The Subfornical Organ

The SFO is composed of a heterogeneous group of neurons that express various receptors involved in cardiovascular function (e.g. Agtr1a), energy metabolism (e.g. leptin receptor), reproduction (e.g. estrogen receptor) and immune function (e.g. tumor necrosis factor-alpha receptor) [61-64], as well as other receptors [21]. The expression of these various receptors is consistent with the involvement of SFO in regulating cardiovascular, metabolic, reproductive, and immune functions. One of the best-studied function of the SFO is its role in regulating body fluid and electrolyte balance as well as autonomic cardiovascular functions. Although ablation of SFO alone in sheep does not affect the drinking behavior induced by systematic infusion of hypertonic saline, ablation of the SFO with the OVLT impairs the drinking behavior induced by systematic infusion of hypertonic saline compared to animals in which only OVLT is ablated [65]. Furthermore, studies using electrophysiology and the expression of activity-dependent immediate early genes – c-Fos labeling have shown that neurons in the SFO are activated by hypertonic saline and ANG II [21, 66-70]. According to a recent study using c-Fos labeling, ~13% of neurons in the SFO respond to systematic infusion of hypertonic saline and ~35% respond to systematic infusion of ANG II [21]. Additionally, optogenetic stimulation of glutamatergic neurons or Agtr1a+ glutamatergic neurons in the SFO triggers drinking and/or a rapid and reversible increase in blood pressure, while optogenetic stimulation of GABAergic neurons suppresses drinking behavior [44, 71, 72]. These studies highlight the prominent role of SFO in regulating body fluid and electrolyte balance as well as autonomic cardiovascular response.

Neurons originating in the SFO project to multiple brain regions in the hypothalamus, thalamus, cortex, and brain stem [28]. Neuronal projections to the OVLT, MnPO, PVN, SON,

and the bed nucleus of the stria terminalis (vBNST) are pivotal to the regulation of drinking behavior, autonomic response to fluid imbalance, and salt appetite. Neuronal projections to the OVLT and MnPO modify drinking behaviors and autonomic regulations of blood pressure since optogenetic stimulation of glutamatergic neurons projecting to the OVLT and/or MnPO promotes drinking behavior and elevates blood pressure, while optogenetic stimulation of GABAergic neurons projecting to the OVLT suppresses thirst [44, 72, 73]. Neuronal projections from the SFO to the vBNST regulate salt appetite, as stimulation of glutamatergic neurons in the SFO projecting to the vBNST increases sodium consumption [72]. Similar to the OVLT, neuronal projections from the SFO to the SON and PVN regulate VP release by increasing the firing rate upon ANG II stimulation [68, 69].

The SFO is also enriched in non-neuronal cell types including tanycytes, pericytes, NG2 glia, oligodendrocytes, microglia, and endothelial cells (Fig. 3). The structure and cellular composition of the SFO is reviewed in several studies [21, 58, 74], but the function of non-neuronal cell types and their contribution to the SFO's function in autonomic cardiovascular functions and body fluid and electrolyte balance remains poorly studied. The presence of ANG II receptors in non-neuronal cell types in the SFO remains to be verified.

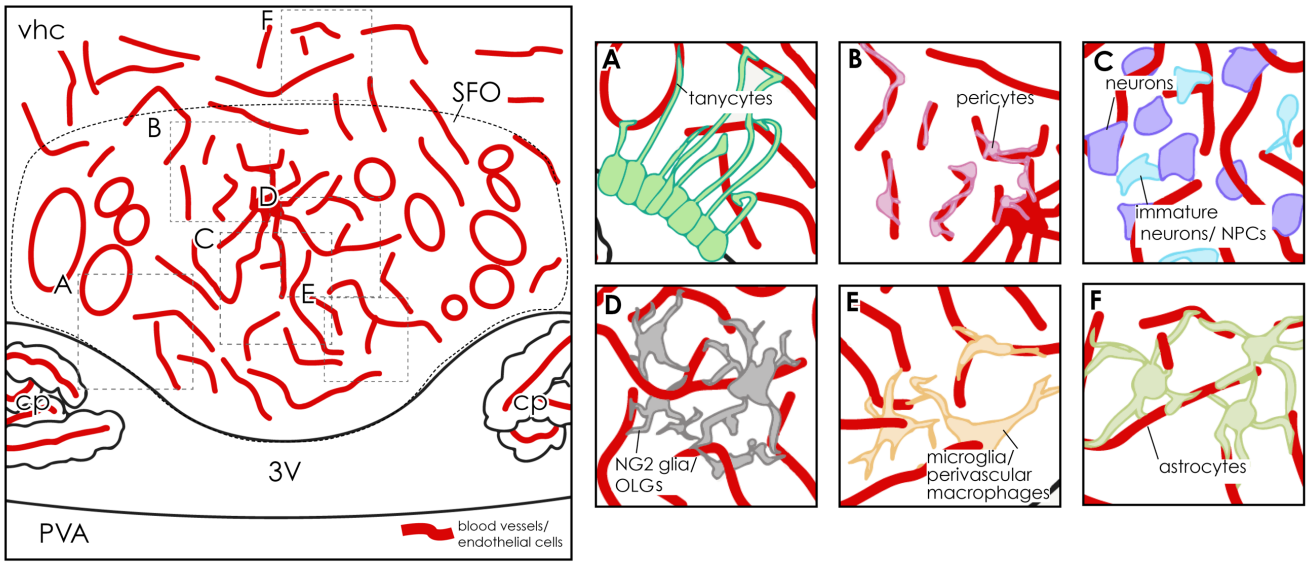

Figure 3. Illustration of the structure and cellular composition of the SFO in coronal plane. Only cells that are identified in previous studies or the present study in the SFO are presented. Astrocytes are sparse in the SFO, and the blood vessels are enwrapped by tanycytes endfeet instead of astrocytic endfeet. Abbreviations: 3V, third ventricle; cp, choroid plexus; NPCs, neural progenitor cells; OLGs, oligodendrocytes; PVA, anterior nucleus of paraventricular thalamus; SFO, subfornical organ; vhc, central hippocampal commissure.

2.2 Neural Stem Cells

Stem cells are defined as cells that are capable of self-renewal and differentiation to other cell types [75]. Stem cells reside in stem cell niches which provide humoral, metabolic, physical, neuronal and paracrine cues to maintain and regulate their stem cell fate [76]. The cell fate of stem cells is regulated by intrinsic signaling pathways, epigenetic regulations, and extrinsic stimuli. NSCs are multipotent stem cells in the nervous system that can give rise to neurons, astrocytes, and oligodendrocytes. In embryonic development, a small number of NSCs known as radial glia constitute the neural tube [77]. These cells undergo rapid symmetric division to expand the NSC pool and asymmetric division to give rise to immediate progenitor cells [77]. Many neurons and glia are not direct progenies of radial glia but are the progenies of immediate progenitor cells [77]. However, radial glia are not presented in the adult brain. Adult NSCs are primarily presented in the SGZ and SVZ and are mostly maintained in a state of reversible cell cycle, or quiescent [78]. Quiescent adult NSCs can give rise to immediate progenitor cells (also known as active NSCs), which further generate neuroblasts and eventually neurons [77].

Importantly, the rate of adult neurogenesis is strongly correlated to the functionality of the brain region which harbors the NSCs. In physiological aging, the number of quiescent NSCs and proliferating progenitor cells in the SVZ and SGZ progressively declines, resulting in fewer newborn neurons in aged animals [79-82]. The progressive decline of cell proliferation in the SVZ and SGZ is accompanied by diminished fine olfactory discrimination and memory formation [80, 83]. This correlation between cell proliferation and functionality also holds true independent of the context of aging as pharmacological suppression of cell proliferation in the SGZ can induce memory deficits [84]. Conversely, increasing the rate of neurogenesis in the

SVZ and SGZ can increase olfactory memory and rescue age-dependent memory formation decline [85, 86].

This correlation between adult NSCs homeostasis and functionality of the adult NSCs niche is also present in the hypothalamus. Central administration of ciliary neurotrophic factor can induce weight loss in obese animals primarily through increasing cell proliferation in the hypothalamus, in which many these proliferative cells express neuronal markers and, in turn, contribute to neurogenesis [12]. Consistent with this finding, the increase in cell proliferation in the hypothalamus induced by a high-fat diet prevents the development of obesity, since the blockage of this cell proliferation accelerates the onset of obesity [87]. Moreover, recently, the hypothalamus has been found to play a pivotal role in regulating the ageing process through hypothalamic NSCs [88, 89]. Similar to the depletion of adult NSCs in the SVZ and SGZ in aged animals, the number of hypothalamic NSCs progressively declines with aging. The number of hypothalamic NSCs is directly correlated to age-related functional decline of the body, as ablation of hypothalamic NSCs accelerates the aging process and shortens lifespan, while implantation of hypothalamic NSCs slows down ageing and prolongs lifespan [88]. In summary, the number of NSCs, the rate of cell proliferation, and the rate of adult neurogenesis in the hypothalamus are linked to the hypothalamic regulation of energy balance and physiological aging.

2.3 Circumventricular Organs: Novel Adult Neural Stem Cell Niches

Increasing evidence has indicated that cells in the CVOs exhibit NSC properties. Subpopulations of cells in CVOs express specific markers commonly found in adult NSCs in the SGZ and SVZ, such as intermediate filaments (e.g. vimentin, nestin, and GFAP) and NSC transcription factors (Sex-determining region Y – box 2 or Sox2) [90]. Cell cultures derived from the OVLT, SFO, ME, and AP can form neurospheres and express neuronal and glial markers when differentiated [90]. Cells obtained from the wall of the third ventricle close to the ME can form neurospheres in cultures, be passaged more than 10 times, express various stem cell markers, and differentiate into several brain cells (e.g. neurons, astrocytes, and oligodendrocytes) in the presence of appropriate growth factors [15, 90]. Nevertheless, *in vivo* and *in vitro* studies have shown that newborn cells in the CVO are more prone to differentiate into glia as opposed to neurons as in the SVZ and SGZ [90, 91]. This tendency to generate glia is potentially due to extrinsic stimulation originating within the CVOs, as heterotopically transplantation of cells derived from the ME and OVLT into the SVZ can integrate into the rostral migratory system and ultimately become mature neurons in the olfactory bulb [92]. This suggests that when exposed to a neurogenic environment, adult NSCs obtained from the CVOs are likely to differentiate into neurons. Lastly, the CVOs contain progenitor cells that are highly proliferative, which are discussed in the following section.

2.4 Progenitor and Highly Proliferative Cells in the Circumventricular Organs

2.4.1 Tanycytes

Tanycytes are specialized ependymal cells found in the CVOs [93]. Tanycytes have a morphology resembling radial glia and are occasionally referred to as hypothalamic radial glia [13]. Tanycytes express high levels of Sox2, the transcription factor essential to maintain self-renewal and pluripotency in stem cells [94, 95]. In the ME, tanycytes are classified into α- and β-tanycytes. α-tanycytes line the lateral ventral walls of the third ventricle and β- tanycytes line the base of the third ventricle. Both α- and β- tanycytes have been shown to have NSC potential, depending on the age of the animal. In postnatal mice, β-tanycytes in the ME are highly proliferative and can give rise to neurons that integrate into the hypothalamic neuronal network that regulate energy balance as well as astrocytes [13]. However, in adult mice, α- but not β-tanycytes are proliferating progenitor cells, as α- tanycytes can give rise to β- tanycytes and differentiate preferentially to astrocytes and a small number of neurons [15]. Moreover, the NSC potential of tanycytes is regulated by both intrinsic genetics and extrinsic stimulation, as both disruption of NFI (nuclear factor I) family of transcription factor regulations and extrinsic stimulation by fibroblast growth factor 2 can promote proliferation and neurogenesis of tanycytes [15, 96].

2.4.2 Neural/Glial Antigen2 Glia

Neural/glial antigen2 (NG2) is a chondroitin sulfate proteoglycan expressed in incompletely differentiated precursors and has been suggested to promote cell proliferation and

migration that is critical to the maturation of immature progenitor cells [97]. NG2 expressing cells represent a heterogeneous population of precursors in the brain, including protoplasmic astrocyte precursors during embryonic development, oligodendrocyte precursors, and pericytes [98-100]. NG2 glia have the potential to become neurons *in vivo* and *in vitro* in both SGZ and SVZ [101-103]. In the hypothalamus, NG2 glia have a high regeneration capacity, comprising more than 80% of the newborn cells [26]. These NG2 glia can continuously give rise to oligodendrocytes and a small number of neurons in the hypothalamus of the adult brain [26]. Consistently, a fraction of NG2 glia in the hypothalamus expresses Sox2 [26]. In the ME, NG2 glia play a key role in regulating energy balance [25]. Specifically, NG2 glia in the ME interact with leptin receptor- containing neurons and are required for leptin sensing and body weight control [25].

2.4.3 Pericytes

Pericytes are elongated cells that wrap around endothelial cells in capillaries, arterioles, and venules. They are multipotent stem cells that can contribute to vasculature repair after injury and vascularization of brain tumors [104]. In models of ischemic strokes, pericytes can start expressing NSC markers such as Sox2 and nestin and differentiate into microglia-like cells with phagocytic activities [105, 106]. Furthermore, pericytes acquire stem cell potential after a stroke, as they can form neurospheres *in vitro* and differentiate into neurons, osteoblasts, and adipocytes [106]. In human studies showed that isolated pericytes with multipotent perivascular mesenchymal stem cell properties are able to differentiate into immature neurons, astrocytes, and oligodendrocytes *in vitro* given the right induction environment [107]. Additionally, pericytes

harvested from human brains can be reprogrammed into induced neurons, or neurons derived *in vitro* from non-neuronal cells, by activating the expression of NSC- like genes [108].

2.4.4 Microglia

Microglia are innate immune cells that reside in the brain. Microglia are not progenitor cells as they cannot give rise to other cell types, but they can rapidly replenish themselves through self-renewal after genetic ablation [109]. This rapid replenishment is mostly derived from local proximal microglia and rarely derived from bone-marrow progenitors [110, 111]. Although microglia are often regarded as long-lived compared to the short-lived macrophages, recent studies have found that microglia have a high proliferation rate, and it takes approximately 96 days for the whole microglia population to renew in the brain of healthy animals [112].

3. Rationale for the Study, Hypothesis, and Specific Aims

Given that the CVOs are potential adult NSC niches, we are interested in studying the adult NSC potential of cells in the OVLT and SFO. We are particularly interested in studying the effect of high salt diet-induced hypertension on cell proliferation and adult neurogenesis in these nuclei, as both the OVLT and SFO are involved in regulating body fluid and electrolyte balance as well as autonomic cardiovascular function.

In the proposed study, we will: (a) identify potential progenitor cells that are undergoing constant proliferation in the OVLT and SFO, (b) investigate if adult neurogenesis is presented in the OVLT and SFO, and (c) examine if cellular proliferation and adult neurogenesis in the OVLT and SFO are affected in a rat model of high salt diet-induced hypertension.

We hypothesize that the OVLT and SFO contain adult NSCs, and that cellular proliferation and adult neurogenesis proceed throughout adulthood and are modified when animals are exposed to high salt diet.

4. Methods

4.1 Animals

Male, 300-550 g of Wistar rats (Charles River Laboratories, Saint-Constant, QC, Canada) were housed individually under a 12h light, 12h dark cycle with lights on at 7 a.m. Before the experiment, rats were handled at 9am and 6pm for three days to reduce the stress associated with injections. Rats were introduced to either high salt water or regular drinking water *ad libitum*. In high salt diet, rats received 2% NaCl (2g of NaCl in 100ml of water) instead of regular water for 7 days, a protocol that induces increases in blood pressure leading to hypertension [113]. All animal procedures were approved by the McGill University Animal Care Committee and were in accordance with the guideline provided by the Canadian Council of Animal Care.

4.2 BrdU Treatment

For intraperitoneal (i.p.) BrdU administration, rats received 6.25 mg/ml of BrdU (Sigma Life Science) dissolved in saline at a dose of 50 mg/kg body weight. Injections were done twice a day, at 9 a.m. and 6 p.m. for 7 days. The first injection was given the day after three days of handling and the first day of high salt diet protocol. For the oral administration, BrdU was added to the drinking water at a concentration of 1.0 mg/ml and supplied *ad libitum*. Drinking water with BrdU was renewed every 3 days to avoid chemical degradation.

4.3 Transcardiac Perfusion

Rats were under anesthetization with 3% isoflurane and perfused with 10 ml phosphate buffered saline (PBS, 300 mOsm/kg for normal water fed rats and 320 mOsm/kg for high salt diet rats, 7.4 pH) followed by 4% paraformaldehyde (PFA) dissolved in PBS. Both PBS and 4% PFA in PBS were at room temperature. Brains were extracted and immersed in 4% PFA for at least 48 hours prior to sectioning. To visualize pericytes using anti- CD13/mAminopeptidase N, rats were perfused with 4% PFA and 0.25% glutaraldehyde in PBS (pH 6.8) at 37°C. Brains were sectioned coronally using vibratome (Leica, VR1200S) into tissue sections of 50 μm thickness. Brain sections were stored in 1X PBS with 0.02% sodium azide at 4 °C prior to immunohistochemistry staining.

4.4 Plasma Osmolality

Blood was collected from the right atrium prior to transcardiac perfusion of PBS or 4% PFA in PBS. Blood samples were placed on ice for at least an hour, then centrifuged at 1,500 rpm for 10 min. Serum samples were collected after centrifugation and blood osmolality was measured using an osmometer in triplicates (Advanced Instrument model 3320).

4.5 Antibodies

Primary antibodies used are indicated in the table below.

Antibody	Animal	Dilution	Company	Catalog Number	Reference
anti-Ki67	rabbit	1:250	Abcam	Ab15580	[114]
anti-BrdU	rat	1:500	Abcam	Ab6326	[115]
anti-vimentin	chicken	1:5000	EMD Millipore	Ab5733	[116]
anti-NG2	rabbit	1: 500	EMD Millipore	Ab5320	[26]
anti- CD13/ mAP-N	goat	OVLT: 1:50 SFO: 1:200	R&D Systems	AF2335	[117]
anti-Iba1	rabbit	1:3000	Wako	019-19741	[118]
anti-Iba1	guinea pig	1:500	Synaptic Systems	234-004	[119]
anti-NeuN	guinea pig	1:500	EMD Millipore	ABN90	[120]
anti-Sox2	rabbit	1:1000	EMD Millipore	AB5603	[121]
anti-HuC/HuD	mice	1:50	Invitrogen	A21271	[122]
anti-doublecortin	guinea pig	1:1000	EMD Millipore	Ab2253	[123]

Table 1. Primary antibodies used and their corresponding animal, dilution, product company, catalogue number, and reference.

Secondary antibody used were Alexa Fluor- conjugated 488, Alexa 568 and Alexa 647 (1:500, Life Technologies). Negative controls for the secondary antibodies were tested using the

same protocol in **Section 4.6** on OVLT and SFO sections and verified no non-specific staining (Data not shown).

4.6 Immunohistochemistry

To reveal the antigens for BrdU, sections were incubated in 1N HCl at 45°C for 12.5 min. Sections were immediately placed on ice after the HCl bath. To neutralize the acidity following the HCl bath, sections were incubated in borate buffer (100 mM, pH 8.5, constitute NaCl and Boric acid, Sigma, B6768) for 30 min at room temperature and then washed with PBS three times. Sections were blocked with 10% normal goat serum in 0.3% Triton X in PBS for 1 hour at room temperature. After blocking, sections were incubated with primary antibodies for 12-24 hours at 4°C. Sections were then washed three times and incubated with corresponding secondary antibodies and DAPI (4′,6-diamidino-2-phenylindole,1:1000, Invitrogen, California, USA) for 2 hours at room temperature. Finally, sections were washed three times and mounted on coverslips with Slow Fade Gold Antifade reagent (Life Technologies).

For co-staining of BrdU and HuC/HuD or Dcx, sections were incubated in citrate buffer (trisodium citrate, pH2) at 75°C for 15 min and placed on ice immediately after the acidic bath. Borate buffer (100mM, pH 8.5) was applied for 30min at room temperature following three washes.

For anti-CD13 staining, rats were perfused with 4% PFA and 0.25% glutaraldehyde. Brain sections were incubated 15 min in 0.1% $NaBH_4$ in cytoskeletal buffer containing: 130 mM NaCl, 10 mM MES, 5 mM EGTA, 5 mM $MgCl_2$, and 5 mM $MgCl_2$, pH 6.3. Antigen blocking was done by incubating in 10% normal donkey serum. Primary antibodies were incubated for 48-72

hours. Secondary antibodies were applied by first using donkey anti-goat consecutively followed by other goat secondary antibodies.

To co-stain Ki67 and NG2 which are both rabbit antibodies, a Double Fluorescent Tyramide Signal Amplification protocol (Perkin Elmer TSA kit, Boston, MA) was used [124]. Brain sections were incubated with anti-Ki67 (1:25000) on the first day and anti-NG2 (1:100) on the second day.

4.7 Image Acquisition and Analysis

Immunohistochemistry sections were imaged using ApoTome.2 microscope (ZEISS) 20X objective, LSM 880 Apo 20x/0.8 or 63x/1.40 oil objectives (Zeiss AG, Oberkochen, Germany). Confocal z-stacks were generated by acquiring 18-22 optical sections with 1.5 µm steps using 20X objective and 20-80 optical sections with 0.2-0.4 µm steps using 63X objective. Images were analyzed with Zen 2 blue (ZEISS). Each rat brain contains 5-6 sections of OVLT and 5-8 sections of SFO (50 µm thick). 4 optimal medial sections were taken from the OVLT and SFO of each rat to quantify the number of BrdU+ cells in the OVLT and SFO. BrdU+ cells were verified by its colocalization with DAPI, and quantification is based on manual counting. The number of BrdU+ cells in the OVLT and SFO are based on the cumulative BrdU+ cells in the 4 optical medial sections. The identity of BrdU+ cells was further verified by analyzing BrdU colocalization with other cellular markers (e.g. vimentin, NG2, CD13, Iba1, Sox2, Dcx, HuC/HuD, and NeuN). A minimum of 5 rats were used for each quantification. Figures were assembled using Adobe InDesign and Illustrator (Adobe Inc).

4.8 Statistics

Statistical analysis was performed using multiple Student's t-tests, 2-way ANOVA, and unpaired Student's t-test. Results are reported as mean plus or minus standard error of the mean (± SEM). Statistical significance was set up as the following: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, non-significant. Graphs were made using Prism 9 (GraphPad Software) and statistics were done using Prism 9 and Excel.

5. Results

5.1 Constant Cell Proliferation in the OVLT and SFO in Adult Rat Brain

To study if cells in the OVLT and SFO undergo constant cell proliferation, Ki67, an endogenous mitotic marker that labels actively dividing cells (G_1, S, G_2, and mitosis) but not quiescent cells (G_0), was employed. Ki67-positive (Ki67+) cells were observed in both the OVLT and SFO of adult rat brains, implying constitutive cell proliferation occurs in the OVLT and SFO of adult rat brains (Fig. 4A & B). In the OVLT, Ki67+ cells appeared more frequently near the ventricular surface as well as in the subpial zone, or the peripheral parenchyma that is close to the pia (Fig. 4A). In the SFO, Ki67+ cells appeared scattered through the parenchyma of the SFO (Fig. 4B). To further validate these results, rats were treated with bromodeoxyurine (BrdU). BrdU is a nucleoside homolog that incorporates into newly synthesized DNA (during S phase), therefore labeling newborn cells [125]. Young adult rats (around 3-month-old, bodyweight > 350g) were treated with BrdU for 7 consecutive days of intraperitoneal (i.p.) injections and sacrificed the day after their last BrdU treatment, labeling cells that underwent S phase during the 7-day BrdU treatment. A profound number of BrdU+ newborn cells in the OVLT and SFO was observed, confirming the presence of cells undergoing constant cell proliferation in the OVLT and SFO in adult rat brains (Fig. 4C & D). In contrast to the Ki67 expression, BrdU+ newborn cells in the OVLT and SFO appeared scattered throughout the parenchyma. This suggests that the BrdU+ newborn cells have migrated during the 7-day BrdU treatment, resulting in a relatively uniform distribution of newborn cells. Together, these observations show that cells in the OVLT and SFO undergo constant cell proliferation in adult rat brain. Note, although Ki67 labels a greater portion of cell cycle than BrdU, Ki67 can only

label actively dividing cells at the moment when the rat was perfused. In contrast, BrdU can label cells that has undergone S phase during the 7-day injection period. Thus, BrdU accumulates in cells that underwent division, labeling more cells than ki67 which is only present in cells dividing at the time of perfusion.

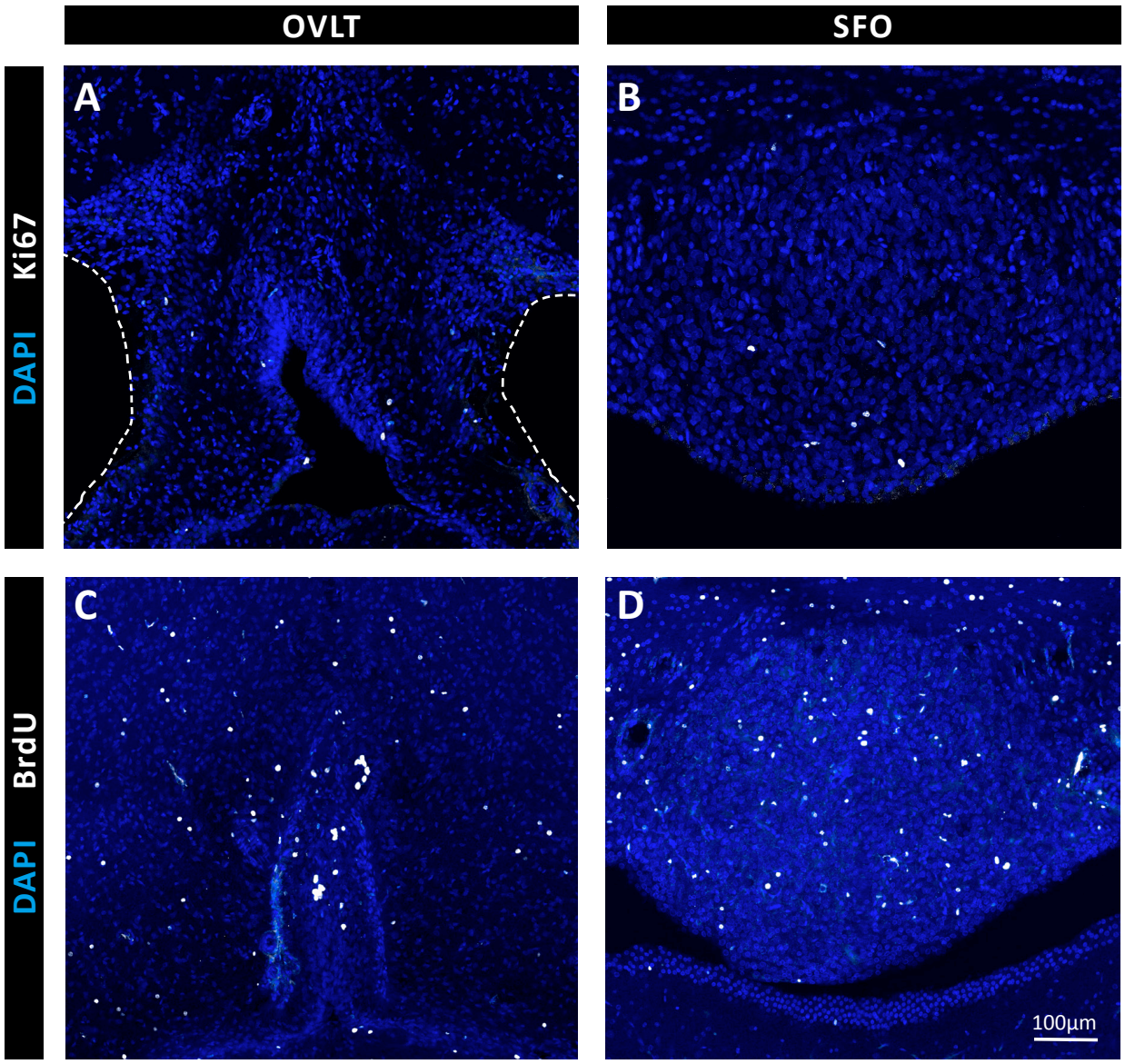

Figure 4. Cell proliferation in the OVLT and SFO in adult rats.

(A, B) Coronal sections of the OVLT and SFO of young adult rats with normal drinking water (and no i.p. injections), labeled for Ki67 (white) and DAPI (blue). White dashed lines delineate the pia matter around the OVLT. **(C, D)** Coronal sections of the OVLT and SFO of young adult rats that were i.p. injected with BrdU for 7 days, labeled for BrdU (white) and DAPI (blue).

5.2 Tanycytes, Pericytes, Microglia and NG2 Glia are Highly Proliferative Cells in the OVLT and SFO

To identify potential progenitor cells in OVLT and SFO, we studied cells that are undergoing constant cell proliferation in the OVLT and SFO. Different cellular markers were used to characterize Ki67+ or BrdU+ newborn cells. We are interested in glia such as tanycytes, NG2 glia, and microglia, which are highly proliferative cell types, as well as pericytes.

We observed Ki67+ tanycytes (Fig. 5A, visualized by vimentin), pericytes (Fig. 5B, visualized by CD13), microglia (Fig. 5C, visualized by Iba1), and NG2 glia (Fig. 5D, visualized by NG2) in the OVLT of untreated rats (rats that did not receive i.p. injection). To further validate our results, we subjected rats to 7 days BrdU i.p. injections. We observed BrdU+ tanycytes (Fig. 5E, I & J), pericytes (Fig. 5F & H), microglia (Fig. 5G), and NG2 glia (Fig. 5H) in the OVLT, validating our previous observation with Ki67. Therefore, tanycytes, pericytes, microglia, and NG2 glia undergo continuous proliferation in the OVLT. To evaluate the cellular composition of proliferating cells in the OVLT, BrdU+ tanycytes, pericytes, microglia, and NG2 glia were quantified (Table 2). Note that 53.12 ± 1.55 % cells proliferating in the OVLT did not express detectable levels of cellular markers and thus their identity remains unknown in the OVLT.

Similarly, we used Ki67 as a marker of proliferating cell in the SFO of untreated rats. We found Ki67+ NG2 glia (Fig. 5L), pericytes (Fig. 5M), and microglia (Fig. 5N) in the SFO, but we did not detect any Ki67+ tanycytes. Next, we administered rats with BrdU i.p. for 7 days and found BrdU+ NG2 glia (Fig. 5K & O), microglia (Fig. 5P & Q (iv)), and pericytes (Fig. 5K & Q (iii)). Consistent with the previous observation, little to no BrdU+ tanycyte was observed in the SFO of young adult rats, implying that tanycytes do not contribute to the proliferating cells in the

SFO in healthy adult rats. To quantify the cellular composition of proliferating cells in the SFO, BrdU+ NG2 glia, pericytes, and microglia were quantified (Table 2). Additionally, there are 44.65 ± 4.61 % of SFO proliferating cell types, whose identity could not be identified using above-described cell markers.

In summary, tanycytes, NG2 glia, microglia, and pericytes are proliferating cells in the OVLT; and NG2 glia, microglia, and pericytes are proliferating cells in the SFO. The observation that little to no tanycytes undergo cellular proliferation in the SFO suggests that tanycytes in the SFO do not serve as progenitor cells, indicating potential functional differences between tanycytes in the OVLT and the SFO.

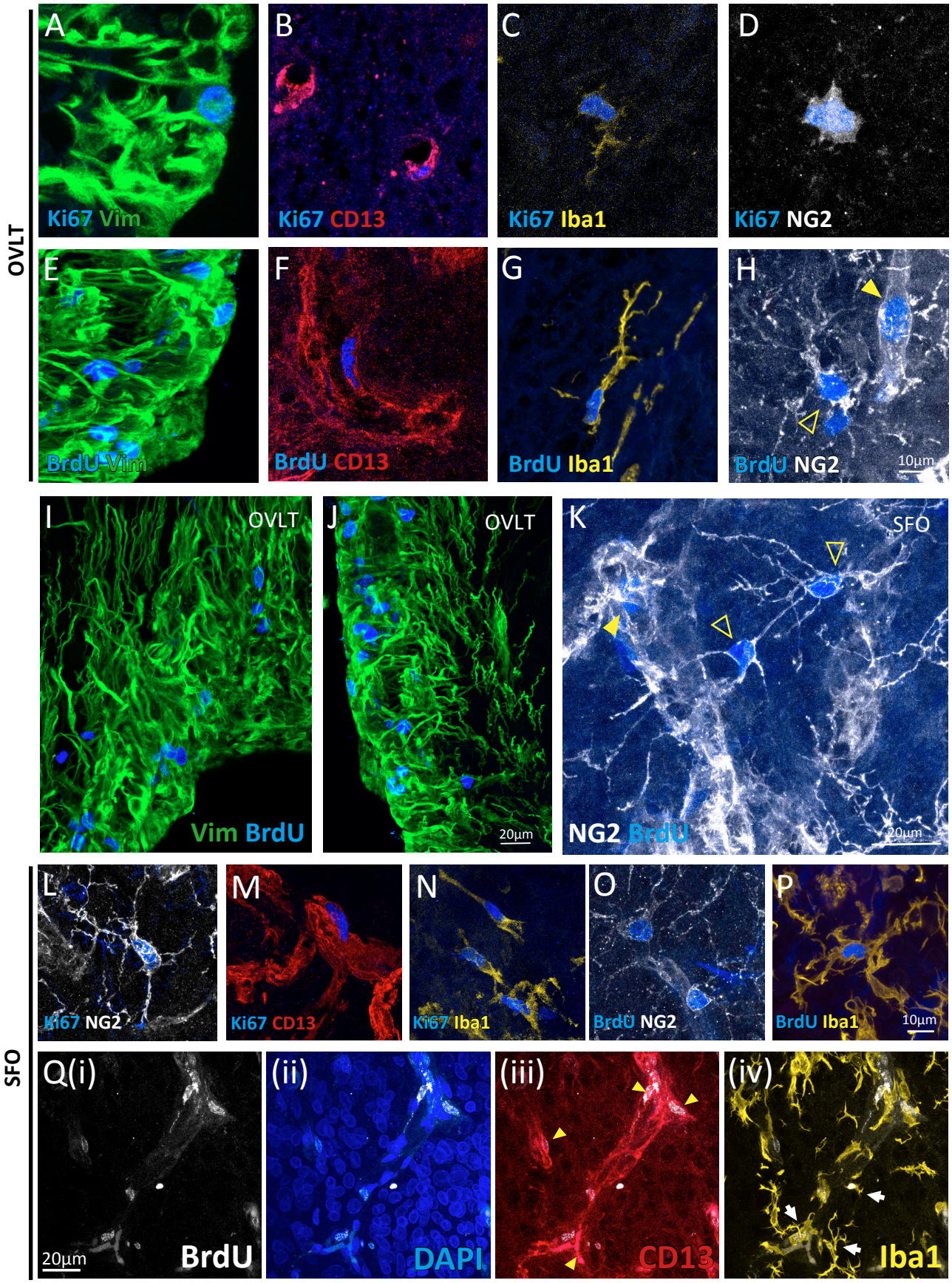
OVLT
A
Ki67 Vim
B
Ki67 CD13
C
Ki67 Iba1
D
Ki67 NG2
E
BrdU Vim
F
BrdU CD13
G
BrdU Iba1
H
BrdU NG2
10μm
I
OVLT
Vim BrdU
J
OVLT
20μm
K
SFO
NG2 BrdU
20μm
SFO
L
Ki67 NG2
M
Ki67 CD13
N
Ki67 Iba1
O
BrdU NG2
P
BrdU Iba1
10μm
Q(i)
20μm
BrdU
(ii)
DAPI
(iii)
CD13
(iv)
Iba1

Figure 5. Cell types constantly proliferating in the OVLT and SFO.

(A-D) High magnification images of Ki67+ tanycytes (A, vimentin, green), pericytes (B, CD13, red), microglia (C, Iba1, yellow), and NG2 glia (D, NG2, white) in the OVLT of young adult rats. **(E-J)** High magnification images of BrdU+ tanycytes (E, I, J), pericytes (F & H, yellow arrowheads), microglia (G), and NG2 glia (H, open yellow arrowheads) in the OVLT of young adult rats treated with 7 days BrdU i.p. injections. **(L-N)** High magnification images of Ki67+ NG2 glia (L), pericytes (M), and microglia (N) in the SFO of young adult rats. **(K, O-Q)** High magnification images of BrdU+ NG2 glia (K, yellow open arrowhead & O), microglia (P & Q iv, white arrows), and pericytes (K yellow arrowheads labeled by NG2 & Q iii, yellow arrowheads labeled by CD13) in the SFO of rats treated with 7 days BrdU i.p. injections.

	OVLT		SFO	
	Absolute value	**Percentage (%)**	**Absolute value**	**Percentage (%)**
Tanycytes	43 ± 1.84	13.14 ± 0.70	0.13 ± 0.13	0.03 ± 0.03
NG2 glia	84.12 ± 5.42	25.52 ± 1.41	159 ± 10.05	40.02 ± 2.92
Pericytes	11.4 ± 1.54	3.55 ± 0.50	29.4 ± 3.64	7.09 ± 0.79
Microglia	19.5 ± 2.50	6.02 ± 0.75	31.33 ± 4.18	7.63 ± 1.31
Others	171.6 ± 8.19	53.12 ± 1.55	186.4 ± 25.17	44.65 ± 4.61
Total	328.63 ± 5.68	100	403.88 ± 15.57	100

Table 2. Quantification of BrdU+ cells in the OVLT and SFO in adult rats.

Absolute values calculated based on the total BrdU+ cells presented in the 4 optimal medical sections of 50 μm thickness of the OVLT and SFO. Percentages are in proportion to the total BrdU+ cells counted in the OVLT and SFO. Numbers are reported in mean ± standard error.

5.3 Adult Neurogenesis is Present in the Rat OVLT and SFO

To examine the adult NSC potential of the rat OVLT and SFO, Sox2 was used as a neural stem cell marker [126]. In the OVLT, Sox2 is highly expressed in tanycytes and in cells located in the subpial zone, consistent with the Ki67 expression pattern observed in the OVLT (Fig. 6A & Fig. 3A). Numerous Sox2+ cells were also found scattered throughout the OVLT, suggesting the presence of hypothalamic NSCs in the OVLT parenchyma (Fig. 6A). In the SFO, Sox2+ cells are highly expressed throughout the parenchyma (Fig. 6B). Notably, Sox2 is also highly expressed in tanycytes in the SFO, which have undetectable levels of other proliferation markers. The observation of Sox2 signal in tanycytes in the SFO resembles the strong Sox2 expression in ependymal cells, which have also been shown to express high levels of NSC markers such as Sox2 but lack NSC functions since they fail to form neurospheres, proliferate, and survive *in vitro* [127, 128].

To examine if proliferating cells can acquire neuronal fate and undergo neurogenesis in the OVLT and SFO of adult rats, Ki67 labeling was used in combination with doublecortin (Dcx), a marker expressed in neural progenitor cells (NPCs) and immature neurons [129]. We found Ki67+ Dcx+ cells with short processes delineated by Dcx, suggesting that NPCs and/or immature neurons are present in the adult OVLT and SFO (Fig. 6C & D). We also analyzed young adult rats treated with BrdU i.p. for 7 days and found BrdU+ Sox2+ Dcx+ triple-positive cells in the OVLT and SFO of these rats, suggesting that a subpopulation of cells can acquire neuronal cell fate (Fig. 6E & F). To study if these NPCs or immature neurons can further mature and become adult neurons in the OVLT and SFO, we used long-term administration of BrdU. To reduce any possible side effects of long-term exposure to high doses of BrdU, we used low concentrations of BrdU (1mg/ml) by adding it to drinking water for 28 or 60 days [130]. BrdU+

cells were observed in the OVLT and SFO of adult rats that received 28 or 60 days of BrdU in drinking water (Fig. 7 & 8). Markers of differentiated and mature neurons, HuC/HuD and NeuN, were used to examine the presence of adult neurogenesis in the OVLT and SFO (Fig. 7C&D). We found BrdU+ HuC/HuD+ neurons in the OVLT and SFO of adult rats treated with 28 days BrdU drinking water (Fig. 8A & B). Similarly, BrdU+ HuC/HuD+ NeuN+ neurons were found in the OVLT and SFO of adult rats treated with 60 days BrdU in drinking water (Fig. 8C&D). These findings reveal the presence of immature and mature neurons in the OVLT and SFO, uncovering the presence of constitutive adult neurogenesis in the sensory CVOs and establishing the OVLT and SFO as potential adult NSC niches. Interestingly, the numbers of BrdU+ cells are smaller in rats treated with 60 days BrdU in drinking water than in rats that received 7 days i.p. injections. Further investigation revealed that all these BrdU+ cells in rats treated with 60 days BrdU express neuronal markers, possibly suggesting only neuronal progeny can be labelled using this method of BrdU administration (more details discussed in **Section 6.3**).

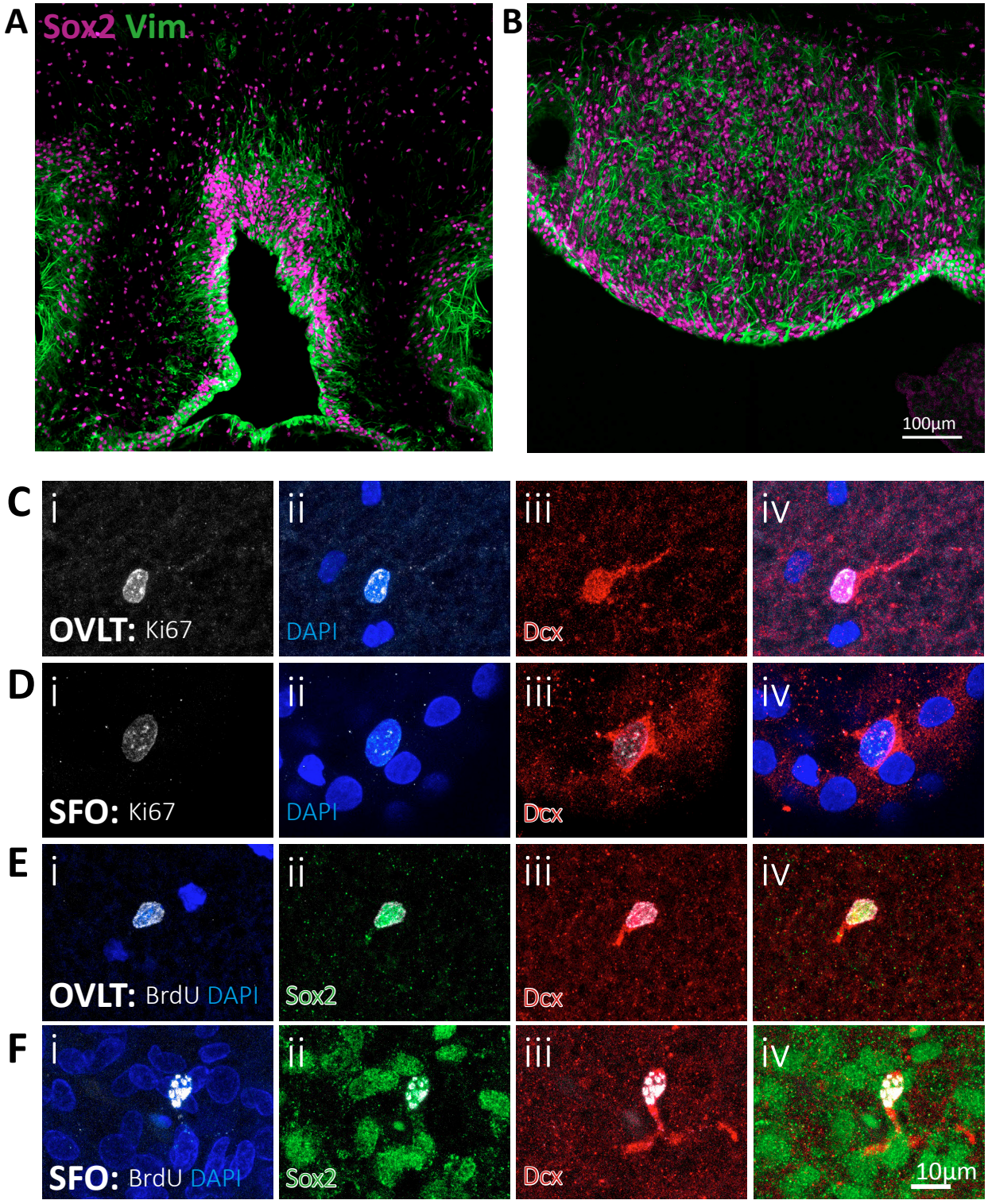
A
Sox2 Vim
B
100µm
C
i
ii
iii
iv
OVLT: Ki67
DAPI
Dcx
D
i
ii
iii
iv
SFO: Ki67
DAPI
Dcx
E
i
ii
iii
iv
OVLT: BrdU DAPI
Sox2
Dcx
F
i
ii
iii
iv
SFO: BrdU DAPI
Sox2
Dcx
10µm

Figure 6. Adult NSC potential of the OVLT and SFO.

(A, B) Coronal sections of the OVLT (A) and SFO (B) of young adult rats were stained with antibodies against Sox2 (purple) and vimentin (green). **(C, D)** High magnification images of Ki67+ (white) Dcx+ (red) immature neurons or NPCs in the OVLT and SFO in young adult rats. **(E, F)** High magnification images of BrdU+ (white) immature neurons or NPCs that co-express Sox2 (green) and Dcx (red) in the OVLT and SFO in young adult rats that received 7 days of BrdU i.p. injections.

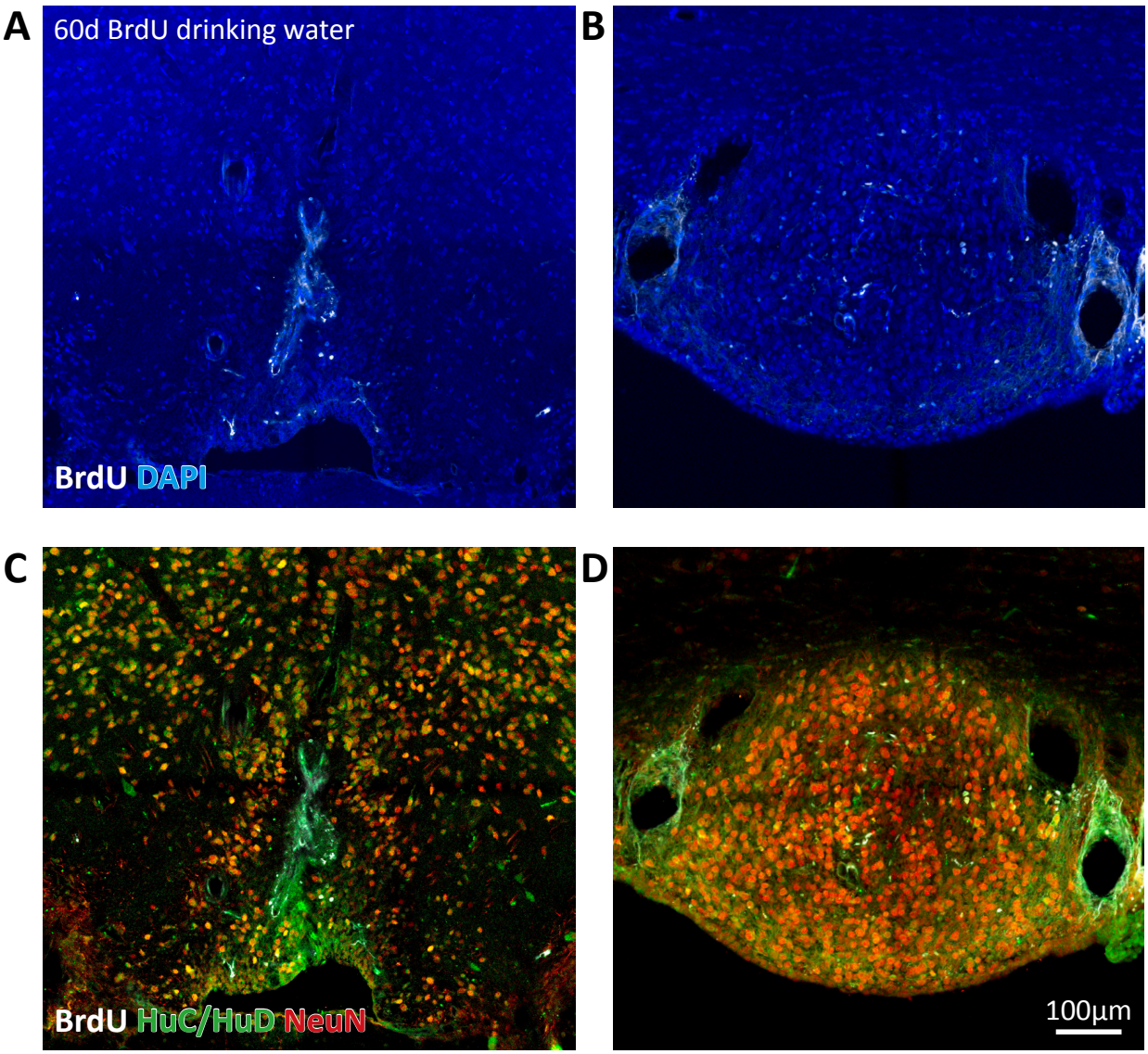

Figure 7. Tracing adult neurogenesis in the OVLT and SFO through long-term administration of BrdU.

(A, B) Coronal sections of the OVLT (A) and SFO (B) in adult rats that received BrdU in drinking water for 60 days, labeled for BrdU (white) and DAPI (blue). **(C, D)** Coronal sections of the OVLT (C) and SFO (D) in adult rats that received BrdU in drinking water for 60 days, labeled for BrdU (white), HuC/HuD (green), and NeuN (red).

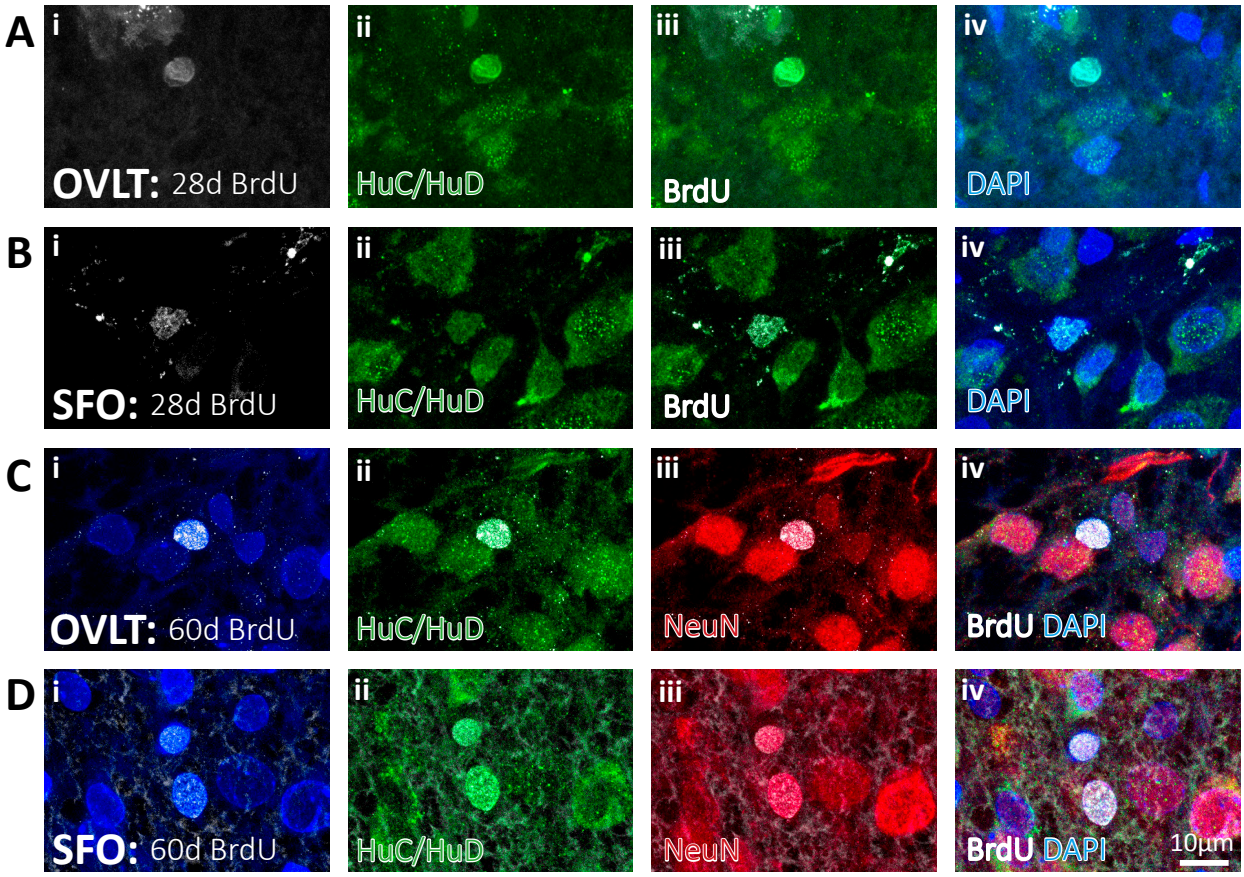

Figure 8. Mature neurons in the OVLT and SFO of adult rats.

(A, B) BrdU+ (white) in neurons expressing HuC/HuD (green) in the OVLT (A) and SFO (B) of rats treated with 28 days BrdU drinking water. **(C, D)** BrdU (white) in mature neurons expressing HuC/HuD (green) and NeuN (red) in the OVLT (C) and SFO (D) of rats treated with 60 days BrdU drinking water.

5.4 Increase in Cell Proliferation in a Model of Acute Hypertension

Because both the OVLT and SFO are involved in regulating cardiovascular functions and maintaining fluid and electrolyte balance, we next examined the effect of high dietary salt-induced hypertension on cell proliferation in these areas. Young adult rats were given 2% NaCl in the drinking water for 7 days (or high salt diet, HSD). Following 7 days of HSD, the rats developed hypernatremia, increased plasma osmolality, and became progressively hypertensive [113]. During the course of HSD, rats were administrated with BrdU i.p. and sacrificed the day after the 7 days HSD. Thus, cells proliferated during the 7 days of HSD incorporated BrdU, allowing quantification of BrdU+ newborn cells. Rats treated with HSD had a significant increase in blood osmolality which is indicative of acute hypertension (Fig. 9G) [113]. Further, rats that received HSD drank significantly more and gained less weight compared to control rats receiving tap water (Fig. 9H, J), consistent with our previous observations in rats subjected to this model of HSD-induced hypertension.

Importantly, there were significant increases in the number of BrdU+ newborn cells in both the OVLT and SFO of rats treated with HSD, implying that HSD stimulates cell proliferation in these areas (Fig. 9A-C). In contrast, the number of BrdU+ newborn cells in ME remained unaffected after HSD, indicating that this increase in cell proliferation is specific to the OVLT and SFO (Fig. 9A & D). A closer examination of cell types proliferating during HSD in the OVLT revealed that the enhanced proliferation is mainly contributed by tanycytes and microglia (Fig. 9E). In contrast, increases in cell proliferation during HSD in the SFO are mainly due to proliferation of NG2 glia and microglia (Fig. 9F). This suggests that OVLT tanycytes and microglia as well as SFO NG2 glia and microglia increase their proliferation in response to HSD.

To study the effect of HSD on adult neurogenesis, rats were subjected to BrdU i.p. injections during the 7 days of HSD. Proliferating hypothalamic NSCs (BrdU+ Sox2+) and immature neurons or NPCs (BrdU+ Sox2+ Dcx+) in the OVLT and SFO were analyzed (Fig. 10A&B). No statistically significant difference in the number of OVLT and SFO BrdU+ Sox2+ cells was observed between HSD and control rats. However, the number of BrdU+ Sox2+ Dcx+ immature neurons or NPCs was significantly increased in the OVLT and SFO in rats fed with HSD compared to rats receiving tap water (Fig. 10C). This suggests that the rate of adult neurogenesis increases in the OVLT and SFO in response to salt-induced hypertension.

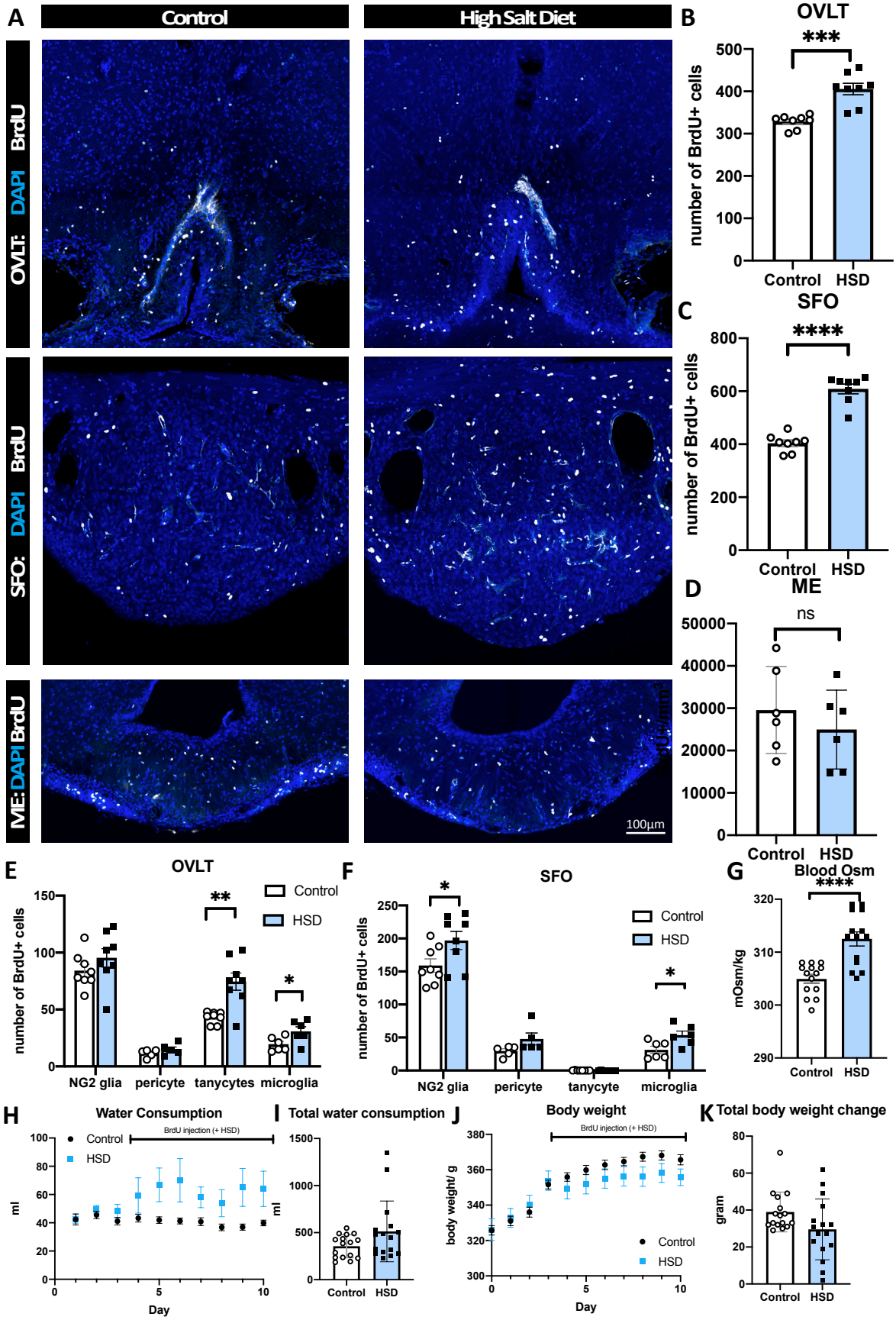
A
Control
High Salt Diet
OVLT: DAPI BrdU
SFO: DAPI BrdU
ME: DAPI BrdU
100µm
B
OVLT
***
number of BrdU+ cells
Control HSD
C
SFO
****
number of BrdU+ cells
Control HSD
D
ME
ns
Control HSD
E
OVLT
number of BrdU+ cells
Control
HSD
**
*
NG2 glia
pericyte
tanycytes
microglia
F
SFO
number of BrdU+ cells
Control
HSD
*
*
NG2 glia
pericyte
tanycyte
microglia
G
Blood Osm
****
mOsm/kg
Control HSD
H
Water Consumption
BrdU injection (+ HSD)
Control
HSD
ml
Day
I
Total water consumption
ml
Control HSD
J
Body weight
BrdU injection (+ HSD)
body weight/ g
Control
HSD
Day
K
Total body weight change
gram
Control HSD

Figure 9. HSD-induced hypertension increases cell proliferation in the OVLT and SFO. **(A)** Coronal sections of the OVLT, SFO, and ME of rats that received 7 days BrdU i.p. injections with or without HSD. **(B)** Quantification of BrdU+ newborn cells in the OVLT of HSD and control rats. Unpaired t-test, $p = 0.000125$, [n=8]. **(C)** Quantification of BrdU+ newborn cells in the SFO of HSD and control rats. Unpaired t-test, $p < 0.000001$ [n=8]. **(D)** Quantification of BrdU+ newborn cells in the ME of HSD and control rats. Unpaired t-test, $p = 0.4377$, [n=6]. **(E)** Quantification of the cell identities of BrdU+ newborn cells in the OVLT of HSD and control rats. Multiple t-tests, [NG2 glia: n=8, p=0.2686; pericyte: n=5, p=0.2437; tanycytes: n=8, p=0.0012; microglia: n=6, p=0.0367]. **(F)** Quantification of the cell identity of BrdU+ newborn cells in the SFO of HSD and control rats. Multiple t-tests, [NG2 glia: n=8, p=0.0442; pericyte: n=5, p=0.1033; tanycytes: n=8, p>0.9999; microglia: n=6, p=0.0242]. **(G-K)** Blood osmolality (Osm) (G), daily water intake (H), total water intake (I), daily body weight change (J) and total body weight change (K) of HSD and control rats. [n=16]. **(H)** Rats that received HSD are likely to drink more compared to control rats ($p<0.0001$) given by a simple main effect analysis of a two-way ANOVA. Day 0 marks the first day of handling. **(I)** No statistically significant difference in total water intake is found between control and HSD using an unpaired t-test ($p=0.0778$). **(J)** Rats that received HSD are likely to lose more weight compared to control rats ($p<0.0001$) given by a simple main effect analysis of a two-way ANOVA. **(K)** There is no statistically significant difference in total body weight change between control and HSD given by an unpaired t-test ($p=0.0668$). * $p<0.05$, **$p<0.01$, ***$p<0.001$, **** $p < 0.0001$.

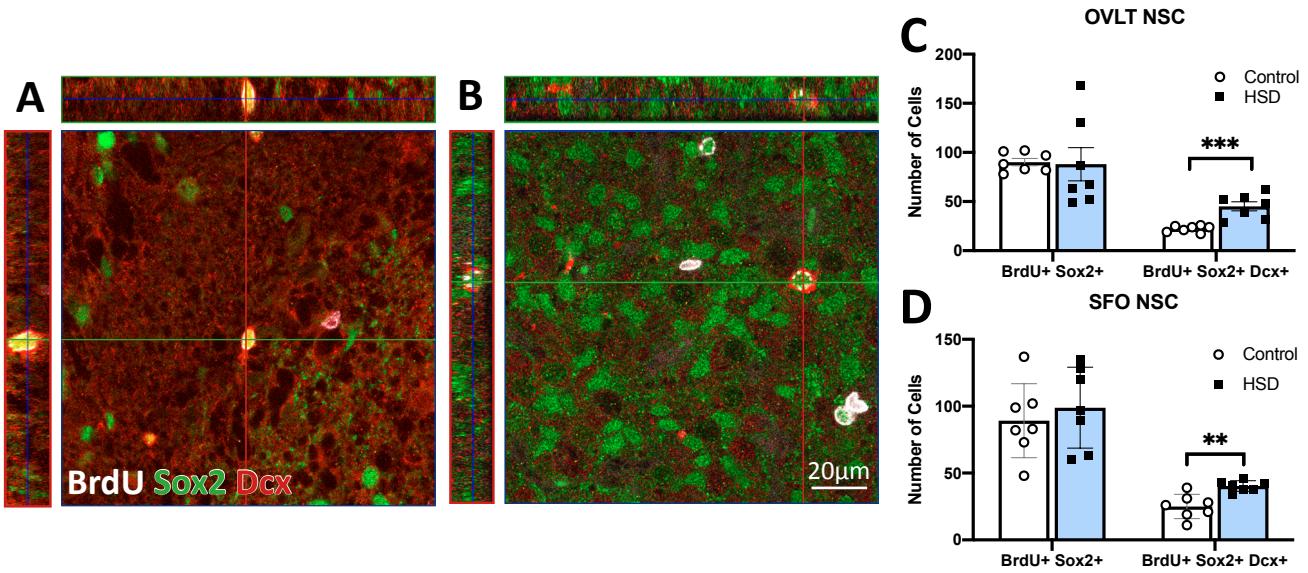

Figure 10. The effect of HSD-induced hypertension on the proliferation of immature neurons or NPCs in the OVLT and SFO.

(A, B) Orthogonal view of BrdU+ (white) Sox2+ (green) and Dcx+ (red) immature neurons or NPCs presented in the OVLT (A) and SFO (B) of young adult rats that received 7 days BrdU i.p. injections.

(C, D) Quantification of proliferating hypothalamic neural stem cells (BrdU+ Sox2+) and immature neurons or NPCs (BrdU+ Sox2+ Dcx+) in rats that received HSD or tap water (control). [n=6; OVLT: BrdU+ Sox2+: p=0.9036, BrdU+ Sox2+ Dcx+: p=0.0004; SFO: BrdU+ Sox2+: p=0.5426, BrdU+ Sox2+ Dcx+: p=0.0015]

6. General Discussion and Results

6.1 Summary of Results

The present study characterized the adult NSC niche potential of the OVLT and SFO. We found continuous cell proliferation taking place in the OVLT and SFO of young adult rats. Our analysis revealed that tanycytes, NG2 glia, pericytes, and microglia proliferate in the OVLT; and NG2 glia, pericytes, and microglia but not tanycytes proliferate in the SFO of adult rats. Moreover, our data suggest that neurogenesis proceeds in both the OVLT and SFO of adult rats, implying that both sensory CVOs represent adult NSC niches. As OVLT and SFO are involved in the regulation of body fluid balance and cardiovascular functions, we examined the effect of HSD-dependent hypertension on cell proliferation in the OVLT and SFO. Using an established rat model of HSD-induced hypertension, we showed that cell proliferation is increased in the OVLT and SFO of rats exposed to HSD. This increase in cell proliferation is mainly contributed by tanycytes and microglia in the OVLT as well as NG2 glia and microglia in the SFO. Lastly, we found that HSD increased the proliferation of NPCs or immature neurons in the OVLT and SFO, therefore accelerating adult neurogenesis. Together, our findings represent the first demonstration that the OVLT and SFO contain highly proliferative cells and NSCs that are responsive to HSD-induced hypertension.

6.2 Limitations of the Study

This study showed the presence of constitutive cell proliferation in young adult male Wistar rats of 300-550g. Since we did not investigate this process in older rats, we cannot conclude that constitutive cell proliferation continues in the OVLT and SFO of rats throughout

adulthood. Likewise, we cannot extend our conclusion to female animals. In previous studies, Sprague Dawley rats were mostly used to study hypertension [131-133], while mice were used to study the NSC potential of cells in the OVLT and SFO [90, 92]. As hypertensive traits and NSC properties may vary between species and breeding, different animal models should be used to verify the result of these studies. Here, we used Wistar rats to study the effect of HSD-induced hypertension on NSCs in the OVLT and SFO, providing additional evidence to the adult NSC niche potential of the OVLT and SFO. Nonetheless, future studies should explore other animal models to gain a more comprehensive understanding of the NSC potential of cells in the OVLT and SFO as well as the physiological significance.

In our study, we used BrdU, and this approach has imposed several limitations on this study. First, the visualization of BrdU requires an antigen-retrieval procedure. This procedure potentially affected the detection of other molecular markers (e.g. NG2, vimentin, CD13 and etc.) using immunohistochemistry. Although the antigen-retrieval procedures were optimized for a milder condition and additional measurements were employed (e.g. using borate buffer to neutralize the acidity, thorough wash with PBS, and increasing the concentration of other antibodies), it is possible that this antigen-retrieval procedure decreased the immunogenicity of other markers. In other words, our values of BrdU+ tanycytes, pericytes, NG2 glia, microglia, and NPCs were potentially underestimated due to the use of BrdU. An alternative approach could have been employed using 5-ethynyl-2-deoxyuridine (EdU), since detecting EdU potentially has a smaller effect on the immunohistochemistry labeling. Another limitation attributable to BrdU is that this substance is frequently reported as cytotoxic to neurogenesis in *in vitro* and developmental studies [134-136]. One study reported the lack of *in vivo* approach to study the cytotoxic effect of BrdU on cell proliferation and adult neurogenesis and therefore

investigated this *in vivo* in the adult rat hippocampus [137]. Specifically, this study examined the effect of different BrdU dosages on the number of newborn cells and immature neurons after 2 hours and 4 days and observed no significant differences, leaving the long-term effect and other cytotoxic indicators undiscussed. The lack of *in vivo* studies analyzing the effect of BrdU on neurogenesis and cell proliferation makes the effect of BrdU application in our study and other studies using BrdU enigmatic. Therefore, it is possible that employing BrdU in our study affected our results, and the actual number of proliferating cells is higher in rats not treated with BrdU. This limitation represents a major weakness of all studies using BrdU.

Furthermore, our quantification of BrdU+ cells in the OVLT and SFO is potentially incomparable to other non-CVO brain regions. Since the central nervous system (CNS) has little *de novo* synthesis of nucleoside, the CNS primarily relies on nucleoside transporters in endothelial cells and epithelial cells of the choroid plexus [138]. This nucleoside transport system also mediates BrdU access into the CNS. However, it remains unknown if BrdU is transported to the CVOs using a similar mechanism to non-CVO brain regions. Given the presence of fenestrated blood vessels, CVOs are highly permeable to blood-borne molecules. Permeability studies using peripheral injections of Evans Blue and FITC-conjugated low molecular mass tracer showed that these tracers are found within the parenchyma of CVOs, but not the parenchyma of nearby tissues, implying a specialized blood-brain barrier that prevents the leakage of blood-borne molecules to nearby brain tissues [20, 21, 139]. Whether BrdU is passively diffused into the OVLT and SFO or transported through nucleoside transporters similar to non-CVO brain regions remains unexplored. If BrdU is passively diffused into the OVLT and SFO, then the OVLT and SFO are presumably exposed to a higher concentration of BrdU, which is i.p. delivered. This might lead to the quantification of BrdU+ cells in the OVLT and SFO

incomparable to other brain regions such as the hippocampus, where the blood-brain barrier is complete. Accordingly, in our study we compared the effect of HSD-induced hypertension on cell proliferation in the OVLT and SFO to that in the ME but not in the hippocampus [140, 141]. An alternative approach that will allow a reliable comparison between the CVOs and non-CVO region is delivering BrdU by intracerebroventricular (i.c.v.) osmotic pump, as was done in several studies [11, 12, 142, 143]. Nevertheless, the use of i.c.v. osmotic pumps would require additional surgical procedure and is more invasive, and thus can potentially have additional effects on cell proliferation.

Furthermore, the long-term effect of HSD-induced hypertension on adult neurogenesis in the OVLT and SFO remains to be explored. Since the HSD-induced hypertension animal model we used is limited to 7 days, we were only able to estimate the effect of HSD on the proliferation of NPCs during these 7 days of HSD. Neurogenesis is a complicated and long process that occurs for 3-6 weeks for a newborn neuron to become fully mature in the SGZ [144]. Therefore, to study how HSD affects neurogenesis in the OVLT and SFO, the long-term effect of HSD on neurogenesis should also be examined.

Lastly, we were unable to identify the lineage of a fraction of proliferating cells in the OVLT and SFO with the array of cellular markers that we used. Accordingly, approximately 53% BrdU+ cells in the OVLT and 45% BrdU+ cells in the SFO remain unidentified. This number is potentially overestimated, since the antigen-retrieval protocol of BrdU may attenuate the signal of some cellular markers. In addition, progenitor cells undergoing differentiation might express a low level of cellular markers which were undetectable using immunohistochemistry. It is also possible that one or more uncharacterized cell types are proliferating in the OVLT and SFO. Future studies should gain a more holistic view of cellular and molecular composition in

the OVLT and SFO through single cell RNA sequencing, enabling a more comprehensive approach to characterize different cell types within the OVLT and SFO.

6.3 Implication on Adult Neurogenesis and Adult Neural Stem Cell Niche

In the study of Ramon y Cajal, he described the adult brain as "something fixed, immutable" and that "nothing may be regenerated" [145]. This had led to the idea of newborn neurons in the adult brain being greatly ignored for approximately half a century, until Altman presented the first evidence of adult neurogenesis in 1962 [146]. Today, although the presence of adult neurogenesis in the human brain remains controversial, the presence of adult neurogenesis in rats, mice, songbirds, and nonhuman primates has been established [147]. Besides the well-characterized SVZ and SGZ, the adult neurogenic potential is also reported in other brain regions including the hypothalamus, striatum, substantia nigra, amygdala, and cortex [148]. Some studies reported that newborn neurons found in these regions are differentiated from migrating NSCs and progenitor cells that typically originate in the SVZ [149-152]. Other studies reported the presence of endogenous adult NSC pool, allowing them to replenish local neurons [153-155]. It is possible that both processes coexist in the adult brain, where there are neurogenic systems originating in the SVZ and SGZ that give rise to migrating NPCs, and local NSCs and NPCs that replenish and maintain local cell populations. The CVOs are one of the novel neurogenic sites. The adult NSC niche potential of CVOs has been studied not only in rodents [15, 90, 92], but also in humans [156]. Specifically, a neurogenic system that rises from the CVOs and continues to the circuitry of the hypothalamus and, to a certain degree, to the limbic system is characterized in the adult human brain [156]. This characterization of the hypothalamic neurogenic system raises the question if adult neurogenesis in the hypothalamus follows a structured way of

migration similar to the rostral migratory stream in the SVZ. Admittedly, research on the neurogenic potential of the hypothalamus and CVOs often focus on a part of the hypothalamus. Future research should consider this possible neurogenic system in the hypothalamus to study neurogenesis in the hypothalamus.

The neurogenic potential of these novel neurogenic sites is linked to the physiological function of these brain regions. For example, neurogenesis in the hypothalamus plays a role in regulating energy metabolism and sexual behavior [12, 87, 157, 158], and adult neurogenesis in the amygdala has been linked to fear conditioning and stress response [159, 160]. Previous studies showed that cell proliferation and neurogenesis in the ME play a role in regulating body weight and energy metabolism [13]. The present study shows that cell proliferation and adult neurogenesis in the OVLT and SFO are affected by HSD-induced hypertension, implying that cell proliferation and adult neurogenesis in the OVLT and SFO are potentially involved in regulating body fluid balance and autonomic cardiovascular response. This increase in cell proliferation is brain function-specific, as the HSD-induced increase in cell proliferation is only presented in the OVLT and SFO, but not in the ME, which is also exposed to the peripheral circulation (Fig. 9A-D). Thus, the present study contributes new evidence supporting the neurogenic potential of CVOs and provides insight into the physiological significance of these neurogenic niches.

The CVOs potentially represent an interesting NSC niche to consider. The stem cell niche is defined as a specialized local environment required to maintain the potency of stem cells and support the differentiation of stem cells [160]. CVOs closely interact with peripheral circulation and the CSF, which potentially provides cues that are not present in other brain regions. Using Ki67, we show that the OVLT has highly proliferative zones at the periventricular parenchyma

and the subpial zone (Fig. 4A). These observations of the proliferative nature of the periventricular space and subpial zone have been reported in other studies. In fact, one of the first reports of the neurogenic potential of the hypothalamus was observed around the third ventricle of the hypothalamus [154]. Later, it was discovered that this proliferative nature around the third ventricle is contributed by tanycytes [15], which is also the case in the OVLT (Fig 5.A, E, I, and J). This periventricular NSC niche potentially receives cues from the CSF, as tanycytes act as the CSF-parenchyma barrier through regulating their well-organized tight junctions at the ventricular surface [93, 161]. Furthermore, the subpial zone is also reported as neurogenic. Human studies have shown that the subpial zone is rich in intermediate filament GFAP and nestin [156, 162]. Additionally, we show that the subpial zone of the OVLT in rats is rich in vimentin (Fig. 6A). All three intermediate filaments are regarded as NSC markers. Animal studies have identified the neurogenic potential of the subpial zone in the adult cerebral cortex and developing cerebellum as well as SGZ [163-165]. Interestingly, the subpial zone contains the Virchow-Robin space, or the perivascular space filled with CSF that allows directional CSF flow for waste elimination [166]. This concept that the brain utilizes the Virchow-Robin space in a systematic way for waste elimination is known as the glymphatic system. The close association between the glymphatic system and the subpial zone suggests that the subpial zone is exposed to potential cues originating from the CSF to regulate NSC cell fate. The CSF contains various growth factors and signaling molecules to regulate neurogenesis and cell proliferation throughout development and into adulthood. During embryonic development, the CSF carries growth factors such as fibroblast growth factors (FGFs), insulin-like growth factors, sonic hedgehogs, retinoic acid, and others [167, 168]. In the adult brain, the CSF contains signaling molecules such as the proteoglycan Glypican-2, whose level in the CSF predicts the neurogenesis rate in the adult

hippocampus through inhibiting FGF2-induced neural proliferation [169]. Thus, the close contact between the CVOs and CSF may provide cues from the CSF to regulate NSC cell fate in the CVOs.

Importantly, the fenestrated blood vessels unique to the brain's CVOs potentially creates a specialized local environment for NSCs in these regions. Vasculature provides a specialized niche to maintain the homeostasis of local stem cells and guides, induces, and specifies the differentiation of organ-specific stem cells (e.g. CNS, lung, liver, pancreas, and adipose tissue) for tissue regeneration and development [170]. In the adult brain, the vascular niche supports the maintenance and proliferation of NSCs. In the SVZ, type B1 stem cells give rise to type C transient amplifying cells and type A neuroblasts, which are all located near the blood vessels [171-174]. In the SGZ, NSCs and their progenitor cells also lie close to the capillaries [175]. This is supported by *in vitro* and *in vivo* studies showing that brain endothelial cells regulate NSCs homeostasis and differentiation through direct contact enabled by membrane bound proteins (e.g. EphrinB2 and Jagged-1) [176, 177], as well as the secretion of growth factors (e.g. Bran-derived nerve growth factor, vascular endothelial growth factor receptor 2 and neurotrophin-3) [178-181]. Interestingly, blood vessels in the SVZ are also specialized compared to the classical blood-brain barrier, as they are not completely enwrapped by pericytes and astrocytic endfeet and display an increased permeability to small molecules originating from the peripheral circulation [174, 182]. In contrast, the CVOs harbor a more permeable blood-brain barrier and are highly vascularized [58], possibly providing a vascular niche for NSCs unparallel to other brain regions. One study shows that NPC marker-expressing cells are often localize next to the vasculature in CVOs [183]. Nonetheless, it remains to be explored how the specialized

vasculature (e.g. exposure to blood-borne molecules) might support and maintains the adult NSC niche in the CVOs.

The present study has examined the neurogenic potential of the OVLT and SFO in the adult rat brain. In our initial experiments aiming to uncover adult neurogenesis, we administrated 2 dosages/day of BrdU (50mg/kg) for 7 days and rats were sacrificed 28 days after the last BrdU injection. While we were able to identify BrdU+ cells after 7 days of injections, we were unable to identify any BrdU+ cells in the OVLT and SFO after 28 days. This implies that cells that uptake BrdU divided, diluting the BrdU concentration, and thus its levels in their progenies were too low to detect. Therefore, we administrated BrdU through drinking water which provides a more continuous way of labeling newborn cells. To our surprise, in the OVLT and SFO, all BrdU+ cells are also HuC/HuD+ in rats that received 28 days and 60 days BrdU drinking water. This suggests that low concentration, prolonged duration of BrdU administration only labels neurons. A possible explanation is that NPCs need to undergo multiple division before becoming neurons, while other cell types are committed to their cell fate once they are generated from their progenitor cells. In other words, NPCs need to undergo more divisions and cycles of S phases to differentiate into neurons, while other cells do not require that many cell cycles. Therefore, only NPCs and their progenies – neurons can accumulate enough BrdU to be visualized, while other cells cannot accumulate enough BrdU to be detected. Thus, our results suggest that NPCs in the OVLT and SFO undergo a series of divisions to differentiate into neurons. This process in which NPCs undergo a stepwise division to commit to the neuronal cell fate is seen in the SVZ and SGZ. In the SVZ, type B stem cells give rise to type C transit amplifying cells, which generate type A neuroblasts, that can differentiate into immature neurons [184]. In the SGZ, SGZ astrocytes give rise to intermediate precursors cells known as type D cells [184]. Type D cells

proliferate and differentiate, progressively generating more differentiated progenies - type D1, type D2, and type D3 cells – that can mature into granule cells [184]. In the OVLT and SFO, NPCs presumably have their own pathways of becoming mature neurons, which remains to be studied.

6.4 Implications of Elevated Cell Proliferation and Adult Neurogenesis in the OVLT and SFO in Response to Acute Hypertension

The physiological significance of the increase in cell proliferation and adult neurogenesis in the OVLT and SFO in rats treated with HSD remains to be elucidated. In rodent models of obesity, it remains controversial whether cell proliferation in the ME contributes to the weight gain or provides resilience to the development of obesity. In one study, ablation of proliferating cells in the ME through X-irradiation in young mice (5.5 weeks) fed with a high-fat diet caused a reduction in the body weight and an increase in energy expenditure [13]. However, another study reported that ablation of proliferating cells in the ME through X-irradiation in adult mice (8-12 weeks) fed a normal chow diet caused a significant increase in body weight [185]. Similarly, the effect of increasing cell proliferation and adult neurogenesis in the OVLT and SFO on the development of hypertension can be either beneficial or detrimental. Similar to ME, where increasing cell proliferation and adult neurogenesis in the basomedial hypothalamus can provide resilience to the development of obesity [12, 87], increases in cell proliferation and adult neurogenesis in the OVLT and SFO can potentially represent a protective mechanism providing resilience to the development of hypertension. Conversely, cell proliferation and adult neurogenesis in the OVLT and SFO can potentially contribute to the development of hypertension. Since lesions of the OVLT attenuate the increase in the mean arterial pressure

induced by HSD, proliferating cells in the OVLT might contribute to neural circuits regulating the autonomic response to hypernatremia [186]. Thus, the functional contribution of enhanced cell proliferation and adult neurogenesis in the OVLT and SFO to high dietary salt-dependent hypertension remains to be explored. One potential way to do this is to induce or inhibit cell proliferation through pharmaceutical approaches and assess how the induction or inhibition of cell proliferation in the OVLT and SFO affect the blood pressure and blood osmolality in animal models of high dietary salt-dependent hypertension.

6.5 Perspectives of Stem Cell Therapy to Treat Hypertension

Understanding the physiological significance of cell proliferation and adult neurogenesis in the OVLT and SFO might be important for developing therapeutics to treat hypertension in the future. Stem cell therapy is one of the most promising strategies for treating and preventing diseases. Clinical studies utilizing stem cell therapy aim to treat a variety of diseases, including but not limited to spinal cord injury [187], diabetes mellitus [188, 189], multiple sclerosis [190], rheumatoid arthritis [191], and others. In animal studies focusing on the hypothalamus, the induction of hypothalamic adult neurogenesis can treat obesity, and the transplantation of immune-resistant hypothalamic NSCs can prolong lifespan [12, 88]. Identifying the role of NSCs in the OVLT and SFO in the development of salt-dependent hypertension might provide a new avenue for treating hypertension by manipulating NSCs in these areas. Existing stem cell therapy treating hypertension focuses on secondary hypertensions such as pulmonary arterial hypertension, which only encompass a small subset of hypertension [192]. However, primary hypertension, which represents most hypertension cases, is associated with a series of metabolic disorders (e.g. abdominal obesity, insulin resistance, and dyslipidemia) which have a central

neuroendocrine origin in the hypothalamus [193]. A stem cell therapy focusing on the hypothalamus can potentially provide a better treatment for primary hypertension and its associated metabolic diseases. Nevertheless, there is still a very long way from the present study to the development of potential clinical treatments. Numerous fundamental questions regarding NSCs in the OVLT and SFO remain to be addressed. What triggers the increase in cell proliferation and adult neurogenesis in animals that receive HSD? Is there an increase in apoptosis in the OVLT and SFO in animals that receive HSD? What cell type(s) do progenitor cells in the OVLT and SFO differentiate into? Do proliferating cells in the OVLT and SFO migrate to other brain regions? What are the active and quiescent NSCs in the OVLT and SFO? Answering these questions will provide the fundamental knowledge that can be used to generate new therapeutic tools for treating diseases and ultimately improving the quality of lives.

7. Bibliography

1. Barinaga, M., *No-new-neurons dogma loses ground.* Science, 1998. **279**(5359): p. 2041-2.
2. Reynolds, B.A. and S. Weiss, *Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system.* Science, 1992. **255**(5052): p. 1707-10.
3. Ray, J., et al., *Proliferation, differentiation, and long-term culture of primary hippocampal neurons.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3602-6.
4. Seri, B., et al., *Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus.* J Comp Neurol, 2004. **478**(4): p. 359-78.
5. Seri, B., et al., *Astrocytes give rise to new neurons in the adult mammalian hippocampus.* J Neurosci, 2001. **21**(18): p. 7153-60.
6. Doetsch, F., et al., *Subventricular zone astrocytes are neural stem cells in the adult mammalian brain.* Cell, 1999. **97**(6): p. 703-16.
7. Fuentealba, L.C., K. Obernier, and A. Alvarez-Buylla, *Adult neural stem cells bridge their niche.* Cell Stem Cell, 2012. **10**(6): p. 698-708.
8. Ma, D.K., et al., *Adult neural stem cells in the mammalian central nervous system.* Cell Res, 2009. **19**(6): p. 672-82.
9. Magavi, S.S., B.R. Leavitt, and J.D. Macklis, *Induction of neurogenesis in the neocortex of adult mice.* Nature, 2000. **405**(6789): p. 951-5.
10. Markakis, E.A., et al., *Novel neuronal phenotypes from neural progenitor cells.* J Neurosci, 2004. **24**(12): p. 2886-97.
11. Pencea, V., et al., *Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus.* J Neurosci, 2001. **21**(17): p. 6706-17.
12. Kokoeva, M.V., H. Yin, and J.S. Flier, *Neurogenesis in the hypothalamus of adult mice: potential role in energy balance.* Science, 2005. **310**(5748): p. 679-83.
13. Lee, D.A., et al., *Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche.* Nat Neurosci, 2012. **15**(5): p. 700-2.
14. Rizzoti, K. and R. Lovell-Badge, *Pivotal role of median eminence tanycytes for hypothalamic function and neurogenesis.* Mol Cell Endocrinol, 2017. **445**: p. 7-13.
15. Robins, S.C., et al., *alpha-Tanycytes of the adult hypothalamic third ventricle include distinct populations of FGF-responsive neural progenitors.* Nat Commun, 2013. **4**: p. 2049.
16. P M Gross 1, A.W., *Peering through the windows of the brain.* J Cereb Blood Flow Metab, 1987. **6**: p. 663-72.
17. Bentivoglio, M., K. Kristensson, and M.E. Rottenberg, *Circumventricular Organs and Parasite Neurotropism: Neglected Gates to the Brain?* Front Immunol, 2018. **9**: p. 2877.
18. Mirzadeh, Z., et al., *Bi- and uniciliated ependymal cells define continuous floor-plate-derived tanycytic territories.* Nat Commun, 2017. **8**: p. 13759.
19. Bourque, C.W., *Central mechanisms of osmosensation and systemic osmoregulation.* Nat Rev Neurosci, 2008. **9**(7): p. 519-31.

20. Prager-Khoutorsky, M. and C.W. Bourque, *Anatomical organization of the rat organum vasculosum laminae terminalis.* Am J Physiol Regul Integr Comp Physiol, 2015. **309**(4): p. R324-37.
21. Hicks, A.I., et al., *Anatomical Organization of the Rat Subfornical Organ.* Front Cell Neurosci, 2021. **15**: p. 691711.
22. McKinley, M.J., et al., *The sensory circumventricular organs of the mammalian brain.* Adv Anat Embryol Cell Biol, 2003. **172**: p. III-XII, 1-122, back cover.
23. Miller, A.D. and R.A. Leslie, *The area postrema and vomiting.* Front Neuroendocrinol, 1994. **15**(4): p. 301-20.
24. d'Anglemont de Tassigny, X., et al., *Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals.* Endocrinology, 2008. **149**(8): p. 3926-32.
25. Robins, S.C. and M.V. Kokoeva, *NG2-Glia, a New Player in Energy Balance.* Neuroendocrinology, 2018. **107**(3): p. 305-312.
26. Robins, S.C., et al., *Evidence for NG2-glia derived, adult-born functional neurons in the hypothalamus.* PLoS One, 2013. **8**(10): p. e78236.
27. Luboshizsky, R. and P. Lavie, *Sleep-inducing effects of exogenous melatonin administration.* Sleep Med Rev, 1998. **2**(3): p. 191-202.
28. McKinley, M.J., et al., *From sensory circumventricular organs to cerebral cortex: Neural pathways controlling thirst and hunger.* J Neuroendocrinol, 2019. **31**(3): p. e12689.
29. Ganong, W.F., *Circumventricular organs: definition and role in the regulation of endocrine and autonomic function.* Clin Exp Pharmacol Physiol, 2000. **27**(5-6): p. 422-7.
30. Johnson, A.K. and J. Buggy, *Periventricular preoptic-hypothalamus is vital for thirst and normal water economy.* Am J Physiol, 1978. **234**(3): p. R122-9.
31. McKinley, M.J., et al., *Efferent neural pathways of the lamina terminalis subserving osmoregulation.* Prog Brain Res, 1992. **91**: p. 395-402.
32. Bourque, C.W., et al., *Neurophysiological characterization of mammalian osmosensitive neurones.* Exp Physiol, 2007. **92**(3): p. 499-505.
33. Ciura, S. and C.W. Bourque, *Transient receptor potential vanilloid 1 is required for intrinsic osmoreception in organum vasculosum lamina terminalis neurons and for normal thirst responses to systemic hyperosmolality.* J Neurosci, 2006. **26**(35): p. 9069-75.
34. Vivas, L., E. Chiaraviglio, and H.F. Carrer, *Rat organum vasculosum laminae terminalis in vitro: responses to changes in sodium concentration.* Brain Res, 1990. **519**(1-2): p. 294-300.
35. Sunn, N., M.J. McKinley, and B.J. Oldfield, *Circulating angiotensin II activates neurones in circumventricular organs of the lamina terminalis that project to the bed nucleus of the stria terminalis.* J Neuroendocrinol, 2003. **15**(8): p. 725-31.
36. Gonzalez, A.D., et al., *Distribution of angiotensin type 1a receptor-containing cells in the brains of bacterial artificial chromosome transgenic mice.* Neuroscience, 2012. **226**: p. 489-509.
37. Lenkei, Z., et al., *Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review.* Front Neuroendocrinol, 1997. **18**(4): p. 383-439.

38. Egan, G., et al., *Neural correlates of the emergence of consciousness of thirst.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 15241-6.
39. Morita, H., et al., *Sequence of forebrain activation induced by intraventricular injection of hypertonic NaCl detected by Mn2+ contrasted T1-weighted MRI.* Auton Neurosci, 2004. **113**(1-2): p. 43-54.
40. McKinley, M.J., et al., *Water intake and the neural correlates of the consciousness of thirst.* Semin Nephrol, 2006. **26**(3): p. 249-57.
41. McKinley, M.J., et al., *Osmoregulatory thirst in sheep is disrupted by ablation of the anterior wall of the optic recess.* Brain Res, 1982. **236**(1): p. 210-5.
42. Thrasher, T.N., L.C. Keil, and D.J. Ramsay, *Lesions of the organum vasculosum of the lamina terminalis (OVLT) attenuate osmotically-induced drinking and vasopressin secretion in the dog.* Endocrinology, 1982. **110**(5): p. 1837-9.
43. Abbott, S.B., et al., *Reciprocal Control of Drinking Behavior by Median Preoptic Neurons in Mice.* J Neurosci, 2016. **36**(31): p. 8228-37.
44. Leib, D.E., et al., *The Forebrain Thirst Circuit Drives Drinking through Negative Reinforcement.* Neuron, 2017. **96**(6): p. 1272-1281 e4.
45. Kinsman, B.J., et al., *Integration of Hypernatremia and Angiotensin II by the Organum Vasculosum of the Lamina Terminalis Regulates Thirst.* J Neurosci, 2020. **40**(10): p. 2069-2079.
46. Miller, R.L., et al., *ENaC-expressing neurons in the sensory circumventricular organs become c-Fos activated following systemic sodium changes.* Am J Physiol Regul Integr Comp Physiol, 2013. **305**(10): p. R1141-52.
47. Gross, D.S., *Distribution of gonadotropin-releasing hormone in the mouse brain as revealed by immunohistochemistry.* Endocrinology, 1976. **98**(6): p. 1408-17.
48. Nagaishi, V.S., et al., *Possible crosstalk between leptin and prolactin during pregnancy.* Neuroscience, 2014. **259**: p. 71-83.
49. Johnson, A.K., J.T. Cunningham, and R.L. Thunhorst, *Integrative role of the lamina terminalis in the regulation of cardiovascular and body fluid homeostasis.* Clin Exp Pharmacol Physiol, 1996. **23**(2): p. 183-91.
50. Herde, M.K., et al., *Gonadotropin-releasing hormone neurons extend complex highly branched dendritic trees outside the blood-brain barrier.* Endocrinology, 2011. **152**(10): p. 3832-41.
51. Sunn, N., M.J. McKinley, and B.J. Oldfield, *Identification of efferent neural pathways from the lamina terminalis activated by blood-borne relaxin.* J Neuroendocrinol, 2001. **13**(5): p. 432-7.
52. Ott, D., et al., *Neurons and glial cells of the rat organum vasculosum laminae terminalis directly respond to lipopolysaccharide and pyrogenic cytokines.* Brain Res, 2010. **1363**: p. 93-106.
53. Samson, W.K. and S.M. McCann, *Effects of lesions in the organum vasculosum lamina terminalis on the hypothalamic distribution of luteinizing hormone-releasing hormone and gonadotropin secretion in the ovariectomized rat.* Endocrinology, 1979. **105**(4): p. 939-46.
54. Bourque, C.W., *Osmoregulation of vasopressin neurons: a synergy of intrinsic and synaptic processes.* Prog Brain Res, 1998. **119**: p. 59-76.

55. Brimble, M.J. and R.E. Dyball, *Characterization of the responses of oxytocin- and vasopressin-secreting neurones in the supraoptic nucleus to osmotic stimulation.* J Physiol, 1977. **271**(1): p. 253-71.
56. Wakerley, J.B., D.A. Poulain, and D. Brown, *Comparison of firing patterns in oxytocin- and vasopressin-releasing neurones during progressive dehydration.* Brain Res, 1978. **148**(2): p. 425-40.
57. Hollis, J.H., et al., *The trajectory of sensory pathways from the lamina terminalis to the insular and cingulate cortex: a neuroanatomical framework for the generation of thirst.* Am J Physiol Regul Integr Comp Physiol, 2008. **294**(4): p. R1390-401.
58. Duvernoy, H.M. and P.Y. Risold, *The circumventricular organs: an atlas of comparative anatomy and vascularization.* Brain Res Rev, 2007. **56**(1): p. 119-47.
59. Naganawa, S., et al., *Appearance of the Organum Vasculosum of the Lamina Terminalis on Contrast-enhanced MR Imaging.* Magn Reson Med Sci, 2018. **17**(2): p. 132-137.
60. Thomas, M.A., et al., *Localization of angiotensin II (AT1)-receptor-immunoreactive fibres in the hypothalamus of rats: angiotensin II-sensitive tanycytes in the ependyma of the third ventricle?* Brain Res, 2003. **967**(1-2): p. 281-4.
61. Song, K., et al., *Mapping of angiotensin II receptor subtype heterogeneity in rat brain.* J Comp Neurol, 1992. **316**(4): p. 467-84.
62. Smith, P.M., et al., *The subfornical organ: a central nervous system site for actions of circulating leptin.* Am J Physiol Regul Integr Comp Physiol, 2009. **296**(3): p. R512-20.
63. Somponpun, S.J., et al., *Estrogen receptor-alpha expression in osmosensitive elements of the lamina terminalis: regulation by hypertonicity.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(3): p. R661-9.
64. Simpson, N.J. and A.V. Ferguson, *The proinflammatory cytokine tumor necrosis factor-alpha excites subfornical organ neurons.* J Neurophysiol, 2017. **118**(3): p. 1532-1541.
65. McKinley, M.J., et al., *Effect of individual or combined ablation of the nuclear groups of the lamina terminalis on water drinking in sheep.* Am J Physiol, 1999. **276**(3): p. R673-83.
66. McKinley, M.J., D.K. Hards, and B.J. Oldfield, *Identification of neural pathways activated in dehydrated rats by means of Fos-immunohistochemistry and neural tracing.* Brain Res, 1994. **653**(1-2): p. 305-14.
67. Oldfield, B.J., et al., *Fos production in retrogradely labelled neurons of the lamina terminalis following intravenous infusion of either hypertonic saline or angiotensin II.* Neuroscience, 1994. **60**(1): p. 255-62.
68. Felix, D. and K. Akert, *The effect of angiotensin II on neurones of the cat subfornical organ.* Brain Res, 1974. **76**(2): p. 350-3.
69. Felix, D. and W. Schlegel, *Angiotensin receptive neurones in the subfornical organ. Structure-activity relations.* Brain Res, 1978. **149**(1): p. 107-16.
70. Anderson, J.W., D.L. Washburn, and A.V. Ferguson, *Intrinsic osmosensitivity of subfornical organ neurons.* Neuroscience, 2000. **100**(3): p. 539-47.
71. Oka, Y., M. Ye, and C.S. Zuker, *Thirst driving and suppressing signals encoded by distinct neural populations in the brain.* Nature, 2015. **520**(7547): p. 349-52.
72. Matsuda, T., et al., *Distinct neural mechanisms for the control of thirst and salt appetite in the subfornical organ.* Nat Neurosci, 2017. **20**(2): p. 230-241.
73. Zimmerman, C.A., et al., *Thirst neurons anticipate the homeostatic consequences of eating and drinking.* Nature, 2016. **537**(7622): p. 680-684.

74. Mark, M.H. and P.M. Farmer, *The human subfornical organ: an anatomic and ultrastructural study.* Ann Clin Lab Sci, 1984. **14**(6): p. 427-42.
75. Ramalho-Santos, M. and H. Willenbring, *On the origin of the term "stem cell".* Cell Stem Cell, 2007. **1**(1): p. 35-8.
76. Li, X. and X. Zhao, *Epigenetic regulation of mammalian stem cells.* Stem Cells Dev, 2008. **17**(6): p. 1043-52.
77. Kriegstein, A. and A. Alvarez-Buylla, *The glial nature of embryonic and adult neural stem cells.* Annu Rev Neurosci, 2009. **32**: p. 149-84.
78. Urban, N., I.M. Blomfield, and F. Guillemot, *Quiescence of Adult Mammalian Neural Stem Cells: A Highly Regulated Rest.* Neuron, 2019. **104**(5): p. 834-848.
79. Tropepe, V., et al., *Transforming growth factor-alpha null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma.* J Neurosci, 1997. **17**(20): p. 7850-9.
80. Enwere, E., et al., *Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory neurogenesis, and deficits in fine olfactory discrimination.* J Neurosci, 2004. **24**(38): p. 8354-65.
81. Kuhn, H.G., H. Dickinson-Anson, and F.H. Gage, *Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation.* J Neurosci, 1996. **16**(6): p. 2027-33.
82. Jin, K., et al., *Neurogenesis and aging: FGF-2 and HB-EGF restore neurogenesis in hippocampus and subventricular zone of aged mice.* Aging Cell, 2003. **2**(3): p. 175-83.
83. Geinisman, Y., et al., *Hippocampal markers of age-related memory dysfunction: behavioral, electrophysiological and morphological perspectives.* Prog Neurobiol, 1995. **45**(3): p. 223-52.
84. Seigers, R., et al., *Methotrexate decreases hippocampal cell proliferation and induces memory deficits in rats.* Behav Brain Res, 2009. **201**(2): p. 279-84.
85. Berdugo-Vega, G., et al., *Author Correction: Increasing neurogenesis refines hippocampal activity rejuvenating navigational learning strategies and contextual memory throughout life.* Nat Commun, 2020. **11**(1): p. 1138.
86. Rochefort, C., et al., *Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory.* J Neurosci, 2002. **22**(7): p. 2679-89.
87. Gouaze, A., et al., *Cerebral cell renewal in adult mice controls the onset of obesity.* PLoS One, 2013. **8**(8): p. e72029.
88. Zhang, Y., et al., *Hypothalamic stem cells control ageing speed partly through exosomal miRNAs.* Nature, 2017. **548**(7665): p. 52-57.
89. Zhang, G., et al., *Hypothalamic programming of systemic ageing involving IKK-beta, NF-kappaB and GnRH.* Nature, 2013. **497**(7448): p. 211-6.
90. Bennett, L., et al., *Circumventricular organs: a novel site of neural stem cells in the adult brain.* Mol Cell Neurosci, 2009. **41**(3): p. 337-47.
91. Zilkha-Falb, R., N. Kaushansky, and A. Ben-Nun, *The Median Eminence, A New Oligodendrogenic Niche in the Adult Mouse Brain.* Stem Cell Reports, 2020. **14**(6): p. 1076-1092.
92. Bennett, L.B., et al., *Heterotopically transplanted CVO neural stem cells generate neurons and migrate with SVZ cells in the adult mouse brain.* Neurosci Lett, 2010. **475**(1): p. 1-6.

93. Langlet, F., et al., *Tanycyte-like cells form a blood-cerebrospinal fluid barrier in the circumventricular organs of the mouse brain.* J Comp Neurol, 2013. **521**(15): p. 3389-405.
94. Zhang, S. and W. Cui, *Sox2, a key factor in the regulation of pluripotency and neural differentiation.* World J Stem Cells, 2014. **6**(3): p. 305-11.
95. Furube, E., et al., *Neural stem cell phenotype of tanycyte-like ependymal cells in the circumventricular organs and central canal of adult mouse brain.* Sci Rep, 2020. **10**(1): p. 2826.
96. Yoo, S., et al., *Control of neurogenic competence in mammalian hypothalamic tanycytes.* Sci Adv, 2021. **7**(22).
97. Stallcup, W.B., *The NG2 proteoglycan: past insights and future prospects.* J Neurocytol, 2002. **31**(6-7): p. 423-35.
98. Nishiyama, A., R. Suzuki, and X. Zhu, *NG2 cells (polydendrocytes) in brain physiology and repair.* Front Neurosci, 2014. **8**: p. 133.
99. Zhu, X., et al., *Age-dependent fate and lineage restriction of single NG2 cells.* Development, 2011. **138**(4): p. 745-53.
100. Stallcup, W.B., *The NG2 Proteoglycan in Pericyte Biology.* Adv Exp Med Biol, 2018. **1109**: p. 5-19.
101. Belachew, S., et al., *Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons.* J Cell Biol, 2003. **161**(1): p. 169-86.
102. Aguirre, A. and V. Gallo, *Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone.* J Neurosci, 2004. **24**(46): p. 10530-41.
103. Aguirre, A.A., et al., *NG2-expressing cells in the subventricular zone are type C-like cells and contribute to interneuron generation in the postnatal hippocampus.* J Cell Biol, 2004. **165**(4): p. 575-89.
104. Ahmed, T.A. and N. El-Badri, *Pericytes: The Role of Multipotent Stem Cells in Vascular Maintenance and Regenerative Medicine.* Adv Exp Med Biol, 2018. **1079**: p. 69-86.
105. Ozen, I., et al., *Brain pericytes acquire a microglial phenotype after stroke.* Acta Neuropathol, 2014. **128**(3): p. 381-96.
106. Sakuma, R., et al., *Brain pericytes serve as microglia-generating multipotent vascular stem cells following ischemic stroke.* J Neuroinflammation, 2016. **13**(1): p. 57.
107. Paul, G., et al., *The adult human brain harbors multipotent perivascular mesenchymal stem cells.* PLoS One, 2012. **7**(4): p. e35577.
108. Karow, M., et al., *Direct pericyte-to-neuron reprogramming via unfolding of a neural stem cell-like program.* Nat Neurosci, 2018. **21**(7): p. 932-940.
109. Bruttger, J., et al., *Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System.* Immunity, 2015. **43**(1): p. 92-106.
110. Zhan, L., et al., *Proximal recolonization by self-renewing microglia re-establishes microglial homeostasis in the adult mouse brain.* PLoS Biol, 2019. **17**(2): p. e3000134.
111. Gomez-Nicola, D. and V.H. Perry, *Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity.* Neuroscientist, 2015. **21**(2): p. 169-84.
112. Askew, K., et al., *Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain.* Cell Rep, 2017. **18**(2): p. 391-405.

113. Choe, K.Y., et al., *High salt intake increases blood pressure via BDNF-mediated downregulation of KCC2 and impaired baroreflex inhibition of vasopressin neurons.* Neuron, 2015. **85**(3): p. 549-60.
114. Olateju, O.I., et al., *Mitogen and Stress-activated Protein Kinase 1 Negatively Regulates Hippocampal Neurogenesis.* Neuroscience, 2021. **452**: p. 228-234.
115. Li, Q., et al., *Ischemic Preconditioning Induces Oligodendrogenesis in Mouse Brain: Effects of Nrf2 Deficiency.* Cell Mol Neurobiol, 2022. **42**(6): p. 1859-1873.
116. Wittmann, G. and R.M. Lechan, *Prss56 expression in the rodent hypothalamus: Inverse correlation with pro-opiomelanocortin suggests oscillatory gene expression in adult rat tanycytes.* J Comp Neurol, 2018. **526**(15): p. 2444-2461.
117. Womack, T.R., et al., *Prostacyclin Promotes Degenerative Pathology in a Model of Alzheimer's Disease.* Front Cell Neurosci, 2022. **16**: p. 769347.
118. Aikawa, T., et al., *ABCA7 haplodeficiency disturbs microglial immune responses in the mouse brain.* Proc Natl Acad Sci U S A, 2019. **116**(47): p. 23790-23796.
119. Chang, D., et al., *Distinct Cellular Profiles of Hif1a and Vegf mRNA Localization in Microglia, Astrocytes and Neurons during a Period of Vascular Maturation in the Auditory Brainstem of Neonate Rats.* Brain Sci, 2021. **11**(7).
120. Lacoste, B., et al., *Sensory-related neural activity regulates the structure of vascular networks in the cerebral cortex.* Neuron, 2014. **83**(5): p. 1117-30.
121. Dai, J., et al., *Olig1 function is required for oligodendrocyte differentiation in the mouse brain.* J Neurosci, 2015. **35**(10): p. 4386-402.
122. Bhattarai, P., et al., *The effects of aging on Amyloid-beta42-induced neurodegeneration and regeneration in adult zebrafish brain.* Neurogenesis (Austin), 2017. **4**(1): p. e1322666.
123. Cho, K.O., et al., *Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline.* Nat Commun, 2015. **6**: p. 6606.
124. Low, K.L., C. Ma, and K.K. Soma, *Tyramide Signal Amplification Permits Immunohistochemical Analyses of Androgen Receptors in the Rat Prefrontal Cortex.* J Histochem Cytochem, 2017. **65**(5): p. 295-308.
125. Kee, N., et al., *The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis.* J Neurosci Methods, 2002. **115**(1): p. 97-105.
126. Ellis, P., et al., *SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult.* Dev Neurosci, 2004. **26**(2-4): p. 148-65.
127. Stratton, J.A., et al., *A tale of two cousins: Ependymal cells, quiescent neural stem cells and potential mechanisms driving their functional divergence.* FEBS J, 2019. **286**(16): p. 3110-3116.
128. Shah, P.T., et al., *Single-Cell Transcriptomics and Fate Mapping of Ependymal Cells Reveals an Absence of Neural Stem Cell Function.* Cell, 2018. **173**(4): p. 1045-1057 e9.
129. Brown, J.P., et al., *Transient expression of doublecortin during adult neurogenesis.* J Comp Neurol, 2003. **467**(1): p. 1-10.
130. Taupin, P., *BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation.* Brain Res Rev, 2007. **53**(1): p. 198-214.
131. Doggrell, S.A. and L. Brown, *Rat models of hypertension, cardiac hypertrophy and failure.* Cardiovasc Res, 1998. **39**(1): p. 89-105.

132. Padmanabhan, S. and B. Joe, *Towards Precision Medicine for Hypertension: A Review of Genomic, Epigenomic, and Microbiomic Effects on Blood Pressure in Experimental Rat Models and Humans.* Physiol Rev, 2017. **97**(4): p. 1469-1528.
133. Lerman, L.O., et al., *Animal Models of Hypertension: A Scientific Statement From the American Heart Association.* Hypertension, 2019. **73**(6): p. e87-e120.
134. Lynn, L.A. and J.P. O'Neill, *The cytotoxic and mutagenic effects of 5-bromodeoxyuridine-plus-black-light treatment in cultured mammalian cells.* Mutat Res, 1982. **94**(1): p. 179-87.
135. Caldwell, M.A., X. He, and C.N. Svendsen, *5-Bromo-2'-deoxyuridine is selectively toxic to neuronal precursors in vitro.* Eur J Neurosci, 2005. **22**(11): p. 2965-70.
136. Sekerkova, G., E. Ilijic, and E. Mugnaini, *Bromodeoxyuridine administered during neurogenesis of the projection neurons causes cerebellar defects in rat.* J Comp Neurol, 2004. **470**(3): p. 221-39.
137. Hancock, A., et al., *Does 5-bromo-2'-deoxyuridine (BrdU) disrupt cell proliferation and neuronal maturation in the adult rat hippocampus in vivo?* Behav Brain Res, 2009. **199**(2): p. 218-21.
138. Parkinson, F.E., et al., *Molecular biology of nucleoside transporters and their distributions and functions in the brain.* Curr Top Med Chem, 2011. **11**(8): p. 948-72.
139. Morita, S. and S. Miyata, *Different vascular permeability between the sensory and secretory circumventricular organs of adult mouse brain.* Cell Tissue Res, 2012. **349**(2): p. 589-603.
140. Mullier, A., et al., *Differential distribution of tight junction proteins suggests a role for tanycytes in blood-hypothalamus barrier regulation in the adult mouse brain.* J Comp Neurol, 2010. **518**(7): p. 943-62.
141. Morita, S. and S. Miyata, *Accessibility of low-molecular-mass molecules to the median eminence and arcuate hypothalamic nucleus of adult mouse.* Cell Biochem Funct, 2013. **31**(8): p. 668-77.
142. Kokoeva, M.V., H. Yin, and J.S. Flier, *Evidence for constitutive neural cell proliferation in the adult murine hypothalamus.* J Comp Neurol, 2007. **505**(2): p. 209-20.
143. McNay, D.E., et al., *Remodeling of the arcuate nucleus energy-balance circuit is inhibited in obese mice.* J Clin Invest, 2012. **122**(1): p. 142-52.
144. Piatti, V.C., et al., *The timing for neuronal maturation in the adult hippocampus is modulated by local network activity.* J Neurosci, 2011. **31**(21): p. 7715-28.
145. Cajal, S.R.y., *Degeneration and Regeneration of the Nervous System* ed. R.M. May. 1928, London: Oxford University Press.
146. Altman, J., *Are new neurons formed in the brains of adult mammals?* Science, 1962. **135**(3509): p. 1127-8.
147. Kumar, A., et al., *ADULT NEUROGENESIS IN HUMANS: A Review of Basic Concepts, History, Current Research, and Clinical Implications.* Innov Clin Neurosci, 2019. **16**(5-6): p. 30-37.
148. Jurkowski, M.P., et al., *Beyond the Hippocampus and the SVZ: Adult Neurogenesis Throughout the Brain.* Front Cell Neurosci, 2020. **14**: p. 576444.
149. Bernier, P.J., et al., *Newly generated neurons in the amygdala and adjoining cortex of adult primates.* Proc Natl Acad Sci U S A, 2002. **99**(17): p. 11464-9.
150. Cao, J., K. Wenberg, and M.F. Cheng, *Lesion induced new neuron incorporation in the adult hypothalamus of the avian brain.* Brain Res, 2002. **943**(1): p. 80-92.

151. Dayer, A.G., et al., *New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors.* J Cell Biol, 2005. **168**(3): p. 415-27.
152. Inta, D., et al., *Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20994-9.
153. Zecevic, N. and P. Rakic, *Development of layer I neurons in the primate cerebral cortex.* J Neurosci, 2001. **21**(15): p. 5607-19.
154. Evans, J., et al., *Characterization of mitotic neurons derived from adult rat hypothalamus and brain stem.* J Neurophysiol, 2002. **87**(2): p. 1076-85.
155. Jhaveri, D.J., et al., *Evidence for newly generated interneurons in the basolateral amygdala of adult mice.* Mol Psychiatry, 2018. **23**(3): p. 521-532.
156. Nogueira, A.B., et al., *Existence of a potential neurogenic system in the adult human brain.* J Transl Med, 2014. **12**: p. 75.
157. Bernstein, P.L., M. Zuo, and M.F. Cheng, *Social condition affects the courtship behavior of male ring doves with posterior medial hypothalamic lesions.* Behav Neural Biol, 1993. **59**(2): p. 120-5.
158. Fowler, C.D., et al., *The effects of social environment on adult neurogenesis in the female prairie vole.* J Neurobiol, 2002. **51**(2): p. 115-28.
159. Shapiro, L.A., et al., *Subventricular zone-derived, newly generated neurons populate several olfactory and limbic forebrain regions.* Epilepsy Behav, 2009. **14 Suppl 1**: p. 74-80.
160. Saul, M.L., et al., *Proliferating cells in the adolescent rat amygdala: Characterization and response to stress.* Neuroscience, 2015. **311**: p. 105-17.
161. Langlet, F., et al., *Tanycytic VEGF-A boosts blood-hypothalamus barrier plasticity and access of metabolic signals to the arcuate nucleus in response to fasting.* Cell Metab, 2013. **17**(4): p. 607-17.
162. Roelofs, R.F., et al., *Adult human subventricular, subgranular, and subpial zones contain astrocytes with a specialized intermediate filament cytoskeleton.* Glia, 2005. **52**(4): p. 289-300.
163. Xue, J.H., et al., *Induced spreading depression evokes cell division of astrocytes in the subpial zone, generating neural precursor-like cells and new immature neurons in the adult cerebral cortex.* Stroke, 2009. **40**(11): p. e606-13.
164. Ponti, G., P. Peretto, and L. Bonfanti, *A subpial, transitory germinal zone forms chains of neuronal precursors in the rabbit cerebellum.* Dev Biol, 2006. **294**(1): p. 168-80.
165. Li, G., et al., *Identification of a transient subpial neurogenic zone in the developing dentate gyrus and its regulation by Cxcl12 and reelin signaling.* Development, 2009. **136**(2): p. 327-35.
166. Jessen, N.A., et al., *The Glymphatic System: A Beginner's Guide.* Neurochem Res, 2015. **40**(12): p. 2583-99.
167. Lehtinen, M.K. and C.A. Walsh, *Neurogenesis at the brain-cerebrospinal fluid interface.* Annu Rev Cell Dev Biol, 2011. **27**: p. 653-79.
168. Zappaterra, M.W. and M.K. Lehtinen, *The cerebrospinal fluid: regulator of neurogenesis, behavior, and beyond.* Cell Mol Life Sci, 2012. **69**(17): p. 2863-78.
169. Lugert, S., et al., *Glypican-2 levels in cerebrospinal fluid predict the status of adult hippocampal neurogenesis.* Sci Rep, 2017. **7**: p. 46543.

170. Rafii, S., J.M. Butler, and B.S. Ding, *Angiocrine functions of organ-specific endothelial cells.* Nature, 2016. **529**(7586): p. 316-25.
171. Shen, Q., et al., *Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells.* Science, 2004. **304**(5675): p. 1338-40.
172. Shen, Q., et al., *Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions.* Cell Stem Cell, 2008. **3**(3): p. 289-300.
173. Mirzadeh, Z., et al., *Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain.* Cell Stem Cell, 2008. **3**(3): p. 265-78.
174. Tavazoie, M., et al., *A specialized vascular niche for adult neural stem cells.* Cell Stem Cell, 2008. **3**(3): p. 279-88.
175. Palmer, T.D., A.R. Willhoite, and F.H. Gage, *Vascular niche for adult hippocampal neurogenesis.* J Comp Neurol, 2000. **425**(4): p. 479-94.
176. Ottone, C., et al., *Direct cell-cell contact with the vascular niche maintains quiescent neural stem cells.* Nat Cell Biol, 2014. **16**(11): p. 1045-56.
177. Ottone, C. and S. Parrinello, *Multifaceted control of adult SVZ neurogenesis by the vascular niche.* Cell Cycle, 2015. **14**(14): p. 2222-5.
178. Leventhal, C., et al., *Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma.* Mol Cell Neurosci, 1999. **13**(6): p. 450-64.
179. Jin, K., et al., *Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo.* Proc Natl Acad Sci U S A, 2002. **99**(18): p. 11946-50.
180. Nolan, D.J., et al., *Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration.* Dev Cell, 2013. **26**(2): p. 204-19.
181. Delgado, A.C., et al., *Endothelial NT-3 delivered by vasculature and CSF promotes quiescence of subependymal neural stem cells through nitric oxide induction.* Neuron, 2014. **83**(3): p. 572-85.
182. Lacar, B., et al., *Gap junction-mediated calcium waves define communication networks among murine postnatal neural progenitor cells.* Eur J Neurosci, 2011. **34**(12): p. 1895-905.
183. Hourai, A. and S. Miyata, *Neurogenesis in the circumventricular organs of adult mouse brains.* J Neurosci Res, 2013. **91**(6): p. 757-70.
184. Ming, G.L. and H. Song, *Adult neurogenesis in the mammalian brain: significant answers and significant questions.* Neuron, 2011. **70**(4): p. 687-702.
185. Djogo, T., et al., *Adult NG2-Glia Are Required for Median Eminence-Mediated Leptin Sensing and Body Weight Control.* Cell Metab, 2016. **23**(5): p. 797-810.
186. Collister, J.P., et al., *OVLT lesion decreases basal arterial pressure and the chronic hypertensive response to AngII in rats on a high-salt diet.* Physiol Rep, 2013. **1**(5): p. e00128.
187. Sykova, E., et al., *Autologous bone marrow transplantation in patients with subacute and chronic spinal cord injury.* Cell Transplant, 2006. **15**(8-9): p. 675-87.
188. Kong, D., et al., *Umbilical cord mesenchymal stem cell transfusion ameliorated hyperglycemia in patients with type 2 diabetes mellitus.* Clin Lab, 2014. **60**(12): p. 1969-76.
189. Cho, J., et al., *A review of clinical trials: mesenchymal stem cell transplant therapy in type 1 and type 2 diabetes mellitus.* Am J Stem Cells, 2018. **7**(4): p. 82-93.

190. Abi Chahine, N., et al., *Autologous Bone Marrow Derived Stem Cells for the Treatment of Multiple Sclerosis.* Int J Stem Cells, 2016. **9**(2): p. 207-212.
191. Ghoryani, M., et al., *The Sufficient Immunoregulatory Effect of Autologous Bone Marrow-Derived Mesenchymal Stem Cell Transplantation on Regulatory T Cells in Patients with Refractory Rheumatoid Arthritis.* J Immunol Res, 2020. **2020**: p. 3562753.
192. Ding, X.F., et al., *Efficacy of stem cell therapy for pulmonary arterial hypertension: a systematic review and meta-analysis of preclinical studies.* Stem Cell Res Ther, 2019. **10**(1): p. 55.
193. Bjorntorp, P., et al., *Hypertension and the metabolic syndrome: closely related central origin?* Blood Press, 2000. **9**(2-3): p. 71-82.