Masterclass in Neuroendocrinology 11

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Glial-Neuronal Signaling in Neuroendocrine Systems

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Masterclass in Neuroendocrinology

Volume 11

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Series Preface

This series began publication as a joint venture between the International Neuroendocrine Federation and Wiley-Blackwell and now is continuing with Springer Nature as publisher for the federation. The broad aim of the series is to provide established researchers, trainees, and students with authoritative up-to-date accounts of the present state of knowledge and prospects for the future across a range of topics in the burgeoning field of neuroendocrinology. The series is aimed at a wide audience as neuroendocrinology integrates neuroscience and endocrinology. We define neuroendocrinology as the study of the control of endocrine function by the brain and the actions of hormones on the brain. It encompasses the study of normal and abnormal function and the developmental origins of disease. It includes the study of the neural networks in the brain that regulate and form neuroendocrine systems. It also includes the study of behaviors and mental states that are influenced or regulated by hormones. It necessarily includes understanding and study of peripheral physiological systems that are regulated by neuroendocrine mechanisms. Clearly, neuroendocrinology embraces many current issues of concern to human health and well-being, but research on these issues necessitates reductionist animal models.

Contemporary research in neuroendocrinology involves the use of a wide range of techniques and technologies, from the subcellular to systems at the whole-organism level. A particular aim of the series is to provide expert advice and discussion about experimental or study protocols in research in neuroendocrinology and to further advance the field by giving information and advice about novel techniques, technologies, and interdisciplinary approaches.

To achieve our aims, each book is on a particular theme in neuroendocrinology, and for each book, we have recruited editors, experts in the field, and they have engaged an international team of experts to contribute chapters in their individual areas of expertise. Their mission was to give an update of knowledge and recent discoveries to discuss new approaches, "gold-standard" protocols, translational possibilities, and future prospects. The authors were asked to write for a wide audience, to minimize references, and to consider the use of video clips and explanatory text boxes; each chapter is peer-reviewed, has a Glossary and a detailed Index.

The Masterclass Series is open-ended; books in the series published to date are: *Neurophysiology of Neuroendocrine Neurons* (2014, ed. WE Armstrong & JG Tasker); *Neuroendocrinology of Stress* (2015, ed. JA Russell & MJ Shipston); *Molecular Neuroendocrinology: From Genome to Physiology* (2016, ed. D Murphy & H Gainer); *Computational Neuroendocrinology* (2016, ed. DJ Macgregor & G Leng); *Neuroendocrinology of Appetite* (2016; ed. SL Dickson & JG Mercer); *The GnRH Neuron and its Control* (2018; ed. AE Herbison & TM Plant); and *Model Animals in Neuroendocrinology* (2019, ed. M Ludwig & G Levkowitz). The first books of the series published by Springer Nature are *Neurosecretion: Secretory Mechanisms* (2020, ed. J Lemos & G Dayanithi); *Developmental Neuroendocrinology* (2020, ed. S Wray & S Blackshaw); *Neuroendocrine Clocks and Calendars* (2020, ed. F Ebbling & H Piggins); and this volume, *Glial–Neuronal Signaling in Neuroendocrine Systems* (ed. JG Tasker, JS Bains, & JA Chowen). In development are *Neuroanatomy of Neuroendocrine Systems* (ed. V Grinevich & A Dobolyi) and *Neuroendocrinology of Pregnancy and Lactation* (ed. P Brunton & D Grattan).

Feedback and suggestions are welcome.

International Neuroendocrine Federation—http://neuroendonow.com/

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Volume Preface

The hypothalamus, comprised of multiple cell populations organized into defined intercommunicating nuclei, plays a critical role in the survival of the organism. The hypothalamus has served as a vanguard for furthering our understanding of glia in the nervous system. These fascinating cells have gone from being thought a mere support for the surrounding neurons to assuming diverse essential functions. Driven by the advent of new experimental tools that provide unprecedented levels of insight into cellular function, the past two decades have seen an explosion of work on glial cells in the nervous system. However, it was the pioneering work from the laboratories of D. Theodosis (France) and G. Hatton (USA) showing the physiological state-dependent plasticity of glial processes in the magnocellular neurosecretory system in the supraoptic and paraventricular nuclei of the hypothalamus that was pivotal in advancing the idea that the glia–neuron relationship is dynamic. The ensuing years have witnessed an explosion of exciting discoveries in glial–neuronal interactions throughout the brain that have built on this classical work. This volume presents a cross-section of our current state of knowledge of the bidirectional glial–neuronal interactions in the hypothalamus.

Part I of the volume addresses the importance of glia during early development. Chapter 1 focuses on microglia in the hypothalamus and their crucial role during embryogenesis. It provides a comprehensive description of key findings on the sexually dimorphic function of microglia in the establishment of hypothalamic metabolic circuitry in the developing hypothalamus.

Part II focuses on the glial–neuronal interactions that control the magnocellular neuroendocrine system. Chapter 2, leveraging in part the structural dynamism of astrocyte processes, describes observations implicating a critical role for these glial cells in regulating spillover of neurotransmitter substances to presynaptic sites, as well as showcasing the pivotal work showing that astrocytes provide an essential co-agonist for neuronal NMDA receptor function. Chapter 3 details the fundamental role of glia–neuron and axon–glia interactions in regulating the diffusion of neuropeptides from the neurohypophysis into the general circulation. Chapter 4 summarizes findings that implicate the gliotransmitter ATP as a regulator of synaptic strength at glutamate synapses on magnocellular neurosecretory cells. Chapter 5 goes on to discuss how the interactions between glia, both astrocytes and microglia,

and neurons are not only important for normal physiological function but are also associated with disease progression.

Part III describes glial–neuronal interactions among cell populations in the hypothalamus that regulate energy balance and metabolism, key neuroendocrine systems currently receiving considerable attention due to the ongoing obesity epidemic. Chapter 6 highlights findings demonstrating that astrocytes are critical players in the regulation of metabolism, including their participation in the central response to metabolic factors such as insulin and leptin and astrogliosis in obesity. Chapter 7 then underscores the importance of bidirectional communication between neurons and microglia in the induction and progression of neuroinflammation associated with obesity and its secondary complications.

Part IV focuses on glial–neuronal interactions that regulate the neuroendocrine response to stress. Chapter 8 compiles state-of-the-art information underscoring the complex dialogue between astrocytes and neuronal dendrites in the regulation of hypothalamic neuroendocrine cells that control the stress response.

Then, Part V turns to the control of reproductive, social, and affective behaviors and function by hypothalamic glial–neuronal interactions, as glial cells in the hypothalamus, both microglia and astrocytes, are also vital in the control of reproductive, social, and affective behaviors, both in health and disease. In Chap. 9, how stress and reproductive hormones influence the microglial control of hypothalamic circuit formation and plasticity is described. The important role of the microglia influence on neural circuitry is explored in the context of the regulation of both normal and dysregulated behaviors. Chapter 10 showcases the critical role of astrocytic plasticity during puberty and the astrocyte regulation of estrogen positive feedback and neuroprogesterone release on the hypothalamic–pituitary–gonadal axis during the estrous cycle.

Finally, Part VI of this volume looks at the essential role of interactions between neurons and glia for the appropriate regulation of the blood–brain barrier, focusing on the primary role of tanycytes in this function in the hypothalamus. Thus, Chap. 11 discusses the ongoing characterization of the diversity of these specialized glial cells and delves into their importance, including structural remodeling of tanycytes in response to nutritional status and other hormonal signals regulating the transport of circulating factors into the hypothalamus and modulation of pituitary hormone secretion. Chapter 12 concludes this overview of glial–neuronal interactions in neuroendocrine systems with a review of the tanycyte control of the hypothalamic–pituitary–thyroid axis, highlighting the tanycyte regulation of thyroid hormone feedback onto thyrotropin-releasing hormone neurons and control of thyrotropin-releasing hormone secretion and access to the anterior pituitary.

Microglia, astrocytes, and tanycytes control multiple structural, functional, and behavioral characteristics of neuroendocrine systems through their bidirectional communication with and reciprocal regulation of neuroendocrine cell populations in the hypothalamus. Glial cells are increasingly recognized as playing a critically active role as partners with neurons in neural development, synaptic regulation and plasticity, hormonal feedback, metabolic and reproductive function, and blood–brain barrier control, with new and surprising physiological functions being characterized

all the time. We have attempted in this collection of papers to provide a snapshot of the broad range of interactions of glial cells with different populations of neuroendocrine cells of the hypothalamus, this is to present an overall picture of the diverse glial–neuronal dynamic in neuroendocrine regulation. In this way, we hope to instill in the reader an appreciation of the rich and varied nature of these glial–neuronal interactions and a recognition of glial cells as critical partners in the control of neuroendocrine function.

New Orleans, USA — Jeffrey G. Tasker
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October 2020

Contents

Part I

Glial–Neuronal Interactions in the Control of Hypothalamic Development

The Role of Microglia in the Developing Hypothalamus

1

Jessica M. Rosin and Deborah M. Kurrasch

Abstract

The hypothalamus is a key component of the limbic system that plays an essential role in regulating physiological homeostasis via the release of trophic hormones that serve to connect the nervous and endocrine systems. The organization and function of the discrete nuclei that comprise the hypothalamus have been well studied, yet the programs that govern their development remain poorly described. This paucity of understanding is especially true for the microglial-neuronal interactions that occur during hypothalamic development and are important for the generation of a fully functioning hypothalamus. Recent scientific advancements have begun to elucidate the intricate programs that drive the invasion and maturation of these specialized glial cells, especially within the embryonic hypothalamus. Broadly, during neurogenesis, macrophages travel from the yolk sac to invade the brain parenchyma, where they transition to become microglia, the first and only glial cell present in the early embryonic central nervous system. These phagocytic immune cells are crucial during embryogenesis for the proper establishment of hypothalamic metabolic circuitry and have been shown to be both sexually dimorphic themselves, as well as contribute to the sexual dimorphism that exists within the hypothalamus. Thus, understanding the molecular nature of microglial-neuronal interactions in the developing hypothalamus is essential to having a comprehensive appreciation for the establishment of this important neuroendocrine region.

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Keywords

Hypothalamus · Embryogenesis · Microglia · Metabolism · Sexual Dimorphism · Neuroendocrine · Development

1.1 Introduction

1.1.1 Hypothalamic Organization and Function in the Neuroendocrine System

The hypothalamus is generated from the ventral portion of the developing secondary prosencephalon and can be found just below the thalamus in the mature brain (Fig. 1.1). The hypothalamus is a key component of the limbic system, playing a role in the regulation of emotions and motivated behaviors. At the same time, the hypothalamus also regulates physiological **homeostasis** by sending hormones into the pituitary gland that then control endocrine signaling. Proper hypothalamic function is required to maintain various homeostatic processes, such as

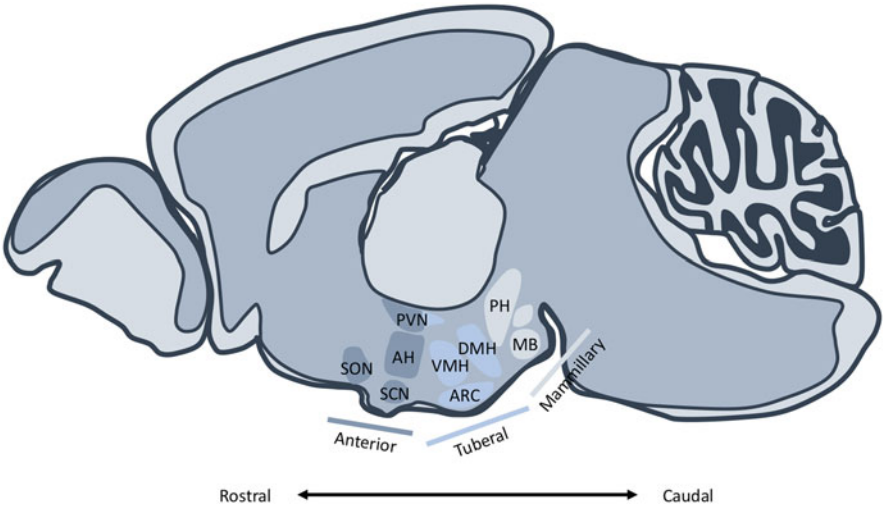

Fig. 1.1 Diagram of the nuclei in the mature hypothalamus as depicted in a sagittal section through the mouse brain. The nuclei comprising the anterior hypothalamus (dark blue, far left) are the supraoptic nucleus (SON), anterior hypothalamic nucleus (AH), suprachiasmatic nucleus (SCN), and the anterior portion of the paraventricular nucleus (PVN). The nuclei comprising the tuberal hypothalamus (blue, middle) are the posterior portion of the PVN, ventromedial hypothalamic nucleus (VMH), arcuate nucleus (ARC), and the dorsomedial hypothalamic nucleus (DMH). The nuclei comprising the mammillary hypothalamus (light blue, right) are the posterior hypothalamic nucleus (PH), and the mammillary body (MB). The rostral-caudal axis is represented below the cartoon diagram of the brain (adapted from Frontiers in Neuroendocrinology, 2019, Vol. 54, Jessica M. Rosin and Deborah M. Kurrasch, Emerging roles for hypothalamic microglia as regulators of physiological homeostasis, Page No. 100748, Copyright (2020), with permission from Elsevier)

fluid-balance, body temperature, energy regulation and metabolism, in addition to mediating the fight-or-flight response, a key physiological response that allows the quick reaction to threats or changes in the environment. The hypothalamus is also involved in behaviors associated with mood, physical and diurnal activity, reproduction, **parturition** and lactation, and parenting and attachment. While the nuclei of the hypothalamus act together to mediate body homeostasis, the hypothalamus is often divided into three regions along the rostral-caudal axis (Fig. 1.1). The rostral-most region is known as the anterior hypothalamus and contains the supraoptic nucleus (SON), suprachiasmatic nucleus (SCN), anterior hypothalamic nucleus (AH), and the anterior portion of the paraventricular nucleus (PVN). The middle region is referred to as the tuberal hypothalamus and is comprised of the posterior portion of the PVN, ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), arcuate nucleus (ARC), and the lateral hypothalamus (LH). Lastly, the most caudal region is termed the mammillary hypothalamus and contains the posterior hypothalamic nucleus (PH) and the mammillary body (MB). Together, these discrete nuclei are comprised of specialized neurons that are generated during embryogenesis and signal both within and outside of the hypothalamus to coordinate body homeostasis. Although the organization and function of each of these discrete neuronal clusters has been studied in-depth, the glial-neuronal interactions that occur during neuroendocrine development to generate a fully functioning hypothalamus are just beginning to emerge.

1.1.2 Microglia Invasion During Hypothalamic Development

Historically, the neuronal and glial lineages of the central nervous system (CNS) have been viewed as playing two separate roles, with neurons processing the information they receive from glial cells, and glia, in contrast, simply acting as support cells that modulate neuronal activity. While these generalized characteristics still hold true today, it is becoming apparent that glia, particularly **microglia**, are more than just support cells, as they are emerging as key players both during the development of the CNS and for proper CNS homeostasis. Recent scientific advancements have begun to elucidate the intricate programs that govern the invasion and development of microglia cells, especially within the embryonic hypothalamus. At present, we know that during early embryogenesis, when neural progenitors are actively dividing to generate neurons, infiltrating **macrophages**, which ultimately transition to become microglia, are the only glial cell type present in the developing CNS. Indeed, the neural progenitors that line the ventricles will go on to give rise to oligodendrocytes and astrocytes (**gliogenesis**) later during the embryonic period (Fig. 1.2). Intriguingly, this suggests that microglia represent a glial population that is present throughout embryonic development and that is in a position to perhaps influence neurogenesis and/or gliogenesis.

During embryogenesis, resident macrophages or microglia comprise approximately 3% of the developing brain (Rosin et al. 2018), and go on to represent 5–15% of the cells in the adult brain, depending on the brain region. Under

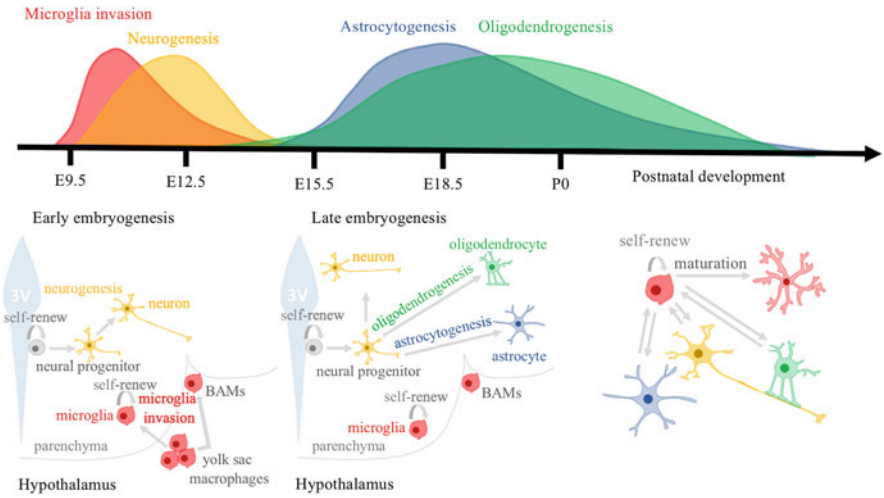

Fig. 1.2 Diagram outlining the developmental programs involved in generating the neurons, oligodendrocytes, astrocytes, and microglia of the embryonic CNS. Top, waves of microglial invasion (red), neurogenesis (orange), oligodendrogenesis (green), and astrocytogenesis (blue) are depicted along an outlined developmental time course. Infiltration of yolk sac macrophages into the CNS parenchyma occurs around embryonic day 9.0 (E9.0) (red). Neurogenesis begins in the developing hypothalamus at E9.5 (orange), while oligodendrogenesis starts at approximately E13 and continues postnatally (green). Astrocytogenesis follows oligodendrogenesis and begins at approximately E13.5 and continues postnatally (blue). Below the development timeline, a schematic diagram shows that during early embryogenesis (left) yolk sac-derived macrophages (red cells) give rise to both microglia, located within the parenchyma, and border-associated macrophages (BAMs), located around the CNS but outside the parenchyma. During these early embryonic time points neural progenitors also generate neurons (yellow cells). During late embryogenesis (middle) neural progenitors give rise to oligodendrocytes (green cells) and astrocytes (blue cells) within the CNS parenchyma, while microglia (red cells) proliferate, differentiate, and self-renew. Postnatally (right) neurons (yellow cells), oligodendrocytes (green cells), astrocytes (blue cells), and microglia (red cells) signal to one another (arrows), which further plays a role in microglial maturation as well as establishment of hypothalamic circuitry

physiological conditions, microglia represent the largest population of immune cells within the **CNS parenchyma**. The **yolk sac** gives rise to the tissue-resident macrophages that travel into the **neuroectoderm** and start to enter the developing CNS at embryonic day 9.0 (E9.0) (Fig. 1.2) via the blood vasculature, just prior to the closure of the **blood–brain barrier** (**BBB**) (Ginhoux et al. 2010; Gomez Perdiguero et al. 2015). As a result of BBB closure, access to the CNS later in development and adulthood is restricted, and microglia become the self-renewing resident macrophages and phagocytic immune cells of the CNS. Classically, microglia are responsible for surveying their environment, responding to neural insults, and disposing of cellular debris. However, the last decade of microglial studies has shown that these non-CNS-derived immune cells have functions in the brain as unique as their origin.

1.1.3 Hypothalamic Gliogenesis

To begin to highlight the differences between microglia and other glial cells in the brain, namely oligodendrocytes and astrocytes, it is important to first appreciate their distinct origins. To start, here is the process of hypothalamic gliogenesis, the development of oligodendrocytes and astrocytes (Fig. 1.2). Since the same progenitor zone that lines the third ventricle of the hypothalamus will give rise to both neurons and glia (Fig. 1.2), upon termination of neurogenesis, gliogenesis begins. In mouse models, gliogenesis is a protracted process that begins during late embryogenesis and continues into the early postnatal period. Very little is known about the molecular programs that govern gliogenesis in the hypothalamus when compared to other brain regions. Gliogenesis begins around E13 with three consecutive but distinct waves of **oligodendrogenesis**, which is then followed by **astrocytogenesis** (Fig. 1.2). Specifically, glial progenitors are generated from sex-determining region Y (SRY)-box 9 (SOX9)-positive progenitor cells that reside along the third ventricle and express oligodendrocyte transcription factor 2 (OLIG2) (Marsters et al. 2016). As these progenitors migrate outwards towards the hypothalamic mantle and away from the ventricle at E13.5, they begin to express platelet-derived growth factor receptor alpha (PDGFRα) and SRY-box 10 (SOX10) and become what is known as **glioblasts** (Marsters et al. 2016). Glioblasts actively proliferate to give rise to oligodendrocyte precursor cells (OPCs), which continue to mature into myelin basic protein (MBP)-expressing oligodendrocytes. Once fully mature, oligodendrocytes begin to myelinate neuronal axons in order to support neurons, insulate neuronal axons, and aid in neuronal signal transduction. Alongside oligodendrogenesis, but just shortly after its start, astrocytes begin to differentiate. Likewise, astrocytes also arise from SOX9-positive (OLIG2-negative) progenitor cells lining the ventricle of the embryonic hypothalamus (Marsters et al. 2016). Astrocytes can also be generated from OLIG2-positive (PDGFRα-negative) glioblasts that go on to express the early astrocyte marker aldehyde dehydrogenase 1 family member L1 (ALDH1L1) (Marsters et al. 2016). In addition to supporting and communicating with neurons (see Chaps. 4, 7, 8 and 10), mature astrocytes are also important for the BBB (see Chaps. 11 and 12), provide nutrients to CNS tissues and maintain extracellular ion and neurotransmitter levels (see Chaps. 2, 3, 6 and 7), and play a role in neuroinflammation and traumatic damage (see Chaps. 5 and 9), both during the repair and scarring process, among other roles. Thus, while microglia arise from a hematopoietic lineage and migrate to the developing brain via blood vessels, oligodendrocytes and astrocytes arise from neural progenitor cells that lie within the ventricular zones of the developing brain.

Microglia, oligodendrocytes, and astrocytes represent a diverse and unique set of support cells that communicate with neurons and one another for the proper establishment and continued function of the hypothalamus and the entire CNS (Fig. 1.2). The early residency of microglia in the developing CNS may simply be a consequence of timing, that is, their unique ontogeny and need to invade the embryonic CNS before BBB closure requires they appear prior to the onset of gliogenesis or it may indicate that these non-CNS-derived glial cells have unappreciated roles during

CNS development. For example, microglia may be involved in signaling to progenitors to switch from neurogenesis to gliogenesis. Accordingly, further exploration of microglial-neural interactions during hypothalamic development requires a deeper knowledge of the similarities and differences of microglia when compared not only to other glial cells in the brain, but also to other resident macrophage populations found in the body.

1.2 Unraveling Hematopoiesis and the Unique Origins of Microglia

To fully appreciate the distinct origins of microglia, some background in **hematopoiesis** is needed. Although the intricately timed and spatially distinct waves of hematopoiesis that generate the **hematopoietic stem cells** (**HSCs**) that emerge from our bone marrow are well defined, the origin and precise sequence of events that generate the different tissue-resident macrophage populations, such as microglia, are more complicated. Despite being classified as hematopoietic-derived cells, tissue-resident macrophages arise during embryogenesis from precursor cells that colonize specific tissues prior to birth. These unique tissue-resident populations can self-renew and are thought to be maintained locally without contributions from adult hematopoiesis. In order to understand the complex origin of these specialized macrophages, studies have employed sophisticated lineage tracing strategies to reexamine the different waves of hematopoiesis.

1.2.1 Hematopoiesis

Using the mouse as a mammalian model to study the hematopoietic programs that generate the different spatial and temporal waves of hematopoiesis, the first hematopoietic progenitors are found at embryonic day 7 (E7.0) in the embryonic yolk sac blood islands. These hematopoietic progenitors in the yolk sac produce early **erythro-myeloid progenitors** (**EMPs**) in what has been termed primitive hematopoiesis (Fig. 1.3). These early EMPs give rise to a population of primitive macrophages that invade the CNS parenchyma to generate microglia (Ginhoux et al. 2010). At around E8.5 there is a second wave of hematopoiesis, known as the transient definitive stage, whereby late EMPs are generated alongside lympho-myeloid progenitors (LMPs) (Fig. 1.3). Late EMPs give rise to the first fetal **monocytes**, which will go on to become embryonic tissue-resident macrophages. In addition to emerging from the yolk sac, EMPs are also found in the placenta and umbilical cord (reviewed in ref. (Dzierzak and Speck 2008)) where they enter the circulation and colonize the fetal liver (Fig. 1.4). The third and final wave of embryonic hematopoiesis is termed definitive hematopoiesis and begins around E10.5. Definitive hematopoiesis generates the first HSCs, which are derived from the hemogenic endothelium (i.e., umbilical arteries, vitelline arteries, placenta, and yolk sac) and the aorta-gonads-mesonephros (AGM) region of the embryo (Fig. 1.3).

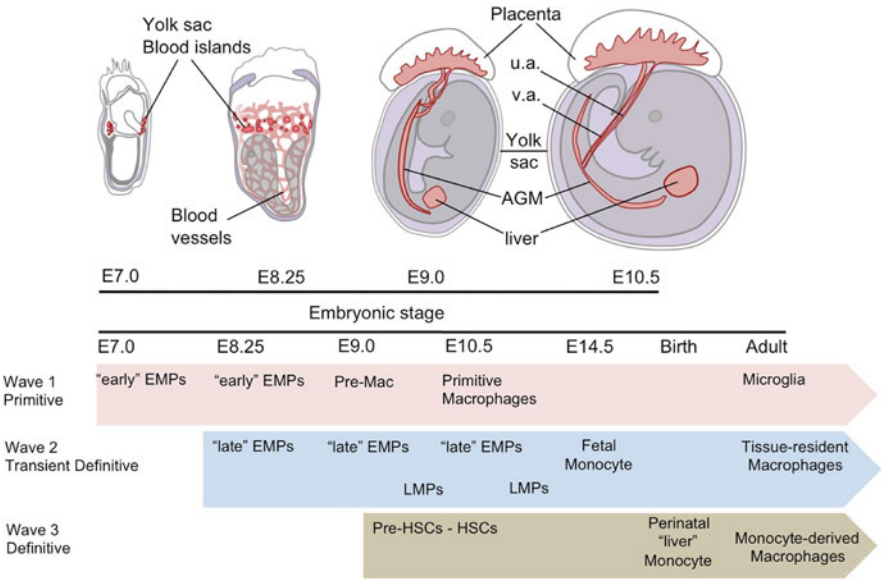

Fig. 1.3 Diagram depicting primitive, transient definitive, and definitive hematopoietic programs that occur in three sequential waves during embryogenesis. Primitive hematopoiesis is the first wave of hematopoiesis and begins at embryonic day 7.0 (E7.0) in the embryonic yolk sac blood islands. Primitive hematopoiesis gives rise to early erythro-myeloid progenitors (EMPs), which will subsequently generate the primitive macrophages that colonize the CNS to generate microglia. The second wave of hematopoietic programming, or transient definitive hematopoiesis, occurs in the hemogenic endothelium (HE) to generate late EMPs, which give rise to the first fetal monocytes. It is these fetal monocytes that travel to specific tissues to differentiate into the tissue-resident macrophage populations that exist outside of the CNS. Transient definitive hematopoiesis also generates lympho-myeloid progenitors (LMPs). The third and final wave of embryonic hematopoiesis is termed definitive hematopoiesis and begins at E10.5 with the generation of the first hematopoietic stem cells (HSCs) that are derived from the HE and the aorta-gonads-mesonephros (AGM) region of the developing embryo. Following E10.5, HSCs can be generated from the HE of the umbilical arteries (u.a.), vitelline arteries (v.a.), placenta, and yolk sac. Together, EMPs, LMPs, and HSCs colonize the fetal liver, which goes on to act as the major source of hematopoietic cells up until late gestation. HSCs have also been shown to generate fetal monocytes that colonize a small proportion of tissue-resident macrophages (reprinted from Cellular Immunology, 2018, Vol. 330, Guillaume Hoeffel and Florent Ginhoux, Fetal monocytes and the origins of tissue-resident macrophages, Page No. 5–15, Copyright (2020), with permission from Elsevier)

Definitive hematopoiesis is responsible for generating the HSCs that colonize the fetal liver, which acts as the major source of hematopoietic cells during embryogenesis, and is the source of the fetal monocytes that colonize a small proportion of tissue-resident macrophages outside of the CNS (Fig. 1.3) (Kieusseian et al. 2012). From E11.5 onwards, the fetal liver is the major source for all hematopoietic lineages that expand during definitive hematopoiesis. Later in life, following HSC migration, the bone marrow becomes the major source of HSCs throughout the body.

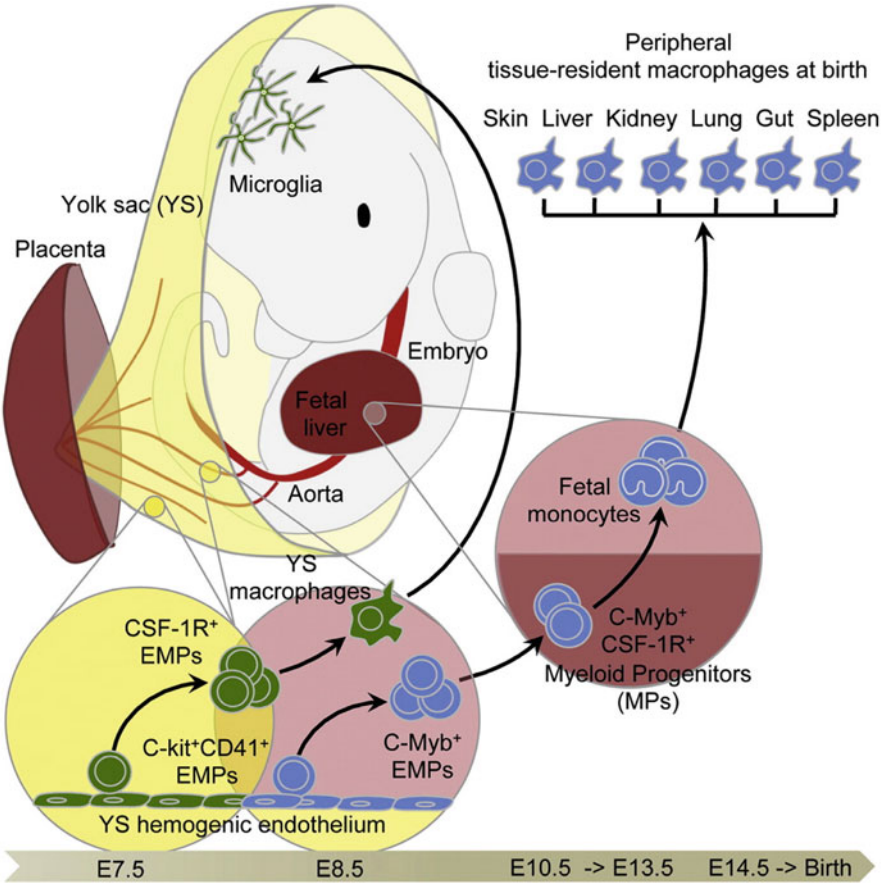

Fig. 1.4 Diagram depicting two distinct developmental programs that generate embryonic tissue-specific macrophages, specifically c-Myb-dependent and independent pathways of tissue-resident macrophage generation. Early embryonic erythro-myeloid progenitors (EMPs) are generated from the yolk sac hemogenic endothelium and give rise to yolk sac macrophages, without a monocytic intermediate phase, which go on to colonize the CNS parenchyma and differentiate into microglia. Later embryonically, EMPs generated from the yolk sac hemogenic endothelium that are c-Myb-positive travel into the fetal liver. These c-Myb-positive cells give rise to fetal monocytes, which differentiate into macrophages that populate tissues comprised of self-renewing tissue-resident macrophage populations, such as the skin, liver, kidney, lungs, gut, and spleen (reprinted from Immunity, 2015, Vol. 42, Guillaume Hoeffel, Jinmiao Chen, Yonit Lavin, Donovan Low, Francisca F. Almeida, Peter See, Anna E. Beaudin, Josephine Lum, Ivy Low, E. Camilla Forsberg, Michael Poidinger, Francesca Zolezzi, Anis Larbi, Lai Guan Ng, Jerry K.Y. Chan, Melanie Greter, Burkhard Becher, Igor M. Samokhvalov, Miriam Merad and Florent Ginhoux, C-Myb+ Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages, Page No. 665–678, Copyright (2020), with permission from Elsevier)

1.2.2 Microglial Origins

Early fate-mapping strategies struggled to distinguish whether microglial cells arose from primitive versus definitive hematopoiesis, and if macrophages generated during fetal development came from EMP or HSC lineages, making the origin of adult tissue-resident macrophages a controversial topic. However, by exploiting cellular dependence on the transcription factor cellular Myb (c-Myb), fate-mapping strategies were able to distinguish from where and when different tissue-resident macrophage population such as microglia originated (Hoeffel et al. 2015).

At around E9.0, blood islands in the yolk sac are populated with yolk sac-derived macrophages. These yolk sac macrophages are generated without passing through a monocytic intermediate phase, and are now appreciated to be the primary source of microglia in the CNS (Ginhoux et al. 2010). In contrast, most other macrophages are derived from precursors that transitioned through a fetal monocytic intermediate phase. Specifically, it appears that EMPs give rise to yolk sac macrophage populations that do not require an intermediate monocytic phase, such as those that go on to become microglia, also colonize the fetal liver following the expression of c-Myb and establishment of blood circulation. The EMPs that give rise to c-Myb-positive fetal liver monocytes will become tissue-resident macrophages that colonize specific tissues throughout the body. Therefore, both microglia and other tissue-resident macrophage populations (e.g., lung) arise from embryonic yolk sac-derived EMPs (Fig. 1.4) (Hoeffel et al. 2015). It is important to note that all these studies have been examined in the rodent embryo and might not fully capture the sequence of development in other species. In fact, studies in zebrafish, which are a vertebrate model used to study human development, show that a secondary wave of hematopoiesis contributes to the microglial population located in the CNS (Xu et al. 2015), demonstrating that studies in rodents might not capture the exact sequence of events that occurs in humans or other animals. In fact, a recent study shows a small population of Homeobox b8 (*Hoxb8*)-positive microglia exist in the brain, suggesting that a small portion of mammalian microglia may arise from the liver (De et al. 2018).

1.2.3 Border-Associated Macrophages

Considering that microglia are located in the parenchyma of the CNS, which is protected by the BBB, under normal steady-state conditions microglia are thought to replenish their populations using self-renewal strategies (Huang et al. 2018). This ability to proliferate is in contrast to other yolk sac-derived macrophage populations, which are replenished from circulating monocytes. Outside of the CNS parenchyma, **border-associated macrophages** (**BAMs**), which also originate from yolk sac-derived macrophages, are located in the meninges and surround other CNS structures (Fig. 1.2). These BAMs have been distinctly classified as meningeal, choroid plexus, and perivascular macrophages (Goldmann et al. 2016). At present, it is not clear if all BAMs arise from a common progenitor and whether microglia

and BAMs even share this common precursor, or alternatively, if each distinct lineage is generated following exposure to specialized signals present in each of their **microenvironments** in and around the CNS. Unlike microglia, macrophages located in the choroid plexus can be replaced by bone marrow-derived cells generated in the adult, suggesting that the choroid plexus is not encompassed by a BBB and remains open to the circulation (Goldmann et al. 2016).

1.3 Local Environmental Signals Shape Microglia Development and Influence Microglial Heterogeneity

Microglia colonization of the brain parenchyma begins around E9.0 and continues well into the second postnatal week, when an adult-type pattern is achieved and microglia adopt a more ramified morphology. Initially, EMPs transition through several immature phases and begin to express colony-stimulating factor-1 receptor (CSF1R), C-X3-C motif chemokine receptor 1 (CX3CR1), and cluster of differentiation 45 (CD45), as they transition to become the mature tissue-resident macrophages of the CNS (Ginhoux et al. 2010). In response to signals in their local microenvironment that macrophages receive during entry into the CNS, such as colony-stimulating factor 1 (CSF1), interleukin 34 (IL-34), and transforming growth factor beta (TGFβ), macrophage signatures are eliminated and microglial signatures are upregulated (Fig. 1.5) (Butovsky et al. 2014). This includes, but is not limited to, the downregulation of the tyrosine phosphatase receptor CD45 and the upregulation of microglia-specific markers, including the purinergic receptor p2y, G-protein coupled, 12 (P2RY12), transmembrane protein 119 (TMEM119), and spalt-like transcription factor 1 (SALL1) (Fig. 1.5) (Butovsky et al. 2014). Identifying the minimal transcriptional profile required to define microglia has been a challenge, since removal of microglia from their surrounding microenvironment results in the rapid loss of both their **epigenetic** and transcriptional identity (Amit et al. 2016). Moreover, bone marrow-derived monocytes do not differentiate into microglia when placed into the CNS (Bennett et al. 2018), suggesting that local cues are insufficient to drive microglial identify. Together, these findings suggest that both the unique origin of microglia and the CNS-specific cues are what compel these unique brain-specific macrophages to become microglia.

1.3.1 Microglial Heterogeneity

Recent scientific advancements in **single-cell RNA sequencing** (Box 1.1) show that local CNS-specific microenvironments also likely contribute to microglial heterogeneity and the CNS colonization pattern they adopt. For example, distinct populations of microglia have been found to localize in diverse regions, such as near neurogenic progenitor niches, surrounding axonal tracts in the corpus callosum, and even within specific layers of the cortex (Cunningham et al. 2013; Hammond et al. 2019). The adaptation of these unique expression signatures depending on the location of these

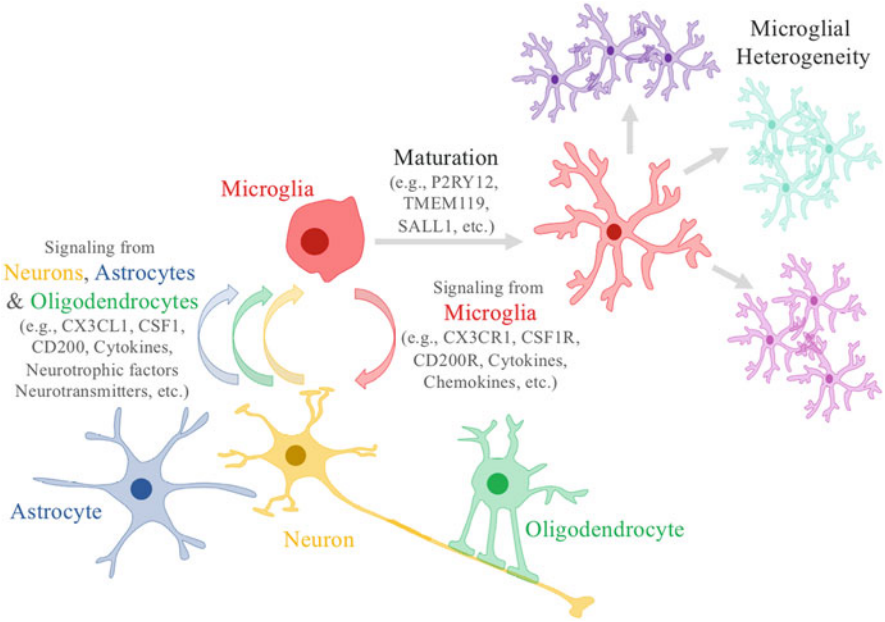

Fig. 1.5 Diagram outlining microglial, neuronal, oligodendrocytic, and astrocytic crosstalk within the CNS during microglia development and differentiation. The diagram highlights examples of signals (e.g., CX3CL1, CSF1, CD200, cytokines, neurotrophic factors, neurotransmitters, etc.) transmitted to microglia (red cell) from neurons (yellow cell), oligodendrocytes (green cell), and/or astrocytes (blue cell). The diagram also highlights examples of signals (e.g., CX3CR1, CSF1R, CD200R, cytokines, chemokines, etc.) transmitted from microglia (red cell) to neurons (yellow cell), oligodendrocytes (green cell), and/or astrocytes (blue cell). This embryonic signaling to and from microglia is thought to be involved in microglia maturation (e.g., expression of P2RY12, TMEM119, SALL1, etc.) and could also aid in driving microglia to become the heterogeneous populations (purple, turquoise, pink microglia) of cells found in the CNS

microglial populations is proposed to occur as a result of transiently expressed CNS signals from nearby cells (e.g., neurons, oligodendrocytes, astrocytes). Signals such as CSF1, IL-34, C-X3-C motif ligand 1 (CX3CL1, also known as fractalkine), and C-X-C motif chemokine ligand 12 (CXCL12) can be released by neural cells and signal to the microglia to influence their cellular **phenotype**, and presumably, behaviors (Fig. 1.5) (Wang et al. 2012). These microenvironments and the bidirectional signaling between microglia, neurons, oligodendrocytes, and astrocytes are likely important for the proper differentiation and maturation of all of these different cell types (Fig. 1.5), a process we are just now beginning to appreciate due to the technological advancements made in single-cell RNA sequencing. Indeed, single-cell RNA sequencing performed on mouse brains across a developmental time course has demonstrated that microglia present in the embryo are the most transcriptionally diverse, becoming more homogeneous as the CNS transitions to the postnatal period (Hammond et al. 2019). Moreover, the adult mouse was found to have the least diverse expression signatures when compared to all other groups, which is only

altered during the aging process or as a result of insult or injury to the brain (Hammond et al. 2019).

At present, it is not clear if CNS-derived signals immediately drive microglial differentiation of the newly infiltrating macrophages in the CNS parenchyma, or if additional exposure time in specific CNS microenvironments is required to induce a macrophage to acquire a microglial phenotype. Interestingly, at E14.5 in the mouse, a distinct population of microglia share an overlapping expression signature with BAMs, including expression of membrane-spanning 4-domains, subfamily A, member 7 (*Ms4a7*) (Hammond et al. 2019), a transmembrane **chemo-sensor** that can regulate immune function. Single-cell RNA sequencing studies also identify embryonic microglia that appear to be in a transitional phase, expressing both macrophage signatures such as C-C motif chemokine receptor 1 (*Ccr1*) and transcripts found in more mature populations of microglia (e.g., *P2ry12*) (Hammond et al. 2019). Considering that embryonic microglia populations are proliferative and quickly expand their numbers to fill the growing brain, it is not surprising that single-cell RNA sequencing experiments have also identified a population of metabolically active and proliferative microglia (Hammond et al. 2019). To date, these single-cell RNA sequencing studies show that sex does not appear to impact the diversity of microglial cell types found or the number of microglia representing each population. However, it is important to note that these studies only examined transcriptional signatures at a limited number of developmental time points. Therefore, future analyses with additional developmental time points, as well as the concomitant assay of protein levels to test whether these altered expression signatures are indeed functional, will be important moving forward.

Box 1.1: Single-Cell RNA Sequencing

Prior to the development of single-cell RNA sequencing technologies, methods such as microarrays and bulk RNA sequencing were used to analyze the expression of transcripts from a large population of cells or specific tissues. Unfortunately, these types of analyses provided RNA expression outputs from a mixed population of cells, thereby lacking the resolution to study cellular phenotypes individually. Considering what is now appreciated regarding cellular heterogeneity, these bulk technologies potentially masked the unique expression signatures found between different cell types within a tissue. Accordingly, advancements in single-cell RNA sequencing technologies have enabled the examination of distinct expression profiles of individual cells, as well as facilitated the uncovering of rare cell types that were unknown within a cellular population. Although the small amount of material available from each cell can sometimes make it challenging to obtain a complete RNA signature for every transcript present in a cell using single-cell RNA sequencing, cluster analysis has allowed for patterns of gene expression to be identified, thereby enabling the grouping of like-cells.

(continued)

Box 1.1 (continued)

Briefly, single-cell RNA sequencing requires the ability to collect cells singly or in a single-cell suspension. Initially, single-cell RNA sequencing was achieved by separating individual cells into separate wells; however, more recent advancements in this technique now allow individual cells in a suspension to be separated using droplets into a microfluidic device. The reverse transcription step can then take place in the droplet, whereby the cellular RNAs are converted to cDNAs. Given that each droplet carries a unique DNA "barcode" (i.e., a unique sequence of nucleotides) that tags these cDNAs after completion of reverse transcription, this microfluidic technology can be used to uniquely label and identify cDNAs derived from a single cell. These droplets can then be mixed together for amplification and sequencing, since the barcoded transcripts from a single cell can be easily separated using bioinformatics tools.

1.4 Microglial Functions During Hypothalamic Development

Early studies in the developing rat hypothalamus used electron microscopy to visualize cells at the ultrastructural level. As part of this work, microglia were clearly identified at E12.0 lining the ventricles and within the median eminence. At the ultrastructural level, these embryonic microglia resembled other phagocytic cells, such as macrophages, and were considered large, irregularly shaped cells with an electron-lucent nucleus and a number of **phagosomes** that could be found in various sizes (Fig. 1.6a) (Rutzel and Schiebler 1980). By the end of the embryonic period, as the hypothalamus continues to mature and transition into the postnatal period, these microglia appeared more dense, with an electron-dense nucleus and chromatin condensation (Fig. 1.6b) (Rutzel and Schiebler 1980). Finally, the mature microglia displayed larger and more distinct processes, fewer ribosomes, and a larger number of phagosomes that were smaller than those observed earlier in development (Fig. 1.6b) (Rutzel and Schiebler 1980). Further characterization of the distribution and morphological heterogeneity of microglia in the mouse brain show that the number of microglia present in the hypothalamus is consistent with that found in other brain regions (Fig. 1.6c) (Lawson et al. 1990); however, the morphology of hypothalamic microglia is very diverse, with the median eminence containing larger, **amoeboid microglia** (Fig. 1.6d, e) as compared to other regions of the hypothalamus, where microglia are more **ramified** in appearance (Fig. 1.6f) (Lawson et al. 1990). Microglia can also be found surrounding vessels in the mediobasal hypothalamus (Fig. 1.6g, h) and appear to contribute to the hypothalamic BBB (Norsted et al. 2008).

Intriguingly, the peptide hormone amylin, which is involved in neurogenesis, axonal fiber outgrowth, and leptin signaling in the hypothalamus, plays a role in the

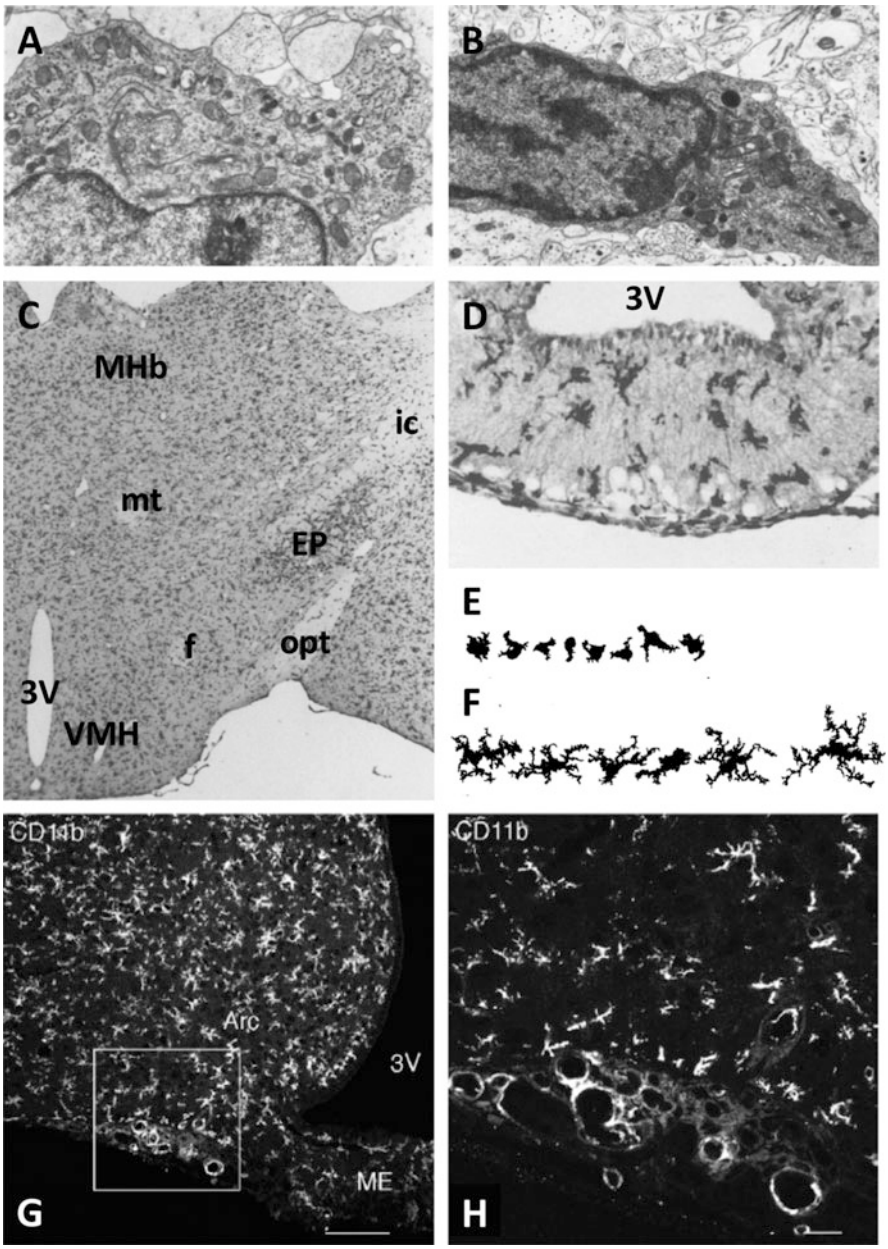

Fig. 1.6 Microglia cells observed at different time points and locations throughout the developing hypothalamus. (**a**) Electron micrograph of an embryonic day 16 (E16.0) microglia observed between the arcuate nucleus and the median eminence in the rat. (**b**) Electron micrograph of a postnatal day 4 (P4) microglia observed in the median eminence in the rat (**a** and **b** reprinted from Cell and Tissue Research, 1980, Vol. 211, H. Rutzel and T. H. Schiebler, Prenatal and early postnatal development of glial cells in the median eminence of the rat, Page No. 117–137, Copyright (2020), with permission from Springer Nature). (**c**) Coronal section through the adult mouse brain labeled with EGF module-containing mucin-like receptor (F4/80) to highlight

proliferation of microglia in the hypothalamic arcuate nucleus (Lutz and Le Foll 2019). Specifically, using bromodeoxyuridine (BrdU), an analog of the nucleoside thymidine that can be used to identify proliferating cells at the time of injection, the birth rates of neurons, microglia and astrocytes were examined in wild-type and amylin knockout mice (Lutz and Le Foll 2019). Although amylin deficiency did not alter neuron or astrocyte numbers in the arcuate nucleus, it did result in reduced numbers of microglia (Fig. 1.7) (Lutz and Le Foll 2019). Considering this phenomenon was observed specifically within the arcuate nucleus, these findings could indicate that hypothalamic microglia respond to local microenvironments to drive proliferation and maturation, with discrete neuronal clusters impacting regional microglial development to match the functional needs of the nucleus. This notion is particularly true in the adult mouse brain, where microglia appear to work together with surrounding hypothalamic neurons to coordinate homeostasis and manage metabolic physiology (discussed in Chap. 8). It is only recently that embryonic microglia have been appreciated to contribute to the development of the same feeding circuitry that they help regulate later in life.

1.4.1 Importance of Embryonic Microglia for the Proper Development of Hypothalamic Circuitry

Although numerous studies have visualized microglia in the developing hypothalamus across a number of time points (Lawson et al. 1990; Norsted et al. 2008; Rutzel and Schiebler 1980), it was unclear whether these hypothalamic microglia played an actual role in the establishment of this brain region or whether they were passive bystanders. Although technologies do not yet exist to eliminate microglia from a targeted brain region, the global depletion of fetal microglia across the entire embryonic CNS by administering to pregnant female mice the CSF1R inhibitor PLX5622 (Box 1.2) results in a hypothalamic-related phenotype. Specifically, offspring born to dams treated with PLX5622 were devoid (>99%) of microglia

Fig. 1.6 (continued) microglia distribution across the medial habenular nucleus (MHb), mammillothalamic tract (mt), ventromedial hypothalamus (VMH), fornix (f), internal capsule (ic), entopenduncular nucleus (EP) optic tract (opt), and surrounding the third ventricle (3V). (**d**) Coronal section through the mouse median eminence labeled with F4/80 to highlight microglia size, shape, and distribution. (**e**) Drawings of F4/80+ microglia observed in the median eminence. (**f**) Drawings of F4/80+ microglia observed in the hypothalamus (**c**–**f** reprinted from Neuroscience, 1990, Vol. 39, L. J. Lawson, V. H. Perry, P. Dri and S. Gordon, Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain, Page No. 151–170, Copyright (2020), with permission from Elsevier). (**g**, **h**) Coronal sections through the rat mediobasal hypothalamus labeled with cluster of differentiation molecule 11B (CD11B) to highlight microglia in the arcuate nucleus (Arc) and median eminence (ME) which can also be seen surrounding vessels. (**h**) High magnification of region of Arc designated by the box in **g** (**g** and **h** reprinted from Journal of Chemical Neuroanatomy, 2008, Vol. 36, Ebba Norsted, Burçak Gömüç and Björn Meister, Protein components of the blood–brain barrier (BBB) in the mediobasal hypothalamus, Page No. 107–121, Copyright (2020), with permission from Elsevier)

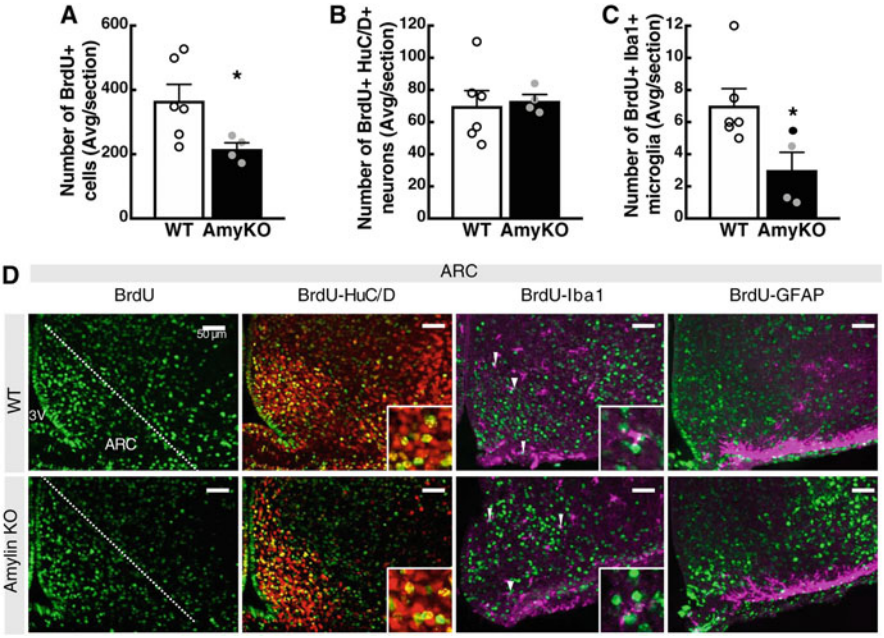

Fig. 1.7 Immunohistochemical analysis and quantification of the number of neurons, microglia, and astrocytes born in the arcuate nucleus. (**a**–**c**) Quantification of the number of Bromodeoxyuridine (BrdU)-positive (**a**) cells that co-localize with neurons (labeled with the neural Hu proteins HuC/D) (**b**) or microglia (labeled with ionized calcium binding adapter molecule 1 (IBA1)) (**c**) in the postnatal day 2 (P2) arcuate nucleus of wild-type (WT) (white bar) or amylin (Amy)-knockout (KO) (black bar) brains. (**d**) Representative immunohistochemical images displaying BrdU-positive cells (green), BrdU (green) and HuC/D (red) double positive neurons, BrdU (green) and IBA1 (purple) double positive microglia, and BrdU (green) and glial fibrillary acidic protein (GFAP) (purple) double positive astrocytes within the arcuate nucleus of WT and Amylin KO mouse brains (reprinted from American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2019, Vol. 316, Thomas A. Lutz and Christelle Le Foll, Endogenous amylin contributes to birth of microglial cells in arcuate nucleus of hypothalamus and area postrema during fetal development, Page No. 791–801, Copyright (2020), with permission from The American Physiological Society)

during embryogenesis and show accelerated weight gain in early postnatal life, from postnatal day 5 (P5) to P15 (Fig. 1.8) (Rosin et al. 2018). Furthermore, immunohistochemical analyses of the brains from these pups showed a reduction in the number of hypothalamic pro-opiomelanocortin (POMC) neurons (Fig. 1.8), one of the two key neuronal populations located in the arcuate nucleus that are responsible for sensing and communicating energy availability (Box 1.3). These findings demonstrated that the loss of microglia during embryogenesis directly affects the development of specific neuronal populations and suggests that microglia are required during development to establish proper hypothalamic feeding circuitry.

Similarly, studies in primates also show that maternal nutrition can impact the development of hypothalamic circuitry, with microglia playing a central role.

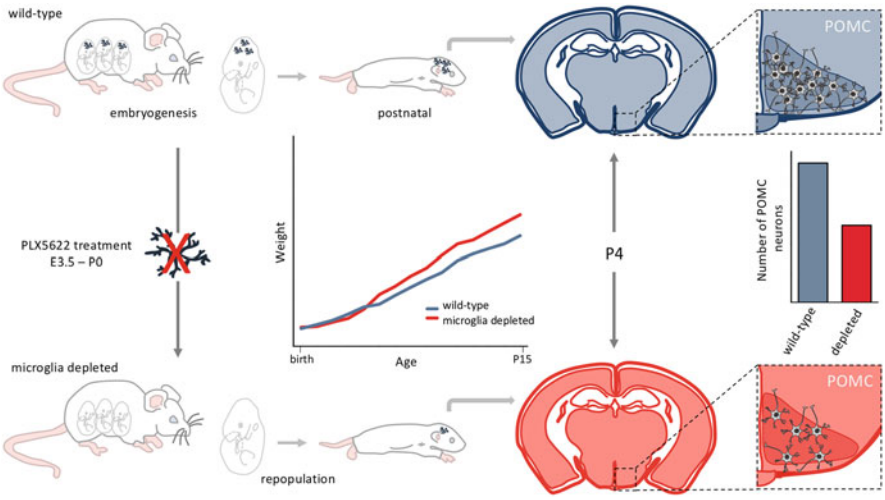

Fig. 1.8 Diagram of embryonic depletion of microglia using the colony-stimulating factor 1 receptor (CSF1R) inhibitor PLX5622 that causes a reduction in pro-opiomelanocortin (POMC) neurons and accelerated weight gain. Pregnant dams are depicted as wild-type (top) or treated with the CSF1R inhibitor PLX5622 (bottom) from embryonic day 3.5 (E3.5) to birth (postnatal day 0 (P0)). Embryos and pups are depicted with (top) or without (bottom) microglia (blue cells). Mouse pups born from female mice treated with PLX5622 during gestation show accelerated weight gain (line graph) from P5 to P15, represented as the red line (microglia depleted) on the graph, as compared to wild-type pups (blue line). Cartoon coronal brain slices through the P4 hypothalamus of control (blue) and microglia-depleted (red) pups illustrate that microglia-depleted pups have fewer POMC neurons (gray cells) in the arcuate nucleus (right side higher magnification image). This is also depicted on the bar graph (right) showing that wild-type pups (blue bar) have a higher number of POMC neurons compared to microglia-depleted pups (red bar) (adapted from Frontiers in Neuroendocrinology, 2019, Vol. 54, Jessica M. Rosin and Deborah M. Kurrasch, Emerging roles for hypothalamic microglia as regulators of physiological homeostasis, Page No. 100748, Copyright (2020), with permission from Elsevier)

Specifically, third trimester fetuses taken from female macaques that were put on a high-fat diet (HFD) for 4 years prior to and during pregnancy show increased POMC mRNA and decreased agouti-related protein (AgRP) mRNA (Grayson et al. 2010). As discussed above and in Box 1.3, the opposing activity of POMC and AgRP/Neuropeptide Y (NPY) neurons is crucial for proper hypothalamic control of feeding, therefore, not surprisingly, the offspring of HFD-treated macaques develop early onset weight gain. Interestingly, further analysis of the brains of fetuses of HFD-treated macaques showed elevated IL-1 and IL-1 type 1 receptor (IL-1R1), and activated microglia in the hypothalamus when compared to fetuses from control fed females (Grayson et al. 2010). However, offspring of female macaques that were treated with HFD for 4 years and then switched to a year of control diet (diet reversed) showed normal melanocortin signaling (Grayson et al. 2010), suggesting that the altered fetal development of the hypothalamic region is the result of HFD consumption during pregnancy and it appears to be independent of maternal obesity

and diabetes. Given the elevated pro-inflammatory signaling as well as the activated state of microglia observed in the fetal brains from mothers on the HFD, it appears that microglia and inflammatory signaling may be playing an unappreciated role in the proper development and function of hypothalamic circuits involved in feeding and energy balance in a non-human primate model.

Box 1.2: Using CSF1R Inhibitors to Deplete Microglia in the CNS

Microglia are dependent on CSF1R signaling for their proliferation, differentiation, and survival (Elmore et al. 2014; Ginhoux et al. 2010). Accordingly, researchers developed CSF1R inhibitors to penetrate the BBB and robustly eliminate microglia from CNS tissues. CSF1R is homologous to other type III receptor tyrosine kinases, including the receptor tyrosine-protein kinase KIT and fms-related tyrosine kinase 3 (FLT3). Therefore, CSF1R inhibitors also bind non-selectively to these receptors. The original CSF1R (and KIT/FLT3) inhibitor, PLX3397, only showed approximately 5% brain penetrance, but yet depleted around 99% of microglia in the adult mouse brain following treatment for 21 days (Elmore et al. 2014). A second CSF1R inhibitor, PLX5622, was generated in order to yield both higher brain penetrance and a shorter duration of exposure to achieve microglial depletion. Indeed, PLX5622 showed a dramatically improved brain penetrance of approximately 20%, likely due to its physiochemical properties such as higher lipophilicity, better cellular permeability, and lower molecular weight compared to PLX3397 (Spangenberg et al. 2019). Moreover, PLX5622 depletes 90% of microglia in the adult mouse brain following treatment for 5 days (Spangenberg et al. 2019). Similarly, treatment of pregnant dams with PLX5622 from E3.5 to E15.5 (12 days) depletes approximately 99% of the microglia present in the E15.5 brain (Rosin et al. 2018). In addition to a higher brain penetrance, PLX5622 also displays preferential binding to CSF1R, with >20-fold selectivity for CSF1R over KIT and FLT3 (Spangenberg et al. 2019). This improved selectivity is thought to occur due to a 2-fluoro substitution on the middle pyridine ring of PLX5622, which helps improve access to the unique space next to Gly-795 present in CSF1R. KIT and FLT3, in contrast, have a bulkier cysteine in this position. Moreover, the terminal pyridine group of PLX5622 stabilizes the allosteric pocket of CSF1R, while binding of PLX5622 to KIT or FLT3 causes steric clash and compromises the optimal fit when binding to these homologous receptors. Together, PLX5622 shows both a higher brain penetrance and a greater specificity for CSF1R when administered using rodent chow (Spangenberg et al. 2019).

Box 1.3: Hypothalamic Circuitry Involved in Feeding and Energy Balance

Briefly, two key neuronal populations located within the arcuate nucleus of the hypothalamus are responsible for sensing and communicating energy availability to induce eating or **satiety** cues (see Chap. 6). Specifically, **anorexigenic** POMC neurons and **orexigenic** AgRP/NPY neurons act in opposition to regulate satiety and appetite, respectively. Therefore, disrupted numbers or signaling from either neuronal population can result in altered feeding, energy availability, and metabolic function. Thus, when homeostatic balance is not achieved, neuroendocrine disruptions that result in diseases such obesity can occur.

1.5 Microglia Are Sexually Dimorphic and Contribute to Sexual Dimorphism Within the Hypothalamus

The neuroendocrine hypothalamus is heavily impacted by sex, and a number of studies now show that microglia have sex-specific roles in the brain. Although no obvious sex differences in microglia numbers, colonization, or transcriptional signatures have been reported in the embryonic mouse brain, the removal of microglia during embryogenesis results in altered behavior in a sex-specific manner. Indeed, depleting microglia during gestation (i.e., exposure to the CSF1R inhibitor PLX5622) results in behavioral deficits in female offspring both during adolescence and into adulthood (Rosin et al. 2018). Specifically, female mice that are depleted of microglia during embryogenesis are hyperactive during adolescence and, in general, are less anxious during adulthood (Rosin et al. 2018). This is consistent with findings from CX3CR1 knockout mice, where hyperactivity and anxiolytic behaviors are only observed in mutant female mice, demonstrating that altering CX3CR1 signaling in microglia accounts for the changes in behavior in female mice (Bolos et al. 2018). Surprisingly, altered behavior was not apparent in male offspring that were depleted of microglia during embryogenesis (Rosin et al. 2018). This suggests a sex-specific requirement for microglia in females for proper development of the embryonic brain. In contrast, the depletion of microglia in the adult brain (i.e., using the CSF1R inhibitor PLX3397) does not result in overt behavioral changes in either sex (Elmore et al. 2015), suggesting a sex-specific requirement for microglia during embryogenesis and early life that influences the proper establishment of specific behavioral circuits.

In contrast to the embryonic brain, striking microglial sex differences are apparent in the postnatal brain. Indeed, sex differences in microglial expansion, morphology, gene expression, inflammatory activation, and response to drugs have been observed postnatally (Caetano et al. 2017; Mirza et al. 2015; Schwarz et al. 2012). For example, at P4, there are more microglia in males compared to females, although

females show a higher number of amoeboid microglia both as juveniles and adults compared to males (Schwarz et al. 2012). Furthermore, following injury, specifically during neonatal hypoxic-ischemic encephalopathy, males show a greater inflammatory response compared to females, which can be observed as increased microglia activation and an upregulation of inflammatory cytokines (Mirza et al. 2015). Similarly, male microglia become hyper-ramified in response to prenatal glucocorticoid exposure, while female microglia decrease in numbers and become less ramified (Caetano et al. 2017). Together, these studies demonstrate that postnatally microglia are sexually dimorphic with regard to their observable features and their response to different challenges or insults.

1.5.1 A Role for Microglia in the Establishment of Sexually Dimorphic Brain Regions

Intriguingly, the **sexual dimorphism** that exists in the brain that contributes to sex-specific behaviors is also impacted by microglia. For example, adult male copulatory behavior, which is associated with the masculinization of the hypothalamic preoptic area, requires microglia. Specifically, inhibiting microglia using minocycline during sexual differentiation of the preoptic area (i.e., PN0 and PN1) prevented estradiol-induced upregulation of prostaglandin E(2) (PGE(2)), the masculinization of dendritic spine density, and the development of copulatory behavior in males (Lenz et al. 2013). Moreover, exogenous treatment with estradiol and PGE (2) masculinizes microglia number and morphology in females. Similarly, deficits in mounting and copulatory behaviors arise in adult male rats when liposomal clodronate is used to temporarily deplete microglia early postnatally (i.e., PN0, PN2 and PN4) (VanRyzin et al. 2016). Together, these studies demonstrate that during development microglia influence the sexual differentiation of the brain and ultimately the establishment of sex-specific behaviors of the neuroendocrine system.

Emerging work in the neuroendocrine field is also finding a unique relationship between microglia and another myeloid-derived cell that can be found in the brain—the **mast cell**. Indeed, interactions between microglia and mast cells during development are important for the proper onset of sexual behaviors that appear during adulthood. Specifically, the masculinizing hormone estradiol acts directly on and through mast cells during early postnatal time points to mediate sexual differentiation of the brain. When newborn female rats are treated with estradiol, an increase in mast cell number as well as the release of histamine from mast cells is observed, which then stimulates nearby microglia to release **prostaglandins** and influence male-typical synaptic patterning of the preoptic area of the developing brain (Lenz et al. 2018). Furthermore, prenatal exposure to allergens can disrupt the development of these adult sexual behaviors through changes to mast cell programs (Lenz et al. 2019), perhaps also via the involvement of microglia. Together, recent scientific advancements in the neuroimmune and neuroendocrine fields are beginning to highlight the importance of mast cell-microglia interactions in driving sexual dimorphism within the developing brain.

1.6 Embryonic Microglia Act as Sensors During Development

Embryogenesis represents a critical developmental window when cells are dividing and differentiating to populate individual organs and specialized niches that control unique functions. This growth is especially true for the CNS, where rapid proliferation of neural progenitors is followed by protracted phases of neurogenesis, gliogenesis, and synaptogenesis. Given this rapid rate of development, it is perhaps not surprising that this embryonic time frame also represents a window of vulnerability whereby the developing CNS is critically sensitive to external insults, such as maternal stress, environmental contaminants, maternal obesity, etc. However, it remains poorly understood as to how these challenges get translated into changes in the developing brain—do they somehow act directly on neurons to change their behavior or are other cells present in the CNS that are primed to sense and react to these insults? It is possible that microglia can sense some of these cues and react by secreting factors and/or signals that then influence the nearby neural progenitors and/or neurons. For example, ethanol exposure during development results in a variety of physiological and behavioral alterations, including neuronal apoptosis in the hypothalamus, by inducing oxidative stress both directly in neurons and via the release of secreted factors from microglia (Boyadjieva and Sarkar 2013). Similarly, environmental chemical exposure during development is proposed to contribute to the higher prevalence of obesity observed in society. Indeed, male offspring from pregnant female mice exposed to diesel exhaust particles (DEP) during gestation displayed weight gain and insulin resistance when challenged with HFD, compared to male offspring not exposed to DEP but receiving a HFD challenge (Bolton et al. 2014). These male mice also showed increased levels of microglial and macrophage activation and presented with anxiety-like behaviors (Bolton et al. 2014). These findings suggest that maternal exposure to air pollution affects the developing brain through sexually dimorphic neuroinflammatory changes that cause an increased susceptibility to diet-induced obesity. Furthermore, recent work on gestational exposure to the plasticizer bisphenol A (BPA), which is known to cause abnormalities in the hypothalamus and the appearance of abnormal behaviors in adulthood, results in elevated microglial numbers in the hypothalamus in addition to increased levels of inflammatory factors such as tumor necrosis factor alpha (TNFα) (Takahashi et al. 2018). These data suggest that BPA may act through microglia to alter hypothalamic neurodevelopmental programs during gestation.

Interestingly, a unique signaling connection between the maternal and fetal brains occurs during pregnancy. Specifically, across pregnancy and especially during late gestation, the maternal brain suppresses its immune response, which appears to be mirrored in the fetus, since both maternal and fetal neuroimmune signaling is dampened just prior to birth. This observation suggests that immune modulation, including cytokine production, in the placenta and fetal brain mimic the immune response of the mother. Considering that the mammalian fetus develops in what is largely considered a sterile environment up until birth, at which point it becomes immediately exposed to a complex array of microbiota, a new avenue of research now seeks to understand the influence of microbiota on brain development. For

example, in mice born under sterile, germ-free (GF) conditions, an increase in the number and size of microglia is observed, with a concomitant decrease in inflammatory cytokines such as TNFα and IL-1β, when compared to newborn mice that are colonized with microbiota at birth (Castillo-Ruiz et al. 2018). Moreover, GF offspring show higher levels of cell death in the PVN of the hypothalamus and lower levels of cell death in the ARC (Castillo-Ruiz et al. 2018), suggesting that exposure to microbiota at birth may indeed influence important neurodevelopmental programs as the fetus transitions from in utero sterile conditions to postnatal microbiota-rich environments. Taken together, microglia can sense external insults that the mother is exposed to during pregnancy and they are finely tuned-in to sense environmental changes that they experience as they transition from embryogenesis to postnatal life.

1.7 Perspectives

The hypothalamus regulates physiological homeostasis by releasing trophic hormones that serve to connect the nervous system to the endocrine system. Despite insight into the role of glial cells in the adult brain, the glial-neuronal interactions that occur during neuroendocrine development (i.e., embryogenesis) are just beginning to emerge. It is becoming widely apparent that all glial cells, and this is especially true for microglia, are more than just support cells, since they are emerging as key players both during the development of the CNS and for proper brain homeostasis. Recent studies now elucidate the intricate programs that govern the invasion and development of microglia cells, especially within the embryonic hypothalamus. Following neurogenesis, infiltrating macrophages will transition to become microglia, the only glial cell present in the developing CNS at this early embryonic time point. Therefore, microglia represent a glial population that is present in the brain longer than any other glial cell.

Microglial development can be further shaped by the local microenvironment, since they receive signals from developing neurons, oligodendrocytes, and astrocytes within the brain parenchyma. Microglia go on to become a heterogeneous population of tissue-resident macrophages within the CNS, with unique morphologies and expression signatures both within specific brain structures, such as the hypothalamus, and also across the entire CNS. These phagocytic immune cells are crucial during embryogenesis for the proper establishment of metabolic circuitry in the hypothalamus and are both sexually dimorphic and contribute to the sexual dimorphism that exists within the hypothalamus. Furthermore, these unique immune cells can mirror the maternal immune system during gestation, quickly responding to changes in their external environment both within and outside of the body, and perhaps be local CNS sensors to impactful insults, including ethanol, air pollution, and the widely-used plasticizer BPA. Together, microglia represent a unique subset of glial cells that play a prominent role in neuroendocrine development.

Future work into the role of embryonic hypothalamic microglia should focus on whether their early appearance in the developing hypothalamus, prior even to the onset of neurogenesis, is merely a coincidence of timing (i.e., invading the CNS prior to BBB closure) or their presence is required to influence normal

neurodevelopment. For example, are embryonic microglia involved in neurogenic programs, which could be why we see a decrease in the number of POMC neurons present in mice devoid of microglia during embryogenesis? Or could embryonic hypothalamic microglia perhaps be involved in the transition from neurogenesis to gliogenesis, helping to signal the termination of neurogenesis and/or the initiation of gliogenesis? Similarly, given the unique relationship between embryonic microglia and altered feeding circuitry, an exciting question will be whether dietary alterations (e.g., HFD) during pregnancy act through microglia to permanently alter the melanocortin system or if microglia themselves change in response to dietary insults, which then affects their signaling cues to neurons later in life. Moreover, while advancements in single-cell RNA sequencing technologies are identifying unique populations of microglia across the embryonic CNS, the changes in transcript levels will need to be confirmed to be functionally relevant using protein-based assays and functional tests. Finally, it is intriguing that microglia are sexually dimorphic and also contribute to sexual dimorphism in the hypothalamus. A further understanding of the contexts in which these cells are susceptible to sex-specific programs that then govern their influence on brain development will be critical to fully appreciate the role of these glial cells in the establishment of sex-specific circuits. Combined, microglia are a unique glial cell that influences development of the neuroendocrine hypothalamus in a variety of ways.

1.8 Key Literature

Butovsky et al. (2014) This paper was the first to identify unique TGF-beta-dependent molecular and functional signatures in microglia.

Elmore et al. (2015) This seminal paper demonstrated that CSF1R signaling is necessary for microglia viability in the adult mouse.

Ginhoux et al. (2010) This was the first paper to show that adult microglia are derived from primitive macrophages in the yolk sac.

Gomez Perdiguero et al. (2015) This seminal paper demonstrated that tissue-resident macrophages originate from yolk sac-derived EMPs.

Hammond et al. (2019) This seminal paper used single-cell RNA sequencing of microglia across the mouse lifespan and in the injured brain to reveal complex cell-state changes.

Hoeffel et al. (2015) This seminal paper showed that C-Myb(+) EMP-derived fetal monocytes give rise to adult tissue-resident macrophages.

Lawson et al. (1990) This seminal paper showed heterogeneity in the distribution and morphology of microglia in the adult mouse brain.

Lenz et al. (2013) This was the first paper to demonstrate that microglia are essential to masculinization of brain and behavior.

Rosin et al. (2018) This was the first paper to demonstrate that depletion of embryonic microglia has adverse sex-specific effects on mice, including accelerated weight gain and disruption of satiety circuitry.

VanRyzin et al. (2016) This paper was the first to show that temporary depletion of microglia early postnatally induces sex-dependent effects on behavior in rats.

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Part II

Glial–Neuronal Interactions in the Control of the Magnocellular Neuroendocrine System

Functional Consequences of Morphological Plasticity in the Adult Hypothalamo-Neurohypophysial System

2

Daniel L. Voisin, Aude Panatier, and Stéphane H. R. Oliet

Abstract

The adult hypothalamo-neurohypophysial system (HNS) comprises the cell bodies of the magnocellular neurons of the hypothalamus, located in the supraoptic and paraventricular nuclei, and their axons that project onto the neurohypophysis, where they release oxytocin and vasopressin directly in the bloodstream. Oxytocin governs parturition and lactation, while vasopressin is the antidiuretic hormone and a vasopressor. The HNS undergoes a remarkable reversible, activity-dependent morphological neuroglial plasticity during lactation and dehydration. Here we summarize how this made it a seminal model to study the physiological contribution of the astrocytic environment to synaptic signaling. We show first that reduction in glial processes modifies local glutamate level at the synapse by reducing its uptake, and thus affects homosynaptic strength. Second, it also changes neurotransmitter diffusion, and as a result contributes to inter-synaptic crosstalk between glutamate and GABAergic inputs through metabotropic and kainate receptors. Finally it hampers the contribution of astrocytes to glutamatergic synaptic communication through D-serine gliotransmission. Astrocytes thus dynamically dictate the tenor of brain signaling.

Keywords

Hypothalamo-neurohypophysial system · Oxytocin · Vasopressin · Astrocyte · Plasticity

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2.1 Introduction: Structural Glial Plasticity in the Adult HNS

2.1.1 Anatomy of the HNS System

The hypothalamo-neurohypophysial system (HNS) is the first identified brain peptidergic system and oxytocin and vasopressin the first peptides to be chemically synthesized in the laboratory. The anatomical organization of the HNS, with neuronal cell bodies clearly identifiable and clustered in small, specific nuclei, together with highly accessible secretory terminals in the neurohypophysis, made it a seminal model to study the synthesis and maturation of peptides, co-synthesis and co-secretion, stimulus-secretion coupling, pulsatility of hormonal release, rhythmogenesis of electrical activity and dendritic release of neurotransmitters. It also proved to be a critical model to study the functional impact of structural glial plasticity (reviewed in Theodosis et al. 2008).

The HNS is made up of the cell bodies of magnocellular neurons located in the hypothalamic supraoptic, paraventricular and accessory nuclei and their axons that project through the internal layer of the median eminence into the posterior pituitary lobe, as illustrated in Fig. 2.1 (for review see Tasker et al. 2017). The supraoptic nuclei are located in the anterior and lateral hypothalamus, bilaterally on either side of the optic chiasm/tract. The paraventricular nuclei are located bilaterally on either side of the third ventricle. Two distinct populations of these neurons synthesize the precursors of the nonapeptidic hormones oxytocin and vasopressin, as shown in Fig. 2.2. The newly synthesized peptides are included in vesicles, where they mature and are transported toward the distal axon terminals that release them directly into the general circulation through exocytosis. Magnocellular neurons possess axons that ramify in numerous terminals in the posterior lobe of the hypophysis and each terminal contains many secretion granules. Magnocellular neurons also send axons to the amygdala and other brain regions involved in emotional, social, and affiliative behaviors. Action potentials from the soma invade the axon, depolarize the terminals, opening voltage gated calcium channels, which triggers exocytosis of neurosecretory granules. Each peptide can also be secreted from the dendrites in the central nervous system, independently of action potential activity, and act in an autocrine or paracrine manner, or through diffusion into the cerebrospinal fluid, contributing to volume transmission.

Magnocellular neurons have a relatively large soma (20–35 μm in diameter) compared to other hypothalamic neurons. They synthesize either oxytocin or vasopressin and also, to a lesser extent, a variety of other peptides and neurotransmitters, such as dynorphin, galanin, ATP (Brown et al. 2013). Most of them possess 2–3 primary dendrites that, in the supraoptic nucleus, ramify in the ventral glial lamina, a region that is rich in dendro-dendritic appositions and makes an important site of reception of synaptic inputs. There are similar numbers of oxytocin and vasopressin neurons in the supraoptic nuclei. The oxytocin neurons tend to cluster rostrally and dorsally in the nucleus, while the vasopressin neurons are preferentially distributed ventrally and caudally, as shown in Fig. 2.2. The paraventricular nuclei contain three times fewer magnocellular neurons than the supraoptic nuclei, but also contain

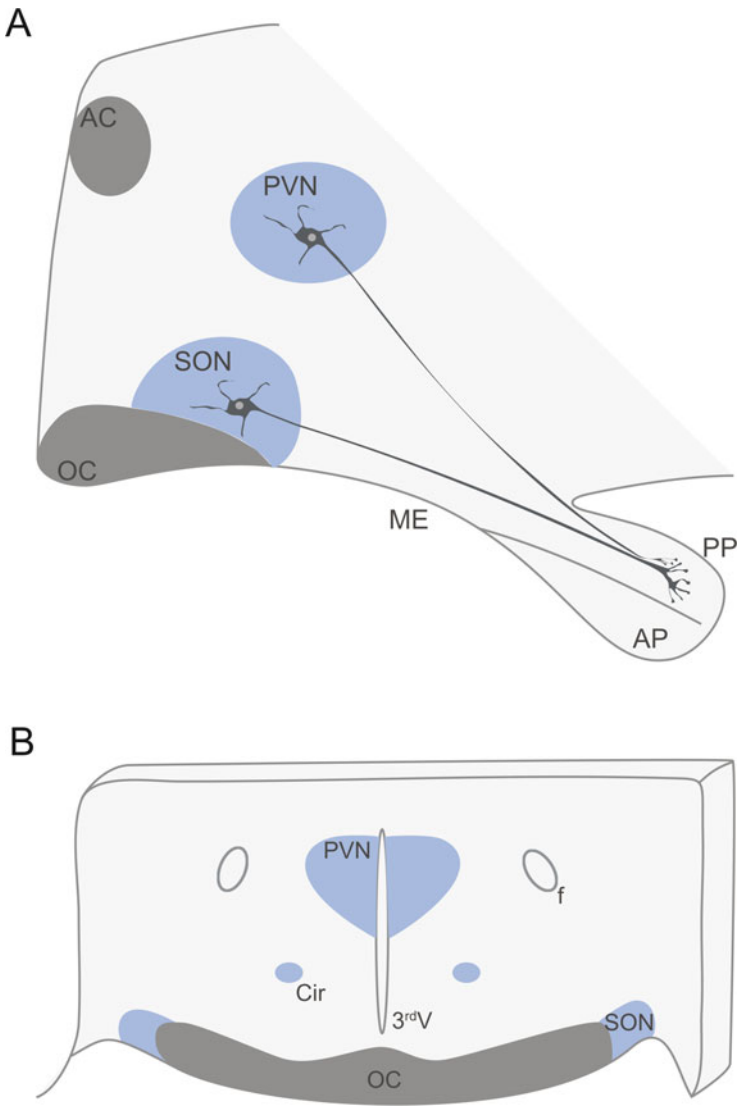

Fig. 2.1 Anatomical organization of the hypothalamo-neurohypophysial system. (**a**) Schematic drawing showing a sagittal view of the location of magnocellular neurons in the rat supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus and their axonal projections into the posterior pituitary (PP). (**b**) Schematic tracing of a coronal section through the hypothalamus showing the location of the SON, PVN, and circularis nucleus (Cir). AC, anterior commissure; AP, anterior pituitary; f, fornix; ME, median eminence; OC, optic chiasma; 3rd V, third ventricle

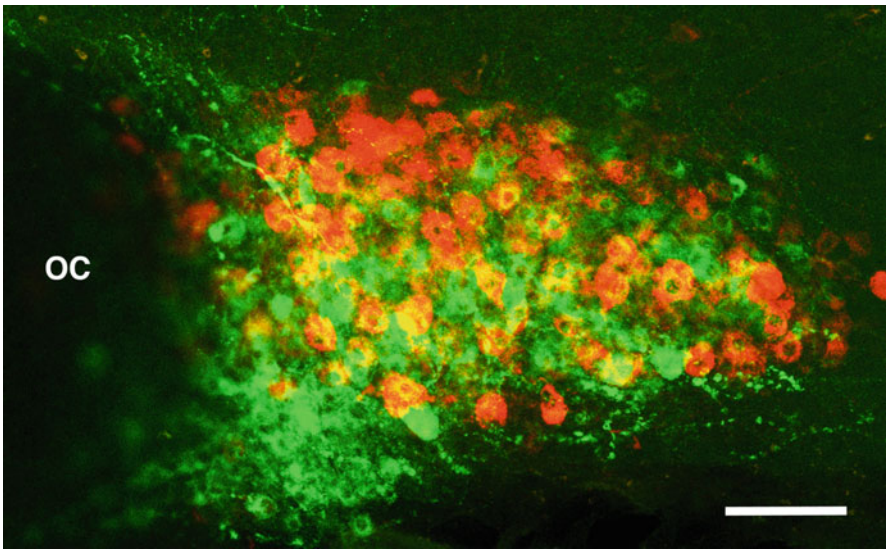

Fig. 2.2 Oxytocin and vasopressin neurons in the supraoptic nucleus. Confocal microscopy image showing the distribution of oxytocin (in red) and vasopressin (in green) neurons in an unfixed 250μm-thick coronal section of the supraoptic nucleus. The labeling results from the expression of a vasopressin-enhanced green fluorescent protein (eGFP) fusion protein and an oxytocin-monomeric red fluorescent protein 1 (mRFP1) fusion protein in a 3.5-month old double-transgenic virgin female Wistar rat. OC, optic chiasma. Calibration bar, 100μm. Courtesy of Drs ZS Thirouin and CW Bourque, McGill University

parvocellular neurons, some of which express oxytocin or vasopressin and project to the external layer of the median eminence and centrally (Armstrong 2015).

The main afferent inputs of the magnocellular neurons are glutamatergic and GABAergic projections that, respectively, provide the fast excitatory and inhibitory synaptic control over oxytocin and vasopressin release, while noradrenergic projections act primarily to modulate these inputs. In normal conditions of secretion, around 45% of HNS synapses are GABAergic, 25 % glutamatergic, and both types are often in close proximity. Around 10% of HNS synapses are noradrenergic.

2.1.2 Functions of Oxytocin and Vasopressin

Oxytocin and vasopressin released in the general circulation are vital for the species and the individual, while their central release governs crucial social behaviors and visceral functions (for review see Tasker et al. 2017).

Oxytocin is present in all mammals. Oxytocin produced by magnocellular neurons and released from the posterior pituitary acts on the uterus and contributes to its contraction during parturition, although it is not absolutely required for successful delivery. Oxytocin released in the general circulation is also the efferent

pathway of the milk ejection reflex, and this function is vital to allow pups to receive milk in response to suckling. Indeed, oxytocin knockout mice are fertile, and females are able to deliver their litters, however, the pups do not successfully suckle and die within 24 h without milk in their stomachs. In the rat, oxytocin is also a natriuretic hormone, regulating sodium excretion from the kidneys. Centrally released oxytocin is involved in the control of visceral functions, including cardiovascular regulation, food intake, sexual functions, inhibition of nociception. It also modulates anxiety and fear and plays a major role in social cognition and affiliative behaviors.

Vasopressin produced by magnocellular neurons and released from the posterior pituitary promotes water reabsorption from the kidney, and this function is vital to maintain body fluid balance. Vasopressin also increases blood pressure through vasoconstriction. Centrally released vasopressin is also an important modulator of diverse social behaviors.

2.1.3 Electrophysiology of Oxytocin and Vasopressin Neurons

Peripheral release of oxytocin and vasopressin is driven by the highly specific electrical activity of each cell population, under the main control of glutamatergic, GABAergic, and noradrenergic afferents (for review see Tasker et al. 2017). Central release of the neuropeptides does not always parallel peripheral release.

Since oxytocin and vasopressin neuron terminals do not sustain intrinsic repetitive action potential discharge, the release of both peptides in the neurohypophysis is mainly determined by the frequency and pattern of action potential discharge initiated at the cell bodies. Different mechanisms contribute to the generation of that electrical activity, including the intrinsic membrane properties of the magnocellular neurons, their synaptic inputs, autoregulation by local release of each peptide and other co-transmitters, factors released by neighboring glial cells, and influence of circulating hormones.

Under normal conditions, oxytocin and vasopressin neurons show a low level of irregular electrical activity (1–3 action potentials/s) that allows a constant and low release of hormone into the general circulation. In response to physiological stimuli, they progressively increase their firing rate and also produce cell-type specific and stereotyped patterns of bursting activity (Poulain and Wakerley 1982). These patterns are important for generating the appropriate secretion of each peptide to meet the current physiological demands. They are related to the frequency facilitation and fatigue properties of oxytocin and vasopressin neuron terminals. In contrast to sustained electrical activity, relatively brief bursts of action potentials facilitate release, whereas pauses between bursts allow for recovery of the mechanisms of exocytosis. Schematically, in response to the asynchronous, phasic bursting activity of vasopressin neurons, vasopressin is released in a tonic fashion into the blood. This allows to precisely adjust water reabsorption from the kidney and corrects changes in osmolality (Bourque 2008). By contrast, during parturition and suckling, oxytocin neurons discharge intermittent high-frequency bursts of action potentials that are synchronized across their entire population, leading to the pulsatile release of

oxytocin that acts on the uterus and the myoepithelial cells of the mammary glands, which facilitates the delivery of pups and promotes ejection of milk, respectively (Poulain and Wakerley 1982).

Somatodendritic release of oxytocin and vasopressin can be regulated independently of that from the axon terminals in the neurohypophysis, which requires refined regulatory mechanisms. Somatodendritic, but not axon terminal release, can be modulated by changes in intracellular calcium concentration by release of calcium from intracellular stores, resulting in priming of dendritic pools of secretory granules for activity-dependent release (Ludwig and Stern 2015). Locally released peptides may provide a local feedback regulation of the electrical activity of magnocellular neurons and synaptic inputs.

2.1.4 Glial Cells of the HNS

Three types of astrocytes are present in the supraoptic nucleus (Theodosis et al. 2008). As illustrated in Fig. 2.3, a large population of astrocytes have a radial glia-like morphology, with cell bodies located along the ventral glia lamina (VGL), long processes traversing the nucleus in the coronal plane, horizontally-oriented processes that form a dense network in the VGL, and a short process oriented toward the pia. They are immunoreactive for glial fibrillary acidic protein (GFAP) and vimentin, an intermediate filament protein of immature glial cells and a marker for radial glia, and they are not dye-coupled. A second population of astrocytes, less numerous than the radial type, is located close to the subarachnoid space. They are

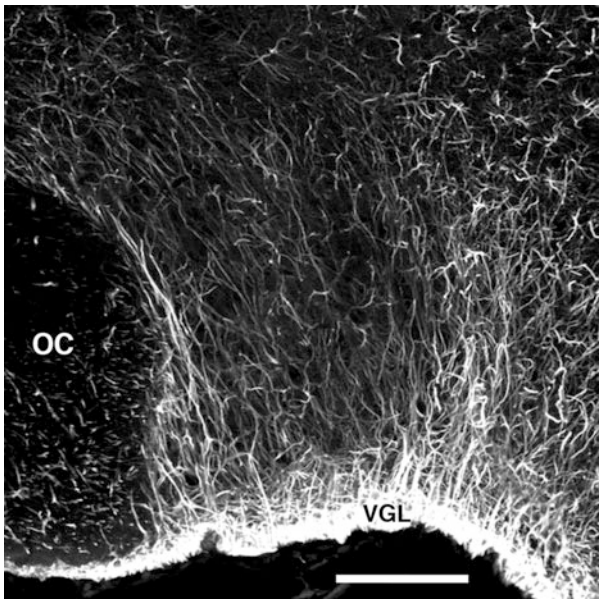

Fig. 2.3 Radial glia-like astrocytes of the supraoptic nucleus. Confocal microscopy image showing immunostaining for glial fibrillary acidic protein (GFAP) in the supraoptic nucleus of a male Wistar rat. Long, thick GFAP-positive fibers coming from the ventral glia lamina (VGL) extend dorsally through the nucleus. OC, optic chiasma. Calibration bar, 100μm. Courtesy of Dr M Prager-Khoutorsky, McGill University

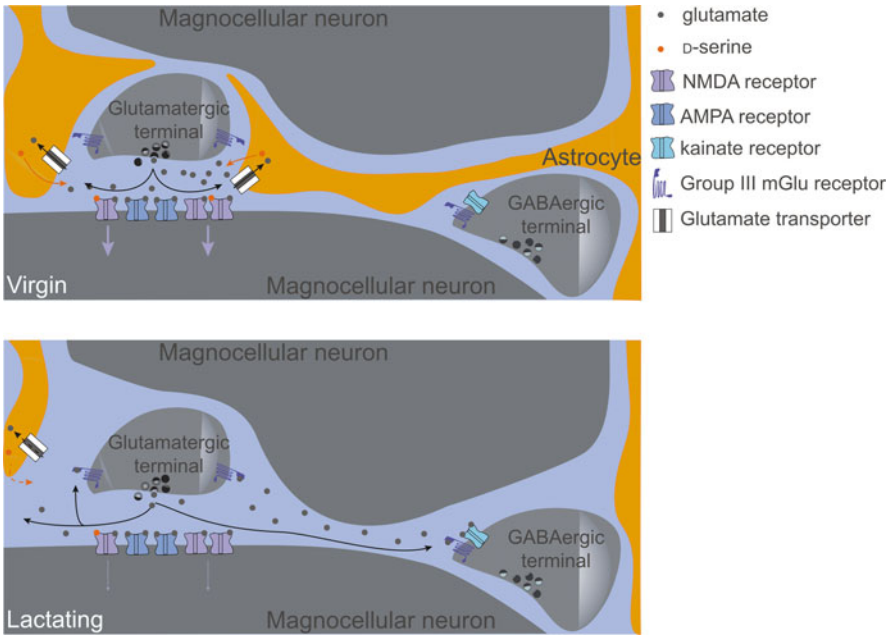

Fig. 2.4 Structural glial plasticity in the adult HNS. During lactation, when the electrical and secretory activities of oxytocin neurons are greatly enhanced, the astrocytic coverage of magnocellular neurons is reduced, compared to virgin animals. Astrocytic processes no longer wrap around synapses and neuron somata, and dendrite surfaces become directly juxtaposed. Consequently, glutamate uptake by astrocytic transporters is reduced, which favors homosynaptic regulation of glutamate release through presynaptic metabotropic glutamate receptors (mGluRs). Diffusion is facilitated, which allows inter-synaptic crosstalk through glutamate actions at mGluRs and kainate receptors expressed by GABAergic terminals. D-serine availability at the synapse is greatly reduced, which impairs NMDA receptor-mediated synaptic transmission and long-term synaptic plasticity. In this oversimplified representation, the extracellular space appears very large, whereas in fixed tissues, it is about 10 nm between contiguous cellular elements. Adapted with permission from Theodosis et al. (2004)

characterized by small and round cell bodies with few processes, show little immunoreactivity for GFAP and are dye-coupled. The third population is made of typical stellate astrocytes, similar to that of most astrocytes in the adult central nervous system, and found scattered in the nucleus. As shown in Fig. 2.4, under basal conditions of secretion, the thin distal astrocytic processes separate the magnocellular neurons, although these are often tightly packed in clusters of cells. There are also astrocyte-like cells (pituicytes) in the neurohypophysis.

The functional significance of each type of supraoptic astrocyte is still unknown. Astrocytes generally contribute to the regulation of the microenvironment in which neurons function. The HNS astrocyte plasmalemma is enriched with glycoproteins that contribute to the molecular composition and complexity of the extracellular space, such as the highly sialylated isoform of the neural cell adhesion molecule PSA-NCAM. By expressing different transporters, HNS astrocytes may be involved

in the clearance of synaptically released neurotransmitters, like glutamate and GABA (Fig. 2.4). Through the interposition of their fine distal processes between all neuronal elements, they make a physical barrier to restrict spillover (i.e. the escape of neurotransmitters from the synaptic cleft) and diffusion of locally released neuroactive molecules into the extracellular space (Fig. 2.4). Finally, HNS astrocytes can detect synaptic activity through the expression of specific receptors at their surface, which may lead to the release of signaling molecules that are collectively named gliotransmitters (Fig. 2.4). These include glutamate, D-serine, ATP, taurine, and cytokines. In the neurohypophysis, processes of pituicytes ensheath neurosecretory axons and may affect neurosecretion by limiting the diffusion of secreted peptides into perivascular spaces and thus, into the general circulation.

2.1.5 Structural Glial Plasticity in the Adult HNS

Magnocellular nuclei undergo extensive and reversible morphological transformations under conditions of intense activity such as lactation, parturition, and chronic dehydration (for review see Miyata and Hatton 2002; Salm 2000; Theodosis et al. 2008). In these conditions, increased numbers of synapses are observed and magnocellular neuronal somatic and dendritic surfaces are no longer separated by astroglial processes, but become directly and extensively juxtaposed as a result of active retraction of astrocytic processes covering their surfaces (Fig. 2.4). The reduction of astrocytic coverage impacts essentially the oxytocin neurons and their synapses. It is rapid, since changes can be detected within a few hours of the onset of stimulation. However, juxtaposed neuronal membranes do not contact each other and the intervening extracellular space remains constant (≈10 nm). Such structural modifications of the astrocytic environment affect the volume and geometry of the extracellular space, as well as its molecular composition. Changes are fully reversible upon cessation of stimulation. Rapid glial plasticity also occurs at the level of the neurohypophysis, including a dynamic change in the interaction between pituicytes and nerve terminals.

The structural glial plasticity implies dynamic cell–cell and cell–matrix interactions, requiring cell surface, extracellular fluid molecules, and soluble factors to come into play. For instance, PSA-NCAM, the expression of which is not related to neuronal activity, still is a required permissive factor for HNS glial plasticity, since specific enzymatic removal of PSA from NCAM in the supraoptic nucleus can inhibit the glial remodeling associated with lactation and chronic dehydration. Other molecules such as glutamate and oxytocin, acting in synergy with sex steroids, provide the signaling message that triggers the mechanisms leading to HNS glial plasticity.

2.1.6 Investigating the Functional Consequences of Structural Plasticity

The adult HNS thus undergoes a remarkable reversible, activity-dependent morphological neuroglial plasticity. In this chapter, we summarize how this made it a seminal model to study the physiological contribution of the astrocytic environment to synaptic strength, inter-synaptic crosstalk, and neuronal signaling through the release of the gliotransmitter D-serine. We also open perspectives related to these discoveries and to future research on neuroglial plasticity in the HNS.

As illustrated in Fig. 2.4, investigating the functional consequences of structural plasticity took advantage of the reversible reduction in the astrocytic coverage of oxytocin neurons that is induced during lactation, when the surfaces of oxytocin neurons become extensively juxtaposed and synapses are no longer tightly ensheathed by astrocytic processes. In normal conditions, the fine distal processes of astrocytes that ensheath synapses contribute to point-to-point synaptic transmission by expressing different transporters that are involved in the clearance of synaptically released neurotransmitters, such as glutamate and GABA. Moreover, by their position, astrocytic processes provide a barrier to prevent neurotransmitter spillover and limit volume transmission. Finally, they may themselves contribute to synaptic transmission by releasing neuroactive molecules, such as glutamate, D-serine, ATP, taurine. The consequences of the absence of glial coverage were investigated in the supraoptic nucleus of lactating animals considering three possibilities: transporters may no longer be available at the synapse; astrocyte processes may no longer hinder diffusion in the extracellular space; the contribution of astrocytes to synaptic communication through gliotransmission may be hampered. Identification of these consequences fueled the now fundamental concept that astrocytes are dynamic partners of brain signaling.

2.2 Contribution of the Astrocytic Environment to Homosynaptic Strength

Glutamate is the main excitatory neurotransmitter in the HNS. Its clearance depends on uptake and diffusion. Uptake of synaptically released glutamate relies essentially on the astrocytic high-affinity glutamate transporter GLT-1. Changes in glutamate uptake may produce local variations of glutamate concentrations close to the release sites, and thus affect synaptic transmission at both post- and presynaptic levels. Additionally, a prolonged alteration in glutamate uptake may lead to excitotoxicity through the chronic activation of glutamate receptors.

Box 2.1: Investigating the Presynaptic Origin of Drug Action Using Paired-Pulse Facilitation

To determine whether presynaptic mechanisms are involved in the action of a drug on evoked postsynaptic currents/potentials, one may use paired-pulse facilitation (PPF). PPF is a form of short-term synaptic plasticity. Two presynaptic spikes are evoked in close succession and the responses of the postsynaptic cell are measured in terms of postsynaptic potentials or currents. PPF is expressed as the amplitude ratio (S2/S1) of the second synaptic response (S2) over the first synaptic response (S1). In PPF, the second postsynaptic response, S2, is larger than the first (S1). In most cases, PPF reflects a presynaptic phenomenon, due to an increase in the number of vesicles released by the presynaptic element, even if postsynaptic contributions must also be considered. Different mechanisms have been proposed to explain the presynaptic facilitation. They converge on a build-up of calcium in the presynaptic terminal that leads to an increased probability of neurotransmitter release upon the second spike.

In presynaptic elements with a low initial probability of release, the first spike causes a small postsynaptic response, but the build-up of calcium in the presynaptic terminal increases the probability of neurotransmitter vesicle release upon the second spike. The second postsynaptic response is thus greater than the first one, showing facilitation. In presynaptic elements with a high initial probability of release, the first spike depletes part of the available readily releasable pool of vesicles and even if calcium concentration is higher after the second pulse than after the first one, less transmitter is available to be released due to the reduced number of readily releasable vesicles. The second postsynaptic response is thus smaller than the first one, showing a depression.

Although not conclusive, a change in PPF ratio after a drug treatment suggests that the drug may be acting at the presynaptic level. If the drug is acting at the postsynaptic level, it may change the amplitude of individual evoked currents, but the release of neurotransmitter should not be affected and neurotransmitter interaction with receptors should be equally affected by the drug during the two pulses, leaving PPF unchanged. If the drug is acting at the presynaptic level, the probability of neurotransmitter release is affected, which alters the facilitation process and thus PPF.

In addition to PPF, one may assess whether presynaptic mechanisms are involved in the action of a drug by analyzing miniature postsynaptic currents or potentials and trial-to-trial fluctuation of the evoked responses using the inverse of the squared coefficient of variation ($1/CV^2$) (CV=SD/mean, coefficient of variation) of the postsynaptic response amplitude.

To test the possibility that a deficiency in the availability of glutamate transporters at the synapse may result from the absence of glial coverage that normally wraps glutamatergic synapses and secondarily affects synaptic transmission, the following

questions were addressed (Oliet et al. 2001): (1) What are the effects of a deficiency in glutamate uptake upon excitatory synaptic transmission? (2) What are the mechanisms of these effects? (3) Do changes in glial coverage affect excitatory synaptic transmission in the same way? (4) Do changes in glial coverage affect glutamate concentration and/or time course in the synaptic cleft?

2.2.1 Role of Glutamate Uptake on Homosynaptic Efficacy

To answer the first question, the effects of inhibiting glutamate transporters in the supraoptic nucleus were measured on glutamate-mediated, evoked excitatory post-synaptic currents (EPSCs) using whole-cell patch clamp recordings. Supraoptic nuclei were taken for virgin rats, in which the glial coverage of synapses is normal. Both a specific inhibitor of GLT-1 or PDC, a broad-spectrum glutamate transporter blocker, inhibited reversibly EPSCs by about 50%, which suggests that the most part of their effect was mediated by inhibition of GLT-1. As illustrated in Fig. 2.5a, analysis of miniature EPSCs (mEPSCs) showed that PDC decreased the frequency, but not the size of these events, suggesting a presynaptic origin of the modulation of synaptic currents by excess ambient glutamate when glutamate transporters are inhibited. This was confirmed by analysis of the paired-pulse facilitation (PPF) ratio of evoked currents that showed an increase when glutamate transporter antagonists were applied (see Box 2.1). It appears therefore that the increase in local glutamate concentration after inhibition of glutamate transport is sufficient to activate presynaptic glutamate receptors controlling neurotransmitter release.

2.2.2 Control of Homosynaptic Efficacy Through Metabotropic Glutamate Receptors

One likely mechanism underlying these effects could be the activation of group III metabotropic glutamate receptors (mGluRs), as they were known to induce presynaptic inhibition of glutamate release from excitatory synapses in the supraoptic nucleus. To test this hypothesis, the effects of blocking glutamate transport on excitatory synaptic inputs were tested in the presence of agonists and antagonists of group III mGluRs. As shown in Fig. 2.5b, the mGluR agonist reduced evoked EPSCs and subsequent addition of the transporter blocker had no further effect on EPSC amplitude, which suggested that the blockade of glutamate transport was occluded by agonist activation of mGluRs. Reciprocally, the mGluR antagonist increased EPSC amplitude, which indicated that mGluRs are tonically activated in normal conditions. Blocking group III mGluRs also prevented the effects of increasing extracellular glutamate with blockade of glutamate uptake. Together, these data show that presynaptic mGluR activation underlies the inhibitory effect on synaptic transmission of increased glutamate concentration resulting from glutamate uptake reduction.

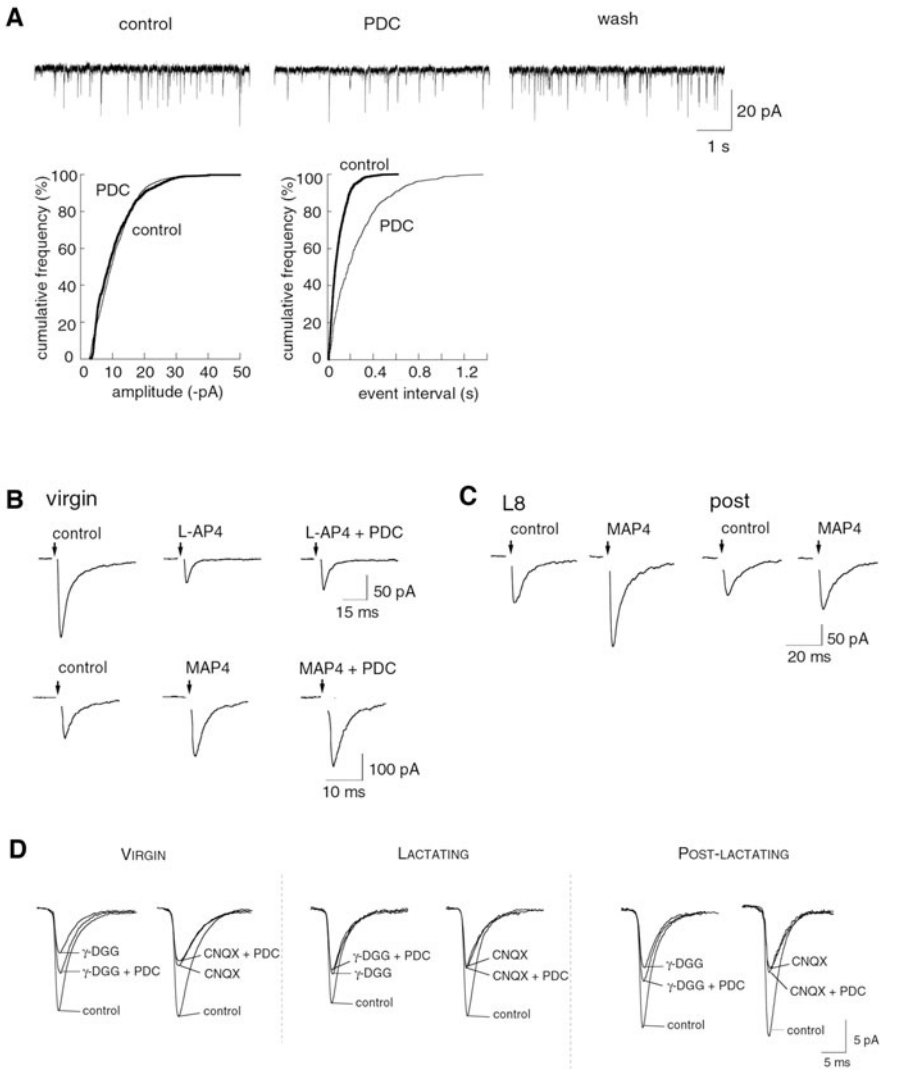

Fig. 2.5 Control of glutamate uptake and synaptic efficacy by glial coverage of neurons. (**a**) Glutamate transporter blockade induces presynaptic inhibition of EPSCs. Top, traces from a recording of a magnocellular neuron showing a reversible reduction in mEPSC activity with application of a glutamate transporter blocker, PDC. Bottom, the corresponding cumulative amplitude distributions obtained in the presence and absence of PDC were not statistically different ($P > 0.05$), whereas the cumulative event interval distribution was significantly shifted to the right with PDC ($P < 0.05$), which corresponds to a reduction in mEPSC frequency. (**b**) Glutamate transporter blockade causes glutamate activation of presynaptic group III mGluRs. Sample traces of evoked EPSCs obtained in virgin rats in the absence and presence of a group III mGluR agonist (L-AP4, upper panel) and antagonist (MAP4, lower panel). Subsequent blockade of glutamate transport with PDC had no effect on the EPSC amplitude under both conditions. (**c**) Reduction in astrocytic coverage affects presynaptic group III mGluR activation. Sample recordings of evoked EPSCs obtained in magnocellular neurons in the presence and absence of the group III mGluR antagonist MAP4 in slices from lactating (L8) and postlactating (post) rats. (**d**) Glial coverage of

2.2.3 Glial Coverage Controls Homosynaptic Efficacy

If the absence of glial coverage in lactating conditions results in a deficiency in the availability of glutamate transporters at the synapse, it should lead to increased glutamate concentration. Therefore, presynaptic mGluRs should be more activated, leading to a decrease of release probability and reduced evoked EPSCs. The effect should be the same as the one induced in virgin rats by the application of a specific antagonist of glutamate transporters. In addition, this transporter antagonist should have either a small or no effect on excitatory synaptic transmission in lactating rats, in which glutamate concentration is already increased. Accordingly, glutamate transporter blockade was less effective in reducing evoked EPSCs in lactating rats, suggesting just such a partial occlusion effect. In postlactating rats, the effects of glutamate transporter blockade reversed to what was found in virgin rats, as expected in a condition when astrocytic coverage reverts back to basal conditions. In addition, antagonizing presynaptic mGluRs induced a larger increase of evoked EPSC amplitude in lactating rats than observed in virgin or postlactating rats (Fig. 2.5c), suggesting that the tonic activation of mGluRs by ambient glutamate is increased in lactating rats. Altogether these data show that presynaptic mGluR activation underlies the inhibitory effect on synaptic transmission resulting from a reduction in glial coverage of synapses in lactating rats.

2.2.4 Glial Coverage Controls Concentration and/or Time Course in the Synaptic Cleft

To test whether changes in glial coverage affect glutamate concentration and/or time course in the synaptic cleft, γ-D-glutamylglycine (γ-DGG), a low-affinity, competitive AMPA receptor antagonist, whose effect is sensitive to the concentration and/or time course of glutamate in the synaptic cleft, was used. γ-DGG reduced the amplitude of miniature EPSCs (mEPSCs) in virgin and postlactating rats by about 50% (Fig. 2.5d). When used in combination with glutamate uptake blockade, the reduction was only by about 35%, as expected from the presence of an increased concentration of glutamate in the synaptic cleft. Accordingly, in lactating rats, γ-DGG only reduced the amplitude of mEPSCs by about 35%, and additional blockade of glutamate reuptake did not change the amount of reduction. The reduction of mEPSCs by a high-affinity, slowly dissociating, competitive AMPA

Fig. 2.5 (continued) supraoptic neurons affects the relative glutamate concentration and/or time course in the synaptic cleft. Examples illustrating the inhibition of mEPSCs observed under different conditions (control, γ-DGG alone, γ-DGG + PDC, CNQX alone, CNQX + PDC) in virgin, lactating, and postlactating animals. γ-DGG is a low-affinity, competitive AMPA receptor antagonist, whose effect is sensitive to the concentration and/or time course of glutamate in the synaptic cleft, which is not the case for CNQX, another AMPA receptor antagonist. Adapted with permission from Oliet et al. (2001)

receptor antagonist was similar in all three conditions, around 55%, and remained unaffected by addition of reuptake blocker, which is consistent with the fact that the effect of the high-affinity AMPA antagonist is not sensitive to the concentration and/or time course of glutamate in the synaptic cleft. These data strongly suggest that changes in glial coverage affected glutamate concentration and/or time course in the synaptic cleft.

2.2.5 Physiological Consequences

Together, these data show that the absence of glial coverage of glutamatergic synapses in lactating rats results in a reduction in the availability of glutamate transporters, leading to a deficiency in glutamate clearance, leading to an increase in local glutamate concentration, which further activates presynaptic mGluRs and causes a reduction in homosynaptic strength. Thus, astrocytes control the level of the negative feedback exerted by ambient glutamate on its own release at HNS excitatory synapses. Similar physiological findings were found in the supraoptic nucleus of chronically dehydrated rats, in which it is known that structural glial plasticity also takes place (Boudaba et al. 2003).

As a consequence, low to moderately active synapses should undergo a clear presynaptic inhibition. At very active synapses, however, the higher firing rate of presynaptic inputs should potentiate the increase in Ca^{2+} in presynaptic terminals, which would overcome presynaptic inhibition and facilitate glutamate release. In other words, increased presynaptic inhibition due to a reduction in glial coverage in lactating animals sets a functional high-pass filter. In the context of parturition and the milk ejection reflex, that could serve to prevent activation of oxytocin secreting neurons in response to low-frequency stimuli that are not directly related to uterine or mammary gland inputs, thus saving oxytocin for its two most important functions in such a context. The high-pass filter function would also result in a decreased synaptic noise, thus increasing postsynaptic membrane resistance and consequently raising the gain of the input--output neuronal response. Whether this would tune the responsiveness of oxytocin neurons to pertinent synaptic inputs remains to be explored (Jourdain et al. 1998).

2.3 Contribution of the Astrocytic Environment to Inter-synaptic Crosstalk

The demonstration that the reduction in glial processes modifies the local glutamate concentration at the synapse by reducing its uptake, and thus affects homosynaptic strength, led to the question whether such remodeling might also change neurotransmitter diffusion, and as a result contribute to inter-synaptic crosstalk. Indeed, astrocyte processes make a physical barrier to diffusion in the extracellular space, hindering the movement of molecules through this space, and changes in their coverage may thus influence heterosynaptic modulation of adjacent synapses.

GABA is the main inhibitory neurotransmitter in the HNS, and GABAergic synapses are often located close to glutamatergic synapses. In addition, it has been shown that presynaptic mGluRs located on inhibitory terminals in the supraoptic nucleus can be activated by changes in ambient glutamate, provided these changes reach a certain magnitude, which is not the case in basal conditions (Piet et al. 2003). However, when activated, presynaptic mGluRs may reduce GABA release. Inter-synaptic crosstalk between glutamatergic and GABAergic synapses can thus be explored in the supraoptic nucleus, provided the extracellular concentration of glutamate reaches sufficient levels to saturate the transporter system and diffuse away to affect adjacent synapses.

To test the possibility that astrocyte processes may no longer hinder diffusion in the extracellular space in lactating rats, the following questions were thus addressed (Piet et al. 2004): (1) Do changes in glial coverage during lactation affect diffusion in the supraoptic nucleus? (2) Do such changes in diffusion facilitate heterosynaptic activity through metabotropic glutamate receptors? (3) Is inter-synaptic crosstalk prevented when diffusion is limited?

Kainate receptors (KARs) are ubiquitous ionotropic glutamate receptors in the central nervous system. Activation of presynaptic KARs may regulate GABA release and this regulation has been shown to be bidirectional in several brain areas, with experimental increase in the concentration of KAR agonist resulting in a switch from facilitation to inhibition of GABA release. The HNS offered thus an excellent model to study the impact of changes in glutamate levels resulting from a reduction in glial coverage on the mechanisms and function of KAR switch upon GABA synaptic release. To address this issue, three steps were followed (Bonfardin et al. 2010): (1) Establishing the presence of functional KARs on GABAergic terminals in the supraoptic nucleus; (2) Showing that physiological enhancement of extracellular levels of glutamate resulting from glial withdrawal can lead to a switch in KAR activity; (3) Identifying the underlying mechanisms for KAR facilitation and inhibition.

2.3.1 Glial Coverage Controls Diffusion in the Extracellular Space

To answer the question whether changes in glial coverage during lactation affect diffusion in the HNS, diffusion parameters in the extracellular space were measured in the supraoptic nucleus. The diffusion parameters in the extracellular space were measured using the real-time TMA^+ (tetramethylammonium) iontophoretic method (Fig. 2.6a). In brief, the extracellular marker TMA^+, to which cell membranes are relatively impermeable, is administered in the tissue by iontophoresis and its concentration is measured at distance (about 100μm) by a selective microelectrode filled with an ion exchanger. The concentration profile depends on the diffusion properties of the extracellular space. The diffusion curves that are obtained can be compared with that obtained in obstacle-free conditions and fitted to extract the following parameters: volume fraction (α), tortuosity (λ), and nonspecific uptake (k'). Volume fraction is the proportion of tissue volume occupied by the extracellular space.

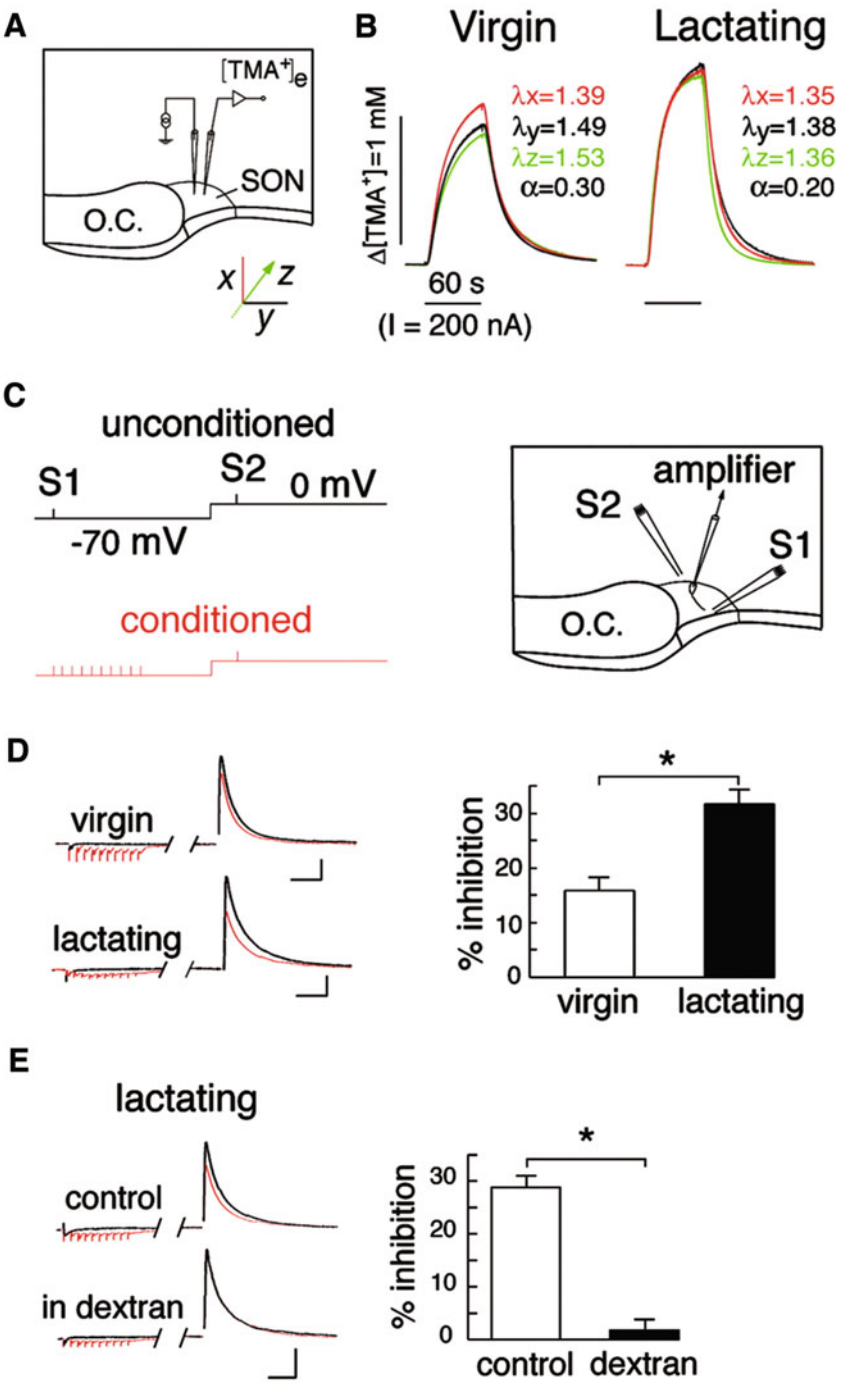

Fig. 2.6 Physiological contribution of the astrocytic environment of neurons to inter-synaptic crosstalk. (**a**) Experimental set-up illustrating the real-time TMA^+ iontophoretic method applied to the supraoptic nucleus (SON) in acute slices. Measurements were made along three perpendicular

Tortuosity is a measure of restriction of diffusion in the extracellular space in comparison with an obstacle-free medium.

Lactation induced changes in diffusion parameters in the supraoptic nucleus, but not in the cortex, used as a control (Fig. 2.6b). In virgin animals, tortuosity was lower in the ventrodorsal axis than in the rostrocaudal and mediolateral axes, showing that the tissue is anisotropic, i.e. with properties that change according to the direction. This likely reflects the ventrodorsal orientation of radial-like glia in the supraoptic nucleus. In contrast, in lactating animals, tortuosity was reduced and similar in the three axes, indicating that the tissue becomes isotropic during lactation. Diffusion is thus enhanced in lactating animals and becomes equivalent in all directions as a result of glial remodeling. Volume fraction was also decreased in the SON from lactating animals, as a probable consequence of the reduction in interneuronal space resulting from the withdrawal of astrocytic processes.

2.3.2 Changes in Diffusion Facilitate Heterosynaptic Activity Through Metabotropic Glutamate Receptors

To ask whether such changes in diffusion facilitate heterosynaptic activity, the presynaptic mGluRs located on inhibitory terminals in the supraoptic nucleus were used as sensors of the spillover of glutamate (Fig. 2.6c). GABA-mediated, evoked inhibitory postsynaptic currents (IPSCs) were measured using whole-cell patch clamp recordings from supraoptic neurons, in response to stimulating an afferent pathway. This was preceded by the stimulation of a second, independent pathway with brief high-frequency trains of stimuli, in order to produce a sustained release of glutamate. Recordings were performed in the presence of a $GABA_B$ receptor antagonist to avoid presynaptic changes that could result from the activation of these receptors by GABA spillover associated with the conditioning train. The conditioning train caused a significant reduction in IPSC amplitude in both virgin and lactating rats, but the reduction was twice as big in lactating (≈30%) as in virgin (≈15%) animals (Fig. 2.6d). Analysis of the paired-pulse facilitation ratio and trial-to-trial fluctuations of the evoked-GABAergic responses (see Box 2.1) showed that

Fig. 2.6 (continued) axes (*x*, *y*, and *z*), as illustrated. O.C., optic chiasm. (**b**) Example of diffusion curves obtained in virgin and lactating rats. Values for tortuosity and volume fraction extracted from three curves recorded along the *x*, *y*, and *z* axes are indicated. (**c**) Experimental set-up illustrating the stimulation paradigm (left) and the position of the stimulating electrodes (S1, S2, right) to measure heterosynaptic depression of evoked IPSCs. (**d**) Sample traces (Left) and summary histogram (right) showing the heterosynaptic depression of evoked IPSCs obtained in virgin ($n = 18$) and lactating ($n = 18$) rats. (scale bars = 200 pA, 40 ms). (**e**) Diffusion in the extracellular space affects glutamate spillover. Left, sample traces obtained from a magnocellular neuron from a lactating rat in the absence and presence of dextran in the extracellular solution. Dextran increases extracellular viscosity and decreases passive diffusion. (Bars = 200 pA, 40 ms). On the right, histogram summarizing the heterosynaptic depression in the absence and presence of dextran. Adapted with permission from Piet et al. (2004), copyright (2004) National Academy of Sciences, U.S.A

the origin of this depression was presynaptic. It was blocked by the mGluR antagonist in virgin as well as in lactating rats, indicating that it also involved mGluRs. Additional experiments involving variations in the delay between the beginning of the conditioning train and the IPSC stimulation, and variations in the stimulation frequency of the conditioning train, showed that reduction in IPSC amplitude was directly related to the amount of glutamate that was released, but this was always larger in lactating animals. These data therefore imply that inter-synaptic crosstalk between glutamatergic and GABAergic afferent inputs depends on the concentration of glutamate reaching the GABAergic terminals. They also show that the action of glutamate at a distance from its release sites is facilitated in the supraoptic nucleus of lactating animals. In other words, the enhanced diffusion in the extracellular space that results from a reduction in glial coverage is paralleled by an increased heterosynaptic activity.

2.3.3 Limiting Changes in Diffusion Affect Glutamate Spillover

If changes in diffusion interfere with inter-synaptic crosstalk, reducing the coefficient of diffusion of glutamate should limit the concentration of the excitatory amino reaching neighboring synapses and, as a consequence, alter heterosynaptic modulation. As illustrated in Fig. 2.6e, adding 5% dextran (40 kDa) in the perfusion solution, which is known to increase viscosity and reduce the coefficient of diffusion of glutamate in the extracellular space, led to an abolition of the heterosynaptic depression of evoked IPSCs measured in the supraoptic nucleus of lactating rats. Together, these data show that the glial environment of HNS neurons is an important regulator of glutamatergic communication between independent synapses by setting the parameters of diffusion in the extracellular space (Piet et al. 2004).

2.3.4 Functional Presynaptic Kainate Receptors on GABAergic Terminals in the HNS

The possibility that glutamate might modulate GABAergic synapses though KARs was also investigated. Kainate was found to increase the mIPSC frequency in supraoptic neurons, without changing their amplitude, which indicates a presynaptic action on GABAergic terminals. The GluK1-specific KAR agonist ATPA had the same effect, and it was blocked by UBP302, a selective antagonist of GluK1-containing KARs. Functional presynaptic GluK1-containing KARs are thus present on GABAergic terminals in the supraoptic nucleus (Fig. 2.7a).

Interestingly, ATPA was less efficient than KA in increasing mIPSC frequency, and the increase in mIPSC frequency triggered by kainate was only partially abolished by UBP302, which suggests that an additional KAR subtype is present on these inhibitory terminals. This was confirmed, since the remaining kainate-induced response was completely blocked by a broad-spectrum AMPA/KA receptor

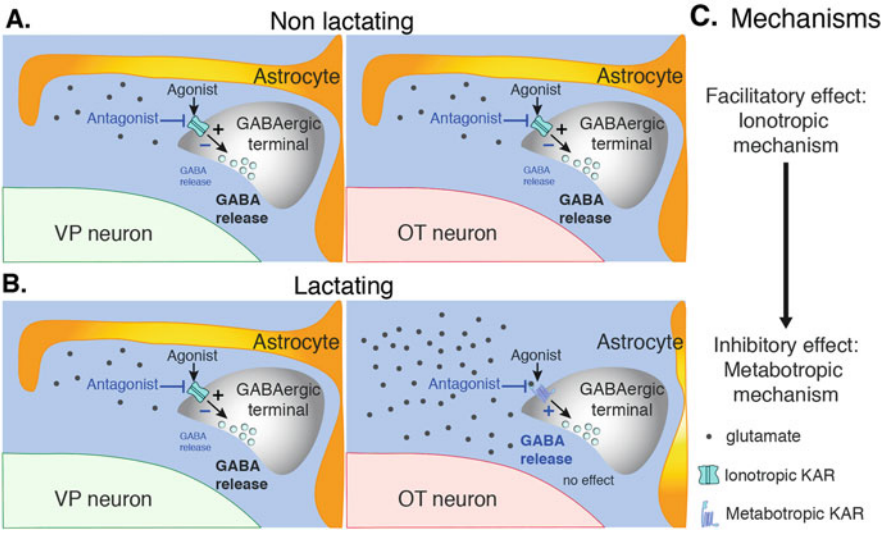

Fig. 2.7 Astrocyte-dependent switch of presynaptic kainate receptor action. (**a**) Presynaptic kainate receptors (KARs), including GluK1-containing KARs, are present on GABAergic terminals synapsing on vasopressin (VP) and oxytocin (OT) neurons in the supraoptic nucleus. In non-lactating rats, they are positively coupled to GABA release onto OT and VP neurons, so that application of KAR agonists facilitates GABA release and application of KAR antagonists inhibits GABA release. Ambient glutamate facilitates GABA release onto OT and VP neurons through GluK1-containing KARs. (**b**) In lactating rats, blocking KARs still has an inhibitory effect on GABA release onto VP neurons, but the effect switches to increased GABA release onto OT neurons. In lactating rats, increased levels of ambient glutamate in the vicinity of GABAergic synapses impinging on OT neurons change the coupling of KARs to GABA release, leading to an inhibition of GABA release. The increased levels of ambient glutamate also occlude the effect of KAR agonists. (**c**) The mechanisms for facilitation and inhibition of GABA released by KARs are mediated through an ionotropic and a metabotropic mode of action of KARs, respectively. It is not known whether the ionotropic and metabotropic actions of KARs are mediated by the same or by two distinct types of GluK1-containing receptors

inhibitor. Two different types of KARs are thus present on GABAergic terminals impinging on magnocellular neurons in the supraoptic nucleus.

In both identified oxytocin and vasopressin neurons, application of the antagonist UBP302 alone induced a reversible reduction in mIPSC frequency, whereas mIPSC amplitude remained unchanged (Fig. 2.7a). Blocking the remaining AMPA/KA receptors did not induce any further reduction in mIPSC activity. Together, these data show that only GluK1-containing KARs are activated by ambient glutamate and they regulate GABA release in a similar manner in both oxytocin and vasopressin neurons.

2.3.5 Changes in Glial Coverage Switches Kainate Receptor Presynaptic Action

Experiments designed to test whether physiological enhancement of extracellular levels of glutamate resulting from glial withdrawal can lead to a switch in KAR activity were performed in the presence of an mGluR antagonist, MAP4, as presynaptic mGluRs are activated by increased levels of ambient glutamate and can control GABA release. In lactating rats, blocking KARs with UBP302 still had an inhibitory effect on mIPSC frequency in vasopressin neurons, but the effect switched to excitation in oxytocin neurons (Fig. 2.7b). This suggests that in lactating rats, increased levels of ambient glutamate in the vicinity of GABAergic synapses impinging on oxytocin neurons change the coupling of KARs to GABA release. The KAR agonist ATPA still facilitated GABA transmission in vasopressin neurons, but was without effect in oxytocin neurons. The hypothesis that this lack of effect of ATPA results from an occlusion by increased ambient glutamate levels was confirmed when slices from lactating rats were incubated with an enzymatic glutamate scavenger to reduce ambient levels of the excitatory amino acid: when scavenging was efficient enough to desaturate the GluK1 receptors, but without affecting their coupling to GABA release, ATPA was found to decrease mIPSC frequency in oxytocin neurons.

To confirm that increasing glutamate concentration switches GluK1-containing KAR activity, extracellular glutamate was experimentally increased in the supraoptic nucleus of non-lactating rats using the broad-spectrum inhibitor of glutamate transporters D,L-TBOA. Under these conditions, the KAR antagonist UBP302 induced a reversible increase in mIPSC activity in both oxytocin and vasopressin neurons. The opposite KAR-mediated regulation of GABA transmission as a function of extracellular glutamate levels was also observed in supraoptic neurons when monitoring IPSCs evoked by afferent input stimulation.

Together, these data establish that lactation induces a selective switch in GluK1 activation in oxytocin neurons, in relation to increased extracellular glutamate levels resulting from a reduction in glial coverage (Bonfardin et al. 2010).

2.3.6 Mechanisms for Facilitation and Inhibition by KARs

On one hand, the facilitation of inhibitory transmission by presynaptic KARs in the supraoptic nucleus was found to be mediated through the ionotropic mode of action of KARs, as it was prevented by philantotoxin-433, a blocker of Ca^{2+}-permeable AMPA / KARs (Fig. 2.7c). Such an effect is likely to be caused by the KAR-induced membrane depolarization and / or the direct permeation of Ca^{2+} ions through KARs. On the other, the inhibitory effect of KAR activation on GABA release was not due to an indirect mechanism involving the activation of other presynaptic receptors, since adding a mixture of antagonists to block $GABA_B$ receptors, adenosine A1 receptors, mGluRs, NMDA receptors, CB1 receptors, nicotinic receptors, dopamine D4 receptors, and adrenergic receptors did not affect the inhibitory action of GluK1-

containing KARs on GABA release. The inhibitory effect appeared to be mediated by a metabotropic mode of action of KARs, as it was prevented in the presence of a phospholipase C inhibitor and unaffected by philantotoxin-433 (Fig. 2.7c).

2.3.7 Physiological Consequences

The reduction in glial processes, by changing neurotransmitter diffusion, contributes to inter-synaptic crosstalk. This has also proven to be the case for endocannabinoids (Di et al. 2013) and ATP (Gordon et al. 2005) in the HNS. Other neuroactive substances that are released in the HNS nuclei such as neurosteroids, oxytocin, and vasopressin would also see their concentration and range of action increase, except, of course those released from the glial processes that have retracted. For somatodendritically released oxytocin that provides a positive feedback regulation of the electrical activity of oxytocin neurons, a facilitated diffusion would have important physiological consequences. After local release, it could reach distant targets and contribute further to the recruitment and synchronization of additional bursting of oxytocin neurons.

Enhanced heterosynaptic inhibition of GABA transmission by glutamate acting on mGluRs or KARs could boost transmission of high-frequency excitatory information, by inhibiting nearby GABAergic synapses and thus creating a local disinhibition. This could favor the excitation of oxytocin neurons during parturition and suckling (Jourdain et al. 1998). In other brain areas where similar glial changes occur, disinhibition could also play a physiological role or be involved in the pathophysiology of neurodegenerative diseases, epilepsy, and ischemia, which are associated with greatly enhanced levels of extracellular glutamate. Under such conditions, the disinhibition may amplify the excitotoxic action of glutamate and further reduce neuronal survival.

2.4 Gliotransmission in the HNS: A Case for D-Serine

Since astrocytic processes are closely juxtaposed to synapses, they are ideally located to detect synaptic activity through the expression of specific receptors at their surface, and in response, to release signaling molecules, named gliotransmitters by analogy to neurotransmitters (see Box 2.2), such as glutamate, D-serine, ATP, and taurine (Savtchouk and Volterra 2018). Among them, the amino acid D-serine (see Box 2.3) is considered as an important co-agonist of the synaptic NMDA receptors that are known to play a key role in synaptic plasticity in the HNS, as well as in the brain and spinal cord. Considering the hypothesis that the retraction of astrocytic processes could hamper the contribution of astrocytes to glutamatergic synaptic communication through D-serine gliotransmission, the consequences of the absence of glial coverage on D-serine availability, NMDA receptor activity and plasticity were investigated in the supraoptic nucleus (Panatier et al. 2006). The step-by-step

approach involved identifying D-serine production in supraoptic astrocytes, demonstrating that D-serine, and not glycine, is the endogenous co-agonist of NMDA receptors in the supraoptic nucleus, and showing that the D-serine level in the synaptic cleft depends on the coverage of synapses, with consequences for long-term synaptic plasticity.

Box 2.2: Is Gliotransmission a Physiological Phenomenon?

Gliotransmission can be defined as the active transfer of information from glia to neurons through neuroactive molecules that activate neuronal receptors located at synaptic and/or extrasynaptic sites. According to the concept of the tripartite synapse, peri-synaptic astrocytic processes are not only structural, but also functional partners of the pre- and postsynaptic neuronal elements, through the release of gliotransmitters.

Astrocytes express a large number of channels, transporters, and receptors, many of which are the same as the ones expressed by neurons. It has been shown that astrocytes may respond to synaptically released neurotransmitters, in acute brain slices as well as in vivo. Although astrocytes are not electrically excitable, they may sense synaptic activity by responding with different intracellular signaling cascades, including cyclic adenosine monophosphate elevations and calcium mobilization from internal stores via G protein-coupled receptor stimulation or calcium influx through transient receptor potential channels. Upon activation, astrocytes may release gliotransmitters (such as glutamate, GABA or ATP) using various mechanisms, including exocytic release from vesicles or release from the cytosol via plasma membrane ion channels and pumps. The molecular mechanisms leading to gliotransmitter release are still intensely disputed. Once released, gliotransmitters produce a variety of effects on synaptic activity, according to the specific receptors involved and their pre- or postsynaptic location.

The concept of gliotransmission has been harshly criticized in recent years based on the general argument that gliotransmission should be defined as the Ca^{2+}-dependent release of neurotransmitters by astrocytes and that no changes in physiologically relevant astrocyte Ca^{2+} have ever resulted in any detectable gliotransmission by astrocytes (see Fiacco and McCarthy 2018). However, gliotransmission appears to be a much more complex phenomenon than originally thought, since it is not triggered by a single neurotransmitter receptor, does not necessarily involve a single Ca^{2+} source or code, and does not necessarily imply neuron-like vesicular transmitter release (Savtchouk and Volterra 2018). Astrocytes do not behave like neurons, and they process information over a much slower time scale. The main function of gliotransmission seems to be to fine-tune neuronal processing according to more general brain states.

(continued)

Box 2.2 (continued)

In the HNS, the amino acid taurine was identified as a putative gliotransmitter in the late 90s (for review see Hussy et al. 2000). Taurine is one of the main osmolytes used by cells to compensate for changes in extracellular osmolarity. It is highly concentrated in supraoptic astrocytes, and released by them in response to hypo-osmotic stimuli through volume-sensitive anion channels. Taurine inhibits magnocellular neuron activity by binding to neuronal glycine receptors. Definite proof that taurine is a gliotransmitter in the HNS came from the work of Choe et al. (2012) showing that supraoptic neurons are unable to release taurine themselves and selective depletion of taurine eliminates the inhibitory tone on glycine receptors, ruling out the possibility that glycine receptors could be activated by other agonists. These data show that, in the HNS, gliotransmission plays an active part in a physiological regulatory loop.

Box 2.3: Is D-Serine a Gliotransmitter?

D-serine is a potent co-agonist at the glycine site (GluN1) of the NMDA receptor. For the receptor to open, either glycine or D-serine must bind to it and glutamate must simultaneously bind to its recognition site on the GluN2 subunits. The synthesis of D-serine requires serine racemase to convert L-serine into D-serine. Early reports identified D-serine as a potential gliotransmitter based on the localization of D-serine and serine racemase in astrocytes. Further and more recent results support the concept that D-serine originates from astrocytes, acts at synaptic NMDA receptors and is taken up by neurons, where it is degraded (reviewed in Papouin et al. 2017).

The concept has been harshly criticized recently by different groups arguing that D-serine is of neuronal and not glial origin, based on the development of serine racemase knockout and conditional knockout mice (Wolosker et al. 2016). According to the alternative hypothesis, astrocytes may still affect D-serine levels by synthesizing L-serine, which shuttles to neurons to fuel the neuronal synthesis of D-serine. According to this view, immunostaining for D-serine in astrocytes would be an artifact resulting from the high concentrations of L-serine in these cells. However, in our hands, not only D-serine, but also serine racemase was found to be exclusively localized in supraoptic astrocytes, and not in magnocellular neurons. This does not preclude, however, the possible presence of D-serine and serine racemase in neurons elsewhere in the brain.

2.4.1 D-serine Is Synthesized and Expressed by Astrocytes in the Supraoptic Nucleus

Several lines of evidence suggested D-serine expression in astrocytes of the supraoptic nucleus. First, immunoblot analysis revealed the high expression of the synthesizing enzyme of D-serine, serine racemase, in the supraoptic nucleus. Second, immunocytochemistry showed that serine racemase was localized throughout the supraoptic nucleus in astrocytic cell bodies and in the neuropil surrounding magnocellular somata (Fig. 2.8a). Third, high levels of D-serine were detected in the supraoptic nucleus by HPLC analysis. Finally, immunofluorescence for D-serine showed a similar distribution to that of serine racemase and double immunofluorescence for D-serine and the astrocytic marker GFAP confirmed the presence of the amino acid in cell bodies and processes of all supraoptic astrocytes. Together, these data identified D-serine production in supraoptic astrocytes.

2.4.2 D-Serine Is the Endogenous Co-agonist of NMDA Receptors in the Supraoptic Nucleus

To demonstrate that D-serine is an endogenous ligand of NMDA receptors in the supraoptic nucleus, NMDA receptor-mediated synaptic responses were recorded from magnocellular neurons in hypothalamic slices treated with D-amino acid oxidase (DAAO), an enzyme specifically degrading D-serine. Due to the high variability in NMDA evoked responses from cell to cell, and since supraoptic neurons are driven by excitatory glutamatergic afferents acting via postsynaptic AMPA receptors and NMDA receptors, the AMPA/NMDA ratio was used to evaluate the contribution of NMDA receptors to EPSCs. The AMPA/NMDA ratio corresponds to the ratio of the peak amplitude of the EPSCs recorded at –60 mV, representing the AMPA receptor-mediated response, over the peak amplitude of the EPSCs recorded at +40 mV in the presence of an AMPA receptor antagonist, revealing the NMDA receptor-mediated response. As illustrated in Fig. 2.8b, the NMDA component of EPSCs was reduced significantly in DAAO-treated slices from virgin animals. Such a reduction was not due to a deleterious action of D-serine oxidation by-products on NMDA receptors since acute application of D-serine restored the AMPA/NMDA ratio to values measured in the absence of the enzyme.

In addition, glycine oxidase (GO), which selectively degrades glycine, was ineffective in altering NMDA receptor-mediated currents, showing that glycine is not an endogenous ligand of NMDA receptors in the supraoptic nucleus. Care was taken to check, using HPLC measurements, that DAAO and GO specifically and efficiently degraded D-serine and glycine, respectively. Together, these data showed that D-serine, and not glycine, is the endogenous co-agonist of NMDA receptors in the HNS, which is consistent with the paucity of glycinergic fibers in this region.

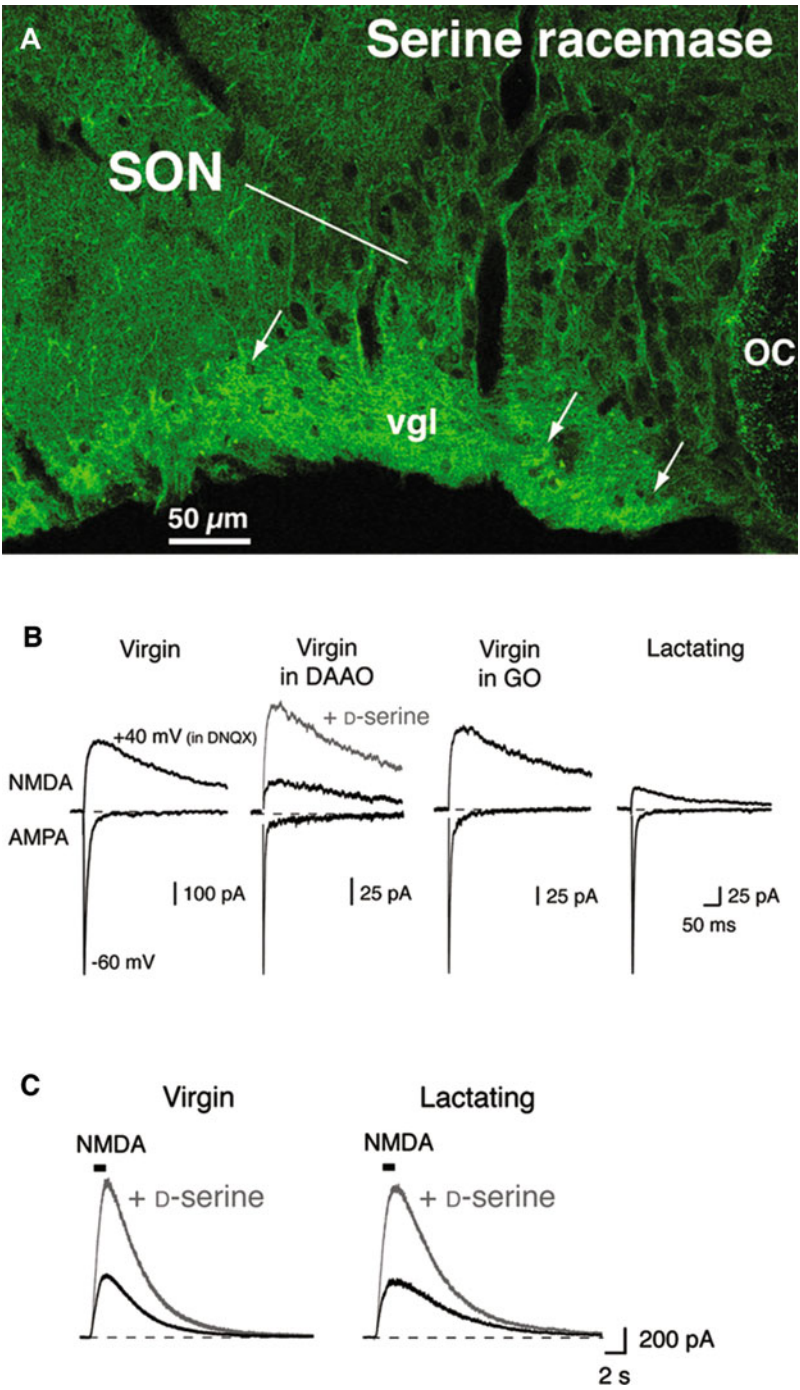

Fig. 2.8 Astrocyte-derived D-serine controls NMDA receptor activity. (**a**) Localization of serine racemase in the rat supraoptic nucleus (SON). Immunofluorescence revealed a strong reaction for serine racemase throughout the nucleus, localized in large processes along blood vessels, in the

2.4.3 Astrocytes Regulate D-Serine Concentration in the Synaptic Cleft

To demonstrate that the D-serine level in the synaptic cleft depends on the coverage of synapses, the AMPA/NMDA ratio was measured in the supraoptic nucleus of lactating rats and found to be larger than that measured in virgin animals and similar to that observed in DAAO-treated virgin slices (Fig. 2.8b). Since AMPA receptor-mediated responses in magnocellular neurons are not altered by lactation (Oliet et al. 2001), such a change in the ratio implied a decrease in NMDA receptor-mediated EPSCs. Pharmacological data obtained using the selective NR2B antagonist ifenprodil showed that this was not due to a switch in NMDA receptor NR2 subunits that are known to confer distinct biophysical properties to NMDA receptors. In addition, NMDA receptor-mediated synaptic responses recorded in slices from lactating rats recovered when the perfusion medium was supplemented with saturating concentrations of D-serine. Together, these data showed that the level of occupancy of the glycine site of synaptic NMDA receptors is critically controlled by the extent of astrocytic coverage of the neuron.

Finally, as shown in Fig. 2.8c, responses elicited by local applications of NMDA, reflecting activation of mainly extrasynaptic NMDA receptors, were enhanced considerably by D-serine and to a similar extent in slices from virgin and lactating rats. These data indicate that the level of occupancy of the glycine site of extrasynaptic NMDA receptors is unsaturated and unaffected by the relative changes in glial coverage. In other words, astrocytes regulate D-serine concentration preferentially in the synaptic cleft.

2.4.4 Astrocytes Control Long-Term Synaptic Plasticity

Glutamatergic synapses in the supraoptic nucleus exhibit activity-dependent long-term synaptic plasticity similar to that prevailing in other brain areas, which could play an important role in the context of physiological responses, like dehydration or lactation, where the activity of presynaptic glutamatergic neurons is strongly

Fig. 2.8 (continued) neuropil surrounding immunonegative magnocellular somata and in the ventral glial lamina (vgl), where the cell bodies of astrocytes (arrows) accumulate. OC, optic chiasma. (**b**) D-serine is the endogenous co-agonist of synaptic NMDA receptors in the supraoptic nucleus. Evoked EPSCs recorded at –60 mV to isolate synaptic AMPA currents and at +40 mV in AMPA receptor antagonist (DNQX) to isolate synaptic NMDA currents in supraoptic neurons. An increase in the AMPA/NMDA ratio was seen in supraoptic neurons from lactating compared to virgin rats and after D-serine degradation with D-amino acid oxidase (DAAO), but not after glycine degradation with glycine oxidase (GO). All recordings were carried out in the presence of bicuculline. (**c**) Astrocyte regulation of D-serine concentration does not occur outside the synaptic cleft. The D-serine-induced enhancement of the amplitude of extrasynaptic responses elicited by local puffs of NMDA (50 μM) was comparable in virgin and lactating rats. Adapted from Panatier et al. (2006)

increased. The induction of long-term potentiation (LTP) and long-term depression (LTD) relies on Ca^{2+} entry through NMDA receptors. Therefore, changes in D-serine concentration in the synaptic cleft resulting from changes in glial coverage should alter the induction of LTP and LTD.

This hypothesis was confirmed, since an experimental protocol that induced NMDA receptor-dependent LTP in control conditions elicited NMDA receptor-dependent LTD in lactating rats (Fig. 2.9). In addition, a stimulation protocol that induced NMDA receptor-dependent LTD in the supraoptic nucleus of virgin animals was ineffective in lactating rats. Finally, when synapses were stimulated strongly enough using high-frequency stimulation of afferent inputs, NMDA receptor-dependent LTP was produced in the supraoptic nucleus of both virgin and lactating rats.

The most likely explanation for this modification of LTP threshold is that a reduction in the number of NMDA receptors activated during the induction protocol, due to a reduced D-serine availability following astrocyte process withdrawal, resulted in a smaller postsynaptic Ca^{2+} rise, which favored phosphatases over kinases, thus reducing the postsynaptic response to glutamate, which manifests as LTD. In hypothalamic slices from lactating rats, application of saturating concentrations of D-serine increased the number of NMDA receptors available for activation and entirely reversed this effect. In other words, astrocytes control synaptic plasticity and its direction in the HNS through the action of D-serine.

2.4.5 Physiological Consequences

These results confirm that glial cells participate in synaptic signaling and show that they can drive the direction of synaptic plasticity. Retraction of astrocytic processes hamper the contribution of astrocytes to glutamatergic synaptic communication through D-serine gliotransmission, thus reducing NMDA receptor activity at synapses in the HNS.

The shift in LTP threshold in HNS neurons of lactating rats should favor potentiation at glutamatergic inputs displaying high activity, and depress synapses exhibiting lower activity. This could favor the excitation of oxytocin neurons during parturition and suckling and reduce their activation in response to other stimuli, thus preserving oxytocin for its primary functions.

2.5 Perspectives

In this chapter, we have summarized how the adult HNS, through its remarkable reversible, activity-dependent morphological neuroglial plasticity, has proved to be a seminal model to study the physiological contribution of the astrocytic environment to synaptic strength, inter-synaptic crosstalk, neuronal signaling and synaptic plasticity. Such a contribution has fueled the now fundamental concept that astrocytes are dynamic partners of brain signaling. That distal processes, as proven by the

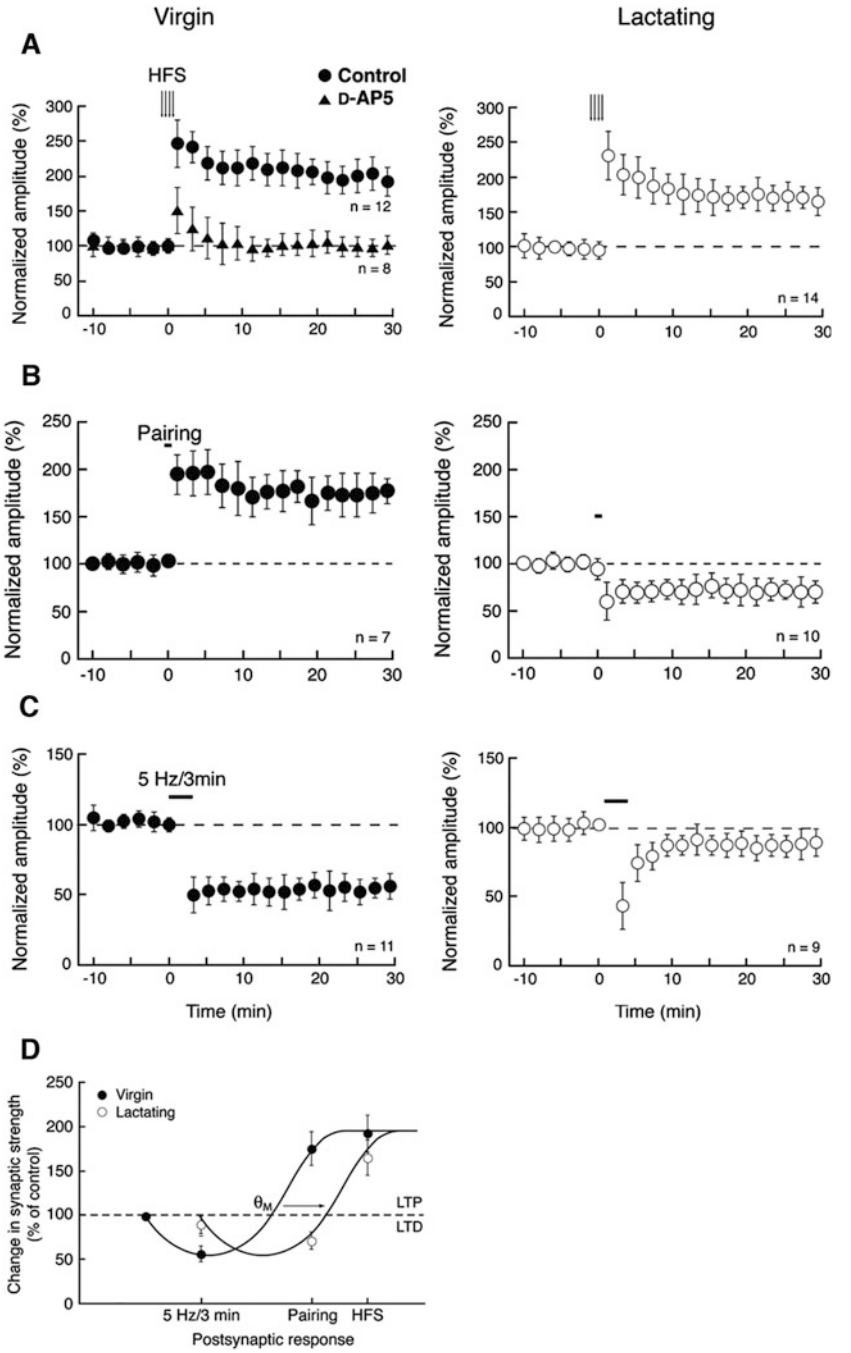

Fig. 2.9 Astrocyte-derived D-serine controls synaptic plasticity. (**a**) High-frequency stimulation (HFS) induced NMDA receptor-dependent long-term potentiation (LTP) of EPSCs in supraoptic neurons of virgin (left) and lactating (right) rats. The LTP was blocked by the NMDA receptor antagonist D-AP5. (**b**) A pairing stimulation paradigm, pairing postsynaptic membrane

effects of their retraction, can play such an important role in the dialog between astrocytes and neurons has been emphasized recently by the fact that small, rapid, and localized Ca^{2+} responses can be regulated in small compartments along the astrocytic processes by minimal synaptic activity (Panatier et al. 2011).

While HNS neuroglial plasticity was initially seen as a possible morphological basis for the synchronization of oxytocin neuron firing during lactation, this proved not to be the case. When glial withdrawal and synaptic remodeling were prevented by removing polysialic acid from the neural cell adhesion molecule using the enzyme endoneuraminidase, parturition and the milk ejection reflex remained normal (Catheline et al. 2006). Neuroglial remodeling thus is not essential to parturition and lactation. It rather seems that remodeling serves to isolate the oxytocin neurons from external excitatory influences, like those carrying stress or osmotic information, in order to preserve oxytocin for parturition and milk ejection.

Still, the high morphological plasticity of astrocytes in the HNS provides a flexible system to adapt and enrich the functioning of magnocellular neurons in response to physiological demand, and future research will need to further address this issue as well as others. For instance, the impact of glial changes on the finely tuned properties of the receptors mediating the astrocyte response to neurons will have to be studied, as well as on how astrocytes encode and integrate incoming inputs from different sources. What are the consequences of astrocytic process retraction on calcium signaling and travel in astrocytes? Are there different second messenger systems involved? Does this influence the mechanisms by which astrocytes release gliotransmitters? D-serine has a very precise site of action in the synaptic cleft, while taurine targets are more diffuse on the neuronal membrane (Deleuze et al. 2005), still the action of these two gliotransmitters is hampered in conditions of astrocyte retraction (Panatier et al. 2011; Choe et al. 2012): how does this work? Might the same astrocyte release these two gliotransmitters? What is the impact of glial morphological plasticity on the coordination of vasopressin neuron firing during osmotic challenges? Finally, we have little information about the plasticity of the different types of astrocytes in the HNS and we need to know the possible interactions they may develop with other non-neuronal cells, such as

Fig. 2.9 (continued) depolarization with stimulation of presynaptic glutamate axons at 2 Hz for 45 s (pairing, bar), potentiated the amplitude of evoked EPSCs in supraoptic neurons of virgin rats (left), but induced a persistent depression of the evoked EPSC amplitude in supraoptic neurons of lactating rats (right). (**c**) Stimulating afferent glutamatergic axons at 5 Hz for 3 min caused long-term depression (LTD) in supraoptic neurons from virgin rats (left), but not in supraoptic neurons from lactating rats (right). (**d**) Curve of astrocyte-dependent shift in plasticity threshold. Points represent percent changes in EPSC amplitude measured 30 min after the induction of LTD, pairing LTP, and HFS-LTP, respectively. These points were positioned arbitrarily on the *x*-axis. A curve was drawn to fit the data obtained in virgin rats (filled circles) and then shifted to fit the points obtained in lactating animals (empty circles). Note that the threshold for LTP induction (θ_M) is shifted toward higher activity values in lactating rats. Data are reported as means ± SEM. Adapted from Panatier et al. (2006)

vascular endothelial cells and microglia, during the process of active morphological plasticity.

2.6 Key Literature

Bonfardin et al. (2010) This study revealed that physiological astrocytic plasticity modifies the mode of action of presynaptic kainate receptors, thereby inversing their coupling with GABA release.

Oliet et al. (2001) This seminal paper demonstrated that astroglial wrapping of neurons, by controlling glutamate clearance by way of the GLT-1 transporter, plays a significant role in regulating the efficacy of glutamatergic neurotransmission.

Panatier et al. (2006) Key paper that revealed that the degree of astrocytic coverage of neurons governs the level of glycine site occupancy on the NMDA receptor, thereby affecting NMDA receptor availability for activation and thus the activity dependence of long-term synaptic changes.

Piet et al. (2004) Established how astrocytes, by hindering diffusion in the extracellular space, regulate intersynaptic communication between neighboring synapses.

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3 Fenestrated Capillary and Dynamic Neuro-Glial-Vascular Reorganization of the Adult Neurohypophysis

Seiji Miyata

Abstract

It is well known that dynamic structural reorganization occurs in the adult mammalian neurohypophysis (NH) in response to chronic physiological stimulation such as osmotic stimulation and lactation. Neurohypophysial glial cells, pituicytes engulf axon terminals and interpose between the axon terminals and fenestrated capillaries under healthy normal conditions, whereas chronic physiological stimulation increases the neuro-vascular contact area via the retraction of pituicyte cellular processes. Recent evidence shows that an activity-dependent shape conversion of perivascular pericytes also participates in increasing the neuro-vascular contact area by extension of the pericyte cellular processes. In addition to the rapid activity-dependent responses of pituicytes and pericytes, angiogenesis and gliogenesis also occur to maintain a proper population density of pituicytes and endothelial cells. I will describe in this chapter how glial–neuronal or axonal–glial interactions modulate neuropeptide diffusion from the NH into the blood circulation. In conclusion, the NH has more dynamic and complicated mechanisms of structural reorganization than we have previously thought.

Keywords

Pituicyte · Pericyte · Neurosecretion · Angiogenesis · Gliogenesis

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3.1 Introduction

The pituitary gland is an endocrine gland weighing five grams in humans that protrudes from the bottom of the hypothalamus at the base of the brain. In 150 AD, the Greek physician Galen first described the pituitary gland, but he misinterpreted its role to drain the **phlegma** (pituita), a cesspool of waste products derived from the brain. From 1928 to 1937, Drs. Ernst and Berta Scharrer opened up an entirely new field in the neurosciences, the field of neuroendocrinology. These two eminent and pioneering scientists hypothesized that nerve cells in the fish brain secreted hormones, which was a revolutionary idea at the time because the scientific dogma was that nerve cells conduct electrical impulses and endocrine cells secrete hormones, and that no cells exist that possess both capabilities. A specialized staining method enabled one to visualize Gomori-positive substances (e.g., neuropeptide–neurophysin complex by Gomori's chrome alum hematoxylin stain) in the supraoptic nucleus (SON), the paraventricular nucleus (PVN), and the neurohypophysis (NH), and the ligation of the pituitary stalk resulted in an accumulation of Gomori-positive substances on the proximal side of the ligation. These Gomori-positive substances were synthesized in the **hypothalamic magnocellular neurons** of the SON and PVN and were transported in the magnocellular axons to the NH. A short time later, the Gomori-positive substances were purified and identified as oxytocin (OXT) and arginine vasopressin (AVP). These neurosecretory cells resemble non-neural endocrine cells since they release peptides into the circulation and control specific physiological responses. But, like classic neurons, they also propagate electrical impulses and release their neuropeptides in response to electrical activity. Thus, the hypothalamo-neurohypophysial system was the first identified peptidergic nervous system in the brain.

3.2 Cellular Components of the Neurohypophysis

3.2.1 Axonal Terminals

The adult NH is largely comprised of glial cells and the axonal terminals of AVP and OXT magnocellular neurons (Fig. 3.1). The terminals of the NH contain both small synaptic vesicles (**SSVs**) that carry classical neurotransmitters such as acetylcholine, glutamate, GABA, and glycine, and large dense-core vesicles (**LDCVs**) that store amines and neuropeptides. Exocytosis of SSVs is triggered rapidly in response to a single action potential, whereas that of LDCVs shows slow release upon repetitive firing of action potentials. Axonal terminals of magnocellular neurons have numerous AVP- and OXT-containing LDCVs and directly contact the outer basement membrane of fenestrated capillaries in the NH. Two types of binding proteins, neurophysin I and neurophysin II, are present in LDCVs by binding to OXT and AVP, respectively. In addition, axonal terminals of magnocellular neurons have many SSVs containing glutamate and ATP.

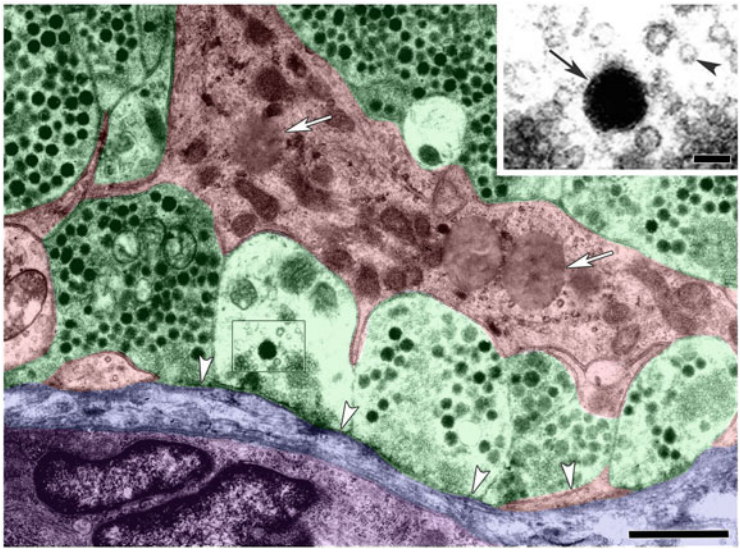

Fig. 3.1 An electron micrograph showing axonal terminals of AVP and OXT magnocellular neurons contacting with the basement membrane or perivascular space of a rat fenestrated capillary. Some axonal terminals (green) make direct contact with the outer basement membrane (open arrowheads), whereas cellular processes of pituicytes (red) engulf axonal terminals and often separate axonal terminals from basement membrane by intervening between them. Pituicytes have lipid droplets (open arrows) that are the intracellular sites for neutral lipid storage. Inset: Higher magnification view shows LDCV (solid arrow) and SSV (solid arrowhead) at top right. Red, pituicytes; green, axonal terminals; blue, perivascular space; purple, endothelial cell. Scale bars = 100 nm (inset at top right), 1 μm (lower right). Micrographs were modified from Miyata et al. (2001) with permission

3.2.2 Pituicytes

Pituicytes are specialized glial cells in the NH that have a star-shaped morphology and form a sponge-like network. They comprise 43% of the total NH cellular population (Virard et al. 2008). Since pituicyte morphology resembles that of another type of glial cell, the astrocyte, they are sometimes called neurohypophysial astrocytes. But, pituicytes differ from astrocytes. First, pituicytes engulf unmyelinated axons and axonal terminals (also called Herring bodies), whereas astrocytes surround mainly somata and dendrites of neurons and sometimes locate closely to presynaptic terminals in the brain. Second, although pituicytes express high levels of an intermediate filament protein, glial fibrillary acidic protein (GFAP), like astrocytes, they also express the intermediate protein, vimentin, and microtubule-associated protein 2D, which are not present in astrocytes (Virard et al. 2008; Matsunaga et al. 1999). Furthermore, S100β, another marker of glial cells, is expressed in most pituicytes, but GFAP is expressed in a subpopulation of these cells, indicating that the pituicyte population is heterogeneous.

3.2.3 Oligodendrocyte Progenitor Cells

Another type of glial cell, oligodendrocyte progenitor cells (**OPCs**), are also present in the adult NH, although fully differentiated oligodendrocytes are absent. OPCs derived from the explants of the adult NH differentiate into myelinated mature oligodendrocytes in the presence of appropriate growth factors. Neurohypophysial OPCs express typical OPC markers, platelet-derived growth factor receptor β and NG2. They can proliferate in vivo and form primary spheres and differentiate into pituicytes and oligodendrocytes in vitro (Virard et al. 2008). These data indicate that OPCs are glial progenitor cells that generate new pituicytes, renew pituicytes, and thus maintain the pituicyte population.

Box 3.1. Oligodendrocyte Progenitor Cells
OPCs express the proteoglycan NG2 and the transcription factor Olig2, and can generate oligodendrocytes in the developing and adult central nervous system. OPCs can generate a subset of astrocytes during perinatal development in vivo and give rise to only one type of astrocyte when cultured in the presence of serum or bone morphogenetic proteins. This type of astrocyte differentiated from OPCs is named the type-2 astrocyte to distinguish it from type-1 astrocytes that generally exist in the central nervous system in vivo and in vitro. Therefore, OPCs are sometimes called oligodendrocyte-type-2 astrocyte (O2-A) progenitor cells. Type-1 and type-2 astrocytes express an intermediate filament GFAP, while the A2B5 antigen is expressed only in type 2, but not type-1, astrocytes.

3.2.4 Microglia

Microglia are resident immune cells in the central nervous system and have functions such as clearing cellular debris and dead cells from tissue through the process of phagocytosis (e.g., cell eating) (see Chap. 1). Microglia are developmentally derived from the embryonic mesoderm, whereas other neuroglia in the central nervous system are derived from neuroectoderm. Microglia sometimes engulf axonal terminals of OXT or AVP-containing neurons, and some phagosomes and secondary lysosomes possess morphologically intact neurosecretory granules and others contain partially destroyed LDCVs in the NH. Microglia are responsible for remodeling of axonal terminal arborization of neurosecretory neurons. In addition, other non-neuronal components such as endothelial cells, pericytes, and fibroblasts are present.

3.3 Fundamental Characteristics of Neurohypophysial Capillaries

3.3.1 Wide Perivascular Space and Thick Basement Membrane

Fenestrated capillaries of the NH largely differ from continuous capillaries present in the general adult brain. Furthermore, the NH has a wide **perivascular space** between the inner and outer basement membranes, and the surface of fenestrated capillaries is complicated or markedly rough (Miyata et al. 2001; Miyata 2017; Nishikawa et al. 2017) (Fig. 3.2). Continuous capillaries in the general brain vasculature have outer and inner basement membranes, which are frequently fused together and are seen as a single layer of basement membrane. The rough profile of perivascular space in the NH results from the irregular alignment of pericyte cellular processes (Nishikawa et al. 2017). Pericytes align parallel to endothelial cells and tightly contact with the endothelial cell layer in continuous capillaries of the general brain vasculature. Neurohypophysial pericytes, however, often elongate their cellular processes into the interstitial space between axonal terminals. Extending cellular processes of pericytes are always covered with the outer basement membrane and this specialized extension is named the "**perivascular protrusion.**" Quantitative ultrastructural analysis estimates that the occurrence of wide perivascular space and perivascular protrusions increases the surface area of neuro-vascular contacts of fenestrated capillaries by approximately 1.5 times. This system resembles microvilli that increase surface area of nutrient absorption without cell-surface enlargement in the gastrointestinal tract.

In addition to increasing the surface area of neuro-vascular contact, perivascular protrusions act as the main diffusion route for low-molecular weight (MW) tracer substances (Nishikawa et al. 2017). In the adenohypophysis, dextran 20,000, corresponding to the size of growth hormone, moves rapidly from the interstitial space between growth hormone-secreting cells to the perivascular space and then diffuses slowly from the perivascular space to the circulation (Lafont et al. 2010). Thus, the perivascular space and protrusions are the main diffusion route of AVP and OXT.

In the NH, moreover, both the inner and outer basement membranes are thicker than those in the continuous capillaries of the general brain vasculature. The expression of collagen IV is higher at the inner basement membrane than the outer one, whereas the expression of laminin is more prominent at the outer basement membrane compared with that of the inner one (Nishikawa et al. 2017). Specialized pericyte alignment and a thick basement membrane result in a complicated profile of perivascular space, which acts to increase the vascular surface area contacting axonal terminals for efficient diffusion of AVP and OXT to the blood circulation.

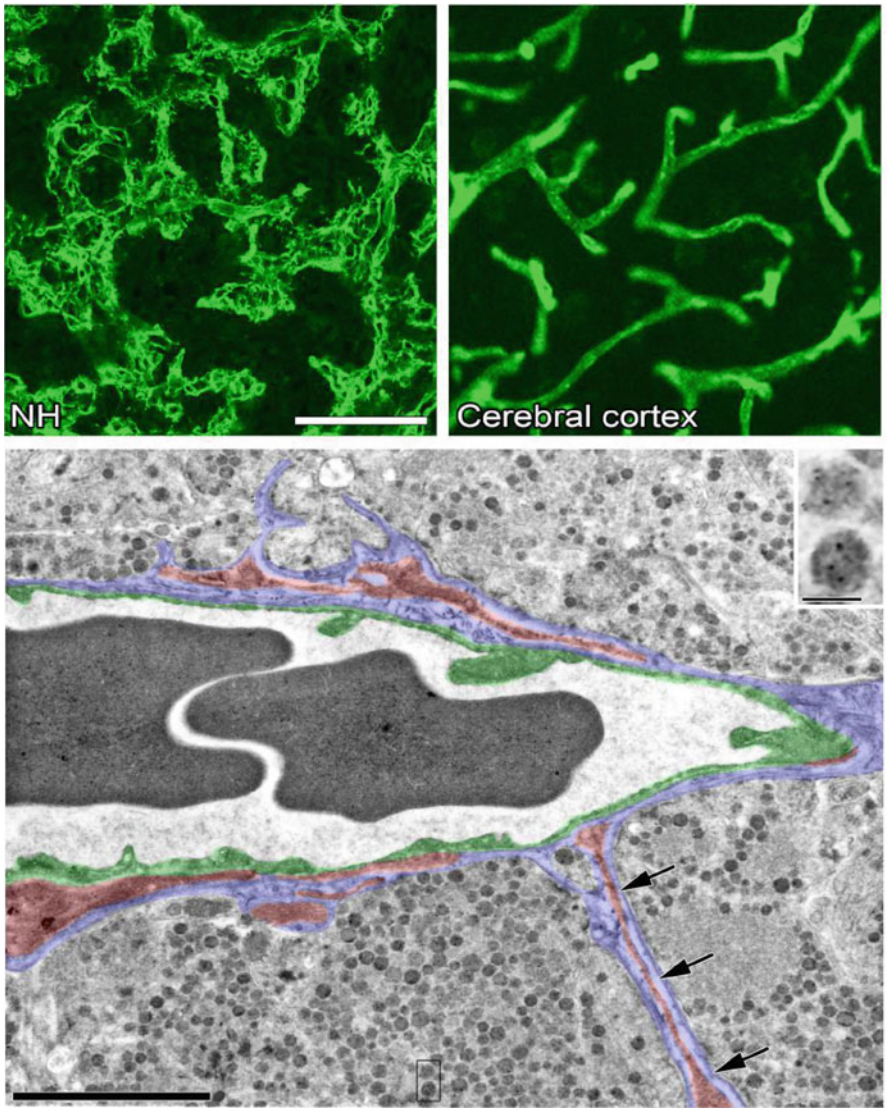

Fig. 3.2 Light and electron micrographs revealing the wide perivascular space and unique alignment of pericyte cellular processes of the adult mouse NH. Laminin is main component of the basement membrane and its immunohistochemistry is useful to visualize the profile of fenestrated capillaries. The profile of the fenestrated capillary in the NH is larger in size and more complicated (upper left) compared with that of the continuous capillary in the cerebral cortex (upper right). Pericytes, vascular mural cells, often localize parallel to the endothelial cell layer and extend short cellular processes into the interstitial space between axonal terminals and sometimes elongate a long cellular process, or perivascular protrusion (arrows in bottom panel), whose inside is occupied with long cellular processes of pericytes. The electron microscope image in the bottom panel shows LDCVs labeled with 5 nm gold particles of neurophysin antibody (see inset at top right of bottom panel). Scale bars = 50 μm (top panels), 1 μm (bottom panel), 100 nm (inset). Micrographs were modified from Miyata (2017) and Nishikawa et al. (2017) with permission

3.3.2 Lack of Endothelial Blood–Brain Barrier

The vascular system of the general adult brain possesses a blood–brain barrier (**BBB**) that precludes free entry of various unnecessary or harmful exogenous substances (e.g., glutamate, GABA, neuropeptides, potassium ions) into the brain parenchyma by an endothelial cellular sheet endowed with tight junctions (Zlokovic 2011; Daneman 2012). However, the lack of typical tight junction proteins (e.g., claudin-1, claudin-5, occluding, ZO-1, ZO-2) means the endothelial BBB is absent in the vasculature of the secretory circumventricular organs (**CVOs**), such as the NH and median eminence (ME) (Nishikawa et al. 2017; Langlet et al. 2013). Exogenous substances in the blood do not cause any damage to the NH even in the absence of the BBB, because the NH lacks neural elements (e.g., somata and dendrites) and also contains cellular elements (pituicytes, microglia) which can phagocytize unwanted material. Thus, fenestrated capillaries in the NH enable AVP and OXT released from axonal terminals to diffuse into the blood circulation.

Box 3.2. Blood–Brain Barrier
In most parts of the body, a narrow space is present between endothelial cells so that blood-derived substances can move readily between the inside and outside of the capillary. In the brain, however, endothelial cells are tightly connected to each other by tight and adherence junctions and most substances, including ions and even water, cannot pass out of the bloodstream. Some substances required by the brain, such as various nutrients, ions, organic anions, and high-MW substances, are transported into the brain from the blood by specific transporters or specialized channels (e.g., aquaporin). To constitute a complete BBB, pericytes envelop and make intimate connections with adjacent endothelial cells and glial cells, astrocytes, to form a layer around brain blood vessels. The BBB protects the brain from unnecessary or harmful exogenous substances in the blood circulation that may cause damage to the brain neurons, and the BBB thereby maintains a constant brain environment.

Box 3.3. Circumventricular Organs
The term "circumventricular organs" was originally coined by anatomist Helmut Hofer in 1958. CVOs are specialized brain regions characterized by dense fenestrated capillaries and act as blood–brain interfaces. The CVOs are classified into the sensory and secretory CVOs on the basis of their main functions. The sensory CVOs, comprised of the organum vasculosum of the lamina terminalis (OVLT), the subfornical organ (SFO), and the area postrema (AP), express a variety of receptors and channels that allow direct sensing of

(continued)

Box 3.3 (continued)
blood-derived signals and send them to other brain regions. The secretory CVOs, including the median eminence (ME) and neurohypophysis (NH), mainly consist of axonal terminals and glia. The secretory CVOs can release neuropeptides from axonal terminals of hypothalamic neurons into the blood circulation.

3.3.3 Size-Limited Vascular Permeability

The fenestrated capillaries of the NH and ME exhibit size-limited permeability. Intravenous administration of fluorescent tracers with low MW, less than 3000 kDa, can diffuse and reach the interstitial space in the NH and ME (Morita and Miyata 2012; Miyata 2015) (Fig. 3.3). Further detailed analysis reveals that low-MW substances are likely to diffuse through perivascular protrusions. High-MW substances, more than 10,000 kDa, can scarcely pass the outer basement membrane to reach the interstitial space in the NH and ME. This size-limited permeability of fenestrated capillaries in the NH and ME is quite reasonable when considering the MW of OXT (MW = 1007), AVP (MW = 1084), and adenohypophysial hormone-releasing hormones, which range from thyrotropin-releasing hormone (MW = 362.4) to growth-hormone releasing hormone (MW = 5040.4). Low-MW soluble substances with a molecular radius less than 3 nm are presumed to move passively through endothelial intercellular clefts in capillaries lacking the endothelial BBB (Komarova and Malik 2010). The theoretical peptide molecular radius is estimated at 1.1 nm (5 kDa), 1.42 nm (10 kDa), 1.78 nm (20 kDa), and 2.4 nm (50 kDa), if the protein has the simplest spherical shape (Erickson 2009). High-MW tracers such as horse radish peroxidase and lectins are incorporated by endocytosis and **transcytosis**, which are often mistaken for passage through the perivascular space and endothelial intercellular clefts, although high-MW substances with more than a molecular radius of 3 nm cannot pass through endothelial intercellular clefts. Thus, the endothelial intercellular cleft in the NH acts as a physical barrier, or "ultrafiltration membrane," that only allows the passage of substances less than approximately 5 kDa.

3.3.4 Dynamics of Capillary Density by Angiogenesis

Another important difference between the vascular system in the NH and other general brain regions is the occurrence of continuous **angiogenesis** in the NH. Angiogenesis is robust during the development and growth of the brain, but it becomes completely quiescent in the mature adult brain. In the NH, however, proliferating endothelial cells have been observed even in healthy normal adult mouse (Furube et al. 2014) (Fig. 3.4). The vascular endothelial growth factor-A

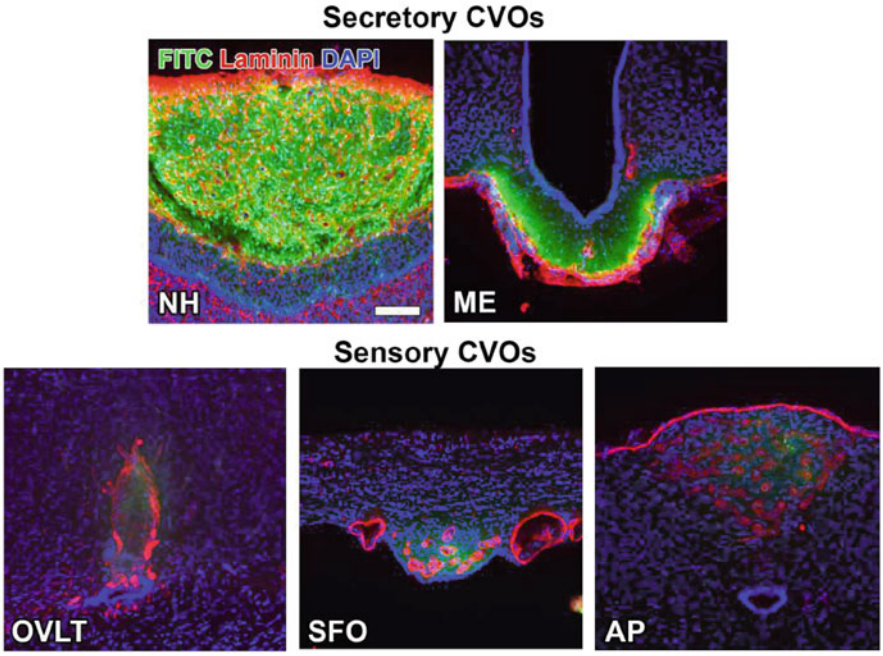

Fig. 3.3 High vascular permeability of fenestrated capillaries in the CVOs of the adult mouse. Fluorescein isothiocyanate (FITC) is able to bind covalently to the primary amine groups of cellular components to form a stable thiourea link and is therefore useful to visualize exact vascular permeability by combing immunohistochemistry. The BBB in continuous capillaries of adult brains generally restricts free movement of substances, but fenestrated capillaries in the CVOs allow vascular permeability of FITC. Low-MW tracer FITC is permeable through fenestrated capillaries in the CVOs, but not through continuous capillaries of other adjacent brain regions. The secretory CVOs including the ME and NH show higher vascular permeability and higher penetration of FITC green fluorescence than the sensory CVOs comprising the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), and area postrema (AP). Scale bar = 50 μm. Photographs were modified from Morita and Miyata (2012) with permission

(**VEGF-A**) and **VEGF receptor-2** are expressed in pituicytes and endothelial cells in the NH, respectively. The inhibition of VEGF signaling decreases the proliferation of endothelial cells in the NH, and a robust increase in proliferation of endothelial cells occurs in the NH after cessation of VEGF inhibition. The inhibition of VEGF signaling also causes a synchronous decrease in the density of AVP- and OXT-containing axonal terminals and endothelial cells (Fig. 3.5). Continuous angiogenesis is also present in the other CVOs under healthy normal conditions. The reader is directed to a review for an in-depth description of continuous angiogenesis in the CVOs (Miyata 2015). Thus, the population of endothelial cells is regulated coordinately with that of axonal terminals in the adult NH in a VEGF-dependent manner.

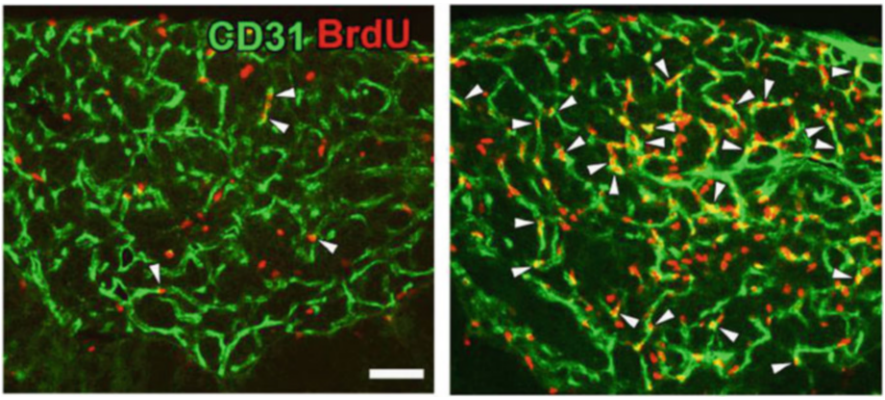

Fig. 3.4 Continuous proliferation of endothelial cells under normal conditions and augmented proliferation after the withdrawal of VEGF signaling inhibition in the mouse NH. The proliferation marker BrdU, a thymidine analog, is detected in CD31-immunopositive endothelial cells under normal unstimulated conditions (left panel). Many more BrdU-labeled proliferating endothelial cells (arrowheads) are detected after the cessation of VEGF signaling inhibition (right panel). Scale bar = 50 μm. Photographs were modified from Furube et al. (2014) with permission

Box 3.4. Angiogenesis

Angiogenesis is the process by which new blood vessels are formed from elaboration of the existing vasculature. The turnover of endothelial cells is normally very low in adulthood, whereas angiogenesis occurs in the female reproductive tract and wound or injury regions. Endothelial "tip cells" lead sprouting vessels by extending filopodia and migrate in response to gradients of vascular endothelial growth factor-A (VEGF-A), while adjacent endothelial "stalk cells" trail the endothelial tip cells to make the trunk of new vessels. VEGF-A and its receptor VEGF receptor 2 (VEGFR2) are predominant angiogenic signaling factors that control the proliferation and sprouting of endothelial cells (Gerhardt et al. 2003).

3.4 Activity-Dependent Structural Reorganization

3.4.1 Activity-Dependent Increase in Neuro-vascular Contacts

Chronic physiological stimulation such as lactation and dehydration not only causes a continuous increase of neural activity in AVP and OXT magnocellular neurons, but also causes structural reorganization of somata and dendrites in the SON and PVN and axonal terminals in the NH (Miyata and Hatton 2002). In the SON and PVN, the structural reorganization is characterized by the formation of multiple synapses of afferent inputs, increased direct neuronal membrane apposition of

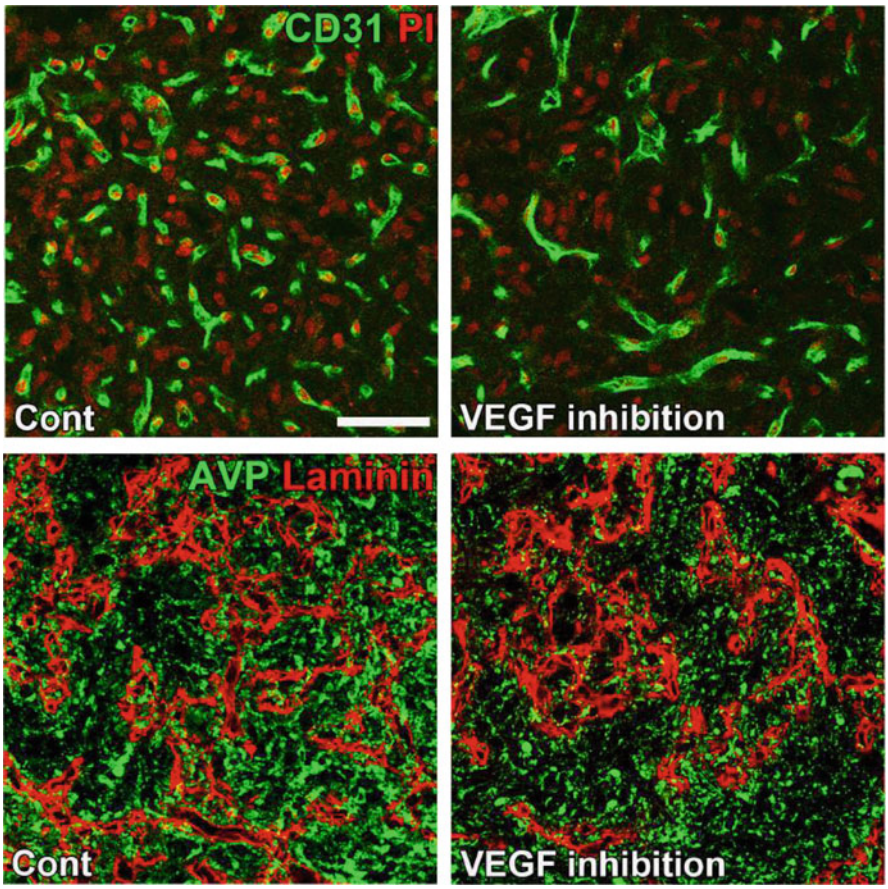

Fig. 3.5 Inhibition of VEGF signaling simultaneously decreases the density of endothelial cells and axonal terminals in the adult mouse NH. The inhibition of VEGF signaling largely decreases the number of CD31-immunolabeled endothelial cells compared with the control NH (upper panels). The inhibition of VEGF signaling also causes a robust reduction in the density of AVP axonal terminals (lower panels). Scale bar = 50 μm. Photographs are modified from Furube et al. (2014) with permission

somata, and dendritic bundling by the retraction of astrocytic cellular processes. The structural reorganization of somata and dendrites in the hypothalamic nuclei is associated with coordinated population activity to respond appropriately to altered physiological circumstances (Tasker et al. 2012) (see Chap. 2).

In addition to the hypothalamic nuclei, chronic physiological stimulation also induces structural reorganization in the NH. Wittkowski and Brinkmann (1974) observed that the relative extent of neuro-vascular contacts was significantly increased by chronic osmotic stimulation. Electron microscopic observation shows that pituicytes generally engulf the axonal terminals of magnocellular neurons and intervene between axonal terminals and the vascular basement membrane under

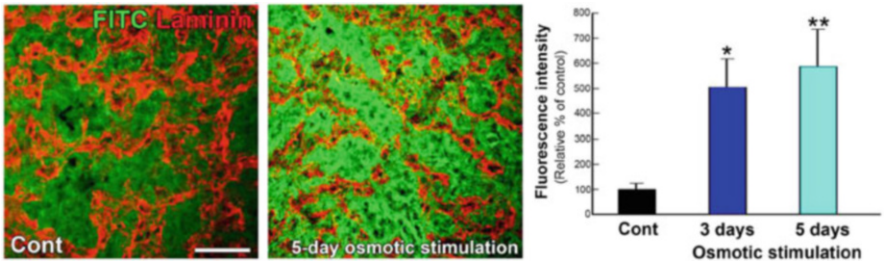

Fig. 3.6 Activity-dependent increase in vascular permeability to low-MW fluorescent tracer FITC in the adult mouse NH. The FITC fluorescence intensity is higher in the NH of an animal that received chronic osmotic stimulation, drinking of 2% NaCl for 5 days (left micrograph), compared to that of the control animal (right micrograph). Quantitative analysis shows that the relative intensity of FITC fluorescence was significantly increased in osmotically stimulated mice compared with the control mice (right graph). Scale bar = 50 μm. * $p < 0.05$; ** $p < 0.01$ by ANOVA with Tukey's post hoc test. Micrographs were modified from Nishikawa et al. (2017) with permission

normal unstimulated conditions (Tweedle and Hatton 1982; Miyata and Hatton 2002; Miyata 2017). Upon chronic osmotic stimulation, however, a reduction in the number of neurosecretory axons enveloped by cellular processes of pituicytes results in an increase in both the length of individual nerve terminals and the number of terminals (Tweedle and Hatton 1982; Miyata et al. 2001). Therefore, the structural reorganization of the NH is caused by the coordinate rearrangement of axonal terminals, the outer basement membrane, and glial cells, rather than enlargement or sprouting of the magnocellular terminals themselves. Furthermore, chronic osmotic stimulation leads to increased vascular permeability of low-MW substances (Nishikawa et al. 2017; Miyata 2017) (Fig. 3.6). Thus, the activity-dependent increase of neuro-vascular contacts contributes to efficiency of AVP and OXT diffusion into the blood circulation.

3.4.2 Dynamic Alteration of Neuro-vascular Contacts by Shape Conversion of Pituicytes and Pericytes

Activity-dependent neuro-vascular structural reorganization is caused by retraction of cellular process of pituicytes (see Sect. 3.4.1). Shape conversion or retraction of cellular process of pituicytes is mediated by β-adrenergic receptors in cultured pituicytes (Rosso and Mienville 2009; Miyata 2017) (Fig. 3.7) and in the isolated NH in vitro (Smithson et al. 1990). Moreover, shape conversion of pituicytes is observed in vivo during chronic osmotic stimulation (Matsunaga et al. 1999) (Fig. 3.7). The reader is directed to reviews for an in-depth description of shape conversion of pituicytes (Rosso and Mienville 2009; Miyata 2017). Taken together, these results indicate that the retraction of pituicyte cellular processes engulfing axonal terminals results in an increase in neuro-vascular contacts in the NH.

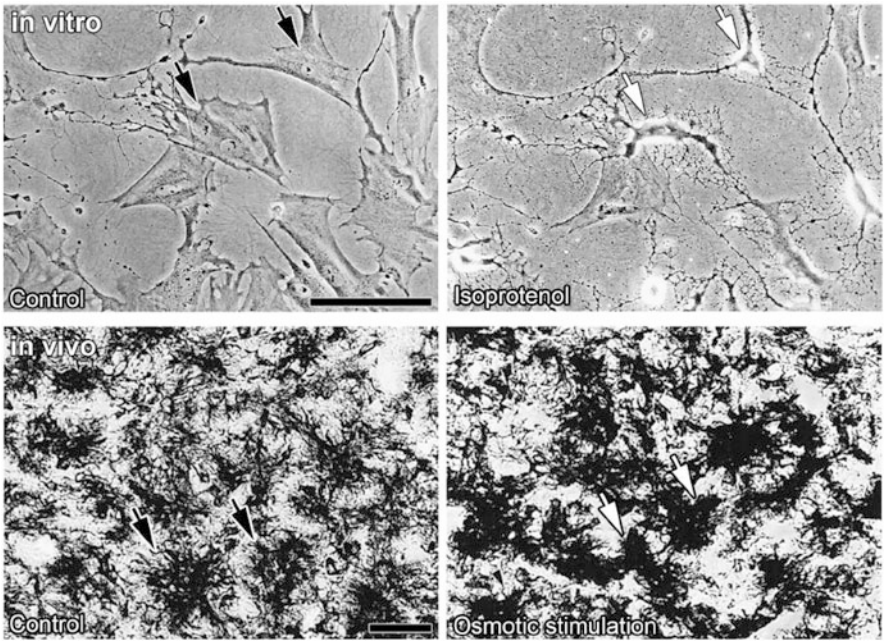

Fig. 3.7 Neurotransmitter-induced shape conversion of cultured pituicytes and activity-dependent morphological change of pituicytes in the adult rat NH. An agonist of adrenergic β-receptors, isoproterenol, changes the shape of cultured pituicytes from flat to stellate (upper panels). Chronic osmotic stimulation via water deprivation causes the retraction of the cellular processes of pituicytes (open arrows), which have well-branched cellular processes (closed arrows) in control osmotic conditions (lower panels). Scale bars = 50 μm. Photographs were modified from Miyata et al. (1999) and Matsunaga et al. (1999) with permission

Until recently, activity-dependent neuro-vascular structural reorganization was considered to be caused simply by the shape conversion of pituicytes. There was no information on changes in perivascular structure, because the fenestrated capillaries were believed to be resistant to change. **Pericytes** are vascular contractile mural cells that modify vascular ultrastructure and alter gene expression in endothelial cells in response to brain microenvironment alterations. Recently, my research group demonstrated dynamic changes of the perivascular space through shape conversion of pericytes in the adult mouse NH (Nishikawa et al. 2017) (Fig. 3.8). Pericytes extend their cellular processes into the extracellular space between axonal terminals so that the surface area of the neuro-vascular contacts is increased (see Sect. 3.3.1). Besides, chronic osmotic stimulation further increased the number of perivascular protrusions by 2.72-fold without changing the density of pericytes, the area of perivascular space and endothelial cells, or the diameter of vessels (Nishikawa et al. 2017).

Platelet-derived growth factor-B (**PDGF-B**) is highly expressed in endothelial cells in the developing brain vasculature. The gradient of PDGF-B is necessary for

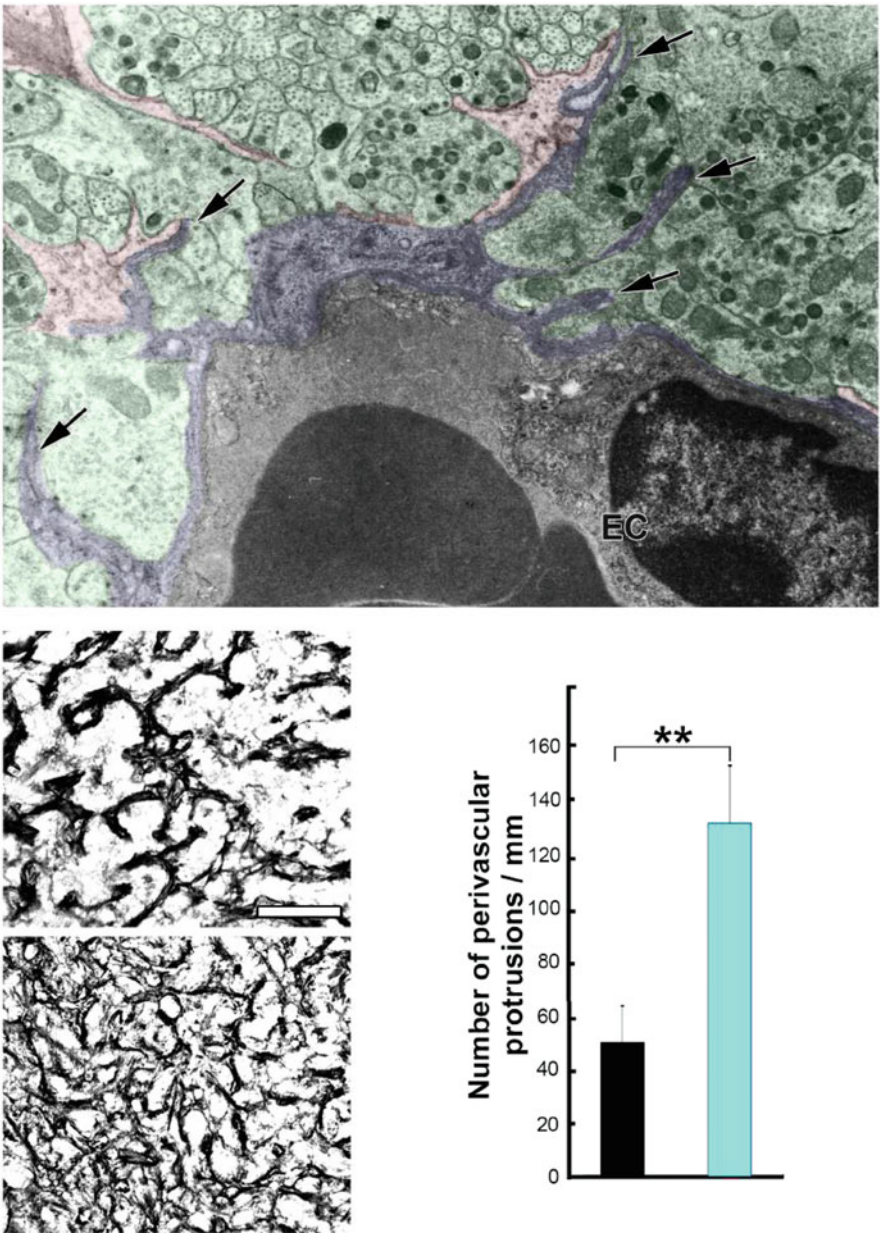

Fig. 3.8 Activity-dependent morphological changes in vascular mural cells, pericytes, in the adult mouse NH. Upper panel: An electron micrograph revealing that chronic osmotic stimulation causes dramatic morphological changes in pericytes (red) that are accompanied by extension of perivascular protrusions (arrows) or expansion of perivascular space (purple). Green, axonal terminals. Left lower panels: Chronic osmotic stimulation increases the fine cellular processes of pericytes. Right lower graph: Quantitative analysis reveals that chronic osmotic stimulation significantly increases the number of perivascular protrusions. * $p < 0.01$, Student's *t*-test. Micrographs were modified from Nishikawa et al. (2017) with permission

attachment to endothelial cells and migration of pericytes. In the adult mouse NH, the **PDGF receptor β (PDGFRβ)** is strongly expressed in pericytes, whereas PDGF-B is present in LDCVs in axon terminals of OXT magnocellular neurons (Nishikawa et al. 2017). This observation indicates that dynamic shape conversion of pericytes is probably caused by activity-dependent release of PDGF-B from axonal terminals of OXT magnocellular neurons. Thus, activity-dependent neuro-vascular reorganization requires shape conversion of both glial cell pituicytes and vascular mural cell pericytes.

3.4.3 Activity-Dependent Change in Glial Proliferation

Chronic osmotic stimulation promotes the proliferation of OPCs and pituicytes, but does not change total cell number due to ongoing apoptosis (Virard et al. 2008). OPCs, but not pituicytes, express PDGFRα in the adult mouse NH and the proliferation of OPCs is mediated by **PDGFRα** signaling, like in other brain regions (Furube et al. 2014). The pituicyte population in the NH is regulated by a proliferation of pituicytes and OPCs and possibly contributes to make a space or "cushion" to maintain neurohypophysial volume regardless of changes in pituicyte and pericyte shape conversion, proliferation of endothelial cells, or changes in capillary density during activity-dependent structural reorganization.

3.5 Perspectives

Table 3.1 summarizes characteristics of cellular components in the adult NH. Pituicytes engulf axonal terminals and interpose between axonal terminals and fenestrated capillaries under healthy normal conditions; neuro-vascular contact is increased during chronic physiological stimulation by the retraction of cellular processes of pituicytes depending on increased demand of neuropeptide secretion. The activity-dependent shape conversion of perivascular pericytes also participates in increasing neuro-vascular contact by extending their cellular processes and increasing the number of perivascular protrusions. In addition to shape conversion of pituicytes and pericytes, angiogenesis and gliogenesis maintain the proper population density of pituicytes and endothelial cells. The expression of cell adhesion molecules, cytoskeletal proteins, receptors, and extracellular matrix, some of which are detected only during early periods of development and are unusual in fully mature cells, enable neurohypophysial reorganization. Recent findings reveal that the NH has more dynamic and complicated mechanisms of structural reorganization than we have thought. Understanding the molecular basis and mechanisms for crosstalk among axonal terminals, pituicytes, OPCs, pericytes, and endothelial cells will be necessary to achieve an eventual comprehensive description of the structural reorganization of the adult NH.

Table 3.1 Proliferative activity, shape conversion ability, and specific protein expression characteristics in each cellular component of the adult rodent NH

	Proliferative activity	Acute shape conversion	Typical protein expression
Pituicyte	+	+	GFAP, Vimentin, S100β, VEGF-A Notch3, MAP2d, Plasminogen
OPCs	+	–	PDGFRα, NG2
Endothelial cell	+	–	VEGFR2
Pericyte	–	+	PDGFRβ, NG2, Tenacin-C
Axonal terminal	–	–	DLL4, MAP1B, tPA, PSA-NCAM, F3, Phosphacan, Neurocan
Microglia	+	–	Iba1, OX42
Basement membrane	–	–	CS-4-PG, CS-6-PG, Collagen IV, Laminin

3.6 Key Literature

Miyata (2015) Review of characteristics of fenestrated capillaries in the CVOs.
Miyata (2017) Review of recent new evidence for structural reorganization of the NH.
Rosso and Mienville (2009) Review mechanisms for shape conversion of pituicytes.
Virard et al. (2008) Provides precise information for glial composition and characteristics in the NH.

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Astrocyte–Magnocellular Neuron Interactions in Hypothalamic Memory

4

Grant R. Gordon, Christopher V. Dayas, and Jaideep S. Bains

Abstract

The magnocellular neuroendocrine cells—located in the supraoptic and the paraventricular nuclei of the hypothalamus—project their axons to the posterior pituitary, where they secrete the neurohormones vasopressin or oxytocin into the systemic circulation. Vasopressin regulates water–salt balance and blood pressure, whereas oxytocin controls key steps in lactation and parturition. These neuroendocrine cells interact with surrounding astrocytes, and this relationship plays an important role in synaptic and circuit plasticity, thereby regulating neuroendocrine output. Noradrenergic and glutamatergic inputs to the magnocellular neurons are essential drivers of neurohormone release, yet there is a surprising reliance on the recruitment of astrocyte-derived ATP in this excitation process. ATP release from these glia scales the strength of excitatory synapses on the magnocellular neurons, effectively increasing their ability to respond to many different afferent glutamate inputs when neurohormone release is needed. This system represents a unique example of how astrocyte–neuron interactions can shape the activity-level of homeostatic neural networks to meet physiological demands.

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Keywords

Magnocellular neuroendocrine cell · Astrocyte · Gliotransmission · ATP · Distributed plasticity · Scaling

4.1 Introduction

The hypothalamus is the major integration and output centre for all incoming neural and blood-borne signals related to the unconscious internal workings of an organism, as well as the conscious behaviours that help an organism accomplish desired homeostatic goals. The hypothalamus is an aggregation of many diverse nuclei in the central basal forebrain. This chapter focuses on the large cell bodied magnocellular neuroendocrine cells (MNCs), which are found primarily in two hypothalamic nuclei: the supraoptic nucleus (SON) and the paraventricular nucleus (PVN). These cells secrete one of two neurohormones (or neuropeptides): vasopressin, which regulates water–salt balance and blood pressure, and oxytocin, which facilitates lactation and parturition in females (see below for other functions of these peptides). As with all neurons in the brain, MNCs interact with surrounding glial cells, such as astrocytes. These interactions, which play an important role in synaptic and circuit homeostasis and actively contribute to long-lasting synaptic plasticity, may be critical for regulating neuroendocrine output of this system. Importantly, a single physiological stress or homeostatic challenge (such as dehydration) increases the efficacy with which the hypothalamus and its associated connections respond to subsequent challenges. This change represents one clear example of plasticity in autonomic circuitry. Effectively, the initial challenge to homeostasis alters neural network function, so that it is better able to meet the demands of subsequent challenges. It is generally accepted that enduring alterations in the output of a neural circuit require similar alterations in the strength of the individual synapses that comprise that circuit. Synaptic plasticity of this type is ubiquitous throughout the nervous system, even in the neural pathways involved in restoring homeostatic set points such as osmotic balance, or the pathways that respond quickly to essential physiological demands such as lactation. In this chapter, we will first outline the MNC system and relevant connections with noradrenergic cell populations in the brainstem. Then we will explore neuron–astrocyte interactions in the hypothalamus, with a particular focus on one type of MNC–astrocyte interaction that helps instill a unique form of long-lasting synaptic strengthening of excitatory glutamatergic synapses. Rather than being synapse specific, astrocytes promote a type of ‘distributed’ plasticity across many synapses, which may help to shape the activity patterns and hormonal outputs of these neuroendocrine cells.

4.1.1 Magnocellular Neuroendocrine Cells (MNCs)

Each MNC is a specialist, synthesizing and secreting either the neurohormone arginine vasopressin (VP) or the neurohormone oxytocin (OT), with only a small fraction of cells making both peptides. VP and OT are each nine amino acids in length, differing in only two residues. VP and OT are translated as a larger (~100 amino acid) prohormone consisting of the hormone proper and neurophysin. During transport, the prohormone is enzymatically cleaved within large dense core vesicles to produce the biologically active hormone, VP or OT, and a physiologically inactive neurophysin peptide. Each MNC sends out a single axon and together most MNC axons course ventrally and emerge from the bottom surface of the forebrain to form the bulk of the posterior lobe of the pituitary gland, also known as the neurohypophysis or neural lobe. Each axon gives rise to thousands of neurosecretory terminals, which are packed with large dense core vesicles containing the neuropeptides to be secreted. Calcium-dependent exocytosis triggers the release of VP or OT into the extracellular space of the gland, which diffuses into perforated capillaries to enter the systemic circulation. MNCs have been termed 'neurosecretory' as they, for the most part, do not communicate via the axon to other neurons, but instead talk to distant targets in the body by secreting hormones directly into the blood. However, not all axons arising from the MNCs target the neurohypophysis. The brain regions targeted most robustly by VP MNCs include the substantia nigra, the nucleus of the tractus solitarius (NTS), the nucleus motoris dorsalis vagus and the nucleus commissuralis. Other areas with weaker VP projections include, but are not limited to, the amygdala and lamina X of the spinal cord. The brain areas innervated most strongly by OT MNCs include the substantia nigra, the NTS, the nucleus commissuralis, the nucleus reticularis lateralis and the parabrachial nucleus. Weaker OT projections include the amygdala, raphe dorsalis and the spinal cord, among others. These projections speak to the varied roles of VP and OT acting as neuromodulators within neural networks (not detailed in this chapter), in addition to their role as systemic hormones, which is briefly described below.

4.1.2 Vasopressin

The primary role of VP is to conserve water at the kidney to reduce urine volume, a process known as antidiuresis. This helps to maintain a constant plasma osmolarity by making the extracellular fluid (ECF) more dilute and helps to increase blood pressure by adding to the total ECF volume. VP acts at the distal and collecting tubule of the nephron in the kidney by binding to G_s-coupled V_2 receptors to help reabsorb water. In addition to its effects on the kidney, VP also combats the low blood pressure resulting from hypovolaemia (low blood volume) by binding to and activating $G_{q/11}$-protein-linked V_{1a} and V_{1b} receptors located on vascular smooth muscle cells that surround large arteries and smaller arterioles. VP-induced increases in free Ca^{2+} here promote vasoconstriction, increasing systemic blood pressure. Thus, there are three principal situations that require the release of VP from the

neural lobe: high Na^+, hyperosmolarity and hypovolaemia. Importantly, though VP MNCs are intrinsically osmosensitive and can alter their excitability in response to changes in the local osmotic environment, MNC activity is also highly influenced by afferent input. In particular, afferent input from the median preoptic nucleus, from the anterior circumventricular organs, which include the subfornical organ and the organum vasculosum of the lamina terminalis, and from peripheral baroreceptors which convey information about systemic pressure/volume through several cardiac-relevant brainstem signalling systems before reaching the VP MNCs. Thus, synaptic input to the VP MNCs is a critical determinant of neurohormone output and acts to help control osmotic and blood pressure homeostasis.

VP MNCs exhibit a phasic bursting pattern of action potential discharge (Arnauld et al. 1974). VP cells burst independently of one another (i.e. neighbours are not synchronized), making the release of hormone at the neural lobe continuous rather than pulsatile. In response to elevations in osmolarity, VP MNC bursts become longer and, if the challenge persists, the bursts become briefer and achieve higher peak frequencies. Intrinsic membrane properties play a critical role in generating the phasic firing observed in VP MNCs (Renaud 1994). At burst initiation, a small but long-lasting depolarizing-after-potential, which succeeds every spike, triggers and works together with a persistent inward current to ramp the cells voltage from rest up to a stable plateau potential. This new, elevated baseline is maintained by Ca^{2+} currents and facilitates the generation of a burst because individual excitatory postsynaptic potentials (EPSPs) can more easily cross spike threshold. This latter point highlights the importance of afferent input to help drive VP release in combination with changes to intrinsic membrane properties.

4.1.3 Oxytocin

Once secreted into the bloodstream, OT has two principal jobs: (1) to trigger milk letdown from the mammary glands, a process termed the milk ejection reflex, and (2) to aid the birthing of new young by stimulating uterine contraction, a process termed parturition. Surprisingly, males possess a similar number of OT MNCs and produce OT in similar quantities, suggesting alternate functions for this peptide. For example, OT can act in conjunction with VP at the kidney to enhance salt excretion. Other actions of OT include sexual arousal, pair bonding, analgesia, anxiolytic effects, positive social interaction effects and the development of trust between individuals. For females, OT is also important for maternal behaviours.

Oxytocin binds to a single receptor, the OT receptor. The OT receptor is functionally expressed in the myoepithelium of the mammary glands and in the myometrium and endometrium of the uterus at the end of gestation. The OT receptor couples to the $G_{q/11}$-protein intracellular cascade, which initiates smooth muscle cell contraction in myoepithelial and myometrial cells. Smooth muscle contraction in the mammary gland increases intra-mammary pressure and leads to ejection of milk, while smooth muscle contraction in the uterus facilitates the birthing process.

The afferent input required for OT secretion and the subsequent initiation of milk letdown stems from the actions of a suckling newborn. After breast sensory afferents project to the spinal cord, some areas thought to be important in the milk ejection reflex include: the dorsal horn, the lateral cervical nucleus and part of the dorsolateral funiculus. The afferent pathways utilized to initiate OT release during parturition begin in the uterus and cervix, where sensory neurons communicate with the NTS via the vagus nerve. Approximately 80% of the projecting axons from the NTS to the OT MNCs are noradrenergic and they facilitate the release of OT through the activation of α_1-adrenoceptors (more below).

OT release from the neurohypophysis requires a transition from a basal action potential firing rate of only a few Hertz to transient, intense bursts of action potentials (1–2 s in duration at 100 Hz), with 5–10-min intervals in response to suckling pups (Lincoln and Wakerley 1974). Simultaneous recordings from all four MNC regions in the hypothalamus show that all OT MNCs burst concomitantly within 400 ms of one another, with relatively long pauses of silence in between the bursts of population activity. Hypotheses for this synchronization include the autocrine and paracrine actions of dendritically released OT, suckling-related afferent information and glutamate interneurons that link the spatially separated MNC nuclei. Coordinated OT cell bursting with intermittent periods of rest is necessary for milk letdown because a concentrated bolus of blood-borne OT is needed to achieve OT receptor activation without causing receptor desensitization. This ensures maximal myoepithelium contraction and mammary gland pressure. Finally, OT receptors in the mammary gland require a large and rapid increase in OT concentration so that more subtle OT release for the purpose of natriuresis does not induce milk letdown.

4.2 Noradrenaline in MNC Output

In the brain, noradrenaline (NA, or norepinephrine) is synthesized by cells that reside in discrete anatomical territories in the brainstem. The ventral noradrenergic column contains the A1 and A5 cell groups, whereas the dorsal noradrenergic column holds the A2, A6 and A7 groups. A significant amount of afferent information related to the control of VP and OT release from the neurohypophysis is relayed through noradrenergic nuclei A1 and A2. The aortic, carotid sinus and vagus nerves are the primary cardiac afferents carrying volume-, pressure- and chemoreceptor-related information to the ventrolateral medulla and the NTS. The vagus nerve also contains an afferent component that relays information to the NTS related to parturition. Interposed amongst the ventrolateral medulla and NTS, and receiving many of the same visceral inputs, are the A1 and A2 noradrenergic cell groups. These cell groups project to the MNCs to regulate the release of VP and OT (Sawchenko and Swanson 1981; Cunningham and Sawchenko 1988) (Fig. 4.1). Each cell group is partial to the MNCs it targets; A1 preferentially innervates VP cells, whereas A2 favours OT cells, yet the total amount of noradrenergic innervation between both cell types is approximately equal. The A2 group also projects to the neurohypophysis, where it directly influences neurohormone release.

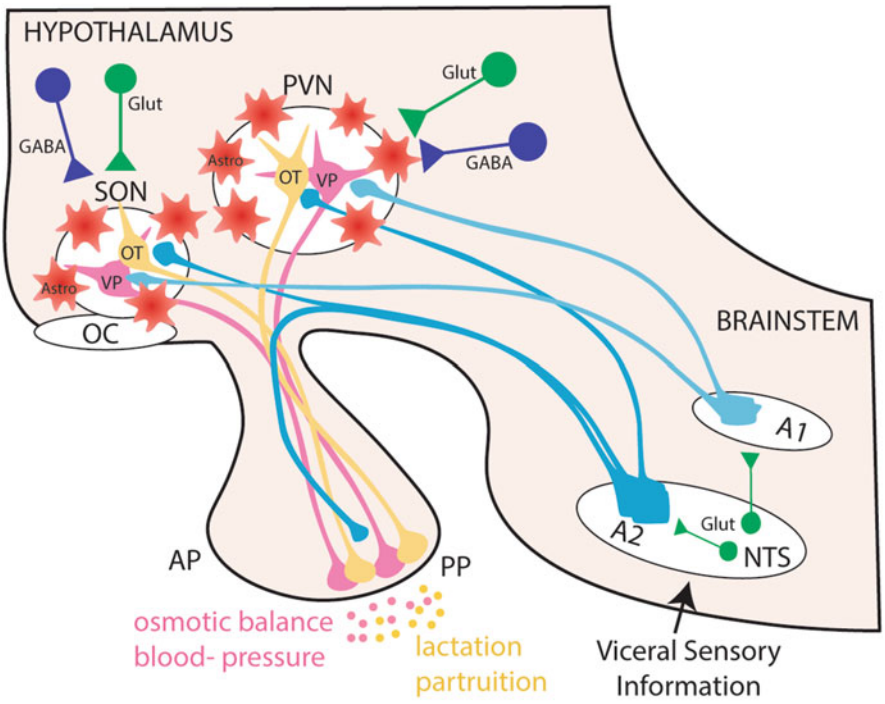

Fig. 4.1 Simplified circuit diagram showing the main MNC groups in the PVN and SON of the hypothalamus, resident astrocytes (red), and some important afferent inputs. MNCs project their axons to the posterior pituitary (PP) gland (or neurohypophysis) where they release vasopressin (VP, pink) or oxytocin (OT, yellow) into the systemic circulation. The brainstem noradrenergic A1 and A2 cell groups (A2 within the NTS) (blue) project to the MNC nuclei and the PP. One important role for these afferents is to excite local astrocytes to release ATP. A driver of the A1/A2 cells is excitatory afferent input from the NTS, which integrates numerous peripheral inputs about physiological homeostasis. Finally, the MNCs are innervated by a variety of long-range and short-range excitatory (Glut) and inhibitory (GABA) neurons that play an important role in controlling MNC output at the posterior pituitary and other brain regions

NA released from the A1 and A2 cell group terminals is a critical mediator of MNC responses (Day et al. 1984, 1990; Shioda and Nakai 1992). Intracerebroventricular injection or direct injection of NA into the MNC nuclei excites OT and VP cells via α_1-adrenoceptor activation (Armstrong et al. 1986; Yamashita et al. 1987; Shioda et al. 1997; Daftary et al. 1998), which can also trigger the release of OT and VP from hypothalamic explants. Central blockade of α_1-adrenoceptors or destruction of noradrenergic terminals by prior injection of the neurotoxin 6-hydroxydopamine eliminates the increase in MNC activity observed in response to physiological challenges to homeostasis. There are three primary methods through which α_1-adrenoceptor activation increases MNC output: first, NA alters MNC excitability by increasing glutamate release onto these cells through actions on intranuclear glutamate interneurons (more below); second, NA

depolarizes MNC membrane potential and third, NA decreases postsynaptic K^+ conductances to facilitate phasic firing. The effects of NA at the level of excitatory synapses directly onto MNCs were detailed further in the early 2000s, where NA was found to induce multiple distinct types of glutamatergic plasticity to increase MNC excitability: (1) NA turns off glutamatergic autoreceptors on presynaptic terminals that normally act to curtail glutamate release; (2) NA causes the synchronous release of multiple glutamate synaptic vesicles, dramatically increasing the amplitude of the synaptic current and thus, the ability of the synapse to bring the MNC to action potential threshold and (3) NA recruits astrocytes to globally increase postsynaptic strength; making numerous incoming excitatory events more effective at firing the MNC. This latter mechanism is described in more detail below with our focus on MNC–astrocyte interactions. To a lesser extent compared to excitation, NA has been found to have some inhibitory influence over MNCs through the activation of adrenoceptors other than α_1. These will not be further described here, as there is a robust excitatory role for α_1-adrenoceptor activation and more details are understood about how these receptors affect synaptic function, cell excitability and the tripartite synapse.

4.2.1 Noradrenaline and ATP

The A1 and A2 noradrenergic cells are not homogenous populations. In the A1 group, several other peptides are co-expressed with NA, including substance P, galanin and neuropeptide Y (NPY). A2 NA cells do not show the same degree of co-expression with other molecules as A1. In addition to neuropeptides, indirect evidence has emerged in support of the idea that A1 activation results in either the co-release of NA and ATP or the exclusive release of ATP. While NA and ATP co-release from nerve terminals may occur in a number of central and peripheral regions, it is now understood that NA is also a potent activator of ATP release from glia. The contribution of ATP is important because ATP can elicit excitatory effects in MNCs, and there is evidence for cooperative and synergistic actions of NA and ATP molecules. Purinergic P2 receptors encompass a number of different ionotropic and metabotropic proteins. P2X receptors are non-selective cation channels, passing Na^+, K^+ and Ca^{2+}, which allows them to not only excite a cell via depolarization, but also to activate Ca^{2+}-dependent signalling pathways. P_2 purinergic receptors are present in MNCs and there are multiple types of ionotropic P2X receptors that are functionally expressed in the PVN and SON (Shibuya et al. 1999). Injection of ATP into the PVN or SON increases plasma VP concentration through P2- rather than P1-receptor activation (Mori et al. 1992). In the SON, direct application of ATP excites VP cells, and P2X receptors mediate TTX-insensitive depolarization of both MNC types (Hiruma and Bourque 1995). The first indirect evidence in support of the combined role of NA and ATP came from measuring an increase in the extracellular concentrations of both NA and a purine metabolite in the SON in response to peripheral haemorrhage (Kendrick and Leng 1988). Additionally, synergistic action of NA and ATP in the hypothalamic explant preparation was demonstrated when VP

and OT release was found to be far greater and more sustained when both molecules were given simultaneously, versus when each molecule was delivered alone (Kapoor and Sladek 2000). Early evidence supported the idea that P2X receptors, PKC activation, gene transcription and sustained postsynaptic Ca^{2+} rises in MNCs were involved. The idea that the P2 receptor activation contributed to the NA effect was supported by earlier in vivo experiments demonstrating that MNC excitation by A1 stimulation could be blocked by the broad-spectrum P2 antagonist suramin (Day et al. 1993), indicating that this effect may result from ATP rather than NA release (Buller et al. 1996). This result could be interpreted as co-transmission of NA and ATP or that noradrenergic terminals exclusively released ATP rather than NA, but other explanations are possible, such as NA triggering the release of ATP from another cell, which then acts to affect MNCs. The difficulty in assuming co-transmission was first made clear by the demonstration that vascular smooth muscle cells release ATP in response to NA within the context of autonomic control of vascular tone (Vizi et al. 1992). This brought to light the possibility of ATP arising from other cellular sources. However, evidence in support of co-transmission from the same sympathetic neuronal population has been provided by detecting simultaneous NA and ATP release from pure neuron cultures (Von Kügelgen et al. 1994). Nevertheless, it is now well appreciated that NA can robustly activate ATP release from other cell types, including astrocytes. While there is no ultrastructural evidence for direct noradrenergic synapses on glia in the PVN and other CNS regions, most noradrenergic varicosities lack postsynaptic specializations, leaving released NA free to activate other cell types such as astrocytes—the so called volume transmission of neuromodulators.

4.3 Glial–Neuronal Interactions in the MNC Nuclei

ATP is the best described gliotransmitter. In its original description, ATP was released from cultured astrocytes to generate calcium waves for the purpose of inter-astrocyte communication (Guthrie et al. 1999). In a clever experiment, the surrounding media from activated astrocytes were used to initiate new calcium waves in naïve cells, an effect that was sensitive to ATP-degrading enzymes and P2-receptor antagonists. Subsequently, others demonstrated that the calcium wave could ‘jump’ a gap between otherwise connected cultured astrocytes and continue unhindered on the other side. Both experiments strongly suggested ATP was released as a diffusible paracrine messenger. From this discovery and many others to follow, it was speculated that glial ATP, once released, might have access to neuronal elements in more intact preparations. The original idea came from creating a simple link between the abundance of ATP release from astrocytes and the notion that ATP can have direct effects on neural P2 receptors/channels. Work conducted outside the hypothalamus demonstrated that ATP released from glial cells was quickly cleaved into adenosine to inhibit neighbouring neurons in the retina and the hippocampus. In the retina, glial cell activation hyperpolarized nearby ganglion cells. The effect was blocked by adenosine receptor antagonists and lessened by

ecto-ATPase inhibitors, suggesting that glial-derived ATP was indeed broken down by enzymes into adenosine. In the CA1 region of the hippocampus (acute slice), glutamate release from afferent stimulation triggered heterosynaptic depression—decreased release probability at synapses distant to the activated region. Part of this effect depended on gap junctional channels and adenosine receptors. Of note, astrocytes are extensively coupled to each other via gap junctions. This finding was further supported using transgenic mice in which astrocyte vesicular release is impaired using the over expression of a dominant-negative SNARE protein. In this mouse, ATP release from astrocytes was impaired and the ensuing adenosine effects on synaptic transmission were absent (Pascual et al. 2005). These experiments collectively showed that: (1) endogenous glutamate initiates ATP release from glia; (2) astrocyte-to-astrocyte signalling may be involved due to necessity of gap junctions and (3) astrocyte-derived ATP is broken down into adenosine to decrease release probability presynaptically at both local and distant synaptic sites.

As purinergic receptors are expressed on neurons, another question was whether ATP itself can affect synaptic efficacy without being degraded into adenosine? One answer hailed from a discovery in the MNCs of the PVN of the hypothalamus, where NA or glutamate triggered the release of ATP from astrocytes, which had direct actions on postsynaptic MNC P2X7 receptors. Activation of P2X7 channels caused a long-lasting enhancement of mEPSC amplitude (Gordon et al. 2005, 2009) (Fig. 4.2). It was postulated that because P2X receptors are Ca^{2+} permeable and can link to phosphoinositide 3-kinase, a critical kinase involved in postsynaptic AMPA receptor insertion, P2X receptors may serve as an aid or alternative to NMDA receptor-mediated plasticity, which also recruits AMPA receptor insertion to increase postsynaptic signal strength. Indeed, the increase in mEPSC amplitude was dependent on postsynaptic Ca^{2+} and PI3K activation. Furthermore, while either NA or glutamate produced the long-lasting increase in mEPSC amplitude, P2X7 receptor antagonists blocked the plasticity, suggesting that the downstream release of ATP was the actual end effector. Finally, application of the P2X 2,4 and 7 agonist BzATP caused an inward current in the MNC and increased mEPSC amplitude.

The ATP effect observed in the hypothalamic MNCs was similar to other 'lower' brain regions in which ATP effects have been described rather than adenosine, such as astrocyte ATP-mediated control of breathing neural circuits in the retrotrapezoid nucleus (Gourine et al. 2010) and 'gliogenic' long-term potentiation in the spinal cord (Kronschläger et al. 2016). The astrocyte-mediated plasticity in MNCs in the PVN can be recruited either with α1-adrenoceptor or group1 metabotropic glutamate receptor (mGluR) activation in astrocytes. Both receptors drive Gq-coupled intracellular signalling cascades that lead to the generation of IP3, which elevates free cytosolic Ca^{2+} via activation of IP3 receptors on the endoplasmic reticulum. This triggers the release of ATP from astrocytes in vitro and in vivo. Two-photon 'uncaging' of IP3 within individual astrocytes was used as a causal test of this hypothesis, representing a critical piece of evidence for the existence of direct astrocyte-to-MNC communication (Fig. 4.3). This technique allows for the liberation of biomolecules, such as IP3, with high temporal and spatial resolution within cells. Notably, in all experiments, the increase in mEPSC amplitude manifested only

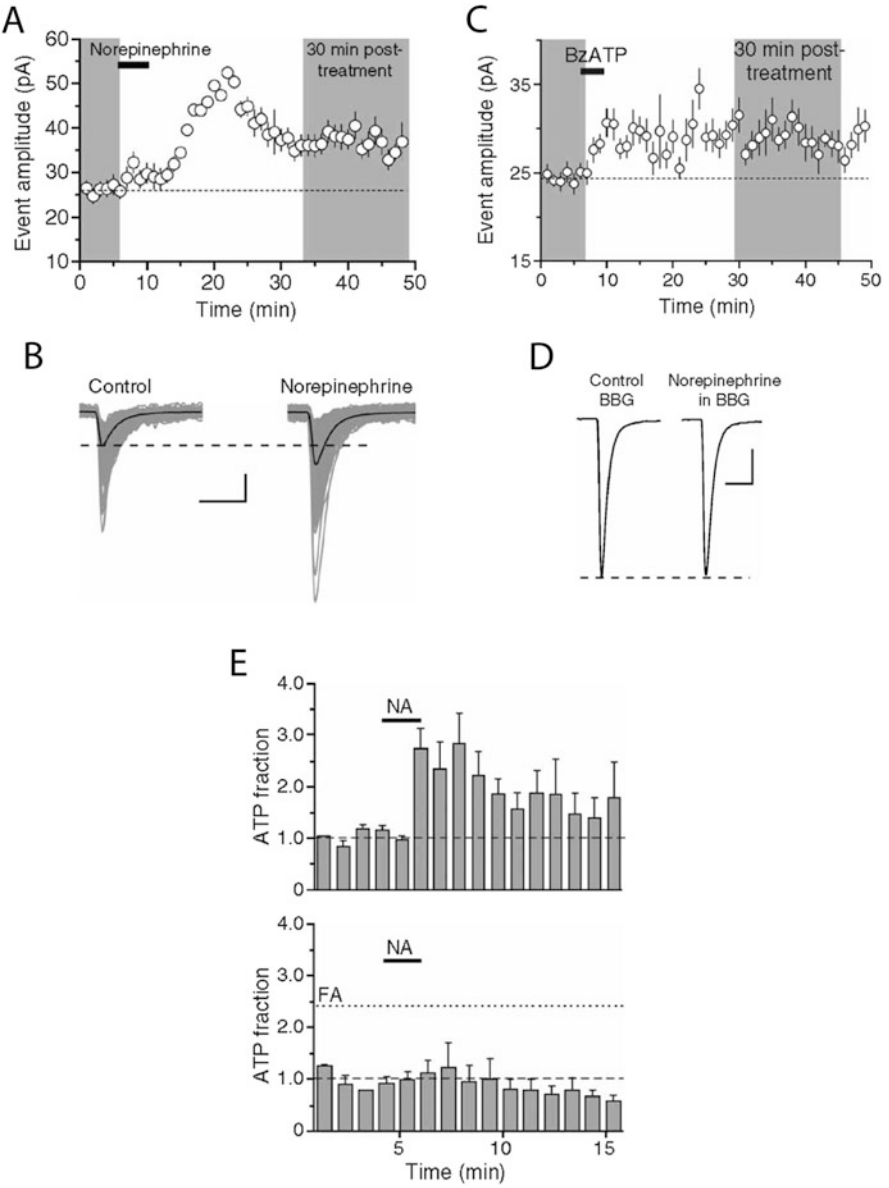

Fig. 4.2 Noradrenaline triggers the release of glial ATP to increase excitatory postsynaptic currents. (**a**) Time course of changes in mEPSC amplitude in response to bath application of NA. The early increase is mediated by a separate presynaptic mechanism. The late sustained phase requires glia (30 min post-treatment). (**b**) Superimposed mEPSC traces from experiment in **a**. Black lines represent the means. (**c**) Time course of the P2X agonist BzATP on mEPSC amplitude, showing an increase. (**d**) The increase in mEPSC amplitude caused by NA is blocked by the P2X7 antagonist BBG. (**e**) Detection of extracellular ATP increase in response to NA applied to cultured hypothalamic glia. The ATP release was blocked by the glio-toxin fluoroacetate (FA). Adapted from Gordon G.R. et al. (2005) Nat Neurosci. Aug;8(8):1078–86

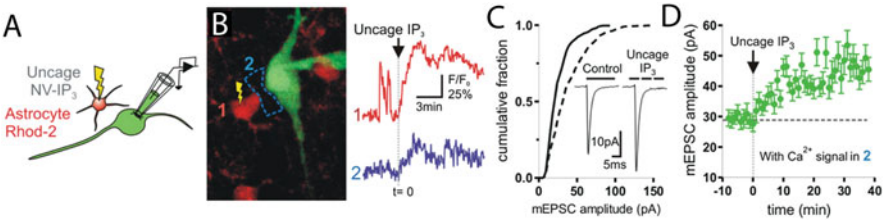

Fig. 4.3 Liberating IP3 in an astrocyte increases synaptic strength in an MNC (**a**) Depiction of IP3/AM uncaging within an astrocyte loaded with caged IP3 and calcium indicator Rhod-2/AM, next to a whole-cell patched MNC. (**b**) Two-photon live-cell image and calcium traces showing IP3 liberation within an astrocyte cause Ca^{2+} elevation in the astrocyte soma (1) and neuropil region (2) next to neuron. (**c**) Cumulative plot of mEPSC amplitude before and after astrocyte IP3 uncaging. (**d**) Time course of MNC mEPSC amplitude increase in response to IP3 uncaging within a single astrocyte. Adapted from Gordon G.R. et al. (2009) Neuron. Nov 12;64(3):391–403

when the astrocyte Ca^{2+} signal was spatially localized to the area immediately adjacent to the neuron under investigation. If this criterion was not met, no plasticity occurred, despite large astrocyte Ca^{2+} transients induced in nearby regions (~10μm or greater from the neuron). For example, in dual MNC recordings, activating an astrocyte via IP3 uncaging close to the dendrite of one MNC generated the increase in mEPSC amplitude, but did not affect synapses in the MNC farther away (Fig. 4.4). It was also demonstrated that the broad-spectrum P2X antagonist PPADS did not disrupt the ability of astrocytes to exhibit a Ca^{2+} signal, or to allow that Ca^{2+} to spread in the local astrocyte network in response to IP3 uncaging. However, in the same experiments, PPADS was able to block the increase in mEPSC amplitude in a nearby patched MNC, despite a sufficient astrocytic Ca^{2+} elevation (Fig. 4.5).

4.4 ATP Release from Astrocytes

Different spatial characteristics of ATP release from astrocytes have been described. ATP release can occur in continuous waves coinciding with Ca^{2+} wave propagation or in isolated bursts that are highly localized and separated by appreciable distances. The mechanisms of ATP release from glial cells are wide and varied, but there is strong support for induction by $G_{q/11}$-coupled metabotropic receptor activation, including purinergic P2Y receptors, group I mGluRs and α_1-adrenoceptors, among others. For Ca^{2+}-dependent ATP release, vesicular release is a key mechanism. In addition to the role for $G_{q/11}$ activation, nitric oxide also increases astrocytic free Ca^{2+} and causes ATP efflux. ATP release can be blocked by the Ca^{2+} chelator BAPTA and botulinum toxin C, suggesting that the process requires vesicles and the associated SNARE protein complex. Incubating astrocytes in bafilomycin A1, a vesicle H^+ transporter inhibitor, attenuates ATP-induced Ca^{2+} waves, further suggesting that astrocytes release ATP via vesicles. Genetic techniques also support vesicular release of ATP from astrocytes; for example, the conditional expression of a dominant-negative SNARE protein selectively in astrocytes blocks the observed

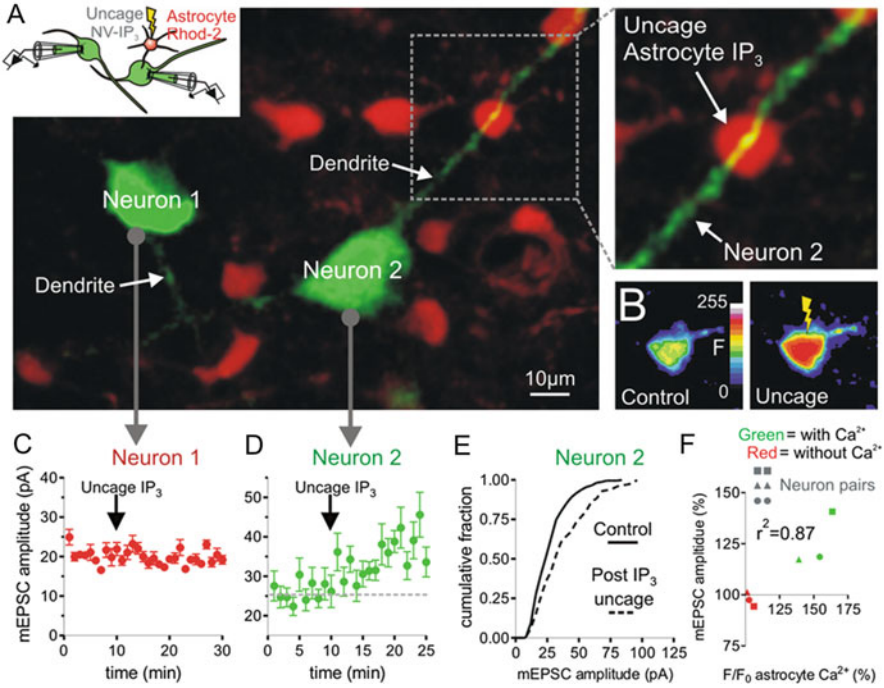

Fig. 4.4 Astrocytes influence nearby MNCs (**a**) Inset: Schematic of experiment. (Left) A stack image showing a pair of recorded neurons (1 and 2, green) with surrounding astrocytes loaded with Rhod-2/AM and caged IP3/AM (red). (Right) Close up shows the dendrite of neuron 2 and the closely associated astrocyte to be activated. (**b**) Pseudocolour images of the Ca^{2+} increase in this astrocyte from IP3 uncaging. (**c**, **d**) Uncaging IP3 within this astrocyte fails to induce plasticity in the more distant neuron 1 (**c**) but does increase mEPSC amplitude in the closer neuron 2 (**d**). (**e**) The cumulative mEPSC amplitude distribution in neuron 2. (**f**) Strong correlation between the astrocyte Ca^{2+} signal localized to only one neuron in a paired neuron recording and the expression of plasticity. Adapted from Gordon G.R. et al. (2009) Neuron. Nov 12;64(3):391–403

effects of ATP/adenosine on synaptic transmission. In contrast to the fast, Ca^{2+} dependent exocytosis observed in neurons that relies on presynaptic voltage-gated Ca^{2+} channels, astrocytes use Ca^{2+} released from intracellular stores to initiate vesicle exocytosis. Thus, IP_3 itself is able to evoke ATP release from astrocytes, which is consistent with the role of α1 adrenergic receptors, group 1 mGluRs and the photolysis of caged-IP_3 in the induction of a purinergic receptor-dependent increase in quantal glutamate transmission onto MNCs. Other studies have shown that neither bafilomycin nor the application of tetanus toxin (another molecule that cleaves SNARE proteins) completely blocked ATP release under certain conditions, suggesting that ATP also can be secreted through alternative routes. These include connexin and pannexin hemichannels, large-pore anion channels, MRP1 transporters and P2X7 channels. For example, use of extracellular ATP biosensors in the brainstem has shown that in response to the stimulation of astrocytes with

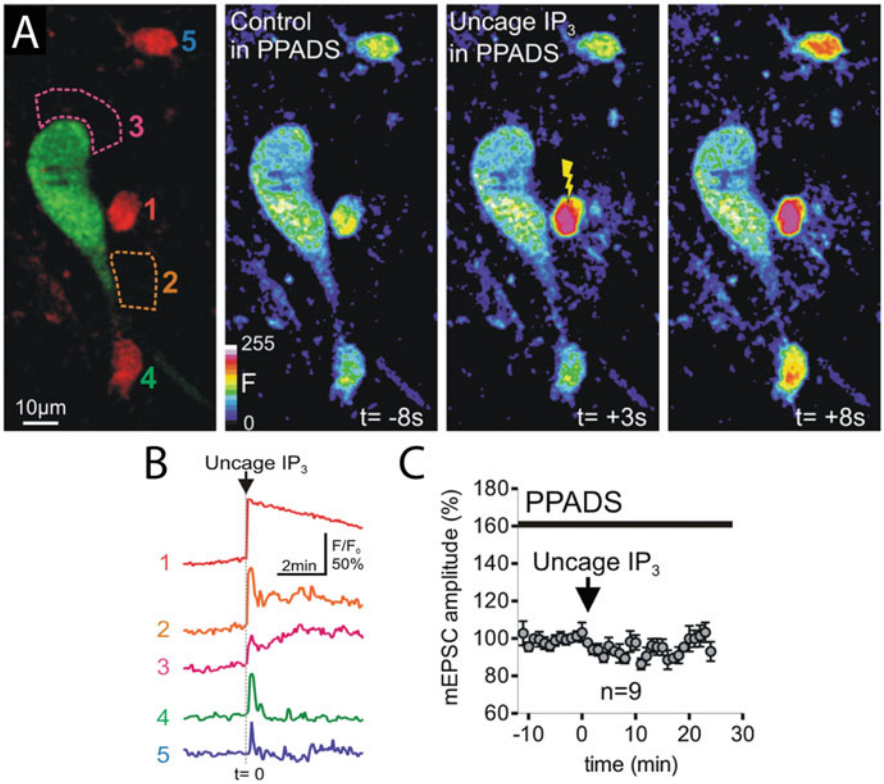

Fig. 4.5 The broad-spectrum P2X blocker PPADS does not affect directly evoked astrocyte Ca^{2+} signals yet blocks the increase in mEPSC amplitude in response to astrocyte stimulation. (**a**) Left: stack image showing a patched neuron (green) and astrocytes loaded with Rhod-2/AM and caged IP3/AM (red). Right: pseudocolour images of Ca^{2+} increases in response to uncaging IP3 within astrocyte 1; Ca^{2+} increases were observed in astrocyte processes around the neuron (2 and 3) and in neighbouring astrocytes (4 and 5) in the presence of PPADS. (**b**) Corresponding astrocyte Ca^{2+} signal traces. (**c**) Running mEPSC amplitude summary of astrocyte IP3 uncaging in PPADS. Adapted from Gordon G.R. et al. (2009) Neuron. Nov 12;64(3):391–403

CO_2, ATP was detected and was sensitive to hemi-channel blockers (Huckstepp et al. 2010). This shows that modes of ATP release other than vesicle exocytosis are possible.

4.5 Structural Remodelling Reveals Astrocyte MNC Interactions

The ***PVN*** and ***SON*** of the ***hypothalamus*** represent an intriguing system to study ***astrocyte***–neuron interactions because the coverage of ***glial*** processes around neuronal somata and synapses is reduced during lactation and dehydration (Tweedle and

Hatton 1977; Hatton and Tweedle 1982; Theodosis et al. 1981). Specifically, the glial cell processes that extend between ***MNC*** cell bodies and around synapses retract, increasing soma appositions and removing glial cell synapse barriers, respectively. Thus, in addition to active glial–neuronal signalling, the MNC nuclei of the hypothalamus provide an excellent example of a unique glial–neuronal interaction that is dynamic and purely physical. This provides a testing ground for a given synaptic effect in the presence and absence of glial processes. The result can be used as evidence for or against the involvement of glia. In a series of seminal electrophysiological experiments in virgin and lactating rats, the concentration of cleft ***glutamate*** and its diffusion in the extracellular space was found to depend on synaptic glial cell coverage (see Chap. 2). In the absence of glial cells, glutamate can access presynaptic ***mGluRs*** lowering release probability. This was not seen in virgin rats, indicating the anatomy of the neuropil plays a critical role in regulating synaptic efficacy (Oliet et al. 2001). Using this model, it was also shown that glial cell diffusion barriers govern heterosynaptic inhibition of GABAergic terminals by glutamate spillover, as well as the threshold for classical synaptic strengthening. With respect to the latter, glutamate afferents, and in particular those originating in the organum vasculosum lateral terminalis, exhibit NMDA receptor-dependent long-term potentiation (LTP) and long-term depression (LTD). Interestingly, the induction of plasticity at these synapses is precisely controlled by the astrocytes that ensheath the synaptic contacts. It was in the SON that astrocytes were first discovered to serve as the source of synaptic D-serine, an amino acid which is an endogenous ligand for the D-serine binding site on the NMDA receptor (Panatier et al. 2006) (Fig. 4.6). When the supply of D-serine from astrocytes is relatively high, there is robust NMDA receptor activation in response to a pairing electrical stimulation protocol of glutamate afferents, resulting in LTP. In contrast, compromising the availability of astrocyte-derived D-serine, either by facilitating D-serine breakdown or by decreasing the physical interposition between glial cells and synapses, causes LTD of synapses in response to the same stimulation parameters. The ability to induce LTP in the absence of glial cells could be recovered by applying a more intense, high frequency electrical stimulation protocol, suggesting that glial cells use D-serine to not only activate NMDA receptors, but also to set the threshold for plasticity in this system. Consistent with the IP3 uncaging data in the PVN, which implicated astrocytes in the P2X-mediated increase in synaptic strength, the plasticity is completely absent in the dehydrated condition, when astrocyte processes are withdrawn (Fig. 4.7). In the dehydrated state, postsynaptic signalling downstream of the astrocyte is still intact. Even though the effects of NA on MNC synapses were absent in the dehydrated condition, direct activation of postsynaptic P2X receptors was still possible to increase the amplitude of mEPSCs. The physiological significance of the changes in astrocyte-mediated synaptic plasticity under these physiological conditions in the magnocellular system is not well understood. See Box 4.1 for ideas on this topic.

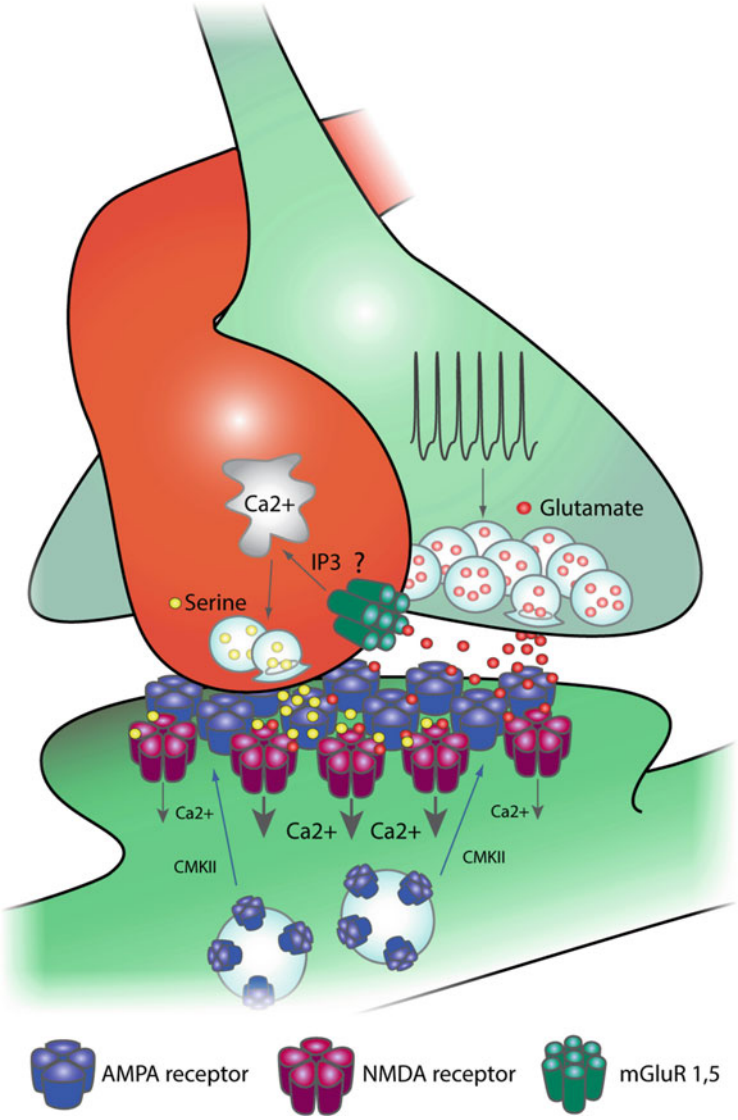

Fig. 4.6 Illustration depicting astrocytes sensing synaptic activity and releasing D-serine, a co-agonist of NMDA receptors. Postsynaptic NMDAR activation triggers AMPA receptor insertion to increase synaptic efficacy

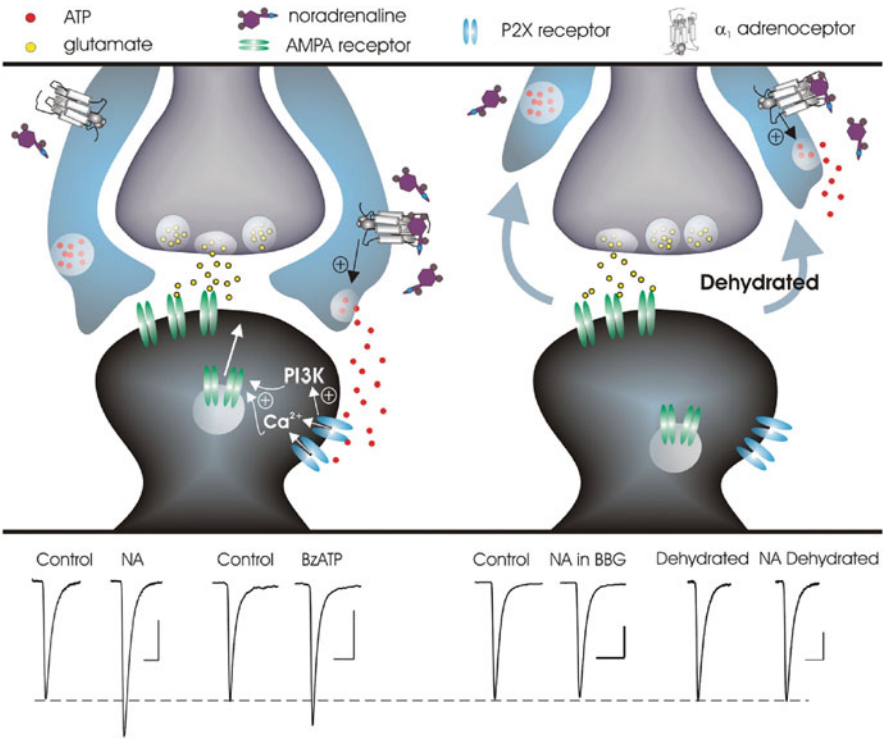

Fig. 4.7 Illustration of the presence vs absence of glial processes in Noradrenaline/ATP plasticity. Left: control state showing that NA triggers the release of glial ATP in response to α1-adrenoceptor activation. ATP acts on postsynaptic P2X receptors to allow Ca^{2+} influx and activate PI3K to cause AMPA receptor insertion. Applying the P2X agonist BzATP mimics the effects of NA. Representative excitatory synaptic currents shown at bottom. Right: During dehydration, when astrocyte processes have retracted from synapses, NA-induced ATP release is no longer able to access postsynaptic P2X receptors to cause AMPA receptor insertion and increase synaptic current amplitude. Furthermore, applying the P2X antagonist BBG in the normal state (no dehydration), also blocks the ability of NA to affect mEPSCs, suggesting the actions of NA are through P2X activation. Representative synaptic current traces below. Adapted from Gordon G.R. and Bains J.S. (2006) J Physiol. Oct 15;576(Pt 2):341–7

Box 4.1

The structural remodelling of the neuropil in the PVN and SON could represent an effective mechanism for selectively inducing or preventing changes in synaptic strength depending on the relative presence or absence of glial cell processes surrounding neurons, respectively. In the retracted state, afferent signals coming from the brainstem or other hypothalamic nuclei would be unable to influence MNC activity if these inputs recruited the release of

(continued)

Box 4.1 (continued)
gliotransmitters from local glia. Thus, the glial–neuronal plasticity described here may be important for MNC system behaviour. Long-term strengthening of excitatory synapses by glial ATP or D-serine may be used as an early signal at the onset of dehydration or lactation to meet the ensuing challenge. Then, only after a persistent challenge do astrocytic processes retract, which may be designed to 'lock' synapses in the potentiated state. It follows then that after retraction, plasticity requiring glial cells will not be induced as easily, while other types of plasticity that do not require glial cells are permissible. These ideas are supported by the observations in dehydrated animals that (1) NA only increases glutamate release probability (which does not require glia) without a change at the postsynapse (which does require glia), resulting possibly from earlier actions of ATP and/or D-serine in the tripartite synapse; and (2) the D-serine-dependent LTP requires much greater afferent activation to cross the threshold for induction, showing that synapses are somewhat 'locked' in the absence of glia.

4.6 ATP Versus Adenosine

The initial step for both heterosynaptic suppression in hippocampal pyramidal neurons and synaptic strengthening in MNCs is the release of ATP from astrocytes. In the former, glia-derived ATP is broken down into adenosine by the action of ecto-ATPases and nucleotidases (referred to collectively as nucleotidases from here on), and adenosine binds to adenosine receptors (A1, A2A, A2B). In the latter, glial ATP must remain intact to bind postsynaptic P2X receptor/channels. One reason this difference may exist is because of the location and or expression level of nucleotidases around MNC synapses versus synapses in cortical regions. The presence of adenosine in the hippocampus does depend on nucleotidase activity, however, the distribution is not uniform but regional. This suggests that the relative presence or absence of these enzymes may dictate to what degree adenosine is utilized as a transmitter over ATP. That nucleotidase activity can change over development and become altered in certain disease states, indicates these enzymes are not static and may change depending on the age and (patho)physiological state of the organism. Furthermore, nucleotidases are pH sensitive. As pH decreases, nucleotidase activity increases; thus, CO_2 levels can dictate the amount of extracellular adenosine and the size of synaptic currents. The higher the CO_2, the lower the pH and the greater the external adenosine concentration. Finally, pH, ATP and adenosine each regulates the diameter of arterioles to change blood flow, and increases in blood flow help clear CO_2; therefore, CO_2, nucleotidase activity, purines and blood flow all regulate each other, likely reaching a steady-state until CO_2 changes from neural metabolism or ATP/adenosine is released in response to neural activity and

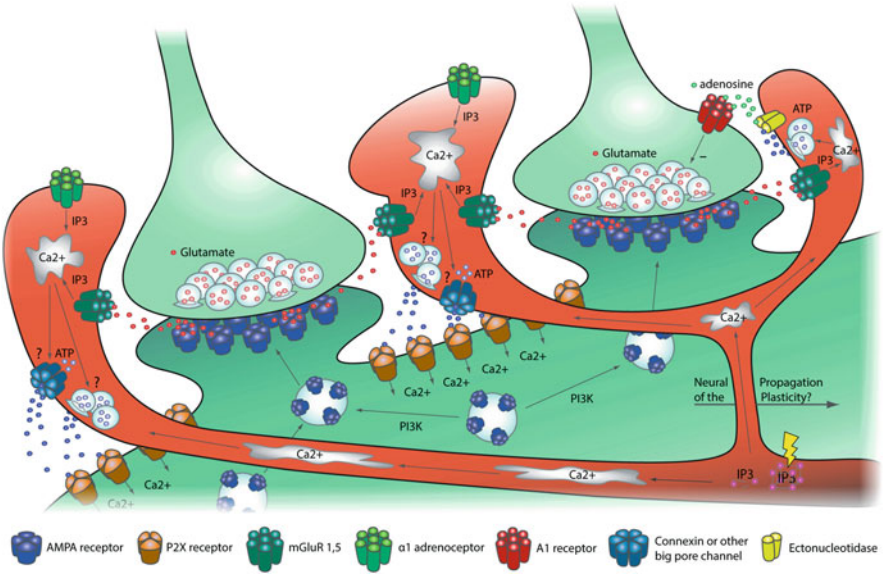

Fig. 4.8 Cartoon depicting the different mechanisms of astrocyte-purine mediated effects on synaptic strength. Gq-coupled receptor activation or IP3 uncaging triggers vesicular ATP release or the opening of large-pore channels to release ATP. ATP can then bind to postsynaptic P2X receptors that are Ca^{2+} permeable. Ca^{2+} influx activates signalling cascades, such as PI3K, that are involved in AMPA receptor insertion to increase the size of the postsynaptic current. If extracellular ATP is broken down by ecto-nucleotidases, adenosine can act on presynaptic A1 adenosine receptors to decrease release probability

the activation of astrocytes. We can extrapolate from this that there is no clear 'rule' stating that all extracellular ATP must be broken down to adenosine or that no ATP shall be broken down. The presence and/or function of nucleotidases in the PVN are still understudied, but the enzymes are present in the neural lobe. We also know that prolonged afferent stimulation in the SON results in the release of endogenous adenosine, which can reduce the synaptic release of both GABA and glutamate, but the source of adenosine remains unknown. Given the glia-derived ATP effects observed in the PVN, brainstem and spinal cord, the neural release of adenosine from sustained synaptic and spiking activity, which taxes the supply of ATP for energy purposes, is the most likely candidate. See Fig. 4.8 for a conceptual diagram of astrocyte ATP-mediated synaptic plasticity.

4.7 Conclusion and Future Perspectives

AMPA receptor insertion is an accepted means for increasing signal strength post-synaptically. This process is thought to occur via spike timing-dependent plasticity where coincident presynaptic release and postsynaptic depolarization provide

sufficient stimulus to relieve the magnesium pore block on NMDA receptors. The Ca^{2+} influx through NMDA channels can promote the insertion of AMPA receptors into the postsynaptic side of the synapse if the Ca^{2+} signal is large enough or the removal of AMPA receptors if the Ca^{2+} signal is sufficiently small. The activation of kinases such as CaMKIIα and PI3-K is necessary for AMPA receptor incorporation into the postsynaptic density. The effects of astrocyte-derived ATP on MNCs in the PVN (as well as gliogenic ATP-mediated LTP in the spinal cord) have provided an additional mechanism through which postsynaptic signal strength can be augmented. By acting at α_1-adrenergic receptors, NA triggers the release of astrocyte ATP into the extracellular space. Liberated ATP binds to postsynaptic P2X channels on MNCs. Evidence suggests that the ionotropic, Ca^{2+}-permeable P2X channel activates PI3-K and leads to the insertion of AMPA receptors through a SNARE dependent mechanism, thus recruiting a similar molecular pathway as NMDA receptors. These MNC experiments provided the first demonstration of a gliotransmitter, ATP, and purinergic P2X receptors linking to AMPA receptor trafficking.

This mechanism raises several questions as to why astrocytes would be involved in this form of synaptic strengthening, why NA does not directly signal to the postsynaptic neuron to promote the insertion of AMPA receptors and why NA utilizes P2X channels as opposed to NMDA receptors via enhanced afferent signalling. Unlike NMDA receptors, P2X channels are not regulated by Mg^{2+} in a voltage-sensitive manner and thus do not require the same local depolarization to become activated. Only a sufficient concentration of ATP is required, suggesting that synapses can undergo strengthening independent of the voltage state of the postsynaptic neuron as well as independent of the timing of afferent inputs. Furthermore, astrocytes occupy distinct, non-overlapping volume domains in the CNS, where each astrocyte essentially controls a sphere of influence. The large scaffolding and signalling capabilities of astrocytic networks present a unique opportunity for incoming afferent signals to influence spatial neuronal 'domains'. Where classical thinking stipulates that the strengthening or weakening of the synapse depends on the specific activity of that synapse, glial networks may provide a means to change the efficacy of groups of synapses and groups of neurons at one time. Outside the hypothalamus, this concept is supported by the demonstration that (1) glia-derived glutamate can activate extrasynaptic NMDARs on multiple neurons simultaneously, thereby synchronizing them; (2) ATP released from glia can trigger depression at synapses distant to the activation area after ATP has been broken down into adenosine and (3) Ca^{2+} signals in astrocytes in vivo in response to physiological stimulations can be robust, developing in many processes and in many astrocyte somata. When combined with the anatomy of the astrocyte, the Ca^{2+} signals can be broadly distributed, creating the possibility for interaction with many synapses simultaneously.

In fact, in MNCs, the ATP-mediated P2X plasticity appears not to be restricted to single synapses (Gordon et al. 2009). Instead, ATP scales the strength of all

spontaneous (or miniature) synaptic currents that can be sampled via the patch pipette. Notably, NA causes widespread increases in astrocyte Ca^{2+}, even more so than mGluR activation, suggesting that NA may be the most potent trigger to activate glial cell networks to control synaptic efficacy more globally. If astrocyte activation is strong enough, this would allow astrocytes to propagate signals to more distant neural sites, influencing synapses away from the source of activation. However, one can imagine a different scenario, in which astrocytes only contribute in part to the spatial distribution of the plasticity signal, and where the neuron is also capable of using its own intrinsic abilities to propagate signals through its processes to spatially distribute the change in synaptic efficacy. In the hypothalamus, focal glutamate uncaging on astrocytes, or IP3 uncaging within a single astrocyte, triggers a distributed and 'multiplicative' increase in synaptic strength, whereby many synapses improve in efficacy by a proportional amount. This was demonstrated by measuring a population of synaptic currents that all scale in amplitude by the same factor. The domain characteristic of astrocytes is very intriguing as it provokes new ways of thinking about synaptic transmission in the brain; one that is not synapse specific but where synapses are controlled more globally in domains of activation. Classical, synapse-specific plasticity and astrocyte-mediated distributed scaling are of course not mutually exclusive and can readily co-exist. Overall, this type of 'distributed' plasticity may be beneficial, so that MNCs do not have to rely on coincident detection to augment hormone output in the face of physiological challenges. If the goal is to dramatically increase neurohormone secretion, not relying on the concerted efforts of afferent inputs in combination with precisely timed postsynaptic spiking could be advantageous, especially when the MNC spike pattern is critical for determining the right amount of hormone release. Enhancing excitability using a diffuse and likely broad-reaching signal, such as astrocyte-derived ATP, may represent an efficient method to help increase the release of vital neurohormones during physiological/homeostatic challenges so that all incoming glutamate inputs can more readily excite neurosecretory cells.

4.8 Key Literature

Day et al. (1993) The first demonstration of ATP mediating part of the effect of noradrenergic input to magnocellular neurons in vivo.

Gordon et al. (2005) The first demonstration of glial derived ATP being partly responsible for the excitatory effect of noradrenaline on MNCs using acute brain slices.

Gordon et al. (2009) A follow up paper from Gordon et al. 2005 showing the same phenomenon by direct astrocyte stimulation with two-photon uncaging. Furthermore, the plasticity appeared "distributed", affecting many excitatory synapses on an MNC.

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Further Recommended Reading

Kronschläger MT, Drdla-Schutting R, Gassner M, Honsek SD, Teuchmann HL, Sandkühler J (2016) Gliogenic LTP spreads widely in nociceptive pathways. Science 354(6316):1144–1148
This paper describes a type of glial mediated LTP in the spinal cord that is similar to that described by Gordon et al. in the PVN, which relies on ATP, P2X7 receptors and D-serine

5 The Multifaceted Roles of Hypothalamic Astrocytes and Microglial Cells in Neuroendocrine and Autonomic Regulation in Health and Disease

Ferdinand Althammer and Javier E. Stern

Abstract

The hypothalamus is a key regulatory brain region that coordinates and modulates various vital processes, including cardiovascular and respiratory functions, metabolism and mood, among others. This complex function is achieved by the concerted action of neurons, astrocytes, and microglia within this nucleus. However, various pathophysiological conditions, particularly those involving a neuroinflammatory process, disturb the normal function and interaction of these various cellular components of the nucleus. Here we provide a broad overview about the origin, development, and function of microglia and astrocytes and discuss their respective roles in the regulation of hypothalamic activity. We further provide insight into how cardiovascular diseases such as heart failure or hypertension affect neuronal and glial activity in the hypothalamus and impose detrimental consequences to the well-being of affected individuals. Finally, we discuss a recently developed technique that allows the three-dimensional reconstruction and analysis of glial cells, thereby providing unprecedented details about glial morphology.

Keywords

Microglia · Astrocytes · Neuroendocrinology · Heart failure · Neuroinflammation · Hypothalamus

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5.1 Introduction: Neuron–Glia Interactions in the Brain

The idea that glial cells (i.e., astrocytes, oligodendrocytes, and microglia) are merely supportive elements within the brain, which provide stability and nutrients to neurons, has been overthrown many years ago. Research in the last two decades has not only revealed many more vital functions of glia, but highlighted various neuron–glia interactions that are crucial for proper signal communication, integration, and maintenance within and across different brain networks. In fact, the intricate interaction between neurons and astrocytes has been deemed so important that scientists coined the term "*tripartite synapse*," which refers to the close interaction between the pre-synapse, post-synapse, and astrocytes.

5.1.1 Astrocytic Diversity

Astrocytes display astonishing variability across different species, brain regions, and even within the same functional units/networks in the brain. In different brain regions and during different times of development, astrocytes display distinct functional properties. The size of astrocytes can even increase with brain size and cognitive abilities. Interestingly, human astrocytes are up to threefold larger and tenfold more ramified than rodent astrocytes (Allen and Eroglu 2017). In addition, recent genetic studies have revealed significant differences in gene expression between rodent and human astrocytes, which could explain why certain unique features of the latter are cell-autonomous. While mouse cortical astrocytes contact approximately 100,000 synapses, their human counterparts have 2,000,000 synaptic contacts.

5.1.2 Astrocytes as Regulators Between Pre- and Post-synapse

Astrocytic processes ensheath synapses and this supportive formation is critical for normal synapse function, brain homeostasis, and neuronal health (Araque et al. 1999). Astrocytes mop up various neurotransmitters from the extracellular space, and the high expression of glutamate transporters in astrocytic processes prevents excessive extrasynaptic accumulation of glutamate, thereby providing protection against excitotoxicity. Furthermore, astrocytes control ionic balance at the synapse through various channels, such as potassium channels, to maintain a healthy/balanced ionic milieu, which is a prerequisite for proper synaptic transmission. While it is certain that astrocytes are equipped with metabotropic and ionotropic receptors, their precise relationship with astrocytic Ca^{2+} transients and subsequent gliotransmission remains controversial (Fiacco and McCarthy 2018; Savtchouk and Volterra 2018). However, it seems that astrocytic Ca^{2+} transients occur in a delayed/slower fashion than in neurons and recent findings highlighted the presence of Ca^{2+} microdomains in astrocytes (Agarwal et al. 2017).

Astrocyte–synapse interaction is critical for normal CNS development, and the main periods of synaptogenesis and axonal formation take place during the second and third postnatal week, after the differentiation of astrocytes is already completed. Early experiments in neuronal cultures showed that the addition of astrocytes increased both the number and strength of synapses and highlighted the important role of neuronal–glial interaction in synapse development. Over the years, these findings could be replicated and turned out to be true for many different species across the animal kingdom, including *C. elegans*, Drosophila, Xenopus, rodents, and humans. Moreover, astrocytes are involved in the formation of many synapse types, including glutamatergic, GABAergic, glycinergic, and cholinergic synapses.

Astrocytes secrete the so-called Thrombospondins, such as TSP1/2 and a protein named SPARCL1/Hevin, which are signals that control glutamatergic synapse formation. A number of astrocyte-derived signals have been identified, such as Gpc4/6 and TNF-α, which regulate AMPA receptor localization to post-synaptic terminals and increase AMPAR levels at existing synapses. Astrocytes also play an important role in the control of synapse numbers: In the developing brain, they directly phagocytose excess synapses via the astrocytic phagocytic receptors Mertk and Megf10 (Chung et al. 2013). Recent studies have corroborated the hypothesis that astrocyte-secreted signals regulate synaptogenic pathways within neurons (Baldwin and Eroglu 2017; Singh et al. 2016), adding another layer of complexity to the intricate neuron–glia interaction. However, mRNA profiling of isolated astrocytes from different brain regions in rodents and humans suggested that not all astrocytes have the same synaptogenic potential (Zhang et al. 2016). For detailed reviews about astrocyte–synapse interactions in the healthy and diseased brain, as well as neuron–glia interactions in the formation of the blood–brain barrier, we refer the reader to the following literature: (Allen and Eroglu 2017; Eroglu and Barres 2010).

Despite the fact that astrocytes have emerged as important communicative elements in neuron–glia communication during the last decade, another key function that they are involved with is the formation of the blood–brain barrier (BBB). The BBB outlines cerebral microvessels and is composed of endothelial cells, astrocytes, neurons, and pericytes (Zhao et al. 2015). The complex interactions between endothelial cells, extracellular matrix, basal lamina, pericytes, and astrocytes comprise a neurovascular unit, which regulates central nervous system (CNS) development and synaptic activity, and can even influence the permeability of the BBB. Within the neurovascular unit, astrocytes are bidirectional communication partners that receive signals from neighboring neurons and respond with the release of neuroactive substances.

5.2 Transfer of Power in the Glial Kingdom: Microglial Cells

While the importance of astrocytes for synapse development and signal integration within a functional brain cannot be questioned, another glial cell type that has been overlooked and underestimated for many years is microglia (see also Chap. 1).

Microglia hide in plain sight and are the secret heroes of the brain immune system, despite being much smaller (hence the name, 5–10 μm diameter) than other glia in the brain. When Pio del Rio Hortega, a student of the famous Santiago Ramon y Cajal, first described microglia around 1920, he could not have foreseen how crucial these small glial cells actually are for normal brain function. During the last decade, microglia have emerged as key players in brain development, synapse formation and pruning, as well as in a plethora of vital immune-related functions. Microglia are specialized immune cells that take up residence in brain parenchyma and are among the first responding cells during injury, cell death, or entry of unwanted intruders that might access the brain due to a compromised blood–brain barrier. Microglia represent a major component in the immune response and participate in the neuroinflammatory response largely via the release of various pro-inflammatory cytokines. Research throughout the last decades has provided compelling evidence that microglia are more than just passive bystanders, and refuted the long-held dogma that microglia represent circulating CNS macrophages that enter the brain to replenish the local pool of immune cells. An overview of the respective roles of astrocytes and microglia in the developing and healthy adult brain can be found on Box 5.1.

5.2.1 Origin of Microglia: A Journey from the Yolk Sac to the Developing Brain

For a long time, it was believed that microglia represent a distinct pool of tissue-specific macrophages that populate the brain parenchyma and gain access to the brain through the BBB. However, recent research showed that microglia develop from c-Kit^{lo}CD41^{lo} progenitors that originate around embryonic day 7.25 (E7.25) in the yolk sac and find their way into the developing brain (Ginhoux et al. 2010). Several studies in zebrafish, mice, and chickens show that microglia are established during early embryogenesis, well before other glial cells such as astrocytes and oligodendrocytes arise. Microglia represent a self-sustainable pool of resident brain immune cells, which are not dependent on circulating macrophages (Ajami et al. 2007, 2011). Microglia in the embryonic, early postnatal, and adult CNS significantly differ in their expression, highlighting that adult brain microglia and microglia during early brain development are in fact functionally distinct entities. Once they reach the developing brain, microglia take over a variety of important functions and actively participate in shaping emerging brain circuits.

5.2.2 Role of Microglia in the Developing Brain: Neuronal Support and Synaptic Pruning

Microglia in the developing brain lack the characteristic ramified extensions of adult microglia (Lawson et al. 1990), which scan the environment for cues of sick neurons and tissue damage in inflammation. On the contrary, microglia in the developing

brain are considerably more proliferative than in the adult and are actively involved in phagocytosis and tissue remodeling. By engulfing neighboring cells in the developing CNS and clearing apoptotic neuronal debris, microglia help to shape neuronal networks (Peri and Nusslein-Volhard 2008). In addition, microglia stimulate neurogenesis and support the survival, proliferation, and maturation of neuronal progenitor cells in the developing brain. During the early postnatal period, crosstalk of microglia and oligodendrocyte precursor cells has been described, suggesting far-reaching and long-lasting consequences of microglial activity in this period. The microglial chemokine receptor CX3XR1 has been highlighted as a key component of the microglia-mediated survival cascade, and microglia lacking CX3XR1 do not produce sufficient amounts of the neurotrophic insulin-growth factor (IGF)1 and subsequently fail to promote postnatal survival. Microglia actively participate in synaptic pruning via CX3XR1, and CX3XR1 knockout mice lacking the receptor in microglia display a significantly higher number of post-synaptic puncta, which is indicative of impaired synaptic pruning. Synapses can be actively tagged for engulfment by microglia via complement protein (C3), allowing a directed C3 receptor-mediated phagocytosis (Stevens et al. 2007). On the other hand, synapses can be protected from excess pruning via CD47, which represents a "don't eat me" signal that stops microglia from engulfing the respective synapses (Lehrman et al. 2018). The reader is referred to Chap. 1 for a detailed description of the role of microglia in the development of the hypothalamus.

5.2.3 Microglia in the Adult Brain: Homeostasis and Immune Response

In the healthy adult brain, microglia extend their tiny processes throughout the extracellular space and constantly scan the environment for various cues. Although the cell bodies of microglia remain in place, their processes are anything but static (Nimmerjahn et al. 2005). Indeed, several studies suggest that microglia can encompass areas more than tenfold the size of their cell bodies and constantly interact with neighboring cells, although the precise underlying receptors, ion channels, and neurotransmitters for these interactions are under debate. Microglia display a remarkable heterogeneity and drastic differences in brain region-specific density, morphology, and genetics.

Upon acute injury within the CNS, microglial processes converge toward the site of injury and, after hours to days, they retract their processes and transform into their activated, ameboid form, which is responsible for mediating the appropriate immune response (Nimmerjahn et al. 2005). The signal-directed movement of microglia to the site of injury, known as chemotaxis, depends on purinergic P2Y12 receptors on microglia that bind to ATP or ADP released by various types of neural cells. In very rare cases, activated microglia can respond to noxious stimuli by transforming into a hyper-ramified phenotype, although the precise underlying mechanisms are currently unclear. It is important to know that at any given point, ramified, primed, reactive, or ameboid microglial phenotypes co-exist, even in the healthy brain,

making it clear that a binary classification of active/inactive microglia is an over-simplification. Reactive microglia secrete various pro-inflammatory cytokines, such as IL-1, TNF-α, IL-18, IL-6, or IL-23, which in turn act on neural cells and promote further neuroinflammation (Prinz et al. 2019). In addition to cytokine release, microglia release reactive oxygen species (ROS) and nitrogen species, which are toxic to both neurons and oligodendrocytes, thereby actively promoting neuroinflammation-induced apoptosis. It was recently discovered that the combined secretion of TNF-α, IL-1α, and C1q by activated microglia turns astrocytes into a neurotoxic (A1) astrocyte phenotype. Astrocytes can release orosomucoid-2 to inhibit microglial activation, block the chemokine receptor type 5 of the microglial membrane, downregulate the inflammatory response, or further promote neuroinflammation and neurodegeneration. This bidirectional interaction allows an efficient and tailored response to various potentially harmful threats that the brain might be exposed to. Another recent study highlighted the "immune memory" of microglia, resulting in smaller volumes of experimental stroke and maintenance of IL-10 expression (Wendeln et al. 2018). For a comprehensive literature about microglial origin, microglial function during disease, and microglia–astrocyte interaction during neuroinflammation, we refer the reader to these reviews: (Liddelow and Barres 2017; Prinz et al. 2019; Prinz and Priller 2014). The respective functional and pathological roles of activated microglia and astrocytes are summarized in Box 5.1.

Box 5.1: Overview of Astrocyte and Microglia Functions in the Developing, Mature, and Diseased Brain

Cell type	Stage	Function	Reference
Microglia	Development	Phagocytosis, tissue remodeling	Peri and Nusslein-Volhard (2008)
Microglia	Development	Stimulation of neurogenesis, proliferation and support of cellular survival	Sierra et al. (2010)
Microglia	Adult brain	Synaptic pruning: engulfment of synapses and regulation of post-synaptic puncta	Stevens et al. (2007)
Microglia	Adult brain	Constant scanning of CNS for unwanted intruders and signs of injury	Prinz et al. (2019)
Reactive microglia	Brain reacting to acute injury/ immune response	Altered synaptic density, neurotoxic effect, neuroinflammation, cytokine release, uncontrolled and excessive synaptic pruning, peripheral macrophages gain access to CNS	Ajami et al. (2011); Ajami et al. (2007); Liddelow and Barres (2017); Liddelow et al. (2017)

(continued)

Box 5.1 (continued)

Astrocytes	Development	Synaptogenesis, axonal formation, regulation of synaptic strength	Zhang et al. (2016)
Astrocytes	Development	Phagocytosis of excess synapses	Chung et al. (2013)
Astrocytes	Development/ Adult brain	Formation of the blood–brain barrier	Zhao et al. (2015)
Astrocytes	Development/ Adult brain	Control of glutamatergic, GABAergic, cholinergic, and glycinergic synapse formation	Ullian et al. (2001)
Astrocytes	Adult brain	Regulation of the tripartite synapse, control of extracellular glutamate/ GABA balance	Araque et al. (1999)
Astrocytes	Adult brain	Active part in neuron–glia communication, *gliotransmission*	Fiacco and McCarthy (2018); Savtchouk and Volterra (2018)
Reactive astrocyte	Brain reacting to acute injury/ immune response	Compromised BBB, altered levels of neurotransmitters, compromised access of circulating signals to the brain, astrogliosis, retraction of astrocytic processes, brain swelling, neuronal death	Liddelow and Barres (2017); Liddelow et al. (2017)

5.3 The Supraoptic and Paraventricular Nuclei of the Hypothalamus: Role in Homeostasis and Emotional Regulation

The supraoptic nucleus (SON) of the hypothalamus is situated at the base of the brain, just slightly above the optic tract, while the paraventricular nucleus (PVN) of the hypothalamus is located on both sides of the third ventricle (Fig. 5.1). Magnocellular neurosecretory cells (MNCs) synthesizing OT and VP are present in both the SON and PVN and release these neuropeptides into the systemic circulation via axonal projections to the posterior pituitary. The estimated total number of MNCs amounts to 100,000 in humans and 10,000 in rats. The SON plays an important role in regulating plasma osmolality, labor, and lactation, and has recently been proposed to be involved in the regulation of context-dependent fear memories (Hasan et al. 2019). While the SON comprises exclusively MNCs, the PVN harbors, in addition to MNCs, parvocellular neurons that project to the median

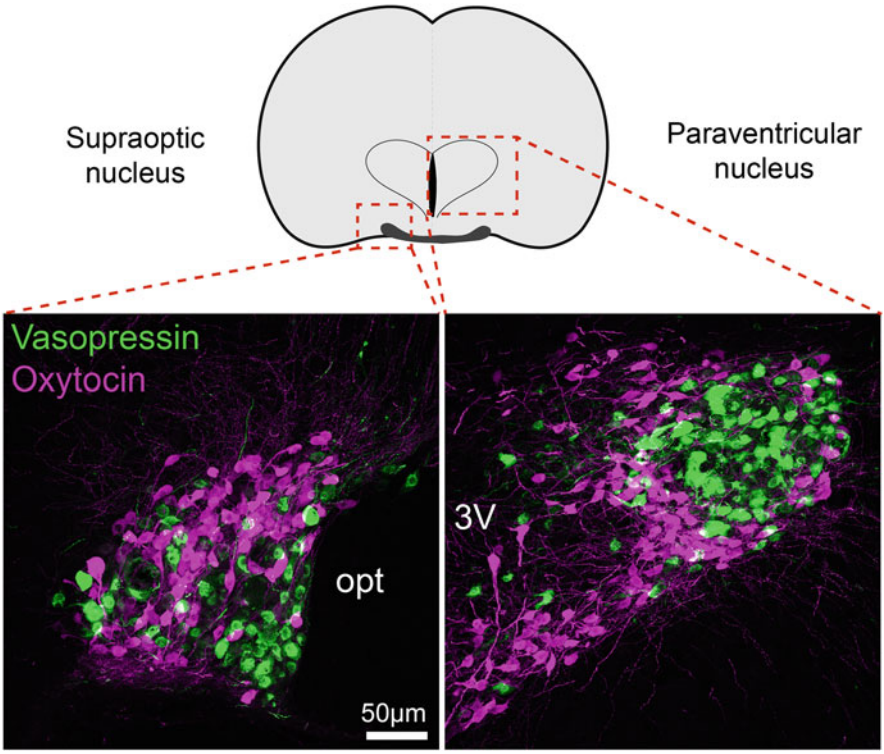

Fig. 5.1 Oxytocin and vasopressin neurons in the rodent hypothalamus. Confocal images show rat vasopressin (green, from vasopressin-GFP transgenic rat) and oxytocin (magenta, immunofluorescence with oxytocin antibody) neurons located in the supraoptic and paraventricular nucleus. The scheme depicts the topographical location of the respective nuclei in the rat brain

eminence and various hindbrain regions (Althammer and Grinevich 2017). The PVN is a key structure for the regulation of sympathetic outflow and cardiovascular control, playing thus a major role in physiological homeostasis and neuroendocrine control (Stern 2015). These actions are mediated via direct innervation of sympathetic-related brainstem and spinal cord neurons. OT and VP neurons in both the SON and PVN have been implicated in emotional regulation and affect a plethora of different behaviors including anxiety, aggression, fear, depression, social behavior, and pair bonding. For comprehensive reviews on the physiological regulation MNCs and emotional control via OT/VP neurons, we refer the readers to the following literature: (Brown et al. 2013)

5.3.1 Somato-dendritic Release of Oxytocin and Vasopressin

Both types of MNCs in the SON and PVN have the ability to release their respective neuropeptides from both dendrites and somata via a process called *somato-dendritic*

release (Ludwig and Leng 2006). Somato-dendritic release of OT and VP mediates multiple and unique functions, which are distinct from those mediated by axonal, systemic release of these neuropeptides. For example, somato-dendritic release of OT and VP serves as an autocrine signal by which magnocellular neurosecretory neurons autoregulate their firing activity and systemic hormone release. Moreover, dendritically-released VP can diffuse in the extracellular space to act as a diffusible, interpopulation signaling molecule, coordinating the activity of sympathetic and neurosecretory PVN neurons (Son et al. 2013), playing thus a critical role in the generation of multimodal homeostatic responses by the PVN; (see Ludwig and Stern (2015) for a review on this subject).

5.3.2 Role of Neuro–glial Interaction in Regulating the Physiological Activity in the SON and PVN

Various forms of complex neuro–glial interactions have been shown to take place in the MNC system, primarily involving bidirectional communication between astrocytes and neurons. Astrocytes express an abundance of G protein-coupled receptors for various neurotransmitters, including glutamate, GABA, and various neuropeptides as well. Thus, astrocytes can readily sense neuronally-derived signals. For example, noradrenaline has been shown to stimulate α_1-adrenoceptors on PVN astrocytes resulting in the release of ATP from the stimulated astrocytes. Astrocytes in the SON and PVN also express high levels of endothelin-B receptors, and have been shown to contribute to endothelin-mediated regulation of MNC firing activity in a nitric oxide-dependent manner (Filosa et al. 2012). Finally, dendritically-released VP from MNCs can evoke Ca^{2+} activity in surrounding astrocytes, a mechanism recently proposed to contribute to ghrelin-mediated modulation of VP activity in a nutritional-dependent manner.

Another key mechanism by which astrocytes influence MNC firing activity is via regulation of the concentration and time course of neurotransmitters in synaptic and perisynaptic areas. This is mediated by the activity of selective and powerful neurotransmitter transporters, particularly for the amino acid transmitters glutamate and GABA (see Chap. 2). Over the past 10 years, our laboratory has focused on a particular aspect of this phenomenon, namely the ability of astrocyte amino acid transporters to restrict the ability of extracellular neurotransmitters to access and activate extrasynaptic, particularly extrasynaptic glutamate NMDA receptors (eNMDARs). Differently from classical synaptic receptors, eNMDARs display a low degree of desensitization and a low affinity for glutamate, mediating a persistent, "tonic" excitatory current that is thought to globally influence neuronal excitability and the overall gain within a network of neurons. In this sense, we showed that activation of eNMDARs by ambient, extracellular glutamate levels results in a persistent inward current, which tonically stimulates SON neuronal activity (Fleming et al. 2011). Moreover, the strength of this tonic excitatory drive is directly regulated by astrocyte GLT1 activity. Thus, pharmacological block of GLT1 activity or glial retraction during dehydration results in an enhanced activation and

contribution of eNMDARs to SON firing activity. We proposed this phenomenon to contribute to the homeostatic increase in neurosecretory firing activity during dehydration.

Importantly, we recently demonstrated that the neuropeptide Angiotensin II (AngII) can directly inhibit the activity of astrocyte GLT1 transporters, resulting in the buildup of extracellular glutamate and activation of SON and PVN neurons (Stern et al. 2016). Finally, we also demonstrated that changes in the expression and/or fraction of GLT1 in the SON and PVN can contribute to exacerbated hypothalamic neuronal activity in prevalent cardiovascular diseases, including heart failure and hypertension (Stern et al. 2016).

In summary, a significant amount of progress has been made regarding the role of astrocytes in the regulation of the magnocellular system, particularly under physiological conditions. Conversely, and compared also to other major CNS regions such as the cortex and hippocampus, much less is known about the contribution of microglial cells in shaping the normal activity of hypothalamic neurons and circuits, an area that clearly deserves to be further investigated. Figure 5.2 shows isolated microglia and astrocytes in high magnification as well as an overview of astrocytes, microglia, and OT neurons in the SON and PVN.

5.3.3 Neuroinflammation in the SON and PVN in Disease Conditions

Neurohumoral activation, a process that involves exacerbated sympathoexcitatory activity, along with elevated circulating levels of neurohormones, including VP, AngII, and endothelins, among others, is a common pathophysiological phenomenon in highly prevalent cardiometabolic diseases, including hypertension, heart failure, diabetes, and obesity. Importantly, a direct correlation between the degree of neurohumoral activation and morbidity and mortality in these diseases is well-established. Thus, elucidating the precise underlying mechanisms mediating neurohumoral activation is of high clinical relevance.

A growing body of evidence supports a critical role for the SON and PVN in the onset and maintenance of neurohumoral activation in these diseases, particularly in hypertension and heart failure. Importantly, neuroinflammation within these hypothalamic nuclei has been identified as a key underlying pathophysiological mechanism. Thus, several studies have found elevated cytokine levels, astrogliosis, microglia activation and infiltration by peripheral immune cells, as well as disruption of the BBB. In extreme cases, a compromised BBB can lead to entry of peripheral macrophages, which further exacerbates neuroinflammation in the brain (Ajami et al. 2007, 2011). Still, the precise mechanisms and cascade of signaling events contributing to neuroinflammation in cardiometabolic diseases remain to be determined.

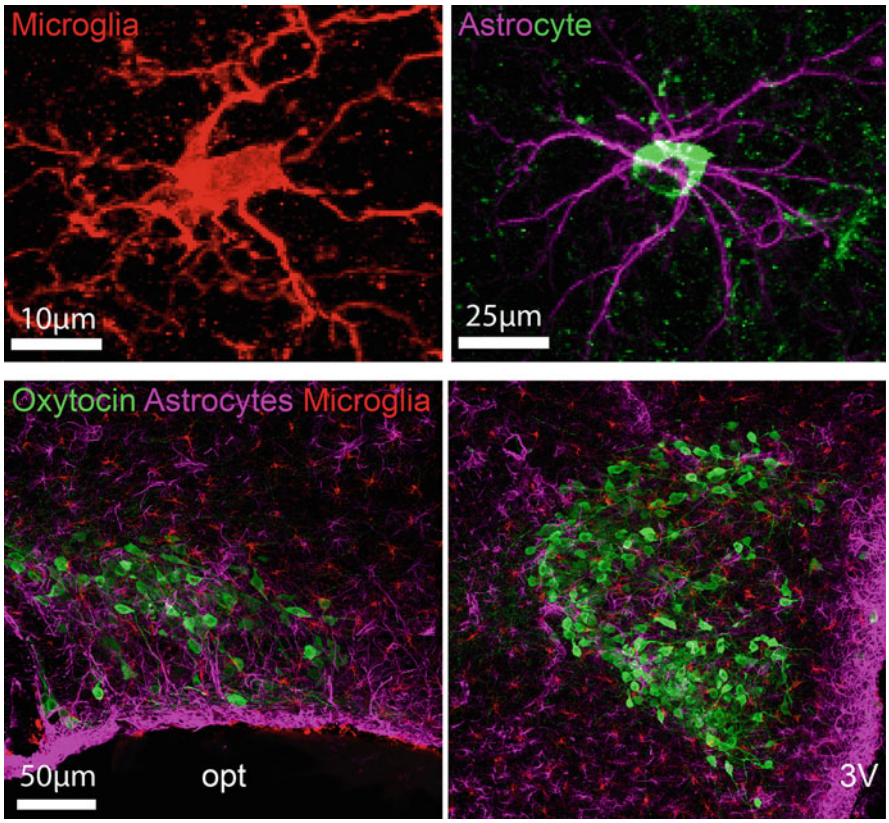

Fig. 5.2 Microglia and astrocytes in the rat hypothalamus. Top confocal images show individual microglia (red, anti-IBA1 immunofluorescence) and astrocytes (green, anti-glutamine synthetase immunofluorescence, and magenta, anti-GFAP immunofluorescence) under high magnification. Bottom panels show the distribution of microglia (red) and astrocytes (magenta) in the oxytocin-expressing (green, anti-oxytocin immunofluorescence) supraoptic (left panel) and paraventricular (right panel) nuclei

5.3.4 Role of the Renin–Angiotensin System (RAS) in Mediating Reactive Astrocytes and Microglia Cell Activation in the SON and PVN

A common denominator in several of these cardiometabolic diseases is activation of the renin–angiotensin system (RAS) (Diaz et al. 2019). Compelling evidence supports that overactivation of both the peripheral and brain RAS in hypertensive and heart failure conditions contributes to increased vascular tone, Na+ retention, volume expansion, and sympathohumoral activation. Indeed, some of the most efficient pharmacological tools available for the treatment of these diseases target either AngII receptors or angiotensin converting enzymes.

Importantly, AngII is also recognized as a potent pro-inflammatory molecule, and a growing body of evidence points to AngII as a candidate mediating hypothalamic neuroinflammation in cardiometabolic disease. AngII receptors in the brain have been assumed to be exclusively expressed in neurons. While still a controversial topic (de Kloet et al. 2015), we recently demonstrated that AngII AT1 receptors are also expressed both in astrocytes and microglial cells of the PVN. Indeed, we reported that AT1 receptor activation in astrocytes inhibits GLT1 transporter activity, resulting in the buildup of extracellular glutamate and activation of eNMDARs in PVN neurons. We proposed this to be one of the key mechanisms by which AngII mediates increased neuronal activity and neurohumoral outflow from the PVN (Stern et al. 2016).

Whether microglial cells can also be directly targeted by the RAS is also a controversial issue, with conflicting results described in the literature. In a recent paper, we demonstrated that AT1 receptor mRNA is present in PVN microglia, and that AT1 receptor stimulation results in microglia activation and oxidative stress (Biancardi et al. 2016). Interestingly, these AngII effects on microglia required Toll-like receptor 4-mediated signaling, supporting a functional interaction between the RAS and innate immune signaling in mediating RAS-dependent neuroinflammation in the PVN. Moreover, we also showed that AngII-mediated signaling contributes to microglia activation and PVN neuroinflammation during hypertension (Biancardi and Stern 2013).

5.3.5 Compromised PVN Blood–Brain Barrier Integrity as Part of the Neuroinflammatory Response During Hypertension

Finally, as stated above, glial cells play critical roles in maintaining the integrity of BBB. A compromised BBB permeability has been described in several neurological disorders that have a strong neuroinflammatory component, such as stroke, traumatic brain injury, hypertension, and dementia, among. Moreover, RAS blockade has been shown to have neuroprotective effects when used in these conditions. Thus, we aimed to investigate whether AngII pro-inflammatory effects in the PVN could also lead to BBB disruption in hypertensive conditions. To this end, we developed a relatively simple imaging approach, based on the intravascular infusion of two fluorescent dyes of different colors (red and green) and molecular sizes (70 kDa and 10 kDa, respectively), to quantitatively assess the level of disruption of BBB permeability, in this case, in a rat model of hypertension. The principle of this approach is that under control conditions with an intact BBB function, both dyes circulate intravascularly, without extravasating into the tissue parenchyma. During pathological conditions involving increased BBB permeability, the small-sized dye (green) will be able to extravasate into the parenchyma, whereas the large-sized dye (red) would still remain intravascularly. Thus, using simple imaging algorithms, it is possible to isolate and quantitatively measure pixels containing small green dye located extravascularly (see Fig. 5.3 and (Biancardi et al. 2014; Biancardi and Stern 2016) for detailed information about this procedure). Using this approach, we found

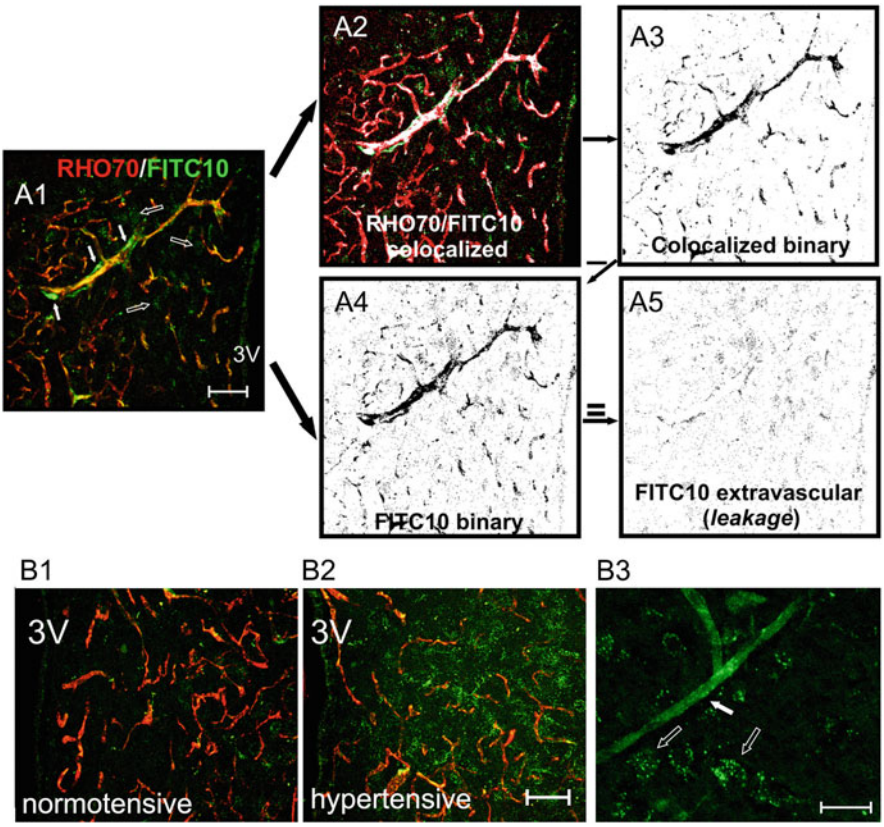

Fig. 5.3 Imaging approach to quantify changes in blood–brain-barrier permeability. (**a1**) Sample of labeling of the PVN microvasculature following the simultaneous intracarotid injection of the small size dextran-FITC 10 kDa (green) and the large size dextran-rhodamine 70 kDa (RHO70, red). (**a2**) The first step in this process consists in detecting individual pixels that contain both signals (green and red, shown in white color). These pixels showing colocalization are isolated and a separate image is generated (**a3**). Concurrently, a binary image containing only the FITC 10 green signal (small dye) is obtained (**a4**). Finally, to isolate and quantify the extravasated FITC 10 signal, image A3 is digitally subtracted from image A4, resulting in a new image containing only the extravascular FITC 10 signal (**a5**), which is then used for densitometry analysis. (**b**) Representative images showing increased extravasated small-size dextran-FITC 10 kDa (green dye) but not large size dextran-rhodamine 70 kDa (RHO70) (red dye) in the PVN of a hypertensive (**b2**), compared to a normotensive (**b1**) rat. A sample of FITC 10 dye located in the PVN parenchyma (empty arrows) around a nearby vessel (filled arrow) is shown in **b3**. Scale bars = 50 μm. 3V: third ventricle. Panels **a** and **b** modified from Biancardi et al., Hypertension 2014 and Biancardi and Stern, J Physiol 2016, respectively)

that elevated circulating levels of AngII during hypertension resulted in the disruption of the BBB integrity, allowing access of circulating AngII to the PVN. Interestingly, the leaked AngII was found to be predominantly bound to microglial cells (Biancardi et al. 2014; Biancardi and Stern 2016).

Finally, it is important to mention that a RAS-mediated neuroinflammatory response is not a phenomenon restricted to the PVN or hypothalamus, given that a contribution of the RAS to neuroinflammation in other brain regions and pathologies such as Parkinson's disease (Rodriguez-Perez et al. 2018) has also been demonstrated recently.

5.4 Experimental Approach to Monitor Microglia Activation During the Neuroinflammatory Response

One of the main challenges in studying microglial morphology and function is choosing a reliable technique that reports structural and functional changes in an unbiased manner. While conventional measurements of cytokine mRNA or protein levels, as well as immunohistochemical staining for specific microglial markers, have been around for quite a while, they provide only indirect evidence of microglial activation. During neuroinflammation, microglia undergo a morphological transition from a highly ramified to a deramified state, retracting their fine processes and experiencing an overall reduction in cell volume (Nimmerjahn et al. 2005). Although it seems evident that a comprehensive knowledge of microglial deramification is of paramount importance, very little is known about the precise series of events that ultimately lead to this phenomenon, highlighting the need for tools that allow a detailed morphometric analysis of microglial remodeling. While classical markers such as increased microglial density, increased expression of ionized calcium-binding adapter molecule 1 (IBA1), and various cytokines are widely used to assess neuroinflammation, they fall short of addressing detailed microglial morphological changes during this pathological process. This is critical because diverse microglial morphometric features are not only associated with diverse microglial functions, but more importantly, they also have been associated recently with different stages in the spatio-temporal progression of the neuroinflammation process (Prinz et al. 2019). In this sense, classical two-dimensional maximum projection analysis is insufficient to provide detailed information about microglial features such as changes in cellular or somatic volume. In fact, it becomes clear that detailed three-dimensional analysis or microglia cell morphology is of paramount importance, especially considering microglial heterogeneity and brain region-specific differences in size, density, and activation stages.

Our lab recently developed a comprehensive glial profiler on the basis of the IMARIS (Bitplane, Oxford Instruments) software, which allows rapid, unbiased, and flexible three-dimensional reconstruction of glial surface and filaments (Althammer et al. 2020). An overview of the individual steps of the three-dimensional reconstruction can be found on Fig. 5.4. The uploaded videos highlight the three-dimensional rotation and the features of the glial profiler. With this approach, we quantified and analyzed microglia and astrocytes in the PVN in rats with heart failure. We found that already 8 weeks after the surgical ligation of the coronary artery, microglia transitioned into a high-activity state and displayed somatic swelling and retraction of their processes. These two microglial alterations were

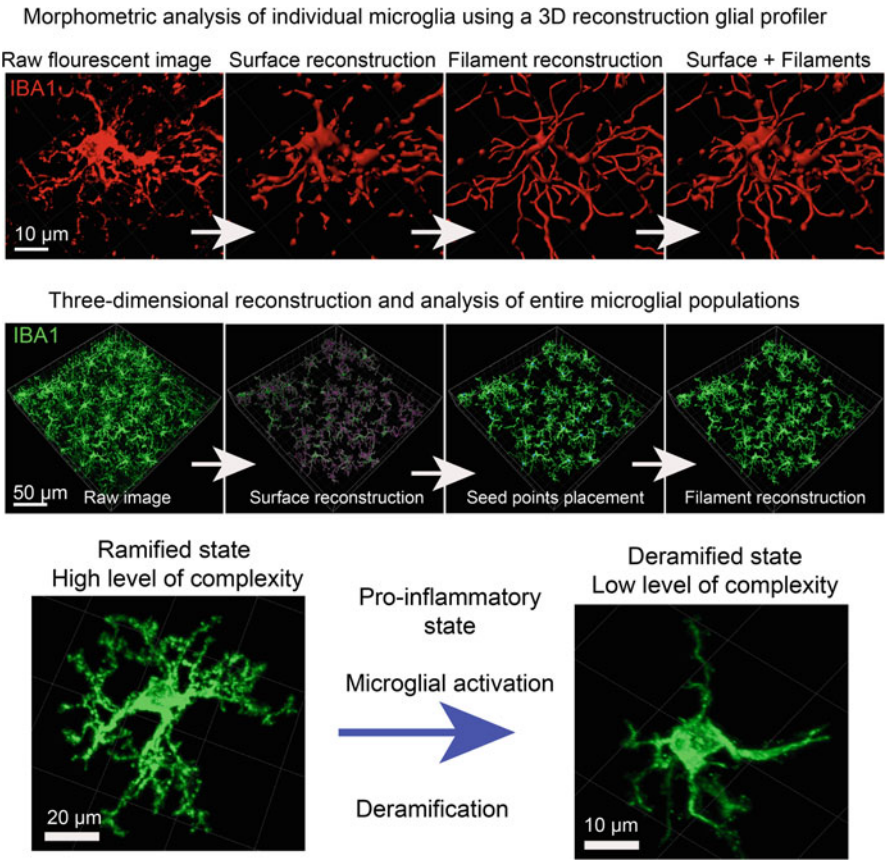

Fig. 5.4 Three-dimensional reconstruction of microglia via IMARIS. Raw z-stacks of fluorescently labeled microglia are used for the three-dimensional reconstruction. Surface and filaments are reconstructed in a two-step process and the final reconstruction can be used to calculate various glial parameters such as surface, cell volume, branches, and complexity. During injury, disease, and neuroinflammation, microglial cells retract their processes and become less ramified, which is usually referred to as *microglial activation.* This term can be slightly misleading, given that microglia are highly active cells even in the basal state. The precise description of the three-dimensional reconstruction of glial cells can be found in Althammer et al. (2020)

highly correlated, indicating that somatic swelling and deramification are processes that occur in parallel within the same microglial cells. In addition, we found convincing evidence for A1 astrocytes 14 weeks after the surgery through genetic quantification of A1 markers via qPCR and assessment of astrocyte morphology using the 3D glial profiler.

Recent technical advances have made it possible to monitor microglial activity, calcium transients, and morphometric changes in vitro and in vivo. While our recently developed glial profiler allows to monitor microglial population shifts and respective changes in microglial morphology, these tools are very useful to study

acute effects of various compounds on microglial activity and function in individual, non-fixed cells. Further improvement of these techniques will be necessary to specifically manipulate microglial function in vivo and in vitro, with methods similar to those that have been used to activate/inhibit both neurons and microglia under various conditions.

5.5 Perspectives

The respective roles of astrocytes and microglia in healthy and diseased brains have been extensively studied (Eroglu and Barres 2010; Liddelow and Barres 2017). However, several questions regarding the transition from healthy to diseased states remain for both cell types and require further studies. For instance, it remains unclear how the still heavily debated concept of *gliotransmission* changes during various disease conditions. How do neurodegenerative diseases, chronic neuroinflammation, and CNS injury affect neuron–glia communication, and ultimately information processing and function in the brain? How exactly does disturbed astrocyte function and signaling translate into impaired network function, which can be observed in various cognitive disorders and neurodegenerative diseases? The recent discovery of A1 astrocytes that become neurotoxic upon induction by activated microglia represented a milestone in neuroinflammation research (Liddelow et al. 2017). However, the precise role and identity of the neurotoxin is currently still unknown (Liddelow and Barres 2017; Liddelow et al. 2017). While most of the work in the field of neuroinflammation has been focused on cognition-related brain areas, including the cortex and the hippocampus, important findings regarding the contribution of astrocytes and microglial cells to altered hypothalamic network functions in disease states has recently started to emerge. These studies provide compelling evidence for a critical contribution of altered astrocyte and microglia function to exacerbated neuronal activity as well as autonomic and neuroendocrine outputs (e.g., neurohumoral activation) in prevalent cardiovascular diseases, particularly heart failure. Moreover, a growing body of evidence supports astrogliosis in the arcuate nucleus as a key mechanism underlying compromised energy-related signaling in the hypothalamus during obesity. Finally, recent clinical studies provide compelling evidence supporting a high degree of comorbidity between cardiometabolic diseases and cognitive impairment and mood disorders (Hammond et al. 2018), with neuroinflammation standing as a potential common underlying mechanism. Thus, changes in hypothalamic neuro–glial communication in disease states could have important pathological impacts beyond autonomic and neuroendocrine regulation. Recent advances in microglia and astrocyte research have made it possible to label and manipulate glial cells in a cell type-specific manner to provide detailed insights into their origin, genetic variability, and functions during health and disease. Understanding the precise molecular architecture and functional roles of glia will help to develop tailored approaches to treat patients suffering from a myriad of cognitive, neurodegenerative, and even developmental diseases that involve one or more types of glial cells.

5.6 Key Literature

Allen and Eroglu (2017) Comprehensive review about the interaction between astrocytes with neurons and synapses.

Althammer et al. (2020) Development of a three-dimensional morphometric glial profiler used to highlight drastic changes to microglial and astrocytic morphology during heart failure.

Araque et al. (1999) Review of the seminal finding that astrocytes are an active participant in the tripartite synapse.

Brown et al. (2013) The most detailed and comphrehensive review about magnocellular neurons, their function, their in- and outputs and modulation of various vital physiological processes.

Eroglu and Barres (2010) The role of glia in synaptogenesis, synapse modulation and synapse elimination.

Liddelow and Barres (2017) The role of reactive astrocytes during neuroinflammation.

Ludwig and Leng (2006) The mechanism of somato-dendritic release of neuropeptides and its consequences on local networks and physiology.

Prinz et al. (2019) Comprehensive overview of microglial concepts, microglial development and microglial function.

Stern (2015) Review about the relationship of somato-dendritic release and neuro-endocrine integration in the hypothalamus and its link to pathophysiological conditions.

Zhao et al. (2015) Fantastic review on the blood-brain barrier and its role in the healthy and diseased brain.

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Part III

Glial–Neuronal Interactions in the Control of Metabolic Function

Control of Systemic Metabolism by Astrocytes in the Brain

6

Ophélia Le Thuc, Tim Gruber, Matthias H. Tschöp, and Cristina García-Cáceres

Abstract

Astrocytes are specialized glial cells that are embedded in a framework of neurons and act as an interface between neurons and the vasculature in the brain. This privileged, interconnecting position has recently been shown to render these cells crucial in the central control of systemic metabolism by allowing them to sense and convey blood-borne information within the brain and, in turn, critically fine-tune properties of neuronal networks that calibrate energy intake and expenditure. For decades, however, these neuronal networks have largely occupied the limelight regarding the study of energy homeostasis. Accordingly, the aim of this chapter is to summarize the paradigm shift currently taking place in studies of the central control of energy balance occurring over the last years, from a rather

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"neurocentric" view towards a more holistic perspective in which the role of other cell types, such as astrocytes, is increasingly appreciated. Finally, we will discuss recent cutting-edge methodological approaches emerging in the field that allow for the study of astrocytes, presently or yet to be conceived, which will provide a further and more complete understanding of the central regulation of energy metabolism.

Keywords

Astrocytes · Gliotransmission · Calcium · Tripartite synapse · Gliosis · Metabolism

6.1 Introduction

Astrocytes are a specialized type of glial cell—also known as "astroglia." To note, the prefix "astro" refers to the star-like shape of these cells. Although neurons have long been the center of attention in neuroscience, it is important to highlight that an intimate and coordinated association between astrocytes and surrounding neurons is required to carry out all biochemical and physiological processes that occur in the brain. Beyond merely supporting neurons, astrocytes have recently started to draw more attention due to their involvement with neurons to ensure brain function in general, such as for the homeostatic regulation of body weight and systemic metabolism (Garcia-Caceres et al. 2019).

6.1.1 A Brief History of Astrocytes

The mammalian brain contains billions of cells, but the existence of cell types distinct from neurons only gained recognition around 1825. At that time, the common hypothesis among neuroscientists was that the primary function of the "mass" of cells, in which the main neural elements were nested, would be to serve as some kind of connective tissue. Accordingly, this mass of non-neuronal cells was conflated and jointly coined "neuroglia" by Rudolf Virchow in the 1850s and was thought to merely fulfill passive functions such as providing structural support. Eventually, between the end of the nineteenth century and the beginning of the twentieth century, Golgi described glia as round cells developing many fine processes in each and every direction. He also already determined using silver chromate staining that glial cells: (1) are diverse; (2) form networks; and (3) direct their glial endfeet to the blood vessels.

In 1895 the term "astrocyte" was introduced by von Lenhossék to describe a subtype of the parenchymal glia. Interestingly, the use of this term was mostly propagated after Ramón y Cajal developed a gold chloride-sublimate staining to label glial-fibrillary acidic protein (GFAP), in both protoplasmic and fibrous astrocytes, which is still to this day the most utilized marker to visualize astrocytes.

In the mid-twentieth century, when A. L. Hodgkin, A. F. Huxley, and others elegantly described neuronal electrical properties, neurons became the predominant focus of investigation in nervous system function, heralding decades of research on the effects of ionic flow through neuronal membranes and action potentials. Consequentially, astrocytes were ignored for several decades, demoted to supporting actors for the neuronal leading roles. In recent decades, this imbalance has gradually diminished, and neurons and glial cells comprising the nervous system are now seen as vital and interacting partners in a sophisticated and well-coordinated communication network. Nowadays, recent advances in biology, optics, genetics, and pharmacology, combined with the use of genetically engineered animals, are establishing new strategies to further investigate the physiology and functional contribution of astrocytes to the regulation of neuronal function in health and disease.

6.2 Biology and Physiology of Astrocytes: Characteristics and Function

Astrocytes were thought to be less relevant than neurons, being considered merely as a scaffold for the latter. Yet, over time, the scientific community has uncovered and attributed an extensive number of functions to astrocytes. We now know that these glial cells are essential for: (a) maintaining homeostasis of the central nervous system (CNS) through modulation of neurotransmitter levels (glutamate–glutamine cycle), maintaining ionic equilibrium (K^+ and H^+ buffering), limiting reactive oxygen species (ROS) (in glutathione recycling), and participating in osmotic regulation; (b) regulating cerebral blood flow by interacting with endothelial cells of microvessels and pericytes that form the blood–brain barrier (BBB); (c) mounting the brain's line of defense together with the meningeal lymphatic system and microglia (another type of glial cells); and (d) proper neuronal function, by performing critical activities such as providing neurons with energy substrates, and more generally by regulating synaptic sculpting (genesis, maturation, elimination), synaptic plasticity and synaptic transmission, which are crucial not solely during development, but also during adulthood (Fig. 6.1).

6.2.1 Astrocytes Are Critical for Energetics of the CNS

The brain is the most energy-demanding organ. While weighing only 2% of the total body mass, the brain requires 25% of the circulating glucose for maintaining its regular function under physiological conditions. Astrocytes, like neurons, uptake and metabolize glucose and other energy substrates to generate energy in the form of ATP, necessary for the normal functioning of the cell. Interestingly, glucose is the preferred energy substrate in the brain, and only astrocytes—during adulthood and under physiological conditions—are able to accumulate and store glucose in the

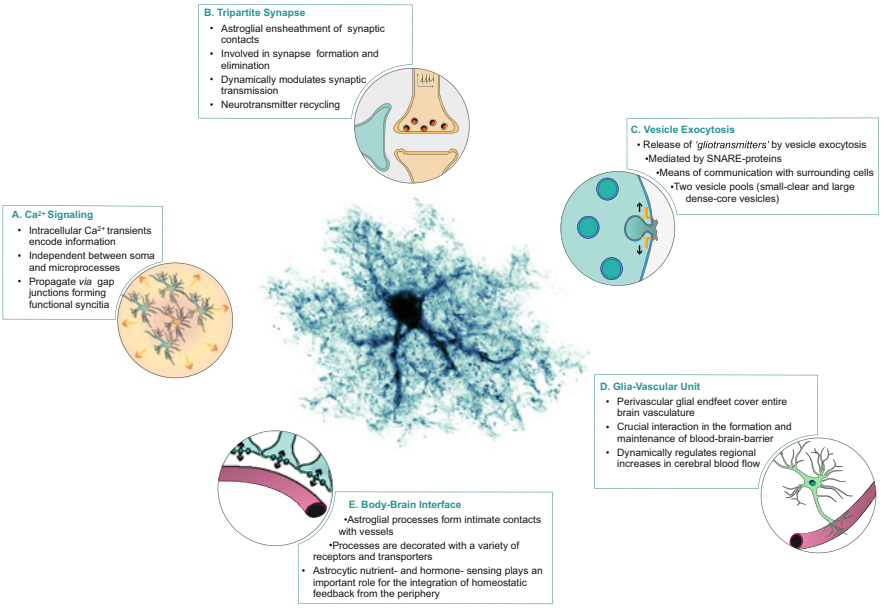

Fig. 6.1 Fundamentals of astrocyte function in the CNS. Astrocytes take on a broad range of functions in the brain and are considered gatekeepers of central nervous system (CNS) homeostasis. (**a**) They encode information by means of intracellular Ca^{2+} signals, which eventually even spread to neighboring astrocytes via gap junctions, forming what resembles functional astrocyte networks. (**b**) Moreover, astrocytes ensheath synaptic connections with their fine processes to form the tripartite synapse, which is the trademark for regulating neuronal transmission. (**c**) In addition, astrocytes contain various types of intracellular vesicles, whose cargo can be released via Ca^{2+}-regulated exocytosis; following fusion with the plasma membrane, these vesicles release their signaling cues, also called "gliotransmitters," to act on surrounding cells. (**d**) Furthermore, astrocytes constitute an integral part of the neuro-glia-vascular unit. By directly sensing neuronal activity, astrocytes in turn guide cerebral blood flow towards activated brain regions to support locally increased energy demands. Astrocytes are also crucial for the development and maintenance of the blood–brain barrier together with endothelial cells and pericytes. (**e**) Lastly, astrocytes take center stage in sensing and integrating homeostatic feedback signals emanating from the periphery. A variety of receptors and transporters are distributed throughout astrocytic processes, which extensively cover the cerebral vasculature. Thus, astrocytes are ideally situated and equipped to detect blood-borne signals and modulate their entry into the brain

form of glycogen, allowing them to secure the energy supply for neurons under conditions of decreasing circulating glucose levels.

In the adult CNS, under certain conditions associated with a glucose deficit, the brain activates one of its complex homeostatic mechanisms to maintain normal neuronal activity. This mechanism consists of astrocytes breaking down their glycogen stores into lactate, which is then provided to neurons to sustain oxidative metabolism. Glycogen is also used to support long-term potentiation in neurons, which experimentally correlates with learning and memory consolidation (Drulis-

Fajdasz et al. 2015). This concept was introduced by Magistretti and Pellerin in 1994 when they proposed the existence of an astrocyte–neuron lactate shuttle for the supply of energy substrates to neurons in an activity-dependent, glutamate-mediated manner (Pellerin and Magistretti 1994). They demonstrated that cortical neurons use lactate or glucose indistinctly to support oxidative metabolism. Yet, other studies have questioned this astrocyte–neuron metabolic pathway and suggest that lactate could have an additional signaling role rather than being solely another energy source for neurons.

Among other energy substrates, astrocytes utilize fatty acids (FA) to generate ATP. Indeed, in the CNS, astrocytes are the major site of FA oxidation. As a matter of fact, it has been reported that astrocytes oxidize FA to meet their energy requirements during low-fat diet intake, whereas they switch their energy metabolism to generate ketone bodies from the excess FA during high-fat diet intake. Once produced, the ketone bodies leave the astrocytes via the monocarboxylate transporter (MCT)-1 and enter the neurons via MCT-2. In neurons, ketone bodies are metabolized by mitochondria as another metabolic fuel source, notably used in states of starvation (Le Foll and Levin 2016; Puchalska and Crawford 2017).

6.2.2 Astrocyte Networks and Diversity: Morphological and Molecular Hallmarks

Astrocyte Networks Each astrocyte occupies a specific territory in non-overlapping domains defined by its finger-like processes that can interact with blood vessels, individual neighboring neurons, synapses, and other cells, thus forming a unique functional network structure. Apart from physically interacting with neurons and other cells, astrocytes are engaged in extensive astrocyte–astrocyte communication through gap-junction channels formed by connexins 43 and 30. These connexins-mediated gap junctions associate astrocytes to form specific astrocytic networks that act as functional metabolic units and are directly involved in activity-dependent trafficking of glucose and its metabolites from blood vessels to neurons imbedded into these astrocytic networks.

Astrocyte Diversity An individual astrocyte is typically recognized as having a stellate-like morphology with fine, rather long, and numerous processes extending from the soma. In the early 1900s, astrocytes were grouped into two main sub-types: fibrous and protoplasmic astrocytes. Fibrous astrocytes are located in the white matter and display the "prototypic" star-like shape attributed to astrocytes, with rather regular contours and processes, whereas the protoplasmic astrocytes are located in the gray matter and characteristically display a more irregular shape that has been referred to as "bushy." We now know that the majority of protoplasmic astrocytes show an extensive and elaborate arborization, which exhibits more of a sponge shape rather than that of a star.

Regardless of astrocytic diversity (Fig. 6.2), most studies are still primarily targeting astrocytes by using GFAP, retaining it as the prevailing astrocyte marker.

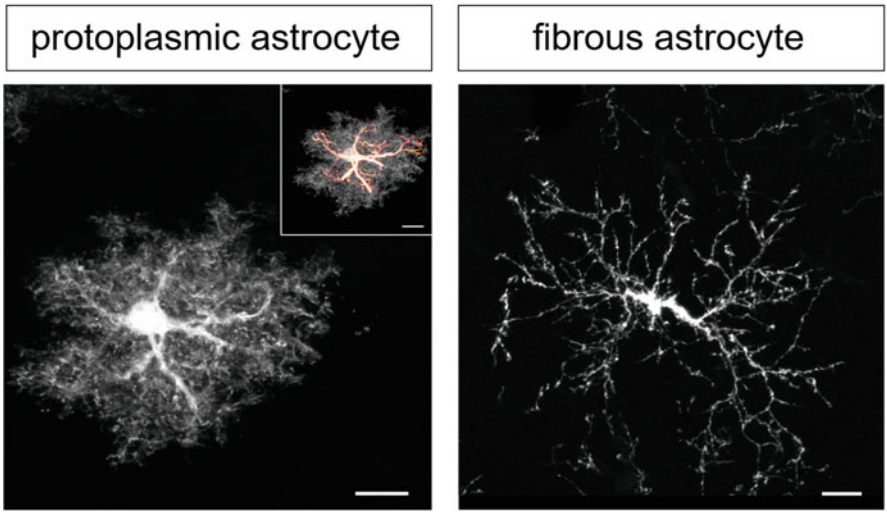

Fig. 6.2 Morphological diversity of astrocytes in the CNS. The central nervous system contains several, morphologically distinct subclasses of astrocytes. The images show two of the major astroglial "morphotypes" visualized in mice expressing enhanced green fluorescent protein (eGFP) under transcriptional control of the GFAP promoter. Protoplasmic astrocytes (found in gray matter) exhibit a round and bushy appearance with highly arborized processes. In contrast, fibrous astrocytes (found in white matter) appear elongated with long and less complex processes. Notably, the morphological complexity of astrocytes remained elusive for a long time since the most common visualization method relied on the marker GFAP (see Table 6.1), which only reveals the primary, star-shaped processes (inset; red). Scale bars: 10 μm

By doing so, these studies might overlook the fact that GFAP only encompasses one of the several astrocyte populations, and there are other astrocyte-specific molecular markers that allow for the visualization of these glial cells (Table 6.1). Unfortunately, so far, no universal marker has been identified to visualize all astrocytes indistinctly.

The variety of available markers supports the heterogeneity of astrocytes, which could also define some functional aspects of these subpopulations. In fact, an individual astrocyte usually does not express only one of these markers, but rather a combination of them (Verkhratsky et al. 2016), which can be influenced by the surrounding micro-environment and neighboring cells. This molecular diversity is indicative of the great heterogeneity characterizing astrocytes, with inter- and intra-regional features, both in their function and phenotype (Ben Haim and Rowitch 2017). Yet, recent evidence indicates that astrocytes can modify and adjust their molecular and functional properties depending on the surrounding neural circuits and stem from the energetic demands of the extracellular space in which they are located (Farmer et al. 2016; Hasel et al. 2017).

Table 6.1 Astrocytic markers

Marker	Function	Properties
GFAP (glial-fibrillary acidic protein)	Structural protein from the intermediate filament system	In the CNS, GFAP is only expressed in a subset of astrocytes, with high regional variability. GFAP expression is upregulated in CNS injury and/or disease
Vimentin	Structural protein from the intermediate filament system	Regarding its expression, reported in both astrocytes (mostly expressed in reactive ones, subsets of both protoplasmic and fibrous astrocytes and immature astrocytes) and tanycytes
GLAST or EAAT-1 (glutamate aspartate transporter-1 or excitatory amino acid transporter-1)	Glutamate transporter	In the CNS, it is expressed in astrocytes primarily to allow for the clearance of glutamate from the synaptic cleft. An extensive expression of this marker has been visualized throughout the brain in mice with green fluorescence protein driven by the GLAST promoter
GLT-1 or EAAT-2 (glutamate transporter-1 or excitatory amino acid transporter-2)	Glutamate transporter	As with the GLAST marker, it has been reported to primarily allow for the clearance of glutamate from the synaptic cleft. It is also expressed in axon terminals
Aldh1L1 (aldehyde dehydrogenase 1 family, member L1)	• Enzyme for folate metabolism • Contributes to nucleotide synthesis and to cell division	This marker together with GLAST has a broader expression than GFAP in the brain. Yet, its expression varies with age, and in the CNS it can also be detected in some oligodendrocytes. It is mostly expressed in the cytosol
S100β (S100 calcium-binding protein B)	Ca^{2+}-binding protein (both buffer and sensor)	Mostly expressed in mature astrocytes and increases under pathological conditions in the CNS
GS (glutamine synthase)	Enzyme allowing the conversion of ammonia and glutamate into glutamine	This marker is mostly cytosolic and expressed in most astrocytes
Cx43 and Cx30 (connexins 43 and 30)	Allow the formation of hemichannels connecting astrocytes and can control the release of small molecules (e.g., ATP) from astrocytes	Cx43 and Cx30 are astrocyte-specific connexins. Cx43 is the major connexin in astrocytes, whereas Cx30 is only expressed in astrocytes from the gray matter in which it is mostly expressed in the astrocytic processes and endfeet

(continued)

Table 6.1 (continued)

Marker	Function	Properties
AQP4 (aquaporin 4)	Plays a key role in water movements across astrocytic plasma	Within the CNS, this aquaporin is solely expressed in astrocytes. AQP4 is mostly expressed in the endfeet
SOX9	Transcription factor Sox9	This marker is highly enriched in astrocytes and exhibits substantial overlap with the GLT-1-expressing astrocyte population, labeling astrocytes outside of the neurogenic regions
ACSA-1 and -2 (astrocyte cell surface antigen-1 or 2)	Glycosylated surface molecule of murine astrocytes at all developmental stages	ACSA-1 and -2 labeling show largely overlapping expression with GLAST-expressing astrocyte population. ACSA-1 and -2 labeling is mostly used in magnetic-based isolation of astrocytes from the CNS

6.2.3 Astrocytic Ca^{2+} Signaling: The Trademark of Astrocyte Communication

The underlying principles of information processing by the brain remain one of the greatest enigmas in neuroscience. Despite being considered electrically silent, astrocytes actually do assist in neural encoding and utilize various mechanisms to propagate information to surrounding astrocytes through astroglial networks by employing a form of **intracellular ion waves**, mainly Ca^{2+} (Rusakov 2015) (Fig. 6.1 and Video 6.1). The Ca^{2+}-dependent propagation of information from a single astrocyte to neighboring astrocytes allows for local and coordinated signaling synchronization with the surrounding cells, including neurons and endothelial cells of the microvasculature (Arcuino et al. 2002; Gordon et al. 2008; Schummers et al. 2008). Likewise, astrocytes can respond in a Ca^{2+}-dependent manner to fluctuations in neuronal activity occurring in the surrounding synapses, and, as these glial cells are also in direct contact with the microvasculature, they consequently act as intermediates in translating neuronal function into changes in the local blood flow (Rossi 2006). Indeed, Ca^{2+} signaling in astrocytes also contributes to neurovascular coupling to regulate local cerebral blood flow by eliciting vasoconstriction or vasodilation of arterioles (Metea and Newman 2006). Interestingly, recent technical advances and improvements in real-time monitoring and manipulation of in vivo changes in Ca^{2+} signaling in astrocytes (Fig. 6.3 and Table 6.2—Ca^{2+} indicators) have revealed (a) that intracellular Ca^{2+} transients and oscillations in these glial cells

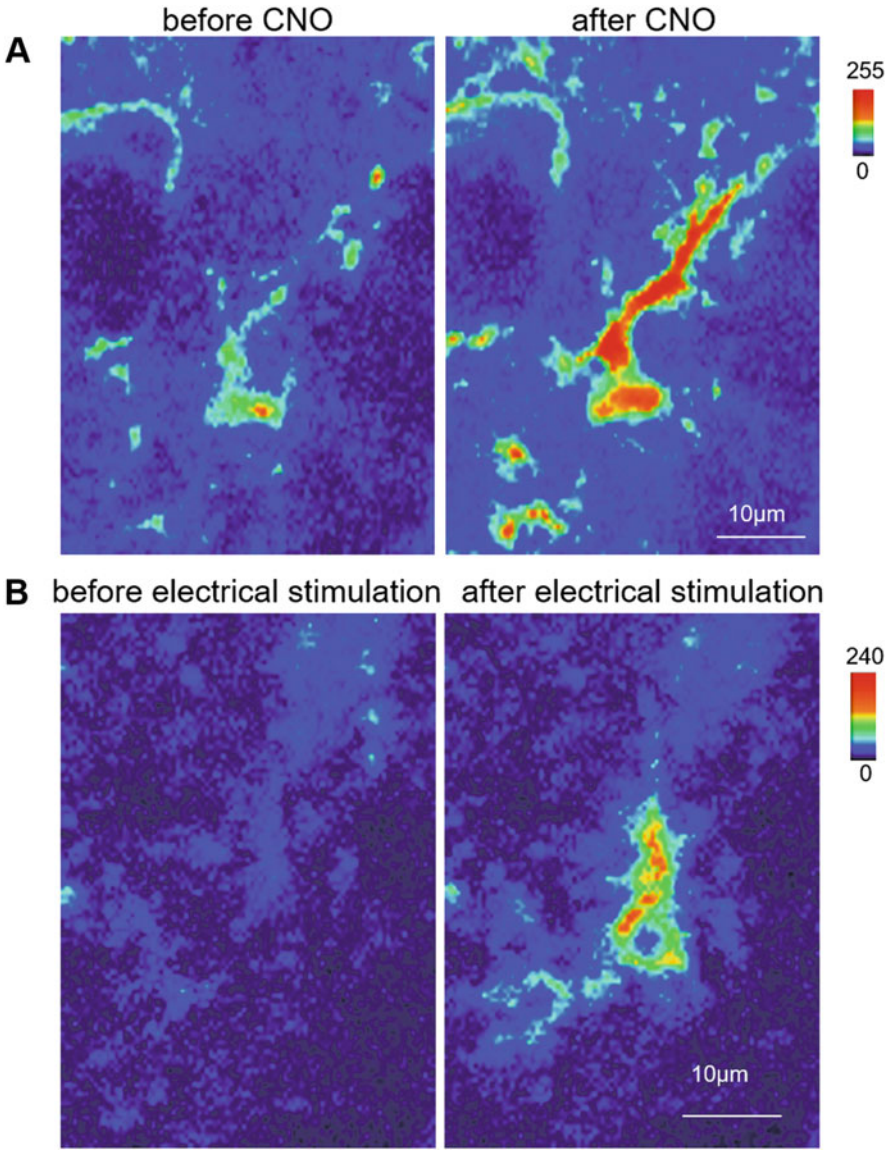

Fig. 6.3 Visualizing astrocyte Ca^{2+} responses to diverse stimuli using genetically encoded Ca^{2+} indicators (GECIs) in Ca^{2+} brain slices. Pseudocolor images representing fluorescence intensities indicative of Ca^{2+} responses in dorsolateral striatum resident astrocytes expressing GCaMP6f (part of the GECIs, coupled with the green fluorescent protein; see Table 6.2) before (left) and after (right) (**a**) application of clozapine-N oxide, an agonist of the hM3Dq DREADD (designer receptors exclusively activated by designer drugs; see Table 6.3) or (**b**) electrical stimulation of corticostriatal axons to evoke astrocyte calcium-dependent signal

Table 6.2 Genetically encoded sensors for Ca^{2+} (GECI) and lactate

GECIs and lactate	Application	Availability	References
GCaMP1-6	Series of fluorescent Ca^{2+} sensors; emission properties change proportionally to intracellular Ca^{2+} concentrations	Cre-lox system, viral vectors, in utero electroporation	Yu et al. (2020) See Fig. 6.3 for the visualization of changes in Ca^{2+} concentrations via GCaMP6f
Lck-GCaMP3-6	Improved variant of fluorescent Ca^{2+} sensor; membrane-tethering of sensor facilitates visualization of Ca^{2+} dynamics even in finer cellular compartments such as in processes		
Laconic	Fluorescent lactate sensor; emission properties change proportionally to intracellular lactate concentrations	Viral vectors	Machler et al. (2016)

differ substantially in timing and amplitude depending on whether changes in the Ca^{2+} waves occur in the astrocyte's soma or its processes (Yu et al. 2020) and (b) the presence of subtle, asynchronous Ca^{2+} dynamics in microdomains of glial processes (Fig. 6.1). Importantly, alterations in astrocytic Ca^{2+} homeostasis have been reported to occur with brain injury, reflecting healing processes or pathophysiology (Hamby and Sofroniew 2010).

6.2.4 Astrocytes: Secretory Cells Within the CNS

At the beginning of the 1900s, a pioneering study of neuroglia by Held revealed the presence of granular inclusions in astrocytes, which hinted towards a putative secretory pathway in these cells. This suggested that astrocytes constitute actively communicating cells that respond and signal to the surrounding cellular partners via the release of diverse chemical substances (Held 1909). A century later, this early hypothesis of a secretory compartment present in astrocytes was ultimately confirmed: astrocytes were shown to release signaling cues, also referred to as gliotransmitters (ATP, glutamate, D-serine), to neighboring neurons and other glial cells to regulate synaptic function, a process currently known as gliotransmission (Araque et al. 2014). Therefore, astrocytes, like neurons, are secretory cells with the ability to send molecules and ions back and forth between themselves and neurons, other glial cells, and blood vessels, and to control all physiological processes in the brain, including the activity and plasticity of local neuronal networks. In fact, neuron-derived transmitters can activate G protein-coupled receptors (GPCRs) in astrocytes, resulting in elevation of intracellular Ca^{2+} concentrations, which induces

a fine-tuned and rapid exocytosis of gliotransmitters in a Ca^{2+}-dependent manner. Upon activation, astrocytes can also release glutamate, which regulates the dynamics of neuronal responses by controlling synaptic strength between neurons (Araque et al. 2014).

Gliotransmitter Release from Astrocytes Astroglial secretion is mainly regulated by cytoplasmic Ca^{2+} and Na^{+} signals, but several alternative pathways by which astrocytes signal to neighboring cells also appear to exist. In fact, a given molecule might be released through several of these pathways, which comprise: (1) vesicle-mediated exocytosis (Box 6.1); (2) diffusion through pores/channels; and (3) extrusion by transporters.

Box 6.1: Astrocytes Release Gliotransmitters via Ca^{2+}-Regulated Exocytosis

Vesicle-mediated exocytosis is primarily induced in response to increases in cytosolic free Ca^{2+}. The coupling of transient changes in intracellular Ca^{2+} and vesicle fusion relies on proteins of the so-called SNARE (soluble N-ethyl maleimide-sensitive fusion protein attachment protein receptor) family (Fig. 6.1). Upon surpassing a given Ca^{2+} threshold, the SNARE complex initiates a dramatic change in its molecular conformation, which ultimately triggers the fusion of vesicles with the astrocytic plasma membrane to release its cargo. The exocytotic machinery of astrocytes generally displays a lower sensitivity compared to neurons, which results in a rather lethargic stimulus-secretion coupling. Not only do exocytotic events in astrocytes occur with quite some delay, their overall number is also substantially lower compared to neurons (maximal secretion: 0.1–2/s in astrocytes and 3–6000/s in neurons) (Verkhratsky et al. 2016).

Types of Vesicles. At the ultrastructural level, two main distinct secretory organelles have been described: (1) synaptic-like microvesicles (SLMV) and (2) large dense-core vesicles (LDCV). SLMVs typically have a diameter of 30–100 nm and contain the aminergic "gliotransmitters" glutamate and/or D-serine (Fig. 6.1). Importantly, they are neither as densely packed nor as numerous as their neuronal counterparts and exist in small groups (ca. 15 vesicles) directly adjacent to neuronal structures. While SLMV appear small and clear (or electron-lucent) under electron microscopy inspection, a separate family of vesicles can be easily distinguished given their larger dimensions and higher electron-density. Referred to as LDCVs, these pools of vesicles typically harbor neuropeptides, hormones, and ATP and are generated at the trans-Golgi network. Astroglial LDCVs have a diameter of 100–600 nm and carry numerous and diverse substances, including neuropeptide Y (NPY), atrial natriuretic peptide (ANP), octadecaneuropeptide (ODN), brain-derived neurotrophic factor (BDNF), and ATP. Intriguingly, the SLMV and LDCV pools engage separate SNARE isoforms and non-overlapping mechanisms for regulated exocytosis.

6.2.5 Astrocytes Regulate Synaptic Plasticity and Transmission

The concept of "tripartite synapse" to define a bidirectional and rapid dialog between neurons and surrounding astrocytes was first used by Araque and colleagues in 1999 (Araque et al. 1999) (Fig. 6.1). Astrocytes project terminal processes to neighboring neurons, and both cell types exchange encoded information in a rapid, plastic, bidirectional manner through an extensive number of receptors, ion channels, and transporters expressed along their membranes. Neuronal activity triggers the release of neurotransmitters in the synaptic cleft that can induce the Ca^{2+}-dependent activation of proximal astrocytes, which in turn secrete gliotransmitters to ultimately modulate neuronal communication (Fig. 6.1). Several studies have also pointed out that the degree of astroglial ensheathment of the neuronal membrane influences the number and type of synapses—in the pre-, post-, and extra-synaptic elements—and shapes the local neuronal networks (Fig. 6.1). The perisynaptic astroglial processes rapidly remodel to strengthen or weaken synapses, which means that astrocytes influence synaptic plasticity. Additionally, and further supporting that astrocytes can influence synaptic events, astrocytes locally control neurotransmitter homeostasis by buffering the concentrations of presynaptically released glutamate and GABA at the synaptic cleft.

Until very recently, technical limitations had impeded further advancement in understanding how astrocytes regulate synaptic activity through the release of gliotransmitters. However, advances in targeting non-neuronal cells based on remotely controlling in vivo astrocyte activity and gliotransmitters release, in combination with novel bioengineered technologies applied in neuroscience (Table 6.3), now allow us to focus on fully understanding the relevance of astrocyte–neuron interactions in the control of brain function.

6.2.6 Astrocytes as Integral Components of the Neuro-Glio-Vascular Unit

Local regulation of cerebral blood flow is a crucial element for brain activity, especially in conditions of increased neuronal firing when vasodilation of the surrounding microvessels is required to respond to neuronal energy demands, also termed functional hyperemia (Fig. 6.1). Astrocytes are thought to be key active regulators of cerebral blood flow, as they are the first barrier line between the blood and neurons. Indeed, in the later part of the 1800s, the neuroanatomists Camillo Golgi and Santiago Ramón y Cajal had already speculated that astrocytes, due to their unique anatomical positioning within the brain, would be perfect candidates to elicit various functions. Localized between the vasculature and neurons, astrocytes are ideally placed to govern the brain–body interface and integrate homeostatic feedback from the periphery. In fact, astrocytes line the entire vasculature of the brain and provide complete blood vessel coverage by morphological specializations called perivascular endfeet (Fig. 6.1). Through both this physical contact and by releasing an array of soluble factors with vasoregulatory properties, astrocytes

Table 6.3 Technical approaches to manipulate astrocyte Ca^{2+}-dependent activity and gliotransmission

	Description	Availability	References
Manipulating Ca^{2+} signaling in astrocytes			
$IP_3R2^{-/-}$ (Itpr2)	Mutant mouse model globally lacking inositol triphosphate receptor 2 (IP_3R2), which is the major Ca^{2+} signaling pathway in astrocytes. Yet, it appears that lack of IP_3R2 only abolishes cytosolic Ca^{2+}, while transients in microprocesses remain unaffected given their independence of endoplasmic Ca^{2+} stores	Global knockout mouse line	Yu et al. (2020)
MrgA1R	Overexpression of a Gq-protein coupled receptor in astrocytes normally only found in nociceptive neurons; application of the agonist peptide (FLRFra) potently evokes Ca^{2+} signaling in astrocytes	Tetracycline-dependent system	Li et al. (2013)
hM3Dq	Overexpression of a permutated muscarinic Gq protein-coupled receptor that triggers Ca^{2+} signaling in astrocytes upon the administration of an inert ligand CNO (clozapine-N oxide)	Cre-lox system, viral vectors, in utero electroporation	Yu et al. (2020) See Fig. 6.3a for the visualization of changes in Ca^{2+} concentrations after activation of the hM3Dq DREADD in astrocytes
ChR2	Overexpression of light-sensitive opsins specifically in astrocytes to induce Ca^{2+} influx via blue light stimulation; caution must be taken into consideration because of major changes in pH and internal Na^+	Cre-lox system, viral vectors, in utero electroporation	Yu et al. (2020)
VIVIT	Overexpression of VIVIT-peptide in astrocytes to inhibit the calcineurin/Ca^{2+}-dependent activation of the transcription factor NFAT (nuclear factor of activated T cells) to ameliorate aspects of inflammatory astrogliosis	Viral vectors	Li et al. (2013)

(continued)

Table 6.3 (continued)

Blocking exocytosis			
LSL-iBOT	Overexpression of Botulinum neurotoxin B specifically cleaving SNARE proteins in astrocytes upon Cre-dependent recombination	Cre-lox system	Sahlender et al. (2014)
tetO-dnSNARE	Overexpression of a non-functional, dominant-negative (dn)SNARE protein, which competitively blocks regulated exocytosis in astrocytes upon doxycycline administration	Tetracycline-dependent system	Sahlender et al. (2014)
Astrocyte–neuron interaction			
NAPA	Neuron–astrocyte proximity assay (NAPA) comprises two-component fluorescent markers and utilizes Forster-resonance energy transfer (FRET) to derive information on astroglia–neuron spatial interactions	Viral vectors	Yu et al. (2020)

contribute to the formation and maintenance of the BBB (Fig. 6.1). Moreover, astrocytes tune the properties of the endothelium in order to regulate the entry of nutrients and hormones. As previously mentioned, by forming the first line of cells behind the BBB, astrocytes are ideally positioned to rapidly sense and adjust to changing levels of nutrients and other factors. Astrocytes are fully equipped to act as putative "metabolic sensors," given that they express a wide array of receptors and transporters distributed throughout their extensive cell surface area. While the BBB-associated astrocytes effectively shield the brain from changes in the blood milieu that could be devastating, they equally hamper the intended delivery of drugs to the brain—including potential candidates for the treatment of brain diseases.

6.2.7 Astrocytes in the Brain Control of Systemic Metabolism

Astrocytes Regulate Glucose Entry into the Brain via Insulin Signaling

As previously mentioned, in order to maintain proper brain function, it is of utmost importance to guarantee a constant and uninterrupted supply of glucose from the periphery to the brain to be used as its major source of energy (Box 6.2). In response to a meal, the body regulates glucose homeostasis to a large extent by secreting insulin from pancreatic β-cells, which is used by the cells of peripheral tissues (e.g.,

liver, adipose tissue, skeletal muscle) to take up glucose to generate energy. Unlike the rest of the body, glucose utilization by the brain was believed to be regulated independently of insulin, attributing the existence of abundantly expressed insulin receptors (IRs) within the brain to other roles of this hormone not related to glucose homeostasis. Yet, if neurons themselves do not rely on insulin signaling to utilize glucose, it remains possible that the entry of glucose into the brain via other cellular components, especially those forming the intricate body–brain interface (endothelial cells, astrocytes, pericytes), might depend on this specific signaling. Indeed, it was recently uncovered that insulin acts in astrocytes as a signal to regulate glucose entry from the periphery into the brain. In fact, the ablation of IRs from astrocytes induces a decrease in the astrocytic uptake of glucose, resulting from a reduced expression of the glucose transporter 1 (GLUT-1), and is associated with a lower glycolytic rate together with a decreased L-lactate efflux (Garcia-Caceres et al. 2016; Hernandez-Garzon et al. 2016). Such changes in cellular energy metabolism in astrocytes promote fatty acid β-oxidation. Aside from the impact of insulin on astrocytic bioenergetics, insulin signaling in astrocytes was reported to be determinant of how these glial cells functionally engage with, and are integrated into, hypothalamic neuronal circuits that are key in the control of metabolism. Specifically, astrocytes lacking IRs failed to properly ensheath pro-opiomelanocortin (POMC)-expressing neurons, which, in turn, rendered this otherwise glucose-responsive population insensitive to elevated blood glucose levels. Therefore, these findings support the notion that, contrary to what was previously assumed, glucose metabolism in the brain involves local insulin-dependent pathways (Fig. 6.1).

Box 6.2: Central Regulation of Glucose Homeostasis

The notion that the brain might be crucially involved in the regulation of blood glucose concentrations actually dates back to 1854, when the French physiologist Claude Bernard induced diabetes simply by puncturing the floor of the fourth ventricle ("piqûre diabétique"). Nowadays, it is well established that intricate glucoregulatory systems exist in the brain, which readily respond to both hypoglycemia and hyperglycemia. Distinct populations of neurons have been described to reside in the hypothalamus and brainstem that are either excited or inhibited by glucose. Such glucoregulatory system is crucially important for surveying circulating levels of glucose and subsequently eliciting immediate counterregulatory mechanisms. Intriguingly, these glucose-sensitive neurons share the molecular machinery that has been described to allow pancreatic β-cells to monitor blood glucose levels. As soon as glucose enters the cells, it gets phosphorylated by a distinct isoform of the hexokinase enzyme (hexokinase IV or glucokinase), which exhibits a relatively low affinity to glucose and is thus well within the range to act as a glucose-sensing enzyme. Lastly, these cells have incorporated the ATP-dependent potassium channel (K_{ATP}), which is a universal sensor linking cellular energy status—for example, impacted by glucose fluxes—with membrane depolarization.

Astrocytes Control Feeding via Leptin Signaling

Leptin is a well-known adipocyte-derived hormone that plays a pivotal role in energy balance and the control of body weight. As its concentration in blood correlates with adiposity, leptin is a reflective measure of energy reserves and provides an anorexigenic feedback signal that is sensed by the brain, in particular at the level of the hypothalamus. Most emphasis has been placed on how leptin affects neuronal activity and how neurons process and convey this information in order to calibrate food intake, energy expenditure, and ultimately body weight. However, it was revealed that astrocytes constitute additional, functionally relevant targets of blood-borne leptin (Kim et al. 2014; Wang et al. 2015). Specifically, Kim and colleagues reported that the inducible loss of the functional long form of the leptin receptor (LepR) impairs astrocyte–neuron spatial interactions in the hypothalamus. Similar to what has been observed in mice deficient in astrocytic insulin signaling, the inducible loss of LepR led to pronounced effects on the synaptic organization of feeding circuits located in the arcuate nucleus of the hypothalamus, namely the anorexigenic POMC neurons and the orexigenic neurons that co-express both Agouti-related peptide and neuropeptide Y (known as AgRP/NPY neurons), which are considered paramount for energy homeostasis (Box 6.3). Interestingly, leptin treatment failed to suppress food intake as efficiently in mice devoid of astrocytic LepR than in control ones. Mice lacking astrocytic LepR showed further feeding alterations such as a potentiated food intake in response to fasting or the fasting-mimicking hormone ghrelin (Kim et al. 2014). Notably, the subsequent study by Wang and colleagues similarly reported that the loss of LepR in astrocytes impairs leptin signaling in the brain, as evidenced by reduced phosphorylation of signal-transducer and activator of transcription 3 (pSTAT3) (Wang et al. 2015). Interestingly, this was observed even when leptin was administered centrally by directly infusing it into the cerebral ventricle, suggesting a central role of astrocytic LepR that is independent of hormonal transport across the BBB. In summary, these studies further support the observation that astrocytes are crucially important for integrating hormonal feedback signals to shape and tune the homeostatic neurocircuits in the hypothalamus (Fig. 6.1).

Box 6.3: Astrocyte–Neuron Interactions in the Arcuate Nucleus of the Hypothalamus

Over the past several decades, substantial research effort has been placed on the mapping of neurocircuitries controlling energy balance and body weight. In the course of this endeavor, two distinct populations of neurons emerged as crucial players, with both of them coexisting within the same brain region, the arcuate nucleus of the hypothalamus (ARC) (Fig. 6.4). By being situated directly adjacent to the median eminence, a circumventricular organ, ARC neurons and surrounding astrocytes have a privileged direct access to circulating feedback signals entering through local fenestrated blood vessels.

(continued)

Box 6.3 (continued)
Thus, ARC neurons can constantly monitor the metabolic state of the body, signaled, for instance, by means of circulating hormones such as ghrelin, leptin, or insulin. Importantly, two populations of ARC neurons can be characterized by their distinct molecular signature, with one subset expressing Agouti-related peptide/neuropeptide Y (AgRP/NPY) and the other population identified by expression of the pro-opiomelanocortin (POMC) precursor neuropeptide. Each set of ARC neurons exerts opposing effects on feeding behavior and energy expenditure and is arranged in an antagonizing relationship to the other. On the one hand, AgRP/NPY neurons get activated in the context of food deprivation and are necessary and sufficient to trigger feeding by increasing the consummatory drive. On the other hand, POMC neurons are activated by signals of energy surplus and reduce food intake while increasing energy expenditure. Intriguingly, the projection patterns of AgRP/NPY and POMC neurons are overlapping and exert opposing effects. Clearly, the circuit of AgRP/NPY and POMC neurons plays an integral role in controlling energy homeostasis. More recently, however, other cell types present in the ARC have stepped into the limelight. Among them, local astrocytes were attributed particular significance given that they show various region-specific properties not found elsewhere in the brain. In response to environmental cues such as nutrients and hormones, for instance, those astrocytes residing in the ARC were shown to undergo rapid changes in morphology and function. This in turn results in profound changes in synaptic function in the local AgRP/NPY and POMC neurocircuit. In summary, the traditional AgRP/NPY and POMC neurocircuit is nowadays known to be structurally and functionally influenced by the local ARC-residing astrocytes.

Other Emerging Roles of Astrocytes in Metabolic Control

Additional studies suggest other functions or have further confirmed the role of astrocytes in the central regulation of whole-body energy metabolism. Interestingly, Gao and colleagues reported the metabolic relevance of the capacity of astrocytes to uptake FA by generating a mouse model deficient in lipoprotein lipase specifically in GFAP-positive astrocytes (Gao et al. 2017b). The authors found that such alteration promotes ceramide accumulation in hypothalamic neurons, which in excessive amounts has been reported to induce detrimental effects in the brain, such as lipotoxicity and neuronal dysfunction (Chaurasia and Summers 2015). A recent study, led by Bouyakdan and colleagues, reported another interesting aspect, namely

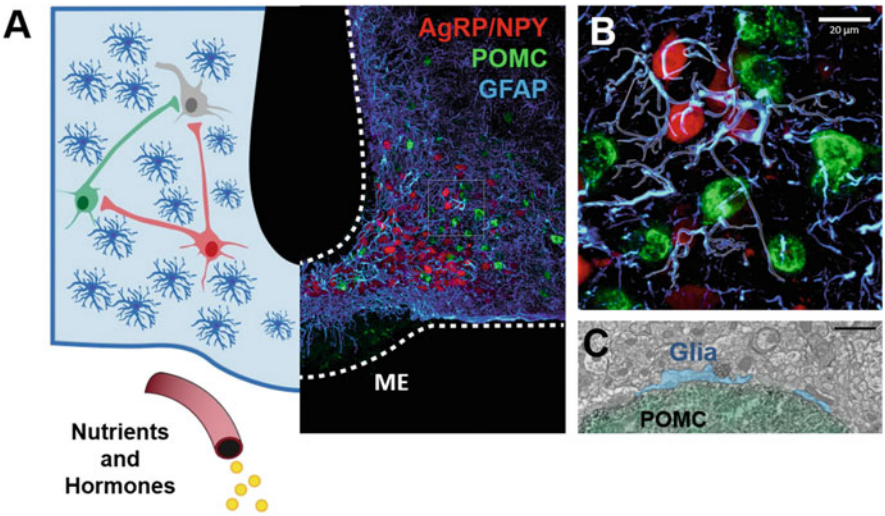

Fig. 6.4 Astrocyte–neuron interactions in the arcuate nucleus of the hypothalamus. (**a**, **b**) The arcuate nucleus of the hypothalamus is ideally located to serve as a metabolic sensing hub (hormones, nutrients) and also hosts two neuronal populations of utmost importance for the control of energy balance and body weight: the neurons expressing the orexigenic neuropeptides Agouti-related peptide and neuropeptide Y (aka. AgRP/NPY neurons, in red) and the neurons expressing pro-opiomelanocortin (POMC neurons, in green), a precursor of anorexigenic neuropeptides (for more details, see Box 6.3). Surrounding these neurons are astrocytes (in blue—confocal microscopy image—scale bar: 20 µm), which are emerging as another cell type that plays a key role in the regulation of the activity of these hypothalamic neuronal circuits. (**c**) Astroglial processes ensheath the surrounding neurons to regulate their activity, as shown in this electron microscopy image where a glial process (in blue) is covering the soma of a POMC neuron (in green). Scale bar: 1 µm

the role of astrocytic endozepines in the central control of energy balance (Bouyakdan et al. 2019). Endozepines are generally defined as endogenous ligands for the benzodiazepine receptor and it was previously shown that central exogenous delivery of a specific endozepine, ODN, both reduced food intake and improved glucose tolerance. In that study, the authors revealed that the deletion, specifically in GFAP-positive astrocytes, of the endogenous acyl-CoA-binding protein (ACBP), from which endozepines can be derived, is sufficient to promote food intake in both males and females (Bouyakdan et al. 2019). Interestingly, by placing ACBP-positive astrocytes in contact with the anorexigenic POMC neurons, the authors were able to show that ODN can activate these neurons. Furthermore, overexpressing ACBP in the ARC decreased food intake and weight gain. These results highlight ACBP as a gliopeptide that plays a central role in the control of energy balance by exercising an anorectic effect through interaction with the melanocortin system (Bouyakdan et al. 2019).

Other studies recently highlighted the active role of astrocytes in metabolic control by using recently developed techniques to manipulate astrocytic activity in the hypothalamus (e.g., chemo- (DREADDs, designer receptors exclusively

activated by designer drugs) and opto-genetics technologies). Specifically, studies employing these techniques have shown that Ca^{2+}-dependent activation of astrocytes located in the mediobasal hypothalamus (MBH) is determinant for the reduction in food intake, both in basal conditions and in a ghrelin-induced food intake paradigm (Yang et al. 2015), which is independent of the emotional state of the animal (Sweeney et al. 2016). Likewise, these studies have allowed for the identification of astrocyte-derived adenosine as the molecule mediating the inactivation of AgRP neurons via adenosine A1 receptors (Yang et al. 2015). Intriguingly, another group using a similar approach recently reported that activation of astrocytes in the ARC of the hypothalamus (located in the MBH) was associated with an increase in food intake (Chen et al. 2016), which is in contradiction to the study published by Yang and colleagues. The authors attributed these food intake-promoting effects of activating astrocytes to a sequential activation of AgRP neurons, while no direct changes in POMC neuronal activity were observed (Chen et al. 2016). Overall these opposing findings suggest that the functionality of astrocytes in the control of metabolism could be determined by the local network in which they are embedded and also highlight the necessity of being extremely cautious with the experimental setup, especially when it comes to using relatively new tools, but also with the conclusions we draw from the results that are obtained.

6.3 Astrocytes in Pathological Conditions

Over the last decades, substantial progress has been made in elucidating the roles of astrocytes in CNS disorders and pathologies. Astrocytes are plastic cells that respond dynamically to environmental stimuli, thereby allowing versatile alterations in their morphological, molecular, and functional properties. However, such alterations vary depending on the nature of the stimulus and can even be accompanied by a conspicuous structural change, which is frequently observed in activated or reactive astrocytes in response to CNS injury and/or disease. Astrocytosis or astrogliosis is known as the characteristic hypertrophic (reactive), and at times proliferative, phenotype that these glial cells adopt, undergoing an increase in GFAP and vimentin expression, associated with alterations in astrocytic Ca^{2+} homeostasis, all of which can reflect either healing processes or pathophysiology. Therefore activated/reactive astrocytes undergo morphological, functional, and molecular changes that occur to a greater or lesser extent depending on the severity and/or nature of stimuli. In severe cases, these modifications observed in astrocytes could even lead to a pronounced overlapping of astrocytic domains and the generation of a dense, narrow, and compact glial scar. The generation of a glial scar is characterized by the accumulation of hypertrophic resident astrocytes and the production, by all CNS cell types, of cytokines, mediators of innate immunity (e.g., toll-like receptor ligands), chemokines, neurotransmitters (e.g., glutamate, noradrenaline), growth factors, hypoxia, and neurotrophic factors, among others. Although many groups have extensively studied the formation of a glial scar in response to a stab wound, it is still unclear if it serves as a defense mechanism or participates in the propagation of

further CNS insult and dysfunction. Initially, a glial scar was thought to be due to a negative and maladaptive response of the CNS, which inhibits axonal regeneration and in turn impedes functional neuronal recovery, contributing to the initiation and progression of neurological complications. However, accumulating evidence rather indicates that the formation of this physical barrier could avoid the propagation of inflammatory factors, for example, from the lesion core to the healthier surrounding tissue. The types of CNS insult able to induce astrogliosis are very heterogeneous. This insult can be mechanical, resulting from a wound injury or stroke, or rather related to neurodegenerative diseases. Signs of astrogliosis have been reported in Alzheimer's, Parkinson's, and Huntington's diseases, amyotrophic lateral sclerosis, multiple sclerosis, dementia, and so on. In these chronic pathologies, the formation of a glial scar is less systematic, although it has also been reported.

Being associated with CNS insult, the presence of astrogliosis is linked to inflammatory states that are more or less pronounced. Interestingly, the laboratory of Ben Barres coined as "A1-reactive astrocytes" a subtype of astrocytes that are rendered reactive via neuroinflammation (Liddelow et al. 2017). These A1-reactive astrocytes have been found in patients with neurodegenerative diseases and are believed to be involved in promoting neuronal and oligodendrocyte cell death through the secretion of a yet-to-be identified toxin and via the loss of many of the normal astrocytic functions (Liddelow et al. 2017). Conversely, aging is also considered a driver of astrogliosis leading to improper astrocyte functionality, which results in defects in the astrocyte ability to properly maintain a healthy CNS environment, affecting their interaction with neighboring cells and ultimately contributing to the development of an inflammatory state associated with aging.

6.3.1 Reactive Astrocytes in Obesity

Astrogliosis is a hallmark of the tissue inflammation and/or injury that underlies neurological diseases. Interestingly, studies have pointed out that obesity might be a brain disease, also showing signs of inflammation and astrogliosis that were until recently solely reported in neurogenerative diseases (Fig. 6.5). In 2005, De Souza and colleagues were the first to demonstrate that obesity, at least in rodents, is associated with an increase in inflammatory signaling in the hypothalamus (De Souza et al. 2005). This led to studies aiming to further understand which aspects of inflammation were involved in the progression of metabolic disease. In 2010, experiments led by Horvath and colleagues demonstrated that diet-induced obese mice exhibited an upregulation of GFAP in astrocytes, particularly in the hypothalamus, which was associated with changes in the physical interactions of astrocytes with endothelial cells and neurons, contributing to alterations of the cytoarchitecture and synaptology of hypothalamic circuits (Horvath et al. 2010). Interestingly, such inflammatory hallmarks, including increased cytokines in the hypothalamus, were detected prior to any changes in peripheral inflammation and body weight gain (Thaler et al. 2012), suggesting their potential role in hypothalamic dysfunction associated with astrogliosis to promote obesity pathogenesis (Fig. 6.5). Further

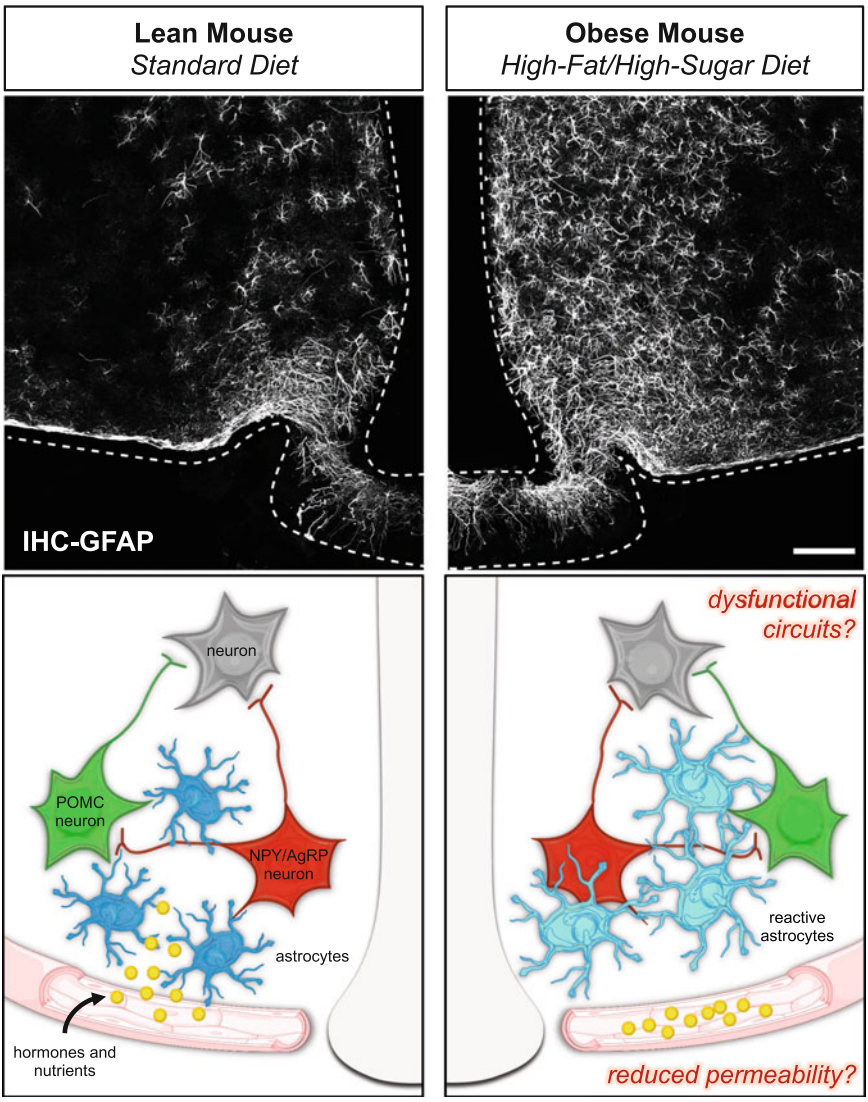

Fig. 6.5 Diet-induced astrogliosis in the arcuate nucleus of the hypothalamus. Consumption of a high-calorie diet can trigger profound changes in the hypothalamic cytoarchitecture, including the rapid upregulation of GFAP (glial-fibrillary acidic protein) in local astrocytes. By acquiring a more "bushy," hypertrophic morphology as a consequence, these now so-called reactive astrocytes are believed to: (a) disrupt the synaptology and function of local neurocircuits in the hypothalamus controlling energy balance and (b) to hamper the entry of homeostatic feedback signals emanating from the periphery. However, more functional studies are yet warranted to support such a claim, which currently remains based mainly on descriptive reports

studies have provided supplementary evidence that confirmed the presence of reactive glia in the hypothalamus in monogenic models of obesity (Buckman et al. 2013; Hsuchou et al. 2009; Pan et al. 2008), but also in response to maternal or neonatal overnutrition (Fuente-Martin et al. 2012; Garcia-Caceres et al. 2011). Importantly, hypothalamic astrogliosis, as detected by magnetic resonance imaging, has also been reported to occur in humans with high body mass index (BMI) (Thaler et al. 2012).

The astrogliosis associated with the consumption of hypercaloric diet was reported to be a reversible event, since the resumption of a normal chow diet restrains hypothalamic astrogliosis in association with a reduction in body weight (Berkseth et al. 2014). Yet, not all calorie-dense diets induce the same changes in hypothalamic glial activity, which indicates a certain heterogeneity in the response of glial cells depending on the composition of the diet.

According to Gao and colleagues, the combination of dietary fat and sugars, but not fat or obesity per se, is a determinant for the induction of microglial activity. Yet, changes in the expression of GFAP did not seem to depend on the combination of high-carbohydrates and high-fat in the diet, but rather solely on increased levels of fat in the diet (Gao et al. 2017a). Overall, these findings suggest the existence of hypothalamic specific responses from the different types of glia to distinct diet components in the context of hypercaloric diets.

In 2014, Morselli and colleagues highlighted a sex discrepancy in the development of hypercaloric diet feeding-associated astrogliosis with the observation that male mice—but not females—exhibited hypothalamic astrogliosis and upregulation of cytokines, despite both sexes exhibiting excessive weight gain (Morselli et al. 2014). Thus, these findings suggest the existence of sexual differences in diet-induced responses of hypothalamic astrocytes. Furthermore, astrogliosis associated with the consumption of a hypercaloric diet was also reported to affect extra-hypothalamic areas such as the hippocampus and the thalamus (Buckman et al. 2013), although the time and the composition of the diet that are needed to induce astrogliosis can differ depending on the brain area. Interestingly, microglia are thought to be involved in some of the astrocytic responses elicited by a hypercaloric diet and are considered the first responders, producing inflammatory factors that would, in turn, simultaneously activate astrocytes and trigger neuronal stress (Thaler et al. 2012; Valdearcos et al. 2014) (see Chap. 7).

Astrocytic Pathways Mediate Hypothalamic Astrocytosis Associated with Diet-Induced Obesity

Recent work by Pfuhlmann and colleagues has demonstrated that hypercaloric diets trigger hypothalamic astrocytosis by activation of the Ca^{2+}/calmodulin-activated serine/threonine phosphatase calcineurin (Pfuhlmann et al. 2018). Conversely, other studies have proposed inhibition of pro-inflammatory pathways in astrocytes as a means to prevent low-grade hypothalamic inflammation, including astrocytosis, associated with the consumption of energy-dense diets and obesity. In this regard, Douglass and colleagues have reported that blocking the IκB kinase (IKK) β/NF-κB pathway, involved in most inflammatory signaling, specifically in astrocytes is

sufficient to attenuate diet-induced astrogliosis, as well as the upregulation of inflammatory factors and the impairment of leptin and insulin sensitivity occurring within the hypothalamus. Importantly, these findings were associated with a decrease in food intake and an increase in energy expenditure in mice fed with a hypercaloric diet (Douglass et al. 2017). Other studies are aligned with these observations underlining the relevance of inhibiting the inflammatory IKKβ/NF-κB pathway in astrocytes to improve whole-body energy homeostasis under obesogenic conditions (Zhang et al. 2017). Interestingly, these studies reported dynamic changes in astrocytic morphology depending on the feeding status of mice. Chronic overnutrition, together with the upregulation of the IKKβ/NF-κB pathway, induced long-lasting shortening of astrocytic processes that was accompanied by glucose intolerance and an increase in blood glucose levels, fat accumulation, and total body weight (Zhang et al. 2017). Furthermore, these authors reported that the IKKβ/NF-κB pathway in astrocytes mediates the astrocytic regulation of extracellular levels of GABA and BDNF (brain-derived neurotrophic factor), which was partially responsible for the metabolic syndrome observed in these mice on a hypercaloric diet (Zhang et al. 2017).

Other signaling pathways in astrocytes have been identified to be involved in the generation of astrogliosis, such as Stat-3 and ErB, but none of these has yet been studied in the context of diet-induced obesity, leaving ample opportunity for further mechanistic understanding in this regard.

6.4 Perspectives

At the end of the nineteenth century, Ramon y Cajal's pioneering studies were the first to reveal that an intimate and coordinated association between glia and neurons is required for normal brain function. Despite the undeniable essential role of astrocytes in the brain, a simplistic view, solely concerned with exploring neuronal activity, prevailed during the previous decades, ignoring the presence and active role of other cells in the brain. This has likely hindered the progression of knowledge towards forming a complete understanding of how the brain controls the many processes that are under its jurisdiction, including the control of systemic metabolism. We now know that the regulation of brain function cannot be operated or explained by neurons alone, and the notion that astrocytes play an important role in metabolic control is currently gaining momentum. Moreover, the implication of astroglia in this process has brought these cells into the spotlight and has resulted in advances in our understanding of their role in the physiological control of metabolism, but also in the pathophysiology of metabolic diseases. However, there is still much to be learned regarding astrogliosis in both diet-induced obesity and dietary challenges. Indeed, scientists need to continue putting effort into identifying new markers and generating new tools that are less invasive and allow higher resolution, which will allow us to abate the difficulties and eventually grant new exciting discoveries. Hence, one continuing challenge is to determine the relationship between the different inflammatory and glial responses in the hypothalamus and

their implication in the perpetuation of weight gain, as well as the associated secondary complications. Understanding these processes may lead to new therapeutic targets to treat CNS diseases, including obesity.

6.5 Key Literature

Araque et al. (1999) [Review discussing the integral role of astrocytes within synapses].

Garcia-Caceres et al. (2016) [Original article reporting the importance of astrocytic insulin signaling for the control of both central glucose sensing and systemic glucose metabolism by modulating the entry of glucose across the blood–brain barrier, depending on the overall metabolic status].

Garcia-Caceres et al. (2019) [Review discussing the importance of non-neuronal partners in the central control of systemic metabolism].

Horvath et al. (2010) [Original article reporting that diet-induced obese mice exhibit hypothalamic astrocytic reactivity which is associated with changes in the physical interactions of astrocytes with endothelial cells and neurons, contributing to alterations of the cytoarchitecture and synaptology of hypothalamic circuits].

Verkhratsky et al. (2016) [Review summarizing the features of astrocytic secretion of signaling molecules].

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Glia-Neuron Communication: Not a One-Way Street

7

Andy Tran, Jim T. C. Chen, and Denise D. Belsham

Abstract

Obesity and its comorbidities are increasing at an alarming rate worldwide. A key mechanism in the development of this disease involves dysregulation of the hypothalamus, which is a key region of the brain responsible for maintaining energy balance. Specifically, energy excess primarily due to elevated levels of fats and lipids induces a neuroinflammatory response in the hypothalamus that impairs key regulatory mechanisms that maintain energy balance. Crucially, the induction and progression of neuroinflammation involves bidirectional communication between resident neurons and immune cells called microglia. This chapter will cover the basics of inflammation, the methods used to study this phenomenon, neuronal and microglial mechanisms underlying neuroinflammation, and the ways in which these two components interact in this pathogenic state, and will provide future perspectives on targeting neuroinflammation to restore hypothalamic function and treating obesity.

Keywords

Obesity · Lipids · Fatty acids · Hypothalamus · Neuroinflammation · Microglia

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7.1 Introduction

Obesity is a pandemic affecting 650 million adults globally, and obesity among children is also growing at an alarming rate (Wrzosek et al. 2018). Insulin resistance, type 2 diabetes, hypertension, and cardiovascular diseases are comorbidities of obesity that increase mortality and place heavy burdens on healthcare systems. Obesity is a chronic condition caused by positive energy balance resulting from increased caloric intake and reduced energy expenditure over time. This often occurs due to long-term consumption of a Western-style diet alongside a sedentary lifestyle. As the central nervous system (CNS) is a major regulator of energy balance, a growing focus of research has been on CNS involvement in the development of obesity and its comorbidities.

One of the primary regions participating in the regulation of energy balance is the hypothalamus. Proper functioning of the hypothalamus requires appropriate communication between hypothalamic neurons and glia, which were originally considered to be the supporting cells of the CNS, but are now known to contribute actively to both physiological and pathophysiological processes (Tasker et al. 2012). Any perturbations to neurons or glia can disrupt the intricate and bidirectional relationship between these cell types, potentially leading to metabolic dysfunction and obesity. In recent years, research has identified hypothalamic neuroinflammation, which involves and affects neurons and glia alike, as a major contributor to the pathogenesis of obesity (Zhou 2018). This chapter will summarize the current understanding of the neuronal and glial mechanisms that contribute to hypothalamic neuroinflammation, and how neurons and glia interact with each other in the pathogenic context of obesity.

7.2 The Arcuate Nucleus of the Hypothalamus Regulates Energy Homeostasis

7.2.1 The Hypothalamus Is the Master Regulator of Homeostasis

The hypothalamus is a small region located near the base of the brain, below the thalamus and above the pituitary gland. The hypothalamus regulates whole-body homeostasis via the autonomic nervous system and the endocrine system, in part by regulating hormone release from the pituitary gland. The hypothalamus receives inputs from the periphery that are then integrated to produce appropriate responses for maintaining homeostasis (Zhou 2018).

Although this chapter focuses on the role of the hypothalamus in regulating feeding behavior and energy homeostasis, one should keep in mind that the hypothalamus regulates nearly all functions critical to survival including water balance, wakefulness, reproduction, stress, and body temperature.

7.2.2 Nuclei of the Hypothalamus

The hypothalamus is divided into distinct regions called nuclei. Some nuclei are responsible for regulating distinct processes. When it comes to energy homeostasis, multiple hypothalamic nuclei are involved: these include the suprachiasmatic nucleus (SCN), arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamus (LH), ventromedial nucleus (VMN), and dorsomedial hypothalamus (DMH) (Box 7.1). These nuclei do not work in isolation and contain interconnected neuronal pathways, forming a complex network controlling different aspects of feeding behavior and energy expenditure.

Box 7.1: The Hypothalamus Is Home to a Diverse Array of Distinct, Specialized Nuclei That Play Critical Roles in Homeostatic and Allostatic Functions of the Body

Hypothalamic nucleus	Primary function(s)
Suprachiasmatic nucleus (SCN)	Circadian rhythmicity of endocrine functions and behaviors
Supraoptic nucleus (SON)	Regulation of blood volume and lactation (through secretion of vasopressin and oxytocin)
Paraventricular nucleus (PVN)	Regulation of blood volume, metabolism, growth (through secretion of CRH, TRH, oxytocin, vasopressin, somatostatin)
Lateral hypothalamus (LH)	Promotes feeding (through orexin production)
Ventromedial nucleus (VMN)	Regulation of satiety, glucose metabolism
Arcuate nucleus (ARC)	Regulation of feeding behavior through integration of peripheral signals and communication to other feeding-related nuclei, as well as growth-related signals
Dorsomedial nucleus (DMN)	Leptin-mediated responses, blood pressure regulation, heart rate regulation

7.2.3 The Arcuate Nucleus of the Hypothalamus Is a Key Center for Regulating Feeding Behavior

Shifts in neuropeptide expression patterns of the ARC can result in profound changes in an organism's fuel status and consumption levels, affirming the ARC as a potent regulator of metabolism and appetite. In the ARC, neurons form opposing orexigenic and anorexigenic circuits that promote or suppress feeding behavior, respectively.

For example, neuropeptide Y (NPY) and agouti-related peptide (AgRP) are secreted by NPY/AgRP neurons to stimulate feeding, with transgenic overexpression of *Npy* causing weight gain and insulin resistance (Ruohonen et al. 2008), and ablation of AgRP neurons causing acute anorexia (Gropp et al. 2005). POMC (pro-opiomelanocortin) neurons in the ARC secrete alpha-melanocyte-stimulating hormone (α-MSH) to promote satiety; unsurprisingly, deletion of MC4R (a target receptor of α-MSH) results in hyperphagia, leading to obesity (Butler et al. 2001). The balance between these two opposing networks is a primary determinant of feeding behavior.

7.2.4 Neurons of the Arcuate Nucleus Detect Nutrients and Whole-Body Energy Status

ARC neurons are adjacent to a permeable, circumventricular organ called the median eminence (ME). In the brain, the ME is a unique region in which the CNS is exposed to peripheral contents due to fenestrations in the endothelium of the vasculature. Terminal portions of neurons in this region extend beyond the protective barriers of the CNS to facilitate privileged access, allowing ARC neurons to directly sense circulating metabolic signals which can then be integrated and conveyed to other feeding-related nuclei (Rodriguez et al. 2010).

In addition to nutrient sensing, nutrients and hormones originating from the periphery are able to physically enter the ARC via the ME. Entry of nutrients such as amino acids, glucose, and lipids and hormones such as insulin, ghrelin, and leptin inform the hypothalamus of peripheral energy status. As such, the permeability of the blood–brain barrier (BBB), particularly near the median eminence, is a significant factor for the hypothalamic control of energy homeostasis.

7.3 The Hypothalamus Contains a Heterogeneous Population of Cells

The "heterogeneity" of the hypothalamus acknowledges not only the different cell types, but also the diversity within each cell type. Although hypothalamic neurons are essential to maintaining energy homeostasis, their proper placement, functions, and activities are maintained by a cast of supporting cells called neuroglia, also referred to as glia. Types of glia include microglia, oligodendrocytes, astrocytes, and tanycytes that perform different functions to ensure proper functioning of the neurons and the overall CNS (Zhou 2018). Although the glia-to-neuron ratios in the brain and in the hypothalamus remain heavily disputed, the fact remains that glia constitute a large portion of our CNS and their functions are critical for brain health and proper neuronal function.

7.3.1 Neurons

The hypothalamus is home to distinct neuronal populations. Each population has unique neuropeptide and receptor expression patterns rendering them uniquely sensitive to certain signals. The regulation of these responses is governed by a combination of population-specific signal transduction components and transcription factors. In addition, there is heterogeneity within neuronal populations. Single-cell RNA sequencing studies have revealed functionally distinct subpopulations of AgRP and POMC neurons, demonstrating the heterogeneous nature of neurons in the hypothalamus (Lam et al. 2017; Chen et al. 2017).

7.3.2 Glial Cells

Figure 7.1 shows an illustration of the different types of glial cells and their relationship to neurons and blood vessels in the brain.

7.3.2.1 Oligodendrocytes

Oligodendrocytes are derived from oligodendrocyte precursor cells, also known as NG2-glia due to their expression of the integral membrane NG2 proteoglycan (Zhou 2018). Oligodendrocytes are the main producers of myelin in the CNS and wrap

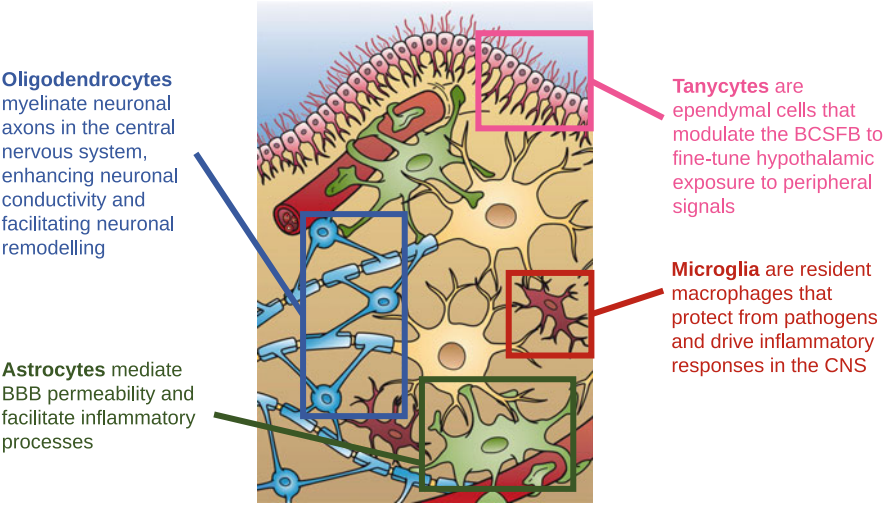

Fig. 7.1 Proper neuronal function is enabled by a repertoire of diverse and specialized glial cells. Oligodendrocytes, tanycytes, astrocytes, and microglia in brain tissues serve unique and specialized functions, ranging from synaptic connectivity maintenance and modification, barrier permeability modulation, nutrient supplies, and immune defense. Depletion of any of these specialized glial cell types can result in profoundly aberrant neuronal signaling and neuroendocrine functions. (Adapted from Wikimedia Commons Dec 2013; Author: Holly Fischer; License: Creative Commons Attribution 3.0 Unported license)

around neuronal axons with myelin sheaths, thereby dynamically and selectively enhancing the conductivity of electrical signals. In addition, new evidence suggests that oligodendrocytes also provide metabolic support to neurons by transferring glycolytic derivatives to neurons through regions of uncompacted myelin. Therefore, damage to oligodendrocytes may lead to neuronal demyelination and starvation, potentially impairing overall neuroendocrine circuitry and hypothalamic responses to energy status (Philips and Rothstein 2017).

7.3.2.2 Tanycytes

Tanycytes are specialized ependymal cells that line the third ventricle and regulate the permeability of the blood–cerebrospinal fluid barrier (BCSFB) and BBB (Rodriguez et al. 2010). As key regulators of the metabolic functions of the hypothalamus, tanycytes regulate hypothalamic exposure to metabolic factors and also regulate the release of hypothalamic neuropeptides into the periphery. Tanycytes pass through the median eminence, allowing them to modulate the degree of CNS exposure to whole body energy status (Zhou 2018).

Box 7.2: The Discovery of Microglia by Pío del Río Hortega

Prior to the identification of glial cells in the early twentieth century, non-neuronal entities were termed "neuroglia," derived in part from the ancient Greek word for "glue." This reflected the notion at the time that neurons of the CNS were held together by a "glue" or matrix (Sierra et al. 2016). Following the discovery that astrocytes were a distinct cellular neuroglial entity and the "second element" of the CNS (with neurons representing the "first element"), the Spanish scientist Santiago Ramón y Cajal (1852–1934) developed a method that robustly visualized astrocytes but poorly stained undefined cells, which he described as the "third element" of the CNS (Sierra et al. 2016).

Pío del Río Hortega (1882–1945), who worked under Cajal, developed a new metallic staining method that was able to distinguish two distinct types of cells that comprise the "third element," one referred to as mesoglia (microglia) and the other as interfascicular glia (oligodendrocytes) (Perez-Cerda et al. 2015). Following this discovery, Hortega described the morphology and functions of microglia. He noted how resting, ramified microglia carried out surveillance functions and were strongly phagocytic once activated. Furthermore, he illustrated the morphological changes that occur when ramified microglia transition to a phagocytic amoeboid upon activation (Perez-Cerda et al. 2015; Ransohoff and Brown 2012).

7.3.2.3 Astrocytes

Stellate-shaped astrocytes are the most abundant glial cell type in the brain and help modulate the permeability of the BBB (Cabezas et al. 2014). They are able to detect

and shuttle nutrients including glucose, lipids, and lactate, as well as hormones such as leptin and insulin, into the hypothalamus. Together, these shuttling mechanisms support neuronal nutritional demands and facilitate hypothalamic sensing of peripheral energy status. The detection of insulin by astrocytes is required for efficient brain glucose uptake, which is critical for neuronal health and hypothalamic control of energy homeostasis (Garcia-Caceres et al. 2016). Similar to microglia, astrocytes can be activated and subsequently recruited to sites of neuronal injury and inflammation through a process known as reactive astrocytosis. In this state, extensive astrocyte-microglia crosstalk occurs to facilitate hypothalamic neuroinflammation (Farina et al. 2007).

7.3.2.4 Microglia

Microglial cells are the resident immune cells of the CNS and play an important role in protecting the CNS from pathogenic insults and injury (Luo and Chen 2012). Unlike peripheral macrophages, which are derived from the monocyte lineage, microglia are derived from the myeloid lineage and become established in the brain early in development (Zhou 2018). Inflammatory stimuli including trauma, cell stress, and pathogenic invasion can lead to microgliosis, a process in which microglia are activated and transition to a phagocytic amoeboid (Zhou 2018). Activated microglia are then recruited to sites of insult where they proliferate to facilitate or resolve inflammation, depending on the context (Sochocka et al. 2017).

7.4 When Things Go Wrong: Nutrient Excess and Neuroinflammation

7.4.1 What Is Inflammation?

Inflammation is a complex and multifaceted process. In general terms, inflammation is the body's defense mechanism against infection, stress, and cellular damage (Chen et al. 2018). The inflammatory response involves coordination and interaction between molecular and cellular components. Molecular components include transcription factors, cytokines, and other signal proteins that are rapidly induced and released into the extracellular environment upon a triggering signal. Cellular components of the immune system include resident cells of the affected tissue, as well as specialized immune cells that can be recruited from more distant sites (Ransohoff et al. 2015). Cytokines and other inflammatory mediators inform surrounding resident cells and immune cells of the nature and location of the insult to orchestrate an appropriate immune response. Pro-inflammatory cytokines can facilitate an immune response by recruiting immune cells, inducing activation and differentiation, and enhancing their phagocytotic and cytotoxic functions (Zhou 2018; Tran et al. 2016). Molecular and cellular components of inflammation coordinate to remove pathogens or stressed and damaged cells via phagocytosis, and directing programmed cell death if needed. If the threat is removed without overly extensive damage to tissues, regulatory mechanisms create a regenerative

environment to promote a return to homeostasis. While inflammation is often associated with pathogenic invasion, many pathologies are described as sterile inflammatory diseases such as ischemia, atherosclerosis, and obesity, as they are not initiated by pathogens (Douglass et al. 2017).

7.4.2 The CNS Has Immune Privilege

The CNS is an immune privileged site, as it is uniquely separated from the immune cells, soluble factors, and pathogens in the periphery. This is due to the presence of the BBB that isolates the CNS from the periphery (Ransohoff et al. 2015). The BBB is comprised of a continuous layer of endothelial cells, astrocytes and pericytes joined together by tight junctions to separate circulating blood in the vasculature from the brain and cerebrospinal fluid (Ransohoff et al. 2015). The semi-permeable barrier prevents infiltration of pathogens and immune cells that circulate the bloodstream but selectively allows nutrients and peripheral hormones to enter the CNS (Ransohoff et al. 2015). As peripheral immune cells do not have access to the CNS under normal conditions, the brain contains its own population of microglia to quickly respond to stress and injury within the brain (Tran et al. 2016; Posfai et al. 2019).

7.4.3 Inflammation Develops in the Periphery and CNS During Obesity Through Different Mechanisms

Inflammation of peripheral tissues, such as adipose tissue, is a key trait of obesity. However, inflammation in obesity is not restricted to the periphery; scientists recently identified regions of the CNS that develop neuroinflammation in the pathogenesis of obesity (Thaler et al. 2012). Obesity-induced neuroinflammation occurs in multiple regions of the CNS, including the cortex, cerebellum, amygdala, hippocampus, and brainstem. Specifically, inflammation in the hypothalamus disrupts homeostatic processes that regulate energy balance. Critically, the timing and mechanisms underlying the onset of inflammation in peripheral adipose tissue are different from those in the hypothalamus.

In the case of adipose tissue, weight gain during the course of obesity is associated with increased lipid storage in adipocytes, leading to hyperplasia. Fat tissue-derived cytokines (adipokines) activate resident macrophages, which will clear dead and dying adipocytes and subsequently secrete inflammatory cytokines to recruit additional leukocytes. Therefore, inflammation in adipose tissue develops over the course of weight gain and is thought to be a consequence, rather than the cause, of obesity (Thaler et al. 2012).

Conversely, hypothalamic neuroinflammation is induced rapidly by excess nutrients, particularly fats and lipids, which is thought to be initially protective (Tran et al. 2016; Dalvi et al. 2017). This neuroinflammation is observed well before weight gain suggesting that unlike in adipose tissue, hypothalamic inflammation is

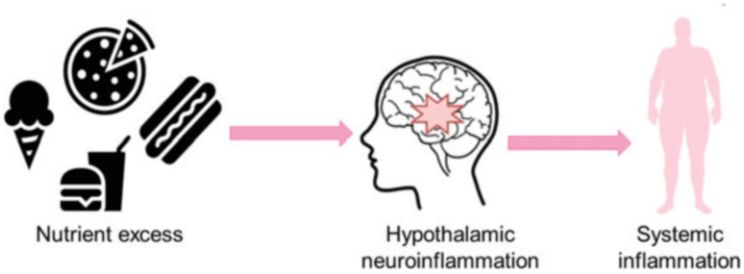

Fig. 7.2 Hypothalamic neuroinflammation precedes systemic inflammation after overconsumption and the pathogenesis of obesity. Unlike systemic inflammation, hypothalamic neuroinflammation can occur prior to the onset of weight gain. While inflammation-induced insulin resistance in peripheral tissues is a key observation in obesity and type 2 diabetes, neuroinflammation-induced leptin resistance and insulin resistance in the hypothalamus may actually be an underlying cause of obesity for two main reasons: the rapid induction of hypothalamic neuroinflammation in response to nutrient excess, and the critical role of the hypothalamus in energy homeostasis

not a consequence of weight gain (Thaler et al. 2012). Although integration of peripheral cues in the hypothalamus depends upon direct sensing of fat and lipid levels in a healthy individual, excess fats and lipids in the body instead rapidly trigger cellular stress and hypothalamic neuroinflammation, perturbing central regulation of energy balance (Fig. 7.2). With longer-term exposure to lipids, this protective response can change to a detrimental chronic neuroinflammatory response.

7.4.4 Hypothalamic Neuroinflammation Impacts Energy Balance

Critically, inflammation impairs pathways used by hypothalamic neurons to sense whole-body energy status from the periphery. For example, the activation of inflammatory pathways directly interferes with leptin and insulin signaling (Zhou 2018; Douglass et al. 2017; van Dijk et al. 2015). When the hypothalamus is unable to reliably detect these anorexigenic signals, the expression of feeding-related neuropeptides also becomes dysregulated. In this pathologic state, an appropriate anorexigenic tone cannot be initiated when it is necessary, such as during energy excess.

Different modes of inflammation have differential effects on hypothalamic control of feeding behavior. Specifically, it is sustained low-grade inflammation that underlies the dysregulation in hypothalamic function leading to weight gain and obesity. Conversely, acute, severe inflammation driven by pathogenic invasion and infection results in a sickness response characterized by temporary anorexia (Burfeind et al. 2016; Thaler et al. 2013).

7.5 Methods Used to Investigate Central Feeding Regulation and Hypothalamic Neuroinflammation

Many models are at our disposal for the study of CNS involvement in obesity. It is important to note that models are not intrinsically good or bad; rather, the appropriateness of a model depends on the research questions being asked as well as the ethical, financial, and logistical constraints of a study. In this regard, each model has its own benefits and drawbacks.

7.5.1 Human Models

Although studying neuroinflammation in humans would be the ideal approach, current methods are limited to non-invasive imaging or the analysis of post-mortem brain samples. Two primary imaging techniques are used to identify neuroinflammation: magnetic resonance imaging and positive electron tomography (Kreutzer et al. 2017). MRI is a common imaging technique that provides finely detailed images of the brain, by relying on the alignment of randomly oriented protons in an external magnetic field and disruption of the aligned protons with a radio frequency. After removing the radio frequency, the protons become realigned with the magnetic field to release a detectable signal. Importantly, regions of inflammation on an image generated by MRI appear as hyperintense regions relative to the surrounding area.

On the other hand, PET is a technique that detects the presence of radioactive ligands. 11[C]-labelled PK11195 or the second generation 11[C]-labelled DPA-713 are specific tracers for translocator protein 18 kDa (TSPO) (Chaney et al. 2018). TSPO is a cholesterol transporter on the outer mitochondrial membrane that is significantly induced in activated microglia (Beckers et al. 2018). Thus, this induction can be used to detect activated microglia and infer neuroinflammation. However, both of these imaging techniques only suggest, rather than definitively identify neuroinflammation.

Direct analysis of hypothalamic tissue can be performed on post-mortem brain slices (Kreutzer et al. 2017; Schur et al. 2015). Staining for markers of activated microglia, such as Iba1, and markers of activated astrocytes, such as increased levels of GFAP, as well as changes in glia morphology, can verify gliosis, often considered a marker of neuroinflammation (Dalvi et al. 2017; Kreutzer et al. 2017). This provides a better measure of neuroinflammation and direct visualization of associated structural and morphological changes in microglia and astrocytes. Compared to using MRI or PET on live humans, post-mortem samples are harder to obtain; as such, these studies tend to have very limited sample sizes. Despite these limitations, these techniques helped establish that hypothalamic neuroinflammation is present in obese individuals (Thaler et al. 2012; Kreutzer et al. 2017).

7.5.2 Monogenic Rodent Models of Obesity

Genome editing techniques, combined with the ease of breeding rodent strains, have allowed for the development of many monogenic models of obesity. These models were instrumental in identifying essential regulators of energy homeostasis, such as leptin and POMC. For example, the ob/ob mouse arose from a spontaneous loss-of-function mutation of the leptin gene (Zhang et al. 1994). This mouse strain displays hyperphagia, rapid weight gain, and poor metabolic control as demonstrated by hyperglycemia and insulin resistance.

Affecting the same pathway, db/db mice and their Zucker fatty rat counterparts are leptin receptor-deficient and phenotypically similar to ob/ob mice (Tartaglia et al. 1995). These models helped establish leptin as an anorexigenic hormone, and continue to be used as models of obesity and type II diabetes. Leptin targets POMC neurons of the hypothalamus; knockout of *POMC*, the precursor of the anorexigenic peptide α-MSH or its target receptors, melanocortin receptor 3 (MC3R) or melanocortin receptor 4 (MC4R) lead to hyperphagia, obesity, and metabolic dysfunction (Lutz and Woods 2012).

Monogenic rodent models have been instrumental to advancing our understanding of the specific molecular regulators of feeding behavior. However, when it comes to obesity in humans, monogenic mutations are rarely the underlying cause; the dominant driving forces behind the obesity epidemic are sociocultural and socioeconomic influences on diet and lifestyle. With the understanding that obesity is often a product of the environment rather than genetics, DIO models are often a more relevant model for the study of human obesity (Lutz and Woods 2012). However, it is important to note that genetics do play a role in the susceptibility to dietary responses in both humans and rodents.

7.5.3 Diet-Induced Obese (DIO) Rodent Models

As the underlying causes of obesity in most humans tend to be environmental with the contribution of individual underlying genetic response to diet (Albuquerque et al. 2017), high-fat diet (HFD)-fed mice and rats are common tools for modeling obesity and its comorbidities, including neuroinflammation, glucose intolerance, and insulin resistance (Thaler et al. 2012). Generally, studies using these models will feed animals of the same strain concurrently for a period of time using either standard rodent chow (containing approximately 10% calories from fat) or a high-fat diet (in which fat contributes up to 60% of the caloric content). HFD-fed rodents gain weight and eventually develop obesity along with accompanying metabolic disorders, including diabetes and insulin resistance.

7.5.4 In Vitro Primary Culture

Due to the heterogeneity and complex architecture within the hypothalamus, in vivo models may not be ideal when studying the molecular properties of hypothalamic neurons. Such investigations may therefore benefit from the use of in vitro hypothalamic models. Primary hypothalamic cultures can be established by extracting hypothalami from animals then dispersing the neurons into culture media. This model retains the cellular heterogeneity of the hypothalamus, with the added benefit of being able to directly assess changes in transcription, protein translation, and the underlying molecular mechanisms of these processes. This is done in the absence of efferent or afferent connections with other CNS regions that may complicate these mechanistic studies. However, primary hypothalamic cultures, mostly derived from immature animals, have a limited lifespan and do not preserve the three-dimensional architecture of the hypothalamus.

7.5.5 Immortalized Cell Lines

The limited lifespan of primary hypothalamic cultures translates to increased costs and labor to continually generate them. Hypothalamic cultures can be immortalized to greatly extend their lifespan and ease of use (Fig. 7.3), while simultaneously reducing the number of animals sacrificed. Cell lines can be established from tumors, or via transfection with SV40 T antigen. However, the inherent heterogeneity of primary cultures and their immortalized derivatives makes it difficult to study the responses and regulatory mechanisms of specific populations of neurons. In this regard, clonal cell lines can provide valuable insight. While a number of clonal neuronal cell lines have been isolated from tumors, they fail to represent the extensive and diverse repertoire of cells in the hypothalamus; this gap has been addressed by the generation of clonal cell lines from heterogeneous immortalized cell lines. Through the transfection of SV40 T antigen, immortalized embryonic and adult cell lines have been established and represent the diverse neuronal populations in the hypothalamus (Tran et al. 2016).

In vitro manipulation of immortalized cell lines is used to dissect the molecular mechanisms that regulate their responses and functions. For example, NPY/AgRP and POMC cell lines have provided insight into the differential mechanisms involved in regulating each of the respective feeding neuropeptides. Furthermore, different clonal NPY/AgRP cell lines revealed differential responses to leptin, highlighting the existence of functionally distinct neuronal subpopulations in the hypothalamus.

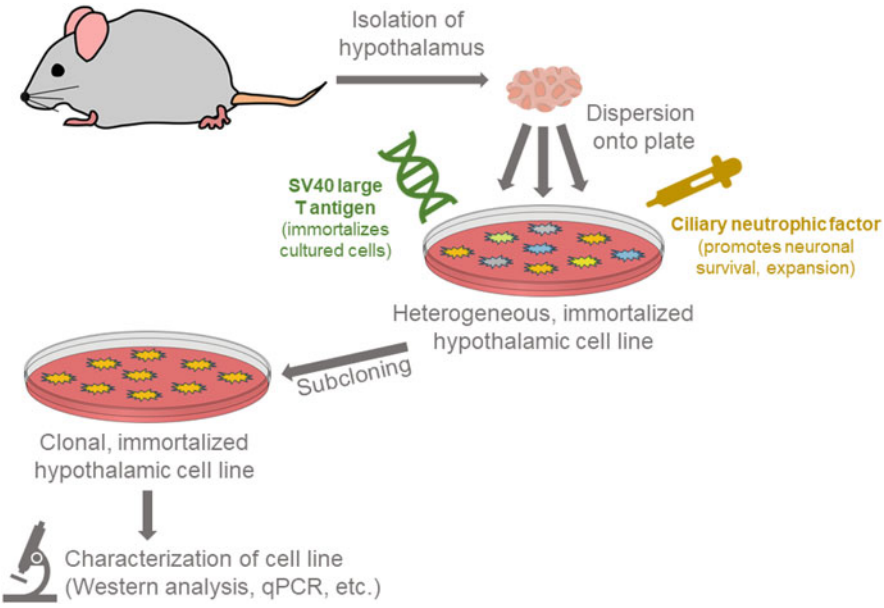

Fig. 7.3 Heterogeneous and clonal immortalized hypothalamic cell lines can be established from dispersion of hypothalamic tissues. Whole hypothalamic tissue from a sacrificed animal can be dispersed into cell culture media, then grow in ciliary neurotrophic factor to promote proliferation of the dispersed cells. Transfection of cultured cells with SV40 large T antigen can induce cell immortalization, cells retain contact inhibition, and remain a monolayer on the plate even when fully confluent (Tran et al. 2016). Subcloning can then isolate an individual immortalized cell from the heterogeneous cell population. The clonal cell line can then be characterized in order to identify the cell type and other characteristics

7.6 Neuroinflammation from the View of the Neuron

As previously mentioned, the ability of the hypothalamus to sense lipids circulating in the periphery is crucial to regulating energy balance, but also puts it at risk of developing neuroinflammation upon exposure to high levels of fat. This neuroinflammatory response involves distinct mechanisms on both sides of the plasma membrane, including the activation of fatty acid receptors on the cell surface as well as signaling via intracellular metabolism of the fatty acids.

7.6.1 Fatty Acids Induce Inflammation Through Cell Surface Receptors

Fatty acids induce inflammation in hypothalamic neurons by activating innate immune receptors, such as toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4), which bind peptidoglycans and lipopolysaccharides, respectively (Zhou

2018; Tran et al. 2016). Surprisingly, although TLR4 mediates the inflammatory effects of the fatty acid palmitate, it was demonstrated not to directly bind TLR4 (Lancaster et al. 2018). The mechanism of activation remains unclear. In addition to toll-like receptors, fatty acids can also bind the G protein-coupled receptors GPR40 and GPR120 to mediate downstream signaling (Moniri 2016).

Activation of the immune receptors by fatty acids leads to the activation of the canonical IKK-β/NF-κB pathway to drive the expression of inflammatory cytokines such as IL-1β, IL-6, and TNFα (Zhou 2018; Tran et al. 2016; Douglass et al. 2017). In addition to inducing inflammation, IKK-β/NF-κB signaling also induces expression of orexigenic *Npy* mRNA, illustrating how palmitate-induced inflammatory signaling cascades could directly dysregulate orexigenic responses to energy excess (Dalvi et al. 2017). Furthermore, the pro-inflammatory cytokine TNFα, which is induced upon palmitate exposure, is able to directly induce *Npy* and *Pomc* mRNA, demonstrating again how neuroinflammation can dysregulate feeding peptide expression (Dalvi et al. 2017).

7.6.2 Bioactive Products of Fatty Acid Metabolism Induce Inflammation

Aside from engaging cell-surface receptors, fatty acids can enter cells where they are metabolized into bioactive intermediates. Using the fatty acid palmitate as an example, the process begins with the conversion of palmitate to palmitoyl-CoA. From here, the condensation of palmitoyl-CoA with serine by serine palmitoyltransferase (SPT) represents the rate-limiting step in a series of enzymatic steps to produce the sphingolipid ceramide (Cruciani-Guglielmacci et al. 2017). Increasing ceramide levels in the hypothalamus via experimental injections or elevated fatty acid accumulation and subsequent de novo ceramide synthesis leads to induction of pro-inflammatory cytokines IL-6 and TNFα (Sergi et al. 2018).

7.6.3 Neuroinflammation Impairs Hypothalamic Insulin and Leptin Signaling and Metabolic Neuropeptide Expression

Activation of inflammatory signaling by palmitate or its metabolites can result in leptin resistance and insulin resistance. This is in part due to the intersection of inflammatory signaling with pathways downstream of the leptin receptor and the insulin receptor. For example, activation of inflammatory c-Jun N-terminal kinases (JNK), a component of the mitogen-activated protein kinase (MAPK) pathway, leads to inhibitory phosphorylation of insulin receptor substrate 1 (IRS-1) and interferes with signaling downstream of the insulin receptor. This contributes to hypothalamic insulin resistance, failure to properly sense whole-body energy status, and inappropriate neuropeptide expression. Inflammatory signaling can further drive neuropeptide expression toward weight gain by interacting with their regulators, such as the transcription factors FOXO1 and suppressor of cytokine signaling

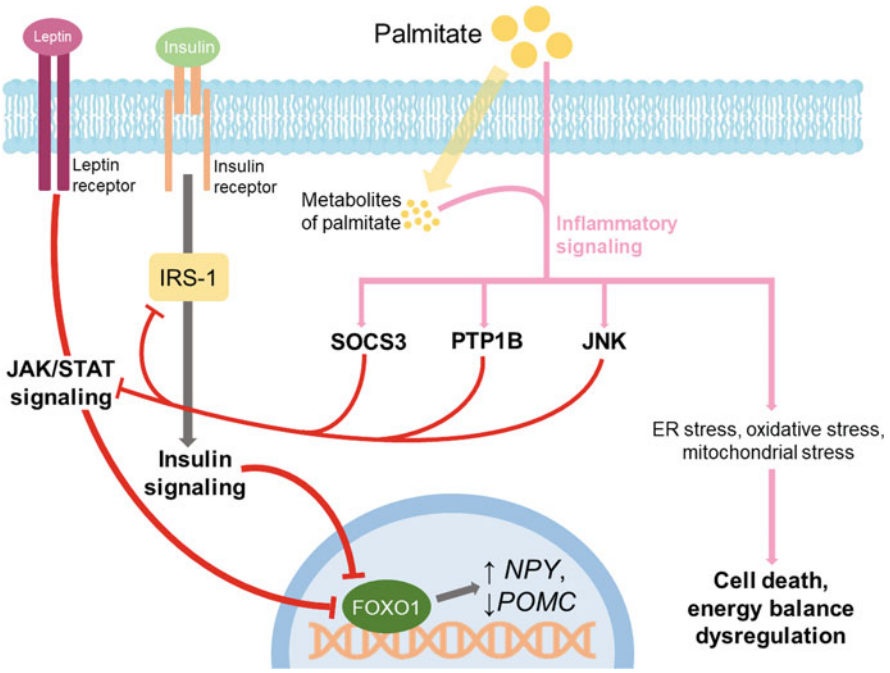

Fig. 7.4 Palmitate and its metabolites induce inflammatory signaling, thereby contributing to cellular stress, insulin and leptin resistance, and dysregulation of feeding-related neuropeptide expression

3 (SOCS3), the kinase mTOR, and the phosphatase PTP1B (Varela and Horvath 2012). In addition to altering neuropeptide expression, cell death is a consequence of prolonged inflammation as it leads to oxidative stress, endoplasmic reticulum stress, and mitochondrial stress (Tran et al. 2016; Baufeld et al. 2016). The resulting loss of neurons also contributes to dysregulated hypothalamic control of energy balance (Fig. 7.4).

7.6.4 Populations of Hypothalamic Neurons Respond Differently to Neuroinflammation

An important concept that has developed in recent years is that not all hypothalamic neurons are affected equally by neuroinflammation, nor are they affected by the same mechanisms. For instance, IKK-β/NF-κB signaling has been mechanistically linked to the induction of *Npy* and mRNA by palmitate, but not to the induction of *Pomc* mRNA, which instead involves JNK signaling and palmitoyl-CoA synthesis (Baufeld et al. 2016; Tse and Belsham 2018). As previously mentioned, prolonged inflammation leads to cell death, and interestingly, chronically elevated levels of fat selectively induces neuronal injury and loss of POMC but not NPY/AgRP neurons

(Thaler et al. 2012). Consequently, this impairs anorexigenic responses that can be generated by the hypothalamus. Together, neuropeptide expression regulated by metabolic signals occurs through population-specific pathways and neuroinflammation negatively impacts the viability of specific neuronal populations to varying degrees.

7.7 Neuroinflammation from the View of the Glia

All inflammatory CNS disorders, including obesity, involve a microglial component that plays a part in the initiation, progression, and/or resolution of the disorder. This section will cover the triggers that activate and recruit microglia to sites of inflammation, and how microglial functions can be modulated by the neuroinflammatory environment.

7.7.1 Microglia Directly Sense Fat to Initiate an Inflammatory Response

Much like peripheral immune cells, microglia residing in the brain express an array of toll-like receptors, including TLR4. Elevated levels of lipids in the hypothalamus induce inflammatory responses in microglia in a TLR4-dependent manner. This process results in the induction of cytokines such as IL-4, IL-6, IL-10, TNFα and other inflammatory mediators such as reactive oxygen species (ROS), nitric oxide (NO), and prostaglandins (Donat et al. 2017). Microglia can be "stress conditioned" to produce beneficial inflammatory responses that dampen neuroinflammation and limit impacts on the CNS (Ransohoff and Cardona 2010). Conversely, maladaptive microglial responses can exacerbate neuroinflammation (Douglass et al. 2017; Donat et al. 2017). While microglia can detect and directly mount immune responses to lipids, bidirectional microglia-neuron interactions can alter the course of hypothalamic neuroinflammation.

7.7.2 Microglia Are First Responders to Sites of Neuroinflammation and Stress

Microglial inflammatory responses are influenced by neurons, which also experience inflammation and cellular stress. Under these conditions, neurons release cytokines and molecules signifying distress to local microglia. Key distress signals include TNFα (which promote pro-inflammatory activation of microglia), CX3CL1 and CCL2/MCP-1 (which recruit microglia to distressed neurons), as well as extracellular nucleotides ATP, ADP, and UDP (which are only released during stressful conditions) (Zhou 2018; Tran et al. 2016; Douglass et al. 2017; Szepesi et al. 2018). Upon activation and arrival, the microglia phagocytose apoptotic cells and debris (Donat et al. 2017; Ransohoff and Cardona 2010). Depending on the

inflammatory context, microglia can secrete factors and cytokines that eventually resolve inflammation and repair damage, or exacerbate inflammation leading to further neuronal injury and loss.

7.7.3 Microglial Activation Exists Along a Spectrum, with Pro-inflammatory M1 and Anti-inflammatory M2 Marking the Two Extreme States

Once activated by inflammatory stimuli, microglia transition from a ramified state characterized by long, fine projections extending from a small cell body (soma) to an amoeboid-like morphology (characterized by expansion of soma and retraction of processes) (Szepesi et al. 2018). As morphological differences between different activation states are subtle, the definitive method to identify a specific activation state is gene expression profiling (Donat et al. 2017; Ransohoff and Cardona 2010). The different microglial activation states are similar to macrophage states in the periphery. This classification scheme describes two primary activation states: M1, which is the classical pro-inflammatory phenotype, or M2, which is an alternative anti-inflammatory phenotype (Fig. 7.5).

However, such a model is oversimplified. Microglia rarely express markers of either state exclusively; in reality, they tend to express a mixture of markers of both states, which places their activation state on a spectrum (as indicated by the changing colors in the figure) that can be biased toward the M1 or M2 state. M1 microglia are characterized by expression of pro-inflammatory cytokines IL-1β, IL-6, and TNFα, inducible nitric oxide synthase (iNOS), and the chemokine CCL2/MCP1, which

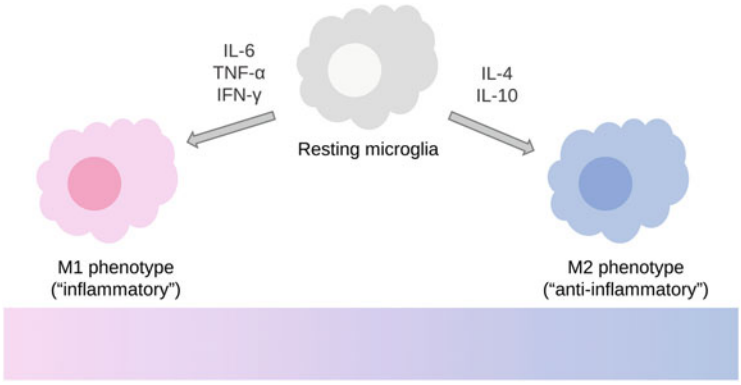

Fig. 7.5 Resting microglia can differentiate into inflammatory or anti-inflammatory phenotypes after exposure to specific cytokines. The phenotypes of differentiated microglia exist along a spectrum ranging between the pro-inflammatory M1 phenotype and anti-inflammatory M2 phenotype (as indicated by the color changes below the diagram). Pro-inflammatory cytokines tend to drive microglia toward an M1-like phenotype, while anti-inflammatory cytokines tend to drive microglia toward an M2-like phenotype. Microglia can thus either exacerbate or resolve neuroinflammation, depending on the inflammatory context of their local environment

helps recruit additional microglia. Conversely, M2 microglia are characterized by expression of anti-inflammatory cytokines IL-4, IL-10, and IL-13, in addition to the enzyme arginase 1 (Arg1) (Cherry et al. 2014). The activation state that microglia initially adopt is not absolute, as they can dynamically shift along the spectrum during the course of inflammation (Donat et al. 2017; Cherry et al. 2014).

7.7.4 M2 and M1 Activation States Are Prevalent in Acute and Chronic States of Neuroinflammation, Respectively

The divergent roles of microglia in hypothalamic inflammation are seemingly contradictory; in the short term, it is likely that through stress conditioning by elevated saturated lipids from the periphery, microglia mediate a protective effect to prevent subsequent injury to the hypothalamus and CNS as a whole (Ransohoff and Cardona 2010). Specifically, direct exposure to high levels of saturated fatty acids subsequently biases an M2 microglia activation state marked by the induction of anti-inflammatory IL-10 and IL-13, thereby dampening the detrimental impact of saturated fatty acids on hypothalamic neurons. However, prolonged exposure polarizes M2 microglia toward the M1 state and induces a pro-inflammatory response in neurons, thereby exacerbating stress and damage in the CNS (Tran et al. 2016; Dalvi et al. 2017; Donat et al. 2017).

7.8 Crosstalk Between Neurons and Glial Cells

The traditional notion that non-active microglia remain quiescent in a healthy CNS has been proven incorrect; even when microglia are "quiescent," they are actively surveying the CNS and engaging with neurons. This allows microglia to gather information on the state of the CNS and detect signs of injury and pathology. Microglia are constantly active in this state as their processes undergo cycles of extension and contraction, to make contact with neighboring neurons. It is estimated that microglia survey the entire brain every 4–5 h (Ransohoff and Cardona 2010).

7.8.1 Neurons in the Healthy CNS Maintain Microglia in a Surveillant, Ramified State

Under normal physiological conditions, neurons express basal levels of "resting" signals that repress microglial activation and maintain microglia in their ramified state. Two of the most studied resting signal axes include CD200-CD200R and CX3CL1-CX3CR1 (Douglass et al. 2017; Ransohoff and Cardona 2010). CD200 is a glycoprotein ubiquitously expressed on the cell membranes of neurons, astrocytes, and oligodendrocytes, whereas the target receptor CD200R is only expressed on microglia (Szepesi et al. 2018). Similarly, CX3CL1 (also known as fractalkine) is a chemokine constitutively expressed by neurons while its receptor, CX3CR1, is

highly expressed on microglia in the CNS. Furthermore, the neuronal integrin CD47 signals via microglial CD172a to prevent microglial activation (Kierdorf and Prinz 2013).

In addition to preventing aberrant microglial activation, the presence of multiple inhibitory signals also serves neuroprotective roles under normal conditions to prevent neuronal stress, injury, and loss. In cases of neuronal distress, it is possible that a down-regulation of inhibitory signals from neurons is a mechanism to activate microglia.

7.8.2 Neuroinflammation Recruits and Modifies Microglial Responses

As previously mentioned, microglia are able to directly sense and become activated by elevated fatty acids. However, microglia are also activated by distressed neurons in this environment. The onset of neuroinflammation alters neuronal activity, neuronal expression of the aforementioned resting signals, as well as the release of neuropeptides, neurotransmitters, and cytokines that can activate and recruit microglia to distressed neurons.

Neuronal distress can lead to the leakage or vesicular release of the nucleoside phosphates ATP, ADP, and UDP into extracellular space, which can be detected by microglial purinergic receptors. ATP and ADP are detected via P2Y12R and P2X4R, while UDP is detected by P2Y6R (Szepesi et al. 2018; Kierdorf and Prinz 2013). As the distribution of ATP and ADP tends to be overwhelmingly intracellular, their presence in the extracellular environment is interpreted by microglia as a danger signal. Detection of these signals by microglia induces activation and chemotaxis to the source of the nucleoside phosphates.

Aside from extracellular nucleoside phosphates, microglia are also modulated by neuronal activity through the expression of various neurotransmitter receptors. The neurotransmitter glutamate is able to inhibit microglial activity via metabotropic G-protein coupled glutamate receptors (mGluRs). Gamma-aminobutyric acid (GABA) is another neurotransmitter that acts on microglial GABA-A and GABA-B receptors to suppress inflammatory cytokine production (Posfai et al. 2019). The neurotransmitters dopamine and glycine also mediate similar inhibitory effects on microglial inflammation through their respective receptors (Szepesi et al. 2018).

Hypothalamic neuropeptides can also regulate microglial activity. Microglial subpopulations are heterogeneous in their ability to respond to certain neuropeptides. For example, microglia express receptors for NPY as well as peptides derived from POMC, such as ACTH and α-MSH. NPY and α-MSH inhibit inflammatory microglial responses by repressing production of TNFα, NF-κB, NO, and IL-1β.

Importantly, neuron-secreted cytokines play a critical role in determining the tasks performed by microglia in the local environment. A major chemotactic factor that recruits microglia is the chemokine CCL2/MCP-1, which binds either CCR2 or CCR4. Another example is the chemokine CCL21, which is expressed exclusively by distressed neurons to recruit CXCR3-expressing microglia (Szepesi et al. 2018).

Upon arrival at sites of inflammation, microglial effector functions are profoundly influenced by the inflammatory milieu; for instance, neuron-derived IL-6, TNFα, and IFNy strongly bias microglia toward the M1 pro-inflammatory state, whereas IL-4 and IL-10 bias them toward the M2 anti-inflammatory state. These activated microglia can in turn modulate the environment, a process that will be discussed in the ensuing section.

In summary, changes in hypothalamic neuron activity, as well as their expression of nucleoside phosphates, neurotransmitters, feeding-related neuropeptides, and cytokines, will modulate inflammatory microglial activation and hypothalamic inflammation overall. Aside from the given examples, microglia express a growing list of receptors for neuron-derived somatostatin, dopamine, bradykinin, serotonin, endothelin-1, histamine, substance P, insulin and leptin, amongst a number of other receptors for peripheral signals. These emerging discoveries suggest that microglial responses are intimately linked to the state of neurons.

7.8.3 Microglia Influence Neuroinflammation and Neuronal Function in the Hypothalamus

Once microglia are activated in their local environment, often in a neuron-dependent manner as previously described, microglia will produce cytokines and other inflammatory mediators that in turn affect the course of inflammation in resident neurons. For example, microglia biased toward the pro-inflammatory M1 state will produce pro-inflammatory TNFα, IL-1β, IL-6, and NO in order to further exacerbate neuroinflammation and neuronal injury. The activity of M1 microglia will dysregulate the expression of hypothalamic neuropeptides, thereby impairing the ability of the hypothalamus to regulate energy balance. Conversely, microglia polarized toward the anti-inflammatory M2 state will express anti-inflammatory IL-4, IL-10, and IL-13; these cytokines not only buffer against neuroinflammation, but can also normalize changes in neuropeptide expression and preserve hypothalamic regulation of energy balance. The course of neuroinflammation is therefore profoundly influenced by microglia-to-neuron communication.

7.8.4 Bidirectional Communication via Extracellular Vesicles

Both neurons and glia produce extracellular vesicles as a means of communication over extended distances (Fig. 7.6). These vesicles contain an assortment of proteins, enzymes, mRNA and microRNA, as well as membrane surface proteins and receptors (Szepesi et al. 2018). Because of the extensive variety of potential contents, vesicles can vary greatly in size, from microvesicles under 100 nm in diameter to apoptotic bodies with a diameter of over 1000 nm. Vesicular contents change during the course of neuroinflammation as both neurons and microglia induce expression of inflammatory mediators. Importantly, vesicular docking and transfer to a recipient cell can alter the cell's gene expression, behavior, and function

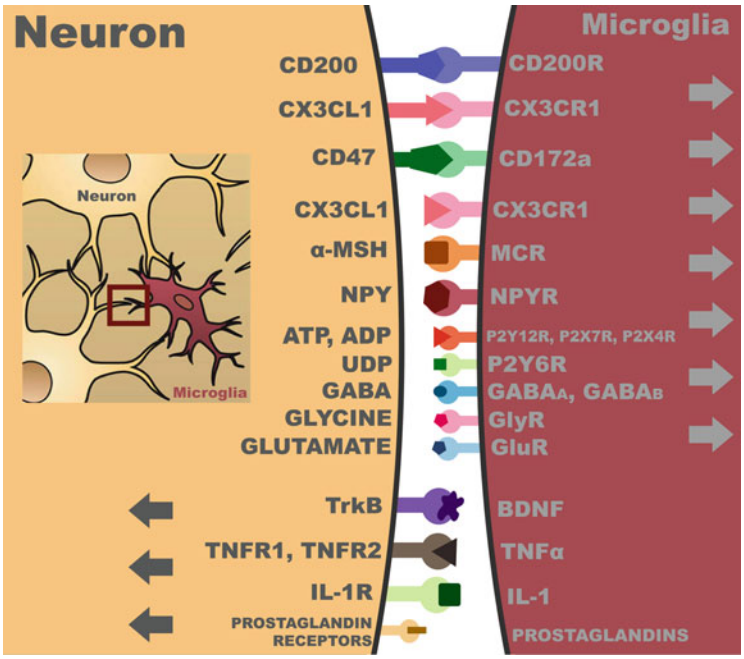

Fig. 7.6 Membrane-bound and soluble ligands facilitate bidirectional communication and influence between neurons and microglia (Adapted from Wikimedia Commons Dec 2013; Author: Holly Fischer; License: Creative Commons Attribution 3.0 Unported license)

by conferring cell surface receptors and internal contents (Szepesi et al. 2018). This mode of communication can induce inflammation in nearby cells when the docking vesicles deliver pro-inflammatory cargo.

7.9 Perspectives: Therapeutic Approaches to Counter Neuroinflammation

Because of extensive research efforts, hypothalamic neuroinflammation has been identified as a key therapeutic target to treat obesity. Options range from general anti-inflammatory compounds to drugs that specifically target neuronal or glial components of hypothalamic inflammation (Fig. 7.7). Recurring inflammatory pathways throughout this chapter are also potential candidates for drug targeting, but drugs that target pathways that are too general may have severe and often dangerous side effects.

Compounds with broad anti-inflammatory properties can relieve hypothalamic inflammation. Many of these compounds are FDA-approved or commonly consumed food products. For example, salicylate is an anti-inflammatory compound in medication such as aspirin and has been demonstrated to normalize the induction

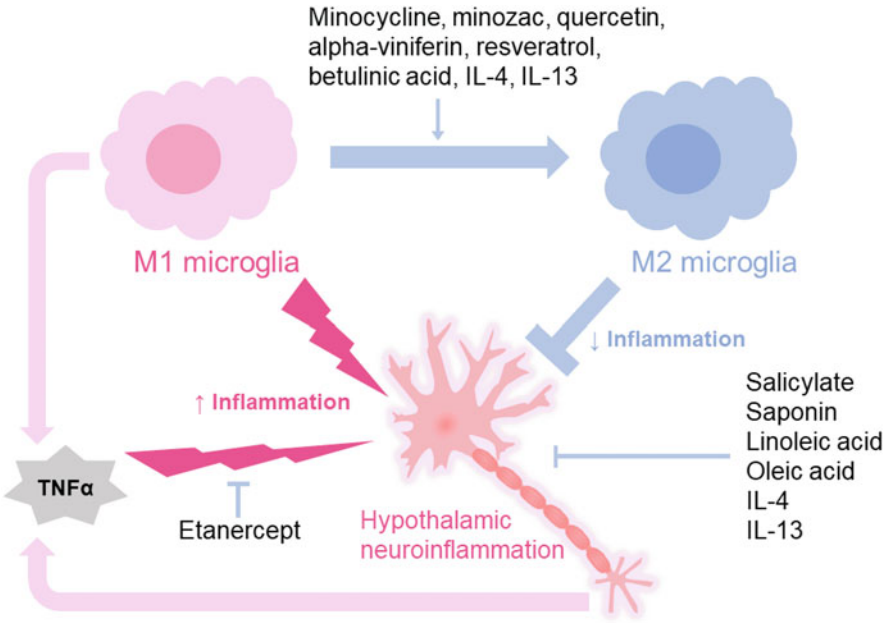

Fig. 7.7 Because hypothalamic neuroinflammation involves a vast amount of cellular and molecular involvement, many processes present as potential drug targets for managing the pathogenesis of obesity. Pro-inflammatory relationships are colored pink, while anti-inflammatory relationships are colored blue

of *Npy* and *Agrp* mRNA by palmitate. Saponin, a compound found in certain teas can reduce inflammatory cytokine production as well as restore leptin sensitivity and *Pomc* mRNA expression. Unlike saturated fatty acids, monounsaturated and omega-3 fatty acids do not induce inflammation. Flaxseed oil (a source of omega-3 α-linolenic acid) and olive oil (which consists primarily of monounsaturated oleic acid) dampen neuroinflammation by reducing inflammatory NF-κB and JNK activation. Treatment with oleate normalizes the induction of *Pomc* and *IL-6* mRNA by palmitate (Tse and Belsham 2018). Furthermore, treatment with IL-4 and IL-13 can mimic the anti-inflammatory actions of M2 microglia, such as reducing inflammatory TNFα expression and normalizing the palmitate-mediated induction of *Npy* mRNA levels.

Another large focus of this chapter is the notion that microglia can be biased toward a pro-inflammatory M1 state or an anti-inflammatory M2 state. Indeed, targeting microglia on the basis of attenuating inflammatory microglial activation and biasing an M2 state is another potential strategy to counter neuroinflammation. Minocycline and minozac are two compounds that dampen pro-inflammatory microglia activation by inhibiting NF-κB and JNK pathways (Donat et al. 2017). Minozac also inhibits the enzymes NADPH oxidase and iNOS to reduce the levels of reactive oxygen and nitrogen species respectively, thereby decreasing oxidative

stress and its contribution to inflammation (Donat et al. 2017). Furthermore, many plant-derived compounds, such as the polyphenols quercetin, alpha viniferin, and resveratrol, as well as the terpenoid betulinic acid, can promote M2 microglial activation. In addition to mediating the anti-inflammatory effects of M2 microglia, IL-4 and IL-13 can also promote M2 microglia polarization. The anti-inflammatory actions of these cytokines on both neurons and microglia highlight their synergistic effects in alleviating inflammation.

A few neuroinflammatory pathways common to both neurons and microglia were discussed in this chapter, including TLR4, IKK-β/NF-κB, and MAPK signaling. These are candidate targets for resolving hypothalamic inflammation, especially considering that these pathways induce inflammation in both neurons and microglia. For example, pharmacologic inhibition of the TLR4-IKK-β/NF-κB axis protects rodents from neuroinflammation and ultimately diet-induced obesity (Dalvi et al. 2017; Milanski et al. 2012). Another strategy is to target inflammatory cytokines downstream of TLR4-NF-κB signaling. For example, blockage of TNFα with antibodies or etanercept reduces hypothalamic neuroinflammation (Dalvi et al. 2017; Donat et al. 2017).

The importance of the hypothalamus in our homeostatic functions, including energy balance, can hardly be overstated. The hypothalamus is a heterogeneous environment, home to diverse glial cell types and neuronal populations. Diet-induced obesity and exposure to elevated lipids have been consistently linked to hypothalamic neuroinflammation, a process that involves both neuronal and microglial components. Many in vivo and in vitro studies on the pathogenesis and development of obesity are shedding light on the complex process of hypothalamic neuroinflammation, and how neuroinflammation can be caused by, and further exacerbate, feeding dysregulation.

This is probably just the beginning to our understanding of how the neurons and glial cells of the hypothalamus engage in extensive crosstalk during the course of neuroinflammation, with many cytokines, peptides, and metabolites playing important roles. A better understanding of the interactions between neurons and microglia that contribute to neuroinflammation can help us develop targeted therapeutics to alleviate neuroinflammation as a means to prevent or treat obesity.

7.10 Key Literature

Levin et al. (2011) Seminal paper that shows how neurons sense nutrients.
Lutz and Woods (2012) An overview of animal models used in obesity studies.
Milanski et al. (2012) Important paper linking inflammation to TLR4 and insulin resistance.
Perez-Cerda et al. (2015) Describes the key glial discovery.
Thaler et al. (2012) Study of microglial actions in the brain.
Zhang et al. (1994) The discovery of leptin.

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Part IV

Glial–Neuronal Interactions in the Control of the Stress Response

Retrograde Signaling Via Dendritic Activation of Glial-Neuronal Circuits

8

Juhee Haam, Zhiying Jiang, and Jeffrey G. Tasker

Abstract

Neuroendocrine cells in the hypothalamus secrete neuropeptides from both their axon terminals and their dendrites to regulate homeostatic function. Vasopressin is expressed in both vasopressin and corticotropin-releasing-hormone neurons and is released dendritically following exposure to homeostatic neuromodulators such as ghrelin and norepinephrine. Dendritically released vasopressin stimulates ATP release from astrocytes, which activates local upstream glutamate and GABA circuits. This *trans*-neuronal-glial retrograde signaling exploits the spatial domain of astrocytes to expand the reach of dendritic volume transmission from neuroendocrine cells to distal presynaptic partners to regulate local synaptic circuit inputs, thus providing a powerful means of neuroendocrine cell autoregulation of hormonal output.

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Keywords

Astrocytes · Tripartite synapse · Gliotransmitter · Vasopressin · Corticotropin-releasing hormone · Ghrelin · Norepinephrine

8.1 Introduction: Gliotransmission

8.1.1 The Tripartite Synapse

First proposed in the 1990s, the model of the **tripartite synapse** (Fig. 8.1) recognizes the essential role of glia in synaptic function by means of interleaving glial processes with presynaptic and postsynaptic neuronal structures (Araque et al. 1999). Since glial cells lack the ability to generate action potentials, they previously have been considered "quiet" cells, whose main role is to provide mechanical and metabolic support for neuronal cells in the brain. An abundance of data collected over the past two decades, however, highlights the many unique roles of glial cells in sensing changes in neuronal activity and influencing neuronal signals by releasing transmitters (termed **gliotransmitters**) such as taurine, ATP, D-serine, and glutamate.

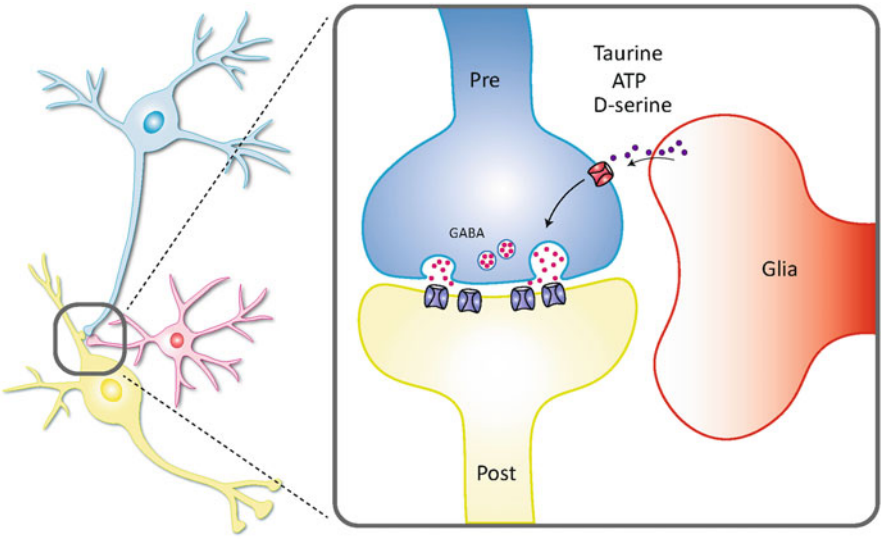


Fig. 8.1 The tripartite synapse and gliotransmission. Astrocyte processes are frequently in close proximity to presynaptic axon terminals and postsynaptic dendrites and dendritic spines, where they can respond to orthograde and retrograde chemical signals from neurons (i.e., neurotransmitters) and release chemical signals (i.e., gliotransmitters) onto neurons. Thus, they can modulate synaptic transmission both pre- and postsynaptically. In the example shown here, gliotransmitters such as taurine, ATP, and D-serine regulate synaptic release at GABAergic synapses

Astrocytes are the most abundant type of glial cell. During the past thirty years, advances in anatomical studies have provided support for the role of astrocytes in synaptic function by demonstrating a close juxtaposition of astrocytic processes to neuronal synaptic structures. Astrocytes extensively cover various parts of neurons, such as dendrites, axon terminals, and somata, with irregular shaped processes. By virtue of their proximity to neurons and neuronal processes, astrocytes can act as a critical modulator of neuronal function. Their ability to maintain homeostasis of the extracellular milieu and to modulate neuronal activity by releasing gliotransmitters has been well documented over the past 20 years (Araque et al. 2014; Sahlender et al. 2014).

The unique anatomical characteristics of astrocytes provide clues as to how they act as the third participant at the synapse, along with the presynaptic axon terminal and the postsynaptic neuronal dendrite, and are indispensable in neuronal circuit signaling. Recent advances in histology and imaging techniques have revealed that a single astrocyte, with its extremely elaborate processes, can contact more than 10,000 synapses in rodents and two million synapses in humans (Bushong et al. 2002; Fields et al. 2015). Astrocytic domains are characterized by unique territories that form a tile-like structure with minimal overlap with their neighboring astrocytes (Bushong et al. 2002), allowing each astrocyte to exercise quasi-exclusive control over neuronal synapses in its spatial domain. By providing extensive coverage of its territory, an astrocyte can effectively control a set of functionally defined synapses. In addition, the dynamic nature of the astrocytic processes offers a powerful means of regulation of synaptic activity relevant to synaptic plasticity. The astrocyte-specific glial fibrillary acidic protein (GFAP) is expressed primarily in the large stem processes of astrocytes, which are altered in response to physiological stimuli, suggesting that the astrocytic stem processes are dynamic. Furthermore, the GFAP-negative fine processes that extend from the stem processes are also highly flexible, showing significant morphological changes on a time scale of seconds to minutes that result from their lack of intermediate filaments and microtubules and low expression of actin filaments (Derouiche and Frotscher 2001).

The morphological flexibility of astrocytes has been particularly well demonstrated in the hypothalamus. In the suprachiasmatic nucleus, which is the circadian clock of the brain, morphological changes occur according to the light/dark cycle within 24 h. In the rostral preoptic area, astrocytes undergo steroid hormone-mediated changes in morphology during the estrous cycle that alter the astrocytic coverage of the surface of gonadotropin-releasing hormone (GnRH) neurons, resulting in corresponding changes in synaptic inputs to the GnRH neurons. In the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus, where magnocellular neurons that release the neurohypophysial hormones oxytocin and vasopressin are located, strong physiological stimuli such as dehydration and lactation induce dramatic changes in the astrocytic coverage, which also has a potent impact on neuronal signaling at excitatory and inhibitory synapses on the magnocellular neurons (described in detail in Chap. 2).

8.1.2 Neuronal Communication with Astrocytes

Unlike neurons, which generate electrical signals, glial cells signal via fluctuations in their intracellular calcium concentration. The seminal work by Kuffler and colleagues in the 1960s demonstrated that glial cells present an increase in intracellular calcium when neighboring neuronal cells are electrically stimulated, providing the first direct evidence that glial cells actively respond to neuronal signals (Orkand et al. 1966).

Astrocytes express a variety of ionotropic and metabotropic receptors that are activated by neurotransmitters. They express high levels of metabotropic receptors, which are G protein-coupled receptors. The activation of metabotropic receptors triggers intracellular G-protein and second-messenger signaling cascades that lead to an increase in the intracellular calcium concentration in astrocytes. Changes in intracellular calcium levels are caused primarily by release from intracellular calcium stores, the endoplasmic reticulum and mitochondria, but calcium can also enter the cell from the extracellular space through store-operated channels or transporters, such as the sodium-calcium exchanger (Verkhratsky et al. 2012). In addition, ionotropic receptors, which are transmitter-activated ion channels, have been shown to play a critical role in mediating fast-acting astrocytic responses to a variety of neurotransmitters. Glutamate receptors were among the first neurotransmitter receptors to be identified in astrocytes. They comprise both ionotropic and metabotropic receptors activated by the release of the excitatory neurotransmitter glutamate from neighboring neurons. Astrocytes also express both metabotropic and ionotropic receptors for the inhibitory neurotransmitter GABA, though GABA's actions in astrocytes are usually not inhibitory. Activation of the metabotropic $GABA_B$ receptors causes an increase in intracellular calcium levels via release from intracellular calcium stores. Activation of the ionotropic $GABA_A$ receptors on astrocytes causes a membrane depolarization due to the high intracellular chloride concentration and reversed chloride concentration gradient, which can subsequently activate voltage-gated calcium channels. In addition to glutamate and GABA receptors, astrocytes express several other types of receptor that can be activated by neurotransmitters such as norepinephrine (NE) and acetylcholine.

There have been significant improvements recently in identifying the source of neurotransmitter-induced calcium responses in astrocytes. Recent advances in calcium indicators have improved labeling of astrocytic processes. While many of the bulk-loaded dye and cytosolic calcium indicators do not effectively label the astrocytic processes with which most synaptic structures are intimately associated, the newer membrane-targeted genetically encoded calcium indicators effectively label fine astrocytic processes (Box 8.1). Furthermore, advances in *in vivo* imaging have enabled us to circumvent issues with altered calcium responses under *in vitro* conditions. With these advances, we now have gained a better understanding of the cellular mechanisms underlying the neuronal activation of astrocytes.

In the hypothalamus, astrocytes respond not only to classical neurotransmitters, but also to various neurohormones. Vasopressin and cholecystokinin, which are neurohormones secreted by neuroendocrine cells, have been shown to activate

hypothalamic astrocytes (Chen et al. 2019; Crosby et al. 2018; Haam et al. 2014). Astrocytes in the hypothalamus also respond to NE (Gordon et al. 2005). When activated, the neurohormone/neurotransmitter receptors on astrocytes trigger a signaling cascade that subsequently induces an increase in intracellular calcium and often the release of gliotransmitters. The gliotransmitters released into the extracellular space can reach neuronal receptors and modulate neuronal circuits relevant to the physiological function of the brain region, which will be discussed in more detail in the next few sections.

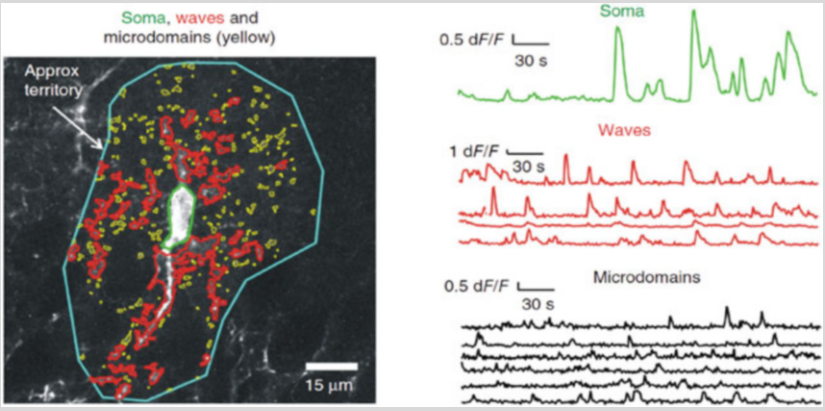
In addition to classical neurotransmitter release from axon terminals, somatodendritic release of neurotransmitters has also been well documented in neuroendocrine cells. Astrocytes that cover dendritic regions can receive dendritically released neurotransmitters and mediate a retrograde feedback signal to a group of neuroendocrine cells that are functionally related (Chen et al. 2019; Crosby et al. 2018; Haam et al. 2014). The hypothalamic astrocyte's capacity to respond to various transmitters, including neuroendocrine hormones, suggests that astrocytes can effectively sense local neuronal activity and participate in the modulation of the relevant neural circuits.

Box 8.1 Measuring glial activity using calcium imaging

Astrocytes use calcium signaling to code information, communicate with other cells, and release gliotransmitter. The activity of glial cells can be measured by using calcium indicators that change their fluorescence intensity with changes in the calcium concentration. Since the early days of chemical calcium indicators, which are loaded into cells using patch-clamp techniques or membrane-permeant acetoxymethyl (AM) esters, there has been great progress in calcium imaging in glial cells. Recent developments in genetically encoded calcium indicators (GECIs), such as GCaMP, have allowed not only the cell type-specific expression of calcium indicators in glial cells, but also the labeling of fine distal processes of glia, which chemical calcium dyes cannot easily reach. With the use of GECIs, calcium fluctuations can be observed not only in the somatic regions but also in fine processes (microdomains, figure below) (Srinivasan et al. 2015). Furthermore, advances in *in vivo* fluorescence measurement techniques such as multi-photon imaging and fiber photometry have allowed calcium measurement in freely behaving animals.

(continued)

Box 8.1 (continued)



Calcium fluctuations can manifest as three predominant subtypes depending on the location within a cell: somatic Ca^{2+} fluctuations (green), Ca^{2+} waves that spread between adjacent areas in processes (red), and microdomains, which are restricted to local spots in processes (yellow territories on the left, black traces on the right) (Adapted from (Srinivasan et al. 2015) with permission).

8.1.3 Astrocyte Communication with Neurons

It is well known that neuronal cells communicate with each other by releasing neurotransmitters at chemical synapses. Astrocytes not only release classical transmitters, such as glutamate and GABA, but also release various types of other transmitters, such as ATP, D-serine, and taurine, which exert diverse effects on other astrocytes and on neighboring neurons (Araque et al. 2014; Sahlender et al. 2014) (see also Chap. 2).

Neurotransmitters released from neighboring neurons bind to their cognate receptors and activate a variety of signal transduction pathways in astrocytes. Among those, an increase in intracellular calcium has been well characterized as the hallmark of astrocytic activation, which triggers the release of gliotransmitters (Araque et al. 2014; Sahlender et al. 2014; Verkhratsky et al. 2012). Extracellular gliotransmitters then activate receptors on neighboring neuronal cells, which triggers the opening of ion channels or a second messenger-mediated response (Araque et al. 2014).

Astrocytes have been shown to communicate with each other to expand their spatial signaling to enable regulation of a large population of neurons when necessary. Astrocytes communicate directly with other astrocytes via gap junctions,

through which second messengers as well as intracellular calcium ions can quickly spread. Glia-to-glia communications also can be achieved through gliotransmitters. Extracellularly released gliotransmitters can activate receptors on neighboring astrocytes in a paracrine fashion to propagate intercellular calcium waves and amplify astrocytic signals.

In the hypothalamic PVN and SON, changes in gliotransmission can happen slowly due to morphological changes in glia or rapidly in response to local signals such as neurotransmitters. Astrocytes present dramatic morphological changes during strong physiological stimuli such as lactation and dehydration, which result in an altered astrocytic coverage of magnocellular neuroendocrine cells. The retraction of astrocytic processes surrounding magnocellular neuroendocrine cells during lactation leads to a reduced capacity for synaptic plasticity at glutamate synapses due to a decrease in the astrocytic release of D-serine, which acts at excitatory synapses as a co-agonist of the NMDA receptor required for synaptic potentiation (Panatier et al. 2006) (see also Chap. 2). In addition, glial retraction after a chronic dehydration regime eliminates taurinergic gliotransmission, which normally activates glycine receptors expressed on magnocellular neurons (Hussy et al. 2001). Finally, as mentioned above, removal of the glial coverage of magnocellular neuronal membranes results in new glutamate and GABA synapse formation (see Chap. 2 for a detailed description).

8.1.4 ATP as a Gliotransmitter Regulating Neuroendocrine Cells

Although used also as a neurotransmitter, ATP has been described as a gliotransmitter in structures throughout the brain. In the PVN and SON, astrocytes release ATP to regulate neuroendocrine cell activity in response to multiple signals from both extrinsic and intrinsic sources. Briefly (see below for a detailed description), the dendritic release of vasopressin from vasopressinergic neuron dendrites activates ATP release from PVN and SON astrocytes, which stimulates recurrent GABAergic circuits to the vasopressin neurons (Haam et al. 2014). Norepinephrine activates alpha1 receptors directly on astrocytes in the SON/PVN to stimulate the astrocytic release of ATP, which controls the membrane trafficking of glutamate receptors in magnocellular neurons (Gordon et al. 2005). Recent evidence suggests that norepinephrine also activates alpha1 receptors on corticotropin-releasing hormone (CRH) neurons in the PVN to cause the dendritic release, interestingly, of vasopressin, which stimulates astrocytes in the PVN to release ATP and recruit recurrent GABA and glutamate circuit inputs to the CRH neurons (Chen et al. 2019).

8.1.5 Trans-astrocyte Inter-neuronal Communication

Bidirectional signaling between neurons and glia has been described in several brain areas to be critical for the proper function of neural circuits. The trans-neuronal endocannabinoid signaling in the hippocampus is an excellent example that shows

neuronal-glial bidirectional communication that is fundamental to synaptic function (Navarrete and Araque 2008). The type 1 cannabinoid receptor is expressed in hippocampal astrocytes and binds endocannabinoid released from neighboring neurons. The astrocytes respond by increasing intracellular calcium levels, which triggers the release of glutamate onto neighboring neurons and the subsequent activation of neuronal NMDA receptors (Navarrete and Araque 2008).

The "sandwich synapse" described by Stanley and colleagues also clearly demonstrates trans-glial neuronal signaling (Rozanski et al. 2013. At this synapse, two dorsal root ganglion neurons communicate with each other through an intercalated satellite glial cell. The stimulation of dorsal root ganglion cell somata induces the neuronal release of ATP to activate neighboring glial cells, which subsequently release a gliotransmitter to activate nearby neuronal cells.

8.2 Neuroendocrine Systems

8.2.1 Dendritic Neuropeptide Release in the Hypothalamic-Neurohypophysial System

The hypothalamic-neurohypophysial system (HNS), first described by German biologists Wolfgang Bargmann and Ernst Scharrer in the 1950s, is an important integrative structure that coordinates cardiovascular and reproductive functions. The HNS consists of magnocellular neuroendocrine cells in the SON and PVN of the hypothalamus that secrete the neuropeptides vasopressin and oxytocin from their axons, which run through the internal zone of the median eminence (ME) and terminate on the capillaries of the posterior lobe of the pituitary gland (Fig. 8.2). In response to hypovolemic and/or hyperosmotic stimuli, vasopressin (a.k.a. antidiuretic hormone) is released into the circulation, where it acts on the vascular smooth muscle to cause vasoconstriction and on the kidneys to regulate water conservation and restore fluid homeostasis. In females, oxytocin is involved in parturition and milk ejection by acting at the uterus and mammary glands, respectively.

In response to stimulation, magnocellular neurons in the HNS release vasopressin and oxytocin from their axonal terminals in the posterior lobe of the pituitary, as well as from their somata and dendrites in the SON and PVN. Large dense core vesicles (LDCVs), the organelles that store neuropeptides, are located throughout the neuroendocrine cell. The HNS is the best-studied model system for the detailed mechanisms of somato-dendritic release due to the relatively homogeneous neuroendocrine cell population in the SON and PVN, the relative lack of interneurons, and the unique anatomy that makes the neuroendocrine cells easily identifiable by antidromic stimulation of the pituitary.

Dendritic release of oxytocin occurs during parturition and lactation and contributes to the bursting activity of the oxytocin neurons and the pulsatile secretion of oxytocin into the blood. Synchronous firing among the oxytocin neurons is facilitated by positive feedback of dendritically released oxytocin and activation of

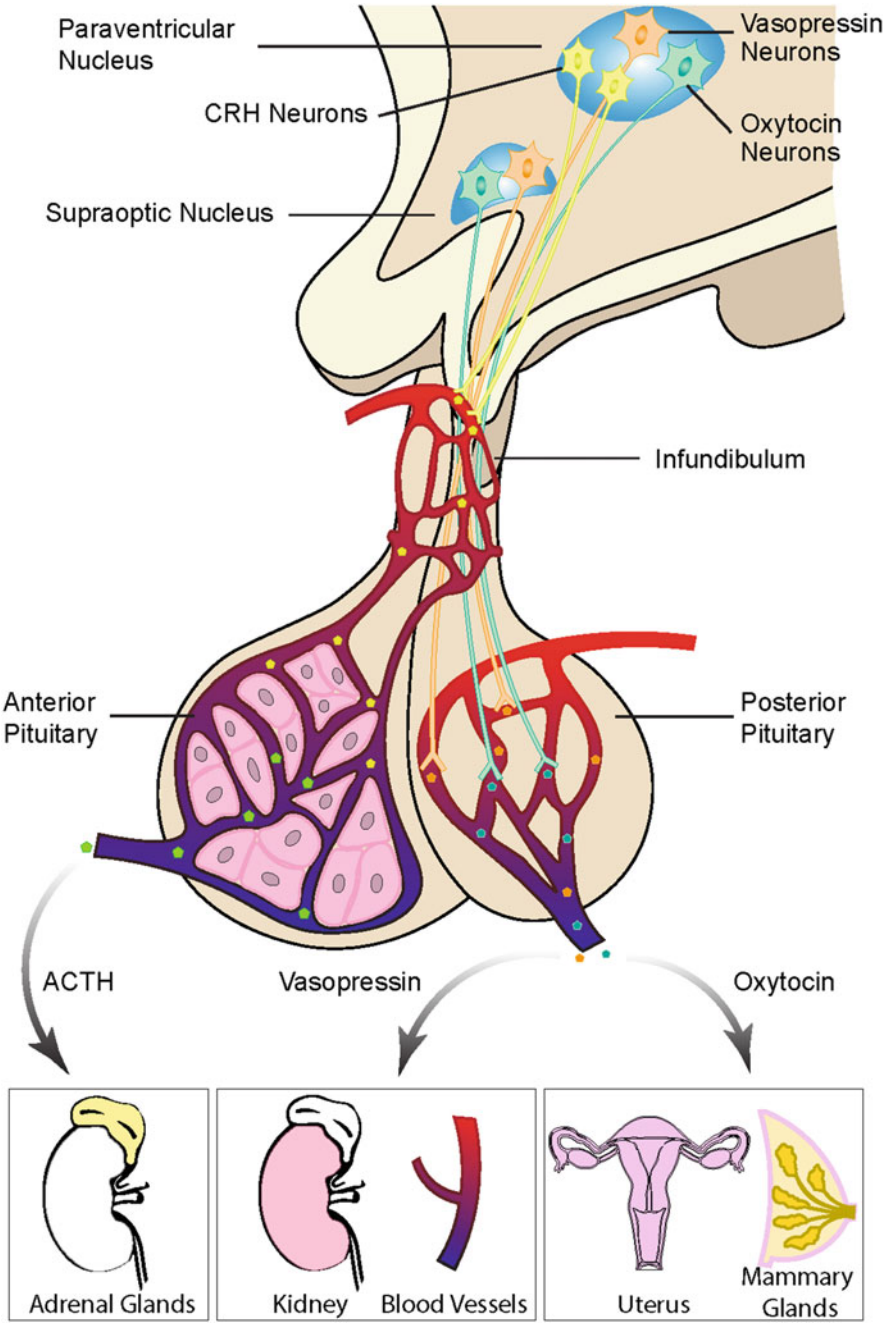


Fig. 8.2 The hypothalamic-neurohypophysial system and hypothalamic-pituitary-adrenal axis. Magnocellular neuroendocrine cells of the hypothalamic-neurohypophysial system reside in the hypothalamic supraoptic nucleus and paraventricular nucleus and secrete vasopressin and oxytocin from their axon terminals into the capillaries of the posterior lobe of the pituitary gland when stimulated. Circulating vasopressin binds to receptors in the kidney to cause water reabsorption and

oxytocin autoreceptors in the SON and PVN (Neumann et al. 1994). Oxytocin secretion into the blood promotes uterine contraction to facilitate the delivery process, and promotes milk let down by stimulating the milk ejection reflex during lactation. Dendritic release of oxytocin also contributes to the induction and maintenance of morphological changes in oxytocin neurons and astrocytes and to neuronal-glial interactions during reproduction (Theodosis et al. 1986). Dendritic release of oxytocin also occurs during exposure to stressors, especially social stressors such as social defeat and resident-intruder stress, and is believed to play a role in the maternal aggression during lactation that is critical for the defense of the offspring (Bosch et al. 2005).

Vasopressin neurons are osmosensitive, and dendritic release of vasopressin occurs in response to hypertonic stimuli. In contrast to the excitatory effect of oxytocin, the activation of autoreceptors on vasopressin neurons leads to a decrease in their firing and modulates their firing mode (Ludwig and Leng 1997). In addition to its autocrine effects, vasopressin can also function as a paracrine signal. Thus, dendritically released vasopressin can also diffuse to neighboring presympathetic neurons in the PVN to coordinate sympathetic outflow with homeostatic hormonal output (Son et al. 2013), or to nearby astrocytes that control local circuit activity in the SON and PVN (Haam et al. 2014).

Somato-dendritic release and axonal release of oxytocin and vasopressin can occur simultaneously or independently of one another. Parturition and suckling, as well as forced swim and shaker stress, increase oxytocin release simultaneously both centrally and peripherally, while social defeat only increases the dendritic release of oxytocin in the SON. Similarly, hyperosmotic stimulation increases both somato-dendritic and axonal release of vasopressin, while social defeat and forced swim selectively promote somato-dendritic vasopressin release (Engelmann et al. 2001; Neumann et al. 1993; Wotjak et al. 2001). The differential axonal and somato-dendritic release of neuropeptides in response to different stressors confers context-specific regulation of homeostasis.

The release of neuropeptide from dendrites and axons has distinct spatio-temporal profiles due to a different release machinery involved. First, axonal release depends on action potential generation, while somato-dendritic release depends on the mobilization of intracellular calcium stores (Ludwig et al. 2002). Thus, differential densities of intracellular organelles that store intracellular calcium, such as the endoplasmic reticulum and mitochondria, and differential expression of ion

Fig. 8.2 (continued) in blood vessels to cause vasoconstriction. Circulating oxytocin binds to oxytocin receptors in the uterus to stimulate uterine contractions and parturition and in the mammillary gland to stimulate milk ejection. CRH neurons of the hypothalamic-pituitary-adrenal axis reside in the hypothalamic paraventricular nucleus and secrete CRH and vasopressin from their axon terminals in the median eminence into the pituitary portal circulation. Portal CRH and vasopressin stimulate ACTH secretion into the general circulation from corticotropes in the anterior lobe of the pituitary gland. Circulating ACTH then stimulates corticosteroid synthesis and secretion from the adrenal cortex into the general circulation, from where it accesses target tissues, including the brain

channels, plasma membrane calcium pumps, and sodium/calcium exchangers all may contribute to the differential release from dendrites and axons. Second, release of neuropeptide depends on the exocytosis of LDCVs, and the proteins involved in exocytosis may be expressed differentially in somato-dendritic and axonal compartments.

Differential dendritic and axonal release is exemplified by the regulation of central oxytocin release by α-melanocyte stimulating hormone (α-MSH) (Sabatier et al. 2003). SON and PVN neurons express high levels of melanocortin-4 receptors and receive inputs from α-MSH neurons in the arcuate nucleus. Central administration of α-MSH increases c-fos expression in oxytocin neurons, but decreases the oxytocin neuron firing rate, selectively promoting the dendritic release of oxytocin. This is caused by the mobilization of intracellular calcium and the release of retrograde-signaling endocannabinoids, which reduce the excitatory synaptic inputs and drive to the oxytocin neurons.

8.2.2 Dendritic Neuropeptide Release in the Hypothalamic-Pituitary-Adrenal System

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for the neuroendocrine component of the stress response. The stress response is a highly conserved, multi-faceted physiological reaction that is activated when an organism is challenged by internal and external disturbances. During the generalized stress response, corticotropin-releasing hormone (CRH) neurons (also referred to as corticotropin-releasing factor (CRF) neurons) located in the PVN, a key nucleus that integrates physiological and psychological afferent information, are activated. The activated CRH neurons release CRH from their axon terminals in the median eminence, which is located at the base of the brain. The secreted CRH transits through the portal circulatory system of the pituitary to the anterior lobe of the pituitary, where it activates the corticotrope cells to secrete adrenocorticotropic hormone (ACTH) into the general bloodstream. The secreted ACTH then acts on cells of the adrenal cortex to cause the synthesis and release of corticosteroids, the primary hormonal product of the HPA (Fig. 8.2). Corticosteroids impact nearly every cell in the body by activating glucocorticoid and/or mineralocorticoid receptors. Corticosteroids also feed back to the brain and pituitary to negatively regulate CRH and ACTH expression and secretion, thereby limiting the duration and impact of the neuroendocrine stress response and returning circulating corticosteroid concentrations to normal, unstressed levels.

In addition to its antidiuretic function, vasopressin also is co-expressed in PVN CRH neurons and potentiates the CRH stimulation of the release of ACTH from corticotropes in the anterior pituitary. Interestingly, the co-expression of vasopressin in CRH neurons is state-dependent and can be enhanced by specific stressors. Systemic stressors such as interleukin-1β (IL-1), lipopolysaccharide (LPS), brain surgery, electric foot shock, and immobilization (both acute and repeated) increase vasopressin expression in the CRH neurons (Bartanusz et al. 1993). Removing

circulating corticosteroid by adrenalectomy in rats induces vasopressin peptide expression in over 70% of CRH neurons in the PVN (Sawchenko et al. 1984). Indeed, transient suppression of corticosteroid release by central CRH immunoneutralization or by peripheral inhibition of corticosteroid synthesis increases vasopressin mRNA expression in CRF fibers in the median eminence (Schmidt et al. 1997). The corticosteroid-sensitive expression of vasopressin in the CRH neurons reveals a plasticity of the HPA in response to different stressors.

The somato-dendritic release of neuropeptide from the CRH neurons in the PVN is less well studied than the dendritic release from the MNCs in the SON and PVN, partly due to the heterogeneous populations of neurons in the PVN. With advances in optogenetics tools, recent studies have demonstrated that selective activation of CRH neurons excites neighboring neurons within the PVN via local CRH release and activation of type 1 CRH receptors (CRHR1), which suggests the possible dendritic release of CRH in the PVN (Jiang et al. 2018). In addition, studies of the mechanisms of NE excitation of CRH neurons suggest that CRH neurons can also release vasopressin from their dendrites to recruit astrocytes to amplify dendritic volume transmission (Chen et al. 2019).

8.3 Ghrelin and Norepinephrine Activate Neuronal-Glial Signaling

8.3.1 Ghrelin Activation of Vasopressin Neurons in the PVN Via a Neuronal-Glial Circuit

Ghrelin is a well-known hunger signal that is released from the gastrointestinal tract in response to a negative energy balance to mediate feeding-related behavior. In addition to a variety of functions in energy homeostasis, ghrelin also regulates fluid balance by modulating signaling involved in osmoregulation, cardiovascular function, and drinking behavior. The ghrelin signaling in the PVN that leads to the activation of vasopressin neurons demonstrates neuronal-glial signaling essential for neuroendocrine function; the signaling is mediated via neuronal activation of astrocytes (vasopressin neurons → astrocytes) and subsequent astrocytic activation of neuronal cells (astrocytes → GABAergic neurons) (Haam et al. 2014). In this section, we will review the vasopressin-expressing MNCs, the synaptic inputs that regulate vasopressin neurons, and the ghrelin signaling that stimulates vasopressin neurons via a neuronal-glial signaling mechanism.

Vasopressin neurons and oxytocin neurons are the two types of MNCs found in the PVN and SON (Fig. 8.2). Although co-expression of oxytocin and vasopressin is observed under certain conditions, such as in response to osmotic challenge, the majority of MNCs typically express only one of the two neuropeptides under baseline conditions, and the neuropeptide expression defines each of the two types of MNCs. In addition to playing roles in social behaviors, oxytocin and vasopressin mediate distinct physiological functions: oxytocin facilitates parturition and triggers milk ejection, while vasopressin regulates osmotic homeostasis by eliciting water

reabsorption in the kidney and vasoconstriction. Oxytocin also plays a role in osmoregulation by regulating sodium excretion in the kidney.

The excitability of MNCs is regulated by glutamatergic and GABAergic synaptic inputs. It has been shown that MNCs in the PVN and SON receive synaptic inputs from local glutamatergic and GABAergic neurons located in or near the nuclei (Boudaba et al. 1996, 1997). Both ionotropic and metabotropic receptors are expressed in MNCs to mediate the glutamatergic and GABAergic synaptic regulation of the MNCs. Unlike glutamate, GABA actions are significantly affected by intracellular ionic concentrations. At excitatory synapses, ionotropic AMPA and NMDA glutamate receptors are permeable to the cations Na^+, K^+, and Ca^{++}, and the opening of the receptor channels by glutamate causes membrane depolarization due to the significantly more positive reversal potential of the cationic synaptic currents (~0 mV) relative to the resting membrane potential. On the other hand, at inhibitory synapses, the ionotropic $GABA_A$ receptors are permeable to Cl^-, and because the equilibrium potential for Cl^- is normally negative to the MNC resting membrane potential, the opening of the $GABA_A$ receptor channels causes influx of Cl^- and hyperpolarization. The intracellular Cl^- concentration is maintained in MNCs by the ionic co-transporters K^+-Cl^- co-transporter 2 (KCC2), Na^+-K^+-Cl^- co-transporter 1 (NKCC1), and Na^+-K^+-Cl^- co-transporter 2 (NKCC2). While KCC2 decreases the intracellular Cl^- concentration by exporting Cl^- ions from cells using the outward K^+ concentration gradient, NKCC1 and NKCC2 increase the intracellular Cl^- concentration by importing Cl^- ions using the inward Na^+ concentration gradient. As the $GABA_A$ receptor-mediated ionic mechanism relies on the intracellular Cl^- concentration, any alteration in the intracellular Cl^- concentration, such as via a change in the expression or function of the Cl^- transporters, can change GABA's actions by altering the magnitude and direction of the Cl^- flow across the membrane. During development, a low level of KCC2 expression contributes to a high intracellular Cl^- concentration and shifts the Cl^- reversal potential positive, which results in the activation of $GABA_A$ receptors causing a membrane depolarization (Ben-Ari et al. 2012). In addition, potent physiological stimuli can alter Cl^- transporter function and increase the intracellular Cl^- concentration, which shifts GABA's actions to less inhibitory or excitatory. The intracellular Cl^- concentration is regulated in a cell type-specific manner and is modulated by signaling molecules, such as brain-derived neurotrophic factor, vasopressin, and oxytocin. Evidence suggests that GABA can have excitatory actions in vasopressin-expressing MNCs due to a low level of KCC2 expression in these cells (Haam et al. 2012; Kanaka et al. 2001). The nature of $GABA_A$ receptor signaling is a function of environmental conditions impacting Cl^- transporter expression and/or function because GABA actions can be either inhibitory or excitatory in vasopressin neurons at baseline under different conditions, and can shift to less inhibitory or excitatory with chronic stimulation of the vasopressin system (Choe et al. 2015; Haam et al. 2012; Kim et al. 2011).

Vasopressin neurons have the capacity to release vasopressin from their dendrites to exert neuromodulatory effects that are distinct from the classical axonal release of the peptide from the posterior pituitary that mediates vasoconstriction and water

reabsorption (Ludwig et al. 2002). Dendritically released vasopressin plays a critical role not only in providing autocrine and retrograde regulation to vasopressin neurons (Ludwig and Leng 1997), but also in recruiting neighboring astrocytes to amplify signals (Haam et al. 2014). When ghrelin activates the type 1a growth hormone secretagogue (ghrelin) receptor (GHS-R1a) expressed in vasopressin neurons, it induces G protein-coupled receptor signaling to trigger dendritic release of vasopressin (Haam et al. 2014) (Fig. 8.3). The dendritically released vasopressin then triggers calcium signaling in neighboring astrocytes by activating astrocytic vasopressin 1a (V1a) receptors (Haam et al. 2014). The V1a receptors are coupled to $G_{\alpha q/11}$ and activate phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to form the second messengers inositol (1,4,5) trisphosphate (IP3) and diacylglycerol (DAG), which subsequently induce Ca^{2+} release from intracellular calcium stores.

The release of gliotransmitter is an important physiological phenomenon that results from an astrocytic calcium response and is responsible for the modulation of multiple neuronal and non-neuronal cells within the spatial sphere of influence of the astrocyte. When ghrelin triggers a calcium response in astrocytes via the dendritic release of vasopressin, the activated astrocytes release ATP, a well-known gliotransmitter (Fig. 8.3). Although ATP was originally known as a source of energy transfer in living cells, more recent studies have identified an exciting role of ATP as a gliotransmitter. ATP has a variety of neuromodulatory functions by acting at purinergic P2X and P2Y receptors, which are ionotropic and metabotropic receptors, respectively. The activation of P2X ionotropic receptors has been shown to stimulate GABAergic synaptic transmission in the hypothalamus (Crosby et al. 2018; Haam et al. 2014; Vavra et al. 2011). In the downstream response of vasopressin neurons to ghrelin, ATP elicits an increase in GABAergic synaptic inputs to the vasopressin neurons by activating P2X receptors to stimulate presynaptic GABAergic neurons (Haam et al. 2014). Since GABA is excitatory in vasopressin neurons under baseline conditions in our hands (Haam et al. 2012; Morton et al. 2014), this increase in GABAergic inputs can activates the vasopressin neurons (Haam et al. 2014).

8.3.2 Norepinephrine Activation of CRH Neurons in the PVN Via a Neuronal-Glial Circuit

Stress-relevant information converges in the PVN, and CRH neurons are positioned to generate rapid and accurate changes in hormonal, autonomic, and behavioral outputs in response to dynamic stress contexts and reward conditions. Abundant evidence indicates the role of brainstem catecholaminergic signaling in the activation of the HPA. CRH neurons in the PVN receive noradrenergic inputs from the dorsal and ventral medulla and express adrenergic receptors. Functionally, selective immunotoxin ablation of noradrenergic inputs from the A1 and A2 catecholaminergic cell groups of the medulla or selective blockade of adrenergic receptors within the PVN diminishes stress-induced activation of ACTH and corticosterone secretion (Ritter et al. 2003), suggesting that NE excites CRH neurons. *In vitro*

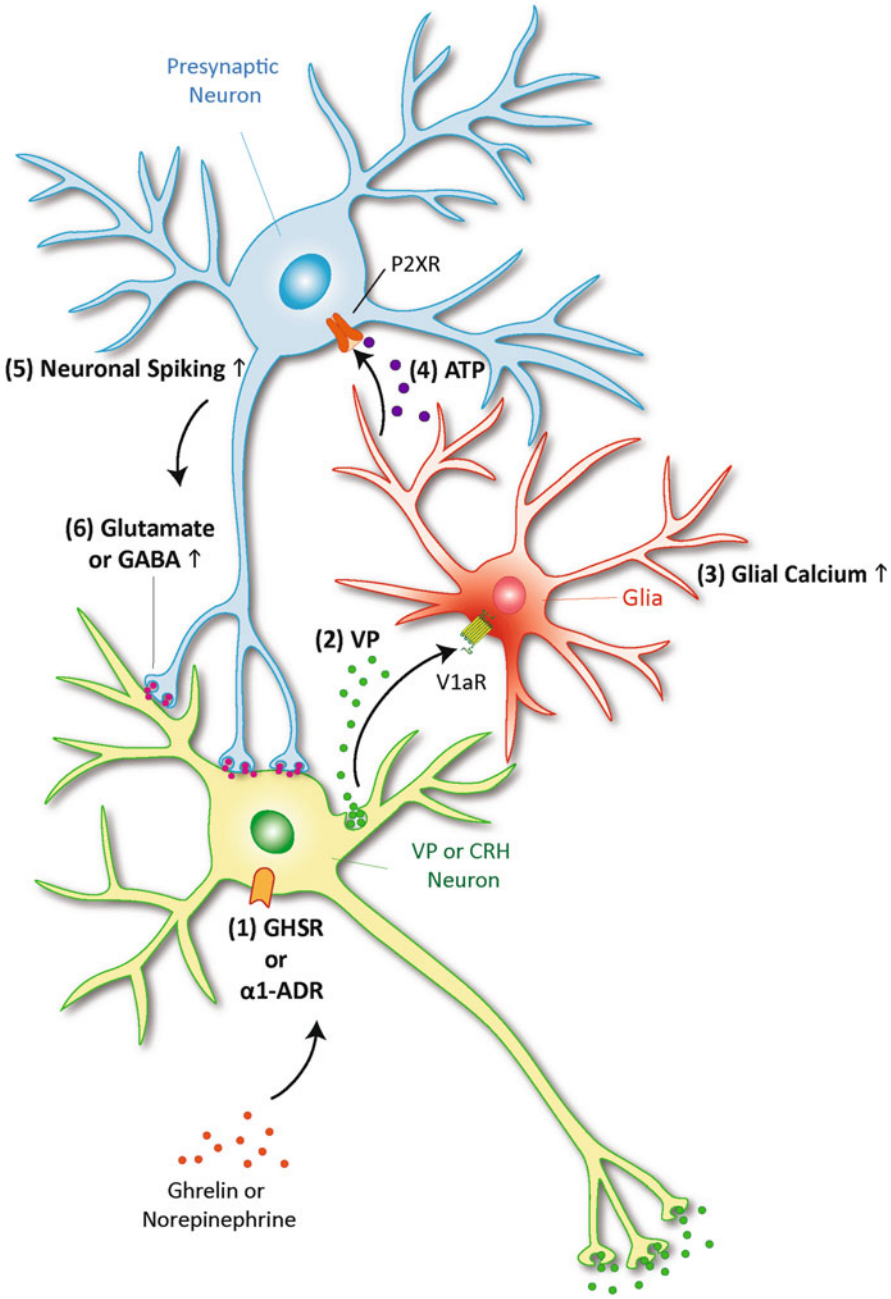


Fig. 8.3 Vasopressin as a retrograde messenger that activates astrocytes in ghrelin and NE signaling. Ghrelin stimulates vasopressin MNCs and norepinephrine stimulates CRH neurons in the PVN (1) to release vasopressin from their dendrites (2). The dendritically released vasopressin activates V1a receptors in astrocytes to trigger an increase in intracellular Ca^{2+} signaling (3). Activated astrocytes release the gliotransmitter ATP onto neighboring glutamatergic and/or GABAergic neurons. (4) ATP activates P2X receptors in the glutamatergic and GABAergic

electrophysiology showed that NE increases action potential firing in CRH neurons (Chen et al. 2019), consistent with studies showing that activation of medullary catecholaminergic inputs to the PVN or microinjection of NE directly into the PVN increases CRH release and elevates circulating ACTH and corticosterone (Itoi et al. 1994; Plotsky 1987).

Despite direct noradrenergic innervation of the CRH neurons in the PVN, early electrophysiological studies indicated that NE activates PVN neurons indirectly, through activation of local synaptic circuits (Daftary et al. 1998; Han et al. 2002). This discrepancy was reconciled with the finding that NE stimulates presynaptic circuits by activating postsynaptic alpha1-adrenoceptors on the CRH neurons and triggering the dendritic release of a retrograde messenger to activate local upstream glutamate and GABA circuits (Chen et al. 2019). In addition to activating both presynaptic glutamate and GABA circuits via an alpha1 receptor-dependent retrograde signaling mechanism in CRH neurons, NE also activates presynaptic alpha2 adrenergic receptors located directly on GABAergic axon terminals to cause a suppression of GABA release, thus causing a complex modulation of GABAergic synaptic inputs to CRH neurons.

Why would NE have opposing effects on synaptic excitation and inhibition in the same population of CRH neurons? It turns out that the differential effects of NE on glutamate and GABA neurotransmission have different concentration sensitivities and may therefore be recruited under different physiological conditions. The NE retrograde facilitation of glutamatergic synaptic inputs and presynaptic suppression of GABAergic synaptic inputs occur at lower concentrations of NE than the retrograde facilitation of GABAergic synaptic inputs. Therefore, at the initiation of the stress response when the NE concentration is low, the NE facilitation of glutamate transmission and suppression of GABA transmission may work together, if GABA is inhibitory (see Chap. 4), to excite the CRH neurons and activate the HPA. At a later phase of the stress response, when the NE concentration reaches its peak, the high-threshold NE facilitation of GABAergic inhibitory transmission may be recruited into the response to counter the excitatory actions of NE and dampen the activation of the CRH neurons and the HPA, which could contribute to the termination of the stress response and the return to homeostasis. The NE activation of GABAergic inputs to the CRH neurons at high concentration could also provide an inhibitory break on HPA activation in the case of extreme or prolonged activation of the ascending noradrenergic afferents. The valence of GABA signaling in CRH neurons can shift from inhibitory to excitatory following acute stress exposure (Hewitt et al. 2009), such that, paradoxically, increased GABA inputs at higher NE concentration could actually contribute to the excitation of the CRH neurons and amplify the activation of the HPA.

Fig. 8.3 (continued) neurons to stimulate spiking (5), which triggers an increase in the GABAergic/glutamatergic synaptic inputs to the postsynaptic vasopressin and CRH neurons, completing the retrograde volume transmission-activated recurrent signaling circuit

Endogenous NE release induced by optogenetic activation of the noradrenergic inputs greatly enhances excitatory synaptic transmission in CRH neurons, similar to the response to exogenous NE application. Interestingly, the effect is only partially suppressed by blocking alpha1-adrenergic receptors or by blocking G-protein signaling in the CRH neurons. Thus, it seems that the noradrenergic afferents that project to PVN CRH neurons co-release glutamate at their synapses (Chen et al. 2019), consistent with the co-expression of the vesicular glutamate transporter 2 with tyrosine hydroxylase in brainstem catecholaminergic neurons (Johnson et al. 2018).

The facilitatory effect of NE on glutamatergic and GABAergic transmission requires the activation of postsynaptic alpha1 adrenoreceptors on the CRH neurons, whereas the effect is generated by presynaptic spike-mediated glutamate and GABA release. This discordance in the pre- vs. postsynaptic sites of action indicates the involvement of a retrograde messenger, which, surprisingly, was found likely to be vasopressin (Chen et al. 2019) (Fig. 8.3). Antagonists of the vasopressin V1a receptor blocked the NE effect and exogenous vasopressin mimicked the NE effect on synaptic glutamate and GABA release. Ghrelin, which activates dendritic vasopressin release from neighboring vasopressin neurons in the PVN (see above), had no effect on the excitatory synaptic inputs to CRH neurons, suggesting that vasopressin release from vasopressin neurons does not spill over onto the CRH neurons. Also, blocking type 1 CRH receptors had no effect on the NE modulation of synaptic inputs, indicating that CRH is not the dendritic messenger. While somewhat surprising, the release of vasopressin from CRH neuron dendrites is not unfounded, since PVN CRH neurons express vasopressin in their somata and axon terminals, and vasopressin and CRH are independently regulated in CRH neurons by chronic stress (Bartanusz et al. 1993; Schmidt et al. 1997).

Chemical inhibition of astrocyte metabolism and genetic impairment of exocytosis specifically in astrocytes blocked the NE modulation of synaptic inputs to the CRH neurons, revealing the involvement of astrocytes in NE signaling. Astrocytes express vasopressin receptors, and both NE and vasopressin evoke V1a receptor-mediated calcium responses in astrocytes, likely via calcium release from intracellular stores. These observations together suggested that NE elicits the dendritic release of vasopressin from CRH neurons, and that this causes astrocyte activation (Chen et al. 2019).

Several gliotransmitters have been identified in the hypothalamus, such as glutamate, D-serine, taurine, ATP, and TNP-α (Theodosis et al. 2008) (see Chap. 2). As described above, the dendritic release of vasopressin from vasopressin neurons stimulates astrocytes to release ATP (Haam et al. 2014). The NE-induced vasopressin release from CRH neuron dendrites also stimulates astrocytes in the PVN to release ATP, which causes action potential generation in upstream glutamate and GABA neurons via ionotropic P2X purinergic receptor activation. Astrocytes can transmit signals to remote sites by propagating calcium waves among multiple astrocytes coupled electrotonically via gap junctions and/or chemically via ATP release. GABAergic neurons presynaptic to the CRH neurons are likely located outside of the PVN (Boudaba et al. 1996), which would necessitate the propagation of the NE-induced signal through a chain of astrocytes. Therefore, the recruitment of

astrocytes by dendritically released vasopressin can greatly expand the spatial domain of the NE-induced retrograde signaling.

8.4 Perspectives

During the past three decades, advances in neuroscience have revealed a significant role for glia in synaptic function. By virtue of their close spatial relationship with neuronal synaptic structures and their expression of a variety of neurotransmitter receptors, as well as their release of gliotransmitters, astrocytes are capable of reading neuronal signals and reciprocally influencing neuronal activity. The hypothalamic PVN and SON are brain regions in which physiological stimuli induce significant changes in astrocyte morphology, which cause important effects on neuroendocrine signaling, as described in Chaps. 2 and 3. Astrocytes in the PVN and SON actively modulate neuronal populations by releasing gliotransmitters in response to external stimuli. Here, we have described a novel form of neuronal-glial signaling in the hypothalamus in which the dendritic release of vasopressin from vasopressin and CRH neurons stimulates astrocytes. The activated astrocytes then transmit signals to local presynaptic GABAergic and/or glutamatergic neurons. In this way, postsynaptic neuroendocrine cells exert a robust control of the activity of their presynaptic partners via retrograde volume transmission, extending their influence on upstream synaptic circuits potentially by the reach of several astrocytic spatial domains.

8.5 Key Literature

Araque et al. (1999) The first paper that proposed the tripartite synapse model.

Bushong et al. (2002) Seminal paper demonstrating the non-overlapping spatial domains of astrocytes to create a tiling effect of astrocyte distribution.

Chen et al. (2019) This study demonstrated stress-induced NE activation of retrograde trans-neuronal-glial signaling that stimulates local glutamate and GABA circuits and elicits an increase in excitatory and inhibitory synaptic inputs to stress-related CRH neurons in the PVN.

Haam et al. (2014) This was the first study to describe retrograde trans-neuronal-glial signaling that activates upstream local circuits via dendritic volume transmission and astrocyte activation to modulate synaptic inputs to the postsynaptic neurons.

Orkand et al. (1966) A seminal study providing the first evidence of a glial calcium response to neuronal signals.

Parpura et al. (1994) One of the pioneering works that demonstrated gliotransmission by showing that calcium elevation in astrocytes triggers astrocytic glutamate release, which subsequently activates NMDA receptors on neurons.

Srinivasan et al. (2015) This study identified three different compartments of astrocytes that present distinct types of calcium fluctuations, including IP3 receptor-independent signaling.

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Part V

Glial–Neuronal Interactions in the Control of Reproductive Function

Microglia, Hormones, and Behavior

9

Jaclyn M. Schwarz and Margaret M. McCarthy

Abstract

The goal of this chapter is to describe brain–immune communication, focusing on microglia, the innate immune cells of the brain, and peripheral immune cells that traffic into and out of the nervous system. We describe how microglia are influenced by circulating hormones across the lifespan. In particular, we focus on the hormones associated with reproduction and stress and how these impact microglia, which in turn influence the establishment and plasticity of neural circuits. We also describe how these interactions between hormones and microglia affect healthy behaviors such as social play and mating and dysregulated behaviors including sickness behaviors, stress, depression, anxiety, and cognitive impairment.

Keywords

Microglia · Sex · Development · Pregnancy · Mating · Social play · Mental health

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9.1 Introduction: Synchronizing Physiology and Behavior with the Internal and the External Environments

The immune and endocrine systems synchronize the function of physiological processes throughout the body. Both systems respond to environmental and internal homeostatic signals that drive this synchronization. Through various mechanisms, neurons in the brain are sensitive to the effects of signaling molecules from the periphery, such that the endocrine and immune systems can significantly affect behaviors including sex behavior, play behavior, cognition, emotion, and motivational states. In addition, many of these behaviors and physiological states are either unique to one sex or are modulated differently in the two sexes in order to assure maximal reproductive success. As a result, the unique interaction of these physiological systems and the brain can influence behavior differentially across the two sexes.

9.2 Brain–Endocrine–Immune Communication: The Molecular and Neural Signals

The immune and endocrine systems communicate with various organs throughout the body primarily via the production of signaling molecules that are released into the bloodstream and act on target tissues. Endocrine glands release hormones that communicate the homeostatic status and the sex or reproductive status of the individual to the brain and other organs throughout the body. Until recently, the immune system was thought to operate independently from the brain; however, we now know that, similar to the endocrine system, the immune system releases signaling molecules, namely **cytokines** and **chemokines**, into the bloodstream that activate immune cells in the brain and the proliferation of peripheral immune cells to maintain homeostasis throughout the body (Fig. 9.1). In addition, there are important neural pathways that communicate the status of the brain to the body and vice versa. These neural pathways include the **vagus nerve** and the **autonomic nervous system**, which use neurotransmitters including acetylcholine, epinephrine, and norepinephrine to modulate immune function, endocrine function, and other bodily functions.

9.2.1 Immune Cells of the Brain: Microglia, Macrophages, and Mast Cells

Various organs in the body have tissue-specific resident macrophages, and the brain is no exception. **Microglia** make up approximately 10% of the cells in the brain and are the resident macrophages and the primary immune cells of the brain (Fig. 9.2).

Despite their relatively uniform appearance (Fig. 9.2), microglia throughout the brain are not homogeneous and it is likely that the specific neural environment in which the cells reside is a primary determinant of the microglial phenotype (de Biase

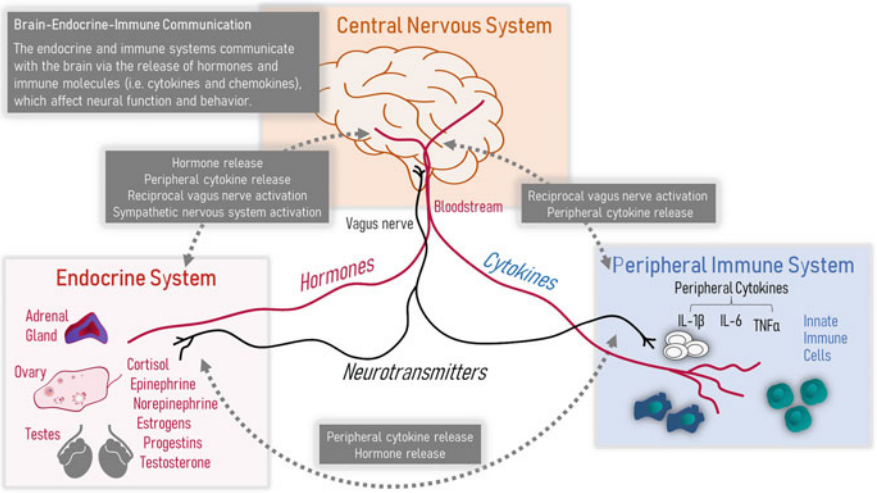

Fig. 9.1 A schematic of the neural and chemical messengers used in brain–endocrine–immune communication. The endocrine and immune systems communicate with the brain via the release of cytokines (e.g., IL-1β, IL-6, and TNFα) and hormones (e.g., cortisol, epinephrine, estrogens, progestins, and testosterone). These secreted molecules communicate information about the periphery and our environment to the brain. In turn, the brain also controls the function of the endocrine and immune systems via the release of neurotransmitters (e.g., acetylcholine and norepinephrine) from the autonomic nervous system. The brain also produces "releasing hormones" that stimulate endocrine glands to produce hormones. These pathways and molecules highlight the reciprocal communication between the periphery and the brain

and Bonci 2019). This in turn influences each cell's signaling profile, independent of their similar origin. Consistent with this idea, microglia across various regions of the brain have distinct responses to the same inflammatory stimulus. Microglia are the primary source and target of cytokines and chemokines in the central nervous system, important for inter- and intracellular communication. They also produce and respond to **prostaglandins** and **endocannabinoids**, two membrane-derived signaling molecules associated with inflammation, but also central to many forms of synaptic communication.

Box 9.1 A Brief History: The Discovery and Description of Microglia

Microglia were first described in depth in a landmark 1932 study by Pio del Rio-Hortega (1932). He was the first to characterize microglia by *describing nine properties of these neural–immune cells that are still valid today*. These properties include: (1) microglia progenitors enter the brain during embryonic development; (2) the invading progenitor cells have an amoeboid morphology and are of a unique mesodermal origin relative to other neural progenitor cells;

(continued)

Box 9.1 (continued)
(3) microglia use blood vessels and white matter tracks to guide them as they seed brain regions; (4) they transform into a branched, ramified morphological phenotype in the more mature brain; (5) in the mature brain, microglia are found evenly dispersed throughout the central nervous system and display uniform morphological characteristics; (6) each cell occupies a unique, defined territory as they perform various sentinel immune and homeostatic functions.; (7) during infection or inflammation, microglia transform into a polarized population of cells that can perform pro- or anti-inflammatory functions; (8) transformed cells can acquire an amoeboid morphology, similar to the one observed early in development; and (9) these cells have the capacity to migrate, proliferate, and phagocytose.

In addition to microglia, there are a few tissue-specific macrophages that reside in surrounding structures just outside the brain. These include the **perivascular macrophages**, which line blood vessels in the brain and the blood–brain barrier,

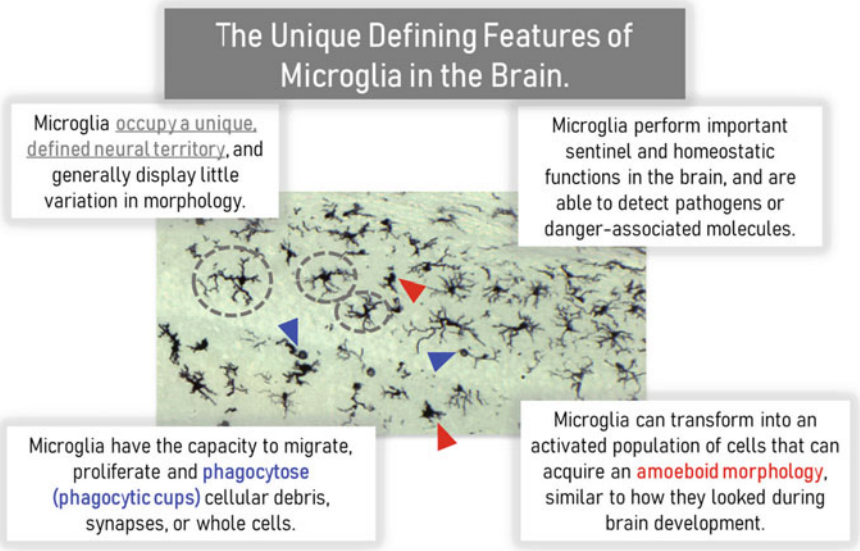

Fig. 9.2 The unique characteristics and important functions of microglia. Pío del Río Hortega was the first to characterize the unique properties of microglia and their function. He noted that microglia typically have a defined (non-overlapping) neural territory (note the dotted gray lines as examples) and display little variation in morphology across different regions of the healthy adult brain. Microglia express the receptors necessary to detect pathogens and other danger-associated molecules, at which point the cells transform into an activated form with an amoeboid morphology and phagocytic capability. Given that microglia are the brain-resident macrophages, they are capable of phagocytosing a number of things, including pathogens, cellular debris, synapses, or entire cells

the **meningeal macrophages**, which reside in the meninges layered on top of the brain, and the **macrophages of the choroid plexus**, which reside in the structure in the brain's ventricles that produces cerebrospinal fluid (CSF). These three types of macrophages that reside at the interface of the central nervous system and the periphery are also highly specified, unique relative to each other and relative to microglia (Fig. 9.3). These three types of macrophages are also key components of the brain's immune system and its response to an immune challenge (Norris and Kipnis 2019; Li and Barres 2018).

Mast cells are also one of the few immune cells that reside in the brain tissue. Unlike microglia, they are localized to specific brain regions, including the preoptic

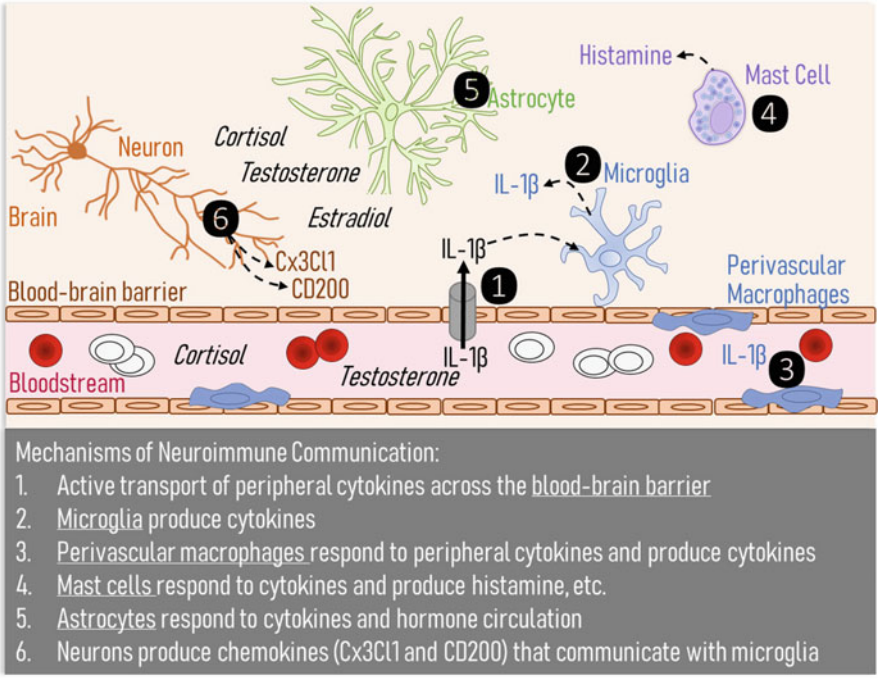

Fig. 9.3 Mechanisms of neuroimmune communication across the blood–brain barrier. Unlike most of the body's organs, the brain exists behind a blood–brain barrier designed to minimize the passage of peripheral cells and pathogens into the delicate neural tissue. However, there are numerous mechanisms by which the immune system can communicate information about the periphery and the immune system to the brain. First, there is active transport of immune molecules across the blood–brain barrier. Second, microglia can produce cytokines in response to peripheral immune activation. Third, perivascular macrophages reside in the vasculature and produce high levels of cytokines that can regulate microglia function. Fourth, mast cells reside in particular brain regions, where they communicate with surrounding neurons via the production of cytokines and histamine. Fifth, astrocytes respond to the production of cytokines and hormones and in turn produce certain cytokines. Sixth, neurons themselves express two types of chemokines, Cx3Cl1 and CD200, the receptors for which are located on microglia. Thus, via these molecules, neurons can communicate directly to the immune cells of the brain

area, pituitary, and indusium griseum, as well as the choroid plexus and meninges (Fig. 9.3). As such, they are an important component of the neuroimmune response, interacting with microglia, macrophages, astrocytes, and neurons in these brain regions and driving subsequent neuroendocrine responses to an immune challenge or stressor. The distribution of mast cells also varies by sex and age, with substantially higher numbers in the brain early in development compared to adulthood, and more in some regions of the male brain than the female (Lenz et al. 2018).

9.2.2 Peripheral Immune Cells Traffic to and from the Brain

Under homeostatic conditions, the healthy brain is devoid of many peripheral immune cells because they are prevented from entering the central nervous system by the presence of the blood–brain barrier. In the meningeal spaces, there are a variety of immune cells that regulate the function of the central nervous system through surveillance and maintenance of homeostasis via cell-to-cell communication. A wide variety of peripheral immune cells, including monocytes and T-cells, can also traffic into the CSF from the circulation, thus monitoring and communicating the status of the brain to the periphery and vice versa (Li and Barres 2018; Norris and Kipnis 2019). Peripheral immune cells that enter the brain are subsequently "washed out" via a special brain lymphatic system that was recently discovered in the central nervous system (da Mesquita et al. 2018). The lymphatic system in the brain is located within the dura and meningeal sinuses, and these structures express the key molecular signatures of other, peripheral lymphatic vessels, suggesting they are both of the same origin and have similar functions. This recent finding has expanded the understanding of brain–immune communication and the mechanisms by which peripheral inflammation can affect neural function and behavior.

9.3 Sickness Behavior: A Coordinated Effect of Immune and Endocrine Function on Behavior

Sickness behavior is a coordinated set of behaviors that manifest in individuals after the immune system has been activated, for example, by a pathogen (Fig. 9.4). Sickness behaviors include lethargy, depression, loss of appetite, sleepiness, hyperalgesia, and difficulty concentrating, all of which drive the affected individual to rest and limit feeding, drinking, or social contact (Dantzer 2006; Hart 1988). Sickness behaviors interfere greatly with our daily lives, but are actually adaptive and relatively conserved across a number of species. These behaviors are coordinated and initiated by cytokines and chemokines that are produced by the immune system in response to a challenge or stressor (Fig. 9.4). The purpose of sickness behaviors is to conserve energy for the high energetic cost of fighting infection. The activation of peripheral immune receptors triggers the immune system to produce pro-inflammatory cytokines (e.g., IL-1β, IL-6, and TNFα), which circulate throughout the bloodstream (Dantzer 2006; Hart 1988). Peripheral cytokines influence the

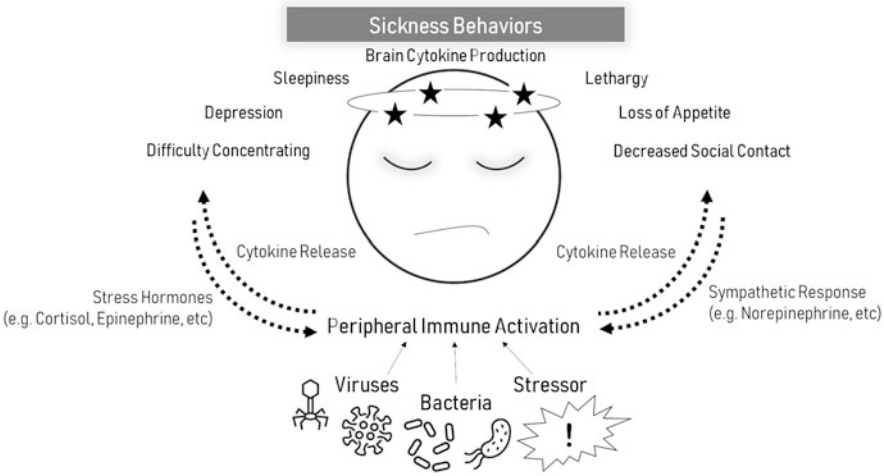

Fig. 9.4 Sickness behaviors are orchestrated by the immune and endocrine systems in response to invasion by a pathogen. When the peripheral immune system detects a pathogen (bacteria or virus) or a stressor, it releases cytokines and chemokines into the bloodstream, mobilizing a coordinated immune response to fight the infection. These immune molecules can be transported across the blood–brain barrier into the brain, where they also have effects on microglia and neurons. Microglia produce their own cytokines and chemokines. Together these immune molecules have a profound effect on behavior, eliciting sleepiness, lethargy, loss of appetite, decreased social contact, difficulty concentrating, and depression. This is an "adaptive" behavioral response to the cytokine production, necessary to conserve energy and fight infections. In turn, the brain initiates the release of hormones necessary to fight the infection, including stress hormones, and activation of the sympathetic nervous system

brain, particularly the circumventricular organs with little or no blood–brain barrier, where they initiate sickness behaviors and an accompanying fever. Fever is initiated by prostaglandins acting on neurons in the preoptic area. Prostaglandins are synthesized by immune cells of the periphery but also centrally, including by microglia. Interestingly, peripheral administration of cytokines (e.g., IL-1β) at very low, undetectable serum levels can initiate sickness behavior, highlighting the sensitivity of and the multiple coordinated mechanisms by which the brain and the immune system communicate to ensure sickness behaviors in the presence of a pathogen (Maier and Watkins 1998). For example, cytokines also activate the vagus nerve, one of the primary nerves of the autonomic nervous system that has both efferent and afferent connections with the brain stem. This reciprocal communication allows the vagus nerve to relay the status of peripheral organs to the brain and vice versa (Bluthé et al. 1994).

The immune system coordinates physiological systems and behavior to divert energy resources away from everyday tasks and towards maintaining body temperature and combating the infecting pathogen (Hart 1988). In addition to the overt sleepiness, nausea, and decreased appetite, sickness behaviors include changes in social and cognitive behaviors (Hennessy et al. 2014; Kelley et al. 2003). For

example, a sick individual will often withdraw or remove themselves from a social group. This helps the individual to stay calm and reduce energy use while minimizing the chances of disease transmission within the group. As a result, behaviors such as playing, grooming, and sexual behavior are reduced across a variety of species during illness. Cognitive function is also impaired during sickness, including reaction time, memory, and verbal abilities. It is not clear, though, whether there is an adaptive function of sickness-induced cognitive impairment (Hennessy et al. 2014). Rather, sickness-induced cognitive impairment may be a neutral response that reflects the sensitivity of the brain to the general effects of immune activation. Importantly, however, this last point also highlights the potential role of immune activation or dysregulation on cognitive dysfunction that is associated with a number of mental health disorders (Dantzer 2006).

Cytokines, including IL-1β, IL-6, and TNFα, can induce the release of hormones from the pituitary and the adrenal gland, resulting in robust activation of the hypothalamic–pituitary adrenal axis during sickness. This is an essential component of the immune response, which stimulates the production of "stress hormones" including **glucocorticoids**, **epinephrine,** and **norepinephrine** that in turn have potent immunomodulatory effects. In general, these three "stress" hormones *suppress* peripheral immune function, but in the brain, particularly following acute stimulation, they can further *induce* the activation of microglia and enhance production of cytokines and chemokines, thereby resulting in the sickness behaviors described above (Nguyen et al. 1998).

Gonadal hormones, including **testosterone**, **estrogens**, and **progestins**, can also impact the immune response and the production of cytokines and chemokines. Males have elevated levels of testosterone and females produce elevated levels of estrogens and progestins, all of which profoundly influence the peripheral immune response. In general, females have more robust immune responses than males, particularly via the activation of toll-like receptor (TLR) 7 and TLR9, innate immune receptors that identify and respond to viruses, although as explained below, it is not that simple. Often sex differences in the immune response are correlated with the levels of circulating androgens in males and estrogens in females, which can inhibit or enhance the immune response, respectively. In contrast, males have higher levels of TLR4, an innate receptor that identifies and responds to bacteria, on certain peripheral immune cells. As a result, stimulation of peripheral immune cells from male rodents results in greater pro-inflammatory cytokine expression, an effect that is reversed by castration and the removal of circulating androgens (Rettew et al. 2008).

Within the brain, males typically have a more robust neural immune response to peripheral TLR4 activation via lipopolysaccharide (LPS) administration. This robust neuroimmune response to TLR4 activation mimics the TLR4 response measured in the periphery. This results in greater cytokine expression in the male brain, more robust changes in body temperature, and more robust sickness behaviors in males than females (Ashdown et al. 2006). In contrast, peripheral stimulation of TLR4 results in elevated levels of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1ra) in females, which inhibits the production of other cytokines in the brain (Ashdown et al. 2006). Notably, these sex differences in brain cytokine production

induced by TLR4 activation are expressed after puberty and are the result of age-related changes in immune function and the combined effect of increasing levels of gonadal hormones at this time.

9.4 Effects of Hormones and Immune Function on Brain Development and Behavior

9.4.1 Sexual Differentiation and Mating Behavior

Sexual differentiation is a developmental process that establishes the sex-specific neural circuits in the brain that are necessary for sex-specific control of physiology and sex-specific expression of behaviors in adulthood (Arnold 2009; Phoenix et al. 1959). Sexual differentiation of the brain occurs during a critical period of early brain development (prenatal in humans and prenatal–postnatal in rodents) (Herman et al. 2000; McCarthy et al. 2017). In males, this process is initiated by the production of testosterone in the testes during embryonic development. In females, this process is initiated in the absence of testosterone exposure. The mechanisms by which particular brain regions are organized in a sexually dimorphic manner involves specific processes that must occur at specific stages of neural development. Sexual differentiation of the brain requires the involvement of and communication among multiple cell types in the brain. This chemical communication has been classified into separate systems, mainly the nervous system (i.e., neurotransmitters), endocrine system (i.e., hormones), and the immune system (i.e., cytokines). However, it has become increasingly clear that there is substantial overlap and intercellular communication between these systems, and this is perhaps most apparent in the cellular process by which sexual differentiation of the medial preoptic area occurs.

The **medial preoptic area** (POA) in the rodent brain is a sexually dimorphic cluster of cells located in the preoptic nucleus (Gorski et al. 1978). The sexually dimorphic nucleus (SDN), a small cluster of cells within the POA, is three to five times larger in males compared to females and is necessary for the appropriate expression of male sex behavior in adulthood, with perhaps its most important role being to promote sexual preference for females (Roselli et al. 2004). In addition to this macroscopic, structural difference in the size of the SDN-POA, neurons throughout the male POA also have two to three times more dendritic spines, indicative of more synaptic connections, than POA neurons in females. Astrocytes in the male POA also have longer processes with more branches compared to astrocytes of the female POA. Masculinization of the POA is dependent on testosterone, which is converted to estradiol in the rodent brain during the critical period of sexual differentiation. A most surprising finding for its time was that these cellular changes are induced by the hormone-mediated synthesis of prostaglandin E2 (PGE2), a common pro-inflammatory signaling molecule of the immune system (Amateau and McCarthy 2004).

Amateau and McCarthy (2004) determined that activation of estrogen receptors in the POA upregulates the production of the rate-limiting enzymes in prostaglandin

production, COX-1 and COX-2, leading to an increase in PGE2 (Amateau and McCarthy 2004). Notably, PGE2 is synthesized by a variety of cells, including microglia and astrocytes, within the POA via activation of glial adenosine receptors by ATP. Astrocytes are also responsive to PGE2; they release glutamate following stimulation with PGE2, which, combined with the highly stellate morphology in males, has the potential for a direct impact on synapses. Thus, astrocytes play a critical role in the establishment of sexually dimorphic synaptic connectivity within the POA (Fig. 9.5). Given what we have discussed thus far, it was perhaps not surprising that microglia, the immune cells in the brain, would also have an

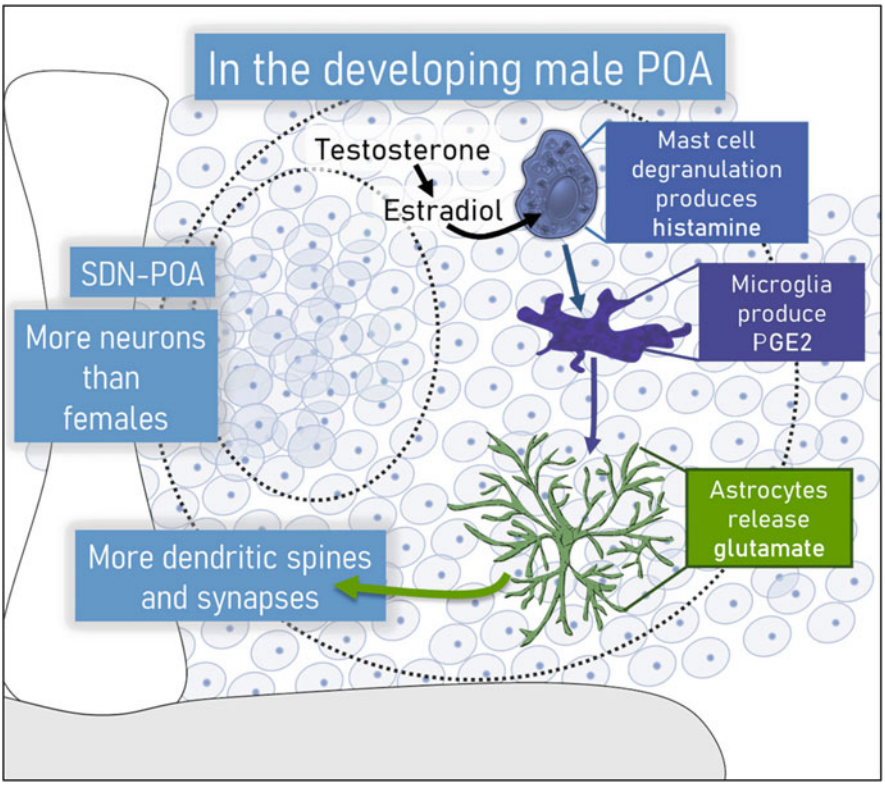

Fig. 9.5 Sexual differentiation of the preoptic area (POA) involves endocrine and immune communication across various neural cell types. The POA is perhaps one of the most sexually dimorphic structures in the brain. First there is a robust sex difference in the number of cells within the sexually dimorphic nucleus (SDN) of the POA. Second, neurons in the male POA have significantly more dendritic spines than neurons in the female POA. Both of these sex differences are established during a critical period of brain development and maintained into adulthood. The process begins when testosterone is secreted by the male testes, which gets converted via an enzyme in the brain to estradiol (an estrogen). Estradiol initiates the degranulation of resident mast cells, which in turn produce histamine. Microglia, the resident immune cells, respond by increasing the production of prostaglandin (PG)E-2. PGE2 stimulates the release of glutamate from surrounding astrocytes, which drives the formation of dendritic spines in the developing male brain

important role in this hormone-mediated, multi-cellular and multi-signaling process of sexual differentiation of the POA.

In the POA, males have twice as many microglia with an amoeboid-like morphology, indicative of activation, as females, and estradiol treatment of female rat pups masculinizes POA microglia number and morphology (Lenz et al. 2013), just as it masculinizes neuronal and astrocyte morphology. Furthermore, inhibiting microglia activation prevents testosterone-induced masculinization of dendritic spines in the POA as well as adult copulatory behavior in males, indicating that microglia are an important cellular mediator of the process of brain sexual differentiation (Lenz et al. 2013). Even more surprising was the subsequent discovery that the greater number of mast cells found in the male POA release histamine, and it is the histamine that activates the microglia and leads to increased PGE2 production (Lenz et al. 2018). Most importantly, and fascinatingly, these data highlight a unique process through which steroid hormones initiate the development of a functional behavioral circuit (male sex behavior) by engaging in a multi-cellular and multi-signaling (hormonal, inflammatory, and neurotransmitter) process during a discrete and critical period of brain development.

9.4.2 Hormones, Immune Function, and Social Behaviors

Decreased social interaction is an important component of sickness behaviors, such that reduced social investigation of a novel conspecific is a common laboratory measure of sickness behavior in rodents (Arakawa et al. 2009). However, the relationship between sickness and social behavior is complex, and data highlight the idea that immune molecules are key modulators of social behavior in a manner dependent upon other hormonal and social factors (Hennessy et al. 2014). For example, an injection with the pro-inflammatory cytokine IL-1β can suppress sexual receptivity behaviors in females, as well as their preference for a gonadally intact male (Yirmiya et al. 1995). In contrast, in males, a dose of IL-1β that is sufficient to suppress general activity has no effect on male sexual behavior with a receptive female (Avitsur et al. 1995). Thus, there is a robust sex difference in how cytokines or sickness may affect the motivation to engage in certain social behaviors, in this case sexual behaviors. That said, when motivation is high enough, females can also overcome sickness behaviors to engage in a social behavior. For example, when a dam is injected with a high dose of lipopolysaccharide (LPS), but her pups are cold due to low ambient temperature, the high dose of LPS has no effect on the maternal behavior she expresses towards her pups (Aubert et al. 1997). Thus, when the motivational state is sufficiently elevated, driven by hormonal changes specific to males (during sex) or females (in postpartum maternal care), even sickness cannot suppress meaningful social behaviors.

A meaningful social interaction can also *reverse* the expression of sickness behaviors. Zebra finches are social animals. When a zebra finch is administered LPS in isolation, it exhibits robust sickness behavior, but when a zebra finch is housed with its social group, the finches show no sickness behavior following the

same dose of LPS administration (Lopes 2014). Infant guinea pigs form strong attachments with their mothers and show robust sickness-like behaviors when isolated from the mother, even at weaning. Yet, when an infant pup is returned to its mother, the sickness behavior is immediately reversed (Hennessy et al. 2014), an effect that may be dependent upon the release of another hormone, oxytocin, that is released in the presence of the attachment figure. The relationship between social behavior and immune activation is complex and influenced by the social nature of the species, the hormonal status, and the sex of the individual.

There is growing evidence that the expression of social behaviors may be influenced by the immune system, even in the absence of overt sickness (Hennessy et al. 2014). Microglia have a critical role in the development of circuits that underlie social behavior. Temporary depletion of microglia from the developing brain (during a "sensitive period" of neurodevelopment) has negative effects on behavior later in life (Nelson and Lenz 2017). Specifically, treatment of neonatal rats with liposomal clodronate, which results in a significant loss of microglia in the forebrain, results in persistent changes in social behavior in both juvenile and adult rats. Notably, these behavioral deficits are observed even after microglia repopulate the brain (Nelson and Lenz 2017; VanRyzin et al. 2016), as well as in the absence of any external immune activation, suggesting that microglia in the *neonatal* brain have an important and active role in the establishment of neural circuits necessary for appropriate social behavior that is not even expressed until later in life. Thus, manipulation of microglia or *activation* of microglia at various critical time points in development may influence the manifestation of certain social behavior deficits later in life, many of which may be associated with mental health disorders.

9.5 Pregnancy, Hormones, and the Maternal Immune System

Pregnancy is associated with significant changes in maternal physiology, including robust changes in the immune system. During pregnancy, immune function is tightly controlled, and even minor disruptions in this process may affect the success of the pregnancy (Colucci 2019). In general, pregnancy suppresses immune function. This immunosuppression is initiated across the **maternal–fetal interface of the placenta** and contributes significantly to maternal health peripartum while also impacting the growth and development of the baby. For example, pregnant women are very susceptible to infection (Fig. 9.6). Late pregnancy is associated with an increased severity and mortality associated with a number of infections, both viral and bacterial (Robinson and Klein 2012), and these effects of pregnancy contribute to an overall female-biased susceptibility to infectious disease. In contrast, pregnancy-induced immunosuppression has the opposite effect for many women who suffer from autoimmune disorders. Patients with rheumatoid arthritis report significant improvement of their symptoms during their pregnancy, particularly in late gestation, and a significant worsening of symptoms shortly after birth. Similar findings have been reported for the autoimmune disease multiple sclerosis (MS), as women

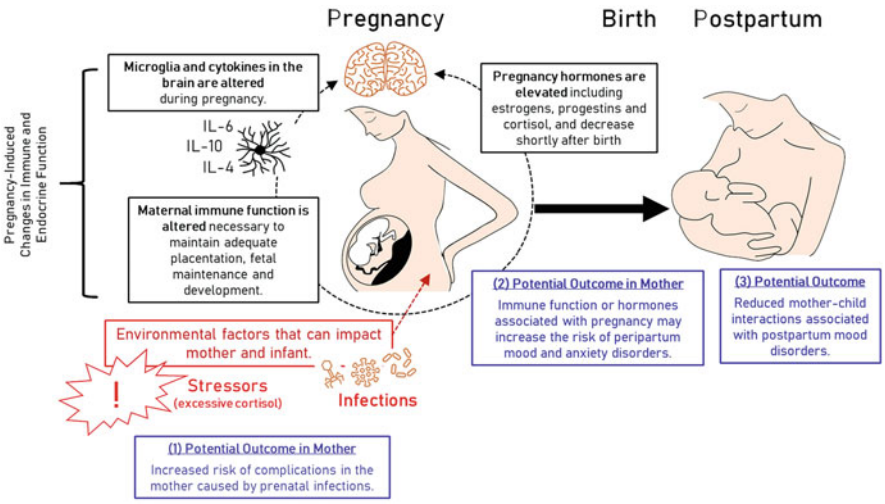

Fig. 9.6 Peripartum changes in immune and endocrine factors may increase the risk of complications and mental health disorders. During pregnancy, the maternal immune system is suppressed in a manner necessary to maintain adequate development of the semi-allogenic fetus. These changes are driven by changes in hormones and cell-to-cell contact within the placenta. These peripheral immune changes, in turn, likely result in changes in cytokine production in the maternal brain. The impacts of these robust and widespread immune changes are many. First, mothers are at a greater risk of complications or death associated with infection. Second, mothers are at increased risk of mood and anxiety disorders during the postpartum period, an effect that may be the result of robust changes in immune and endocrine function around birth. Third, mothers with postpartum mood disorders are more likely to exhibit decreased mother–child interactions, which may have negative consequences for the baby

have significantly reduced rates of MS relapse during pregnancy and a significant increase in relapse in the first three months postpartum.

Very few studies have investigated whether immune function in the brain is also altered during pregnancy, or whether changes in peripheral immune function during pregnancy can impact neural function or behavior. As female rodents approach reproductive age, they have more microglia than males across a number of brain regions that are associated with cognitive function (Schwarz et al. 2012). These brain regions include the hippocampus, parietal cortex, and amygdala, which contain significantly more microglia with thick, highly branched processes, characteristic of an "activated" morphology, in the female brain than in the male brain (Fig. 9.6). One might hypothesize that microglia respond to peripheral immune and endocrine factors that are altered by pregnancy and, as a result, female microglia may be more likely to "communicate" pregnancy-induced changes in peripheral immune function into mood-related or anxiety-related behaviors (Klein and Schwarz 2018; Sherer et al. 2017). In fact, it has been proposed that pregnancy may be a primary reason why there is a strong sex bias in the susceptibility of women to mood- and anxiety-related disorders relative to men. A systematic review found that certain types of

perinatal depression are associated with altered cytokine production in the periphery, which would suggest that women diagnosed with perinatal depression are experiencing dysregulation of the immune system (Osborne and Monk 2013). This review also reveals that, to date, very few studies have systematically examined whether there is a correlation between peripartum depression and peripheral cytokine production, how this correlation might be related to changes in hormones throughout the peripartum period, or whether there are differences in how the immune system changes during and after pregnancy in women with associated depression or anxiety.

A few studies have sought to examine the impact of pregnancy and parturition on cytokine expression in the female brain using a rodent model. Female rats have elevated levels of IL-6 and IL-4 in the hippocampus and prefrontal cortex immediately after birth (Haim et al. 2017; Sherer et al. 2017), which is consistent with the idea that microglial function is modulated towards the greater production of **Th2-type** or anti-inflammatory cytokines, just as peripheral immune function is attenuated during pregnancy (Fig. 9.6). There is a decrease in the density of microglia in the CA1 and dentate gyrus of the hippocampus in female rats on the day of birth. In contrast, microglia are increased in their activation or density within the CA3 of the hippocampus. Though the postpartum changes in cytokine production were consistently found in the prefrontal cortex, there were no changes in the density of microglial cells in this brain region, suggesting that the resident microglia became more activated following birth. IL-4 is a classic "Th2-type" cytokine; however, there is very little understanding about how IL-4 might impact microglial function, neural function, or behavior. In contrast, IL-6 is well-known as a potent neuromodulator that can have both pro-inflammatory and anti-inflammatory effects in the brain. The dysregulation of IL-6 in particular has been associated with a number of mood disorders, most notably depression (Hodes et al. 2015; Köhler et al. 2017). Postpartum female rats exhibit a decrease in sucrose consumption despite an overall increase in fluids, and this decrease in preference can be extended weeks postpartum by chronic stress in pregnancy (Haim et al. 2017). This would suggest that pregnancy-induced changes in microglia and the immune molecules they produce are correlated with a change in mood or reward processes immediately postpartum, an effect that can be further influenced by stress hormones (Fig. 9.6). Notably, however, these data only indicate that microglia are changed on the day of birth, which suggests, but does not prove, that microglia are also altered during pregnancy. We have yet to determine the full extent to which microglia are altered during pregnancy or by pregnancy hormones, and for how long these changes may persist postpartum, which could have significant implications for better understanding the cellular basis of peripartum mood and anxiety disorders.

9.6 Interaction of Hormones and Immune Cells in Mental Health Disorders

9.6.1 Stress, Anxiety, and Depression

Similarly to pathogens, stress can induce a range of sickness behaviors and associated inflammatory responses that affect our health (Kiecolt-Glaser et al. 2010). Sickness behaviors are considered adaptive; however, the adaptive role of sickness behaviors and associated inflammation may be less clear in the context of stress, in the absence of overt sickness (Fig. 9.3). Yet it is likely that the acute, pro-inflammatory response to stress drives a change in behavior that is caused by a full-body shift in energy needs caused by the release of stress hormones, including glucocorticoids (Hennessy et al. 2014). Stress, in particular psychosocial stress, is capable of producing endocrine and immune dysregulation and increased inflammatory cytokine signaling in the brain. These physiological and immune alterations contribute to the development of mental health disturbances, including anxiety and depression. As mentioned earlier, immune dysregulation induces adaptive, anhedonic, and withdrawal behaviors, but in the case of chronic stressors, this behavioral response associated with depression or anxiety is maladaptive.

Chronic stress promotes the upregulation of pro-inflammatory cytokines via the induction of NF-κB in peripheral immune cells and microglia. It is also possible that the stress or anxiety signals from neurons can indirectly stimulate the release of central pro-inflammatory cytokines via their release of danger signals, or danger-associated molecular patterns (DAMPs), including HSP72 or HMGB1, that activate immune cells in the periphery and the brain (Fleshner et al. 2017). The evidence from preclinical models of stress-induced anxiety and depression indicate that the ability of peripheral immune cells to produce IL-6, and the continuous upregulation of white blood cells and monocytes, is actually a risk factor for how stress can impact the subsequent risk of depressive-like behaviors in an individual (Hodes et al. 2015).

Several studies in humans and non-human animals have reported sex differences in the hormonal, behavioral, and neural responses to stress, and often these sex differences are the direct result of gonadal hormone effects on the production and secretion of stress hormones (i.e., glucocorticoids and epinephrine) (Viau and Meaney 1991). In general, females have higher circulating baseline levels of adrenocorticotropic hormone (ACTH) and glucocorticoids and more robust increases in glucocorticoids after exposure to a stressor (Handa et al. 1994). There are also well-documented sex differences in the expression of the glucocorticoid receptors (GR) throughout the brain; GR expression is higher in females than in males, with gonadal hormones directly mediating GR expression and function in the brain. In turn, females are more likely to exhibit depressive-like behaviors following chronic stress, an effect that is not seen in males (Bourke and Neigh 2011). It is likely that interactions among the sex of an individual, the age of an individual, the individual's peripheral immune response, the associated endocrine stress response, and the reciprocal effect that stress and its duration has on immune function in the periphery and the brain are sex-specific. These interactions require greater

consideration. The sex-specific interactions of these factors are important for understanding gender biases in mental health disorders that have known or suspected immune etiologies and links to stress or trauma, including anxiety, depression, and post-traumatic stress disorder (PTSD) (Rincón-Cortés et al. 2019).

9.6.2 Immune Activation, Cognitive Impairments, and Developmental Disorders

The developing brain is particularly vulnerable to activation of the peripheral immune system. Microglia seed the developing brain and spinal cord beginning around embryonic day 9.5 in the rodent and week 8 of gestation in humans (Ginhoux et al. 2013; Male and Rezaie 2001). As stated earlier, microglia have an active role in the formation of neural circuits that control behavior (Stevens and Schafer 2018). As such, activation of the immune system and the subsequent activation of microglia in the developing brain may divert the function of these neural cells during sickness so that they are unable to perform their important functions associated with the formation of functional neural circuits in the brain as they respond to an ongoing immune challenge. Evidence suggests that microglial dysregulation, specifically, has significant consequences on brain development and the risk for **neurodevelopmental disorders**, including ASD and schizophrenia (Frick and Pittenger 2016). As a result, understanding the consequences of immune dysregulation and associated microglial activation in the developing brain is necessary when investigating the relationship between environmental exposures and the risk for developing ASD or schizophrenia.

Cognitive impairments are a symptom of sickness behavior but also many mental health disorders that are also associated with the dysregulation of the immune system, including autism, schizophrenia, and depression (Dantzer 2006; Frick and Pittenger 2016). While the adaptive role of this association is not quite clear, it likely highlights the sensitivity of neurons to an increase in cytokines and chemokines. In addition, microglia and astrocytes, which can also respond to immune activation, are required for various synaptic functions including synaptic pruning, synapse formation, and synaptic transmission (Stevens and Schafer 2018). Despite the well-known interactions among various cytokines and chemokines in the peripheral nervous system, it remains unclear how the upregulation of immune molecules at even moderate levels can impact the function of microglia and neurons in the brain. Importantly, however, this converging evidence highlights the potential role of immune activation or dysregulation on cognitive dysfunction that is often associated with mental health disorders.

Moreover, sex bias in the prevalence of a number of neurodevelopmental disorders, particularly those considered to be the result of “mis-wiring” in the brain, including autism spectrum disorders, dyslexia, and generalized learning disorders, suggests that gonadal hormones, particularly in males, may influence the immune response to an early-life challenge and the subsequent risk of neurological disorder (McCarthy 2019; Osborne et al. 2018). Consistent with this idea,

Werling et al. (2016) reanalyzed published transcriptomes and found that the relative gene expression from microglial and reactive astrocytes was significantly greater in brains of fetal males compared to females. More strikingly, when they compared the transcriptomes from adult post-mortem samples, men that had been diagnosed with ASD had elevated markers of inflammation compared to men without an ASD diagnosis (Werling et al. 2016). Unfortunately, women with ASD were not included in the study, presumably due to their relative infrequency. Males are more likely to be diagnosed with ASD and a number of neurodevelopmental disorders that are often diagnosed early in development. As mentioned earlier, males are also exposed to elevated levels of testosterone during development, which is necessary to shape neural circuits that control the male gonads and program the ontogeny of appropriate male social and sexual behaviors. In contrast, the female brain undergoes an active process of feminization that programs the ontogeny of appropriate female social and sexual behaviors. One working hypothesis is that, early in life, males may be more vulnerable to the effects of any immune activation, either as a result of their genetic makeup, the influence of perinatal testosterone exposure, or greater maternal–fetal immune conflict caused by the maternal exposure to the fetal male's antigenic Y chromosome. Even slight differences in the immune response, the associated neuroendocrine and stress response, or the disruption of homeostasis in males may thus place them at greater risk of perturbations in the ongoing development of neural circuits following early-life immune activation (McCarthy 2019). Despite the widespread understanding of sex differences in peripheral immune function in adults, it is still unclear how the sex of a developing fetus or neonate may influence subsequent immune responses and the risk of neurodevelopmental disorders that involve the expression of appropriate social and cognitive behaviors.

9.7 Perspectives

Our increasingly sophisticated knowledge of the nervous system has required a more complete integration of other physiological systems, most notably the endocrine and immune systems. Surprising discoveries such as the steroidogenic capacity of the brain itself, and the active role of the innate immune cells of the brain in sculpting neural circuit formation and modulating adult behaviors, have forced researchers to rethink how the brain controls behavior. The richness of reproduction provides ample biological evidence for the integration of these systems with a complexity driven by evolutionary pressures both common and unique across species. Exploiting this richness reveals fundamental principles of normal processes and highlights nodes of vulnerability for dysregulation and potential therapeutic intervention.

In reviewing the current state of the art of our understanding of neuroimmunology, endocrinology, and behavior, many unanswered questions are revealed. The importance of cell-to-cell communication is evident, but how that signaling is regulated is often obscure due to the inherent difficulties in measuring fast acting membrane-derived signaling molecules. The criticality of sex as a

biological contributor to healthy and dysregulated brain development is also evident, but precisely how sex modulates the many complex processes and interactions is only beginning to be understood. How chromosome complement interacts with gonadally and locally derived steroids is essentially completely unknown. Moreover, how the environment and experience, both of which vary for males and females from the moment they are born, impact developmental trajectories is also inadequately understood and only rarely incorporated into experimental designs.

Of all the organs in the body, the brain is uniquely designed to incorporate internal and external signals into its design. We are all born with a fully functioning heart, liver, lungs, and kidneys; however, our developing brains are but a "work in progress" waiting to adapt and grow in response to the internal and external stimuli received. Thus, what we must continue to recognize is that those stimuli are multifactorial and include hormones, immune mediators, and epigenetic modifiers at the least. Turns out, the most complex organ of the body, the brain, is more complex than we ever imagined.

9.8 Key Literature

Amateau and McCarthy (2004) The first paper to identify the role of an immune molecule (PGE2) in the establishment of functional brain circuits during development.

Dantzer (2006) A seminal review focusing on the role of cytokines in sickness behavior and its relation to mental health.

de Biase and Bonci (2019) A key paper describing the various phenotypes of microglia throughout the brain.

del Rio-Hortega (1932) Pío del Río Hortega was the first to describe key characteristics of microglia in the brain.

Fleshner et al. (2017) A comprehensive review on how stress impacts immune signaling throughout the body and brain.

Ginhoux et al. (2013) A comprehensive review on the ontogeny of microglia from the researchers who first identified their progenitor source.

Gorski et al. (1978) The first paper to identify a sex difference in brain structure.

Hart (1988) Benjamin Hart was the first scientist to identify and consider sickness behavior and its causes.

Hennessy et al. (2014) An interesting, more recent review on the relationship between sickness behavior and social behavior.

Li and Barres (2018) A good review of the immune cells that reside in or traffic to and from the brain.

Maier and Watkins (1998) An early review of brain–immune communication and its impact on behavior.

McCarthy (2019) A recent review discussing how sex differences in immune function may have a fundamental role in the sex-biased risk of neurodevelopmental disorders.

Miller et al. (2009) Andrew Miller was one of the first psychiatrists to consider a role for cytokines in mental health.

Phoenix et al. (1959) The first study to show that prenatal testosterone exposure can organize developing neural structures underlying sex behavior.

Schwarz et al. (2012) One of the first papers to quantify sex differences in microglia number and morphology throughout development.

Sherer et al. (2018) The first review written to consider the role of the immune system during pregnancy and its impact on peripartum behaviors.

Stevens and Schafer (2018) A good review on the role of microglia in brain development.

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Hypothalamic Astrocytes and the Role of Neuroprogesterone in Estrogen Positive Feedback

10

Paul Micevych and Margaret Mohr

Abstract

Astrocytes are critical for the CNS control of female reproduction by mediating estrogen positive feedback—an event through which the hypothalamus controls the surge release of luteinizing hormone (LH) to induce ovulation and the formation of the corpus luteum. Estradiol controls physical interactions between astrocytes and gonadotropin-releasing hormone (GnRH) neurons, controlling synaptic input and peptide release. Importantly, estradiol stimulates astrocytes to release neuroprogesterone, which activates kisspeptin neurons that control GnRH neuronal activity. Like estrogen positive feedback, estradiol-induced neuroprogesterone synthesis appears only in females after puberty. Coincident with the maturation of the reproductive system during puberty, a new population of astrocytes is added to the hypothalamus. It is thought that during estrogen positive feedback, estradiol induces progesterone receptors in kisspeptin neurons, which are activated by estradiol-facilitated neuroprogesterone release from newly born hypothalamic astrocytes. Kisspeptin release then triggers GnRH release, inducing the surge secretion of LH.

Keywords

Progesterone · Astrocytes · Astrogenesis · Puberty · Kisspeptin

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10.1 Introduction

10.1.1 Overview

Glia can be considered the Rodney Dangerfield of the nervous system. They get no respect, at least until relatively recently. Glia remain vastly underappreciated, even though various luminaries of neuroscience, including Rudolf Virchow, Pedro and Santiago Ramon y Cajal, Stephen Kuffler, and Pio del Rio-Hortega, examined and studied glia. For many years glia, including astrocytes, oligodendrocytes, and microglia, were considered the stroma (i.e., the connective tissue) of the nervous system. It was thought that glia *only* provided structural support. In fact, this is how glia got their name: from the Greek word for glue. Astrocytes, especially, were thought to be the passive stroma, while neurons were the parenchyma, i.e. the functional part of the brain. These ideas were based on observations that electricity was the main biophysical feature of brain function, and while neurons use electrical events to convey information, astrocytes are not electrically excitable cells. Astrocytes, it was noted, surround neuronal cell bodies and terminals, and are partially responsible for the blood–brain barrier by covering brain capillaries with their "endfeet." Importantly, astrocytes maintain the extracellular environment (e.g., controlling $[H^+]$, detoxifying NH_3, scavenging free radicals, and buffering K^+). Another glial cell, the oligodendrocyte, forms myelin that insulates large axons, allowing for more rapid conduction of action potentials. Microglia, the macrophage of the CNS, have also been shown to remodel synapses.

The passive function of glia was not substantively questioned until the realization that devastating nervous system diseases target glia. The most prominent of these is multiple sclerosis (MS), in which the loss of the myelin sheath around axons leads to disrupted neuronal function. Astrocytes also have been implicated in the etiology of various nervous system pathologies including ischemia, neurodegenerative diseases, and trauma (Anderson et al. 2003; Burda and Sofroniew 2014).

10.1.2 Tripartite Synapse: Great Awakening of Glia Research

Arguably the "great awakening" to the importance of glia to brain function was the concept of the **tripartite synapse,** the realization that "in addition to the information flow between the pre- and post-synaptic neurons, astrocytes exchange information with the neuronal synaptic elements, respond to synaptic activity, and regulate synaptic transmission" (Araque et al. 1999). Morphological observations of the tripartite synapse—of astrocytic elements in association with chemical synapses and pre- and post-synaptic neuronal elements—were made dating back to the beginnings of ultrastructural examination of the nervous system (Fig. 10.1). While the morphological component of the tripartite synapse was noted early on, the functional component of the tripartite synapse was more difficult to demonstrate. To be fully realized, the idea of the tripartite synapse needed to encompass several functional aspects of astrocytes, evidence of which took longer to obtain because of

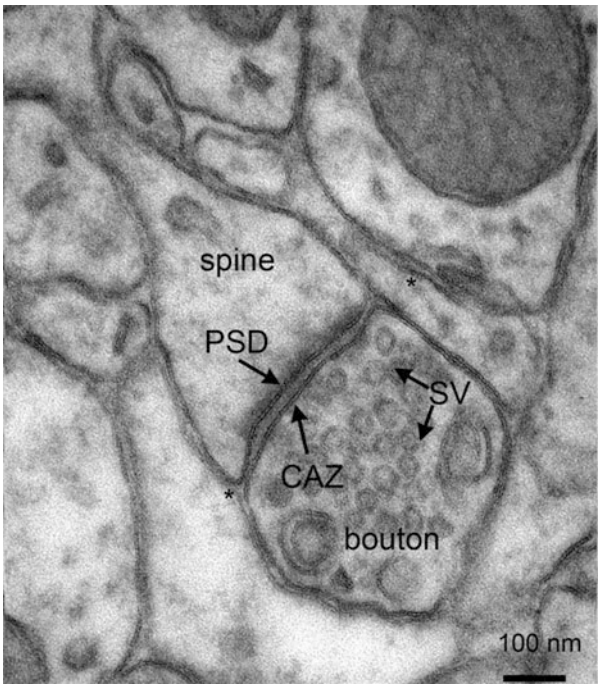


Fig. 10.1 Electron micrograph of a tripartite synapse in the rat cortex. Note the astrocyte endfeet (*) in close juxtaposition with pre- and post-synaptic terminals. CAZ, cytomatrix at the active zone; PSD, post-synaptic density; SV, synaptic vesicles. Taken from: Dieterich DC, Kreutz MR. 2016. Proteomics of the synapse—a quantitative approach to neuronal plasticity. Mol. Cell. Proteomics 15, 368–381. (https://doi.org/10.1074/mcp.R115.051482)

technical limitations and a neuron-centric perspective of the nervous system. This included the demonstration that astrocytes are "excitable" cells that respond to chemical stimuli, which they shape. Moreover, astrocytes communicate with neurons through the release of intercellular messengers, sometimes referred to as gliotransmitters (neurotransmitter-like molecules released by glial cells), including glutamate, D-serine, ATP, adenosine, GABA, tumor necrosis factor-α (TNFα), prostaglandins, peptides, and steroids.

10.1.3 Astrocyte Excitability: Increasing Intracellular Calcium

When appropriate tools became available, the idea that astrocytes were "non-excitable" compared with neurons was shown to be demonstrably mistaken. Neuronal excitability had long been defined as electrical signals generated across the plasma membrane, but the discovery of G-protein-coupled receptors (GPCRs) expanded the definition of excitability—a definition that eventually encompassed astrocytes. Astrocytes contain GPCRs, which are activated by a wide variety of ligands including hormones, peptides, and neurotransmitters. Upon activation, many of these GPCRs stimulate phospholipase C and the formation of inositol (1,4,5)-triphosphate (IP_3), which increases free intracellular calcium concentrations ($[Ca^{2+}]_i$) through activation of IP_3 receptors on intracellular Ca^{2+} stores (Pasti et al. 1997). Increasing

intracellular Ca^{2+} can initiate a multitude of signaling pathways and is considered a hallmark of glial excitability.

10.1.4 Astrocyte Diversity

Astrocytes make up a large (perhaps the largest) and diverse cell population of the nervous system. In fact, the idea that there are different types of astrocytes originated with Ramon y Cajal. The earliest distinction was fibrous vs. protoplasmic astrocytes. Protoplasmic astrocytes usually occur in gray matter, unlike fibrous astrocytes that are often found in white matter. Moreover, fibrous astrocytes, in distinction to protoplasmic, have an abundance of an intermediate filament, glial fibrillary acidic protein (GFAP). Soon, other biochemical differences were also recognized. Over the years, investigators have characterized astrocytes in various regions of the CNS as radial glia, cells with two processes forming endfeet at the ventricular wall and the pial surface, usually during development. In adulthood, cells with similar features are tanycytes in the median eminence (see Chaps. 11 and 12), pituicytes in the neurohypophysis (posterior pituitary) (see Chap. 3), Müller glia in the retina, and Bergmann glia in the cerebellum. Significantly, throughout life, radial glial cells appear to be progenitors for glia (oligodendrocytes and astrocytes) and neurons (Kriegstein and Alvarez-Buylla 2009). Additionally, modified astrocytes that line the ventricles and subretinal space are named ependymocytes, choroid plexus cells, and retinal pigment epithelial cells. Gomori astrocytes comprise another subtype of astrocyte that are iron-rich, have particularly high glucose metabolism, and are found mostly in the arcuate nucleus of the hypothalamus. Accumulating evidence suggests that the brain functions through concerted activity of a neuron–glia network, overthrowing the classically accepted paradigm that brain function results *exclusively* from neuronal activity.

The heterogeneity of astrocytes has been extensively and recently reviewed (Khakh and Deneen 2019). Morphological reconstructions and RNA sequencing have recently supplemented classic ultrastructural and immunocytochemical analyses to demonstrate the diversity of astrocyte function in terms of brain region, physiology, and pathology. Continued advances in neuroscience and access to new tools will undoubtedly uncover new roles of astrocytes and novel ways of categorizing this diverse cell population. In line with the idea of a diversity of astrocytes, we have demonstrated that hypothalamic astrocytes are physiologically distinct in males and females and appear to be sexually differentiated by the perinatal gonadal steroid environment (Kuo et al. 2010).

10.2 Hypothalamic Glia Aid in Homeostasis

In the hypothalamus, uncovering the role of astrocytes dates back to the late 1970s and studies on the surge release of oxytocin and vasopressin (Tweedle and Hatton 1977). Investigators observed changes in astrocyte covering of magnocellular

neurons in response to physiological stimuli such as dehydration, parturition, and lactation (see Chaps. 2 and 3). **Glial ensheathment** was inversely related to synaptic input and the juxtaposition of adjacent magnocellular neuron plasma membranes (Theodosis and Poulain 1984). It appears that astrocytic processes interposed between neurons prevent electrical interactions between magnocellular neurons, which may influence the synchronous firing needed to release oxytocin and/or vasopressin.

In addition to regulation of lactation (oxytocin) and plasma osmolarity (vasopressin), technical advances have begun to uncover roles of astrocytes in other aspects of neuroendocrinology, including homeostatic regulation of metabolism, providing metabolic cues to initiate puberty, and developmental origins of structural and functional sex differences. Given the location of many of these hypothalamic glial cells so close to the blood–brain barrier, they are well positioned to relay a whole host of signals from the periphery into the brain. The use of transgenic mouse models to study astrocyte physiology has begun to uncover roles for astrocytes in relaying metabolic cues that help initiate the onset of puberty and regulate adult reproductive function. Deletion of insulin receptors specifically in astrocytes delayed puberty in males and females, leading to lifelong reproductive dysfunction in females (Manaserh et al. 2019). Glial cells residing at the edge of the blood–brain barrier transport and release a wide variety of substances that are related to metabolic signals. Significantly, obesity has been associated with astrogliosis in the hypothalamus (Horvath et al. 2010). Astrogliosis is a cellular and proliferative response to CNS pathology including neurodegenerative disease, trauma, infection, ischemia, and inflammation, which has now been suggested as a mechanism involved in obesity. In terms of sexual differentiation, masculinization of the rodent preoptic area during development requires steroid hormone-dependent signaling between neurons, astrocytes, and microglia, highlighting the importance of glial steroid signaling in development (Lenz et al. 2013).

10.3 Astrocytes and Reproduction: GnRH Neurons

10.3.1 Interactions with GnRH Neurons

Some of the best-characterized actions of astrocytes are in the regulation of reproduction. The hypothalamus controls the development of ovarian follicles, ovulation, and the formation of the corpus luteum through its control of the release of gonadotropins (**luteinizing hormone, LH** and follicle-stimulating hormone, FSH) from the anterior pituitary. This is accomplished through the release of **gonadotropin-releasing hormone (GnRH)**. Astrocytes have been observed to interact with GnRH neurons in a manner similar to their interaction with magnocellular neurons. Glia cover large parts of the surface of GnRH neurons and this ensheathment increases following a loss of estradiol (ovariectomy), suggesting GnRH neuronal inactivity is due at least partly to glial ensheathment that prevents synaptic input. Indeed, the glial apposition is negatively correlated with the density of synaptic input

to GnRH neurons. Replacement of gonadal steroids in ovariectomized rodents partially reversed the glial covering and reduction in synaptic input, demonstrating the plasticity of astrocyte morphology in response to steroid hormone stimulation. Indeed, astrocytes in the rostral preoptic area that are juxtaposed to GnRH neurons exhibit hormonally-mediated fluctuations in morphology such that the amount of GnRH surface area covered by astrocytic processes decreases just before the initiation of the preovulatory GnRH/LH surge, likely allowing increased synaptic input to GnRH neurons.

Astrocytic apposition to hypothalamic arcuate nucleus (ARH) neurons also changes during the estrous cycle and this covering is correlated with gonadal hormone levels (Olmos et al. 1989). The increase of astrocyte covering in the ARH from the morning to the afternoon of proestrus is mimicked by estradiol treatment. This suggests that the functions of cells in the ARH and the preoptic area are distinct. As we discuss, it is clear that neurons within the ARH restrain GnRH release and those in the preoptic area drive the GnRH surge.

GnRH neurons send their axons to the **median eminence**, where they release GnRH into the portal circulation. At the level of the GnRH nerve terminals in the median eminence, tanycytes and astrocytes are dynamically regulated in a manner consistent with a role in modulating GnRH release. Morphological analyses show that classical astrocytes reside in an intermediate position within the median eminence and strongly contact GnRH nerve terminals. Tanycytes, on the other hand, form a single layer beneath the ependyma of the ventrolateral and ventral aspects of the third ventricle and extend their processes laterally into the mediobasal hypothalamus and to the pial surface of the median eminence, and are classified by anatomical location, gene expression, and projection pattern. Similar to the astrocyte coverage of GnRH cell bodies that prevents synaptic innervation of GnRH neurons, tanycyte ensheathment of the GnRH terminals in the median eminence restricts access of GnRH nerve terminals to the hypophyseal portal capillaries, and restricts GnRH release. Retraction of tanycyte endfeet allows for GnRH release (Ojeda et al. 2008). Recent advances in single-cell sequencing (see Box 10.1) reveals that tanycytes are transcriptionally similar to radial glial cells but distinct from ependymal cells. The ensheathment of GnRH nerve terminals by glia in the median eminence is a dynamic process that, as expected, fluctuates throughout the estrous cycle.

10.3.2 Chemical Communication with GnRH Neurons

In addition to providing a physical barrier, astrocytes and tanycytes also release an assortment of factors that promote GnRH release. Ojeda (1994) first proposed that glial cells are directly involved in providing signals to GnRH neurons including prostaglandins, transforming growth factor-α and -β (TGFα/β), and nitric oxide (Galbiati et al. 1996; Bellefontaine et al. 2011). Additionally, the epidermal growth factor (EGF) family is important in glia-to-GnRH neuron and glia-to-glia communication that results in GnRH secretion (for review see (Ojeda et al. 2010)). Much of

this work was done using astrocyte-conditioned media and GT1-7 cells as an in vitro stand-in for GnRH neurons. These experiments demonstrated that TGF-β1 and the progesterone metabolite 3α,5α-THP (3α,5α-tetrahydro progesterone) stimulate the expression and release of GnRH by transducing an estradiol signal (Galbiati et al. 1996). Undoubtedly, these signals have a role in GnRH physiology, but today it is clear that the most important stimulatory signal to GnRH neurons is **kisspeptin** (Han et al. 2005), discussed in more detail in Sect. 10.5.

10.4 Astrocytes and Reproduction: Neurosteroids

10.4.1 Neurosteroids: an Overview

The gonads produce the bulk of circulating sex steroid hormones, but it is important to recognize that the brain itself synthesizes steroid hormones, or **neurosteroids**, which regulate a variety of brain functions, from reproduction to cognitive performance (reviewed in (Ratner et al. 2019)). Given the vast array of functions that are controlled by steroid signaling, it is not surprising that there is evidence of neurosteroid signaling throughout most of the brain. Altering neurotransmitter levels can affect neurosteroid production (Uzunov et al. 1996; Kuo et al. 2010), suggesting that under normal physiological conditions the brain balances the levels of these chemical messengers through a dynamic interaction between neurons and glial cells. Progesterone and progesterone metabolite interactions with GABA, glycine, and *N*-methyl-D-aspartate (NMDA) receptor function have been studied. Initially, these studies by classical neuroscientists assumed that the gonads were the source of gonadal steroids that were modified in the CNS. Neuroendocrinologists, on the other hand, had already demonstrated that steroids were synthesized *de novo* from cholesterol in the brain and designated these as neurosteroids, as opposed to neuroactive steroids, which are metabolites of steroids of gonadal/adrenal origin. Indeed, enzymes required for steroid synthesis are prominent throughout the brain, in a variety of species (Corpechot et al. 1981; Zwain and Yen 1999; Saldanha et al. 2000). Moreover, the synthetic capacity for neurosteroids is independent of steroids originating in the periphery. Of the cell types in the CNS capable of steroidogenesis, including neurons, astrocytes and oligodendrocytes, astrocytes are the most steroidogenic (Zwain and Yen 1999). More recently, microglia have been reported to have steroid-converting enzymes, but not the enzyme needed to produce steroids *de novo*, and thus they convert circulating steroids but do not synthesize neurosteroids (Gottfried-Blackmore et al. 2008).

Neurosteroids have an important function in the central regulation of reproduction. Often, neurons contain the enzymes needed to convert steroid hormones that act on local neural circuitry. For example, there is an established role for neuroactive estradiol, which is converted from testosterone by the enzyme aromatase that affects copulatory performance and sexual motivation in male birds (Cornil et al. 2018; de Bournonville et al. 2019). In other cases, the cell type responsible for the production of the neurosteroid is unknown, but is probably the astrocyte that synthesizes

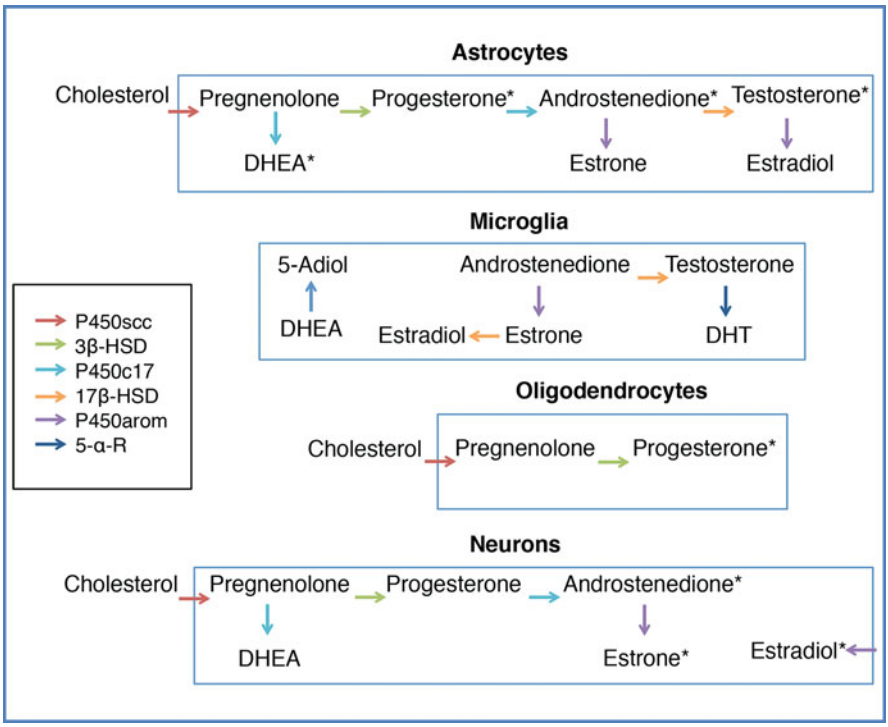


Fig. 10.2 CNS cell types involved in sex steroid synthesis. The arrows represent the enzymes found in these cell types. Asterisks indicate the steroid most synthesized by that cell type. Based on data in Zwain and Yen 1999; Gottfried-Blackmore et al. 2008

pregnenolone and progesterone, which in turn can be converted to allopregnanolone, a neuroactive progesterone metabolite (Zwain and Yen 1999). Progesterone itself is a reproductively critical neurosteroid that astrocytes synthesize from cholesterol (reviewed in (Micevych et al. 2017)) (Fig. 10.2).

10.4.2 Neuroprogesterone and Estrogen Positive Feedback

Progesterone, synthesized *de novo* in the hypothalamus, referred to as **neuroprogesterone,** is required for an LH surge and normal estrous cyclicity. Female rats that are ovariectomized and adrenalectomized—a treatment that at one point was thought to eliminate all sources of steroid hormones—do not show a surge in LH when treated with estradiol and a 3β-HSD inhibitor, which blocks the conversion of pregnenolone to progesterone (Micevych and Sinchak 2008b). Similarly, gonadally-intact female rats treated with a P450scc/CYP11A1 inhibitor, which blocks the conversion of cholesterol to pregnenolone, do not cycle (Micevych and Sinchak 2008a). In ovariectomized-adrenalectomized rats, estradiol treatment

increases levels of progesterone in the hypothalamus with a concomitant increase of 3β-HSD mRNA, supporting the idea that progesterone is produced locally in the hypothalamus from cholesterol. Adult hypothalamic astrocytes are the source of estradiol-induced neuroprogesterone.

The importance of neuroprogesterone in female reproduction has been demonstrated *in vivo* (Micevych and Sinchak 2008b), while years of work using primary hypothalamic astrocyte cultures have begun to shed light on the cellular mechanisms controlling neuroprogesterone synthesis. Perhaps the most startling discovery was that estradiol acted on estrogen receptor alpha (ERα) at the cell membrane of the astrocyte in females (Kuo et al. 2009). The estradiol membrane-initiated signaling involves transactivating a metabotropic glutamate receptor-1a, leading to phosphorylation of steroidogenic acute regulatory protein (StAR) and translocator protein (TSPO), which mediate the rate-limiting step of steroidogenesis—transport of cholesterol into the mitochondrial matrix. This estradiol-augmented neuroprogesterone synthesis, controlled by hypothalamic astrocytes, reveals heterogeneity in astrocytes between sexes.

The signaling between astrocyte-derived neuroprogesterone and kisspeptin neurons was established through *in vitro* studies using immortalized adult female kisspeptin neurons and primary astrocyte cultures (Mittelman-Smith et al. 2015, 2018). Estradiol, through a classic nuclear action, induces progesterone receptor (PGR) in kisspeptin cells and, through estradiol membrane-initiated signaling, upregulates kisspeptin expression. Some of the PGRs are trafficked to the membrane and, when stimulated, increase intracellular levels of free calcium, indicating that PGR will rapidly stimulate kisspeptin cells. In astrocyte/kisspeptin cell co-culture, estradiol treatment augments the estradiol-induced kisspeptin expression and kisspeptin release. While there is relatively little information about membrane PGR signaling, emerging data are congruent with the idea that PGR activates Src kinase in kisspeptin cells. *In vivo*, an Src inhibitor prevents the estradiol- or the estradiol + progesterone-induced LH surge. These results are consistent with earlier studies showing that progesterone coupled to BSA, which does not penetrate the cell membrane, induces GnRH release (Ke and Ramirez 1987).

10.4.3 Neuroprogesterone: Sex Differences

The rodent sex differences in estradiol facilitation of progesterone synthesis in hypothalamic astrocyte mirrors the rodent sex difference in estrogen positive feedback signaling, which is present in adult females and absent in males of any age. Only astrocytes from post-pubertal female rodents are capable of estradiol-induced neuroprogesterone synthesis, while hypothalamic astrocytes in males do not elevate neuroprogesterone synthesis when treated with estradiol (Kuo et al. 2010). When differences between sexes are noted, it is useful to determine whether they are due to differences in gonadal hormones or are the result of chromosomal differences (i.e., XX vs. XY). Using the four-core genotypes mouse model, in which gonadal sex can be delineated from chromosomal sex, it was found that only astrocytes from gonadal

females elevate neuroprogesterone synthesis when treated with estradiol (Kuo et al. 2010). In other words, regardless of the chromosomal sex, mice with testicles prevented hypothalamic astrocytes from responding to estradiol and facilitating neuroprogesterone synthesis. This is a classic mechanism of defeminization of the hypothalamus. Underlying these differences in neuroprogesterone synthesis between male and female astrocytes are: (1) increased levels of membrane ERα in females, and (2) greater amounts of intracellular calcium release in response to estradiol in female astrocytes compared with male astrocytes. These studies have highlighted that gonadal steroid hormone exposure can functionally change astrocyte signaling, demonstrating that hypothalamic astrocytes are shaped by ovarian hormone exposure in a way that prepares them for their crucial role in estrogen positive feedback.

10.5 Neurosteroids, Kisspeptin, and the LH Surge

10.5.1 Overview of Estrogen Positive Feedback

As with all physiological and behavioral events controlled by the brain (i.e., all of them), an extensive network is involved. The brain regulates female reproduction and the key to understanding this process is **estrogen positive feedback**. Gonadal steroids, in both sexes, feed back onto the pituitary and hypothalamus to regulate the release of the pituitary gonadotropins, LH and FSH (follicle-stimulating hormone). When gonadal steroid secretion is low, negative feedback restrains the release of GnRH into the hypothalamo-hypophyseal portal system maintaining a relatively constant LH and FSH release. In one of the most robust physiological sex differences, in females, as estradiol from developing follicles rises to peak levels on proestrous (in rodents) and mid-menstrual cycle (in primates), instead of constraining GnRH release, estradiol *stimulates* a surge of GnRH release that induces a surge of LH, causing ovulation of the dominant ovarian follicle. Thus, in females, there are two modes of estradiol signaling to the hypothalamus: negative and positive feedback. Both of these types of feedback have been extensively studied. From these investigations, the neuropeptide kisspeptin has emerged as a critical modulator of GnRH release in both negative and positive feedback (extensively reviewed in (Kauffman and Smith 2013)).

10.5.2 Kisspeptin: Estrogen Negative and Positive Feedback

Hypothalamic kisspeptin neurons form two populations: an anterior group along the rostral third ventricle (RP3V, rostral periventricular zone of the third ventricle that contains the **AVPV—anteroventral periventricular nucleus**) in rodents and in the preoptic area of monkeys, and a posterior group in the ARH. These two populations have distinct roles in regulating the secretion of GnRH that controls LH release from the pituitary. The RP3V kisspeptin neurons regulate the surge release of GnRH and

positive feedback, while the ARH kisspeptin neurons control GnRH pulse generation and estradiol negative feedback (Mittelman-Smith et al. 2012; Clarkson et al. 2017). The ARH neurons express neurokinin B and dynorphin as well as kisspeptin and, thus, are designated KNDy neurons. In keeping with their roles in GnRH regulation, estradiol inhibits the expression of kisspeptin in KNDy neurons while in the RP3V population, estradiol stimulates kisspeptin expression.

10.5.3 Estrogen Positive Feedback: Integration of Estradiol and Progesterone Signaling in Kisspeptin Neurons

Both estrogen positive and negative feedback affect GnRH neurons, which interestingly do not express reproductively relevant estrogen receptors. Thus, another cell is required to transduce steroid information into signals to which GnRH neurons respond. In terms of estrogen positive feedback, there is increasing evidence that this intermediary is the kisspeptin neuron:

1. Kisspeptin neurons express ERα and PGR (Smith et al. 2005; Clarkson et al. 2008; Mittelman-Smith et al. 2015, 2018).
2. Estradiol and progesterone increase kisspeptin expression (Smith et al. 2005; Mittelman-Smith et al. 2015).
3. Electrophysiological studies demonstrate that kisspeptin is arguably the most potent excitatory agent of GnRH neurons, which express the kisspeptin receptor (Han et al. 2005).
4. Deletion of PGR from kisspeptin neurons abrogates estrogen positive feedback and the LH surge (Stephens et al. 2015).

As mentioned earlier, the brain is not the passive recipient of peripheral sex steroid information. Rather, the brain is actively involved with providing its own steroid signaling required for estrogen positive feedback, which in spite of the name requires both estradiol and progesterone (see Box 10.2). Peripheral estradiol originates in granulosa cells of ovarian follicles, and rises during the diestrus (follicular phase in primates), stimulating the expression of PGR in the hypothalamus (Blaustein and Turcotte 1989). As peripheral estradiol levels peak, they stimulate ERα on astrocyte cell membranes, inducing the synthesis of neuroprogesterone in astrocytes (reviewed in (Micevych et al. 2017). Briefly, estradiol-activated ERα transactivates mGuR1a, leading to a G-protein-coupled receptor-induced signaling cascade involving phospholipase C (PLC), inositol trisphosphate (IP_3)-induced release of intracellular calcium and activation of a calcium-sensitive cAMP protein kinase that phosphorylates StAR and TSPO, increasing the shuttling of cholesterol from the outer to the inner mitochondrial matrix, where cholesterol is converted to pregnenolone by the enzyme P450scc/CYP11A1. In the smooth endoplasmic reticulum, 3β-HSD converts pregnenolone to progesterone, which subsequently diffuses out of the astrocyte to act on kisspeptin neuron PGR (Mittelman-Smith et al. 2017).

Estradiol of ovarian origin and progesterone from astrocytes converge to up-regulate both kisspeptin expression and release, which stimulates GnRH release, thus inducing the LH surge. Significantly, estrogen positive feedback and the estradiol augmentation of progesterone synthesis are both sexually dimorphic and appear in adulthood—that is, after puberty.

10.5.4 Development of Estrogen Positive Feedback in the Female Rat

While it is customary to say that estrogen positive feedback does not occur until after puberty in the female rodent, the reality is more nuanced. During the infantile period, in distinction to the situation in adults, high estradiol doses *suppress* circulating levels of LH, demonstrating that estrogen negative feedback mechanism(s) is/are present early in life. Estrogen positive feedback develops gradually. Estradiol stimulation of LH release appears at postnatal day (PND) 16, then from PND 16 to PND 20, the amount of estradiol required to elicit a surge in LH is twice as high as the proestrous (natural) levels of estradiol. At approximately PND 22, the LH surge can be induced with proestrus levels of estradiol, however the timing of the surge is different than it is in mature animals, occurring 54 h after estradiol administration. By PND 30, animals show a robust LH surge, with an amplitude that is indistinguishable from that of adult proestrus animals. An important site for the hypothalamic control of the LH surge is the sexually dimorphic AVPV. Because puberty marks the shift to an adult pattern of the LH surge, it is critical to know how the AVPV is structurally remodeled during puberty.

10.6 Anteroventral Periventricular Nucleus (AVPV) and Estrogen Positive Feedback

10.6.1 Morphological Sex Differences of the AVPV: When Do They Emerge?

The AVPV is uncommon in that it is a female-biased brain structure, i.e., larger and contains more cells in females compared with males (Fig. 10.3). Many sexually dimorphic brain structures, such as the medial amygdala, the central part of the medial preoptic nucleus (MPNc), and the bed nucleus of the stria terminalis, are male-biased. In 1982, Bleier and colleagues first documented a female-biased sex difference in cell distribution and density in what would later be named the AVPV in young adult rats, guinea pigs, hamsters, and mice. In adulthood, the AVPV is roughly 1.6 times larger in females compared with males. Soon after, investigators found that treating neonatal or perinatal females with testosterone eliminated the sex difference in AVPV volume in adulthood.

These results are consistent with the general hypothesis of the formation of sex differences in the brain in which gonadal hormones act during the critical perinatal

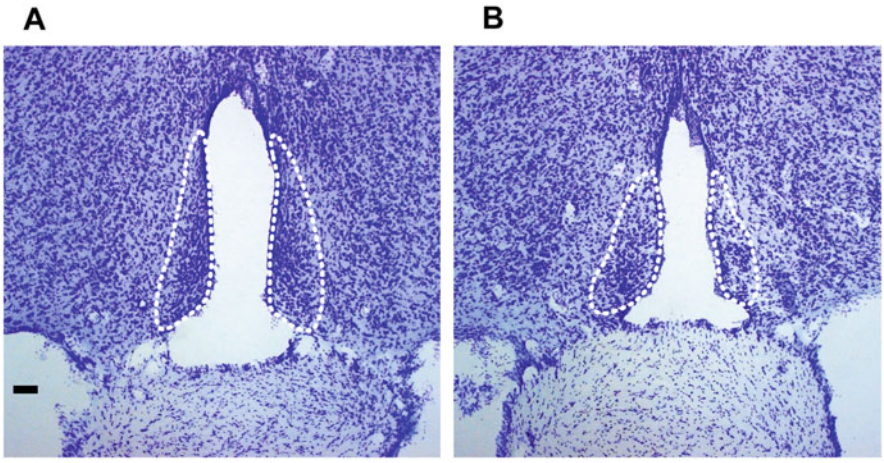


Fig. 10.3 Morphological sex differences in rat AVPV. Photomicrograph demonstrating that the (**a**) female AVPV is greater in volume and cell number compared with the (**b**) male AVPV. Dashed lines indicate AVPV boundaries. Scale bar = 100μM

period to induce sexual differentiation of brain structures. Distinctly, gonadal hormones act on the AVPV during the *pubertal* period, rather than the perinatal period, to induce sexual differentiation of the AVPV (Davis et al. 1996). During puberty, the AVPV becomes morphologically different in female and male rats, both in volume (apparent at PND 30–40) and length (PND 60–80). In this report, cell addition to the AVPV was not considered as a feasible mechanism to cause these volumetric differences because, at the time, neurogenesis was only thought to occur in discrete regions of the brain. Thus, there were several other explanations offered. First, perinatal androgens induce cell death in the male AVPV. This was an interesting interpretation of the results, because the sex difference of the MPNc, a male-biased region, arises due to the testosterone restraint of apoptosis. Second, the authors attributed the volumetric differences that arise after puberty to be the result of increased diameter of neurons, glia, or changes in the nature or density of synaptic contacts within the AVPV.

Even though there is now strong evidence that cell addition contributes to the AVPV sex difference, cell death as a mechanism contributing to AVPV sex differences must not be overlooked. In transgenic mouse studies, cell death was determined to be a cellular mechanism important for the morphological sex difference of the AVPV. Over-expression of Bcl-2 (an anti-apoptotic protein) and a knockout of Bax (critical protein for cell death) eliminated sex differences in cell number and volume of the female mouse AVPV. In males, over-expression of Bcl-2 caused an increase in AVPV cell density to female levels. Significantly, the highly sexually dimorphic population of dopaminergic neurons within the AVPV is unaffected by these genetic manipulations. Thus, these dopaminergic cells, and conceivably others, acquire sexual dimorphisms through processes that are independent of

cell death. The dopaminergic population of cells in the AVPV is of particular interest because kisspeptin is co-expressed in a vast majority of these cells.

10.6.2 The Addition of Cells to the AVPV During Puberty

Because the effects of perinatal gonadal hormones on the gross morphology of the AVPV are not apparent until after puberty, when gonadal hormone secretion rises, and cell death does not affect the dopamine/kisspeptin population within the AVPV, it makes sense to consider that gonadal hormones might promote the addition of new cells to the AVPV during puberty. Indeed, sexually dimorphic brain regions have a sex-biased addition of new cells and gonadal hormones drive these sex differences (Ahmed et al. 2008). Specifically, more cells are added to the female rat AVPV during puberty compared with the male. Prepubertal gonadectomy abrogates the sex difference of cell addition to the AVPV. The opposite pattern is true in the medial amygdala and the MPNc, where more cells are added in males to these male-biased brain regions during puberty. Thus, hormonally-mediated cell addition is an intriguing mechanism to form and maintain morphological sex differences throughout puberty and adulthood. An enticing idea is that this cell addition during puberty also contributes to physiological and behavioral sex differences.

10.6.3 Estrogen Positive Feedback and Cell Proliferation in the AVPV

Starting at puberty, when estrogen positive feedback develops, and extending into adulthood, a significant number of cells are added to the female rat AVPV: ~10–15% are neurons, ~25% are microglia, and the largest population are astrocytes, comprising ~20–40% of the new cells (Mohr et al. 2017). The percentage of pubertally born cells that differentiate into neurons and astrocytes in the AVPV is similar to phenotyped pubertally born cells in limbic and hypothalamic regions of male hamsters (Mohr and Sisk 2013), suggesting that pubertal neuro- and glio-genesis contributes to the pubertal maturation of the brain (Box 10.3). Pubertally and adult-born cells, which are predominately glia, are not only active during an estradiol and progesterone-induced LH surge, but inhibiting the birth of these cells also blunts and delays the LH surge. Thus, cells are added to the AVPV during puberty and early adulthood and are functionally incorporated into neural circuits involved in estrogen positive feedback and generation of the GnRH/LH surge, implying that postnatal cell addition contributes to the maintenance of functional sexual dimorphisms (Mohr et al. 2017).

10.6.4 Newborn Astrocytes: Source of Neuroprogesterone?

Astrocytes within the AVPV play an important role in estrogen positive feedback during the preovulatory LH surge. Estrogen positive feedback involves a tango among hypothalamic astrocytes in the AVPV, which synthesize neuroprogesterone in response to estradiol. This response is sexually differentiated (see above) and developmentally regulated. Only post-pubertal female astrocytes respond to estradiol: neither neonatal nor adult male astrocytes increase progesterone synthesis in response to estradiol stimulation (Kuo et al. 2010). Blocking steroidogenesis in the hypothalamus of gonadally-intact, cycling rats or deleting PGR from kisspeptin cells in ovariectomized, estradiol-primed mice prevents the LH surge, indicating that CNS derived neuroprogesterone is a key component of the estrogen positive feedback surge mechanism (Stephens et al. 2015; Micevych et al. 2017).

Astrocytes harvested from neonatal mice do not synthesize neuroprogesterone in response to estradiol administration and maturation in vitro does not make them competent to respond to estradiol. The development of estradiol-induced progesterone signaling occurs across puberty (Mohr et al. 2019). Prepubertal female rats do not respond to estradiol treatment with an increase in hypothalamic progesterone synthesis, while female rats at the start of puberty have an intermediate neuroprogesterone response, and adult females respond to estradiol with robust hypothalamic progesterone synthesis. This is similar to the developmental time course of the estrogen positive feedback-triggered LH surge (Mohr et al. 2019). It is tempting to speculate that the birth of estrogen-sensitive astrocytes in the AVPV during puberty contributes to the gain of function in brain circuits that control estrogen positive feedback.

10.6.5 Future Studies: Inhibiting Astrogenesis to Study Estrogen Positive Feedback Signaling

Since the inception of neuroendocrinology, investigators have used strategies of removing organs (usually gonads), lesioning brain areas, blocking receptors and, more recently, preventing the expression of various proteins to understand physiological functions and/or behaviors. Following this logic, the best way to determine if newly born astrocytes in the AVPV/preoptic area help drive estrogen positive feedback would be to remove newly born astrocytes from this region and measure the LH response to estradiol. Earlier studies teased at this by removing pubertally or adult-born cells using a mitotic inhibitor, which blunted and delayed the estradiol- and progesterone-induced LH surge (Mohr et al. 2017). A majority of the pubertally or adult-born cells in the AVPV/preoptic area are principally astrocytes, a result that strongly implies that newborn astrocytes are involved in signaling needed for the LH surge. The LH surge might have been more severely diminished if the LH surge paradigm had *not* included a peripheral progesterone injection. If neuroprogesterone acts to time the LH surge, much like progesterone's role in lordosis (Mittelman-Smith et al. 2017), then the fact that the LH surge was also delayed might be more

evidence that newborn astrocytes are making neuroprogesterone involved in the LH surge. An important caveat of these studies is that, in addition to presumptive newborn astrocytes, newborn neurons and newborn microglia were also eliminated, precluding a formal proof that it was the loss of astrocytes that suppressed the LH surge.

While specifically inhibiting newborn astrocytes is a simple idea, there are various technical considerations that are roadblocks to its successful completion. A significant issue is that transgenic mice that would make this a straightforward experiment often use a tamoxifen-inducible Cre system. Tamoxifen is a selective estrogen receptor modulator (SERM) that targets endogenous estrogen receptors, acting both as an agonist and an antagonist. Disentangling these effects from the tamoxifen-induced loss of newly born astrocytes is highly problematic. Another confound is that GFAP-expressing cells may be stem cells that line the third ventricle and are the progenitor pool for *both* new astrocytes and neurons. While these neural stem cells express GFAP only for a short period of time, transgenic models utilizing the GFAP promoter may not be any more selective than inhibiting all dividing cells (Mohr et al. 2017). With that said, the GFAP-TK transgenic mouse, in which mitotically active GFAP-expressing cells are killed by ganciclovir, could present a tractable model—with sufficient controls. Such controls should include single-cell sequencing to determine the phenotypic makeup of cells affected by this manipulation.

10.7 Perspectives

While new tools to study astrocytes continue to be developed, new roles of astrocytes will continue to emerge, leading to advances in therapeutic treatments available for various disorders and pathologies. An important corollary to this is that unless a more holistic appreciation of the CNS emerges and replaces the neuron-centric view of the nervous system, our understanding of brain functions will remain rudimentary. The neuron-centric view of the nervous system has perpetuated an incomplete notion that electrical activity is the only important signal worth measuring. Over the past ~20 years, tools to understand the significance of astrocytes have been made available and progress elucidating glial function has been remarkable. These studies have shown that astrocytes specifically, and glia in general, are active participants in brain function. In neuroendocrinology, as elsewhere, without an appreciation of the role of astrocytes in information processing, even the most detailed analysis of neuronal morphology, connections, and activity will not produce a complete understanding of brain function. We discussed that the role of astrocytes in the neuroendocrine hypothalamus is to physically restrain neuronal excitability by ensheathing neurons and processes, and to release diffusible signals to modulate the expression and release of neuropeptides—all in response to physiological signals including plasma osmolarity and hormones. Astrocytes sense and relay information, but as has been convincingly demonstrated by the tripartite synapse, they also actively participate in chemical transmission. As we focus on these important

cells, we are beginning to see all the ways they influence brain function. In the regulation of classical estrogen positive feedback, astrocytes are a critical player. Their participation in brain steroidogenesis demonstrates, from a neuroendocrine view, that neither astrocytes nor the brain are passive recipients of steroid hormone information.

Box 10.1 Paradigm-shifting technique: How single-cell sequencing has changed our view of glia

The advent of single-cell sequencing has allowed us to view the brain in ways that were not possible before. Now, we have the ability to know which genes are expressed in an individual cell. Until recently, bulk sequencing was used to inform about transcriptome changes in brain regions (via micropunch, with no discrimination between neurons, astrocytes, oligodendrocytes, etc.) or in cells sorted based on expression of a particular gene or cell surface protein (using fluorescent reporters or fluorescent antibodies against cell surface proteins, respectively, and sorting using a flow cytometer or magnetic beads; alternatively, immunolabeling or fluorescent in situ hybridization and laser capture microdissection could be used). In all of these approaches, the cells are grouped, providing only a zoomed-out snapshot of what is happening. Bulk sequencing of astrocytes has limited our understanding to the transcriptome changes within a particular brain region in cells that express only one specific gene. With single-cell sequencing, each cell is barcoded and individually sequenced, providing a comprehensive overview of the molecular identity of that individual cell. Thus, we now have a more comprehensive understanding of what makes an astrocyte an astrocyte, and we are finding out that using a single specific gene to target astrocytes has its limitations. For example, in the case of the male mouse medial posterodorsal amygdala, a brain region involved in inter-male aggression, not all astrocytes express the gene for glial fibrillary acidic protein (GFAP), a traditional marker of mature astrocytes (Wu et al. 2017).

Box 10.2 Landmark discovery: Hypothalamic neuroprogesterone synthesis as a key component of estrogen positive feedback

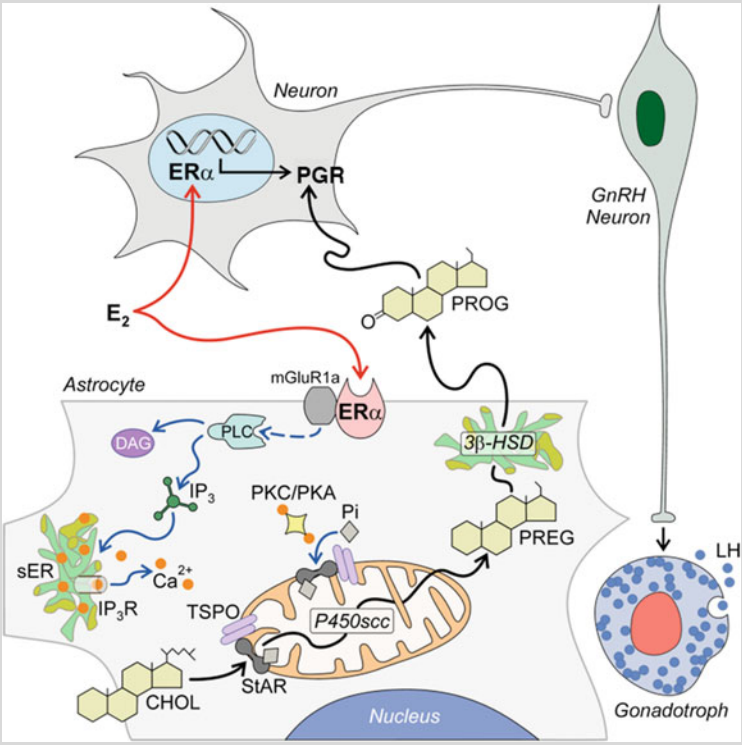
The importance of progesterone signaling in estrogen positive feedback has been known for decades (Chappell and Levine 2000). In 2003, a landmark discovery was made showing that *the brain* was the source of progesterone required for the LH surge. Blocking progesterone synthesis with a 3β-HSD inhibitor prevented the LH surge in ovariectomized/adrenalectomized (ADX) female rats. When another set of gonadectomized/ADX rats were given the same amount of estradiol that induced the surge, neuroprogesterone synthesis

(continued)

Box 10.2 (continued)
was increased in the female hypothalamus, but not in males. In other words, ovarian estradiol induces neuroprogesterone synthesis to help stimulate kisspeptin neurons, leading to the GnRH/LH surge. Neuroprogesterone is also involved in normal estrous cyclicity: blocking *central* steroidogenesis caused ovary-intact rats to remain in constant estrous. Through more than a decade of work using primary glia cell culture (>95% astrocytes), the cellular mechanisms controlling neuroprogesterone synthesis by hypothalamic astrocytes have been elucidated. As shown in the figure, ovarian estradiol (E_2) acts at the nucleus in kisspeptin neurons to upregulate estrogen receptor-alpha expression, whereas it has more rapid effects to stimulate neuroprogesterone synthesis by binding to membrane-associated ERα. By binding to the membrane ERα/mGluR1a complex, estradiol activates the PLC pathway, leading to increased intracellular calcium and activation of PKA. PKA signaling causes the phosphorylation of steroidogenic acute regulatory protein (StAR) and translocator protein (TSPO), which shuttle cholesterol into the inner mitochondrial membrane, where P450scc (CYP11A1) enzyme converts it to pregnenolone. The enzyme 3β-HSD converts pregnenolone to progesterone, which binds to PGR in nearby kisspeptin neurons, including at the cellular membrane. Kisspeptin neurons need both estrogen and progesterone stimulation to activate GnRH neurons and induce a surge of LH secretion from the anterior pituitary, which then circulates to the ovaries to initiate ovulation.

(continued)

Box 10.2 (continued)

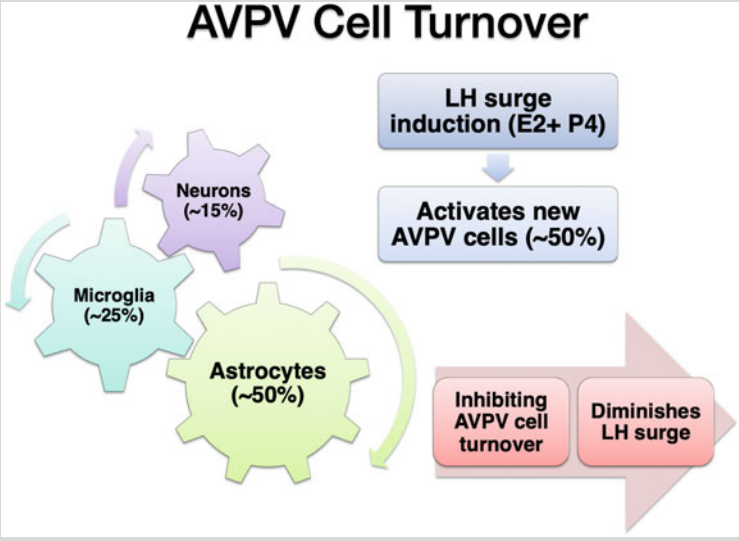


Box 10.3 Cell turnover in the AVPV: clue to the maintenance of estrogen positive feedback?

Neuro- and glio-genesis occur in sexually dimorphic brain regions during puberty in a sexually dimorphic manner: in male-biased regions, males have more pubertally born cells, whereas in the AVPV, a female-biased region, females have more pubertally born cells. Gonadal hormones drive these sex differences in cell generation. The majority of the newborn cells in AVPV are glia; nearly half of the newborn cells are astrocytes. Importantly, these cells show signs of increased activity during an estradiol- and progesterone-induced LH surge (i.e., newborn cells express Fos, an immediate early gene protein marker of activity in the brain). Most importantly, and what demonstrates that the newborn AVPV cells have a functional significance, the inhibition of mitosis in these cells diminishes the LH surge. In other words, without

(continued)

Box 10.3 (continued)
newborn AVPV cells, the majority of which are astrocytes, estrogen positive feedback is defective. Thus, the addition of new cells to sexually dimorphic brain regions during puberty helps shape functional sex differences (Ahmed et al. 2008; Mohr et al. 2016, 2017).



10.8 Key Literature

Cashion et al. (2003) Key paper that demonstrated the rhythmic and plastic nature of the astrocytic coverage of GnRH neurons underlying the astrocytic control of synaptic inputs over the estrous cycle.

Chaban et al. (2004) This paper was one of the first to show that estrogen causes rapid intracellular signaling in astrocytes through a membrane-initiated mechanism.

Kuo et al. (2009) This study elucidated the mechanism of intracellular calcium mobilization by activation of a membrane ERα/metabotropic glutamate receptor type 1a in hypothalamic astrocytes.

Micevych et al. (2003) Landmark study showing that estrogen-facilitated hypothalamic progesterone synthesis is a prerequisite for the LH surge and a necessary component, therefore, of the signaling that leads to ovulation in females.

Micevych et al. (2007) A follow-up to the Micevych et al. (2003) study that demonstrated that hypothalamic astrocytes are the source of neuroprogesterone that is synthesized by a rapid estradiol-mediated increase in intracellular calcium in post-pubertal females.

Mohr et al. (2017) Seminal paper demonstrating that newborn brain cells, predominantly astrocytes, are required for the estrogen positive feedback signal that triggers the LH surge.

Mohr et al. (2019) This study confirmed that the hypothalamus is incapable of responding to estradiol with neuroP release prior to pubertal maturation, an observation orignally made using primary hypothalamic astrocytes.

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Part VI

Glial–Neuronal Interactions that Link Brain and Periphery

Unveiling the Importance of Tanycytes in the Control of the Dialogue Between the Brain and the Periphery

11

Sreekala Nampoothiri, Manon Duquenne, and Vincent Prevot

Abstract

Tanycytes, a specialized type of ependymoglial cell, modulate an interesting cycle of communication between the brain and the periphery for energy homeostasis and reproduction. While most circulating factors do not enter the brain because of the blood–brain barrier (BBB), homeostatic signals from the periphery converge on the hypothalamus—a brain region influencing feeding and energy expenditure—through a privileged route that bypasses the brain barrier. Research on the why's and the wherefore's of this peculiar brain–periphery communication has made substantial progress in the recent past. One of the compelling revelations has been the ability of tanycytes to adapt their physiology based on the metabolic status of an individual—a key to understanding how tanycytes regulate the transfer of peripheral metabolic hormones into the brain and communicate reciprocally with brain neuronal networks for the regulation of food intake and energy homeostasis. It has been shown that tanycytes adapt by altering the functional and structural organization of the blood–hypothalamus barrier under different metabolic conditions. Moreover, attempts have been made to decode the plasticity and the diversity of tanycytes by morphological and transcriptomic analyses, primarily at the single-cell level. Tanycytes also possess neural stem cell properties as a result of their putative descent from radial glial cells, and may promote hypothalamic neurogenesis in response to dietary or reproductive signals. Thus, tanycytes form the gatekeepers of metabolic signals connecting the loop of behavior, hormonal changes, signal transduction, and neuronal activation. In this chapter, we assess recent advances in the understanding of

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tanycytic plasticity and function in the hypothalamus and the associated molecular mechanisms, with special emphasis on the techniques and experimental models implemented to achieve these objectives.

Keywords

Tanycytes · Plasticity · Energy homeostasis · Blood–brain barrier · Brain · Periphery · Leptin · Hormones · Single-cell · Neural stem cell · Hypothalamus · Dietary · Reproductive · Neurons

11.1 Structural Organization of Tanycytes in Direct Brain–Periphery Communication

Tanycytes have been traditionally classified into four subtypes (α1, α2, β1, and β2) based on their dorsoventral position along the third ventricle (Prevot et al. 2018) (Box 11.1). Depending on the hypothalamic structure adjacent to the ventricle harboring tanycytes, the wall of the third ventricle can be divided into the median eminence (ME), the arcuate nucleus (ARH), the ventromedial nucleus (VMH), and the dorsomedial nucleus (DMH) of the hypothalamus, respectively (Fig. 11.1). Interestingly, the ME, a circumventricular organ (CVO) with fenestrated endothelial capillaries, permits the passage of polypeptide hypothalamic hormones to trigger changes in brain function without disrupting the BBB. The ARH is a major sensing site for circulating metabolic signals and is positioned next to the ME, which is ideally composed to convey these signals to the ARH neurons. The cell bodies of tanycytes that line the floor of the third ventricle extend processes toward the external zone of the ME, where the tanycyte endfeet contact the vascular wall of the pituitary portal blood system to facilitate tight interactions with the neurosecretory terminals. Hence, tanycytes sense the nutrient status of the organism and integrate signals from peripheral hormones, including adipocyte-derived leptin and gut-derived ghrelin, to regulate calorie intake, glucose metabolism, and energy expenditure. Any interruption in the passage of these signals to ARH neurons results in metabolic disorders such as obesity (see for review Prevot et al. 2018).

However, the current scientific consensus suggests that tanycytes possess distinct genetic, morphological, and functional properties that depend on their spatial distribution and intercellular interactions and influence brain–periphery communication. At the outset, tanycytes can be divided according to their ability to contact fenestrated vessels, or BBB capillaries, via their endfeet (Fig. 11.1). While the first population is mainly contained in the ME and overlaps with the currently classified β-tanycytes, the second mostly lies along the lateral walls of the third ventricle and corresponds to α-tanycytes. Furthermore, tanycytes in contact with ME capillary loops in the ventral ARH tend to oscillate between two phenotypes based on the permeability of the capillary endothelium, which, in turn, is dependent on the energy status of the individual (Langlet et al. 2013). Uniciliated tanycytes in the floor and biciliated tanycytes in the lateral wall of the third ventricle present another degree of

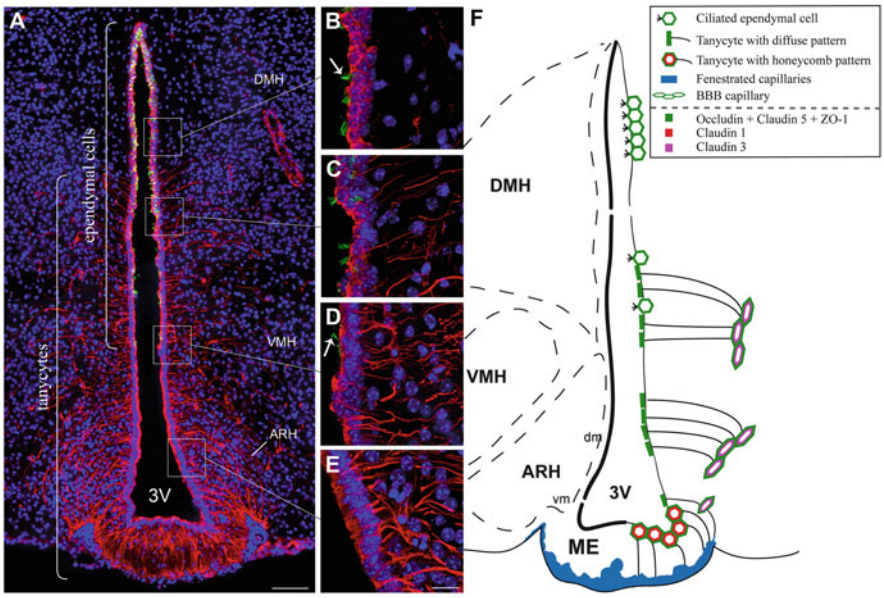

Fig. 11.1 Structural organization of tanycytes and ependymocytes in the 3rd ventricle region of the hypothalamus. (**a**) Representative immunofluorescence image of glu-tubulin (green) and vimentin (red) along the 3rd ventricle at a low magnification. (**b**, **c**) Immunoreactivity of glu-tubulin demonstrating cilia (green, arrows) along the dorsomedial nucleus of the hypothalamus (DMH) and (**d**) ventromedial nucleus of the hypothalamus (VMH). No glu-tubulin immunoreactivity is seen in vimentin-labeled tanycytes of the (**a**) median eminence (ME) or (**e**) arcuate nucleus of the hypothalamus (ARH). Sections are counterstained with Hoechst (blue) to visualize cell nuclei and determine the morphological boundaries of each hypothalamic structure. Scale bars: (**a**) 100 μm; (**b**–**e**) 20 μm. (**f**) Schematic representation of the tight junction protein distribution in the 3rd ventricle of the hypothalamus (Prevot et al. 2018). *ME* median eminence, *ARH* arcuate nucleus of the hypothalamus, *dm* dorsomedial, *vm* ventromedial. Adapted with permission from Prevot et al. (2018)

diversity in comparison with the adjacent cuboidal ependymal cells with tufts of motile cilia driving the flow of cerebrospinal fluid (CSF) (Conductier et al. 2013; Mirzadeh et al. 2017).

Similar to astrocytes, tanycytes interact with neuroendocrine and non-neuroendocrine neural networks (Prevot et al. 2018). For instance, β1-tanycytes specifically interact with the neuroendocrine axons of the gonadotropin-releasing hormone (GnRH) neurons that control the hypothalamic-pituitary-gonadal (HPG) axis, while β2-tanycytes interact with other neuroendocrine neuronal populations, including thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone neurons of the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary-adrenal (HPA) axis, respectively. Although inferential and speculative evidence on tanycytes has been presented in abundance, our understanding of their precise nature and function is far from fulfillment.

11.2 Plasticity of Tanycytes Regulates the Transport of Circulating Factors Across the Blood–Hypothalamus Barrier

Tanycytes adapt their physiology based on their location, the cells they interact with, and the organism's metabolic status, which consequently alter the functional and structural organization of the blood–hypothalamus barrier at the ME-ARH. In light of this, tanycytes along the floor and lateral walls of the infundibular recess have gained particular attention due to the intriguing morphological link of the CSF with the pars tuberalis and the ME vasculature, suggesting the plausible involvement of tanycytes in the transport of circulating factors across the blood–hypothalamus barrier (BHB), as well as in the neuroendocrine control of pituitary function.

11.2.1 Differential Distribution of Tight Junction Proteins Defines the Physical Barrier Between Tanycytes and the CSF

It has been shown that circulating molecules of a size less than or equal to 40 kDa can extravasate freely and rapidly through the fenestrated microvessels of the ME *in vivo*. Electron microscopy and immunofluorescence studies show that capillary fenestration is restricted to the ME, and tight junction protein complexes around tanycytic cell bodies form a physical barrier at the ventricular wall. Hence, the transported molecules are sequestered within the ME, preventing their diffusion from the CSF to other brain regions.

Box 11.1 Early morphological observations leading to the classification of tanycytes and beyond

Though Santiago Ramón y Cajal found cells resembling embryonic radial glial cells representative of tanycytes as early as 1909, there was little consensus on the terminology of tanycytes until Horstmann (1954) coined the term for elongated ependymal cells stained by Cajal's gold sublimate method. Later, several studies employed Golgi's impregnation method to observe morphological differences along the entire length of elongated tanycytes, which prompted scientists to divide them into several categories. A great wealth of information regarding tanycyte morphology has emerged from ultrastructural electron microscopic studies. Immunogold labeling, which is very beneficial in the localization of target markers in cells and tissues, was used for ultrastructural visualization of tanycytes, providing an excellent insight into structural and functional connections within the tissue or cellular microenvironment.

In 1973, Akmayev et al. divided tanycytes into α- (lining the inferolateral walls of the third ventricle) and β-tanycytes (lining the floor of the third ventricle close to the ME) on grounds of morphological, topographical, and

(continued)

Box 11.1 (continued)
metabolic criteria by enzyme-histochemical and histoautoradiography analysis of deafferented rat MBH. Tanycytes were found to form an integral part of the ependyma of the floor and the ventral part of the third ventricle and were further categorized into subgroups: (1) α1 tanycytes facing the ventromedial nucleus; (2) α2 tanycytes projecting toward the arcuate nucleus; (3) β1-tanycytes in the lateral extensions of the infundibular recess, and (4) β2-tanycytes lining the floor of the third ventricle close to the ME. Though the arbitrary nature of this classification has been recognized since its inception, it has been in use ever since to describe tanycyte morphology. Recent studies have raised concerns and probed into the exigent need for a more stringent classification of tanycyte subpopulations.

Tight junctions represent a structural barrier with molecular complexes in the epithelial and endothelial sheets (Figs. 11.1b, 11.2). This barrier checks the free movement of ions and molecules while maintaining adhesion between the adjacent cells. The tight junction protein complex is composed of the transmembrane and membrane-associated constitutive tight junction proteins zonula occludens 1 (ZO1), occludin, and claudins, which give tanycytes their polarity to generate spatial cues essential for the transport of molecules along the basal (endfeet)-apical (cell body) axis, but also likely along the converse route. Both claudin 3 and 5, the other members of the claudin transmembrane protein family, are readily expressed in BBB microvessels, while tanycytes of the ME have been found to express only claudin 5. Hence, tight junction molecular complexes are well organized in the form of a continuous belt around the cell bodies of the tanycytes lining the ventral part of the third ventricle. On the contrary, ARH tanycytes exhibit a disorganized expression pattern of occludin, zonula occludens-1 (ZO-1) and claudin-5, and are characterized by the absence of claudin 1 expression. Interestingly, claudin-1 is specifically expressed in tanycytes contacting fenestrated vessels, but not BBB vessels. The peripheral and central injections of Evans blue dye corroborates that functional tight junctions characterize only tanycytes of the ME, whereas tanycytes at the ARH form a permeable layer (see for review Prevot et al. 2018).

11.2.2 Diet-Induced Changes in Tanycyte Plasticity

Recent findings demonstrate that the structural organization of tanycytes and the tight junction complex organization are dependent on the nutritional status of an individual (Langlet et al. 2013) (Fig. 11.2). Fasting for 24 h strikingly augments the fenestration of ME capillary loops within the ME and the ventromedial (vm) ARH, but not in the ARH BBB vessels. Moreover, a concomitant rise in the organization of tanycytic tight junction complexes in both the ME and the vmARH, together with the expression of claudin 1 by all vmARH tanycytes, prevents the access of blood-

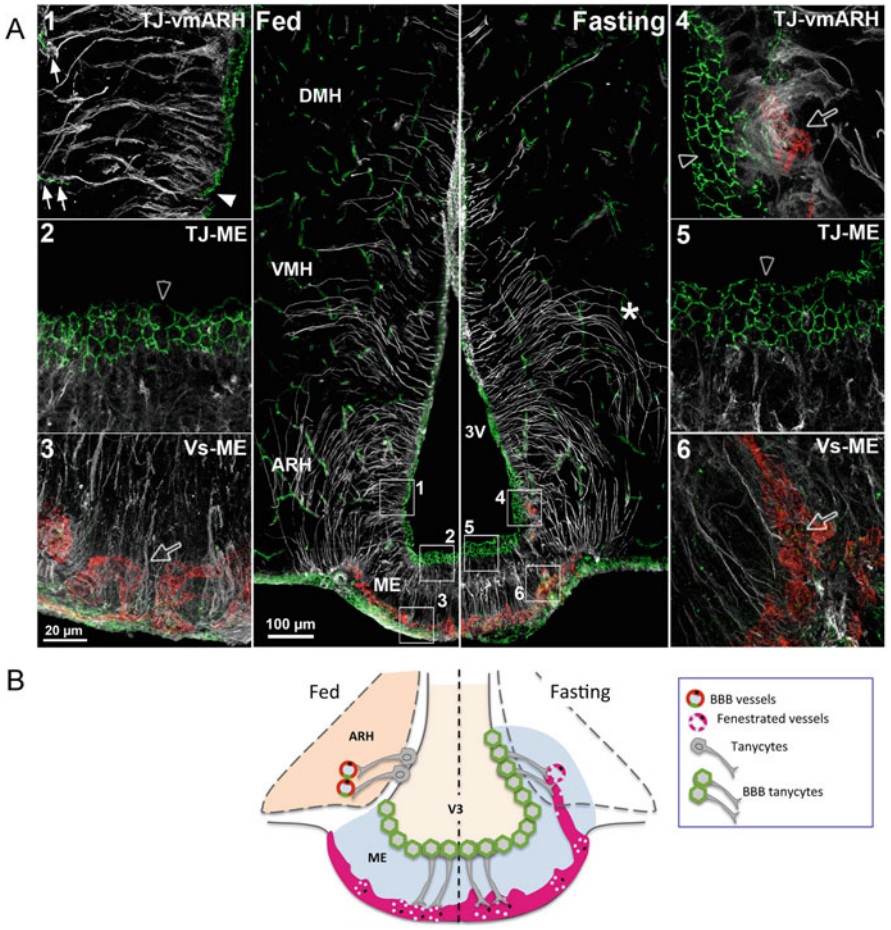

Fig. 11.2 Diet-induced changes in tanycyte plasticity. (**a**) Immunoreactivity of vimentin (white), zonula occludens-1 (ZO-1, green), and plasmalemmal vesicle-associated protein 1 (PV1) (red) in coronal sections of the hypothalamic 3rd ventricle region in fed and fasting mice. Fasting induces fenestration in the median eminence (ME) microvessel loops that reach the ventromedial arcuate nucleus (vmARH) and affect tight junction complex reorganization in ARH tanycytes. The tight junction (TJ) complexes interacting with ZO-1^{+} blood vessels exhibit a diffuse pattern (arrowhead, inset 1), while the TJ complexes vessels display a honeycomb pattern (empty arrowheads, insets 2, 4, and 5) when interacting with PV1 (clone MECA-32)-immunoreactive vessels (empty arrows, insets 3, 4, and 6) [adapted with permission from Langlet et al. 2013]. (**b**) Schematic representation of the structural changes in the tanycytic barrier in response to the direct access of blood-borne molecules to the vmARH. Reproduced from Prevot et al. 2018, with permission. TJ, tight junction; Vs, vessels

borne molecules extravasating from the newly permeable vessels to the CSF. This phenomenon is, however, reversed upon refeeding. The transient rise or dip in the blood glucose level during the fed or fasted state is perceived by tanycytes by virtue

of their glucose-sensing properties. While most circulating factors, with exceptions such as glucose that use specific transporters, do not cross the BBB to access the brain, specific hypothalamic nuclei that are vital for the control of food intake and body weight, such as the ARH, gain direct access to circulating homeostatic factors by a privileged route that bypasses brain barriers. Though the ability of critical metabolic substrates, such as circulating glucose, leptin, and ghrelin, to enter the ARH and modify neuronal circuits to meet physiological demands in response to changes in homeostatic status is just beginning to be explored, it represents a key question in order to advance our knowledge regarding mechanisms pertaining to feeding and energy metabolism, as well as their coordination with fertility.

11.3 Monitoring Tanycyte-Mediated Shuttling of Homeostatic Signals Between the Brain and the Periphery

Structural alterations to the blood–CSF barrier control the direct access of circulating factors to the vmARH in the wake of acute nutritional challenges. The shuttle of these homeostatic signals depends on the expression of tight junction proteins by the ME/vmARH tanycytes, which not only precludes the diffusion of solutes via the paracellular cleft but also regulates cellular polarity and intracellular trafficking. This role of ME/vmARH tanycytes in conveying blood-borne molecules into the CSF by transcytosis has long been suspected (Anand Kumar and Knowles 1967). However, the precise mechanism as to how these homeostatic signals gain access to deep hypothalamic neurons to modulate energy balance is still under investigation.

11.3.1 Tanycyte-Mediated Transport of Leptin and Other Peripheral Signals Across the Blood–CSF Barrier

Since the control of many physiological functions requires continued dialogue between the periphery and the hypothalamus, studying the access of peripheral signals to the hypothalamus is essential for understanding the proper functioning of implicated neural circuits. Leptin is a 16 kDa hormone that is mainly secreted by adipose tissue in proportion to the fat mass and signals satiety in the brain. Obesity is associated with functional leptin resistance, where the actions of this hormone are dysregulated and fail to signal food intake status. Thus, several studies have focused on understanding leptin transport between the blood and the brain under different metabolic conditions (Di Spiezio et al. 2018; Kleinert et al. 2018; Faouzi et al. 2007; Balland et al. 2014; Yoo et al. 2019; Harrison et al. 2019). The choroid plexus appears to be a likely target for leptin transport to the brain due to its high expression of leptin receptor (LepR). A selective decrease in LepR expression in vessels and the choroid plexus was shown to increase body weight because of a hypersensitivity to reward (Di Spiezio et al. 2018). In mice, leptin transport through the BBB appears to be unaffected either at a short (20 days) (Kleinert et al. 2018) or long (20 weeks) (Harrison et al. 2019) period after the initiation of a high-fat diet regime. These

findings, which are in contrast to what has been observed in overweight and obese humans (Caro et al. 1996; Schwartz et al. 1996), seem to limit the influence of altered blood-borne leptin transport into the brain in the development of leptin resistance associated with pathologies, such as obesity and diabetes. However, it has been shown in minipigs that the transport of circulating leptin into the CSF is altered as early as four weeks after initiation of a high-fat diet (Chmielewski et al. 2019), a phenomenon that also occurs in mice following eight weeks of high-fat diet (Balland et al. 2014).

Interestingly, when leptin is administered peripherally in a wild-type mouse, it activates leptin-sensitive hypothalamic neurons along a ventrodorsal gradient over time (Faouzi et al. 2007). Thus, it could be hypothesized that leptin extravasates from the fenestrated vessels and is taken up by ME tanycytes to reach hypothalamic targets. In vitro and in vivo studies show that leptin is captured by tanycytes and shuttled by transcytosis via clathrin-coated vesicles before being released into the CSF. This process might be dependent on LepR due to the rapid activation of phospho-signal transducer and activator of transcription 3 (pSTAT3) after peripheral leptin injection, while injection of a point-mutated leptin does not produce the same effect. After eight weeks of a high-fat diet regime, tanycyte-mediated transport of leptin is blunted and limits the access of this hormone's satiety signal to the mediobasal hypothalamus. However, chronic activation of the ERK signaling pathway in tanycytes restores leptin transport and stimulates mediobasal hypothalamus neuron activation, demonstrating tanycyte-mediated transport of leptin (Balland et al. 2014) (Fig. 11.3). Nevertheless, a recent study presents controversial findings suggesting a lack of LepR expression in tanycytes, which would imply that tanycyte-

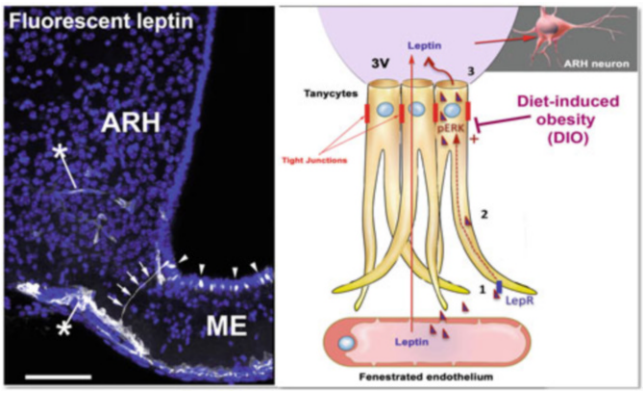

Fig. 11.3 Transport of leptin from the periphery into the CSF via the ME. Left: Representative image displaying tanycytic processes (as indicated by arrows) and cell bodies (arrowheads) labeled with fluorescent bioactive leptin injected intravenously (25 nmoles per animal; white label) in wild-type mice (asterisks indicate the BBB vessels of the ARH). Nuclei of cells are counterstained with Hoechst. Scale bar: 50 μm Right: Schematic illustration of the passage of leptin from fenestrated pituitary portal blood vessels to the CSF of the 3rd ventricle as well as hypothalamic neurons expressing leptin receptor (LepR) Reproduced from Prevot et al. 2018, with permission

mediated transport of circulating leptin is independent of LepR (Yoo et al. 2019). However, the low level of expression of LepR in tanycytes possibly led to this inference. Highlighting tanycytes as a shuttle for peripheral hormones, including leptin and ghrelin (Prevot et al. 2018), has given rise to a growing interest in the study of production and transport of these signals by tanycytes. A recent study shows that in response to fasting, tanycytes produce fibroblast growth factor 21 (FGF21), which maintains body lipid homeostasis (Geller et al. 2019). Whether they can also transport the blood-borne FGF21 released by the liver into the brain remains unexplored. Finally, pharmacological compounds, such as the GLP1 receptor agonist Semaglutide, represent promising candidates that may cross these glial cells to signal in the hypothalamus (Gabery et al. 2020).

11.3.2 Strategies to Monitor and Study Tanycyte-Mediated Transport of Metabolic Signals

11.3.2.1 Characterization of Tanycytes by Neuroanatomical Methods

When studying hypothesis-driven mechanisms, the first step is to identify whether the candidate factors and their cognate receptors are expressed in the region of interest. This is usually accomplished by using classical neuroanatomical approaches such as immunohistochemistry (IHC) or *in situ* hybridization (ISH). These methods generally involve: (i) preparation of the brain; (ii) cutting sections of the brain; (iii) labeling and staining of the sections: (iv) mounting the sections onto microscopic slides; and (v) microscopic analysis.

Immunohistochemistry (IHC)

The morphological characteristics of tanycytes have been confirmed and extended by immunohistochemical studies, revealing differences between tanycyte subtypes and their functions. Immunohistochemistry involves the application of specific antibodies to visualize the exact position and distribution of the protein of interest in a given tissue while retaining the tissue's microstructure. Initial studies on rats presenting anti-glial fibrillary acidic protein-peroxidase-anti peroxidase (anti-GFAP-PAP) staining of ME established a connection between tanycytes on the floor of the third ventricle and the portal vessels (see for review Rodríguez et al. 2005). However, GFAP is not readily expressed in tanycytes of all species, including mice and humans (Prevot et al. 2018). In contrast, vimentin and nestin, two other intermediary cytoskeletal proteins, are considered reliable tanycyte markers in the adult brain across species (Pellegrino et al. 2018). Fluorescence IHC applied to the tuberal region of the hypothalamus has demonstrated that DARPP-32 is exclusively expressed in tanycytes, while GFAP is largely seen in astrocytes of the internal layer of the ME, beneath the tanycyte cell bodies, as well as in other hypothalamic nuclei (Meister et al. 1988). Only a few rare tanycytes express GFAP (Meister et al. 1988). Electron-microscopic IHC has revealed that glucose transporter-1 (Glut1), a BBB marker, is expressed in α1-, α2-, and β1-tanycytes, but not in β2-tanycytes. A similar electron microscopic analysis using colloidal gold labeling has demonstrated

that, unlike β2-tanycytes, β1-tanycytes express insulin-like growth factor-binding protein. Somatostatin 2 (sst 2) variant sst2(a) protein receptor generates a strong immunoreaction in α tanycytes, with moderate reactivity in β-tanycytes. Immunostaining with molecular markers of endocytosis and transcytosis processes (clathrin, caveolin-1, Rab4, and ARF6) also demonstrates a marked distinction between the four tanycyte subtypes. β2-tanycytes express caveolin-1 at the apical pole of the cell as well as in the endfeet in contact with the portal capillaries, while β1-tanycytes express caveolin-1 only at the endfeet. However, α1 and α2 tanycytes do not express the caveolin-1 protein. On the other hand, α1- and α2-tanycytes express clathrin protein in the whole basal process, including in their perivascular endings, with restricted or region-specific expression in β1-and β2-tanycytes. Rab4, a protein involved in endocytosis and transport of synthesized proteins, is highly expressed in the basal processes of β2-tanycytes and the ependymal cells of the choroid plexus, with little or no immunoreactivity in α1-, α2-, and β1-tanycytes and no Rab4 immunoreactivity in ciliated ependymal cells (see for review Rodríguez et al. 2005).

Immunohistochemistry for junction proteins and plasmalemmal vesicle-associated protein 1 (PV1) expressed in fenestrated microvessels also reveals differences among tanycyte subtypes and their role in brain–periphery communication. Immunoreactivity for vimentin, ZO-1, and PV1 (using a rat monoclonal antibody to a mouse-specific endothelial 50–60 kDa antigen, clone MECA-32) in coronal sections of the hypothalamic tuberal region in fed and fasted mice has shown that tanycytic tight junction complexes exhibit a diffuse pattern when interacting with ZO-1-positive blood vessels, and a honeycomb pattern when interacting with MECA-32-positive vessels (Fig. 11.2a) (Langlet et al. 2013). β1- and β2-tanycytes are strongly immunoreactive for α-catenin throughout the cellular processes and endings, while α2 tanycytes are weakly or non-reactive and α1-tanycytes are only reactive at the ventricular pole of the cell. Only the processes and endings of β1-tanycytes are immunoreactive for N-cadherin (Rodríguez et al. 2005). Though immunostaining studies have provided useful insights into the morphology of tanycytes, it is worth noting that the common cytoskeletal markers used to identify glial cells (GFAP, vimentin, and nestin), which are predominantly enriched in the cell body and major processes of glia, may not provide an appropriate morphological representation of tanycytes. Their protein expression may also vary under different physiological conditions, leading to inconclusive inferences. These limitations may be overcome by using tracer molecules to monitor the transport of circulating factors directly (Sect. 11.3.2.2) or by using cre-inducible reporter mice to target tanycytes selectively (Sect. 11.7.3).

More recent immunofluorescence and electron microscopy analyses intriguingly suggest that subpopulations of tanycytes may express gap junctions, as suggested by the presence of connexin-43 (Cx43) immunoreactivity (Szilvásy-Szabó et al. 2017; Recabal et al. 2018). Cx43-immunoreactivity appears to be much more abundant in α tanycytes than in ME and vmARH tanycytes (β), suggesting that tanycytes that compose the wall of the third ventricle and that contact BBB microvessels may be endowed with functional gap junctional coupling.

In Situ Hybridization (ISH)

In addition to conventional immunostaining, non-isotopic ISH with RNA probes has been used to label mRNA in specific populations of tanycytes. Labeling of tanycyte mRNA with RNA probes for glutamate/aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1) showed that GLT-1 is strongly expressed by α tanycytes situated in the dorsolateral walls of caudal tuberal and mammillary recess portions of the third ventricle, whereas GLAST is expressed by β-tanycytes of the ventral floor and lateral walls in the tuberal and mammillary recess portions of the third ventricle (see for review Prevot et al. 2018; Goodman and Hajihosseini 2015). RNAscope®, a novel RNA ISH technology, promotes molecular detection of target RNA within intact cells. This technology, with its proprietary probe design, allows the amplification of target-specific signals as well as the validation of the site of expression of novel transcriptomic biomarkers. A recent study detected robust Rax mRNA expression in tanycytes and used it as a marker to distinguish LepR expression in tanycytes from that in other cell types, including neurons, astrocytes, and endothelial cells (Yoo et al. 2019).

11.3.2.2 Tracers

The intracerebroventricular or peripheral administration of tracer molecules and fluorescently labeled bioactive substances has been very helpful in understanding the communication between tanycytes in the ME milieu and the perivascular space of the portal vessels, as well as in the subarachnoid CSF. Early studies demonstrating the barrier between ME and ventricular CSF used horseradish peroxidase (HRP) as a tracer. When injected into the ventricular CSF, HRP enters the hypothalamus but does not cross the ME owing to the tight tanycyte layer. Later, the functionality of the tight junctions joining ME tanycytes at their apices was demonstrated by the fact that molecules as small as 1 kDa, such as Evans blue dye, cannot diffuse through the cellular sheet, irrespective of the site of injection (i.e., peripheral, into the blood compartment, or central, in the cerebral ventricles) (Langlet et al. 2013). However, the paracellular diffusion of the dye to the ARH through dmARH tanycytes is indicative of the absence of a tight physical barrier between the dmARH and the CSF (Prevot et al. 2018; Rodríguez et al. 2005).

Another method that allows the study of putative state-dependent morphological plasticity of tanycytes is the injection of a tracer dye via a patch pipette into individual tanycytes during patch-clamp recordings in living brain slices (Szilvásy-Szabó et al. 2017; Recabal et al. 2018). Lucifer Yellow and biocytin are the two most often used tracers. However, they differ in their gap junction permeability due to differences in their charge. Lucifer yellow is highly anionic or negatively charged, whereas biocytin is a positively charged fluorescent dye. The difference in the net charge of the tracers is a useful parameter for studies concerning the cell–cell coupling network.

11.3.2.3 Microdialysis

Microdialysis enables sampling and collection of low-molecular-weight substances from the interstitial space of the ventricles in the brain, allowing the measurement of

neurotransmitters, peptides, and hormones in animals. A probe with a semi-permeable membrane is introduced into the brain, and samples can be collected and analyzed after their diffusion and equilibration with the extracellular medium (Chefer et al. 2009). Microdialysis cannulae were stereotaxically implanted for the simultaneous measurement of ARH and VMH glucose levels in fed and fasting rats to demonstrate how BHB plasticity modulates the access of blood-borne metabolic factors to the ARH (Langlet et al. 2013). The flux in the glucose concentration flowing into and out of the probe suggested that fasting-induced structural changes at the BHB open a privileged route by which circulating glucose reaches glucose-sensing ARH neurons, bypassing BBB as well as the blood–CSF barrier.

Microdialysis is also a promising tool for assessment of the transport of circulating polypeptide hormones into the CSF, both in anesthetized and freely moving rodents (Kleinert et al. 2018). This type of information is usually accessible only in bigger pre-clinical animal models, such as minipigs (Chmielewski et al. 2019) or humans via lumbar puncture (Caro et al. 1996; Schwartz et al. 1996).

11.4 Comprehending the Interface Between Tanycytes and Neurosecretory Axons

The ME acts as an endpoint of the neurosecretory axons of main neuroendocrine axes such as the HPG, HPT, and HPA axes. The axons of GnRH neurons controlling fertility and gonadal hormone production, as well as TRH neurons controlling the thyroid axis, are closely apposed and ensheathed by tanycyte endfeet in the ME.

11.4.1 Structural Remodeling of Tanycytes Based on GnRH Levels

The ovarian cycle affects the structural plasticity at the interface of ME tanycytes and neurosecretory GnRH nerve terminals, which in turn controls the access of GnRH neurons to the portal vasculature (see for review Prevot et al. 2018; Chachlaki and Prevot 2020). During the diestrus stage with low gonadotropin levels, tanycytes ensheath GnRH-secreting nerve terminals, which creates a diffusion barrier that prevents the access of GnRH to the blood vessels and hampers its release into the pituitary portal circulation. On the other hand, the preovulatory stage of proestrus causes a structural remodeling of tanycytes, where tanycytes no longer surround the GnRH axons and allow direct contact between neurosecretory GnRH axons and the basal lamina of pituitary vessels. These changes are mediated by the release of the chemotrophic factor semaphorin 3A (Sema3A) and its action on its receptor neuropilin 1 on GnRH neurons. Immunopanning methods used to purify vascular endothelial cells of the ME and experiments coculturing these cells with isolated tanycytes have demonstrated that fenestrated ME endothelial cells promote acute reorganization of the actin cytoskeleton in tanycytes by releasing the highly diffusible and labile transmitter nitric oxide (NO). Given this observation, preincubation of ME for 30 min with NO, prostaglandin E2 (PGE2), or the NO precursor L-arginine

induces movement and reconfiguration of tanycytes, resulting in an enhanced GnRH release. On the other hand, local infusion of inhibitors of nitric oxide synthase (NOS) or cyclooxygenase (COX, an enzyme involved in prostaglandin synthesis) into the median eminence arrests the ovarian cycle either in the diestrus or estrus phase when GnRH release is low and GnRH neuroendocrine terminals are enclosed by tanycyte endfeet. Furthermore, studies aimed at understanding the effect on the GnRH nerve terminal microenvironment of aging, a physiological condition where both GnRH release and the responsiveness of the GnRH neural network to estrogens are diminished, suggest that alterations of the relationship between neuroendocrine terminals and tanycyte processes may contribute to the senescence of the HPG axis.

11.4.2 Tanycytes and the Control of Thyrotropin-Releasing Hormone

The production of thyroid hormones (triiodothyronine or T3 and L-thyroxine or T4) is regulated by crosstalk between the hypothalamus, pituitary, and thyroid gland, initiated by the hypothalamic release of TRH. Tanycytes seem to play a critical role in this regulation due to their participation in several mechanisms. The first one is based on the tanycytic expression of two thyroid hormone transporters, MCT8 and OATP1C1, allowing these glial cells to take up T4 from the circulation or CSF. T4 can then be converted to active T3 by deiodinase 2 enzyme (Dio2), which is expressed in tanycytes across species (see for review (Prevot et al. 2018)). T3 is important for negative feedback on neural TRH production. Retrograde transport of T3 from TRH nerve terminals in the median eminence to TRH cell bodies in the paraventricular nucleus of the hypothalamus inhibits TRH transcription (Fekete and Lechan 2014). β2-tanycyte endfeet are in direct contact with TRH neuronal terminals in the external layer of the ME, creating a microenvironment conducive to exchanges and interactions between TRH neurons and tanycytes. Tanycytes highly express TRH-degrading ectoenzyme (Trhde), suggesting that tanycytes can impact TRH bioavailability before its secretion into the blood circulation. Elevated intracellular calcium levels through the Gαq/11-coupled pathway in ME tanycytes promote the outgrowth of tanycytic processes ensheathing TRH neuroendocrine terminals and an upregulation of Trhde activity (Müller-Fielitz and Schwaninger 2019). Recently, Fekete and colleagues (Farkas et al. 2020) also demonstrated that tanycytes can produce endocannabinoids such as 2-arachinodonoylglycerol (2-AG), and modulation of endocannabinoid production in ME explants can regulate TRH secretion. In return, TRH neurons may also secrete glutamate that acts on tanycytes via AMPA and kainate receptors. Together, these results suggest the existence of a microcircuit between β2 tanycytes and hypophysiotropic cannabinoid receptor 1-expressing TRH neurons that could be implicated in the regulation of TRH release (see Chap. 12 for a detailed description of tanycyte–TRH neuron interactions).

11.5 Transcriptional Reprogramming and Tanycyte-Mediated Energy Homeostasis

The efficient switch between anabolic and catabolic states to maintain energy homeostasis determines physiological robustness. The aberrant expression of genes in response to varying metabolic states (such as the feed-fast cycle) may be associated with the onset of metabolic or aging-associated diseases such as obesity and diabetes, severely affecting organismal survival. Though these studies are in their infancy, the plasticity of tanycytes at the BHB is perceived to be driven by the coordination between gene expression and metabolic status of the individual, enabling tanycytes to adapt their physiology based on the feeding conditions. Thus, analyzing varying levels of transcript abundance with the changing metabolic states could explain how transcriptional reprogramming is essential for the regulation of metabolic pathways to maintain energy balance.

Though all tanycytes express common genes such as DARPP-32, GPR50, Ppp1r1b, Rax, Dio2, Slc16a2, vimentin and nestin, each tanycyte subtype is also deemed to express its own molecular signature gene(s) associated with its physiological function in the regulation of energy homeostasis (Prevot et al. 2018). For instance, β-tanycytes at the blood–brain interface dynamically regulate the access of nutrients and hormones to the brain and the secretion of neuropeptides into the hypothalamo-hypophysial vascular system in the ME, while α-tanycytes are the modulators of neuronal activity. Together, α- and β-tanycytes are chemosensitive and diet-responsive cells. Reclassification of tanycytes based on their localization and projection in the hypothalamic regions and their transitioning topology with varying energy status would provide a better understanding of how tanycytes regulate the transfer of peripheral metabolic hormones into the brain and reciprocally communicate with brain neuronal networks for the regulation of food intake and energy homeostasis. It is anticipated that the selective isolation of tanycytes by fluorescence-activated cell sorting (FACS) and studying their transcriptome at the single-cell level may lead to the discovery of novel subtypes, where each subtype may show a varying degree of diversity depending on the metabolic status of the individual.

11.5.1 Selective Isolation and Molecular Profiling of Tanycytes by State-of-the-Art Fluorescence-Activated Cell Sorting

For the selective isolation of tanycytes, TAT-Cre recombinant protein is stereotactically infused into the third ventricle of tdTomato$^{loxP/+}$ reporter mice (see Sect. 11.7.3.2). TAT-Cre is a recombinant protein containing Cre recombinase, which penetrates cells and catalyzes the site-specific recombination event between two loxP DNA sites (Peitz et al. 2002). This induces Cre-mediated expression of the red fluorescent protein tdTomato in cells that line the third ventricle border (i.e., tanycytes and ependymal cells) (Langlet et al. 2013). The identity of targeted cells is confirmed by testing for the expression of vimentin in tdTomato-positive cells.

After TAT-Cre infusion, ME is microdissected and dissociated to obtain a single-cell suspension. tdTomato-positive cells are then isolated from tdTomato-negative cells by FACS (Fig. 11.4a, b). The gating intervals for scatterplot is specifically designed to select the population of tdTomato-positive cells, excluding autofluorescent cells from wild-type controls and a population of specifically negative cells (Fig. 11.4c). Real-time polymerase chain reaction (RT-PCR) analysis is used to verify the identity and purity of the sorted cells. To verify that tdTomato-positive cells are representative of tanycytes, they must express high levels of the transcript for the tanycyte marker DARPP-32, while transcripts for the fenestrated endothelial cell marker MECA-32 and the neuronal cell markers NPY and POMC must not be detected. Transcriptomic analyses of FACS-isolated tanycytes have suggested that fasting induces VEGF-A in tanycytes (Langlet et al. 2013) (Fig. 11.4d). Moreover, it has also been possible to isolate ME endothelial cells as well as GnRH neurons using FACS, leading to the following findings: (i) confirmation of the expression of

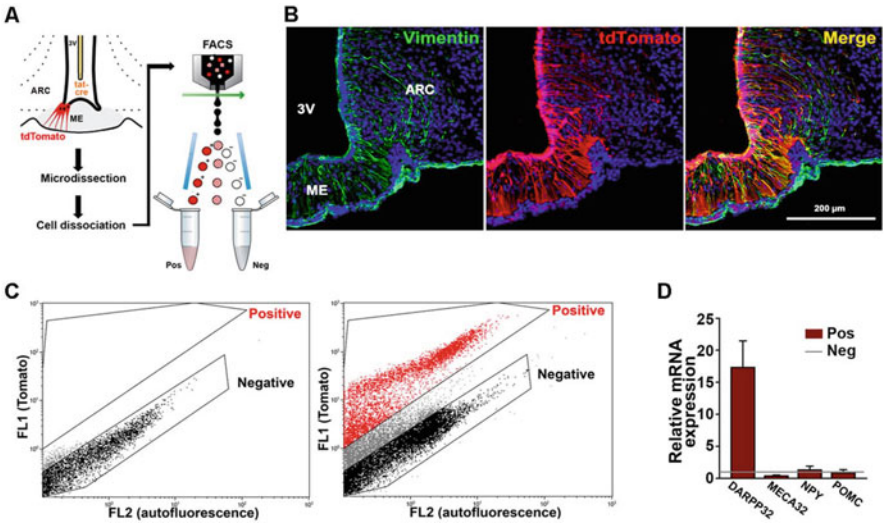

Fig. 11.4 Selective isolation of tanycytes by FACS (**a**) Schematic representation of TAT-Cre recombinant protein infusion into the 3rd ventricle of tdTomato$^{loxP/+}$ reporter mice, which drives the Cre-dependent expression of the red fluorescent protein tdTomato in cells that line the 3rd ventricle (i.e., tanycytes and ependymal cells). This is followed by the microdissection and the dissociation of the ME. tdTomato-positive (Pos) cells are FACS isolated from tdTomato-negative (Neg) cells. (**b**) The expression of vimentin (green) in tdTomato-positive cells validates the targeting of tanycytes. (**c**) Scatterplots (FL1 vs. FL2) showing tdTomato-specific fluorescence (FL1) and autofluorescence (FL2) of cell suspensions from wild-type (left panel) and tdTomato-positive animals (right panel). The gating intervals are designed in a way to choose the population of Pos cells (red), excluding autofluorescent cells and the Neg cells (black). (**d**) Real-time polymerase chain reaction (RT-PCR) analysis confirms the identity and purity of FACS-isolated cells. Pos cells express high levels of the tanycytic marker DARPP-32 gene, while PV1/MECA-32 (a marker for the fenestrated endothelial cells), NPY and POMC (neuronal markers) are not expressed. *ME* median eminence. Adapted from Langlet et al. 2013, with permission

Sema7A and Sema3A transcripts in tanycytes and fenestrated endothelial cells of the ME, respectively; (ii) selective variation of Sema7a during the estrus cycle in tanycytes, and (iii) expression of estrogen receptor alpha and progesterone receptor transcripts in fenestrated endothelial cells and tanycytes, showing that gonadal steroids can directly regulate non-neuronal cells in the ME (see for review Prevot et al. 2018). A similar strategy can be applied for FACS isolation of tanycytes targeted by AAV-Dio2-iCre-2A-GFP (see Sect. 11.8.3.3 for virus-induced *in vivo* models), where the microdissection and dissociation of ME generates a cell suspension with green fluorescent protein (GFP)-positive and negative cells. Thus, FACS-based isolation of cells delivers an opportunity to perform molecular profiling of subpopulations of tanycytes under different metabolic and pathological conditions.

11.5.2 Defining Energy-Regulated Transcriptional Dynamics in Tanycytes at the Single-Cell Level

To refine tanycyte classification in a way that better reflects their complex biology, it is necessary to implement more powerful tools for the analysis of the transcriptional profile of individual cells. Recently, single-cell RNA sequencing (scRNA-seq) in dissected mediobasal hypothalamus has been used to characterize the genetic signature of hypothalamic neural cells (Chen et al. 2017; Campbell et al. 2017). A high-throughput Drop-seq method sequenced more than 14,000 single cells dissociated from hypothalamic tissues. Using semi-supervised clustering analysis, the study identified 45 cell clusters, including tanycytes and their four subtypes with distinct gene expression. Among all the clusters, a Sox9+ and Rax+ cell cluster—transcriptionally distinct from ependymocytes and other glial cell types—corresponded to tanycytes. A deeper characterization of transcriptional heterogeneity of tanycytes unveiled four known tanycyte subpopulations (Chen et al. 2017). Another study by Campbell et al. also used Drop-seq to analyze more than 20,000 single cells obtained from mediobasal hypothalamus (ARH-ME region) and revealed two clusters for tanycytes (Campbell et al. 2017) (Fig. 11.5). While the data confirm four tanycyte subtypes, a new tanycyte marker gene, small proline-rich protein SPRR1A, was interestingly identified with a restricted pattern of expression in the dorsolateral region of the ME, where tanycytes are thought to form a diffusion barrier (currently classified as β1-tanycytes). This suggests the presence of an additional tanycyte subgroup with special diffusion barrier properties. Nevertheless, these studies have applied single-cell sequencing to the entire mediobasal hypothalamus, while tanycytes represent a confined cellular population in a pool of other cellular populations in the region. Thus, studies involving the single-cell sequencing of selectively isolated tanycytes are warranted to delve deeper into novel tanycyte subtypes and their specific markers. This will be made possible by using existing as well as developing genetic tools for delineating, labeling, and tracing tanycyte subpopulations.

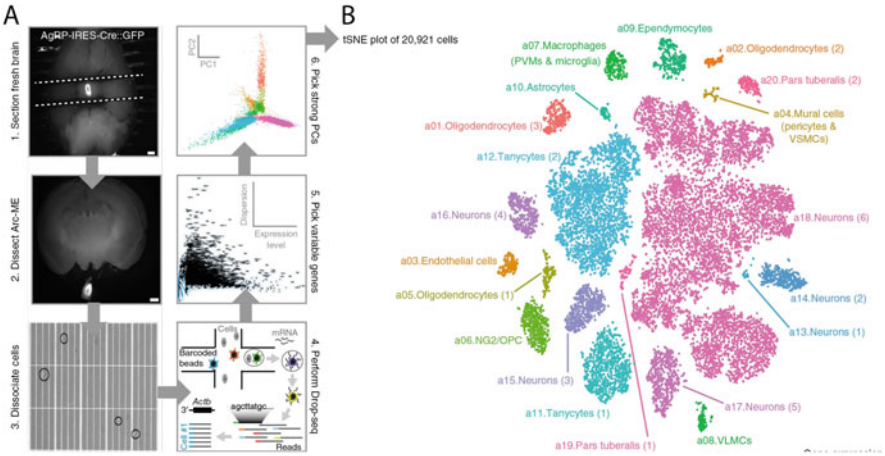

Fig. 11.5 Single-cell transcriptomics of arcuate-median eminence complex. (**a**) Schematic representation summarizing the workflow involving the dissection and the dissociation of arcuate-median eminence region (Arc-ME) from fresh brain sections, and the implementation of Drop-seq method to detect dynamic expression changes at the single-cell level across Arc-ME cellular populations. After correcting for batch effects and eliminating the most variable genes, principal component (PC) analysis is performed followed by dimensionality reduction using spectral t-distributed stochastic neighbor embedding (tSNE) and density-based clustering of 20,921 cells. (**b**) tSNE plot showing 50 transcriptionally distinct Arc-ME cellular populations, including two clusters for tanycytes. *PC* principal component; Reproduced from Campbell et al. 2017, with permission

11.5.3 Other Potential State-of-the-Art, Next-Generation Sequencing Methods for Transcriptomic Profiling of Tanycytes

The first set of single-cell studies involving tanycytes has already added a new layer of information to the existing knowledge. More recently, single nuclei RNA-Seq (sNuc-Seq) was introduced, which uses isolated nuclei instead of whole cells to profile gene expression (Habib et al. 2016). Using droplet microfluidic technology, this method enables the profiling of thousands of nuclei at low cost with high throughput. Nuclei can typically be quickly and easily isolated from lightly fixed, frozen tissues or archived tissues without the prolonged incubations and processing time necessary for the isolation of single cells. Perturbations in transcriptome due to long isolation procedures may be relatively reduced with the isolation of nuclei. This method is particularly appropriate for cells difficult to isolate or for archived tissues. Like drop-seq, sNuc-seq also uses droplet microfluidics for the encapsulation of single nuclei instead of single cells with uniquely barcoded beads. The transcriptomes of single nuclei can be analyzed and unique cell types identified after downstream processing and sequencing, providing a potential next-generation sequencing method to study the tanycyte epigenome. A recently published method,

"DroNc-Seq," also uses single nuclei, as a proxy for whole cells that cannot be readily dissociated or are fixed, to produce single nuclear transcriptomes (Habib et al. 2017). It is also feasible to perform single-cell multi-omic analyses, which allow the investigation of the genome, transcriptome, and epigenome altogether to uncover the true level of heterogeneity within cells.

Amid the recent stir involving scNGS analyses, the utility of bulk RNA sequencing in comparing metabolic and pathophysiological conditions cannot be ruled out. Though scNGS offers the possibility of noise reduction by tracing the reads back to the cell of origin, it comes with a steep monetary cost compared to traditional bulk NGS. Since bulk RNA-seq measures the average expression level for each gene across the entire population of input cells, it may be extremely useful for comparative transcriptomics of tanycytes to determine differential gene expression under various metabolic conditions.

It is worth considering that transcriptional reprogramming typically initiated by transcription factors in tanycytes may not be sufficient to regulate metabolic pathways and maintain energy balance. For instance, transcript abundance and translatability during fasting ought to be rapidly downregulated post-feeding, and vice versa. This posits a role for microRNA (miRNA)-based post-transcriptional control of transcriptional mechanisms in establishing oscillatory gene expression that harmonizes physiological transitions. Moreover, hypothalamic neural stem cells (NSCs) (potential tanycytes, see Sect. 11.6) have been shown to possess an endocrine function of secreting exosomal miRNAs for the control of systemic aging, as evidenced by the reduced expression of over 20 miRNA species in the CSF of mid-aged mice. Thus, it will be interesting to invoke the less-explored domain of miRNA-mediated regulation of exclusive dynamic oscillatory gene expression in tanycytes. Small-seq is a ligation-based method that enables the capture, sequencing, and molecular counting of small RNAs, including microRNAs (miRNAs), fragments of tRNAs, and small nucleolar RNAs (snoRNAs), from individual mammalian cells (Hagemann-Jensen et al. 2018).

Given the roles of small RNAs to modulate gene expression post-transcriptionally, for example, via miRNA-mediated degradation of target transcripts, it would be interesting to extend the investigation by measuring both small RNAs and mRNAs at the genome scale in tanycytes to decipher the mechanisms underlying intercellular miRNA and mRNA heterogeneity. Wang and colleagues developed single-cell microRNA-mRNA co-sequencing to reveal non-genetic heterogeneity and mechanisms of microRNA regulation (Wang et al. 2019). Using this approach, it could be envisaged that co-sequencing miRNA and mRNA profiles from the same individual tanycytes could empower studies relating post-transcriptional cell-to-cell variability in terms of varying miRNA–target interactions. This information might be extrapolated to address unresolved questions concerning tanycyte heterogeneity and their plasticity in response to feeding fluctuations. Moreover, differentially expressed microRNAs in healthy and diseased states could be exploited for targeted gene therapy of metabolic diseases.

11.6 Exploring the Neural Stem Cell Nature of Tanycytes and Their Role in Hypothalamic Neurogenesis

Besides morphological and transcriptomic plasticity, tanycytes also possess NSC properties. They express a variety of neural stem/progenitor cell (NSC/NPC) markers such as nestin, vimentin, Sox2, brain lipid-binding protein, GLAST, Musashi-1, and GFAP, which is evolutionarily conserved (Pellegrino et al. 2018). Tanycytes must proliferate, self-renew, and differentiate into neuronal, astroglial, and oligodendroglial lineages to qualify as bona fide NSCs. Though tanycytes have long been suspected of possessing NSC-like properties due to their morphological and molecular resemblance to radial glial cells, the recent use of transgenic mouse lines expressing reporter genes under the control of tanycyte promoters has opened a novel arena for researchers to fate-map tanycytes and study their multipotency *in vivo* (see for review Goodman and Hajihosseini 2015).

11.6.1 Discovery of Potential NSC Nature of Tanycytes

A range of *in vitro* and *in vivo* approaches have been used to demonstrate the NSC property of tanycytes. However, determining the neurogenic/gliogenic potential of tanycytes has been a gradual process. Initially, CVOs in adult rats and mice were found to be rich in nestin+, GFAP+, vimentin+ cells expressing Sox2 and the cell cycle-regulating protein Ki67 (Pellegrino et al. 2018). In culture, these cells proliferate as neurospheres and express neuronal (doublecortin+, β-tubulin III+) and glial (S100β+, GFAP+, RIP+) phenotypic traits (see for review Goodman and Hajihosseini 2015). Labeling of the third ventricle of adult rat brain with Dil, a membrane stain, demonstrated neurogenesis in the ependymal layer of the third ventricle. The isolation and the in vitro floating neurosphere culture of Dil + NPCs revealed that these NPCs are essentially derived from cells at the ependymal layer of the third ventricle. These neurospheres self-renew and differentiate into a variety of cell types, including neurons and glia (Xu et al. 2005).

Several studies demonstrated the proliferative capacity of hypothalamic neural stem cells (see for review Goodman and Hajihosseini 2015; Prevot et al. 2018). In vivo administration of bromodeoxyuridine (BrdU), a structural analog of thymidine that is incorporated into the DNA of dividing cells, reveals the proliferative property of adult hypothalamic stem/progenitor cells and their ability to exhibit constitutive neurogenesis and gliogenesis. Generally, BrdU is injected intraperitoneally at an interval optimized to detect temporal changes (twice a day for a long period, i.e., 9 days) or saturation kinetics (every 2 h for 12 h to 48 h) of cell division. The animals are allowed to survive for days to weeks after the last BrdU injection, and BrdU+ cells are visualized and quantified using a confocal microscope (Lee et al. 2012). Alternatively, BrdU can be applied continuously via drinking water (as a 1 mg/ml solution containing 0.25 mg/ml glucose) instead of multiple daily intraperitoneal injections to avoid potential stress to the animals. However, fresh BrdU solution must be supplied every 72 h, and the drinking bottles must be protected from light. It

is also important to evaluate the stability of BrdU in this paradigm, which can be done by finding if the BrdU solution after 72 h contains enough nucleotide analog to label a significant number of cells in the SGZ and SVZ of wild-type mice (Haan et al. 2013).

If tanycytes are neural stem cells, they must be able to generate other cell types in the hypothalamus. Injecting an adenoviral green fluorescent protein (GFP) construct into the third ventricle to label tanycytes and ependymal cells probed this property (Xu et al. 2005). Using this technique, a small number of GFP-containing neurons were detected in the paraventricular nucleus (PVN) and the lateral hypothalamus (that were identified as orexinergic), suggesting that migration and differentiation of progenitor cells from the ependymal layer occurred (Xu et al. 2005). GFP tracing indicated that neural progenitor cells might have migrated from the third ventricle to the hypothalamic parenchyma, where they integrate into neural networks by forming synapses. However, in most in-bred laboratory animal models, the PVN lacks tanycytes *in vivo* and neurons in the PVN, in all likelihood, are not derived from tanycytes. Thus, the combination of BrdU administration, nestin/GFAP immunohistochemistry, and GFP tracing suggests that at least some tanycytes might be NPCs in the ependymal layer of the third ventricle. More recently, Rax1 residual cells in the maturation phase of hypothalamic differentiation in mouse embryonic stem cell (mESC) cultures have been shown to have characteristics similar to ventral tanycytes, suggesting the successful induction of Rax1 tanycyte-like cells from mESCs (Kano et al. 2019).

However, the precise location, identity, and the potential of adult hypothalamic stem/progenitor cell(s) to form neurospheres, a hallmark of NSCs, remained elusive for a long time and continue to remain controversial. Studies to date could only raise the possibility that the adult hypothalamus contains NSCs in a niche near the third ventricle. However, tanycytes, ependymocytes, subventricular astrocytes, and parenchymal glial cells all reside near the third ventricle, and each is a potential adult stem and progenitor cell candidate, warranting lineage tracing and fate-mapping studies (see Sect. 11.6.2). One way to avoid the potential pitfall of cellular non-specificity is by isolating tanycytes expressing stable fluorescent reporters by FACS and evaluating whether single fluorescent cells regenerate neurospheres that can be differentiated into reporter-positive cells that coexpress markers of neuronal and glial lineages.

The most persuasive evidence for hypothalamic NSCs originates from exploiting the Cre-lox labeling approach (see Sect. 11.7.3.1) to trace the lineage of new daughter cells. In these studies, Cre-recombinase expression is activated by a suitable promoter (such as Sox2 or Nestin) in mouse strains with a specific fluorescent protein gene behind a loxP-flanked stop cassette in the ROSA26 locus. The stop cassette is excised in the NSCs expressing the specific promoter gene, resulting in the expression of the fluorescent protein in all daughter cells. These animal models have been used to study the age and diet-dependent effects of ME neurogenesis (Lee et al. 2012).

11.6.2 Lineage Tracing and Fate-Mapping of Tanycytes

The heterogeneity of tanycytes in terms of NPC marker expression, proliferation, and the fate of their progeny can be defined by fate-mapping and lineage tracing experiments. Fate-mapping involves studies that determine the embryonic origin of the specific tissue at a certain stage in development, whereas cell lineage studies determine the relationships between cells at each stage of division. In the adult rat, fate-mapping experiments of the hypothalamic ventricular wall first showed that proliferating ependymal cells, including but not exclusively tanycytes, can give rise to progeny that migrate along tanycyte processes (Pérez-Martín et al. 2010). In the early postnatal stage, the base of the ME harboring β2 tanycytes is particularly enriched with $BrdU^+$ ependymal layer cells representing the proliferative zone. Fate-mapping of tanycytes using inducible nestin promoter-driven reporter expression demonstrates that young postnatal tanycytes generate neurons in the ME/vmARH (Lee et al. 2012). Breeding transgenic Nestin:CreERT2 driver10 and ROSA26stopYFP reporter11 to induce selective fate-mapping of tanycytes and their progeny reveals that β2-tanycytes directly give rise to neurons *in vivo*. Moreover, nestin-creERT2:R26YFP or:R26RFP double transgenic mice at late postnatal stages exhibit no YFP/RFP expression and successfully fate maps tanycytes only in the early postnatal stage (Haan et al. 2013). In addition to neurons, tanycytes also tend to generate glial cells (but to a lesser extent) that populate the arcuate, ventromedial, dorsomedial, lateral, and posterior nuclei of the hypothalamus. Because nestin is expressed in all tanycytes, the fate-mapping of nestin+ cells provides an overview of the neuro- and gliogenic potential of tanycytes at the level of the whole population.

Interestingly, lineage tracing of tanycyte subpopulations reveals that not all tanycytes are equal in their fate. GLAST::CreERT2 mice offer captivating evidence that GLAST+ α-tanycytes in the lateral wall of the third ventricle self-renew to give rise to different tanycyte subclasses, parenchymal astrocytes, and neurons, where the long tanycyte processes potentially provide a migration route for newborn cells (Robins et al. 2013). This neurogenic property of adult α-tanycytes was studied by a suitable genetic label, where adult GLAST::CreERT2 mice were crossed with Cre reporter mice ubiquitously expressing either β-gal or GFP (z/EGmice) after Cre-mediated recombination. Cre-recombinase activity was restricted to α-tanycytes, particularly in α2-tanycytes. No expression was detected in β-tanycytes. Such experiments benefit from double-label analyses with nestin or co-labeling with GFAP to confirm the reporter activity in the defined tanycyte subset.

The impact of tanycytes on the dynamics of neurogenesis during late postnatal and adult life can be determined by stage-specific lineage tracing to induce the constitutive expression of a traceable marker gene within tanycytes at desired time points. One such marker gene is fibroblast growth factor (FGF) 10 (Goodman and Hajihosseini 2015). FGF10 expression is restricted to the population of tanycytes in the ME/vmARH that coexpress nestin, Sox2, brain lipid-binding protein, and Musashi-1, but lack GFAP and GLAST. Stage-specific lineage tracing from P28 and later (late postnatal to adult life) using the FGF10-creERT2 line demonstrated that FGF10-expressing tanycytes, and possibly also their parenchymal descendants,

continually add new neurons that mostly populate the ARH and VMH, but not GFAP+ progeny. To lineage-trace FGF10+ cells, FGF10CreERT2/+ mice carrying a knock-in CreERT2-IRES-YFP cassette in exon 1 have been used. Therefore, based on the ability of GLAST+ α tanycytes to produce β2-tanycytes (vmARH tanycytes), and the exclusively neurogenic nature of β-tanycytes that do not form neurospheres *in vitro* and are highly enriched in doublecortin (a microtubule-associated protein expressed in neuronal progenitors), one may speculate that GLAST+ α tanycytes are NSCs, whereas β-tanycytes are more committed NPCs.

Gene expression profiling approaches also help in the fate-mapping of selected tanycyte subpopulations to refine our knowledge of the complexity of the hypothalamic niche. Sonic hedgehog (Shh) controls the proliferation and cell fate of NPCs during development of the nervous system. Interestingly, ARH tanycytes are derived from the Shh-expressing floor-plate during embryogenesis (Mirzadeh et al. 2017) and then preserve Shh expression through adulthood, while Shh expression is absent in the ME. However, two membrane proteins involved in Shh signal transduction have been detected in rat ME tanycytes, which potentially means that the tanycyte subtype facing the ARH directs the glial and neuronal cell fate of contiguous tanycyte populations via Shh secretion, analogous to the cell-fate properties of floor-plate-derived Shh during embryogenesis. Also, ME and vmARH tanycytes have recently been shown to express Fezf2, an evolutionarily conserved, forebrain-enriched zinc finger transcription factor dynamically active during mouse hypothalamic development and that is involved in modeling the developing diencephalon (Mirzadeh et al. 2017). Studies conducted both in zebra fish and mice suggest that the level of expression of Fezf2 in NSCs could tightly regulate their level of quiescence by patterning directional Notch signaling among neighboring NSCs, with high Fezf2 expression characterizing quiescent NSCs and low Fezf1 expression proliferative ones (see for review Prevot et al. 2018).

Despite the accumulating evidence suggesting that tanycytes possess neurogenic properties, these studies have been challenged by the questions: (i) Do tanycytes possess distinct pools of NPCs with varying proliferation and lineage dynamics in response to intrinsic and extrinsic cues? (ii) Do lineage relationships link different tanycyte subtypes, as recently found for NSCs in the SVZ? and (iii) What is the extent of activity of adult tanycytes? The advent of novel gene expression profiling methods to better characterize the molecular heterogeneity of tanycytes and the development of new tools for the isolation and fate-mapping of selected tanycyte subpopulations will enable us to refine our understanding of the complexity of the hypothalamic niche.

11.7 Experimental Models to Delineate the Pleiotropic Nature of Tanycytes

The intricate nature of tanycyte-mediated brain–periphery communication warrants the use of multifaceted experimental approaches involving fundamental in vitro and ex vivo experimental models to test the proposed candidate mechanisms followed by *in vivo* validation using genetically modified animal models.

11.7.1 *In Vitro* Primary Culture of Tanycytes

In vitro primary culture of tanycytes has greatly helped in comprehending the fundamental mechanisms underlying tanycyte plasticity and tanycyte behavior in different hormonal contexts (Prevot et al. 2003). The successful preparation of primary cultures of tanycytes depends on the precision of ME dissection as well as the age of the animals used for culture. Unlike astrocyte culture prepared from tissues of 2- or 3-day-old pups, tanycytes are cultured using tissues from 10-day-old pups. Tanycyte cultures are characterized by the presence of cells immunoreactive for the tanycytic markers DARPP-32 (Meister et al. 1988) and G protein-coupled receptor 50 (GPR50), and the absence of GFAP immunoreactivity, which is characteristic of astrocytes. By using primary cultures of tanycytes, the role of the TGFα and TGFβ signaling pathways in PGE2-dependent cytoskeletal dynamics, causing tanycytic process retraction, was confirmed (Prevot et al. 2003). The effect of Sema7a on the growth of tanycytic endfeet via the integrin β1 signaling pathway was also shown by using the in vitro primary culture of tanycytes. The communication between the endothelial cell and tanycyte processes and their role in directing the access of GnRH neurons to the pericapillary space of the ME was also first discovered in vitro. In that study, tanycytes were co-cultured with fenestrated endothelial cells of the ME that were isolated by an immunopanning technique, resulting in an acute cytoskeleton remodeling in tanycytes via endothelial nitric oxide signaling. Exposure to estradiol facilitates the cytoskeletal remodeling in the co-cultures by inducing the expression of a dominant-negative endothelial nitric oxide synthase (NOS) in the primary ME endothelial cells (Prevot et al. 2018; Chachlaki and Prevot 2020).

Primary tanycytes can also be used to study transcytosis mechanisms using a time-lapse technique following incubation with a fluorescent compound (Balland et al. 2014). They also serve as tools to assess intracellular changes (e.g., glucose concentration, cellular metabolism) in response to a modulation of their environment (Geller et al. 2019). Finally, using co-immunoprecipitation and multiple staining, cultures of tanycytes allow the investigation of protein–protein interaction processes and new receptor function, in a primary, i.e., untransfected, system. For example, they have been used to study endogenous activity of tyrosine kinase receptors of the EGFR family (also named ErbB receptors) (Prevot et al. 2003).

11.7.2 Living ME Explants and Brain Slices

Live hypothalamic explants containing the ME serve as models to study the function of molecules involved in the structural plasticity of the ME by using ex vivo treatments and ultrastructural analyses. This model demonstrated that the structural alteration favoring the direct access of GnRH neurons to the ME is a rapid process. Treating hypothalamic explants from diestrus animals with L-arginine, a well-known activator of NOS, mimics the proestrus state in a diestrus explant within 30 min of treatment (see for review Chachlaki and Prevot 2020). A similar activity was

observed when acute hypothalamic explants were treated with PGE2 or Sema3A. Strikingly, Sema7A treatment of proestrus ME explants generates the opposite effect on ME structural dynamics, switching from the proestrus to the diestrus state (Prevot et al. 2018).

Living brain slices can also be prepared for electrophysiological and calcium imaging experiments. Recording the response of tanycytes and ependymal cells in living hypothalamic brain slices to changes in extracellular K^+ concentration established the importance of the interaction of tanycyte processes with neuroendocrine axons. For intracellular recording, a microelectrode is inserted into a single cell to measure its electrical activity as currents pass through the cell membrane to generate changes in transmembrane voltage. When applied onto living hypothalamic slices, fura-2AM, a fluorescent membrane derivative of the ratiometric calcium indicator Fura-2, is seen to readily load into tanycytes. Real-time fluorescence imaging using this tool showed that the direct exposure of tanycytes to ATP via a puffer pipette, as well as the release of ATP from tanycytes via glucose stimulation, induces a robust Ca^{2+} response (Frayling et al. 2011). A similar approach demonstrated the responses of tanycytes to L-amino acids (Lazutkaite et al. 2017). Recording the membrane potential in hypothalamic slices from wild-type and *Gfap*::*Cre*; $Cx43^{loxP/loxP}$ mouse lines revealed the role of Cx43 gap junctions in the spread of calcium waves in tanycytes. More specifically, the genetic depletion or pharmacological inhibition of Cx43 elicited a reduction in transmembrane currents in α-tanycytes, implying the significance of gap junctions formed by Cx43, particularly in α-tanycytes (Recabal et al. 2018). Another interesting approach to specifically study intracellular calcium signaling in tanycytes is to obtain acute brain slices from mice that express the calcium sensor GCamP6s18 or GCaMP3 in tanycytes by injecting AAV-CAG-GCamP6s or AdV-pTSHR-GCaMP3 into the lateral ventricle (Müller-Fielitz and Schwaninger 2019).

11.7.3 *In Vivo* Selective Targeting of Tanycytes

11.7.3.1 CreErT2 Models

The Cre/lox site-specific recombination system has emerged as an important tool for the generation of conditional somatic mouse mutants. This method allows the control of gene activity in space and time in almost any tissue of the mouse, opening new avenues for studying gene function and for establishing sophisticated animal models. Activation of ligand-dependent Cre recombinases by injecting tamoxifen into the animal has been one of the early breakthroughs in terms of in vivo inducibility (Feil et al. 2009). Tanycytes express glial markers, such as GLAST (encoded by the Slc1a3 gene) and GFAP (encoded by Gfap), and tamoxifen-inducible CreERT2 lines are available for both genes, which have been used to target tanycytes as well as astrocytes. For the visualization of the detailed morphology of tanycytes, injecting tamoxifen (i.p.) in inducible reporter mice to selectively express the red fluorescent tandem dimer Tomato (tdTomato) protein in tanycytes appears to be a valuable option. The specificity of this approach relies on the expression of a CreERT2 allele

driven by the GLAST/excitatory amino acid transporter 1 locus, which is known to be selectively expressed in glial cells (Slezak et al. 2007). Injecting tamoxifen into these mice activates Cre recombinase and excises a floxed stop sequence ($STOP^{LoxP/LoxP}$) that lies upstream of a tdTomato coding sequence. The successful recombination in the subset of astrocytes and tanycytes causes a high level of tdTomato expression, thereby filling the entire cytoplasm of the cell at the recombination site.

Apart from GLAST, the other CreERT2 transgenic mice models with tanycyte-specific promoters, such as nestin and Fgf10 (Pérez-Martín et al. 2010; Lee et al. 2012; Haan et al. 2013), have been used to temporally induce DNA recombination in tanycytes. These transgenic models were among the first to be used for the lineage tracing of tanycytes by breeding them with the reporter mice encoding fluorescent proteins followed by tamoxifen injection to activate recombination in tanycytes at different time points. Fluorescent protein expression in the recombined tanycytes aids in monitoring their division and the differentiation of their progeny. Similarly, crossing the Rax-CreERT2 mouse line with the Cre-dependent Ai9 tdtomato reporter line shows the distribution of Rax in the brain and suggests a role of Rax as a tanycyte marker based on the tdTomato expression in tanycytes and not in the dorsally located ependymal cells lining the third ventricle (Pak et al. 2014). However, the use of RaxCreT2 to target tanycytes in adults may be limited by the fact that its ability to mediate efficient gene recombination with a single dose of 4-hydroxytamoxifen (4-OHT) is much lower than in infantile (P7) and juvenile (P28) mice. To enhance gene recombination efficiency, repeated injections 4-OHT, which has strong pro-estrogenic actions, are often used, including during early postnatal development (i.e., when hypothalamic neuronal circuits are developing). The presence of a robust tdtomato expression in radial Müller glia, as well as fibrous pituicytes, also interferes with the selective targeting of tanycytes. Despite the limitations, Rax-CreERT2 mice have been recently crossed with $LepR^{lox/lox}$ mice for the generation of tanycyte-specific LepR-knockout mice (Yoo et al. 2019).

In addition to the non-specificity of CreERT2 animal models in targeting tanycytes, recent studies have highlighted other caveats involving the interference of tamoxifen with estrogen receptors resulting in: (i) the alteration of the chromatin architecture (Zhou et al. 2019) and (ii) the self-regulation of metabolic activity (Liu et al. 2018), making it a not-fully appropriate model for studies concerning tanycyte-mediated metabolic functions.

11.7.3.2 TAT-Cre Injections in the Third Ventricle to Selectively Target Tanycytes In Vivo

Innovative alternative approaches to the use of CreERT2 have been designed and implemented to selectively target tanycytes. The unique position of tanycytes in the brain can be exploited to implement tools for genetic manipulations. Tanycytes can be selectively targeted by stereotaxically injecting TAT-Cre fusion protein into the third ventricle of $tdTomato^{loxP/+}$ reporter mice. TAT-Cre infusion in the ventricular system of Cre reporter line mice (Ai9 mice) showed DNA recombination in ependymal cells, including tanycytes and common cuboidal ependymal cells, as well as in the choroid plexus (Langlet et al. 2013). Intriguingly, the recombinant

protein does not cross the ependymal layer and does not target neural cells in the parenchyma. However, a few neurons, most likely in contact with the CSF, may sometimes be observed (Conductier et al. 2013).

This approach was first used to isolate tanycytes by FACS to study the tanycyte gene expression profile (Langlet et al. 2013). tdTomato reporter mice infused with TAT-Cre in the third ventricle were sacrificed after two days, and the mediobasal hypothalami were microdissected to obtain a single-cell suspension for FACS. Quantifying DARPP32, a tanycyte marker, gives a measurement of tanycyte enrichment. TAT-Cre may also be used to delete gene expression in tanycytes in Cre-dependent mice. The main advantages of using TAT-Cre include: (i) the ability to modulate the function of tanycytes rapidly and efficiently; (ii) deletion of the gene by the direct infusion of Tat-Cre in Cre-dependent mice without having to cross mouse lines; and (iii) very efficient and quick recombination in less than 24 h of administration making it ideal for labeling, neuroanatomy, and cell sorting sample preparations. However, it may be necessary to allow one week of recovery with anti-inflammatory treatment before starting physiological studies due to the surgical infusion of TAT-Cre. TAT-Cre may also target other ependymal cells along the ventricle, as well as the choroid plexus, and the specificity must be adjusted by carefully considering the infusion conditions and the area of dissection for the isolation of tanycytes. Since TAT-Cre induces DNA recombination close to the site of injection, parameters such as the flow rate, the site, and the volume of injection must be optimized for the efficient targeting of tanycytes. Of note, TAT-Cre acts rapidly, but is also rapidly cleared due to its peptide nature. Thus, TAT-Cre efficiency depends on the state of chromatin structure at the time of the injection, as it has to target the pair of loxP sites to complete the reaction in a short time.

11.7.3.3 Viral Based Methods

Another method to selectively target tanycytes involves the administration of DNA vectors based on recombinant adeno-associated virus (rAAV) 1/2 into the lateral ventricle of mice. The serotypes 1 and 2 are barely known to diffuse into the parenchyma. Injecting the rAAV vector expressing the Cre recombinase into the lateral ventricle of Ai14 reporter mice induces the expression of the reporter tdTomato in the ependymal layer, suggesting that the rAAV1/2-based vector is trapped in the ependymal layer of the ventricular system. The promoter of Dio2, a gene mainly expressed in tanycytes, can also be used to drive expression from an rAAV-based vector. Administration of AAV-Dio2-iCre-2A-GFP into Ai14 reporter mice led to the expression of tdTomato in cells lining the ventral third ventricle and extending processes into the parenchyma, the typical morphology of tanycytes (Müller-Fielitz and Schwaninger 2019). Introduction of AAV-CAG-GCamP6s and an adenoviral construct AdV-pTSHR-GCaMP3, expressing the calcium sensor GCamP6s18 and GCaMP3 in tanycytes, respectively, into the lateral ventricle enables the measurement of intracellular Ca^{2+} concentration (see for review Müller-Fielitz and Schwaninger 2019). Adenoviruses expressing the TSHR promoter have also been used recently to express a Ca^{2+}-permeable version of

channelrhodopsin2 (CatCh) in tanycytes, thus enabling their selective activation by light. Photostimulation of tanycytes was shown to evoke long-lasting Ca^{2+} waves in living brain slices loaded with Rhod-2 AM (an alternative calcium indicator to Fura2-AM) and to trigger depolarization of arcuate nucleus neurons, as assessed by whole-cell patch-clamp electrophysiology studies (Bolborea et al. 2020). Acute activation of calcium transients in tanycytes *in vivo* also appears to promote food intake during the resting period (i.e., during daylight in mice).

11.8 Perspectives

Since research on tanycytes has made progress only during the past ten years, much remains to be uncovered concerning their changing morphological behavior and the factors regulating these changes. The use of state-of-the-art systems neuroscience approaches along with *in vivo* genetic manipulations is crucial to delineating the multifaceted mechanistic activity of tanycytes for the control of physiological systems. The selective manipulation of tanycytes will undoubtedly open prospects for developing novel, specific, and tailor-made therapies for metabolic diseases such as obesity and type 2 diabetes. Transcriptomic analysis at the single-cell level may further revolutionize the existing information on tanycyte-mediated brain–periphery communication.

Given that most metabolic diseases display marked sex differences, it is time to incorporate sex as a factor into studies pertaining to tanycyte physiology and function. For instance, obesity is a pandemic affecting 35% of the population in Western societies, including both men and women; however, there is a marked sex difference, as about twice as many women as men suffer from severe obesity (Leeners et al. 2017). A study also implied that female mice could survive significantly longer than male mice under fasting conditions (Jikumaru et al. 2007). Considering the dire need to address the sex-specific differences in energy homeostasis, more studies are warranted that can lead to novel revelations in the direction of sex-specific differences in tanycyte behavior.

11.9 Key Literature

Balland E et al. (2014) Definitive evidence of the idea that the ME permits the entry of peripheral leptin into the hypothalamus via tanycytic ERK signaling.

Bolborea M et al. (2020) This study provides the first demonstration that optogenetic stimulation of tanycytes modulates the electrical activity in arcuate NPY and POMC neurons in living brain slices.

Campbell JN et al. (2017) This study identifies 50 transcriptionally distinct Arc-ME cell populations using the Drop-seq single-cell sequencing method.

Langlet F et al. (2013) This study shows that fasting alters the structural organization of the blood-hypothalamus barrier via a VEGF-dependent mechanism.

Lee DA et al. (2012) One of the first studies showing that median eminence neurogenesis involving tanycytes may have important implications for central regulation of metabolism *in vivo*.

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Tanycyte Regulation of Hypophysiotropic TRH Neurons

12

Ronald M. Lechan and Csaba Fekete

Abstract

Tanycytes lining the third ventricle are emerging as an integral component of the hypothalamic regulatory system. Their functions, including the regulation of the hypothalamic-pituitary-thyroid (HPT) axis, extend far beyond their role as barrier cells. This chapter summarizes the various mechanisms by which tanycytes contribute to the control of the HPT axis. These cells modulate thyroid hormone negative feedback regulation of hypophysiotropic thyrotropin-releasing hormone (TRH) neurons by regulating local T3 availability. In addition, tanycytes control the availability of TRH transport to the anterior pituitary by at least three different mechanisms. These include degradation of TRH immediately following its release from hypophysiotropic TRH axon terminals, repositioning of tanycyte end feet processes between the TRH axons and the fenestrated capillaries of the portal system, and inhibiting the activity of TRH terminals *via* glutamate signaling and the endocannabinoid system.

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Keywords

CB1 · Diacylglycerol lipase α (DAGLα) · End feet retraction · Endocannabinoids · Hypophysiotropic · Hypothalamic-pituitary-thyroid axis (HPT) · Median eminence · NF-κB · Pyroglutamyl peptidase II (PPII) · Tanycytes · Thyroid hormone · Thyroid hormone transporters · Thyrotropin-releasing hormone (TRH) · Thyrotropes · Thyroxine (T4) · Tri-iodothyronine (T3) · Type 2 iodothyronine deiodinase (D2)

12.1 Introduction

The thyroid hormones, T4 (thyroxine) and T3 (tri-iodothyronine), orchestrate a wide variety of biological processes to support peripheral tissues by affecting protein synthesis and/or altering the metabolic activity of cells. They also have a particularly important role in brain development and maintenance of normal neurological function through effects on neuronal proliferation, organization, arborization, synapse formation, migration, and myelination (Bernal 2002). The effects of thyroid hormones occur through transcriptional control of hundreds of TH-dependent genes mediated through thyroid hormone receptors, but also through a variety of non-genomic actions such as stimulation of oxidative phosphorylation and modulation of ion channels (Bassett et al. 2003; Wrutniak et al. 1998). Thus, the maintenance of normal thyroid hormone levels is paramount to normal physiological function that depends upon a complex interplay between the hypothalamus, anterior pituitary, and thyroid gland, as well as other factors that influence the function of these organ systems.

Circulating thyroid hormone levels are controlled by the hypothalamic-pituitary-thyroid (HPT) axis. The classic view of this neuroendocrine axis is that it is comprised of a cluster of hypophysiotropic neurons in the parvocellular subdivision of the hypothalamic paraventricular nucleus (PVN) that produce the tripeptide, thyrotropin-releasing hormone (TRH), thyrotrophs in the anterior pituitary that produce thyroid-stimulating hormone (TSH), and the thyroid gland that produces thyroid hormone. TRH is released from axon terminals in the external zone of the median eminence and conveyed to the anterior pituitary by way of the portal vessels to stimulate the synthesis, release, and biological activity of TSH, which is released into the systemic circulation and acts on the thyroid gland to increase the synthesis and release of thyroid hormone. A simple negative feedback loop is proposed where circulating levels of thyroid hormone are then kept in check by their negative feedback effects on both hypophysiotropic TRH-producing neurons in the PVN and thyrotrophs in the anterior pituitary. Thus, when circulating thyroid hormone levels are increased, TRH and TSH gene expressions are decreased in hypophysiotropic neurons and thyrotrophs, respectively, whereas the converse is true in association with hypothyroidism. Over the past two decades, however, it has become increasingly apparent that the mechanisms involved in the regulation of the HPT axis are vastly more complicated, as indicated in Fig. 12.1, and involve a

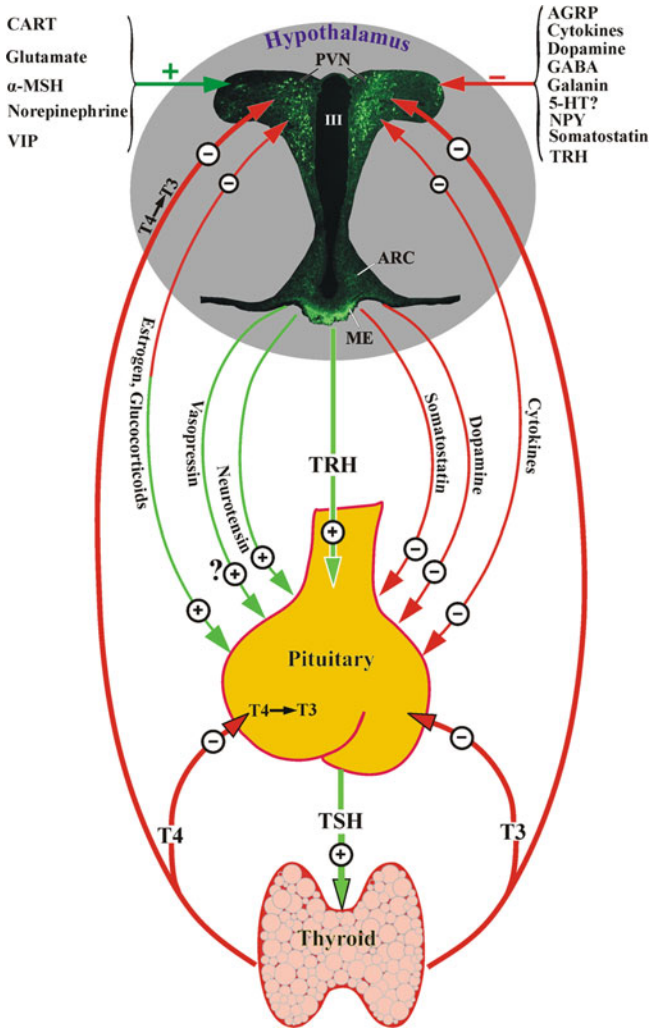

Fig. 12.1 The hypothalamic-pituitary-thyroid axis, a classic neuroregulatory control system involved in the secretion of thyroid hormone. The bold red lines denote the negative feedback loop of thyroid hormone on TRH secretion from the hypothalamus and on TSH secretion from the anterior pituitary. In addition, both hypothalamic TRH neurons and anterior pituitary thyrotropes are acted upon by numerous other neuronal inputs and/or circulating regulatory factors that are activated under specific physiological or pathological conditions. ARC = arcuate nucleus, ME = median eminence, PVN = paraventricular nucleus, III = third ventricle. Reprinted with permission from Lechan et al. (2009)

number of neuronal inputs to the hypophysiotropic TRH neurons and other circulating factors. Since the original observation by Tu et al. (1997) that tanycytes express one of the highest concentrations of type 2 iodothyronine deiodinase (D2) in the central nervous system, an enzyme critically involved in the conversion of

thyroxine (T4) to the more potent, biologically active thyroid hormone, tri-iodothyronine (T3), it has become apparent that regulation of the HPT axis also involves this unusual cell type. This chapter will focus on the role of tanycytes in the regulation of the HPT axis with emphasis primarily on hypophysiotropic TRH. An expanded discussion on the complexity of the central regulation of hypophysiotropic TRH, including regulation by neuronal inputs, involvement of the autonomic nervous system and other circulating hormones, is reviewed elsewhere (Fekete and Lechan 2014; Joseph-Bravo et al. 2016).

12.2 Tanycyte D2 in Feedback Regulation of the Hypothalamic-Pituitary-Thyroid Axis

12.2.1 Anatomy of Tanycytes

Tanycytes are specialized, elongated ependymal cells of glial origin that line the floor and ventrolateral walls of the third ventricle between the rostral and caudal limits of the hypothalamic median eminence (Fig. 12.2). The anatomy of tanycytes has been reviewed recently in detail by Rodriguez and colleagues (2019). Characteristics of tanycytes include apical, villi-like protrusions that extend into the cerebral spinal fluid (CSF) and a basal process that ramifies into the underlying neuropil, terminating on blood vessels in the adjacent arcuate and ventromedial nuclei and on or near fenestrated capillaries of the primary portal plexus in the external zone of the median eminence. Tanycytes comprise a heterogenous group of

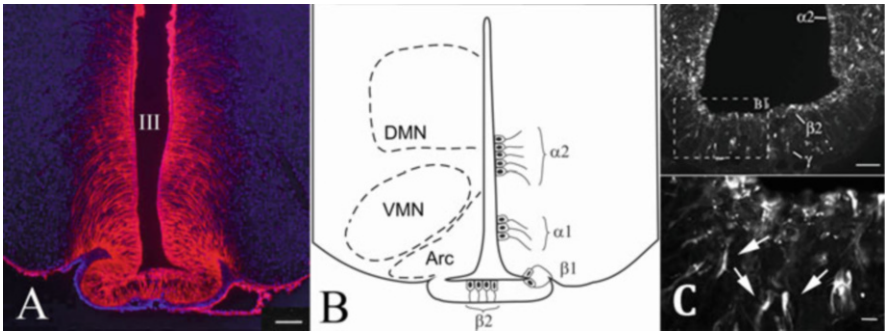

Fig. 12.2 Organization of tanycyte subtypes in the mediobasal hypothalamus. (**a**) Vimentin-immunolabeled (red) coronal section with DAPI counterstaining (blue) shows the distribution of tanycytes and their processes. (**b**) Schematic diagram illustrating the location of α1, α2, β1, and β2 tanycyte subtypes in the wall and floor of the third ventricle. (**c**) The γ-tanycyte subtype in the median eminence. Low power magnification of the median eminence demonstrating the location of α2, β2, and γ tanycytes by in situ hybridization histochemistry. Inset is shown under high magnification below. Arrows point to γ tanycytes. Scale bar = 200 μm in **a**, 100 μm in top part of **c** and 25 μm in bottom part of **c**. Arc = arcuate nucleus, DMN = dorsomedial nucleus, VMN = ventromedial nucleus, III = third ventricle. Modified with permission from Fekete and Lechan (2014), Wittmann et al. (2017)

cells and, according to the nomenclature of Akmayev (Akmayev and Fidelina 1976), include β1- and β2-tanycytes that line the floor and lateral extensions of the third ventricle, and α1- and α2-tanycytes that line the walls of the third ventricle and project into the hypothalamic arcuate, ventromedial, and dorsomedial nuclei (Fig. 12.2). However, additional tanycyte subtypes have also been proposed, including γ tanycytes, small cells present within the substance of the median eminence and infundibular stalk (Wittmann et al. 2017), and parenchymal tanycyte-like cells in the hypothalamic arcuate nucleus (Jourdon et al. 2016; Wittmann and Lechan 2018).

12.2.2 Physiologic Significance of Tanycyte D2 in Regulation of the HPT Axis

As noted in the Introduction, circulating thyroid hormone has an essential role in the regulation of the HPT axis. The major secretory product of the thyroid gland is T4, but it must be converted to T3 within the brain to exert its negative feedback effects on hypophysiotropic TRH neurons. Circulating levels of T3, alone, are not sufficient to regulate hypophysiotropic TRH neurons, as only thyrotoxic levels of T3 can restore TRH mRNA synthesis to euthyroid levels in hypothyroid animals devoid of T4 (Kakucska et al. 1992). In addition, T4 is transported into the brain much more efficiently than T3 (Hagen and Solberg 1974). The mechanism involved in the conversion of T4 to T3 within the brain requires the enzyme D2, as is schematically illustrated in Fig. 12.3. Curiously, hypophysiotropic TRH neurons do not express D2 (Riskind et al. 1987; Tu et al. 1997), indicating that the conversion of T4 to T3 must occur elsewhere in the CNS before T3 is delivered to these neurons. While in most parts of the brain D2 has a uniform distribution, the hypothalamus is devoid of D2 expression except in the mediobasal hypothalamus, where high concentrations of D2 mRNA and D2 protein are expressed in both α- and β-tanycytes (Fig. 12.4), located throughout the rostral-caudal limits of the median eminence (Tu et al. 1997; Diano et al. 2003). In addition, tanycytes express high levels of MCT8 and OATP1c1 thyroid hormone transporters (Roberts et al. 2008). Therefore, this anatomy raised the possibility that thyroid hormone activation in tanycytes may have a key role in the feedback regulation of hypophysiotropic TRH neurons. Unlike D2 activity in other regions of the brain, such as the cerebral cortex, where D2 activity is increased by hypothyroidism or iodine deficiency and reduced by hyperthyroidism (Anguiano et al. 1995), this phenomenon is not observed in the mediobasal hypothalamus where tanycytes reside (Tu et al. 1997; Diano et al. 1998). Whereas hypothyroidism results in a more than four-fold increase in D2 activity in the cortex, it has no effect on D2 activity in the mediobasal hypothalamus (Leonard et al. 1981; Serrano-Lozano et al. 1993; Diano et al. 1998). In accordance, the cortical T3 concentration is unaltered when the peripheral T4 concentration changes, even over a wide range, whereas the hypothalamic T3 concentration changes in parallel with the circulating T4 levels (Broedel et al. 2003). The relatively stable level of D2 activity in the mediobasal hypothalamus under basal conditions and with changes in circulating thyroid hormone, therefore, allows hypophysiotropic TRH neurons to sense any changes in T4 output by the thyroid gland, whereas if hypothalamic T3 concentrations were stable,

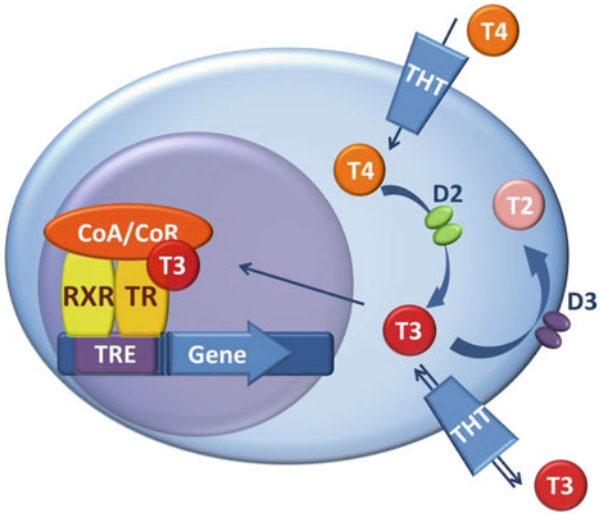

Fig. 12.3 Thyroid hormone action and its regulation by thyroid hormone transporters and deiodinases. As the first step of thyroid hormone action, type 2 deiodinase (D2) activates T4 by converting it to T3. Type 3 deiodinase (D3) catalyzes the inactivating pathway by degrading T3 to T2 and converting T4 to the inactive reverse T3. The expression of the two main deiodinases, D2 and D3, varies according to cell type. T3 can either bind to a regulatory region of thyroid hormone-responsive genes, or be transported out of the cell *via* a thyroid hormone transporters (THT). Reprinted with permission from Mohacsik et al. (2018)

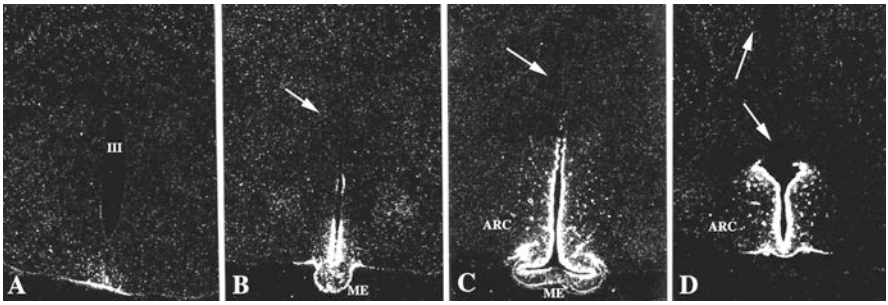

Fig. 12.4 Rostral-caudal distribution of type 2 iodothyronine deiodinase (D2) mRNA in the mediobasal hypothalamus. Dark-field micrographs of D2 mRNA labeled by in situ hybridization histochemistry. In the most rostral section (**a**), hybridization is present in the external zone of the median eminence (ME), but is absent from walls of the third ventricle (III). In more caudal sections (**b–d**), intense hybridization is seen over cells lining the floor and lateral walls of the third ventricle and in the arcuate nucleus (ARC) and ME. Hybridization is absent in the dorsal portions and roof of third ventricular wall (arrows, **b–d**). Original magnification x40. Reprinted with permission from Lechan et al. (2009)

the sensitivity of the feedback regulatory mechanism would be reduced. Further support for the importance of hypothalamic-derived expression in contributing to the feedback regulation of hypophysiotropic TRH is suggested in studies by Mohacsik et al. (2016a) in chicken embryos showing that feedback inhibition of hypophysiotropic TRH is responsive to T3 but not T4 until postnatal day 2, when the developmental increase in tanycyte D2 becomes maximal.

In addition to tanycytes, D2 is also expressed throughout the brain in astrocytes. However, based on the studies by Fonseca et al. (2013), it is unlikely that astrocyte-derived D2 contributes to feedback regulation of the HPT axis. This conclusion is based on the observation that transgenic mice with near-complete loss of D2 selectively in astrocytes, but not in tanycytes (astro-D2 KO mouse), maintain normal T4-dependent negative feedback and thyroid economy. In particular, astro-D2 KO mice have normal serum levels of TSH, T4, and T3, thyroid morphology, TRH gene expression in the PVN and maintain responsiveness of the HPT axis to the exogenous administration of T4 or T3. Presumably, astrocytic D2 primarily functions to maintain normal levels of T3 in the brain as noted previously, as it is increased or decreased, respectively, in association with hypothyroidism or hyperthyroidism, and facilitates neuronal integrity in response to brain injury Fonseca et al. 2013 and Arrojo Drigo et al. 2013.

The location of D2-expressing tanycytes along the base and walls of the third ventricle led to the hypothesis that tanycytes are in a strategic position to extract T4 from the bloodstream by tanycyte end feet processes terminating on portal capillaries or blood vessels in the arcuate nucleus or from the CSF *via* apical specializations after T4 has traversed the choroid plexus (Fekete and Lechan 2007). T4 can then be converted to T3 in the tanycyte cytoplasm and released back into the CSF (Fekete and Lechan 2007). T3 released into the CSF could then diffuse into the substance of the brain by volume transmission, moving between ependymal cells lining the third ventricle, and provide a source of T3 to hypophysiotropic neurons that just underlie the third ventricle. T3 could also be released into the portal system for conveyance to anterior pituitary thyrotrophs, contributing to the regulation of TSH secretion.

Anatomical observations by Sanchez et al. (2009), however, have led to an alternative hypothesis. In these studies, double immunofluorescence-labeled sections for pro-TRH and vimentin, a marker of tanycytes, demonstrated close juxtapositions between the cytoplasmic extensions of β2-tanycytes and TRH axon terminals in the external zone of the median eminence (Fig. 12.5). By confocal microscopy, TRH axon terminals were found to be intertwined with tanycyte end feet processes in a dense plexus that appeared to follow the course of the end feet processes to its point of termination near portal capillaries. Thus, it is also feasible that the T3 released from tanycyte end feet processes could be taken up by TRH-containing axon terminals in the median eminence and then transported back to hypophysiotropic cell bodies in the hypothalamic paraventricular nucleus. In support of this is the observation that the majority of axon terminals in the external zone of the median eminence, including axon terminals of hypophysiotropic TRH neurons, contain the MCT8 thyroid hormone transporter (Kallo et al. 2012). Further support is given by autoradiographic evidence for axonal transport of T3 within the

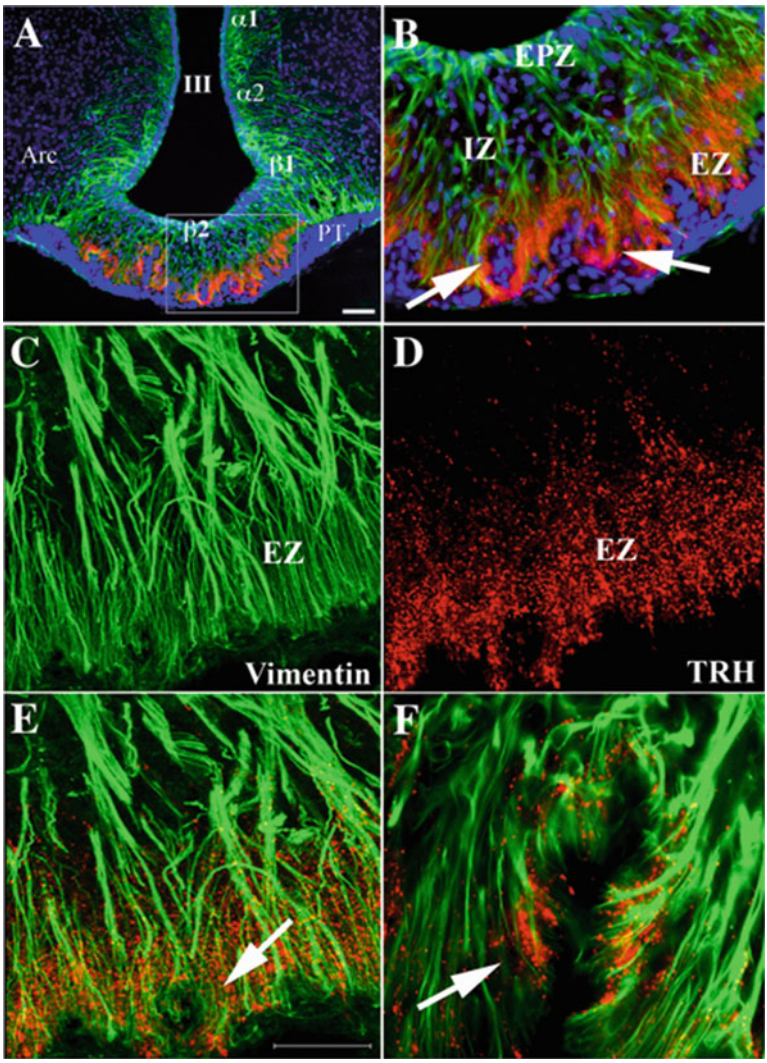

Fig. 12.5 Association between tanycytes and TRH-containing axon terminals in the median eminence. (**a**) Low-powered magnification of a triple-labeled section showing vimentin immunofluorescence (green) labeling tanycytes, TRH immunofluorescence (red) labeling TRH axons, and DAPI labeling (blue) of cell nuclei. TRH axon terminals are associated primarily with β2 tanycyte cytoplasmic processes. (**b**) High magnification of insert in (**a**). (**c** and **d**) Confocal images of β2 tanycyte end feet processes (**c**) and TRH axon terminals (**d**). (**e** and **f**) Merged images of (**c**) and (**d**). TRH axon terminals establish close appositions to tanycyte end feet processes in the external zone of the median eminence (**e**) and around portal capillaries (**f**). III, third ventricle, Arc, arcuate nucleus, EPZ, ependymal zone of the median eminence; EZ, external zone of the median eminence; IZ, internal zone of the median eminence. Arrows in (**b**), (**e**), and (**f**) point to portal capillaries. Scale bar = 200 μm in A and 50 μm in E. Reprinted with permission from Sanchez et al. (2009)

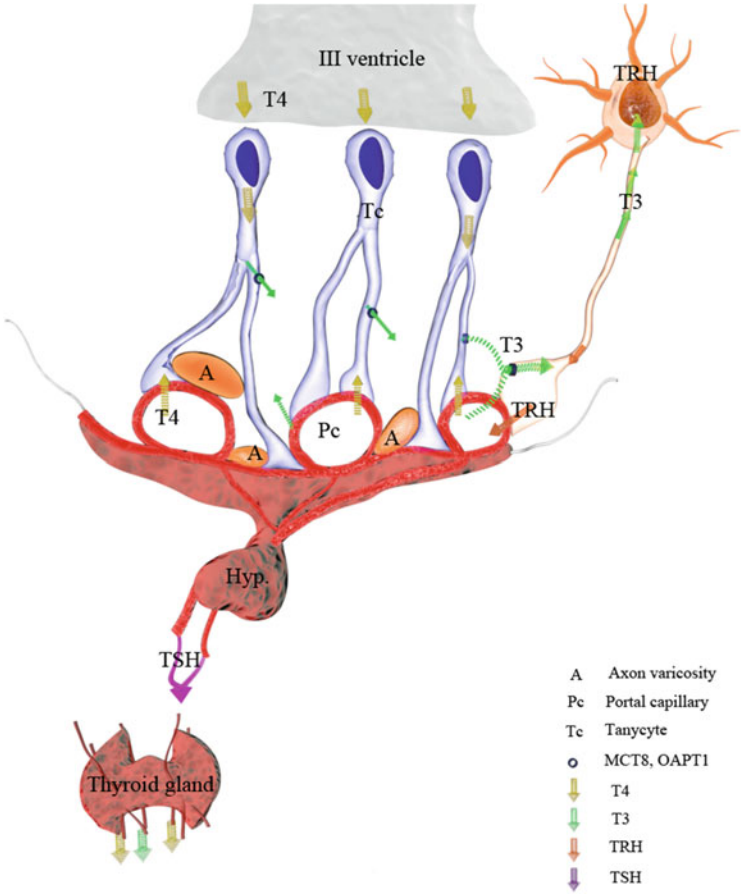

Fig. 12.6 Schematic illustration of a proposed mechanism whereby tanycytes are involved in the negative feedback regulation of hypophysiotropic TRH neurons. T4 is transported to tanycytes by uptake either from the CSF or from the vascular system, where it is converted to T3 and transported *via* specific thyroid hormone transporters (MCT8, OAPT1) to TRH axon terminals that are closely juxtaposed to tanycyte basal processes. There it is conveyed via retrograde transport to the TRH cell bodies in the PVN to regulate the synthesis and ultimately release of TRH into the portal system. Reprinted with permission from Fekete and Lechan (2014)

brain following intravenous administration of radiolabeled T3 (Dratman et al. 1987) and other bioactive molecules such as neurotrophins (von Bartheld et al. 1996; Watson et al. 1999). This mechanism is somewhat more tenable than the mechanism proposed above by volume transmission, as it would explain discrete regulation of hypophysiotropic TRH neurons in the PVN without affecting other TRH populations that are not involved in regulation of the HPT axis but also express thyroid hormone receptors (Lechan et al. 1994). A schematic representation of the proposed mechanisms is provided in Fig. 12.6.

12.2.3 Tanycyte Regulation of Hypophysiotropic TRH Neurons During Infection

D2 expression by tanycytes may also have an important role in altering regulation of the HPT axis in association with infection, prolonged critical illness and fasting, each of which is associated with a decline in circulating thyroid hormone levels yet low TRH gene expression in hypophysiotropic neurons (Fekete and Lechan 2014). Following the systemic administration of bacterial endotoxin (LPS), D2 mRNA and activity are increased in tanycytes by ~400% in a rather rapid and dramatic fashion (Fig. 12.7), differing from the more gradual increase seen in other regions of the brain (Fekete et al. 2004, 2005). The rise in tanycyte D2 is not dependent on the LPS-induced decline in circulating thyroid hormone levels, as the administration of LPS to T4-clamped animals that are kept euthyroid also produces a four-fold increase in tanycyte D2 activity, yet increased D2 activity in the cortex is prevented (Fekete et al. 2004, 2005). Therefore, it is hypothesized that the increased D2 activity of tanycytes results in central hypothyroidism by increasing the concentration of T3 in the mediobasal hypothalamus, thereby inhibiting hypophysiotropic TRH neurons *via* an ultrashort negative feedback mechanism.

Direct evidence that increased D2 activity in tanycytes in response to LPS increases T3 signaling in the mediobasal hypothalamus has been demonstrated

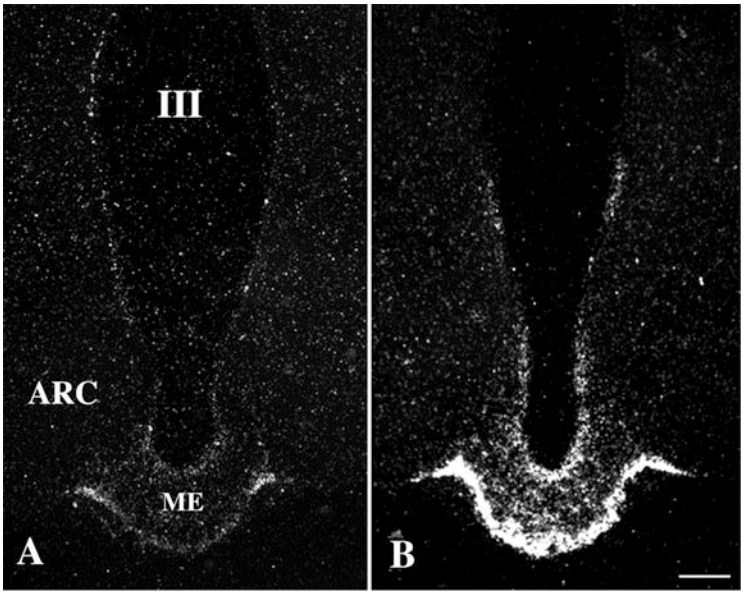

Fig. 12.7 Change in D2 expression with immune challenge. Underexposed in situ autoradiograms showing D2 expression in (**a**) control animal and (**b**) following LPS administration. D2 mRNA expression in tanycytes showed a marked increase in the LPS-treated animals. ARC = arcuate nucleus, ME = median eminence; III = third ventricle. Scale bar = 200 μm. Modified with permission from Fekete et al. (2004)

recently by Mohacsik et al. (2016b). Utilizing a novel thyroid hormone action indicator mouse model (THAI-Mouse) that allows assessment of thyroid hormone signaling in live animals by bioluminescence, an increase in luciferase expression was observed in the mediobasal hypothalamus with endotoxin administration shortly following the increase in D2 expression and preceding the decline of hypophysiotropic TRH mRNA in the PVN. In contrast, luciferase expression in the pituitary gland decreased, providing support for tissue specific regulation of T3 availability by LPS. The hypothesis is also supported by the observation that the LPS-induced inhibition of hypophysiotropic TRH neurons is abolished in D2 *knock out* (KO) mice (Freitas et al. 2010).

One mechanism hypothesized to cause the increase in tanycyte D2 activity in response to LPS is a direct action of LPS on tanycytes *via* their expression of Toll-like receptor 4 (TLR4), as TLR4 KO mice do not show alteration in thyroid function in response to LPS (Hoshino et al. 1999; Rocchi et al. 2007). It is also of interest that the D2 gene contains two, high affinity binding sites for NF-κB, which increases D2 promoter activity (Zeold et al. 2006) and is the main second messenger utilized by TLR4 (Zhang and Ghosh 2001). However, we have only been able to identify evidence for NF-κB signaling in α-tanycytes and significantly later than the rise in tanycyte D2 mRNA (Sanchez et al. 2008, 2010), making this an unlikely early mechanism. However, evidence for NF-κB signaling and a rise in TSHβ mRNA in TSH-producing cells of the pituitary pars tuberalis (Sanchez et al. 2010), a structure that lies directly below the median eminence, may signify an important role for the pars tuberalis. TSH derived from the pars tuberalis has a distinct glycosylation pattern that allows for a local, paracrine action but results in its inactivation when entering the circulation, hence preventing any activating effects on the thyroid gland (Nakao et al. 2008). As tanycytes express TSH receptors and their processes terminate on the surface of the pars tuberalis, TSH derived from the pars tuberalis could induce D2 expression in tanycytes by increasing intracellular cyclic AMP (Murphy et al. 2012). In fact, this mechanism is utilized in photoperiodic animals to increase the conversion of T4 to T3 in tanycytes to increase T3 concentrations in the mediobasal hypothalamus, contributing to the regulation of energy homeostasis and reproductive function (see Ebling and Lewis for review (Ebling and Lewis 2018)). Increased circulating levels of glucocorticoids are not involved in the regulation of D2 by LPS in tanycytes as a corticosterone clamp in adrenalectomized animals does not prevent the LPS-induced increase in D2 activity (Sanchez et al. 2008).

12.2.4 Tanycyte Regulation of Hypophysiotropic TRH Neurons During Fasting

Fasting is also associated with central hypothyroidism characterized by suppression of circulating thyroid hormone levels and hypophysiotropic TRH synthesis (Fekete and Lechan 2014). Fasting also results in an approximately two-fold increase in D2 mRNA in tanycytes and D2 activity in the mediobasal hypothalamus (Diano et al. 1998). Again, the increased D2 activity is not due to the decline in circulating thyroid

hormone levels as T4 treatment does not prevent the fasting-induced increase in mediobasal hypothalamic D2 activity (Diano et al. 1998). Rather, it is believed that the fasting-induced decline in circulating leptin and/or increase in circulating glucocorticoid levels may be responsible (Coppola et al. 2005). Nevertheless, it is not clear whether the two-fold increase in D2 activity is sufficient to induce enough of an increase in T3 in the mediobasal hypothalamus to induce central inhibition of HPT axis when the circulating T4 level falls by approximately 50%. It is unlikely, however, that tanycyte-generated T3 is acting directly on hypophysiotropic TRH neurons under these circumstances, as fasting similarly suppresses TRH mRNA in hypophysiotropic neurons in transgenic mice lacking the β2 isoform of the thyroid hormone receptor (TRβ2), believed to be primarily responsible for the feedback effects of thyroid hormone on hypophysiotropic TRH secretion (Abel et al. 2001). Rather, tanycyte-generated T3 may exert effects on neuronal populations in the adjacent hypothalamic arcuate nucleus, which have known, direct projection pathways to TRH neurons in the PVN and express thyroid hormone receptors (Fekete and Lechan 2014). A similar mechanism has been described for seasonal breeders such as the Syberian hamster and Japanese quail, in which long photoperiods increase T3 in the mediobasal hypothalamus as the result of increased tanycyte D2 activity, increasing appetite to increase body weight and promoting reproductive function partly through neuronal networks in the mediobasal hypothalamus that include kisspeptin and RF amide-related peptide neurons (Barrett et al. 2007; Murphy et al. 2012).

An alternative hypothesis to consider is that tanycyte-generated T3 exerts indirect effects on neurons in the adjacent arcuate nucleus that project to hypophysiotropic TRH neurons in the PVN through the generation of retinoic acid (RA). T3 potently induces retinaldehyde dehydrogenase 1 (Raldh1) in tanycytes, which is required for RA synthesis (Stoney et al. 2016). Whereas T3 does not significantly alter the expression of agouti-related peptide (AGRP), neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) or pro-opiomelanocortin (POMC) genes (Ross et al. 2009), whose peptides have been shown to contribute to the regulation of hypophysiotropic TRH neurons during fasting through direct, afferent projections (Fekete and Lechan 2014), RA significantly upregulates the expression of AGRP in hypothalamic cultures (Stoney et al. 2016).

12.3 Tanycyte Regulation of TRH Degradation

In addition to modulating feedback regulation of hypophysiotropic TRH neurons by regulating the concentration of T3 in the mediobasal hypothalamus, tanycytes can also regulate the amount of TRH reaching the anterior pituitary through the production of the TRH-degrading enzyme, pyroglutamyl peptidase II (PPII, also referred to as TRH-degrading ectoenzyme or TRH-DE). PPII is a membrane-bound peptidase with a large extracellular C-terminal region that contains the exopeptidase and catalytic motifs that are selective for TRH (Charli et al. 1998) and is present in all tanycyte subtypes (Sanchez et al. 2009). However, it is particularly prominent in

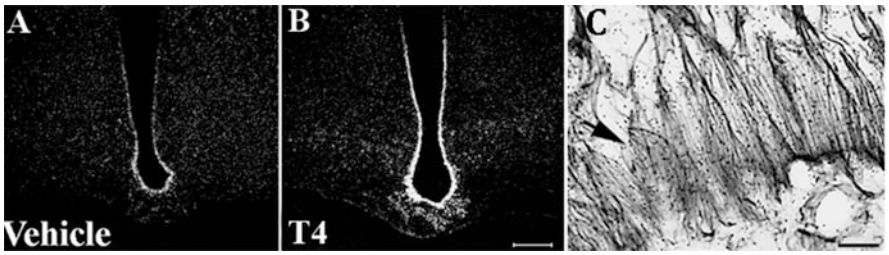

Fig. 12.8 Distribution of PPII mRNA in the median eminence. In situ hybridization histochemistry in (**a**) euthyroid and (**b**) hyperthyroid animals. PPII mRNA increased following the systemic administration of T4 in both tanycyte cell bodies and cytoplasmic processes in the median eminence (**b**). (**c**) Co-localization of PPII mRNA and vimentin-immunoreactivity in tanycyte basal processes (arrow) in the external zone of the median eminence. Scale bar = 200 μm in **b** and 50 μm in **c**. Reprinted with permission from Sanchez et al. (2009)

association with basal processes of β2-tanycytes (Fig. 12.8). Therefore, the close juxtaposition of TRH-containing axon terminals in the external zone of the median eminence with the processes of β2-tanycytes places these two structures in a unique anatomical position to allow PPII to regulate the amount of TRH released from the hypophysiotropic axon terminals into the periportal capillary spaces of the median eminence before transport to the anterior pituitary. Tanycyte PPII is also highly regulated by thyroid hormone, such that the peripheral administration of T4 markedly increases PPII mRNA and activity in tanycytes (Sanchez et al. 2009), whereas methimazole-induced hypothyroidism markedly reduces tanycyte PPII mRNA levels (Lazcano et al. 2015), further suggesting the potential role of PPII in modulating the amount of TRH released into the portal circulation and transported to the anterior pituitary. As PPII expression is upregulated only by the systemic administration of T3, but not by T4 in D2 KO mice (Marsili et al. 2011), it is likely that local conversion of T4 to T3 by D2 activity in tanycytes regulates the HPT axis not only *via* the feedback inhibition of TRH synthesis, but also *via* T3-induced upregulation of PPII expression.

In support of the importance of tanycyte PPII in the regulation of the HPT axis is that Hermodice carunculata protease (HcPI), a specific inhibitor of PPII activity, increases the recovery of TRH following its release from median eminence explants, and inhibition of PPII *in vivo* with N-1-carboxy-2-phenylethyl (N^{im}-benzyl)-histidyl-βNA (CPHNA) increases circulating TSH levels following the administration of exogenous TRH (Sanchez et al. 2009). In addition to tanycytes, the anterior pituitary also contains PPII. However, downregulation of anterior pituitary PPII activity by antisense oligonucleotides or inhibitors does not affect TRH-induced TSH secretion in primary cultures of anterior pituitary cells, but does increase TRH-induced prolactin secretion (Cruz et al. 1991, 2004). These observations suggest that the effects of PPII inhibition on the HPT axis are primarily *via* inhibition by tanycyte-derived PPII, and are supported by the absence of PPII in the thyrotropes of the anterior pituitary (Bauer et al. 1990). Furthermore, as the systemic administration of T4 to hypothyroid mice results in a 50% decrease in TSH within 5 hours

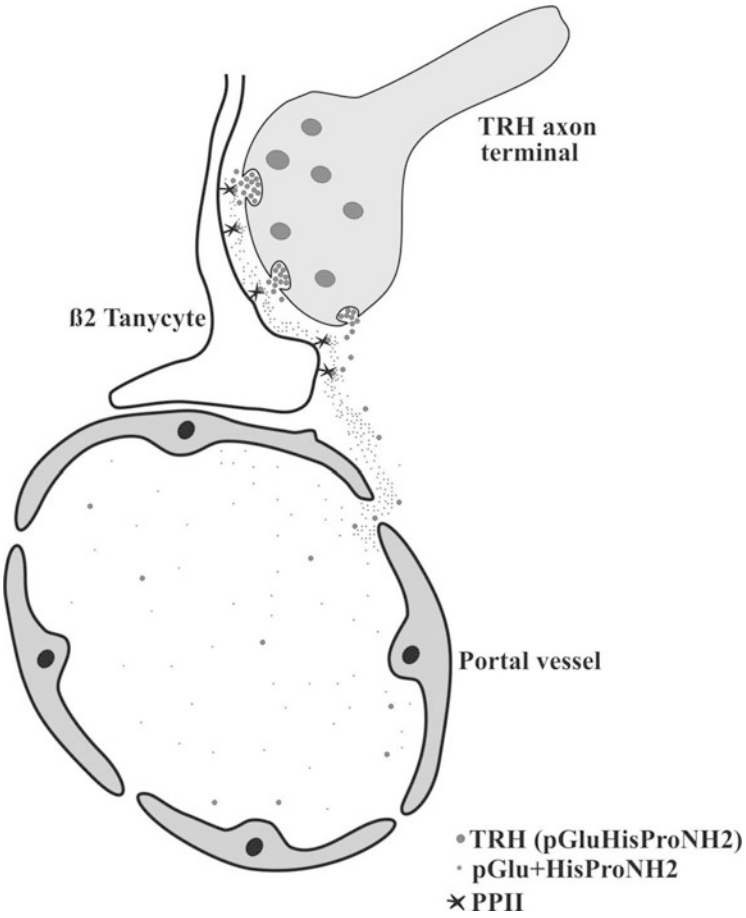

Fig. 12.9 Proposed mechanism for the role of β2 tanycyte PPII in the median eminence. Once released from axon terminals, TRH is degraded by increased tanycyte PPII in the periportal space in response to elevated circulating levels of thyroid hormone, which reduces the amount of active TRH transported in the portal system and, consequently, the secretion of TSH. The increase in tanycyte PPII occurs following active transport of T4 (or T3) into tanycytes by thyroid hormone transporters and the conversion of T4 to T3 in the tanycytes by type 2 iodothyronine deiodinase. Modified with permission from Sanchez et al. (2009)

that is associated with a significant increase in tanycyte PPII mRNA but no significant change in hypophysiotropic TRH mRNA, it is hypothesized that TRH inactivation may be occurring in the median eminence even before the feedback effects of T4 on hypophysiotropic TRH neurons occur (Marsili et al. 2011). A summary of the proposed mechanism of how PPII contributes to the feedback regulation of hypophysiotropic TRH neurons is illustrated in Fig. 12.9.

It is of interest that fasting also increases tanycyte PPII mRNA and activity (Lazcano et al. 2015). Given that fasting suppresses the HPT axis, resulting in a

decline in circulating thyroid hormone levels, this observation would seem incongruent with the mechanisms proposed above, as a decline in thyroid hormone levels would normally inhibit PPII expression. As noted previously, however, fasting also upregulates D2 activity in tanycytes (Diano et al. 1998), which may lead to a local increase in mediobasal hypothalamic T3 levels.

12.4 Tanycyte Regulation of TRH Release

12.4.1 Regulation by Tanycyte End Feet Retraction

There is strong evidence in the literature to support an important role of tanycytes in the regulation of reproductive function. Most convincing, however, is evidence of anatomical ensheathment of GnRH-containing axon terminals by tanycyte end feet processes, indicating that tanycytes can regulate GnRH secretion to the anterior pituitary by modulating access of the GnRH axon terminals to the portal vessels. Thus, during diestrus in the rat, when GnRH output to the pituitary is very low, GnRH axon terminals in the median eminence are fully ensheathed by tanycyte end feet processes. The morphological change in tanycytes is mediated by transforming growth factor beta 1 (TGFβ1) and Sema7A, preventing GnRH from reaching the portal blood (Parkash et al. 2015). Conversely, during the preovulatory surge of proestrus, when there is an increase in GnRH secretion, estrogen binds to alpha-type estrogen receptors on tanycytes and induces retraction of tanycyte foot processes through prostaglandin E2-dependent production of TGFβ1, allowing GnRH axon terminals to have direct contact with the fenestrated portal capillary system (Prevot et al. 1999). Systemic changes in thyroid hormone levels also promote retraction of tanycyte end feet processes and that can be replicated by implanting T3 directly into the hypothalamus (Yamamura et al. 2006).

Given the close anatomical association between tanycyte end feet processes and TRH-containing axon terminals described above, it is not surprising that similar mechanisms are in play to regulate the secretion of TRH from hypophysiotropic axon terminals. Muller-Fielitz et al. (2017) showed that activation of TRH receptor 1 (TRHR1) selectively on β-tanycytes using a pharmacological dose of agonist alters the size of the tanycyte end foot processes, thereby mitigating TRH secretion into the portal capillary system. The proposed mechanism involves elevation of intracellular calcium through a $G\alpha_{q/11}$-coupled pathway. If TRHR1 is blocked with a non-selective antagonist of TRH, midazolam, or if TRHR1 KO mice are studied, the intracellular calcium increase in β-tanycytes in response to a TRH receptor agonist is inhibited. In addition, when $G\alpha_{q/11}$ proteins are deleted specifically in tanycytes of transgenic mice, TRH or TRH analogues are similarly unable to increase intracellular calcium, which is associated with an inability of tanycyte end feet to expand. Furthermore, chemogenetic activation of PVN neurons in which the modified acetyl choline receptor expression was driven by a short TRH promoter increased the size of tanycyte end feet (Muller-Fielitz et al. 2017). In addition, not only was the response abolished in $G\alpha_{q/11}$-KO mice, there was also a greater increase

in circulating levels of TSH. Thus, a new mechanism is proposed that may contribute to regulation of the HPT axis, namely the TRH-induced expansion of tanycyte end feet processes, which impedes the transport of TRH into the portal capillary system. Subsequent studies by Farkas et al. (2020), however, found that the β-tanycytes do not express TRH receptors and TRH has no effect on the membrane potential and intracellular Ca^{2+}levels of these cells, questioning whether TRH has direct effects on β-tanycytes.

12.4.2 Other Paracrine Regulation

Recent studies by our groups demonstrated a bidirectional communication between hypophysiotropic TRH axons and the tanycytes in the median eminence (Farkas et al. 2020). Hypophysiotropic TRH neurons express vesicular glutamate transporter 2 (VGLUT2), indicating their glutamatergic phenotype (Hrabovszky et al. 2005), while β-tanycytes express AMPA and kainite receptors and glutamate transporters (Farkas et al. 2020), suggesting that TRH axons may influence the tanycytes *via* glutamate release. Indeed, glutamate administration causes a marked depolarization of β-tanycytes that can be prevented by combined inhibition of AMPA and kainite receptors and glutamate transporters. Furthermore, optogenetic activation of the TRH axons in the median eminence induces a biphasic depolarization of β-tanycytes. The initial, faster phase of this depolarization can be prevented by inhibition of AMPA and kainite receptors and glutamate transporters, demonstrating the importance of glutamate in the communication between TRH axons and the tanycytes.

Wittmann et al. (2007) have also demonstrated that a large number of hypophysiotropic axon terminals in the external zone of the median eminence contain the type 1 cannabinoid receptor (CB1). Subsequent studies demonstrated that the majority of hypophysiotropic TRH neurons express CB1 mRNA and contain punctate CB1-immunoreactive signal in their axon varicosities in the external zone of the median eminence (Farkas et al. 2020), further suggesting that TRH axons may be sensitive to endocannabinoid signaling. In addition, β-tanycytes appear to produce endocannabinoids, as indicated by the presence of the endocannabinoid synthesizing enzyme, diacylglycerol lipase α (DAGLα), in tanycyte cell bodies and processes in close association with TRH-containing axon terminals in the median eminence (Farkas et al. 2020). The physiologic importance of this association is suggested by the ability of a CB1 antagonist or DAGLα inhibitor to stimulate TRH release in median eminence explants, indicating a tonic, endocannabinoid-induced inhibition of TRH release in the median eminence by β-tanycytes (Farkas et al. 2020). Inhibition of glutamate signaling markedly decreases the 2-AG content of the median eminence, where the tanycytes are the only endocannabinoid synthesizing cells. Thus, these studies demonstrate the existence of a microcircuit in the median eminence in which the activity of TRH axons stimulates endocannabinoid synthesis in tanycytes, while the tanycytes restrain the delivery of TRH to the anterior pituitary *via* the endocannabinoid system (Fig. 12.10). This

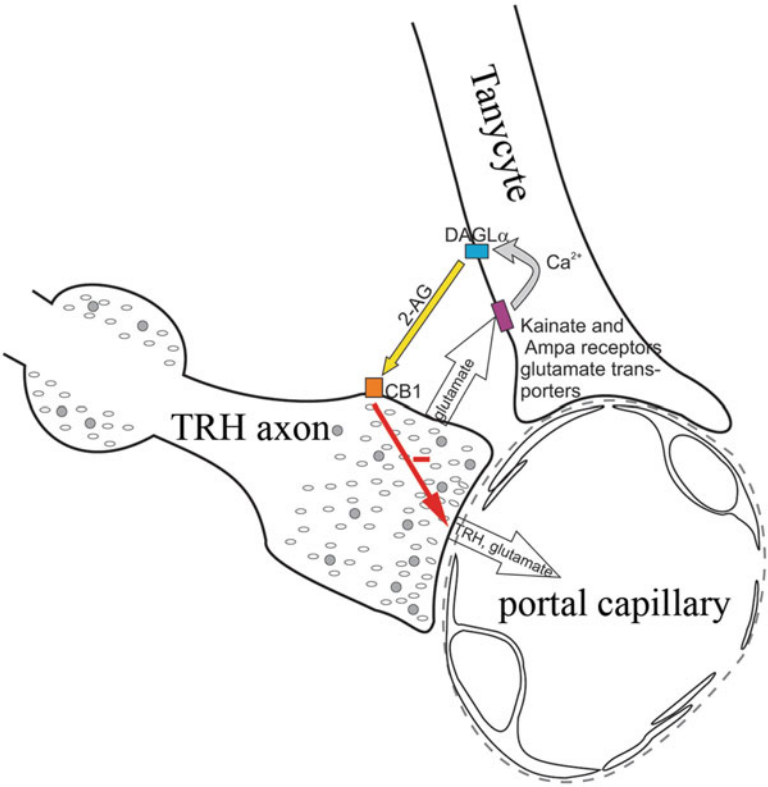

Fig. 12.10 Schematic illustration of the neuro-glial microcircuit in the external zone of the ME. Axon terminals (AT) of the hypophysiotropic TRH neurons are closely associated with the end feet processes of tanycytes (T) in the vicinity of fenestrated capillaries (FC) of the hypophyseal portal circulation. The TRH axons release glutamate that stimulates the DAGLα activity of tanycytes, and thereby 2-AG synthesis in these cells by acting through kainite and AMPA receptors and glutamate transport, and by the resulting increase in intracellular Ca^{2+}. The released endocannabinoids bind to the CB1 expressed on hypophysiotropic TRH axons and inhibit the amount of TRH released into the portal capillary. Reprinted with permission from Farkas et al. (2020)

interaction may have importance in the regulation of pulsatile secretion of TRH and gives an extra flexibility to the control of TRH release.

12.5 Perspectives

The importance of tanycytes in neuroendocrine regulation is becoming increasingly recognized, and in particular, its role in contributing to regulation of the thyroid axis. Tanycyte-neuronal interactions appear to be an essential part of the mechanism that

maintains normal, circulating thyroid hormone levels and alters thyroid hormone levels in response to environmental stressors through ultrashort feedback regulation of hypophysiotropic TRH neurons. This occurs as a result of tanycyte generation of T3 and by controlling the amount of TRH transported to anterior pituitary thyrotrophs. It is uncertain whether any of these mechanisms serve a redundant purpose or are independently essential to assure tight control of circulating thyroid hormone levels, but they indicate a high complexity of regulation of the HPT axis that goes well beyond classic descriptions of a simple negative feedback loop.

Box 1 Novel methods that can be applied to study the function of tanycytes

In addition to the classical morphological tools such as immunocytochemistry and in situ hybridization, a wide array of methods are now being applied to further elucidate the neurobiology of tanycytes. For example, the availability of Cre-LoxP technology has made possible cell type-specific modulation of gene expression. Already, two mouse lines expressing tamoxifen-inducible Cre recombinase have been used to target tanycytes, including the Rax-CreERT2 mouse and Glast-CreERT2 mouse. Theoretically, Nestin-CreERT2 mice could also be used for the same purpose, but this mouse line frequently induces a high level of non-tamoxifen dependent recombination (personal observations). As TAT peptide increases the cellular uptake of Cre recombinase, intracerebroventricular administration of TAT-Cre fusion protein could also be used to deliver Cre recombinase into tanycytes without the need to establish Cre-expressing mouse lines. Another important marker of glia activation is the increase of intracellular Ca^{2+} level that can be studied using Ca^{2+}-sensitive dyes. Tanycytes can be loaded with membrane-permeable Ca^{2+}-sensitive dyes, such as Fluo4 AM, simply by immersion of a tissue slice into the dye-containing solution. Changes in the fluorescence of the dye can be then studied by confocal microscopy. Optogenetics together with patch clamp electrophysiology are also powerful tools that can be used to study tanycyte activation as described in this chapter and reported by Farkas et al. (2020). Further insight into the working of tanycytes can be achieved by transcriptome analysis following isolation of tanycyte cell bodies by laser capture microdissection and next generation sequencing or TaqMann PCR, or analyzing cells of the mediobasal hypothalamus by single-cell sequencing.

12.6 Key Literature

Farkas et al. (2020) Demonstration of the existence of a microcircuit in the median eminence in which the activity of TRH axons stimulates endocannabinoid synthesis in tanycytes, while in turn tanycytes, via endocannabinoids, restrain the delivery of TRH from axon terminals to the anterior pituitary.

Fekete et al. (2004) First report demonstrating that endotoxin causes a marked increase in D2 exclusively in tanycytes, raising the possibility that upregulation of tanycyte D2 is responsible for suppression of the thyroid axis associated with the nonthyroidal illness syndrome.

Mohacsik et al. (2016a) Direct evidence that increased D2 activity in tanycytes in response to LPS increases T3 signalling in the mediobasal hypothalamus.

Sanchez et al. (2009) First report demonstrating the presence of PPII in tanycytes and its regulation by thyroid hormone, providing a new mechanism for feedback regulation of the thyroid axis.

Tu et al. (1997) First report demonstrating that tanycytes express one of the highest concentrations of type 2 iodothyronine deiodinase (D2) in the central nervous system, raising the possibility that tanycyte D2 contributes to feedback regulation of the thyroid axis.

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Correction to: Control of Systemic Metabolism by Astrocytes in the Brain

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Glossary

A1/A2 Catecholaminergic cell groups in the caudal ventrolateral medulla and nucleus of the solitary tract, respectively, that express the neurotransmitter noradrenaline and project to various targets, including neuroendocrine cells in the hypothalamus

Adenosine triphosphate (ATP) A compound that mediates the transfer of energy in energy-dependent cellular processes. Also acts as a signaling molecule by activating purinergic receptors expressed on other cells and has been shown to play a role as a neurotransmitter and a gliotransmitter

Adenosine A nucleoside that is the product after complete ATP hydrolysis, as well as a transmitter acting on P1 purinergic receptors

Adrenoceptors, adrenoreceptors, or adrenergic receptors A class of receptors that bind noradrenaline and adrenaline

Amoeboid microglia Microglia with short and thick processes and in general a rounded shape

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor The primary glutamate-gated excitatory ion channel (passes the cations Na^+ and K^+) expressed postsynaptically at glutamate synapses

Anorexigenic Causing anorexia or a loss of appetite

Anorexigenic signal Signal that blocks food intake

Anteroventral periventricular nucleus (AVPV) Brain region that plays a crucial role in estrogen positive feedback signaling

Astrocyte endfeet Astrocyte specialized processes extending from the soma that are in contact with the basement membrane, which encircles the endothelial cells and pericytes of blood vessels

Astrocyte A subtype of glial cell with a star-shaped cell body that helps neurons maintain homeostasis and plays important roles in the blood–brain barrier, brain development, maintenance of extracellular ion concentrations, regulation of synaptic communication and immune response; most abundant type of glial cell found in the brain

Astrocyte–neuron lactate shuttle Model that theorizes that neurons are mostly oxidative and astrocytes mostly glycolytic cells and that astrocytes accumulate

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glucose that they process to generate lactate, which is then transferred from astrocytes to neurons where the lactate is further oxidized to generate energy

Astrocytic processes Fine, lamellate branches of astrocytes that ensheathe neuronal elements, including synapses. Distal astrocytic processes contain little cytoplasm and lack most intracellular organelles

Astrocytogenesis The generation of astrocytes from neural progenitor cells

Astroglia ensheathment Wrapping of cellular components by astrocytic processes

ATP Adenosine triphosphate The main substrate for performing cellular work and that is also released as a transmitter acting on purinergic P2 receptors

Autonomic nervous system A division of the peripheral nervous system that includes both the sympathetic and parasympathetic nervous systems to unconsciously control peripheral organ function and bodily functions, including but not limited to heart rate, digestion, respiratory rate, and immune function

Blood–brain barrier (BBB) A semipermeable barrier of endothelial cells, pericytes, and astrocytes, separating the circulating blood from the brain and the cerebrospinal fluid. It represents a highly selective barrier to the passage of cells, particles, and large molecules from the peripheral circulation into the brain

Border-associated macrophages (BAMs) Unique and distinct populations of macrophages that reside along CNS borders

CaMKIIα Ca^{2+} calmodulin-dependent protein kinase II is a protein kinase important for long-term plasticity at glutamate synapses at the postsynaptic site

Cannula A tube inserted into the body to deliver, remove, or collect fluid samples

Central nervous system The portion of the nervous system that includes the brain and spinal cord

Chemokines Small cytokines originally named for their ability to direct the function and chemotaxis of immune cells to sites of immune activation

Chemo-sensor A sensory receptor that detects and transduces a chemical signal into a neural response

Circumventricular organs Brain structures characterized by extensive and highly permeable capillaries, which are in contact with blood and cerebrospinal fluid

Clonal cell line A population of identical cells originally derived from a single cell

CNS parenchyma Functional tissue in the CNS that is comprised of neurons and glial cells

Corticotropin-releasing hormone (CRH) A peptide hormone produced in the hypothalamus and released into the pituitary portal circulation to trigger the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland

Co-transmission When a nerve terminal releases not one but multiple neurotransmitters

Cre-LoxP recombination system Genetic tool to investigate genes of interest in a particular tissue, cell, and/or time. Cre-recombinase protein recognizes and excises floxed gene loci (i.e., located between two LoxP sites) to delete, insert, or translocate a gene of interest at specific sites in the cellular DNA

Cytokines Small signaling molecules secreted from immune cells that direct immune function

Deiodinase Selenium-containing enzymes that catalyze the activation or inactivation of thyroid hormone

D-Serine A co-agonist of the NMDA receptor that binds to GluN1 subunits, and also a ligand for the GluD2 ionotropic glutamate receptor. This amino acid is released from astrocytes during the induction of long-term plasticity

Endocannabinoids Lipid-based, endogenous cannabinoid signaling molecules, including arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol, that act as retrograde neurotransmitters and bind to type 1 and type 2 cannabinoid receptors (CB1 and CB2)

Endothelial cells The main cell type lining the lumen of blood vessels

Ependymoglial cells Bipolar cells in contact with ventricular surface with processes that extend to basal lamina

Epigenetic Non-genetic influences on gene expression

Epinephrine A hormone produced primarily by the adrenal glands that is an essential component of the stress response, mobilizing the availability of glucose and stimulating the activation of the sympathetic nervous system to increase heart rate and respiratory rate

Erythro-myeloid progenitors (EMPs) A second lineage of progenitor cells with hematopoietic potential that exist in the yolk sac and can generate both erythroid and myeloid lineages

Estrogen positive feedback Elevated levels of estradiol that induce a surge release of luteinizing hormone that causes ovulation

Estrogens A class of steroid hormones that are known primarily as a female sex hormone given its primary production in the ovaries and its impact on female reproductive function. It also influences the function of other physiological functions, including immune function and neural function

Excitatory postsynaptic potential/current (EPSP/C) The voltage (potential) or current measured postsynaptically using electrophysiology when an excitatory neurotransmitter such as glutamate is released from a nerve terminal and binds to a postsynaptic neuron, activating ligand-gated ion channels

Fenestrated capillaries Capillaries with pores that allow the diffusion of small molecules into and out of the blood

GABA Gamma aminobutyric acid, the main inhibitory neurotransmitter in the central nervous system, released at GABAergic synapses

GFAP (Glial Fibrillary Acidic Protein) An intermediate filament protein that is commonly used to identify astrocytes, although not all astrocytes express GFAP. GFAP immunoreactivity is visible in cell bodies and in thick primary and secondary processes of astrocytes

Ghrelin A peptide hormone that is predominantly produced in the gut and triggers neurobehavioral signals to stimulate feeding behavior

Glia/Glial cell Non-neuronal cells found in the nervous system that comprise astrocytes, oligodendrocytes, Schwann cells, microglia, satellite cells, tanycytes, and ependymal cells

Glia-derived When transmitter molecules are released from astrocytes (gliotransmitters)

Glial ensheathment The act whereby glial cells use their processes to cover nearby neurons

Glioblast Cell that is able to differentiate into different types of neuroglia

Gliogenesis Development of non-neuronal glial cells from multipotent neural stem cells

Gliosis A non-specific change occurring in glial cells (e.g., number, morphology, molecular appearance) upon central nervous system insult. Gliosis has been described as a reactive state of microglia and astrocytes

Gliotransmission Active and rapid transfer of information from glial cells to surrounding cells (i.e., neurons) through the release of neuroactive molecules, e.g., via regulated exocytosis of gliotransmitters (molecules released by glial cells)

Gliotransmitter A signaling molecule released by glial cells that modulates neighboring glial or neuronal cells

Glucocorticoids A class of steroid hormones that bind to glucocorticoid receptors. They are produced in the adrenal glands and are an essential component of the stress response, mobilizing the availability of glucose in the body and inhibiting the function of physiological processes involved in homeostasis, including immune function

Glutamate receptors Membrane-bound receptors that result in postsynaptic excitation of cells and include the ionotropic NMDA, kainate and AMPA receptors, and the metabotropic mGlu1-8 receptors

Glutamate transporters Specialized carrier molecules in the cell membrane that allow the transport of glutamate across a membrane either into cells or cytoplasmic vesicles

Glutamate An amino acid and the main excitatory neurotransmitter in the central nervous system, released from glutamatergic synapses

Gonadotropin-releasing hormone (GnRH) A peptide hormone released from the hypothalamus that stimulates the release of gonadotropins from the anterior pituitary gland

$G_{q/11}$ A subtype of alpha subunit for g-protein signalling that is responsible for activating the diacylglycerol and IP3-Ca^{2+} pathways

Hematopoiesis The generation of blood cells

Hematopoietic stem cells (HSCs) Immature cells or blood stem cells that can develop into different blood cells

Heterosynaptic Referring to a synapse that is different from, and usually adjacent to the stimulated or active synapse

Hippocampus Brain region in the temporal lobe and a part of the limbic system that is responsible for the formation of different types of declarative memory

HNS (hypothalamo-neurohypophysial system) Magnocellular neuroendocrince cells of the hypothalamus located in the supraoptic and paraventricular nuclei and that project their axons to the neurohypophysis, where they release directly in the bloodstream the peptidergic hormones oxytocin and vasopressin

Homeostasis Stability in body equilibrium and physiological processes

Hyperphagia An abnormal increase in food intake

Hypophysiotropic That which acts on the pituitary gland

Hypothalamic magnocellular neurons Neuroendocrine cells of the HNS that extend their axons down the pituitary stalk to contact fenestrated capillaries in the neurohypophysis and collateral projections to central targets such as the central amygdala

Hypothalamic paraventricular nucleus (PVN) A collection of cells in the hypothalamus surrounding the third ventricle. Resident populations of cells include the magnocellular neuroendocrine cells that express either oxytocin (OT) or vasopressin (VP), the parvocellular neuroendocrine cells that express corticotropin-releasing hormone (CRH) or thyrotropin-releasing hormone (TRH), and preautonomic neurons that project caudally to the brainstem and spinal cord

Hypothalamic Supraoptic Nucleus (SON) A collection of cells in the hypothalamus close to the optic chiasm. This nucleus exclusively contains magnocellular neuroendocrine cells expressing vasopressin (VP) or oxytocin (OT)

Hypothalamic-pituitary-thyroid (HPT) axis Interactive neuroendocrine system involved in the regulation of circulating thyroid hormone levels

Hypothalamus A collection of brain nuclei at the base of the brain just dorsal to the pituitary gland, responsible for neuroendocrine and autonomic homeostatic control of the organism

Immortalized Proliferating indefinitely

Innate immune receptor Germline-encoded receptors that are able to recognize evolutionarily conserved molecular patterns such as bacterial lipopolysaccharide or peptidoglycan

Insulin/leptin resistance Improper or attenuated responses to insulin/leptin characterized, in part, by reduced activity of signaling pathways downstream of the respective receptors

Intracerebroventricular An invasive technique of injecting substances directly into the cerebrospinal fluid in cerebral ventricles to bypass the blood–brain barrier

Ionotropic Ion-passing channels

IP3/inositol triphosphate Intracellular lipid signalling molecule that actuates Ca^{2+} release from internal stores after gating IP3 channels/receptors

KARs (kainate receptors) A family of cationic glutamate receptor channels with different permeabilities to Ca^{2+} ions. Presynaptic kainate receptors may control glutamate or GABA release through a metabotropic mechanism

Kisspeptin A peptide that regulates the release of GnRH

Long-term depression (LTD) A type of long-lasting synaptic plasticity whereby the amplitude of the EPSP/C is decreased following repetitive stimulation

Long-term potentiation (LTP) A type of long-lasting synaptic plasticity whereby the amplitude of the EPSP/C is increased following repetitive stimulation

LPS (Lipopolysaccharides) Component of the cell wall of gram-negative bacteria that can stimulate the release of inflammatory cytokines

Luteinizing hormone (LH) A gonadotropin released by the anterior pituitary gland that causes ovulation and the formation of the corpus luteum in females and testosterone production in males

Macrophage Phagocytic immune cell that plays a role in the detection, engulfment, and clearing of apoptotic cells and cells infected by pathogens

Macrophages of the choroid plexus Immune cells that reside in the choroid plexus of the brain, a structure that produces cerebrospinal fluid (CSF). These cells monitor the CSF for pathogens or metabolic products

Magnocellular neuroendocrine cell (MNC) A large neuron that secretes a peptide/hormone primarily into the blood circulation to affect target cells or target tissues outside the nervous system. The large majority of MNCs reside in the hypothalamic paraventricular and supraoptic nuclei, approximately half of which secrete oxytocin and the other half vasopressin into the bloodstream via axonal projections to the posterior pituitary gland

Mast cells Immune cells derived from myeloid stem cells that contain granules filled with histamine and heparin, and are best known for their role in allergic responses. They are an important component of the immune response and can influence neural function

Median eminence Important midline structure located in the basal hypothalamus ventral to the third ventricle comprising a functional component of the hypophyseal portal system that connects the hypothalamus with the pituitary gland. It is here that all hypophysiotropic hormones are secreted into the portal blood and conveyed to the pituitary gland

Meningeal macrophages Immune cells that reside in the lymphatic system of the brain located in the meninges. These cells survey and clear the meningeal lymphatic system of pathogens or cell debris

mEPSC A 'miniature' excitatory postsynaptic current that occurs when neurotransmitter is released spontaneously in the absence of a presynaptic action potential

Metabotropic receptors G protein-coupled receptors that stimulate intracellular second messenger cascades

mGluR (metabotropic glutamate receptor) mGluRs are G-protein coupled receptors coupled to phospholipase C and intracellular calcium signaling (Group I) or negatively coupled to adenylyl cyclase (Groups II and III). mGluRs can control neuronal excitability by modulating K^+ and Ca^{2+} conductances via a large variety of intracellular messengers. mGluRs couple to a variety of different intracellular g-protein cascades in neurons and glial cells

Microglia The primary innate immune effector cell that resides in the brain and the spinal cord (CNS). These phagocytic immune cells, often refered to as the resident macrophages of the CNS, are responsible for immune surveillance, being the first responders to injury in the CNS, and are the main producers of

pro-inflammatory cytokines in the brain. They also participate in basic neural functions

Monocyte White blood cell, or leukocyte, that is involved in the innate immune system and can differentiate into macrophages or dendritic cells

Neurodevelopmental disorders Disorders that have their origins in development and affect the developing nervous system, including but not limited to autism spectrum disorders, schizophrenia, learning disabilities, motor disabilities, attention and hyperactivity disorders, and many communication disorders

Neuroectoderm Also known as neural ectoderm, is comprised of cells derived from the ectoderm and is a tissue formed early in the development of the nervous system

Neuro-glio-vascular unit Functional unit of neurons, astrocytes, pericytes, and endothelial cells working together

Neuroinflammation The brain's immune response to acute injury or intruders, which is characterized by a concerted action of microglia and astrocytes, accompanied by release of pro-inflammatory cytokines that can lead to neuronal loss in extreme cases

Neuronal encoding Describes how neurons represent information through electrical activity

Neuronal plasticity The ability of the nervous system to modify its phenotype in response to changes in internal or external environment. Neuronal plasticity allows adaptation to changing environmental conditions and physiological demands

Neuroprogesterone Progesterone that is synthesized de novo within the brain that plays a critical role in the luteinizing hormone surge

Neurosteroids Steroid hormones that are synthesized de novo within the central nervous system

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) Transcription factor involved in the regulation of inflammatory responses

Nitric oxide (NO) A gaseous signaling molecule that can activate the cGMP/PKG pathway

NMDA receptors Glutamate-gated excitatory ion channels permeable to Na^+, K^+ and $Ca2^+$ primarily expressed postsynaptically at glutamate synapses. These cationic-receptor channels, highly permeable to Ca^{2+} ions, are blocked by Mg^{2+} ions at voltages close to the resting potential and opened with depolarization, which confers strong voltage dependence. Their activation requires the presence not only of glutamate but also of a co-agonist (glycine or D-serine)

Noradrenaline/Norepinephrine (NA/NE) Catecholamine neurotransmitter/neuromodulator released in the central and peripheral nervous system from noradrenergic nerve terminals. This neurotransmitter has a variety of functions in the stress response, behavioral arousal, and the sympathetic nervous system

Nucleotidases/ecto-ATPase Extracellular enzymes that hydrolyze ATP into ADP, AMP and adenosine

Nucleus tractus solitarius (NTS) Large brainstem nuclei receiving and integrating peripheral visceral inputs concerning body homeostasis and containing the A2 noradrenergic cell group

Oligodendrogenesis The generation of oligodendrocytes from neural progenitor cells

Orexigenic Increasing appetite

Orexigenic signal Signal that promotes food intake

Oxytocin (OT) A nonapeptide hormone produced in MNCs of the hypothalamus and secreted from the posterior pituitary gland that has peripheral (parturition, lactation-milk ejection reflex, natriuresis) and central (regulation of visceral functions, anxiety, fear, social cognition, and affiliative behaviors) functions

P1 receptors Purinergic receptors that primarily bind adenosine

P2 receptors Purinergic receptors that primarily bind ATP

P2X receptors Ionotropic P2 receptors/channels

P2X7 receptor A specific type of non-selective cationic P2 receptor/channel that is gated by high concentrations of ATP

Paracrine regulation Form of cell signaling in which one cell secretes a substance that affects a nearby cell

Paraventricular nucleus of the hypothalamus (PVN) Bilateral basal forebrain nucleus located in the medial hypothalamus that contains three major neuron types: magnocellular neuroendocrine cells, parvocellular neuroendocrine cells, and parvocellular preautonomic cells

Parturition Expulsive phase of child birth

Parvocellular neuroendocrine cells Neurosecretory cells in the hypothalamus, including the CRH neurons, that secrete peptide hormones into the pituitary portal circulation to regulate secretion from the anterior lobe of the pituitary gland

Pericyte Contractile mural cells of the microvasculature that wrap around endothelial cells and are involved in the control of cerebral blood flow

Perinatal depression A type of mood disorder that presents in women around the time of birth, either during pregnancy (prepartum) or after birth (postpartum). The symptoms include sadness, anxiety, irritability, sleep disturbances, decreased energy, and changes in appetite.

Periphery Regions of the organism that are outside of the central nervous system

Perivascular macrophages Immune cells that reside in the vasculature of the brain, within the blood–brain barrier that can respond to pathogens and initiate an inflammatory immune response that can be transmitted to the microglia residing in the brain

Perivascular space Fluid-filled space that surrounds some blood vessels and varies in dimension according to the type of blood vessel (also known as Virchow–Robin)

Phagocytosis Ingestion of pathogens or debris by phagocytic cells

Phagosome A vacuole that contains phagocytosed particles enclosed within a cell membrane and located within the cytoplasm of a cell

Phenotype A set of observable characteristics that can result from the interaction between genotype (genetics) and environment

PI3-K Phosphoinositide 3-kinases are a class of kinases (protein that tags phosphate groups on other proteins) involved in growth, proliferation and differentiation

Platelet-derived growth factor receptor (PDGFR) β and PDGFRα Specific cellular markers for pericytes and OPCs, respectively

Progestins A class of steroid hormones that are known primarily as a female sex hormone given its production in the ovaries and its impact on female reproductive function and its ability to support pregnancy. It also influences the function of many other physiological functions, including immune function and neural function

Prostaglandins Physiologically active lipid compounds that have hormone-like effects. Signaling molecules derived by enzymatic conversion of lipid molecules in the cell membrane. Prostaglandins are found in many tissues throughout the body and direct inflammation

Pyroglytamyl peptidase II (PPII) Membrane-bound metallopeptidase that inactivates TRH in the extracellular space

Ramified microglia Microglia with long and thin processes that branch out and are dynamic for surveying their surrounding

Reactive oxygen species (ROS) Highly oxidative free radicals that participate in cell signaling in homeostasis, but can damage cellular structures when dramatically elevated

Satiety The state of feeling sated or full

Semi-supervised clustering A type of clustering analysis that aims to organize "similar" data items (genes) in a cluster, which is guided by limited supervision from an available source of external knowledge

Sexual dimorphism Observable differences in appearance or characteristics between males and females of the same species

Sickness behavior A coordinated set of adaptive behaviors that occur in response to immune activation, most often associated with a pathogen. These behaviors include, but are not limited to, lethargy, sleep, hyperalgesia, loss of appetite, social withdrawal, and depression. These behaviors are directed by immune activation and are highly conserved across animal species

Single-cell RNA sequencing RNA expression profiles of individual cells

SNARE proteins A complex of proteins involved in vesicle exocytosis, such as associated with the release of transmitters

Somato-dendritic release Neuropeptide (e.g., oxytocin or vasopressin) or neurotransmitter (e.g., dopamine) release from the somatic and/or dendritic compartment of a neuron that mediates autocrine and paracrine interactions

Sphingolipid A category of lipids containing a sphingosine backbone

Stereotactically Employing a three-dimensional coordinate system mainly used to locate brain structures

Supraoptic nucleus of the hypothalamus (SON) Bilateral basal forebrain nucleus located in the basolateral hypothalamus that contains magnocellular neuroendocrine cells that secrete oxytocin or vasopressin

Synapse An inter-neuronal junction by which neurons communicate via electrical or a chemical neurotransmission

Synaptic plasticity When synapses undergo increases or decreases in the strength of communication between neurons

Tanycytes Specialized cells that line the floor and ventrolateral walls of the third ventricle between the rostral and caudal limits of the hypothalamic median eminence

Taurine Amino acid that is one of the main osmolytes used by cells to compensate for changes in extracellular osmolarity; serves as gliotransmitter in the HNS

Testosterone A steroid hormone that is known primarily as a male sex hormone given its primary production in the testes and its impact on male reproductive function. It also influences the function of many other physiological functions, including immune function and neural function

Th2-type Refers to a type of immune response that is named for the T helper cells (T_h cells), which are an important component of the adaptive immune response that, as opposed to a Th1-type response, is anti-inflammatory in nature and includes the production of cytokines and immune signals that either dampen or stimulate an alternate immune response

Third ventricle One of the four cavities of the ventricular system within the brain that is filled with cerebrospinal fluid

Transcytosis Transportation of macromolecules across the cell. A method of transcellular transport in which a cell enfolds extracellular large substances by endocytosis and then the vesicle moves across the cell to release the large substances at the opposite cell membrane by exocytosis

Tripartite synapse A synaptic complex that includes and mediates intercommunication between a presynaptic neuron, a postsynaptic neuron, and a glial cell to control synaptic function. This is a twist from the original concept of the synapse only considering neuronal pre- and post-synaptic elements. In the tripartite synapse, astrocyte processes ensheathe the neuronal axons and spines in the synapse and functionally influence synaptic activity

Two-photon microscopy Type of optical microscopy in which fluorescence of a fluorophore or uncaging is achieved by the simultaneous absorption of two photons

Uncaging Using a particular wavelength of light to liberate ions or small molecules bound to a larger molecule by a photo-labile bond

Vagus nerve The tenth cranial nerve of the peripheral nervous system and the longest nerve of the autonomic nervous system that controls parasympathetic innervation of peripheral organs, but also relays information about peripheral organs back to the brain

Vasopressin (VP) A nonapeptide hormone produced in magnocellular neuroendocrine cells (MNCs) in the hypothalamus and secreted from the posterior pituitary

gland that mediates a variety of functions in fluid homeostasis, vasoconstriction, stress, and social behavior. Also known as the antidiuretic hormone (ADH) because it increases water reabsorption in the kidney and hence decreases urine output

Vascular endothelial growth factor receptor (VEGFR)2 When activated, this receptor is a positive regulator of angiogenesis by facilitating proliferation of endothelial cells

Ventral glia lamina (VGL) Ventral part of the supraoptic nucleus along the base of the brain, where the cell bodies of radial glia-like astrocytes are lined up

Yolk sac Membranous sac that is formed from cells of the hypoblast (tissue from the inner cell mass) that is attached to the embryo

Index

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Masterclass in Neuroendocrinology 11,
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