Masterclass in Neuroendocrinology 12

Valery Grinevich
Árpád Dobolyi *Editors*

Neuroanatomy of Neuroendocrine Systems

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Neuroanatomy of Neuroendocrine Systems

Springer

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Series Preface

This series began 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, and also includes the study of behaviours and mental states that are influenced or regulated by hormones. In addition, it includes the understanding and study of peripheral physiological systems that are regulated by neuroendocrine mechanisms. While neuroendocrinology embraces many issues of concern to human health and well-being, research in reductionist animal models is required to fully understand these issues.

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

To achieve our aims, each book focuses on a particular theme in neuroendocrinology. For each book, we recruit editors, who are leaders in their field, to engage an international team of experts to contribute chapters in their individual areas of expertise. The mission of each contributor is to provide an update of current knowledge and recent discoveries, and to discuss new approaches, 'gold-standard' protocols, translational possibilities, and future prospects. Authors are asked to write for a wide audience, to use references selectively, and to consider use of video clips and explanatory text boxes; each chapter is peer-reviewed and has a Glossary. In all of these efforts, we are guided by an Advisory Editorial Board.

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)
- *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. JR Lemos & G Dayanithi)
- *Developmental Neuroendocrinology* (2020, ed. S Wray & S Blackshaw)
- *Neuroendocrine Clocks and Calendars* (2020, ed. FJP Ebling & HD Piggins)
- *Glial-Neuronal Signaling in Neuroendocrine Systems* (2021, ed. JG Tasker, JS Bains, & JA Chowen)
- *Neuroanatomy of Neuroendocrine Systems* (2021, ed. V Grinevich & A Dobolyi).

In development are *Neuroendocrinology of Pregnancy and Lactation* (ed. P Brunton & D Grattan) and *Neuroendocrine-Immune System Interaction* (ed. JP Konsman & T Reyes).

Feedback and suggestions are welcome.

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Preface

Neuroendocrine systems represent a critically important regulatory element of our physiology as they master regulate hormonal responses in the body. The hypothalamus connects hormonal responses via the pituitary with behavioural responses exerted by the nervous system. Our understanding of the neuroendocrine system increased tremendously in recent years due to the appearance of novel experimental methods, including different transgenic animals, opto- and chemogenetics, calcium imaging, and fMRI technologies. The use of these advanced tools revealed the functional circuits responsible for hypothalamic regulations as well as fine morphological details of the system, which turned out to be crucial for its proper function. The immense new knowledge on the neuroanatomy of the neuroendocrine system has to be integrated into the established regulatory framework controlling the hormonal status and the emerging novel, mostly behavioural, actions of the hypothalamic systems controlling them. This book addresses this gap in a well comprehensible manner.

The book is written for students, trainees, established researchers, and teachers. The authors are outstanding scientists with world-leading expertise in their respected research fields. This authority guarantees a high quality of the chapters of the book entitled *Neuroanatomy of Neuroendocrine Systems*. The book contains a comprehensive description of the neuroanatomy of hypothalamic neuroendocrine systems in a way that major recent research advances in the field will be covered. First, the hypothalamus and the pituitary will be introduced including ontogenesis, hypothalamic stem cells, and evolutionary aspects with a separate chapter on invertebrate neuroendocrinology. The human hypothalamus will be presented in particular detail using state-of-the-art imaging techniques. The next part of the book will contain chapters about the traditional hypothalamo-hypophyseal systems, such as the magnocellular neuroendocrine cells, emphasizing similarities and differences between oxytocinergic and vasopressinergic neurons, the hypothalamic neuron types regulating different pituitary hormones including gonadotropin, corticotropin, and thyrotropin. Newly established direct neuronal regulatory functions of brain projections of neurons in the neuroendocrine system will also be covered. Subsequently, complex hypothalamic functions will be addressed, such as the control of circadian rhythm, metabolism, and appetite in relation to specific peptidergic circuits. In addition, to cover the neuroanatomy of the different neuroendocrine

systems, the book also aims to present the neuroendocrine functions closely connected to the structures. Lastly, the book presents the fine organization of neuroendocrine systems and their cytological plasticity. The latest technologies in neuroendocrinology research will be emphasized in each chapter. Thereby, the book will be able to raise awareness within the neuroendocrine community regarding leading-edge research questions addressed by advances in neuroanatomical tools.

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Part I

Structural Components of the Neuroendocrine Systems

Ontogenesis of Hypothalamic Neurons in Mammals

1

Sebastien G. Bouret and Françoise Muscatelli

Abstract

The hypothalamus is an essential component of brain circuits that control critical physiological functions. It plays a particularly important role in regulating energy balance and feeding behaviors. Accumulating evidence suggests that perturbations in hypothalamic development greatly contribute to obesity and metabolic diseases in later life. This chapter will discuss the timelines during which hypothalamic neurons develop, paying particular attention to neurons producing agouti-related peptide/neuropeptide Y, pro-opiomelanocortin, and oxytocin, because of their documented role in feeding regulation. It will also describe hormonal, molecular, and cellular factors related to the development of these neuronal systems. Finally, it will review the role of genetic and nutritional factors in hypothalamic development.

Keywords

Hypothalamus · Development · Pro-opiomelanocortin · Oxytocin · Agouti-related peptide · Neuropeptide Y · Obesity

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Abbreviations

AgRP	agouti-related peptide
AN	accessory nucleus
ARH	arcuate nucleus of the hypothalamus
ATG	autophagy-related protein
AVP	arginine vasopressin
BNST	bed nucleus of the stria terminalis
CTR	calcitonin receptor
DIO	diet-induced obesity
DMH	dorsomedial nucleus of the hypothalamus
E	embryonic day
ERK	extracellular signal-regulated kinase
GHSR	growth hormone secretagogue receptor
GLP1	glucagon-like peptide 1
GLP1-R	glucagon-like peptide 1 receptor
HFD	high-fat diet
LepR	leptin receptor
LHA	lateral hypothalamic area
MC4-R	melanocortin 4 receptor
MRI	magnetic resonance imaging
MTII	melanotan II
NPY	neuropeptide Y
OT	oxytocin
OTR	oxytocin receptor
P	postnatal day
POMC	pro-opiomelanocortin
PVH	paraventricular nucleus of the hypothalamus
PWS	Prader-Willi Syndrome
RAMP	receptor activity-modifying protein
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
STAT	signal transducer and activator of transcription
VMH	ventromedial nucleus of the hypothalamus
Y1R	neuropeptide Y receptor 1
αMSH	alpha melanocyte-stimulating hormone

1.1 Introduction

The hypothalamus is an essential component of neuroendocrine and pre-autonomic circuits that regulate a variety of physiological and behavioral functions such as feeding behavior and energy homeostasis. Classical experiments using physical lesions of specific hypothalamic nuclei and, more recently, studies using genetic

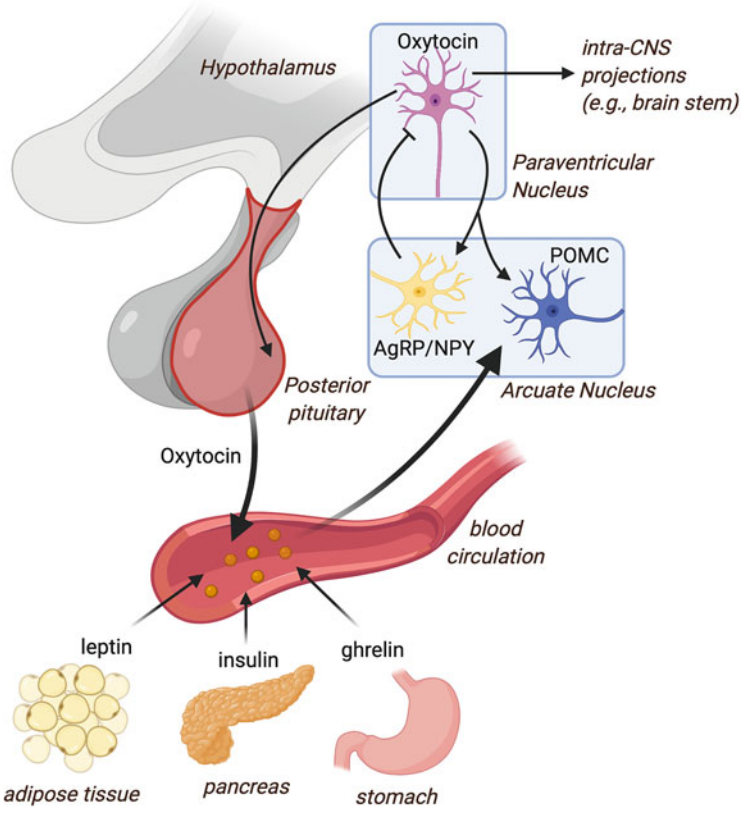
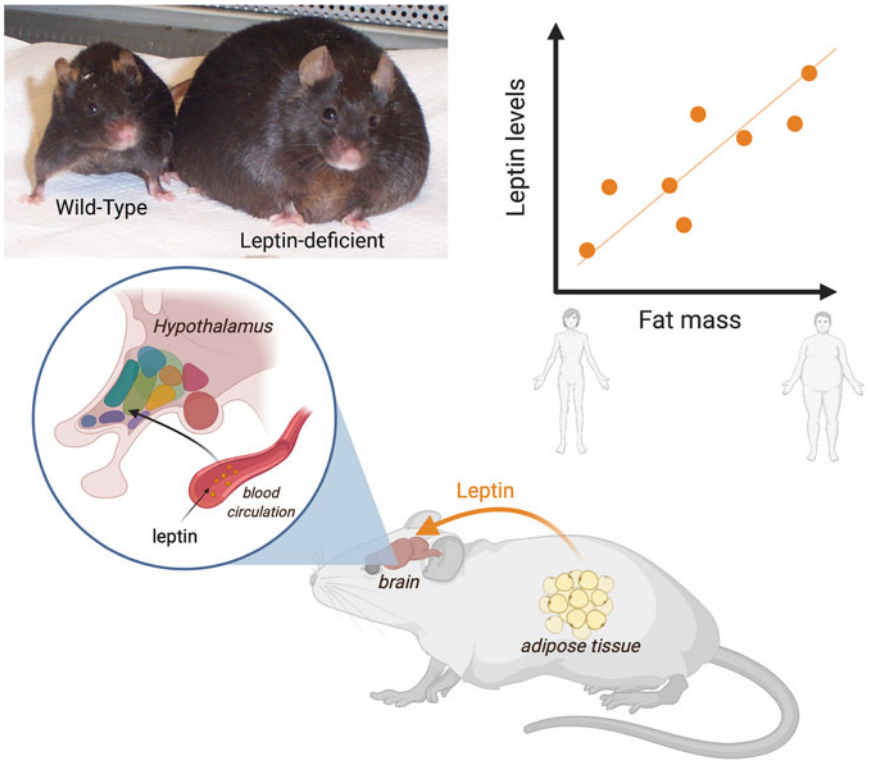


Fig. 1.1 Anatomy of neuroendocrine systems involved in feeding and body weight regulation. Circulating hormones that reflect peripheral energy status, such as insulin, leptin, and ghrelin, act directly on metabolically-relevant neurons within the arcuate nucleus of the hypothalamus, in particular, neurons containing pro-opiomelanocortin (POMC) or co-expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY) to regulate energy balance and glucose homeostasis. These neurons send in turn extensive projections to other parts of the hypothalamus, including oxytocin (OT) neurons of the paraventricular nucleus

neuron-specific approaches have revealed that the hypothalamic regulation of energy homeostasis involves a distributed and interconnected neural network that contains specialized neurons located in the arcuate nucleus (ARH), the ventromedial nucleus (VMH), the dorsomedial nucleus (DMH), the paraventricular nucleus (PVH), and the lateral hypothalamic area (LHA) (for review, see chapter from Gruber et al.). The ARH is the primary site for integrating endocrine signals such as leptin, insulin, and ghrelin (Fig. 1.1). The best-characterized ARH neuronal populations are neurons that co-express agouti-related peptide (AgRP) and neuropeptide Y (NPY) and neurons that produce pro-opiomelanocortin (POMC) (Fig. 1.1). AgRP/NPY neurons are orexigenic, which means that they increase appetite and induce hyperphagia, while POMC neurons are anorexigenic, meaning that they decrease appetite. Both



Box 1.1 Leptin was discovered by positional cloning of the *obese* (*ob*) gene. Mutation of the *ob* gene, for example in leptin-deficient *ob/ob* mice, results in profound obesity. Plasma leptin levels correlate positively with fat mass, meaning that the higher the fat mass, the higher the plasma leptin level. It is secreted in the blood by fat cells and acts through the brain, and particularly the hypothalamus, to mediate its anorexigenic effects

NPY/AgRP- and POMC-containing neurons send extensive axonal projections to other hypothalamic nuclei, including the PVH, DMH, and LHA, which in turn project to other intra- and extra-hypothalamic sites to regulate feeding. Projections to the PVH are of particular importance because this nucleus is the most thoroughly characterized hypothalamic interface between the endocrine, autonomic, and somatomotor systems that influence feeding behavior and energy metabolism (Sawchenko 1998; Sawchenko and Swanson 1983). At the core of PVH neuroendocrine and somatomotor circuits are oxytocin (OT) neurons, which are located in magnocellular neurosecretory and parvocellular parts of the PVH (Fig. 1.1). In short, magnocellular OT neurons send axons directly to the posterior pituitary to release OT peripherally, whereas parvocellular OT neurons send ubiquitous projections within the CNS, including the brain stem, to exert central actions (Lee et al. 2009) (see chapter from Althammer et al.) (Fig. 1.1). Of note, magnocellular OT neurons are also found in the supraoptic (SON) and accessory nuclei (AN) of the

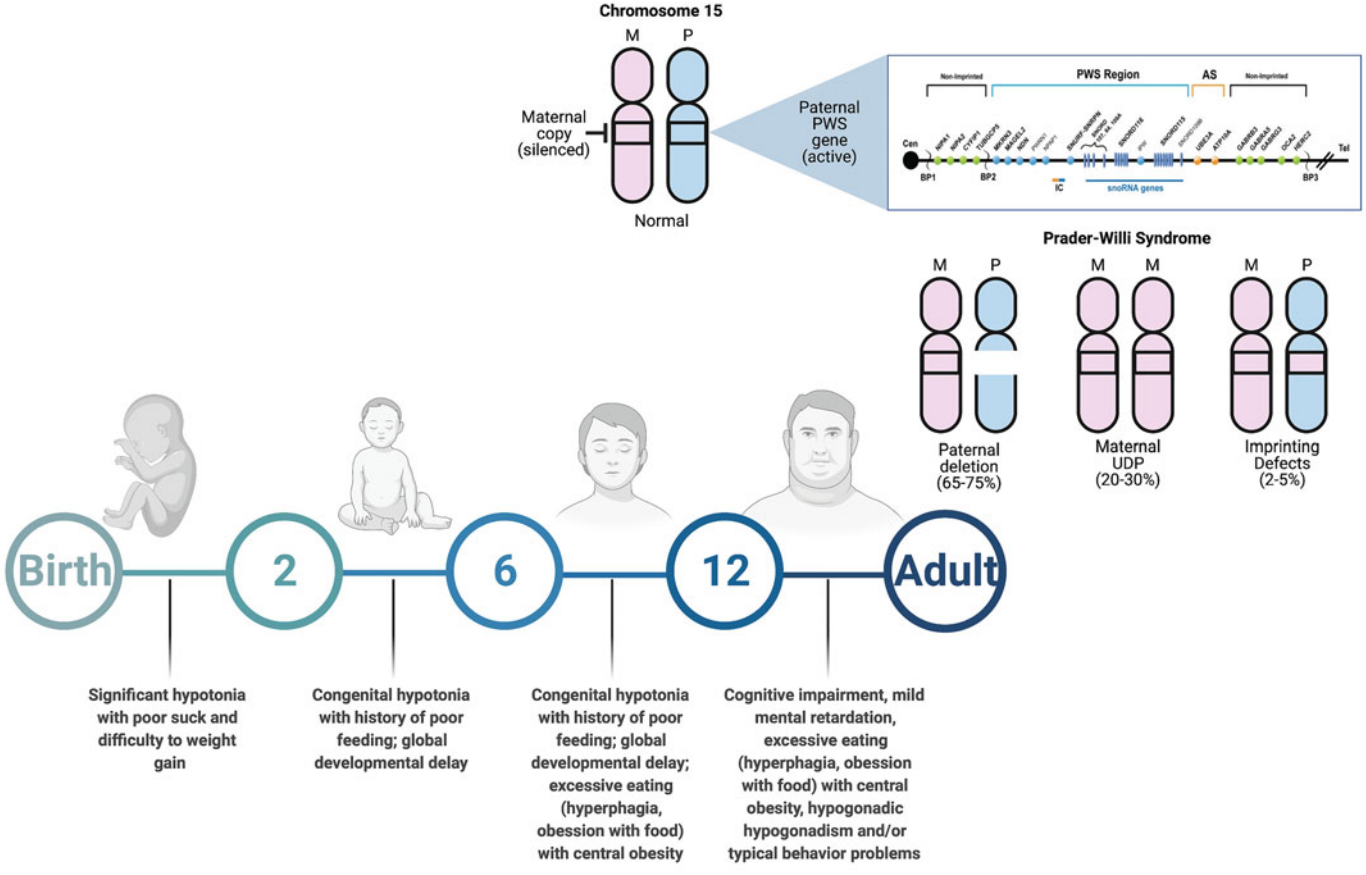
hypothalamus and OT neurons have been reported in the medial amygdala and the bed nucleus of the stria terminalis (BNST) (Jurado 2020). The molecular signature of magnocellular *versus* parvocellular OT neurons remains largely unknown but their differences are based on their morphology (magnocellular OT neurons are large whereas parvocellular OT neurons are smaller), location, afferent and efferent projections, and electrophysiological activity (see chapter from Althammer et al.). In addition to their role in feeding regulation, OT neurons are well known to play an essential role in other physiological processes, such as lactation and uterine contraction, and in social cognition and behavior (see chapter from Althammer et al.). OT neurons are innervated by arcuate POMC and AgRP/NPY neurons, and recent studies have shown the importance of OT neurons in mediating the effects of AgRP neurons on feeding behavior (Atasoy et al. 2012). In this chapter, we will describe the major steps and factors underlying the ontogenesis of POMC, AgRP/NPY, and OT neurons.

1.2 Major Stages of Hypothalamic Development

Hypothalamic development begins soon after the formation and closure of the neural tube, where a tight spatial and temporal regulation of transcription factors and signaling molecules shapes hypothalamic morphogenesis and cellular specification. These early phases of hypothalamic development have been elegantly reviewed by Diaz and colleagues (Diaz and Puelles 2020). The hypothalamus then develops in 4 well-defined phases: (1) neurogenesis, (2) migration, (3) axon growth, and (4) synapse formation.

1.2.1 Neurogenesis

The hypothalamic primordium arises from cells located in the ventral tube of the diencephalon at embryonic day 9 (E9) in the mouse and E10 in the rat. Cells that compose hypothalamic nuclei are primarily derived from precursor cells located in the proliferative zone, which is in the inner and the lower portion of the third ventricle and is also known as the neuroepithelium of the third ventricle (Sauer 1935). During early stages, the neuroepithelium is a one-cell-thick layer but thickens as proliferation progresses. A key event in the formation of hypothalamic neurons is the terminal mitosis, *i.e.* the withdrawal of dividing neuronal precursor cells from the mitotic cycle. The birth of hypothalamic cells was first characterized using the thymidine incorporation assay. This empirical approach uses a radioactive nucleoside, [^{3}H]thymidine, which is incorporated in the nuclear DNA during the S-phase of the cell cycle. By injecting pregnant rats with [^{3}H]thymidine at various stages of embryonic development, Altman and Bayer (1986) and Ifft (1972) reported that the majority of cells located in the hypothalamus were born between E13 and E15 in rats. Using a similar approach, Shimada and Nakamura found that most neurons in the mouse hypothalamus were born between E10 and E14 (Shimada and Nakamura



Box 1.2 Summary of the genetic and expression map of the Prader-Willi Syndrome (PWS) region (source https://www.ncbi.nlm.nih.gov/books/NBK1116/). Deletions and other abnormalities of this region located on chromosome 15 result in PWS, which is a rare genetic disease. The phenotype of patients with PWS is complex and includes distinct phases ranging from difficulties feeding prenatally and at birth to hyperphagia and behavioral problems during adulthood

1973). More contemporary non-isotopic methods that use the thymidine analog bromodeoxyuridine 5-bromo-2′-deoxyuridine (BrdU) confirmed that the vast majority of mouse hypothalamic neurons are born between E10 and E16, with a sharp peak of neurogenesis occurring at E11–E12 (Ishii and Bouret 2012; Padilla et al. 2010). Neurons in the DMH and PVH and the LHA are born between E12 and E14 in mice. The ARH and LHA exhibit a more extended neurogenic period. Many neurons in these nuclei are born at E10–E12, but some neurons are generated as late as E16 in both mice and rats (Brischoux et al. 2001b; Croizier et al. 2010; Ishii and Bouret 2012). VMH neurons are also born during a relatively long neurogenic period. Many neurons in these nuclei are born on E12 in mice and E13 in rats, but some neurons are generated as late as E16 in mice and E15 in rats (Ishii and Bouret 2012; McClellana et al. 2006).

1.2.2 Neuronal Migration

Following a final mitotic event, a cell must migrate (or not) to a destination among potential hypothalamic destinations to join particular cell groups and form a nucleus. Two types of migration occur during hypothalamic development: radial migration, in which cells migrate toward the surface to form the mantle layer, and tangential migration, in which neurons move in trajectories that are parallel to the ventricular surface. One of the best-characterized migration routes is that of neurons located in the VMH (McClellana et al. 2006). Although VMH cells undergo final mitotic divisions as early as E10, the earliest sign of cytoarchitectonic boundaries visible in Nissl-stained sections is not seen until E16–E17 in mice and E18–E19 in rats (Coggeshall 1964; Hyyppä 1969). To form the VMH, postmitotic neurons migrate radially away from the third ventricle guided by radial glial processes and tangential to such fibers, often along with neuronal processes (Rakic et al. 1994). A new prosomeric model of hypothalamic development based on the antero-posterior and ventro-dorsal axis replaces the columnar morphological model (Diaz and Puelles 2020). In contrast to the "inside-out" pattern of other brain structures such as the cortex, hypothalamic neurons are born in an "outside-in" pattern, which means that the earliest-born cells in the hypothalamus migrate the farthest from the ventricle. For example, LHA neurons located next to the third ventricle are generated after those located close to the cerebral peduncle (Brischoux et al. 2001a; Croizier et al. 2011; Risold et al. 2009).

1.2.3 Axon Growth

Differentiated neurons must send out axonal processes to other target neurons to convey neuronal information and control behavior. Some hypothalamic neurons, such as ARH neurons, have relatively short axons and connect primarily to neurons within the hypothalamus (Bouret et al. 2004a). Other hypothalamic neurons, such as LHA and PVH neurons, send long axons to distant targets, including the brainstem

or the cortex (Saper et al. 1979; Swanson and Kuypers 1980). In part because of their importance in appetite regulation, the first systematic studies that examined the development of hypothalamic feeding projections examined the ontogeny of projection pathways from the ARH. Axonal tracing experiments revealed that ARH axons develop postnatally during distinct temporal domains (Bouret et al. 2004a). On postnatal day 6 (P6), ARH projections extend through the periventricular zone of the hypothalamus to provide inputs to the DMH, followed by inputs to the PVH between P8 and P10. ARH projections to LHA develop a bit later, with the mature innervation pattern first apparent on P12. The pattern of ARH axonal projections achieves a distribution resembling that seen in the adult around P18 (Bouret et al. 2004a). In contrast to the development of projections from the ARH, efferent projections from the DMH to the PVH and LHA are fully established by P6 (Bouret et al. 2004a). Also, projections from the VMH form prior to those from the ARH. By P10 VMH axons provide strong inputs to the LHA whereas, at this age, the LHA is almost devoid of axons from the ARH (Bouret et al. 2004a). In addition, LHA neurons send axonal projections embryonically soon after their birth and differentiation, *i.e.*, around E11–E12 (Croizier et al. 2011). Similarly, the neurohypophyseal pathway from the PVH to the median eminence and projections from magnocellular neurons to the posterior pituitary appear to be primarily formed prenatally (Daikoku et al. 1984; Makarenko et al. 2000; Wu et al. 1997). Together, these anatomical observations indicate that hypothalamic axon growth is a dynamic and relatively long developmental process that starts during mid-gestation and continues well past the second week of postnatal life.

1.2.4 Synapse Formation

The formation of synapses follows the development of axon projections. The gold standard method to visualize synapses is electron microscopy. This technique was used very effectively by Matsumoto and colleagues in the 1970s, who reported a gradual increase in the number of synapses in the ARH from birth to adulthood (Matsumoto and Arai 1976). Ultrastructural analysis of synapses within the rat ARH revealed very few axodendritic or axosomatic synapses on P5. In contrast, by P20 (*i.e.*, just before weaning), about one-half of the synapses found in adult animals are already formed. The number of synapses found in the ARH continues to increase after weaning to reach an adult-like pattern by P45 (Matsumoto and Arai 1976).

1.3 Ontogenesis of POMC and AgRP/NPY Neurons

1.3.1 Timelines of POMC and AgRP/NPY Neuronal Development

Birth dating approaches using BrdU indicated that the majority of POMC and AgRP/NPY neurons in the mouse ARH are born primarily at E11–E12 (Khachaturian et al. 1985; Padilla et al. 2010) (Fig. 1.2). However, some POMC neurons, which are

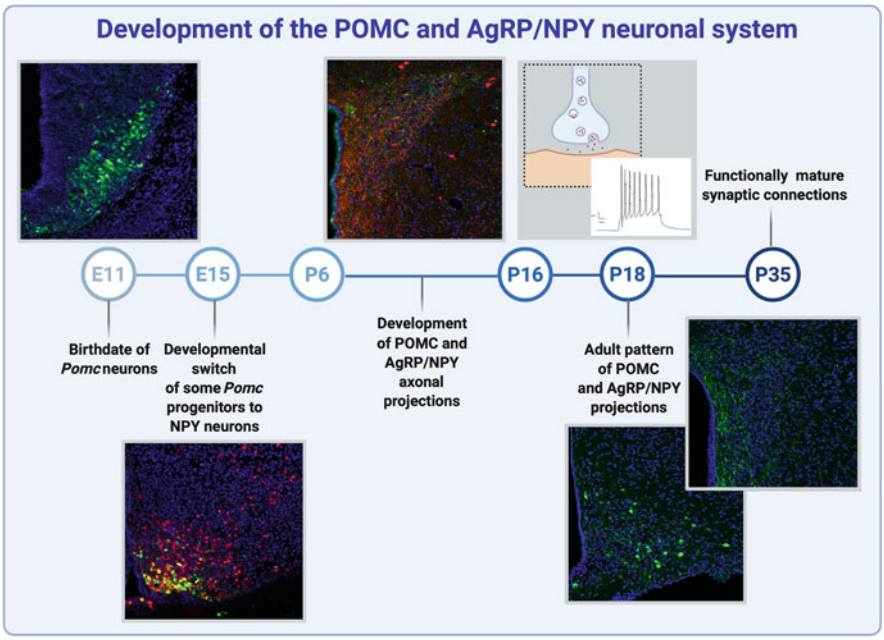


Fig. 1.2 Important periods of POMC and AgRP/NPY neuronal development. The development of POMC and AgRP/NPY neurons begins with neurogenesis followed by neuronal specification during the embryonic life. In contrast, POMC and AgRP/NPY axon growth and synapse formation occur postnatally

located more laterally in the ARH, are generated as late as E13. Gene expression studies showed that neurons in the presumptive ARH begin to express *Pomc* mRNA at E10–E12, whereas *Npy* mRNA expression is not observed until E14 (Padilla et al. 2010). These observations are consistent with the early determination of *Pomc* cell fate. Intriguingly, genetic cell lineage tracing studies revealed that only a portion of embryonic *Pomc*-expressing precursors adopts a POMC fate in adult mice. Half of the *Pomc*-expressing precursors acquire a non-POMC fate in adult mice, and nearly one-quarter of the mature NPY neurons in the ARH share a common progenitor with POMC cells (Padilla et al. 2010; Diaz and Puelles 2020). These data show the unique property of *Pomc*-expressing progenitors with respect to giving rise to antagonistic neuronal populations. The development of axonal projections from POMC and AgRP/NPY neurons occurs significantly later. Using immunohistochemical techniques Grove and colleagues reported that projections immunopositive for AgRP/NPY are immature at birth and develop mainly during the second week of postnatal life in rats (Grove et al. 2003). The same temporal pattern was observed for the development of POMC projections in mice (Nilsson et al. 2005b; Diaz and Puelles 2020).

The POMC-derived peptide αMSH and AgRP modulate the activity of the melanocortin 4 receptor (MC4R). Whereas αMSH activates MC4R, AgRP acts as

an endogenous inverse agonist of MC4R, which means that it suppresses constitutive MC4R activity and simultaneously antagonizes the effects of αMSH. *Mc4r* mRNA is first expressed at E12 in the proliferative zone surrounding the lower portion of the third ventricle (also known as the neuroepithelium), and its expression peaks at E16 (Mountjoy and Wild 1998). These findings are particularly interesting because, as described above, it is known that neurons that compose various hypothalamic nuclei in adults are primarily derived from precursors that originate from this proliferative zone, raising the possibility that MC4R could be involved in hypothalamic neurogenesis. However, further studies are needed to determine the developmental stage during which MC4R becomes functional. Nevertheless, the fact that peripheral injection of the MC4R agonist melanotan II (MTII) reduces milk intake and body weight as early as during the first two weeks of postnatal life suggests that MC4R receptors are present and functional in the hypothalamus at this stage (Glavas et al. 2007). Consistent with this idea, *in situ* hybridization analysis showed that *Mc4r* mRNA is abundant in the hypothalamus, especially in the PVH at P10. That peripheral injection of MTII induces strong induction of cFos immunoreactivity (a marker of neuronal activation) in the PVH at P5–P15 further supports the functionality of MC4R in the PVH during early postnatal life (Glavas et al. 2007). Similar to MC4R, NPY receptors are present and functional soon after birth. As early as P2, a low density of NPY Y1 receptors is detected in neuronal cell bodies in the rat ARH, DMH, and PVH (Grove et al. 2003). However, Y1R was not found in axons in these regions at this age. The density of Y1R in ARH, DMH, and PVH cell bodies and fibers increased at P5–P6 and peaked around P15–P16. The finding that microinjection of NPY directly into the PVH at P2 resulted in increased milk and water intake suggests that NPY receptors may be present and functional in the PVH before innervation of this nucleus by ARH AgRP/NPY fibers (Capuano et al. 1993). Electrophysiology can also be used to study when functional synapses are forming. Using this approach, Melnick and colleagues showed an age-dependent increase in the electrophysiological response of specific sets of PVH neurons to melanocortins, with a maximal response observed at P28–P35 (Melnick et al. 2007). These results suggest that synapses between POMC and AgRP axons and PVH target neurons are not structurally and functionally mature until puberty (Diaz and Puelles 2020).

1.3.2 Hormonal Factors That Influence POMC and AgRP/NPY Neuronal Development

1.3.2.1 Leptin

The discovery of leptin led to a paradigm shift in understanding how food intake and body weight can be powerfully and dynamically regulated by hormonal signals (Kojima et al. 1999; Nakazato et al. 2001; Zhang et al. 1994) (for more information see Box 1.1). In 1994, Friedman and colleagues used positional cloning and found that the *ob* gene encodes the hormone leptin, which is secreted by the adipose tissue in proportion to its mass (Zhang et al. 1994). Subsequently, other groups reported

that leptin administration reduces body weight and food intake in leptin-deficient mice and humans (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995).

Box 1.1 The discovery of leptin and how it revolutionized our understanding on how peripheral factors control feeding through brain mechanisms

Leptin is a 16-kDa protein secreted by fat cells that acts as a crucial signal for body energy stores. It is found in the blood circulation in proportion to fat mass and functions to reduce feeding behavior and promote energy expenditure. Early studies in two mutant strains of mice, the obese mouse (*ob/ob* mouse) and the diabetic mouse (*db/db* mouse) supported the concept of peripheral control of feeding and adiposity Coleman 1973; Coleman and Hummel 1969). The *ob/ob* mouse is characterized by obesity, increased adiposity, hyperglycemia, and hyperinsulinemia (Bray and York 1979). In 1994 Zhang and colleagues used positional cloning to identify the *obese* (*ob*) gene as the affected mutation in this mouse and showed that it encoded the hormone leptin, which is synthesized and secreted by white adipose tissue (Zhang et al. 1994). Shortly thereafter, the *db* locus was identified as encoding the long form of the leptin receptor (LepRb) (Chen et al. 1996; Lee et al. 1996). In the subsequent years there has been explosive progress that demonstrated that leptin primarily acts on the brain to mediate its effects on feeding and energy balance. Key observations include that central injections of leptin reduce body weight in *ob/ob* mice, but not in *db/db* mice (Halaas et al. 1997; Pelleymounter et al. 1995). Moreover, brain-specific deletion of leptin receptors results in a phenotype that is a virtual carbon copy of whole-body leptin receptor-deficient db/db mice (Cohen et al. 2001; McMinn et al. 2005), whereas transgenic brain-specific reconstruction of leptin receptors in *db/db* mice ameliorates obesity (de Luca et al. 2005; Kowalski et al. 2001). The hypothalamus has traditionally been the focus of studies on obesity, owing not only to its central role in neuroendocrine functions and feeding behavior, but also to the fact that it contains the highest density of leptin receptors of any brain region (Caron et al. 2010). Accordingly, leptin acts directly on neurons located in various parts of the hypothalamus, to induce its effects on feeding and energy balance regulation.

A few years later, Ahima and colleagues reported that circulating leptin levels exhibit a distinct surge between P8 and P12 in mice (Ahima et al. 1998), yet exogenous leptin does not modulate food intake, growth, or energy expenditure at this developmental stage (Ahima and Hileman 2000; Mistry et al. 1999; Proulx et al. 2002; Schmidt et al. 2001). Instead of regulating food intake and body weight acutely, leptin appears to be an important neurodevelopmental factor that influences hypothalamic development. Axonal labeling of ARH axons combined with

immunohistochemical analyses showed that POMC and AgRP neuronal projections are disrupted in leptin-deficient (*ob/ob*) mice (Bouret et al. 2004b). The exact sites of action for the developmental effects of leptin include at least a direct action on ARH neurons, because leptin induces neurite extension from isolated organotypic explants of the ARH *in vitro* (Bouret et al. 2004b). Remarkably, leptin appears to exert its developmental action on POMC neural projections during a discrete developmental critical period: exogenous leptin treatment up to P28 rescues AgRP projections in *ob/ob* mice (Bouret et al. 2004b; Kamitakahara et al. 2017). In contrast, leptin treatment of *ob/ob* mice after P28 is relatively ineffective because it does not increase the density of either POMC or AgRP fibers in the PVH to levels that are characteristic of wild-type mice (Bouret et al. 2004b; Kamitakahara et al. 2017). Together, these observations suggest the existence of a critical period for the neurotrophic effect of leptin on POMC and AgRP/NPY circuits that closes around puberty. More in-depth studies have examined leptin receptor signaling pathways that mediate the axonotrophic effect of leptin. The leptin receptor exists in several alternatively spliced isoforms, of which only the long form (LepRb) associates with Janus kinase 2 to mediate intracellular signaling. LepRb initiates multiple intracellular signal transduction pathways upon leptin binding that result in the activation of STAT family transcription factors, extracellular signal-regulated kinases (ERK), and phosphoinositol-3 kinase/Akt. During development, POMC and AgRP/NPY neurons express LepRb (Caron et al. 2010), and leptin administration in mouse neonates results in the activation of major LepRb signaling pathways, including STAT3, ERK, and Akt (Bouret et al. 2012; Caron et al. 2010). Disruption of POMC and AgRP axonal projections is observed in mice or rats that lack functional LepRb signaling (*db/db* mice and *fa/fa* rats, respectively) (Bouret and Simerly 2007; Bouret et al. 2012). Moreover, lack of functional LepRb→STAT3 signaling *in vivo* (*s/s* mice) or *in vitro* results in a reduced density of POMC fibers without altering the development of AgRP projections, showing the importance of this signaling pathway, specifically in the development of POMC neural projections (Bouret et al. 2012). However, not all LepRb signaling pathways play a role in the formation of ARH projections. For example, mice that lack LepRb→ERK signaling (*l/l* mice) display comparable densities of POMC- and AgRP- axons in the PVH compared to wild-type mice (Bouret et al. 2012).

1.3.2.2 Ghrelin and Growth Hormone

Ghrelin is a 28-amino acid hormone peptide that is produced mainly by the stomach and is an endogenous ligand for the growth hormone secretagogue receptor (GHSR). It is one of the most potent orexigenic signals, and exerts its action on food intake by stimulating AgRP/NPY neurons that in turn inhibit POMC neurons (Cowley et al. 2003; Tschöp et al. 2000). Although the marked orexigenic effect of ghrelin is not yet present before weaning in mice or rats (Piao et al. 2008; Steculorum et al. 2015), ghrelin in early postnatal life does have a lasting developmental effect on the hypothalamic circuits involved in energy homeostasis, and influences body weight in adulthood (Steculorum et al. 2015). Mice injected with an anti-ghrelin compound during neonatal life display increased densities of POMC- and AgRP-containing

axons innervating the PVH. These structural alterations are accompanied by long-term metabolic defects, including elevated body weight, fat mass, and hyperglycemia (Steculorum et al. 2015). However, if adult mice are treated with the anti-ghrelin compound, it does not alter POMC and AgRP circuits (Steculorum et al. 2015). These findings suggest that, similar to leptin, the developmental action of ghrelin on arcuate projections is restricted to a neonatal critical window. The site of action for the developmental effects of ghrelin is likely to include direct action on arcuate neurons because direct exposure of isolated ARH explants to ghrelin inhibits axonal outgrowth (Steculorum et al. 2015). It also interacts with LepRb→STAT3 signaling to block the neurotrophic effect of leptin (Steculorum et al. 2015).

Ghrelin is a potent stimulator of growth hormone secretion (Tolle et al. 2001). Based on the documented finding that ghrelin influences hypothalamic development and can interact with leptin receptor signaling (Steculorum et al. 2015), it is not surprising that deletion of the growth hormone receptor in *Leprb*-expressing cells also alters the development of POMC and AgRP neuronal circuits (Wasinski et al. 2020). In addition, selective loss of the growth hormone receptor in AgRP neurons affects AgRP axonal projections without affecting POMC circuits (Wasinski et al. 2020) demonstrating a cell-autonomous effect of growth hormone on AgRP neuronal development.

1.3.2.3 GLP1

The incretin hormone glucagon-like peptide 1 (GLP1) is secreted postprandially by intestinal enteroendocrine cells to promote satiety and glucose-induced insulin release (Drucker 1998). Administration of the GLP1-R agonist exendin-4 during the first week of postnatal life decreases the density of NPY fibers innervating the PVH and has a protective effect against both age-related and diet-induced obesity (Rozo et al. 2017). Moreover, genetic deletion of *Glp1r* in *Sim1* neurons of the PVH reduces AgRP and NPY projections while it increases POMC projections to the PVH (Rozo et al. 2017).

1.3.2.4 Amylin

Amylin is a hormone produced by pancreatic β-cells and is co-released with insulin in response to caloric intake. The amylin receptor comprises the core calcitonin receptor (CTR), which heterodimerizes with one or several receptor activity-modifying proteins (RAMP-1, -2, and -3). The primary role of amylin in adults is to reduce food intake by promoting meal-ending satiation and maintaining glucose homeostasis. During development, amylin is detected in the blood circulation of embryos, where it appears to act through RAMP1-3 to influence neurogenesis of POMC neurons (Li et al. 2020). During postnatal life, amylin continues to be secreted in the blood circulation (Abegg et al. 2017), and loss of *amylin* or *Ramp1/3* disrupts the development of POMC and AgRP projections to the PVH (Lutz et al. 2018).

1.3.3 Molecular Programs of POMC and AgRP/NPY Neuronal Development

1.3.3.1 Transcription Factors

Homeobox genes belong to a class of transcription factors that play important roles in regionalization, patterning, and cell differentiation during embryogenesis and organ development. The homeobox genes orthopedia (*Otp*), *Nkx2.*1 and *Bsx* are highly expressed in the ventral hypothalamus during embryonic development. Loss of function studies indicated that while *Nkx2.1 and Otp* and are essential for the normal morphological development of the hypothalamus, including the ARH, *Bsx* is not required (Acampora et al. 1999; Kimura et al. 1996; Wang and Lufkin 2000). The homeobox gene *Nkx2-1*, also known as thyroid transcription factor 1 (*Ttf-1*), plays a particularly important role in ARH specification. Ablation of *Nkx2-1* impairs the formation of the ventral hypothalamic primordium resulting in the absence of ARH formation (Kimura et al. 1996; Marín et al. 2002). However, the VMH, DMH, and LHA are present. Expression of *Nkx2-1* in postmitotic cells suggests that it plays a further role in differentiation and maintenance of ARH neurons in the ventral portion of the hypothalamus (Sussel et al. 1999; Yee et al. 2009). Deficiency in *Nkx2.1* prior to the onset of *Pomc* expression markedly reduces *Pomc* cell numbers (Orquera et al. 2019). However, the number of NPY neurons was not affected in *Nkx2.1* knockout mice, and POMC neuronal cell number was not affected if *Nkx2.1* deletion occurred in postmitotic *Pomc* neurons (Orquera et al. 2019). The LIM-homeodomain transcription factor Islet 1 (*Isl1)* is up-regulated in hypothalamic *Nkx2.1* progenitor cells at E10, *i.e.,* just before the onset of neuropeptide expression (Lee et al. 2016; Nasif et al. 2015). Consistent with the role of *Isl1* in the phenotypic determination of ARH neurons, loss-of-function studies revealed that *Isl1* promotes the terminal differentiation of *Pomc*, *Agrp,* and *Npy* expression (Lee et al. 2016; Nasif et al. 2015). In contrast, deletion of the transcription factors *Dlx1/2* in *Nkx2.1-expressing progenitors* increased *Agrp* expression without affecting *Pomc* expression (Lee et al. 2018). More in-depth molecular studies revealed that *Dlx1/2* controls *Agrp* expression by binding to and repressing the expression of the homeodomain transcription factor orthopedia (*Otp*) that is also known to influence ARH morphogenesis (Acampora et al. 1999; Lee et al. 2018; Wang and Lufkin 2000). Thus, the loss of *Otp* in *Agrp* neurons results in a dramatic reduction in the number of *Agrp* mRNA-expressing cells (Hu et al. 2020). Notably, *Otp* expression is absent in the hypothalamus of *Isl1* knockout embryos (Nasif et al. 2015) suggesting that, in addition to *Dlx1/2*, *Isl1* is also required for the expression of *Otp* in the future ARH. Sonic hedgehog (*Shh*), SIX Homeobox 3 (*Six3*), and the retinal anterior neural fold homeobox (*Rax*) were also identified as critical regulators of ventral hypothalamus development and POMC development. *Shh* signaling increases *Nkx2-1* expression (Manning et al. 2006) and deletion of *Shh* in *Nkx2-1* progenitors affects the development of POMC neurons (Shimogori et al. 2010). *Six3* is a regulator of forebrain development, including the hypothalamus (Lagutin et al. 2003), and is required for *Shh* expression (Geng et al. 2008). Finally, *Rax* is also important for the formation of the ventral neural tube. Mice lacking *Rax* in *Six3*-expressing cells do

not show ventral hypothalamic *Nkx2.1* expression and never express POMC (Lu et al. 2013).

The Oligodendrocyte transcription factor family (*Olig1* and *Olig2*) are basic helix-loop-helix (bHLH) transcription factors highly expressed in the periventricular regions of the brain such as the hypothalamus (Takebayashi et al. 2000; Zhou et al. 2000). Despite its name, *Olig* is expressed not only in oligodendrocytes but also in neural cell progenitors. Lineage tracing experiments indicate that a number of POMC and NPY cells derive from *Olig1* progenitors (Peng et al. 2012). The majority of *Olig1* progenitors also express Bone morphogenetic protein receptor 1A (Bmpr1A) (Peng et al. 2012) and when Bmpr1A is deleted in *Olig1*-expressing cells, it decreased and increased the number of POMC and AgRP neurons, respectively (Peng et al. 2012). Neurogenin 3 (*Ngn3)* is another bHLH transcription factor expressed in hypothalamic progenitors, and it plays an opposite role in the specification of *Pomc* and *Npy* neurons: while it promotes the embryonic development of *Pomc* neurons, it inhibits *Npy* neuronal development (Anthwal et al. 2013; Pelling et al. 2011). However, not all POMC neurons are derived from *Ngn3* progenitors. Using a mouse model of *Mash1* deficiency, McNay and colleagues further reported that this bHLH transcription factor has a pro-neural function and acts upstream of *Ngn3* to regulate neurogenesis in the ventral hypothalamus (McNay et al. 2006). Loss of *Mash1* blunts *Ngn3* expression in ARH progenitors and is associated with a dramatic reduction in the number of POMC and NPY neurons (McNay et al. 2006). The Notch signaling pathway also appears to mediate its developmental effects on POMC and NPY neuronal development through Mash1. Mice lacking Notch signaling in *Nkx2.1*-expressing cells display an increased number of POMC and NPY neurons associated with an induction in *Mash1* expression (Aujla et al. 2013; McNay et al. 2006). In addition, mice with a constitutively active Notch1 intracellular domain show a complete loss of POMC and NPY neurons (Aujla et al. 2013), mirroring the effects of *Mash1* deficient mice (McNay et al. 2006).

As described in 3.1, *Pomc*-expressing progenitors in the ARH have the unique property of differentiation into functional mature NPY neurons (Padilla et al. 2010). Recent work in our laboratory investigated the molecular mechanisms involved in this developmental switch and identified miR-103/107 as candidates that may be involved in the maturation of *Pomc* progenitors. Loss of the microRNA(miRNA)-processing enzyme *Dicer* increases the proportion of *Pomc* progenitors acquiring an NPY phenotype (Croizier et al. 2018). Moreover, silencing of miR-103/107 specifically decreases the number of *Pomc*-expressing cells and increases the proportion of *Pomc* progenitors differentiating into NPY neurons (Croizier et al. 2018). Postnatal maintenance of *Pomc* and *Npy* peptidergic identity also depends on the expression of the transcription factor T-box 3 (*Tbx3*) (Quarta et al. 2019). Because the majority of miRNAs exert their effects on gene expression by targeting transcription factors, it could be interesting to study whether there is a link between miRNAs, *Tbx3,* and *Pomc* and *Npy* gene expression.

1.3.3.2 Axon Guidance Molecules

Axons grow by sending out a highly plastic and sensitive structure called a "growth cone," which travels toward the target and trails behind it the elongating neurite. As described in 3.2. metabolic hormones, including leptin, are critical factors influencing initial POMC and AgRP/NPY axon outgrowth. Growing POMC axons must then choose a path to follow and decide the direction to go on this path to innervate the proper nucleus (*e.g.*, the PVH). The pathways are defined by cell–cell interactions and diffusible chemorepulsive and chemoattractive cues (Tessier-Lavigne and Goodman 1996). The diffusible axon guidance cues semaphorins are highly expressed in the PVH during development, and POMC neurons express the semaphorin receptors neuropilin 1 and 2 (van der Klaauw et al. 2019). Supporting a role for neuropilins/semaphorins in POMC axon guidance, a loss of neuropilin 2 receptors in POMC neurons specifically disrupts the development of POMC axonal projections to the PVH (van der Klaauw et al. 2019). These structural alterations are accompanied by metabolic dysregulation, including increased body weight and glucose intolerance (van der Klaauw et al. 2019). Notably, exome sequencing experiments identified variants in the semaphorin and neuropilin families associated with severe obesity in humans (van der Klaauw et al. 2019) demonstrating the translational importance of these findings. The formation of POMC neuronal connectivity also involves cell–cell contact proteins. Supporting this idea, POMC neurons exhibit enriched expression of the *Efnb1* (EphrinB1) and *Efnb2* (EphrinB2) genes during postnatal development, and loss of *Efnb1* or *Efnb2* in *Pomc*-expressing progenitors decreases the amount of excitatory glutamatergic inputs (Gervais et al. 2020). In addition, mice that are deficient in *contactin*, a cell adhesion molecule involved in the formation of axonal projections, have a reduced density of POMC fibers in the PVH during postnatal development (Nilsson et al. 2005a).

1.3.4 Cellular Factors Underlying the Development of POMC and AgRP/NPY Neurons

1.3.4.1 Autophagy

Axonal growth involves a dynamic remodeling of cytosolic structures and requires protein degradation and turnover to replace damaged organelles and proteins. The maintenance of cell function and growth is achieved with autophagy, which is an important cellular degradation system that engulfs parts of the cytoplasm and organelles within double-membrane vesicles, known as autophagosomes, to turnover and recycle these cellular constituents (Klionsky 2007). This cellular process is also critical in the supply of nutrients for survival during starvation. Constitutive autophagy is detected in the hypothalamus, including in ARH POMC neurons, during critical periods of axon growth and development (Coupe et al. 2012). Loss of the autophagy-related protein (*Atg*) gene *Atg7* disrupts the maturation of POMC axonal projections and causes lifelong metabolic perturbations (Coupe et al. 2012). As described above, leptin is a critical neurotrophic factor for POMC circuits, and

direct crosstalk between leptin and autophagy during perinatal life has been described (Park et al. 2020a). Supporting a role for autophagy in mediating the trophic effects of leptin, the loss of autophagy in POMC neurons exacerbates the metabolic and neurodevelopmental deficits observed in leptin-deficient mice (Park et al. 2020a).

1.3.4.2 Primary Cilia

Another cellular signaling system that plays an important role in brain development and function is the primary cilium, which is an organelle found at the cell surface of most mammalian cells, including hypothalamic neurons (Fuchs and Schwark 2004). For example, during embryonic development, primary cilia are important mediators of sonic hedgehog signaling, which is a critical regulator of the ventral patterning of the hypothalamus (Carreno et al. 2017). Moreover, a strong interaction between primary cilia and autophagy has been reported, including in the developing hypothalamus (Lee et al. 2020). Cilia begin to be observed in hypothalamic neurons as early as on E12, and the number and length of primary cilia gradually increase thereafter to reach an adult-like pattern at P14 (Lee et al. 2020). Disruption of cilia formation in developing POMC neurons, but not in adult POMC neurons, increases body weight, fat mass, and food intake, reduces energy expenditure, and alters glucose homeostasis during adult life (Lee et al. 2020). Neuroanatomically, a transient reduction in the number of POMC neurons was observed in mutant mice at weaning, but POMC cell numbers were normal in adult mice. This reduction in POMC cell numbers during the pre-weaning period is attributed to a decrease in neurogenesis during embryonic development (in opposition to an effect on apoptosis) and the adult normalization in POMC cell number to a compensatory increase in neurogenesis after weaning (Lee et al. 2020). Loss of primary cilia also alters axonal and dendritic growth, resulting in a reduced density of POMC fibers innervating the PVH, DMH, and LHA (Lee et al. 2020). It also blocks the ability of leptin to promote the development of POMC projections in *ob/ob* mice (Lee et al. 2020). Because a sub-population of *Pomc* progenitor cells also give birth to NPY neurons (see above and (Padilla et al. 2010)), it is not surprising that mice lacking primary cilia from embryonic POMC neurons also display a reduction in NPY cell numbers and NPY fibers innervating the PVH (Lee et al. 2020).

1.4 Ontogenesis of OT Neurons

1.4.1 Timelines of OT Neuronal Development

1.4.1.1 Molecular and Technical Considerations

The OT gene encodes for pre-pro-OT-neurophysin I, a pre-pro-hormone that is cleaved by different enzymes to give rise to different OT intermediate forms and neurophysin I, and then to the mature, amidated form of OT (Gimpl and Fahrenholz 2001; Grinevich et al. 2015) (Fig. 1.3). It is also important to keep in mind that OT and vasopressin (AVP) are similar nonapeptides, differing in two amino acids, and

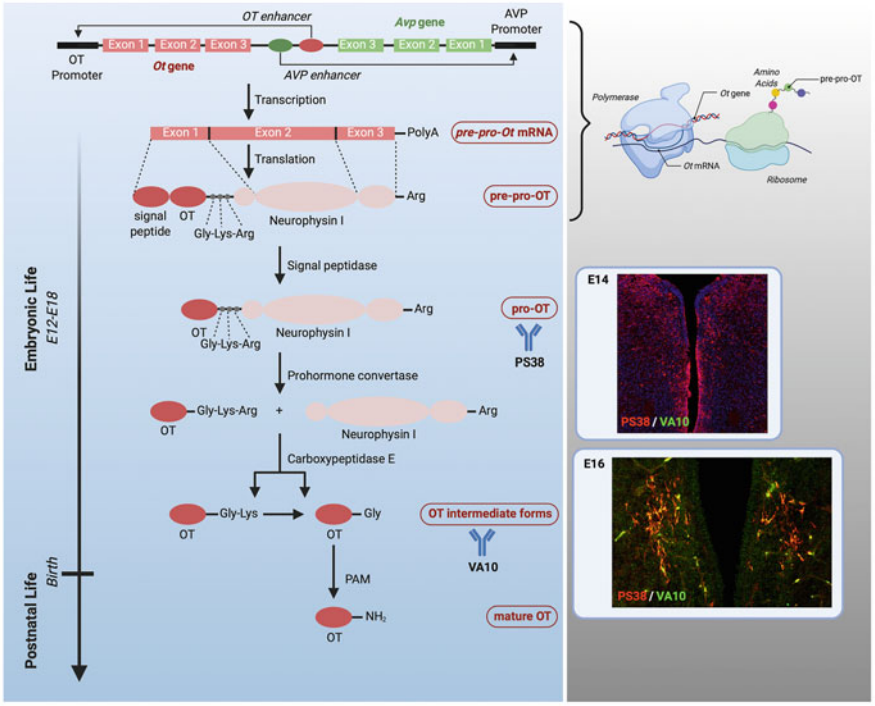


Fig. 1.3 Maturation sequence of oxytocin. Left panel: genomic structure of OT which is contiguous to the AVP gene. The transcription, translation, and maturation steps of OT are developmentally regulated. Right panel: images showing hypothalamic sections derived from E14 and E16 embryos and labeled with PS38 and VA10 antibodies that recognize pro-OT and the intermediate forms of OT, respectively. Note that while intermediate forms of OT are detected at E16, they are not found in the hypothalamus at E14 where only pro-OT is detected

that both AVP and OT neurons are present in the PVH, supraoptic nucleus (SON) and accessory nucleus (AN) of the hypothalamus. The genes encoding AVP and OT are contiguously located in the same chromosomal region and they share common regulatory sequences (Lee et al. 2009) (Fig. 1.3). Like OT, AVP is produced from a precursor protein. Pre-pro-AVP is processed into AVP, neurophysin II, copeptin, and signal peptide (Gimpl and Fahrenholz 2001; Grinevich et al. 2015). Physiologically, the function of AVP neurons is different from that of OT neurons: vasopressin primarily controls fluid balance and blood pressure. Notably, AVP can bind to oxytocin receptors (OTR) and *vice versa* OT can bind to vasopressin receptors, to trigger agonist or antagonist effects depending on the context, although this topic remains a subject of debate.

OT can be detected using different techniques that detect the transcript (mRNA) or the various forms of OT peptide using antibody-based approaches such as radioimmunoassay, enzyme immunoassay, and immunohistochemistry. However, the specificity of the antibodies is not always well established, especially with regard

to their ability to identify different forms of OT. Using the well-characterized antibodies developed by Harold Gainer's laboratory that can specifically detect the immature, intermediate, or mature form of OT (Alstein et al. 1988; Miriam Altstein et al. 1988), studies have identified developmental periods during which OT neurons are found and become mature. More recent work using genetic neuronal labeling and light sheet microscopy followed by 3D reconstructions generated a comprehensive map of OT/AVP neurons and projections from early development to adulthood (Jurado 2020) (https://kimlab.io/brain-map/ot_wiring).

1.4.1.2 Embryonic Development

In rodents, the SON and PVH begin to appear very early. As early as E12, two groups of cells are identified in mouse: one near the third ventricle and another that moves lateral to the pial surface to give rise to the SON (Dongen and Nieuwenhuys 1989). By E14, the SON and PVH are settled (Nakai et al. 1995), while the AN is not distinguishable until later, probably due to its small size and relatively small number of cells (Altman and Bayer 1986). At this stage, an antibody recognizing Neurophysin-I (the carrier protein for OT) shows a positive immunosignal, consistent with the expression of the OT pro-hormone, which is not detected earlier at E12 (Fig. 1.3). Furthermore, an antibody recognizing the intermediate forms of OT (VA10) shows a positive immunosignal of the OT intermediate forms at E16, but not at E14 (Grinevich et al. 2015) (Fig. 1.3). There is dynamic expression of OT and AVP in the developing hypothalamus (Jurado 2020): OT- and AVP-positive neurons can be found in caudal nuclei such as the PVH and SON at E16, but nuclei located in more rostral parts, such as the BNST, remained unlabeled. At birth, whereas the PVH and SON contain neurons co-expressing OT and AVP, SCN neurons express AVP exclusively. The number of OT and AVP neurons increases throughout development in all hypothalamic nuclei. In general, developmental maturation of AVP precedes that of OT, with the expression of the mature form of AVP occurring embryonically, when the mature form of OT is not detected (Jurado 2020).

1.4.1.3 Postnatal Development

Although the OT pro-hormone and intermediate forms are detected during embryogenesis, the mature form of OT is only detected after birth in rodents (Miriam Altstein et al. 1988) (Fig. 1.3). The biological significance of this earlier production of immature forms of OT is unknown, although a functional role of these forms during embryonic development has been suggested (Tribollet et al. 1989). Recently, a transient peak of OT neuron activation has been reported within the first three hours of postnatal life (Hoffiz et al. 2021), but the functional consequences of this peak are not clear. An important maturation step occurs during the first two weeks of postnatal life as evidenced by a progressive maturation of morphological and electrophysiological properties of OT neurons (Fig. 1.4). It was also reported that SON and PVH neurophysin neurons showed dramatic changes in perikaryon size during postnatal development in mice, with a plateau at P5 until P10, followed by a dramatic increase in size that reaches adult values. The late maturation of OT *versus* AVP neurons is also supported by the observation that although AVP neurons

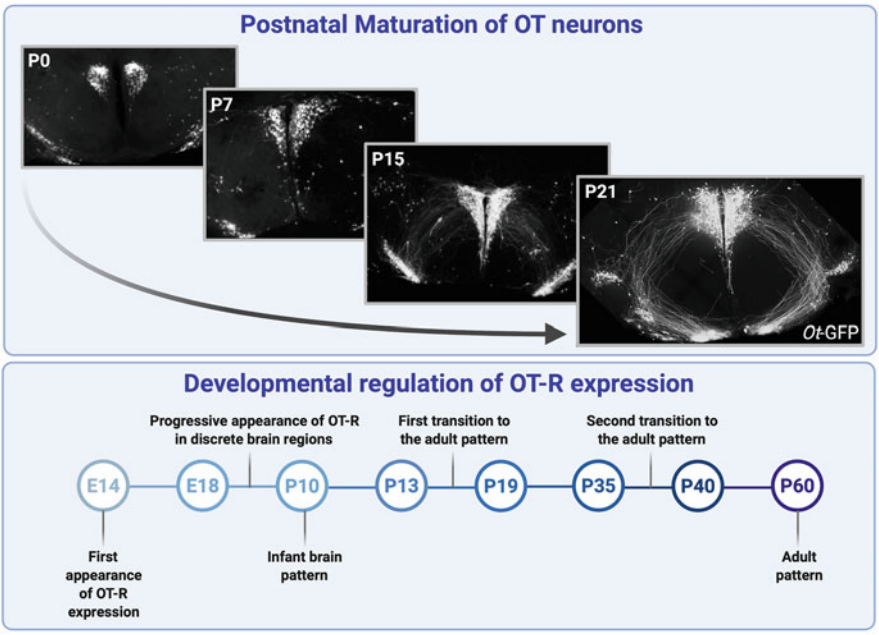


Fig. 1.4 Developmental regulation of OT and OTR. Upper panel: Postnatal maturation of OT neurons. These images show EGFP labeling of OT-neurophysin pre-prohormone in OT-EGFP transgenic mice at P0, P7, P15, and P21. Lower panel: ontogenesis of OTR expression in the developing brain

display long neuritic processes in the PVH at P5, OT neurons only have short processes at this age (Godefroy et al. 2017).

1.4.2 Development of OT Projections

In the classical view, magnocellular OT neurons project to the posterior pituitary to release OT into the blood circulation and parvocellular OT neurons innervate various hindbrain structures, to modulate various aspects of autonomic functions such as breathing, feeding, or cardiovascular responses. Over the past ten years, novel data challenged this view with (1) the demonstration of axon collaterals that innervate various forebrain areas, including the hippocampus, cortex, lateral septum, olfactory nucleus, nucleus accumbens, and amygdala (Althammer and Grinevich 2017; Eliava et al. 2016), and (2) the characterization of somato-dendritic secretion, allowing an OT release in the SON and PVH and possibly in nearby brain areas (see chapter from Brown et al.). Importantly, a functional relationship has also been revealed between parvocellular and magnocellular OT neurons (see chapter from Althammer et al.). A subset of about 30 parvocellular OT neurons have been found to terminate onto magnocellular OT neurons of the SON and neurons of deep layers of the spinal cord,

where they are involved in autonomic functions and the modulation of nociception (Eliava et al. 2016; Jurek and Neumann 2018).

Although central and posterior pituitary projections of OT neurons in adult rodents are well characterized (see https://kimlab.io/brain-map/ot_wiring/), there is a gap in knowledge of when these OT projections develop. Using a neurophysin antibody (which detects both AVP and OT), Silverman *et al.* showed the presence of neurophysin-immunopositive fibers in the posterior pituitary as early as E14 (Silverman et al. 1980). Later on, André Calas' group employed the axonal tracer DiI (which labels PVH fibers independently of their neuropeptide content) and found that the PVH projects to the pituitary at E17 (Makarenko et al. 2000), suggesting that OT neurons in the PVH might project to the posterior pituitary later than OT neurons of the SON. Additional evidence indicated the morphological and electrophysiological immaturity of magnocellular OT neurons at birth and that these neurons continue to develop progressively during the first two postnatal weeks (Widmer et al. 1997).

1.4.3 Developmental Dynamic of Brain OT Receptors

To understand the biological function of OT, it is important to have a comprehensive anatomical map of the cells that express oxytocin receptor (OTR). There is one isoform of OTR, which is a G-protein coupled seven-transmembrane receptor (GPCR). The function of OTR is pleiotropic and *Otr* mRNA is expressed not only in the brain but also in several peripheral tissues (Sun et al. 2019; Yoshimura et al. 1996). The distribution of OTR expression has been examined using different experimental approaches, such as receptor binding of radiolabeled OT on tissue sections, *in situ* hybridization and transcriptomic analysis, and the use of transgenic mice expressing a fluorescent marker under the control of the OTR promoter. More recently, Yongsoo Kim's lab used a fluorescent reporter mouse model (*i.e.*, OTR Venus mice) and established a publicly available brain-wide map of the OTR in mice during postnatal development (https://kimlab.io/brain-map/OTR/) (Newmaster et al. 2020). There is, however, a lack of specific antibodies against the OTR (Grinevich et al. 2015; Vaidyanathan and Hammock 2017).

Comparative analysis of OTR distribution in rodents (*i.e.*, prairie voles, rats and mice) revealed species, sex, and developmental differences in OTR location throughout the brain (see for review Vaidyanathan and Hammock 2017). In the rat brain, OT-binding sites are first detected at E14 in what will become the vagal motor nucleus. *Otr* expression then follows a differential time course, depending on the brain structure considered, with some transient expression detected in several brain regions during the early postnatal period (Tribollet et al. 1989; Yoshimura et al. 1996) (Fig. 1.4). A similar radioligand binding approach was performed in the mouse brain and reported the first detection of OT-binding sites at E16 (Tamborski et al. 2016), with a peak around P14 followed by a decrease in OT-binding sites in all brain regions thereafter (Hammock and Levitt 2013). A strong transient expression of OTR is detected particularly in different cortical regions during postnatal development (Newmaster et al. 2020). Studies in prairie voles also showed a dynamical

expression profile of OTR. To summarize, in rodents (*i.e.*, mice, rats, and prairie voles), *Otr* mRNA and OT binding sites are detected in embryos, even though the mature form of OT is not produced at this stage, and the highest expression of OTR is detected around P14. The distribution of OTRs in the developing brain appears different from that of adult brains with three expression profiles: (1) groups of neurons with early constant expression, where OTR expression begins to be detected during development and is maintained throughout life, (2) sites with transient expression where OTR is observed only during a restricted developmental period and its expression decreases to below the level of detection after that, and (3) another group of neurons with later constant expression, where OTR expression begins to be detected during puberty and is maintained throughout life (Vaidyanathan and Hammock 2017) (Fig. 1.4). OTR expression also appears sexually dimorphic from the early embryonic stages, with a greater *Otr* mRNA expression found in female compared to male brains (Tamborski et al. 2016). OTR is also strongly detected in the peripheral tissues of neonatal mice and prairie voles. In particular, OTR is transiently expressed in the oro-facial region of mouse with marked sex and species differences (Greenwood and Hammock 2017).

1.4.4 Molecular Determinants of OT Neurons

The signaling molecules and transcription factors involved in the determination and differentiation of OT neurons are not well known. As described in 3.3.1, the early patterning of hypothalamus depends on a cascade of transcription factors. The bHLH-PAS (basic helix-loop-helix PER-ART-SIM) transcription factor *Sim1* is expressed in the incipient PVH, SON, AN from E10 (Caqueret et al. 2006) where it dimerizes with *Arnt2* (Hosoya et al. 2001; Michaud et al. 2000). A key downstream target of *Sim1*/*Arnt2* is *Brn2*, a POU domain transcription factor required for *Ot* expression as well as for the expression of *Avp* and corticotropin-releasing factor (Nakai et al. 1995; Schonemann et al. 1995). In a parallel or convergent *Sim1*/*Arnt2* pathway, *Otp* is also necessary for expression of *Brn2* (Caqueret et al. 2006), which is still expressed at E15 with *Nkx2.2*. All of these transcription factors are required to define the prospective PVH domain at E12. However, the factors that will subsequently specify the parvocellular and magnocellular OT neurons have not been identified. The ablation of *Brn2* results in a loss of all neurons of the PVH, SON, and presumably of AN (Nakai et al. 1995; Schonemann et al. 1995). Importantly, a lack of axonal projections of magnocellular OT and AVP neurons to the pituitary has been reported in *Brn2* and in *Arnt2* knockout mice (Hosoya et al. 2001; Schonemann et al. 1995), causing a progressive loss of pituicytes (*i.e.*, pituitary astrocyte-like glial cells). These results suggest a role of OT and/or AVP in the formation of the neurohypophysis. Consistent with this hypothesis, the neurovascular interface in the neurohypophysis does not form in zebrafish lacking OT (Gutnick et al. 2011).

1.5 Consideration of Species Differences in POMC, AgRP/NPY, and OT Neuronal Development

There are marked differences in the normal ontogeny of hypothalamic development between rodents and human and non-human primates. First, the regional development of the rodent hypothalamus proceeds on a timeline of days in rodents *versus* weeks to months in human and non-human primates. Second, although rodents exhibit considerable postnatal hypothalamic development, human and non-human primates undergo considerably more prenatal maturation of hypothalamic circuits. For example, although the hypothalamus is not mature until after weaning in rodents, hypothalamic neurogenesis and axon growth occur primarily during intrauterine life in primates, including humans. In macaques, hypothalamic neurogenesis occurs in the first quarter of gestation (Keyser 1979; van Eerdenburg and Rakic 1994). Reports on human fetal chemoarchitecture and cytoarchitecture have also suggested that early hypothalamic neurogenesis is limited to the ninth and tenth weeks of gestation (Ackland et al. 1983; Bugnon et al. 1982; Burford and Robinson 1982; Koutcherov et al. 2002; Mai et al. 1997). Studies from Kevin Grove and colleagues in Japanese macaques reported that *Pomc* and *Npy* mRNA-containing neurons are found in the ARH of NHP at gestational day (G) 100, but only a few NPY/AgRP fibers and no POMC fibers are detected in the PVH at this age (Grayson et al. 2006). The density of NPY/AgRP fibers innervating the PVH markedly increased at G130 and G170, but POMC fibers only begin to be found in the PVH at G170 (Grayson et al. 2006). In human fetuses, OT is detected as early as 14 weeks of gestation, and adult-like levels of immunoreactive cell numbers are found in the PVH by 26 weeks of gestation (Goudsmit et al. 1992). NPY-immunoreactive fibers are detected in the ARH and the PVH of human fetuses as early as 21 weeks of gestation (Koutcherov et al. 2002).

1.6 Pathological Conditions Associated with Disrupted Development of POMC, AgRP/NPY, and OT Neurons

Obesity is a health condition characterized by an excessive accumulation and storage of fat in the body. It has reached alarming rates worldwide and is associated with several life-threatening diseases, including hypertension and type 2 diabetes. Obesity is determined by genetics and obesogenic environments, such as diets rich in fat and/or sugar. In this section, we will give an example of one genetic disorder (Prader-Willi Syndrome) and one environmental condition (maternal obesity) that have been associated with perturbations in the development of the hypothalamic POMC, AgRP/NPY, and OT systems.

1.6.1 Prader-Willi Syndrome

Prader-Willi syndrome (PWS) is a rare genetic disorder characterized by a variety of neuroendocrine and behavioral dysregulations, including hyperphagia, which can lead to life-threatening obesity. It affects ~1 in 25,000 births (Whittington et al. 2001) and is caused by loss of expression of imprinted, paternally inherited genes on chromosomes 15q11q13 (for more information see Box 1.2).

Box 1.2 Prader-Willi syndrome
Prader-Willi Syndrome (PWS) is a multigenic disorder caused by loss of expression of imprinted, paternally inherited genes on chromosomes 15q11q13. It affects ~1 in 25,000 birth and has a population prevalence of ~1 in 50,000 (Whittington et al. 2001). It was first described in 1956 by endocrinologists Prader, Labhart, and Willi (Prader et al. 1956). Clinically, PWS is characterized by a range of behavioral, physical, and physiological symptoms. It includes diminished fetal activity, hypotonic and feeding problems in infancy, small hands and feet, delayed developmental milestones, characteristic faces, and cognitive impairments (Holm et al. 1993). PWS is also characterized with morbid obesity and severe hyperphagia, a tendency to develop diabetes in adolescence and adulthood, hypogonadotropic hypogonadism, short stature, and sleep disturbances (Holm et al. 1993). These later observations suggest that a dysregulation of neuroendocrine systems may be the basis of some of the symptoms of PWS.

Among the genes inactivated in PWS, *MAGEL2* is of particular interest because it is highly expressed in the hypothalamus, including during perinatal development (Kozlov et al. 2007; Lee et al. 2000, 2003; Maillard et al. 2016). The most common hypothesis is that hypothalamic and pituitary dysfunction is responsible for many of the features of this syndrome. Consistent with the idea that PWS results in structural and functional brain alterations, magnetic resonance imaging (MRI) analyses have shown a reduction in brain volume in individuals with PWS (Mercer et al. 2009; Miller et al. 2007). In addition, functional imaging (fMRI) has shown an altered response of the brain in patients with PWS to metabolic cues, such as glucose and food stimuli (Holsen et al. 2006; Shapira et al. 2005). However, the study of changes in specific neural systems using MRI and fMRI has been limited, in part, by instrument resolution. Nevertheless, the development of pre-clinical animal models, such as mice deficient in *Magel2*, has been instrumental in studying the role of *Magel2* in POMC, AgRP, and OT neuronal maturation. The *Magel2*-null mouse model developed by Francoise Muscatelli's group showed an altered onset of suckling activity and subsequently impaired feeding, leading to 50% of neonatal lethality (Schaller et al. 2010). Immunohistochemical approaches further reported that *Magel2*-null mice display a dramatic reduction in the number of neurons expressing the mature form of OT in the PVH (Schaller et al. 2010). However,

this reduction in the number of OT neurons appears to be the consequence of an alteration in the OT neuropeptide maturation process *versus,* for example, an effect on OT neurogenesis or cell death. Indeed, a similar number of immunolabeled neurons were observed when antibodies against OT pro-hormone or OT intermediate forms were used (Schaller et al. 2010). Remarkably, a single subcutaneous injection of OT in *Magel2*-null mice at birth is sufficient to rescue the neonatal suckling deficiencies that cause neonatal death and ameliorate social and cognitive behavior in adults (Meziane et al. 2015; Schaller et al. 2010). These findings support a role for OT in feeding systems during early development. *Magel2*-null mice also display a reduction in the number of POMC-positive cells, accompanied by reduced POMC axon densities in the PVH (Maillard et al. 2016; Mercer et al. 2013; Pravdivyi et al. 2015). However, the number of AgRP/NPY neurons in the infundibular nucleus (the human equivalent of the mouse ARH) (Goldstone et al. 2002, 2003) is not altered in PWS patients. In addition, the development of AgRP projections is not affected in *Magel2*-null mice (Maillard et al. 2016). As in *Magel2*-null mice, a reduction in the number of OT neurons is found in the PVH of patients with PWS (Bochukova et al. 2018; Swaab et al. 1995). Together, these observations show that *Magel2* deficiency causes alterations of the POMC and OT anorexigenic systems. Therapies involving intranasal sprays of OT in neonates and infants (ClicicalTrials.gov: NCT04283578) or treatment with MC4R agonists such as setmelanotide (ClicicalTrials.gov: NCT02311673) are therefore currently being tested in clinical trials to treat PWS patients.

1.6.2 Maternal Obesity

In the USA, epidemiological studies have estimated that more than half of women are obese or overweight when they conceive (Johnson et al. 2006). This disturbing observation highlights the importance of evaluating the outcomes of maternal obesity in the offspring. Maternal high-fat diet (HFD) feeding during pregnancy in rodents is a useful experimental approach for studying the mechanisms underlying maternal obesity. Similar to what is observed in humans, offspring born to obese females fed a HFD (45–60% of calories from fat) during gestation only or during both gestation and lactation become progressively overweight, hyperphagic, and glucose intolerant, and they display an increase in adiposity (Chen et al. 2009; Kirk et al. 2009). These metabolic alterations are associated with a disrupted development of POMC and AgRP/NPY projections to the PVH (Haddad-Tóvolli et al. 2020; Kirk et al. 2009; Park et al. 2020b; Vogt et al. 2014). Notably, maternal consumption of HFD during lactation (but not during pregnancy) appears sufficient to cause obesity and diabetes and to alter the development of POMC and AgRP projections (Vogt et al. 2014) showing the importance of postnatal nutrition, specifically, in hypothalamic programming. Animals born to obese dams also display a dramatic reduction of OT cell numbers (Buffington et al. 2016) and altered *Otr* gene expression and histone binding at the *Otr* promoter (Glendining and Jasoni 2019). The model of diet-induced obesity (DIO) developed by Barry Levin also provides a valuable tool

for studying obesity, in part because Levin's DIO rats share several features with human obesity, including polygenic inheritance (Levin et al. 1997). In outbred Sprague-Dawley rats fed a moderate-fat, high-energy diet, about one-half develop DIO, whereas the remaining rats are diet resistant (DR), gaining no more weight than chow-fed controls. Therefore, this animal model is particularly well suited for studying the relative contribution of genetic *versus* environmental factors in metabolic programming. Animals born to genetically obesity-prone DIO dams display a reduced density of POMC and AgRP fibers innervating the PVH (Bouret et al. 2008). In addition, a significant remodeling of synapses onto POMC neurons has been observed in DIO rats, particularly in response to nutritional challenges (Horvath et al. 2010). DIO rats fed a chow diet display increased inhibitory inputs to POMC neurons compared to obesity-resistant rats. In addition, DIO rats fed a high-energy diet display a loss of synapses onto POMC neurons, whereas high-fat feeding in control obesity-resistant rats causes an increase in POMC synaptic coverage (Horvath et al. 2010).

The precise mechanisms that underlie obesity-induced alterations in hypothalamic development remain elusive. However, several studies have indicated that abnormal leptin and insulin signaling during postnatal development may represent a likely cause for the HFD- and DIO-induced alterations in hypothalamic development. For example, DIO rats and animals born to obese dams display an abnormal organization of projections derived from the ARH that appear to be the result of the diminished responsiveness of ARH neurons to the trophic actions of leptin during critical periods of postnatal development. Moreover, animals born to obese dams display central leptin resistance and leptin sensitivity was improved by the endoplasmic reticulum stress-relieving drug tauroursodeoxycholic acid, which normalized metabolic and neurodevelopmental deficits in these animals (Park et al. 2020b). At the cellular level, a reduction in cilia length and frequency has been reported in the ARH of pups born to obese dams suggesting that alteration of this cellular system critical for hypothalamic development and leptin signaling could contribute to obesity-induced perturbations of hypothalamic development (Lee et al. 2020). Changes in insulin signaling could also mediate the neurodevelopmental effects of maternal obesity. Mothers fed a HFD and their offspring are hyperinsulinemic, and deleting the insulin receptor in POMC neurons prevents the diet-induced disruption of POMC projections (Vogt et al. 2014). More recently, Dearden and colleagues reported that offspring of obese dams display a reduction in POMC cell numbers that is likely to result from a diminished neurogenic action of insulin during embryonic development (Dearden et al. 2020). Similarly, amylin, which is co-released with insulin by pancreatic β-cells, appears to be involved in the nutritional programming of melanocortin circuits. Offspring of obese dams are hyperamylinemic from embryonic age throughout adulthood. Amylin fails to activate hypothalamic amylin receptor signaling if animals are born to obese mothers, and it was associated with an inability of amylin to promote POMC neurogenesis (Li et al. 2020). Similarly, knocking down CTR in the ventromedial part of the hypothalamus of DR rats (a region that encompasses the ARH + VMH) alters the development of POMC circuits (Johnson et al. 2016). In contrast, neonatal amylin

treatment in DIO rats enhances STAT3 signaling in the ARH accompanied by a restoration of AgRP and POMC fibers (Johnson et al. 2016). A potential link between maternal gut microbiota and offspring brain development has recently been suggested (Vuong et al. 2020). Interestingly, maternal high-fat feeding causes microbiota dysbiosis in the offspring, and selective re-introduction of *Lactobacillus reuteri* restores OT levels and social deficits in animals born to obese mothers (Buffington et al. 2016), raising the possibility that this commensal strain could also be involved in the metabolic neuroprogramming of obesity.

One caveat to keep in mind when using animal models of maternal high-fat feeding is that these animals are often not only obese, but they are also hyperglycemic and diabetic. This complication could make it difficult to differentiate the detrimental effects of maternal obesity *per se* as opposed to maternal diabetes. Nevertheless, manipulating glucose and insulin levels without an alteration of the diet can be performed experimentally by injecting streptozotocin, a pancreatic beta-cell toxin. Using this approach, we found that maternal diabetes alone (*i.e.*, without maternal obesity) can cause a reduction in the density of POMC- and AgRP-containing projections to the PVH (Steculorum and Bouret 2011).

1.6.2.1 Perspectives

It is now clear from different fields of neuroscience research, such as autism and schizophrenia, that neurodevelopmental alterations can cause progressive and life-long disorders. In rodents, hypothalamic development is initiated during mid-gestation and continues during the postnatal period under the influence of complex interplay of genetic, molecular, cellular, endocrine, and nutritional factors (Fig. 1.5). These developmental windows represent important periods of vulnerability during which perturbations in the perinatal environment may lead to abnormal hypothalamic development and lifelong metabolic diseases. Therefore, it will be critical to have a comprehensive knowledge of factors that are detrimental or beneficial for hypothalamic development. The marked species difference in terms of hypothalamic developmental trajectories is important to consider as rodents and humans may exhibit different periods of vulnerability to developmental insults or different responses to therapeutic interventions based on the temporal and regional maturation patterns of the hypothalamus. Nevertheless, the mature hypothalamus, whether from rodents or primates, can still exhibit neuroplastic responses, although the degree and the nature of hypothalamic remodeling may differ between adults and neonates. For example, leptin administration to adult mice has little effect on hypothalamic axon growth (Bouret et al. 2004b), but results in rapid changes in the synaptic organization (Pinto et al. 2004). Another salient example of adult neuroplasticity is the neurogenesis that has been described in the adult hypothalamus, albeit to a lesser degree than that observed in the fetal hypothalamus (Kokoeva et al. 2005, 2007; McNay et al. 2012). Together, these data illustrate that although neonatal life represents an important period for shaping hypothalamic circuits, there are periods of opportunity beyond this developmental window to remodel at least some components of hypothalamic circuits.

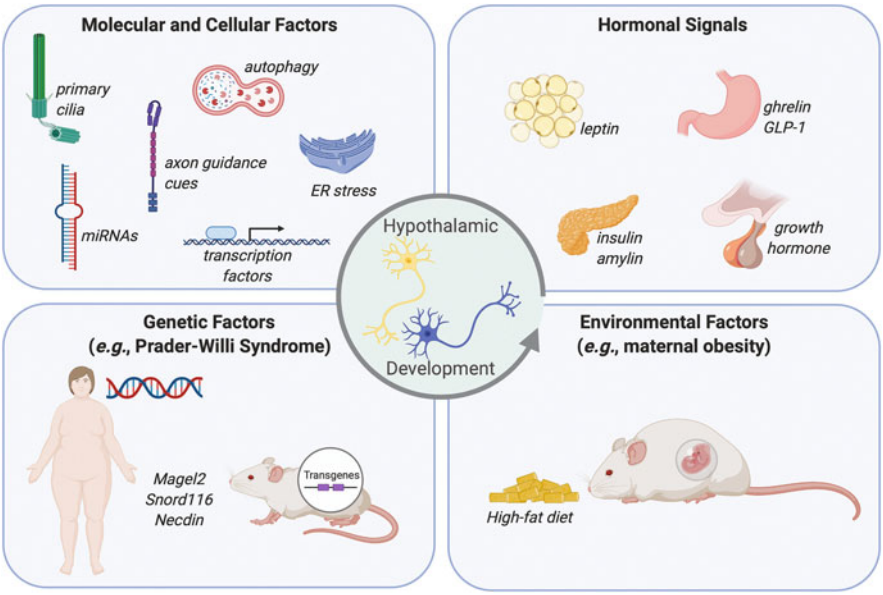


Fig. 1.5 Developmental factors regulating hypothalamic neuroendocrine pathways. Hypothalamic development involves cell-intrinsic cellular and molecular factors, endocrine signals, and genetic and environmental factors

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2 Advances in MRI-Based Anatomy of the Human Hypothalamus and Effects of the Hypothalamic Neuropeptide Oxytocin on Brain BOLD Signals

Christina Mueller, Melanie Spindler, Svenja Caspers, and René Hurlemann

Abstract

In humans, the hypothalamus makes up less than 1% of the total brain volume. Yet, this small structure is involved in various metabolic, behavioral, and endocrine processes, with damage leading to disorders in these domains. For instance, central and peripheral effects, including diverse social functions, are related to the nonapeptide oxytocin, which is synthesized in the supraoptic and paraventricular

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nuclei of the hypothalamus. To evaluate the role of distinct hypothalamic nuclei on behavior, it is necessary to study them in the living individual.

Here we describe magnetic resonance imaging as the neuroimaging method of choice for the investigation of hypothalamus anatomy and function and its neuropeptide oxytocin in vivo in humans. Due to its small size, hypothalamus imaging is faced with unique difficulties, but recent technical and computational interdisciplinary advances have expanded the possible uses of MRI to elucidate the role of the hypothalamus and its components in health and disease. Furthermore, we give an introduction on how neuroimaging techniques can be used to identify neural effects of an endogenous substance like oxytocin and provide an overview of neuroimaging findings concerning the impact of oxytocin on widespread neural networks. In addition, connections between brain responses and behavior are drawn to decipher the role of oxytocin in functions including fear response, attachment, and trust.

Keywords

Oxytocin · Supraoptic nucleus · Paraventricular nucleus · Segmentation · Pharmacological challenge · Social cognition · Allostasis

Abbreviations

AI	Anterior insula
ASL	Arterial spin labeling
AVP	Arginine vasopressin
BOLD	Blood oxygen level-dependent
CSF	Cerebrospinal fluid
FA	Fractional anisotropy
fMRI	Functional magnetic resonance imaging
HPA	Hypothalamus-pituitary-adrenal axis
IU	International units
MD	Mean diffusivity
MFG	Middle frontal gyrus
MRI	Magnetic resonance imaging
MTT	Mammillothalamic tract
NAcc	Nucleus accumbens
OT	Oxytocin
PET	Positron emission tomography
PVN	Paraventricular nucleus
qMRI	Quantitative magnetic resonance imaging
rCBF	Regional cerebral blood flow
SCR	Skin conductance response
VTA	Ventral tegmental area

2.1 Introduction

Magnetic resonance imaging (MRI) is a non-invasive tool for generating an image of the brain and skull (Box 2.1). In the past several years, advances in magnetic field strength and the development of different MR sequences have allowed improved image quality and new areas of application, thereby further enhancing its value for diagnostic and research purposes.

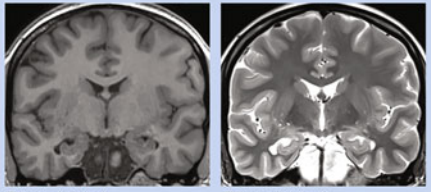
Box 2.1 Magnetic Resonance Imaging (MRI)

MRI is based on the properties of hydrogen atoms, which are present in all tissue types. Hydrogen atoms have a single proton, which in the normal, non-magnetic state, rotates around its axis. If a patient is placed in a strong magnetic field such as the MR scanner, the protons align either against or with the main magnetic field and rotate (or precess) about the field axis. When further energy in the form of radiofrequency pulses is added, the protons change their energy state, synchronize, and spin together ("Resonance"). With the RF pulse turned off again, the protons return to their initial state in the main magnetic field, releasing energy, which is detected in the receiver coil. Signal changes over time can be described as T1 (spin-lattice) and T2 (spin-spin) relaxation. T1 and T2 relaxation occurs faster and more rapidly in fat and more slowly in water, which results in the tissue contrasts seen in MR images. Differences in T1 and T2 relaxation are measured by changing how quickly the radiofrequency pulses are sent and how quickly the return signal is received. In structural (anatomical) MRI, a single 3D image with the high spatial resolution is obtained for assessment of brain anatomy and pathology. Most structural images are created as a mixture of different tissue contents, thus providing no quantitative information about the underlying tissue. In contrast, quantitative MRI (qMRI) describes a spectrum of techniques used for mapping tissue characteristics, including, for example, diffusion parameters, fat, iron, and water fractions. In contrast to conventional structural MRI, qMRI sequences produce standardized images with meaningful gray values comparable across different sites and studies characterizing the underlying tissue. Types of qMRI include diffusion-weighted imaging and relaxometry. Diffusion-weighted imaging is grounded on the random motion of water molecules to estimate diffusion properties in tissue and is a routine sequence in clinical practice to illustrate, for example, edema and lesions (Alexander et al. 2007). Diffusion in the brain is thought mainly to follow the axonal pathways (axial diffusivity) and to occur less across the axon (radial diffusivity), as myelination restricts free movement of water molecules. The most common diffusion parameters used to describe brain tissue are fractional anisotropy (FA, a measure of directionality of diffusion) and mean diffusivity (MD, a measure of amount of diffusion). By contrast, relaxometry MRI

(continued)

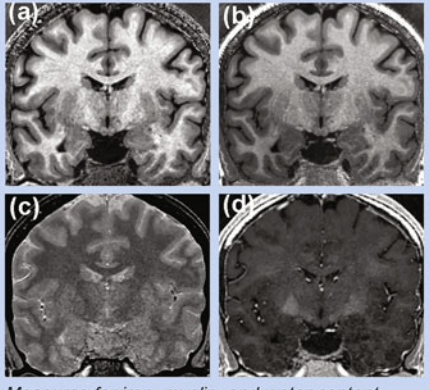
Structural

Conventional

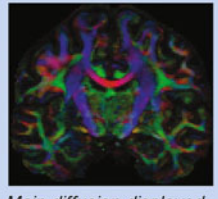


T1-weighted *T2-weighted*

Quantitative

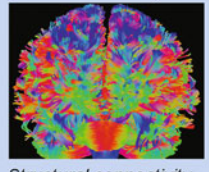


Measures for iron, myelin, and water content based on (a) Magnetization Transfer Saturation, (b) Longitudinal Relaxation Rate, (c) Proton Density, (d) Effective Transverse Relaxation Rate.



Main diffusion displayed along the left/right (red), inferior/superior (blue), and anterior/posterior (green) axis.

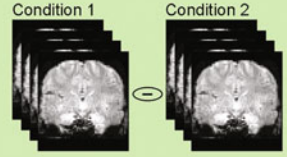
Connectivity



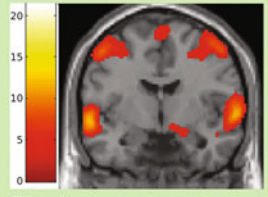
Structural connectivity visualized as diffusion tractography streamlines.

Functional

Contrasts

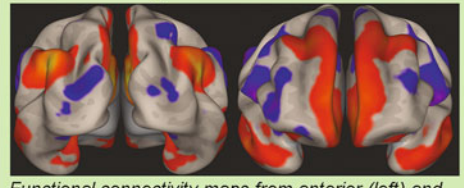


Activation



The obtained pattern of activity reflects the difference between conditions 1 and 2.

Connectivity



Functional connectivity maps from anterior (left) and posterior (right) view. Orange regions show positive functional coupling, purple regions a negative functional coupling.

Fig. 2.1 Overview of different structural and functional modalities obtained from a 3 T magnetic resonance imaging (MRI) scanner (Siemens MAGNETOM Prisma). Left: Structural approaches of MRI are displayed, used to infer brain morphology and underlying tissue properties. Right: Principles of functional MRI are shown. First, contrasts are computed based on the study design. Then, differences in brain blood oxygenation level-dependent signals related to these conditions are computed to infer brain region activation or connectivity between brain regions

Box 2.1 (continued)
techniques are based on quantitative determination of T1 and T2 relaxation maps to produce images sensitive to iron, fat, and water. They are employed less frequently in clinical and research settings but nevertheless provide further information about the underlying tissue relevant, for e.g., aging and specific diseases (Lee et al. 2013; Ward et al. 2014; Möller et al. 2019). Another common tool in MRI is functional MRI (fMRI). Here, low spatial resolution is accepted in favor of the higher temporal resolution for blood-oxygen-level-dependent (BOLD) imaging over a certain time course. Changes in the oxygen saturation of the blood are used to infer brain activity. A single BOLD response does not carry meaningful information about brain activity. By contrasting BOLD responses of different groups (e.g., patients vs. healthy controls) or different conditions (e.g., drug vs. placebo) differences in activation patterns emerge that can be related to the research object under investigation. This enables analyses of brain activation or functional connectivity, (a measure used to describe activation patterns of brain regions in relation to each other). Brain activation and functional connectivity can be investigated in either resting state (without stimulation, only lying relaxed in the scanner and letting one's mind wander) or in an environment where the participant is asked to perform specific tasks during scanning to evaluate neural correlates, e.g., cognitive functions. These can be combined with prior drug administration to examine cognitive or neural effects of the compound. Still, it is important to note that functional connectivity does not imply that functionally connected brain regions are also structurally (anatomically) connected via fiber pathways. To investigate structural connectivity, diffusion tractography is performed, which will not be further discussed in this chapter. An overview of the methods discussed above is given in Fig. 2.1.

MRI is the first choice for diagnostic imaging and detailed examination of the hypothalamic region, a small, central brain structure involved in a variety of behavioral and bodily functions. Due to its small size, high-resolution techniques with appropriate tissue contrasts are critical to accurately evaluate hypothalamus anatomy, location, and characterization of lesions in this area. Therefore, in the past several years, the development of stronger MRI machines and advanced imaging techniques that enable finer resolution led to a more accurate representation of the hypothalamus, benefitting both clinical assessment and research. This structural imaging with high spatial resolution provides the basis of MR examination and serves as a reference for functional imaging. To locate brain regions in functional images, they are registered to the anatomical image (co-registration), where the regions of interest are defined and transferred onto the functional images. Functional MRI (fMRI) can detect changes in brain activity and functional coupling between brain regions, providing insights into the processes and functions in the living brain. To study the effects of neuropeptides on such processes, patterns of brain activity

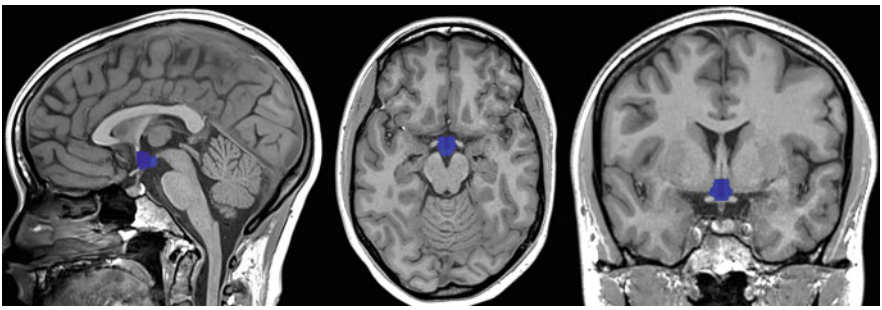


Fig. 2.2 Manual segmentation (blue) of the hypothalamus in orthogonal views of a T1-weighted image (left: sagittal, middle: axial, right: coronal)

under the influence of the neuropeptide of interest can be compared with brain patterns without that influence. Furthermore, fMRI can establish inferences between brain responses and behavior and thereby help to decipher the role of neuropeptides in humans. Structural MRI, on the other hand, can advance our knowledge about the anatomy and pathology of brain regions. In the case of the hypothalamus, image segmentation is performed, which involves a variety of manual, semi-automated, or automated procedures to accurately trace the hypothalamus based on anatomical knowledge and image contrast visible in T1- and T2-weighted images (Figs. 2.1, 2.2). Image segmentation is a common tool for surgical planning, quantitative analyses of the underlying structure, visualizations, and for delineation of small, highly interindividual regions. With segmentations based on high-resolution structural imaging, even small brain structures such as the hypothalamus can be reliably identified.

2.2 MRI Anatomy of the Hypothalamus

Located centrally in the diencephalic part of the brain, the hypothalamus is positioned anterior to and below the thalamus, superior to the pituitary, and lateral to the third ventricle on both sides (Fig. 2.3). The hypothalamus is composed of approximately 15 nuclei that are largely functionally separable (Baroncini et al. 2012). In the last few years, its multifaceted role sparked interest in investigating specific hypothalamic subregions to achieve a greater understanding of hypothalamic functioning in health and disease. Therefore, hypothalamus segmentation is often accompanied by parcellation, the process of defining subregions within the hypothalamus that are thought to represent specific (groups of) nuclei and/or functions. Instead of drawing inferences from overall hypothalamic anatomy, analyses of tissue volume, microstructure, or neural activation are performed in the hypothalamic subunits of interest to allow for functionally meaningful interpretability of the results. In the past several years, abnormalities of hypothalamic subunits have been associated with, e.g., obesity (Spindler et al. 2020), anorexia nervosa (Florent et al. 2020), frontotemporal

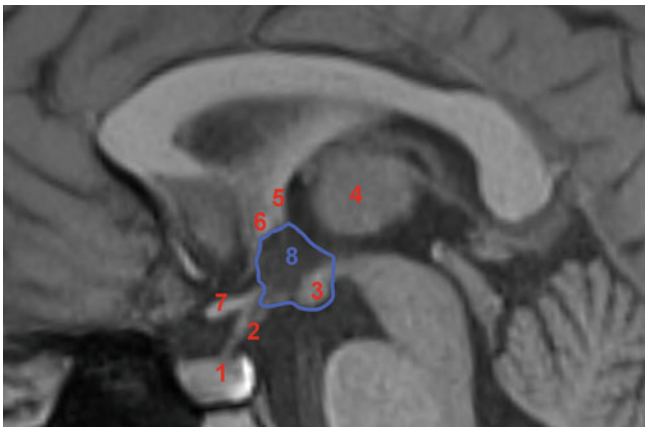


Fig. 2.3 Sagittal section of a T1-weighted MRI of the diencephalon. The outline of the hypothalamus is depicted in blue. 1: Pituitary gland (the posterior pituitary shows a characteristic bright signal); 2: Pituitary stalk; 3: Mammillary body; 4: Thalamus; 5: Fornix; 6: Anterior Commissure; 7: Optic Tract; 8: Hypothalamus

dementia (Piguet et al. 2011; Bocchetta et al. 2015), schizophrenia (Goldstein et al. 2007) and mood disorders (Schindler et al. 2012).

2.2.1 Structural Imaging

2.2.1.1 Manual to Semi-Automated Techniques

Due to the small and highly individual shape of the hypothalamus, manual to semi-automated techniques for its segmentation are currently the most widely used, providing the most accurate delineation of the hypothalamus. Here, T1- and sometimes T2-weighted images are displayed in orthogonal views (axial, sagittal, and coronal slices of the brain), and the hypothalamic outline is traced by a trained examiner (Fig. 2.2). Still, in conventional anatomical MRI, the hypothalamus lacks morphological detail, such that lateral borders are often approximated and cannot be reliably identified. Besides, it is not possible to distinguish individual nuclei due to low contrast and small size/limited spatial resolution of the hypothalamic region. Therefore, histological examinations serve as a gold standard for hypothalamic anatomy and are extrapolated to MRIs (Baroncini et al. 2012; Makris et al. 2013). By employing anatomical landmarks, the hypothalamus is segmented and sectioned into subunits that are thought to reflect the anatomical outline of groups of nuclei. Landmarks include, for example, the anterior commissure, optic tract, hypothalamic sulcus and fornix. Here, segmentation usually starts in coronal view with the first slice where the anterior commissure appears continuous and ends with the last slice where the mammillary bodies are visible. This way, depending on the segmentation protocol, anterior-to-posterior, inferior-to-superior, or medial-to-lateral, segments can be formed to divide the hypothalamus into subunits (for a detailed segmentation

protocol, see Bocchetta et al. (2015)). Still, differences between segmentation techniques and individual raters who perform the segmentations (inter-rater variability) reduce inter-study comparability of landmark-based segmentations. Also, the hypothalamus is mainly surrounded by other gray matter structures, thereby complicating its reliable segmentation, especially at the lateral borders, due to weak tissue contrast. Thus, these procedures require trained examiners and time to perform the segmentations. Still, visibility of anatomical landmarks also depends strongly on the imaging protocol, leading to more conservative or liberal applications of landmarks and size differences of the obtained hypothalamus masks within and across studies. Reported sizes of the entire hypothalamus range between approximately 0.6 and 3.6cm^3, with most recent MRI studies reporting it to be around 1cm^3, which can be explained by methodological advances in the field (Stephan et al. 1981; Schindler et al. 2013). Additionally, there is missing consensus about the number and exact positions of subregions, ranging from two (anterior vs. posterior hypothalamus (Piguet et al. 2011)) to up to six subregions (Lemaire et al. 2011), with most studies reporting 4 or 5 subregions (for an overview see Spindler et al. (2020)). Therefore, studies investigating structure-function relationships in the hypothalamus were often restricted to small sample sizes and could only be compared to a limited extent.

2.2.1.2 Automated and Interdisciplinary Procedures

To tackle the above-mentioned shortcomings of manual segmentation, Rodrigues et al. (2020) provided the first approach for automated hypothalamus segmentation based on advances in machine learning techniques using structural MRI. Building on those achievements, Billot et al. (2020) recently developed an automated segmentation and parcellation approach of the hypothalamus by employing a convolutional neural network trained on a set of manual parcellations. A convolutional neural network is an artificial neural network that can be trained on pre-existing data, for e.g., medical image analysis and image classification. Here, manual parcellations were obtained following a previously published protocol based on anatomical landmarks from histological examinations and included five hypothalamic subdivisions per hemisphere (anterior-superior, anterior-inferior, superior tuberal, inferior tuberal, posterior) (Makris et al. 2013; Bocchetta et al. 2015). Based on this dataset, the convolutional neural network learned to segment the hypothalamus automatically. They included extensive data augmentation techniques (modifications of brightness and shape of the existing data to increase the amount of data) to obtain a high level of agreement between automated and manual parcellations without prior pre-processing of the data. With this approach, hypothalamus segmentation at the subunit level can be performed on large datasets where manual procedures would be unfeasible. For automated parcellation, a single T1-weighted image in 1 mm isotropic resolution is needed as input, which makes the approach applicable to a large range of studies.

In another recent study, Neudorfer et al. (2020) created a high-resolution (0.25 mm and 0.5 mm isotropic voxel size) hypothalamic atlas based on 990 MRI scans from the Human Connectome Project (Van Essen et al. 2012). By registration

of the individual participants' images to the atlas template, this atlas can be used for fine-grained localization of individual hypothalamic nuclei and surrounding structures on different MRI modalities.

2.2.1.3 Pitfalls and Limitations

Hypothalamic gray matter is traversed by white matter tracts and adjacent to cerebrospinal fluid (CSF), inducing partial volume effects. Partial volume effects are defined as the presence of multiple tissue types in a single voxel due to limited spatial resolution and can introduce bias, e.g., in volume estimation. One major white matter bundle that passes through the hypothalamus is the fornix, which predominantly connects the hippocampus with the mammillary bodies. Additionally, originating in the superior edge of the mammillary bodies, the mammillothalamic tract (MTT) connects the mammillary bodies with the thalamus (Saeki et al. 2001). Here, the tract passes outside the hypothalamus but cannot reliably be excluded from the mammillary bodies that are commonly included in the posterior hypothalamic subunit. Lastly, the optic tracts also border anterior and lateral hypothalamic tissue. As the hypothalamus, together with the median eminence, forms the border of the third ventricle and surrounds it laterally, medial nuclei touching the ventricle are either only partially included or introduce CSF in the hypothalamus mask. Hence, both crossing white matter and CSF can induce partial volume contaminations that introduce bias in both structural and functional examinations of the hypothalamus (Alexander et al. 2001; Dukart and Bertolino 2014). For detailed hypothalamic analyses, it is desirable to minimize these influences. On the basis of T1-weighted images only, white matter exclusion, especially related to the fornix and MTT, is difficult and results in incomplete and/or unreliable exclusion of the fibers due to low contrast. In Billot et al. (2020), parts of the fornix were identified and excluded, whereas the mammillary end of the MTT was included in the posterior subunits. Additionally, a common pitfall of machine learning applications is that most are black boxes. This means that in the case of Billot et al. (2020), it is still unknown how the classifier achieved a decision concerning each voxel belonging to the hypothalamus or not (e.g., fornix, bordering regions). It is therefore unclear which voxels were effectively used to determine the exact location of the hypothalamus, its subunits and the fornix, and whether the decision was based on image contrast and anatomy or rather on other previously learned features, such as shapes and size ratios (Lapuschkin et al. 2019). This way, individual healthy or pathological variations in nucleus size, hypothalamus, or fornix shape can be overlooked and lead to misrepresentations of the generated subunits, as the algorithm will produce subunits with similar ratios and orientations learned from the training dataset. This is also especially relevant in atlas-based approaches, such as in Neudorfer et al. (2020), where the individual brain is mapped onto an atlas template. Here, especially when investigating a small brain structure such as the hypothalamus, pinpoint registration is critical to achieve a good overlap of the individual with the template structure, but registration performance can vary depending on the algorithm used or the individual shape of the brain structure (e.g., due to lesions or tumors). Hence, to grasp possible structural interindividual

differences in hypothalamic microstructure, parcellation protocols taking advantage of the underlying tissue information can be a useful addition to atlas- and landmark-based parcellation techniques and are discussed in the following section.

2.2.2 Data-Driven Approaches

It is unclear whether subdivisions based on landmarks truly reflect functional architecture. Therefore, when additional MRI information is available, data-driven methods can be used to delineate hypothalamic subunits based on microstructural or functional properties. Recent approaches have been developed based on information derived from functional or quantitative MRI sequences to categorize voxels based on similarity measures. For example, functional MRI measures complemented structural approaches for hypothalamic segmentation and parcellation (Osada et al. 2017; Ogawa et al. 2020). They showed that nuclei of the hypothalamus could be localized by using functional connectivity. This way, voxels exhibiting similar functional connectivity during rest are grouped together, whereas voxels with dissimilar functional connectivity patterns are separated.

Another data-driven approach for hypothalamus parcellation employs clustering of voxels based on quantitative information about the underlying tissue. In 2013, Schönknecht and colleagues used a k-means clustering algorithm to divide the hypothalamus into three subunits based on their principal diffusion direction derived from diffusion tensor imaging (Box 2.1) (Schönknecht et al. 2013). They argue that the resultant subunits represent groups of nuclei involved in the same major fiber systems incorporated in the hypothalamic structure.

Still, fiber systems in the hypothalamus are largely overlapping, and crossing fibers cannot be resolved using the principal diffusion direction. Due to the crossing fibers problem, in the last years, clustering based on diffusion data has been advanced by introducing more encompassing models describing the diffusion process and different diffusion compartments in each voxel beyond the simple tensor model (Tuch 2004; Aganj et al. 2010). In 2020, Spindler et al. applied these more advanced methods to define four hypothalamic subunits based on diffusion properties in each voxel, comprising anterior-superior, anterior-inferior, intermediate, and posterior subdivisions, each displaying different microstructural characteristics in the hypothalamus (Spindler et al. 2020). Still, one limitation of data-driven approaches based on functional and diffusion-weighted imaging is that spatial resolution is often low, which leads to only few datapoints being available and high risk of partial volume contaminations due to insufficient exclusion of white matter and CSF. Additionally, there are differences in acquisition strategies in both diffusion and functional MRI. Therefore, standardized MRI techniques with spatial resolution comparable to conventional structural imaging could be a useful addition to enable more detailed hypothalamic volumetry. Research on different cortical and subcortical brain areas has shown that segmentation with quantitative MRI based on relaxometry showed considerable advantages regarding segmentation accuracy compared to segmentation on T1-weighted images alone (Carey et al. 2017). In

contrast to conventional anatomical MRI, quantitative MRI sequences based on relaxometry produce standardized images with meaningful gray values and comparable spatial resolution. Hence, they could be used to quantify hypothalamic microstructure and subunits, thereby enhancing inter-study comparability and interpretational power. A first approach to implement such a standardized procedure was made by Spindler and Thiel (in press), where quantitative MRI sequences of iron, fat, and water fractions were probed for hypothalamic segmentation. By employing different qMRI techniques, automated CSF and fiber extraction from the hypothalamus was achieved, addressing the problem of residual white matter and fluid confounds in the hypothalamus. Another advantage of quantitative imaging is the possibility of linking tissue microstructure to behavior or endocrine function to identify functional correlates of hypothalamic structure in health and disease.

2.3 Functional Anatomy of the Hypothalamus and Its Nuclei

The hypothalamus serves as the central hub between the endocrine and nervous systems. A variety of conditions, including affective disorders such as major depression and bipolar disorder (Bao and Swaab 2019), eating and metabolic disorders (Seong et al. 2019), cognitive deficits (e.g., frontotemporal dementia) (Piguet et al. 2011) and endocrine abnormalities (Yu 2014), can affect the hypothalamus. It is also susceptible to inflammatory diseases, tumors, and granulomatous diseases that in turn lead to disturbed behavior and metabolic dysfunction (Asa and Mete 2019). As most hypothalamic disorders are related to their endocrine functions, the regulatory impact of the hypothalamus on the endocrine system and associated key functions are briefly introduced in the following.

Endocrine control of the hypothalamus is achieved by its secretory products and complex interactions with the pituitary gland (or hypophysis). The pituitary is an endocrine gland that is divided into two distinct parts: the anterior lobe (adenohypophysis) and the posterior lobe (neurohypophysis), which are attached to the hypothalamus via the pituitary stalk. The pituitary lobes are functionally separable and exchange information with the hypothalamus via separate routes.

2.3.1 Anterior Route

The hypothalamus is connected to the anterior pituitary mainly by hypophysial-portal vessels passing through the infundibular stalk, where hormones synthesized in the hypothalamus are transported to regulate hormone secretion of the adenohypophysis (Miyata 2017). Hormones secreted in the adenohypophysis are involved in many physiological systems, including body homeostasis, energy usage and stress. Large hypothalamic projections to the adenohypophysis belong to the hypothalamic-hypophyseal-adrenal (HPA) axis, which is critically involved in orchestrating physical and emotional stress responses via adrenal secretion of stress hormones known as glucocorticoids (primarily cortisol) (Hellhammer et al. 2009). Here,

corticotropin-releasing hormone neurons in the paraventricular nucleus (PVN) of the hypothalamus cause synthesis of cortisol, which in turn exerts negative feedback on the pituitary. Dysregulation of the HPA axis is, for example, associated with a higher risk for major depression (Bao and Swaab 2019). Other hypothalamic nuclei related to the adenohypophysis mainly include the arcuate nucleus, preoptic area and ventromedial hypothalamus (Saper and Lowell 2014).

2.3.2 Posterior Route

In contrast, the main agents of the hypothalamo-neurohypophyseal system are the two nonapeptides, oxytocin (OT) and arginine vasopressin (AVP) (Miyata 2017). These are primarily synthesized in magnocellular neurons of the supraoptic nucleus and PVN and transported to the neurohypophysis via the pituitary stalk, where they are released into the systemic blood circulation to act on peripheral organs, contributing to reproduction-related functions (OT) and water balance (AVP). In addition, smaller, parvocellular neurons of the PVN transport OT to other brain regions via somatodendritic release, including forebrain regions such as the central nucleus of the amygdala, septal nuclei and olfactory bulbs (Leng et al. 2015). This way, OT expresses widespread behavioral effects, including effects on social and sexual behavior (Neumann and Landgraf 2012). Lesions and disorders of the posterior pituitary can result in central diabetes insipidus or disorders related to OT deficiency (Daubenbüchel et al. 2016). In patients with diabetes insipidus, AVP secretion is altered, leading to abnormalities in thirst and water intake control. The characteristically hyperintense (bright) signal in T1-weighted MRI of the healthy neurohypophysis (Fig. 2.2) is often absent in diabetes insipidus patients, which is believed to stem from a failure of AVP storage (Bonneville et al. 2006).

2.4 Neuroimaging Effects of the Hypothalamic Neuropeptide Oxytocin

2.4.1 How to Study the Effects of Oxytocin in the Human Brain

In the second part of this chapter, we are focusing on the effects of one specific hypothalamic peptide on the brain and behavior and ways to study it. Much of what is known about the effects of OT on the brain is derived from animal studies. Though animal research has advanced our knowledge indubitably and is indispensable, there are inevitable limitations when translating such findings into the human domain, particularly in a clinical context. Therefore, human studies are needed as well to gain insights into the mechanisms of OT in the human brain. A brief history of OT research in humans is provided in Box 2.2.

Box 2.2 A Brief History of Oxytocin Research in Humans

The neuropeptide oxytocin (OT) has gained specific attention over the last decades and has become one of the most extensively researched neuropeptides (see Marsh et al. (2020)). The peripheral effects of the hormone OT, including the control of labor and lactation, have been clinically employed since the 1950s. However, central effects of OT were first demonstrated in the 1980s with the work of Keith Kendrick and Barry Keverne, who showed that central release of this hormone in sheep during birth and lactation could promote the formation of mother-infant bonds (Keverne and Kendrick 1992). In 1992, Thomas Insel and Lawrence Shapiro discovered different distributions of OT receptor densities in two vole species—the monogamous prairie and the polygamous montane voles (Insel and Shapiro 1992). These variations in receptor expressions are assumed to determine the social bonding and affiliative behaviors in these rodents. This and other groundbreaking studies have sparked an enthusiasm around this molecule that is still present today. Its impact on the human brain and behavior has only been researched for the last two decades, but the number of studies carried out has grown exponentially since, with less than 500 studies published on the platform PubMed in 2005 and more than 1000 in 2020. Early studies on the effects of OT in social cognition have triggered a media hype around the so-called "love hormone" or "cuddle chemical" (e.g., Coghlan 2010; Martin-Du Pan 2012). Most studies have focused on the effect of intranasally administered synthetic OT on human pair bonding and related affiliative social behaviors, trust and cooperation and fear and stress processing. Initial results of early studies in humans have given rise to some optimism regarding potential applications of OT. For the first time, an endogenous compound has been identified that has the potential to modulate social behavior and therefore appears to be a promising candidate for the treatment of social impairments in psychiatric disorders such as schizophrenia and autism spectrum disorders. As to the present day, there is no pharmacological treatment available to target the social dysfunctions in these disorders specifically.

Having said this, some difficulties await researchers who want to study the effects of endogenous substances on the human brain in general and OT in particular. The most obvious obstacle compared to animal research is that the armamentarium is restricted to non-invasive methods to monitor the brain. Thus, a substance of interest cannot be directly injected into a specific brain region. With rare exceptions (e.g., epilepsy patients undergoing presurgical evaluation), it is unethical to insert an electrode into neurons and record their activity or manipulate gene expression inside the brain and observe behavioral changes. Under normal circumstances, it is thus generally not possible to directly measure brain activity, and indirect techniques are required that enable the inferring of information about the living brain. Fortunately, there are some neuroimaging techniques available to study the working human brain,

such as positron emission tomography (PET, Box 2.3), electroencephalography (EEG), magnetoencephalography (MEG) and two forms of functional MRI (Box 2.1). With a major focus on fMRI for studying OT effects in the brain, the following sections will introduce evidence obtained so far using this technique.

Box 2.3 Neuroimaging Labeling Methods

1. Positron Emission Tomography.

Positron emission tomography (PET) is a neuroimaging technique that measures metabolic activity in cells or tissues (Granov et al. 2013). PET scans can be used to diagnose various diseases with characteristic metabolic changes, including cancer or Alzheimer's Disease. Moreover, by providing information about the binding of drugs, PET imaging can help to optimize clinical treatment by relating drug binding (receptor occupancy) and drug effects in patients and thereby confirm specific targets (Farde 1996).

To uncover the metabolic activity, a radioactive compound is used that can be detected by PET scanners. Those radio nuclides used in PET scanning are typically isotopes with short half-lives. These radio nuclides are incorporated either into compounds normally used by the body, such as glucose (or glucose analogs), water or ammonia, or into molecules that bind to receptors or other sites of drug action. Such labeled compounds are known as radiotracers. The radioactive tracer is injected into the bloodstream, where it can diffuse through the blood-brain barrier and bind to neuroreceptors and transporter vesicles or is metabolized by endogenous enzymes (Dierckx et al. 2021). The tracer will then decay and emit a positron. Through the resulting annihilation of the positron, a pair of annihilation gamma photons is produced. These typically move in different directions, at roughly 180 degrees. These photons can be detected by the scanning device. The data acquired during the total duration of the scan is integrated into a three-dimensional image representing the temporal and spatial distribution of radiotracer concentration in brain tissue.

The development of appropriate radiotracers is very challenging as they have to fulfill some very specific properties such as high target specificity and a small range of molecular weight and molecular affinity. There exist suitable ligands for several neurotransmitters or neuropeptides, even for specific receptor subtypes such as for D2/D3 dopamine receptors or for selective serotonin transporters. Unfortunately, there is currently no selective radiotracer available for tracking oxytocin (OT) in the human brain in vivo. There have been several attempts in mammals, including rats, mice, and pigs. However, the radioligands were lacking receptor affinity and sufficient brain concentrations (e.g., Wenzel et al. 2016; Vidal et al. 2017) until the work in 2018 by Beard et al. who could demonstrate direct nose-to-brain uptake of intranasally administered oxytocin in the rat brain with their newly developed tracer (Beard et al. 2018).

(continued)

Box 2.3 (continued)

2. Arterial Spin Labeling Magnetic Resonance Imaging.

Arterial Spin Labeling (ASL) is a magnetic resonance imaging (MRI) technique that aims to quantify cerebral blood flow (CBF) or tissue perfusion (Williams et al. 1992). Tissue perfusion refers to the process of transporting oxygen and nutrients to tissue by blood flow. For this technique, the arterial cerebral blood water protons are labeled shortly before they enter the tissue we want to measure (Petcharunpaisan et al. 2010). In contrast to other tracing techniques such as Positron Emission Tomography, ASL is completely non-invasive. Instead of injecting radioactive tracers into the blood, the arterial blood water is magnetically tagged directly before it enters the tissue of interest, typically in the neck. Similar to the general principle of MRI, a 180-degree radiofrequency pulse is applied that inverts the protons in the flowing arterial blood water. When the magnetically labeled blood is reaching the tissue a difference in net magnetization can be detected by the scanner. This difference is proportional to the local CBF and thus can be used as a measure of relative CBF. The spin inversion is only transient (1–2 s) and decays with the longitudinal relaxation rate T1 (Wolf and Detre 2007). Additional to each tag image, a control image without a magnetic tracer is required. By subtracting the labeled images from the control images, the static tissue signal is discarded, and the perfusion signal remains. Multiple tag and control image pairs are acquired and averaged to derive CBF maps. Typically, an ASL acquisition takes between 1 and 5 min with one tag and control image pair every 4 s.

To study the effect of an endogenous compound, some knowledge of the anatomical distribution of the corresponding system is required. In animal studies, loci with high expression of OT receptors were found in the nucleus accumbens, olfactory bulb, amygdala, and dorsal vagal nucleus (Winterton et al. 2020). However, as to the aforementioned restrictions to non-invasive procedures in humans, there are some difficulties delineating receptor distribution in humans compared to animals. Since behavioral disparities between species are assumed to be mirrored in varying receptor distributions, translating findings from animals to humans is not trivial. In humans, first inferences were derived from post-mortem brains, but the significance of these findings is limited due to a static snapshot of a dynamic system in only a limited sample of human brains. In an attempt to indirectly outline the distribution of OT pathways in the living human brain, distribution maps of OT pathway gene mRNA were created based on the distribution of OT signaling genes messenger RNA (Quintana et al. 2019). OT receptors were highly expressed in central regions, olfactory regions, the hippocampus, parahippocampal gyrus, amygdala and the medial and superior temporal pole. These maps correspond to brain areas extracted from fMRI studies associated with anticipatory, appetitive, and aversive cognitive states. Although the effects of OT on the BOLD response are

reliant on the local expression of the OT signaling genes, they are not restricted to such areas. Due to the highly interconnected nature of the human brain, neurochemically active compounds can exert effects within brain regions without corresponding receptors by affecting connected brain areas with high expression of receptors that carry forward the effects (functional connectivity).

Furthermore, some idea of the concentration of OT in the brain is needed. However, OT levels in the blood carry only limited information about central levels of OT. One way of studying the effect of an endogenous substance like OT is to manipulate the levels of that substance within the brain by exogenously administering it and then observing changes within the brain and behavior. This is called a pharmacological challenge. For this purpose, the substance of interest has to reach the brain reliably. Until recently, it was assumed that OT could not readily pass the blood-brain barrier in significant amounts; intravenous or other classical routes of administration thus did not appear a viable option to influence central levels of OT. The discovery of a protein in mice that transports OT mainly unidirectionally from the blood to the brain and from the intestinal tract to the blood has reopened the potential of other routes of administration, including oral and intravenous routes, for further investigation (Yamamoto and Higashida 2020). A relatively novel approach is to bypass the blood-brain barrier by intranasal application of neuroactive substances. For this process, a substance is dissolved in a saline solution and administered as a nasal spray. Born et al. provided evidence that intranasal administration of neuropeptides elevated their concentrations in the CSF (Born et al. 2002). Due to the absence of a selective radiolabeled OT ligand in humans, there is no direct way to examine the penetration into the central nervous system and subsequent distribution of synthetic OT. Striepens et al. (2013) were the first to show that intranasal OT could increase the concentration of OT in the CSF, but the relation between CSF concentrations and concentrations within specific brain regions is unclear. Another indirect approach is arterial spin labeling MRI (ASL MRI, Box 2.3).

With this technique, changes in regional cerebral blood flow (rCBF) can be detected as a surrogate measure of neuronal activity. The work by Paloyelis et al. (2016) could demonstrate that intranasal administration of OT induced changes in rCBF in the human brain in various brain regions that overlap with regions expressing OT receptors and are among core regions of emotional and social cognition networks, including the amygdala, hippocampus, caudate nucleus, ventral striatum and pallidum, septal and hypothalamic nuclei and the anterior cingulate cortex. Although still indirect, this work provides further evidence that intranasal OT reaches the human brain despite changes in plasma levels. With all that in mind, the findings of studies examining the effect of OT on the brain will be described in the following sections.

2.4.2 Oxytocin, the Prosocial Hormone

2.4.2.1 Fear and Stress Processing

Although central effects of OT have been researched in humans since the 1980s (e.g., Fehm-Wolfsdorf et al. 1984), the initial study that inspired OT research in human subjects was the study by Kosfeld et al. (2005). This work showed an increase of trust towards other unknown players in an economic game paradigm following the administration of intranasal OT and led the way towards a plethora of studies on the prosocial neuropeptide OT. The first study investigating the neural effects of OT on the human brain followed shortly and was published by Kirsch et al. (2005). Based on results in animal studies, Kirsch et al. expected to find a reduction of responses to fear-associated stimuli in the amygdala, a key region of fear processing. To investigate this effect, the authors let the subject self-administer intranasal OT or placebo and then, after a short waiting period, the subject underwent fMRI while performing an experimental task. This task involved social (faces) and nonsocial (scenes) threatening stimuli, and the subjects had to match them with an identical target scene. Compared with placebo, they found a strong reduction in amygdala activity. This effect was more pronounced for socially than nonsocially relevant stimuli. Additionally, they investigated changes in functional connectivity of the amygdala with other brain regions. A decrease in coupling was found between the amygdala and the periaqueductal gray and the reticular formation, two brainstem regions prominent for their role in the fear response. The dampening of amygdala activity in response to threatening stimuli has become the best-replicated OT finding in humans.

Eckstein et al. (2015) focused on another important aspect of fear processing: fear learning. To study this aspect, fear is experimentally evoked and then potentially revoked. Obviously, this can only happen in a constrained and controlled manner to prevent any harm to the participants and infringement of ethical guidelines. Accordingly, the authors used a Pavlovian fear conditioning paradigm to study the effects of OT on fear extinction. In that study, the participants were randomly assigned to one of two groups: one group received intranasal OT and the other group placebo. Subjects were presented with pictures of faces or houses. For one part of these pictures, the participants simultaneously received a mild, non-painful electric shock for 70% of the time (CS+; fear-associated stimulus) to achieve fear conditioning. For the other fraction of the picture set, no shocks were administered (CS−; non-fear-associated stimulus). After this conditioning procedure, the participants received either intranasal OT or a placebo. After a 30-minute break, the extinction procedure inside the MRI started. For this purpose, the identical picture set was shown to the participants, but this time without applying any electric shock. Simultaneously, skin conductance responses (SCRs) were recorded. SCRs are considered a physiological marker of arousal and can thereby indicate the intensity of the experienced fear response. During the extinction phase, when presented with the conditioned stimuli that had been associated with electric shocks, there was at first a greater increase in SCRs under OT that was followed by a stronger decline compared to placebo. The increase in SCRs during the early extinction phase was mirrored by elevated activity

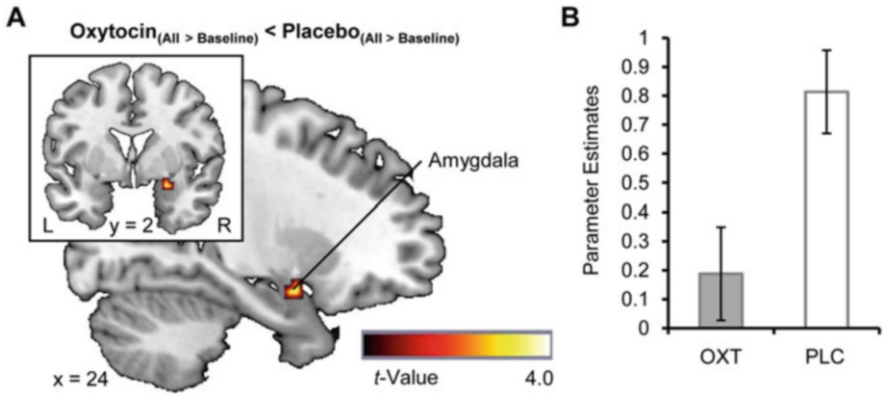


Fig. 2.4 Effects of oxytocin on amygdala activation. (**a**, **b**) Decreased response in the right amygdala. L, left; OXT, oxytocin; PLC, placebo; R, right. Reprinted from Eckstein et al., 2015, with permission from Elsevier

in prefrontal brain areas and connectivity to the posterior cingulate cortex and the precuneus. These areas have previously been related to human fear of extinction. In contrast, OT dampened amygdala activity during the entire extinction procedure (Fig. 2.4). By this twofold mechanism of action, OT is assumed to facilitate fear extinction in humans. These are only two of many studies demonstrating potentially anxiolytic effects of OT in humans, mediated by a downregulation of amygdala response.

2.4.2.2 Attachment and Human Pair-bond

Following the work on voles and other non-human mammals, studies of neural correlates of affiliative behavior were carried out in humans. In one experiment, heterosexual pair-bonded male volunteers were asked to rate photographs of 3 women: their female partner, another highly familiar woman and an unknown woman regarding their attractiveness (Scheele et al. 2013). OT increased the neural response to the partner stimuli compared to unfamiliar women in several brain reward regions, including the ventral tegmental area (VTA) and the nucleus accumbens (NAcc). These results were mirrored by elevated ratings of the partner's face following intranasal OT administration. This effect was absent for familiar women. The increase in NAcc activity for the partner compared to a familiar woman points towards a pair-bond specificity of this oxytocinergic effect. This lines up with the finding in voles that the OT receptor density was significantly higher in the monogamous compared to the polygamous species (Insel and Shapiro 1992). Additionally, OT administration reduced VTA activation to the faces of other women. By this dual mechanism, OT could contribute to maintaining long-lasting pair relationships in men by increasing the reward value of their female partner and decreasing the value of interactions with unfamiliar women. In a later study (Scheele et al. 2016), a similar OT-mediated mechanism has been observed in women who viewed pictures of their male partners.

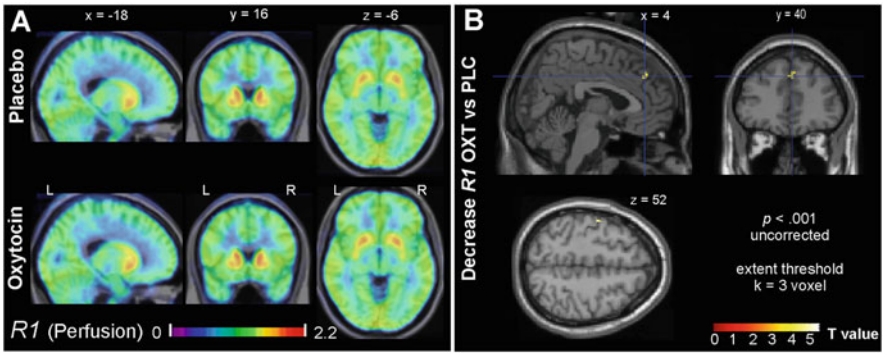


Fig. 2.5 Perfusion effects of oxytocin. (**a**) Relative radioligand delivery rate, R1, reflecting perfusion or blood flow, under oxytocin and placebo. (**b**) Change in R1 at the level $p < 0.05$. Under oxytocin, increased perfusion was observed in the following regions: left anterior insula, subgenual and posterior cingulum, nucleus accumbens and fusiform gyrus; right superior parietal gyrus and medial frontal gyrus. Lower blood flow under oxytocin occurred bilaterally in the dorsomedial prefrontal cortex, expanding into the dorsal cingulum and bilaterally into the inferior frontal gyrus, pars opercularis. *OXT* oxytocin, *PLC* placebo. Adapted from Striepens et al., 2014, with permission from Elsevier

Despite the role in maintaining romantic relationships, there is some evidence that OT might also be implicated in the formation of human pair bonds. In a positron emission tomography (PET, Box 2.3) study, healthy male volunteers rated the attractiveness of photographs of unfamiliar female faces once after administering placebo and once after OT (Striepens et al. 2014). Administration of OT boosted the ratings of the unfamiliar females. This behavioral effect correlated with increased perfusion in the striatum encompassing the caudate and NAcc, centers of the neurocircuitry of reward (Fig. 2.5). Thus, OT could facilitate human pair-bond formation by promoting the perceived attractiveness of unfamiliar females.

It is known that touch can increase endogenous OT levels, e.g., in romantic relationships and between parents and infants. Nevertheless, until recently, it was unclear how OT, in turn, influences the perception of the touch. Scheele et al. conducted an experiment in which heterosexual males were made to believe that they were either touched by a male or female experimenter while they were actually touched by the same female experimenter (Scheele et al. 2014). The experimenter stroked the volunteers' shinbone in a standardized fashion while they were lying in the MRI scanner. Intranasal OT enhanced the pleasantness ratings of female touch, and this was paralleled by an increased neural response in several brain regions involved in social touches such as the insula, precuneus, posterior-anterior cingulate cortex and the orbitofrontal cortex. In this experiment, OT might have increased the reward value of social touch, and this could be one mechanism promoting the rewarding experience of physical intimacy in a romantic relationship. This reinforcing mechanism could contribute to forming attachment as well as the maintenance of romantic pair bonds in humans. The same experiment was carried out by Kreuder et al. (2017), but this time with heterosexual couples. The couples

believed they would be either touched by their partner or an unfamiliar person of the opposite sex, whereas they were always touched by the same person. Behaviorally, OT amplified the pleasantness ratings if the participants believed they were touched by their romantic partner which was mirrored by an increased BOLD response in the NAcc. The NAcc activation was even related to the volunteers' assessment of their relationship quality. As mentioned before, the NAcc is part of the neurocircuitry of reward, and the enhanced response could have increased the reward value of the partner's touch while simultaneously reducing the reward value of a stranger's touch.

The same group carried out a study examining the effect of OT on partner support when experiencing experimentally induced pain (Kreuder et al. 2019). Specifically, 97 heterosexual couples participated in this study, with one partner being scanned and receiving either OT or placebo intranasally. The pain induction was achieved by the application of brief electric shocks, the intensity of the shocks being individually determined before the scanning session. The shocks were applied to the left, non-dominant hand while the participants were instructed that their right hand was either held by their romantic partner, a stranger of the opposite sex, or a rubber hand as the non-support control condition. In reality, the hand was always held by the same male experimenter wearing a cotton glove to control for possible skin and temperature differences. The volunteers then rated the unpleasantness of the shocks. OT significantly decreased the unpleasantness ratings of the shocks regardless of the support condition but had no effect when no shock was applied. Notably, the stronger the couples rated their romantic love, the stronger was the pain-relieving effect in the OT group, but not the placebo group. At the neural level, OT enhanced the beneficial effect of partner support relative to no support in the left anterior insula (AI) by significantly reducing the response to the electric shocks and elevating the response in the right middle frontal gyrus (MFG). This latter effect correlated with the unpleasantness ratings of shocks under partner support. Additionally, under OT, an increase in functional coupling between AI and MFG was observed. The AI is involved in the integration of salience information about stimuli, including nociceptive processing. The pain-relieving effect was also present, although weaker, for stranger support, which indicates a general role of social support for the experience of noxious stimuli by reducing the salience of such stimuli. The work reported here points towards an integral role of OT in human affiliative behaviors and corresponding neural mechanisms.

2.4.2.3 Interpersonal Trust and Cooperation

In this section, some experiments examining OT's effect on interpersonal trust and cooperation will be presented. At the beginning of this chapter, the study by Kosfeld et al. (2005) and their finding of increased trust towards others following OT administration was mentioned. The experimental paradigm used in this study was the so-called trust game, a well-established task in research on the edge of the disciplines of economy and neuroscience. In this trust game, two players are interacting anonymously with the objective of a monetary gain. The generated profit will usually be disbursed for the participants to take home, giving them an objective

incentive. This game involves two roles: one investor and one trustee. In the first step, the investor chooses the amount of money s/he wants to invest in the unknown trustee, keeping in mind that transferring money is an act of trust and bears risks. The invested amount is tripled and added to the trustee's account. The trustee has two options now: s/he can return the investor's trust and share the money equally between the two players or s/he can retain the whole amount. The latter option is usually referred to as a breach of trust. The game is designed as a one-shot game, meaning that the decisions made in one round will not directly influence the next. The investors in the study by Kosfeld et al. transferred a significantly higher amount, which was interpreted as increased trust towards others. Baumgartner et al. (2008) aimed to establish whether OT influences the player's behavior when the trust is breached and how this is moderated by the neurocircuitry of trust. In this study, the participants played as investors, and the trustee's responses were taken from a pilot study and adjusted in a way that the investor's trust was breached about 50% of the time. After half of the rounds, the investor received feedback about the proportion of rounds s/he invested and the trustees' transferring behavior. As might be expected, the volunteers in the placebo group adapted their behavior when they received the feedback about the betrayal of trust by a decrease in transfers. Astonishingly, this was not true for the OT group as they did not change their behavior following feedback. This sustained trusting response was mediated by a reduced activation of the amygdala and connected brainstem effector sites known to be implicated in fear and stress responses. This diminished fear response compared to placebo might have facilitated overcoming the risk of further betrayal. The neural process was accompanied by a downregulation of activity in the caudate nucleus, a brain region engaged in reward learning related to feedback processing and behavioral adaptation. Interestingly, a reduction of caudate activation in the trust game has been previously reported when a player perceived the partner as good or trustworthy (Delgado et al. 2005). The authors interpret this finding as an implicit assumption of their game partner's trustworthiness.

Another paradigm to test reciprocal human cooperation with an unknown partner is the so-called Prisoner's Dilemma game. In this game, two players can either cooperate or defect and, as in the trust game, receive a monetary payoff based on the interaction of their respective choices. The highest payoff can be received if the first player chooses to cooperate, but the second player defects or vice versa, but only the defecting player will get the payoff, while the other cooperating player will receive nothing. The second highest payoff will be obtained if both players collaborate, followed by the option that both players opt to defect. Chen et al. (2016) utilized this task inside a scanner after administering intranasal OT to healthy men. OT attenuated the neural response to unreciprocated cooperation in the amygdala. Betrayal as a form of negative social interaction can evoke a stress or fear reaction that would be reflected in an increased amygdala response. Although OT did not significantly change the behavioral outcomes in this study, the attenuation of amygdala activity might contribute to maintaining a cooperative, trusting style of interaction despite the experience of betrayal. The ability to trust is crucial for every society, as without trust, social cooperation or cohesion would not be possible. The

reported studies might propose one mechanism by which OT secures cooperation and bonds between individuals and within a society.

2.4.3 Making the Story Complicated: Heterogeneity of Oxytocin Effects

2.4.3.1 Dose-Response Relationship

Most studies have used similar doses of OT, mainly 24 international units (IU), but only very few studies have systematically tested the dose-response relationship to OT in humans; one might intuitively assume that the higher the dose, the stronger the effect, but this does not necessarily hold true. Accordingly, one study testing different doses of OT has actually found a smaller OT effect on the cortisol stress response with a higher dose of 48 IU compared to the standard dose of 24 IU (Cardoso et al. 2013). Since the dampening of amygdala responses in men following OT administration has been the most reliable neuroimaging finding, Spengler et al. (2017) selected this region to address the question of dose-response relation of intranasal OT. While lying in the MRI, the subjects performed a facial emotion recognition task with face photographs of different emotional valence and intensity, ranging from happy over neutral to low- and high-intensity fear. The participants then had to classify the depicted emotion. The different doses of OT (12, 24, and 48 IU) and latencies (15–40, 45–70, and 75–100 min) elicited divergent neural responses.

Both 12 IU and 24 IU decreased the amygdala response to highly fearful faces compared to neutral faces, but only the 24 IU dose achieved a significant reduction with the strongest effect in the time window between 45 and 70 min. Surprisingly, the 48 IU dose even exerted opposite effects on the amygdala activity and resulted in an amplification (Fig. 2.6).

This points to an optimal dose and latency range of the anxiolytic effects of OT in the form of an inverted-U shape, with lower as well as higher doses leading to the absence of the desired effect or even the opposite effect. One potential explanation for the increase in amygdala activity following the higher dose is an interaction with a related neuropeptide system—the vasopressin system. At higher doses, the OT receptors might be saturated, causing OT to bind to vasopressin receptors and producing opposing effects. This study presents a first glimpse into the prevailing heterogeneity of the neurobehavioral effects of OT in humans.

2.4.3.2 Non-prosocial Effects of Oxytocin

Since the pioneering work of Kosfeld et al. (2005), there have been several attempts to replicate the trust-enhancing effects of OT, but with mixed results. However, most replication studies differ in one or more key aspects from the original. Declerck et al. (2020) attempted to replicate the initial study accurately. As in the original study, participants had minimal contact with each other prior to the experiment while waiting in a common room, but without knowing they would play with each other or who exactly they were playing with. This replication study extended this

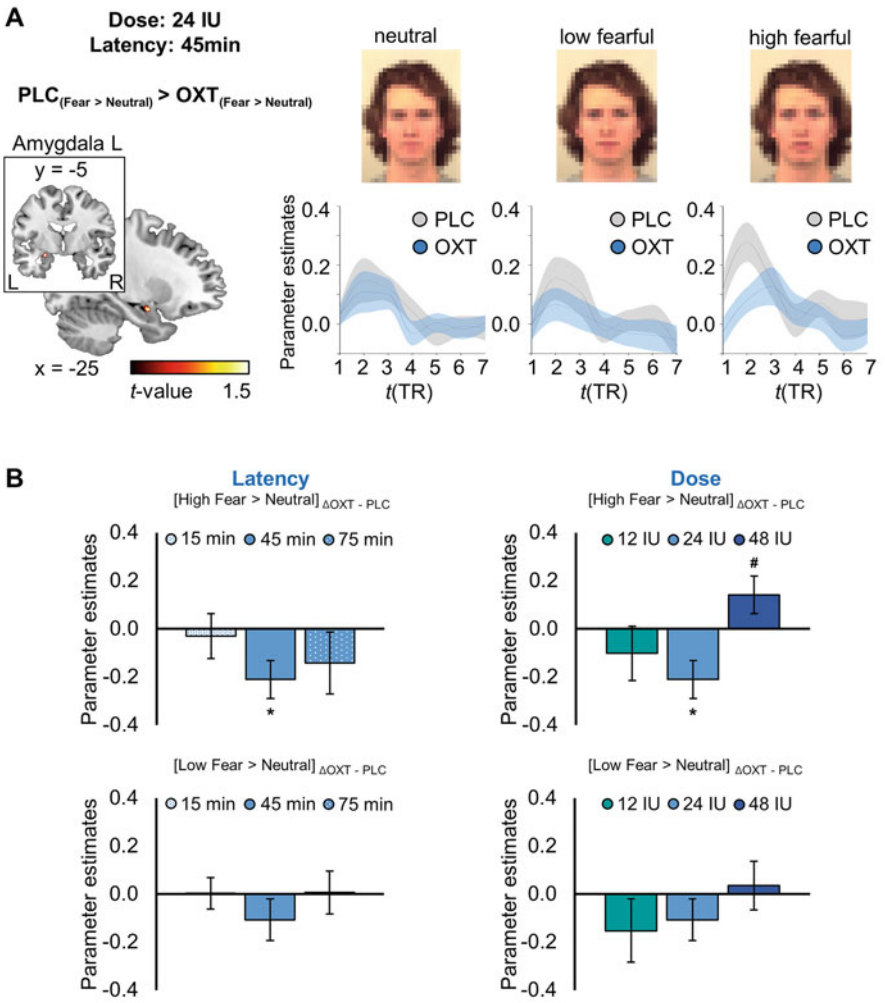


Fig. 2.6 Dose- and latency-dependent effects of oxytocin (OT) on the left amygdala during an facial emotion recognition task. During the task, participants viewed face pictures depicting the emotions of fear (low or high intensity), happiness (low or high intensity), or no emotion. (**a**) Diminished amygdala response to fearful faces following 24 international units (IU) administered 45 min prior to the task onset. The strongest effect of OT for high fearful faces. (**b**) OT effect on amygdala activation to low- and high-intensity fearful faces following three different doses (12 IU, 24 IU and 48 IU) and dose–test latencies (15 min, 45 min and 75 min), respectively. The largest decrease in amygdala response occurred after 24 IU administered 45 min prior to the task onset. L, left; PLC, placebo; R, right; TR, repetition time. Reprinted from Spengler et al., 2017, with permission from Elsevier

condition with a no-contact condition where players had not met before. In contrast to the original work, no effect of OT was found on trusting behavior in the trust game in the minimal contact condition. In the no-contact condition, they observed an increase in trust in only a subsample of participants with a low disposition to trust. This study points towards two important developments in the OT field: first, initial and groundbreaking results have sometimes failed to be replicated. The underlying reasons are manifold and may include methodological shortcomings such as small sample size and deficient test-retest reliability. Second and maybe more importantly, there are various factors moderating the effects of OT, including interindividual differences (e.g., the personality trait of readiness to trust strangers) and contextual factors. Similarly, in a complex experimental setting investigating trust in the context of social value representations in the amygdala, Liu et al. (2019) demonstrated that distinct neural and behavioral effects of OT depend on personality traits. They selected two groups of participants based on their social reference point, either prosocials or individualists. Both groups were given intranasal OT, but OT enhanced trusting behavior only in individualists and increased the amygdala response, assumed to represent social value representation. In a similar manner, in the social touch study by Scheele et al. (2014), OT increased pleasantness ratings only if the male participants assumed they had been touched by a female experimenter. The following section explores some of these contextual factors that have led to great heterogeneity in OT findings in the past decade and challenge the view of OT as a purely prosocial and/or anxiolytic neuropeptide.

In addition to the absence of prosocial effects or the existence of those effects limited to certain subgroups, OT can even act in the opposite direction by enhancing aggressive or antisocial behaviors. Lambert et al. (2017) employed another economic game similar to the trust game, with an aggressive style among players yielding the highest payoff, but only if the opponent does not exhibit aggression. In that case, a cooperative style would be preferable. Social cues, i.e., angry or neutral faces, were presented to indicate a threatening or safe decision environment. Neural activation patterns were similar to previous studies, i.e., increased activation in the NAcc and a decrease in the amygdala following OT administration. However, under OT, participants flexibly employed a behavioral response style to maximize their own personal gain based on the social cues—aggression or cooperation. Thus, OT did not enhance prosocial behavior *per se,* but would, depending on the context, also promote antisocial behavior. Several animal and human behavioral studies have shown antisocial and aggressive effects of OT, but these effects have rarely been investigated with fMRI. As such, the results from two behavioral studies should be briefly mentioned. Shamay-Tsoory et al. examined the effects of OT on reactions in situations when the participants either gained or lost more money than a fake participant in an economic game (Shamay-Tsoory et al. 2009). Contrary to their hypotheses, OT did not decrease but rather increased envy and schadenfreude (gloating). Another study investigated the effect of OT on inclinations of violence towards intimate partners (DeWall et al. 2014). In the subgroup of participants with a tendency to physical aggression, OT increased such inclinations.

2.4.3.3 Anxiogenic Effects of Oxytocin

A growing body of research from human as well as animal studies has shown that OT does not always dampen stress and fear responses but can also have anxiogenic effects. One paradigm inducing social stress that is suitable for an MRI is the Montreal Imaging Stress Task. In this task, participants are instructed to carry out mental arithmetic and receive feedback that their performance is considerably inferior compared to others. OT significantly enhanced the perception of social stress, while these increased levels of perceived stress were related to elevated activity in the anterior cingulate cortex and precuneus (Eckstein et al. 2014). The anterior cingulate cortex has previously been implicated in the sensation of social stress, the precuneus in self-referential thinking. Another example of the anxiogenic effects of OT is provided in the study by Eckstein et al. (2016). In Sect. 2.3.2.1, we described the effects of OT on fear extinction following Pavlovian fear conditioning (Eckstein et al. 2015). Using the same paradigm but shifting forward the time of OT administration prior to the fear conditioning, OT produced faster task-related responses and enhanced SCRs to fear-associated stimuli without dampening of the amygdala, but with increased activity in the right subgenual anterior cingulate cortex, and for social stimuli, in the left posterior midcingulate cortex.

2.4.3.4 Sex Dimorphic Effects

In pharmacological research, the issue of sex-specific effects has gained attention over the past several years (Bolea-Alamanac et al. 2018). Biologically, it is more complicated to examine drug-specific effects in females than in males as the menstrual cycle, with its phases of changing levels of hormones or hormonal contraceptives, must be considered. This is especially true when researching hormones. Thus, more resources, including money, time, and personnel, are necessary to represent drug effects during the different phases adequately. Consequently, in pharmaceutical research often only male samples are used in order to avoid potential interactions with the estrous cycle. This holds true for OT as the vast majority of studies employed only male participants. Studies that did use female or mixed samples have often shown distinct effects for men and women. Corresponding to the experiments done in male volunteers on the reward value of their female partner's face described in Sect. 2.4.2.2, Scheele et al. (2016) carried out a similar experiment in pair-bonded females. Half of the female volunteers were taking hormonal contraceptives. Parallel to the results in men, OT increased the perceived attractiveness of their partner's face compared to other men's, augmented by heightened neural responses in reward-related regions comprising the NAcc. Interestingly, these OT effects were altered in women using hormonal contraceptives. This is one further example of how hormone interactions can influence oxytocinergic effects.

Another study examined the kinetics of OT effects on amygdala activation in females and compared the results to the study by Spengler et al. (2017) reported previously. Lieberz et al. (2019) used the same fMRI task and tested three different doses of OT (6,12, and 24 IU). Contrary to the results in men, OT significantly elevated amygdala reactivity to highly fearful and somewhat fearful faces,

independent of dosing in the administered range. Accordingly, OT does not seem to exert the same anxiolytic properties in women. Likewise, an increased striatal response to highly and low happy faces was found but were absent for the male comparison group. Baseline differences in striatal activation between males and females disappeared following OT. These findings support the view of sexually dimorphic effects of OT in some functions and brain areas that are not caused by sex-specific dose-response functions. This dimorphism potentially results from interactions with gonadal hormones and dynamic patterns of OT receptor expression corresponding to the estrous cycle.

2.4.3.5 Social vs. Nonsocial

One aspect that has often been highlighted is the social specificity of OT effects. For example, Hurlemann et al. (2010) examined learning performance on a feedback-guided item-category association task under the influence of OT. The feedback was provided either socially as smiling or angry faces or nonsocially as green or red lights. Providing social feedback generally improved the learning performance, and this effect was more pronounced under OT. In the memory domain, OT selectively facilitated memory recognition for face stimuli but not for nonsocial stimuli (Rimmele et al. 2009). Furthermore, the pain-relieving effects of OT in the presence of social support have been reported in an earlier section (Kreuder et al. 2019). Without social support, OT failed to produce the same effect on heat-induced pain (Zunhammer et al. 2016). Likewise, the attenuation of the neural response to unreciprocated cooperation or breach of trust was present only for human partners and not when facing a computer (Baumgartner et al. 2008; Chen et al. 2016). Moreover, the anxiolytic effect of OT on the amygdala was more pronounced for social, compared to nonsocial threats (Kirsch 2005).

2.4.4 Attempts to Reconcile Conflicting Findings

What are the implications of these context-dependent and sometimes conflicting findings for the future research of this neuropeptide? Has scientific progress reached a deadlock? Or is there a way to reconcile these ambiguous findings? In this section, we will explore two overarching theories that try to find patterns and unite several—though not all—conflicting findings.

2.4.4.1 The Social Salience Hypothesis

The first metatheory concerning OT is the social salience hypothesis (Shamay-Tsoory and Abu-Akel 2016), which postulates that OT enhances the salience of social stimuli by orienting attention, depending on context factors. OT has been shown to affect several attentional subprocesses at the early stages of attention processes, orienting focus towards social cues. Considering the inconsistent findings on the perception of and neural response to stress and threat, the authors propose that OT reduces the stress response in positive, supportive contexts by increasing the salience of safety signals. On the other hand, when in an unpredictable and

threatening situation, OT may exert the opposite effect. Regarding the discrepancy between the prosocial and non-prosocial or even antisocial behavioral effects of OT, the authors suggest that rather than generally shifting the behavior towards sociality, the direction of effect may depend on the context: when an individual is in an aggressive or competitive situation, OT may enhance aggressive or competitive behavior patterns. When in a cooperative, emotionally positive situation, OT may promote prosocial behaviors, respectively. However, not only situational factors but also the relationship between parties concerned as well as other interindividual factors may have an influence on the directionality. Accordingly, the attachment style of the caregiver impacts the formation of infant-caregiver bonds. For instance, blood OT levels are higher in mothers with a secure attachment style than in mothers with an insecure attachment style (Strathearn et al. 2009) and saliva levels of OT correlated between caregivers and infants with higher levels of OT in parents and infants that showed higher affect-synchrony (Feldman et al. 2010). These early attachment styles can therefore influence the development of the OT system and subsequently affect the formation of social relationships and social behaviors throughout the lifetime. This perspective might contribute to the explanation of heterogeneous findings since no two humans will share all the social and biological factors causing the unique development of an individual's OT system and the subsequent interaction with the environment. There has been some evidence that the effects of OT depend on baseline capabilities promoting social salience, especially in individuals with impaired or reduced social functioning, but not in already socially optimally functioning ones. This points to a potential for enhancing social cognition in patients with psychiatric disorders that are characterized by deficits in social functioning. However, this theoretical framework of social salience also implies caution, as contextual factors might determine whether the administration of OT will have beneficial or detrimental effects.

2.4.4.2 The Allostatic Model

Another attempt at a more holistic model of OT action is the allostatic theory (Quintana and Guastella 2020). Quintana and Guastella argue against a pure social specificity of OT and for a more general role in preserving a biological equilibrium. Allostasis is the process of keeping a biological system stable by anticipating changes in the environmental conditions and consequently adjusting behavior and/or physiology before the system loses its balance. In contrast, homeostasis by that definition refers to adaptation processes after changes in the environment have occurred. The prediction of future needs and fluctuations in the environment is based on the integration of prior knowledge and experiences with currently available information.

This theory takes a phylogenetic and ontogenetic view of OT into perspective. Evolutionarily, OT-like peptides date back at least 600 million years, with the precursors to the mammalian OT emerging prior to the separation of vertebrates from invertebrates. Even in invertebrates with relatively unsophisticated nervous systems such as the roundworm, evidence has been found that the OT homolog supports association learning of a particular environment with aversive properties. In

humans, the heightened response to Pavlovian fear conditioning to social fear signals (Eckstein et al. 2016) described earlier is accordingly interpreted as an enhanced adaptive learning process to dynamic environmental demands. Moreover, brain networks integral for learning processes exhibit high expression of OT receptors. In less complex invertebrates, OT triggers tissue contractions. This role of OT is highly conserved in mammals, including humans. OT-mediated smooth muscle contractions can be seen during birth, lactation, digestion, etc.; all are states the authors consider to be conditions of changing demands that require adjustment of physiological processes. In more complex organisms like mammals and particularly humans, the role of OT has extended from basic peripheral functions into the central nervous system (e.g., bonding behavior). Taken together, OT signaling might promote survival and thereby provide an evolutionary advantage by facilitating behavioral flexibility.

The second line of argumentation refers to the dynamic role of OT during human development. First, OT neurons are produced during the embryonic stage of development, with altering patterns of receptor expression being specific for different developmental phases. The varying manifestations of the OT system correspond to changing functional demands. During fetal development, OT may facilitate childbirth through its analgesic properties and protection against hypoxia, support fluid expulsion from the lungs, and help cope with the birth trauma. In newborns, OT might increase the chances of survival by promoting infant-caregiver bonds and social learning. In early adult life, the oxytocinergic stimulation of human bonding with allies and potential mates can serve a clear evolutionary purpose. In the postpartum phase, OT, in turn, facilitates bonding with the infant. In montane voles, which otherwise exhibit very limited affiliative behaviors, the OT receptor distribution changes within 24 h after giving birth (Insel and Shapiro 1992). Differences between species in their bonding habits and the upbringing of their offspring are paralleled by varying neuronal OT patterns. The downside of this unique level of adaptation in humans might be the extreme vulnerability of the OT system to disruptions in early life. Such disruptions could cause impairments in learning, prediction, and response mediated by the OT system and may even lead to psychiatric disorders (e.g. Quattrocki and Friston 2014).

This theory does not contradict the role of OT in social behavior but shifts the focus from a purely social role to a more general one of promoting allostasis. The imperative role of social behavior for humans could explain the abundant findings within the social domain. On the other hand, the allostatic theory could account for situations when an individual's own survival and adaptation cannot be achieved by prosocial responses.

2.5 Perspectives

MRI-based methods offer various ways to deepen and advance our understanding of the neuroanatomy of the hypothalamus and the effects of the hypothalamic peptide OT on the human brain. Overall, interest in MRI-based hypothalamic anatomy and

function has seen a rise in the past several years, which can be attributed to the development of advanced imaging techniques, modeling approaches and automatization. In the future, these will allow for deeper investigation of hypothalamic structure-function relationships and linking neuroimaging and machine learning methods could be key to large-scale investigations of the hypothalamus. With that aim, training of neural network architectures could be extended to cover different imaging modalities, and additional information about underlying tissue properties in the hypothalamus could be incorporated to achieve a better understanding of hypothalamus anatomy and function.

Moreover, fMRI can help to decipher the role of OT in social cognition with task- and functional connectivity-based measures. Evidence from fMRI and other neuroimaging disciplines indicates a moderating role of OT for various functions in humans, including fear and stress processing, attachment and pair bonding, and cooperation and trust. Nevertheless, the precise neural mechanisms underlying these functions are still a matter of debate, and reproducibility has often failed to be achieved. The field of OT research hence finds itself at a critical stage. Thus, some challenges are awaiting in the OT field: foremost, establishing overarching theories and conducting sufficiently powered studies explicitly designed to test theory-driven hypotheses. Such theories should incorporate linking central and peripheral effects of OT as they are unlikely to be independent of each other and decipher the role of oxytocin beyond social cognition. Methodologically, developing a selective radioligand for OT in humans would be highly desirable to retrace the path of intranasal OT to the brain and investigate other potential routes of administration in order to manipulate central levels of OT reliably and efficiently. A profound understanding of the neurobiological mechanisms of the neuropeptide OT is required to develop OT into an effective therapy for social impairments in psychiatric disorders that is still desperately needed.

Key Literature

Baroncini et al. (2012) In this paper, detailed hypothalamic anatomy is assessed based on histology and visibility in magnetic resonance images.

Billot et al. (2020) This paper demonstrates the use of a neural network architecture for detailed hypothalamus segmentation and parcellation.

Kosfeld et al. (2005) This study inspired oxytocin research in human subjects.

Kirsch et al. (2005) This was the first paper to investigate effects of oxytocin on human BOLD response.

Quintana & Gustella (2020) This is currently one of the leading overarching theories on the role of oxytocin in humans.

Spengler et al. (2017) This was the first paper to investigate dose-response relationships for oxytocin in the human brain.

Payoelis et al. (2016) This was the first study to show the temporal dynamics and sites of action of oxytocin in the human brain.

Neudorfer et al. (2020) This was the first paper to generate a detailed morphological atlas of the hypothalamus and surrounding structures at the nucleus level.

Winterton et al. (2020) This current review provides insights of theoretical considerations and critical methodological advances in the oxytocin field.

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3 Generation of Hypothalamus and Adenohypophysis from Human Pluripotent Stem Cells

Daisuke Hagiwara, Hidetaka Suga, and Hiroshi Arima

Abstract

The hypothalamus orchestrates various essential physiological and behavioral processes via neuropeptide secretion. The adenohypophysis (anterior pituitary) is a major center for peripheral endocrine organs, which secretes systemic hormones responding to hypothalamic neuropeptides as releasing factors. This functional connection between the hypothalamus and adenohypophysis is indispensable for the endocrine system and homeostasis. Pluripotent stem cells are promising tools for studying the process of human organ development, disease modeling, and regenerative medicine. Differentiation methods derived from pluripotent stem cells have been studied over the last quarter of a century. Recent studies have succeeded in the differentiation into hypothalamus and adenohypophysis from mouse and human pluripotent stem cells by a three-dimensional floating culture method of embryonic bodies. The induced hypothalamic-like progenitors generate hypothalamic neurons such as vasopressin neurons. In the induction of adenohypophysis, Rathke's pouch-like structures are self-organized as seen in embryogenesis in vivo, and functional anterior pituitary hormone-producing cells are subsequently differentiated.

Keywords

Hypothalamus · Adenohypophysis · Pituitary · Embryonic stem cell · induced pluripotent stem cell · Differentiation

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Abbreviations

ACTH	adrenocorticotropic hormone
AVP	arginine vasopressin
BIO	6-bromo-3-[(3E)-1,3-dihydro-3-(hydroxyimino)-2H-indol-2-ylidene]-1,3-dihydro-(3Z)-2H-indol-2-one
BMP	bone morphogenetic protein
CPH	congenital pituitary hypoplasia
CRH	corticotropin-releasing hormone
DAPT	(2S)-*N*-[*N*-(3,5-Difluorophenacetyl)-l-alanyl]-2-phenylglycine tert-butyl ester
ES	embryonic stem
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
GATA2	GATA-binding factor 2
gfCGM	growth factor-free chemically defined medium
GH	growth hormone
iPS	induced pluripotent stem
IRX3	Iroquois homeobox gene 3
KSR	knockout serum replacement
LCA	large-cell aggregate
LH	luteinizing hormone
LHX3	LIM-homeobox 3
NKX2.1	NK2 homeobox 1
OTP	orthopedia homeobox
OTX2	orthodenticle homeobox 2
PAX6	paired box 6
PI3K	phosphoinositide 3-kinase
PIT1	pituitary transcription factor 1
PRL	prolactin
Rock	Rho-associated kinase
SAG	smoothened agonist
SF1	steroidogenic factor 1
SFEBq	serum-free culture of embryoid body-like aggregates with quick re-aggregation
SHH	sonic hedgehog
SIX3	SIX homeobox 3
SOX1	SRY-box transcription factor 1
TBX19	T-box transcription factor
TSH	thyroid-stimulating hormone
VAX1	ventral anterior homeobox 1

3.1 Introduction

The hypothalamus and pituitary gland are essential for fundamental physiological processes such as stress responses, growth, sexual development, reproduction, regulation of food intake and energy expenditure and circadian rhythms. In the central part of the brain, hypothalamic neurons integrate afferent information from the periphery and respond by releasing neuropeptides and neurotransmitters. The pituitary gland consists of the adenohypophysis (anterior pituitary gland) and neurohypophysis (posterior pituitary gland). Some of the hypothalamic neuropeptides reach the adenohypophysis through the pituitary portal vein and regulate anterior pituitary hormones, including adrenocorticotropic hormone (ACTH), growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Through the regulation of these anterior pituitary hormones, the adenohypophysis, in turn, stimulates or inhibits hormone secretion from peripheral endocrine glands such as the adrenal cortex, thyroid, and gonads. The neurohypophysis contains axon terminals of hypothalamic neurons producing arginine vasopressin (AVP) and oxytocin, the somas of which are located in the supraoptic and paraventricular nuclei in the hypothalamus.

Dysfunction of the hypothalamus and pituitary gland causes various systemic symptoms (Schneider et al. 2007). Currently, the only available treatment for hypothalamic and pituitary disorders is hormone replacement therapy, which is not able to fully and precisely meet the demands of hormone requirements, which may change from moment to moment under various physiological conditions. Indeed, adrenal crisis (a life-threatening condition due to glucocorticoid deficiency) occurs in a substantial proportion of patients with hypopituitarism (deficiency of pituitary hormones), and also adrenal crisis-associated death could occur even in educated patients treated with glucocorticoid replacement therapy (Hahner et al. 2015). Moreover, patients with ACTH-dependent adrenal insufficiency (glucocorticoid deficiency) have higher risks of diabetes mellitus, hypertension, hyperlipidemia, depression, and anxiety, presumably due to glucocorticoid overreplacement (Stewart et al. 2016). Furthermore, adipsic patients with diabetes insipidus (polyuria due to antidiuretic hormone AVP) exhibit severe fluctuations in serum sodium levels, resulting in poor prognosis (Arima et al. 2014). These still unsatisfactory results of conventional hormone replacement therapies are attributed to the lack of a hormonal feedback mechanism, which is an essential feature of the endocrine system.

To overcome the difficulties in the current treatment for hypothalamic and pituitary disorders, high expectations have been growing for regenerative medicine using hypothalamic and pituitary hormone-producing cells differentiated from human embryonic stem (ES) and induced pluripotent stem (iPS) cells. Theoretically, ES/iPS cells are able to differentiate into all types of cells in our body. In addition, these pluripotent stem cells provide an unlimited cell source because of their self-renewal properties. Since recent studies have succeeded in the differentiation into hypothalamic neurons and adenohypophysis cells from mouse and human ES/iPS cells (Wataya et al. 2008; Suga et al. 2011; Dincer et al. 2013; Wang et al. 2015; Merkle et al. 2015; Ozone et al. 2016; Zimmer et al. 2016; Lund et al. 2016;

Yamada-Goto et al. 2017; Ogawa et al. 2018; Kano et al. 2019; Mitsumoto et al. 2019; Kasai et al. 2020), regenerative medicine is coming closer to reality in the neuroendocrine field. Furthermore, these differentiated hypothalamic neurons and adenohypophysis cells can also be used for developmental basic research and disease modeling. In this chapter, we introduce achievements to date of differentiation into hypothalamic neurons and adenohypophysis cells from mouse and human ES/iPS cells.

3.2 Three-Dimensional Culture Method for Embryoid Body

Organ formation during embryogenesis consists of complicated and sophisticated processes with various local interactions among distinct cells and tissues. To imitate these developmental processes during embryogenesis, a three-dimensional culture is an ideal approach. An efficient three-dimensional culture method has been established for selective neural differentiation from ES cells, which is called "serum-free culture of embryoid body-like aggregates with quick re-aggregation (SFEBq)" (Watanabe et al. 2005; Eiraku et al. 2008). Dissociated ES/iPS cells are autonomically and quickly aggregated in low-cell adhesion well plates in the differentiation medium (Fig. 3.1).

The SFEBq method is appropriate for differentiation into various ectodermal derivatives from ES/iPS cells. In the SFEBq method, the ES/iPS cell aggregates exhibit self-organization (Sasai et al. 2012) and spontaneous formation of a highly ordered structure and patterning. This floating culture has revealed intrinsic programs driving locally autonomous modes of organogenesis and homeostasis.

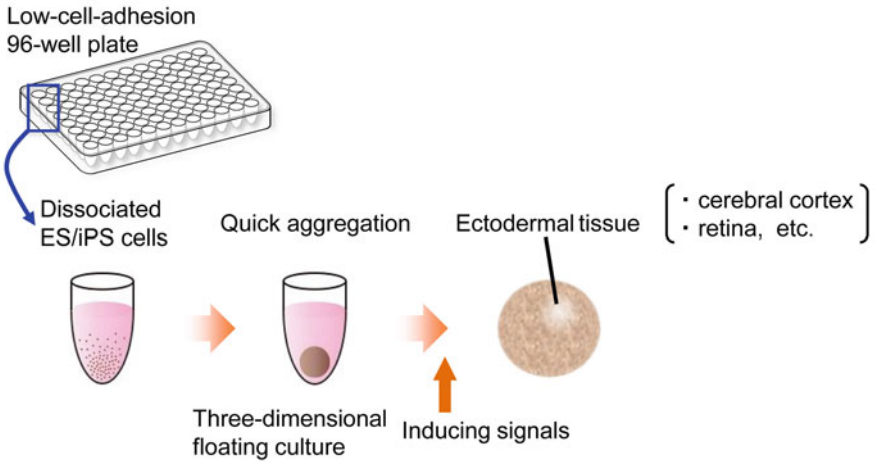

Fig. 3.1 Schema of SFEBq method. Dissociated ES/iPS cells are seeded into the low-cell-adhesion 96-well plate and quickly aggregated. Using inducing signals in the culture medium, the aggregates differentiate into aimed ectodermal tissues. Reproduced from Suga (2019), with permission

Based on the SFEBq method, cortex neurons (Eiraku et al. 2008; Danjo et al. 2011; Kadoshima et al. 2013), the optic cup (Ikeda et al. 2005; Osakada et al. 2008; Eiraku et al. 2011; Nakano et al. 2012), cerebellar neurons (Muguruma et al. 2010) and hippocampal neurons (Sakaguchi et al. 2015) have been generated from ES cells.

3.3 Mouse ES Cells as a Pioneer of a Human Model

Fundamental processes in differentiation studied in mouse ES cells can apply to human ES cells. For example, the retinal differentiation process in human ES cells (Nakano et al. 2012) has been established based on a previous report using mouse ES cells (Eiraku et al. 2011). Furthermore, the duration of mouse fetal development is approximately 20 days, which is considerably shorter than the 300 days of human fetal development. Since numerous trials and errors are required to establish a novel differentiation method, we should take advantage of mouse ES cells as a first step.

3.3.1 Hypothalamic Neuron Differentiation from Mouse ES Cells

SFEBq-cultured ES cells spontaneously differentiate into neural progenitors of the rostral forebrain and efficiently generate telencephalic progenitors (Watanabe et al. 2005). In the SFEBq method, ES cell aggregates are cultured in serum-free medium containing knockout serum replacement (KSR) without major exogenous inductive factors such as fibroblast growth factor (FGF), bone morphogenetic protein (BMP) or Wnt. However, the serum-free medium used in the original SFEBq culture still includes some exogenous signals that might affect the differentiation pathway. In particular, KSR, widely used for the maintenance and differentiation of ES cells, contains bioactive growth factors, including a high concentration of insulin and lipid-rich albumin purified from bovine serum.

Wataya et al. have established the differentiation method for hypothalamic neurons from mouse ES cells using a growth factor-free, chemically defined medium (gfCDM). Strict removal of exogenous patterning factors during early differentiation steps induces efficient generation of rostral hypothalamic progenitors ($Rax^+/Six3^+/Vax1^+$) in mouse ES cell aggregates. The addition of insulin to the SFEBq/gfCDM culture strongly inhibits differentiation into Rax^+ hypothalamic progenitors from mouse ES cells via the PI3K/Akt pathway. The ES cell-derived Rax^+ progenitors generate rostral-dorsal hypothalamic precursors ($Pax6^+/Nks2.1^-$) and subsequently AVP neurons that efficiently release the hormone upon stimulation. Besides, rostral-ventral hypothalamic precursors ($Pax6^-/Nks2.1^+$) and neurons are differentiated from ES cell-derived Rax^+ progenitors by treatment with the Sonic hedgehog (SHH) (Wataya et al. 2008).

ES cell differentiation into hypothalamic progenitors in SFEBq/gfCDM culture is summarized in Fig. 3.2. When cultured in the chemically defined medium without additional growth factors, including insulin, FGF, BMP, Wnt, and retinoic acid, ES cells frequently differentiate into $Sox1^+$ naïve neuroectodermal cells and subsequent

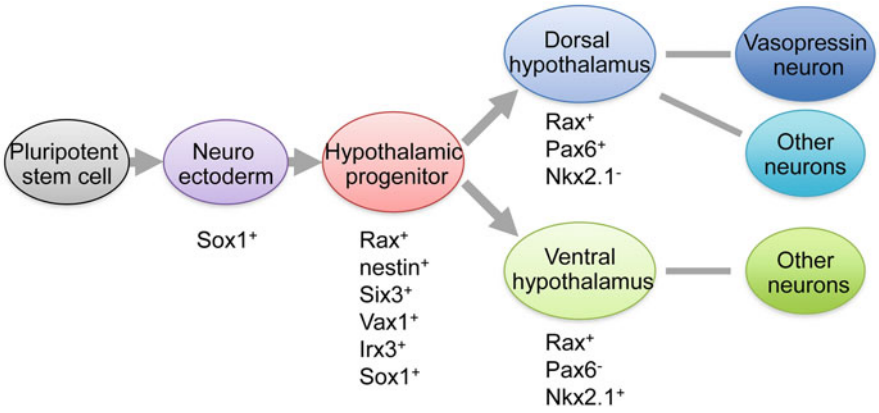

Fig. 3.2 Schema of hypothalamic differentiation. When cultured in the chemically defined medium without additional growth factors, pluripotent stem cells frequently differentiate into $Sox1^+$ naïve neuroectodermal cells and subsequent rostral hypothalamic progenitors (Rax^+/$nestin^+$/$Six3^+$/$Vax1^+$/$Irx3^+$). Without SHH treatment, these rostral hypothalamic progenitors have the characteristic of dorsal hypothalamic progenitors ($Pax6^+$/$Nks2.1^-$), while SHH treatment promotes differentiation into ventral hypothalamic progenitors ($Pax6^-$/$Nks2.1^+$). Dorsal hypothalamic progenitors generate AVP neurons. SHH-treated ventral hypothalamic progenitors give rise to neurons characteristic of the ventral hypothalamus. Modified from Suga (2019), with permission

rostral hypothalamic progenitors (Rax^+/$nestin^+$/$Six3^+$/$Vax1^+$/$Irx3^+$). Without SHH treatment, these rostral hypothalamic progenitors have the characteristics of dorsal hypothalamic progenitors ($Pax6^+$/$Nks2.1^-$), while SHH treatment promotes differentiation into ventral hypothalamic progenitors ($Pax6^-$/$Nks2.1^+$). ES cell-derived dorsal hypothalamic progenitors generate AVP neurons, presumably via Otp^+/$Brn2^+$ intermediate precursors. SHH-treated ES cell-derived ventral hypothalamic progenitors give rise to neurons characteristic of the ventral hypothalamus (e.g., $SF1^+$ glutamatergic neurons in the ventromedial hypothalamic nucleus, A12 dopaminergic neurons, and neuropeptide Y/agouti-related peptide neurons in the arcuate nucleus).

3.3.2 Adenohypophysis Differentiation from Mouse ES Cells

The principal aspect of the SFEBq method is replicating the embryonic differentiation environment and imitating complicated and sophisticated processes during embryogenesis. Therefore, a detailed understanding of developmental biology is essential for the establishment of differentiation methods from ES cells.

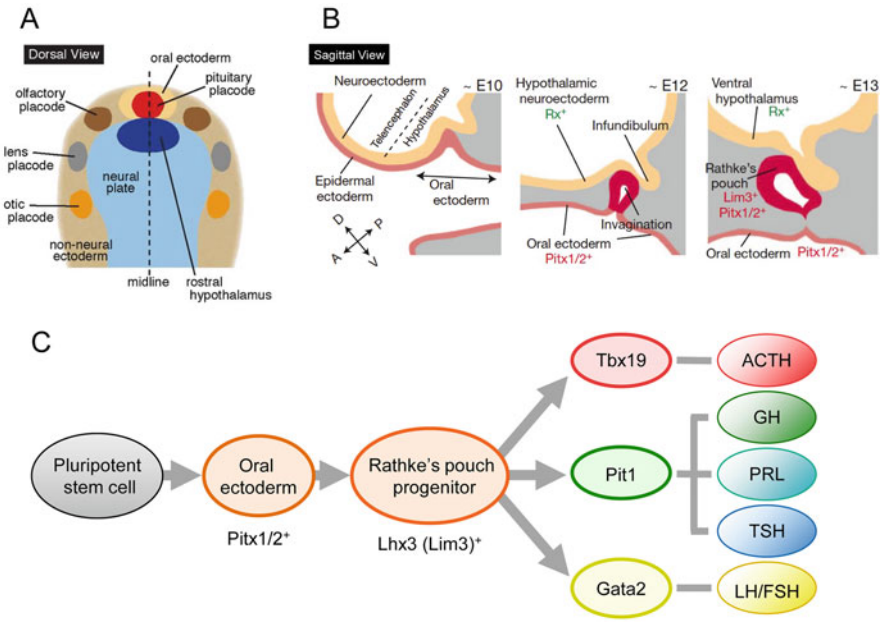

Fig. 3.3 Diagram of adenohypophysis differentiation. (**a**) Dorsal view of neural plate and placodes. The adenohypophysis anlage (pituitary placode) is located in the non-neural ectoderm adjacent to the hypothalamus anlage (rostral hypothalamus). (**b**) Sagittal view of adenohypophysis embryogenesis. The thickened placode invaginates and subsequently detaches from the oral ectoderm, leading to the formation of a hollowed vesicle termed Rathke's pouch. The epithelial cells of Rathke's pouch express Lim3 (also called Lhx3). (**c**) Schema of adenohypophysis differentiation and subsequent generation of anterior pituitary hormone-producing cell lineages. $Lhx3^+$ Rathke's pouch progenitors (adenohypophysis progenitors) are derived from the oral ectoderm ($Pitx1/2^+$) and subsequently generate several lineages of anterior pituitary hormone-producing cells. Among them, the ACTH-producing cell lineage requires a transcription factor Tbx19. GH-, PRL-, and TSH-producing cell lineages are differentiated from $Pit1^+$ intermediate precursors. The third lineage differentiates into LH- and FSH-producing cells. Modified from Suga (2019), with permission

Box 3.1 Adenohypophysis Development In Vivo

The adenohypophysis anlage originates as a placode in the non-neural ectoderm adjacent to the hypothalamic anlage situated in the top of the anterior neural plate (Fig. 3.3a). The adenohypophysis placode and hypothalamic anlage interact with each other during early development. In particular, the thickened placode invaginates and subsequently detaches from the oral ectoderm, leading to the formation of a hollowed vesicle termed Rathke's pouch (Fig. 3.3b) (Zhu et al. 2007). The molecular nature of local inductive interaction underlying the initial phase of adenohypophysis formation has been

(continued)

Box 3.1 (continued)
intensively investigated, revealing that FGF, BMP, and SHH signals are involved as important factors (Takuma et al. 1998; Brinkmeier et al. 2007). Also, epithelial cells of Rathke's pouch express Lhx3 (also called Lim3). $Lhx3^+$ adenohypophysis progenitors generate several lineages of anterior pituitary hormone-producing cells (Fig. 3.3c). Among them, the ACTH-producing corticotroph lineage requires a transcription factor, Tbx19. GH-, PRL-, and TSH-producing cell lineages are differentiated from $Pit1^+$ intermediate precursors. The third lineage differentiates into LH- and FSH-producing cells. Lhx3 knockout mice reveal that Lhx3 is essential for these anterior pituitary hormone-producing cell lineages (Sheng et al. 1996).

3.3.2.1 Two-Layer Formation In Vitro as the First Step of Adenohypophysis Differentiation

As discussed above, the formation of Rathke's pouch is attributed to interactions between the hypothalamus and neighboring oral ectoderm. By inducing the hypothalamus and oral ectoderm simultaneously within the same ES cell aggregate *in vitro* to recapitulate these developmental processes in embryos, Suga et al. have established the differentiation method for adenohypophysis from mouse ES cells (Suga et al. 2011).

In the ES cells cultured by the SFEBq method for hypothalamic differentiation (Wataya et al. 2008), the expression of an oral ectoderm marker Pitx2 is low. Since oral ectoderm and hypothalamic progenitors are adjacent, a slight shift in the positional information is expected to promote the simultaneous generation of both tissues within the same aggregate in SFEBq culture. A key positioning factor for the oral ectoderm is BMP4. BMP4 treatment increased Pitx2 expression in SFEBq-cultured ES cells, whereas the BMP4 antagonist dorsomorphin suppresses the generation of the oral ectoderm. However, treatment with exogeneous BMP4 in contrast inhibits hypothalamic differentiation even at a low dose. An optional condition is a large-cell aggregation (LCA) culture (Fig. 3.4a; starting differentiation culture at 10,000 cells per aggregate, instead of 3000 in the original SFEBq culture). In the LCA-SFEBq culture, both the oral ectoderm ($Pitx1/2^+$) and hypothalamic progenitors (Rax^+) are differentiated simultaneously within the same aggregate (Fig. 3.4b, b′) as a result of the moderate elevation of endogenous BMP4 (Suga et al. 2011).

3.3.2.2 Self-Formation of Rathke's Pouch In Vitro

SHH signals are known to provide positional information to adjust towards the midline *in vivo* (Zhu et al. 2007). During embryogenesis, Rathke's pouch receives SHH signals from neighboring tissues and develops in the middle of the rostral head ectoderm. Also in vitro, treatment of smoothened agonist (SAG), a hedgehog agonist induces multiple oval epithelial clusters in the LCA-SFEBq-cultured mouse EB cell aggregates (Fig. 3.4c, c′). These oval structures are located between the oral

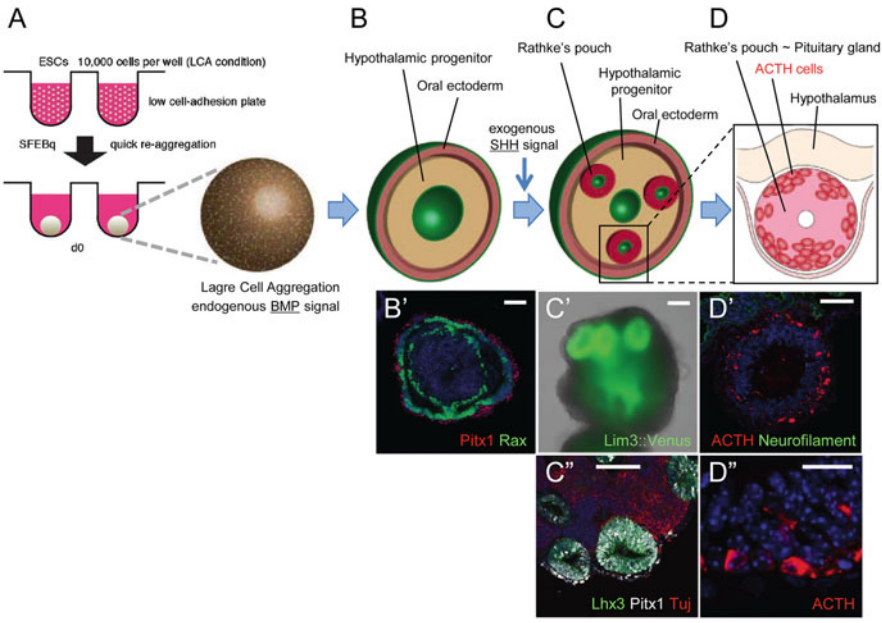

Fig. 3.4 *In vivo* differentiation into adenohypophysis from mouse ES cells. (**a**) Schema of LCA-SFEBq method. (**b**, **b′**) Two-layer formation of the oral ectoderm and hypothalamic progenitors in the LCA-SFEBq-cultured aggregates (**b**). Immunostaining of the aggregates (**b′**, scale bar 100 μm). (**c**–**c′**) Self-formation of Rathke's pouches (**c**). Bright field images (**c′**, scale bar 100 μm) and immunostaining of the aggregates with Rathke's pouches (**c″**, scale bar 100 μm). (**d**–**d″**) Generation of ACTH$^+$ cells in the differentiated adenohypophysis (**d**). Low-power field view (**d′**, scale bar 50 μm) and high-power field view (**d″**, scale bar 20 μm) of ACTH$^+$ area. Modified from Suga (2019), with permission

ectoderm and hypothalamic neurons (Fig. 3.4c″). Rathke's pouch marker Lim3 (also called Lhx3) is expressed in epithelial cells comprising these oval structures. The long axis of the Lim3$^+$ (Lhx3$^+$) pouches reaches a diameter of 150–200 μm, comparable to that of early Rathke's pouch *in vivo*. Interactions between the oral ectoderm and hypothalamic neurons are critical for *in vitro* induction of Rathke's pouch. Neither isolated surface ectoderm nor hypothalamus alone generates Lim3$^+$ (Lhx3$^+$) pouches (Suga et al. 2011).

3.3.2.3 Generation of Multiple Endocrine Lineages

During early adenohypophysis development, Lhx3$^+$ adenohypophysis progenitors commit to several hormone-type-specific lineages (Davis et al. 2011). Among them, the ACTH-producing corticotroph lineage requires the transcription factor Tbx19 (Lamolet et al. 2001), the expression of which is inhibited by Notch signaling (Zhu et al. 2006; Kita et al. 2007). Treatment with the Notch inhibitor DAPT increases Txb19 expression in SAG-treated LCA-SFEBq-cultured ES cell aggregates.

Substantial numbers of ACTH$^+$ cells accumulate in the Tbx19$^+$ domains of DAPT-treated pouch tissues (Fig. 3.4d–d″) (Suga et al. 2011).

Previous reports have shown that canonical Wnt signaling promotes Pit1 expression (DiMattia et al. 1997; Olson et al. 2006). Consistent with these findings, treatment with the Wnt agonist BIO increases Pit1 expression, leading to subsequent GH$^+$ and PRL$^+$ cell differentiation in the SAG-treated LCA-SFEBq-cultured ES cell aggregates. Head mesenchyme has been suggested to promote adenohypophysis development *in vivo* (Gleiberman et al. 1999). In the SAG-treated LCA-SFEBq-cultured ES cell aggregates with conditioned medium of PA6 stromal cells, LH$^+$, FSH$^+$, and TSH$^+$ cells are successfully differentiated (Suga et al. 2011).

Positive and negative feedback systems are characteristic of endocrine cells. The complete recapitulation of these properties is the ultimate goal for endocrine regenerative medicine. To investigate in vitro functionality, a corticotropin-releasing hormone (CRH) loading test has been performed on induced ACTH$^+$ cells generated in SAG+DAPT-treated LCA-SFEBq-cultured aggregates of mouse ES cells. From the SAG+DAPT-treated LCA-SFEBq-cultured aggregates, substantial amounts of ACTH are secreted, comparable to peripheral ACTH levels in mice. ACTH secretion from the adenohypophysis is negatively regulated by the downstream glucocorticoid hormone. Consistent with this hormonal regulation in vivo, ACTH release *in vitro* after CRH stimulation is suppressed by glucocorticoid pre-treatment. These findings demonstrate that the ES-cell-derived endocrine cells in the adenohypophysis actively secrete ACTH and respond to both positive and negative regulators that work for endocrine homeostasis in vivo (Suga et al. 2011).

3.3.2.4 Functional Rescue in Hypophysectomized Model Animals

In hypophysectomized mice with the SAG+DAPT-treated LCA-SFEBq-cultured aggregates transplanted into the kidney capsule, CRH loading induces a substantial elevation of blood ACTH levels and downstream glucocorticoid hormone corticosterone, indicating that ACTH from the graft sufficiently induced the downstream hormone. Even without CRH loading, the basal levels of ACTH and corticosterone are also increased after transplantation, suggesting that partial recovery of blood ACTH has a moderate but biologically significant effect. With hormonal recovery, the transplanted hypophysectomized mice exhibit higher spontaneous locomotor activities and survive longer. Although CRH, secreted from the hypothalamus, should be diluted at the peripheral site, mouse ES cell-derived adenohypophysis tissues improve survival and spontaneous activities even when transplanted into the kidney capsule (Suga et al. 2011).

3.3.3 Application to Human ES/iPS Cell Culture

Although human ES cells are vulnerable to apoptosis upon cellular detachment and dissociation, it has been demonstrated by Watanabe et al. that Y-27632, a selective inhibitor of Rho-associated kinase (Rock), markedly diminishes dissociation-induced apoptosis of human ES cells, which enables human ES cells to aggregate

in the SFEBq method (Watanabe et al. 2007). Based on differentiation methods into hypothalamic neurons (Wataya et al. 2008) and adenohypophysis (Suga et al. 2011) from mouse ES cells using the SFEBq culture, differentiation methods have been established for human hypothalamic neurons (Merkle et al. 2015; Ogawa et al. 2018) and adenohypophysis cells (Ozone et al. 2016) from human ES cells. Very recently, a functional hypothalamic-adenohypophysis unit has been generated using human iPS cells (Kasai et al. 2020).

3.3.3.1 Hypothalamic Neuron Differentiation from Human ES Cells

Merkle et al. have reported hypothalamic neuronal differentiation from human pluripotent stem cells using the SFEBq method, although hormonal secretion from differentiated hypothalamic neurons has not been demonstrated (Merkle et al. 2015). Recently, Ogawa et al. established a differentiation method for functional hypothalamic neurons from human ES cells (Ogawa et al. 2018).

In the development of hypothalamic neurons from mouse ES cells, strict removal of exogenous patterning factors is essential in the SFEBq/gfCDM culture (Wataya et al. 2008); however, human ES cells fail to aggregate and die within several days in this method. A small amount of KSR as well as Rock inhibitor Y-27632 enables human ES cells to aggregate in the gfCDM, although the cells differentiate towards the telencephalon under these conditions. Therefore, by the slight modification of positional information with BMP4, SAG, and Akt inhibitor, SFEBq/gfCDM-cultured human ES cell aggregates differentiate into rostral hypothalamic progenitors (Rax^+). Furthermore, the tuning of SHH signals induces dorsal ($Pax6^+$/$Nks2.1^-$) or ventral ($Pax6^-$/$Nks2.1^+$) hypothalamic progenitors. Via Otp^+/$Brn2^+$ intermediate precursors, dorsal hypothalamic progenitors generate AVP neurons that efficiently release the hormone upon stimulation (Ogawa et al. 2018). A schematic summary of human ES cell differentiation into hypothalamic neurons is shown in Fig. 3.5.

3.3.3.2 Adenohypophysis Differentiation from Human ES/iPS Cells

As demonstrated in the adenohypophysis differentiation method from mouse ES cells (Suga et al. 2011), the stages of differentiating pluripotent stem cells into adenohypophysis are as follows; (1) simultaneous induction of neighboring hypothalamic neuroectoderm and oral ectoderm, (2) self-formation of adenohypophysis anlage (Rathke's pouch) as a result of interaction between two layers of the hypothalamic neuroectoderm and oral ectoderm, (3) generation of multiple endocrine lineages from $Lhx3^+$ adenohypophysis progenitors, and (4) differentiation into functional anterior pituitary hormone-producing cells. By following the above steps and mimicking the differentiation method in mice (Suga et al. 2011), Ozone et al. have established the adenohypophysis differentiation method from human ES cells (Ozone et al. 2016).

In SFEBq-cultured human ES cell aggregates with gfCDM/KSR/Y-27632 medium, the addition of SAG, BMP4, and FGF2 to the differentiation medium induces hypothalamic neuroectoderm and oral ectoderm, following self-formation of $Pitx1^+$/$Lhx3^+$ Rathke's pouch-like oval structures (Fig. 3.6a). These structures

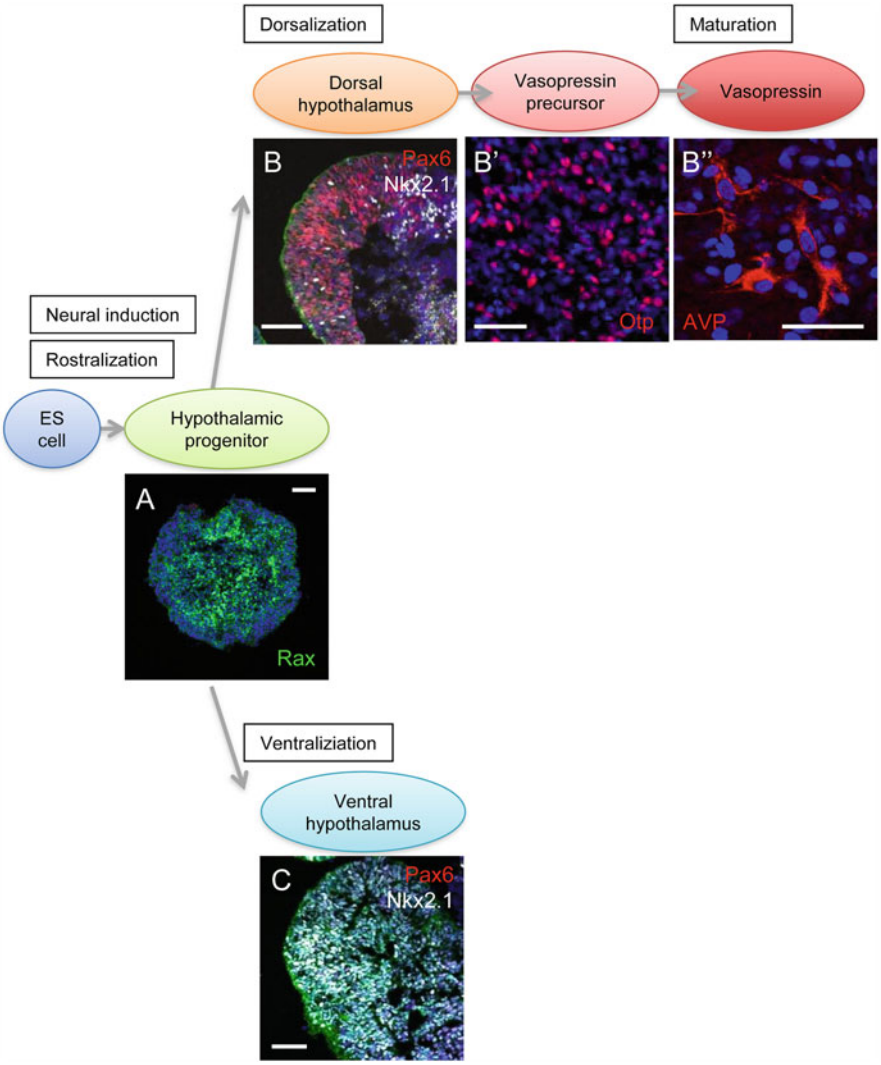

Fig. 3.5 Human ES cell differentiation into hypothalamus. Schematic summary of development from human ES cells to AVP neurons. Hypothalamic progenitors expressing Rax (**a**). Turning of SHH signals induces dorsal hypothalamus ($Pax6^{+}/Nks2.1^{-}$, **b**), the subsequent generation of AVP precursor cells (Otp, **b′**) and AVP neurons (**b″**), or ventral hypothalamus ($Pax6^{-}/Nks2.1^{+}$, **c**). Scale bars 50 μm. Modified from Suga (2019), with permission

subsequently differentiate into all lineages of anterior pituitary hormone-producing cells (Fig. 3.6b, c–c″, d–d″, e–e′). Among them, human ES cell-derived ACTH-producing corticotrophs and GH-producing somatotrophs have been demonstrated feedback systems. Moreover, electron microscopy reveals secretory granules characteristic of endocrine cells stored in the cytoplasm of these cells (Fig. 3.6f).

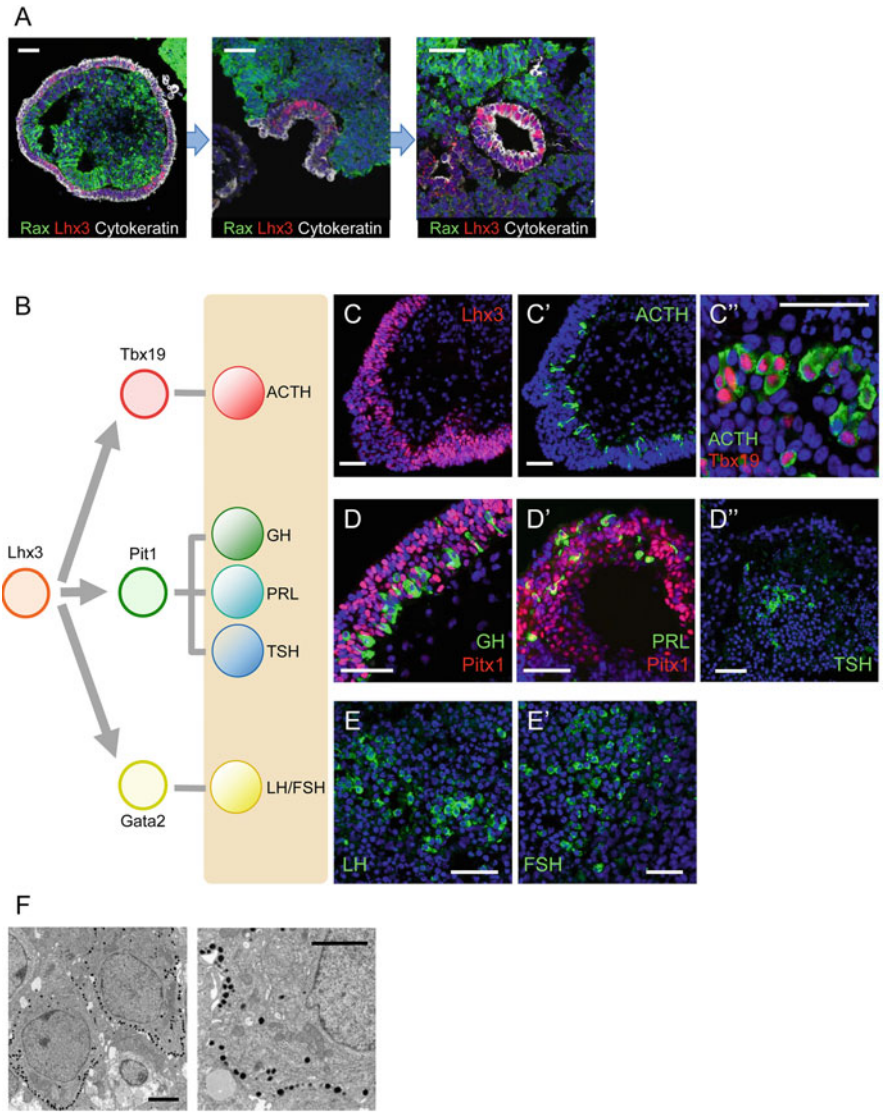

Fig. 3.6 Applied adenohypophysis differentiation in human ES cells. (**a**) Self-formation of Rathke's pouch-like structures. Scale bars 50 μm. (**b**) Schema of differentiation into multiple lineages of anterior pituitary hormone-producing cells. (**c**–**c**″) Corticotroph. Scale bars 50 μm. (**d**–**d**″) Somatotroph (**d**), lactotroph (**d**′), and thyrotroph (**d**″). Scale bars 50 μm. (**e**, **e**′) Gonadotrophs. Scale bars 50 μm. (**f**) Secretory granules characteristic of endocrine cells in electron microscopy. Scale bars 2 μm. Modified from Suga (2019), with permission

Furthermore, transplantation with human ES cell-derived adenohypophysis tissues into the kidney capsule improved survival and spontaneous activities in hypophysectomized mice (Ozone et al. 2016).

By following the above differentiation method with slight modifications, Kasai et al. have succeeded in generating a functional hypothalamic-adenohypophysis unit from human iPS cells (Kasai et al. 2020). This hybrid organoid exhibits simultaneous differentiation and maturation of the hypothalamic neurons and anterior pituitary hormone-producing cells within the same aggregates. Therefore, ACTH secretion capacity is comparable to that *in vivo* since CRH from the hypothalamic area regulates ACTH-producing cells in analogy with the hypothalamic-pituitary axis (Kasai et al. 2020).

3.4 Perspectives: Applications of Human ES/iPS Cell-Derived Hypothalamic Neurons and Adenohypophysis

3.4.1 In Vitro Human Model of Development and Disease

Human iPS cells are promising tools for studying the process of human organ development and its disorders. Recently, Matsumoto et al. have established a disease model of congenital pituitary hypoplasia (CPH) using iPS cells derived from patients with a heterozygous mutation in the orthodenticle homeobox 2 (OTX2) gene. The patient-derived iPS cells retain the potential to differentiate into the oral ectoderm but exhibit a severely impaired adenohypophysis differentiation. OTX2 in the hypothalamus is essential for progenitor cell maintenance by regulating Lhx3 expression in the ectoderm via FGF10 expression in the hypothalamus (Matsumoto et al. 2020).

iPS cell lines from patients with various hereditary diseases have been generated so far. Regarding hypothalamic and pituitary diseases, besides CPH described above, iPS cell lines from patients afflicted with familial neurohypophysial diabetes insipidus (Yoshida et al. 2020b) and multiple endocrine neoplasia type 1 (Yoshida et al. 2020a) have recently been generated.

3.4.2 Transplantation of Human ES/iPS Cell-Derived Hypothalamic Neurons and Adenohypophysis

ES cell-derived ACTH-producing cells function with hormonal regulation and improve survival and spontaneous activities in hypophysectomized mice even ectopically transplanted in the kidney capsule (Suga et al. 2011; Ozone et al. 2016). These findings raise the possibility of simple grafting in a peripheral site; however, physiological CRH release from the hypothalamus does not directly affect these peripheral grafts. Therefore, orthotopic transplantation into the sella or hypothalamus is one of the future candidates.

There are several challenges for regenerative medicine using human ES/iPS cell-derived hypothalamic neurons and adenohypophysis. First, the differentiation method still needs to be optimized: the maturity of ACTH- and GH-producing cells and the differentiation of other lineages are not enough. Also, xeno-free culture

systems are required for clinical application. Second, ensuring safety is essential for clinical use. Contamination of immature cells increases the risk of tumorigenesis. Therefore, purification methods for target cells need to be developed. Third, ethical issues should be considered appropriately, even though human iPS cells have fewer issues than ES cells in general.

Key References

Watanabe et al. (2005) The first paper to introduce a method of serum-free suspension culture (SFEBq; serum-free floating culture of embryoid body-like aggregates with quick re-aggregation).

Wataya et al. (2008) This paper demonstrates that strict removal of exogeneous patterning factors during early differentiation step induces hypothalamic progenitors in mouse ES cells.

Suga et al. (2011) Innovative work on self-formation of Rathke's pouch-like adenohypophysis progenitors and subsequent differentiation into functional adenohypophysis from mouse ES cells.

Ozone et al. (2016) This paper illustrates the differentiation method into adenohypophysis from human ES cells.

Kasai et al. (2020) Seminal work generating hypothalamic-adenohypophysis functional units from human iPS cells.

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The Neurohypophysis and Urophysis: Ancient Piscine Neurovascular Interfaces

4

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Abstract

Vertebrate homoeostasis is regulated by secretion of neurohormones from specialized neuroendocrine neurovascular interfaces such as the hypothalamic–neurohypophyseal system (HNS). Fish are shown to possess an additional caudal neurosecretory system (CNSS), which is termed urophysis, due to its anatomical location at the caudal spinal cord and its structural similarity to the hypophysis gland. The urophysis is a vascularized gland-like structure, which is interfaced by exceptionally large neurons termed Dahlgren cells. In contrast to the well-studied HNS of fish and mammals, the development and function of the urophysis/CNSS are not well understood, and related research has strongly declined in the last three decades. In this chapter, we summarize the main knowledge regarding the evolution, development and structure of the two neuroendocrine interfaces. Additionally, we describe the main knowledge regarding their regulatory and functional roles in fish homoeostasis. Where applicable, a general comparison to non-piscine vertebrates is described.

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Hypothalamus · Neurohypophysis · Neurovascular · Urophysis · Urotensin · Zebrafish · Dahlgren cell

4.1 Introduction

Neuroendocrine regulation of homoeostasis in most vertebrates is mainly orchestrated by the hypothalamus, a brain region whose neurons either affect the anterior pituitary gland by means of a vascular portal system, or directly form neurovascular interfaces with the capillary network of the posterior pituitary gland, the neurohypophysis, to release neurohormones into the circulation (Wircer et al. 2016; Biran et al. 2018). Interestingly, piscines uniquely possess an additional homoeostatic neurovascular interface known as the caudal neurosecretory system (CNSS, Fig. 4.1). In 1914, Dahlgren identified huge secretory cells residing in the spinal cord of elasmobranchs (Dahlgren 1914). A few years later, Speidel performed a systematic analysis of the caudal spinal cord of various fish species and identified the cells of Dahlgren in 26 out of 30 species he examined. Moreover, Speidel found that in more evolved fishes, Dahlgren cells innervate a vascularized glandular structure that shares high structural homology to the neurohypophysis (Speidel 1922). This glandular structure was later termed the urophysis. These discoveries initiated a great deal of research which resulted in the identification of novel

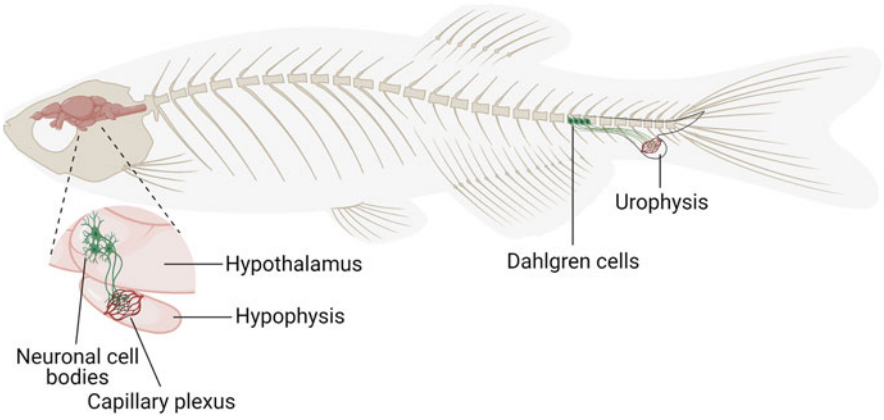

Fig. 4.1 Schematic representation of the neurohypophysis and urophysis in fish. The neurohypophysis is located in the posterior pituitary of the zebrafish brain, with axonal projections coming in from the hypothalamus. The axonal projections are interspersed within the vascular plexus of the posterior pituitary (magnified schema). The urophysis is located on the caudal region of the spinal cord with projections coming in from the Dahlgren cells located along the spine

neuropeptides affecting blood pressure—the urotensins, which were later shown to be functionally important in other vertebrates. Importantly, while the neurohypophysis releases its neuropeptides to the adenohypophysis and to the general circulation, vascular drainage of the urophysis delivers caudal neurohormones into the kidney, liver and swim bladder (Bern 1985). Despite the above findings, in the last twenty years there has hardly been any published information concerning the urophysis. This might be due to the uniqueness of the urophysis to fish species and the failure to identify a robust physiological function, which could be directly attributed to the CNSS (Bern 1985). In this chapter, we review some of the major findings regarding the piscine neurohypophysis and urophysis and their suggested neuroendocrine physiological functions in fishes.

4.2 The Hypothalamo–Neurohypophysis

The hypothalamo–neurohypophyseal system (HNS) is a neurosecretory interface, which is conserved across all vertebrate organisms. The fish HNS comprises two distinct populations of neurons that secrete the arginine-vasopressin-like (AVPL) and oxytocin-like (OXTL) neuropeptides, also known as arginine-vasotocin and isotocin, respectively, in all fish species other than zebrafish. In the interest of simplicity, we will henceforth refer to the piscine neurohypophyseal neurohormones as OXTL and AVPL. These cells reside in the piscine neurosecretory preoptic region (NPO) and posterior tuberculum (PT) of the fish diencephalon and project their axons onto the posterior pituitary, also known as the neurohypophysis, where they secrete their neurohormone cargo through a neuroendocrine–vascular interface (Biran et al. 2018). The termini of these neurons have distinctive swellings along their length which serve as synaptic release sites for their neurohormones (Tweedle et al. 1989). Upon their release, OXTL and AVPL are taken up by the fenestrated, i.e. permeable, capillary plexus of the neurohypophysis. The vasculature of this particular region is an extension of the cerebral vascular network; however, it possesses distinct qualities that allow for its selective permeability. Together, they also represent one of the key neurovascular interfaces, which will be discussed in length in a later section.

In addition to these components, the neurohypophysis also contains specialized astrocyte-like cells called pituicytes (Anbalagan et al. 2018; Chen et al. 2020). The pituicytes extend processes that engulf the secretory axonal termini, likely to act as a regulatory barrier to neurosecretion (Miyata 2017), like the glia of the fish urophysis, which were named urocytes (Kriebel 1980),

4.2.1 Evolution and Ontology of the Neurohypophysis

Box 4.1 The historical tale of the neurohypophysis
The hypothalamo–neurohypophyseal system has long posed enigmatic questions regarding its existence, and later its true function. The Dutch physiologist Van Rijnberk stated in 1901 that the posterior pituitary is a functionless rudimentary organ (Described in: Hackenberg and Etminan 2003). In 1908, Herring (Herring 1908) alluded to the presence of nerve fibres and neuroglia in the posterior pituitary, and described what would later be referred to as Herring bodies. A year later, Blair-Bell described the effects of pituitary extracts on atonic uteri during labour (Bell 1909); this work was in line with that of others, suggesting the physiological effects of pituitary extracts (Von den Velden 1913). However, the concept of neurosecretion in the neurohypophysis was first suggested in 1917, by Speidel (Scharrer 1987). This idea was carried forward by the seminal work of Ernst Scharrer in 1928 who described the histology of the European Minnow nucleus magnocellularis preopticus and revealed vacuoles/vesicles in nerve-gland cells in the diencephalon that may secrete hormones into the neurohypophysis (Scharrer 1928). In 1940, Ernst and Berta Scharrer published their neurosecretion concept of the hypothalamo–neurohypophyseal system (HNS), which was shown to be conserved across multiple vertebrate species (Scharrer and Scharrer 1940). The functionality of the HNS as a pathway for the secretion of neurohormones was finally accepted unanimously in 1949 after neurosecretory material in the hypothalamic neurons and the neurohypophysis were shown to be one and the same (Bargmann and Hild 1949).

The pars nervosa of the posterior pituitary is a conserved structure across all vertebrates, including over 34,000 piscine species. The structure and morphology of the HNS vary from the most primitive fishes, Cyclostomes, through to the higher vertebrates, including humans, however, the basic components of the system seem to remain constant.

Primitive fish, or the Elasmobranchs, lack a clear demarcation between the magnocellular (i.e. larger cells) and parvocellular cells (i.e. smaller cells) of the preoptic nuclei. Within the same class, we see larger cells, indistinguishable from each other, in the subclass of Holocephali. In more evolved fishes, the presence of two distinct populations of cells, the parvocellular and the magnocellular neurons, is noted from pre-teleosts through to the advanced teleosts (Wircer et al. 2016; Perks 1969).

As in pre-teleost bony fish, primitive teleosts such as the European eel (*Anguilla anguilla*) present a neurohypophyseal structure that is a thickened extension of the infundibular stalk. In advanced teleosts, this structure is better represented as a pituitary core, surrounded by adenohypophyseal tissue. Axonal innervations pass through the hypophyseal tract into the pars nervosa, characterized by bead-like

droplets along their length, carrying neurosecretory material. Multiple studies have shown the presence of granules or elementary vesicles in these swellings, containing neurohormones (Holmes and Knowles 1960; Navone et al. 1989; Anbalagan et al. 2019). While some researchers reported that the sizes of these elementary vesicles were similar to those found in the preoptic nucleus, other works demonstrated that two distinct elementary vesicles containing two different peptides may be present in the pars nervosa (Knowles et al. 1966; Leatherland and Dodd 1967).

Within the pars nervosa, the axonal termini are distributed amongst a dense capillary network that conveys neurosecretory material into the systemic circulation (Anbalagan et al. 2019). Within the purview of vascular structures of the pars nervosa, pre-teleostean fish, such as the longnose gar (*Lepidosteus osseus*), seem to possess a vascular portal system, while the teleostean structure lacks it (Sathyanesan and Chavin 1967).

The presence of neurohypophyseal glia, the pituicytes, is consistent across teleostean species. A striking result regarding these cells was observed in a few unrelated teleosts, European eel, European conger (*Conger conger*) and goldfish (*Carrassius auratus*) (Knowles and Vollrath 1966; Leatherland 1972). These studies showed that axonal bundles carrying neurosecretory material terminated not only around the dense capillary plexus, but also on the surface of the pituicytes (Knowles and Vollrath 1965). In primitive fish like the Cyclostomes, the pituicytes were described as being derived from the ependymal cells of the ventricles which proliferated into the pars nervosa (Green and Maxwell 1959). These ependymal cells were also found to be present in the Elasmobranchs, the spiny dogfish (*Squalus acanthias*), and were then described as "parenchymatous pituicytes" (Van de Kamer and Verhagen 1955).

4.2.2 Development of the Neurohypophysis

The neurohypophysis is formed as an invagination of the diencephalon floor, deepening to form the infundibular cavity. In zebrafish (*Danio rerio*), precursor cell clusters on both sides of the diencephalon merge to form a pituitary cluster at about 28 h post-fertilization (Glasgow et al. 1997; Chapman et al. 2005). Within 36 h post-fertilization, cell bodies from the NPO generate axonal convergence along the midline at the developing neurohypophysis (Gutnick et al. 2011). Structural analysis of the HNS in the adult European eel shows that axonal fibres projecting into the neurohypophysis are separated into bundles by the radial pituicytes (Knowles and Vollrath 1965). This suggests the possibility that the pituicytes reside in the pars nervosa during, and perhaps prior to axonal enervation.

Over the next 36 h, i.e. 72 h post-fertilization, the embryonic neurohypophysis undergoes vascularization, probably from angiogenic cues released by the axonal termini and astroglia in the region (Gutnick et al. 2011). The hypophyseal artery and vein grow into the developing region, conceivably from existing cerebral vasculature. Thus, endothelial vessels in the ventral diencephalon sprout towards the palatocerebral artery from 48 h post-fertilization, giving rise to the hypophyseal artery. At the same time, the primary head sinus sprouts bilaterally towards the

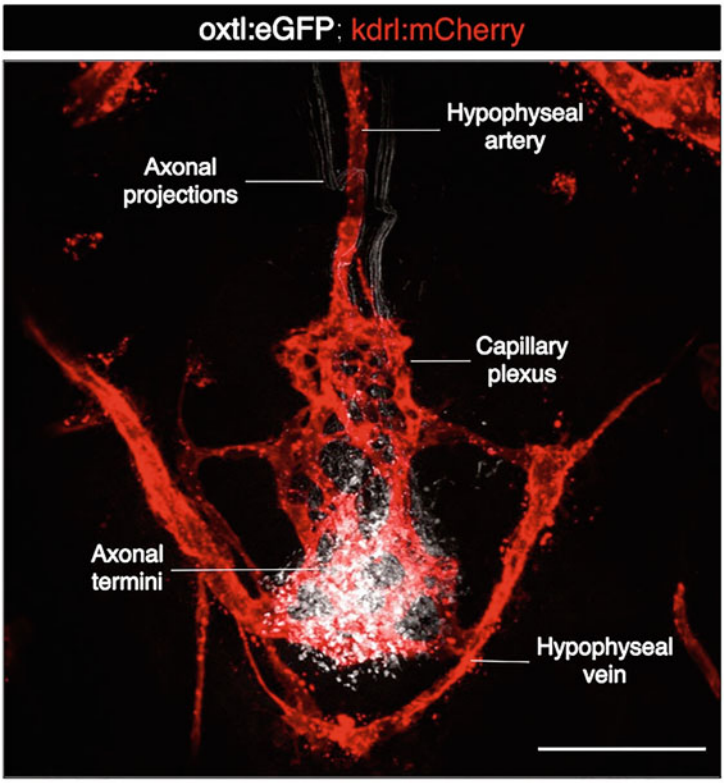

Fig. 4.2 Neurohypophysis in juvenile zebrafish. A confocal microscope image of a transgenic juvenile zebrafish (30-day old) in which both the hypothalamo–neurohypophyseal oxytocin neurons and blood vessels are genetically tagged with fluorescent proteins. The image shows the hypophyseal capillary plexus (in red) which is innervated by hypothalamic axonal projections (in grey) forming multiple neuro-vascular interfaces through which the oxytocin neurohormone is released into the peripheral blood circulation. *Oxtl* oxytocin-like, *kdrl* vascular endothelial growth factor receptor kdr-like

midline giving rise to the hypophyseal vein. By 72 h post-fertilization, these vascular branches fuse to create a loop-like structure dubbed the hypophyseal capillary (Gutnick et al. 2011). Thereafter, the hypophyseal capillary makes tight connections with the axonal termini, and along with the resident pituicytes, forms the basis of a functional neurovascular interface of the neurohypophysis (Fig. 4.2). As the animal develops further, the density of the axonal projections increases along with the complexity of the vasculature, forming an anterior and posterior capillary plexus with dense innervation of nerve fibres, and numerous pituicytes (Anbalagan et al. 2018; Gordon et al. 2019).

The adult neurohypophyseal structure bears clear differences in the structure of the pars nervosa (as outlined in the previous section) between the different classes of fish as well as in the interaction with intermediate lobes and the adenohypophysis parts of the pituitary. Non-teleostean fish, from the Elasmobranchs up to the

Holosteans, show the presence of neuronal projections from the pars nervosa projecting into the intermediate lobe of the pituitary. Remarkably, in teleosts the neural tissue invaginates into parts of the intermedia, extending all the way into the adenohypophysis (Perks 1969).

4.2.3 Neurohypophyseal Function

As in other vertebrates, the Piscine neurohypophyseal system is the primary region of secretion of two major homeostatic hormones, OXTL and AVPL. Together, these two neuropeptides regulate the homeostatic responses to various internal and external physiological challenges, ranging from water balance to social behaviour.

4.2.3.1 Osmoregulation

In mammals, AVP was first identified as an antidiuretic hormone, maintaining water balance in the organism by affecting water reabsorption rates from the kidney (Baratz and Ingraham 1959). AVP in the mammalian kidneys acts on AVP-V2 receptor to increase the expression of aquaporins in the membrane of nephron tubule cells, thereby increasing water reuptake rates. In teleost species, the V2 receptor was shown to be expressed in the nephros and the gills. For example, freshwater eels exposed to salt water had a marked increase in plasma AVPL levels. This result was also replicated when the freshwater eels were injected with saline solution intraperitoneally (Warne and Balment 1995).

Interestingly, the mRNA of avplv1 receptor was found to be expressed in the gills of the freshwater eel and the density of its expression was found to change depending on the osmolarity of the environment, salt water inducing increased expression of the receptor compared to freshwater (Balment et al. 2006). This suggests that osmoregulation in teleosts is mediated by coordination between neurohypophyseal AVPL and expression of its receptors in the gills.

These effects, while predominantly studied in the context of AVPL, were also observed in the case of OXTL. Studies in banded houndshark (*Triakis scyllium*) identified an increase in plasma OXTL after exposure of freshwater fish to salt water (Hyodo et al. 2004). OXTL has also been implicated in regulating ionocyte differentiation in zebrafish, thus affecting ion exchange to maintain optimal internal salt balance (Chou et al. 2011).

In the elasmobranch dogfish (*Scyliorhinus canicular),* perfusion of AVPL into *in situ* preparations of the kidney showed a marked antidiuretic effect. This study also implicated the addition of AVPL in decreased glomerular filtration rates, a possible mechanism by which the dogfish acclimatizes to reduced salinity (Wells et al. 2002).

4.2.3.2 Reproduction

OXT has been widely studied in mammalian models in the context of pregnancy, childbirth and lactation (Russell et al. 2003). In mammals, oxytocin receptors are widely found in both female and male reproductive tissues, affecting uterine contractions during labour, menstrual cycles and ovulation, milk ejection reflex

during lactation, sperm shedding from the testis and ejaculation (Burbach et al. 2006). In piscine species, however, OXTL has been implicated to play a major role in courting behaviour, egg-laying and sexual health of teleosts (Altmieme et al. 2019; Piccinno et al. 2014; Viveiros et al. 2003).

Male zebrafish subjected to female pheromones demonstrated increased courtship behaviour, which was inhibited following administration of OXTL and AVPL antagonists; it was, therefore, suggested that triggering the release of these neurohormones stimulates the central behavioural pathways, thereby increasing the possibility of reproductive success (Altmieme et al. 2019).

The two neurohypophyseal peptides also play a key role in the release of oocytes from fish females through their action on the smooth muscle cells of the ovarian wall. Ovarian wall contractility of gilthead seabream (*Sparus aurata* L.) was shown to be induced by *in vitro* administration of OXTL in vitellogenic non-spawning females (Piccinno et al. 2014). Similarly, when exposed to OXTL *in vitro*, testes slices of the African catfish (*Clarias gariepinus*) increased semen release to the media (Viveiros et al. 2003). These data demonstrate the involvement of OXTL and AVPL peptides in the regulation of reproductive functions at both central and gonadal levels in both mammals and fishes.

4.2.3.3 Behaviour

AVPL and OXTL exert major effects on mammalian behaviour, specifically in the context of social behaviour and dysfunction. In non-human mammalian models, these peptides allowed for better social recognition (Ross et al. 2009; Veenema et al. 2012), maternal behaviour (Bosch and Neumann 2012), and conversely played a role in cognitive impairments (Abramova et al. 2020).

Some of the mammalian phenotypes are recapitulated in fish shoaling and mating behaviours. In the case of the goldfish, these two peptides were shown to act in a conflicting fashion. While OXTL increased the tendency to social approaching, AVPL inhibited it (Thompson and Walton 2004). Additional complexity is added by the demonstration that HNS hormones also alter sex-specific social tendencies, as seen in the case of *Porichthys notatus* where the acoustic behavioural responses decreased in males and females only on exposure to AVPL and OXTL, respectively (Goodson and Bass 2000a, 2000b). Within the purview of hierarchical behaviour, exposing shoaling *Neolamprologus pulcher* cichlids to OXTL increased their awareness towards the dominant individuals (Reddon et al. 2012; Balshine et al. 2014).

Zebrafish are a social species in that they display collective behaviour in the formation of small, loose groups, known as shoals (Robinson et al. 2019; Miller and Gerlai 2012; Suriyampola et al. 2016). The absence of OXTL-mediated signalling was shown to reduce their shoaling tendencies, the converse of which was true when they were exposed to the peptide (Landin et al. 2020). Zebrafish OXTL receptor regulates memory recognition of familiar vs novel conspecifics (Ribeiro et al. 2020a, 2020b). The zebrafish receptor is also involved in the perception of biological motion, but not conspecific shape—two specific visual features that zebrafish use to appraise and react to social cues (Nunes et al. 2020).

It can be proposed that the effect of OXTL on fish behaviour is context dependent, as put forward in Ramsey's analysis of the social salience hypothesis that oxytocin expression allows improved cognitive processing in social contexts (Ramsey et al. 2019). The same can also be said in the case of AVPL expression in teleosts. Much like OXTL, the effects of AVPL on their behaviour seem to be context dependent. For example, intraperitoneal injection of AVPL into bluehead wrasse (*Thalassoma bifasciatum*) was shown to decrease aggression in territorial males while increasing it in non-territorial males (Semsar et al. 2001). Cohesively, the administration of Manning compound (AVPL receptor antagonist) was seen to inhibit these behavioural effects.

A key feature that should be noted is the differential effect of centrally and peripherally released hormones. In white perch (*Morone Americana*), intracerebroventricular administration of AVPL peptide showed strong activation of circuits involved in mating behaviour while circulating intraperitoneal AVPL injection had negligible effects on this behaviour (Salek et al. 2002).

Finally, although the classical effects of hormones, including OXT, is to activate or facilitate specific behavioural responses in an acute manner, OXT can have organizational effects on the developing social brain as well. Thus, pharmacological treatment of neonatal rats with OXT had long-term effects on behaviour in the adult (Noonan et al. 1989). Recently, it has been shown that OXTL can shape the structure of the developing forebrain as well as the functional connectivity of the so called social decision making network (SDMN) in zebrafish (Nunes et al. 2021). Thus, perturbation of zebrafish OXTL neurons during early but not late development disrupts the behavioural display of social drive in the adult, affecting the neurodevelopment of specific dopaminergic clusters associated with visual processing and reward (Nunes et al. 2021). Taken together, these data suggest that OXTL in fish regulates complex social behaviours including the ability to assimilate and process more social cues and information.

4.2.4 Neurovascular Interface

The teleost HNS neuronal populations are mostly investigated for their roles in the central regulation of homeostatic processes. However, the role of HNS vasculature and non-neuronal cells in this regulation is less clear. Importantly, understanding the mechanism through which the HNS exerts its systemic influence requires us to elaborate on a crucial topic—the neurovascular interface.

As described earlier, the axonal fibres in the neurohypophyseal tissue form direct contact with the capillary plexus. This capillary plexus in adult zebrafish arises from a simple loop-like structure of the embryonic HNS (Gutnick et al. 2011). While this capillary is an extension of the cerebral vasculature, it lacks a blood–brain barrier and instead, the vasculature of this region is highly fenestrated, i.e. permeable, allowing the exchange of blood-borne proteins and hormones between the brain and the peripheral circulation (Gordon et al. 2019; Anbalagan et al. 2018).

Functionally, the presence of fenestrated capillaries in the neurohypophysis is of great significance as it allows for the HNS to respond to peripheral stimuli and

allows the direct release of neurohormones into the blood circulation. This versatile structure is maintained by factors released by the resident pituicytes. We have recently shown that several angioactive ligands released by the pituicytes inhibit the formation of tight junctions between the vascular endothelia while maintaining the endothelial cell fenestrations (Anbalagan et al. 2018). Electron microscopy images of this region in adult zebrafish demonstrated the presence of neurosecretory termini seated near the basement membrane of the vasculature with pituicyte processes ensheathing them (Anbalagan et al. 2018, 2019).

4.3 Urophysis

Box 4.2 The urophysis—an underexplored neuroendocrine interface
The first indication of a caudal secretory system came from Weber in 1827 (Weber 1826) when dissection of the carp spinal cord indicated a caudal structure at the termini. This was further investigated almost a century later when Dahlgren described large cells along the spine of skates, which secreted granules into the blood (Dahlgren 1914). In 1925, Favaro (1925) showed the morphological similarities between a caudal bulge of teleosts and that of the neurohypophysis. Enami and Imai (1955) showed the conserved anatomical organization of this structure across fish species. By this point, it had become evident that this caudal neurosecretory system existed only in fishes, and it was suggested that it could serve as a neuroendocrine interface. A seminal study concerning the caudal neurosecretory system (CNSS) was the 1959 description of Dahlgren cells and their axonal projections into the vasculature of the urophysis (Enami 1959). The presence of neurosecretory products released in the urophysis was elucidated in 1969 where urophyseal extracts were shown to be functionally significant in blood pressure (Bern and Lederis 1969) and later in maintaining osmolality (Loretz and Bern 1981). By the 1990s (Conlon et al. 1996), urotensin II had been identified in species that lacked a caudal neurosecretory system, indicating a conserved role for the ancient hormone.

Fishes are unique amongst vertebrates, due to the presence of an additional neurosecretory organ associated with the spinal cord at its caudal end (Fig. 4.1). Importantly, the posterior pituitary interface bears a strong resemblance to the previously described caudal neurosecretory system in fish (Kriebel 1980). As demonstrated in the *Pomolobus aestivalis*, the urophysis consists of an axonal-vascular entanglement termed the neurohaemal zone/urophysis, which is roughly comparable to the hypophyseal neurovascular interface. Axonal fibres terminating in the urophysis are surrounded by fenestrated capillaries, with a predominant perivascular space. The ultrastructure of this region also shows the presence of neurosecretory granules contained in axonal swellings, dubbed Herring bodies, similar to what is observed in the pars nervosa (Kriebel et al. 1979). In 1914, Dahlgren identified giant

neurosecretory cells located at the distal end of the spinal cord of skates (*Rajidae*) (Dahlgren 1914) and in 1927 Weber found these localized swellings in the posterior end of the spinal cord (Weber 1927). This was later shown to generate a neurovascular structure, which was designated urophysis/urohaemal-organ, and the giant neural secretory cells were later termed Dahlgren cells (Enami 1959). Dahlgren cells are large magnocellular neurons projecting into the urophysis through thick non-myelinated axon endings. The axon endings are rich in secretory granules and have an intimate relationship with endothelial cells for the transfer of neurosecretory products (Holmgren 1964). The structural anatomical assembly of Dahlgren cells with the urophysis is referred to as the CNSS. The simplest organized form of the urophysis was commonly found in elasmobranchs (*Chondrichthyes*) whereas the highest organized form was found to be present in all bony fishes (*Osteichthyes*). Teleosts develop a discrete CNSS which shows a structural analogy to the cranial HNS (Bern 1985; Winter et al. 2000). The piscine CNSS is located at the distal end of the spinal cord, and in teleosts it spans the last three vertebrae (Holmgren 1964). The urophysis resides at the end of the spinal cord, posterior to the last spinal nerve (Fig. 4.1). In some species, the urophysis is innervated by means of a stalk through which the nerves and ependymal fibres enter, while in other species innervation is more diffused (Bern and Takasugi 1962). The urophysial outpouching structure is covered by meninges that arise from the end of the spinal cord and was shown to be populated by glial cells. Furthermore, ependymal and glial fibres from the spinal cord and vasculature generate an anatomical network (Amin et al. 1992; Fridberg 1962).

4.3.1 Evolutionary Aspects of Urophysis

Understanding the evolutionary aspects of Dahlgren cells and urophysis can give additional insights regarding piscine evolution (Fig. 4.3). More evolved fish display elongation or extension of Dahlgren cell terminals innervating a distinct neurohaemal organ which reflects a well-developed urophysis. Elasmobranchs are known as primitive ancestral fish, and exhibit more dispersed Dahlgren cells with shorter axons and a less anatomically discrete urophysis (Fridberg and Bern 1968; Qureshi et al. 1978). Accordingly, the neurosecretory Dahlgren-like cells of less evolved fish are widely distributed and form a diffuse neurohaemal zone. For example, in the neurohaemal zone of elasmobranchs small terminals of the Dahlgren cells are connected to the ventral part of the spinal cord and directly contact the capillary bed (Bern and Hagadorn 1959). Traces of such arrangement of Dahlgren cells were also noticed in some cyprinids, where the processes of Dahlgren cells terminate at the ventral part of the spinal cord and come close to the meningeal sheath to contact the blood vessels (Fridberg 1962). The anatomical isolation of Dahlgren cell terminals from the spinal cord occurs in the course of evolution. It was proposed that the isolation of Dahlgren cell terminals occurs in three stages: (i) In elasmobranchs and the early developmental stage of CNSS in some teleosts the terminals of Dahlgren cells are present within the spinal cord. (ii) In elasmobranchs

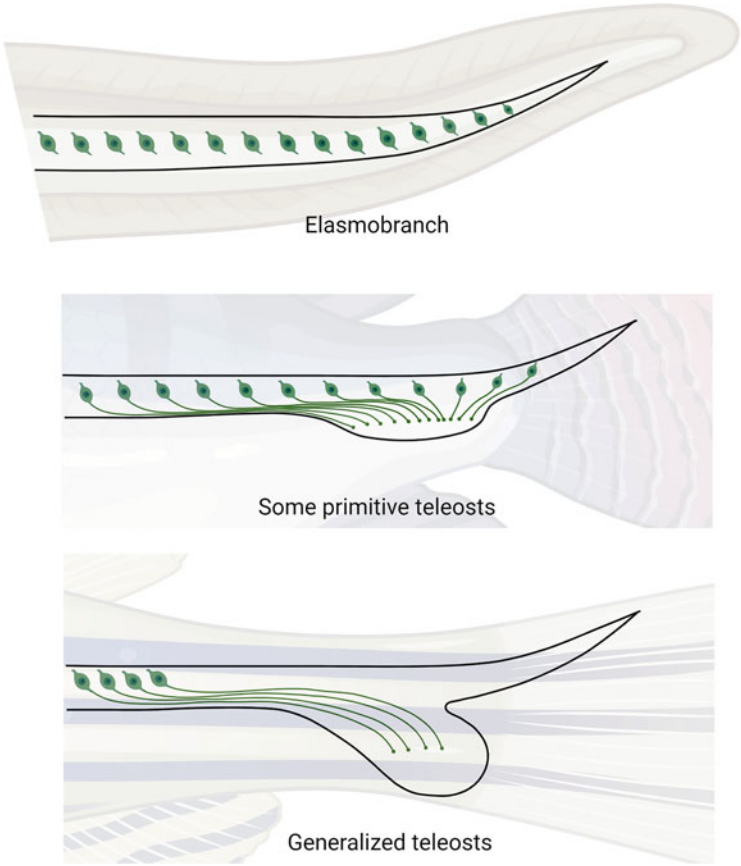

Fig. 4.3 Evolution of the piscine urophysis. Schematic representation of the urophyseal structure in fish showing the evolution of the caudal neurosecretory system from primitive fish (Elasmobranch) to the teleosts. Dahlgren cells evolve through the piscine phylogenetic tree to send projections from the caudal spinal cord into the neurohaemal interface of the urophysis

as well as in the intermediate developmental stage of CNSS in teleosts the nerve terminals penetrate the meningeal sheath. (iii) In more evolved teleost species, Dahlgren cell terminals move out from the spinal cord to penetrate the urophysis and terminate at the capillary bed, resulting in a lobular structure of the urophysis (Fig. 4.3) (Saenko 1978).

An evolutionarily related change in Dahlgren cell morphology was suggested for the evolution of teleosts from primitive fish (Speidel 1922). In early evolved piscines, the cells are small and similar to other nerve cells, without any morphological resemblance to Dahlgren cells. The second group of fish species, which are more evolved, possess small to moderate-sized cells, with limited resemblance to Dahlgren cells. The third and most evolved species show large-sized Dahlgren cells with modified morphology and are commonly seen in most teleost species

(Speidel 1922). Notably, in the early developmental stages of teleosts, moderately sized cells are present which later develop into large-sized Dahlgren cells in the CNSS of mature fish (Cioni et al. 2000). This developmental differentiation of Dahlgren cells from neuronal populations of teleost embryos further supports an evolutionary speciation process. In this view, small cells of the spinal cord initially served as specialized nerve cells in primitive piscines and later evolved into large glandular cells in teleosts.

4.3.2 Ontogenesis of CNSS

Embryonic development of Dahlgren cells and the urophysis was studied through various immunoreactive, histological and microscopy studies. Histological studies demonstrated that morphogenesis of the urophysis is initiated in the early larval stages, however, its mature organ form is finalized only after several months from hatching (Cioni et al. 2000; Fridberg 1962; Imai 1965; Sano and Kawamoto 1959). In chum salmon (*Oncorrhynchus keta*), immunohistochemistry of Urotensin1 (UI) and Urotensin2 (UII) localized in immature Dahlgren cells (i.e. appearing as agranular ovoidal cells) and fibres near the caudal region of the neural tube of forty-day-old embryos before hatching. Two weeks from hatching, the UI- and UII-positive cells and fibres increase in number, however, pronounced capillary formation is only detected in 3-month-old larvae and the maturation of the CNSS is finalized 5 months after hatching (Oka et al. 1993). It is interesting to note that although the HNS develops earlier than the CNSS, synthesis of UI and UII is identified in the embryonal CNSS before its appearance in the HNS (Oka et al. 1993). It was suggested that the urophysis differentiates from the meningeal tissue of the spinal cord and that Dahlgren cells originate from embryonic neuroectodermal cells, which differentiate first at the anterior region, gain secretory properties and migrate to the caudal region (Fridberg 1962; Fridberg and Bern 1968). A later study in chum salmon demonstrated that Dahlgren cells originate from neuroblasts and differentiate in the lateral plate of the caudal neural tube (Oka et al. 1993).

In Nile tilapia (*Oreochromis niloticus*), UI and UII immunoreactive perikarya and fibres were identified for the first time only in four days post-hatching larvae. At this stage, two bundles of neurosecretory fibres were observed at the future site of the urophysis. The initial differentiation of the tilapia urophysis is observed near the caudal region at 24 days post-hatching. The budding urophysis comprises a ventral swelling of the spinal cord in association with protruding dilated vessels. Further development occurs through increasing the number of neurosecretory terminals and branching of blood vessels. Meanwhile, neurosecretory cells rise in number and start to differentiate morphologically. The mature or fully formed urophysis is observed in four-month-old juveniles (Cioni et al. 2000). Obviously, additional work is needed with transgenic marker lines that will help to clearly uncover the embryonal origins of the CNSS. Nonetheless, it seems that functional speciation of Dahlgren cells begins at the initiation of hatching and free swimming and requires several months to reach the mature CNSS organ formation.

4.3.3 Physiology

The CNSS serves as the main neuroendocrine site for the synthesis of several neuropeptides with key importance in the homeostatic regulation of physiological functions. Nonetheless, although it has been recognized for more than a century, an exclusive critical role of the CNSS in physiological homoeostasis has yet to be elucidated. The CNSS is known as the major site for synthesis and release of urotensins (Ichikawa et al. 1982; Pearson et al. 1980). These neuropeptides show close similarities with other cortistatin and somatostatin peptides expressed by the central nervous system and other tissues of higher vertebrates, from reptiles and birds to mammals and humans (Lu et al. 2008; Vaudry et al. 2010). From an evolutionary perspective, this signifies the functional importance of these urotensins. The CNSS also produces and secretes additional neuropeptides such as corticotrophin-releasing factor (CRF), parathyroid hormone-related protein (PTHrP), OXTL and AVPL (Gozdowska et al. 2013; Ingleton et al. 2002; Lederis et al. 1982). Little is known regarding the functional and physiological importance of their secretion from the CNSS, however, they were found to be involved mainly in osmoregulation, reproduction and blood circulation.

4.3.3.1 Osmoregulation

UI and UII exert a direct effect on ion transport through epithelial cells in the kidney, which support their involvement in osmoregulation (Loretz et al. 1983; Marshall and Bern 1979; Ashton 2006). The importance of CNSS as an osmoregulatory structure is supported by: (i) the urophysis displays structural modifications with respect to the osmotic stress, (ii) urophysectomy affects the osmotic balance and (iii) urotensins secretion from the urophysis result in altered renal function of fish (Berlind 1973; Chan 1975). Several studies demonstrated that the urophysis undergoes structural and secretory modifications in response to altered salinity. Bonefish (*Albula vulpus*) raised in ponds with fluctuating salinity display increased intracellular cytoplasmic invagination and a higher level of secretory product was measured in their urophysis than in bonefish collected from open sea (Fridberg et al. 1966a). Cytological variations and altered urophyseal secretion were also detected in euryhaline brook trout (*Salvelinus fontinalis*) exposed to variable ion concentrations. Brook trout raised in a freshwater environment have an irregular shape of nucleus, elongated endoplasmic reticulum and Golgi bodies with reduced secretory granules. When maintained for a few days in deionized water, the cell organelles were shown to be highly developed, with increased numbers of secretory granules. Nonetheless, prolonged exposure to deionized water does not lead to increased neurosecretory activity, including changes in secretory granules. When exposed to 25% sea water for 24 h, brook trout exhibited increased secretory activity in the cells while prolonged exposure to increased salinity reduced the secretory activity in the urophysis (Chevalier 1976). These findings support the involvement of the CNSS in the homeostatic regulation of osmotic stress, mainly in response to acute environmental fluctuations. The Mozambique tilapia (*Oreochromis mossambicus*) is a hardy euryhaline fish that can grow in variable salinities from freshwater to sea water

(Chourasia et al. 2018). Freshwater-adapted tilapia that were urophysectomized and exposed to brackish water displayed significantly increased Na^+, K^+ and Ca^{++} in their blood than sham-operated controls. Contrastingly, sea water-adapted urophysectomized fish exhibited decreased Na^+ and K^+ in the bloodstream compared to sham-operated control fish. These results indicate that the urophysis has a role in maintaining osmotic balance in the fish (Baldisserotto et al. 1994). Similar results were obtained in urophysectomized Mozambique tilapia exposed to water containing 1.7% NaCl (Takasugi and Bern 1962). However, as treated fish exhibited increased mortality and weight loss with high level of serum chloride that was not identified in the later experiment, it was suggested that the lack of calcium in NaCl salinated water increased the osmotic stress (Baldisserotto et al. 1994; Takasugi and Bern 1962). Molecular analysis of urotensin expression in the euryhaline flounder (*Platichthys flesus*) suggested that UII is highly important for water and electrolyte homoeostasis and has an active role in preventing dehydration and salt deposition in high salinity conditions such as haemodilution in freshwater conditions (Lu et al. 2006). Urotensins were found to affect ion absorption in the urinary bladder of fish. Urinary bladders of longjaw mudsuckers (*Gillichthys mirabilis*) were exposed in vitro to physiological doses of UII, which directly altered ion transport in surface epithelia, a known component of osmoregulation. Moreover, India ink injection into the caudal vein demonstrated a direct but separate connectivity of the urophysis to the kidney and urinary bladder, further supporting a direct effect UII on the urinary bladder (Loretz and Bern 1981). It was also demonstrated by similar means that UII stimulates the absorption of Na^+ and Cl^- ions in the posterior intestine in 5% sea water-adapted longjaw mudsuckers (Loretz et al. 1983). These studies suggest that the CNSS directly modulates the main tissues known to be involved in water and electrolyte homoeostasis in fish both under baseline and osmotic stress conditions.

4.3.3.2 Reproduction

Analysis of urophysis protein extracts and molecular gene expression analysis of piscine CNSS during reproductive cycle and spawning period has demonstrated a role for the CNSS in fish reproduction. UII was found to be increased in the blood of white suckers (*Catostomus commersoni*) three months prior to the spawning period and it declined by half during and after spawning (Lederis 1973). Analysis of CNSS structure during the goldfish reproductive cycle demonstrated that the size of Dahlgren cells is altered with respect to ovarian development. Dahlgren cell size increases towards spawning initiation and decreases at the end of spawning season (Chen and Mu 2008). Importantly, while several studies demonstrated that urophysial extracts can modulate the contraction of ovary, oviduct and sperm ducts in some bony fishes (Berlind 1972; Lederis 1970), only one report demonstrated the direct effect of UII on ovarian smooth muscle contraction (Leonard et al. 1993). Urophysial extracts were found to be inefficient for spawning induction in several teleost species, further supporting their role in gonadal contraction and not as gonadal maturation factors (Behr et al. 2000). Gonad-localized and follicular-stage dependent UI levels were identified in the ovary of olive flounders (*Paralichthys olivaceus*), supporting the involvement of urotensins in piscine

ovarian development (Zhou et al. 2019), however, the possible connection and interaction between gonadal and CNSS urotensins remains to be determined.

4.3.3.3 Other Physiological Roles

Urotensins were reported to play a role in the stress regulation and muscle contraction of fishes. Dahlgren cell structure and its peptide secretion varied with temperatures. The firing frequency of Dahlgren cells was shown to increase with temperature, suggesting the role of the urophysis in thermoregulation. The response to thermal stress was suggested to be mediated through the transient receptor potential cation channel family (TRPs) (Yuan et al. 2020b). CNSS expression of UI, UII and corticotropin-releasing hormone (CRH) as well as plasma cortisol, CRH but not UII were shown to increase in olive flounders on exposure to acute hypothermal stress, returning to baseline levels following 8 days of adaptation (Yuan et al. 2020a). Chronic but not acute hyperthermal challenge led to increased expression of CNSS CRH and UI but not UII (Yuan et al. 2020a). In addition, the urophysis was suggested to be involved in the regulation of blood circulation, vascular smooth muscle contraction and the digestive system of fish (Fridberg 1962; Lederis 1977). Overall, current literature suggest pleiotropic functions of CNSS, which is not surprising considering the expression of multiple neurohormones in this structure. Further research regarding urophysial functions in homeostatic fish physiology is needed.

4.4 Conclusions and Outlook

The importance of the HNS and its related neurohormones in the regulation of homoeostatic and physiological functions is obvious given its structural and functional evolutionary conservation. Nonetheless, the existence of the CNSS in fishes as well as its evolvement in piscine species support an unidentified but highly important urophysiological role(s).

Some of the failures in underpinning major CNSS functions may be explained by the 2–3 weeks required for full regeneration of this system following complete removal of all CNSS neurohemal components (Fridberg et al. 1966b). Paradoxically, this very rapid regeneration further supports the high importance of the CNSS in fish physiology. Importantly, new and relevant pharmacological and genetic tools have been developed for the urotensin system (Lescot et al. 2008; Zhang et al. 2018) and some were also developed for non-neuronal components of the HNS (Anbalagan et al. 2018; Gordon et al. 2019). These tools may prove valid for studying both neural and non-neural components of the CNSS aiming to identify specific physiological functions of this system.

While the HNS is fully functional during early embryonal stages, CNSS components begin to differentiate at later developmental stages and its structural establishment occurs only several months later. This suggests that the CNSS functions are of importance to adult fish physiology and possibly connected to sexual maturation. As described above, euryhaline fish exhibit more developed

CNSS anatomy, which further supports this concept. Nonetheless, the ability of urophysial extracts and hormones to modulate water and electrolyte homoeostasis, as well as the CNSS anatomy, has led to an inherent bias as most fish species used to study this system were euryhaline, making at least some of the findings questionable with regard to stenohaline piscines.

Finally, much effort has been invested in recent years in understanding the regulatory mechanisms of HNS neuropeptide secretion. However, the anatomical location and complex connectivity of the HNS with additional brain centres hinder these efforts. The close morphological, cellular and structural similarities between HNS and CNSS and the ability to analyze CNSS ex vivo make the CNSS a potentially unique model for the study of neurohormone secretion.

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Cytoskeletal Organization and Plasticity in Magnocellular Neurons

5

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Abstract

Magnocellular neurons are neuroendocrine cells that produce and secrete vasopressin and oxytocin. These neuropeptides are synthesized in the somata of magnocellular neurons, which are located in the hypothalamic supraoptic and paraventricular nuclei and send their axons to the neurohypophysis, where vasopressin and oxytocin are secreted into the circulation. Magnocellular neurons feature classical actin and microtubule cytoskeletal networks that mediate trafficking of vasopressin- and oxytocin-containing vesicles and other cargoes to different cellular compartments, and are also involved in the regulation of peptide secretion from axonal terminals in the neurohypophysis. In addition, recent studies revealed specialized actin and microtubule networks that are present exclusively in magnocellular neurons and are not found in any other neuronal types investigated. These unique cytoskeletons are involved in the regulation of magnocellular neuron firing activity in response to osmotic stimuli. Modulating the density of actin and microtubule network changes in the activity of magnocellular neurons. Moreover, recent studies showed that actin and microtubule cytoskeletons are modified following chronic exposure to high dietary salt, contributing to the enhanced activation of magnocellular neurons in this condition.

Keywords

Cytoskeleton · Actin · Microtubules · Vasopressin · Oxytocin · Super-resolution microscopy · Osmosensing · TRPV1 channels · Salt-loading

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5.1 Introduction

The cytoskeleton is a complex array of filaments that is present in most cells. The term cytoskeleton, meaning cellular skeleton (cyto is cell), was proposed in 1903 by Nikolai Koltsov, who pioneered the idea that the shape of cells is determined by subcellular structures or tubules. All eukaryotic cells contain three main cytoskeletal filaments: microfilaments, intermediate filaments, and microtubules. Microfilaments or filamentous actin (F-actin) are composed of monomers of globular actin (G-actin) assembled into a ~ 7 nm double helix structure. Actin filaments are mostly involved in cell motility, cell division, endo- and exocytosis, and cell contractility. Intermediate filaments are composed of a family of proteins sharing a common structure. Different family members display cell specificity, e.g., neurons express neurofilaments and glia cells express glial fibrillary acidic protein (GFAP). Intermediate filaments form rope-like fibers of 8–12 nm diameter and their primary function is to stabilize cell shape and create cell cohesion. Microtubules are the largest of the cytoskeletal filaments, produced by assembly of α- and β-tubulin monomers to form a hollow tube with an outer diameter of ~25 nm and an inner diameter of ~13 nm. In addition to regulating cell shape, microtubules play a key role in intracellular transport and cell division. A salient feature of actin filaments and microtubules, but not of intermediate filaments, is that they can be very dynamic, capable of undergoing rapid polymerization (growth) and depolymerization (shortening) by addition or removal of subunits (monomers or dimers). Notably, actin and microtubule filaments display polarity, and the addition or removal of new subunits mostly occurs at the barbed or "plus" end, while the "minus" end is typically less dynamic and sometimes anchored into other subcellular structures.

While actin filaments and microtubules are present in all eukaryotic cells, including neurons, intermediate filaments display cell specificity, and most neurons express neurofilaments (Bomont 2021). The organization of cytoskeletal filaments in neurons has been extensively studied for many decades. Dysfunctions of microtubules have been considered a common feature associated with pathogenesis of neurodegenerative diseases (Sferra et al. 2020). All three types of cytoskeletal filaments were described in magnocellular neurosecretory neurons in the early 1970s, in studies that utilized classical electron microscopy (Flament-Durand 1971). Most of the work investigating microtubules in magnocellular neurons focused on their role in the transport of neuropeptides in dense core vesicles, and demonstrated that disruption of microtubules with microtubule-depolymerizing drugs blocks dense core vesicle trafficking (Flament-Durand and Dustin 1972; Flament-Durand and Distin 1972). While studies investigating the ultrastructural organization of actin cytoskeleton focused on magnocelluar synaptic terminals located in the neurohypophysis (Alonso et al. 1981), more recent research also examined the role of the actin cytoskeleton in the regulation of somato–dendritic and synaptic release of vasopressin and oxytocin (Tobin and Ludwig 2007b; Anbalagan et al. 2019).

Understanding of the cytoskeletal ultrastructure in magnocellular neurons has rapidly evolved in recent years, due to the development of new microscopy

technologies, such as super-resolution microscopy (Box 5.1). These novel approaches provided new insights into the understanding of cytoskeletal organization in different neuronal subtypes, including magnocellular neurons. Comparative analyses of cytoskeletal networks in neurons from different brain areas in situ revealed that magnocellular neurons feature unique actin and microtubule structures that are not found in other neuronal or non-neuronal cell types. Moreover, these studies demonstrated that in addition to the classical roles of microtubules in intracellular trafficking and actin in the regulation of synaptic release, these unique cytoskeletal networks play distinct roles in regulating the electrical activity of magnocellular neurons.

Box 5.1 Analyzing cytoskeletal networks in fixed brain tissue in situ using super-resolution imaging

Analysis of cytoskeletal networks by light microscopy, such as confocal imaging, is limited by the spatial resolution of this approach, defined by the diffraction laws of light and thus preventing distinguishing objects located within less than a few hundred nanometers apart. Since the dimensions of individual cytoskeletal filament range between 7 and 25 nm, analysis of dense cytoskeletal networks in neurons using this approach represents a significant challenge. Super-resolution microscopy refers to imaging techniques designed to bypass the limited resolution of light microscopy by implicating image processing strategies to generate super-resolved images, optical imaging schemes that overcome the diffraction limit, and sample manipulations (Jacquemet et al. 2020).

Super-resolution approaches include single-molecule localization techniques, which are based on detecting the fluorescence of an individual molecule and implicating image processing to calculate the precise location of each molecule. These techniques include Stochastic Optical Reconstruction Microscopy (STORM) (Rust et al. 2006), Photoactivated Localization Microscopy (PALM) (Betzig et al. 2006), and DNA-Point Acquisition in Nanoscale Topography (PAINT) (Jungmann et al. 2014). Other super-resolution techniques involve specialized imaging devices to bypass the diffraction limit by implicating detector arrays (e.g., Zeiss Airyscan (Huff 2015)), patterned illumination (Structured Illumination microscopy (York et al. 2012)), or modification of illumination beam size (Stimulated emission depletion (STED microscopy) (Hell and Wichmann 1994)). A complementary approach that does not require specialized microscope set-ups or imaging protocols is Expansion microscopy, which includes magnifying the sample itself by physically expanding it after embedding into a hydrophilic gel. This approach also results in increased resolution when combined with common light microscopic techniques (Jurriens et al. 2021).

(continued)

Box 5.1 (continued)

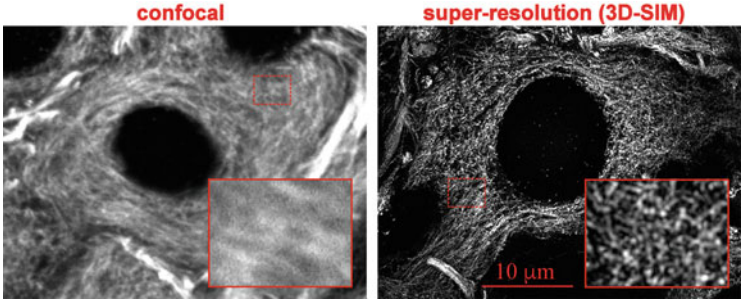
Over the last decade, development of super-resolution microscopy facilitated new discoveries previously unattainable using conventional light microscopy. However, the current application of super-resolution microscopy to the reconstruction of 3-dimensional (3D) specimens is limited to thin samples. 3D reconstruction of thick samples using super-resolution fluorescence microscopy remains challenging due to high levels of background noise contaminating the single-molecule images, light scattering, as well as fast photobleaching of fluorescent probes when imaging optical sections, due to illumination of molecules located within the whole imaging volume. Despite these difficulties, several recent studies succeeded in adapting super-resolution to imaging of spines and synaptic proteins in fixed sections in situ (Dani et al. 2010) and in vivo (Ter Veer et al. 2017). Notably, imaging cytoskeletal elements in neurons in a thick sample presents additional challenges because the high density of cytoskeletal proteins in the sample creates even stronger out-of-focus fluorescence and further reduces the signal-to-noise ratio, while filaments that are located very close to each other are therefore harder to resolve. Thus, in most cases, it is impossible to distinguish between individual filaments and microtubule or actin bundles.

Hence, analyzing subcellular structures using super-resolution microscopy in brain tissue (in vivo or in situ) represents a major challenge, mostly due to the thickness of the tissue. Thus, most studies examining the organization and function of neuronal cytoskeletons have been conducted in cultured neurons (Leterrier 2021). Several limitations should be considered when interpreting these studies. Neurons in culture adhere to the culture dish and thus flatten, losing their 3D morphology. In addition, while neurons in vivo are embedded in extracellular matrix proteins and interact closely with other cell types (e.g., astrocytes, microglia, and endothelial cells), cultured neurons are surrounded by an artificial environment. From a functional point of view, cultured neurons are typically prepared from embryonic brain tissue and even when matured in culture they receive very few, if any, synaptic inputs. Thus, their functional profile appears to resemble that of developing neurons rather than adult mature neurons. It remains unclear whether cytoskeletal patterns found in cultured neurons are also present in adult neurons in vivo.

Analyzing the organization of cytoskeletal networks in neurons embedded within their natural environment (tissue) remains challenging. Some super-resolution approaches, such as 3D structured illumination microscopy and Airyscan, are more compatible with thick samples. These approaches allow only 1.7–2.0-fold improvements in the resolution, however, since these increases are in all three dimensions, they result in five- to eightfold increased volumetric resolution as compared to a confocal microscope. Moreover, these techniques can be used to analyze relatively thick fixed tissue sections when

(continued)

Box 5.1 (continued)
imaging objects that are located within ~10 μm from the surface of the section. In addition to implementing optical and computational approaches to enhance the imaging resolution, high-quality tissue preservation has a key importance. In contrast to cultured cell samples growing as a monolayer and undergoing instantaneous fixation upon fixative administration, optimal fixation of the brain tissue requires transcardial perfusion of the animal. Therefore, the time until the cell within the tissue is fixed may vary considerably, depending on the quality of perfusion and specific fixation protocols. Recently developed fixation protocols enable maximal preservation of intracellular structures in native tissue and allow visualization and detailed characterization of cytoskeletal networks in fixed samples in situ using 3D structured illumination microscopy and Airyscan (Hicks et al. 2020; Barad et al. 2020; Prager-Khoutorsky et al. 2014). Moreover, recent studies advanced methodologies to adopt single-molecule localization super-resolution approaches to imaging in brain sections that may also be beneficial for analyzing cytoskeletons (German et al. 2017).



Box 5.1 Microtubules in magnocellular neurons are visualized using confocal imaging (left) and super-resolution with 3D structured illumination microscopy (3D SIM, right). Insets show magnified areas (4x3 μm) outlined by small red squares on the corresponding images, illustrating that confocal imaging fails to resolve dense cytoskeletal arrays that can be visualized with 3D SIM

5.2 Unique Actin Filament Networks in Magnocellular Neurons

Magnocellular neurosecretory neurons feature two distinct types of actin networks: a thin layer of actin filaments located beneath the plasma membrane (subcortical layer), and an array of comet-like structures occupying the cytoplasm of the neurons (Fig. 5.1). The subcortical layer of actin filaments was described over a decade ago both in situ, in coronal hypothalamic slices containing supraoptic nucleus (Tobin and Ludwig 2007b), and in magnocellular neurons acutely isolated from rat brain (Zhang

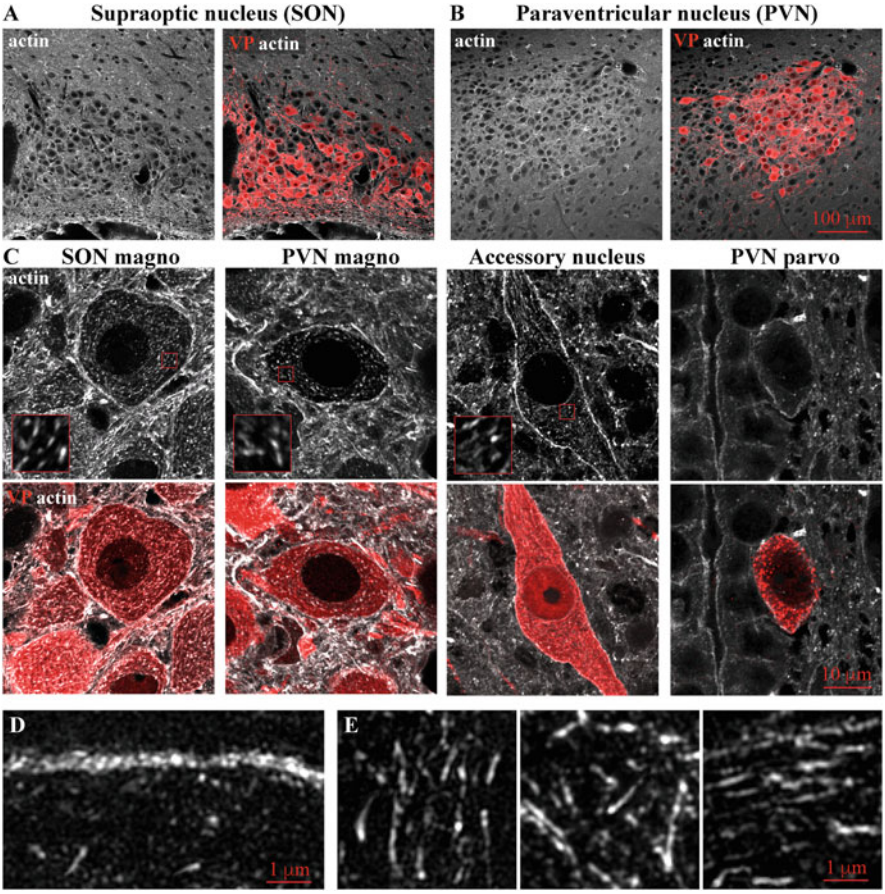


Fig. 5.1 Magnocellular neurons feature unique actin cytoskeletal networks. Double immunostaining of β-actin (white) and vasopressin (VP, red) in adult rat brain sections analyzed by confocal imaging (**a**, **b**), and super-resolution with (**c**) Airyscan and (**d**, **e**) 3D structured illumination microscopy (SIM). Low magnification images of supraoptic (**a**) and paraventricular (**b**) nuclei. (**c**) High magnification images of magnocellular neurons from the supraoptic nucleus (SON magno), paraventricular nucleus (PVN magno), accessory nucleus and a parvocellular vasopressin neuron from the paraventricular nucleus (PVN parvo). Note that magnocellular neurons in all three nuclei feature a prominent subcortical layer of actin. In addition, magnocellular neurons comprise an array of short comet-like actin filaments in their soma. Insets in (**c**) show magnified areas (3x3 µm) outlined by small red squares on the corresponding images, illustrating cytoplasmic actin comets. SIM images of the subcortical actin layer (**d**) and cytoplasmic comet-like actin structures (**e**) in magnocellular neurons. Adapted with permission from Barad et al. (2020)

and Bourque 2008). A recent study utilizing novel light microscopy-based imaging methodologies with improved resolution (Box 5.1), enabled a more detailed characterization of this cytoskeletal network in vasopressin magnocellular neurons in situ (Barad et al. 2020), (Fig. 5.1c, d). Magnocellular neurons are located in the hypothalamic supraoptic nuclei (SON), within the magnocellular part of the

paraventricular (PVN) nuclei, and in smaller numbers in accessory neurosecretory nuclei (Bourque 2008; Voisin and Bourque 2002; Gottlieb et al. 2006; Tasker et al. 2017). The subcortical actin layer outlines neuronal somata and extends into dendrites. Similar organization of a subcortical actin layer was found in neurons from other brain areas such as hippocampal neurons and neurons in the hypothalamic arcuate nucleus, while cortical neurons lack this actin structure. Notably, this actin cortical layer does not appear to be a distinct feature of vasopressin neurons, but rather of magnocellular neurons, since this layer was not found in parvocellular vasopressin neurons of the PVN or vasopressin neurons located in the suprachiasmatic nucleus (Barad et al. 2020). High-resolution analysis using super-resolution microscopy (Box 5.1) revealed that in magnocellular neurons, the subcortical actin structure comprises a ~ 0.3-μm thick actin layer (Fig. 5.1d). Yet, the thickness of this subcortical actin layer varies between neuronal subpopulations, and is completely undetectable in some types of neuron while it appears to be denser and wider in magnocellular neurons. These features do not appear to be related to the large size of magnocellular neurons, since other neuronal types that have comparable soma size (e.g., hippocampal pyramidal neurons), contain significantly smaller subcortical actin layer (Barad et al. 2020). The following section will discuss a potential functional role of this prominent actin layer in the regulation of magnocellular neuron activity in response to osmotic stimuli and neuropeptide release (5.5).

In addition to the subcortical actin layer, magnocellular neurons feature a unique cytoskeletal structure comprised of an array of short actin filaments sparsely distributed throughout the entire volume of the perinuclear cytoplasm (Fig. 5.1c). These structures resemble comet tail-like filaments of about 1 μm long (Fig. 5.1c, e), and are found in magnocellular neurons from supraoptic, paraventricular, and accessory nuclei. Notably, an examination of other brain areas (e.g., cortex, hippocampus, and hypothalamic suprachiasmatic and arcuate nuclei) revealed that these comet-like actin structures are not present in any other neuronal type and thus appear to be a unique cytoskeletal network featured by magnocellular neurons. Remarkably, the comet-like actin filaments are not similar to the organization of classical actin networks present in other cell types, such as stress fibers found in fibroblasts, endothelial and epithelial cells (Tojkander et al. 2012), or actin-rich structures forming filopodia and lamellipodia in motile cells and neuronal growth cones (Lehtimäki et al. 2016).

Interestingly, actin comets in magnocellular neurons display resemblance to filamentous actin structures propelling endocytic vesicles (Collins et al. 2011; Svitkina 2018) and comet tails forming after infection by a certain genus of bacteria (Cameron et al. 2001; Svitkina 2013). Once bacteria gain entry into the cytoplasm, they promote the polymerization of actin comet tail-like filaments from their surface, thereby pushing and propelling bacterial movement throughout the cell (Cameron et al. 2000). A recent study investigating ultrastructural architecture of actin structures in cancer cells using electron microscopy revealed similar actin comet structures that appear to be involved in trafficking of clathrin-coated vesicles (Collins et al. 2011; Svitkina 2018). The dynamic properties of actin comets in

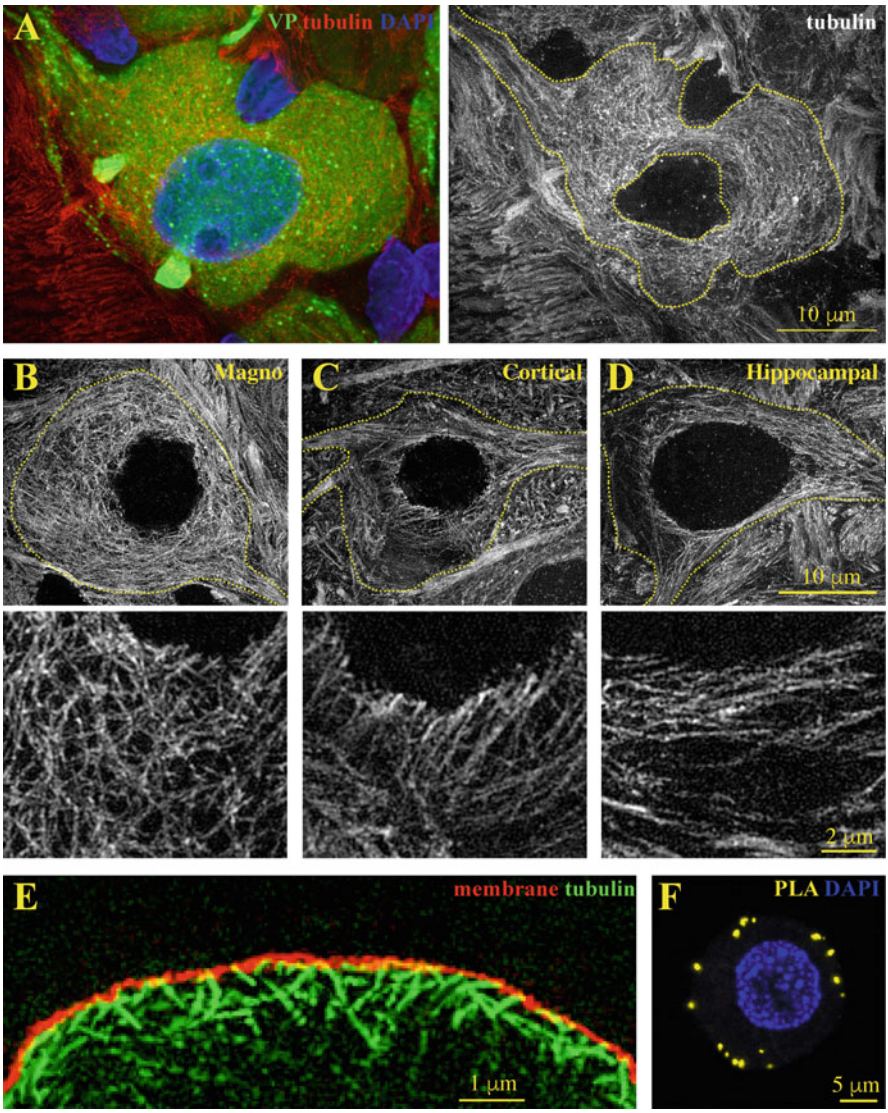


Fig. 5.2 Magnocellular neurons feature a unique interweaved microtubule scaffold. (**a**–**d**) Immunostaining of brain sections from adult rats analyzed by super-resolution 3D structured illumination microscopy (SIM). (**a**) Triple staining for α-tubulin (red or white), vasopressin (VP, green), and DAPI (blue) showing a magnocellular neuron in the supraoptic nucleus. To delineate the perimeter of an individual neuron, vasopressin labelling was used to trace a yellow dotted line along the cell perimeter and this line was superimposed on the corresponding tubulin image showing tubulin signal alone (white, left panel). (**b**–**d**) SIM images showing microtubule organization in magnocellular (**b**), cortical (**c**), and hippocampal (**d**) neurons. Lower panels beneath each neuron show higher magnification of microtubule networks in the soma of the corresponding neuron. Note that magnocellular neurons comprise a remarkably dense and interweaved microtubule scaffold that fully occupies the soma of these cells (**b**). In contrast, microtubules are sparser and organized as linear arrays in the soma of cortical (**c**) and hippocampal (**d**) neurons. (**e**) SIM imaging to visualize submembrane microtubules (α-tubulin, green) in respect to the plasma membrane (DiI,

magnocellular neurons are not known, but it is conceivable that these structures might be involved in trafficking of secretory vesicles, and therefore are unique to magnocellular neurons which are specialized in the transport and secretion of neuropeptides. Although previous studies observed subcortical actin layers in both vasopressin and oxytocin magnocellular neurons, further research is required to investigate whether actin comet-like structures are also present in oxytocin neurons.

5.3 Unique Network of Microtubules in Magnocellular Neurons

In addition to actin networks, magnocellular neurosecretory neurons feature a unique scaffold of microtubules in their soma (Fig. 5.2). Characterization of microtubule organization in magnocellular vasopressin neurons using super-resolution imaging in situ (Box 5.1) revealed that microtubules create a remarkably dense and complex three-dimensional network of filaments that occupies the entire cytoplasm of neuronal somata (Prager-Khoutorsky et al. 2014) (Fig. 5.2a, b). This dense microtubule scaffold extends from the nucleus to the cell surface, where microtubule ends contact the plasma membrane at multiple points (Fig. 5.2e) and interact with the transient receptor vanilloid type 1 (TRPV1) channels (Fig. 5.2f). Magnocellular neurons from both supraoptic and paraventricular nuclei feature this dense somatic microtubule network (Hicks et al. 2020). However, this structure was not found in neurons in other brain areas, including cortex, hippocampus, and cerebellum, nor in other hypothalamic areas, such as arcuate and suprachiasmatic nuclei (Fig. 5.2b–d), (Hicks et al. 2020; Prager-Khoutorsky et al. 2014). While the microtubule density was significantly higher in the somata of magnocellular neurons, as compared to neurons from other brain areas, microtubule density and organization in dendrites were found to be similar in neurons from different brain areas (Prager-Khoutorsky et al. 2014). These studies focused mostly on vasopressin magnocellular neurons, and further research is required to examine whether this unique microtubule network is also found in oxytocin magnocellular neurons.

In addition to the remarkable density of microtubules in the soma of magnocellular neurons, the organization of this network is strikingly different from microtubule networks described in other neuronal and non-neuronal cells (Prager-Khoutorsky et al. 2014). Somatic microtubules in magnocellular neurons comprise a complex array of interweaved filaments, in sharp contrast to the classical pattern of centrosome-divergent microtubules present in non-neuronal cells (Luxton and Gundersen 2011; Stiess and Bradke 2011). Moreover, this interweaved scaffold

Fig. 5.2 (continued) red), showing microtubules extending to and establishing contacts with the plasma membrane. (**f**) Confocal image showing an in situ proximity ligation assay (PLA) to visualize sites where tubulin and TRPV1 interact at the nanoscale (<40 nm, yellow spots, DAPI blue). Note that multiple sites of tubulin–TRPV1 interactions are observed at the cell surface, where transduction occurs. Adapted with permission from Prager-Khoutorsky et al. (2014)

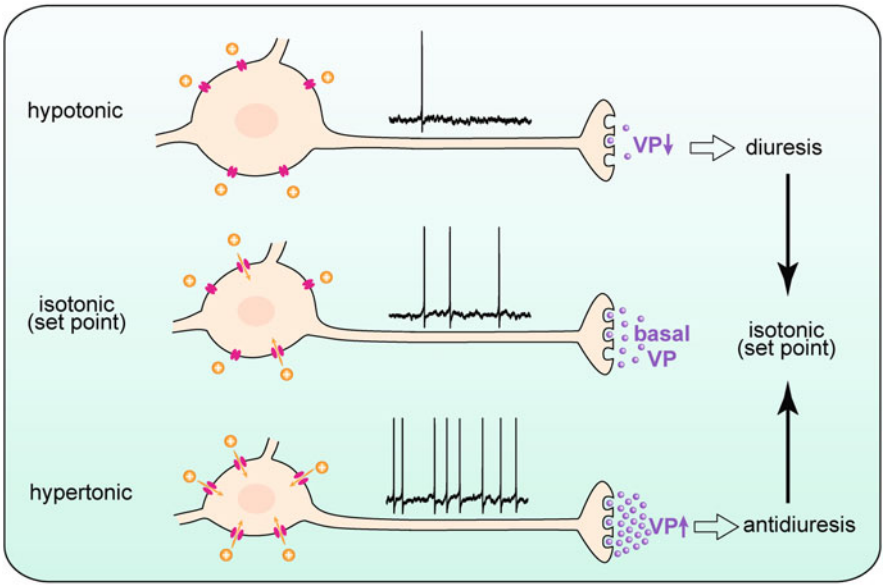


Fig. 5.3 Magnocellular neurons are intrinsically osmosensitive. Changes in osmolality cause inversely proportional changes in cell volume. Hypertonicity-evoked shrinking activates transduction channels, which are non-selective cation channels (a variant of the transient receptor potential vanilloid receptor 1, ΔN-TRPV1). This causes depolarization and increases the action potential firing rate of magnocellular neurons, leading to enhanced vasopressin (VP) release from magnocellular axon terminals in the neurohypophysis. Elevated VP levels in the circulation stimulate water reabsorption in the kidney (antidiuresis) to restore extracellular fluid osmolality toward the set point. Hypotonic stimulus inhibits the transduction channels that are open under the basal isotonic condition (set point), leading to hyperpolarization and a decrease in the firing rate of magnocellular neurons. This causes a reduction in VP release and promotes diuresis

is dramatically different from the rectilinear network of parallel microtubule filaments observed in soma, axons, or dendrites of other neuronal types in vitro or in situ (Fig. 5.2b–d), (Stiess and Bradke 2011). While in non-neuronal cells the most prominent role of microtubules is mediating the segregation of chromosomes during cell division, in postmitotic mature neurons microtubules are recognized mostly for their role in intracellular trafficking. Specifically, in neurons microtubules are organized as an array of parallel bundles (like a railway track network) mediating the transport of cargoes within the cell to deliver neurotransmitter-containing vesicles, endoplasmic reticulum, mitochondria, and other essential elements to and from distant neuronal compartments. This microtubule-based transport is essential for the steady supply of newly synthesized proteins to nerve terminals and other distant locations as well as for the removal of damaged proteins and organelles for degradation or recycling. This microtubule-based transport mechanism is essential for all neurons, including magnocellular neurons, and previous work has demonstrated that interfering with the microtubule system in magnocellular neurons perturbs the delivery of dense core vesicles (Flament-Durand and Dustin 1972;

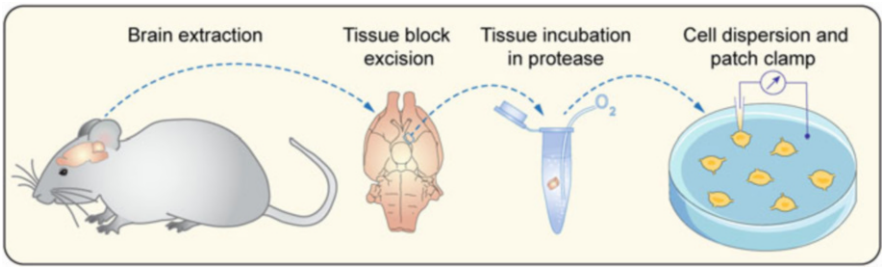
Flament-Durand and Distin 1972). Consistent with this idea, a recent study used super-resolution analysis to show that the organization of microtubules in processes of magnocellular neurons is similar to that in other neuronal types (Prager-Khoutorsky et al. 2014), while only the somatic dense interweaved microtubule scaffold is a specialized network featured by magnocellular neurons. The functional significance of this unique microtubule scaffold in magnocellular neurons is discussed in the Sect. 5.5.

Box 5.2 Investigating intrinsic neuronal properties by patch clamp recordings from acutely isolated neurons

Different strategies can be used to determine the responsiveness of neurons to specific stimuli. These methods include electrophysiological recordings, immunostaining to detect expression of activity-dependent immediate-early genes (e.g., c-Fos), and functional imaging (e.g., MRI). However, most of these methods do not establish whether the stimulus directly affects neuronal activity or the stimulation effect is caused indirectly, via another cell type or synaptic inputs from a different brain area. For example, osmosensitive neurons are cells that change their action potential firing frequency in response to alterations in blood osmolality (the total solute concentration in the blood plasma). Previous studies using MRI, c-Fos, and electrophysiological recording in vivo or in vitro from acute brain slices demonstrated that osmosensitive neurons are present in several brain areas (Bourque 2008; Oldfield et al. 1994; Egan et al. 2003). Moreover, previous studies, using electrophysiological recordings in which synaptic transmission was blocked with pharmacological agents, suggested that osmosensitive neurons are present in the *organum vasculosum laminae terminalis,* subfornical organ, supraoptic and paraventricular nuclei, the medial preoptic area, and the caudal part of the nucleus tractus solitarius (Vivas et al. 1990; Bourque et al. 1994; Mason 1980; Bourque 1989; Sibbald et al. 1988; Izawa et al. 2000). However, since astrocytes can also respond to osmotic perturbation and affect the activity of local neurons (Choe et al. 2012), chemical blockade of synaptic transmission is not sufficient to prove that the activation of neurons is mediated directly by osmotic stimulus and therefore that the neurons are intrinsically osmosensitive. Thus, to demonstrate that a neuron is intrinsically sensitive to a certain stimulus, such as osmolality, it is required to stimulate neurons acutely isolated from the tissue in a preparation that contains no synaptic contacts or influences from other cell types (e.g., glial cells). The preparation of acutely isolated neurons includes extracting blocks of tissue from the specific brain area of interest, triturating the tissue, and then dissociating cells on a petri dish. Incubating the tissue with low doses of a protease solution to digest the extracellular matrix helps to loosen cell connections within the tissue and avoid damaging neurons during the tissue dispersion. Patch clamp

(continued)

Box 5.2 (continued)
recordings performed on acutely isolated neurons from specific brain regions demonstrated that neurons in the *organum vasculosum lamina terminalis* (Ciura and Bourque 2006; Ciura et al. 2011), subfornical organ (Anderson et al. 2000), as well as magnocellular neurons in the supraoptic nucleus (Oliet and Bourque 1992, 1993a; Zhang et al. 2007; Prager-Khoutorsky et al. 2014) are intrinsically osmosensitive. The recent developments of transgenic rats expressing green fluorescent protein (Ueta et al. 2005) under the vasopressin promoter and red fluorescent protein under the oxytocin promoter (Katoh et al. 2011) allow identification of vasopressin and oxytocin neurons and examination of their intrinsic properties.



Box 5.2 For the preparation of acutely isolated neurons, the brain is extracted and small blocks of tissue containing the area of interest are excised and placed in an oxygenated protease solution. The block is then triturated and plated on a petri dish, and electrophysiological patch clamp recordings are performed from isolated neurons within a few hours of cell plating.

5.4 Osmotic Control of Magnocellular Neurons

Magnocellular neurons play a key role in the regulation of body fluid homeostasis, and their activity is modulated by changes in blood osmolality (Bourque 2008). Electrical activity of magnocellular neurons is tightly coupled to the secretion of vasopressin and oxytocin from their nerve terminals, located in the neurohypophysis, into the circulation (Bourque 1991; Brown 2016). Systemic increases in blood osmolality elevate the firing rate of magnocellular neurons, leading to enhanced hormonal secretions and increasing circulating levels of hormones (Bourque and Renaud 1984; Poulain and Wakerley 1982). The activity of magnocellular neurons is regulated by both extrinsic and intrinsic factors. Extrinsic factors regulate the activity of magnocellular neurons via synaptic projections from other osmoregulatory nuclei (*organum vasculosum laminae terminalis,* subfornical organ, and the medial

preoptic area), contributing to the firing activity of magnocellular neurons (Brown et al. 2013; Brown 2016). In addition to synaptic inputs, glial cells also contribute to the regulation of magnocellular neurons' activity in response to osmotic stimuli (Tasker et al. 2012). For example, local secretion of taurine by astrocytes mediates hyperpolarization providing inhibitory tone in magnocellular neurons in resting and hypoosmotic conditions (Choe et al. 2012; Brown 2016; Hussy et al. 2000).

In addition to the regulation mediated by afferent projections from other osmoregulatory areas and from local glial cells, a striking feature of the magnocellular vasopressin and oxytocin neurons is their ability to respond to changes in extracellular fluid osmolality in the absence of glial cells and synaptic contacts (Oliet and Bourque 1993a, 1993b). Thus, magnocellular neurons are intrinsically osmosensitive (Box 5.2). The activity of isolated magnocellular neurons is increased by hypertonicity and inhibited by hypotonicity (Oliet and Bourque 1993b), (Fig. 5.3). These changes are mediated by the modulation of activity of a non-selective cation channel formed by an N-terminal truncated variant of the transient receptor vanilloid type 1 (ΔN-TRPV1) channel (Zaelzer et al. 2015; Sharif-Naeini et al. 2008). This modulation of neuronal activity in response to osmolality is a mechanical process associated with changes in the cell volume. Exposure to a hypertonic extracellular environment causes water to flow out of the cell to compensate for the increased concentration of solutes in the extracellular fluid, leading to cell shrinkage, which results in the activation of ΔN-TRPV1, membrane depolarization, and an increased firing rate. A hypotonic extracellular environment causes water to move into the cell to balance the elevated concentration of solutes inside the cell, resulting in cell swelling and causing the closure of ΔN-TRPV1 channels, which are open under basal conditions. This, in turn, leads to hyperpolarization and decreases the firing rate of the neurons (Fig. 5.3).

Notably, the changes in cell volume of magnocellular neurons are directly coupled to their firing activity and do not depend on changes in the solute concentration or ionic strength. Experiments on acutely isolated magnocellular neurons demonstrated that changing cell volume by applying positive or negative pressure via a patch pipette causes changes in neuronal activity equivalent to those induced by hypo- and hyperosmolality (Zhang et al. 2007). Moreover, hypertonicity-induced activation of magnocellular neurons can be reversed by increasing the cell volume with a positive pressure applied through the patch pipette, and hypotonicity-induced inhibition of firing activity can be reversed by decreasing the cell volume by applying suction via patch pipette (Zhang et al. 2007). Overall, these findings demonstrated that modulation of the firing rate of magnocellular neurons in response to changes in extracellular osmolality is a mechanical process coupled to changes in cell volume (Prager-Khoutorsky 2017; Prager-Khoutorsky and Bourque 2015). Importantly, while exposure of other cell types to extracellular environments with altered osmolality induces compensatory adaptive changes in cell volume (regulatory volume decrease or regulatory volume increase (Strange 2004; Lang 2007)), magnocellular neurons do not undergo these compensatory volume regulation mechanisms. Instead, they display stable changes in cell volume for as long as the extracellular osmolality deviates from the basal condition (Zhang and Bourque

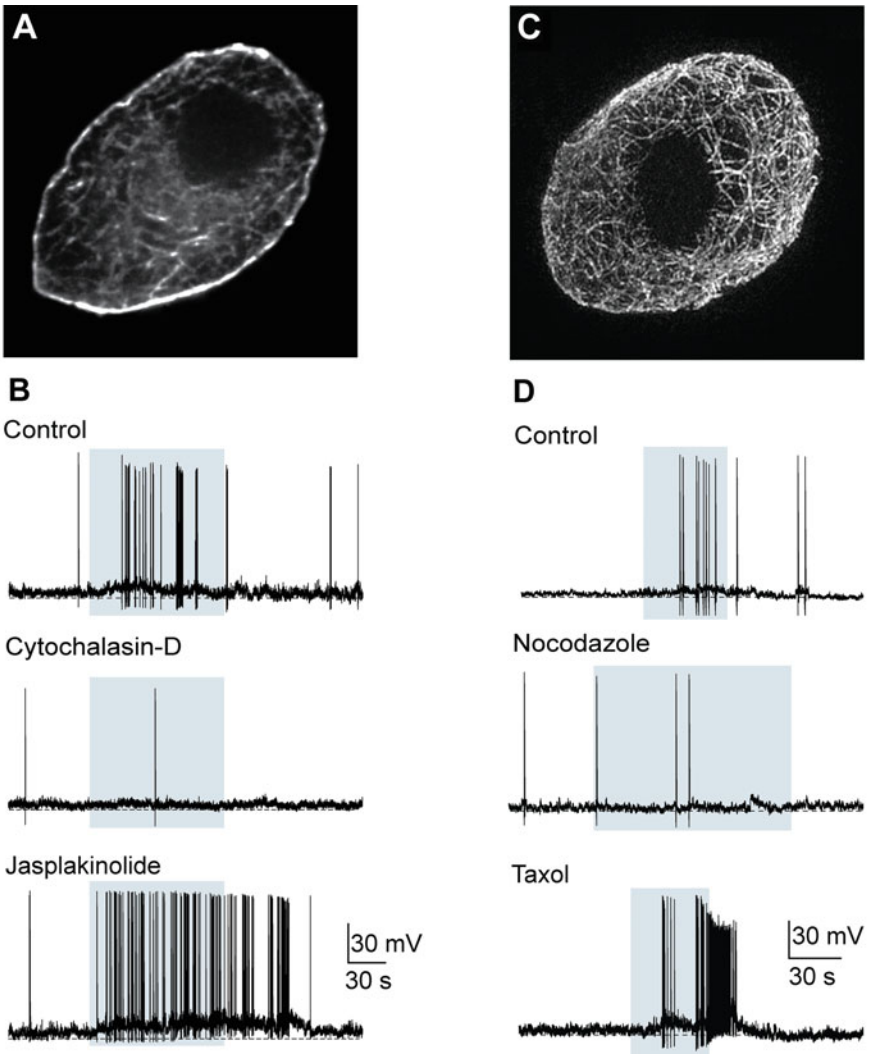


Fig. 5.4 Actin and microtubules are essential for osmotic activation of magnocellular neurons. (**a**) Super-resolution imaging with Airyscan showing the organization of actin cytoskeleton in an acutely isolated magnocellular neuron from rat supraoptic nucleus. (**b**) Whole-cell current clamp recordings from an acutely isolated magnocellular neuron. Hyperosmotic stimulation (shaded area) causes depolarization and an increase in the action potential firing rate in control magnocellular neurons (top panel). The hypertonicity-induced responses are suppressed by pre-treating the neurons with the actin-depolymerizing agent cytochalasin D (middle panel) and enhanced in the cell pre-treated with the actin-stabilizing drug jasplakinolide (bottom panel). Adapted with permission from Prager-Khoutorsky and Bourque (2015). (**c**) Super-resolution imaging with 3D SIM showing the organization of microtubules in the soma of an acutely isolated magnocellular neuron from rat supraoptic nucleus. (**d**) Whole-cell current clamp recordings from an acutely isolated magnocellular neuron. Suction-induced cell shrinkage (shaded area) causes depolarization and an increase in the action potential firing rate in control magnocellular neurons (top panel). The mechanically induced responses are suppressed by pre-treating neurons with microtubules-disrupting agent nocodazole (middle panel) and enhanced in cells pre-treated with microtubule-stabilizing drug taxol (bottom panel). Adapted with permission from Prager-Khoutorsky et al. (2014)

2003). This feature is vital, as in conditions such as dehydration, when blood osmolality is increased, the activity of magnocellular vasopressin neurons and thereby the secretion of vasopressin should remain elevated to promote antidiuresis in order to potentiate renal water retention until the plasma osmolality returns to the physiological set point.

Since maintaining changes in cell volume is critical for the function of magnocellular neurons, it is plausible that magnocellular neurons possess a specialized intracellular apparatus to achieve these stable changes in cell shape. Thus, unique cytoskeletal networks described in the previous section might be essential for providing mechanical stability to withstand compression caused by hypertonicity-induced shrinkage or to maintain cell integrity in response to hypotonicity-induced swelling. Moreover, since the activation of magnocellular neurons in response to hypertonicity is mediated by the mechanical activation of ΔN-TRPV1 channels (Zaelzer et al. 2015), the intracellular cytoskeletal apparatus appears to be a key candidate that transduces forces generated during changes in cell volume into the activation of these channels. Consistent with this idea, the next section will discuss recent studies showing that both actin and microtubule networks play important roles in the regulation of activity of magnocellular neurons in response to osmotic stimuli.

5.5 Cytoskeletal Networks Regulate the Activity of Magnocellular Neurons

Studies conducted on acutely isolated magnocellular neurons (Box 5.2, Fig. 5.4a) revealed that their activation in response to osmotic stimuli is abolished when the neurons are pre-treated with a drug that depolymerizes actin filaments (cytochalasin D). Whole-cell patch clamp recordings from magnocellular neurons treated with cytochalasin D show that while these neurons shrink following bath application of hypertonic saline or suction applied via patch pipette, this shrinking does not induce depolarization or an increase in the firing rate observed in intact neurons (Fig. 5.4b) (Zhang et al. 2007). Conversely, treating isolated magnocellular neurons with jasplakinolide, a drug that stabilizes actin filaments and promotes their polymerization, facilitates the activation of the neurons in response to hypertonicity- or suction-induced shrinking (Fig. 5.4b) (Zhang et al. 2007). These findings indicate that actin cytoskeleton plays an important role in control of the intrinsic osmosensitivity of magnocellular neurons, and the gain of the neuronal activation by osmotic stimuli can be bidirectionally modified by modulating the actin cytoskeleton (Prager-Khoutorsky and Bourque 2010).

Notably, angiotensin II, an excitatory vasoactive neuropeptide that promotes the activation of magnocellular neurons during hypotension (Nicolaidis et al. 1983) and hypovolemia (Ishibashi et al. 1985; Potts et al. 2000), increases subcortical actin density in acutely isolated magnocellular neurons (Zhang and Bourque 2008). The effect of angiotensin II on actin involves activation of phospholipase C and calcium-dependent form of protein kinase C (Zhang and Bourque 2008; Bansal and Fisher

2017). Moreover, the stimulatory effect of angiotensin on magnocellular neuron activity is eliminated by disrupting actin filaments with cytochalasin D (Zhang and Bourque 2008). These findings suggest that angiotensin II-mediated increases in the subcortical actin layer underlie the increased osmoresponsiveness of magnocellular neurons and vasopressin release under conditions such as hypotension, hypovolemia, and dehydration (Prager-Khoutorsky and Bourque 2010).

In addition to intrinsic responses to osmotic stimuli, the actin cytoskeleton plays a role in regulating hormonal secretion from magnocellular neuron terminals (Anbalagan et al. 2019) and somato-dendritic release (Tobin and Ludwig 2007a, b). The subcortical actin layer is located between the plasma membrane and secretory vesicles, surrounding these vesicles, and it thus has been described as a barrier restricting the docking of vesicles to release sites and preventing their fusion with the plasma membrane. According to this model, a small and transient disassembly of subcortical actin network is required to enable peptide release. Consistent with this model, a bath treatment of acute sections containing supraoptic nucleus with high K^+-induced membrane depolarization and caused a depolymerization of the subcortical actin layer in the soma and dendrites of magnocellular neurons (Tobin and Ludwig 2007b). In addition, the release of oxytocin and vasopressin in response to high-K^+-induced depolarization can be modified by treating acute brain sections with actin-modifying drugs. Specifically, bath application of low doses of the actin-depolymerizing agent latrunculin increases, while stabilizing actin filaments with jasplakinolide inhibits the release of the peptides triggered by high-K^+-induced depolarization (Tobin and Ludwig 2007b). Importantly, a complete disassembly of the subcortical actin cytoskeleton blocks peptide secretion induced by high K^+, suggesting a more complex and dynamic role for the actin cytoskeleton. It appears that in addition to creating a barrier preventing secretory vesicles from docking to the plasma membrane, actin is also required for exocytosis of docked vesicles (Tobin and Ludwig 2007a; Tobin et al. 2012).

Notably, the mechanism by which actin regulates peptide release is thought to be different in axon terminals, since disrupting the actin cytoskeleton had no effect on high-K^+-induced secretion from axon terminals located in the neurohypophysis (Tobin and Ludwig 2007b). Previous studies reported that two distinct networks of actin filaments are found in neurohypophysial synaptic terminals (Alonso et al. 1981), similarly to other central synapses (Bleckert et al. 2012; Nelson et al. 2013). One actin network resembles the subcortical actin layer found in the soma and is associated with the plasma membrane; and the second cytoplasmic actin pool is associated with secretory vesicles in nerve terminals. These distinct actin networks appear to be involved in the docking and fusion of secretory vesicles with the plasma membrane and membrane internalization by endocytosis to recycle membrane after the release. A recent study analyzing neurohypophysial magnocellular synapses in vivo using super-resolution imaging confirmed these observations, demonstrating that actin filaments form a cage-like structure surrounding and interacting with peptide-containing vesicles (Anbalagan et al. 2019). Moreover, this study demonstrated that actin assembly/disassembly dynamics, regulated by the Robo-

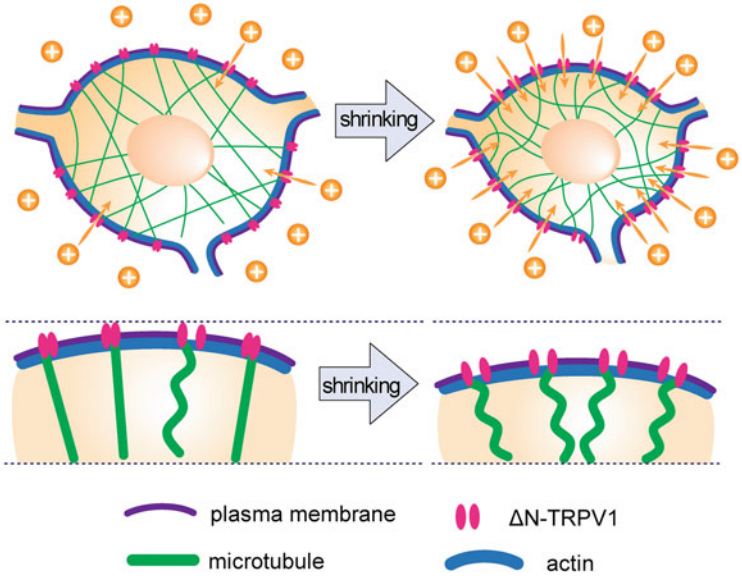


Fig. 5.5 Mechanism of mechanical activation of magnocellular neurons by hypertonicity. Microtubules extend to the plasma membrane, where they interact with the transduction channels (ΔN-TRPV1) on the surface of magnocellular neurons. At rest (left), while many transduction channels are attached to microtubules, only a few are activated because of lack of sufficient pushing force. In response to hypertonicity- or mechanically induced cell shrinking, the plasma membrane shifts inward (right), leading to microtubule compression. As a result, microtubules push back and activate the transduction channels. Subcortical actin does not interact directly with the transduction channels but may serve as a flexible layer to maintain membrane elasticity and prevent it from slackening. In response to cell shrinking, this elastic actin layer transmits the changes in cell volume into the adequate movement of the plasma membrane, resulting in microtubule compression. Adapted with permission from Prager-Khoutorsky et al. (2014)

Cdc42 pathway, is essential to maintain a functional pool of peptide-containing vesicles available for hormonal secretion.

In addition to actin, microtubules play a key role in the regulation of magnocellular neuron activity in response to changes in osmolality (Prager-Khoutorsky et al. 2014). A recent study demonstrated that microtubules interact with the ΔN-TRPV1 channels on the surface of magnocellular neurons from the supraoptic nucleus (Fig. 5.2f). Microtubules can bind the ΔN-TRPV1 directly via two highly conserved β-tubulin-binding domains located on the C-terminus of the channel (Goswami et al. 2007), and this interaction is critical for the osmotic and mechanical activation of the channels (Prager-Khoutorsky et al. 2014). Acutely isolated magnocellular neurons preserve the dense complex microtubule scaffold in their soma (Fig. 5.4c), and treating isolated neurons with the microtubule destabilizing drug nocodazole reduces the number of ΔN-TRPV1-microtubule interaction sites on the cell surface and abolishes shrinking-induced neuronal excitation (Fig. 5.4d). Conversely, treating isolated magnocellular neurons with taxol, a

drug that stabilizes microtubules and promotes their polymerization, elevates microtubule density, increases the number of ΔN-TRPV1-microtubule interactions and potentiates the activation of the neurons in response to hypertonicity- or suction-induced shrinking (Fig. 5.4d). Moreover, a specific disruption of these interactions by infusing isolated neurons with peptides mimicking the channel's β-tubulin binding sites blocks shrinking-induced activation of magnocellular neurons (Prager-Khoutorsky et al. 2014). Remarkably, there is a direct relation between the density of ΔN-TRPV1-microtubule interaction sites and the degree of the shrinking-induced activation of magnocellular neurons. These findings indicate that microtubules play a key role in the regulation of intrinsic mechano- and osmosensitivity of magnocellular neurons, and the sensitivity of this process can be increased or decreased by increasing or decreasing microtubule stability and the density of ΔN-TRPV1-microtubule complexes (Prager-Khoutorsky 2017; Prager-Khoutorsky and Bourque 2015).

Based on these studies, a model describing the role of actin and microtubule cytoskeletal networks was postulated (Fig. 5.5). According to this model, somatic microtubules are directly connected to the transduction channels (ΔN-TRPV1) on the surface of magnocellular neurons. During hypertonicity- or suction-induced cell shrinking, the plasma membrane moves inward leading to compression of microtubules attached to the channels. As a result, compressed microtubules push back onto the channels leading to their opening, thereby causing depolarization and an increase in the firing rate of the neuron (Fig. 5.5). Single-channel cell-attached recordings from acutely isolated magnocellular neurons confirmed that a brief positive pressure pulse applied to the plasma membrane underneath the patch pipette triggers rapid activation of channels in this membrane portion, supporting the model that ΔN-TRPV1 is activated directly by the application of the pushing force via the attached microtubule filament (Prager-Khoutorsky et al. 2014). Future studies should examine how hypertonicity-induced cell shrinking modifies the microtubule network leading to the activation of ΔN-TRPV1 channels and study how hypotonicity-induced cell swelling affects microtubules and their interactions with ΔN-TRPV1 to inhibit channel activity and reduce neuronal activation.

While the interaction of microtubules with TRPV1 channels was characterized in vivo and in vitro (Prager-Khoutorsky et al. 2014; Goswami et al. 2007), the mechanism by which the actin cytoskeleton regulates the osmotic activation of the channels in magnocellular neurons is less clear, as there is no evidence supporting the idea that actin can directly interact with TRPV1 channels (Goswami et al. 2004; Goswami and Hucho 2008). Thus, the effect of the actin network on the activity of transduction channels is likely to be indirect. It is plausible that the subcortical actin layer forms a scaffold beneath the plasma membrane that provides rigidity and/or mechanical support to the plasma membrane during shrinking, or transduces forces essential for compression of microtubules or the gating of the transduction channels (Fig. 5.5).

In summary, both actin and microtubule networks are critical for the intrinsic osmoresponsiveness of magnocellular neurons, and destabilizing these cytoskeletal networks prevents the activation of these neurons by osmotic stimuli. Furthermore,

stabilizing or increasing the density of each one of the cytoskeletal networks enhances the activation of magnocellular neurons by hypertonicity.

The following section will discuss how chronic modulations of these cytoskeletal networks might contribute to changes in the activation of magnocellular neurons. In addition to osmosensitivity, actin networks play a key role in the regulation of somato-dendritic and synaptic release. The organization and function of cytoskeletal networks may vary in different cellular compartments, and future studies should decipher the molecular apparatus differentially controlling these cytoskeletal networks.

5.6 Remodeling of Actin and Microtubule Networks in Response to Chronic Osmotic Stress

The hypothalamic neurohypophysial system has a remarkable capacity to undergo robust structural and functional plasticity under conditions that require high and sustained hormonal release. These conditions include parturition and lactation associated with increased oxytocin release, and dehydration and chronically elevated dietary salt intake associated with enhanced release of vasopressin. Under these conditions, the hypothalamic neurohypophysial system undergoes adaptations to allow the efficient, sustained release of neurosecretory hormones while avoiding depletion of stores. These adaptations include retraction of glial processes to allow direct somatic and dendritic membrane appositions as well as rearrangements of synapses and changes in synaptic properties (Miyata 2017; Hatton 2004; Tasker et al. 2012; Theodosis et al. 2008; Stern et al. 2000; Brussaard and Herbison 2000; Di et al. 2019; Tasker et al. 2020).

Recent studies demonstrated that both actin and microtubule networks in vasopressin magnocellular neurons undergo plastic changes in response to chronic increases in dietary salt intake (Hicks et al. 2020; Barad et al. 2020). In these studies, rats were subjected to seven days of salt-loading, when their drinking solution was replaced by 2% NaCl. This model has been used for many decades and is extensively characterized (Choe et al. 2015; Ludwig et al. 1996; Jones and Pickering 1969; Fujio et al. 2006; Li et al. 1998). Rats exposed to salt-loading gradually increase their fluid intake (Hicks et al. 2020; Barad et al. 2020) and develop chronic conditions including hypernatremia (Li et al. 1998) and increased plasma osmolality, and they become progressively hypertensive (Choe et al. 2015). The increase in mean arterial pressure in animals subjected to salt-loading is attenuated by vasopressin receptor 1 antagonist, suggesting that increased secretion of vasopressin contributes to increases in blood pressure and hypertension in this condition (Choe et al. 2015). Moreover, a recent study using patch clamp recording from acute slices containing supraoptic nucleus demonstrated that hypertonicity-induced responses are potentiated in magnocellular vasopressin neurons from salt-loaded rats (Levi et al. 2021). Furthermore, this study showed that salt-loading treatment increases the intrinsic osmosensitivity of magnocellular vasopressin neurons as well as the density of actin and microtubule networks in acutely isolated magnocellular vasopressin

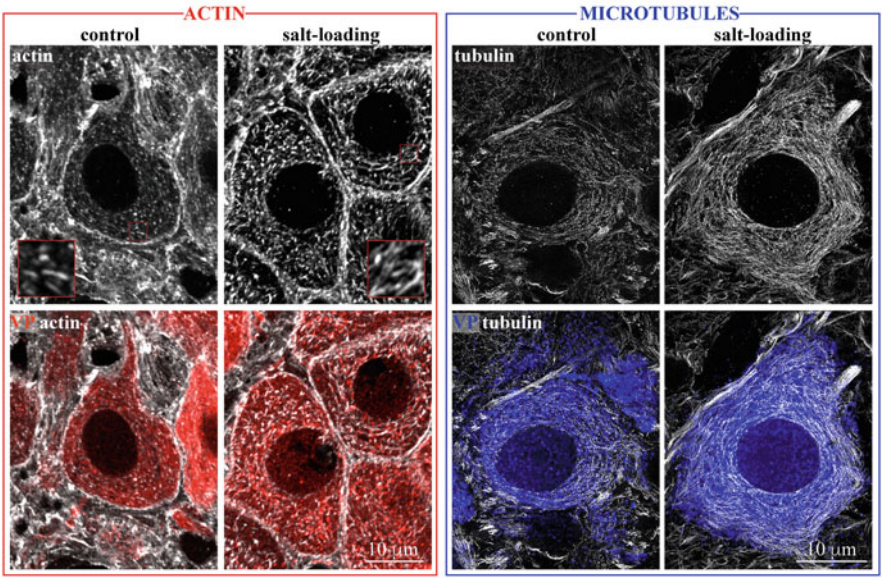


Fig. 5.6 The effect of salt-loading on the organization of actin and microtubule networks in magnocellular neurons. Brain sections containing supraoptic nucleus of control rats and rats exposed to seven days of salt-loading were immunolabeled for β-actin and vasopressin (white and red, left panels) and α-tubulin and vasopressin (white and blue, right panel). Super-resolution imaging with Airyscan (actin panels) and 3D structured illumination microscopy (microtubule panels) show actin and microtubule networks in magnocellular neurons. Insets on actin panels show magnified areas (3x3 µm) outlined by small red squares on the corresponding images, illustrating cytoplasmic actin comets. Note that the thickness of the subcortical actin layer and the density and length of actin comet-like structures are increased following salt-loading. Likewise, the density of microtubules in magnocellular neurons is increased following salt-loading. Notably, these changes in the cytoskeletal networks occur only in magnocellular neurons while the organization of actin and microtubule networks remains unchanged in neurons from other brain areas. Adapted with permission from Hicks et al. (2020) and Barad et al. (2020)

neurons. Consistent with these findings, recent studies reported that the density of actin and microtubule networks is increased in magnocellular vasopressin neurons in situ (Hicks et al. 2020; Barad et al. 2020). Notably, these increases in the density of actin and microtubule networks were observed in magnocellular vasopressin neurons located in the supraoptic, paraventricular, and accessory nuclei (Fig. 5.6). However, the organization and the density of actin and microtubule networks in other brain areas including cortex, hippocampus, arcuate, and suprachiasmatic nuclei, as well as the parvocellular division of paraventricular nucleus, remain unchanged following salt-loading, suggesting that modulation of cytoskeletal networks is limited to magnocellular neurons.

Detailed analyses of the organization of actin networks revealed that the density and thickness of subcortical actin layer are increased in vasopressin magnocellular neurons following salt-loading. Likewise, the density as well as the length of comet-

like actin filaments is increased in this condition (Barad et al. 2020). Remarkably, while magnocellular neurons encompass a somatic microtubule network that is 2.5-fold denser than in other neuronal types (Prager-Khoutorsky et al. 2014), the density of this network further increases following salt-loading by additional ~50% (Hicks et al. 2020).

Previous works have shown that the gain of mechanical and osmotic activation of magnocellular neurons scales in proportions with the density of subcortical actin layer and microtubules and ΔN-TRPV1-microtubule interactions (Zhang et al. 2007; Prager-Khoutorsky et al. 2014; Prager-Khoutorsky and Bourque 2010). Thus, changes in the density of these cytoskeletons in salt-loading can underlie enhanced osmoresponsiveness in this condition. Notably, enhancing the density of the subcortical actin layer or stabilizing microtubules and their interactions with ΔN-TRPV1 channels can potentiate the osmotic activation of vasopressin neurons, leading to enhanced vasopressin release, renal fluid retention and vasoconstriction, and eventually contributing to elevated blood pressure and hypertension in salt-loading.

5.7 Conclusions and Perspectives

The organization and function of the cytoskeleton in magnocellular neurons have been studied for several decades and actin and microtubule networks have been shown to play traditional roles in trafficking and synaptic release, similarly to other neuronal subtypes. In addition to these classical neuronal cytoskeletons, recent studies revealed unique actin and microtubule networks present exclusively in magnocellular neurons and not found in any other neuronal types investigated. Actin networks comprised the subcortical actin layer located beneath the plasma membrane and an array of cytoplasmic comet-like actin filaments. The microtubule network comprised a highly dense and complex scaffold of filaments occupying the entire soma of magnocellular neurons and extending to the plasma membrane where they interact with ΔN-TRPV1 channels. Both actin and microtubule networks are essential for the osmotic activation of magnocellular neurons. The proposed mechanism that underlies the gating of the ΔN-TRPV1 channels by hypertonicity-induced shrinking includes inward movement of actin-supported plasma membrane that compresses underlying microtubules, leading to push activation of ΔN-TRPV1 channels (Fig. 5.5). The activation of the transduction channels causes depolarization and increases the firing rate of magnocellular neurons. Modulation of the stability of these cytoskeletal elements in magnocellular neurons causes proportional changes in the sensitivity of the neuronal activation in response to mechanical and osmotic stimuli. Moreover, recent studies suggest that modification of the subcortical actin and somatic microtubule networks in response to chronic exposure to high dietary salt contributes to the enhanced activation of magnocellular neurons in this condition.

The functional role of comet-like actin filaments in magnocellular neurons remains elusive. It is conceivable that these structures are involved in the trafficking of vasopressin-containing secretory vesicles. Conditions associated with an

increased demand for hormonal release, such as salt-loading (Dunn et al. 1973; Ludwig et al. 1996), require facilitated transport of vasopressin and upregulation of the trafficking machinery to support this massive secretion to adjust to the hydration status of the organism.

Future studies should focus on deciphering the molecular apparatus that underlies unique cytoskeletal networks in magnocellular neurons, as well as signalling pathways that regulate the chronic remodeling of these networks in conditions such as salt-loading. This knowledge is essential for understanding the magnocellular neurons' physiology in healthy organisms as well as in pathological conditions associated with aberrant regulation of body fluid homeostasis.

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Venkatesh et al. (2020) This review discusses the role of actin in organelle trafficking and docking of vesicles in synapses.

Bomont (2021) This review discusses classical as well as novel roles of intermediate filament in health and disease.

Jacquemet et al. (2020) This review provides an overview of a variety of super-resolution approaches emphasizing the pros and cons of each method.

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Part II

Neuroanatomy of Hypothalamo-Hypophyseal Systems

6 Neuroanatomical and Functional Relationship Between Parvocellular and Magnocellular Oxytocin and Vasopressin Neurons

Ferdinand Althammer, Javier E. Stern, and Valery Grinevich

Abstract

Hypothalamic neuroendocrine cells that synthesize oxytocin (OT) and vasopressin (AVP) can be categorized into two major cell types, namely magnocellular and parvocellular neurons. In addition to the previously known differences in morphology, connectivity, and electrophysiological properties, recent studies highlight fundamentally different functions and genetic compositions of these cells. Parvocellular OT neurons have recently been implicated in pain perception and processing, regulation of OT release during fear, and promotion of social behavior in female rats following gentle touch. Despite the vast knowledge of parvocellular OT neurons, surprisingly little is known about parvocellular AVP cells. The activity of AVP receptor-expressing presympathetic cells in the paraventricular nucleus of the hypothalamus is regulated by somato-dendritically released AVP from nearby magnocellular AVP cells. However, the contribution of actual parvocellular AVP neurons to this phenomenon remains questionable. Here we summarize the current body of knowledge about the neuroanatomy and functional relationship of the magnocellular and parvocellular OT and AVP systems. In addition, we discuss several controversial topics including the post-synaptic location of OT receptors, various modes of OT release, and

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misconceptions/fallacies that might have led to oversimplified models of the OT system.

Keywords

Parvocellular · Magnocellular · Oxytocin · Vasopressin · Somato-dendritic release · Anatomy · Projections

Abbreviations

AC	Auditory cortex
AN	Accessory nuclei
AON	Anterior olfactory nucleus
Arc	Arcuate hypothalamic nucleus
AVP	Arginine-vasopressin
BLA	Basolateral amygdala
BNST	Bed nucleus of stria terminalis
BS	Brainstem
CB	Cerebellum
CeA	Central amygdala
CRH	Corticotropin-releasing hormone
HC	Hippocampus
HDB	Horizontal limb of diagonal band nucleus
iCj	Island of Calleja
LC	Locus coeruleus
LS	Lateral septum
magnAVP neuron	magnocellular vasopressin neuron
magnOT neuron	magnocellular oxytocin neuron
MC	Motor cortex
MeA	Medial amygdala
NAcc	Nucleus accumbens
OB	Olfactory bulb
OT	oxytocin
parvAVP neuron	parvocellular vasopressin neuron
parvOT neuron	parvocellular oxytocin neuron
PC	Piriform cortex
PFC	Prefrontal cortex
PLC	Prelimbic cortex
PV	Paraventricular thalamus
PVN	Paraventricular nucleus of the hypothalamus
RGC	Retina ganglion cells
RMg	Raphe magnus nucleus
RVLM	Rostral ventrolateral medulla
SC	Spinal cord

SCN	Suprachiasmatic nucleus
SON	Supraoptic nucleus
SSC	Somatosensory cortex
Tu	Olfactory tubercele
vDB	Ventral diagonal band of Broca

6.1 The Rodent Oxytocin System: Cell Types, Function, and Mode of Release

6.1.1 Oxytocinergic Cell Types

Oxytocin (OT)-ergic neurons can be categorized into two major types: magnocellular (magnOT) and parvocellular (parvOT) cells (Althammer and Grinevich 2017; Swanson and Sawchenko 1980, 1983). They differ in size, shape, anatomical location, function, projection sites, mode of release, and electrophysiological properties. While there has been recent speculation about the potential existence of additional oxytocinergic cell types based on genetic cluster analysis (Romanov et al. 2017), thus far no concrete functional evidence has been provided to corroborate these findings. Moreover, due to the fact that concrete genetic profiles for magnOT and parvOT neurons are currently missing, it is not possible to genetically target and reliably manipulate these two OT-ergic cell types.

Currently available techniques aimed to discriminate between the two cell types make use of cell-type-specific projections sites (i.e., parvOT neurons projection to the supraoptic nucleus (SON)), which can be exploited via virus-based approaches; patch clamp recordings and analysis of afterhyperpolarization/LTD and Flourogold-labeling of magnOT neurons. For a comprehensive description of available techniques see (Althammer and Grinevich 2017).

MagnOT cells are large neuroendocrine cells with a diameter of somas of 20–30 μm, which can be found in the supraoptic (SON), paraventricular (PVN), and accessory (AN) nuclei of reptilian, avian and mammalian hypothalamus (Grinevich and Polenov 1994; Knobloch and Grinevich 2014). The rat hypothalamus comprises approximately 7600 OT cells (Althammer and Grinevich 2017), while the vast majority (>99%) are magnOT neurons. MagnOT neurons release OT into the peripheral circulation (blood stream) and therefore—by definition—all magnOT cells send one axon to the posterior lobe of the pituitary. In addition to peripheral release, most—if not all—magnOT neurons project collaterals from axons of the hypothalamic-neurohypophysial tract to various forebrain regions (Zhang et al. 2021). To this day, more than 50 forebrain regions have been identified as targets for magnOT neurons (Knobloch et al. 2012; Mitre et al. 2016).

ParvOT cells are smaller neurons with a diameter of somas of 10–20 μm and are located mainly in selective subdivisions of the caudal PVN ((Swanson and Kuypers 1980; Swanson and Sawchenko 1983). ParvOT neurons project to the brainstem and

spinal cord and are involved in food intake regulation (Blevins et al. 2004), autonomic functions, such as breathing (Mack et al. 2002), erection and copulation (Melis et al. 1986), cardiovascular reactions (Petersson 2002), gastric reflexes (Sabatier et al. 2013) and pain perception (Rash et al. 2014). All of these projections arise from a small population of parvOT neurons residing within the PVN. While it is well established that parvOT neurons synapse onto magnOT neurons located in the SON to control activity-dependent release of OT into the systemic circulation (Eliava et al. 2016; Hasan et al. 2019), it was recently demonstrated that parvOT neurons tightly control magnOT activity within the PVN as well. While parvOT neurons have been underappreciated for most of the twentieth century, they recently emerged as key regulators of the OT system. In fact, the latest research suggests that somatosensory information first converges on parvOT neurons, which, upon activation, subsequently activate the much larger population of magnOT neurons. This mode of action allows a fine-tuned and effective global activation of the OT system, with coordinated release and context-dependent activity patterns of magnOT subdivisions.

Within the past 5 years, parvOT neurons emerged as new players in modulation of the OT system and it became evident that this small subpopulation of cells plays a vital role in somatosensory signal integration during social interaction (Tang et al. 2020), coordination of nociceptive response both on a central and a peripheral level (Eliava et al. 2016) as well as context-dependent activation of fear-sensitive OT-ergic engram cells in the hypothalamus (Hasan et al. 2019). In fact, these studies suggest that parvOT neurons might be master regulators that tightly control and orchestrate magnOT neuron activity under various conditions. Given the types of scenarios described (fear, pain, and social interaction), it seems reasonable to suspect that the coordination of magnOT release by parvOT neurons might be the general rule rather than the exception. The different projection sites of parvOT and magnOT are depicted in Fig. 6.1.

6.1.2 Functional Relationship and Mode of Release of parvOT and magnOT Neurons

The role of PVN → SON projecting parvOT neurons has been studied extensively (Althammer and Grinevich 2017; Eliava et al. 2016; Hasan et al. 2019; Tang et al. 2020). However, it is far from clear whether all parvOT neurons synapse onto magnOT neurons to coordinate their activity (i), whether all magnOT neurons receive synaptic innervation by parvOT neurons (ii), whether PVN → SON and PVN → PVN projecting parvOT neurons represent overlapping or distinct entities (iii) and how somatodendritic release of OT within the SON and PVN contributes to the activation of magnOT neurons that might or might not receive innervation by parvOT neurons (iv).

To better understand the functional relationship of magnOT and parvOT neurons it is required to have a close look at their electrophysiological characteristics and modes of neuropeptide release, as summarized below.

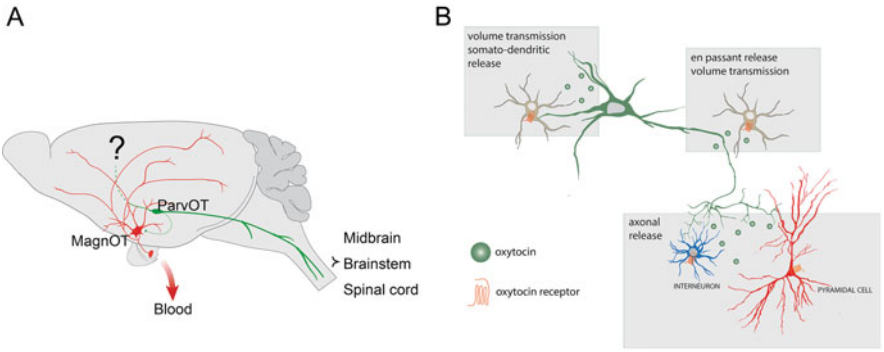

Fig. 6.1 Projection sites and modes of the release of magnOT and parvOT. (**a**) Different projection sites of magnOT and parvOT neurons. (**b**) Magnocellular neurons release OT and AVP from somas and dendrites, from axons passing by (*en passant*) and from long-range axons. OTRs have been found in various neuronal cell types, including GABAergic interneurons (Huber et al. 2005) and pyramidal cells (Lin et al. 2017). The precise pre- and postsynaptic mechanisms, as well as the location of OTRs remain elusive. The brain scheme depicts the currently known magnOT (red) and parvOT (green) neuron interconnectivity within the PVN and SON and their distinct projections to the pituitary, forebrain, midbrain, brainstem, and spinal cord. The green dashed line and the question mark highlight potential, but not yet confirmed, parvOT projections to the forebrain. Modified from Grinevich and Ludwig 2021

6.1.3 Electrophysiological Properties of magnOT and parvOT Neurons

By measuring the electrophysiological properties of blindly recorded cells in the PVN, Tasker and Dudek (1991) first identified two clearly distinct types of neuron by their distinct membrane properties and anatomical peculiarities. Given their blind approach, these cells most likely comprised both AVP and OT cells. Each of these types displayed unique, characteristic features, which allowed precise discrimination. Neurons named type I were characterized by the absence of low-threshold depolarizing potentials, which was found in type II cells. In contrast, type II neurons generally showed relatively small low-threshold depolarizations, which generated one to two action potentials. Furthermore, type I neurons had a significantly shorter membrane time constant (the time it takes the membrane to repolarize after a small current injection of fixed amplitude and duration) than those of type II. Based on the finding that type I neurons were found both in the SON and PVN, the authors concluded that they are most likely magnocellular neurons. Distinctly, type II neurons were found only in the PVN (especially in the caudal part) and therefore most probably belonged to parvocellular neurons, which was recently confirmed (Eliava et al. 2016). Usually, neurons receive an injection of an −100 pA current to hyperpolarize the neuron membrane (reaching −100 mV) before each step. These steps start at 0 pA and increase by 20 pA, reaching +60 pA. To discriminate between parvOT and magnOT, the hyperpolarizing notch and the T-outward rectification (membrane allowing outward current to flow more easily) are measured. Finally,

based on these values and the shape of the action potential it is possible to clearly identify magnOT and parvOT neurons. This protocol is well-established and has been used by several groups (Chu et al. 2013; Luther et al. 2002; Luther and Tasker 2000; Stern et al. 2000; Tang et al. 2020; Yuill et al. 2007). While the original studies by Tasker and Dudek were conducted blind, seminal works by William E. Armstrong and Javier E. Stern provided insights about individual properties of OT and AVP neurons and described various regulatory mechanisms (Du et al. 2015; Roper et al. 2003; Shevchenko et al. 2004; Stern and Armstrong 1995; Stern and Zhang 2005; Teruyama and Armstrong 2007).

6.1.4 The Different Modes of OT Release

There is very little evidence that magnOT neurons form true, functionally relevant synapses with other neurons. Although magnOT neurons project axons to almost the entire rodent forebrain, there has been no report about actual synapse formation from magnOT axons, except the synaptic contact found in the central nucleus of amygdala (Knobloch et al. 2012). In addition to the well-described somatodendritic release of OT and AVP (Landgraf and Neumann 2004; Ludwig and Leng 2006; Tobin et al. 2012), which takes place in the PVN and SON, magnOT neurons engage in volume transmission or *en passant* release (although not confirmed functionally), which is likely the synapse-independent, diffuse release of a small number of large dense core vesicles (LDCVs, please see below), containing OT, within a target region (Chini et al. 2017). For this mode of release, no synapse formation is required and the bulk of release neuropeptide diffuses to its target site with a clear concentration gradient. This phenomenon partly explains the occasionally observed delays (up to 90 s) of OT-ergic action after evoking instantaneous release via optogenetics (Hasan et al. 2019; Knobloch et al. 2012). On the other hand, parvOT neurons have been reported to form true synapses in various structures including the SON, PVN, brainstem and spinal cord (Buijs 1983; Buijs and Van Heerikhuize 1982; Swanson and Sawchenko 1983). This issue has been addressed in more detail in our recent review (Grinevich and Neumann 2021). However, it seems that the function of these synapses is the facilitation not of OT release, but rather of glutamate, which is co-released with OT (Knobloch et al. 2012; Hasan et al. 2019). Whether or not parvOT neurons engage in volume transmission is currently unknown. The potential forms of OT release within the central nervous system (CNS) are summarized in Fig. 6.1.

6.1.5 Controversy Over OTR Activation at Pre- and Postsynaptic Sites

For several decades, it seemed clear that OT-ergic activation of neuronal circuits follows the classical cascade of Ca^{2+}-mediated exocytosis and downstream OT signaling (Burbach et al. 2001). Briefly, OT is packed into large-dense core vesicles (LDCVs)—each of which can hold up to 85,000 molecules of OT (Morris 1976;

Nordmann and Morris 1984). LDCVs are transported to the readily releasable pool of vesicles along the axonal terminals and synaptic vesicle fusion and SNARE-mediated exocytosis takes place in a Ca^{2+}-dependent manner. It was assumed that secreted OT binds to postsynaptic OTRs, triggering a postsynaptic G-protein-dependent signaling cascade involving various G-protein subtypes/pathways (Gaq, Ga11, Gi/o, and β-arrestin) (Chini et al. 2017).

Despite the vast knowledge about synthesis and release, precise mechanisms by which OT targets and activates cells still remain largely elusive. Several seminal papers showed that OT can act on oxytocin neurons themselves (both in the SON and PVN), in a postsynaptic manner. Already in the 1980s several groups showed that OT acts in an autocrine manner (Freund-Mercier and Richard 1984; Moos et al. 1984; Moos et al. 1989; Moos and Richard 1989) and that this process involves calcium release from intracellular thapsigargin-sensitive calcium stores. Finally, Brussaard showed that within OT neurons, OT can also postsynaptically modulate the potency of GABAergic synapses (Brussaard et al. 1996). Although some groups demonstrated the presence of OTR-immunoreactivity at extra-hypothalamic postsynapses (Mitre et al. 2016), there are currently no reports supporting their functional role. Thus, beyond known postsynaptic actions of OT on OT neurons themselves as a mechanism to autoregulate their activity, there are currently no convincing reports further supporting postsynaptic actions in extra-hypothalamic regions.

On the contrary, some papers have been published that suggest presynaptic expression of OTRs (Dolen et al. 2013; Hung et al. 2017; Mairesse et al. 2015). The proposed mechanism includes activation of OTRs on presynaptic neurons by neighboring (or the same) cells, which may lead a subsequent release of conventional neurotransmitters or neuromodulators (such as glutamate or serotonin), which in turns activate the postsynaptic cell in addition or instead of direct "postsynaptic" OT action on OT-sensitive neurons.

6.1.6 The Rodent AVP System: Receptor Subtypes and Islands of AVP Expression

The total number of AVP neurons in the rodent brain (~7500) (Rhodes et al. 1981) is comparable to the number of OT neurons (~7600) (Althammer and Grinevich 2017). While OT neurons in the rodent brain are almost exclusively located in the PVN, SON, and accessory nuclei of the hypothalamus, AVP neurons are also found in various extra-hypothalamic forebrain nuclei, including the bed nucleus of stria terminalis (BNST) and medial nucleus of the amygdala (De Vries et al. 1984). In addition, scattered OT neurons have been found within neighboring areas, such as the BNST of mice (Duque-Wilckens et al. 2020) (Fig. 6.2). While OT acts on only one G-protein coupled receptor, AVP targets three distinct AVP receptor subtypes. These three subtypes of vasopressin receptors are known as V_1, V_2, and V_3 (or V_{1b}). V_2 receptors are present in the renal collecting duct, where AVP regulates water excretion through the insertion of Aquaporin-2 channels into the apical plasma

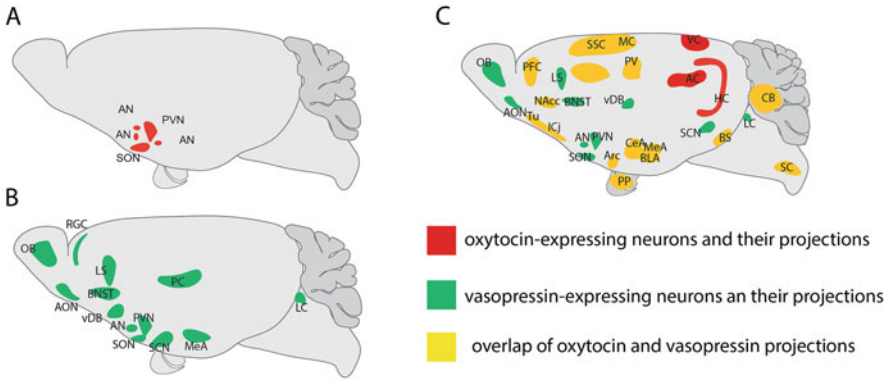

Fig. 6.2 OT and VP systems in the rodent brain—synthesizing nuclei and distinct/overlapping projection sites. Brain schemes highlight the location of OT-synthesizing nuclei (**a**), AVP-synthesizing nuclei (**b**), as well as their projection sites (**c**). All abbreviations of brain structures can be found in the list of abbreviations. Modified from Grinevich and Ludwig 2021

membrane of the principal cells of renal collecting ducts. V_1 receptors are located in the vascular bed, kidney, bladder, spleen, and hepatocytes, as well as the brain (Holmes et al. 2003). The difference between OT and AVP-synthesizing nuclei in the brain as well as their overlapping and distinct projection sites are shown in Fig. 6.2.

In transgenic rats that express the AVP-GFP fused protein under the control of the AVP promoter (Ueta et al. 2005), a GFP signal was also found in neurons of the olfactory bulb, where it modulates the processing of olfactory social signals (Tobin et al. 2010). Very recently, the same group also showed that a small fraction of ganglionic cells in the retina expresses AVP and, through its projections to the suprachiasmatic nucleus (which also contains AVP cells), modulates circadian rhythmicity (Tsuji et al. 2017). The low density and sparse innervation of axonal AVP projections in many brain regions make it technically difficult to dissect the origin of respective axons. A study by Scott Young III and colleagues showed that magnocellular AVP (magnAVP) neurons of the PVN project to CA2 of the dorsal hippocampus (Smith et al. 2016). Moreover, an elegant study of Hernandez and colleagues (Hernandez et al. 2015) combined extracellular recording of CA2, juxtacellular labeling and anatomical reconstructions demonstrated various extrahypothalamic AVP projections of magnAVP cells to numerous forebrain regions, including the preoptic area, suprachiasmatic nucleus, lateral habenula, and the amygdala (for details, please see respective chapter of Limei Zhang and co-authors in this book).

Notably, the forebrain projections of magnOT and magnAVP neurons largely overlap, suggesting simultaneous action of both neuropeptides on the same brain regions and probably on the same cells, which require further investigations (Dumais and Veenema 2016; Grinevich and Stoop 2018; Stoop 2012), especially taking into

consideration the affinity of each neuropeptide for the other receptor (Chini et al. 2008).

6.1.7 Parvocellular AVP Neurons: An Overlooked Cell Type?

While the projections, properties, and functions of parvOT neurons have been extensively studied, very little is known about the respective role of parvocellular AVP (parvAVP) neurons. Early studies identified AVP as a regulator of the hypothalamic-pituitary-adrenocortical axis and showed that AVP can potentiate the stimulatory effect of corticotropin-releasing hormone (CRH) on adrenocorticotropin (ACTH) cells of the anterior pituitary (Whitnall 1993). Later, Greti Aguilera and her colleagues showed that the synthesis of AVP in CRH neurons is triggered by chronic stress, which coincided with the downregulation of CRH expression in these cells. It was proposed that AVP substitutes CRH as the main factor in maintaining the release of adrenal corticosteroids under chronic stress and inflammatory conditions (Grinevich et al. 2001, 2002, 2003). A similar mechanism has been also observed in lactating rats, which exhibit a blunted CRH response that is partly compensated by enhanced synthesis of AVP in CRH neurons, which results in increased neuronal sensitivity (Walker et al. 2001). While it seems possible that parvAVP cells are involved in the stress-induced regulation of the CRH system, especially under chronic inflammatory stress, no concrete evidence confirming this theory has been presented yet. In addition, this particular line of research has been discontinued and thus the role of AVP in CRH neurons should be re-visited with the implementation of novel genetic and functional techniques developed during the last two decades. A good example of re-visiting an old research question with new methods is the recent study led by Yoichi Ueta that investigated the role of cisplatin in the activation of AVP neurons (Akiyama et al. 2020).

In contrast, the intricate interaction between neurosecretory magnAVP networks and preautonomic neurons in the PVN has been intensively studied. The seminal work of Javier Stern lab (Son et al. 2013) has convincingly demonstrated that activity-dependent AVP release from magnocellular neurosecretory neurons stimulated neighboring presympathetic neurons (within the range of 100 μm), thereby mediating interpopulation crosstalk. Moreover, the described mechanisms seem to play a pivotal role in the AVP-dependent polymodal neurohumoral response to a hyperosmotic challenge. This mechanism seems to be distinct to parvOT neurons, which synaptically innervate magnOT neurons in the SON (Eliava et al. 2016). On contrary, magnocellular AVP neurons extend their dendrites to the parvocellular compartments of the PVN containing various parvocellular cells, and this interpopulation crosstalk is mediated by the dendritic release of AVP from magnAVP neurons. In contrast to the conventional synaptic release of a signaling molecule from axonal terminals that act in a temporally and spatially constrained manner, dendritically released AVP acts in a "volume transmission" manner (Son et al. 2013), and the underlying mechanisms regulating release from the somatodendritic compartment differ significantly from those mediating axonal

release of the same peptide (Pitra et al. 2019). This involves diffusion of the neuropeptide in the extracellular space in a rather non-specific manner, "bathing" a mixed population of functionally distinct neighboring neurons within the PVN. In this signaling modality, the specificity of communication is determined by the presence/absence of specific receptors for the released signaling molecule. As demonstrated by Son et al. (2013) a specific population of neurons enriched with V2a receptors is parvocellular presympathetic neurons that project to the neurons of the rostral ventrolateral medulla (RVLM), a structure intimately associated with sympathetic regulation of the cardiovascular system (Guyenet 2006). Using dual-patch recordings and photolytic uncaging, the authors demonstrated that dendritically released AVP acts on V1a receptors located on neighboring parvocellular presympathetic PVN neurons. The V1aR-mediated depolarization and firing discharge of presympathetic neurons was shown to directly influence sympathetic outflow to the cardiovascular system and to specifically participate in the coordination of sympathetic and neurosecretory responses to a systemic osmotic challenge (Son et al. 2013). Importantly, the interpopulation crosstalk between magnAVP and parvo-presympathetic-V1a receptor-expressing neurons may also play an important role in prevalent cardiometabolic diseases, including hypertension, heart failure, and diabetes, in which an exacerbated neurohumoral activation state, which is characterized by elevated neurosecretory and sympathetic outflows, is known to influence prognosis, morbidity and mortality in these conditions (Althammer et al. 2020; Biancardi et al. 2011; Potapenko et al. 2011).

Taken together, the current body of knowledge does not provide evidence for a clear magno/parvo distinction based on projections, functions, and input for AVP neurons. In fact, the unique interaction of parvOT and magnOT neurons seems to be a unique feature of the OT system. Figure 6.3 provides an overview of the limited insight on the interaction between magnAVP, parvAVP, and presympathetic neurons.

6.1.8 Conclusion and Outlook

Recent publications on OT-ergic transmission in the CNS suggest that the classical projections from parvOT and magnOT as well as the presumed modes of release from these axons may be outdated and may have to be overthrown. The lack of clear evidence for postsynaptic receptors, the absence of true synapses in magnOT axonal terminals, the contribution of glial cells in the modulation of OT's effect on neuronal circuits and the discrepancy between magnOT and parvOT projection suggest an intricate interaction of OTergic circuits. Figure 6.4 summarizes the latest findings on OTergic modulation of neuronal and glial circuits and provides an overview of the respective modes of action and release from parvOT and magnOT neurons.

A reliable discrimination of parvOT and magnOT neurons based on their genetic profiles has not yet been achieved. Genetic analysis of OT neurons resulted in four different clusters, although it is not clear if parvOT and magnOT neurons are exclusively represented within those genetic subgroups of OT neurons. In a recent

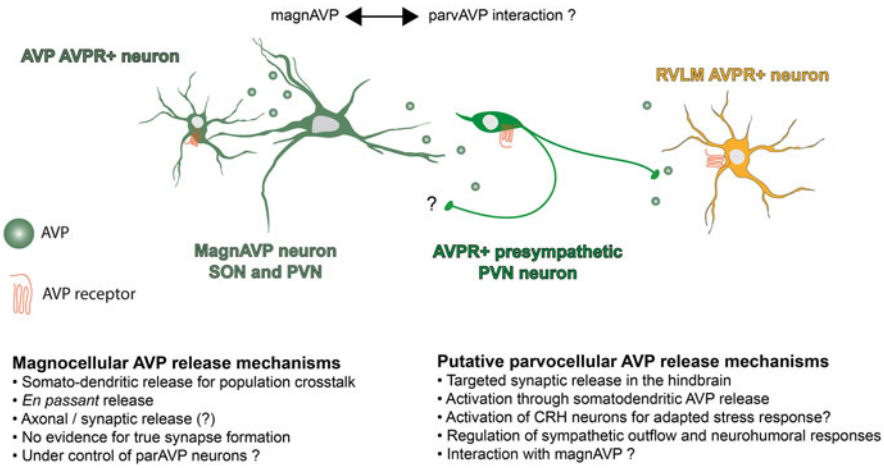

Fig. 6.3 Modes of release and interaction between parvAVP and magnAVP cells. Somatodendritically released AVP from SON and PVN magnAVP cells activates nearby AVPR-positive neurons. Within the PVN, somatodendritic release coordinates interpopulation crosstalk by activation of presympathetic neurons that project to the RVLM to coordinate cardiovascular responses

study (Lewis et al. 2020), the lab of Gul Dölen reports autism risk genes to be enriched in parvOT neurons, which have been genetically dissected based on anatomical location and Flourogold (FG) labeling. Intriguingly, the group reported that 34% of all OT neurons were parvocellular, which is in stark contrast (1–5%) to what has been previously reported in rats. While this discrepancy can partially be attributed to a species-dependent difference in the composition of the OT-ergic system and technical limitations with the use of Flourogold as a marker of magnocellular (neuroendocrine) neurons, further identification of genetic markers discriminating OT cell types will be essential to dissect phenotypes of OT neurons, which can be not limited to only parvocellular or magnocellular cells.

Within the last few years, it has become evident that the classical view on magnOT and parvOT projections is outdated. It seems that magnOT do not project exclusively to the pituitary and forebrain regions and that innervation by parvOT neurons is not confined to hindbrain structures. We now know that parvOT neurons project to OT neurons within the PVN (Tang et al. 2020) and SON (Eliava et al. 2016) and that magnOT neurons innervate the VTA (Hung et al. 2017). Furthermore, a very recent study (Oti et al. 2021) proposes that magnOT neurons also project to the spinal cord, based on the size of LDCVs (75–100 nm), typical of magnOT cells, although no research contradicts the possibility of LDVC presence in axonal terminals of parvOT neurons. Although all of these new findings argue for the long-held dogma on non-overlapping projection sites of parvOT and magnOT neurons, they stimulate further studies focused on compartmentalized subdivisions

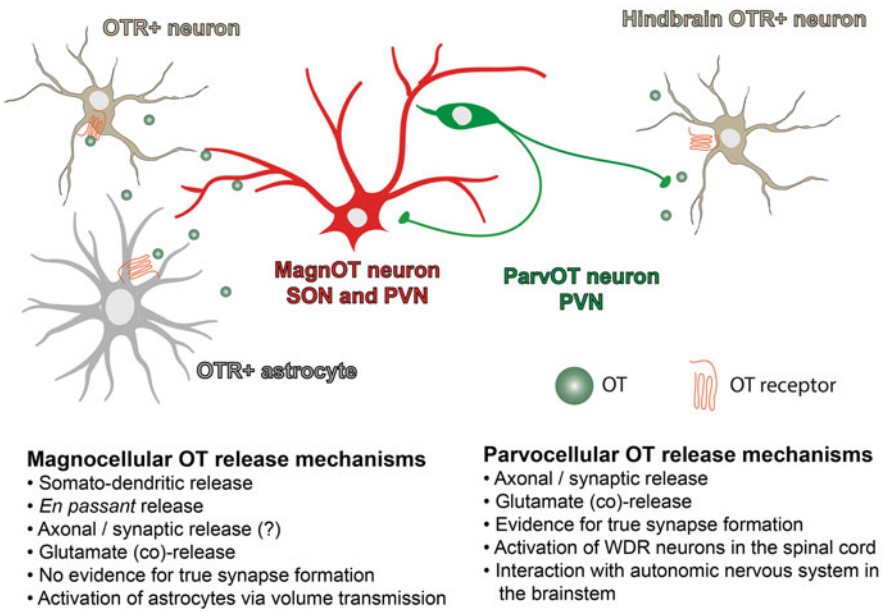

Fig. 6.4 Modes of release and interaction between parvOT and magnOT cells. ParvOT neurons act as hypothalamic master cells and project onto magnOT neurons in the PVN and SON to coordinate their activity. Somato-dendritic release from magnOT neurons provides a feedback mechanism between magnOT neurons. ParvOT neurons form clear synapses with other neurons in the PVN and spinal cord, while secretion from magnOT via volume transmission or *en passant* release activates nearby astrocytes and neurons. WDR: Wide dynamic range neurons

of each OT cell type linked to the specific regulation of distinct brain regions and respective behaviors.

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7 Fine Chemo-anatomy of Hypothalamic Magnocellular Vasopressinergic System with an Emphasis on Ascending Connections for Behavioural Adaptation

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Abstract

This chapter is complementary to Chap. 6 and presents an overview of recent research progress concerning the fine chemo-anatomy of hypothalamic vasopressinergic magnocellular neurons (AVP-magnocells), and their ascending projections to the central nervous system (CNS). Arginine vasopressin (AVP) is released from "dual" neurosecretory and synaptic terminals emanating from AVP-magnocells, not only to the median eminence and posterior pituitary gland but also to multiple extrahypothalamic destinations, especially limbic regions, influencing emotional responses during stress coping and motivational behaviour. Having been fortunate enough to witness important discoveries during the last decade concerning the role of this neurosecretory cell type in CNS neurotransmission, we are aiming to: (a) highlight the crucial findings that integrate endocrine secretion and neurotransmission at a single cell level; (b) challenge, in the light of the new observations, some of the long-standing dogmas concerning the fine chemo-anatomy of hypothalamic neurons based on

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these new findings and (c) fit recent discoveries into basic principles for understanding how this ascending and descending dual neurosecretory and neurotransmission system allows mammals to prioritize actions for survival and reproduction.

Keywords

Juxtacellular labelling · VGLUT · VGAT · Synaptic release · Electron microscopy · Social behaviour

7.1 Introduction

Arginine vasopressin (AVP), also called antidiuretic hormone (ADH), is synthesized mainly in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei by a type of cell with large somata (diameters around 20–35 μm) that are traditionally referred to as magnocellular neurosecretory neurons (they will be referred to as *AVP-magnocells* henceforward). These AVP-magnocells, with their main signalling molecule contents, *vasopressin and glutamate*, are part of the intricate neurobiological mechanisms that mediate fundamental allostatic/homeostatic physiological functions.

Figure 7.1 summarizes the gross chemo-anatomical aspects of the two sub-systems of hypothalamo-hypophysial neuroendocrine control centres, i.e. the hypothalamo-hypophysiotropic system and the hypothalamo–neurohypophysial system, to illustrate the main themes of this chapter. The AVP-magnocells (green cells), located mainly in paraventricular and supraoptic nucleus, containing arginine vasopressin (AVP), oxytocin (OXT) and glutamate (symbolized in greenish generic cells and axons), send their projections to the posterior lobe of the pituitary gland (neurohypophysis), through the hypophysial stalk (also called infundibulum), where the neurohormones are released from the nerve endings to the capillaries derived from the inferior hypophysial artery. The ascending projections of the magnocells are symbolized by green lines projecting into CNS.

This chapter does not cover the whole literature on the involvement of vasopressinergic/glutamatergic pathways in sensorimotor and cognitive processing, since there are recent and excellent reviews on this broader subject (Armstrong 2004; Stoop 2014; Bester-Meredith et al. 2015; Brown et al. 2020). Rather, we focus on recent developments regarding the fine chemo-neuroanatomy, ascending projections and mechanisms whereby vasopressin–glutamatergic pathways modulate neuronal integration in cortical and subcortical brain regions known to be relevant for behavioural adaptation. Before going into the fine chemo-anatomy of AVP-magnocells, we remind our readers that the two well-established physiological actions of vasopressin are *antidiuresis* through increased water reabsorption by the kidney and a pressor action due to *vasoconstriction* of blood vessels. The removal of the posterior lobe of the pituitary gland (also called neurohypophysis) or lesions in the SON and PVN result in diabetes insipidus, the disease characterized by polyuria

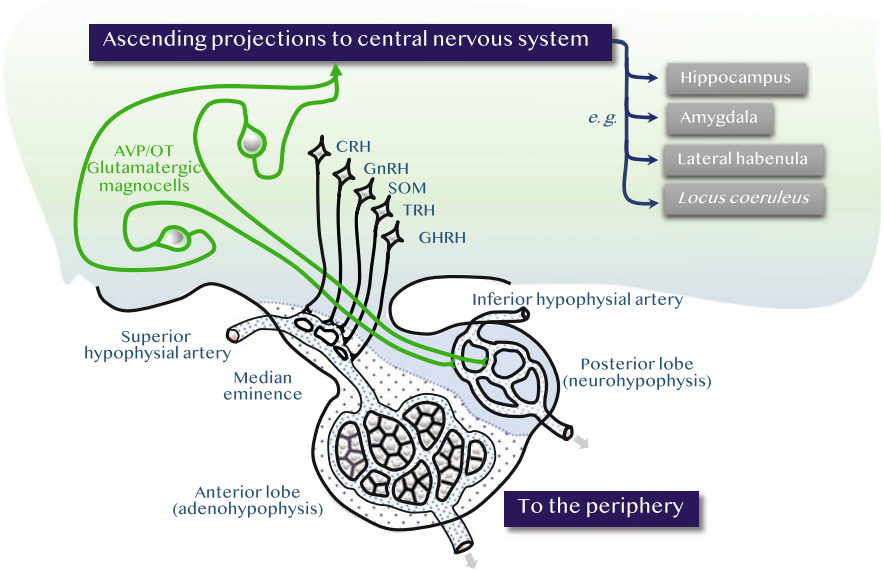

Fig. 7.1 Schematic drawing showing the gross chemo-anatomical components of the hypothalamo–hypophysis (also called pituitary gland) neuroendocrine control centres and their anatomical relationship. Two neuroendocrine sub-systems can be coarsely classified. The first is the hypothalamic–hypophysiotropic system, where neurosecretory neurons located in the hypothalamus produce releasing- or release-inhibiting hormones, (e.g. corticotropin-releasing hormone, CRH; gonadotropin-releasing hormone, GnRH; somatostatin, SOM; thyrotropin-releasing hormone, TRH; growth hormone-releasing hormone, GHRH), send their axons to the hypophysial blood vessels and release hormones into the portal circulation. Hypophysiotropic hormones are then transported to the anterior lobe of the pituitary gland, also called adenohypophysis, to stimulate or inhibit the release of the tropic hormones (e.g. adrenocorticotropin, gonadotropins, prolactin, TSH or GH), from various secretory cells. The second system is the hypothalamic–neurohypophysial system, symbolized by elements within the shaded area. Neurosecretory magnocellular neurons (magnocells) located mainly in paraventricular and supraoptic nucleus, containing arginine vasopressin (AVP), oxytocin (OT) and glutamate (symbolized by greenish generic cells and axons), send their projections to the posterior lobe of the pituitary gland (also called neurohypophysis), through the hypophysial stalk (also called infundibulum), where the neurohormones are released from the nerve endings to the capillaries derived from the inferior hypophysial artery. AVP magnocells also project to median eminence (not shown). The ascending projections of the magnocells are symbolized by green thick lines to the central nervous system (glutamatergic pathways). (Adapted but largely modified from Greger and Windhorst (1996))

(increased urination) and polydipsia (increased thirst sensation). It is well-established that vasopressin binds three distinct receptors (Chap. 8). Secretion of vasopressin is controlled by many hormonal and neural factors. The most important are plasma osmolarity and circulating blood volume (Dunn et al. 1973).

Since the 1950s, the concept that hormone secretion from the pituitary gland is governed by the hypothalamus became established through Geoffrey Harris's notion of hypothalamic releasing factors. It then became natural to think that hormones

released from the pituitary might also act on the brain to induce behavioural responses that were congruent with their peripheral actions (Harris 1948; Leng 2018). It is in this spirit we present to the readers some case studies.

7.2 The Endocrine–Neuronal–Glutamatergic Nature of AVP-Magnocells

> Mother Nature does not, in general, allow her secrets to be revealed with ease.
>
> She usually sides with the hidden flaw, the confounding variable or the unwarranted assumption. In most areas of scientific endeavour, she has drawn on her replete bag of tricks and strewn them liberally along the paths to discovery.
>
> Glenn I. Hatton

AVP-magnocells, together with oxytocinergic magnocells (Chap. 8), were the first known central neural peptidergic cells of the mammalian brain (Bargmann and Scharrer 1951). The discovery of AVP-magnocells and their continuous study in the last seven decades, from hypothalamic–neurohypophysial system (HNS)-centred research to the molecular features of the magnocells and more recently to their ascending connections, have greatly enhanced our understanding of this important peptidergic system. The AVP-magnocells, compared to previously *known* neurons or endocrine cells, possess some unique features, making them unlike endocrine cells, discovered earlier in the twentieth century, in the adrenal medulla, pancreatic islets, gut and anterior pituitary, which also secrete hormones into the general circulation under the influence of other hormones and neuronal inputs. The AVP-magnocell fulfils all the criteria to be called a "neuron", i.e., it has synaptic inputs from other neurons of the brain and emits long axonal processes branching at targeting regions within the internal medial eminence and then in the neural lobe (Fig. 7.1), as well as making ascending projections within the central nervous system. Figure 7.1 illustrates AVP's neurosecretory functions (see the Fig. 7.1 legend for full details).

L-Glutamate, the main excitatory neurotransmitter of the brain, influences virtually all neurons, including the hypothalamic neuroendocrine neuronal populations. However, during the intense investigation of the HNS during the second half of the twentieth century, the release of glutamate from neurohypophysial neuroendocrine cells was not a focus of experimental inquiry. There is a historical reason for the apparent neglect of the dual nature of vasopressin/glutamate co-release. Until the end of the twentieth century, the identification of glutamatergic neurons had only been inferential. This was because the five-carbon amino acid glutamate, unlike GABA, acetylcholine, catecholamines and other neurotransmitters, is ubiquitous to all cells, due to its vital role in cell metabolism, e.g. it is a precursor of GABA and other essential molecules, also being indispensable for cell proliferation, immune function and for acid–base balance. It was around the turn of the century that the identification of the vesicular glutamate transporters, VGLUT1 and VGLUT2, which selectively accumulate L-glutamate into synaptic vesicles, provided the first definitive markers

of glutamatergic neurons (Ni et al. 1994; Aihara et al. 2000). Evidence indicated that neither VGLUT1 nor VGLUT2 bind other amino acid transmitters (Ziegler et al. 2002). Hence, a novel and valuable tool expanded our understanding of the brain's secrets, and of the AVP-magnocells in particular.

In 2002, Herman and colleagues published a pioneering paper on the distribution of vesicular glutamatergic transporter mRNA in rat hypothalamus (Ziegler et al. 2002). They reported for the first time that both PVN and SON host abundant VGLUT1 and VGLUT2 mRNA-expressing cell populations (Fig. 7.3 of (Ziegler et al. 2002)). Hrabovszky, Liposits, and colleagues performed a demonstrative experiment to show either AVP-magnocells or OT-magnocells or both expressed VGLUT2 (Hrabovszky et al. 2007). They injected retrograde tract-tracer Fluorogold (FG) into the systemic circulation. This was taken up by the axon terminals of the neuroendocrine magnocells, as they are in close contact with the basal lamina of the capillaries in the neurohypophysis, and retrogradely transported to the perikarya. Simultaneously, glutamatergic perikarya of the hypothalamus were visualized by the radioisotopic in situ hybridization detection of VGLUT2 mRNA. The results of these dual-labelling studies established that the majority of neurons accumulating FG in PVN and SON also expressed VGLUT2 mRNA (Fig. 7.2). The definitive demonstrations of AVP-magnocell coexpression of VGLUT2 at the single cell level were published in 2020 by the Zhang laboratory (Zhang et al. 2020).

7.3 One AVP-Magnocell Has *(Not)* Only One Axon?

> Science is based on experiment, on a willingness to challenge old dogma, on an openness to see the universe as it really is. Accordingly, science sometimes requires courage - at the very least the courage to question the conventional wisdom.
>
> Carl Sagan

Most of us who have taught neuroanatomy and neurophysiology know a principles of the neuron doctrine, established by Santiago Ramón y Cajal (Cajal 1954), one of the parents of modern neuroscience, is that "*one mature neuron has only one axon*". This canonical rule has served for neuronal classifications, such as projection neurons and interneurons, since then. However, Santiago Ramón y Cajal studied mostly the neocortical and archicortical regions, and most of the fine neuroanatomical investigations published in the twentieth century followed suit. Thus, many neuroscientists and neuroendocrinologists consider this to be a general truth. This perspective, and the general idea that the AVP-magnocells were dedicated solely to their neurohypophysiotropic role, impeded, to a large degree, the ascertainment of the dual axonal projection system of the AVP-magnocell.

Since the late 1970s, three seminal works, using immunohistochemistry with anti-neurophysin (Brownfield and Kozlowski 1977; Swanson 1977) and anti-vasopressin (Buijs 1978) antibodies, had already observed phenomena suggesting possible ascending projections from the rat PVN lateral magnocellular division, as well as the intermediate nucleus which contains AVP-magnocells. Figure 7.3a shows two photomicrographs published in 1977 by Brownfield and Kozlowski, with

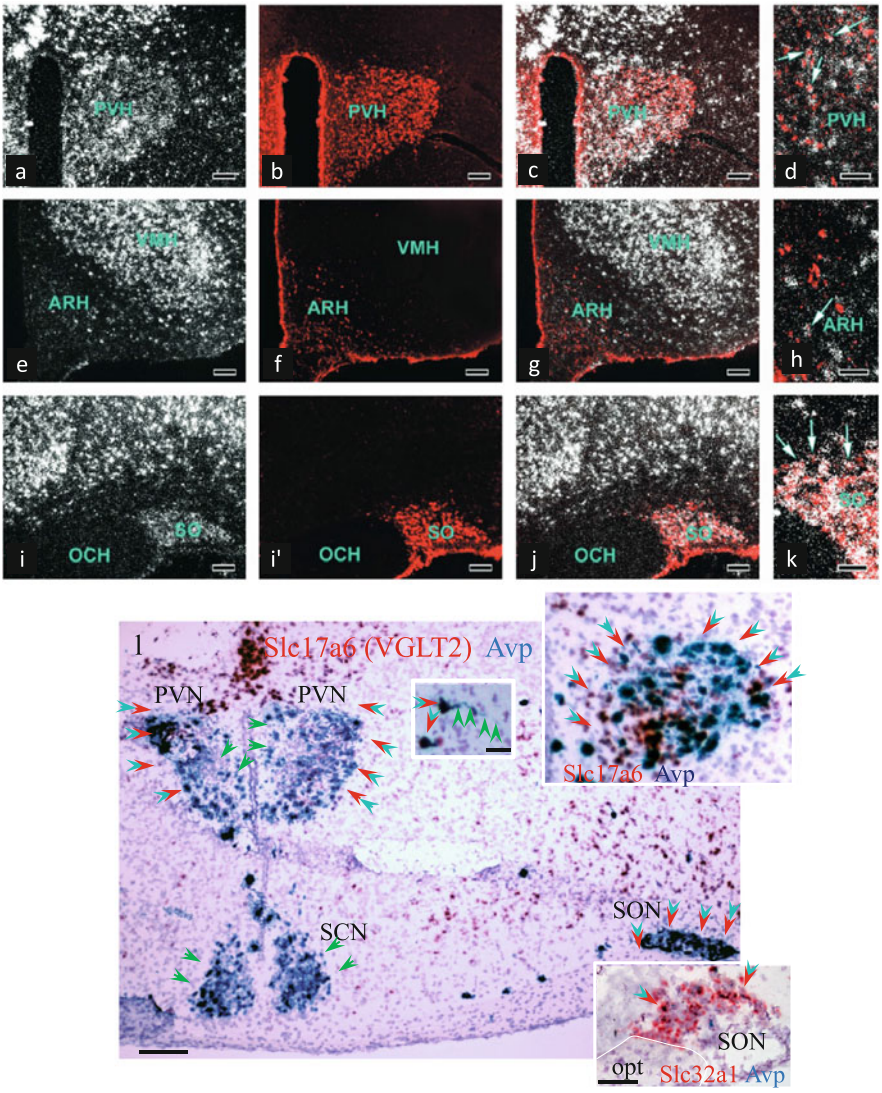

Fig. 7.2 Hypothalamic AVP-magnocells co-express VGLUT2 mRNA (see the text for details). Panels **a**, **e** and **h** show the VGLUT2 ISH in PVN, ventromedial hypothalamic nucleus (VMH) and SON. Panels **b**, **f**, **i** show FG immunohistochemistry also in the above nuclei (F: VMH as negative control), FG was injected into the systemic circulation. Panels **c**, **d**, **g**, **h**, **j** and **k** show the overlapping of the two markers in PVN and SON but not in VMH, which does not host magnocellular neurosecretory cells projecting to the neurohypophysis capillaries. Panel **l** and insets show VGLUT2-mRNA (*Slc17a6*) and *Avp* overlapping at single cell level using the RNAscope technique. Note that in SON there is an intermingling of cells expression *Slc32a1* (mRNA for VGAT) and AVP. **m**–**p**: EM photomicrographs taken from neurohypophysis showing: (M) four axon terminals (AT1–4) with variable contents of peptidergic large dense-core vesicles (DV) (AT1 and AT4 mainly DVs) and small clear vesicles (SV, AT2 and AT3, mainly); (**n**–**p**) shows pre-embedding colloidal-gold labelling for VGLUT2, followed by silver intensification, revealing the preferential distribution of the immunocytochemical signal in axonal profiles dominated by SVs. Arrowheads indicate basal lamina. Pit: pituicyte, PCS: perivascular space. Panels **a**–**k** and **m**–**p**, adapted from Hrabovszky and Liposits (2007), with permission. Panel **l** adapted from Zhang et al. (2020) with permission

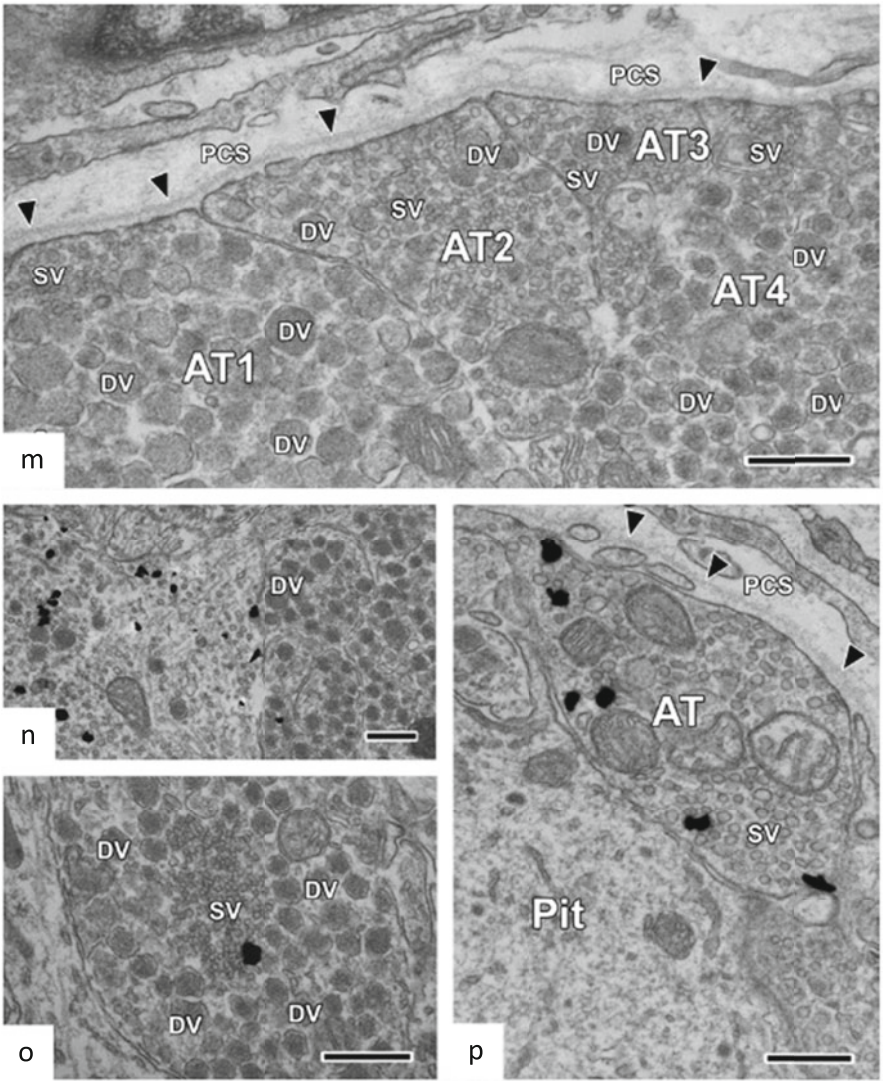

Fig. 7.2 (continued)

immunohistochemistry against the peptide neurophysin, a fragment of the same precursor for vasopressin. In this inspired but rather overlooked study, the authors named the ascending tract they observed the hypothalamo–choroidal tract (HCT, Fig. 7.3a′), in contrast to the *Tract of Greving* (R. Greving was the first anatomist to describe this tract, coining the name *tractus paraventricularis-cinereus*, in a series of papers published in the early twentieth century). Hence, the tract also bears the name *Tract of Greving,* TG, (see Greving (1923, 1926, 1928) for original references in

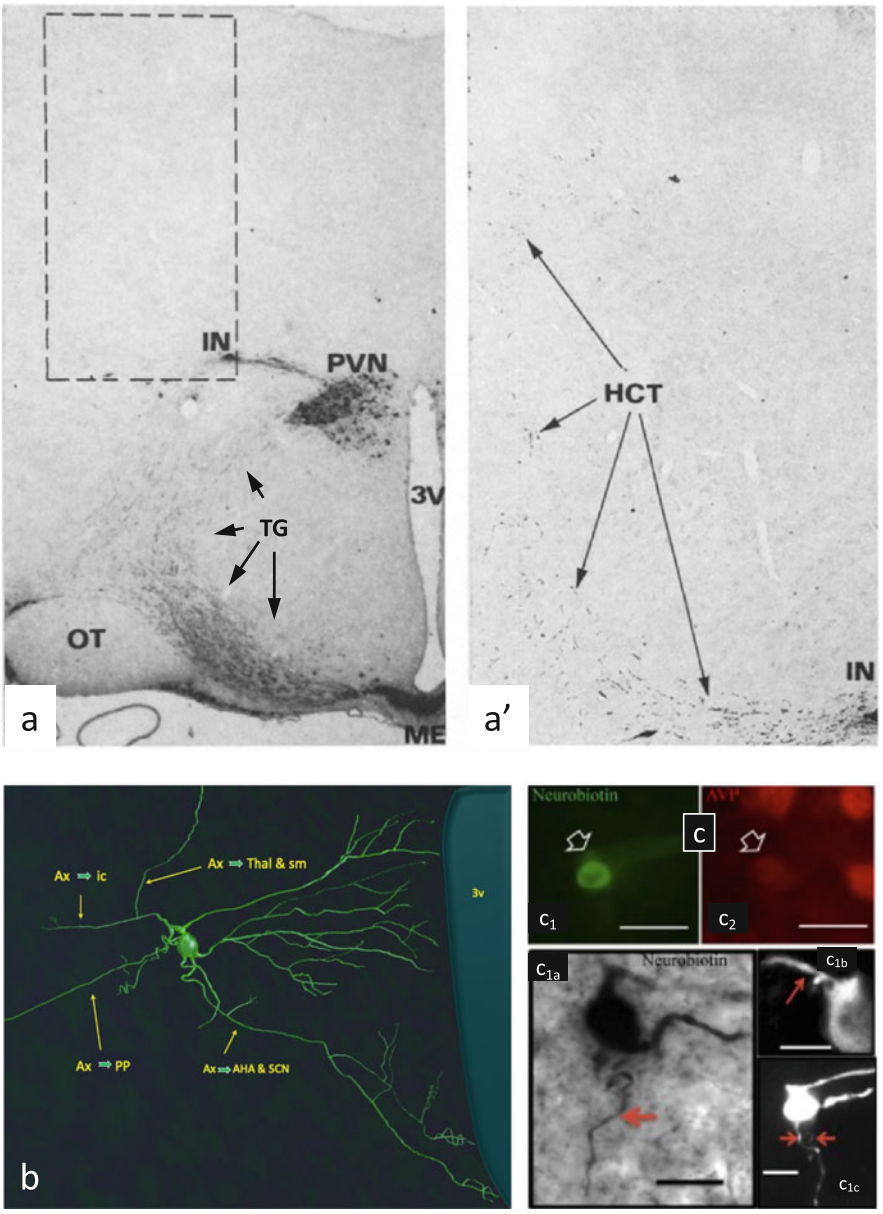

Fig. 7.3 Ascending projections to central nervous system emanate from AVP-magnocells. (**a** and **a'**) Panels from one of the earliest studies reporting that ascending neurophysin immunopositive fibres *seemed to be* emanating from hypothalamic paraventricular nucleus (PVN) and the intermediate nucleus (IN). Note that with this method, the origin of the fibres cannot be unequivocally determined. (**b**) A computer-aided 3D reconstruction of an in vivo juxtacellularly labelled AVP-magnocell from a young male rat revealing multi-axonal nature (indicated by arrows; Ax: axon; ic: internal capsule; Thal: thalamus; sm: stria medularis; PP: posterior pituitary gland; AHA: anterior hypothalamic area; SCN: suprachiasmatic nucleus). (**c**s) Panels show the fluorescence histochemistry of neurobiotin labelling of the soma section (**c**1, green) and vasopressin

German). Brownfield and Kozlowski, the authors of the original publication in 1977, speculated that neurophysin immunopeptide fibres carried vasopressin to the choroid plexus to regulate brain interstitial–ventricular cerebrospinal fluid dynamics. This concept attracted little notice in subsequent years, perhaps because visualization of AVP itself was not possible at the time. Following the identification of vasopressinergic neurons in the bed nucleus of stria terminalis and central amygdala in the 1980s (Caffe and van Leeuwen 1983; van Leeuwen and Caffe 1983; DeVries et al. 1985; Caffe et al. 1987) and the sex-steroid dependency on its immunohistochemical detection for vasopressin antigen level (DeVries et al. 1985), it was concluded that the vasopressinergic innervations in the intracerebral cortical regions (*archicortex* mainly, i.e. olfactory cortex and hippocampal formation), limbic regions and other brain stem come from the bed nucleus of stria terminalis, central amygdala and the parvocellular division of the PVN. This concept dominated the field for some three decades. In 2009, the multi-axonal feature of AVP-magnocells and the possibility of ascending projections resurged with an electrophysiological report from Inyushkin and Dyball in Cambridge, demonstrating the bi-axonal feature of the AVP-magnocells in the supraoptic nucleus (SON), one to the neurohypophysis and one toward regions of the brain stem (Inyushkin et al. 2009). In the fall of 2012, two independent reports (Cui et al. 2013; Zhang and Hernandez 2013) were published demonstrating that the AVP-magnocells serve as a source for vasopressinergic innervation within the hippocampal formation. Zhang's and Hernández's study in the rat investigated the pattern of innervation of the hippocampus by AVP+ axons including cellular and subcellular targets as well as the origin and pathways of these AVP+ fibres by using tract tracing, cutting the fixed rat brains in several oblique angles and with subsequent immunocytochemistry. This traditional anatomical method and 3D reconstruction in continued serial sections allowed us to connect three main tracts from hypothalamic AVP-magnocellular nuclei to the hippocampus in wild-type rats (Zhang and Hernandez 2013) (for the description of the other reports see Sect. 7.5).

What is the *fine structure* of a single AVP-magnocell (including soma and dendrites) and where do its axon(s) originate? It is important to recall at this point that the relatively understudied fine anatomy of subcortical neurons, compared with neocortical and archicortical neurons, makes this question both fundamental and paradigmatic for understanding the general organization of the brain. In vivo juxtacellular recording and labelling, processing methods in combination with

Fig. 7.3 (continued) immunoreactivity (**c**1, red). **c**1a: DAB developed main cell body with the axonal process indicated with an orange arrow (**c**1a). The panel **c**1b and **c**1c are adjacent sections with soma and proximal dendrites pictured under fluorescence microscopy, with arrows indicating origin of other two axonal processes. Panel **a** was modified from Brownfield and Kozlowski (1977). TG: tract of Greving. HCT: hypothalamo–choroidal tract (the authors of the original publication interpreted the neurophysin immunopositive fibres carried vasopressin to choroid plexus to regulate brain interstitial–ventricular cerebrospinal fluid dynamics). Panel **c** was modified from Hernandez et al. (2015), with copyright held by the authors

anatomical reconstruction, provided a golden opportunity to study this question. One of the main advantages of in vivo juxtacellular labelling for single neuron reconstruction is that one can generally unequivocally connect the neurobiotin processes (the main ones, at least), emitted from the labelled soma in wild-type animals under physiological conditions (except the anaesthesia for craniotomy). The first AVP-magnocell cell our laboratory successfully recorded and labelled is presented in Fig. 7.3, panels b and c. In this interesting but perhaps ungainly-appearing neuron, one can observe several processes, straight or curly, emitted from soma, and including long-range projections. This neuron from a young male rat's hypothalamic PVN was identified as an AVP-magnocell, since its soma was identified as AVP-immunopositive and possessed a main axon joining the *Tract of Greving*. A surprising characteristic was revealed with this combination of in vivo juxtacellular labelling and fine anatomical study, that there are at least two long-range projection processes emitted from the cell, one from its soma and another from a primary dendrite. The soma was located in the PVN lateral magnocellular division, with its long axis 30° oblique to the midline. The soma gave rise initially to two short and thick primary dendrites, which branched proximally. The bottom dendrite branched extensively until the fifth order of branches—all directed medially reaching the wall of the third ventricle (3v), indicating possible dendritic release (Brown et al. 2020) directly into the ventricular space, as suggested earlier (Brownfield and Kozlowski 1977). The top dendrite emitted two secondary branches, medial and lateral. The medial branch was similar to the bottom group. The lateral branch curled up proximally near the soma but gave rise to another main axon (Fig. 7.3, C1a, orange arrow). The main axon coursed laterally, passing on top of the fornix (fx), turned ventrally and then medio-posteriorly. One of the ventrally directed axons coursed further ventrally along the periventricular region, reaching the suprachiasmatic nucleus, where neurobiotin-labelled axonal processes were found. We continued our endeavour to label the single AVP-magnocells in vivo, even with a rather low experimental success rate (in 155 experiments, only six well-labelled cells were identified to be AVP-magnocells). However, the hard work paid off—the reward brought by the discovery was unexpected: each of the six juxtacellularly-labelled AVP-magnocells possessed the main axons joining the Tract of Greving, as well as emitting one or two axons in other directions within the brain (see Hernandez et al. (2015) for detailed experimental procedures and descriptions of the fine anatomy of the labelled magnocells). Recently, the Gao laboratory in Hangzhou published a comprehensive study using viral tracing and whole-brain imaging, reconstructing the three-dimensional architecture of the hypothalamic–neurohypophysial system and confirming the collaterals of VP-magnocells within the brain (Zhang et al. 2021).

7.4 Ascending Projections of AVP-Magnocells

> In order to know how the brain processes information, we need a complete description of the structure of nerve cells and the dynamic characteristics of the connections between them. . . . Without such painstaking research there will never be full understanding of the brain.
> Colin Blackmore

Continuing the glutamatergic AVP-magnocell theme, Hrabovszky and Liposits presented electron microscopic evidence that small, clear vesicles are present in magnocells' axon terminals, together with the large dense-core vesicles, in the neurohypophysis (Fig. 7.2m–p). They also observed that following hormonal and homeostatic challenges of the magnocellular system, VGLUT2 mRNA expression is increased. A specific role for glutamate release from *neurohypophyseal* terminals, however, was not readily apparent. Thus, speculation about the purpose of glutamate release from magnocells for a possible dual hormonal and neuronal function for AVP-magnocells began to emerge. Specifically, an interesting possibility presents itself*:* could this apparent *secondary* glutamate liberation in neurohypophysis be *primary* in some other region? This speculation was soon grounded in experimental evidence. Figure 7.4 is taken from the lateral habenula, an epithalamic structure relevant for processing of "disappointment" that its global activation is related to psychomotor deficiency (see also Sect. 7.9 of this chapter for an example of functional implications of AVP-magnocells to lateral habenula pathway). The coloured panels show a double immunofluorescence reaction against vasopressin (red) and VGLUT2 (green), demonstrating co-localization within axon terminals (Fig. 7.4, panels a and bs). The photomicrographs show the peroxidase-diaminobenzidine vasopressin immuno-electron microscopy reaction, with axon terminals containing vasopressin making Gray type I asymmetric synapses (presence of postsynaptic density, PSD, arrowheads) indicating that they are glutamatergic. Small clear vesicles can be clearly seen and from the colour panels it can be deduced that at least some must contain VGLUT2. Large dense-core vesicles with vasopressin immunopositivity can be seen docked at the presynaptic membranes (Fig. 7.4c and d).

Conventional immunohistochemical, electrophysiological and ex vivo labelling methods cannot demonstrate the long-range extra-neurohypophysial projecting axons of AVP-magnocells. This is due to the fact that (1) the large cell size (soma and processes) impedes ex vivo brain slice-based methods of detecting long-range projections, (2) the magnocells within the magnocellular hypothalamic nuclei (i.e. PVN and SON), are densely packed and the usual immunoreaction yields very strong labelling that makes cell borders, and those between axons and dendrites, difficult to discern and (3) VP parvo- and magnocell populations are intermingled (Fig. 7.5a, inset). Applying the technique of juxtacellular labelling and post hoc processing, however, it is feasible to identify the final anatomy of individual VP-magnocells unequivocally (Fig. 7.6, see legend for full description of the fluorogold retrograde and juxtacellular anterograde tracing methods used to

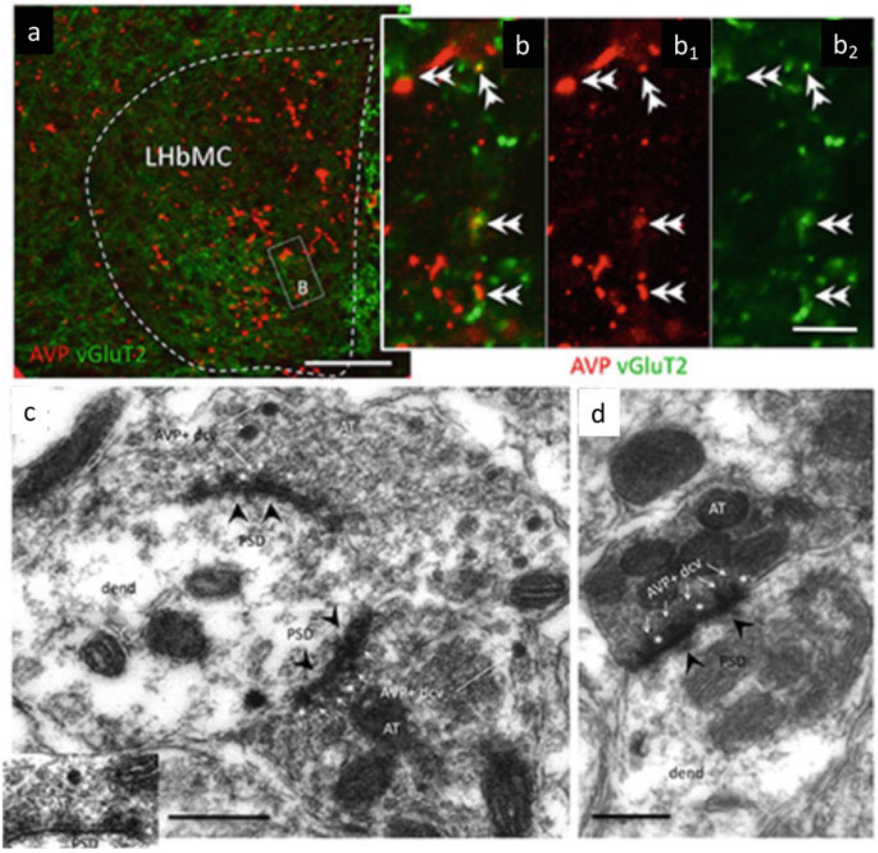

Fig. 7.4 Most AVP+ axon terminals co-expressed vesicular glutamate transporter 2 (VGLUT2) and established Gray type I synapses onto habenular neuron dendrites. **a** and **b**s: Representative confocal photomicrographs of double immunofluorescence AVP (red) and VGLUT2 (green) centred at the medio-central lateral habenular (LHbMC) subnucleus. Double arrowheads indicate the double-labelled axon terminals. **c** and **d**: Electron microscopy photomicrographs showing the axon terminals (AT) containing AVP+ dense-core vesicles (dcv, thin white arrows) established Gray type I synapse (postsynaptic densities, PSD, were indicated with black arrowheads) onto habenular neuron's dendrites (dend). Asterisks are put adjacent to AVP+ dcv, which showed docking onto presynaptic membranes. Scale bars: **a**: 50 μm; **b**: 5 μm; **c**, **d**: 500 nm (Taken from Zhang et al. (2016) with copyright held by authors)

demonstrate the hypothalamic origin of AVP axons in habenula). Connection between AVP-magnocells of PVN and nerve terminals in other brain areas has been unambiguously demonstrated, through the employment of techniques such as juxtacellular labelling, optogenetics and ultrastructural analysis, showing that AVP-magnocells have extensive ascending projections (Fig. 7.7).

As will be discussed in the following sections, the demonstration of dual projections from AVP-magnocells of the paraventricular nucleus (PVN) to both

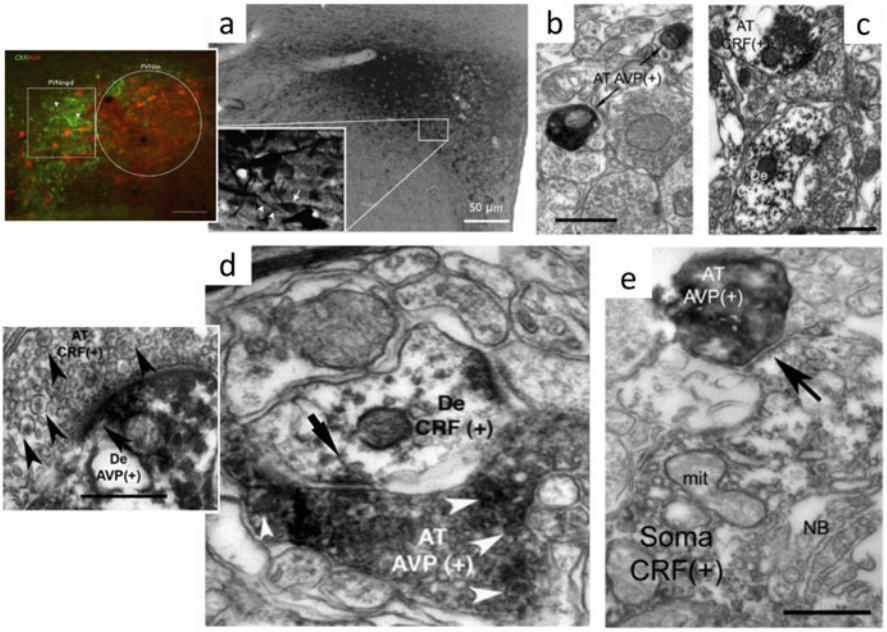

Fig. 7.5 Reciprocal synaptic connections between AVP-magnocells and corticotropin-releasing hormone (CRH) synthesizing neurons in the paraventricular nucleus, lateral magnocellular division and medial parvocellular division (PVN_{mpd} and PVN_{lmd}, respectively). (**a**) Double peroxidase-DAB reaction prepared for electron microscopy (with nickel and without nickel) after immunofluorescence reaction and examination (inset) with AVP (labelled in red) and corticotropin-releasing hormone (CRH, labelled green) antibodies and corresponding secondary antibodies bound with fluorochromes. The white line-delineated square was taken and re-embedded in resin for electron microscopy examination. (**b** and **c**) Examples of AVP-DAB-nickel labelled, and CRH-DAB labelled profiles. (**d**) AVP-immunopositive axon terminal (AT, AVP+) making an asymmetric synapse (Gray type I, black arrow indicates postsynaptic density, PSD, an electron microscopic feature for excitatory synapse), onto a dendritic profile (De) CRH+. Inset shows the opposite case, a CRH+ AT making a Gray type I synapse onto a AVP+ dendritic profile. Arrowheads indicate immunopositive large dense-core vesicles. Panel (**e**) shows a case of an AVP+ AT making a synapse onto a CRH+ soma. NB, Nissl body; mit, mitochondrion (The above panels are adapted from reference (Zhang et al. 2010) with permission). (**f** and **g**)

the posterior pituitary (hormonal) and to the amygdala, hippocampus, habenula and locus coeruleus provides a neuroanatomical basis for understanding how vasopressinergic cells integrate homeostatic and allostatic regulation. Reflexive endocrine control of the internal milieu (homoeostasis) and neuronal control of drives that promote homeostasis (e.g. thirst) occur at the level of the hypothalamus and hypophysis. At the same time, through projections to extrahypothalamic regions, these responses are linked to appetitive/rewarding aspects of thirst and allostatic regulation of complex behaviours such as escape and fear responses. Integration of the two types of responses further allows developmental

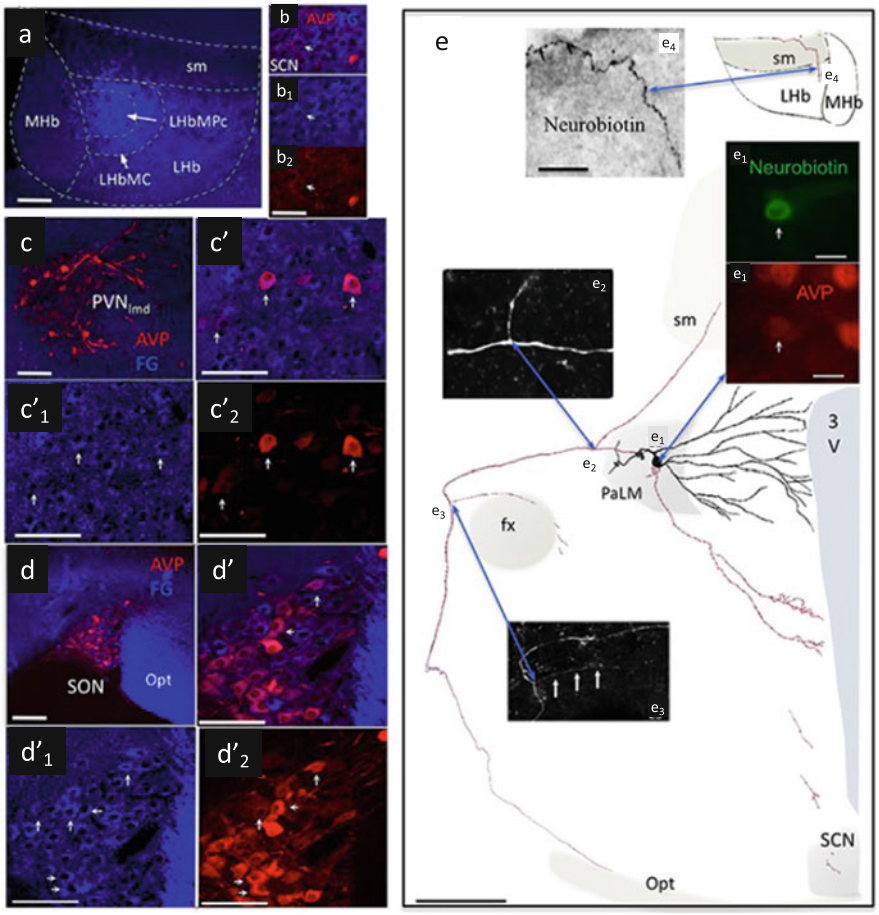

Fig. 7.6 AVP-containing magnocellular neurosecretory neurons serving as one of the sources of AVP+ axons in LHb. (**a**) Fluorogold (FG) retrograde tracer was injected into the medio-central subnucleus of the lateral habenula (LHbMC). (**b**) In the hypothalamic suprachiasmatic nucleus (SCN, panels b′a–b), only sparse double-labelled cellular components were found. (**c**) Numerous FG+/AVP+ somata were found in hypothalamic paraventricular nucleus (PVN). (c′) shows a magnification of the region and (c′a) and (c′b) are the separated channels of the c'. Arrows indicate some double-labelled cells. (**d**), (d′), (d′a), and (d′b): same cases of cs but in the hypothalamic supraoptic nucleus (SON). (**e**) Camera lucida reconstruction of an in vivo juxtacellularly labelled AVP+ magnocellular neuron. The soma and dendrites are represented in black and axonal segments are represented in red. AVP-containing nature was ascertained by AVP immunoreaction (e1, lower panel) in combination with neurobiotin histochemistry (e1, upper panel). The soma gave rise initially to two short thick primary dendrites, which branched proximally. The main axon coursed laterally, passing the fornix (fx), and turned ventro-caudally towards the posterior pituitary gland. Two main collaterals emanated from this axon (e2, e3). The first collateral (e2) coursed dorsomedially, joining the stria medullaris (sm). Neurobiotin-labelled processes were found inside the lateral habenula e4) [The panel (**e**) was modified from (Hernandez et al. 2015), with copyright held by authors]. 3V: third ventricle; Opt: optic tract; SCN: suprachiasmatic nucleus; PaLM: paraventricular lateral magnocellular. Scale bars: a, c′, c′ a–b, and d′, d′ a–b: 100 μm; b′ a–b: 20 μm; e: 250 μm; e1: 20 μm, and e4: 50 μm

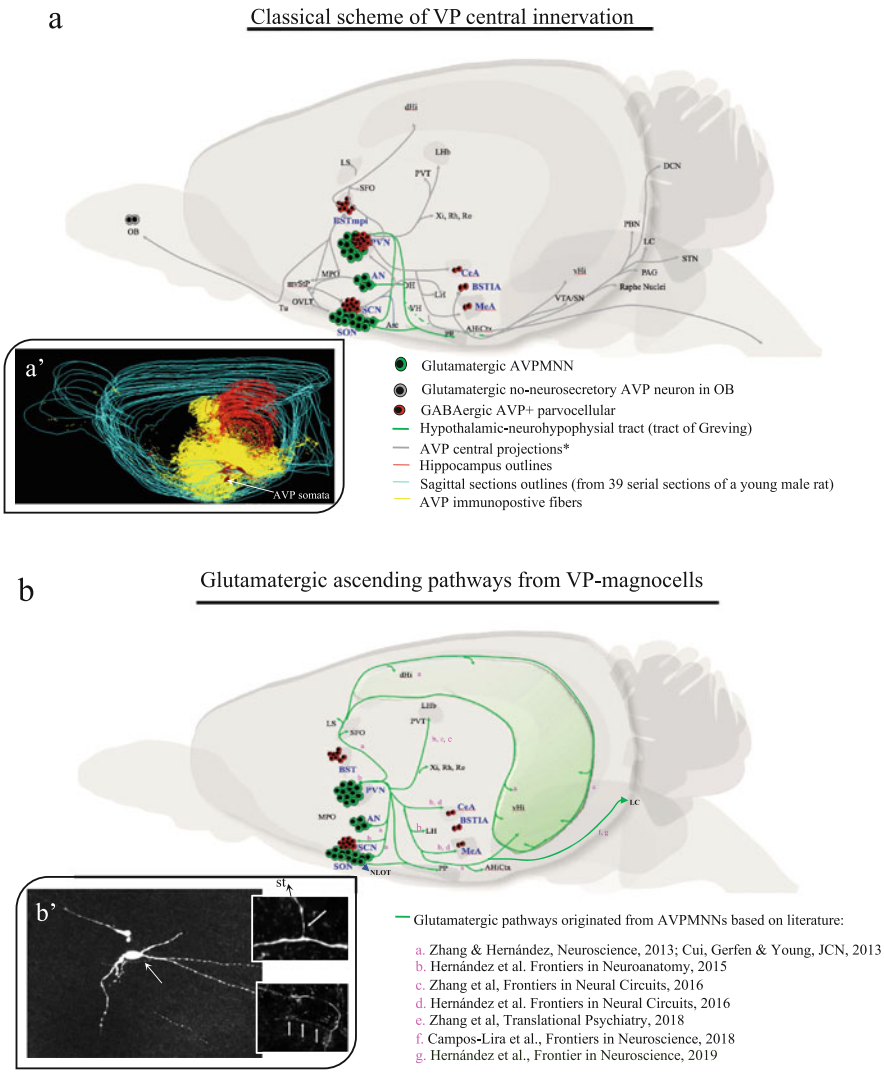

Fig. 7.7 Major central vasopressin-containing nuclei and pathways in the rodent brain. **a**: Classical scheme of AVP central innervation. **a**': Computerized 3D "one-to-one" mapping to visualize the AVP-immunopositive fibre distribution and cell bodies of a young male rat. **b**: Central projections of AVP-magnocellular neurosecretory neurons. Recent additions to the literature on AVP neurosecretory system central projections. **b**': an in vivo juxtacellularly labelled AVPMNN, with white arrows indicating the central branches of the main axons. PVN: hypothalamic paraventricular nucleus; SON: supraoptic nucleus; SCN: suprachiasmatic nucleus; AN: accessory nuclei (which includes nucleus circularis and the posterior fornical nucleus); BSTmpi: bed nucleus of stria terminalis, medial posterior internal division; BSTIA: BST, intra-amygdala division; CeA: central amygdala; MeA Medial Amygdala; LS: lateral septum nuclei; dHi: dorsal hippocampus; vHi: ventral hippocampus; LHB: lateral habenula; PVT: paraventricular thalamic nucleus; OB: olfactory bulb; Tu: olfactory tubercle; OVLT: organum vasculosum of lamina terminalis; mvStP: medial ventral striatal-pallidum region; MPO: medial preoptic nuclei; SFO: subfornical organ; Xi, Rh; Re: thalamic xiphoid, rhomboid and reuniens nuclei; AHi: amygdalohippocampal area; VTA/SN: ventral tegmental area/substantia nigra; PAG: periaqueductal grey; STN: solitarii tractus nucleus; LC: locus coeruleus; PBN: parabrachial nuclei; NLOT: nucleus of lateral olfactory tract (modified from Zhang and Eiden (2019) with permission)

environmental inputs, such as maternal deprivation, to have life-long effects on stress responding and anxious behaviour through long-term plasticity of AVP-magnocells.

7.5 The Projections of AVP-Magnocells to Hippocampus and Social Behaviour

It has been long accepted, from the presence of vasopressin receptors and pharmacological evidence of exogenous vasopressin action, that vasopressinergic innervation of the hippocampus exists. However, the origin of vasopressin nerve terminals remained unclear for a long time. We investigated in the rat the pattern of innervation of the hippocampus by AVP immunoreactive axons (AVP+ axons), including cellular and subcellular targets and the origin and pathways of these AVP+ fibres through tract tracing and immunocytochemistry (Fig. 7.8). Zhang and Hernandez reported a preferential innervation of the ventral hippocampus with the highest density of AVP+ axon terminals in the CA2 region (Zhang and Hernandez 2013). Similar findings were adduced in CA2 of mouse (Cui et al. 2013). AVP+ fibres in the rat were shown to reach the hippocampus through three main pathways and to originate primarily from the magnocellular division of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. The existence of two types of

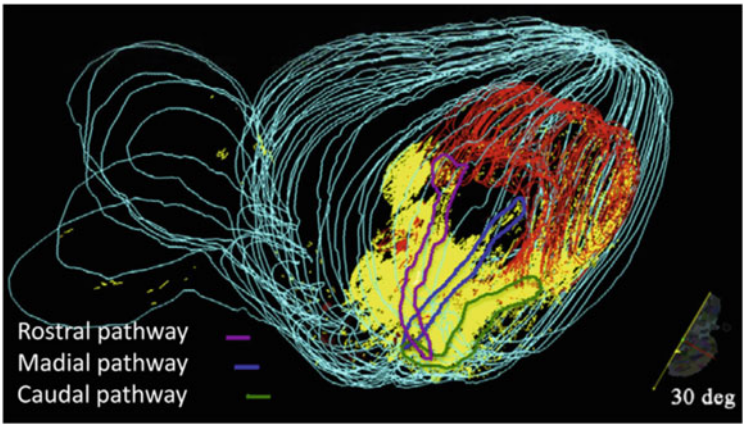

Fig. 7.8 Computerized 3D "one-to-one" mapping of AVP-ir fibres in sagittal sections (hypothalamic PVN and SON as the medial border, extending to stratum oriens of ventral CA1 as the lateral border). Three pathways are delineated as follows: the rostral pathway (purple outline, hypothalamo-septo-fimbria-dorsal hippocampus pathway); the medial pathway (blue outline, hypothalamo-internal capsule-fimbria pathway); and the caudal pathway (green outline, hypothalamo-amygdala-ventral hippocampus pathway). Bright red lines delineate the hippocampus; yellow lines denote the AVP+ fibres reproduced "one-to-one" under microscope using Neurolucida workstation and software for digitalization and Neurolucida Explorer for visualization. The turquoise lines are the outlines of the sagittal sections (modified from Zhang and Hernandez (2013) with permission)

AVP+ axons and terminals was demonstrated by pre-embedding immunoelectron microscopy. One type was characterized by large varicosities, enrichment in dense-core vesicles and type I synapses. The second type of bouton was smaller, containing mainly small clear vesicles and making type II symmetric synapses onto interneuronal dendrites. Oriens-Lacunosum Moleculare (O-LM) interneurons were postulated as one of the primary targets (Zhang and Hernandez 2011, 2013).

Expression of the vasopressin 1b receptor (Avpr1b) in the anterior pituitary, and its function in corticotrop regulation, was discovered by Ferenc Antoni (Antoni 1984). At this site, AVP synergizes with corticotropin-releasing factor to stimulate production and release of adrenocorticotropin hormone (Antoni et al. 1984; Antoni 1993). The Young lab sought to explore possible Avpr1b function in the brain. Wersinger et al. created a total knockout (KO) of the Avpr1b in mice and uncovered a phenotype including reduced social memory and social aggression (Wersinger et al. 2002, 2007). Over the course of several studies, they demonstrated that these behavioural deficits did not result from impairments in spatial memory, sexual behaviour or predatory or defensive behaviours (Wersinger et al. 2004, 2007; Caldwell et al. 2008; DeVito et al. 2009). Young et al. ultimately showed that the Avpr1b is prominently expressed in pyramidal neurons of the dorsal CA2 region of mouse and rat hippocampi, however, at quite low levels (Young et al. 2006). These studies led them to hypothesize that the dorsal CA2 was necessary for proper association of olfactory sensory input with event representation (Young et al. 2006). As mentioned above, it was subsequently shown that AVP+ axons in the CA2 innervating Avpr1b expressing neurons originate in magnocells of the PVN (Cui et al. 2013; Zhang and Hernandez 2013).

Further studies in the Young lab and elsewhere confirmed and supported the original hypothesis of the physiological function of PVN vasopressinergic innervation of CA2 of hippocampus. Lesions (Stevenson et al. 2011; Stevenson and Caldwell 2014) or inactivation (Hitti and Siegelbaum 2014) of neurons within the dorsal CA2 led to decreased social memory. Pagani et al. were able to restore (rescue) social aggression in Avpr1b KO mice by focal viral expression of the receptor in the dorsal CA2 (Pagani et al. 2015). They also showed, in collaboration with the Dudek's lab, that vasopressin enables significant potentiation of excitatory synaptic responses via Avpr1b activation in CA2, but not in CA1, or in hippocampal slices from Avpr1b KO mice (Pagani et al. 2015). A final piece of evidence for the role of AVP innervation of the CA2 in social memory was provided by optogenetic activation to excite vasopressinergic fibres arriving in dorsal CA2 from the PVN. Stimulation of those fibres robustly enhanced social memory, making it more stable and less prone to degradation by a competing social stimulus (Smith et al. 2016), and perhaps enabling the establishment of social structures with multiple individuals.

There is still much to examine, of course, with regard to activation of dorsal CA2 pyramidal neurons and its modulation by AVP. The neuroendocrine role of the PVN (and SON) in stress response seems straightforward, when considering AVP secretion into portal and general circulation. Concomitant release of AVP in the hippocampus is a parallel AVP-mediated stress response with somewhat more complex downstream physiological effects. Stimulating pyramidal neurons of the dorsal CA2

leads to enhanced social memory and aggression, enabling the individual to encode the repeated appearance of another individual in order to launch, or inhibit, a behavioural course of action.

7.6 AVP-Magnocell Projection to Amygdala and Fear-Related Behaviour

The amygdala is a complex region consisting of several nuclei subserving important roles in the integration of fear and anxiety responses (Davis and Whalen 2001; LeDoux 2007). In particular, the central nucleus of the amygdala, which receives dense inputs from diencephalic and cortical regions, is the major output region of the amygdala (LeDoux 2007). The central amygdala is mainly GABAergic and has been shown to have a critical role in the physiological and behavioural responses to fearful and stressful stimuli (Penzo et al. 2015). Several studies have described AVP innervation of the amygdala (Buijs 1978, 1980; Caffe and van Leeuwen 1983; Rood and De Vries 2011; Hernandez-Perez et al. 2018). Figure 7.9 shows an anatomical inventory of the AVP immunoreactive fibre distribution in amygdala. In rats subjected to early-life stress (maternal separation), there is an increase in the AVP fibre density (Fig. 7.9) (Hernandez et al. 2016a, 2016b). Thick and thin fibres are seen in the central amygdala (Fig. 7.10c) that on morphological grounds are likely to emanate from separate sources. Figure 7.10f shows an example of a thick axon terminal making an asymmetric synapse (postsynaptic density is labelled by arrowheads) onto a dendrite in CeA. The hypothalamus is one source of some of those fibres (Hernandez et al. 2016a, 2016b). Figures 7.10 a and b show thick axons that emanate from hypothalamus and enter the amygdalo–hippocampal cortex (Hernandez et al. 2015). The hypothalamic origin of AVP fibres in hypothalamus has been confirmed by juxtacellullar labelling of hypothalamic neurons in PVN and identification of axonal labelled processes in the amygdala (Hernandez et al. 2015) and by identification of labelled neurons in PVN and SON after the injection into central amygdala of the retrograde tracer Fluorogold (Fig. 7.10e) (Hernandez et al. 2016a, 2016b). The behavioural role of this innervation of the CeA by magnocellular vasopressinergic fibres of hypothalamic origin has been investigated. For instance, the maternally separated rats, which have an increased density of AVP fibres in amygdala (Hernandez et al. 2016a, 2016b), display increased anxiety behaviour in the elevated plus maze (EPM) test after water deprivation (Fig. 7.10). Interestingly, the Avpr1a receptor (expressed mainly in GABAergic neurons in CeA) (Fig. 7.10h) has been shown to participate in this AVP-mediated behaviour, since the infusion AVP in CeA increased anxiety while the infusion of a pharmacological antagonist of the Avpr1 reversed the anxiogenic effects of AVP. The results mentioned above suggest that the hypothalamic–amygdalar pathway is plastic in development and can shape the responses of the adult animal in a state-dependent manner, probably shaping a more cautious phenotype in the animals that were subjected to a stressful situation in early life.

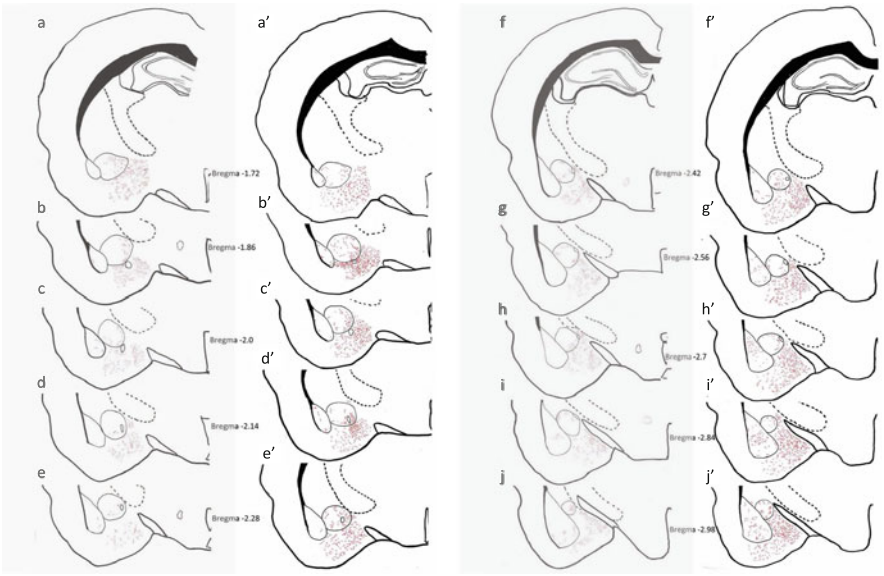

Fig. 7.9 Anatomical charting of AVPir + fibre distribution in amygdala in both control (**a**–**j**) and maternal separation (MS) male adult rat (MS, **a**'–**j**'). Chartings of coronal sections at 10 rostro-caudal levels with line drawings referenced with microscopic observation, representing AVP fibre distribution through the entire amygdaloidal complex. Note the remarkable increase in AVP innervation densities in all regions of the amygdala as a function of MS (Adapted from Hernandez et al. (2016a, b), with copyright held by authors)

7.7 AVP-Magnocell Projections to Lateral Habenula: Interplay with Sex Steroids, Amines and Motivated Behaviour

As mentioned earlier, AVP terminals are found in lateral habenula, a nucleus critical for the processing of aversive stimuli in mammals including mice, rats and monkeys (Hikosaka et al. 2008). In 2015, AVP-magnocell projections to the lateral habenula were noted by Hernandez et al. (2015). The notion that these neurons might be involved in the regulation of behaviour in a manner integrating responsiveness to homeostatic drives such as thirst and other survival priorities conditioned by threat, reproductive opportunity etc. was examined in subsequent experiments. This working hypothesis was reinforced by simultaneous ongoing work connecting thirst and behavioural motivation through CNS projections of AVP-magnocells in other brain regions including amygdala (see Sect. 7.6). Indeed, evidence was accrued that thirst is associated with modulation (suppression) of neuronal output from the lateral habenula, reported as altered neuronal activation in the form of Fos expression, and that active stress-coping behaviours are simultaneously altered in a manner consistent with direct modulation of lateral habenular output via the VP projections to it (Fig. 7.11) (Zhang et al. 2016). Follow-on investigations from these experiments

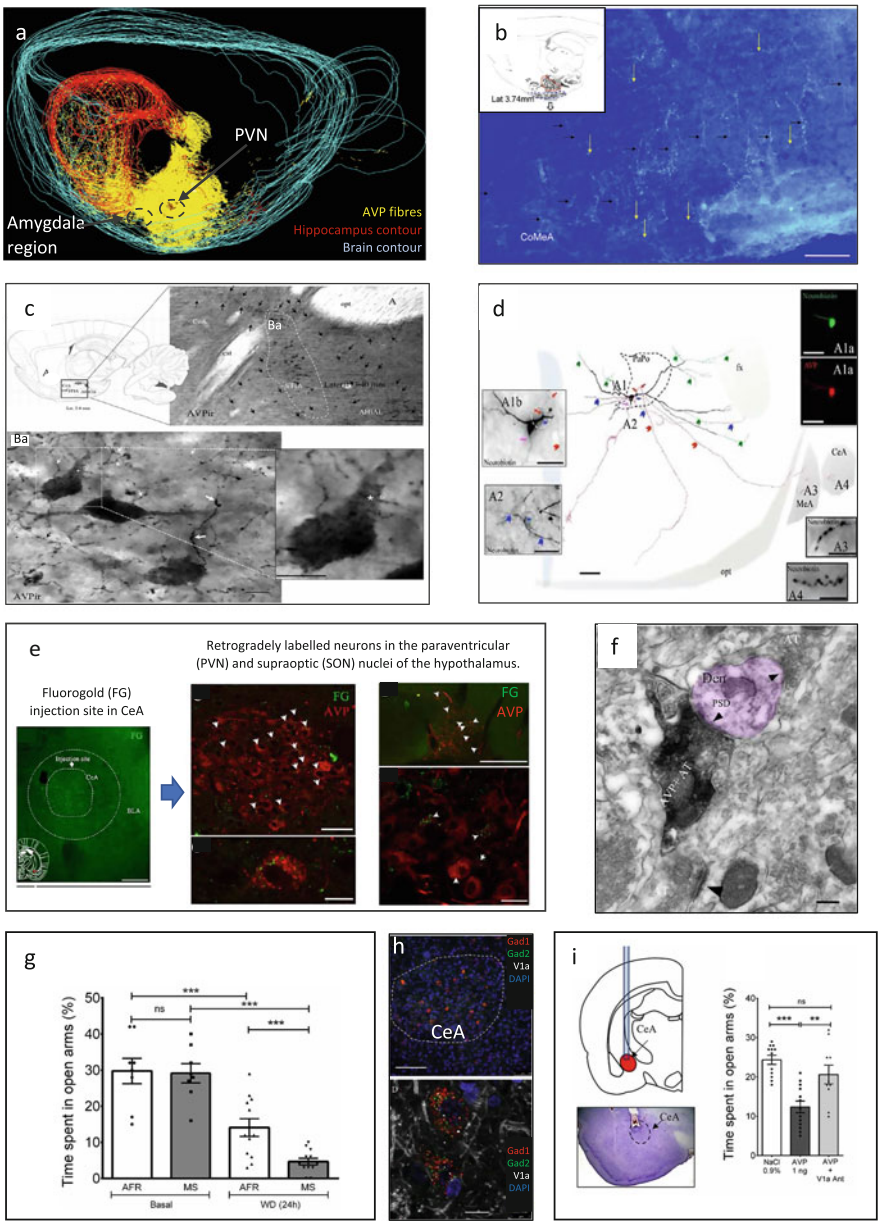

Fig. 7.10 Hypothalamic vasopressinergic magnocellular neurons innervate central amygdala. (**a**) Tracing of the AVP-immunopositive fibres (yellow traces) by *Neurolucida* showing a ventral pathway by which axons from the hypothalamic PVN and SON reach the hippocampus, during their trajectory through amygdala, some fibres were observed to bend orthogonally (black arrows in panel **b**). Panel **c** shows immunohistochemistry of vasopressin in the region of the central amygdala (CeA). Notice in the magnified region the presence of AVP axons of different calibres. Thick arrows indicate large-diameter axons, while thin arrows indicate small-diameter axons, some of which were observed to emerge from local neurons (asterisk). Panel d shows a neuron juxtacellularly (anterogradely) labelled in the posterior division of the PVN, the vasopressinergic phenotype was

revealed a confluence of vasopressinergic and other inputs onto a novel cell type in the medial lateral habenula, the GERN (GABA and oestrogen receptor-expressing neurons). Water deprivation was again used as both a tool to enhance AVP production in AVP-magnocells, and to provide a stimulus to behavioural modification relevant to the dual homeostatic and allostatic roles of magnocell projections to posterior pituitary, and to the extrahypothalamic brain, respectively. GERN responds to gonadal steroid status in male mice because testosterone conversion to oestrogen occurs in AVP-magnocell input to these ER-bearing cells (Zhang et al. 2018). Remarkably, castration of male mice results in a virtually complete loss of AVP immunoreaction of LHbC, where GERNs are located, and upon hormonal replacement therapy (HRT), restoration of AVP-immunopositive fibres was observed. Coincident with this reversible loss and restoration of AVP of LHbC, gonadectomy increases freezing (immobility) in response to predator (cat) presentation, as well as immobility in the forced swim test, and the latter is reversed upon HRT. The reversibility of the loss of vasopressinergic immunoreactivity of LHbC upon castration, then HRT, strongly implies a level of hormone-dependent neuronal plasticity within the AVP-magnocell projection system that is truly remarkable and worthy of further investigation. It will be of interest first to know how this dramatic regulation occurs, whether via vesicular transport control, vasopressin biosynthesis, or both; and second, whether this level of control of neurotransmission is unique to AVP-magnocells or occurs in other regulatory peptide-containing projections within the CNS. The influence of AVP-magnocell projections on GERN function provides insight into the palimpsest of the endocrine and neuroendocrine upon the neuronal, in the translation of homeostatic drives to motor outputs required to seek water and

Fig. 7.10 (continued) assessed by immunohistochemistry (green and red insets show the co-localization of AVP-immunoreactivity and neurobiotin label). In this same **d** panels, micrographs A1 and A2 show the emergence of axon-like processes and in A3 and A4 some neurobiotin labelled processes that were found in medial (MeA) and central amygdala are shown. Panel E shows retrogradely labelled neurons in the paraventricular (PVN) and supraoptic (SON) nuclei after the injection of fluorogold (FG) in the CeA. Panel **f** shows an AVP+ axon terminal (AVP+ AT) making an asymmetric synaptic contact with a dendrite (Den) in the CeA; notice the postsynaptic density (PSD) which is a characteristic of excitatory synapses. Panel **g** shows the anxiety-like behaviours evaluated by the elevated plus maze, comparing control (AFR: animal facility reared) and maternally separated (MS: maternal separation 3 h daily during the first two postnatal weeks) rats. Under basal conditions, there were no differences in behaviour, while after 24 h of water deprivation, MS rats, which were previously shown to develop a potentiated hypothalamic vasopressinergic system (Zhang et al. 2012) and increased density of AVP+ fibres in amygdala (Hernandez et al. 2016a, b) showed diminished time spent in the open arms of the maze, indicating increased anxiety-like behaviour. Panel **h**: in situ hybridization using RNAscope multiplex technique shows that CeA neurons express mRNAs coding the glutamate decarboxylase 1 and 2 (Gad1 and Gad2, key enzymes for GABA synthesis) co-express the Avpr1a, a receptor for vasopressin. Panel I shows that the direct infusion of AVP in the CeA decreases the time spent in the open arms of the elevated plus maze (EPM), and the coadministration of AVP and Manning compound (an Avpr1a receptor antagonist) inhibits the anxiogenic effect of AVP infusion in CeA. Panels **a** and **b** are reproduced with permission from Zhang and Hernandez (2013), panel **c** from Hernandez et al. (2016a, b), panels **d**–**i** from Hernandez et al. (2015)

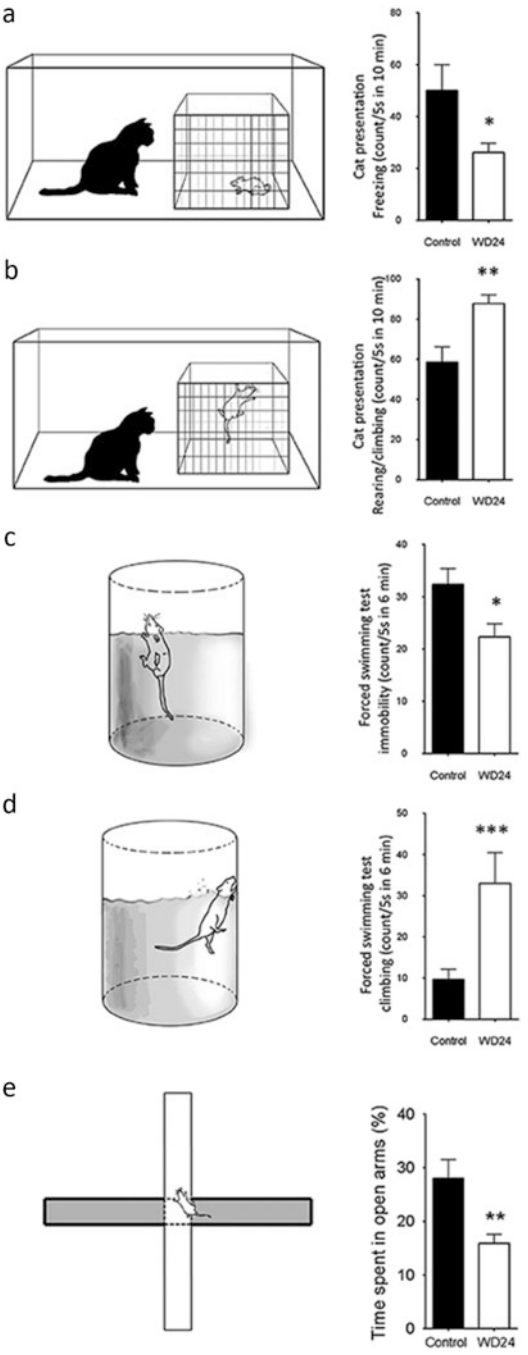

Fig. 7.11 Twenty-four hours of water deprivation (WD24) promoted active stress coping during innate fear processing (cat exposure) and behavioural despair (forced swimming test, FST). WD24 is a potent physiological stimulus to increase metabolic activities of AVP-containing magnocellular neurosecretory neurons in SON and PVN. Upon cat exposure, rats expressed innate fear-related passive (freezing), and active (rearing, climbing and displacement) behaviours (**a**, **b**). Rats from WD24 group showed a significant reduction of freezing counts (**a**) and increased climbing and rearing behaviours (**b**). Similar observations were obtained during FST for behavioural despair (**c**, **d**). For locomotor control, we performed the elevated plus maze (EPM) test on both groups (**e**). The WD24 rats showed normal locomotion patterns but a reduced percentage of time spent in open arms (Mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). ((Zhang et al. 2016), with copyright held by authors)

food, and the restriction or delay of these drives based on environmental contingencies relevant to survival (allostatic regulation).

7.8 AVP-Magnocell Projections Synaptically Innervate the Locus Coeruleus and 24-h Water Deprivation Lead to Enhancement of Memory and Spatial Learning

Two recent studies (Campos-Lira et al. 2018; Hernandez-Perez et al. 2019) provided the first evidence that AVP-magnocells project to the pontine locus coeruleus (LC)-norepinephrine (NE) system, establishing Gray type I asymmetric synapses onto tyrosine hydroxylase immunopositive dendrites, with AVP+ large dense-core vesicles docked onto presynaptic membrane. Upregulation of AVP-magnocells by 24 h deprivation modulates a range of salient brain functions, including memory, spatial learning and response to stress. Water deprivation enhances performance in the Morris water maze (MWM) concomitantly with enhanced activation of LC neurons during the conduct of the MWM test, while increased Fos expression was found in LC and some of its efferent regions such as the hippocampus and prefrontal cortex, suggesting that AVP-magnocell projections to LC could integrate homeostatic responses modifying neuroplasticity (Hernandez-Perez et al. 2019).

7.9 AVP-Magnocells Are Vulnerable to Early-Life Stress

Instinctual behaviours, such as water and food intake, fight–flight stress response and sexual behaviour, are determinants of survival, both at individual and species levels. These essential behaviours are directly controlled by hypothalamic homeostatic circuits, in which neuropeptides and neurosteroids are critically involved. Evolutionary conservation of the hypothalamus attests to its critical role in the control of fundamental behaviours (Elmquist et al. 2005; Swanson 2012; Saper and Lowell 2014). Several recent studies have found that the hypothalamus is particularly susceptible to early stress, induced either by endocrine imbalances or by psychological stressors such as neonatal maternal separation (MS). As previously reported, in response to either maternal hyperthyroidism (Zhang et al. 2008, 2010) or neonatal maternal separation (MS) (Hernandez et al. 2012; Zhang et al. 2012), the rat hypothalamic vasopressin system becomes permanently upregulated, showing enlarged volume of the hypothalamic paraventricular and supraoptic vasopressin nuclei and increased cell number, with an increased sensitivity to acute stressors or anxiogenic conditions in adulthood. Another recent study (Irles et al. 2014) showed that the life-long consequence of neonatal maternal separation may be imprinted in changes in cell density in several hypothalamic regions, through the modification of the activities of pro- and anti-apoptotic factors during development. Moreover, the AVP innervation to amygdala, a brain limbic region, which exerts regulatory functions on food intake, sexual behaviours, aggression and fear processing, is remarkably increased in neonatal maternal separated rats (Hernandez et al. 2016a,

2016b). These observations clearly demonstrate that the stress of maternal separation in early life has a reorganizing effect on this subcortical structure (Fig. 7.9).

The AVP-magnocells have been shown to undergo plastic changes in response to various stimuli, including dehydration, ageing and sodium depletion. State-of-the-art transcriptomic and proteomic methods have been used by Murphy and colleagues, among others, to evaluate the changes in the PVN and SON nuclei under such challenged states and some interesting results have emerged, showing the complexity and finesse of the regulation of these neurons. For instance, the depletion of sodium by means of furosemide induces a decrease in the activity of the paraventricular and supraoptic nuclei. Potential genes regulating such changes were investigated using RNA sequencing (Dutra et al. 2021). Interestingly, sodium depletion induced a decrease in the expression of the Caprin2 and Opn3 genes in both SON and PVN, while dehydration increased expression of these same two genes in the PVN and SON (Loh et al. 2017). The genes upregulated by sodium depletion were very different between both hypothalamic vasopressinergic nuclei, suggesting a differential role of both nuclei in the integration of the response to homeostatic perturbations. Ageing is also a challenge that impacts the functioning of the vasopressinergic system, elderly people being more susceptible to hydro-electrolytic alterations and having a diminished capacity to cope with dehydration. Comparing vasopressinergic transcriptomic and peptidergic changes in response to dehydration of adult and aged rats, there were no differences in the basal or dehydrated levels of circulating AVP, however, under basal and dehydrated conditions the aged rats had an increased transcription of the AVP gene in the SON associated with decreased methylation of the gene. Moreover, the dehydration-induced increase in some previously identified regulatory factors involved in the response of the SON to hyperosmotic challenges was blunted in aged rats (Greenwood et al. 2018). This last example indicates that in aged individuals, the SON and PVN vasopressinergic neurons have a diminished response capacity upon homeostatic challenges at the genome, transcriptome and peptidome levels. However, the behavioural consequences of these plastic changes in gene expression remain to be elucidated. It should also be noted that the bulk SON sequencing carried out includes transcriptome information from every cell type in this nucleus, not only the oxytocinergic and vasopressinergic magnocells, but also the surrounding glia, microglia, some interneurons, and vessels and the blood therein. We await with great interest the inevitable single cell RNAseq analysis of the euhydrated and challenged SON, which will be highly informative regarding the transcriptomic responses of these different cell types. Further, it is to be expected that the magnocells themselves will exhibit an intrinsic diversity with respect to basal gene expression patterns and responses to physiological cues that will have important functional implications.

7.10 Conclusion

Vasopressinergic systems of the brain are among the most consequential circuits governing brain-mediated physiological homeostatic responses and behaviours. They are also high-value targets for translational/therapeutic intervention in human CNS diseases, including anxiety-related, depressive, endocrine and addictive disorders. Vasopressin is secreted from the brain to affect kidney and cardiovascular function. Vasopressin is also secreted in the brain, where it acts as a neurotransmitter. Some the co-authors of this chapter were among the first to show that the very same vasopressin neurons that release vasopressin *from* the brain also release vasopressin, via a separate branching axonal projection system, *within* the brain. This links the activities of vasopressin as a hormone, to its activity in modulating behaviours associated with conditions of thirst and salt imbalance. This finding allowed us to manipulate vasopressin levels physiologically (e.g. by water deprivation) and then to show how thirst affects rodent responses to threat, stress and other challenges. In addition, our laboratories pioneered the discovery that stress during early life affects the vasopressin system and alters the ability to respond to stress during adulthood, again by the integration of the hormonal and neurotransmitter properties of vasopressin. This allowed a landmark contribution to the regulatory peptide literature: that gonadal/sex hormone status profoundly affects behaviour associated with aversive stimulation at the level of the epithalamus (lateral habenula) by modulation of the input from multiple neuropeptides as well as biogenic amine inputs as a function of systemic testosterone/local oestrogen levels.

Overall, two critical questions remain, pointing the way towards future research on vasopressinergic magnocellular neurons. The first is understanding how vasopressin actually acts at the post-synapse in hippocampus, in habenula, in amygdala and in locus coeruleus, four principal target areas of vasopressinergic innervation of the extrahypothalamic brain. The second is exploring whether or not simultaneous vasopressinergic innervation of these highly disparate brain regions results in brain *synchrony* required for full physiological response, homeostatic and allostatic, to environmental perturbogens such as salt imbalance, gonadal hormone fluctuation and contingent inputs from systems such as the orexigenic hypothalamus, and pain and arousal projections of brain stem. As it has allowed pioneering insights into the roles of peptidergic dual projections from and within the brain, the VP magnocellular system is likely to be paradigmatic in revealing the answers to these two questions as well.

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Buijs, R. M. (1978). "Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord." Cell Tissue Res 192(3): 423–435. *Seminal paper in which, by means of newly developed immunohistochemistry against AVP and careful observation, the author traces the pathways of axons innervating extra-neurohypophysial regions of the rat brain.*

Cui, Z., C. R. Gerfen and W. S. Young, 3rd (2013). "Hypothalamic and other connections with dorsal CA2 area of the mouse hippocampus." J Comp Neurol 521(8): 1844–1866. *The authors made use of genetics and classical tracing studies to show that the hypothalamic vasopressinergic neurons innervate the CA2 region of the hippocampus, a region previously unidentified to receive innervation from the hippocampus. Simultaneously with this publication, the Zhang group published a detailed immunohistochemical study (see below) showing the synaptic innervation of diverse hippocampus regions by AVP fibres originating in AVP-magnocells.*

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with axons containing AVP/glutamate and aromatase (enzyme that converts androgens to oestrogens).

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Neuroanatomy of the GnRH/Kisspeptin System

8

Daniel J. Spergel

Abstract

This chapter summarizes the current body of knowledge regarding the neuroanatomy of the gonadotropin-releasing hormone (GnRH)/kisspeptin system controlling fertility. It focuses on contributions made using recent techniques, including cell-type-specific, promoter-driven labeling with green fluorescent protein (GFP) and other fluorescent biomarkers, tissue clearing, expansion microscopy, optogenetics, and viral tracing, to the anatomical characterization of hypothalamic GnRH and kisspeptin neurons as well as to the identification of their synaptic and non-synaptic inputs and outputs in transgenic mice and rats. Among the major findings are that GnRH neurons possess structures, termed "dendrons," exhibiting properties of both dendrites and axons, that inputs to the GnRH neuron soma-proximal dendritic zone and to GnRH neuron distal dendrons from kisspeptin neurons differentially control pulsatile and surge GnRH secretion, and that GnRH and kisspeptin neurons receive inputs from neurons in multiple hypothalamic and extra-hypothalamic areas that convey endocrine, metabolic, and environmental (including circadian, pheromonal, and social behavior-related) signals known to impact fertility.

Keywords

GnRH neurons · Kisspeptin neurons · Promoter-driven labeling · Optogenetics · Viral tracing · Neural circuits · Fertility

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8.1 Introduction

Pulsatile secretion in both sexes, and surge secretion in females at proestrous, of gonadotropin-releasing hormone (GnRH, a.k.a. GnRH1 or luteinizing hormone-releasing hormone [LHRH]) from GnRH neurons, resulting from the release of kisspeptin (a.k.a., Kiss1 or metastin) from kisspeptin neurons onto GnRH neurons, are essential for reproduction in mammals (reviewed by Herbison 2016, 2018; Matsuda et al. 2019; Spergel 2019a, b). Immunocytochemical, retrograde labeling, and secretion studies showed that, in adult rodents, GnRH neurons, which are depicted schematically in Fig. 8.1, number around 800, have somata that are 10–25 μm in diameter, are oval, bipolar, pyriform, or spindle-like in shape, and are located mainly in the preoptic area (POA) of the hypothalamus, as well as in the medial septal nucleus (MS) and diagonal band of Broca (DBB). GnRH neurons project to the median eminence (ME), where they release GnRH into the hypothalamo–hypophyseal portal circulation, and to the organum vasculosum of the lamina terminalis (OVLT), which may play a role in the control of the proestrous GnRH/luteinizing hormone (LH) surge (Naik 1975; Sarkar et al. 1976; Wenger et al. 1979; Bennett-Clarke and Joseph 1982; Kelly et al. 1982; King et al. 1982; Piva et al. 1982; Sheward et al. 1985; Wray and Hoffman 1986; Schwanzel-Fukuda et al. 1987; Merchenthaler et al. 1989; Wu et al. 1997; Glanowska et al. 2012). From the hypothalamo–hypophyseal portal circulation, GnRH binds to its receptors on gonadotrophs in the anterior pituitary, stimulating the synthesis and secretion into the general circulation of LH and follicle-stimulating hormone (FSH), which bind to their receptors in the ovaries and testes. LH and FSH are required for gonadal development and maintenance and for gametogenesis in both sexes; for synthesis and secretion of estradiol (E, a.k.a. E_2) and progesterone (P) as well as for ovulation in females; and for synthesis and secretion of testosterone (T) in males (reviewed by Kaprara and Huhtaniemi 2018). Like GnRH neurons, kisspeptin neurons (in adult rodents), which are also depicted schematically in Fig. 8.1, have somata that are ~10–25 μm in diameter and are oval, bipolar, pyriform, or spindle-like in shape, but they are located mainly in the arcuate nucleus (ARC; a.k.a. ARH or ARN) and rostral periventricular area of the third ventricle (RP3V, where females have a more than ten-fold higher number of kisspeptin neurons than males), which includes the anteroventral periventricular nucleus (AVPV) and the preoptic periventricular nucleus (PeN; a.k.a. periventricular nucleus of the hypothalamus [PeV] or periventricular nucleus of the hypothalamus preoptic part [PVpo]), as well as in the dorsomedial hypothalamic nucleus (DMH), medial amygdala (MeA), and other brain areas. RP3V and MeA kisspeptin neurons project to GnRH neuron dendrites and somata in the POA, while ARC and RP3V kisspeptin neurons, which are interconnected, as shown in Fig. 8.10, project to GnRH neuron dendrons (see below) at or near the border of the ME, and to GnRH neuron axon terminals within the ME (Brailoiu et al. 2005; Clarkson and Herbison 2006; Clarkson et al. 2009; Uenoyama et al. 2011; Yip et al. 2015, 2021; Pineda et al. 2017). GnRH neurons also project to kisspeptin neurons in the ARC and RP3V, indicating reciprocal connectivity between GnRH neurons and kisspeptin neurons (Kalló et al. 2013; Yip et al.

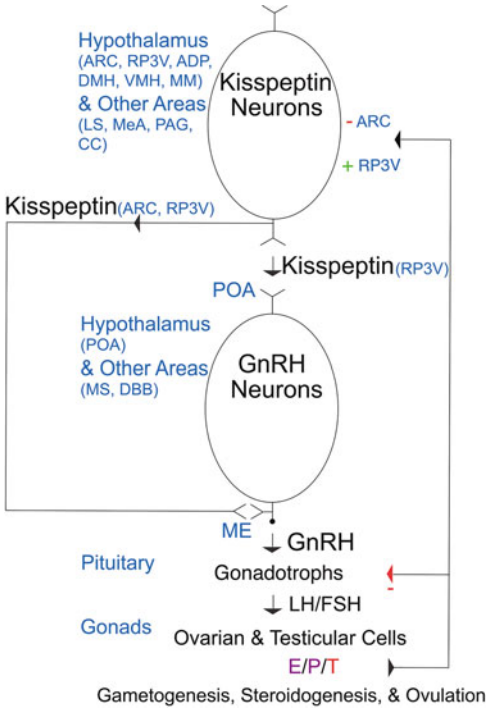

Fig. 8.1 Schematic diagram of the GnRH/kisspeptin system in mice. GnRH and kisspeptin neurons are located in the hypothalamus and other brain areas as indicated. Kisspeptin released from ARC kisspeptin neurons onto GnRH neuron axon terminals in the ME results in pulsatile GnRH secretion into the hypothalamo–hypophyseal circulation in both sexes. In females, kisspeptin released from kisspeptin neurons in the RP3V onto GnRH neuron somata in the POA and axon terminals in the ME results in surge GnRH secretion from GnRH neuron axon terminals in the ME into the hypothalamo–hypophyseal circulation. Following both pulsatile and surge GnRH secretion, GnRH binds to GnRH receptors on pituitary gonadotrophs to stimulate the synthesis and secretion of LH and FSH into the general circulation. LH and FSH, which are required for gametogenesis, steroidogenesis, and ovulation, then bind to receptors in the gonads to stimulate the synthesis and secretion of E, P, and T, which in turn exert negative (E, P, and T) or positive (E and P) feedback effects on GnRH neurons (via kisspeptin neurons) and gonadotrophs depending on the sex and estrous cycle stage of the animal. For simplicity, connections among and between ARC and RP3V kisspeptin neurons (see Fig. 8.10), which may be required for pulsatile and/or coordinated kisspeptin release, as well as those from MeA kisspeptin neurons to GnRH neurons, which may mediate olfactory control of the gonadotropic axis, have been omitted in this figure. Abbreviations are explained at their first occurrence in the main text. Modified from Spergel (2019b), with permission

2015). In addition, GnRH and kisspeptin neurons project to other neurons having various reproduction-related functions in these and other brain areas (Boehm et al. 2005; Yoon et al. 2005; Clarkson et al. 2009; Sotonyi et al. 2010; Wen et al. 2011; Yeo and Herbison 2011; Hellier et al. 2018).

This chapter discusses how cell-type-specific, promoter-driven labeling with green fluorescent protein (GFP) and other fluorescent proteins has helped to identify GnRH and kisspeptin neurons in live tissue and to characterize their anatomy and physiology, as well as how viral tracing, tissue clearing, expansion microscopy, and optogenetics have been used to reveal the projections and connections of GnRH and kisspeptin neurons. The use of these techniques has added to information obtained from earlier immunocytochemical studies that employed GnRH or GFP antibodies in combination with antibodies against various neurotransmitters/neuropeptides and the presynaptic vesicle marker synaptophysin (Rajendren and Li 2001; Rajendren 2002; Campbell et al. 2003; Yoon et al. 2005; Wintermantel et al. 2006; Campbell and Herbison 2007). This chapter briefly describes these techniques, discusses recent improvements that address some of their pitfalls, and suggests how these techniques may lead to future progress in reproductive neuroendocrinology research.

8.2 GnRH Promoter-Driven GFP Labeling to Identify and Anatomically Characterize GnRH Neurons

In addition to allowing the identification of GnRH neurons in live brain tissue for physiological studies (e.g., patch-clamp electrophysiological recording of GnRH neuron activity in brain slices), GnRH promoter-driven GFP (or other fluorescent protein, e.g., mCherry) labeling of GnRH neurons in GnRH-GFP transgenic, as well as in GnRH-Cre transgenic and knock-in, mice and rats (Spergel et al. 1999; Suter et al. 2000; Kato et al. 2003; Yoon et al. 2005; Wolfe et al. 2008; Raftogianni et al. 2018; Yip et al. 2021) has been used to further characterize the anatomy of GnRH neurons and to reveal their inputs and outputs. In this approach, either a GnRH promoter fragment, a bacterial artificial chromosome (BAC) containing the GnRH promoter and additional DNA elements, or a knock-in, in which a sequence encoding an internal ribosome entry site (IRES) and Cre recombinase has been incorporated into the endogenous GnRH coding locus, drives the expression of GFP (Fig. 8.2 and Box 8.1), or of Cre recombinase, which can mediate GFP (or mCherry) expression in GnRH neurons in animals that are doubly transgenic for GnRH promoter-driven Cre and Cre-dependent GFP (or mCherry). Immunocytochemical staining of GFP-expressing cells with a GFP antibody and a GnRH antibody considered the "gold standard" for identifying GnRH neurons confirmed high fidelity GFP expression in GnRH neurons, i.e., a high percentage of GFP-immunopositive cells that are GnRH-immunopositive and a high percentage of GnRH neurons that are GFP-immunopositive, in the POA, MS, and DBB in those animals, with the BAC transgenic and the Cre knock-in lines exhibiting the highest fidelity of expression (Spergel et al. 1999; Suter et al. 2000; Kato et al. 2003; Yoon et al. 2005; Wolfe et al. 2008; Raftogianni et al. 2018; Yip et al. 2021). Hence, the GFP-expressing cells were shown to be GnRH neurons, and GFP could be used to reliably label GnRH neurons in the POA, MS, and DBB for anatomical studies.

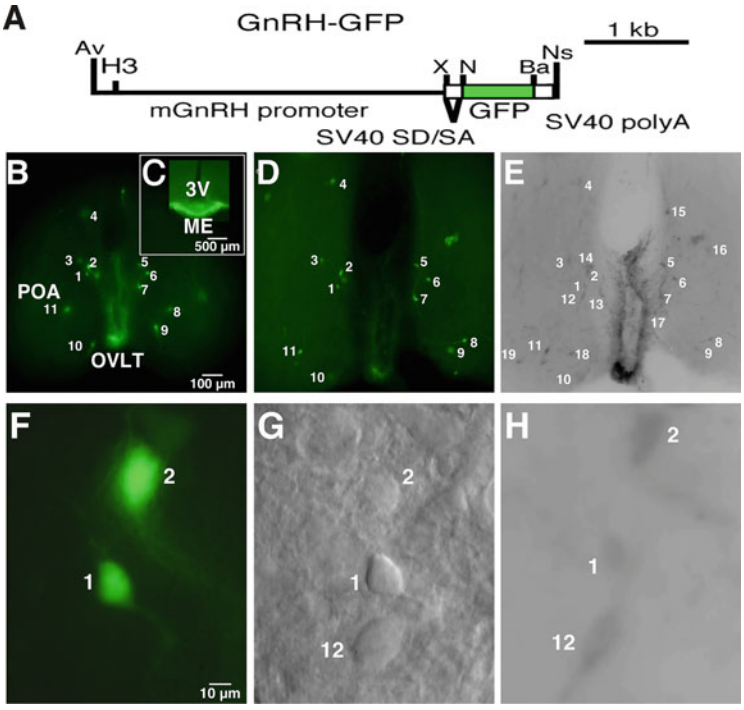

Fig. 8.2 GnRH promoter-driven GFP labeling of GnRH neurons. GFP reporter gene and GFP-expressing neurons in 300 µm-thick brain slices from GnRH-GFP transgenic mice. (**a**) GFP reporter gene expressed under the control of a 3.5 kb fragment of the mouse GnRH (mGnRH) promoter used to generate GnRH-GFP transgenic mice. Restriction sites for cloning (Av, AvrII; H3, HindIII; X, XhoI; N, NotI; Ba, BamHI; Ns, NsiI) and regulatory elements of the minigene [SV40 SD/SA, SV40 splice donor/splice acceptor intron; SV40 polyA, SV40 polyadenylation signal] are indicated. (**b**) GFP-expressing neurons, numbered 1–11, in the POA (at the level of the OVLT) of a live coronal slice from a postnatal day 25 (P25) male GnRH-GFP mouse. (**c**) GFP-expressing axon terminals in the ME from the same mouse as in **b**. (**d**) Same slice as in **b** after fixation, which produced additional fluorescence, and after mounting, which flattened the tissue and thereby changed the relative positions of the neurons. (**e**) Same slice as in **b** and **d** after immunostaining for GnRH. GnRH-immunopositive neurons are numbered 1–19. The gray levels in this panel and in **h** (below) have been inverted to aid the reader in visualizing the GnRH immunostained neurons, which appear as dark spots. Note that all fluorescent neurons in **b** and **d** are GnRH-immunopositive in **e** and that the number of GnRH-immunopositive neurons is larger than the number of fluorescent neurons. Scales in **d** and **e** are the same as in **b**. (**f**) High-magnification fluorescence image of GFP-expressing neurons 1 and 2 in **b**, **d**, and **e**. (**g**) Infrared differential interference contrast (IR-DIC) image of neurons 1, 2, and 12 in **b**, **d,** and **e**. (**h**) High-magnification fluorescence image of neurons 1, 2, and 12 after GnRH immunostaining. Neurons 1 and 2 fluoresce and contain GnRH, whereas neuron 12 contains GnRH but does not fluoresce green in **b**, **d**, and **f**. Scales in **g** and **h** are the same as in **f**. Abbreviations are explained at their first occurrence in the main text except for those in **a**. Modified from Spergel et al. (1999), Copyright [1999] Society for Neuroscience, with permission

Box 8.1 Cell-Type-Specific, Promoter-Driven Labeling with GFP or Other Fluorescent Proteins

Cell-type-specific, promoter-driven labeling with green fluorescent protein (GFP) or other fluorescent proteins involves the creation of a synthetic gene (a transgene) containing the promoter elements of a gene required to encode a peptide, such as GnRH or kisspeptin, that is expressed in a particular cell population, fused to a gene encoding a fluorescent protein or a recombinase (such as Cre or Flp recombinase, which can induce the expression of a fluorescent protein via the Cre/*LoxP* or Flp-*FRT* system, respectively). Using traditional transgenesis, the transgene is then microinjected into fertilized zygotes (a.k.a. pronuclei), or using an embryonic stem cell-based approach is inserted into embryonic stem (ES) cells at a specific site in the genome and subsequently injected into blastocyst-stage embryos, resulting in the integration and stable germ line transmission of the exogenous gene and its expression in the targeted cell population (Gordon et al. 1980; Gordon and Ruddle 1981; Gossler et al. 1986). Due to the random nature of exogenous DNA integration, pitfalls of traditional transgenesis may include variation in copy number and chromosome position effects at the site of integration, resulting in variation in transgene expression between different founder lines (Hogan 1983). The ES cell approach overcomes these pitfalls but requires the generation of transgenic embryonic stem cells, involving electroporation/transfection, selection, and screening, plus an additional generation of breeding to ensure germline transmission in the chimeric founders, making the ES cell-based approach laborious and expensive. Recently developed hybrid approaches, including Pronuclear Injection-based Targeted Transgenesis (PITT), improved PITT (i-PITT), and Efficient additions with ssDNA inserts-clustered regularly interspaced short palindromic repeats (Easi-CRISPR) that involve injection of transgenic DNA into pronuclei, like traditional transgenesis, but direct integration to a specific site, like the ES cell-based approach, may prove to be more advantageous (Ohtsuka et al. 2010; Ohtsuka et al. 2015; Miura et al. 2018).

Confocal microscopy of biocytin-filled GnRH neurons from adult male and female GnRH-GFP mice, along with electron microscopy using pre-embedded, silver-enhanced immunogold labeling for both GnRH and GFP, revealed that the dendrites of most GnRH neurons are long (in some cases extending >1 mm from the soma), and that GnRH neuron cell bodies and dendrites are covered with spine-like protrusions (having a mean density of 0.4 spines/μm), indicating that GnRH neurons receive excitatory input. GnRH neurons were also shown to form multiple close appositions (membrane specializations including punctae and zonula adherens but not gap junctions) with dendrites of other GnRH neurons as well as with afferent axon terminals (Campbell et al. 2005, 2009), suggesting a mechanism of GnRH neuron synchronization (Campbell et al. 2009). Further examination of spines of

GFP-labeled GnRH neurons in GnRH-GFP mice and rats showed that the number and size of spines on the somata and proximal dendrites of GnRH neurons increases throughout pubertal development, suggesting that excitatory input to GnRH neurons increases at puberty (Cottrell et al. 2006; Li et al. 2016). Also, dual-label immunofluorescence experiments for GFP and the immediate early gene c-Fos in brain sections from female GnRH-GFP mice showed that positive feedback levels of E stimulate a robust increase in somatic and dendritic spine density specifically in those GnRH neurons that are activated at the time of the GnRH/LH surge in pubertal and adult females, which may be one mechanism by which steroids modify GnRH neuron activity to produce the surge (Chan et al. 2011).

8.2.1 GnRH Promoter-Driven Labeling to Determine the Inputs of GnRH Neurons

GFP-labeled GnRH neurons in GnRH-GFP mice were shown to receive inputs from kisspeptin neurons and from GABAergic and glutamatergic neurons, some of which may also be kisspeptin neurons. Epifluorescence microscopy of brain sections of male and female GnRH-GFP mice immunostained for kisspeptin (kisspeptin-10) and GFP demonstrated close appositions between kisspeptin fibers, likely originating from kisspeptin neurons in the RP3V, that reached adult-like levels at the time of puberty onset (Clarkson and Herbison 2006), and GnRH neuron somata in the POA, supporting the idea that kisspeptin (further discussed below) acts as a neuroendocrine switch that provides the increased excitatory input required for the onset of puberty (Han et al. 2005). Confocal microscopy of brain sections of male and female GnRH-GFP mice immunostained for GFP and either vesicular GABA transporter (VGAT) or vesicular glutamate transporter 2 (vGluT2) revealed that GnRH neurons (mostly their proximal dendrites) receive contacts from GABAergic and glutamatergic neurons, respectively, and that in females at the time of the proestrous GnRH/LH surge, when there are positive feedback levels of E, a subset of GnRH neurons that does not get activated receives an increased number of GABAergic contacts (Moore et al. 2018a). This suggests that cyclic fluctuations in steroid hormone feedback over the female estrous cycle result in plastic changes in GABAergic inputs to a subpopulation of GnRH neurons; however, the exact role of the subpopulation with respect to driving changes in GnRH/LH surge secretion requires further investigation (Moore et al. 2018a).

GnRH neurons were also shown to receive inputs from neurons expressing peptide transmitters other than kisspeptin. Light, epifluorescence, confocal, and/or electron microscopy of brain sections of GnRH-GFP mice and/or rats immunostained for GFP and the appetite-regulating peptides neuropeptide Y (NPY), agouti-related peptide (AgRP), β-endorphin, melanin-concentrating hormone (MCH), and galanin-like peptide (GALP) provided anatomical evidence for the roles of these peptide transmitters in the modulation of GnRH/LH secretion and reproduction. Light, epifluorescence, and electron microscopy of brain sections of GnRH-GFP mice immunostained for GFP and the orexigenic peptides neuropeptide

Y (NPY) and agouti-related peptide (AgRP), and the enzyme dopamine-β-hydroxylase (DBH) revealed that NPY and AgRP-expressing fibers from NPY/AgRP neurons in the arcuate nucleus (ARC), as well as NPY and DBH-expressing fibers from the brainstem, make axo-somatic contacts onto GnRH neurons that may mediate the chronic inhibitory effects of NPY/AgRP on both GnRH/LH secretion and reproduction associated with malnutrition, obesity, and diabetes (Turi et al. 2003). Epifluorescence and confocal microscopy of brain sections of GnRH-GFP mice corroborated the finding that NPY/AgRP-expressing fibers contact GnRH somata and processes, and showed that fibers expressing the orexigenic peptide β-endorphin, presumably from pro-opiomelanocortin (POMC) neurons in the ARC, and MCH fibers from MCH neurons in the DMH, lateral hypothalamus, and/or zona incerta (ZI), do so as well (Ward et al. 2009; Wu et al. 2009).However, optogenetic experiments suggest that ARC NPY/AgRP neurons inhibit GnRH neurons indirectly, via kisspeptin neurons, rather than directly (Padilla et al. 2017). Light and electron microscopy of brain sections of male GnRH-GFP rats immunostained for GFP and the appetite-regulating peptide galanin-like peptide (GALP) revealed that GALP-containing nerve terminals of GALP neurons, whose somata are located in the ARC and whose expression of GALP is up-regulated by the satiety-inducing hormone leptin, make axo-somatic and axo-dendritic synaptic contacts onto GnRH neurons (Kumano et al. 2003; Takenoya et al. 2006). Taken together with an earlier finding that intracerebroventricular GALP stimulates cFos expression in GnRH neurons as well as GnRH-mediated LH secretion (Matsumoto et al. 2001), this suggests that GALP neurons provide direct input to GnRH neurons and may increase GnRH/LH secretion, perhaps in response to leptin (Takenoya et al. 2006).

It should be noted that all of the neuropeptide-containing neurons (including kisspeptin neurons) from which GnRH neurons receive input appear to contain GABA or glutamate in their axon terminals as well (Cravo et al. 2011; van den Pol 2012; Moore et al. 2018a, b).

GnRH neurons may also receive input via the bloodstream. Juxtacellular filling of GFP-labeled GnRH neurons with neurobiotin showed that a subpopulation of GnRH neurons located in the rostral POA exhibits extremely complex branching dendritic trees that fill the OVLT, which is beyond the blood-brain barrier, and would allow GnRH neurons to sense molecules circulating in the bloodstream (Herde et al. 2011).

8.2.2 GnRH Promoter-Driven Labeling to Determine the Outputs of GnRH Neurons

GnRH neurons project not only to the ME and OVLT but also to other brain areas, where they may modulate the activities of neurons involved in various reproduction-related functions. Using confocal microscopy of combined GFP-immunostaining of GFP-labeled processes of GnRH neurons and tyrosine hydroxylase (TH)-immunostaining of TH neurons in the ARC of female GnRH-GFP mice, Mitchell et al. (2003) showed that GnRH neurons directly contact tuberoinfundibular

dopaminergic (TIDA) neurons at proestrus and estrus, consistent with the hypothesis that GnRH neurons may inhibit TIDA neurons at proestrus and estrus, which may result in increased prolactin secretion required for lactation (Mitchell et al. 2003). Along these lines, although not involving GnRH promoter-driven labeling of GnRH neurons, it should be noted that Sotonyi et al. (2010) used correlated light and electron microscopy of GnRH immunostaining of GnRH neurons and GFP-immunostaining of ARC POMC neurons of female POMC-GFP mice (with POMC promoter-driven GFP labeling of POMC neurons) to show that GnRH neuronal axon terminals directly contact ARC POMC neuronal somata, providing anatomical evidence for the idea that GnRH neurons may modulate feeding, energy expenditure, and glucose homeostasis. In a similar vein, Wen et al. (2011) generated transgenic mice expressing yellow fluorescent protein (YFP) or GFP in GnRH receptor (GnRHR) neurons, used epifluorescence and confocal microscopy to map the distribution of YFP-immunoreactive GnRHR neurons in fixed brain sections, and performed confocal Ca^{2+} imaging in live brain slices with the Ca^{2+} indicator Fura Red/AM to confirm that GFP-expressing GnRHR neurons respond to GnRH. They found that GnRHR expression was initiated only after postnatal day 16 in brain areas that influence sexual behaviors and process olfactory and pheromonal cues, and that responses to GnRH were similar within, and different between, brain areas, suggesting that GnRH acts peri- and post-pubertally to differentially influence brain functions to affect reproductive success (Wen et al. 2011).

8.2.3 Viral Tracing, Tissue Clearing, Expansion Microscopy, and Channelrhodopsin-Assisted Circuit Mapping of GnRH Neurons

In addition to GnRH promoter-driven GFP labeling, more recent approaches, including viral tracing, tissue clearing, expansion microscopy, and channelrhodopsin-assisted circuit mapping, have helped to delineate the projections and inputs of GnRH neurons. Morphological reconstructions, silver-enhanced immunogold labeling and electron microscopy, electrophysiology in brain sections/slices of GnRH-GFP mice, as well as viral tracing coupled with tissue clearing (Box 8.2) in brain sections of GnRH-Cre mice and rats injected with a recombinant adeno-associated viral (AAV) vector containing a Cre-dependent sequence encoding mCherry-linked channelrhodopsin (to target membrane-docked mCherry to Cre-expressing GnRH neurons) (Herde et al. 2013; Moore et al. 2018b; Yip et al. 2021), revealed that GnRH neurons exhibit structures up to 4 mm in length that project from the POA to the ME before branching into multiple short axons responsible for GnRH secretion. These structures, termed “dendrons,” were earlier considered to be GnRH neuron dendrites (Campbell et al. 2005, 2009; Cottrell et al. 2006; Chan et al. 2011) but are in fact structures that function simultaneously as an axon and dendrite (Figs. 8.3 and 8.4). Dendrons have a spike initiation site and

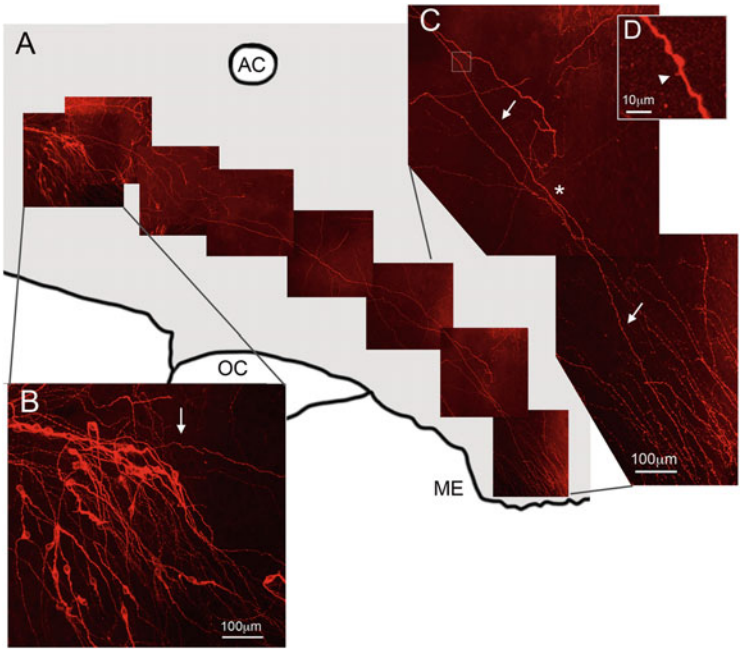

Fig. 8.3 Reconstruction of a single GnRH neuron process projecting from the rostral POA (rPOA) to the median eminence (ME) following GnRH neuron-selective viral tracing with Cre-dependent AAV-ChR2-mCherry and CLARITY (CLear lipid-exchanged Acrylamide-hybridized Rigid Imaging/immunostaining/in situ-hybridization-compatible Tissue hYdrogel) tissue clearing (Chung et al. 2013). (**a**) The position of the GnRH neuron in the sagittal plane is shown in consecutive confocal images. Total length, 3720 μm. (**b**) Magnification of rPOA GnRH neurons expressing mCherry with the traced process indicated by the white arrow. (**c**) High-magnification image of the distal section of the process (white arrows) projecting to the ME. The location of the first axon branch point is indicated with an asterisk. (**d**) High-magnification image of the inset in **c** showing a segment of the traced process elaborating a dendritic spine (white arrowhead). AC, anterior commissure; OC, optic chiasm. Reprinted from Moore AM, Prescott M, Czieselsky K, Desroziers E, Yip SH, Campbell RE, Herbison AE, Synaptic innervation of the GnRH neuron distal dendron in female mice. Endocrinology 2018;159(9):3200–3208. doi: https://doi.org/10.1210/en.2018-00505. Reprinted by permission of Oxford University Press on behalf of the Endocrine Society

conduct action potentials (like axons), while also exhibiting spines (based so far only on morphology and not yet also on their molecular components, e.g., scaffold proteins) along their entire length and a high density of synaptic inputs at the border of the ME (like dendrites). They express GABA, glutamate, and kisspeptin receptors, and would control GnRH secretion from the short axons into the pituitary portal system to regulate fertility (Herde et al. 2013; Iremonger et al. 2017; Moore et al. 2018b).

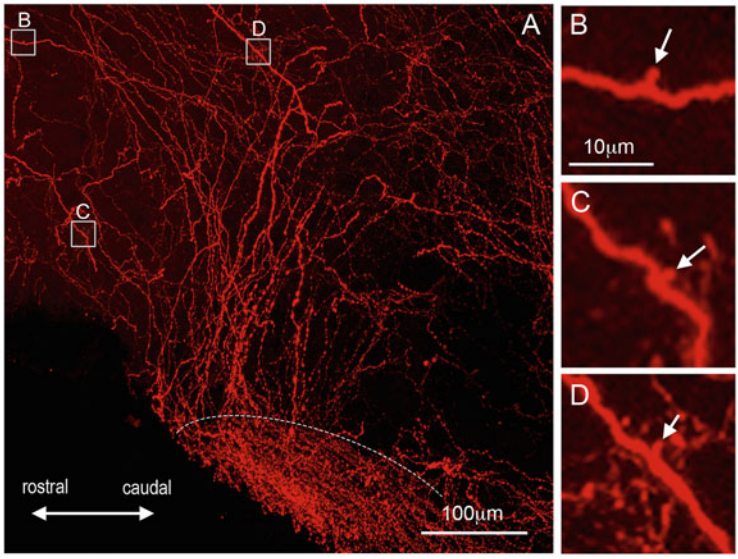

Fig. 8.4 GnRH neuron dendrons expressing ChR2-mCherry. (**a**) Sagittal view of GnRH neuron dendrons projecting from the rPOA to the ME. The dashed white line indicates the external zone of the ME, which contains large numbers of terminal endings. Insets (**b**–**d**) in (**a**) show three different dendrons. (**b**–**d**, right panels) High-magnification images (corresponding to insets **b**–**d** in **a**) of the three dendrons, which exhibit spines (arrows). Reprinted from Moore AM, Prescott M, Czieselsky K, Desroziers E, Yip SH, Campbell RE, Herbison AE, Synaptic innervation of the GnRH neuron distal dendron in female mice. Endocrinology 2018;159(9):3200–3208. doi: https://doi.org/10.1210/en.2018-00505. Reprinted by permission of Oxford University Press on behalf of the Endocrine Society

Box 8.2 Tissue Clearing and Expansion Microscopy

Tissue-clearing methods such as CLARITY (CLear lipid-exchanged Acrylamide-hybridized Rigid Imaging/immunostaining/in situ-hybridization-compatible Tissue hYdrogel) enable three-dimensional (3D) imaging of intact brain tissue (rather than brain sections), long-range projections, local circuit wiring, cellular relationships/interactions, and subcellular structures by transforming it into a nanoporous hydrogel-hybridized form (crosslinked to a three-dimensional network of hydrophilic polymers) that is fully assembled but optically transparent and macromolecule-permeable (Chung et al. 2013). Expansion microscopy involves isotropic swelling (4–5 times) of fixed tissue specimens in which fluorophores are linked to a swellable polymer, enabling ~70 nm resolution with conventional microscopes of putative synaptic contacts between neurons (Chen et al. 2015; Wassie et al. 2019).

Using expansion microscopy (Box 8.2) of synaptophysin-immunoreactive presynaptic terminals apposing GnRH neurons in fixed brain sections of adult female GnRH-GFP mice and rats, as well as in vivo chemogenetics (a technique that targets expression of designer receptors exclusively activated by designer drugs in specific neurons, providing the ability to modulate neuronal firing for several hours with the single administration of a designer drug; reviewed by Sternson and Roth 2014) and optogenetics (Box 8.3), the distal dendron was shown to exhibit the highest density of synaptic inputs to a GnRH neuron (Fig. 8.5) and to mediate both pulsatile and surge GnRH/LH secretion, whereas the soma-proximal dendritic zone was shown to mediate surge, but not pulsatile, GnRH/LH secretion (Wang et al. 2020; Yip et al. 2021). Based on the degree of overlap of their synaptophysin-immunoreactive appositions with GFP-labeled GnRH neuron distal dendrons as determined using expansion microscopy, non-kisspeptin neurons appear to make synaptic appositions at the distal dendron, whereas ARC kisspeptin neurons make abundant close but non-synaptic appositions that may provide input to GnRH dendrons via short-distance volume transmission, which may synchronize their activation (van den Pol 2012; Liu et al. 2021). RP3V kisspeptin neurons, in contrast, appear to make synaptic appositions (and thereby provide classical synaptic input) at the GnRH neuron soma-proximal dendritic zone (Yip et al. 2015; Piet et al. 2018; Liu et al. 2021). However, whether RP3V kisspeptin neurons, like ARC kisspeptin neurons, also make non-synaptic appositions at the GnRH neuron distal dendron is less clear.

Box 8.3 Optogenetics (Including Channelrhodopsin-Assisted Circuit Mapping)

Optogenetics uses light to control cellular activity via a genetically encoded light-sensitive protein (usually an ion channel), such as channelrhodopsin, for excitation, or halorhodopsin, for inhibition (reviewed by Lim et al. 2013; Han et al. 2018). Because light can be delivered with great spatial and temporal precision to particular neurons and their projections when the light-sensitive protein is expressed under the control of a cell-type-specific promoter, optogenetics can be used for neural circuit mapping (channelrhodopsin-assisted circuit mapping) and for elucidating physiological functions (including pulsatile and surge GnRH/LH secretion required for fertility) and behaviors (including sexual behavior).

GnRH neurons receive inputs from, and transmit their output to, neurons in multiple hypothalamic and extra-hypothalamic areas. Using the genetic transsynaptic retrograde and anterograde tracer barley lectin (BL) to identify neurons that are presynaptic or postsynaptic, respectively, to GnRH neurons in GnRH-BL-IRES-GFP mice, Boehm et al. (2005) found that approximately 800 GnRH neurons communicate with approximately 50,000 neurons in 53 different brain areas, including those that process odors and pheromones, with some connections exhibiting sexual dimorphism, indicating that GnRH neurons integrate information from

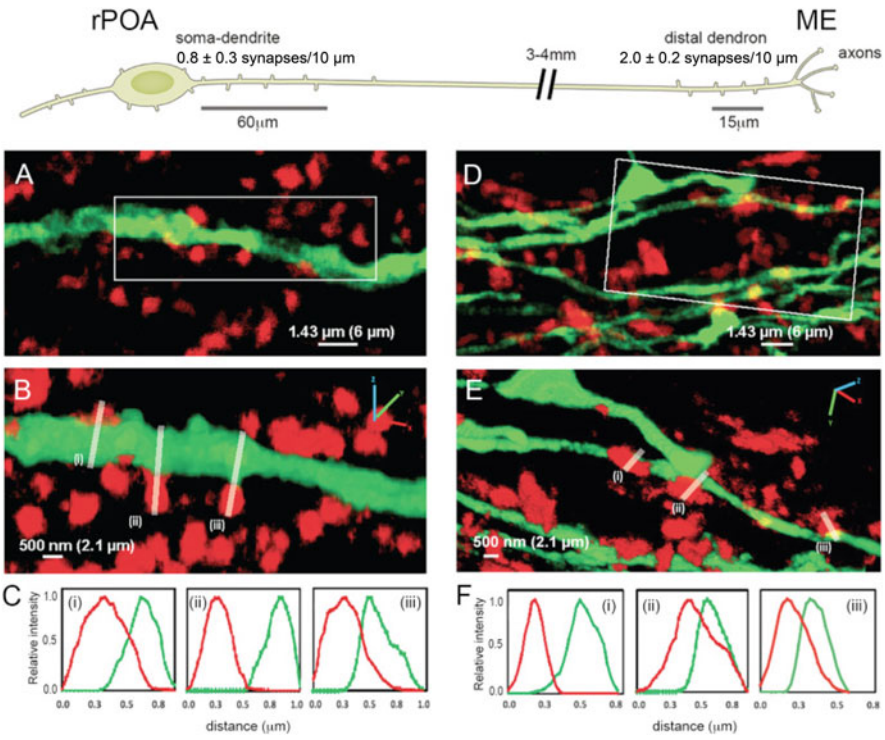

Fig. 8.5 Expansion microscopy of synaptic appositions at the GnRH neuron proximal dendrite and distal dendron. Schematic diagram of a GnRH neuron with its soma-proximal dendrites located in the rostral preoptic area (rPOA) and its distal dendron and short axon branches in the median eminence (ME). Synaptic density analysis was performed on 60 µm-lengths of proximal dendrite and 15 µm-lengths of distal dendron in four diestrous female GnRH-GFP mice. Synaptic densities were found to be higher at the distal dendrons than the proximal dendrites, as indicated. (**a**) Expansion microscopy view of a proximal dendrite (green) with surrounding synaptophysin-immunoreactive puncta (red). (**b**) Rotated 3D reconstruction with white lines indicating three appositions that were examined. (**c**) The side-on relative fluorescence intensity profiles are shown for the three appositions. (1) and (3) represent synaptic appositions, whereas (2) indicates apposing profiles with no overlap that do not represent a synapse. (**d**) Expansion microscopy view of distal dendrons (green) with surrounding red synaptophysin-immunoreactive puncta. (**e**) shows rotated 3D reconstruction with white lines indicating three appositions that were examined. (**f**) The relative fluorescence intensity profiles are shown for the three appositions. (2) and (3) represent synaptic appositions, whereas (1) indicates apposing profiles with no overlap that do not represent a synapse. Scale bars show pre-expansion values with expanded size in brackets. X-axis plots show pre-expansion values. Modified from Wang et al. (2020), with permission

multiple sources and modulate a variety of brain functions. Subsequently, Leshan et al. (2009) used a similar approach to reveal the direct innervation of GnRH neurons by ventral premammillary nucleus (PMV) LepRb neurons, which sense metabolic and sexual odorant cues, consistent with a role for PMV LepRb neurons in regulating the reproductive axis in response to metabolic and odorant stimuli.

Using viral tracing (Box 8.4) by injecting pseudorabies virus (Ba2001 PRV) into the brains of GnRH-Cre mice to identify neurons that are presynaptic to GnRH neurons, Yoon et al. (2005) found that multiple neuronal networks are connected to GnRH neurons, consistent with the results obtained by Boehm et al. (2005) using BL, and include networks that process odors but not pheromones (Figs. 8.6 and 8.7). Using a similar approach, Wintermantel et al. (2006) reported that GnRH neurons in the POA receive direct projections from estrogen receptor alpha (ERα)-expressing neurons in the RP3V that mediate the proestrous GnRH/LH surge and that were shown by Smith et al. (2005) and Dubois et al. (2015) to be kisspeptin neurons. However, employing a combinatorial genetic transsynaptic tracing strategy to analyze the connectivity of individual kisspeptin neurons with the GnRH neuron

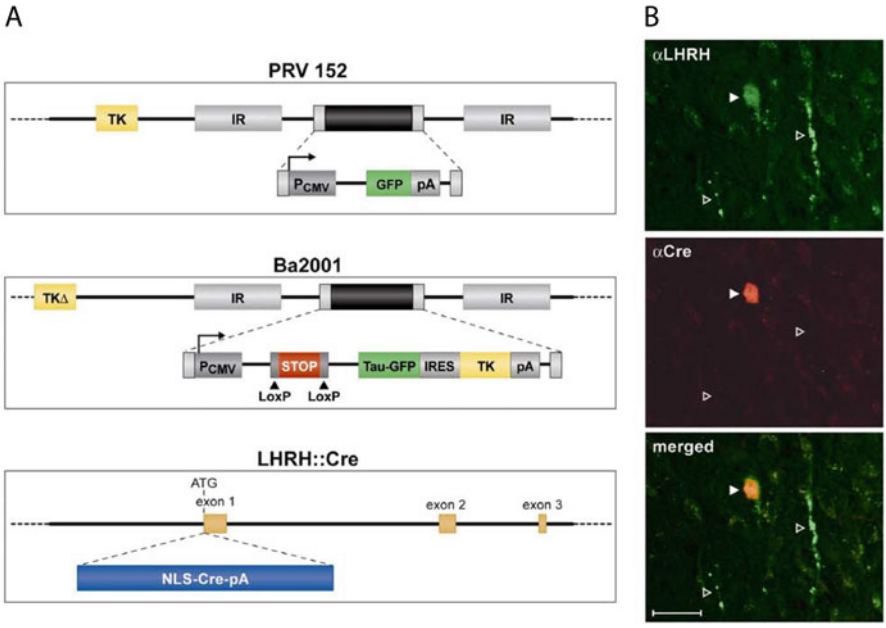

Fig. 8.6 Recombinant viral and transgenic mouse gene constructs for Cre recombinase-dependent viral tracing in GnRH neurons. (**a**) Cre-independent PRV152 virus expresses GFP under the control of the cytomegalovirus (CMV) promoter, permitting visualization of infected neurons. Cre-dependent Ba2001 PRV virus requires Cre-mediated recombination to excise a stop (STOP) cassette, leading to expression of thymidine kinase (TK; essential for viral replication in non-mitotic cells such as neurons) and the neuronal tracer TAU-GFP (τGFP), and thus, viral transfer (retrograde spread from postsynaptic to presynaptic neurons) only from primary infected neurons expressing Cre recombinase. GnRH-Cre (a.k.a. LHRH::Cre) transgenic mice were generated in which Cre recombinase is specifically expressed in GnRH neurons. A Cre-containing cassette including a nuclear localization signal (NLS) and a polyadenylation (pA) sequence was inserted into the start codon of the GnRH gene by homologous recombination of a BAC, RP23- 22 J8, containing 137 kb of 5′ upstream and 73 kb of 3′ downstream sequences around the GnRH coding sequence. IR, inverted repeat. IRES, internal ribosome entry site. (**b**) Double immunostaining with antibodies against GnRH (αGnRH; green) and Cre (αCre; red) shows specific expression of Cre in GnRH neurons of GnRH-Cre mice. Scale bar, 20 μm. Modified from Yoon et al. (2005), with permission

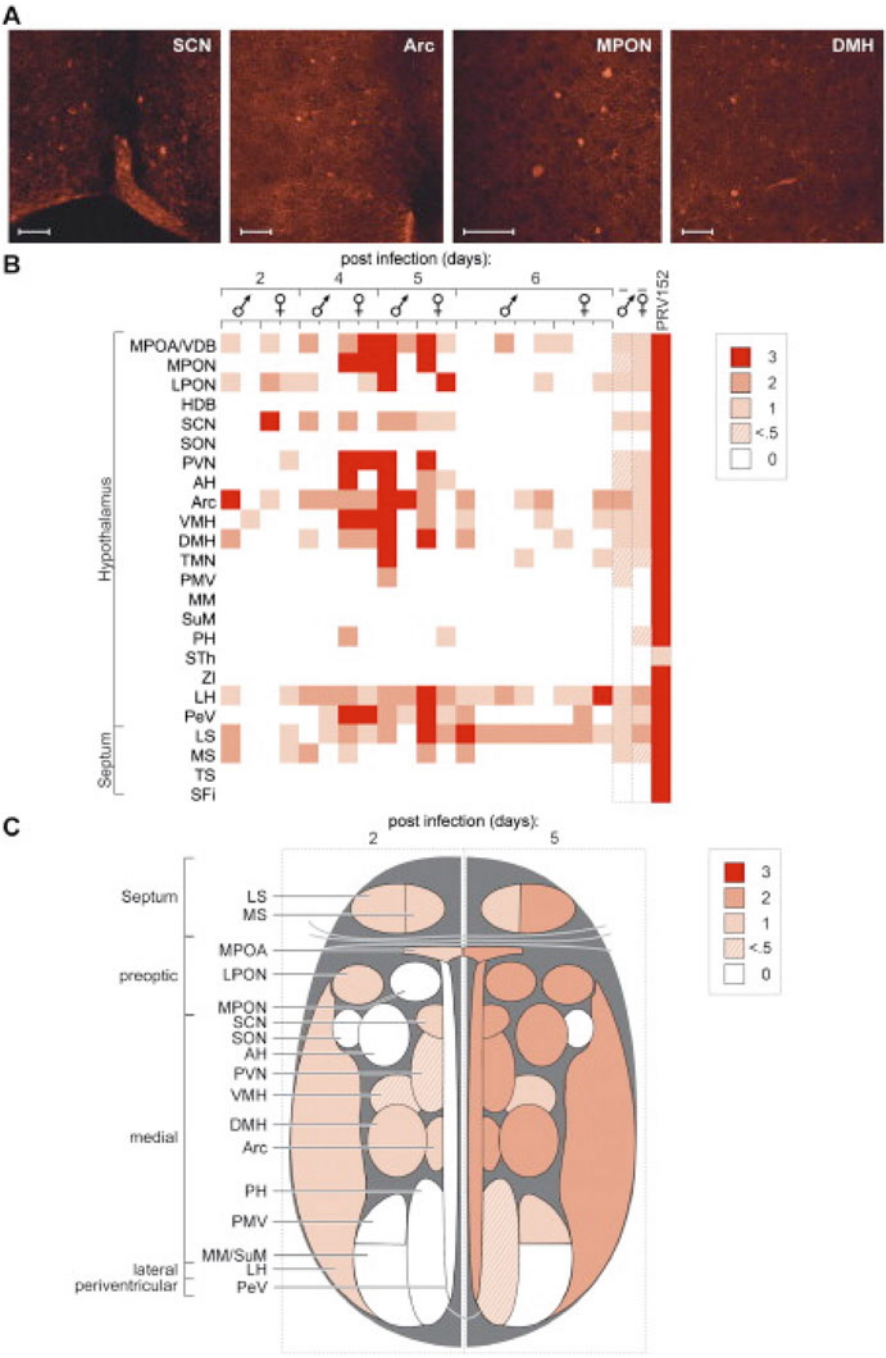

Fig. 8.7 Hypothalamic and septal inputs to GnRH neurons revealed by transfer of Cre-dependent Ba2001 PRV and Cre-independent PRV152. (**a**) Example of GFP expression (GFP immunoreactivity, red) in Ba2001 PRV-infected hypothalamic nuclei: suprachiasmatic nucleus (SCN), arcuate nucleus (Arc), medial preoptic nucleus (MPON), and dorsomedial hypothalamus (DMH). Scale bar,

population in female mice with single-cell resolution, Kumar et al. (2014, 2015) found that only subsets, rather than a majority, of RP3V and ARC kisspeptin neurons are synaptically connected with GnRH neurons, that all kisspeptin neurons within the RP3V connected to GnRH neurons are E-sensitive that most of these express tyrosine hydroxylase (TH), and that the neural circuits between ARC kisspeptin and GnRH neurons are fully established and operative before birth. It should also be noted that, using the Cre-dependent PRV viral tracing approach, Campbell and Herbison (2007) found that GnRH neurons in the POA receive direct projections from serotonergic neurons in the raphe nuclei and from noradrenergic neurons in the locus coeruleus and solitary tract nucleus (NTS).

Box 8.4 Viral Tracing

Viral tracing involving Cre-dependent Bartha 2001 (Ba2001) pseudorabies virus (PRV) has been used to identify neurons making direct presynaptic connections onto GnRH and kisspeptin neurons (Yoon et al. 2005; Yeo et al. 2019). In this approach, Ba2001 PRV is activated only after infecting a Cre-expressing GnRH or kisspeptin neuron in vivo. This allows the Ba2001 PRV to replicate and to pass in a retrograde manner to the primary afferents of

(continued)

Fig. 8.7 (continued) 50 μm. (**b**) Recording of GFP-labeled structures in the hypothalamus and septum resulting from stereotaxic injections of Ba2001 PRV and PRV152. The presence of GFP-positive cells in each structure was displayed on a graph according to the intensity of the labeling, the gender of the injected animals, and the tracing period after viral infection. The color of each box represents the relative level of GFP expression in hypothalamic and septal areas, from 0 to 3: 0—no GFP staining, 1—less than five faintly labeled neurons in the field, 2—less than five strongly labeled neurons per field or more than five weakly labeled neurons per field, 3—more than five strongly labeled neurons. From left to right, columns represent data from 20 individual GnRH-Cre homozygous mice injected with Ba2001 PRV, with adjacent columns representing data after 2, 4, 5, and 6 days of infection with Ba2001 PRV from two (first 12 columns) or four (next eight columns) individual mice of the same sex. Data from each sex were compiled and the mean labeling for males and females is shown on the following two columns. The same color scheme is used to display the mean value, with the addition of a hatched color to indicate areas showing GFP-positive neurons in at least at one animal but with a mean value lower than 0.5. The last column on the right shows mean values for their labeling in all areas traced by PRV152 injection, based on the data from four different infections (two males and two females). (**c**) Mean values of GFP labeling data obtained after 2 and 5 days of infection with Ba2001 PRV are displayed on a simplified horizontal map of the hypothalamus. Relative GFP expression levels are indicated with the same color code as above, on symmetrically outlined nuclei; left shows results at day 2 postinfection and right at day 5. *AH* anterior hypothalamic area, *HDB* horizontal limb of the diagonal band of Broca, *LH* lateral hypothalamic area, *LPON* lateral preoptic nucleus, *LS* lateral septal nucleus, *MM* medial mamillary nucleus, *MPOA* medial preoptic area, *MPON* medial preoptic nucleus, *PH* posterior hypothalamic nucleus, *PVN* paraventricular nucleus, *SuM* supramammillary nucleus, *SFi* septofimbrial nucleus, *STh* subthalamic nucleus, *TMN* tuberomammillary nucleus, *TS* triangular septal nucleus, *VDB* vertical limb of the diagonal band of Broca, *ZI* zona incerta. Other abbreviations are explained at their first occurrence in the main text. Reproduced from Yoon et al. (2005), with permission

Box 8.4 (continued)
a neuron, and subsequently to their own afferents, in a time-dependent manner. The retrograde chain of infection can be followed by evaluating GFP expression, as the unconditional Ba2001 PRV also expresses GFP in each cell it infects. Monosynaptically-restricted rabies virus (RV) tracing, which avoids multi-synaptic spread (reviewed by Saleeba et al. 2019), has been used to further distinguish between the primary and secondary afferents of GnRH and kisspeptin neurons (Moore et al. 2019; Yeo et al. 2019).

Employing viral tracing along with imaging of dendritic spines in adult female GnRH-GFP mice, Moore et al. (2015) found that projections from ARC GABAergic neurons heavily contact and even bundle with GnRH neuron dendrites, and that the density of fibers apposing GnRH neurons, as well as GnRH dendritic spine density, is even greater in prenatally androgenized (PNA) adult mice that exhibit a polycystic ovarian syndrome (PCOS) phenotype (increased LH pulse frequency and impaired progesterone negative feedback). This suggested the existence of a robust GABAergic circuit originating in the ARC that is enhanced in PCOS and may underpin the neuroendocrine pathophysiology of the syndrome, which is the leading cause of female infertility. Subsequently, using channelrhodopsin-assisted circuit mapping (Box 8.3), by injecting Cre-dependent AAV-ChETA (a channelrhodopsin variant)-eYFP into the ARC of VGAT-Cre mice and then optogenetically photostimulating ARC GABA neuron fibers projecting into the rostral POA, where many GnRH neurons are located, Silva et al. (2019) showed that high-frequency activation of ARC GABA neuron fibers in the rostral POA elicits LH secretion (a proxy for GnRH neuron stimulation). However, the LH response to such optogenetic activation (as well as to injection of GnRH) was blunted in PNA VGAT-Cre mice compared to non-PNA VGAT-Cre mice, which may reflect a history of high-frequency GnRH/LH secretion and reduced LH stores in PNA VGAT-Cre mice, but also raises questions about impaired function within the ARC GABA neuron population and the involvement of other circuits (Silva et al. 2019).

Using whole-mount immunocytochemistry (with enhanced antibody penetration and tissue clearing) as well as optogenetics in brain slices of triple transgenic male Preproglucagon (GCG)-Cre/Channelrhodopsin 2 (ChR2)/GnRH-GFP mice, Vastagh et al. (2020) showed that axons of glucagon-like peptide-1 (GLP-1) neurons originating in the NTS innervate about a quarter of GnRH neurons in the POA, forming either single or multiple contacts on GnRH dendrites and somata. When optogenetically activated, the axons of the GLP-1 neurons increased the firing rate of GnRH neurons, an effect that was prevented by pretreatment with the GLP-1 receptor antagonist, Exendin-3-(9-39). Their findings support the idea that GLP-1 neurons, along with other neurons including NPY/AgRP, POMC, MCH, and GALP neurons (as discussed in Sect. 8.2.1), may relay metabolic signals to GnRH neurons and link metabolism with reproduction.

8.3 Kisspeptin Promoter-Driven GFP Labeling to Identify and Anatomically Characterize Kisspeptin Neurons

As with GnRH neurons, labeling with kisspeptin promoter-driven Cre-dependent GFP (or with other fluorescent proteins such as YFP or tdTomato) has been used to identify and characterize kisspeptin neurons anatomically as well as physiologically in Kiss1-CreGFP (Gottsch et al. 2011; Navarro et al. 2011; Qiu et al. 2016), Kiss1-Cre;Rosa26 (R26)-GFP (Cravo et al. 2011), Kiss1-Cre;R26-τGFP (Mayer et al. 2010; de Croft et al. 2012), Kiss1-Cre;R26-τYFP (Mayer et al. 2010), and Kiss-Cre;R26-tdTomato (Yeo et al. 2016) mice (Fig. 8.8). Similar to the approach used for validating GnRH-GFP mice and rats as transgenic models, colocalization of GFP, YFP, tdTomato or Cre expression with kisspeptin in Kiss1-CreGFP, Kiss1-Cre;R26-τGFP, Kiss1-Cre;R26-τYFP, and Kiss-Cre;R26-tdTomato mice was confirmed using a kisspeptin antibody or *Kiss1* riboprobe considered to be "gold standards" for labeling kisspeptin neurons. The cells that expressed GFP, YFP, or tdTomato (hereafter referred to as kisspeptin neurons) were shown to be located in the ARC and RP3V, confirming previous immunohistochemical findings, as well as in the anterodorsal preoptic nucleus (ADP), DMH, and ventromedial hypothalamic nucleus (VMH), mammillary nucleus (MM), lateral septum (LS), medial amygdala (MeA), periaqueductal gray (PAG), and cerebral cortex (CC; Mayer et al. 2010; Cravo et al. 2011; Gottsch et al. 2011; de Croft et al. 2012; Yeo et al. 2016; Pineda et al. 2017).

8.3.1 Kisspeptin Promoter-Driven GFP Labeling to Determine the Inputs of Kisspeptin Neurons

Immunostaining brain sections of Kiss1-Cre;R26-GFP mice for GFP and β-endorphin, Cravo et al. (2011) found that subsets of ARC and RP3V kisspeptin neurons are innervated by β-endorphin-immunoreactive fibers of POMC neurons, which may participate in the metabolic regulation of fertility.

8.3.2 Kisspeptin Promoter-Driven GFP Labeling to Determine the Outputs of Kisspeptin Neurons

Immunostaining brain sections of Kiss-Cre;R26-tdTomato mice for tdTomato, Yeo et al. (2016) showed that ARC kisspeptin neurons project to the RP3V as well as the anterior hypothalamic area (AHA), lateral hypothalamus, median preoptic nucleus (MPN), medial preoptic area (MPA), medial preoptic nucleus, periventricular preoptic nucleus, and ventral tuberomammillary nucleus, whereas RP3V kisspeptin neurons project to the ARC, lateral preoptic area (LPA), MPN, MPA, and OVLT.

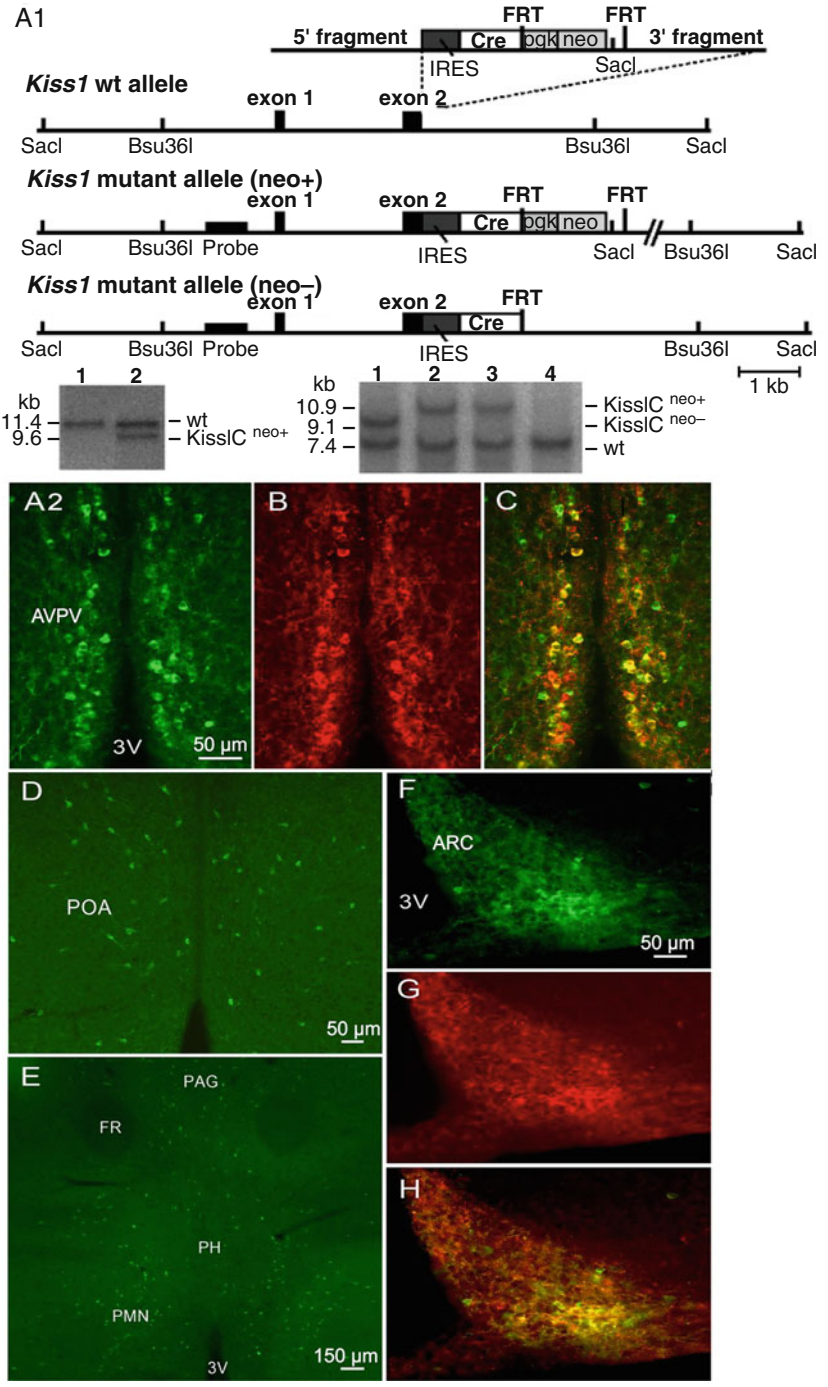

Fig. 8.8 Kisspeptin promoter-driven GFP labeling of kisspeptin neurons. (**a1**) Targeting strategy for expressing Cre recombinase under the control of the Kiss1 promoter to generate Kiss-IRES-Cre mice that could be crossed with Cre-dependent GFP (ROSA26-CAGS-GFP) mice to obtain Kiss-

8.3.3 Viral Tracing, Tissue Clearing, Expansion Microscopy, and Channelrhodopsin-Assisted Circuit Mapping of Kisspeptin Neurons

Viral tracing and channelrhodopsin-assisted circuit mapping (along with tissue clearing and expansion microscopy, as discussed in this section and above in Sect. 8.2.3) have helped to delineate the projections of kisspeptin neurons to GnRH neurons and other neurons as well as the inputs to kisspeptin neurons. Injecting a Cre-dependent recombinant adenovirus encoding farnesylated enhanced green fluorescent protein into the ARC or RP3V of adult male and female Kiss-Cre mice, Yip et al. (2015) showed that (axonal) fibers of ARC kisspeptin neurons project widely but do not directly contact GnRH neuron somata or proximal dendrites. In contrast, they identified RP3V kisspeptin fibers in close contact with GnRH neuron somata and dendrites in both sexes. They also observed kisspeptin fibers from both the RP3V and ARC in close contact with distal GnRH neuron processes in the ARC and in the lateral and internal aspects of the ME, GnRH neuron axon terminals in close contact with the proximal dendrites of ARC kisspeptin neurons in the ARC, and ARC kisspeptin fibers contacting RP3V kisspeptin neurons in both sexes (Figs. 8.9 and 8.10). Channelrhodopsin-assisted circuit mapping by expressing and

Fig. 8.8 (continued) GFP mice. The targeting vector, the wild-type (wt) alleles, and the targeted allele (mutant allele) before (neo+) and after (neo-) removal of the neomycin selection cassette are shown from top to bottom. Restriction sites used for Southern blot analysis, as well as the location of the probes, are indicated. Black boxes represent exons. The inserted cassette is composed of an internal ribosomal entry site (IRES) followed by the coding sequence for Cre recombinase (Cre) and a phosphoglycerate kinase promoter-driven neomycin resistance cassette flanked by Flp recombinase recognition (FRT) sites. Southern blot analysis of ES cell DNA after digestion with SacI. The expected fragment sizes detected by the probe are indicated (wt, 11.4 kb; mutant, 9.6 kb). Clone 2 carries the mutant KissI allele (KissIC^{neo+}). Southern blot analysis of DNA digested with Bsu36I from wt and heterozygous mutant mice before and after removal of the neomycin selection cassette. The expected fragment sizes detected by the probe are indicated (wt, 7.4 kb; mutant allele I KissIC^{neo+}, 9.1 kb; mutant allele II KissIC^{neo-}, 10.9 kb). Mice 2 and 3 carry mutant allele I (KissIC^{neo+}), as shown in lanes 2 and 3 of the Southern blot, whereas mouse 1 carries mutant allele II after Flp recombinase mediated excision of the neomycin cassette (KissIC^{neo-}), as shown in lane 1 of the Southern blot. Reprinted from Mayer et al. (2010), with permission. (**a2–c**) Distribution and correlation of GFP expression with kisspeptin immunoreactivity in Kiss-GFP mice obtained by crossing homozygous Kiss-IRES-Cre mice with homozygous Cre-dependent GFP (ROSA26-CAGS-GFP) mice. GFP-expressing (**a2**) and kisspeptin-immunoreactive (**b**) cells in the AVPV of adult female mouse with overlay in (**c**). (**d**) GFP-expressing cells in the POA of an adult male mouse. (**e**) Distribution of GFP-expressing cells in the posterior hypothalamus (PH) and adjoining brain regions including the PAG and premammillary nuclei (PMN) in an adult female mouse. FR, Fasciculus retroflexus. Other abbreviations are explained at their first occurrence in the main text. (**f** and **g**) Distribution of GFP cells (**f**) and kisspeptin immunoreactivity (**g**) in the ARC of an adult female mouse, with overlay in (**h**). Scale bar, 50 μm (**a2**, **d** and **f**) and 150 μm (**e**). Reprinted from de Croft S, Piet R, Mayer C, Mai O, Boehm U, Herbison AE, Spontaneous kisspeptin neuron firing in the adult mouse reveals marked sex and brain region differences but no support for a direct role in negative feedback. Endocrinology 2012;153(11):5384–5393. doi: https://doi.org/10.1210/en.2012-1616. Reprinted by permission of Oxford University Press on behalf of the Endocrine Society

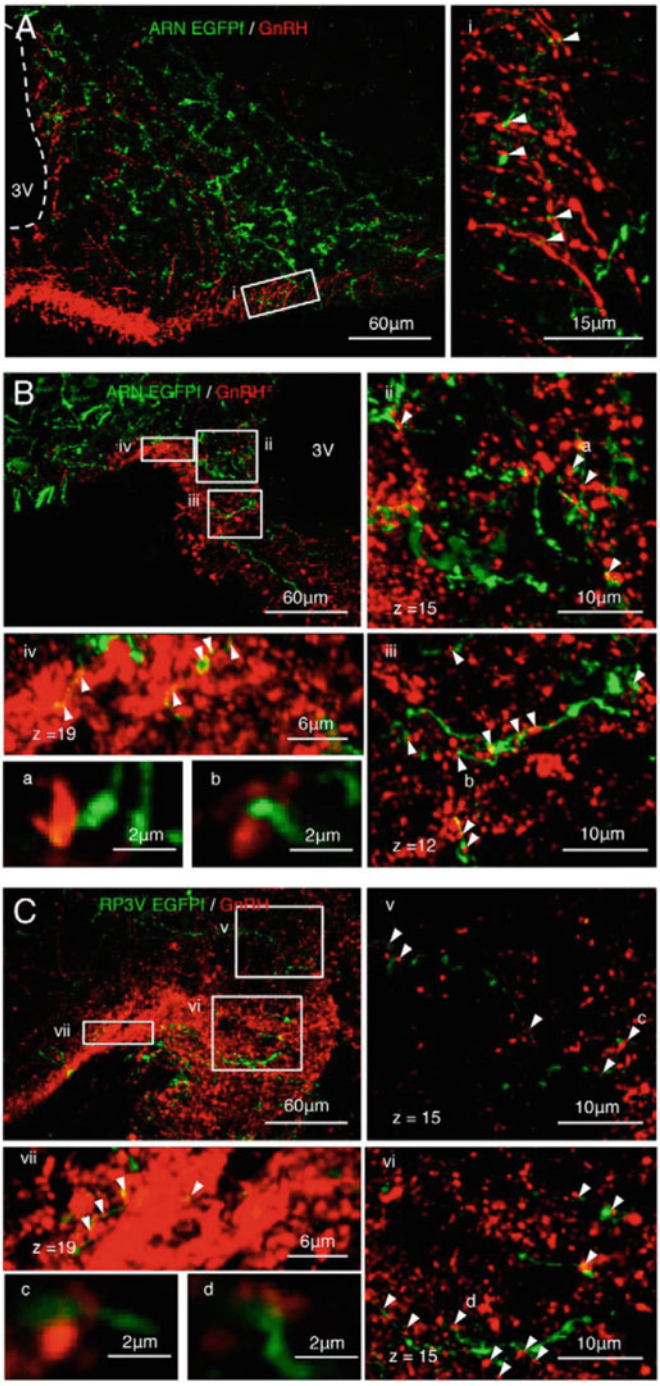

Fig. 8.9 Kisspeptin neuron appositions onto GnRH neuron projections within the ME in brains of adult Kiss-Cre mice stereotaxically injected with a Cre-dependent recombinant adenovirus encoding farnesylated enhanced green fluorescent protein (EGFPf) into the ARC or RP3V and

photostimulating channelrhodopsin at particular frequencies to activate ARC and RP3V kisspeptin neurons, and expressing and photostimulating archaeorhodopsin or halorhodopsin at particular frequencies to inhibit them, revealed that ARC and RP3V kisspeptin neurons form neural circuits with GnRH neurons that drive pulsatile and surge GnRH/LH secretion, respectively (Fig. 8.11) (Han et al. 2015, 2020; Qiu et al. 2016; Piet et al. 2018). Using high- frequency optogenetic stimulation of channelrhodopsin, Qiu et al. (2016) found that ARC kisspeptin neurons locally release excitatory (neurokinin B, NKB) and inhibitory (dynorphin, Dyn) neuropeptides, which synchronizes ARC kisspeptin neuron firing for pulsatile release of kisspeptin onto GnRH neurons, which in turn drives pulsatile GnRH/LH secretion. They also found that ARC kisspeptin neurons release glutamate onto RP3V kisspeptin neurons, which stimulates the burst firing of RP3V kisspeptin neurons and the release of kisspeptin onto GnRH neurons, which in turn excites GnRH neurons and stimulates GnRH/LH surge secretion at proestrous. Expressing and optogenetically photostimulating channelrhodopsin in RP3V kisspeptin neurons revealed that they also impinge on nitric oxide-synthesizing neurons in the VMH to elicit sexual behavior (Hellier et al. 2018).

Moore et al. (2019) used monosynaptic rabies virus-mediated tracing (injection of Cre-dependent AAV viruses encoding TVA [an avian receptor protein]/GFP and optimized rabies glycoprotein into Kiss1-Cre;R26-tdTomato mice followed 7 days later by the EnVA [avian sarcoma leucosis virus glycoprotein]-pseudotyped rabies glycoprotein-deleted virus containing the fluorescent reporter mCherry) combined with tissue clearing and multiple-label immunofluorescence to delineate the inputs of kisspeptin neurons. They found that ARC kisspeptin neurons receive over 90% of their input from hypothalamic nuclei in both male and female mice, with the greatest input coming from non-kisspeptin-expressing ARC neurons, including POMC neurons. They also detected significant female-dominant sex differences in afferent

Fig. 8.9 (continued) that were later sectioned, and then immunostained for GnRH. Confocal images showing EGFPf-positive ARC kisspeptin axonal projections (green) closely apposed to GnRH neuron processes (red) near the pial surface of the base of the brain (**a**) and in the lateral palisade zone (LPZ) of the external ME and medial part of the internal zone of the ME (**b**) in a representative female mouse. The hydrophobic nature of the farnesyl group restricts the trafficking of the enhanced green fluorescent protein (EGFP) to the cell membrane, effectively labeling the distal kisspeptin axonal projections in association with GnRH neurons. (**c**) Representative confocal images showing close apposition of EGFPf-positive RP3V kisspeptin axonal projections to GnRH neuron processes (red) in the LPZ of the external ME and medial part of the internal zone of the ME of a female mouse. Panels i, ii-iv, and v-vii are high-magnification images from corresponding boxed areas in panels **a**, **b**, and **c**. Panels **a**–**d** show single confocal slices (1 μm optical thickness) displaying examples of close apposition by the absence of black pixels between green and red signals. z refers to the number of confocal optical images in the z-plane acquired at 0.5 μm intervals. 3 V, third ventricle. Reprinted from Yip SH, Boehm U, Herbison AE, Campbell RE, Conditional viral tract tracing delineates the projections of the distinct kisspeptin neuron populations to gonadotropin-releasing hormone (GnRH) neurons in the mouse. Endocrinology 2015;156 (7):2582–2594. doi: https://doi.org/10.1210/en.2015-1131. Reprinted by permission of Oxford University Press on behalf of the Endocrine Society

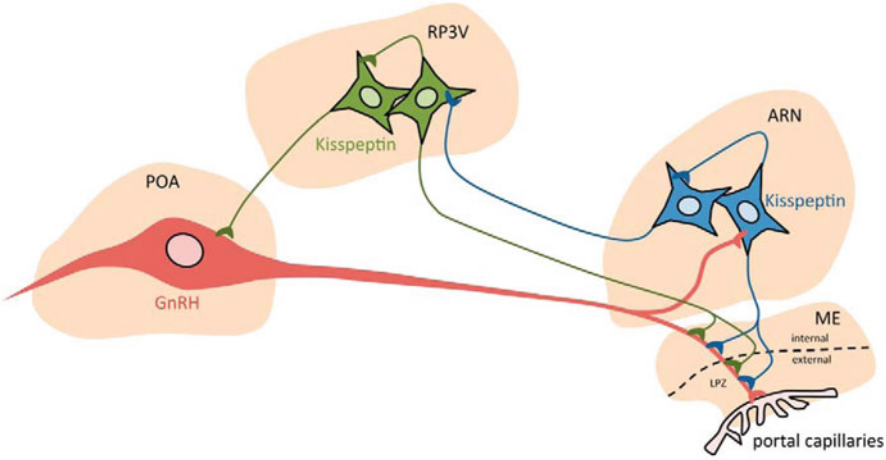

Fig. 8.10 Schematic diagram illustrating the connections between RP3V and ARC (a.k.a. ARN) kisspeptin neurons (green and blue, respectively) and GnRH neurons (red). RP3V kisspeptin neurons project to and contact GnRH neuron somata and proximal dendrites in the POA and to GnRH neuron distal processes in the ARC, internal ME, and lateral palisade zone (LPZ) of the external ME. In contrast, ARC kisspeptin neurons project to distal GnRH neuron processes in the ARC, internal ME, and LPZ of the external ME but do not contact GnRH neuron somata or processes in the POA. ARC kisspeptin neurons also project to the RP3V and contact RP3V kisspeptin neurons. GnRH neurons reciprocally contact ARC kisspeptin neurons and interconnectedness between kisspeptin neurons is evident in both the RP3V and ARC. Reprinted from Yip SH, Boehm U, Herbison AE, Campbell RE, Conditional viral tract tracing delineates the projections of the distinct kisspeptin neuron populations to gonadotropin-releasing hormone (GnRH) neurons in the mouse. Endocrinology 2015;156(7):2582–2594. doi: https://doi.org/10.1210/en.2015-1131. Reprinted by permission of Oxford University Press on behalf of the Endocrine Society

input from E-sensitive hypothalamic nuclei critical for reproductive endocrine function and sexual behavior in mice, indicating that ARC kisspeptin neurons, along with RP3V kisspeptin neurons (Hellier et al. 2018), may coordinate sex-specific behavior and gonadotropin release. Injecting Ba2001 PRV into the brains of Kiss-Cre;R26-tdTomato mice to identify neurons that are presynaptic to kisspeptin neurons, as well as using monosynaptic rabies virus-mediated tract tracing to further distinguish primary from secondary inputs to kisspeptin neurons, Yeo et al. (2019) found that multiple neuronal networks provide primary (direct) synaptic input to ARC kisspeptin neurons in mice, including ARC POMC neurons, RP3V kisspeptin neurons, vasopressin neurons in the supraoptic nucleus, and unidentified neurons in other regions including the MPA, MPN, paraventricular nucleus, MeA, interpeduncular nucleus, PMV, VTM, PAG, and dorsal raphe nucleus (Fig. 8.12).

Channelrhodopsin-assisted circuit mapping/activation by expressing and optogenetically photostimulating channelrhodopsin in ARC agouti-related peptide (AgRP) neurons revealed that ARC kisspeptin neurons also receive inhibitory input from AgRP neurons, consistent with the hypothesis that AgRP neuron activation

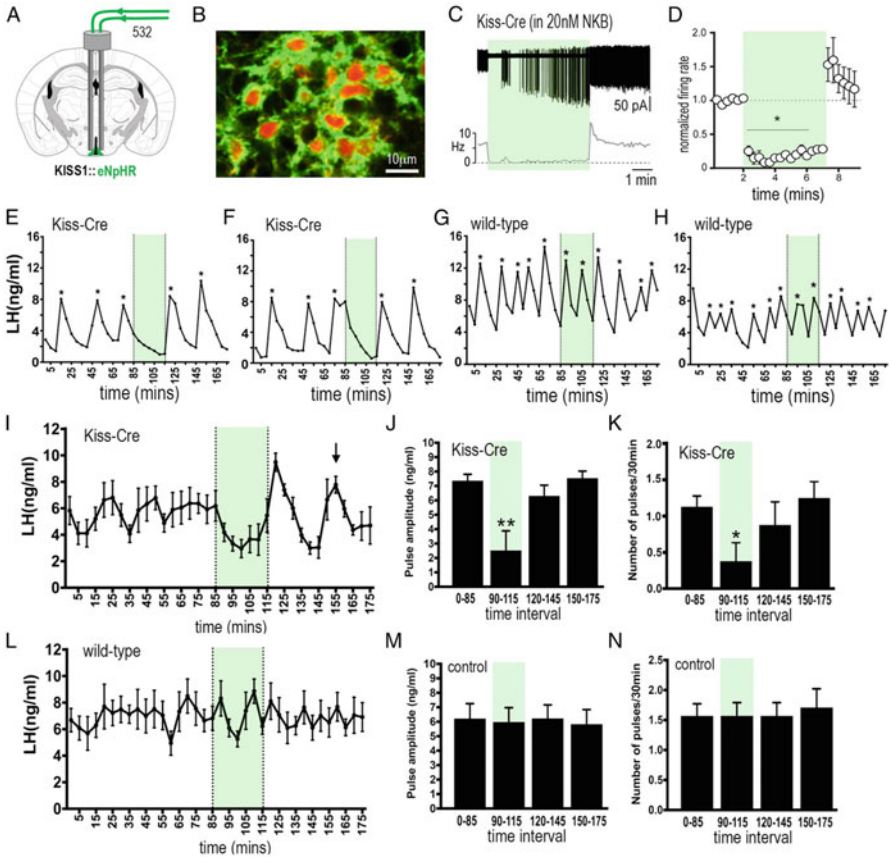

Fig. 8.11 Halorhodopsin (eNpHR3.0) inhibition of ARC kisspeptin neurons suppresses LH pulse amplitude and frequency in gonadectomized female mice. (**a**) Kiss-Cre mice injected with Cre-dependent eNpHR3.0-eYFP AAV into the ARC (green) and implanted with bilateral optic fibers in the ARC. (**b**) Fluorescence image of ARC kisspeptin neurons expressing eNpHR3.0 (green) and a kisspeptin reporter (tdTomato, red). (**c**) Action potential firing in an eNpHR3.0-expressing ARC kisspeptin neuron in the presence of 20 nM neurokinin B (NKB). The neuron responds to green light illumination with an abrupt decrease in firing followed by a sharp rebound activation immediately after the light is switched off. (**c**, top) Action potential firing. (**c**, bottom) Action potential frequency before illumination, during illumination, and after termination of illumination. (**d**) Mean (± SEM) normalized firing rate of ARC kisspeptin neurons ($n = 6$) responding to green light. $^*P < 0.05$ compared with baseline, Friedman test. (**e**–**h**) Pulsatile LH secretion in eNpHR3.0 Kiss-Cre (**e** and **f**) and wild-type (**g** and **h**) gonadectomized female mice. Green light illumination is indicated by green shading. LH pulses are indicated by asterisks. (**i**) Mean (± SEM) LH levels in Kiss-Cre mice ($n = 8$) showing the suppression of LH secretion during green light illumination and the subsequent rebound of LH and resetting of the pulse generator upon termination of green light illumination to evoke a subsequent LH pulse in all mice (arrow). (**j** and **k**) Mean (± SEM) LH pulse amplitude and pulse frequency before (0–85 min), during (90–115 min; green shading), and after terminating (120–145 and 150–175 min) green laser illumination. $^*P < 0.05$, $^{**}P < 0.01$ versus 0–85 min, ANOVA with post hoc Dunnett's test; $n = 8$. (**l**–**n**) Basal (± SEM) LH levels and LH pulse amplitude and frequency in control AAV-injected wild-type mice ($n = 7$). Reproduced from Clarkson et al. (2017), with permission

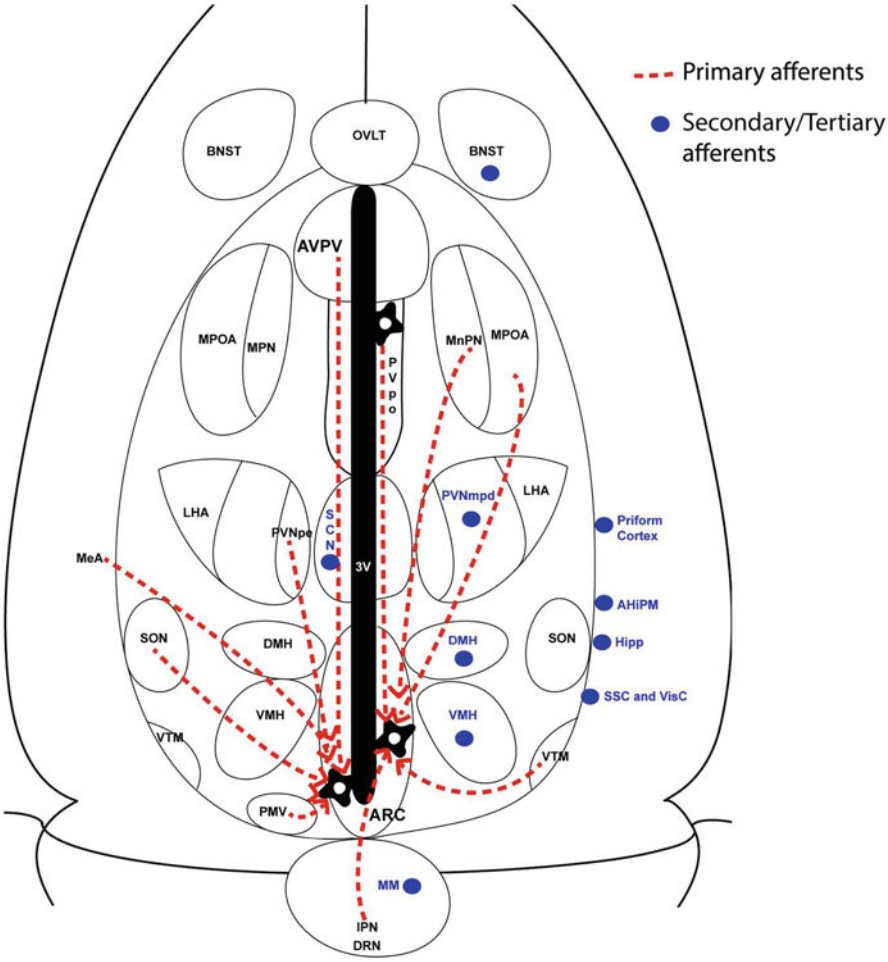

Fig. 8.12 Schematic diagram of neuronal inputs to ARC kisspeptin neurons. Red dashed lines represent primary afferents to mid-caudal ARC kisspeptin neurons, whereas blue circles represent secondary afferents. Two cells (instead of one cell) representing mid-caudal ARC kisspeptin neurons were drawn for better visibility of the primary afferents. *AHiPM* amygdalohippocampal area, *BNST* bed nucleus of stria terminalis, *DRN* dorsal raphe nucleus, *Hipp* hippocampus, *IPN* interpeduncular nucleus, *LHA* lateral hypothalamic area, *MM* mammillary nucleus, *MPOA* medial preoptic area, *MnPN* or *MPN* median preoptic nucleus, *PMV, PVNmpd* paraventricular nucleus (medial posterodorsal part), *PVNpe* paraventricular nucleus (periventricular part), *SCN* suprachiasmatic nucleus, *SON* supraoptic nucleus, *SSC* somatosensory cortex, *VisC* visual cortex, *VTM* ventral tuberomammillary nucleus. Other abbreviations are explained at their first occurrence in the main text. Reproduced from Yeo et al. (2019), with permission

during starvation contributes to infertility by inhibiting kisspeptin neurons and GnRH secretion (Padilla et al. 2017). Conversely, channelrhodopsin-assisted circuit mapping/activation by expressing and optogenetically activating channelrhodopsin

in PMV pituitary adenylate cyclase activating polypeptide (PACAP) neurons showed that ARC and RP3V kisspeptin neurons receive direct contact from PMV PACAP neurons, and that a subset is excited by them, supporting the hypothesis that PMV PACAP neurons relay nutritional state information via kisspeptin neurons to regulate GnRH secretion (Ross et al. 2018). However, other neuronal inputs to RP3V kisspeptin neurons (except those from ARC and other RP3V kisspeptin neurons [Yip et al. 2015; Qiu et al. 2016]) have yet to be reported.

8.4 Perspectives

The techniques discussed in this chapter, along with the genetically encoded fluorescent biomarkers upon which they depend, have greatly enriched our understanding of the GnRH/kisspeptin system. Because of these techniques, we now know that GnRH neurons possess dendrons in addition to dendrites and axons, that inputs to the GnRH neuron soma-proximal dendritic zone and to GnRH neuron distal dendrons from kisspeptin neurons differentially control pulsatile and surge GnRH secretion, and that GnRH and kisspeptin neurons receive inputs from neurons in multiple hypothalamic and extra-hypothalamic areas that convey endocrine, metabolic, and environmental (including circadian, pheromonal, and social behavior-related) signals known to affect fertility. Improvements in these techniques may help to provide further insights into the GnRH/kisspeptin system. One example is promoter-driven labeling with mGreenLantern, a recently engineered GFP variant that exhibits significantly greater brightness compared to other GFPs due to its enhanced folding efficiency and solubility. mGreenLantern not only retains strong fluorescence after tissue clearing and expansion microscopy, but it also facilitates neuronal imaging without the need for GFP immunocytochemistry (Campbell et al. 2020), which may help reveal additional morphological features of the GnRH/kisspeptin system. Another example is a monosynaptic viral tracing method involving complementation of glycoprotein gene-deleted rabies of the SAD B19 strain with its glycoprotein, B19G, or a codon-optimized version of the transmembrane/cytoplasmic domain of B19G and the extracellular domain of rabies Pasteur virus strain glycoprotein (oG), which has been shown to significantly increase tracing efficiency (Kim et al. 2016), and when applied to the GnRH/kisspeptin system may provide additional insights into its connections. Further identification and characterization of the neural circuits that convey endocrine, metabolic, and environmental signals to GnRH and kisspeptin neurons will likely spur the development and use of new approaches to control fertility and treat reproductive disorders.

Key References Boehm et al. (2005) First use of a genetic transsynaptic tracer to identify neurons that are presynaptic or postsynaptic to GnRH neurons

Han et al. (2015) First report showing that activation of ARC kisspeptin neurons generates pulsatile GnRH/LH secretion

Herde et al. (2013) First description of GnRH neuron dendrons

Mayer et al. (2010) First report showing kisspeptin promoter-driven GFP labeling of kisspeptin neurons, which together with immunocytochemistry and viral tracing has been used to characterize the anatomy of kisspeptin neurons and to reveal their inputs and outputs

Moore et al. (2018b) First description of the synaptic innervation of GnRH neuron distal dendrons

Spergel et al. (1999) First report showing GnRH promoter-driven GFP labeling of GnRH neurons, which together with immunocytochemistry and viral tracing has been used to characterize the anatomy of GnRH neurons and to reveal their inputs and outputs

Wang et al. (2020) First demonstration, using expansion microscopy, chemogenetics and optogenetics, that different dendritic domains of the GnRH neuron underlie the pulse and surge modes of GnRH/LH secretion

Yeo et al. (2019) First mapping of neuronal inputs to kisspeptin neurons

Yip et al. (2015) First mapping of the projections of kisspeptin neurons to GnRH neurons

Yoon et al. (2005) First use of viral tracing to identify neurons that are presynaptic to GnRH neurons

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9

Corticotropin-Releasing Hormone in the Paraventricular Nucleus of the Hypothalamus—Beyond Hypothalamic–Pituitary–Adrenal Axis Control

Simon Chang and Jan M. Deussing

Abstract

Corticotropin-releasing hormone (CRH) is the master regulator of the hypothalamic–pituitary–adrenal (HPA) axis. CRH is highly expressed in parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN). PVN^{CRH} neurons are primarily recognized for their role in launching the endocrine stress response. These neurons receive multiple inhibitory and excitatory afferents monitoring external environmental threats and internal physiological states. The integrated information is translated into hormonal, autonomic and behavioural responses aiming at maintaining homeostasis and improving chances of survival. The regulation of the HPA axis is closely associated with glucocorticoid-mediated feedback mechanisms but, in recent years, it has become evident that CRH and its high-affinity CRH receptor type 1 are constituents of a microcircuit within the PVN directly involved in HPA axis regulation. Furthermore, our perception of CRH^{PVN} neurons is currently changing as we have witnessed several exciting studies demonstrating that PVN^{CRH} neurons directly engage in rapid behavioural responses in reaction to stressful stimuli beyond their classical role attributed to neuroendocrine regulation.

Keywords

Corticotropin-releasing hormone · Corticotropin-releasing factor · Hypothalamic–pituitary–adrenal axis · Paraventricular nucleus · Parvocellular neuron · Stress · Hypothalamus

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9.1 Introduction

The discovery of corticotropin-releasing hormone (CRH; also designated corticotropin-releasing factor, CRF) as the principal regulator of the hypothalamic–pituitary–adrenal (HPA) axis was a major breakthrough in neuroendocrinology (Vale et al. 1981). CRH is part of a neuropeptide family comprising urocortin 1 (UCN1), UCN2 and UCN3. CRH is synthesized as a precursor and matures into its 41-amino acid biologically active form via proteolytic processing and C-terminal amidation en route to its storage and release sites at axon terminals. The physiological activity of CRH and related peptides is conveyed by two heptahelical receptors from the family B1 of secretin-like G protein-coupled receptors (GPCRs)—CRH receptor type 1 (CRHR1) and CRHR2 (Deussing and Chen 2018).

Intracerebroventricular application of CRH in rodents promotes behavioural and autonomic reactions reminiscent of a response to natural threats. CRH treatment induces general arousal and anxiogenic behaviour in various behavioural paradigms (Dunn and Berridge 1990). Simultaneously, CRH activates physiological reactions, such as increased heart rate, blood pressure, plasma glucose and oxygen consumption, which are indicative of augmented sympathoadrenal outflow (Brown et al. 1982; Fisher and Brown 1991).

Soon after their discovery, clinical observations supported an involvement of CRH and CRHR1 in stress-related diseases, including mood and anxiety disorders. Patients suffering from major depression present with HPA axis disturbances such as elevated plasma cortisol and adrenocorticotropic hormone (ACTH) levels as well as impaired negative feedback regulation (Holsboer 2000). Post mortem studies showed increased levels of CRH in the cerebrospinal fluid, an upregulation of CRH in the PVN and a compensatory reduction of CRHR1 binding sites in the prefrontal cortex of suicide victims (Arato et al. 1989; Nemeroff et al. 1988; Raadsheer et al. 1994). Remarkably, successful antidepressant treatment can restore HPA axis function and CSF levels of CRH (Ising et al. 2007). A wealth of preclinical and clinical findings has implicated CRHR1 as a promising target for the next generation of antidepressants and anxiolytics (Holsboer 1999; Sanders and Nemeroff 2016). However, after an initial successful study, all subsequent clinical trials failed to demonstrate sufficient efficacy and this has stalled any further development of CRHR1 antagonists (Griebel and Holsboer 2012; Zobel et al. 2000). Nevertheless, the CRHR1 remains an interesting target and the implementation of personalized approaches might help to revisit potential therapeutic strategies based on the CRH/CRHR1 system (Spierling and Zorrilla 2017).

Genetic mouse models underscore the implication of the CRH/CRHR1 system in anxiety-related behaviour (Timpl et al. 1998; Smith et al. 1998), which is independent of the HPA axis disturbances present in CRHR1 knockout mice (Muller et al. 2003). Importantly, mouse models have also revealed that the system is more complex than originally anticipated. For example, CRHR1 is capable of modulating anxiety-related behaviour bidirectionally depending on its cellular localization in glutamatergic or dopaminergic neurons (Refojo et al. 2011; Henckens et al. 2016). Moreover, the effects of CRH are influenced by the individual's previous

experience. Severe stress exposure can, for example, switch the response to CRH from appetitive to aversive (Lemos et al. 2012).

The current progress in basic neuroscience research provides refined tools for in-depth analysis and manipulation of complex ligand/receptor systems from the molecular to the neurocircuit level. CRH effects are traditionally segregated functionally and spatially: in the context of the HPA axis, CRH is regarded as a classical hypothalamic releasing hormone while CRH is considered as a neuromodulator when engaged in neurotransmission and interneuronal communication. Along these lines, it has been a long-standing perception, virtually a dogma, that hypothalamic CRH primarily regulates the activity of the neuroendocrine stress system whereas the modulation of stress-related behaviours is attributed to extrahypothalamic CRH sources.

In this chapter we will focus on the hypothalamic CRH/CRHR1 system, its distribution, physiology and regulation. We will particularly highlight recent findings which provide ample evidence for the convergence of neuroendocrine, autonomic and behavioural responses to stress onto CRH-related neurocircuits within the paraventricular hypothalamic nucleus.

9.2 Hypothalamic Expression of CRH

The antibody-based detection of neuropeptides is frequently hindered by their comparably low baseline expression accompanied by rapid clearance from the neuronal soma via axonal transport in large dense-core vesicles. Experimentally, this can be overcome using a colchicine pretreatment, which blocks vesicular transport and allows visualization of peptide accumulation in the soma (Merchenthaler et al. 1982; Cummings et al. 1983). Application of colchicine, however, is itself a stressor and might also affect the expression of stress-responsive neuropeptides such as CRH (Alonso et al. 1986). Therefore, mRNA in situ hybridization (ISH) has proven to be a valuable and sensitive complementary approach to address the spatial CRH expression pattern in the brain at baseline and following stress (Keegan et al. 1994).

In Vivo Access to CRH Neurons

In the past decade, we have witnessed the establishment of rodent genetic tools, i.e., CRH reporter mice and rats, which provide a previously unmet level of sensitivity to understand peptide expression and distribution of CRH^+ neurons in the rodent brain. Direct reporters have been developed, for example, by integrating a fluorescent protein into the CRH gene. Thus, reporter gene expression reflects the current state of CRH production. However, the relatively low expression level usually requires amplification by antibody staining (Kono et al. 2017; Alon et al. 2009). Indirect reporter mice are

(continued)

based on the expression of Cre recombinase under the control of the CRH promoter. CRH^+ neurons can be visualized by breeding general Cre reporter mice or by local application of viral vectors expressing Cre-dependent reporters (Taniguchi et al. 2011; Krashes et al. 2014; Pomrenze et al. 2015; Itoi et al. 2014). Indirect reporters provide the highest sensitivity, as the reporter is usually driven by a strong promoter. However, this approach cannot discriminate between current and legacy expression, which is caused by any transient activation of the reporter, e.g., during developmental stages resulting in permanent reporter gene expression. In addition, a significant time lag between induction of CRH expression and detection of the indirect reporter has to be considered in experiments addressing induction of de novo expression of CRH. It is of note that the regulatory elements of the CRH gene are not yet fully understood. Thus, knock-in strategies have proven their superiority compared to transgenic strategies involving short promoter fragments or even bacterial artificial chromosome (BAC)-based constructs when carefully comparing the exogenous with the endogenous expression pattern (Chen et al. 2015; Dedic et al. 2018a).

Within the hypothalamus, CRH expression is dominated by the PVN but cells expressing CRH are also found in the lateral (LPOA) and medial preoptic area (MPOA), the lateral (LH) and dorsomedial hypothalamus (DMH), the perifornical area (PFA) and in scattered neurons of the posterior periventricular zone and the suprachiasmatic nucleus (SCN) (Keegan et al. 1994; Merchenthaler et al. 1982; Cummings et al. 1983). Reporter mice confirmed previously identified hypothalamic expression and identified additional CRH^+ neurons in the anterior (AHA) and posterior hypothalamic area (PHA), the ventromedial hypothalamus (VMH) as well as the arcuate nucleus (Arc) (Walker et al. 2019; Peng et al. 2017) (Fig. 9.1).

Hypothalamic CRH neurons display rather small somatic volumes with simple dendritic branches and present only limited numbers of spines (Wang et al. 2021). A molecularly more comprehensive characterization has been obtained by single-cell RNA sequencing, demonstrating that hypothalamic CRH is present in different inhibitory and excitatory neuronal populations (Romanov et al. 2017b; Kim et al. 2020). CRH was primarily found in GABAergic neurons either positive for LIM homeobox 6 or G-protein coupled receptor 15-like. In another study, CRH expression defined a subcluster of neurotensin-positive GABAergic neurons in the hypothalamus (Mickelsen et al. 2019). In addition, CRH was identified in two populations of glutamatergic neurons, confirming previous results that had shown that PVN^{CRH} neurons co-express the vesicular glutamate transporter 2, similar to CRH neurons in the piriform cortex (Dabrowska et al. 2013; Dedic et al. 2018b). Further evidence for the PVN-restricted presence of CRH in glutamatergic neurons originates from conditional CRH knockout mice using the Dlx5/6-Cre driver, which directs Cre-mediated recombination to forebrain GABAergic neurons. These knockout mice lack CRH in the entire hypothalamus but spare CRH expression in the PVN

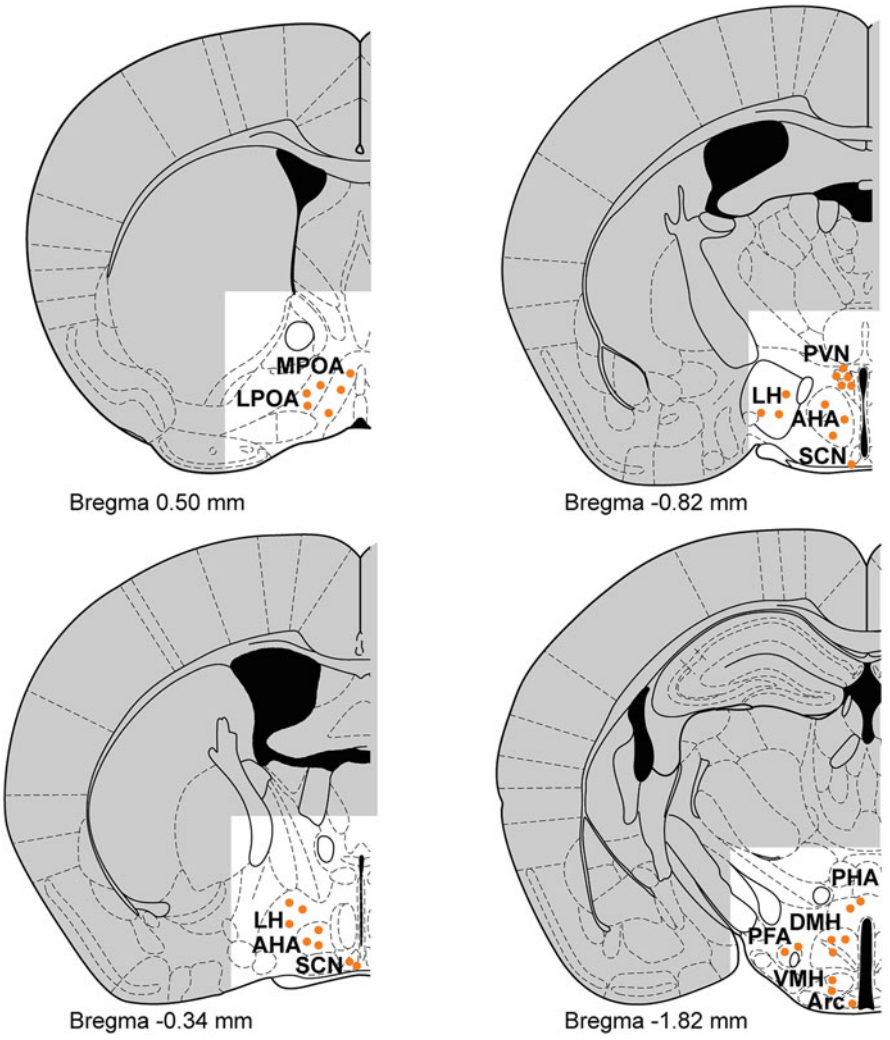

Fig. 9.1 CRH-expressing neurons in the murine hypothalamus. Representative coronal brain sections covering the murine hypothalamus. CRH-expressing somata are illustrated as filled orange circles. CRH neurons in extrahypothalamic areas are not depicted. Abbreviations: *AHA* anterior hypothalamic area, *DMH* dorsomedial hypothalamus, *LA* lateral hypothalamus, *LPOA* lateral preoptic area, *MPOA* median preoptic area, *PFA* perifornical area, *PHA* posterior hypothalamic area, *PVN* paraventricular nucleus of the hypothalamus, *SCN* suprachiasmatic nucleus

and thus exhibit normal HPA axis function (Dedic et al. 2018b). Reporter mice in combination with immunohistochemistry revealed that PVN^{CRH} neurons are unique with regard to their co-expression of other peptides. About 30% of CRH neurons contain neurotensin and 20% enkephalin, while only a small fraction of parvocellular PVN^{CRH} neurons is also positive for cholecystokinin, galanin or

vasoactive intestinal polypeptide (Ceccatelli et al. 1989). There is no overlap with thyrotropin-releasing hormone or somatostatin and only limited co-expression with oxytocin and arginine vasopressin (AVP) (Wamsteeker Cusulin et al. 2013). Under conditions of low circulating corticosterone, however, the overlap with AVP increases significantly, which is in line with the potentiation of ACTH secretion by AVP co-release (Gillies et al. 1982; Rivier and Vale 1983; Muller et al. 2000).

The widespread distribution of CRH in different populations of hypothalamic neurons is in accordance with observations in the hippocampus and cortex suggesting that the production of CRH reflects a functional modality that is acquired by different types of neurons, rather than a classifier defining neuronal identity (Gunn et al. 2019; Kubota et al. 2011; Romanov et al. 2017a).

9.3 Connectivity of Hypothalamic CRH Neurons

The afferent and efferent connections of the vast majority of CRH^{+} neurons in the hypothalamus have not been explored yet using modern anterograde and retrograde tracing tools. Only parvocellular PVN^{CRH} neurons have been studied in greater detail in this regard. PVN^{CRH} neurons project to the external zone of the median eminence to release their peptide cargo to the portal vasculature (Lennard et al. 1993). Whole-brain mapping of afferents of PVN^{CRH} neurons by rabies virus-mediated trans-synaptic retrograde tracing using CRH-ires-Cre mice revealed that PVN^{CRH} neurons integrate information from a plethora of different stress- and reward-related brain areas (Fig. 9.2). PVN^{CRH} neurons receive excitatory inputs from several stress-related brain areas, such as the prefrontal cortex (PFC), paraventricular thalamus (PVT), ventral hippocampus (vHPC) and parabrachial nucleus (PBN), to rapidly activate PVN^{CRH} neurons. At the same time, several nuclei, such as the lateral septum (LS), raphe magnus nucleus (RMg) and bed nucleus of the stria terminalis (BNST), send direct long-range GABAergic inputs onto PVN^{CRH} neurons. Together, these presynaptic stress and reward circuits provide the means to bidirectionally modulate dynamics and plasticity of PVN^{CRH} neurons (Fig. 9.2) (Yuan et al. 2019).

Efferent projections of PVN^{CRH} neurons have been characterized by injecting AAVs expressing a Cre-dependent anterograde tracer into the PVN of CRH-ires-Cre mice. As expected, this approach revealed massively labelled axon terminals within the median eminence but also moderate to dense projections in multiple sites throughout the brain. Abundant PVN^{CRH} fibres were identified in the cingulate cortex, anterior and medial amygdala, LS, subnuclei of the BNST and nucleus accumbens, as well as in multiple intrahypothalamic sites (Zhang et al. 2017). In contrast, Fuzesi and colleagues detected projections of PVN^{CRH} neurons to the LH only, which target an electrophysiological defined population of LH neurons (Fuzesi et al. 2016). Whether these neurons are identical to a population of hypocretin (HCRT)-expressing neurons, which were identified by retrograde trans-synaptic rabies tracing as monosynaptically innervated by PVN^{CRH} neurons, remains to be investigated (Li et al. 2020).

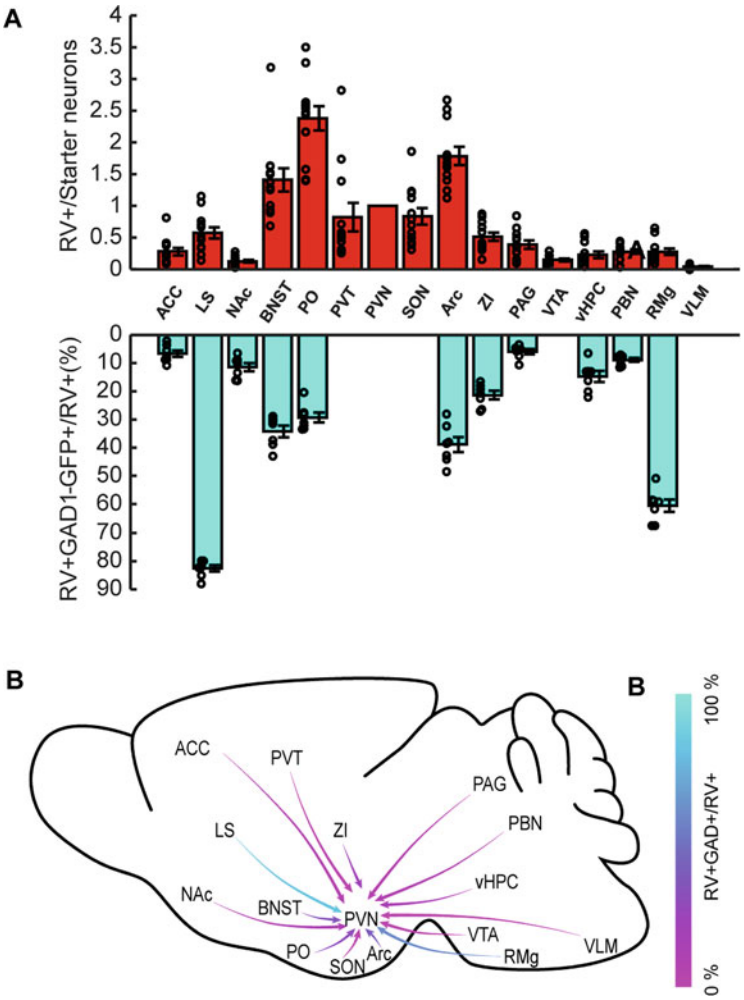

Fig. 9.2 Presynaptic partners of PVN^{CRH} neurons. Rabies virus (RV)-mediated trans-synaptic retrograde tracing. (**a**) Upper bar graph illustrates brain-wide distribution of neurons labelled by retrograde trans-synaptic tracing. Lower bar graph illustrates the proportion of rabies virus and glutamate decarboxylase 1 (GAD1)-positive neurons in each input nucleus. (**b**) A whole-brain model of selected monosynaptic afferents onto PVN^{CRH} neurons. Colours of the arrow encode the proportion of PVN^{CRH}-projecting GABAergic neurons in each input nucleus. Blue, 100%, magenta, 0%. (Modified with permission from Yuan et al. 2019)

9.4 CRH—Master Regulator of the HPA Axis

CRH controls the daily rhythm of ACTH and glucocorticoid secretion and regulates the stress-induced activation of the HPA axis (Herman et al. 2003). CRH is synthesized in parvocellular neurons in the dorsomedial aspect of the PVN, which integrate excitatory and inhibitory afferents to convey a net secretory signal to the anterior pituitary (Herman et al. 2003). CRH is stored in large dense-core vesicles (LDCVs) and transported to nerve terminals located in the external zone of the median eminence (Merchenthaler et al. 1984). Exocytosis and release of LDCV content is regulated by the formation of a SNARE complex, which allows fusion with the cell membrane (Pang and Sudhof 2010). In the median eminence, CRH is co-localized with the calcium-sensing protein secretagogin (SCGN), which has been found in neuroendocrine cells including parvocellular neurons of the PVN (Mulder et al. 2009). SCGN directly interferes with CRH release, thus limiting hormonal responses to stress (Romanov et al. 2015). After its release, CRH reaches the anterior pituitary via the hypothalamic–pituitary portal vasculature, binds to CRHR1 present on corticotropes and triggers the secretion of ACTH into the circulation. In turn, ACTH stimulates the synthesis and release of glucocorticoids from the zona fasciculata of the adrenal gland. Cortisol (in primates) and corticosterone (in rodents) are the key effectors of the stress response and are indispensable for successful recovery and adaptation to internal or external threats to homeostasis (de Kloet et al. 2005). Glucocorticoid effects are mediated via two nuclear receptors: the glucocorticoid and the mineralocorticoid receptor. These play also a fundamental role in negative feedback inhibition of the HPA axis to keep glucocorticoid levels in a tolerable range involving genomic and non-genomic mechanisms (Tasker et al. 2006). In addition, HPA axis activity is controlled on the level of the PVN by changes in neuronal plasticity. Plasticity is shaped by afferents of local stress-responsive GABAergic neurons (Herman et al. 2002) and by long-lasting suppression of N-methyl-D-aspartate (NMDA) receptors, which converts parvocellular neurons into a primed state and thereby increases hormonal responses to a novel stressor (Kuzmiski et al. 2010; Bains et al. 2015). PVN^{CRH} neurons show tonic activity in the absence of external threat stimuli. PVN^{CRH} neurons adapt to homotypic stressors but this adaptation is not mediated by negative feedback of corticosterone. Although negative corticosterone feedback suppresses ACTH secretion, it has only a minor effect on CRH neuron activity. Accordingly, corticosterone inhibits the tonic activity of PVN^{CRH} neurons but not stress-induced activity (Kim et al. 2019b).

9.5 A CRH-CRHR1 Microcircuit Within the PVN Controls HPA Axis Activity

The establishment of BAC-transgenic CRHR1-GFP reporter mice revealed potentially CRHR1-expressing neurons in the PVN (Justice et al. 2008). These CRHR1-GFP neurons are responsive to CRH applied by bath application but also to local CRH release induced by photo-stimulation, indicating the presence of functional

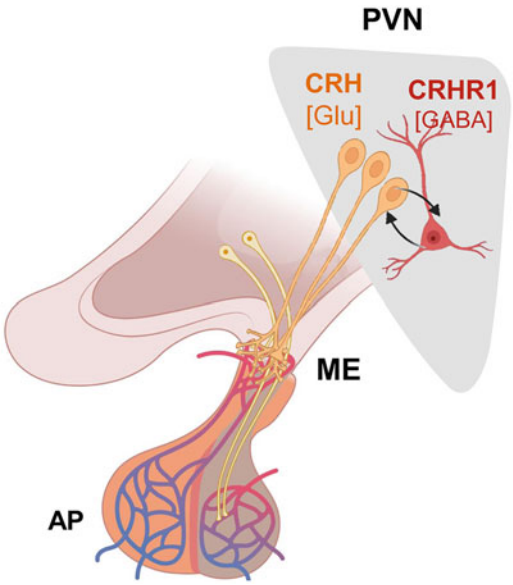

Fig. 9.3 The intra-PVN CRH/CRHR1 microcircuit. Parvocellular CRH neurons signal to CRHR1 neurons in a short negative feedback microcircuit. Released CRH activates recurrent inhibition via neighbouring GABAergic CRHR1^{+} neurons. Abbreviations: *AP* anterior pituitary, *ME* median eminence, *PVN* paraventricular nucleus of the hypothalamus

CRHR1. These neurons resemble a unique population of PVN neurons as they do not express any classical markers of magnocellular or parvocellular neurons but rather possess characteristics of preautonomic neurons that project to brainstem nuclei (Ramot et al. 2017). The majority of PVN CRHR1-GFP neurons are inhibitory, making local GABAergic synapses within the PVN. Additionally, glutamatergic CRHR1-GFP neurons exist and make long-range projections to the LS, BNST, periaqueductual grey (PAG), parabrachial nucleus (PB) and the nucleus of the solitary tract (NTS). Interestingly, a significant portion of CRHR1-GFP neurons express GABAergic as well as glutamatergic markers. PVN^{CRH} neurons make only partially synaptic contacts with CRHR1-GFP neurons but signalling seems to be also possible by CRH release involving volume transmission (Ramot et al. 2017). CRHR1-GFP neurons are positively regulated by glucocorticoids, while low glucocorticoids, as present in CRHR1-knockout mice or adrenalectomized mice, downregulate GFP expression. CRHR1 in the PVN is co-expressed with Sim1, allowing conditional PVN-specific inactivation using Sim1-Cre driver mice. Basal corticosterone levels are unaffected in CRHR1$^{CKO\text{-}Sim1}$ mice compared to control mice. However, chronic social defeat stress resulted in decreased basal corticosterone levels after the end of the stressor. These chronically stressed mice also showed reduced anxiety-related behaviour (Ramot et al. 2017). Selective ablation of PVN^{CRHR1} neurons by selective expression of diphtheria toxin resulted in HPA axis hyperactivity due to reduced feedback inhibition of PVN^{CRH} neurons (Jiang et al. 2018). These results revealed an intra-PVN CRH-CRHR1 microcircuit (Fig. 9.3) introducing a previously unrecognized level of HPA axis activity (Jiang et al. 2019).

9.6 PVN^{CRH} Neurons Are Activated by Aversive Stimuli and Regulate Stress-Induced Behaviours

Parvocellular PVN^{CRH} neurons have classically and almost exclusively been acknowledged for their role in orchestrating the neuroendocrine stress response via the HPA axis. PVN^{CRH} neurons have been demonstrated to control autonomic outflow. For example, PVN^{CRH} neurons project to sites controlling autonomic function and selective stimulation of PVN^{CRH} terminals in the NTS increases blood pressure (Wang et al. 2019). Early experiments involving electrical stimulation suggested that these cells may also regulate complex behaviours but they gained only limited attention (Kruk et al. 1998). Mice show an immediate reaction to acute stressors, e.g., a foot-shock, reflected by the expression of multiple behaviours, which differ in their duration depending on the encountered stressor and the animals' environmental context (Fuzesi et al. 2016). Interestingly, instantaneous optogenetic inhibition of PVN^{CRH} neurons following an acute stressor switched the pattern of stress-induced behaviours from self-grooming to rearing and walking. Accordingly, photoactivation of PVN^{CRH} neurons had the opposite effect, reflected by increased grooming and decreased rearing behaviour. These behavioural alterations were independent of the corticosterone surge induced by optogenetic stimulation of PVN^{CRH} neurons. Moreover, the clear context-dependence of stress-induced behavioural profiles was blunted by stimulation of PVN^{CRH} neurons. Double retrograde tracing using retrobeads and fluorogold revealed that individual PVN^{CRH} neurons project to the median eminence but at the same time send axon collaterals to other brain structures, particularly the LH. Photo-stimulation of those PVN^{CRH} fibres present in the LH had behavioural consequences similar to those seen with direct stimulation of PVN^{CRH} neurons (Fuzesi et al. 2016).

The response of PVN^{CRH} neurons has been addressed in detail by in vivo calcium imaging using fibre photometry. GCaMP6s expressing PVN^{CRH} neurons are immediately activated by a broad array of exteroceptive (e.g. forced swimming, predator odour) and interoceptive (e.g. gastric malaise, food deprivation) stressors/aversive stimuli (Fig. 9.4). On the contrary appetitive or rewarding stimuli such as accessible food or sweet solution rapidly suppressed the activity of PVN^{CRH} neurons (Kim et al. 2019a; Yuan et al. 2019). PVN^{CRH} neurons also responded to social stimuli (Fig. 9.4). Depending on the stimulus, neurons were either suppressed, e.g. when a female mouse was presented with a pup, or activated, e.g. when a mouse was attacked by an aggressive intruder. These bidirectional changes in PVN^{CRH} neuron activity suggest that these neurons convey information with regard to the valence of the encountered stimulus. Accordingly, optogenetic activation of these PVN^{CRH} neurons induces place aversion while optogenetic inhibition of the same neuronal population promotes place preference. Furthermore, photo-stimulation or -inhibition is able to blunt natural preferences (e.g. to food) or aversions (e.g. to LiCl injection), respectively (Kim et al. 2019a).

Moreover, when a rewarding stimulus is presented in conjunction with a stressor, the stress response of PVN^{CRH} neurons is significantly decreased. Similarly, rewarding sucrose-solution is able to diminish signs of a stress response that was artificially

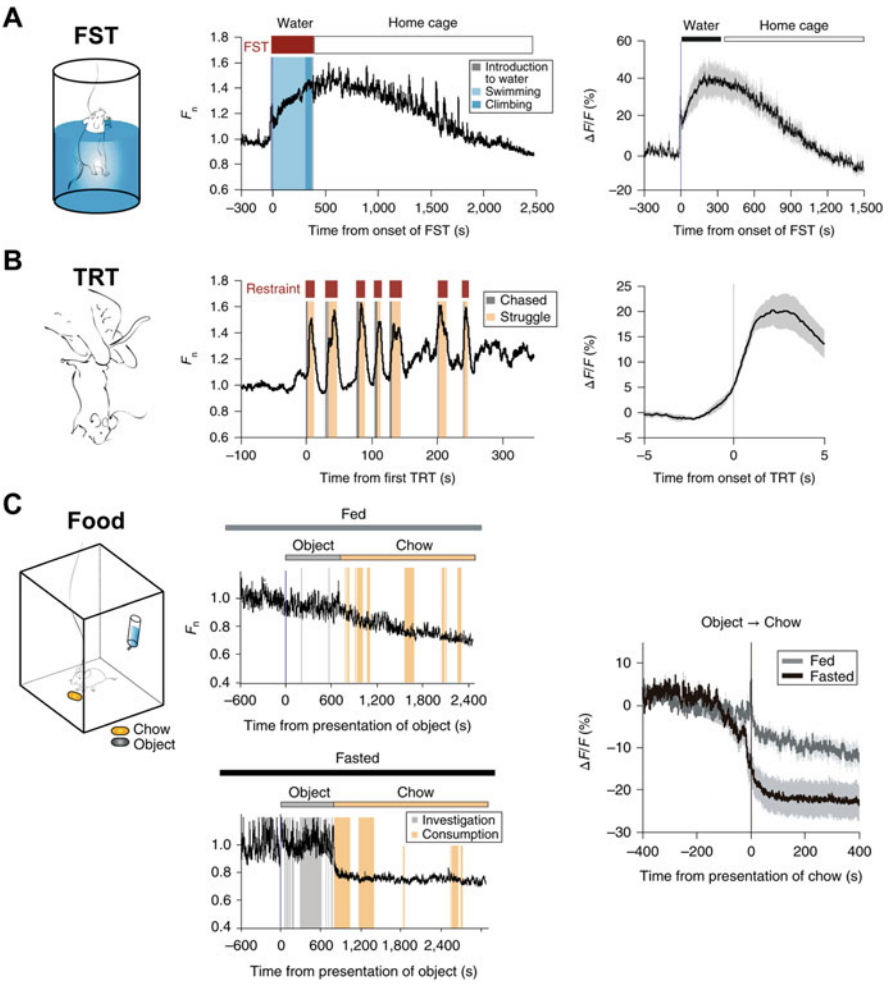

Fig. 9.4 Activation and Inhibition of PVN^{CRH} neurons. GCaMP6s was selectively expressed in PVN^{CRH} neurons and activity was recorded by fibre photometry. (**a**) Cartoon illustrating the forced swim test (FST) and a representative trace illustrating an increased GCaMP6 signal recorded from PVN^{CRH} neurons during FST (red bar, above) and decreased activity while back in home cage (white bar). Behavioural epochs, swimming (light blue) and climbing (blue) are annotated in colour-coded shaded bars. The plot shows combined data from all animals tested aligned to the start and end of FST, and the following rest in the home cage. (**b**) Cartoon illustrating the tail restraint-test (TRT) and a representative trace showing increased GCaMP6 signal from PVN^{CRH} neurons during restraint (red bars, above). Colour-coded shaded bars depict the periods during which mice were chased by a hand (grey) and struggled (beige). The plot shows a peri-event time histogram plot across all tested animals aligned to the start of TRT. (**c**) Cartoon illustrating presentation of freely accessible chow in a chamber. Representative traces showing GCaMP6 signal from PVN^{CRH} neurons of ad libitum-fed and 22-h fasted animals exposed to a non-food object (grey bar), followed by chow pellet (orange bar). Shaded bars depict the epochs during which mice investigated the non-food object (grey) and consumed the chow pellet (orange). The plot across all animals was aligned to the introduction of chow. (Modified with permission from Kim et al. 2019a)

induced by direct chemogenetic stimulation of PVN^{CRH} neurons, i.e., reducing the elevated self-grooming, anxiety and corticosterone release. Mechanistically, repeated stress upregulates glutamatergic neurotransmission and induces NMDA receptor-dependent burst firing. In this context, reward consumption is able to rebalance synaptic homeostasis by increasing inhibition and decreasing excitation resulting in abrogation of burst firing (Yuan et al. 2019).

PVN^{CRH} neurons have been further interrogated with respect to their role in innate defensive behaviours using a looming shadow paradigm as threat (Daviu et al. 2020). This advancing threat leads to an activation of PVN^{CRH} neurons and induces escape behaviour. Optogenetic inhibition switches defensive behaviours from escape to freezing, suggesting that PVN^{CRH} neurons control the balance between passive and active response strategies. Interestingly, PVN^{CRH} neurons generate a preparatory signal anticipating escape behaviour, i.e., CRH neurons are activated before the initiation of escape behaviour. Furthermore, this anticipatory signal is sensitive to stressful stimuli that have high or low levels of controllability. Stressors with high outcome control increase PVN^{CRH} anticipatory activity and thus escape behaviour. In contrast, stressors that do not allow control prevent the occurrence of anticipatory activity and subsequent escape behaviour (Daviu et al. 2020).

Another intriguing finding is the capacity of PVN^{CRH} neurons to transmit signals of distress among individuals (Sterley et al. 2018). Exposure to acute stress alters the short-term plasticity of PVN^{CRH} neuron afferents at glutamatergic synapses. Interestingly, similar changes occur at the synaptic level when naïve mice interact with a previously stressed cage-mate. The transmission of synaptic changes does not even require direct interaction between individuals but can be transferred via currently unknown chemosensory signals (Sterley et al. 2018).

9.7 Hypothalamic CRH Promotes Hyperarousal and Anxiogenic Behaviour

Conditional and constitutive CRHR1-knockout mice consistently exhibit reduced anxiety-related behaviour (Timpl et al. 1998; Muller et al. 2003; Smith et al. 1998). Surprisingly, constitutive CRH knockout mice did not recapitulate the anxiety-related phenotype of CRHR1-mutant mice (Muglia et al. 1995; Muglia et al. 2001). The underlying reasons for the observed discrepancy are unclear but different hypotheses have been put forward: (1) Early inactivation of CRH during embryonic development might induce compensatory mechanisms, including the functional substitution by UCNs or other yet undiscovered family members. (2) The constitutive disruption of CRH might entail pleiotropic effects, which together with the chronic corticosterone deficit mask the consequences on anxiety-related behaviour. (3) CRHR1 possesses to some extent tonic activity independent of ligand-based receptor activation. (4) CRH activity is only relevant under conditions of severe stress. From genomic data, there is no trace of unidentified family members in mammals and it seems unlikely that UCNs can compensate for the CRH loss as their expression is spatially more restricted and no compensatory upregulation has

been observed in CRH knockout mice. Moreover, CRHR1 knockout mice are prone to similar pleiotropic effects including a severe corticosterone deficit. Beside the observation that CRHR1 antagonists are still able to block some of the stress-induced behavioural effects in CRH knockout mice (Weninger et al. 1999), there is no experimental evidence for a constitutively active CRHR1.

The generation of a conditional CRH allele amenable to Cre-mediated inactivation allowed contesting some of the postulated explanations for the absence of any anxiety-related phenotype in constitutive CRH knockout mice (Zhang et al. 2017; Dedic et al. 2018b). Combination of the conditional CRH allele with Dlx5/6-Cre driver line results in the deletion of CRH from forebrain GABAergic neurons, including anxiety- and fear-related brain regions such as the central amygdala (CeA) and BNST, while preserving CRH expression in the PVN and thus leaving the HPA axis intact. However, anxiety-related behaviour was unaffected in $CRH^{CKO\text{-}Dlx5/6}$ mice. Interestingly, and in support of a specific role for CRH under conditions of severe or enduring stress, CRH deletion from forebrain GABAergic neurons conferred resilience to chronic social defeat stress (Dedic et al. 2019). Temporally controlled CRH deletion from long-range GABAergic projection neurons of the CeA and BNST using the tamoxifen-inducible Camk2a-CreERT2 driver line resulted in increased anxiety-related behaviour. This is in accordance with results obtained by selective deletion of CRHR1 from dopaminergic neurons in the ventral tegmental area which is the target region of CeA and BNST CRH^{+} neurons (Refojo et al. 2011). Restricted deletion of CRH from the small population of CRH in glutamatergic neurons mainly in the piriform cortex did not affect anxiety-related behaviour or the response to chronic stress (Dedic et al. 2018b).

The first conditional knockout mice targeting CRH expression in the PVN have been generated by breeding floxed CRH mice to the Sim1-Cre driver line. Sim1-Cre-mediated deletion of CRH in the hypothalamus is not as profound as in constitutive knockout mice. PVN CRH levels are reduced by 70% resulting in decreased basal, diurnal and stress-induced plasma corticosterone levels. Accordingly, the chronic corticosterone deficit results in adrenal atrophy. $CRH^{CKO\text{-}Sim1}$ mice showed markedly reduced anxiety-related behaviour in the open field, hole board, elevated plus maze and dark/light box tests compared to control mice. These behavioural alterations occurred independent of the chronic corticosterone deficit as corticosterone substitution was not able to fully restore normal anxiety related-behaviour in $CRH^{CKO\text{-}Sim1}$ mice (Zhang et al. 2017).

Another line of evidence for a direct involvement of CRH itself in PVN-controlled stress-related behaviours has been demonstrated recently. Restraint stress induces hyperarousal and insomnia, which is accompanied by activation of PVN^{CRH} neurons, as indicated by stress-induced co-expression of the immediate early gene cFos. Restraint stress specifically activates a population of PVN^{CRH} neurons that innervate wake promoting HCRT neurons in the LH. Accordingly, optogenetic stimulation of LH-projecting PVN^{CRH} neurons elicits hyperarousal and wakefulness. In contrast, chemogenetic suppression and ablation of PVN^{CRH} neurons attenuates wakefulness and locomotor activity (Ono et al. 2020). To test the direct impact of CRH on stress-induced arousal, CRH was selectively disrupted

in the PVN using CRISPR-Cas9-mediated inactivation (Li et al. 2020). Similar to the ablation of LH HCRT neurons, downregulation of CRH expression in the PVN was sufficient to block the stress-induced hyperarousal. In this context, it is of interest that GABAergic neurons in the SCN—the organism's central circadian clock—negatively regulate the activity of PVN^{CRH} neurons, which in turn positively regulate wake promoting HCRT neurons (Ono et al. 2020).

Taken together, these results demonstrate that the constitutive deletion of CRH might have been hampered by compensatory and pleiotropic effects due to early deletion throughout the brain, which has been unmasked by conditional strategies of CRH inactivation. In addition, it has become apparent that the function of CRH in parvocellular PVN neurons extends beyond the simple regulation of HPA axis activity but is an integral part of PVN's capability to orchestrate stress-induced behaviours.

9.7.1 Perspectives

CRH in parvocellular neurons of the PVN is well known for its role in activating and controlling HPA axis activity. In particular, genomic and non-genomic glucocorticoid-driven mechanisms promote negative feedback inhibition and tightly regulate HPA axis function. Only recently, with the advent of CRHR1 reporter mice, an intra-PVN CRH/CRHR1 system has been identified and characterized. This microcircuit represents an immediate response system providing another level of neuroendocrine control over the HPA axis. To what extent this microcircuit is also involved in stress-induced behaviours remains to be further investigated. Functional interrogation of PVN^{CRH} neurons is complicated by the inseparability of their neuroendocrine and behavioural functions. In this regard, it will be of utmost importance to better understand to what extent PVN^{CRH} neurons projecting to the median eminence simultaneously send axon collaterals to brain regions relevant to behavioural stress responses.

Fostered by the availability of optogenetic and chemogenetic tools, in recent years we have seen an increasing number of studies focussing on the behavioural stress response conveyed by PVN^{CRH} neurons. These studies have demonstrated that PVN^{CRH} neurons are activated immediately and even anticipatorily upon external threats. Conversely, inhibition of PVN^{CRH} neurons, e.g., by appetitive stimuli, is able to attenuate the stress response. PVN^{CRH} neurons encode a broad spectrum of properties allowing for bidirectional control of behaviour, including selection of suitable innate defensive behaviours or social transmission of distress signals. These findings suggest that PVN^{CRH} neurons control the transition to a state that is permissive for motor action enabling the engagement in stress-related behaviours (Daviu and Bains 2021). It is highly likely that PVN^{CRH} neurons are not a homogenous population but might comprise functionally distinct populations, which could be addressed by applying intersectional approaches in the future. Furthermore, it would be highly relevant to better understand the stimuli that trigger neuropeptide release from CRH neurons. With the establishment of G-protein coupled receptor-

activation based sensors, in vivo monitoring of CRH release might be within reach in the near future. Finally, it is remarkable that hypothalamic neurons outside of the PVN have largely been neglected in the past with regard to their physiology although there is evidence that they also contribute to the neuroendocrine, autonomic and behavioural stress response.

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Multifactorial Regulation of the Activity of Hypophysiotropic Thyrotropin-Releasing Hormone Neurons

10

Patricia Joseph-Bravo, Lorraine Jaimes-Hoy, Adair Rodríguez-Rodríguez, Marco Parra-Montes de Oca, Rosa María Uribe, and Jean-Louis Charli

Abstract

Hypophysiotropic neurons of the paraventricular nucleus of the hypothalamus that express thyrotropin-releasing hormone (TRH) control the synthesis and release of thyrotropin, the pituitary hormone that regulates the synthesis and release of thyroid hormones. Thyroid hormones are pleiotropic hormones with multiple functions involved in growth, development, and energy homeostasis. The TRH neuroendocrine cells receive neuronal inputs from different parts of the brain, as well as local and hormonal signals, and integrate and transduce the information as a hormone (TRH) output. They are glutamatergic neurons; most co-express cocaine- and amphetamine-regulated transcripts; however, their transcriptomic characterization is still in its infancy but suggests functional diversity. Transcription of the *Trh* gene is rapidly but transiently increased by multiple signals, some of which also cause the release of TRH. This review summarizes the basic mechanisms involved in the generation of TRH in hypophysiotropic neurons and turnover in median eminence, and recapitulates the multiple factors that regulate *Trh* synthesis and the amount of TRH that reaches thyrotropes, the physiological conditions and environmental stressors that alter TRH neurons and thyroid axis status during development and in adult animals, as well as critical sex differences.

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Keywords

TRH · TRH-DE · Hypophysiotropic neurons · Tanycytes · Stress · Sex differences · HPT · Prolactin

List of Abbreviations

2-AG	2-arachinodonoylglycerol (Italics are used for gene or mRNA names, and capital letters, for peptides/proteins)
3V	Third ventricle
αMSH	α-melanocyte stimulating hormone
a	Anterior
A2 or 6	Group 2 or 6 noradrenergic neurons
ACTH	Adrenocorticotropin hormone
ADRB3	Beta 3 adrenergic receptor
AgRP	Agouti related protein
Arc/ARC	Hypothalamic arcuate nucleus
BAT	Brown adipose tissue
BDE-209	Decabromodiphenyl ether
BNST	Bed nucleus of the stria terminalis
C1–3	Catecholaminergic neurons
CART	Cocaine and amphetamine regulated transcript
CB1R	Cannabinoid receptor 1
CPE	Carboxy-peptidase E
CRE	Cyclic AMP response element
CREB	cAMP-response element binding protein
CRH	Corticotropin releasing hormone
DA	Dopamine
DAGLα	Diacylglycerol lipase α
DBH	Dopamine beta-hydroxylase
DEX	Dexamethasone
DIO1	Deiodinase type 1
DIO2	Deiodinase type 2
DIO3	Deiodinase type 3
DMH	Dorsomedial hypothalamic nucleus
E	Embryonic day
E2	17β-oestradiol
EDCs	Endocrine disrupting chemicals
EM66	Secretogranin II-derived peptide
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERα or βb	Estrogen receptor α or β
fc	Fenestrated capillaries
GABA	γ-aminobutyric acid

GAD	Glutamic acid decarboxylase
GATA2	GATA-binding factor 2
GC	Glucocorticoid
GLRA1,2,3	Glycine receptor alpha 1, 2 or 3
GLRB	Glycine receptor beta
Glu	Glutamate
GluR	Glutamate receptor
GLYT2	Glycine transporter 2
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HDAC2 or 3	Histone-deacetylase 2 or 3
HLHP-2	Helix-loop-helix protein 2
HPA	Hypothalamic-pituitary adrenal
HPT	Hypothalamic-pituitary-thyroid
Jun	AP-1 transcription factor subunit p39
KLF10	Krüppel like factor 10
KO	Knockout
LC	Locus coeruleus
LH	Lateral hypothalamus
LXR	Liver X receptor
m	Medial
MAPK	Mitogen-activated protein kinase
MBH	Mediobasal hypothalamus
MC4R	Melanocortin 4 receptor
MCT8	Monocarboxylate transporter 8
ME	Median eminence
MS	Maternal separation
n	Undetermined position
NCoR	Nuclear receptor co-repressor
NE	Norepinephrine
NPY	Neuropeptide Y
NTS	Nucleus of solitary tract
p	Posterior
PAM	Peptidylglycine alpha-amidating monooxygenase
PBDEs	Poly-brominated diphenyl ethers
PC	Prohormone convertase
pCREB	Phosphorylated cAMP-response element binding protein
PKA	Protein kinase A
PKAc	Catalytic subunit of PKA
PLZF	Promyelocytic leukemia zinc finger protein
PND	Postnatal day
PNMT	Phenylethanolamine n-methyl transferase
POMC	Proopiomelanocortin
PRL	Prolactin

pSTAT3/5	Phosphorylated signal transducer and activator of transcription 3/5
PVN	Hypothalamic paraventricular nucleus
QC	Glutaminyl cyclase
RMg	Raphe magnus
rT3	Reverse T3
RVLM	Rostral ventrolateral medulla
RXR	Retinoid X receptor
SCN	Suprachiasmatic nucleus
scRNAseq	Single cell RNA sequencing
SIM2	Single-minded homolog 2
SOCS-3	Suppressor of cytokine signaling-3
STAT3	Signal transducer and activator of transcription 3
T2	3,5-diiodothyronine
T3	3,3′,5 triiodo-L-thyronine
T4	Thyroxine
TBBPA	Tetrabromobisphenol A
TBT	Tributyltin
TCS	Triclosan
TH	Thyroid hormone
THR	Thyroid hormone receptor
THRE	Thyroid hormone response element
THRα or β	Thyroid hormone receptor α or β
TIDA	Tuberoinfudibular dopaminergic
TPO	Thyroid peroxidase
TRH	Thyrotropin releasing hormone
TRH-DE	TRH-degrading ecto-enzyme
Trhr, TRH-R1	TRH receptor-1
TSH	Thyrotropin
TSHR	Thyrotropin receptor
UCP-1	Uncoupling protein 1
VGLUT2	Vesicular glutamate transporter 2
VLPAG	Ventrolateral periaqueductal gray
VMH/VMN	Hypothalamic ventromedial nucleus
Y1 or Y5 receptor	NPY receptor 1 or 5

10.1 Introduction

Although evidence of hypothalamic control of anterior pituitary hormones began to accumulate in the 1940s, it was not until 1969 that the structure of a hypothalamic factor that induces the secretion of thyrotropin was solved (reviewed in Joseph-Bravo et al. 2015a); it received the name thyrotropin-releasing hormone (TRH) (Boler et al. 1969; Burgus et al. 1969). The hypothalamus has long been considered the center of homeostasis. Localized at the base of the brain, it contains nuclei that contact many areas in the brain; among these hypothalamic nuclei, the paraventricular nucleus (PVN) contains neurons whose axon terminals do not make synaptic contacts with other neurons but instead approximate fenestrated capillaries. Some of the neurons with a small somatic size, called parvocellular, project their axons to the median eminence, where they secrete releasing or inhibiting factors near portal vessels that transport them to the anterior pituitary to control synthesis and release of adenohypophysial hormones; they are called hypophysiotropic neurons (Watts 2015). Hypophysiotropic PVN neurons that express TRH control the synthesis and release of thyrotropin (TSH), the pituitary hormone that regulates the synthesis and release of thyroid hormones (TH, T4, and T3) from the thyroid gland. These neuroendocrine cells receive neuronal inputs from different parts of the brain, as well as local and hormonal signals, and transduce the integrated information as a hormone (TRH) output that controls the function of the hypothalamic-pituitary-thyroid (HPT) axis (Fig. 10.1). To study the hypophysiotropic TRH neurons it is necessary to consider the physiology of the HPT axis, including the efficient feedback mechanisms common to all neuroendocrine axes, provided by the hormones released by the target organ (in this case T4 and T3) that maintain axis homeostasis (Fekete and Lechan 2014; Hoermann et al. 2015; Joseph-Bravo et al. 2015a, b). In this chapter, we review the mechanisms that determine TRH metabolism and the activity of the hypophysiotropic TRH neurons, including findings that appeared after recent comprehensive reviews (Joseph-Bravo et al. 2015a, b, 2016; Chatzitomaris et al. 2017; Rodríguez-Rodríguez et al. 2019; Charli et al. 2020).

10.2 Hypophysiotropic TRH Neurons

TRH is a peptide formed of three amino acids with the NH and COOH terminal ends modified: pyroglutamyl-histidyl-prolinamide. Soon after its discovery (detailed in Joseph-Bravo et al. 2015a), specific antibodies recognizing TRH allowed its detection by immunohistochemistry in nerve terminals of the median eminence, as well as in neurons of the PVN, various nuclei of the hypothalamus, brain regions, and spinal cord; it was necessary to use colchicine to prevent axonal transport and observe the peptide in cell bodies (Lechan and Jackson 1982). Elucidating the mode of TRH synthesis was a challenge that was overcome by the use of recombinant DNA techniques. These permitted characterization of the mRNA sequence encoding the protein precursor of TRH (proTRH) (Lechan et al. 1986) and the identification of the

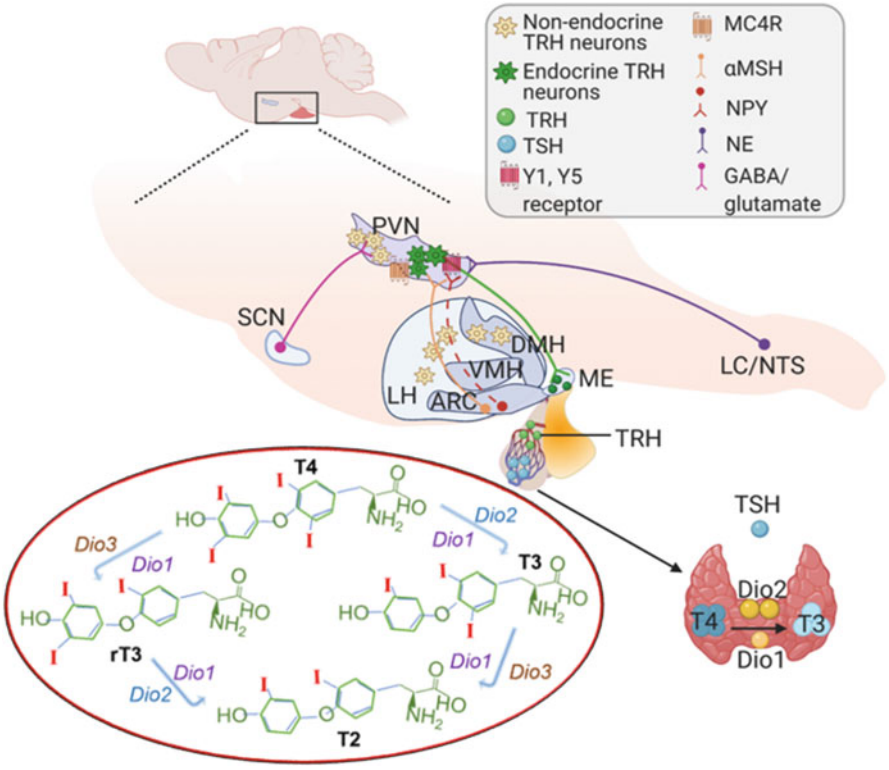

Fig. 10.1 Hypophysiotropic TRH neurons regulate the hypothalamic-pituitary-thyroid axis. In rats, hypophysiotropic TRH neurons are localized in mid-caudal PVN and receive projections from two leptin-responsive neuronal populations located in the ARC (αMSH/CART and NPY/AgRP), from the suprachiasmatic nucleus, and from adrenergic afferents from the brainstem. Hypophysiotropic TRH neurons express αMSH receptor (MC4R) and NPY receptors (Y1, Y5). Nerve terminals of hypophysiotropic TRH neurons reside in the ME, from where TRH is released next to portal vessels that transport it to the pituitary. and stimulates synthesis and release of TSH; TSH regulates in the thyroid synthesis and secretion of T4; T4 is partially deiodinated to T3 in thyroid and in target tissues. TH processing by deiodinases and their chemical structure are depicted on the left; light blue circles represent iodine atoms. Figure created with BioRender.com, modified from Joseph-Bravo et al. (2015b, 2017)

gene (Lee et al. 1988). These discoveries led to complementary immunohistochemical approaches with proTRH antibodies, and in situ hybridization with nucleotide probes against proTRH mRNA, which revealed rat PVN parvocellular and some magnocellular cell bodies containing *Trh* mRNA/proTRH in the PVN and in neurons of other hypothalamic nuclei (Lechan et al. 1987).

The neuroanatomical localization of TRH hypophysiotropic neurons was evidenced by the systemic administration of fluorogold into rats. The tracer was taken up by the median eminence and transported retrogradely to neurons in the PVN that express *Trh* mRNA (Fekete et al. 2000). Hypophysiotropic TRH neurons

are thus the parvocellular cells localized in the mid and caudal zones of rat PVN. In mice, TRH neurons are localized only in the middle zone of PVN (Kádár et al. 2010). Within the mid and caudal areas of the PVN, there are also parvocellular neurons that are not hypophysiotropic, though their efferents have not been characterized. In rats, the anterior or rostral PVN contains a population of parvocellular TRH neurons that send projections into various hypothalamic and extrahypothalamic nuclei (Wittmann et al. 2009). TRH is also expressed in a few magnocellular neurons located in the mid-PVN. These neurons project to the neurohypophysis where TRH modulates oxytocin and vasopressin release (Ciosek and Izdebska 2009). This heterogeneity has to be taken into account whenever data from the whole PVN are obtained.

Additional neurotransmitters have been characterized in TRH neurons of the PVN. Eighty percent of hypophysiotropic TRH neurons express cocaine- and amphetamine-activated transcript (CART) in the rat PVN (Fekete et al. 2000), and many (not quantified) in the mouse PVN (Kádár et al. 2010). Vesicular glutamate transporter 2 is expressed in the hypophysiotropic TRH neurons, suggesting that these neurons use glutamate as neurotransmitter (Hrabovszky et al. 2005; Farkas et al. 2020). Several other peptides and neurotransmitters have been reported in parvocellular TRH neurons (Table 10.1), but a careful assignment to hypophysiotropic or non-hypophysiotropic type is lacking (Table 10.1). The variable match between parvocellular TRH neurons and some neuronal inputs (Table 10.2) suggests heterogeneity of TRH hypophysiotropic neurons, with subsets being responsive to specific stimuli (Sánchez et al. 2001). The potential heterogeneity and limited information about the proteome of hypophysiotropic TRH neurons complicates the adscription of a specific *Trh* cluster from published transcriptomes (Box 10.1, Fig. 10.2) (Chen et al. 2017; Zeisel et al. 2018) to hypophysiotropic TRH neurons. Furthermore, a discrepancy between the TRH cluster ascribed to the PVN in the study of Zeisel et al. 2018 and the chemical identity of hypophysiotropic neurons based on immunohistochemistry and in situ hybridization warrants further study.

Box 10.1. Can Non-spatial Information Provide Spatial Information About Molecular Marker Expression in the Hypothalamus? Retrieving Spatial Information with New Generation Tools

The brain is one of the organs with the most cellular diversity per unit volume. Obtaining spatial definition is always a challenge, and this is not an exception for the paraventricular nucleus of the hypothalamus, a nucleus that shows marked morphological differences in its neuronal populations, along with the expression of a large repertoire of neuropeptides. This information has been retrieved for many years with classical techniques as immunohistochemistry and in situ hybridization, methods that allow a high spatial definition of the molecular architecture. The incorporation of diverse fluorescent markers allowed the detection of more than one molecular marker from the same cell

(continued)

Table 10.1 Partial transcriptome/proteome of parvocellular PVN TRH neurons

Protein	Gene name in rodents	Localization[a]	Species	References[b]
BDNF	*Bdnf*	nPVN	Rat	Smith (1995)
CART	*Cartpt*	mPVN (80%)	Rat	Fekete (2000), Broberger (1999)
pCREB	*Creb1*	a, m, pPVN (20–40%)	Rat	Sotelo-Rivera (2017), Campos (2020)
		Not detected	Mouse	Campos (2020)
DIO3	*Dio3*	nPVN	Human	Alkemade (2005)
		ME (27%)	Rat	Kalló (2012)
EM66	*Scg2*	pPVN, ME	Rat	El Yamani (2013)
FOS	*Fos*	a, m, pPVN	Rat	Sánchez (2001)
GHSR	*Ghsr*	nPVN	Rat	dos-Santos (2018)
GLRA1,2,3, GLRB	*Glra1,2,3, Glrb*	nPVN	Mouse	Varga (2019)
GR	*Nr3c1*	mPVN	Rat	Cintra (1991), Ceccatelli (1989)
HLHP-2	*Nhlh2*	nPVN (41%)	Mouse	Jing (2004)
KLF10	*Klf10*	nPVN	Rat	Martínez-Armenta (2015)
Kv1.2, 1.3, 4.2, 4.3	*Kcna3, Kcna2, Kcnd2, Kcnd3*	mPVN	Rat	Lee (2012)
MC4R	*Mc4r*	nPVN	Rat	Harris (2001)
MCT8	*Slc16a2*	nPVN	Human	Alkemade (2005)
			Rat	Kalló (2012)
Nesfatin-1	*Nucb2*	nPVN (small %)	Rat	Kohno (2008)
PC1, PC2	*Pcsk1, Pcsk2*	a, mPVN	Rat	Sánchez (1997)
PLZF	*Zbtb16*	pPVN	Mouse	Cheng (2020)
SIM2	*Sim2*	a, mPVN	Mouse	Goshu (2004)
SOCS-3	*Socs3*	mPVN (10%)	Rat	Harris (2001)
pSTAT3, pSTAT5	*Stat3, Stat5a, Stat5b*	nPVN	Rat	Huo (2004), Perello (2010), Campos (2020)
		Not detected	Mouse	
THRα1, α2, β2 THR1α, α2, β1, β2	*Thra, Thrb*	nPVN	Human	Alkemade (2005)
			Rat	Lechan (1994)
VGLUT2	*Slc17a6*	nPVN, ME	Rat	Hrabovszky (2005)

List is presented in alphabetical order of peptide/protein names
[a]In parenthesis: percentage of TRH neurons or terminals expressing the gene or protein
[b]To save space, "et al." was omitted from references

Table 10.2 Direct neuromodulatory afferents to parvocellular PVN TRH neurons

Molecules	Origin	Localization[a]	Species	References[b]
AgRP	ARC	mPVN	Rat	Légrádi (1999), Fekete (2002a)
CART	ARC	a, m, pPVN	Rat	Fekete (2000)
CART	C1–C3	mPVN	Rat	Wittmann (2004a)
CRH		pPVN	Rat	Liao (1992)
DBH only	A2, A6	mPVN (36.5%)	Rat	Füzesi (2009)
GAD	ARC (10%)	a, mPVN	Rat	Fekete (2002b)
Galanin		a, mPVN	Rat	Wittmann (2004b)
GLYT2	RMg, VLPAG	a, m, pPVN	Mouse	Varga (2019)
NPY	ARC	m, pPVN	Rat	Toni (1990a), Liao (1991), Légrádi (1998)
NPY	RVLM, C1–C3	mPVN	Rat	Toni (1990a), Liao (1991), Wittmann (2002)
PACAP	C1	mPVN	Rat	Légrádi (1997)
PNMT	C1–C3	mPVN (63.5%)	Rat	Légrádi (1997), Wittmann (2004a), Füzesi (2009)
POMC/α MSH	ARC	a, m, pPVN	Rat	Liao (1991), Fekete (2000)
Somatostatin		pPVN	Rat	Liao (1992)
TRH		mPVN	Rat	Toni (1990b)
VGLUT2		a, m, pPVN (100%)	Rat	Wittmann (2005)

The list is presented in alphabetical order
[a]Percentage of TRH neurons contacted
[b]To save space, "et al." was omitted from references

Box 10.1 (continued)
and subject. With the accessibility of new technologies, such as powerful computers and practically unlimited data-storage units, hypothalamic investigations are starting to take advantage of bioinformatics and sequencing technologies, delivering massive amounts of information. This is the case for single-cell RNA sequencing (scRNAseq) (Fig. 10.2a), a technique that allowed more information about cell expression in the hypothalamus to be obtained than all the information gathered in the past ~60 years. scRNAseq shows that TRH neuron phenotypes of the hypothalamus are diverse (Fig. 10.2b). With this technique, a high level of mRNA counts per cell can be retrieved, surpassing classical histological methods allowing the detection of heterogeneity among individual cells. However, many cells (and their transcriptome) may be underrepresented, as just a small percentage of the

(continued)

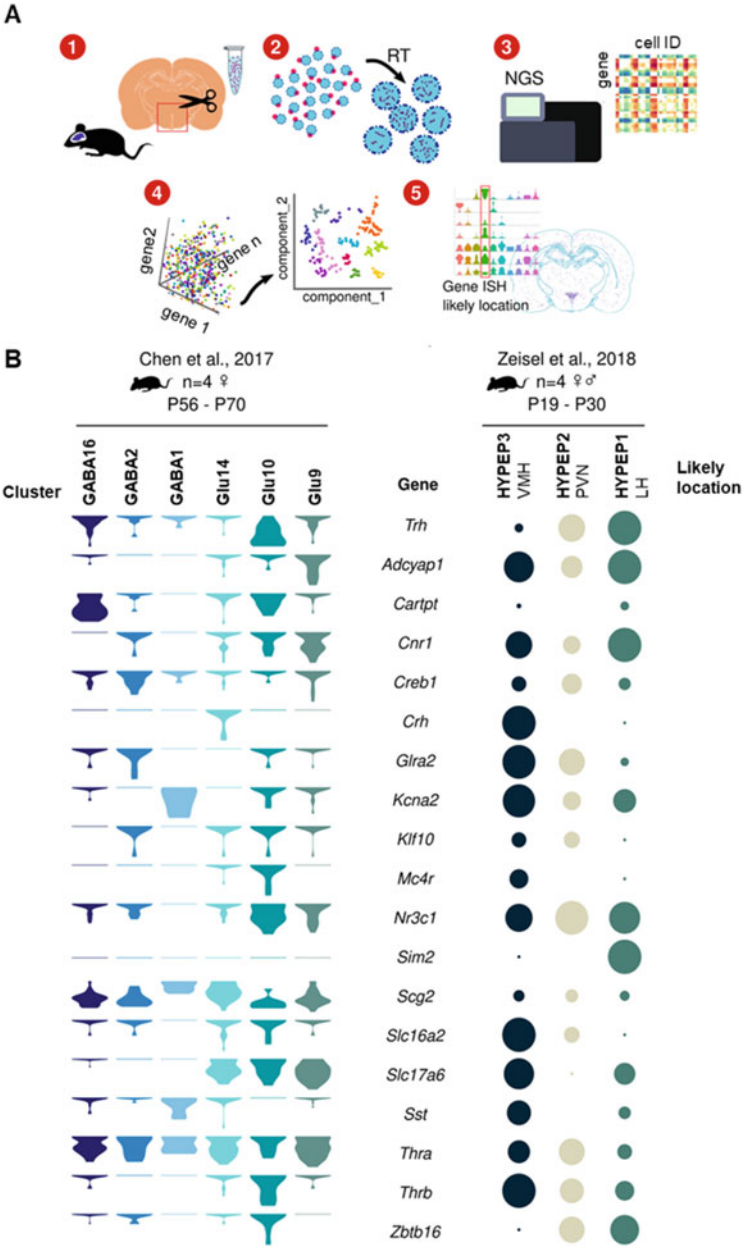
A
1
2
RT
3
NGS
gene
cell ID
4
gene2
gene n
gene 1
component_2
component_1
5
Gene ISH
likely location
B
Chen et al., 2017
n=4 ♀
P56 - P70
Zeisel et al., 2018
n=4 ♀♂
P19 - P30
Cluster
GABA16
GABA2
GABA1
Glu14
Glu10
Glu9
Gene
HYPEP3 VMH
HYPEP2 PVN
HYPEP1 LH
Likely location
Trh
Adcyap1
Cartpt
Cnr1
Creb1
Crh
Glra2
Kcna2
Klf10
Mc4r
Nr3c1
Sim2
Scg2
Slc16a2
Slc17a6
Sst
Thra
Thrb
Zbtb16

Fig. 10.2 Single-cell RNA sequencing provides a large quantity of data from a few samples; diverse types of hypothalamic TRH neurons have been identified. (**a**) To identify multiple types of cells: (1) Hypothalamus is dissected and cells disaggregated. (2) Each cell is conjugated with a barcoded bead and cDNA libraries are generated in situ. (3) Beads are subjected to next-generation DNA sequencing to obtain a matrix of gene counts per cell. (4) Each cell is represented as a vector in a space with multiple dimensions (each gene corresponds to one dimension). Dimensionality is reduced by computational analysis, resulting in grouping of cells with similar expression patterns along two components. Each group of cells or cluster represents a particular cell type or status. Clusters can be refined by subsequent analysis . (5) Cell identity and location can be confirmed with a subsequent analysis of markers with highest expression for each cluster and in situ hybridization experiments. (**b**) Chen et al. (2017) detected six *Trh* expressing clusters; violin plots refer to gene expression in log2 of the transcript count per million along the *x*-axis. Zeisel et al. (2018) identified three clusters of *Trh* expressing neurons; circles radius represent the expression value relative to peptidergic glutamatergic clusters; they also mapped the likely location in the hypothalamus with global transcriptome expression and Allen Mouse Brain Atlas. Data to generate figure **b** were obtained from two public datasets: Gene Expression Omnibus, with accession number GSE87544, and from curated data downloaded from http://mousebrain.org/downloads.html. Data were filtered using dplyr in R and LoomPy in an instance of JupyterLab. Graphs were made using ggplot2 library in R

Box 10.1 (continued)
cells are sampled, and the final decision about cell types (as clusters) depends on how the clustering algorithms are refined with different information, such as previous findings and brain expression maps. It is likely that soon enough a detailed map of the hypothalamic cell types will be prepared based on transcriptomics.

10.3 Setting the Concentration of TRH That Reaches the Anterior Pituitary; a Multi-level Task

10.3.1 TRH-Gene Transcription

The *Trh* gene has been sequenced in several species and the consensus sequences of response elements to various transcription factors have been identified in the promoter region (Hollenberg et al. 1995; Díaz-Gallardo et al. 2010a, b; Cote-Vélez et al. 2011; Guo et al. 2004) (Fig. 10.3). Initial work centered on recognizing the binding sites for thyroid hormone receptors (THR), to explain the negative feedback TH exerts on the axis; TH inhibits *Trh* transcription in vivo, exclusively on hypophysiotropic neurons of the PVN (Dyess et al. 1988; Sugrue et al. 2010). Due to the low concentrations of receptors in cells, and even lower concentrations of their transcripts, the mode of synthesis was first studied with cells transfected with *Thr* and *Trh*-gene promoter linked to a reporter such as luciferase; of the two receptors THRα and THRβ, products of independent genes, THRβ binds either as a monomer, homodimer or heterodimer with retinoid acid receptor X (RXR), to the DNA sequence called Site 4, containing half of a TH-response element (THRE), previously identified in other T3-regulated genes; increased transcription was detected in absence of T3 but repressed in its presence (Hollenberg et al. 1995). Studies with KO mice allowed NCoR to be defined as the nuclear repressor involved (Astapova and Hollenberg 2013), and the isoform THRβ2 as the one responsible for feedback (Abel et al. 2001). The model of unliganded THR bound to DNA having an opposite effect to that when THR is bound to T3 is now questioned, since in transfected cells reporter transcription lacks the constraints of chromatin, and transfection can generate receptor concentrations higher than that normally found in tissues. More sensitive methodologies such as chromatin immunoprecipitation-DNA sequencing (Nakato and Sakata 2020) have recently been applied to liver of hypo- and hyperthyroid mice; various THRE with different affinities for THRβ1 (the receptor found in liver) and for either T3-positively or -negatively regulated genes have been identified together with chromatin remodeling changes (Ramadoss et al. 2014; Grøntved et al. 2015). An analysis of the problems encountered can be found in various articles (Vella and Hollenberg 2017; Flamant et al. 2017; Sasaki et al. 2018).

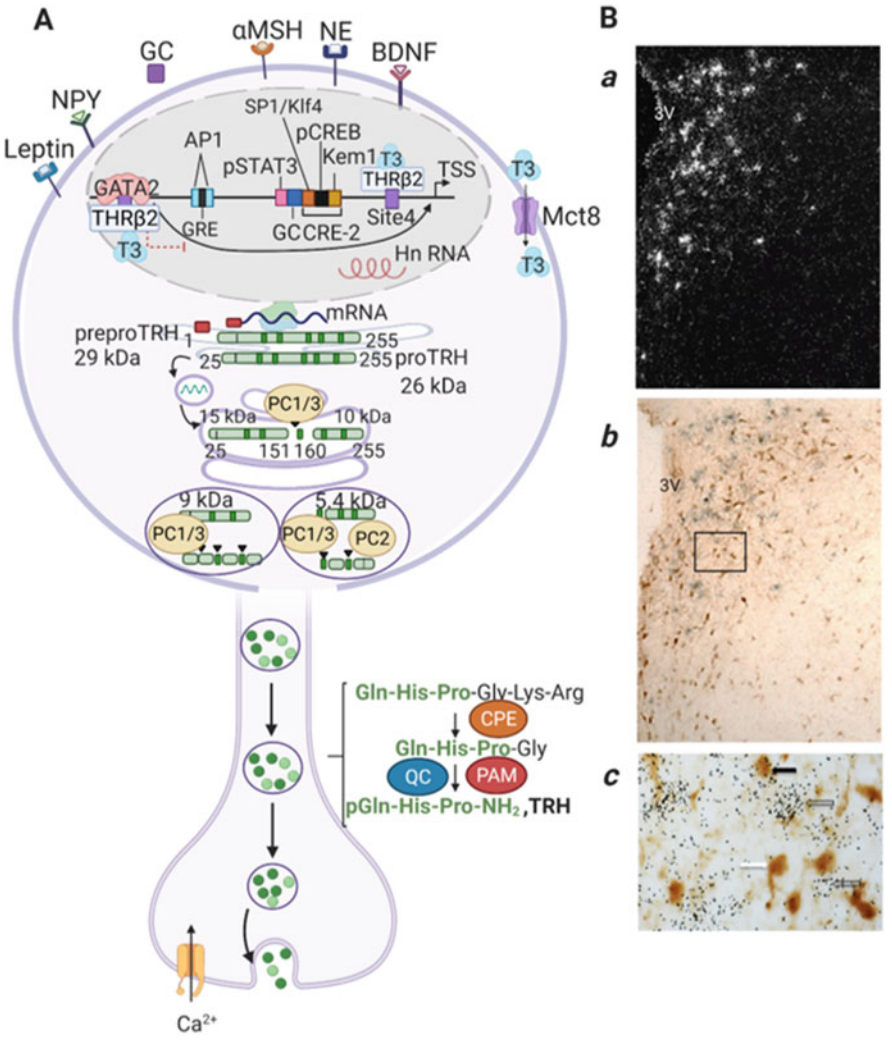

Fig. 10.3 Regulation of *Trh* transcription and proTRH processing. (**A**) Schematic representation of a TRH hypophysiotropic neuron. Identified receptors are depicted in the membrane, and some of the regulatory elements that modulate Trh transcription (mentioned in Sect. 10.3.1) are marked in the design of the *Trh*-promoter region. During translation and ER translocation, the signal sequence (red) of preproTRH is cleaved. ProTRH undergoes processing by PC1/3, PC2, CPE, QC and PAM to TRH (Sect. 10.3.2). TRH is released into the extracellular space in a calcium-dependent manner. Figure modified from Joseph-Bravo et al. (2016), Perello and Nillni (2007), created with BioRender.com. (**B**) Co-expression pSTAT3 and *Trh* mRNA in neurons at the parvocellular mid-level of the PVN of an adult male control rat, revealed by a combination of radioactive in situ hybridization (for *Trh* mRNA) and chromogenic immunohistochemistry (for pSTAT3) on rat brain slices (Bregma -1.9; Paxinos and Watson 2004). (**a**) Dark field 5X micrograph of *Trh* mRNA-positive neurons (white dots). (**b**) Bright field 5× micrograph of pSTAT3-positive cells (brown staining). (**c**) 40X magnification of rectangle in **b**); white arrow: pSTAT3-positive cell (brown color); black arrow: co-expression of *Trh* mRNA (silver grains) and pSTAT3; empty arrow: pSTAT3 negative *Trh* mRNA-positive neuron

Findings on liver samples with THRβ1 still might not be representative of regulation of TRH in PVN or TSH in adenohypophysis, since THRβ2 is the isoform involved in the negative feedback of *Trh* and *Tshb* transcription, and recent studies have identified three aminoacids present in helix 10 of THRβ2 that are responsible for the formation of a homodimer, fully functional for transcription repression of *Tshb* (Pinto et al. 2017). This supports chromatin-immunoprecipitation analyses performed on stable cell lines expressing *Trh*, showing that T3 promotes THRβ and histone-deacetylase 3 (HDAC3) binding to *Trh* promoter; binding of HDAC3 was transient (15–60 min) (Ishii et al. 2004). Similar experiments with primary cultures of rat hypothalami showed THRβ2 and HDAC2 bound to a fragment of *Trh* promoter containing Site 4 (–242/+34). It remains to be determined whether chromatin compaction due to deacetylation expels the receptor and other factors from chromatin (Díaz-Gallardo et al. 2010a; Ishii et al. 2004; Sotelo-Rivera et al. 2017). Recently, the relevance of THR binding to Site 4, and to an equivalent site in *Tshb*, has been questioned since these are near the transcription start site; instead, it is proposed that binding of transcription factor GATA2 to site –357/–352 increases *Trh* transcription, and that THRβ2-bound T3 binds to GATA2 in a tethering fashion, repressing *Trh* transcription (Kuroda et al. 2020, Fig. 10.3). Thus, the whole picture of how T3 regulates *Trh* transcription remains incomplete.

Other response elements that bind transcription factors that respond to extra- and intra-cellular signals are depicted in Fig. 10.3. Foot-printing analyses demonstrated that in primary hypothalamic neurons cAMP analogs promote nuclear proteins binding to an extended area that includes cAMP, Krüppel, KEM and SP-1 recognition elements (Díaz-Gallardo et al. 2010a, b; Cote-Vélez et al. 2011; Pérez-Monter et al. 2011). Upstream of this extended CRE the binding site for STAT3 has been detected (Guo et al. 2004). The element that binds glucocorticoid receptor (GR) is a half site that is stabilized as a heterodimer with c-JUN or c-FOS; this site is called composite GRE and its activity depends on the bound partner (Díaz-Gallardo et al. 2010b; Cote-Vélez et al. 2008). Furthermore, various transcription factors may form heterodimers with THR like RXR, LXR, to name a few. The multiple combinations of transcription factors able to bind THRE, CRE, STAT, or GRE sites are likely to contribute to the multifactorial regulation of *Trh* transcription (Joseph-Bravo et al. 2015b, 2016; Kouidhi and Clerget-Froidevaux 2018).

10.3.2 Pre-pro-TRH Translation and Processing

The primary transcript, a heterologous RNA (2.6 Kb) that contains three exons and two introns, is processed to a mature mRNA that encodes preproTRH (Lechan et al. 1986). As for all secretory proteins, after translation and translocation into the lumen of the endoplasmic reticulum, the propeptide is transported to the Golgi apparatus where it may be cleaved and sorted into secretory granules in the trans-Golgi (Fig. 10.3) (Perello and Nillni 2007). The sequence glutamine-histidine-proline-glycine that leads to TRH is repeated five times in rat and six times in human proTRH; processing enzymes convert it to pGlu-His-ProNH_2, which is the

active form. All enzymes and cofactors are inside the secretory granule and processing continues inside it during its transport through the axon; the processed peptides are enriched at the nerve terminal. In contrast to the extensive knowledge of POMC tissue-specific processing (Cawley et al. 2016; Harno et al. 2018), the functions of the diverse cryptic peptides derived from TRH precursors have been only partially characterized (Nillni 2010).

The expression of processing enzymes is regulated by many factors, including TH (Nillni 2010) and the concentration of Cu^{++} ions, which are a cofactor of the enzyme peptidyl glycine alpha-amidating monooxygenase (PAM) (Giraud et al. 1992). Furthermore, processing of neuropeptides can be affected by altered calcium homeostasis or REDOX state at the level of the endoplasmic reticulum (stressed ER), disrupting the adequate folding of the precursors necessary for their transport to the trans-Golgi; for example, fasting or increased inflammatory markers induce changes in ER molecules involved in folding; their effects have been demonstrated, for example, in an inadequate processing of POMC, decreasing αMSH levels (Cakir and Nillni 2019).

10.3.3 Signals on Hypophysiotropic TRH Neurons Soma Regulate Synthesis and Release of TRH

Immunocytochemical analyses have revealed nerve terminals containing diverse neurotransmitters and neuropeptides in close apposition to the cell body of TRH neurons, suggesting a rich innervation (see Table 10.2), although it is not always clear that they make contact on hypophysiotropic neurons; use of CART coexistence could aid this characterization (Fekete et al. 2000). Furthermore, the expression of neurotransmitter or peptide receptor(s) in the TRH neurons suggest a functional interaction. For example, αMSH nerve terminals contact TRH neurons in the PVN (Fekete et al. 2000), and TRH neurons express melanocortin receptor 4 (MC4R) (Fekete and Lechan 2014). Nerve terminals containing other Arc peptides such as NPY or AgRP (Fekete and Lechan 2014) are observed on TRH cell bodies in the PVN and the administration of these peptides in appropriate physiological conditions modifies *Trh* expression (Fekete and Lechan 2014). These somatic inputs have the potential to modify the rate of action potential generation by the TRH neurons, although this has been seldom studied. One of the few studies that monitored the spiking activity of the TRH neurons demonstrated a rapid inhibitory effect of glucocorticoids, mediated by endocannabinoid-induced inhibition of glutamate release from neurons contacting parvocellular TRH neurons in the PVN (Di et al. 2003). Most evidence about TRH neurons activity is indirect, either being gained in vitro or using serum TSH concentration as a surrogate measure of TRH release. The in vitro data suggest TRH is released by exocytosis, in response to action potentials that open voltage-dependent Ca^{2+} channels (Joseph-Bravo et al. 1979). The coexistence of neuropeptides from distinct precursors in hypophysiotropic TRH neurons (including CART), as well as the existence of two different types of secretory granule containing TRH from the first precursor cleavage (differing in

cryptic peptides) (Perello and Nillni 2007), leave unanswered questions as to how secretion is regulated. Action potential frequency dependency differs for secretory granules containing peptides and for neurotransmitter vesicles (van den Pol 2012) but, the characteristics of secretory granules exocytosis differing in peptide content are still unknown, which opens many questions regarding their function.

10.3.4 In the Median Eminence, Hypophysiotropic TRH Varicosities/Nerve Endings and Tanycytes Form a Functional Unit That Controls TRH Output into Portal Vessels

In the external layer of the median eminence, nerve varicosities and terminals of TRH neurons are localized next to other nerve terminal types, cells of the portal capillary vessels and various glial cell types including tanycytes (Fig. 10.4a, Box 10.2) (Müller-Fielitz et al. 2017; Rodríguez-Rodríguez et al. 2019). Tanycytes express TH transporters, deiodinase 2 (DIO2) and TRH-degrading ectoenzyme (TRH-DE), important elements in regulating HPT axis activity (Fig. 10.4b). In the median eminence, T4 transported from the circulation into tanycytes is deiodinated

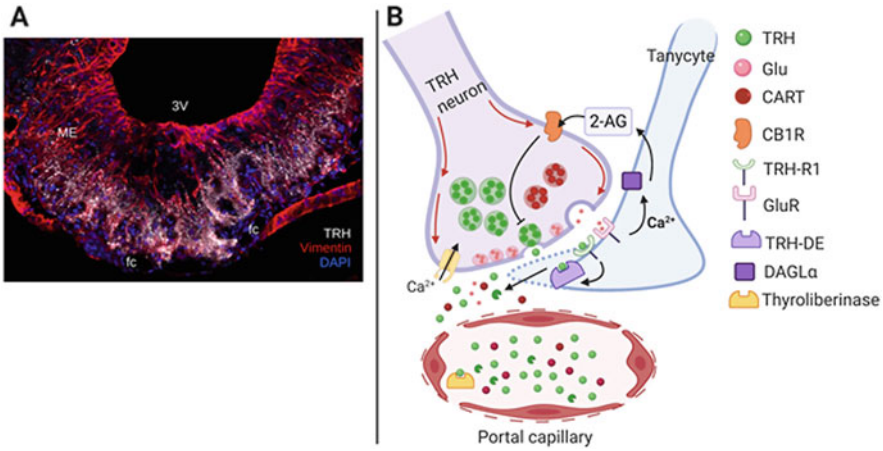

Fig. 10.4 End-feet of tanycytes of the median eminence control TRH release and extracellular turnover. (**a**) Photomicrograph showing the alignment of vimentin-expressing tanycytes of the ME along the 3V and immunodetection of TRH in terminal buttons reaching the external zone of the ME, a region irrigated by fc that allow TRH transport to the anterior pituitary. (**b**) Diagram of elements involved in controlling TRH release and turnover in the external layer of the ME. Released TRH released into the extracellular space may reach the capillary vessels and travel to the adenohypophysis, or it may interact with TRH-R1 localized on β2-tanycytes and increase the activity of TRH-DE, which inactivates TRH, and modify the position of tanycyte end feet, limiting TRH diffusion. In addition, when Glu, a co-transmitter in TRH neurons, binds to GluR in tanycytes, it increases the cytosolic Ca^{2+} concentration and the activity of DAGLα, leading to the synthesis of 2-AG. 2-AG feeds back onto TRH neurons through CB1R and inhibits TRH release. The amount of TRH that reaches anterior pituitary thyrotrophs may also be reduced by the activity of thyroliberinase, which is present in the portal capillary. Figure created with BioRender.com

to T3 by DIO2 (Fekete and Lechan 2014) and T3 is transported to the extracellular compartment of the median eminence. T3 feedback effect on TRH synthesis is proposed to be due to T3 captured by TRH nerve terminals and retrograde axonal transport to the nucleus at the level of the cell body where it inhibits transcription (Fekete and Lechan 2014). β2-tanycytes may also control the amount of TRH released from nerve terminals, based on the activity of TRH neurons. Optogenetic activation of TRH neurons in the median eminence promotes glutamate release, which enhances intracytosolic calcium concentration and diacylglycerol lipase α activity (DAGLα) in tanycytes leading to the synthesis of 2-arachinodonoylglycerol (2-AG), which feedbacks onto TRH neurons through cannabinoid receptors 1 (CB1R), thereby inhibiting TRH release (Fig. 10.4b; Farkas et al. 2020).

TRH-DE is an ectopeptidase with high specificity towards TRH, present on the surface of tanycytes, that cleaves the pyroglutamyl residue and inactivates TRH in the extracellular space after its release from nerve terminals of TRH neurons. Although the portal capillaries are fenestrated (they have transcellular pores that allow diffusion of macromolecules, including TRH released from nerve terminals), the amount of TRH that enters the lumen of the portal vessels is likely to be limited by TRH-DE activity. T3 enhances *Trhde* expression, probably contributing to a decrease in serum TSH concentration (Sánchez et al. 2009). In addition, TRH released into the median eminence interacts with TRH-receptor 1 (TRH-R1) also localized on β2-tanycyte surface and increases the activity of TRH-DE in tanycytes and the size of tanycyte end-feet, likely reducing TRH flux into the portal vessels (Müller-Fielitz et al. 2017).

Therefore, both the somatic and terminal compartments of hypophysiotropic TRH neurons dictate TRH output into portal capillaries. Furthermore, TRH output may also be further reduced by the activity of a serum isoform of TRH-DE (thyroliberinase) in the portal circulation, which is regulated by multiple factors (Charli et al. 2020).

Box 10.2. Tanycytes

Brain ventricles are lined with a simple layer of ependymocytes that allow a free exchange of molecules between cerebrospinal fluid and parenchymal tissue (neurons and other glial cells). However, selected portions of the basal medial hypothalamus have a modified type of ependymal cell with an elongated cell body whose shape resembles that of radial glia. These glial cells are called tanycytes; in contrast to ependymocytes, tanycytes are joined through tight junctions that limit the passage of substances from brain parenchyma to cerebrospinal fluid by pericellular diffusion. Cytoplasmic tanycyte extensions establish contact with neurons of nearby hypothalamic nuclei or basement membranes surrounding capillaries; various subtypes are distributed according to their dorsoventral position in the third ventricle wall. The most basic classification divides tanycytes into four subtypes: α1 tanycytes, adjacent to

(continued)

Box 10.2 (continued)
the ventromedial nucleus of the hypothalamus and part of the dorsomedial nucleus; α2 tanycytes, which mostly contact the arcuate nucleus parenchyma; β1 tanycytes, which localize to the lateral zone of the median eminence, and contact the external limiting membrane of the brain and the portal capillaries; and finally, in the ventral part of the third ventricle/parenchyma interface, β2 tanycytes, which send end feet that approximate portal capillaries in the medial zone of the median eminence. Multiple cellular properties explain the influence of tanycytes in hypothalamic signaling (Rodríguez-Rodríguez et al. 2019). They serve as a bidirectional conduit for the transfer of signaling molecules from the systemic circulation and the hypothalamus. In addition, tanycytes can sense metabolites and hormones from the cerebrospinal fluid, peripheral circulation, or arcuate nucleus, process and transmit this information to adjacent hypothalamic nuclei, through the release of multiple types of signals. Tanycytes are dynamic; they can retract their body to turn the blood–brain barrier leaky and regulate the secretion of neurohormones. Finally, tanycytes are progenitors that can differentiate into neurons and integrate into hypothalamic circuits (Goodman and Hajihosseini 2015; Prevot et al. 2018; Ebling and Lewis 2018; Bolborea et al. 2020).

10.4 Regulation of the Activity of Hypophysiotropic TRH Neurons; Impact on Thyroid Axis Function

10.4.1 Feedback Regulation of HPT Axis Occurs at Multiple Levels

As the activity of TRH hypophysiotropic neurons triggers the activity of the HPT axis, regulation of TRH-neuronal activity has been studied in many paradigms that show alterations in the circulating levels of TSH or TH. HPT axis activity is also regulated at additional levels of the axis and at targets. TH are pleiotropic hormones with many effects in almost every cell; they regulate basal metabolic rate, energy expenditure, thermogenesis, and autonomic function, to mention just a few examples (Fliers et al. 2014; Mullur et al. 2014; Giammanco et al. 2020).

The negative feedback effects of TH provide a good example of their concerted action on the HPT axis at all levels; T3 inhibits *Trh* and TSH synthesis, stimulates degradation of released TRH, TH-metabolism, and cellular transport in a tissue-specific manner (Mendoza and Hollenberg 2017; Nillni 2010; Joseph-Bravo et al. 2016; Sánchez et al. 2009; Lazcano et al. 2015); at the peripheral level, deiodinases have an exquisite control of the cellular and circulating concentration of T3 (Bianco et al. 2019). Low T3 or T4 and high TSH serum concentrations are the clinical references that define the hypothyroid condition, with the opposite for hyperthyroidism: high serum T3 and low serum TSH concentrations. These pathological

conditions are accompanied by disturbances that affect the function of many organs. Hypothyroid individuals present fatigue, weight gain, constipation, dry hair and skin, cardiovascular risk factors, dyslipidemia and increased atherosclerosis, which are many of the symptoms related to metabolic syndrome (Sanchez Jimenez and De Jesus 2020; Biondi and Cooper 2019).

10.4.2 Hypophysiotropic TRH Neurons and Nutrient Status

Alterations of nutrient status during fasting and food restriction, or during hypercaloric diet intake, change HPT axis activity by the concerted action of circulating leptin concentrations and the activity of the Arc-peptidergic neurons (Fekete and Lechan 2014; Joseph-Bravo et al. 2017). Neurons of the Arc, with a "loose" blood–brain barrier, sense hormones and metabolites that reflect the nutritional status of the organism. Two main populations of Arc neurons are those synthesizing POMC or NPY, which have opposite effects on food intake (anorectic and orexigenic respectively) and on PVN *Trh* expression (POMC neurons stimulate while NPY neurons inhibit) (Fig. 10.1; Fekete and Lechan 2014). Leptin is a hormone released by adipose tissue that stimulates the synthesis of TRH directly in PVN neurons via STAT3 activation, and indirectly through activation of αMSH (a product of POMC processing) release and CREB activation (Perello et al. 2010) (Figs. 10.3 and 10.5). Fasting produces tertiary hypothyroidism, with decreases in *Trh* expression in the PVN, TSH serum and pituitary concentrations, and circulating thyroid hormones concentrations, in a sex-specific manner (Joseph-Bravo et al. 2020). The activity of the HPT axis is inhibited by fasting because of the low levels of leptin and the high levels of corticosterone that stimulate tanycyte DIO2 activity (Fig. 10.4, Box 10.2), increasing levels of T3 in PVN neurons (Coppola et al. 2005; Fekete and Lechan 2014). In contrast, pituitary DIO2 is inhibited by fasting, decreasing available T3, and DIO1 activity is reduced in liver decreasing circulating T3. Caloric restriction, even as low as 20% of normal consumption (Uribe et al. 2014), or protein malnutrition (Pałkowska-Goździk et al. 2017), also diminish PVN *Trh* expression and HPT axis activity. Other nutritional deficiencies (Joseph-Bravo et al. 2017), or pathological conditions such as non-thyroidal illness syndrome or cachexia present tertiary hypothyroidism caused also by increased DIO2 activity in tanycytes due to increased interleukins and nuclear factor κB (Fliers and Boelen 2020).

10.4.3 Hypophysiotropic TRH Neurons and Thermogenesis

As well as their role in controlling basal metabolic rate, TH are crucial in thermogenesis (Mullur et al. 2014). In response to cold exposure, TRH neurons are activated, *Trh* synthesis and release, as well as TSH and T4 release, are stimulated within minutes (Uribe et al. 1993; Zoeller et al. 1995); the increase in *Trh* expression and TRH release is rapid and transient (30–60 min); *Trh* mRNA levels peak at 1 h,

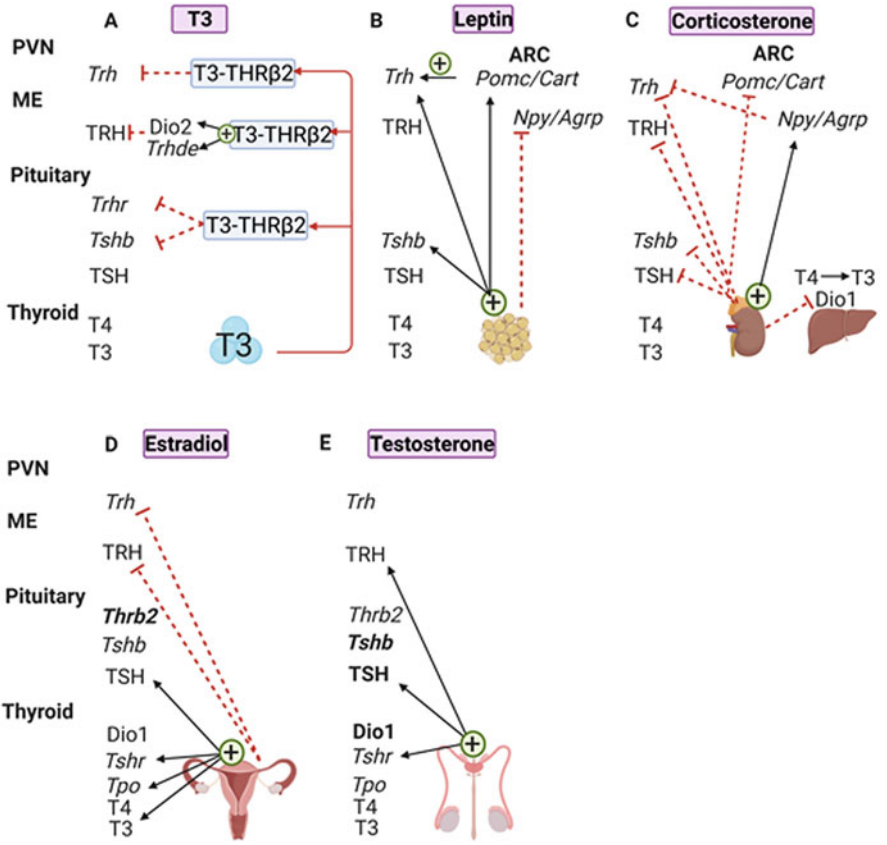

Fig. 10.5 Hormonal regulation of HPT axis activity occurs at various levels. (**a**) T3 exerts negative feedback through THRβ2 inhibiting of *Trh* synthesis and TRH output (stimulation of ME TRH-DE activity), reducting TRH signaling at the pituitary by inhibiting *Trhr* and *Tshb* expression and TSH synthesis. (**b**) Leptin stimulates *Trh* transcription directly (see Fig. 10.3) or, via regulation of ARC neuropeptides, and stimulates pituitary *Tshb* expression. (**c**) Corticosterone inhibits the expression of *Trh* in the PVN and of *Pomc* in the ARC and increases ARC-*Npy* expression. At the pituitary level, corticosterone decreases *Tshb* mRNA levels. The serum T3 concentration is also reduced by inhibition of hepatic Dio1. (**d**) E2 administration to ovariectomized rats reduces *Trh* synthesis in the PVN and secretion from the ME by as yet unidentified mechanisms; in contrast, E2 stimulates *Tshr*, and *Tpo* expression in the thyroid, increasing TSH and T3 concentrations in serum. (**e**) In orchidectomized rats, treatment with testosterone increases TRH secretion, TSH concentration in serum and *Tshr* mRNA levels in the thyroid gland. Continuous red arrows in panel **a** refer to T3 negative feedback; black arrows indicate a stimulatory effect, dashed red lines indicate inhibition. Figure created with BioRender.com

normalizing by 2 h of exposure at 5 °C, even if cold stress is extended (Uribe et al. 1993; Zoeller et al. 1995). The thermogenic effect of HPT axis activation is co-regulated by the concerted action of the sympathetic system that activates DIO2, which transforms T4 to T3 in the main thermogenic organ, the brown adipose

tissue (BAT) (Nedergaard and Cannon 2018). In BAT, the uncoupling protein 1 (UCP-1) produces heat by uncoupling ATP synthesis from oxidative phosphorylation in the mitochondria; T3 stimulates the synthesis of UCP-1, DIO2 and the adrenergic receptor ADRβ3 (Bianco et al. 2019). During cold stress, the increase in serum TSH concentration is amplified when TRH-DE activity is reduced by the injection of a specific inhibitor (Sánchez et al. 2009) or after ablation of tanycytes that does not change PVN *Trh* expression (Yoo et al. 2020), suggesting that tanycyte TRH-DE activity is limiting TSH release during a cold stress.

10.4.4 Hypophysiotropic TRH Neurons and Stress

10.4.4.1 Acute Stress

An adequate response of the HPT axis to metabolic cues, in time and intensity, guarantees energy homeostasis. Situations of stress, however, can affect this response (Joseph-Bravo et al. 2015b). Acute psychological stress such as restraint inhibits PVN *Trh* expression, pituitary TSH release and lowers the serum concentration of T3 because of inhibited DIO1 in liver (Bianco et al. 1987); since in vitro or in vivo, corticosterone administration increases *Trh* expression within 1 h, inhibition of *Trh* expression by acute stress is probably caused by neuronal inputs or by corticosterone-induced endocannabinoid inhibition of TRH neuron activity, as mentioned in Sect. 10.3.3 (Cote-Vélez et al. 2008; Di et al. 2003; Sotelo-Rivera et al. 2014).

Other types of acute stress inhibit the activity of the HPT axis (Joseph-Bravo et al. 2015b) and may also affect its response to acute cold exposure. If an animal is exposed to a short period of stress just before cold exposure, the expected increase in PVN *Trh* expression or serum TSH concentration in response to cold is not observed, an effect mimicked by injection of corticosterone 30 min before cold exposure (Sotelo-Rivera et al. 2014). The mechanism involved has been unraveled at the molecular level: cold increases the amount of pCREB in TRH neurons of the PVN in control rats but not in those injected with corticosterone; in hypothalamic cell culture, forskolin, a drug that activates protein kinase A (PKA) by releasing the catalytic subunit (PKAc), increases phosphorylation of CREB, increases pCREB in TRH neurons and pCREB binding to CRE (Díaz-Gallardo et al. 2010b; Sotelo-Rivera et al. 2014); this effect is not detected if cells are co-incubated with forskolin + dexamethasone (DEX, an analog of corticosterone that specifically binds GR). Nuclear transport of PKAc and of GR is repressed in cells incubated with forskolin and DEX, compared to the drugs alone, suggesting an interaction that impedes each other transport; in cells incubated with forskolin and DEX, physical interaction of PKAc with GR is demonstrated by co-immunoprecipitation. Since coincubation of forskolin and DEX does not stimulate binding to *Trh* promoter of a transcription factor that would recruit deacetylases and impede the binding of GR or pCREB to their response elements (Sotelo-Rivera et al. 2017), the data support a model whereby activated GR traps PKAc in the cytosol, impeding its transport to the nucleus and GR or PKAc binding to *Trh* promoter. If forskolin is added 1 h before

DEX, no interference with *Trh* expression is observed (Sotelo-Rivera et al. 2014). Timing is of utmost importance since it takes few minutes for PKAc to translocate to the nucleus after its activation whereas ligand-bound GR might take longer (Gervasi et al. 2007; Vandevyver et al. 2012; Sotelo-Rivera et al. 2017; Kim and Iremonger 2019); new techniques such as functional fluorescence microscopy imaging (Krmpot et al. 2019) and combined optogenetic approaches (Nomura et al. 2020), reduce calculated time. However, what remains irreducible is the time for a neuronal signal to activate PKA compared to that required for GR to be activated in the brain in vivo, since approximately 20–50 min are required for corticosterone to enter the brain (Joëls et al. 2012; Kim and Iremonger 2019); this is due to the time taken for CRH release, ACTH release from pituitary, ACTH signaling at the adrenal cortex and induction of synthesis and release of corticosterone since, in contrast to neurotransmitters or peptide hormones, steroids are not stored in granules and are synthesized in response to stimuli (Payne and Hales 2004); depending on the stressor, an increased serum concentration of corticosterone may be detected 15 min later, with a peak at 20–60 min (Koolhaas et al. 1997). The blunting effect of a previous corticosterone injection on the response of the hypothalamus-pituitary-adrenal (HPA) axis to a stressor was first reported as restraint-induced expression of PVN *Crh* requiring at least 30 min but no more than 180 min of corticosterone injection previous to the stressor to repress the response (Osterlund and Spencer 2011); in vitro also, DEX must be added before forskolin to stimulate *Crh* expression (van der Laan et al. 2009). GR interference on CREB phosphorylation holds for PKA and not for other kinases such as MAPK or ERK (Cote-Vélez et al. 2008).

10.4.4.2 Chronic Stress

The effects of chronic stress on the HPT axis depend on the type of stress, since the axis habituates to a homotypic stressor as daily intermittent restraint (Uribe et al. 2014). Chronic stress also inhibits the response of the HPT axis to acute cold exposure (Castillo-Campos et al. 2020) but in contrast to the response to an acute stressor, which is attenuated after homotypic chronic stress (as intermittent restraint) and hyperactivated after a heterotypic one (as daily variable stress), the HPT axis response to cold is similarly blunted with both types of stress. The neuronal circuits involved in controlling CRH neurons in these two types of stressor have been characterized and involve GABAergic and glutamatergic neurons projecting from the bed nucleus of stria terminalis to the PVN (Radley and Sawchenko 2015) but whether these circuits participate in the inhibitory effect of chronic stress on the cold-response of hypophysiotropic TRH neurons to cold remains to be elucidated.

10.4.5 Hypophysiotropic TRH Neurons and Exercise

Another energy-demanding situation that activates the HPT axis is physical activity. Exercise requires an adequate supply of fuel to active tissues such as muscle, provided by endogenous reserves through glycolysis and lipolysis; TH regulates several of the enzymes involved in these reactions as well as the supply of metabolic

substrates (Mullur et al. 2014; Klieverik et al. 2009). TH are also involved in the adequate functioning of skeletal and cardiac muscle and in respiration, which explains why hypothyroid individuals have a low exercise performance (Ylli et al. 2020). Increased serum TSH and TH concentrations are detected in animals or humans if taken during or at short times after exercise, before exhaustion or when the individual achieves a negative energy balance (Ylli et al. 2020; Chatzitomaris et al. 2017). Activation of HPT axis activity also occurs in rats undertaking voluntary exercise; *Trh* expression in the PVN increases, compared to pair-fed controls, proportionally to the amount of exercise performed and to the loss of fat mass (Uribe et al. 2014); likewise, serum T4 concentration increases during treadmill exercise (Fortunato et al. 2008). In humans, only peripheral hormones can be quantified, and results are controversial, depending on the time of sampling and nature of exercise; increased serum TSH, T4, or T3 concentrations are detected after submaximal exercise (running or swimming) (Ylli et al. 2020). A recognized feature of chronic exercise is the loss of adipose tissue. Two weeks of voluntary wheel running, which is a non-stressful form of exercise for rodents, diminish fat depots in abdominal and subcutaneous regions, more in males than in females, and increase *Trh* expression in PVN of males, and of TSH concentration in serum of females; these responses are curtailed by chronic restraint stress (Parra-Montes de Oca et al. 2019) and by chronic variable stress (CVS) (Parra-Montes de Oca unpublished) which reduce the quantity of exercise performed and block increased *Trh* expression in the PVN and fat loss.

The inhibitory effect of either acute or chronic stress on the response of PVN TRH neurons or TH concentration to energy demands could explain some of the symptoms of subclinical hypothyroidism, such as fatigability, cold intolerance, and low exercise performance. The efficient mechanisms of HPT axis regulation allow a rapid return to homeostasis; only drastic or pathological situations cause low detectable serum concentrations of TH, in particular T3 (McAninch and Bianco 2014). Knowledge of the mechanisms involved in regulating PVN TRH neurons has allowed a better understanding of situations that stimulate the axis in a transient manner. One can envisage that HPT axis intermittent activation promotes the signals required by energy demands, and that repression of this response produces situations of energy deficit that may be felt as hunger or fatigue, causing increased food consumption and diminished physical activity and eventually leading to obesity and metabolic syndrome.

10.4.6 Sex Differences in the Activity of Hypophysiotropic TRH Neurons

Energy balance depends on mechanisms that regulate food intake, metabolic substrate distribution, and the different components of energy expenditure, including basal metabolism, physical activity, and thermogenesis, all of which are profoundly affected by HPT axis function (Mullur et al. 2014). Energy homeostasis is regulated differently in males and females; during exercise, for example, males first utilize

glycogen reserves while females utilize fat first (Mauvais-Jarvis 2015). Fat distribution also differs, males storing more fat in abdominal depots which promotes the development of metabolic syndrome while females store more in subcutaneous spaces which secrete higher quantities of leptin than abdominal depots (Mauvais-Jarvis 2015; Chusyd et al. 2016).

Parameters of the HPT axis activity and some of those involved in its regulation show sexual dimorphism in their basal state (Fig. 10.5d, e); in some cases, a direct effect of sex hormones has been identified. In the hypothalamus, sex steroids regulate the neuroendocrine and autonomic activity of the PVN; androgen (AR) and estrogen (ER) receptors are expressed in various hypophysiotropic parvocellular neurons and in neurons that project to the brainstem (Bingham et al. 2006), though co-expression of either kind of ER (α or β) with TRH is scant (Suzuki and Handa 2005); using in situ hybridization for *Trh*, which is more sensitive than immunocytochemistry for the TRH precursor, we also detected few cells co-expressing ERα (Uribe et al. 2009), but ERβ has yet to be studied. *Trh* expression in the PVN of ovariectomized rats is higher than in controls in the caudal part of the PVN and is decreased by estradiol administration in a dose-dependent manner (Uribe et al. 2009); in these animals, serum concentration of T3 increases in a dose-dependent manner; in contrast, serum TSH concentration is not affected by the higher dose and serum concentrations of T3 and 17β-oestradiol (E2) correlate positively, whereas both correlate negatively with PVN *Trh* mRNA levels. This illustrates the concerted response of the HPT axis and suggests that the negative effect of E2 on *Trh* expression could in fact be due to the T3 feedback effect. The response of serum TSH to E2 could be explained by the higher levels of pituitary TRH receptors (Donda et al. 1990; Minakhina et al. 2020). Furthermore, E2 directly stimulates the activity of thyroid peroxidase (TPO) (Lima et al. 2006), an enzyme involved in TH synthesis. The direct effect of testosterone on PVN *Trh* expression has not been studied, though it has positive effects on other parameters. The TRH content of median eminence decreases in orchidectomized rats but normalizes after testosterone replacement (Pekary et al. 1990), and testosterone induces the expression of *Tshb* in pituitary (Christianson et al. 1981; Ross 1990) and of *Thsr* in the thyroid gland (Banu et al. 2001).

Previous sections described how HPT axis activity responds to situations that alter energy homeostasis or stress. Most work was performed in males, but a few studies compared males and females within the same experimental paradigm. For example, HPT axis inhibition in response to starvation occurs more intensely and earlier in males than in females and PVN *Trh* expression and TSH serum concentration are reduced in males after 24 h of starvation, whereas in females these changes take place only after 48 h of fasting and to a lesser degree (Joseph-Bravo et al. 2020). The response to voluntary exercise also differs; *Trh* expression in PVN increases in males compared to pair-fed controls proportionally to running but not in females, which show increased TSH serum concentration instead; and females lose less fat than males, in spite of their exercising three times more than males (Parra-Montes de Oca et al. 2019). These results suggest that PVN TRH neurons in females are not activated as in males, but more work is needed to be able to elucidate the mechanism

involved; whether it relates to the effects of estradiol on metabolism (Xu and López 2018) awaits resolution.

10.4.7 Developmental Programming of Hypophysiotropic TRH Neurons

Thyroid hormones play a crucial role in fetal and postnatal neurodevelopment and metabolism (Mullur et al. 2014; Moog et al. 2017a); therefore, any insult that may affect serum TH concentration of the mother, such as maternal disease and diet, exposure to toxins, or disrupting chemicals may have many deleterious effects in offspring development (Mughal et al. 2018; Miranda et al. 2020). Effects may be long-lasting and sex-specific, affecting different tissues depending on their developmental window and time of perturbation (Fall and Kumaran 2019; Miranda et al. 2020).

Before the onset of thyroid function in the human and rat fetus, which occurs around 16–20 weeks of gestation and at embryonic (E) day 17.5, respectively, the fetus is completely dependent on the maternal supply of TH; however, a significant transfer of TH from the mother to the fetus persists after the onset of the fetal thyroid function (Moog et al. 2017a; Morreale de Escobar et al. 1987). While hypothalamic TRH neurons appear by E13 in the rat (Markakis and Swanson 1997), TRH is mainly produced postnatally, showing a peak at day 21 after birth (Burgunder and Taylor 1989). Gestation, lactation, and adolescence are thus critical developmental windows that can alter the function of the HPT axis.

10.4.7.1 Nutrition

Maternal obesity is associated with decreased hypothalamic *Trh* expression only in rat male pups, and increased hypothalamic *Dio2* expression only in female pups (Dias-Rocha et al. 2018). In non-human primates, maternal obesity induced by a pregestational high-fat diet decreases hypothalamic *Trh* expression of the fetus at the beginning of the first trimester (Suter et al. 2012).

Maternal protein or energy restriction during gestation/lactation programs the adult rat offspring for thyroid dysfunction, provoking low TSH in vitro TRH-induced release (Lisboa et al. 2008), low body weight, and a decreased resting metabolic rate (Ayala-Moreno et al. 2013). Inadequate micronutrient intake during gestation and lactation is another factor implicated in the development and HPT axis function of the progeny. In particular, necessary elements for TH synthesis (iodine) or enzyme function (e.g., Se for deiodinases and Zn for various metalloenzymes and transcription factors), if insufficient in the mother's diet during gestation or suckling periods, produce deleterious effects (see reviews Rezaei et al. 2019; Hubalewska-Dydejczyk et al. 2020). Zn deficiency in the mother's diet and during adolescence decreases TRH-DE activity in median eminence and in pituitary and increases TSH concentration in serum with no effect on TH levels (Alvarez-Salas et al. 2015). During pregnancy, adequate iodine intake is required to meet maternal and fetal needs and to account for increased maternal losses; iodine deficiency in early life

impairs cognition and growth (Pearce et al. 2016). Excessive iodine intake during pregnancy and lactation increases the mother's susceptibility to thyroid dysfunction; this can affect cognitive development of the offspring and increases the risk of infant hypothyroidism induced by an excessive concentration of iodine in breast-milk (Pearce et al. 2016; Farebrother et al. 2019). Maternal ingestion of high concentrations of iodine alters the function of the HPT axis of male rat offspring in adulthood; *Trh* and *Tsh* expression in the hypothalamus and pituitary are increased, along with elevated TSH secretion.

During the suckling period pups are also susceptible; overnutrition programs adult male rats for central hypothyroidism, reflected by decreased mRNA levels of *Trh* in the PVN and protein expression in the hypothalamus, and lower basal and TRH-stimulated TSH secretion (Aréchiga-Ceballos et al. 2014; Lisboa et al. 2015). Neonatal and postnatal exposure to a diet high with soy content leads to increased hypothalamic *Trh* expression in adult mice, although TH levels are not affected (Cederroth et al. 2007). In contrast, undernourishment during suckling mice alters postnatal development and long-term hypothalamic gene expression, including that of *Trh,* expression of which is decreased in 21-day-old male and female offspring (Kaczmarek et al. 2016).

10.4.7.2 Stress

Fetal exposure to synthetic GC as DEX or postnatal chronic stress results in behavioral and metabolic disturbances later in life and permanent alterations of gene expression of neuropeptides. Sex-specific effects are observed in adult rats exposed to DEX during late gestation; core body temperature is reduced in females, but not males, and although preproTRH-ir fibers are reduced in the PVN of both male and female offspring, only adult females present a reduced number of preproTRH-ir neurons in the PVN as well as mRNA levels (Carbone et al. 2012). The higher susceptibility of females to prenatal GC exposure is confirmed in guinea pigs (Moisiadis et al. 2017). Postnatal stress, such as maternal separation (MS) during the suckling period or childhood maltreatment, is associated with reduced thyroid activity (decreased serum levels of TSH and/or T3) in male rats, adolescents and non-pregnant women (Jaimes-Hoy et al. 2016, 2019; Sinai et al. 2014; Machado et al. 2015), increasing their risk of exhibiting subclinical hypothyroidism during pregnancy (Moog et al. 2017b) and putting the child at risk of adverse neurodevelopmental outcomes. In contrast to the inhibitory effects of GC administration during gestation on *Trh* expression in females, MS increases it; PVN-*Trh* expression in males is not affected but the expression of its degrading enzyme is increased, resulting in a low TSH serum concentration in adult male rats. MS affected not only the basal activity of the HPT axis but its response to various challenges such as food deprivation or cold exposure is blunted in males, but not in females (Jaimes-Hoy et al. 2016, 2021). The response to hypercaloric palatable diet and psychological stress (restraint) is also modified, depending on whether the diet is started in puberty or adulthood. For example, restrained MS male rats exposed to a high-fat/high-carbohydrate diet from puberty have increased *Trh* expression in PVN and decreased concentrations of TSH and TH in serum, whereas females do have

increased PVN-*Trh* mRNA but serum TH levels are also increased, suggesting that males are more susceptible to interference with the adaptive response of this neuroendocrine axis to a metabolic stressor (Jaimes-Hoy et al. 2019).

10.4.7.3 Tobacco and Endocrine Disrupting Chemicals

Prenatal and infant exposure to toxins or pollutants may have persistent effects throughout life. Tobacco smoking during pregnancy/lactation exerts numerous short- and long-term adverse effects on the neonate's health, increasing the risk of developing obesity, hypertension and metabolic and lung-related diseases, including altered thyroid function and development (Banderali et al. 2015; Miranda et al. 2020). Maternal nicotine exposure leads to microgliosis in the PVN (Younes-Rapozo et al. 2015), reduced hypothalamic TRH content (Younes-Rapozo et al. 2013) and secondary hypothyroidism is induced in the PVN of male offspring at adulthood, with low serum levels of TSH and TH. This is due in part to in vivo TRH-TSH suppression and decreased sensitivity to TRH (Miranda et al. 2020).

Plastics and other chemicals that have contaminated water and soil produce substances that affect the endocrine system, called endocrine-disrupting chemicals (EDCs) (Gore et al. 2019). EDCs may act alone or in combination, impairing estrogenic/androgenic and thyroid function, the latter acting at multiple levels of the HPT and gonadal axes; dysfunction of these axes have been associated with obesity, reproductive alterations, breast, ovarian and thyroid cancer, hypothyroidism and cognitive impairment (Mughal et al. 2018). Perinatal exposure to triclosan (TCS), a common chemical present in household and personal products, reduces expression levels of *Trh*, *Thra* and TH transporters in the brains of mouse offspring (Tran et al. 2020). In gestating mice dams, acute treatment on the day of delivery with the organotin tributyltin (TBT) dose-dependently increases *Trh* transcription in pups' hypothalamic, independent of T3 and mediated by hypothalamic overexpression of *Rxra*; in contrast, chronic exposure of dams to the flame-retardant tetrabromobisphenol A (TBBPA) during late gestation diminishes *Trh* and *Mc4r* transcription in pups' hypothalami in the absence of T3 (Mughal et al. 2018). Polybrominated diphenyl ethers (PBDEs), used as flame retardant additives, have been banned in several countries but persist in the environment (Sharkey et al. 2020); they reduce whole-body T4 content accompanied by down-regulation of *Trh* and *Tshb* in offspring (Han et al. 2017), which may contribute to the associated neurodevelopmental alterations.

The list of endocrine disruptors will probably keep growing, and with uncontrollable stress situations cause long-term effects on HPT axis function, but most worrying are those affecting the gestation period, which might be the basis of the increasing number of patients with diseases related to thyroid effects on brain development.

10.5 Hypophysiotropic TRH Neurons Regulate Prolactin Secretion

Soon after its discovery, it was shown that TRH stimulates prolactin (PRL) secretion either in vivo or in vitro (Jacobs et al. 1971; Tashjian et al. 1971) although controversies about male responses soon appeared (Watanobe et al. 1985). PRL secretion is controlled directly by hypothalamic tuberoinfundibular dopamine neurons, which exert an inhibitory drive, but also by various hypothalamic neurons releasing stimulatory factors (Grattan 2015). During suckling, dopamine (DA) release into portal blood is inhibited while TRH biosynthesis in the PVN and release from the ME are stimulated (Fink et al. 1982; Uribe et al. 1993; Van Haasteren et al. 1996; Sánchez et al. 2001), implicating the hypophysiotropic PVN TRH neurons. Furthermore, TRH antisera inhibit suckling-induced PRL release (de Greef et al. 1987), and studies with TRH and TRH-R1 KO mice have shown that TRH is necessary to maintain maximal prolactin output in lactating mice (Rabeler et al. 2004; Yamada et al. 2006).

TSH is not released by suckling, nor is PRL by cold exposure (Uribe et al. 1993; Van Haasteren et al. 1996; Sánchez et al. 2001), suggesting that post-secretory processes likely refine the specificity of TRH action in the anterior pituitary. Although the hypophysiotropic TRH neurons regulating prolactin may differ in part from those controlling TSH secretion (Sánchez et al. 2001), there is no evidence yet for an anatomical pathway that may segregate TRH released by each kind of hypophysiotropic neuron. The stimulus-dependent response may originate from other aspects of PRL secretion control. CART, co-expressed in TRH hypophysiotropic neurons, is upregulated by 1 h exposure to cold but not by suckling (Sánchez et al. 2007). Since CART inhibits prolactin release in vitro and cold exposure does not induce the release of PRL, CART may serve as a modulator of TRH actions in these physiological circumstances, stimulating TSH release while blocking prolactin release (Sánchez et al. 2001, 2007; Raptis et al. 2004).

DA withdrawal during suckling, or in primary cultures of hypophysial cells, potentiates TRH-induced PRL secretion (Martinez de la Escalera and Weiner 1992). In lactotrophs the intensity of TRH action is under TRH-DE control: in primary cultures of female rat anterior pituitary cells, *Trhde* is expressed in some lactotrophs, and inhibition of *Trhde* expression or activity enhances TRH-induced prolactin secretion (Bauer et al. 1990; Cruz et al. 2008). In anterior pituitary cells, TRH-DE activity is rapidly enhanced by the removal of DA and addition of TRH (Bourdais et al. 2000). These results suggest that lactotrophs TRH-DE activity is controlled by signals that shape PRL secretion in response to TRH and that TRH-DE regulation may in turn alter PRL release.

Finally, although many studies have shown that TRH acts directly on lactotrophs, it is relevant to note that numerous TRH fibers enter the Arc (Péterfi et al. 2018). TRH terminals abut on tuberoinfudibular dopaminergic (TIDA) neurons in rats (Lyons et al. 2010) and humans (Dudas and Merchenthaler 2020), coincident with the expression of the TRH receptor in Arc (Heuer et al. 2000), but the TRH neurons of origin are not known. In hypothalamic slices, TRH provokes a transition from

phasic to tonic firing of the TIDA neurons that control PRL secretion (Lyons et al. 2010). The relative importance of direct and indirect control of prolactin secretion by TRH remains to be elucidated.

10.6 Perspectives

In this review, we have attempted to present a summary of the characteristics of TRH hypophysiotropic neurons. These parvocellular neurons are mixed with multiple cell types in the PVN, including various subtypes of TRH neurons, making this characterization daunting. We have information about a small set of genes; although single-cell transcriptomes of hypothalamic TRH neurons have been published, a definitive assignation of a TRH cluster to the hypophysiotropic neurons is lacking.

Delineation of the neuronal circuits that modulate hypophysiotropic TRH neurons activity under different paradigms is not complete. Advances have been made in defining how they sense metabolic information, including the involvement of Arc inputs. However, knowledge of the circuits involved in other events is only partial. Afferents from the suprachiasmatic nucleus contact PVN TRH neurons (Kalsbeek et al. 2000), but the input and target neuronal types involved are still unknown. Cold exposure activates noradrenaline neurons that contact hypophysiotropic TRH neurons, but whether these inputs arise from locus coeruleus, nucleus tractus solitarius or other noradrenergic nuclei is still under investigation. How chronic stress affects the activity of TRH neurons, and whether it depends on the type of stress as for CRH neurons (Radley and Sawchenko 2015) is also unknown. Another unknown is the identity of the neuronal circuit that activates the hypophysiotropic TRH neurons in response to suckling.

We also reviewed the most important data on TRH mode of synthesis, release, and inactivation. Knowledge of these metabolic steps has been used to obtain subrogate measures of TRH neurons activity, since quantification of TSH or TH serum concentrations does not precisely reflect changes in TRH release into portal blood nor the activity of HPT axis as peripheral metabolism of TH is strictly regulated in a time- and tissue-dependent manner (Bianco et al. 2019). Together, these measurements allowed the identification of an array of regulators of TRH metabolism and neuronal activity that are intimately linked to the activity of the whole HPT axis since basal levels of *Trh* expression depend on TH feedback and nutrition status. In addition, TRH neurons and HPT axis respond to energy-demanding situations according to previous exposure to stress (immediate or during development).

The design of experiments that analyze the activity of the TRH neurons must take into account various considerations mentioned in Box 10.3, as well as conditions of stress and metabolic changes imposed by the paradigm (Castillo-Campos et al. 2020). Consideration of stress conditions during the experiment and at sacrifice is essential since even taking rats from a cage causes the immediate release of corticosterone, which depends on the order of animal removal from its cage (Ferland and Schrader 2011). Another factor is the animal species under study; the rat was the

preferred animal for research in physiology, but since the development of transgenesis the mouse became a common object of study; because, these species differ in many ways (Ellenbroek and Youn 2016), extrapolating results from one species to another could produce false hypotheses. For translational studies, it is relevant to note that most work on rodents is performed during the light period that corresponds to the inactive period of rodents, with low serum corticosterone and high thyroid hormone concentrations, according to circadian status. Another very important task is a comparison of results from males and females, ideally within the same experiment to control most variables.

Finally, until recently many studies have been limited to measuring activity at one (or sometimes a few) sampling time; it is like a snapshot that does not explain the processes under study, which are dynamic, and causal relationships have been difficult to investigate. However, the development of genetic techniques in the recent past, including transgenesis, CRISPR-Cas9, chemogenetic and optogenetic methods, provide an ample portfolio of tools that will undoubtedly allow precise monitoring of TRH neurons activity in vivo, using for example real-time calcium fiber photometry recordings, and manipulation of their activity with chemical and optical tools (Müller-Fielitz et al. 2017; Farkas et al. 2020).

The information gathered so far on TRH neuron activity could help to better diagnose subclinical hypothyroidism and suggests that it is relevant to consider the stress level of the patient. It appears that measurements of serum TSH, TH and cortisol concentrations before and after a bout of exercise or cold exposure may be more appropriate than evaluation only at basal state, which utility is limited for diagnosis and treatment (Biondi et al. 2019).

Box 10.3. Tools to Study Activity of TRH Neurons

To evaluate the activation of TRH neurons, measurement of the levels of mRNA has been used as a good index since they are rapidly increased in response to stimulation; an increase can be detected at 45–60 min (Uribe et al. 1993; Zoeller et al. 1995), near the time required to measure immediate early gene expression, such as *c-fos*. In many events, the same effector stimulates both synthesis and release; for example, cold-induced activation of noradrenaline neurons signals to TRH hypophysiotropic neurons, and increases release as well as the synthesis of TRH (Perello et al. 2007). However, not all effectors affect both processes; for example, acute corticosterone administration increases *Trh* mRNA levels but decreases TRH release through the endocannabinoid pathway at PVN level (Sotelo-Rivera et al. 2014; Di et al. 2003). The activity of the hypophysiotropic neurons can be studied either through sampling most TRH neurons in a large volume of the PVN or with cellular resolution. For rapidly sampling the activity of most hypophysiotropic TRH neurons, a common strategy is to measure *Trh* mRNA levels in punches of the PVN with RT-PCR. Alternatively, levels of proTRH measured by

(continued)

Box 10.3 (continued)
Western blotting, or levels of TRH by RIA, may be used to infer the status of TRH biosynthesis in the PVN. For these strategies to be most specific for hypophysiotropic neurons, an important consideration is the adequate dissection of the PVN. Reports on single-cell transcriptomes that do not report how dissection was performed may have limited usefulness; likewise, analysis of the whole hypothalamus to deduce regulation of HPT axis activity is meaningless. Within the hypothalamus, apart from the PVN, several nuclei express TRH (Joseph-Bravo et al. 2015b). Caudal to the PVN, the dorsomedial hypothalamus contains a large population of non-endocrine TRH neurons that are activated for example by exercise, like those of PVN (Uribe et al. 2014); this localization is essential to consider when injecting directly into the PVN, as well as the size of cannulas. In the ventrolateral directions, the lateral hypothalamus expresses many TRH neurons too. In the PVN proper, many TRH neurons of the anterior PVN are not hypophysiotropic, and project to other hypothalamic nuclei and brain areas (Wittmann et al. 2009), whereas TRH magnocellular neurons located in mid-PVN can only be differentiated from the hypophysiotropic neurons under the microscope. Finally, other parvocellular TRH neurons of mid-caudal PVN (in the rat) are not hypophysiotropic (Simmons and Swanson 2009) although a good correlation has been obtained between punches and histochemical data in various paradigms. On the other hand, immunohistochemical and/or in situ hybridization techniques generate cellular resolution, but to guarantee that data refers to hypophysiotropic parvocellular TRH neurons, additional information, such as CART expression (Fekete et al. 2000) is required. A direct measure of PVN TRH neuron (identified afterward by immunohistochemistry or single-cell transcriptomics) activity can be obtained by electrophysiology in hypothalamic slices (Di et al. 2003). The distinct localization of the PVN and median eminence along the anteroposterior axis in coronal slices allows separate quantifications of PVN *Trh* expression and processed TRH in nerve endings. Techniques designed to measure in vivo release of TRH are cumbersome and not precise. Measuring TRH content in median eminence extracts, less than 2 h after a stimulus, may indicate release if the content is reduced, although processing could also be affected. At the nerve terminal of TRH neurons, cleaved products of TRH precursor are enriched (Lechan et al. 1987), thus for TRH quantification adequate antibodies are required, which usually recognize pGlu and $ProNH_2$ moieties, so those raised against precursor forms will not recognize it. Another indirect measure of TRH release is the measurement of serum TSH concentration. The i.v. injection of anti-TRH antibodies can reduce serum TSH concentration, which validates this approach in short-term studies. However, secretion of TSH is also regulated by other hypothalamic (SRIF) or peripheral influences (TH, corticosterone). The development

(continued)

Box 10.3 (continued)
of recombinant DNA techniques and the creation of transgenic mice have provided information on the role of multiple molecules involved in signaling and transcription of *Trh*, or of the elements involved in the HPT axis as deiodinases. The most interesting data are those obtained with conditional transgenesis, so that expression of the gene of interest is only altered in a specific tissue and/or in a defined window of time, which avoids the problems produced by indirect effects through other cell types (Fonseca et al. 2013) and/or the lack of that protein during development.

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Part III

Hypothalamic Control of Neuroendocrine Functions

Circadian Control of Neuroendocrine Systems

11

Ruud M. Buijs, Eva Soto-Tinoco, and Andries Kalsbeek

Abstract

Neuroendocrine systems together with the autonomic nervous system serve to synchronize physiological processes that keep the body in balance with the environment. Such a process, also called homeostasis, often is thought to keep the conditions in the body constant in a changing environment. The present paper discusses how the brain controls hormone secretion and how the suprachiasmatic nucleus(SCN), the brain's biological clock, influences this process, illustrating that the internal conditions are far from stable but vary with a precise daily rhythm. As a result of this, hormone levels may vary by a factor 10 or more over the day–night cycle, but at a given hour may vary by less than 5% from 1 day to another. Clearly, the SCN influences a vast neuronal network within the hypothalamus, thus controlling a circadian rhythm in hormone secretion. These changing levels in circulating hormones need to be carefully tuned with the autonomic output to the organs to achieve the optimal physiological conditions needed at that time point. Particular emphasis will be paid to the rhythms of melatonin, corticosterone, and luteinizing hormone, of which the last one, even though in rats it only occurs once every 4–5 days, is also driven by the SCN. Finally, attention will also be given to the need of the SCN to be informed about the actual circulating concentration of the hormones, in order to adjust the hormonal levels to the levels appropriate to the time of the day.

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Keywords

Suprachiasmatic nucleus · Melatonin · Corticosterone · Luteinizing hormone · Autonomic nervous system · Circadian

11.1 Introduction

A neuroendocrine system can be defined as a group of neurons that produce the same hormone and release that hormone into the circulation under the influence of other brain areas. A target of a neuroendocrine system is defined as any structure in the body that expresses the adequate receptors for the released hormone. These receptors can be located in many tissues of the body or may be present only in other endocrine organs, such as the anterior pituitary. Consequently, the result of the activation of these neuroendocrine systems is the liberation of hormones that, directly or indirectly, influence various functions in the body. The neuroendocrine systems we will discuss in the present chapter are hypothalamic systems that release their hormones directly into the circulation, either via the median eminence or via the pituitary neural lobe.

Classical hormones released from peripheral tissues, such as insulin, adrenalin, and cholecystokinin, respond either to circulating factors, to other hormones or to tissue-specific stimuli. Meanwhile, neuroendocrine systems are directly influenced by many brain regions and respond to neuronal stimuli resulting from emotions or stimuli from the body sensed by the brain. In this chapter, however, we will pay special attention to the circadian system since it is a unique brain system that influences all, or nearly all, neuroendocrine systems.

Since it is essential for preparing the body for the daily cycle of activity and inactivity, the circadian system is of great importance for all neuroendocrine systems. As indicated before, the hormones of the neuroendocrine systems are essential for adapting the organs of the body for their functions; therefore, the activity-inactivity cycle is one of the main determining variables that requires different levels of hormonal action. These different levels of hormonal action can be obtained by changing hormone concentrations and receptor sensitivity. As we will see for many hormones, both possibilities take place.

Considering that the Suprachiasmatic Nucleus (SCN), the master clock that synchronizes our behavior with the light–dark cycle, activity changes such as food and fluid intake may influence several hormonal rhythms. In the present chapter, we will not take into consideration these behavior-induced hormonal changes, but instead, we will pay attention to hormonal changes that are directly influenced or induced by the SCN.

11.2 The Circadian System

The circadian system comprises the central biological clock, the suprachiasmatic nucleus (SCN), located at the base of the hypothalamus, as well as numerous peripheral clocks located in other brain areas and peripheral tissues. The SCN is constituted of approximately 20,000 neurons, accompanied by glial cells, that have an endogenous activity rhythm. Recent studies have demonstrated that the unique ability of the SCN to generate and sustain an autonomous rhythm, even in vitro, depends not only on the interaction of the different neuronal populations with each other but also on their interaction with the glial cells in the SCN (Brancaccio et al. 2019; Freeman and Herzog 2011).

The release of SCN neurotransmitters into target areas in the hypothalamus transmits the rhythm in neuronal activity to brain areas involved in the control of behavior and different neuroendocrine functions. These hypothalamic projections allow the SCN to influence all aspects of body homeostasis by synchronizing behavior with the functionality of the organs via autonomic and hormonal outputs.

The hypothalamic location of the SCN provides another functional advantage. Positioned just above the optic chiasm, the SCN receives direct light input from the retina, allowing it to synchronize its endogenous circadian rhythmicity with the exact 24 h period of the environmental light–dark cycle (Jones et al. 2018). This synchrony is essential because the daily change in light and darkness is the only reliably constant in the universe and thus, the main synchronizer of all activity.

However, optimal synchronization cannot take place without the SCN being informed about the actual situation in the body. The SCN has been shown to have extensive reciprocal contacts with several brain areas, with afferent connections informing the SCN about the actual physiological conditions of the body. This feedback information results in an adaptation of the output of the SCN (via its efferent projections), that accommodates the physiology to meet the particular needs of the body at any given time of the day (Buijs et al. 2017, 2019).

For example, the SCN has a reciprocal interaction with the arcuate nucleus, also located in the hypothalamus. The arcuate nucleus is an important circumventricular organ that receives information from the circulation via the median eminence, which possesses fenestrated capillaries. This characteristic places the arcuate nucleus, as well as the other circumventricular organs, in a privileged position, allowing them to continuously monitor circulating information (Gropp et al. 2005; Dietrich et al. 2015; Buijs et al. 2017).

The interaction between the SCN and the arcuate nucleus is essential for organizing the daily rhythm in body temperature, which is strongly associated with the animal's metabolic conditions. On the one hand, the SCN imposes a rhythm on the activity of arcuate α-MSH neurons, whose activity is essential for maintaining a high temperature at the end of the activity phase. On the other hand, vasopressin (VP) projections from the SCN to one of the primary brain areas involved in temperature regulation, the medial preoptic area, are essential for the decrease in temperature at the beginning of the sleep phase (Guzmán-Ruiz et al. 2014, 2015). A more pronounced drop in temperature only at the beginning of the inactive period

occurs under fasting conditions. This temperature drop depends on the presence of the SCN; without the SCN, the temperature remains high at any given time of the circadian cycle, even under fasting conditions (Liu et al. 2002). This observation, together with the data showing that SCN-VP is essential for the temperature drop in the early day period, indicates that the fasting information needs to reach the SCN. Therefore, the connection with the arcuate nucleus is the most logical underlying anatomical basis for the transmission of metabolic information to the SCN (Buijs et al. 2017).

11.3 Rhythmic Secretion of Melatonin: A Reflection of SCN Activity

Several hormones show a precise circadian rhythm directly driven by SCN neuronal activity. For example, melatonin secretion is directly induced by the neuronal activity of glutamatergic SCN neurons connected with pre-autonomic neurons in the paraventricular nucleus (PVN), which, via autonomic sympathetic output, drives melatonin secretion from the pineal gland (Teclemariam Mesbah et al. 1999; Perreau-Lenz et al. 2004) (Fig. 11.1). Surprisingly, these glutamatergic SCN neurons are always active and provide a constant stimulus for melatonin secretion. Nevertheless, we know that even in constant-dark conditions, melatonin secretion occurs only in the subjective dark period, earning the name "the hormone of darkness." This raises the question of what are the exact mechanisms for the stimulation and inhibition of melatonin secretion. A series of studies by Perreau-Lenz et al. (2004,

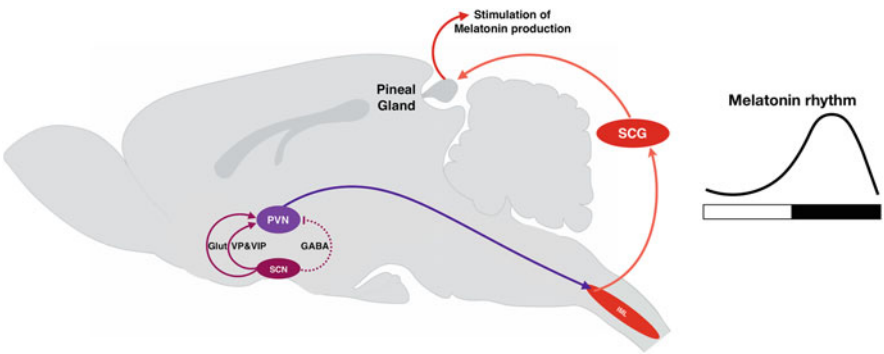

Fig. 11.1 Circadian control of melatonin secretion. Glutamatergic (Glut) projections from the SCN constantly stimulate a specific set of pre-autonomic neurons in the paraventricular nucleus of the hypothalamus (PVN). Vasopressin (VP) and Vasoactive Intestinal Peptide (VIP) also have a stimulatory effect over these pre-autonomic neurons in the PVN. Specifically, these PVN neurons are connected with sympathetic motor neurons located in the intermediolateral column (IML) of the spinal cord, that project to the superior cervical ganglion (SCG). Sympathetic postganglionic noradrenergic neurons projecting to the pineal stimulate the release of melatonin. During the day or subjective day, GABAergic projections from the SCN are activated and override the stimulatory input to the pre-autonomic neurons in the PVN, thereby preventing the melatonin release

2005) showed that these pre-autonomic PVN neurons also receive input from GABAergic SCN neurons, and their light or subjective day-induced inhibitory activity prevents the activation of the sympathetic output to the pineal. Therefore, the rhythm of these GABAergic neurons is essential for rhythmic melatonin secretion. This "simple" control of melatonin secretion might be related to the fact that melatonin is not (or only very little) influenced by other hormones or behaviors in all organisms. Consequently, these observations indicate that a diminution of nightly melatonin secretion could be almost completely ascribed to a decreased activity of the SCN's glutamatergic neurons. Such diminished melatonin secretion is, for example, observed in older people and people with Alzheimer's disease (Mirmiran et al. 1989; Uchida et al. 1996), indicating a lower activity of the glutamatergic neurons of the SCN. This concurs with the diminished SCN activity found in *post mortem* hypothalamic tissue of these subjects (Swaab 2004).

In this regard, it is interesting that melatonin secretion is also diminished in people with hypertension (Brugger et al. 1995; Zeman et al. 2005), indicating that high blood pressure may also interfere with SCN neuronal activity. This idea was corroborated in a study that evaluated *post mortem* tissue from hypertensive people and observed substantial reductions in SCN neuronal activity (Goncharuk et al. 2001).

In search of further mechanistic explanations for those observations, we demonstrated in rodents that the SCN is sensitive to increases in blood pressure (BP) (Buijs et al. 2014; Romo-Nava et al. 2017; Yilmaz et al. 2018, 2019). The nucleus of tractus solitarius (NTS) transmits information about BP increases directly to the SCN. This feedback serves to reduce BP to normal levels, which are determined by the time of the day. A clear illustration of the importance of the SCN in the control of BP is that when a stressful stimulus is given to an SCN lesioned animal, there is an exacerbated increase in BP compared to a sham-operated animal (Buijs et al. 2014; Romo-Nava et al. 2017).

The observations that hypertensive and obese people have a more disturbed sleep–wake pattern than non-hypertensive people (Gangwisch et al. 2005, 2006) provides further support for the hypothesis that alterations in our biological clock might be at the core of the recent surge in diabetes and hypertension (Kreier et al. 2003). In agreement, it was recently shown that the *post mortem* brains of type 2 diabetes patients also show the diminished activity of the SCN (Hogenboom et al. 2019). Together, these observations raise the question of whether these SCN changes in the *post mortem* hypertensive or diabetic human brain are a cause or consequence of hypertension and diabetes. Disturbed sleep–wake rhythms in these patients, together with the above-detailed observations that the SCN is sensitive to feedback, suggest that behavioral changes, and consequently changes in physiology, may be responsible for the observed alterations in the SCN. Considering that, as shown above, the biological clock plays an essential role in determining the setpoints of our physiology, any long-term disturbance in the activity of the SCN may have severe repercussions for our health. Recent shifts in human behavior, such as being active and eating during the night and the resulting changes in the SCN, may start a

vicious downward spiral. Therefore, we emphasize that changes in our behavior that are incompatible with the signals of the SCN may result in disease.

The studies mentioned above indicate that disturbances in SCN neuronal activity induced by aging, disease, medicines or other factors may, in the long term, result in important deviations from the normal physiology. However, the good news is that changes in the patient's physiology due to side-effect action of medicines on the SCN may be reversed by melatonin 'treatment'. An example of this is the second-generation antipsychotic (SGA) Olanzapine that is associated with adverse cardio-metabolic side effects that contribute to premature mortality in patients (Lieberman et al. 2005). Surprisingly enough, melatonin treatment in patients taking SGAs largely diminished these side effects, while maintaining the beneficial effects of the SGAs (Romo-Nava et al. 2014). In animal studies, initiated to find a mechanistic explanation for this observation, it was shown that Olanzapine activates areas of the limbic system as well as the SCN. Through this SCN activation, the hypothalamic output to the parasympathetic system is activated (Romo-Nava et al. 2017).

The selective coordination of the autonomic nervous system in different compartments of the body is an important output mechanism of the SCN (Kreier et al. 2002, 2006) and alterations of this output may, in time, promote the development of the metabolic syndrome (Kreier et al. 2003). In this regard, even with short-term Olanzapine treatment, the parasympathetic activation induces adiposity and increases circulating adiponectin (Togo et al. 2004). Consequently, the increased parasympathetic activity induced by Olanzapine favors the appearance of adverse cardio-metabolic effects such as obesity and changes in plasma lipids, insulin, and glucose (Lieberman et al. 2005). These disturbances are similar to those observed in the metabolic syndrome, where in the long term a compensatory increase in sympathetic cardiovascular tone gives rise to hypertension. However, the observed activation of the SCN by Olanzapine in rats is effectively prevented by treating these animals with melatonin (Romo-Nava et al. 2017), in line with the well-known inhibitory effect of melatonin on SCN activity. In agreement, two other studies also showed that melatonin mitigated Olanzapine-induced cardio-metabolic effects in patients diagnosed with schizophrenia and bipolar disorder (Modabbernia et al. 2014; Mostafavi et al. 2014).

11.4 Corticosterone

The secretion of cortisol in humans and corticosterone in rodents shows a precise circadian rhythm, with higher circulating levels anticipating the activity period. Despite what most handbooks still say, several studies have demonstrated that the circadian peak in corticosterone is not driven by adrenocorticotropic hormone (ACTH), but rather by the sympathetic innervation of the adrenal (Engeland and Arnhold 2005). This could already be deduced from the early studies of Berson and Yalow (1968), demonstrating that the blood levels of ACTH in humans hardly show a rhythm, in contrast to cortisol, which shows a high-amplitude rhythm. Similarly to humans, ACTH does not show a pronounced rhythm in rodents, indicating that the

direct sympathetic drive to the adrenal is responsible for the circadian peak in corticosterone, and making ACTH a permissive factor. Indeed, it has been shown that the corticosterone peak is driven by SCN neurotransmitters influencing the PVN pre-autonomic neurons that project to the sympathetic autonomic neurons innervating the adrenal gland (Buijs et al. 1999; Ishida et al. 2005; Kalsbeek et al. 1996).

Likewise, the stress response is under circadian control, with lower corticosterone responses to stressors presented at the beginning of the activity period and higher responses to stressors presented before the resting period (Buijs et al. 1993). However, different types of stressors can have different circadian patterns. For instance, a metabolic stressor in the form of a hypoglycemic stimulus (insulin) does not induce an increased corticosterone response at the beginning of the resting phase as high that observed after an emotional stressor (a new cage). In the beginning of the active phase, the reverse happens, with a low corticosterone response after the stress of a new cage and a high corticosterone response after the hypoglycemia-induced stress (Kalsbeek et al. 2003). These observations show the complexity (and logic) of the influence of the circadian system on the neuroendocrine responses. Moving into a new cage early in the sleep phase is more disturbing than when it occurs at the beginning of the active phase; thus, it evokes a higher corticosterone secretion. On the other hand, hypoglycemia is more disturbing when being active than when being inactive; hence, it evokes a higher corticosterone response in the active period.

Several observations indicate that the SCN has an inhibitory role in the secretion of corticosterone. First, compared to intact animals, SCN-lesioned animals respond with much higher corticosterone levels when challenged with a stressor. Second, compared to intact animals, SCN-lesioned animals show much a higher basal corticosterone level (Buijs et al. 1997; Kalsbeek et al. 2003). However, the circadian peak of corticosterone in intact animals is higher than the levels in undisturbed SCN-lesioned animals, indicating that the SCN also has a stimulatory influence on corticosterone secretion (Kalsbeek et al. 1996).

Which SCN transmitter is responsible for this stimulation of corticosterone secretion has not been determined. However, the inhibitory influence of the SCN on corticosterone secretion is mediated by the VP neurons of the SCN. The release of VP from the SCN starts at ZT18 and it peaks at ZT6, and thereafter VP release decreases (Schwartz and Reppert 1985). This VP release from SCN terminals at pre-autonomic neurons of the PVN is responsible for inhibiting corticosterone secretion (Fig. 11.2); a timed infusion of VP antagonists demonstrated that only early day infusions of the antagonist could increase corticosterone levels. In agreement with its release pattern, VP only inhibits corticosterone secretion in the early light period (Kalsbeek et al. 1996).

This study also revealed a stimulatory SCN input that exists only from the end of the activity period until the end of the light period. The interaction between the unknown stimulatory SCN input and the VP inhibitory input shapes the circadian peak in corticosterone (Kalsbeek et al. 1996). Recent studies indicate that the unknown stimulatory input could be the SCN Vasoactive Intestinal Peptide (VIP)

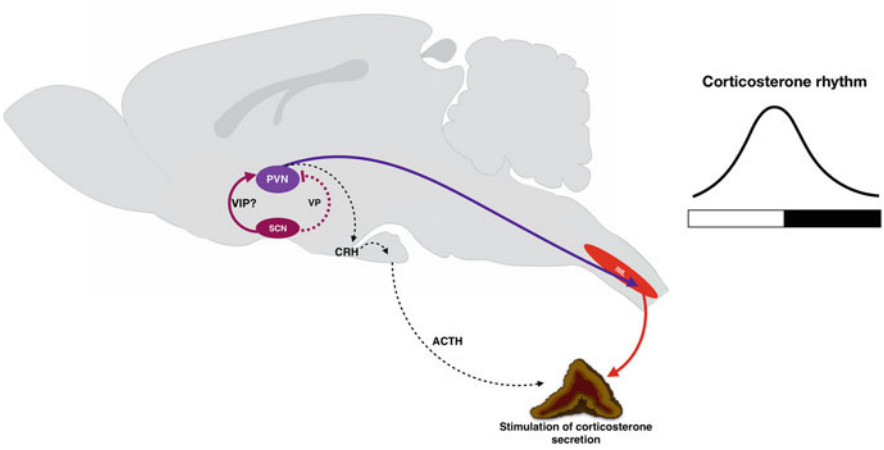

Fig. 11.2 Circadian control of corticosterone secretion. Vasopressin (VP) and other (VIP?) neurons from the Suprachiasmatic Nucleus (SCN) project to pre-autonomic neurons in the paraventricular nucleus of the hypothalamus (PVN). These pre-autonomic neurons project to the sympathetic motor neurons located in the intermediolateral column (IML) of the spinal cord that project to the adrenal and stimulate corticosterone secretion (and are thus different from those that stimulate melatonin secretion). An unknown stimulatory input from the SCN (VIP?) activates those pre-autonomic neurons, resulting in the peak of corticosterone secretion just before the activity period. In addition, Corticotrophin Releasing Hormone (CRH) is produced in the PVN and is released into the median eminence to reach the anterior pituitary, where it stimulates the release of adenocorticotropic hormone (ACTH) into the circulation. The occupation of ACTH receptors in the adrenal cortex is necessary to obtain corticosterone release by the adrenal cortex. From other brain areas, there are also stimulatory inputs to the PVN provoking corticosterone secretion. VP released from SCN terminals strongly inhibits these adrenal connecting pre-autonomic neurons in the PVN during the early morning, resulting in very low corticosterone levels

neurons (Mazuski et al. 2020), suggesting that the activity of those VIP neurons should be high from the middle of the light period to the beginning of the dark period.

11.4.1 Corticosterone Negative Feedback

To adjust the circulating level of corticosterone, it is essential to precisely monitor its concentration and transmit this information to the brain areas involved in releasing ACTH and corticosterone. How circulating corticosterone may enter the brain is still under discussion. However, there is some evidence that in the blood–brain barrier (BBB), multidrug resistance P-glycoprotein (MDR) plays a role in transporting corticosterone into the brain (Karssen et al. 2001).

The negative feedback of corticosterone is proposed to occur at the PVN level, where Corticotrophin Releasing Hormone (CRH) neurons control the secretion of ACTH from the pituitary. These CRH neurons express glucocorticoid receptors (GR) and diminish their activity and CRH production under the influence of

glucocorticoids. However, as we have seen, the control of glucocorticoid secretion occurs mainly via the activation of pre-autonomic neurons in the PVN projecting to the adrenal. These neurons do not express GR and thus are not directly sensitive to glucocorticoid feedback (Leon-Mercado et al. 2017). Moreover, since glucocorticoids do not easily penetrate the BBB, the question is: If there is a fast release of corticosterone, is there also a fast feedback?

To answer this question, we need to focus our attention on those areas where the brain can rapidly monitor the circulating concentration of corticosterone: the four sensory circumventricular organs (CVOs), which possess a more permissive BBB. These structures are the Organum Vasculosum of the Lamina Terminalis (OVLT), the Subfornical Organ (SFO), the Median Eminence (ME)-Arcuate nucleus complex (ARC), and the Area Postrema (AP). The OVLT and SFO are mainly involved in the surveillance of the mineral balance of the body (Gizowski et al. 2016; Gizowski and Bourque 2020; Mimee et al. 2013), whereas the ME-ARC and AP are important for monitoring the metabolic condition (Langlet et al. 2013; Larsen et al. 1997).

Two of the CVOs, the OVLT and ME-ARC, have extensive reciprocal interaction with the SCN, while for the SFO and AP, this has not been demonstrated, but all have elaborate connections with the PVN. Of these four CVOs, only the ARC has a high concentration of GR, making it the logical candidate for corticosterone's fast feedback upon its secretion from the adrenal. Using microdialysis probes inside the ARC and infusing specific GR and mineralocorticoid receptor (MR) agonists and antagonists at different times of the day, it was demonstrated that when systemic corticosterone levels are low, the *MR* has a vital role in the negative feedback. In contrast, when circulating corticosterone concentrations are high, the *GR* is essential for negative feedback. Notably, the increase or suppression in circulating corticosterone levels by MR or GR (ant)agonists in the ARC took place without any change in circulating ACTH (Leon-Mercado et al. 2017), confirming that the hypothalamic output to the adrenal via the ANS executed those changes. This observation illustrates that the brain's CVOs play a crucial role in sensing circulating molecules and signal those levels to regulatory centers in the brainstem and hypothalamus, to adjust not only metabolic conditions but also hormonal levels.

11.5 Luteinizing Hormone

Probably for no other hormone, the timing of secretion is so crucial as that for the Luteinizing Hormone (LH). In addition, perhaps no other hormone is under the influence of so many different factors as the LH. In rodents, there is extensive experimental evidence that the SCN drives the preovulatory LH release, while there is also preliminary evidence that this SCN action is accompanied by its simultaneous influence on the ovary via the autonomic nervous system to induce ovulation (Silva et al. 2020). The involvement of the SCN in the LH surge was first indicated by Everett and Sawyer, who were able to postpone the LH surge in female rats by 24 h with an injection of Nembutal, provided that the injection was given at a crucial moment before ovulation. This pioneering study indicated the circadian

control of ovulation (Everett and Sawyer 1950). Legan and Karsch (1975) provided another piece of evidence when they showed that ovariectomized-estrogen-treated animals show a surge in LH every day (Legan and Karsch 1975), instead of only once every 4–5 days. These experimental conditions also provide an excellent experimental model in which to study how the SCN can influence LH secretion.

In addition to the gonadotrophin-releasing hormone (GnRH) neurons and the circadian system, several other systems are involved in the daily control of LH secretion. Ovulation takes place once every 28 days in humans, raising the question of whether the SCN is still involved in the organization of the menstrual cycle. Despite the monthly cycle, much evidence indicates that similar mechanisms of control exist for human ovulation. Spontaneous initiation of the preovulatory LH surge in women generally occurs in the morning together with the cortisol peak, indicating the importance of the SCN in the timing of human ovulation (Cahill et al. 1998; Kerdelhue et al. 2002).

The SCN involvement in the control of human ovulation was challenged by the observation that the amplitude and frequency of pulsatile LH secretion did not vary over a 24 h period in premenopausal women studied under constant laboratory conditions (Klingman et al. 2011). However, the conditions used in this study, constant light and constant activity for 32 h, could be enough to disrupt the ovulatory cycle (Scarinci et al. 2019). For instance, the ovulatory cycle is modulated by melatonin and melatonin secretion certainly will be disrupted by the constant light conditions used in that study. Moreover, women living under normal LD conditions were lacking as controls, meaning that very little can be concluded from this study.

The complexity of the LH surge timing becomes evident when we consider the contribution of different SCN neuronal populations to the ovulatory cycle. SCN-VP neurons project to the medial preoptic area, where (even in SCN-lesioned animals) VP infusion can induce an LH surge (Palm et al. 1999). Moreover, in SCN-intact, but ovariectomized, estradiol-treated animals, VP could induce this LH surge only within a specific time window (Palm et al. 2001). Both studies show the importance of VP stimulation for the LH surge. After discovering a Kisspeptin population in the medial preoptic area, it became clear that the SCN-VP projections to these Kisspeptin neurons (Vida et al. 2010) underlie the effects of VP on LH secretion (Fig. 11.3).

Besides the influence of VP, Vasoactive Intestinal Peptide (VIP) neurons of the SCN are also involved in controlling the LH surge. The SCN-VIP neurons directly project to GnRH neurons located in the rostral medial preoptic area (Van Der Beek et al. 1997). These VIP neuronal terminals preferentially appose GnRH neurons that show activation (measured by c-Fos) during an LH surge (Van Der Beek et al. 1994). In agreement with this, the LH surge is diminished or prevented by an injection of VIP antiserum (which neutralizes the effects of VIP) (Van Der Beek et al. 1999). Therefore, just like the VP neurons, the VIP projections from the SCN serve to stimulate the GnRH neurons. The timing between the activation of these two neuronal populations is probably essential for an accurate control of ovulation. Moreover, also prokineticin neurons in the SCN may be involved in controlling

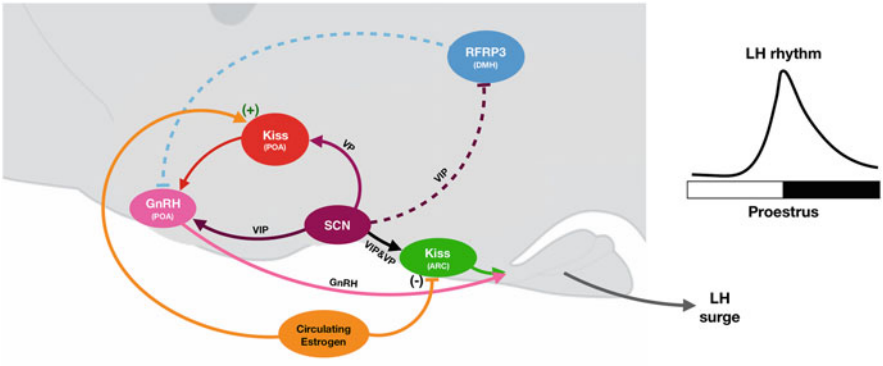

Fig. 11.3 Circadian control of the LH surge. At the center, the suprachiasmatic nucleus (SCN) has projections to several neuronal populations important for the LH surge. With vasopressin (AVP) it stimulates Kisspeptin (Kiss) neurons in the medial preoptic area (POA). With VIP it stimulates Gonadotropin-Releasing Hormone (GnRH) neurons in the POA, while inhibiting RFamide-related peptide 3 (RFRP3) neurons in the dorsomedial hypothalamus (DMH). With both, VIP and AVP, the SCN targets Kiss neurons in the arcuate nucleus (ARC). Kiss neurons in the POA stimulate GnRH neurons in the POA for the release of LH, while RFRP3 neurons inhibit GnRH neurons, and therefore prevent the LH surge. The SCN inhibits the inhibition of the RFRP3 neurons over the GnRH neurons, allowing the LH surge to take place. Lastly, circulating estrogen modulates both populations of Kiss neurons in opposite ways, activating the POA population while inhibiting the ARC population. Just before ovulation, the estrogen levels drop, resulting in an increase of Kisspeptin activity in the ARC which promotes, via its terminals in the median eminence, the final activation of GnRH terminals for the release of LH

the LH surge since receptors for this peptide are present on estradiol-activated neurons in the medial preoptic area (Xiao et al. 2014).

Apart from daily rhythms, other conditions influence the LH surge, such as seasonal or metabolic influences. The seasonal influence on reproduction will hardly play a role in most humans, except when we consider the shortage of food, which may be strongly seasonal in some cultures. On the other hand, many studies illustrate how metabolic conditions play an important role in the functioning of the reproductive cycle. In these studies, the arcuate nucleus appears as an important brain area able to influence the LH surge. As mentioned before, the arcuate is involved in monitoring the metabolic state of the animal via the sensing of circulating metabolites. Arcuate nucleus kisspeptin neurons, Agouti-related peptide (AgRP) and Pro-opioid Melanocortin (POMC) neurons project to the medial preoptic area (MnPO), to the dorsomedial nucleus of the hypothalamus (DMH) and to the PVN (Padilla et al. 2019), all of which structures are involved in the processing of reproductive and metabolic information.

As described above, the arcuate has bidirectional connections with the SCN that are essential for the organization of many circadian rhythms (Yi et al. 2006; Buijs et al. 2017). Such reciprocal connections also exist between the DMH and the SCN (Acosta-Galvan et al. 2011), demonstrating the importance of the interaction between time and metabolism. Furthermore, several physiological studies have

shown the importance of the interaction of the SCN, arcuate and DMH with the medial preoptic area, and emphasized the importance of these areas for controlling reproduction and temperature regulation (Buijs et al. 2017; Guzmán-Ruiz et al. 2015; Padilla et al. 2019).

The SCN and arcuate coordinate the diurnal temperature decrease in the MnPO. The MnPO receives SCN and ARC efferents that influence the temperature. During the night, an SCN-mediated activation of arcuate nucleus α-MSH neurons (Guzmán-Ruiz et al. 2014) sustains high body temperature during the night. In the last part of the dark phase, vasopressin is released from SCN terminals, having a hypothermic effect in the MnPO. This hypothermic effect of vasopressin is counteracted by α-MSH activity in the arcuate as long as it is night. At the onset of the light phase, the SCN inhibits the activity of the arcuate α-MSH neurons (Guzmán-Ruiz et al. 2014). Without α-MSH thermogenic counteraction, vasopressin is able to exert its hypothermic effect and the temperature drops at the beginning of the light period. For more details see Guzmán-Ruiz et al. (2015).

Interestingly, Kisspeptin neuronal populations located in both the medial preoptic area and arcuate nucleus are strongly under the influence of the gonadal hormones estrogen and testosterone. These steroid hormones strongly stimulate Kisspeptin production in the neurons of the medial preoptic area, while inhibiting the production of Kisspeptin in the arcuate nucleus (Smith et al. 2006). Interestingly, both kisspeptin populations have an important stimulatory role on LH secretion (Estrada et al. 2006), indicating that estrogen changes just before ovulation also need to be timed precisely, making the SCN control of the autonomic innervation of the ovary essential. This may be reflected in the way both populations of Kisspeptin neurons influence the GnRH neurons: the medial preoptic area Kisspeptin population mainly influences the GnRH cell bodies, while the arcuate population is better positioned to influence the GnRH axons terminating in the median eminence (Matsuyama et al. 2011; Yip et al. 2021). This suggests that when estrogen levels drop just before ovulation, the Kisspeptin arcuate neurons are stimulated, which then stimulates the GnRH terminals for the final release to induce the LH surge (Fig. 11.3). In addition, this decrease in estrogen and the consequent increase in Kisspeptin activity in the arcuate may also account for the temperature increase after ovulation. It has been demonstrated that the activity of arcuate Kisspeptin-Neurokinin B neurons induces an excess release of Neurokinin B in the medial preoptic area, leading to the activation of the parasympathetic outflow to the blood vessels of the skin, which results in vasodilation and the feeling of hot flushes (Padilla et al. 2018; Rometo et al. 2007; Mittelman-Smith et al. 2012a, b). The same neurons are also important for the control of metabolism (Padilla et al. 2019); again, evidencing a tight coupling between temperature, reproduction, and metabolism.

A similar interaction occurs between the SCN and the DMH, which is also an area where circadian, metabolic, and temperature information is integrated. Here, another population of RF-amide neurons, RFRP-3 (RF-amide-related peptide 3), regulates GnRH neuron activity and gonadotropin secretion. RFRP-3 is known to exert an inhibitory role over the GnRH signaling, although that depends on the species studied.

In female Syrian hamsters (*Mesocricetus auratus*), RFRP-3 neurons have close appositions with SCN derived VP and VIP fibers (Russo et al. 2015, 2018), suggesting that the SCN could also be involved in coordinating the inhibitory functions of RFRP-3 neurons. Indeed, VIP suppresses RFRP-3 neuronal activity only when injected in the evening, therefore removing its inhibitory influence over the GnRH neurons. Together, these data indicate that the SCN can stimulate GnRH secretion by direct projections to the GnRH neurons and indirectly through the inhibition of RFRP-3 neurons, both actions carried on by VIP projections (Russo et al. 2015, 2018). These examples illustrate that the SCN orchestrates the optimal timing of such an important event as ovulation via multiple targets. Moreover, the SCN-VIP neurons receive dense input (feedback) from the DMH-RFRP-3 neurons (Acosta-Galvan et al. 2011) that is essential for the organization of locomotor activity of the animal, which is another behavior that shows profound changes around ovulation in many animal species.

In addition, since there are multi-synaptic connections from the SCN to the ovary (Gerendai et al. 2000) and disruption of the autonomic output to the ovaries disrupts the onset of ovulation (Ramírez et al. 2017), it is likely that the SCN is also involved in the additional autonomic control of ovulation. (Buijs and Kalsbeek 2001)

11.6 Conclusion/Perspective

These examples illustrate the extensive possibilities of the SCN to modulate/influence essential physiological functions of the body. It is established that the SCN employs a wide network of hypothalamic systems that influence the secretion of hormones to target the organs of the body. However, these hormonal actions on the organs are far from sufficient. Therefore, via the same hypothalamic systems, the SCN also changes the autonomic output, thus targeting neuronally the same organs that are reached by circulating hormones. The apparent need of the SCN to influence and synchronize these two systems indicates the urgency for a better understanding of their interaction, not only because it gives a better understanding of how the SCN can synchronize functions in our body, but more because it is essential to understand how the autonomic nervous system sensitizes our organs for the circulating hormones.

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The Neuroanatomical Organization of Hypothalamic Feeding Circuits

12

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Abstract

Based on early experimental lesion findings, the hypothalamus was historically identified as fundamental for balancing energy intake versus expenditure. Research over the last decades has identified considerable detail of the functional specialization of the hypothalamic neurocircuitry, and how it integrates multiple energy status signals and issues output commands for controlling endocrine and

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behavioral responses that collectively govern energy balance. This knowledge must now be harnessed to develop therapeutics to counter disorders of energy homeostasis; i.e., the current obesity pandemic demands acquiring further understanding of the functional and neuroanatomical organization of feeding circuits with the help of new advances in the modern systems neuroscience methodologies. This chapter reviews the current understanding of the anatomical and functional organization of hypothalamic feeding circuits while covering some more recent conceptual and technological milestones in the research of energy homeostasis.

Keywords

Hypothalamus · Arcuate nucleus · Energy homeostasis · Feeding behavior · Central melanocortin system · Melanocortin-4 receptor · Oxytocin · Astrocytes

List of Abbreviations

α-MSH	α-melanocyte-stimulating hormone
aBNST	Anterior bed nucleus of the stria terminalis
AgRP	Agouti-related peptide
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ARC	Arcuate nucleus
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
ChR2	Channelrhodopsin 2
CRACM	Channelrhodopsin-assisted circuit mapping
CRH	Corticotropin-releasing hormone
DMH	Dorsomedial hypothalamus
DYN	Dynorphin
f	Fornix
GABA	γ-amino butyric acid
GFAP	Glial-fibrillary acidic protein
GLAST	Glutamate-aspartate transporter
HCRT	Hypocretin
ip.	Intraperitoneal
ir	Immunoreactivity
LepRb	Leptin receptor b
LHA	Lateral hypothalamic area
MC4R	Melanocortin-4-receptor
MCH	Melanin-concentrating hormone
ME	Median eminence
NMDA	*N*-methyl-D-aspartate receptor
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius

opt	Optic tract
ORX	Orexin
OT	Oxytocin
OT-R	Oxytocin receptor
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
SCN	Suprachiasmatic nucleus
SF-1	Steroidogenic factor 1
Sim1	Single-minded 1
Tbx3	T-box 3
TRH	Thyrotropin-releasing hormone
VMH	Ventromedial hypothalamus

12.1 Introduction: Eating to Live Versus Living to Eat

The drive to consume food is absolutely essential and considered the sine qua non for animal survival. As a consequence, powerful regulatory mechanisms evolved that balance food intake against competing behaviors (such as drinking, mating, sleep, play and exploration, avoidance of predators) as well as calibrating the intake of calories to meet energetic needs. Over the course of evolution, mammals, including *Homo sapiens*, faced an environment in which food availability was scarce and energy expenditure and the risk of predation were high. Based on this, it has been widely assumed that continuous exposure to such selection pressure strongly biased the emergence of biological processes that favor the accumulation and conservation of body fat as energy reserves (e.g., the "thrifty gene hypothesis" (Neel 1962) and more recently the "drifty gene hypothesis" (Speakman 2007)). However, over the last century these ancient and biologically ingrained survival mechanisms have become a major metabolic liability as our environment transformed to grant virtually unlimited access to energy-dense, palatable foods with a high risk of being overconsumed. Thus, in contrast to what occurred over our evolutionary history, many human societies now face the constant risk of caloric intake chronically surpassing energy needs. One key consequence of this mismatch between our innate homeostatic systems and the environment we experience is that the number of obese people worldwide now exceeds those who are underweight (NCD Risk Factor Collaboration 2017). Notably, this steep rise in the incidence of obesity in most developed cultures entails serious implications, as it associates with multiple metabolic comorbidities that significantly reduce life-quality and -expectancy (e.g., type-2-diabetes mellitus, cardiovascular disease, certain cancers, and many more). The rate at which this obesity pandemic is accelerating across the globe necessitates

developing a deeper comprehension of appetite regulation and identifying tangible, therapeutically relevant components of the metabolic homeostatic system.

12.2 Brain Control of Energy Balance: A Historical Perspective

The brain, and particularly its basal brain regions including the hypothalamus, has historically been appreciated to have a prominent role in regulating food intake and body weight. As early as 1840 it was known that hypothalamic damage could lead directly to morbid obesity (Mohr 1840), but it was not until a century later that elegant brain-lesioning studies in animal models began underscoring the importance of the hypothalamus (e.g., targeted ablation of specific hypothalamic nuclei proved sufficient to dramatically and bi-directionally alter food intake and body weight (Hetherington and Ranson 1940; Anand and Brobeck 1951)). These early reports suggested that distinct subregions of the hypothalamus had pivotal roles in the regulation of energy intake and storage, with a "hunger center" (lateral hypothalamic area; LHA) driving food intake and body weight gain and an opposing "satiety center" (ventromedial hypothalamus; VMH) responsible for reducing intake and causing weight loss (Fig. 12.1a). Based on these pioneering results, researchers were prompted to identify signal(s) putatively informing the hypothalamus about the animal's feeding status and the amount of energy stored as fat.

At the same time that the cataloging of the role of specific hypothalamic regions was being pursued, a spontaneous gene mutation in an inbred mouse strain was

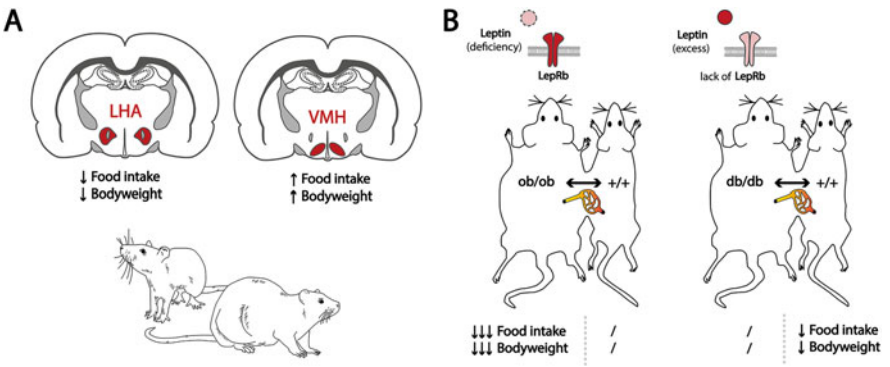


Fig. 12.1 The discovery of an adipose-to-hypothalamus axis controlling food intake and bodyweight. (**a**) Electrolytic lesioning (in red) of two distinct hypothalamic nuclei oppositely affects feeding and adiposity. (**b**) Depiction of parabiosis experiments in which pairs of mice were surgically conjoined in order to share a single circulatory system; the hyperphagic obesity in leptin-deficient *ob/ob* mouse mutants was readily corrected upon pairing with a lean wildtype mouse. Conversely, mutant mice lacking leptin receptors (*db/db*) were unresponsive and actually suppressed food intake in their parabiosed lean wildtype partners. LHA, lateral hypothalamic area; VMH, ventromedial hypothalamus; *ob/ob*, mutant mouse homozygously lacking the *obese* allele, thus lacking adipose tissue and circulating leptin; *db/db*, mutant mouse homozygously lacking a functional leptin receptor; +/+, wildtype mouse

serendipitously found to cause voracious feeding (hyperphagia) and massive obesity, with the affected alleles tellingly being termed *ob/ob* for obese (Coleman 1978). Strikingly, a reversal of both the hyperphagia and obesity could be achieved by surgically fusing the blood circulations of obese mutant mice with that of lean control mice ("parabiosis" experiments), thereby elegantly demonstrating that the mutation caused the loss of a blood-borne satiety signal (Fig. 12.1b). In 1994, Jeffrey Friedman and colleagues succeeded in identifying the gene product, a 16-kDa peptide hormone primarily produced in white adipose tissue (Zhang et al. 1994), which was soon given the name "leptin" (Greek: λεπτός (leptos) for "thin"). Soon thereafter, the cognate leptin receptor (LepR), as well as its *db* mutation (*db* for diabetic) was identified. The LepR has six variants in mice. The major signaling form, LepRb, is long and primarily expressed by neurons of the hypothalamus, where leptin exerts the preponderance of its metabolic effects (Elmquist et al. 1998). Strikingly, the ablation of LepRb exclusively in neurons in the hypothalamus is sufficient to increase body weight and adiposity (Cohen et al. 2001; Dhillon et al. 2006; Ring and Zeltser 2010; Xu et al. 2018). Conversely, the localized restoration of hypothalamic LepRb in otherwise leptin-receptor-deficient mice (de Luca et al. 2005; Coppari et al. 2005) and rats (Morton et al. 2003) greatly ameliorates their obesity. In humans, congenital leptin deficiency, or congenital leptin receptor deficiency, comparably results in hyperphagic obesity at a young age (Read more about leptin biology in Box 12.1). To summarize, leptin signaling constitutes a prototypical, highly conserved mechanism for mammalian adipose-to-hypothalamus communication, and one which is indispensable for proper energy homeostasis (Farooqi and O'Rahilly 2014).

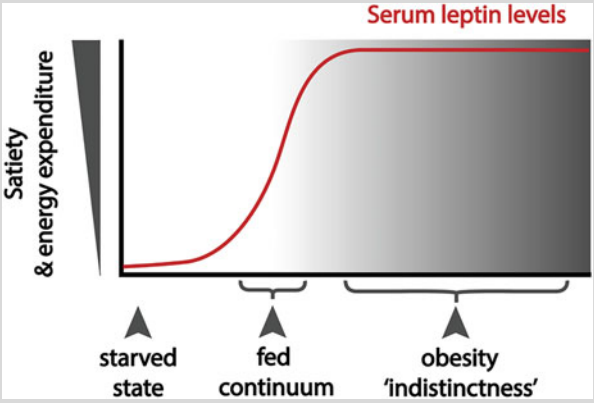
Box 12.1. Leptin Biology and the Concept of "Leptin Resistance"

Circulating leptin has a high positive correlation with adipose tissue mass and thus reliably reflects stored energy (Considine et al. 1996). Accordingly, circulating leptin levels drop profoundly in the face of chronic calorie deprivation and starvation. Low leptin signaling in turn elicits powerful adaptive responses including compensatory hunger and reduced energy expenditure (Rosenbaum and Leibel 2014). Consistently, monogenic mice that lack either leptin or leptin receptors develop obesity because they lack the signal in the hypothalamus that would otherwise suppress food intake and body weight. In contrast, rather than having leptin deficiency, most obese humans actually present with high circulating leptin, levels that are in direct proportion to their adipose mass. Consistent with this, most obese humans fail to respond to exogenous leptin with a reduction in appetite and body weight, a phenomenon conceptualized as an obesity-associated state of "leptin resistance" (=hypothalamic desensitization toward excess hormone levels; (Myers et al. 2012)). Nevertheless, recent elegant studies using pharmacological antagonism implied that sensitivity to endogenous leptin is well retained in diet-

(continued)

Box 12.1 (continued)
induced obesity and supports the notion that the consistently elevated leptin levels in obese individuals saturate the leptin receptors, rendering additional exogenous leptin ineffective (Ottaway et al. 2015).

Current thinking is that leptin's biological effects exhibit a profoundly asymmetric dose-response curve, with powerful catabolic effects elicited in its deficient-to-low state and an early plateauing that prevents further homeostatic adjustments in the face of high-to-obese levels (see Box Figure below).



Hypothetical dose-response curve for leptin action at different stages along the energy homeostasis continuum. Notably, biological effects already max out at a well-fed state and only marginal effects can be achieved when further elevating leptin levels

It is important to note that the brain regions sensitive to leptin also integrate a plethora of other metabolic feedback signals including the opposing paradigmatic "hunger" signal, ghrelin. Produced by gastric X cells in the stomach, the peptide hormone ghrelin is released in concert with increased hunger sensations, and its exogenous administration robustly stimulates feeding via a hypothalamus-centered mechanism as first described by Heiman and Tschöp (Tschop et al. 2000). Ghrelin is a 3.24 kDa peptide hormone that, in order to become functionally active, is required to undergo post-translational modification, namely the enzymatic addition of octanoic acid to a serine residue. The pharmacological inhibition of the enzyme responsible (ghrelin O-acyltransferase) recently emerged as a promising mechanism to lower biologically active, acylated levels of the "hunger signal" ghrelin (Kirchner et al. 2009; Barnett et al. 2010).

Throughout the last two decades, numerous research groups have participated in unfolding the underlying principles of the complex adipose/gut-to-brain crosstalk while starting to implement increasingly innovative technologies. Ultimately, the discovery of leptin and ghrelin catapulted the study of energy homeostasis into a new era, based on modern molecular genetics and systems neuroscience.

12.3 The Neuronal Blueprint of Hypothalamic Feeding Circuits

Although numerous sites in the brain influence metabolic activity throughout the body, the hypothalamic arcuate nucleus (ARC) is pivotal in this regard. The ARC is a narrow, elongated area at the base of the hypothalamus and adjacent to the third ventricle (see Fig. 12.2a). ARC neurons have the highest concentration of both leptin and ghrelin receptors (see Fig. 12.2b), and they express receptors for numerous other metabolic hormones as well. The ARC abuts the median eminence (ME), a circumventricular brain area characterized by relatively large openings ("fenestrations") in its local capillaries that allow for direct neurohemal exchange. As a consequence of these leaky capillaries, circulating factors such as hormones and metabolites are granted high accessibility to this brain area, enabling nearby ARC neurons to rapidly monitor blood-borne metabolic cues. Thus, the ARC-ME complex forms a major homeostatic control hub composed of specialized cell types, many having dedicated roles in the detection, integration, processing, and central propagation of feeding-related peripheral information.

POMC Neurons One of the most extensively studied ARC neuronal populations produce the pro-opiomelanocortin (POMC) precursor peptide inside their relatively large cell bodies. Predominantly found in the lateral portion of the ARC, ARC^{POMC} neurons are activated by signals of energy surplus (e.g., leptin, insulin, glucose) and conversely are inhibited by signals of energy deficit (e.g., ghrelin). Activation of ARC ^{POMC} neurons leads to a reduction of food intake and an increase in energy expenditure. Rather than occurring acutely, these actions require rather prolonged and sustained ARC^{POMC} activation (Aponte et al. 2011; Fenselau et al. 2016). Consistent with more gradual effects, the artificial increase in hypothalamic POMC tone, e.g. by neuronal POMC overexpression upon viral gene transfer, attenuates obesity in mice or rats only over protracted periods of time (Mizuno et al. 2003; Li et al. 2003). Thus, the ARC^{POMC} population appears to serve as a long-term, slow-onset integrator within the energy regulatory system. The major signaling molecule utilized by ARC^{POMC} neurons for mediating these effects is α-melanocyte-stimulating hormone (α-MSH), an anorexigenic neuromodulator derived from the POMC precursor peptide. Strikingly, the pharmacological blockade of α-MSH signaling in the hypothalamus abolishes most of leptin's metabolic effects; i.e., ARC ^{POMC} neurons are a major downstream mediator of leptin (Seeley et al. 1997; Marsh et al. 1999). ARC^{POMC} neurons propagate leptin signaling via long-range axonal projections to numerous brain regions. One of the more important of these is the hypothalamic paraventricular nucleus (PVN), where ARC^{POMC} axons release α-MSH onto second-order neurons that express the cognate MC4R (melanocortin-4 receptor). This $ARC^{POMC} \rightarrow PVN^{MC4R}$ anatomical connection is known as the "central melanocortin system," and this circuit is now known to be a highly conserved core element of central energy balance regulation. Accordingly, loss-of-function mutations of either the *Pomc* or *Mc4r* gene result in massive hyperphagic obesity in both mice (Yaswen et al. 1999; Huszar et al. 1997) and humans (Krude et al. 1998; Farooqi et al. 2003). While such null mutations in single

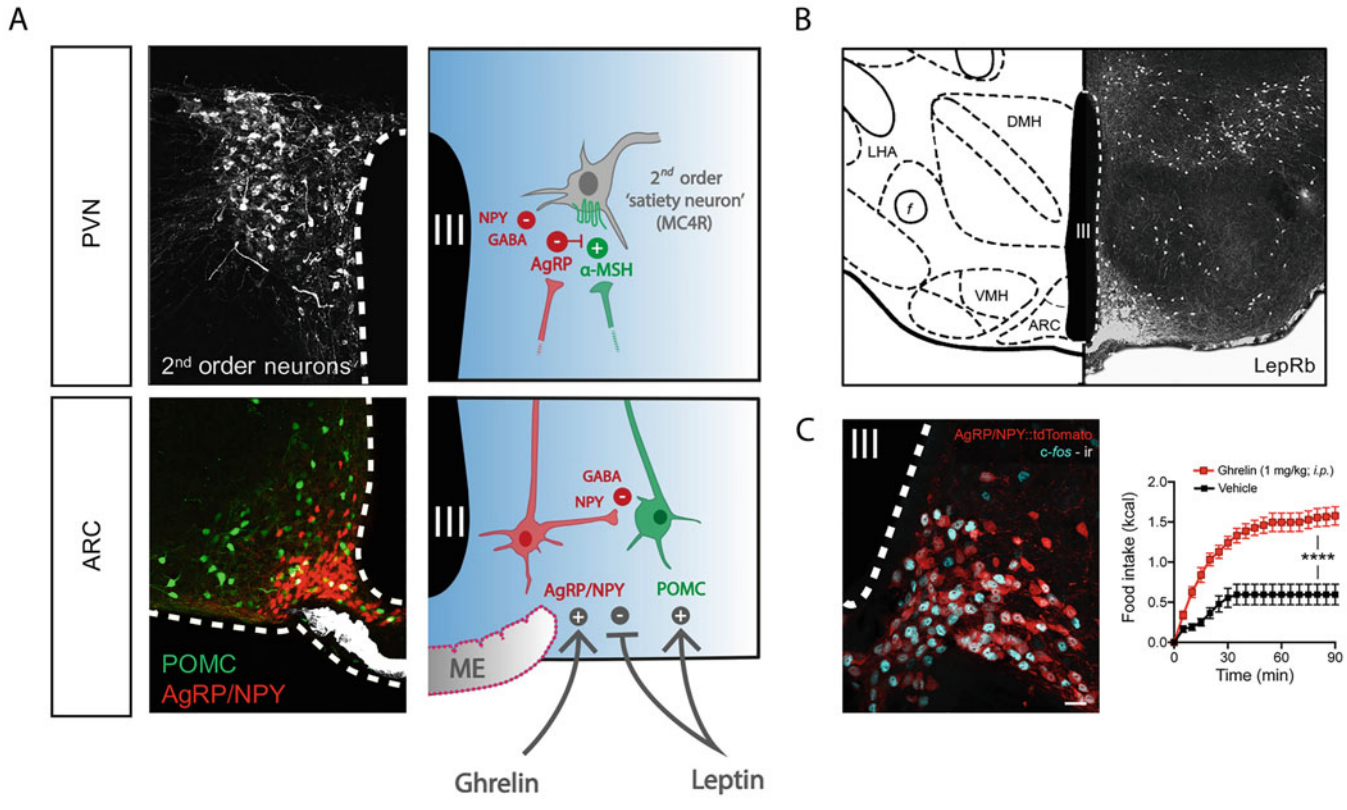


Fig. 12.2 Wiring diagram of the central melanocortin system in the hypothalamus. (**a**) Coronal view of the melanocortinergic ARC → PVN circuit visualized by confocal microscopy of dual reporter mice expressing separate fluorescent proteins in ARC^{POMC} and ARC$^{AgRP/NPY}$ neurons, respectively, in combination

with a mirrored schematic depiction to the right. (**b**) Expression pattern of the long-form LepRb throughout the hypothalamus in the coronal view; a particularly high density of $LepRb^{+}$ neurons can be found within the ARC. (**c**) Confocal micrograph showing prominent c-*fos* induction in $ARC^{AgRP/NPY}$ neuronal nuclei upon peripheral injection of Ghrelin (1 mg/kg; i.p.) indicating intense and very specific neuronal activation of this particular population; the same ghrelin injection triggers a significant and immediate feeding response in mice ($n = 11$; $p^{****} < 0.0001$). Scale bar: 20 μm. DMH, dorsomedial hypothalamus; VMH, ventromedial hypothalamus; LHA, lateral hypothalamic area; ARC, arcuate nucleus; III, third ventricle; f, fornix; ir, immunoreactivity; i.p., intraperitoneal

genes are extremely rare, more widespread allelic variants within the *Mc4r* gene are strongly associated with altered eating, predisposition to weight gain, and ultimately obesity in humans (2–6% prevalence, depending on sample population), further underscoring the significance of this pathway. While anatomical, functional, and genetic data have converged at this $ARC^{POMC} \rightarrow PVN^{MC4R}$ connection, it is important to note that the ARC^{POMC} population (ca. 9000 cells in mice) (Lemus et al. 2015) displays a substantial degree of functional heterogeneity and anatomical segregation. Viral tracing studies have revealed that ARC^{POMC} neurons residing in more rostral regions of the ARC near the retrochiasmatic area project predominantly to pre-autonomic hindbrain areas and are sensitive to estrogens and leptin; in contrast, more caudal populations of ARC^{POMC} neurons primarily innervate other hypothalamic areas such as the PVN, and they consist of subsets that are sensitive to leptin, glucose, insulin, and serotonin, among other effectors (Toda et al. 2017).

AgRP/NPY Neurons Intermingled within the ARC are neurons that are characterized by their distinct expression of Agouti-related peptide (AgRP; an inverse agonist of MC3/4R that increases food intake) and neuropeptide Y (NPY; another powerful orexigenic signal). These $ARC^{AgRP/NPY}$ neurons functionally oppose the anorexia-promoting effects of ARC^{POMC} neurons, and they are activated by signals of energy deficit (e.g., fasting, ghrelin; see Fig. 12.2c). Located in the most medial portion of the ARC, $ARC^{AgRP/NPY}$ neurons are in close apposition to the fenestrated capillaries of the ME, with 60–70% of them being formally considered outside of the blood-brain barrier (Olofsson et al. 2013). This unique and privileged anatomical feature is believed to render them first-line responders to blood-borne signals biasing energy intake. In contrast to ARC^{POMC} neurons, which predominantly release peptidergic neuromodulators such as α-MSH, $ARC^{AgRP/NPY}$ neurons release both peptidergic signals (AgRP and NPY) and rapidly acting ionotropic GABAergic neurotransmitters, to exert immediate inhibitory action on target neurons (Tong et al. 2008; Wu et al. 2009). As occurs on MC4R with leptin, AgRP impacts food intake only after an extended period. In contrast, feeding is almost instantaneously induced in response to fast-acting NPY and GABA (Krashes et al. 2013). $ARC^{AgRP/NPY}$ neurons target PVN^{MC4R} neurons, where the release of AgRP, NPY, or GABA exerts inhibitory action (Cowley et al. 1999). Although there are relatively few $ARC^{AgRP/NPY}$ neurons (ca. 8000 neurons in mouse) (Lemus et al. 2015), either optogenetic or chemogenetic activation of this small population triggers an immediate and voracious feeding response; conversely, their selective ablation by diphtheria toxin leads to cessation of feeding and death by starvation within days (Luquet et al. 2005). Furthermore, the robust food intake elicited by $ARC^{AgRP/NPY}$ stimulation suppresses rival motivational activities based on thirst, innate fear, and social interaction (Burnett et al. 2016) while enhancing behaviors including risk-taking and decision-making (Padilla et al. 2016). Intriguingly, the prioritization of food intake over other drives upon $ARC^{AgRP/NPY}$ stimulation, rather than potentiating the rewarding properties of food, seems to be an attempt to alleviate the uncomfortable feelings associated with energy deficit. Thus, and contrary to expectations, $ARC^{AgRP/NPY}$ activity transmits a negative valence, as

was elegantly demonstrated by combining conditioned flavor preference tests with bi-directional $ARC^{AgRP/NPY}$ modulation (Betley et al. 2015). Thus, the same $ARC^{AgRP/NPY}$ stimulation in the absence of food triggers repetitive and compulsive behaviors in mice, reminiscent of behavioral abnormalities in psychiatric conditions such as anorexia nervosa (Dietrich et al. 2015). Thus, determining how $ARC^{AgRP/NPY}$ neurons align environmental cues with motivational and emotional states may serve as an important entry point to better understand the intersection of deranged metabolism and maladaptive behaviors (for more recent discoveries on $ARC^{AgRP/NPY}$ modulation, see Box 12.2).

In summary, diverse approaches have consistently and cogently established the indispensability of $ARC^{AgRP/NPY}$ neurons for appetite regulation while highlighting an intriguing dichotomy of ARC^{POMC} and $ARC^{AgRP/NPY}$ neurons, which exhibit overlapping projection patterns that often target the same second-order neurons, such as PVN^{MC4R} neurons (Fig. 12.2a).

Box 12.2

Beyond the slow detection of homeostatic signals by ARC neurons, ARC cells are traditionally construed as internally orientated sensory neurons, directly assessing slow alterations of the metabolic milieu (hence being termed "first-order" neurons). By detecting and integrating nutrient- and energy-related signals from the circulation, ARC cells were historically believed merely to translate the level of a hormone, for example, into cellular activity in a manner independent of top-down cognitive control. However, this long-held assumption was recently challenged when optical in vivo recordings were employed for population-specific Ca^{2+} imaging in freely moving mice. Strikingly, these studies revealed that both ARC^{POMC} and $ARC^{AgRP/NPY}$ neurons rapidly and robustly respond to food-related sensory cues including smell, taste, and vision (Chen et al. 2015). Of note, these changes in neural activity precede any nutrient ingestion, thus challenging the view that ARC neurons solely constitute homeostatic rheostat modules. A more recent study has identified even more complexity by demonstrating that a prior period of free access to palatable, calorie-dense food greatly blunts the change in $ARC^{AgRP/NPY}$ neural activity elicited by the presentation of a standard chow pellet, compared to what occurs in palatable-food naive mice. Thus, this perturbed sensory integration at the level of the ARC directly encodes the experience-dependent devaluation of chow diet following rewarding foods in mice and might be manifested as the challenge of obese patients to adhere to healthier but less tasty food choices (Mazzone et al. 2020).

Thus, aided by cell-specific imaging technology, the prescience of the classic behaviorist I.P. Pavlov is apparent, since he demonstrated the importance of anticipation in feeding regulation in the late nineteenth century. After years of research centered at reflex-driven homeostasis, these intriguing

(continued)

Box 12.2 (continued)
findings are now reemphasizing the concerted action of homeostatic and cephalic/anticipatory mechanisms (see review Ramsay and Woods 2014), and importantly, that they are manifested within conserved ARC sensory neurons.

12.3.1 The Neural Connectivity of Food Intake: Mapping Structure onto Function

ARC Hypothalamic feeding circuits strongly bias consuming more food. Accordingly, the local stimulation of $ARC^{AgRP/NPY}$ neurons still evokes robust feeding behavior despite concomitantly activating ARC^{POMC} neurons (Atasoy et al. 2012). Perhaps due to their temporally slow action pattern, this finding unequivocally demonstrated that ARC^{POMC} activation is insufficient to overcome the vigorous feeding response to ARC $^{AgRP/NPY}$-stimulation. Given that ARC^{POMC} neurons express NPY-Y1 and $GABA_A$ receptors and are directly modulated by neighboring $ARC^{AgRP/NPY}$ neurons (Cowley et al. 1999), this further suggests that inhibition of ARC^{POMC} neurons does not constitute a functionally relevant target for acute $ARC^{AgRP/NPY}$ stimulation of feeding. Thus, the actual downstream site(s) of the diametrically opposed ARC populations posed a pivotal question, and several groups have now employed advanced optogenetic approaches, including ChR2-assisted circuit mapping (CRACM), to address it. By individually stimulating distinct terminal fields of axons that emanate from either ARC ^{POMC} or $ARC^{AgRP/NPY}$ neurons, this method provides temporal and regional control over transmitter release in select brain areas, enabling interrogation of their respective functional relevance in freely behaving animals. CRACM studies have found that $ARC^{AgRP/NPY}$ neurons consist of multiple distinct subpopulations, each having unique axon trajectories. The respective destination of these trajectories depends on each $ARC^{AgRP/NPY}$ neuron's topographical location along the rostro-caudal axis of the ARC, as elucidated by using rabies virus-assisted monosynaptic retrograde tracing (SADΔG-mCherry(EnvA)). Although photostimulation of several of the different individual target regions of $ARC^{AgRP/NPY}$ axons was sufficient to elicit a feeding response, the penetrance and strength of these separate output circuits varied. Robust feeding occurred following stimulation of $ARC^{AgRP/NPY}$ fibers in the anterior bed nucleus of the stria terminalis (aBNST), the LHA, or the PVN (Atasoy et al. 2012; Betley et al. 2013). Despite some degree of redundancy in the wiring of $ARC^{AgRP/NPY}$ neurons, these functional mapping studies provide a window into the spatial specialization of feeding-sufficient projections. In particular, these CRACM studies reinforced the concept that the PVN is a pivotal downstream center for reducing energy intake.

PVN The PVN is one of the most neuron-dense, structurally complex regions of the brain. Lying bilaterally adjacent to the dorsal portion of the third ventricle, the PVN

is crucially involved in orchestrating diverse neuroendocrine, autonomic, and behavioral responses for maintaining general homeostasis. Accordingly, human genetic defects that interfere with PVN development, such as haploinsufficiency of *Single-minded 1* (Sim1), or PVN-directed lesions in animal models, have severe consequences, including massive obesity (Cox and Sims 1988; Michaud et al. 2001; Faivre et al. 2002; Tolson et al. 2010). While a detailed elaboration on the complex PVN cytoarchitecture is beyond the scope of this chapter (for further information see Swanson and Sawchenko 1980; Biag et al. 2012), we highlight here that the PVN has several subdivisions harboring distinct types of neurons, which—besides their anatomical location and size—can be further characterized by the receptors they express and the neuropeptide(s) and other transmitters they produce (Li et al. 2019). Many neuropeptides produced by PVN neurons are powerful suppressants of food intake when centrally administered, including oxytocin (OT; for more information, see Box 12.3), arginine vasopressin (AVP), corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), dynorphin (DYN), and brain-derived neurotrophic factor (BDNF). As discussed above, the PVN is also a major target of both ARC^{POMC} and $ARC^{AgRP/NPY}$ neurons, while being particularly enriched in MC4R. Elegant studies using bi-directional genetic manipulations unambiguously determined that PVN^{MC4R} neurons are the critical relay within the melanocortin circuit for maintenance of normal food intake (Balthasar et al. 2005). The genetic deletion of MC4R in the PVN is sufficient to evoke severe hyperphagia; conversely, the PVN-specific restoration of the receptor in mice otherwise devoid of MC4R abolished their heightened drive to feed. PVN^{MC4R} neurons that reduce food intake express glutamate as a transmitter, but do not express CRH, TRH, OT, AVP, or DYN. Thus, glutamatergic PVN^{MC4R} neurons represent a separate, non-peptidergic, and fairly small population within the PVN, as was later corroborated based on transgenic $MC4R^{2a\text{-}Cre}$ reporter mice (Garfield et al. 2015). Despite constituting only a small fraction of overall PVN neurons, this subset receives particularly dense input from $ARC^{AgRP/NPY}$ neurons. 83% of PVN^{MC4R} neurons receive direct $ARC^{AgRP/NPY}$ innervation whereas unidentified, MC4R-negative PVN neurons have far fewer such connections (20%) (Atasoy et al. 2012; Krashes et al. 2014). However, the electrophysiological role of ARC^{POMC} neurons in reducing food intake remained largely elusive given the temporal disconnect of MC4R and the absence of fast-acting neurotransmitters. This conceptual chiasm was recently bridged by the discovery of a population of ARC glutamatergic neurons that are genetically marked by expression of the oxytocin receptor ($ARC^{OT\text{-}R}$ neurons) (Fenselau et al. 2016). As corroborated by single-cell RNA-seq, this cell cluster—as opposed to non-glutamatergic ARC^{POMC} neurons—is highly enriched for transcripts required for fast synaptic transmission, e.g., complexin-1 and synaptotagmin-1. Importantly, photostimulation of $ARC^{OT\text{-}R}$ axons in the PVN evoked excitatory postsynaptic potentials (EPSCs) in PVN^{MC4R} neurons, and also rapidly reduced food consumption, unlike ARC^{POMC} stimulation. To dissect how these different neurons interact to regulate feeding, the authors combined ex vivo and in vivo experiments and identified an intriguing mechanism that coordinates “slow” and “fast” ARC neurons at the level of the PVN; i.e., the

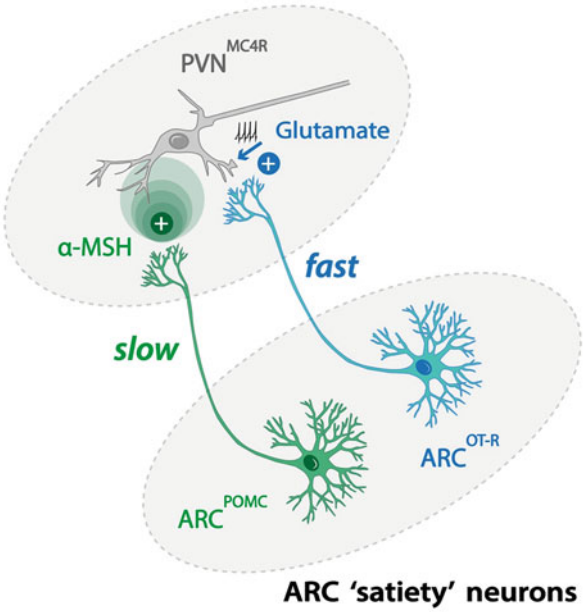


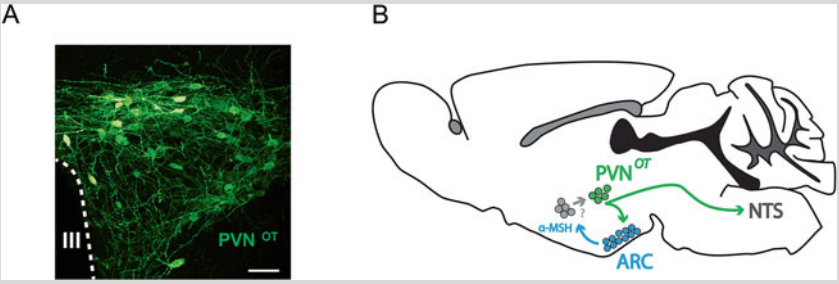
Fig. 12.3 An update on the central melanocortin circuit. The puzzling fact that ARC ^{POMC} neurons reduce feeding only over protracted periods of time, and thus do not constitute the direct counterpart of ARC$^{AgRP/NPY}$ neurons, remained a significant gap in the conceptual framework of the central melanocortin circuit. The recent discovery of an additional fast-acting "satiety" neuron within the ARC closed this gap and provided the mechanism through which PVN^{MC4R} neurons receive direct, glutamatergic input in addition to the slower "metabotropic" modulation, which is mediated by α-MSH. Intriguingly, these fast-acting glutamatergic neurons in the ARC are characterized by the expression of OT-R

release of α-MSH from ARC^{POMC} neurons primes PVN^{MC4R} neurons to potentiate the glutamatergic input from ARC^{OT-R} axons, for instance by increasing the AMPAR-to-NMDAR ratio (Fig. 12.3). A previous report had already demonstrated that PVN neurons possess specific mechanisms to integrate and modulate incoming synaptic inputs over extended periods of time. Mediated by the voltage-gated sodium channel Nav1.7, neurons of the PVN exhibit a peculiar summation of excitatory inputs, while genetic abrogation of this particular mechanism leads to obesity (Branco et al. 2016). Thus, Fenselau and colleagues added a previously elusive component to the melanocortin circuit: the fast-acting, glutamatergic ARC^{OT-R} neuron (Fig. 12.3).

Box 12.3. The Role of Oxytocin (OT) in the Regulation of Food Intake
Besides its well-recognized role as a circulating hormone that coordinates social behavior and reproductive physiology, the hypothalamic neuropeptide

(continued)

Box 12.3 (continued)
OT has emerged as an important regulator of metabolism. While the global loss of OT or OT-R causes late-onset obesity in mice, both central and peripheral administration of OT reduces calorie intake and body weight and thus has potential as an anti-obesity/anti-diabetic agent (McCormack et al. 2020). OT is produced in both magno-and parvocellular neurons of the PVN (see Chap. 6), and parvocellular OT neurons facilitate the effects of reducing food intake by targeting pre-autonomic regions in the brainstem, including the nucleus tractus solitarius (NTS), which is enriched in OT-R (Ho et al. 2014; Ong et al. 2017). Interestingly, a subset of parvocellular PVN^{OT} neurons simultaneously project to both the NTS and the medial ARC via bifurcating axon collaterals (Maejima et al. 2014, 2016; Csiffary et al. 1992). Consistent with this observation, the neurons in the ARC that highly express OT-R are robustly excited by OT (Fenselau et al. 2016), and intra-ARC injection of OT reduces feeding (Maejima et al. 2014). Conversely, anorexigenic α-MSH derived from ARC^{POMC} neurons activates PVN^{OT} neurons and induces central OT release (Olszewski et al. 2001; Maejima et al. 2017); yet, there is clear evidence for an indirect mechanism, since PVN^{OT} neurons do not express MC4R (Balthasar et al. 2005; Garfield et al. 2015). On the basis of these observations, a reciprocal ARC<>PVN feeding network is assumed, that putatively operates by involving OT as a major signaling modality. Lastly, the VMH, another important feeding-regulatory area, is one of the most OT-R enriched areas in the brain (Gould and Zingg 2003). While there is ample evidence suggesting that the hypothalamic OT system is pertinent to metabolic homeostasis, the exact neural pathways and mechanisms remain incompletely understood.



The hypothalamic OT system in the control of feeding. (**a**) Confocal micrographs of PVN ^{OT} neurons expressing green fluorescent protein. (**b**) Schematic depiction of a reciprocal ARC <> PVN pathway in which parvocellular PVN ^{OT} neurons send collateral projections to both the NTS and the ARC. In turn, α-MSH releases from ARC ^{POMC} neurons activates PVN ^{OT} neurons presumably via an indirect mechanism as they lack MC4R. Scale bar: 50 μm

VMH, DMH, LHA, and SCN Anatomically, the VMH is located in the mediobasal hypothalamus and consists of two clearly demarcated, elliptical cell groups surrounded by a cell-poor, dendrite-rich zone. The majority of neurons in

the VMH are glutamatergic excitatory neurons (Ziegler et al. 2002; Tong et al. 2007) that form synaptic connections with other hypothalamic regions, including ARC^{POMC} neurons (Sternson et al. 2005). While the VMH consists of a conglomerate of heterogeneous cell types, most VMH neurons express the transcription factor steroidogenic factor 1 (SF-1), and it has become a specific genetic marker of VMH neurons (Cheung et al. 2013). The VMH is highly sensitive to subtle changes in fuel availability. Subsets of VMH neurons strongly respond to hyper- and hypoglycemia, respectively, and some neurons are sensitive to insulin (Klockener et al. 2011). In contrast to virtually every other brain area, the VMH has the capacity to readily utilize fatty acids for fuel (Wang et al. 1994; Le Foll et al. 2014), and the intracellular balance of β-oxidation versus fatty acid synthesis in the VMH has been reported to be an important determinant for fasting- and ghrelin-induced feeding—states when circulating fatty acid levels are typically high (Lopez et al. 2008). Thus, these metabolic specializations and dynamics render the VMH an ideal sensor of whole-body energy fluxes.

DMH The dorsomedial hypothalamus (DMH) resides astride the VMH. DMH neurons impact autonomic functions and behavior while being sensitive to several signals that reduce food intake such as cholecystokinin (Chen et al. 2008) and leptin (Rezai-Zadeh et al. 2014). DMH neurons expressing LepRb have a GABAergic phenotype and are rapidly activated by food-associated sensory cues. In turn, DMH^{LepRb} neurons give rise to axonal projections that synapse onto $ARC^{AgRP/NPY}$ neurons. As expected, photostimulation of these inhibitory $DMH^{LepRb} \rightarrow ARC^{AgRP/NPY}$ neurons electrically silences these neurons and reduces food intake (Garfield et al. 2016). Thus, this particular microcircuit might well contribute to the rapid modulation of "first-order" ARC neurons by cephalic-anticipatory mechanisms (see Box 12.2).

LHA The more lateral portion of the medial hypothalamus, the LHA, was historically termed a "hunger center" because its electrical stimulation evokes feeding and its ablation induces severe anorexia, aphagia, and weight loss (Anand and Brobeck 1951). The use of modern, more precise technology such as opto- and chemogenetics and virus-based transsynaptic tracing (Sakurai et al. 2005; Kampe et al. 2009) has revealed that the LHA has a pivotal role in numerous regulatory systems, including drug addiction and food reward, food and mate seeking, reinforcement learning and sleep-wakefulness (Berthoud and Munzberg 2011). The LHA is diffusely organized and harbors heterogeneous cell types. Two types of LHA neurons are characterized by the expression of orexigenic neuropeptides: melanin-concentrating hormone (MCH; for more information, see Chap. 13) or orexin/hypocretin (ORX/HPCRT) (Tsujino and Sakurai 2013). Consistent with the importance of the LHA for diverse emotional and motivational systems, its neurons are highly interconnected with numerous cortico-limbic structures. Major afferent input to the LHA comes from the pre-frontal cortex (PFC), a region typically associated with higher-order brain functions; by giving rise to a descending pathway that is relayed in the lateral septum, these PFC neurons ultimately provide top-down

control over LHA activity and food-seeking behavior (Carus-Cadavieco et al. 2017). In turn, LHA neurons innervate major cortico-limbic areas. Perhaps most notably, $LHA^{ORX/HPCRT}$ neurons project to both dopaminergic and non-dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain as well as their principal target region, the nucleus accumbens. Through these circuits, orexin-mediated mechanisms profoundly modulate dopaminergic signaling along the mesolimbic pathway in order to facilitate generalized "wanting" or "seeking" behaviors directed toward environmental incentives and contingencies (Harris et al. 2005; Narita et al. 2006; Leinninger et al. 2009).

SCN In most vertebrates, feeding behavior follows an approximately 24-h diurnal rhythm. Animals experience metabolic fluxes in a predictable, time-of-day-dependent manner and adjust their bodily processes accordingly (sleep-wakefulness, endocrine secretions, autonomic nerve activity, food intake). While virtually every tissue possesses its own intrinsic circadian clock in the form of a cell-autonomous transcription-translation feedback loop (Hardin and Panda 2013), the ultimate master pacemaker resides in the hypothalamus just above the optic chiasm and is called the suprachiasmatic nucleus (SCN). It has two bilateral subdivisions each containing circa 10,000 relatively small neurons (diameter: 10 μm) in the mouse brain (Abrahamson and Moore 2001). The neurons within the core of the SCN become light-entrained by inputs from specialized cells in the retina, and they in turn orchestrate diverse physiological processes including those related to feeding behavior, energy expenditure, and nutrient partitioning (for further information see Chap. 11).

12.3.2 The Neural Development of Hypothalamic Feeding Circuits

The development of hypothalamic feeding circuits follows an intricate process that takes place in late gestational or early postnatal life. It involves a concerted, spatiotemporally regulated array of mechanisms including waves of neuro- and glio-genesis, axon growth and pathfinding, synaptogenic versus synaptoclastic activity, cell migration, and others (Bouret 2010a). Multiple environmental factors can influence hypothalamic wiring during critical periods of neural development and have a lasting impact on the functionality of these feeding networks. As an example, perinatal over- or under-nutrition in rodents and humans is associated with adverse health effects and metabolic dysregulation in later life (Dearden and Ozanne 2015). Leptin has a pivotal role in mediating axonal outgrowth from ARC neurons during brain development, and this is an essential process in the formation of the central melanocortin system (Bouret et al. 2004). Thus, and in addition to its metabolic functions in adulthood, early postnatal surges in circulating leptin are a prerequisite for proper hypothalamic neural development and connectivity.

Despite a certain degree of synaptic plasticity within these established brain networks influencing food intake and metabolism (Pinto et al. 2004), the connectivity diagram of feeding behavior ultimately consists of hardwired components. It is

important to note, however, that maintaining this circuit involves a highly orchestrated process that governs rigorous fate commitment of hypothalamic neurons, the maintenance of their neuropeptidergic identity, and their overall functional role. Recently, the transcription factor T-box 3 (Tbx3) has emerged as a key component of the intracellular machinery directing cellular identity of hypothalamic melanocortin neurons in the ARC (Quarta et al. 2019). Tbx3, whose haploinsufficiency causes obesity in humans (Linden et al. 2009), is enriched in the ARC (Eriksson and Mignot 2009) and modulated by metabolic stimuli (Knight et al. 2012). Importantly, loss of Tbx3 function in hypothalamic ARC neurons causes severe hyperphagic obesity in mice as a consequence of an impaired melanocortin circuitry. These abnormalities include a reduction in both ARC^{POMC} neuron number and their projections as assessed by $\alpha\text{-MSH}^{+}$ fiber density in the PVN. In agreement with a reduced melanocortinergic tone, the central administration of a sub-effective dose of α-MSH was able to normalize food intake in mice devoid of hypothalamic Tbx3. Thus, loss of Tbx3 appears to perturb the developmental program that gives rise to the formation of melanocortin ARC neurons. However, its adult-onset deletion further reveals a pertinent role in already-mature ARC cells, since loss of Tbx3 distorts peptidergic expression profiles, and ultimately cell identity, of mature ARC neurons. In summary, considerable insights have been accumulated in recent years addressing many of the remaining questions regarding the role of hypothalamic neurons and their cell-fate determining molecular cogs.

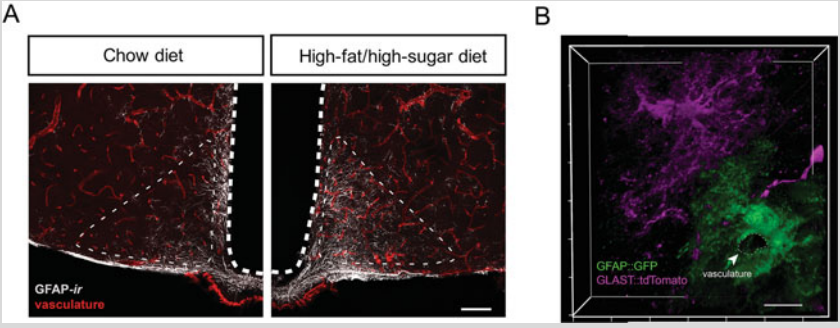
In parallel to this progress, however, we are currently experiencing a profound paradigm shift as the limelight of metabolic research is increasingly taken by classes of cells other than neurons, i.e., by astrocytes (for more information see Box 12.4).

Box 12.4. Astrocytes and Other Non-neuronal Brain Cells in Energy Balance Regulation

It has become increasingly evident that confinement of consideration to exclusively neuronal cells would lead to an insufficient understanding of energy homeostasis. Hence, the rather restrictive "neurocentric" standpoint has given way to a more holistic and integrated perspective (Garcia-Caceres et al. 2019). Astrocytes, the star-shaped and most abundant glial subclass, were recently identified as metabolic sensors in the hypothalamus. By occupying a strategic position within the brain parenchyma, namely the space between vasculature and neurons, astrocytes are ideally situated to sense and moderate the milieu internal and external of the brain. Hypothalamic astrocytes modulate the action of several major hormones including leptin (Kim et al. 2014) and ghrelin, which alters the activity of $ARC^{AgRP/NPY}$ neurons through the release of the gliotransmitter adenosine (Yang et al. 2015). Astrocytes also mediate glucose transport into the brain by an insulin receptor-dependent process; consequently, mice devoid of astroglial insulin signaling exhibited severely perturbed glucose-sensing by hypothalamic

(continued)

Box 12.4 (continued)
neurons as well as impaired glucose tolerance and suppression of feeding following a glucose bolus (Garcia-Caceres et al. 2016). The consumption of a hypercaloric diet dramatically and almost instantaneously elicits a hypertrophic, reactive phenotype, which is particularly evident in the ARC-ME complex, with those astrocytes that are in close proximity to local microvessels being particularly affected (Horvath et al. 2010; Thaler et al. 2012). This structural reorganization, which is driven by the astroglial upregulation of the brain injury-marker GFAP (glial-fibrillary acidic protein; an intermediary filament of the cytoskeleton), precedes any change in body weight; moreover, this early event of diet-induced "reactive astrogliosis" is accompanied by an increased production of pro-inflammatory cytokines in the hypothalamus as well as profound changes in the synaptology of the melanocortin system (Horvath et al. 2010). Given the striking regional confinement of this phenomenon, the existence of specific diet-responsive astrocyte subpopulations is assumed. Indeed, emerging data on general astroglial biology increasingly indicates that astrocytes display substantial functional diversity and inter- and intra-regional heterogeneity. Accordingly, it has been shown that astrocytes adopt distinct functional roles, depending on the neuronal network they are embedded in, thus establishing an additional layer of information processing superimposed onto the basic neurocircuitry (for review see Ben Haim and Rowitch 2017). Finally, endothelial cells within the hypothalamus also exhibit a significant vulnerability toward obesogenic diets, and the hypothalamic angioarchitecture is profoundly remodeled by hypercaloric diets as demonstrated in both mice and humans ("hypothalamic microangiopathy"; (Yi et al. 2012). The pathophysiological relevance of these gliovascular dysfunctions, which occur intriguingly early in the disease process, are currently under intense investigation.



Gliovascular remodeling upon obesogenic diet exposure. (**a**) Confocal micrographs of coronal mouse brain sections showing that high-fat/high-sugar feeding increases the number of GFAP$^+$ reactive astrocytes as well as microvascular density within the ARC ("hypothalamic astrogliosis" and "hypothalamic microangiopathy," respectively). (**b**) High-magnification confocal image of two individual astrocytes each identified by the separate

(continued)

Box 12.4 (continued)
expression of the astroglial marker proteins, GFAP (green; GFP reporter) or GLAST (magenta; tdTomato reporter). Astrocytes are a heterogenous, functionally diverse class of cells that occupy strictly separated domains, infiltrate the local parenchyma with their extensive processes, and cover traversing microvessels with peri-vascular endfeet (white arrow) by which they regulate blood-brain-barrier properties, vascular homeostasis, and cerebral blood flow. Scale bar: 100 and 10 μm. GFAP, glial-fibrillary acidic protein; GLAST, glutamate-aspartate transporter; III, third ventricle

12.4 Brain-Targeted Precision Poly-pharmacology for the Treatment of Obesity

Energy homeostasis is the product of complex processes that involve a plurality of peripheral signals informing dispersed brain control centers about the energy status of the organism. The escalating prevalence of the worldwide obesity pandemic has demanded acquiring a deeper understanding of this intricate interplay. Focusing on processes in the brain, it was hoped that new insights would pave the way for the development of safe and efficacious anti-obesity therapeutics. The rationale to target the brain for fighting human obesity is underscored by large genome-wide association studies that successfully mapped most genetic risk variants for increased body mass index to genes with primary functions within the central nervous system (Willer et al. 2009; Locke et al. 2015). Thus, based on substantial genetic and experimental evidence, obesity is now widely regarded as a "brain disease" rather than a derangement of peripheral metabolism; accordingly, more and more therapeutic strategies are aiming above the neck.

With this end in mind, early pharmacological interventions intending to manipulate more generic aspects of neurotransmission turned out to entail serious psychiatric side effects (Dietrich and Horvath 2012). In order to safely bypass these risks and to provide an answer for the enormous complexity and redundancy inherent to the gut-brain axis, other investigators started to diversify pharmacological approaches, and this has led to the emergence of targeted precision medicine. The field was significantly advanced by early attempts to combine several afferent signals in one preparation, with the goal of conveying convergent and more complete metabolic information, and a series of compelling examples documented the superiority of such poly-pharmacological strategies over single medicines (Tschop et al. 2016). These approaches have now pushed prior limits by chemically merging the individual effects of select metabolic hormones within single molecules. These rationally designed chimeras of two or even three peptide hormones synergistically integrate respective action profiles (unimolecular poly-agonism), and several of such promising drug candidates have now already entered first clinical testing.

12.5 Conclusion and Outlook

Averting the obesity pandemic will become possible only if biomedical innovation is combined with the necessary degree of pragmatic thinking. Historically, the process of drug development has been concerned with a combination of the achievement of hard clinical endpoints with safety and in order to prevent, for example, the threat of leg amputation due to a diabetic ulcer or similar tragedies. With this in mind, a more academic and philosophical point of view must consider that the designated drug target, e.g., the hypothalamic feeding networks, are in fact tightly enmeshed with several other "survival circuits," and targeting one circuit will potentially influence another. Recent research has revealed that these ancient, evolutionarily conserved brain systems are heavily interconnected and influence each other reciprocally. By this crosstalk, these systems jointly evaluate various homeostatic imbalances, calibrate the animal's internal affective state accordingly, and set the necessary motivational scheme in order to facilitate the execution of goal-directed purposive behaviors for the alleviation of the imbalance (e.g., caloric deficit > hunger > attentiveness > food- seeking behavior > ingestion of food). Such a mutually inhibitory relationship of competing schemata and drives was suspected by the German philosopher Friedrich Nietzsche, who when considering the fundamental impulses of men, stated that "*each one of them would have been only too glad to look upon itself as [. . .] the legitimate lord over all the other impulses.*" *(Beyond Good and Evil, 1886).* The remarkable increase in sophistication of modern systems neuroscience technology now allows for the probing of these historical philosophical assumptions, e.g. by combining micro-endoscopic Ca^{2+} imaging with optogenetic spiral stimulation of defined neuronal ensembles in behaving animals. As an example, Jennings and colleagues recently demonstrated that caloric reward and social reward are encoded by strictly separate neuronal ensembles in the orbitofrontal cortex (OFC). Intriguingly, the selective activation of the OFC subnetwork identified as responding to social cues was found to potently inhibit feeding (Jennings et al. 2019). By further deciphering the cellular mechanisms underlying these competing internal states (or "impulses"), neuroscience research might soon be able to provide a much more profound understanding of the brain. These insights might also aid more pragmatic intents such as the design of brain-targeted medicines and programmatic behavioral treatments to precisely and safely reverse homeostatic imbalances (e.g., obesity, hyperglycemia, or hypertension) and maladaptive behaviors (e.g., hyperphagia and sedentary lifestyle, anorexia, and compulsive behaviors).

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Horvath et al. (2010) The first demonstration that hypothalamic astrocytes in the ARC undergo dramatic changes in their morphology when mice are exposed to obesogenic diets ("reactive astrogliosis"); it is interesting to note that these changes have now repeatedly been shown to occur at remarkable rapidity even preceding any change in body weight; this study further suggested for the first time that reactive astrocytes alter the synaptology of the central melanocortin system.

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Melanin-Concentrating Hormone, Neuropeptide E-I, and MCH Receptor 1

13

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Abstract

Melanin-concentrating hormone (MCH) and neuropeptide E-I (NEI) are neuropeptides produced from the pro-melanin-concentrating hormone gene, which are found in many vertebrates playing prominent roles in maintaining the homeostatic balance. While the hypothalamus is its primary site of synthesis, cells synthetize MCH in other areas of the brain and multiple peripheral tissues. Its receptor, MCH receptor 1 (MCHR1), is also found in the brain and peripheral tissues. In addition to neuromodulatory actions, MCH also plays substantial neuroendocrinological roles, including interactions with sex steroids, growth hormone, cortisol/corticosterone, thyroid hormones, prolactin, vasopressin, and oxytocin. These roles are mediated by direct innervation of hypophysiotropic neurons located in multiple brain areas, direct action of MCH and NEI in the adenohypophysis and release of MCH in the bloodstream through the

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neurohypophysis. In this chapter, a detailed description of the MCH peptidergic system is provided, focusing on the distribution of MCH synthesis, its differential processing during lactation, the peripheral tissues where MCH or MCHR1 are produced, and the principal hormonal axes that are influenced by MCH.

Keywords

Hypothalamus · Neuropeptides · Sex steroids · Growth hormone · Cortisol · Thyroid · Prolactin

Abbreviations

α-MSH	α-melanocyte-stimulating hormone
aa	Amino acid(s)
Acb	Nucleus *accumbens*
ACTH	Adrenocorticotropic hormone
Arc	Arcuate nucleus
CART	Cocaine- and amphetamine-regulated transcript
CORT	Cortisol/Corticosterone
CRF	Corticotropin-releasing factor
E2	Estrogen/Estradiol
EB	Estrogen benzoate
ERα	Estrogen receptor α
FSH	Follicle-stimulating hormone
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
HPA	hypothalamic-pituitary-adrenal axis
HPG	hypothalamic-pituitary-gonadal axis
HPS	hypophyseal portal system
HPT	hypothalamic-pituitary-thyroid axis
IHy	Incerto-hypothalamic area
LH	Luteinizing hormone
LHA	Lateral hypothalamic area
MCH	Melanin-concentrating hormone
MCHR1	Melanin-concentrating hormone receptor 1
MCHR2	Melanin-concentrating hormone receptor 2
ME	Median eminence
mfb	Medial forebrain bundle
MGOP	Melanin gene overprinted polypeptide
MPOA	Medial preoptic area
NEI	Neuropeptide E-I
NGE	Neuropeptide G-E
NH	Neurohypophysis

OT	Oxytocin
OTR	Oxytocin receptor
OVX	Ovariectomy/Ovariectomized
P4	Progesterone
PC	Prohormone convertase(s)
PMCH	Pre-pro-melanin-concentrating hormone
PPD	*Postpartum* day
PRL	Prolactin
PRLR	Prolactin receptor
PVH	Paraventricular hypothalamic nucleus
T3	Triiodothyronine
T4	Thyroxine
TH	Tyrosine hydroxylase
TIDA	Tuberoinfundibular dopaminergic
TM	Transmembrane
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
VP	Vasopressin
ZI	Zona incerta

13.1 Introduction

Melanin-concentrating hormone (MCH) and neuropeptide glutamic acid-isoleucine (neuropeptide E-I, NEI) are two neuropeptides found in tissues from multiple vertebrate species. Processed from a single precursor, these neuropeptides act in a large number of functions, ranging from the promotion of motivated behaviors to metabolic energy expenditure, autonomic control, and ventricular ciliary beating. Central to MCH and NEI functions are their interactions with multiple neuronal, neurohormonal, and hormonal systems. This feature has remained conserved across vertebrate evolution and is tied directly to the discovery of MCH and its related proteins.

Numerous species of fish and amphibians rely on skin color changes to better blend into their environment. These changes happen through the translocation of melanin and other pigments in special cells found in the skin of those animals. In 1931, Hogben and Slome hypothesized that a "pigmentary effector system" formed by two antagonistic factors—darkening (or *B* factor) and clearing (or *W* factor)—controls changes in skin pigmentation. These factors would be released in the circulatory system to perform their respective function at the skin level. In the 1950s, α-melanocyte-stimulating hormone (α-MSH) was identified as the *B* factor, as it promotes the darkening of fish scales, but the identity of the *W* factor would remain a mystery for another three decades.

In 1983, Kawauchi et al. isolated a heptadecapeptide from the chum salmon hypophysis that performs the role expected for the hypothetical *W* factor. When applied to melanophores, that peptide promoted the congregation of melanin

molecules within cells, resulting in visible pallor. Given its intracellular effect, the peptide was named *melanin-concentrating hormone*. While scale color alterations have limited translational potential to mammals, it was also established that teleost MCH could modulate the release of adrenocorticotropic hormone (ACTH), a neuroendocrine role of great scientific interest. The discovery that hypothalamic extracts of rats display activities similar to salmonid MCH led to the search of a mammalian homolog, culminating in 1989 on the discovery of the gene and protein (Nahon et al. 1989; Vaughan et al. 1989).

The identification of both the gene and protein unleashed over three decades of intense interrogation of mammalian MCH. Some of the remarkable advances include the mapping of MCH and NEI in the central nervous system of rats (Bittencourt et al. 1992), the discovery of its orexigenic properties (Qu et al. 1996), the identification of its first and second receptors (see recommended literature for references), the mapping of MCH receptor 1 (MCHR1) (Hervieu et al. 2000; Saito et al. 2001), the description of electrophysiological properties of MCH neurons and MCH (Gao and van den Pol 2001; van den Pol et al. 2004), the implication of MCH in sleep modulation (Verret et al. 2003), the identification of MCHR1 as a ciliary receptor (Berbari et al. 2008), the publication of a comprehensive transcriptome of MCH neurons (Mickelsen et al. 2017), and the discovery of volume transmission as part of MCH communication (Noble et al. 2018).

Among the roles played by MCH, we now know that it has a significant relationship with multiple neuroendocrine systems, including oxytocin (OT), vasopressin (VP), prolactin (PRL), sex steroids, cortisol/corticosterone (CORT), and thyroid hormones. This chapter will detail the neuroanatomy of MCH neurons and their relationship with endocrine systems, including the emerging perspective that MCH may constitute a hypophysiotropic and neurohypophyseal hormone in mammals.

13.2 Genes, Proteins, and Phylogenetic Origins

In most vertebrates, the MCH peptidergic family comprises a single gene (*Pmch*) that encodes a full-length peptide precursor (PMCH), which is proteolytically processed to generate three peptides—MCH, NEI, and neuropeptide glycine-glutamic acid (neuropeptide G-E, NGE). The mammalian *Pmch* gene is formed by three exons and is located downstream of multiple promoter motifs, including an AP-1 site, an interferon-ɣ responsive element, and a glucocorticoid response element. The sequence of MCH is encoded predominantly by Exon 3, the most conserved among different species (Fig. 13.1) (Breton et al. 1993; Viale et al. 1997).

The complete mammalian precursor is 165 amino acid (aa) residues long. At the N-terminal sits a 21 aa signal peptide that initiates the posttranslational processing of PMCH and is cleaved after translation. An 87 aa-long structural chain separates the bioactive component of PMCH from the signal peptide. Prohormone convertases (PCs) cleave at residue Lys^{109} to release the bioactive C-terminal, while PC2 cleaves the Lys^{129}-Lys^{130} dibasic pair to form NGE. The remaining NEI-MCH peptide is

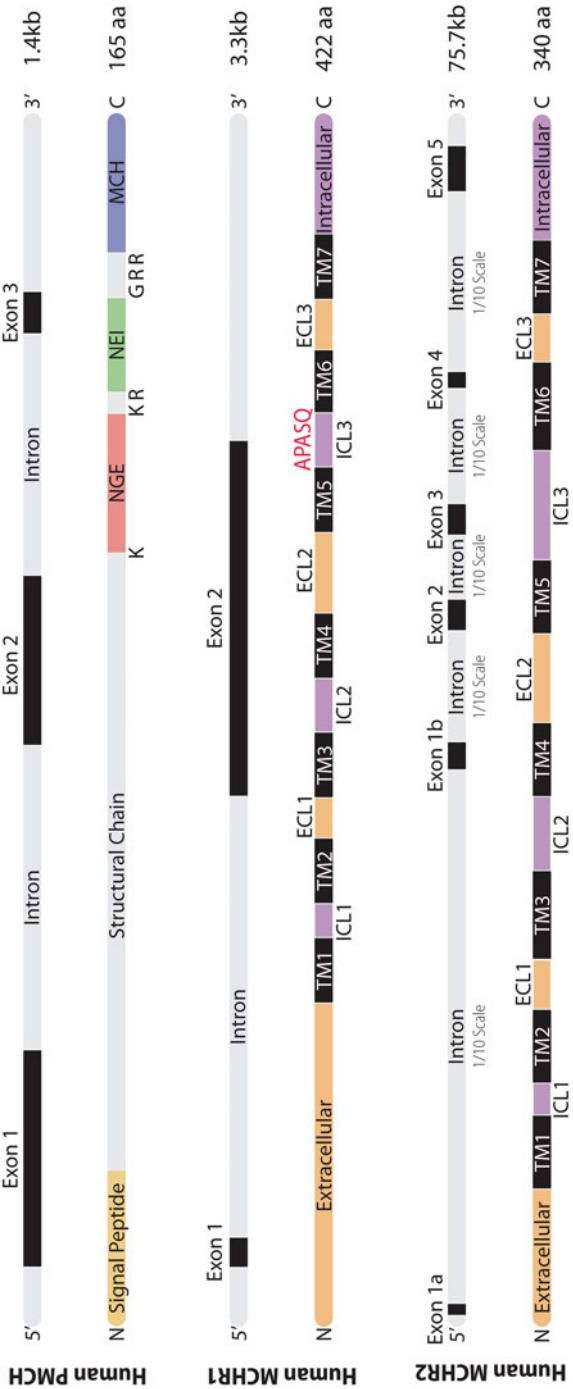

Fig. 13.1 Genome structure of the mammalian MCH peptidergic family. Human PMCH is formed by the expression of three exons, resulting in a 165-aa protein. Cleavage (K, R) and amidation sites (G) at which proteolytic processing occurs are indicated. Human MCHR1 is a 7-TM GPCR, with a long N-terminal extracellular tail, formed from the expression of two exons. The ciliary targeting sequence of MCHR1 is indicated by 3 letters within the third intracellular loop. Human MCHR2, on the other hand, is substantially larger, containing 5 exons with alternative splicing of the first exon. Introns in the MCHR2 gene are represented on a 1:10 scale due to their large size. Abbreviations: *ECL* extracellular loop, *ICL* intracellular loop, *MCH* melanin-concentrating hormone, *MCHR1* MCH receptor 1, *MCHR2* MCH receptor 2, *NEI* neuropeptide E-I, *NGE* neuropeptide G-E, *PMCH* pro-melanin-concentrating hormone, *TM* transmembrane

Fig. 13.2 Proteins of the mammalian MCH peptidergic family. Hexagons represent individual amino acid residues. Blue indicates negatively charged residues, and red indicates positive residues. Mammalian MCH is a nonadecapeptide with a cyclic structure due to a cysteine bridge between residues MCH^{7} and MCH^{16} (black dotted line). While positively charged residues are found in and near the ring portion, negative residues are found in the N-terminus. A chevron indicates residue MCH^{17}, which potentiates the binding of MCH to its receptors. Neuropeptide E-I and NGE are linear peptides with a predominant anionic character and have no known receptor or mechanism of action. Abbreviations: *MCH* melanin-concentrating hormone, *NEI* neuropeptide E-I, *NGE* neuropeptide G-E

cleaved at the Arg^{145}-Arg^{146} dibasic site by multiple PCs, including PC1/3, PC2, PACE4, PC5/6-A, PC5/6-B, and PC7. Amidation at the last residue of NEI produces mature NEI, a 13 aa-long linear peptide, while mature MCH is 19 aa residues-long, with a cyclic conformation resulting from a disulfide bridge formed between cysteine residues (Fig. 13.2) (Viale et al. 1999b).

Mature MCH exerts its activity through two known G protein-coupled receptors (GPCRs) called MCHR1 and MCHR2. The *Mchr1* gene encodes MCHR1 and comprises two exons: the first exon encodes a small portion of the N-terminus of the receptor (27 aa), while the second exon encodes the remaining 326 residues, for a final size of 353 residues (Fig. 13.1). The mature protein displays the characteristic seven transmembrane domains typical of GPCRs, three consensus sites for asparagine-linked glycosylation in the extracellular N-terminus, and two phosphorylation sites for protein kinase A, six for protein kinase C, and one for protein kinase CK2, allowing MCHR1 multiple levels of phosphorylation (Saito et al. 2013). In its final conformation, a central hydrophobic region separates hydrophilic pockets between transmembrane (TM) domains 3 and 7 and TM domains 4, 5, and 6 (Macdonald et al. 2000).

In addition to MCHR1, a second paralog, MCHR2, is found in most vertebrates (Tan et al. 2002). Its encoding gene, *Mchr2*, is substantially more complex than *Mchr1*, with six exons and five intronic sequences of variable length. The first two exons, E1a and E1b, are splice variants, with E1a expression generating a putatively truncated version of MCHR2 in the N-terminus. When E1b is expressed, MCHR2 is 340 residues in length, with typical GPCR features, including seven transmembrane domains, two N-linked glycosylation sites, a DRY motif located at the end of TM3, and a potential palmitoylation site in the C-terminal region (Fig. 13.1).

Both MCHR1 and MCHR2 bind MCH with high selectivity in the nanomolar range and do not respond to other MCH peptidergic family elements (NEI, NGE, Melanin Gene Overprinted Polypeptide—MGOP), natriuretic peptides, opioids, or

melanocortinergic peptides. Likewise, there is no binding to somatostatin or somatostatin-like peptides, despite MCH receptors sharing some sequence similarity with somatostatin receptors. The activation of MCHR1 has a predominantly inhibitory character, with multiple intracellular mechanisms engaged. These mechanisms include the inhibition of forskolin-mediated cAMP production and activation of mitogen-activated protein kinases, preferentially through Gi/Go, and an increase in intracellular Ca^{2+} mediated by Gq (for a review of MCH receptor functions, see Presse et al. 2014). Activation of MCHR1 also leads to its rapid internalization (Saito et al. 2004). On the other hand, activation of MCHR2 has a more limited intracellular effect, increasing intracellular levels of Ca^{2+} and IP_3 production through Gq.

An important aspect of the MCHR1 neurobiology is its translocation to the primary cilium (Berbari et al. 2008). Primary cilia are single non-motile microtubule-based organelles found in cells through the neuroaxis. The interior of primary cilia (axoneme) is gated from the rest of the cell, allowing the primary cilia to have different membrane and axoneme compositions compared to the cytoplasm and cellular membrane, in a mechanism that depends on specialized transport proteins. Primary cilia are mainly considered sensory structures, harboring multiple membrane receptors that can bind to neuroactive substances in the surrounding extracellular space.

A short consensus sequence within the third intracellular loop of MCHR1 is responsible for its targeting to the primary cilium in a transport process mediated by proteins of the BBSome complex (Berbari et al. 2008; Nagata et al. 2013). Ciliary MCHR1 is present in multiple cell culture models and is widespread in the brain of rats and mice. In the absence of primary cilia, as is the case with some cellular lineages, MCHR1 is found in the somatic membrane but displays attenuated action. Electrophysiological studies suggest MCHR1 is also located in the presynaptic membrane, but direct visualization of synaptic MCHR1 is still lacking.

Activation of ciliary MCHR1 by MCH leads to primary cilia shortening, in a process dependent on Gi/o but independent of cell cycle and receptor internalization. This process has been observed in multiple cellular models and, more recently, in hippocampal slices of rats and mice (Hamamoto et al. 2016; Kobayashi et al. 2020; Tomoshige et al. 2017). While the functional significance of ciliary shortening has not been established, it has been suggested to play a role in desensitizing primary cilia to external stimuli. Furthermore, altered ciliary length and morphology have been observed in a significant number of neurological diseases, warranting further investigation.

Present evidence suggests that the founder genes of *Pmch*, *Mchr1,* and *Mchr2* originated in phylostratum 11, at the time of vertebrate divergence (for a detailed review, see Diniz and Bittencourt 2019). This idea is supported by immunohistochemical studies using antibodies directed to salmon MCH that revealed MCH-like immunoreactivity in neurons of lampreys, and potential homologs of *Mchr1* and *Mchr2* that have been identified in the genome of *Petromyzon marinus*. In broad terms, the MCH gene family has remained well conserved throughout vertebrate evolution, with substantial conservation of sequence and structure for both MCH and

its receptors, contrasted only by a remarkably low similarity between MCHR1 and MCHR2. There are, however, notable exceptions for both MCH and its receptors.

At the time of the teleost divergence, a retroposition event likely led to the formation of two *Pmch* orthologs in this clade: *pmcha* and *pmchb* (the nomenclature used in this chapter follows that established in Diniz and Bittencourt (2019) and may not match that used in older works found in the literature). Teleost *pmcha* retained more similarities to the *Pmch* founder gene, although it was subject to a less strict selective pressure which resulted in substantial variability among teleost species. On the other hand, *pmchb* diverged into coding a shorter 17 residues-long MCH_B with residue substitutions in the N-terminal, bioactive ring and C-terminus, which is remarkably conserved between teleost species. In salmonids, a whole-genome duplication event led to the formation of a total of four *Pmch* paralogs (*pmcha1, pmcha2, pmchb1,* and *pmchb2*). The peptide isolated by Kawauchi et al. (1983) during the original discovery of MCH corresponds to MCH_B. The second exception concerns the MCH receptors in the Glires (Lagomorpha + Rodentia) clade, where a frameshift mutation in the *Mchr2* gene resulted in an inactive truncated protein, as observed in Lagomorpha species. The lack of functional MCHR2 ultimately led to the loss of *Mchr2* in rodents, including some of the most common laboratory models. This has severely impacted our understanding of MCH function in primates, where both functional receptors are found.

13.3 MCH/NEI Neurons

Melanin-concentrating hormone and NEI extensively colocalize within the rodent nervous system, a property believed to be shared by other species (Bittencourt et al. 1992; Bittencourt 2011; Diniz et al. 2019). There are two main exceptions to that rule: the preoptic cluster (described in detail in the next section), where MCH immunoreactivity is present in neuronal somas in the absence of NEI, and the interanterodorsal thalamic nucleus of rats, where MCH^+ fibers are found but only scattered NEI immunoreactivity has been reported. The importance of those differences is not known, in part due to a lack of understanding of NEI mechanisms of action. Even less is known about NGE, the third neuropeptide derived from PMCH, as its constitutive synthesis is yet to be demonstrated in mammals. Therefore, for the remainder of this chapter, the term *MCH neuron* will be used to describe neurons that synthesize both MCH and NEI, unless otherwise stated.

Within the neuron, MCH and NEI occupy different subcellular compartments (Fig. 13.3a–a'). Immunoreactivity to MCH is found predominantly within the Golgi apparatus's saccules, with a preference for its trans face, resulting in a honeycomb pattern when observed under high-resolution optical microscopy. Outside the soma, MCH immunoreactivity forms discrete puncta that extend into proximal branches, suggesting its packaging in vesicles for transport. On the other hand, NEI is a diffuse immunoreactive signal that fills the soma, proximal dendrites, and the axon, while sparing the nucleus, which led us to hypothesize that MCH and NEI may be released

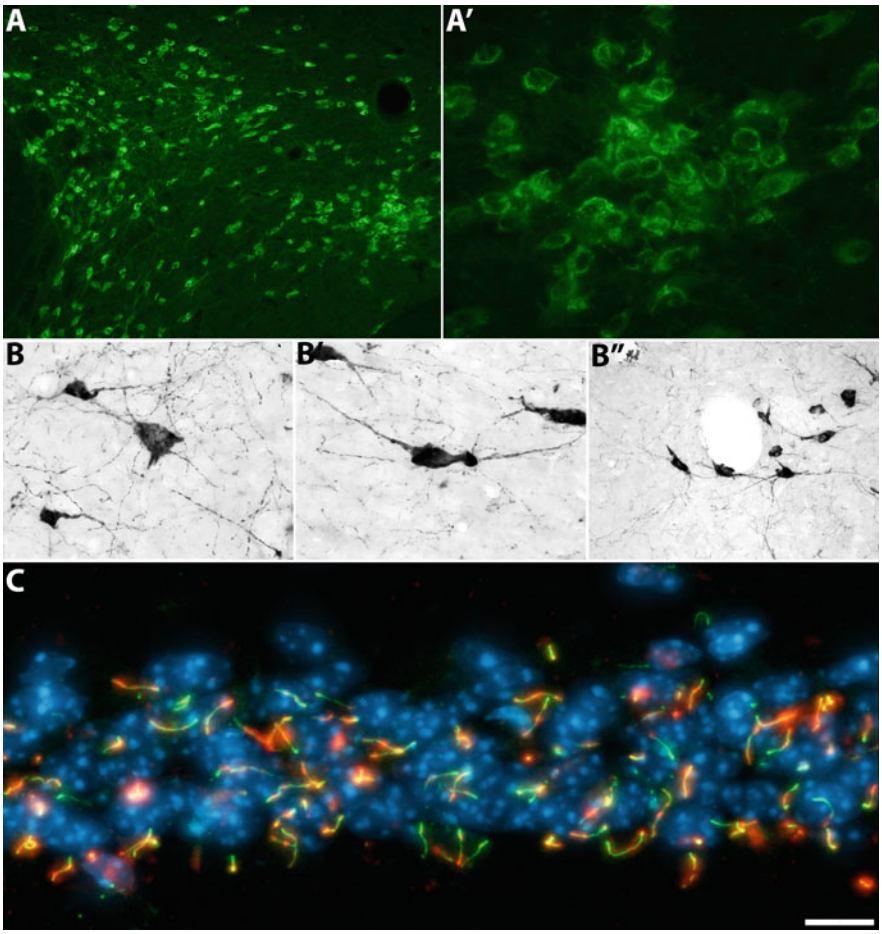

Fig. 13.3 Morphological properties of MCH and MCHR1. Mouse photomicrographs illustrating the morphological diversity of MCH and MCHR1 labeling in rodents. (**a**) Immunolabeled MCH neurons are found widespread throughout the lateral hypothalamus; (**a'**) Upon closer examination, it is possible to observe MCH labeling restricted to the soma surrounding, but not within, the nucleus. Neurons MCH^+ can be categorized based on their morphology, including: (**b**) multipolar; **b**′) bipolar; and **b**″) crescent, when they are associated with blood vessels. (**c**) Immunolabeling of MCHR1 (red) is found in adenylate cyclase 3 (AC3—green)-positive cilia in multiple areas, including the pyramidal layer of CA1, illustrated here. Scale bar: **a**—120 μm; **a'**—30 μm; **b**, **b**′—20 μm; **b**″—40 μm; **c**—10 μm

through different intracellular mechanisms, based on the cellular compartment they occupy.

In addition to MCH and NEI, MCH neurons synthesize many other neuroactive substances, although some aspects of this are not fully understood. Melanin-concentrating hormone neurons express the mRNA for genes involved in the synthesis of GABA, including *Gad1* and *Gad2*, suggesting MCH neurons are

predominantly GABA inhibitory in nature. Recent studies, however, revealed that MCH neurons do not express the vesicular GABA transporter gene (*Slc32a1*), rendering them virtually unable to transport and release GABA vesicles through known mechanisms, despite having the machinery to synthesize it. On the other hand, MCH neurons express the vesicular glutamate transporters (*Slc17a6* and *Slc17a8*), which indicates MCH neurons are predominantly glutamate excitatory in nature, as previously demonstrated for a subgroup of septum-projecting neurons (Chee et al. 2015; Mickelsen et al. 2017). More studies are necessary to fully understand the neurotransmitter identity of MCH neurons.

In addition to neurotransmitter machinery, MCH neurons produce several other neuroactive substances. Integral colocalization has been reported between MCH and α-dystrobrevin, a protein associated with the structural integrity of muscle fibers also found in glial cells and, more rarely, in neurons. Nesfatin-1 is an 82-aa peptide with anorexigenic activity when injected centrally, and reports indicate that 90% of MCH neurons are Nesfatin-1 positive. The cocaine- and amphetamine-regulated transcript (CART) is a neuropeptide found in multiple brain areas that is upregulated following administration of some abuse substances, and it colocalizes with MCH in a region-dependent manner that ranges between 66–90% of tuberal MCH neurons. Other markers found in MCH neurons include the expression of mRNA for the synthesis of galanin (55%), a polypeptide found in several brain areas and implicated in multiple functions through its hyperpolarizing character; pronociceptin (35%), precursor of a 17-aa neuropeptide implicated in pain processing and fear; proenkephalin (30%), a precursor of peptides implicated in nociception; and thyrotropin-releasing hormone (TRH, $<10\%$), a hypophysiotropic hormone (Mickelsen et al. 2017). Other instances of co-expression include acetylcholinesterase in the absence of choline acetyltransferase, purposely for the modulation of cholinergic transmission, secretogranins, and the monocyte chemoattractant protein 1/chemokine ligand 2 (see recommended literature).

Contrasting to their substantial neurochemical heterogeneity, MCH neurons are remarkably uniform in terms of electrophysiological properties. In awake animals or slices, MCH neurons are predominantly quiescent. In these conditions, lateral hypothalamic area (LHA) neurons have low resting membrane potential (-61.3 ± 0.9 mV) and shallow spontaneous spike frequency (0.15 ± 0.1 Hz). These neurons also show spike frequency adaptation, with interspike interval increased by over 70% between the first and second halves of long current injections (van den Pol et al. 2004). Mature MCH neurons hyperpolarize in response to GABA and depolarize in response to glutamate, while immature MCH neurons can show depolarizing responses to GABA during the development period. These neurons undergo a progressive reduction in excitability in immature animals, reaching their most quiescent levels at four weeks of age in mice and seven weeks of age in rats (Li and van den Pol 2009; Linehan et al. 2018). While only occasional spikes are observed during slow-wave sleep, MCH neurons discharge at their maximum rate during paradoxical sleep showing a phasic firing pattern, and during the exploration of novel objects (Blanco-Centurion et al. 2019; Hassani et al. 2009).

A large number of post- and pre-synaptic mechanisms controls the electrophysiological behavior of MCH neurons (for a detailed review, please see Diniz and Bittencourt 2017). Postsynaptically, multiple neurochemical messengers mediate depolarization through transient receptor potential channels and sodium-calcium exchangers. Depolarizing agents include VP through the V1a receptor; OT through its receptor (OXTR), orexins through subtype 2 of their receptor; glucose, which gains entry to MCH neurons through the glucose transporter 3 and shunts ATP-sensitive K^+ channels; insulin, through the insulin receptor; and ATP, possibly through the purinergic receptor P2. Multiple mechanisms mediate postsynaptic hyperpolarization, including acetylcholine through the muscarinic cholinergic receptor; serotonin through a still unidentified receptor; norepinephrine and dopamine through the $\alpha 2_A$ adrenergic receptor; neuropeptide Y through its receptor; and nociceptin-1 mediated by the nociceptin/orphanin receptor, in an effect that is also mediated by dynorphin action on the K-opioid receptor. Hyperpolarization often occurs through G protein-coupled inwardly rectifying K^+ channels and voltage-dependent calcium channels. Most of the messengers that act postsynaptically on MCH neurons also act presynaptically to modulate both GABAergic and glutamatergic transmission into MCH neurons.

Morphologically, MCH neurons display a wide range of characteristics. These neurons can be classified into three main types depending on their morphology: multipolar, bipolar, and crescent, and that morphological classification has been well conserved, at least in mammals (Fig. 13.3b–b"). Multipolar neurons typically have 3–5 primary dendrites and no particular orientation, with medium and large cell bodies. Bipolar neurons have their longer axis preferentially oriented mediolaterally, with two or three primary dendrites and substantial branching in a short distance from the soma. Crescent neurons are a subtype of bipolar neurons found associated with the wall of blood vessels. The two primary dendrites of crescent neurons envelop blood vessel walls, and fibers from those neurons are often found within the wall of blood vessels. Ultrastructurally, MCH neurons display invaginated nuclei and well-developed Golgi apparatus and rough endoplasmic reticulum. No direct membrane apposition is found between MCH neurons and other MCH neurons or surrounding unlabeled cells in the LHA. The general appearance of MCH neurons has been described as similar to that of parvocellular hypophysiotropic neurosecretory neurons (Bittencourt et al. 1992; Diniz et al. 2019).

Axons from MCH neurons are thin (0.1–0.2 μm in diameter) and predominantly unmyelinated, containing small electron-translucent (30–60 nm in diameter) and large dense-core vesicles (80–150 nm). Immunoreactive material is found within the large dense-core vesicles but not in the small translucent ones. The primary contacts formed by MCH neurons are asymmetric and end on the dendritic shafts of unlabeled neurons, while axosomatic contacts are uncommon (Bittencourt et al. 1992).

13.4 Distribution

13.4.1 MCH in the Nervous System

In most early vertebrates, MCH neurons occupy a predominantly medial position within the diencephalon, often in the vicinity of the third ventricle (for a detailed description of MCH in multiple species, see Diniz and Bittencourt 2019). In these species, MCH neurons are found in the dorsomedial hypothalamic nucleus and the periventricular area, with neurons in lamprey reported to contact the interior of the third ventricle. In teleosts, MCH_B neurons drifted ventrally, occupying the lateral tuberal nucleus, a brain structure strongly associated with the hypophysis, reflecting the intimate association between MCH_B and neurosecretion, while MCH_A neurons retained their position adjacent to the ventricles. In sauropsids (reptiles and birds), there was a minor lateralization event, with some MCH neurons found in the lateral hypothalamus.

There is substantial variation in the distribution of MCH neurons between species, including phylogenetically close ones. In all mammalian species, the largest group of MCH neurons is found in the tuberal hypothalamus, predominantly in the lateral zone, intermingled with the crossing fiber groups of the medial forebrain bundle (*mfb*). These neurons form a shell that envelops orexin neurons, located in the core of the LHA. Interspecies variations include the mediolateral and the dorsoventral extensions of the MCH group of cells. In addition to the lateral zone, MCH neurons are also found in the dorsomedial area of the medial zone of the hypothalamus and, in some cases, in the periventricular zone. Neurons extend into the mammillary hypothalamus, often ending anterior to the medial mammillary nucleus. Rodents have been the most extensively examined species in MCH distribution and will be used in this section as the prototypical distribution.

Projections from MCH neurons are found widespread throughout the neuroaxis (Fig. 13.4). All major neuronal groups receive at least some MCH^+ fibers, except for some brainstem motor nuclei. A detailed description can be found elsewhere (Bittencourt et al. 1992; Bittencourt and Diniz 2018; Diniz et al. 2019), but some of the densest areas of innervation include the medial septal nucleus, the dorsal hippocampus, and all hypothalamic zones. Individual targets of innervation relevant to the neuroendocrine role of MCH include both internal and external layers of the median eminence (ME) and the neurohypophysis (NH) (Fig. 13.5), in addition to central projections that will be detailed in the appropriate sections. In the NH, MCH^+ axons processes and their swellings—predominantly Herring bodies—are identified in proximity to other axonal terminals and the basal membrane of fenestrated capillaries characteristic of this area, suggesting direct release of MCH in NH blood vessels to reach the general circulation. On the other hand, no immunoreactivity to MCH is found among specialized adenohypophyseal cells, as expected for a hypophysiotropic hormone released in the portal circulation (Fig. 13.6).

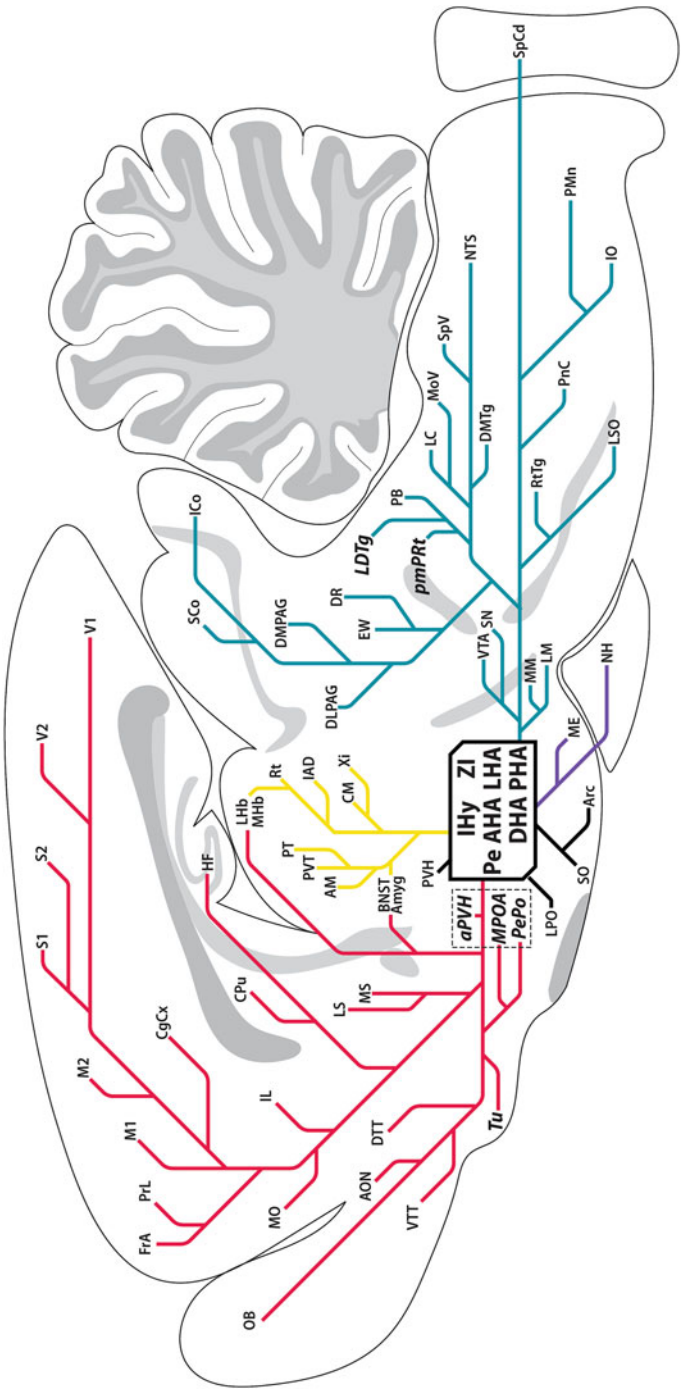

Fig. 13.4 Schematic representation of the main sites of innervation by MCH neurons in the rodent brain. Bold letters indicate structures where MCH neurons are found, a dotted box indicates the group of MCH neurons detectable only in lactating animals, and lines represent projections. There are four main projection routes (indicated by different colors), in addition to intra-hypothalamic connections (indicated in black). **Ascending pathway (red)**: through the ascending pathway, projections from MCH neurons reach the preoptic area, all structures of the basal telencephalon, the olfactory bulb and other olfaction-associated areas, the septal area, caudate-putamen, hippocampus, and multiple areas of the cortical mantle. **Periventricular pathway (yellow)**: the periventricular pathway turns dorsal and allows the innervation of multiple thalamic and epithalamic nuclei. **Hypophyseal pathway (purple)**: fibers in the hypophyseal pathway reach the

external and internal layers of the median eminence and the neurohypophysis. **Descending pathway (green)**: fibers in the descending pathway run throughout the rostrocaudal extent of the mesencephalon, pons, and medulla, ending within the spinal cord. Areas innervated by the descending pathway include the periaqueductal gray matter, the colliculi, raphe nuclei, and olivary areas. Abbreviations: *AHA* anterior hypothalamic area, *AM* anteromedial thalamic nucleus, *Amyg* amygdaloid complex, *AON* accessory olfactory nucleus, *aPVH* anterior part of the paraventricular hypothalamic nucleus, *Arc* arcuate nucleus, *BNST* bed nucleus of the stria terminalis, *CgCx* cingulate cortex, *CM* centromedial thalamic nucleus, *CPu* caudate-putamen, *DHA* dorsal hypothalamic area, *DLPAG* dorsolateral part of the periaqueductal gray matter, *DMPAG* dorsomedial part of the periaqueductal gray matter, *DMTg* dorsomedial tegmental nucleus, *DR* dorsal raphe nucleus, *DTT* dorsal tenia tecta, *EW* Edinger-Westphal nucleus, *FrA* frontal association cortex, *HF* hippocampal formation, *IAD* interanterodorsal thalamic nucleus, *ICo* inferior colliculus, *IHy* incerto-hypothalamic area, *IL* infralimbic cortex, *IO* inferior olivary complex, *LC* locus coeruleus, *LDTg* laterodorsal tegmental nucleus, *LHA* lateral hypothalamic area, *LHb* lateral habenular nucleus, *LM* lateral mammillary nucleus, *LPO* lateral preoptic area, *LS* lateral septal nucleus, *M1* primary motor cortex, *M2* secondary motor cortex, *ME* median eminence, *MHb* medial habenular nucleus, *MM* medial mammillary nucleus, *MO* medial orbital cortex, *MoV* motor nucleus of the trigeminal nerve, *MPOA* medial preoptic area, *MS* medial septal nucleus, *NH* neurohypophysis, *NTS* nucleus of the solitary tract, *OB* olfactory bulb, *PB* parabrachial nucleus, *Pe* preoptic nucleus, *PePo* periventricular preoptic nucleus, *PHA* posterior hypothalamic area, *PMn* paramedian reticular nucleus, *pmPRt* paramedian pontine reticular formation, *PNC* caudal pontine reticular nucleus, *PrL* prelimbic cortex, *PVH* paraventricular hypothalamic nucleus, *PVT* paraventricular thalamic nucleus, *PT* paratenial thalamic nucleus, *Rt* reticular thalamic nucleus, *RtTg* reticular tegmental nucleus, *S1* primary somatosensory cortex, *S2* secondary somatosensory cortex, *SCo* superior colliculus, *SN* substantia nigra, *SO* supraoptic nucleus, *SpCd* spinal cord, *SpV* spinal trigeminal nucleus, *Tu* olfactory tuberculum, *V1* primary visual cortex, *V2* secondary visual cortex, *VTA* ventral tegmental nucleus, *VTT* ventral tenia tecta, *Xi* xiphoid nucleus, *ZI* zona incerta

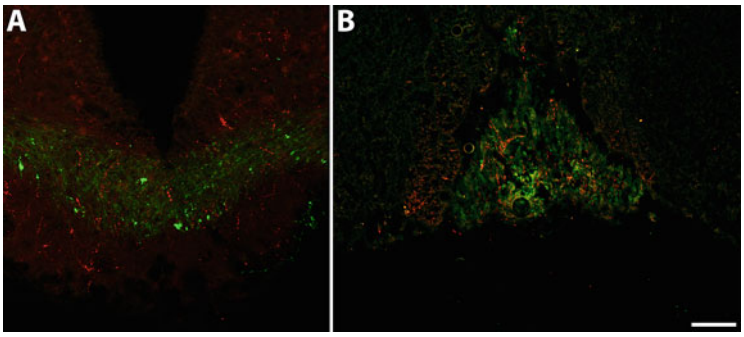

Fig. 13.5 Innervation of MCH in the median eminence and neurohypophysis. Rat photomicrographs illustrating the presence of MCH^+ fibers (red) in neuroendocrine areas. (**a**) MCH^+ axons can be found both in the internal layer of the median eminence, indicated in green by the presence of OT^+ fibers, and in the adjacent external layer. (**b**) Both MCH^+ and OT^+ fibers are also found in the neurohypophysis, indicating MCH fibers course through the internal layer and into the neurohypophysis, where they contact blood vessels. Scale bar: 200 μm. The photomicrograph in B has been reproduced from Costa et al. (2019)

13.4.1.1 Lateral Zone of the Tuberal Hypothalamus

As previously mentioned, the LHA is the leading site of MCH synthesis. Neurons in the LHA are predominantly multipolar and intensely stained, and are found intermingled with the *mfb*, where multiple varicose MCH^+ fibers are found. Dendrites from MCH neurons often protrude transversally to the *mfb*, allowing these neurons to tap into the most extensive fiber bundle crossing the anteroposterior extent of the neuroaxis. These neurons form a continuous mass of neurons, but they can be divided into three groups for didactic purposes. The first group, and the largest, is found in the area between the internal capsule and the fornix, with a large number of neurons forming a triangular shape between the internal capsule and the overlying zona incerta (ZI). The second group of neurons is found within the space between the optic tract and the ventral internal capsule. These neurons are large and strongly labeled, comprising the magnocellular group of MCH neurons. The third group is composed of neurons within or closely associated with the perifornical nucleus, displaying a characteristic centrifugal arrangement surrounding the fornix. Neurons in the perifornical group are often found in posterior levels of the tuberal hypothalamus compared to the other two groups. Some neurons of the perifornical area project to the ME and NH (Cvetkovic et al. 2003). The MCH neurons of the LHA are strongly associated with integrative functions.

13.4.1.2 Zona Incerta

Closely associated with the LHA is a narrow band of MCH neurons located in the ZI. These neurons are separated from the LHA by a narrow band of stain-free neuropil, and they form a compact layer of 3 to 4 cells, with their main axis oriented horizontally following the substantial mediolateral extent of the ZI. Little is known

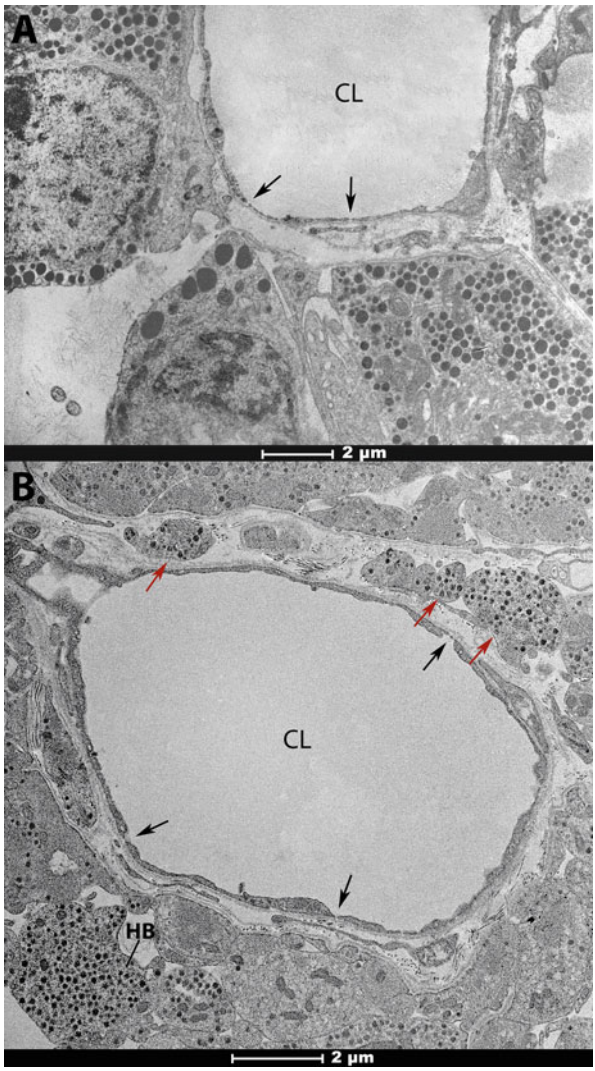

Fig. 13.6 Ultrastructural aspects of MCH immunolabeling in the hypophysis. Transmission electron microscopy of rat hypophyses subject to gold-conjugated immunolabeling for MCH. (**a**) Adenohypophysis. Although electrodense vesicles are abundantly found adjacent to capillary lumina (CL), no MCH immunolabeling is found; (**b**) Neurohypophysis. Secretion granules containing MCH immunoreactive material are observed in Herring Bodies (HB) and axonal processes (red arrows) in proximity to fenestrated capillaries. Note the endothelial pores (black arrows). Scale bars are indicated directly in the figure

about the specific functions of these neurons due to the difficulties associated with separating them from the adjacent LHA.

13.4.1.3 Medial Zone of the Tuberal Hypothalamus

MCH neurons in the medial zone of the hypothalamus are concentrated in the dorsal area, including the anterior hypothalamic area posterior to the paraventricular hypothalamic nucleus (PVH) and the dorsomedial hypothalamic area. These neurons are predominantly multipolar and are often continuous with LHA neurons, forming a single sheet of neurons that blankets the dorsal hypothalamus. No specific functions have been assigned to this specific group of neurons.

13.4.1.4 Incerto-hypothalamic Area

Dorsal to the dorsomedial hypothalamic group is a second, smaller group found in the incerto-hypothalamic area (IHy), a poorly differentiated zone located between the hypothalamus and the ZI (Sita et al. 2003, 2007). Neurons in the IHy can be differentiated from their ventral counterparts by their clear bipolar shape and mediolateral orientation. Neurochemically, the IHy can be identified by the presence of tyrosine hydroxylase (TH)-positive neurons of the dopaminergic group A13 that are found intermingled with MCH neurons, often forming extensive somatic contacts in the absence of colocalization. While the function of the dorsomedial hypothalamic area MCH neurons is poorly understood, the IHy MCH neurons are implicated in integrating energy status for the modulation of hormone secretion and sexual behavior, and projections of some of these neurons target the ME and NH (Cvetkovic et al. 2003).

13.4.1.5 Anterior Periventricular Nucleus

Some neurons in the dorsomedial hypothalamus extend towards the periventricular nucleus. This trend is particularly evident in rats, as MCH neurons are found clearly within its anatomical limits. In this species, a cluster of neurons is concentrated in the third ventricle's dorsal pole, merging with the adjoining dorsomedial area and IHy. These neurons extend projections into the lumen of the third ventricle, possibly contacting the cerebrospinal fluid.

13.4.1.6 Preoptic Cluster

Exclusively in lactating females, a small but well-defined group of MCH neurons is found in the medial preoptic area (MPOA), preoptic periventricular nucleus, and the anterior part of the PVH. These neurons have been described in multiple strains of rats and mice, although they are substantially more challenging to detect in mice due to lower levels of mRNA expression and protein synthesis and incongruence between Cre activation and gene expression (Alvisi et al. 2016; Beekly et al. 2020; Costa et al. 2019; Diniz et al. 2019; Knollema et al. 1992). These neurons express *Gad67* mRNA in rats, but there is no colocalization between MCH and the VGLUT2 or VGLUT3 genes in mice, with contradictory reports about Slc32a1 expression (Beekly et al. 2020; Teixeira et al. 2020), raising essential questions about their neurotransmitter profile. These neurons are also negative for kisspeptin, OT, and TH (Rondini et al. 2010; Teixeira et al. 2020).

While *Pmch* mRNA is not detected in the preoptic cluster in cycling virgin females and pregnant dams, expression starts at shallow levels on the fifth *postpartum* day (PPD) and increases as lactation progresses, reaching its maximum levels around the 15th–16th PPD, coinciding with the pups' eruption of the incisors and their transition into solid foods. Expression of *Pmch* and MCH immunoreactivity remain elevated through the late lactation period, either slowly decreasing towards the 26th PPD when pups are kept with the mothers or rapidly fading when pups are weaned on the 22nd PPD.

Preoptic cluster MCH neurons are intrinsically linked with the lactation process and the offspring, as the number of MCH neurons in this area is positively correlated

with *postpartum* litter size, and the number of cells in multiparous dams is lower compared to primiparous dams (what may be linked with maternal memory) (Ferreira et al. 2017; Teixeira et al. 2020). The mechanism that tethers preoptic MCH neurons and the offspring is not fully understood. While tactile stimulation of the nipples by the pups leads to ample synthesis of the early activation protein FOSB in the hypothalamus, including the preoptic area, this marker is not found colocalized with MCH neurons. A neurohormonal mechanism, however, seems more plausible, as preoptic MCH neurons contain both prolactin (PRL) and estrogen/estradiol (E2) receptors and respond to PRL through the expression of *Stat5* (Alvisi et al. 2016; Teixeira et al. 2020).

Preoptic MCH neurons have been implicated in maternal behavior. Intranuclear injections of MCH into the MPOA of rat dams in the fifth or sixth PPD decrease appetitive components of maternal behavior, including retrieval, licking, and nest building, while sparing consummatory aspects of behavior. Constitutive deletion of MCHR1 has a similar effect, decreasing nesting, maternal aggression, and pup retrieval (Alachkar et al. 2016; Benedetto et al. 2014). Preoptic MCH neurons may also be involved in the release of MCH in the bloodstream. Peripheral injection of a retrograde tracer in the blood leads to labeled MCH^+ neurons in all areas of the preoptic cluster, suggesting these neurons have open terminals in the NH (Costa et al. 2019).

13.4.1.7 Other Clusters

Smaller numbers of neurons are found in several brain areas, including the olfactory tubercle, the dorsomedial part of the tuberomammillary nucleus, the posterior hypothalamic area, the paramedian pontine reticular formation, and the laterodorsal tegmental nucleus (the latter found exclusively in female rats) (Bittencourt et al. 1992; Rondini et al. 2007). Little is known about these additional groups.

13.4.2 MCH in the Periphery

Expression of *Pmch* mRNA or MCH immunoreactivity has been detected in multiple peripheral tissues, but MCH and NEI are not fully processed outside of the brain, resulting in the production of a peptide containing both MCH and NEI epitopes (Viale et al. 1997). This allows the discrimination between MCH produced in the brain and NEI-MCH produced by peripheral tissues. Structures that include *Pmch*-expressing cells are the heart, lungs, stomach, intestine, pancreas, adrenal glands, testis, ovary, and immunological cells (Fig. 13.7). In the gastrointestinal tract, *lamina propria* cells of the mucosal plexus of the duodenum and the antral portion of the stomach express *Pmch* mRNA (Hervieu et al. 1996). In the testis, both *Pmch* expression and MCH immunoreactivity are found in Sertoli cells surrounding seminiferous tubules (Hervieu and Nahon 1995). In the pancreas, it is expressed in pancreatic islets (Pissios et al. 2007). Finally, expression of *Pmch* has been reported in splenocytes, thymocytes, lymphocytes, PBMCs, granulocytes, and $Th2^+$ human

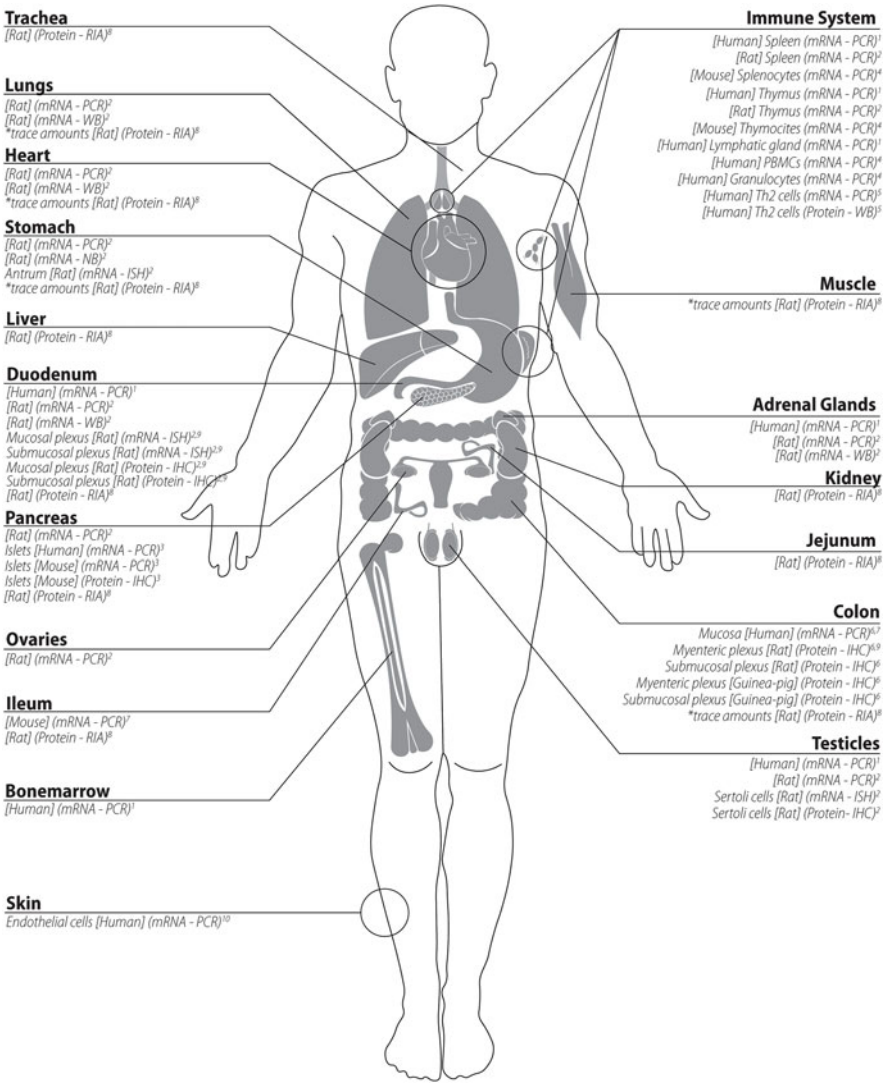

Fig. 13.7 Reports of *Pmch* expression and MCH synthesis in peripheral tissues of mammals. Reports are organized by organ/system, tissue, target, and technique. References: 1—Viale et al. (1997); 2—Hervieu and Nahon (1995); 3— Pissios et al. (2007); 4—Verlaet et al. (2002); 5—Sandig et al. (2007); 6 —Kokkotou et al. (2008); 7—Kokkotou et al. (2009); 8—Lelesz et al. (2016); 9—Hervieu et al. (1996). Abbreviations: *IHC* immunohistochemistry, *ISH* in situ hybridization, *PCR* polymerase chain reaction, *RIA* radioimmunoassay, *WB* western blotting

cells (Sandig et al. 2007; Verlaet et al. 2002). The role played by peripheral NEI-MCH is poorly understood.

13.4.3 MCH in the Circulation

Free mammalian MCH has been found in blood derivatives of rodents and humans. Detection has been performed with both enzymatic and radioimmunoassay methods using commercial and custom kits. Methodological differences make it challenging to directly compare different works, especially when considering that the MCH antibody used in commercial kits may react in a non-specific manner with proteins found in blood from *Pmch* knockout animals (Waters and Krause 2005). In this section, a brief description of each work will be provided.

Naufahu et al. (2017)—This work is possibly the most impactful publication regarding the presence of MCH in the bloodstream, considering that the authors developed an in-house radioimmunoassay and extensively characterized it, although they did not evaluate their test against a sample of *Pmch* KO animals (the gold standard of specificity validation). Perhaps even more important is the fact that this test only binds to NEI-MCH (the peripheral form of MCH) at supraphysiological levels, suggesting the values observed in this study correspond to brain-originated MCH released into the bloodstream. In this cross-sectional study, levels of MCH were determined in the plasma of over 230 adults and compared to body metabolic and morphological properties. Fasting plasma MCH levels were found in the range between 19.5 and 70.4 pg/ml (19.5 pg/ml is the lower limit of detection for the test). Complex relationships were found between circulating levels of MCH and body parameters. Males with BMI > 30 have higher average levels of MCH than males with BMI < 20. Males display a positive correlation between BMI and MCH, while females display the opposite correlation. In older individuals, there is an increase in MCH after eating, while younger individuals show a correlation between MCH and insulin area-under-the-curve. Leptin and MCH are positively correlated in lean males while negatively correlated in males with excess fat.

Schmidt et al. (2015)—In this work, sera from patients with major depressive disorder or controls were investigated using a commercial fluorescence immunoassay kit. While baseline levels of MCH do not differ between unmedicated patients and controls, female patients treated with mirtazapine showed a decrease in MCH levels during treatment.

Carnier et al. (2010)—In this work, sera from post-pubertal obese adolescents undergoing interdisciplinary treatment were investigated using a commercial radioimmunoassay kit. Baseline MCH was found to be 10.65 ng/ml, being upregulated to 12.25 ng/ml after short-term therapy and downregulated to 9.90 ng/ml after long-term therapy. After long-term therapy, MCH and leptin were found to be inversely correlated.

Gavrila et al. (2005)—In this work, sera from 108 healthy individuals were investigated using a commercial radioimmunoassay kit. Baseline serum levels of MCH were 97.8 ± 22.8 pg/ml, with average values significantly lower for men than

women. Levels of MCH were positively correlated with BMI, fat mass, and percentage of fat, while negatively correlated with lean mass. In this same study, fasting for two days increased serum MCH levels.

Sun et al. (2004)—In this work, sera from male and female Wistar rats were analyzed using a commercial competitive immunoassay from kit. Levels of MCH were non-significantly decreased in rats following lesions of the PVH or ventromedial hypothalamic nucleus, and non-significantly increased in lactating rats on the 12th PPD ($n = 5$; 17.6 ± 0.6 ng/ml) compared to nonlactating controls ($n = 5$; 13.7 ± 3.6 ng/ml).

Stricker-Krongrad et al. (2001)—In this work, plasmas from 20 lean and 20 obese Zucker rats were analyzed using a commercial competitive immunoassay kit. Plasma levels of MCH were 7.2 ± 0.8 ng/ml in lean animals and 12.5 ± 1.3 ng/ml in obese animals, a statistically significant difference.

Bradley et al. (2000)—In this work, plasmas from male Sprague-Dawley animals were analyzed using a commercial radioimmunoassay kit. Plasma levels of MCH were found to range between 54 and 397 pg/ml.

13.4.4 MCHR1 in the Brain

As is the case with MCH, most of the anatomical mapping of MCHR1 has been performed in rodents, with brief descriptions of other species available in the literature (Fig. 13.8) (Chee et al. 2013; Diniz et al. 2020; Hervieu et al. 2000; Saito et al. 2001). The presence of $MCHR1^+$ cilia in some relevant neuroendocrine populations is illustrated in Fig. 13.9.

13.4.4.1 Neocortex

There is ample expression of *Mchr1* mRNA and MCHR1 synthesis in the rat and the mouse cortical mantle, including layers II, III, IV, V, and VI. Primary cilia containing MCHR1 are abundantly found in all layers except for layer I, mimicking the pattern of gene expression. Synthesis of MCHR1 is found throughout the neocortex with minimal variation between areas.

13.4.4.2 Olfactory Areas

Olfactory areas display some of the densest concentrations of $MCHR1^+$ primary cilia in the mouse brain. Vast numbers of labeled cilia are found in the granular, internal plexiform, mitral, and glomerular cell layers. In the latter, labeled cilia are strongly associated with TH^+ glomerular cells and, to a lesser extent, with calretinin-positive cells. Additional sites include layer 2 of the piriform cortex, medial part of the anterior olfactory nucleus, dorsal and ventral *tenia tecta*, dorsal and intermediate endopiriform nucleus, and olfactory tubercle.

13.4.4.3 Hippocampal Formation

The hippocampal formation displays a very characteristic pattern of MCHR1 immunoreactivity, with dense ciliary labeling found in pyramidal cells of CA1, CA2, and,

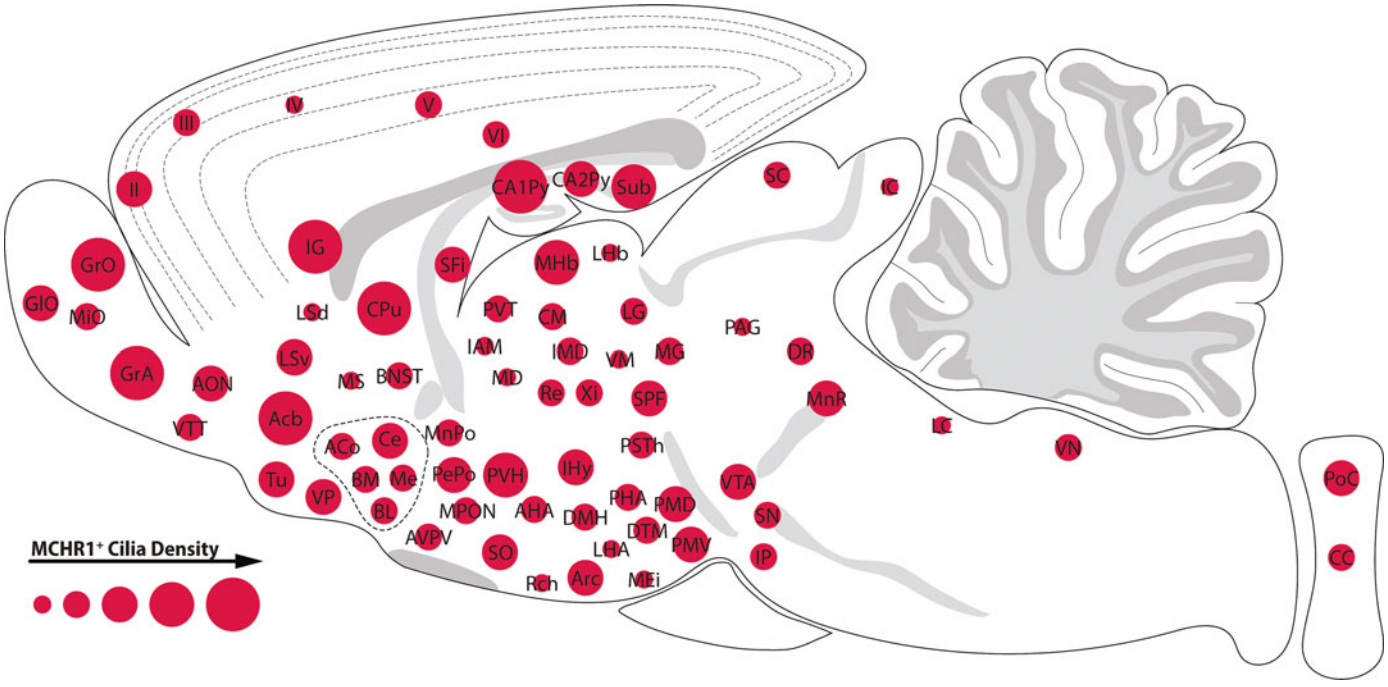

Fig. 13.8 Schematic representation of the distribution and density of ciliary MCHR1 in the mouse brain. The size of red circles indicates the relative density of MCHR1$^+$ cilia in the brain. The area marked by a discontinuous line represents the amygdaloid complex. Abbreviations: *II* cortical layer II, *III* cortical layer III, *IV* cortical layer IV, *V* cortical layer V, *VI* cortical layer VI, *Acb* nucleus *accumbens*, *ACo* anterior cortical nucleus of the amygdala, *AHA* anterior hypothalamic area, *AON* anterior olfactory nucleus, *Arc* arcuate nucleus, *AVPV* anteroventral preoptic nucleus, *BL* basolateral nucleus of the amygdala, *BM* basomedial nucleus of the amygdala, *BNST* bed nucleus of the stria terminalis, *CA1Py* pyramidal layer of the CA1 field of the hippocampus, *CA2Py* pyramidal layer of the CA2 field of the hippocampus, *CC* central column of the spinal cord, *Ce* central nucleus of the amygdala, *CM* centromedial thalamic nucleus, *CPu* caudate putamen, *DMH* dorsomedial hypothalamic nucleus, *DR* dorsal raphe nucleus, *DTM* dorsal tuberomammillary nucleus, *GlO* glomerular layer of the olfactory

bulb, *GrA* granular layer of the accessory olfactory bulb, *GrO* granular layer of the olfactory bulb, *IAM* interanteromedial thalamic nucleus, *IG* induseum griseum, *IMD* intermediodorsal thalamic nucleus, *IP* interpeduncular nuclei, *LC* locus coeruleus, *LG* lateral geniculate nucleus, *IHy* incerto-hypothalamic area, *LHA* lateral hypothalamic area, *LHb* lateral habenular nucleus, *LSd* dorsal part of the lateral septal nucleus, *LSv* ventral part of the lateral septal nucleus, *MD* mediodorsal thalamic nucleus, *Me* medial nucleus of the amygdala, *MG* medial geniculate nucleus, *MHb* medial habenular nucleus, *MiO* mitral layer of the olfactory bulb, *MnPo* median preoptic nucleus, *MnR* median raphe nucleus, *MPON* medial preoptic nucleus, *MS* medial septal nucleus, *PAG* periaqueductal gray matter, *PePo* preoptic periventricular nucleus, *PHA* posterior hypothalamic area, *PMD* dorsal premammillary nucleus, *PMV* ventral premammillary nucleus, *PoC* posterior column of the spinal cord, *PSTh* parasubthalamic nucleus, *PVH* paraventricular hypothalamic nucleus, *PVT* paraventricular thalamic nucleus, *RCh* retrochiasmatic nucleus, *Re* nucleus reuniens, *SN* substantia nigra, *SPF* subparafascicular nucleus, *Sub* subiculum, *Tu* olfactory tubercle, *VM* ventromedial thalamic nucleus, *VN* trigeminal nuclei, *VTA* ventral tegmental area, *VTT* ventral tenia tecta, *Xi* xiphoid nucleus

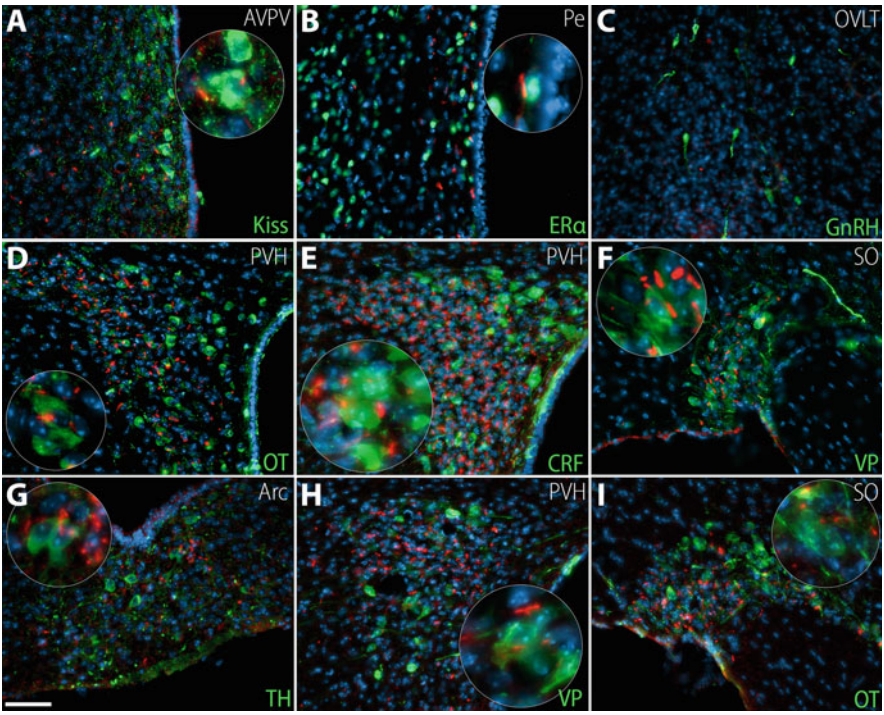

Fig. 13.9 Presence/absence of ciliary MCHR1 in neuroendocrine areas. Mouse photomicrographs showing the co-distribution between ciliary MCHR1 (red) and neuroendocrine populations (green) in the rodent brain. Highlights of the proximity between labeled cilia and neurons are provided in higher magnification circles, when applicable. Scale bar: 50 μm. Abbreviations: *Arc* arcuate nucleus, *AVPV* anteroventral preoptic area, *CRF* corticotropin-releasing factor, *ERa*—estrogen receptor α, *GnRH* gonadotropin-releasing hormone, *Kiss* kisspeptin 1, *OT* oxytocin, *OVLT organum vasculosum* of the *lamina terminalis*, *Pe* periventricular hypothalamic nucleus, *PVH* paraventricular hypothalamic nucleus, *SO* supraoptic nucleus, *TH* tyrosine hydroxylase, *VP* vasopressin. Adapted with permission from Diniz et al. (2020)

to a lesser extent, CA3. Primary cilia immunoreactive to MCHR1 in CA1 are substantially longer than those in CA3, and they undergo ciliary shortening in response to MCH while those in CA3 do not. Fasting in adult mice also reduces the length of CA1 MCHR1-positive cilia, while CA3 cilia remain unchanged (Kobayashi et al. 2020). Minimal numbers of MCHR1$^+$ cilia are found in the *oriens* and *radiatum* strata, and almost no immunoreactivity is found in the dentate gyrus, despite the vast numbers of primary cilia in that structure. The subgranular zone of the dentate gyrus is one of the main sites of differences between rats and mice, with rats displaying a small but well-delimited layer of MCHR1+ cilia that is absent in mice.

13.4.4.4 Subcortical Telencephalic Structures

MCHR1$^+$ cilia are abundantly found in subcortical structures. In both rats and mice, the nucleus *accumbens* (Acb) is among the densest areas of *Mchr1* mRNA expression and MCHR1$^+$ cilia. In the mouse caudate-putamen matrix, a mediolateral gradient is observed, with the highest density of positive cilia found closer to the wall of the lateral ventricles, while no labeling is found in the striasomes. No labeling is observed in the rat caudate-putamen, making this structure the second major dimorphic area between rats and mice. Moderate labeling is observed in the ventral pallidum and medial part of the globus *pallidus*, while sparse labeling is found in the lateral globus *pallidus* and the central part of the lateral septal nucleus. In the amygdaloid complex, only scattered MCHR1$^+$ cilia are found in the basolateral, basomedial, medial, and central nuclei.

13.4.4.5 Thalamus

Several thalamic areas display moderate numbers of MCHR1$^+$ cilia, including the *paratenial* nucleus, paraventricular thalamic nucleus, medial thalamic nuclei, and medial habenular nucleus. In the paraventricular nucleus, MCHR1$^+$ cilia are often found co-distributed with calretinin. While no immunoreactivity is detected in the ZI proper, many TH$^+$/MCHR1$^+$ neurons are found in the IHy.

13.4.4.6 Hypothalamus

In the hypothalamus, small but dense clusters of MCHR1$^+$ cilia are found in the preoptic periventricular nucleus, PVH, supraoptic nucleus, and arcuate nucleus (Arc). In the preoptic hypothalamus, MCHR1 positive cilia are associated with kisspeptin neurons and, to a lesser extent, estrogen receptor α (ERα)-positive cells, but not with gonadotropin-releasing hormone (GnRH) cells (Fig. 13.9). In the PVH, MCHR1$^+$ cilia are co-distributed with OT and corticotropin-releasing factor (CRF), while in the supraoptic nucleus MCHR1$^+$ cilia are often found adjacent to VP$^+$ neurons (Fig. 13.9). Positive cilia are associated with TH neurons in the Arc, but no exact co-distribution is found with αMSH or CART. Moderate numbers of labeled cilia are found in the MPOA, anterior hypothalamic area, and posterior hypothalamic area. The dorsomedial and ventromedial hypothalamic nuclei are mostly devoid of labeling.

13.4.4.7 Brainstem

Scattered labeled cilia are found in the dorsal midbrain, including the area surrounding the mesencephalic aqueduct and the superior colliculus, with a slight preference for the *stratum opticum* in the latter. Ventrally, MCHR1 is found in the ventral tegmental area, *paranigral* nucleus, parabrachial pigmented area, interpeduncular nucleus, and *pars compacta* of the *substantia nigra*. In the midbrain-pons transition area, labeled cilia are observed in the dorsal and medial raphe nuclei. In the posterior brainstem, small numbers of MCHR1 positive cilia are found in select sensory nuclei, including the ventral cochlear nucleus and the nucleus of the solitary tract.

13.4.4.8 Spinal Cord

In the spinal cord, MCHR1$^+$ cilia are present in the dorsal grey column, comprehending the area of Rexed laminae II and III, while only scattered in the area surrounding the central canal.

13.4.5 MCHR1 in the Periphery

In addition to the brain, *Mchr1* expression and MCHR1 immunoreactivity have been found in multiple peripheral tissues, allowing MCH, either released in the circulation or produced locally at the periphery, to influence a large number of physiological systems (Fig. 13.10). These systems include: musculoskeletal—skeletal muscle, tongue, bones; cardiorespiratory—heart, lung, trachea; digestive—esophagus, stomach, small intestine, duodenum, colon, liver; urogenital—kidney, prostate, uterus; immune—splenocytes, lymphocytes, thymocytes, PBMCs, granulocytes; and endocrine—hypophysis, cortex and medulla of the adrenal gland and thyroid gland. The presence of *Mchr1* mRNA has also been reported in adipocytes, the skin, and the placenta (Bradley et al. 2000; Chung et al. 2012; Hill et al. 2001; Hoogduijn et al. 2002; Kokkotou et al. 2008, 2009; Saito et al. 1999; Segal-Lieberman et al. 2006; Takahashi et al. 2001; Verlaet et al. 2002). More recently, both *Mchr1* mRNA and MCHR1 immunoreactivity have been reported in the rat mammary gland. Positive signals are found in the skin covering the glands in both virgin and lactating females, but MCHR1 is found in the parenchyma exclusively in lactating animals, with maximal expression in samples collected on the 19th PPD. Immunoreactivity was found to be associated with alveolar secretory cells, suggesting an active role of circulating MCH in milk ejection, which may explain why MCHR1 ablation or inactivation mid-lactation results in decreased milk production (Alachkar et al. 2016; Battagello et al. 2020).

13.5 Interactions Between MCH and Hypophysiotropic Hormonal Systems

13.5.1 Sex Steroids

There is a complex relationship between MCH, NEI, and luteinizing hormone (LH) that is highly dependent on the hormonal *milieu* of the animal (for an in-depth discussion, see Naufahu et al. 2013). These interactions include both direct and indirect MCH and NEI actions over the release of GnRH and direct and indirect actions of sex steroids over MCH neurons. The high complexity of these interactions has led to a large body of studies in the literature that is difficult to interpret and, at times, contradictory. A schematic representation of MCH in the hypothalamic-pituitary-gonadal (HPG) axis is provided in Fig. 13.11.

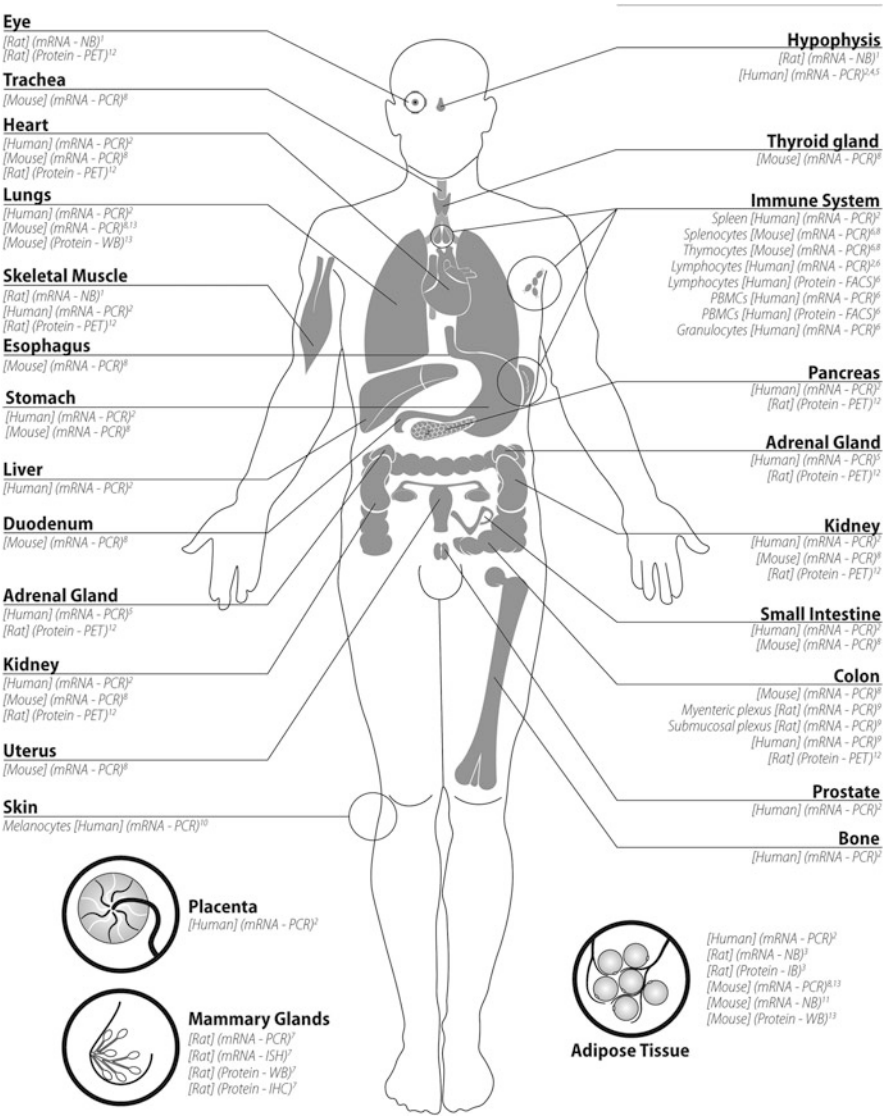

Fig. 13.10 Reports of *Mchr1* expression and MCHR1 synthesis in peripheral tissues of mammals. Reports are organized by organ/system, tissue, target, and technique. References: 1—Saito et al. (1999); 2—Hill et al. (2001); 3—Bradley et al. (2000); 4—Segal-Lieberman et al. (2006); 5—Takahashi et al. (2001); 6—Verlaet et al. (2002); 7—Battagello et al. (2020); 8—Chung et al. (2012); 9—Kokkotou et al. (2008); 10—Hoogduijn et al. (2002); 11—Bradley et al. (2002); 12—Philippe et al. (2016); 13—Balber et al. (2019). Abbreviations: *FACS* fluorescence assisted cell sorting, *IB* immunoblotting, *IHC* immunohistochemistry, *ISH* in situ hybridization, *NB* northern blotting, *PCR* polymerase chain reaction, *PET* positron emission tomography, *RIA* radioimmunoassay, *WB* western blotting

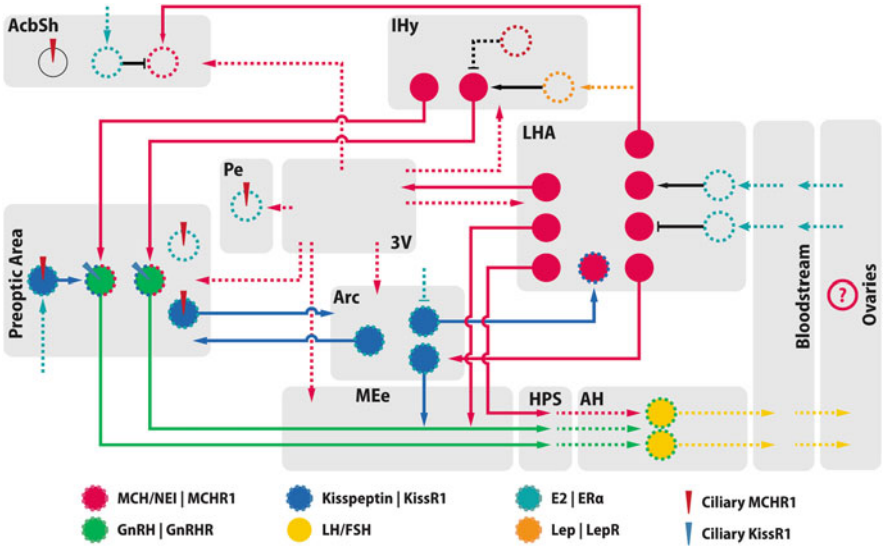

Fig. 13.11 The role of MCH in the hypothalamic-pituitary-gonadal (HPG) axis. Schematic representation of the main components of the HPG axis and their relationship with MCH neurons. For clarity, not all elements of the HPG system are represented . Circles represent cells and their neuromodulator/hormonal content, broken circles represent receptors in the surface of the cells or of unknown subcellular location, continuous lines indicate wired projections, broken lines represent humoral communication or unknown pathways, thin wedges represent ciliary receptors, and black drawings represent cells or relationships that have not been determined. Elements drawn out of gray boxes may originate from multiple or undetermined areas. A question mark is used in cases where MCH or MCHR1 has been reported in peripheral structures but there were no dedicated confirmatory studies. Abbreviations: *3V* third ventricle, *AcbSh* shell of the nucleus *accumbens*, *AH* adenohypophysis, *Arc* arcuate nucleus, *E2* estrogen/estradiol, *ERa* estrogen receptor α, *FSH* follicle-stimulating hormone, *GnRH* gonadotropin-releasing hormone, *GnRHR* GnRH receptor, *HPS* hypophyseal portal system, *IHy* incerto-hypothalamic area, *KissR1* kisspeptin receptor 1, *Lep* leptin, *LepR* leptin receptor, *LH* luteinizing hormone, *LHA* lateral hypothalamic area, *MCH* melanin-concentrating hormone, *MCHR1* MCH receptor 1, *MEe* median eminence, external layer, *NEI* neuropeptide E-I, *Pe* periventricular hypothalamic nucleus

13.5.1.1 The Actions of MCH/NEI on LH Release

Available evidence suggests NEI has a positive effect on the release of LH. When injected intraventricularly in rats, NEI leads to a transient increase in LH concentration in the blood as soon as 10 min and persisting up to 90 min (study endpoint), both in males and in ovariectomized (OVX) females treated with estrogen benzoate (EB) and progesterone (P4) (Attademo et al. 2004). The actions of MCH, on the other hand, are less straightforward. Injection of MCH into the MPOA and the ME leads to an increase in LH secretion in OVX females treated with high levels of EB. Accordingly, immunoneutralization of MCH in the MPOA leads to a decrease in LH secretion in OVX females in the absence of hormonal supplementation. The addition of P4, however, abolishes the inducing effect of MCH over LH release in the MPOA and leads to an impaired surge in LH when MCH is injected into the IHy

(Gonzalez et al. 1997; Murray et al. 2000a, 2006). Intraventricular injections of MCH in OVX females treated with low levels of EB lead to decreased LH secretion (Tsukamura et al. 2000). These results indicate that MCH action over LH secretion depends on the site of action and the hormonal status of the animals, including circulating leptin. The immunoneutralization of MCH in the MPOA blocks the increase in LH secretion caused by leptin injection in the IHy (Murray et al. 2000b).

13.5.1.2 GnRH Neurons as Mediators of MCH Action Over LH Release

GnRH neurons in the medial septal nucleus, *organum vasculosum* of the *lamina terminalis* and preoptic area receive extensive contacts (60%–90%) from NEI^+ and MCH^+ fibers both in rats and mice of both sexes. Contacts between MCH^+ fibers and $GnRH^+$ neurons have also been observed in the human infundibular nucleus, although these contacts appear to be less extensive ($17.7 \pm 3.3\%$). The existence of MCH synapses onto GnRH neurons has been confirmed through electron microscopy (Skrapits et al. 2015; Ward et al. 2009; Williamson-Hughes et al. 2005; Wu et al. 2009). Expression of *Mchr1* mRNA has been reported in approximately half of the GnRH population, although no $MCHR1^+$ cilia are found in those cells (Diniz et al. 2020; Williamson-Hughes et al. 2005). A subset of kisspeptin-sensitive GnRH neurons of the medial septal nucleus and diagonal band of Broca respond postsynaptically to MCH, suggesting MCH and kisspeptin signals converge onto those neurons (Wu et al. 2009).

Given that we detected ciliary MCHR1 closely associated with kisspeptin neurons, MCH may act synaptically on GnRH neurons and through volume transmission over kisspeptin neurons to perform its complex regulation of LH secretion. In addition to action over GnRH somas in the basal forebrain and preoptic hypothalamus, NEI^+ fibers have been identified in close apposition to $GnRH^+$ fibers coursing through the external layer of the ME, raising the possibility that MCH and NEI also modulate LH secretion by altering the release of GnRH into the hypophyseal portal system (HPS) through axo-axonal contacts. This would explain why incubation of ME of proestrus female rats with 10^{-10} or 10^{-9} M of MCH leads to increased GnRH in the media after 30 min (Attademo et al. 2006; Chiocchio et al. 2001; De Paul et al. 2009; Ward et al. 2009; Williamson-Hughes et al. 2005).

13.5.1.3 The Direct Action of MCH and NEI on Gonadotropes

Both MCH^+ and NEI^+ varicose fibers are found in the external lamina of the ME, with dense fiber plexuses found near blood vessels, leading to the suggestion that MCH and NEI are released in the HPS. The addition of NEI to isolated hypophyses increases LH release in the culture media after 1 hour, remaining high for up to 5 hours (study endpoint). Likewise, the incubation of isolated hypophyses obtained from proestrus females with MCH leads to increased LH and follicle-stimulating hormone (FSH) in the culture media. These secretory changes are accompanied by the development of the rough endoplasmic reticulum and Golgi apparatus, accompanied by a reduction in secretory granules in the presence of vesicle exocytosis. These results strongly suggest that both MCH and NEI act directly on gonadotropes to promote LH release and possibly FSH (Chiocchio et al. 2001; De Paul et al. 2009).

13.5.1.4 Sex Steroids Actions on MCH Neurons

The orexigenic (but not locomotor) effect of intraventricular MCH in OVX females is suppressed in EB-supplemented animals compared to controls, suggesting EB influences orexigenic circuits of MCH in a specific manner (Messina et al. 2006; Santollo and Eckel 2008). This effect is at least partially mediated by the action of MCH in the Acb. While activation of MCHR1 in the Acb leads to an increase in feeding in males, only OVX females without treatment displayed a similar response, while supplementation with EB abolished that effect. Given that *Mchr1* mRNA and estrogen receptor 1 (*Esr1*) mRNA are co-distributed in the shell of the Acb, it is likely that both MCH and E2 signaling converge in the Acb to modulate the orexigenic effect of MCH (Terrill et al. 2020).

Hyperestrogenemia has temporally sensitive effects on the expression of *Pmch* mRNA in the LHA. Implantation of E2 pellets in male mice upregulates *Pmch* expression after 48 hours but downregulates it after 22 days and prevents the increase in *Pmch* transcription secondary to caloric restriction (Morton et al. 2004; Mystkowski et al. 2000; Tritos et al. 2004). Physiological single injections of EB or an ERα agonist promote a decrease in the total number of MCH neurons in the LHA 9 and 6 hours after injection, respectively (Santollo and Eckel 2013). In OVX cynomolgus monkeys, acute injections of EB promote a rise in MCH and NEI in the hypothalamus within 72 hours post-administration (Viale et al. 1999a). This effect seems to be mediated through a polysynaptic circuit since MCH neurons of the LHA lack ERα, despite the presence of both markers in the lateral hypothalamus (Muschamp and Hull 2007; Santollo and Eckel 2013). The exception to this is the preoptic cluster, where 70% of MCH neurons colocalize with ERα, allowing E2 to act directly on those neurons (Teixeira et al. 2020).

13.5.2 Growth Hormone

Evidence indicates that both MCH and NEI act as pro-growth hormone (GH) hypophysiotropic hormones (Fig. 13.12). The addition of MCH and NEI in nanomolar concentrations leads to an increase (62% and 124%, respectively) in GH secretion when applied to human fetal hypophyseal cells and mouse hypophyses. A similar phenomenon has been reported in the teleost *Cichlasoma dimerus*, suggesting a pro-GH action of MCH has been conserved in vertebrate evolution (Pérez-Sirkin et al. 2012; Segal-Lieberman et al. 2006).

13.5.3 Cortisol

As is the case with sex steroids, there is a complicated relationship between MCH and the physiological machinery involved in modulating CORT release, with conflicting reports in the literature as the precise role of MCH (Fig. 13.13). Most works point to a pro-secretion role for MCH in the basal release of CORT, while it suppresses release in stress conditions.

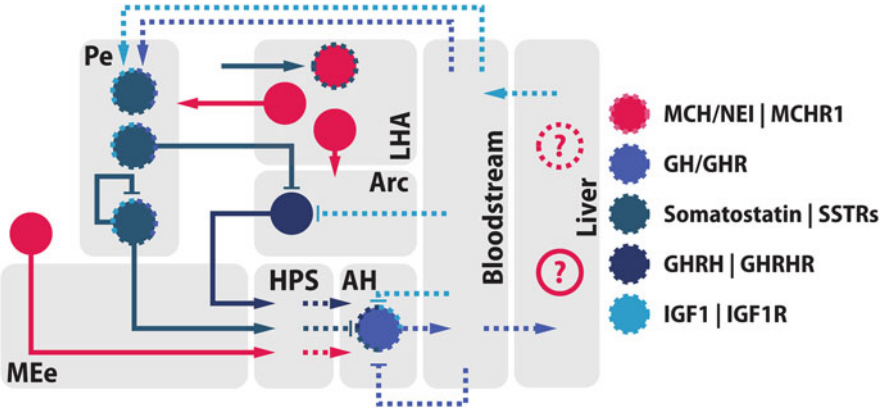

Fig. 13.12 The role of MCH in the hypothalamic-pituitary-somatotropic axis. Schematic representation of the main components of the axis and their relationship with MCH neurons. For clarity, not all elements of the system are represented. Circles represent cells and their neuromodulator/hormonal content, broken circles represent receptors in the surface of the cells or of unknown subcellular location, continuous lines indicate wired projections, broken lines represent humoral communication or unknown pathways. Elements drawn out of gray boxes may originate from multiple or undetermined areas. A question mark is used in cases where MCH or MCHR1 has been reported in peripheral structures but there were no dedicated confirmatory studies. Abbreviations: *AH* adenohypophysis, *Arc* arcuate nucleus, *GH* growth hormone, *GHR* GH receptor, *GHRH* growth hormone-releasing hormone, *GHRHR* growth hormone-releasing hormone receptor, *HPS* hypophyseal portal system, *IGF1* insulin-like growth factor 1, *IGF1R* insulin-like growth factor 1 receptor, *LHA* lateral hypothalamic area, *MCH* melanin-concentrating hormone, *MCHR1* MCH receptor 1, *MEe* median eminence, external layer, *NEI* neuropeptide E-I, *Pe* periventricular hypothalamic nucleus, *SSTR* somatostatin receptor

13.5.3.1 The Effect of MCH/NEI on CRF Neurons

The addition of MCH or NEI to hypothalamic explants raises the level of CRF in the medium (56% over baseline and 134% over baseline, respectively), suggesting MCH promotes the release of CRF (Jezová et al. 1992). This effect is likely mediated by the substantial population of CRF neurons in the PVH associated with MCHR1^{+} primary cilia. However, it should be noted that a different study found no increase in CRF release after incubation of rat hypothalamic explants with synthetic rat MCH (Navarra et al. 1990).

13.5.3.2 The Effect of MCH/NEI on ACTH Release

Intraventricular injection of nanomolar concentrations of MCH in male Wistar rats was reported to result in a transient rise in plasma ACTH (approximately 44% over baseline) 10 min after injection that disappears after 20 min. Intranuclear injection of MCH into the PVH has a more intense effect, increasing plasma ACTH by 133% over baseline after 10 min. Intraventricular injection of 3 μg of MCH 2 hours after the beginning of the light phase elevates plasma ACTH within 15 min of injection, but not at 30 min. Intraventricular injection of 50 μg of MCH in rats leads to an increase in plasma ACTH levels (200–300% of baseline) as soon as 5 min after

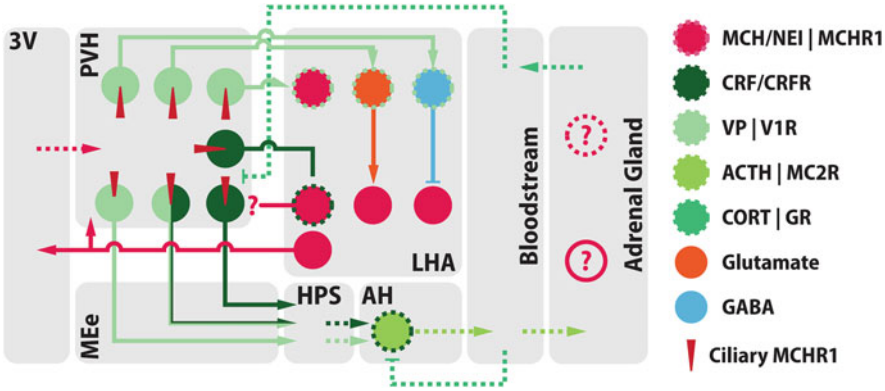

Fig. 13.13 The role of MCH in the hypothalamic-pituitary-adrenal (HPA) axis. Schematic representation of the main components of the HPA axis and their relationship with MCH neurons. For clarity, not all elements of the HPA system are represented. Circles represent cells and their neuromodulator/hormonal content, broken circles represent receptors in the surface of the cells or of unknown subcellular location, continuous lines indicate wired projections, broken lines represent humoral communication or unknown pathways, and thin wedges represent ciliary receptors. A question mark is used in cases where MCH or MCHR1 has been reported in peripheral structures but there were no dedicated confirmatory studies, or when the exact role of MCH neurons over other neurons has not been determined. Abbreviations: *3V* third ventricle, *ACTH* adrenocorticotropic hormone, *AH* adenohypophysis, *CORT* cortisol/corticosterone, *CRF* corticotropin-releasing factor receptor, *CRFR* CRF receptor, *GR* glucocorticoid receptor, *HPS* hypophyseal portal system, *LHA* lateral hypothalamic area, *MC2R* melanocortin receptor 2, *MCH* melanin-concentrating hormone, *MCHR1* MCH receptor 1, *MEe* median eminence, external layer, *NEI* neuropeptide E-I, *PVH* paraventricular hypothalamic nucleus, *V1R* vasopressin receptor 1, *VP* vasopressin

injection and remained elevated up to 17 min (experiment endpoint) (Kennedy et al. 2003; Smith et al. 2006, 2009). It should be noted, however, that other researchers found a decrease in plasma ACTH levels following intraventricular injections of nanomolar concentrations of MCH in male Wistar rats during the light phase or after stress in the dark phase, and a decrease in plasma ACTH levels following handling stress both 45 and 90 min after stimulation (Bluet-Pajot et al. 1995; Ludwig et al. 1998), or no alteration whatsoever after intracerebroventricular infusion of MCH or NEI at 10 μg/h for 24 h in adult OVX ewes (Parkes 1996).

13.5.3.3 The Effect of MCH/NEI on CORT Release

Intranuclear injections of picomolar concentrations of MCH into the PVH of male rats increase circulating CORT (66% over baseline) after 10 min (Kennedy et al. 2003). Accordingly, intraventricular injections of 3 μg of MCH 2 hours after the beginning of the light period substantially elevates circulating levels of CORT after 15 min, remaining elevated up to 60 min (study endpoint) (Kennedy et al. 2003; Smith et al. 2006). On the other hand, no alterations in circulating CORT were found following continuous injection of MCH or NEI intraventricularly in OVX ewes, and intraventricular injections of MCH in rats were found to decrease levels of circulating CORT following handling stress (Ludwig et al. 1998; Parkes 1996).

13.5.3.4 Interactions Between MCH and CORT in Teleosts

Synthetic salmon MCH at picomolar concentrations reduces ACTH release by isolated hypophyses of stressed trout and inhibits the induced secretion of ACTH by CRF, and removed hypophyses from stressed trout chronically injected with salmon MCH release less ACTH *in vitro* (Baker et al. 1985, 1986). Immunoneutralization of MCH in isolated trout hypothalamus also indicates a depressive role for MCH over ACTH release (Baker et al. 1985, 1986; Green et al. 1991). Injection of MCH in trout reduces the stress-induced rise in plasma CORT during the first hour, but not the total rise over the experiment (Gilchriest et al. 2001). As a side note, the tilapia homolog of mammalian NGE was reported to stimulate ACTH release from hypophyses *in vitro* (Gröneveld et al. 1996).

13.5.4 Thyroid Hormones

13.5.4.1 The Actions of MCH on Thyroid Hormones

Evidence in the literature points to MCH having an effect on the release of thyroid hormones, both centrally and peripherally (Fig. 13.14). Administration of 1 μM MCH or NEI reduces the basal production of TRH (73% and 40%, respectively). Accordingly, intraventricular injection of nanomolar concentrations of MCH in male rats depresses plasma thyroid-stimulating hormone (TSH) at 10 (29% reduction from

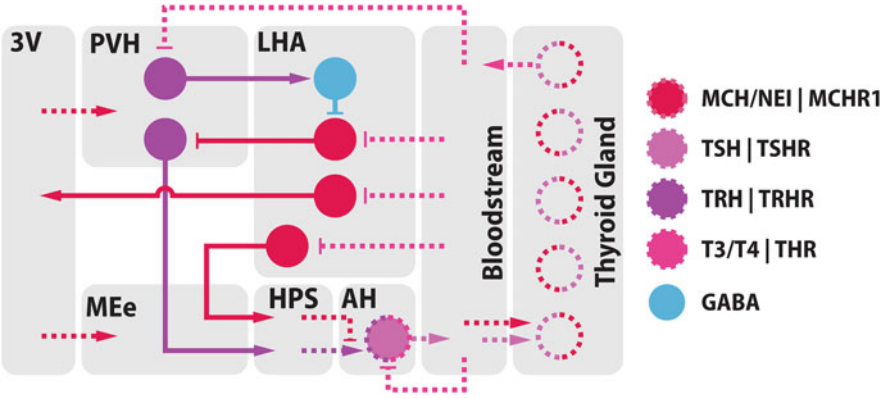

Fig. 13.14 The role of MCH in the hypothalamic-pituitary-thyroid (HPT) axis. Schematic representation of the main components of the HPT axis and their relationship with MCH neurons. For clarity, not all elements of the HPT system are represented. Circles represent cells and their neuromodulator/hormonal content, broken circles represent receptors on the surface of the cells or of unknown subcellular location, continuous lines indicate wired projections, and broken lines represent humoral communication or unknown pathways. Abbreviations: *3V* third ventricle, *AH* adenohypophysis, *HPS* hypophyseal portal system, *LH* lateral hypothalamic area, *MCH* melanin-concentrating hormone, *MCHR1* MCH receptor 1, *MEe* median eminence, external layer, *PVH* paraventricular hypothalamic nucleus, *T3* triiodothyronine, *T4* thyroxine, *THR* thyroid hormone receptor, *TRH* thyrotropin-releasing hormone, *TRHR* thyrotropin-releasing hormone receptor, *TSH* thyroid-stimulating hormone, *TSHR* thyroid-stimulating hormone receptor

baseline) and 60 (53% reduction from baseline) minutes. At the level of the adenohypophysis, MCH counteracts the TRH stimulation of TSH release without inducing a reduction in basal release (Kennedy et al. 2001). There is also evidence for a peripheral action of MCH, as *Mchr1* mRNA is found in the thyroid gland, with expression levels that are substantially higher than that of other peripheral tissues. In *Mchr1* KO animals, T4 secretion in response to TSH is reduced compared to WT mice, and circulating levels of thyroxine (T4), triiodothyronine (T3), and rT3 are all significantly depressed (Chung et al. 2012).

13.5.4.2 The Effect of Thyroid Hormones on NEI Synthesis and Release

There is an intricate pattern of interaction between NEI content, time of day, and circulating thyroid levels. While LHA levels are unaltered, NEI content in the perifornical nucleus decreases after 24 days of altered thyroid hormone levels, regardless of the time of the day when the measurement has taken place. Other areas with altered NEI levels include the *organum vasculosum* of the *lamina terminalis*, anteroventral periventricular nucleus, preoptic hypothalamus, PVH, and ME (Ayala et al. 2011, 2013). There is also an effect of TRH on MCH cells that occurs indirectly, through the excitation by TRH of GABA neurons presynaptic to MCH neurons in the LHA (Zhang and van den Pol 2012).

13.5.5 Prolactin

13.5.5.1 The Action of MCH on PRL Release

When injected into the ventricle, 1 μg of MCH leads to a decrease in the levels of 3,4-dihydroxyphenylacetic acid in the ME and an increase in serum PRL, as expected from an inhibitory action over tuberoinfundibular dopamine neurons (TIDA) in the Arc (Yang and Shieh 2005). This effect is likely to be the result of a direct action of MCH on TIDA neurons, as there is extensive presence of ciliary MCHR1 in TH^+ neurons of the arcuate nucleus (Fig. 13.15). Considering that primary cilia are specialized in detecting signals in the surrounding space, and the permeability of the Arc to bloodstream originated signals, it is possible that MCH originated in both the hypothalamus and the bloodstream may inhibit TIDA neurons to disinhibit the secretory activity of lactotropes in the adenohypophysis. It should be noted that some authors did not detect changes in basal plasma levels of PRL in male rats following intraventricular injection of nanomolar concentrations of MCH (Bluet-Pajot et al. 1995).

13.5.5.2 The Action of PRL on MCH Neurons

There is an ambivalent response of MCH to PRL depending on the brain area. Hyperprolactinemia leads to decreased *Pmch* expression (approx. 33%) in the lateral hypothalamus compared to sham-operated controls (Garcia et al. 2003). The opposite is observed in the preoptic cluster, however, as suppression of PRL secretion through bromocriptine decreases the number of MCH neurons in the lactating MPOA, and *Stat5* knockout animals also display fewer MCH neurons in that area

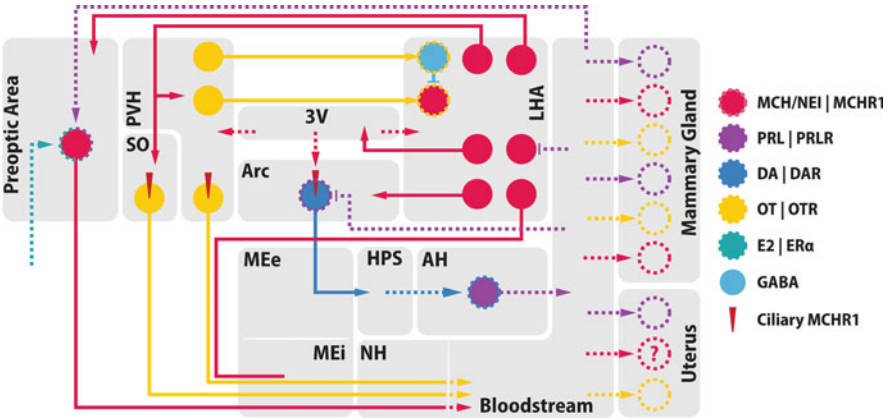

Fig. 13.15 Interactions between MCH, prolactin and oxytocin during lactation. Schematic representation of the main components involved in the hormonal control of lactation processes. For clarity, not all elements are represented. Circles represent cells and their neuromodulator/hormonal content, broken circles represent receptors on the surface of the cells or of unknown subcellular location, continuous lines indicate wired projections, broken lines represent humoral communication or unknown pathways, and thin wedges represent ciliary receptors. A question mark is used in cases where MCH or MCHR1 has been reported in peripheral structures but there were no dedicated confirmatory studies. Abbreviations: *3V* third ventricle, *AH* adenohypophysis, *Arc* arcuate nucleus, *DA* dopamine, *DAR* dopamine receptor, *E2* estrogen/estradiol, *ERa* estrogen receptor α, *HPS* hypophyseal portal system, *LHA* lateral hypothalamic area, *MCH* melanin-concentrating hormone, *MCHR1* MCH receptor 1, *MEe* median eminence, external layer, *MEi* median eminence, internal layer, *NEI* neuropeptide E-I, *NH* neurohypophysis, *OT* oxytocin, *OTR* OT receptor, *PRL* prolactin, *PRLR* PRL receptor, *PVH* paraventricular hypothalamic nucleus, *SO* supraoptic

(Kokay et al. 2020; Teixeira et al. 2020). The action of PRL on preoptic MCH neurons likely results from a direct mechanism: approximately 60% of the *Pmch*-expressing neurons in the MPOA and periventricular nucleus also express the PRL receptor gene (*Prlr*), and more than 90% of neurons in the area colocalize with pSTAT5 after an acute PRL injection. Furthermore, treatment of animals with PRL resulted in a robust synthesis of pSTAT5 in MCH neurons (Kokay et al. 2020; Teixeira et al. 2020).

13.6 Interactions Between MCH and Neurohypophysial Hormonal Systems

13.6.1 Vasopressin

13.6.1.1 Neuroanatomical Substrate

Mouse VP neurons of the PVH synthesize a gene reporter linked to *Mchr1* expression and are co-distributed with MCHR1$^+$ primary cilia, albeit to a less extent to VP neurons in the supraoptic nucleus (Fig. 13.13) (Chee et al. 2013; Diniz et al. 2020). This indicates that VP neurons, or at least a subpopulation of those neurons, can

respond to extracellular MCH. Conversely, MCH neurons have V1a receptors. Through these receptors, VP causes a postsynaptic excitatory effect on MCH neurons, depolarizing them and increasing spike frequency under current clamp. Vasopressin also enhances both excitatory and inhibitory synaptic transmission into MCH neurons (Yao et al. 2012).

13.6.1.2 Physiological Actions of MCH

Despite the anatomical substrate, there is no evidence for physiological interactions between MCH and VP. Administration of 1 μM MCH to hypothalamic explants of adult male Wistar rats does not affect VP release, and continuous intraventricular injection of human MCH in OVX ewes does not change circulating levels of VP (Kennedy et al. 2003; Parkes 1996). There is also no evidence of MCH action at the NH level since the addition of nanomolar or micromolar concentrations of MCH to dissociated rat hypophyses results in no change in VP release (Parkes and Vale 1993). The physiological relevance of MCH-VP interactions therefore remains unclear. It is noteworthy that there is some degree of functional overlap between the two populations. Intraventricular injection of microgram concentrations of MCH in male Long-Evans rats significantly increases water intake in the absence of food within 2 hours of intervention. While central MCH promotes water ingestion, administration of an MCHR1 antagonist alone does not influence water intake, suggesting MCH is not constitutively part of the water intake circuitry (Clegg et al. 2003; Morens et al. 2005). There may also be interactions between MCH and VP in the CORT secretion actions of VP, but further experiments are necessary.

13.6.1.3 Physiological Actions of NEI

Administration of NEI to dissociated hypophyses of adult rats reduces VP secretion after 1 (57 ± 10% of the baseline) and 3 hours (68 ± 11%), suggesting a direct role for NEI in modulating the release of VP by terminals in the NH (Parkes and Vale 1993).

13.6.2 Oxytocin

13.6.2.1 Neuroanatomical Substrate

Mouse oxytocinergic neurons of the PVH synthesize a gene reporter linked to *Mchr1* expression and are substantially co-distributed with $MCHR1^+$ primary cilia (Chee et al. 2013; Diniz et al. 2020). This indicates that OT neurons, or at least a subpopulation of those neurons, can respond to extracellular MCH. Fibers immunoreactive to both MCH and NEI have been found in moderate densities in areas rich in OT and VP neurons, such as the PVH and the SO, and close to OT fibers coursing through the internal layer of the ME towards the NH (Costa et al. 2019). These internal layer MCH fibers form axon terminals, with large dense- cores vesicles immunoreactive to MCH found apposed to other local axons. These terminals are also found in the NH, where OT^+ fibers release their contents into the bloodstream.

Finally, the presence of MCH neurons in the anterior PVH during lactation could allow for the paracrine-like release of MCH to act in nearby OT neurons (Fig. 13.15).

Conversely, approximately 60% of MCH neurons have been reported to express the gene for the OT receptor (*Oxtr*), through which application of OT to current-clamped MCH neurons leads to depolarization and a substantial increase in firing rate. Oxytocin also enhances inhibitory synaptic communication of MCH neurons, but unlike VP, it does not change excitatory activity. This effect seems to be mediated by a very small population (0.7 ± 0.4% of the total OT population) of OT neurons in the PVH (Sanathara et al. 2018; Yao et al. 2012).

13.6.2.2 Physiological Actions of MCH and NEI

Addition of nanomolar and micromolar concentrations of MCH to dissociated rat hypophyses leads to an increase in OT secretion after 3 hours (188 ± 29%), suggesting MCH facilitates the release of OT by terminals in the NH. The addition of NEI in a similar experimental design has an even more intense pre-release effect, increasing the release of OT after 1 (245 ± 89%) and 3 (209 ± 64%) hours (Parkes and Vale 1993). Melanin-concentrating hormone neurons have also been implicated as mediators of some of the central actions of OT (Phan et al. 2020; Sanathara et al. 2018, 2020).

13.7 Perspectives

While the role of MCH as a hormone in teleosts is unquestionable, its status as a central neuromodulator in mammals has been favored in the literature, fueled mainly by the discovery that its role in adaptive color change is not a universal feature in early vertebrates but an acquisition in the teleost lineage (Baker and Bird 2002), and the attention given to the orexigenic actions of MCH, which has attracted much of the research into this neuropeptide. However, based on past and recent developments in the field and an overarching view of the literature, it is our opinion that the roles of hypophysiotropic hormone, neurohypophysial hormone, and neuromodulator for MCH should be treated with equal importance.

Hormones are a large class of chemical messengers with diverse compositions, functions, and origins. The term "hormone" was coined by Ernest Starling in a series of lectures to the Royal College of Physicians in 1905 (Starling, 1905 *apud* Hirst 2004) following his discovery of secretin in 1902 (Bayliss and Starling 1902). *Per* the original definition, hormones are messengers that "have to be carried from the organ they are produced to the organ which they affect by means of the bloodstream" (Starling, 1905 *apud* Hirst 2004), drawing from the concept of a chemical reflex, happening independent of the nervous system. This definition still holds, but modern concepts have expanded endocrine communication to include other modalities, including paracrine, autocrine, and intracrine communication.

To account for some particularities, hormones produced by neurons (and some specialized cells) are categorized as neurohormones. Within the nervous system, two classes of neurohormones are found: hypophysiotropic neurons and hypophyseal

hormones. Hypophysiotropic hormones consist of a highly specialized class of hormones produced by neurons in the hypothalamus, with axonal projections that reach the external layer of the ME to release these hormones in the HPS. From there, hypophysiotropic hormones reach the adenohypophysis (endocrine in origin) to modulate the activity of somatotropes, lactotropes, gonadotropes, corticotropes, and thyrotropes. On the other hand, neurohypophyseal hormones are not released in the HPS but directly into the general circulation. These hormones are produced by a specialized class of neurons, magnocellular secretory neurons of the PVH and supraoptic nucleus. Axons from these neurons travel through the internal layer of the ME and the hypophyseal stalk to reach the NH, where the neurohypophyseal hormones are released in blood vessels.

Melanin-concentrating hormone fits the criteria of both a hypophysiotropic and a neurohypophyseal hormone, while NEI can be classified as a hypophysiotropic hormone based on current data. Immunoreactive fibers for MCH and NEI are found in the external layer of the ME forming varicose buttons in the neighborhood of blood vessels. This allows for the release of MCH and NEI in the HPS. In the adenohypophysis, MCH promotes the release of LH and FSH, while NEI promotes LH release by gonadotropes; MCH and NEI promote the release of GH by somatotropes; and MCH counteracts the TRH-dependent increase in TSH release by thyrotropes. Since MCH also acts on hypophysiotropic neurons that will ultimately modulate the action of adenohypophyseal cells, one may ask why a direct hypophysiotropic effect is beneficial. While speculative, we believe a direct role over adenohypophyseal cells allows MCH and NEI to bypass possible antagonistic signals being integrated by hypophysiotropic neurons.

Several lines of evidence also support the neurohypophyseal role of MCH: (1) MCH neurons send axons through the internal layer of the ME and into the NH; (2) MCH neurons are labeled following injection of a retrograde tracer in the bloodstream; (3) MCH is found in the plasma and serum, and its mature form matches that produced in the brain, but not the NEI-MCH form produced in the periphery; (4) MCHR1 receptors are widely found in peripheral tissues, allowing for action away from the organ where MCH is produced; (5) MCHR1 is found in several targets under strong endocrine regulation, including the mammary glands. Taken together, these data indicate the existence of a complete MCH endocrine axis that can affect multiple systems.

Melanin-concentrating hormone has been implicated in over 12 families of functions, including feeding and water consumption, energy balance, sleep, learning, mood, sexual behavior, maternal behavior, sensory integration, ciliary beating, immunological function, and cardiorespiratory function. Understanding this neuropeptidergic system is essential for understanding vertebrate physiology, both in natural and in pathological conditions. The central roles of MCH as a neuromodulator, however, have overshadowed the hormonal aspect of MCH and NEI, and much remains to be elucidated about these hormones' role in the periphery. In part, the challenges associated with understanding the hormonal aspects of MCH and NEI derive from the often contradictory or hard to replicate results available in the literature. The development of new techniques and increased transparency in the

reporting of materials and methods should ease some of those challenges and accelerate profoundly necessary discoveries. Several outstanding questions remain unanswered, including:

- Neuropeptide E-I plays multiple roles in modulating neuroendocrine functions, including potent actions at the level of the adenohypophysis that, in some cases, surpass MCH actions. However, there is no known NEI receptor or mechanism of action.
- The tilapia homolog of NGE has been reported to promote the release of ACTH. It is still unknown whether NGE is constitutively produced in mammals and if it has any actions.
- Synaptic roles for MCH have been extensively described, but direct visualization of MCHR1 in the synapse has proven challenging.
- MCHR1 has been identified in multiple peripheric tissues where no role for it has been described.
- Both MCH and MCHR1 have been identified in reproductive organs, but their exact role in reproductive physiology through these organs is poorly understood.
- MCH neurons in the brain respond to the lactation period and project to the NH, and MCHR1 is found in the mammary gland, but there is no known mechanism of MCH action on milk secretion.
- MCH plays a role in modulating sex steroids, but this modulation depends on the hormonal milieu of the animal and appears contradictory in some studies. The exact role of MCH in the presence of sex steroids needs further investigation.
- Opposing actions of MCH have been reported in the release of CORT, and no single model of action has been produced.
- Vasopressin neurons of the PVH express *Mchr1* and contain ciliary MCHR1, but no direct interaction between MCH and VP has been demonstrated.
- While progress has been made in understanding the role of MCH in central actions of OT, there are substantial gaps in our understanding of how these two neurohormones interact peripherally.

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Qu et al. (1996)—Initial description of the orexigenic role of MCH.

Saito et al. (1999)—One of multiple papers describing MCHR1 as the specific MCH receptor.

Verret et al. (2003)—Discovery of an MCH role in sleep architecture.

Van den Pol et al. (2004)—Description of neurophysiological properties of MCH neurons.

Berbari et al. (2008)—Identification of MCHR1 as a ciliary receptor.

Mickelsen et al. (2017)—Transcriptome of MCH neurons, revealing major neurochemical properties.

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Neuroanatomy of Tuberoinfundibular Peptide 39 Related to Neuroendocrine and Behavioral Regulations

14

Árpád Dobolyi and Ted B. Usdin

Abstract

Tuberoinfundibular peptide of 39 residues (TIP39), also referred to as parathyroid hormone 2 (PTH2), is the endogenous ligand for the parathyroid hormone 2 receptor. TIP39 is synthesized by neurons in three small and distinct brain regions. These neurons project to discrete regions distributed throughout the brain, with highest abundance in the hypothalamus, lateral septum, medial prefrontal cortex, amygdala, periaqueductal gray, nucleus of the solitary tract, locus coeruleus, and spinal cord dorsal horn. Neurons that express the PTH2 receptor are present in each of the regions to which TIP39 neurons project. Experiments have been carried out to evaluate the potential contribution of TIP39-PTH2 receptor signaling to functions thought to be influenced by circuits in regions with high TIP39/PTH2 receptor density. Current evidence supports a role for this peptide/receptor system in multiple homeostatic responses or adaptations, including to nociceptive stimuli, changes in environmental temperature, threat, maternal function, and social awareness.

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Keywords

Neuropeptide · Hypothalamus · Nociception · Maternal · Lactation · Social · Body temperature

14.1 Introduction of Tuberoinfundibular Peptide of 39 (TIP39)

The identification of TIP39, now also designated parathyroid hormone 2 (PTH2), followed from the discovery and characterization of the parathyroid hormone 2 receptor (PTH2R). The PTH2R was discovered in a screening project aimed at identifying new G-protein coupled receptors (GPCRs). At the time of this project GPCRs selectively activated by secretin, vasoactive intestinal polypeptide (VIP) and parathyroid hormone (PTH) had been identified. Their sequences were homologous and while their predicted 7-transmembrane architecture was like that of other GPCRs their amino acid sequences had little similarity to the majority of known GPCRs. The originally described large GPCR group, which includes the beta-adrenergic receptors and channel rhodopsin as prototypical members, was referred to as the rhodopsin-like receptors and now as GPCR family A. The secretin/VIP related receptors are now designated family B. A fragment of the PTH2R was identified as a novel receptor-like sequence in a collection of PCR products generated using primers designed to recognize common sequences in two regions of the secretin, VIP and PTH receptors with brain-derived cDNA libraries as templates. A full-length receptor cDNA was isolated using the PCR fragment as a probe. The novel receptor cDNA was expressed in tissue culture cells. The cells were exposed to a series of potential receptor ligands selected on the basis of similarity to peptides that specifically activated the secretin, VIP and PTH receptors. PTH, and only PTH, caused a robust increase in cAMP production in cells expressing the novel receptor. Since a receptor for PTH was already characterized the new receptor was designated the PTH2 receptor (Usdin et al. 1995).

Several observations lead to the idea that PTH was not in fact the endogenous physiological ligand for the PTH2 receptor and to a search for that ligand. First, as described in detail below, the PTH2 receptor is abundantly expressed by neurons within the brain (Usdin et al. 1996) while attempts to detect PTH in the brain were unsuccessful. Second, while PTH is a potent activator of the human PTH2 receptor, PTH has very low potency on the rat PTH2 receptor. And third, when compared to an activity in crude hypothalamic extracts that was immunologically distinct from PTH, PTH based peptides were weak partial agonists at the rat PTH2 receptor (Usdin 1997). Using selective stimulation of cAMP production in cells that expressed the cloned PTH2 receptor as an assay a peptide was chromatographically purified from bovine hypothalamus. The sequence of this peptide was determined, and it was chemically synthesized and determined to have a pharmacological profile consistent with that of a physiological ligand for the PTH2 receptor (Usdin et al. 1999), which includes potent activation of the PTH2 receptor from multiple species and little efficacy at the PTH1 or other receptors (Fig. 14.1). The peptide was initially called

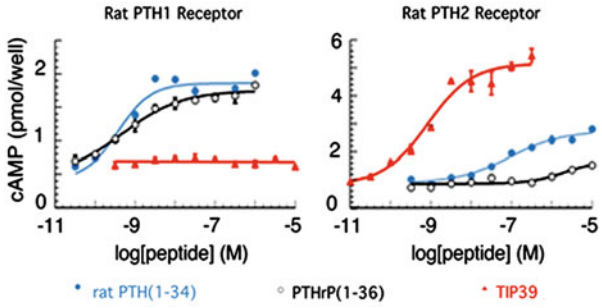

Fig. 14.1 Activation of rat parathyroid hormone 1 (PTH1) and PTH2 receptors. cAMP accumulation is shown in relation to increasing concentrations of PTH, PTH-related peptide, and tuberoinfundibular peptide of 39 residues (TIP39) in COS7 cells expressing the rat PTH1R (A) and the rat PTH2R (B), respectively. The figure was taken from our previous publication (Dobolyi et al. 2012)

Tuberoinfundibular Peptide of 39 Residues (TIP39) and is referred to as this in the original series of publications characterizing its distribution and effects. An international committee on nomenclature subsequently designated it as PTH2 on the basis of its role as a ligand for the PTH2 receptor, which is how it is referred to in databanks (UniGene at http://www.ncbi.nlm.nih.gov/sites/entrez, Mm.207078 for the mouse and Hs.339845 for the human gene) and more recent publications. However, this name is also used for a second form of PTH found in fish that more closely resembles mammalian PTH than does TIP39 (Gensure et al. 2004).

Box 14.1 The Parathyroid Hormone Peptide Family

Mature TIP39 is a secreted peptide of 39 amino acid residues. It is processed from a precursor of approximately 100 residues (depending upon the species). TIP39 is a member of a small peptide family composed of parathyroid hormone (PTH), parathyroid hormone-related peptide (PTHrP), and TIP39 (Usdin et al. 1999). Mature PTH and PTHrP are also polypeptides of about 100 residues. They are products of separate genes but they activate the parathyroid hormone 1 receptor (PTH1 receptor) with equal potency (Gensure and Juppner 2005). Their first 34 or 36 residues are sufficient for high-affinity binding and full efficacy at the PTH1 receptor, and they share twelve of these amino acids (Gillespie and Martin 1994). TIP39 contains only four of the residues that are common to PTH(1–34) and PTHrP(1–36), as well as several additional similar residues (Usdin et al. 1999). However, TIP39 has a backbone structure that can be nearly superimposed on that of PTH (Piserchio et al. 2000). The three peptides of the parathyroid hormone peptide family are members of a larger family that also includes secretin, VIP, calcitonin, gastric

(continued)

Box 14.1 (continued)
inhibitory polypeptide, growth hormone-releasing hormone, pituitary adenylate cyclase-activating polypeptide, and glucagon.

In cells that express the receptors for TIP39 (Goold et al. 2001; Della Penna et al. 2003) and related peptides, both the production of cyclic AMP (cAMP) and an increase in cytoplasmic calcium concentration through a phospholipase C/protein kinase C mechanism via G-protein (Gs and Gq) dependent mechanisms have been demonstrated. The current understanding of TIP39's physiological role is described in the text. PTH and PTHrP act on the same PTH1 receptor through which they are critical regulators of calcium homeostasis and skeletal development and growth, respectively (Rizzoli et al. 1992; Martin et al. 1997).

14.2 Neuroanatomy of TIP39 Cell Groups

14.2.1 TIP39-Expressing Neurons in Adult Males

TIP39 neurons have a highly restricted localization, first described in adult male rats. Developmental stage-dependent and sexually dimorphic expression patterns (Dobolyi et al. 2010 are described in later sections. TIP39 mRNA-expressing neurons and TIP39-immunolabeled neurons had the same distribution pattern: they are present in two brain sites in adult male rats (Dobolyi et al. 2002, 2003b), the medial paralemniscal nucleus (MPL) in the lateral pons and the periventricular gray of the thalamus (PVG) (Fig. 14.2). The latter area may also be referred to as the subparafascicular area because it includes the subparafascicular thalamic nucleus.

Periventricular thalamic TIP39 neurons constitute the largest TIP39 cell group in the brain of young adult rats and mice (Dobolyi et al. 2003b; Faber et al. 2007). TIP39 neurons in the PVG appear rostrally ventral to the central median nucleus of the thalamus, dorsal to the posterior hypothalamic nucleus, and medial to the parvicellular ventral posterior nucleus of the thalamus and mostly medial to the magnocellular subparafascicular nucleus. Additional TIP39 neurons are situated more laterally, ventral to the fasciculus retroflexus. Caudally, TIP39 cells disappear as the PVG becomes the periaqueductal gray of the midbrain at the level of the posterior commissure. In sagittal sections, the distribution of periventricular TIP39 neurons has a sigmoid shape with a rostroventral to posterodorsal orientation, which means that a few TIP39-positive neurons appear in the rostroventral part of the cell group, the density of cells increases postero-dorsally, and finally, only few TIP39 neurons are present in the postero-dorsal extension of the cell group. TIP39 neurons in the PVG are intermingled with tyrosine hydroxylase (TH)-containing neurons corresponding to the A11 dopaminergic cell group. However, no TIP39/tyrosine hydroxylase double-labeled cells were detected in the area (Dobolyi et al. 2010).

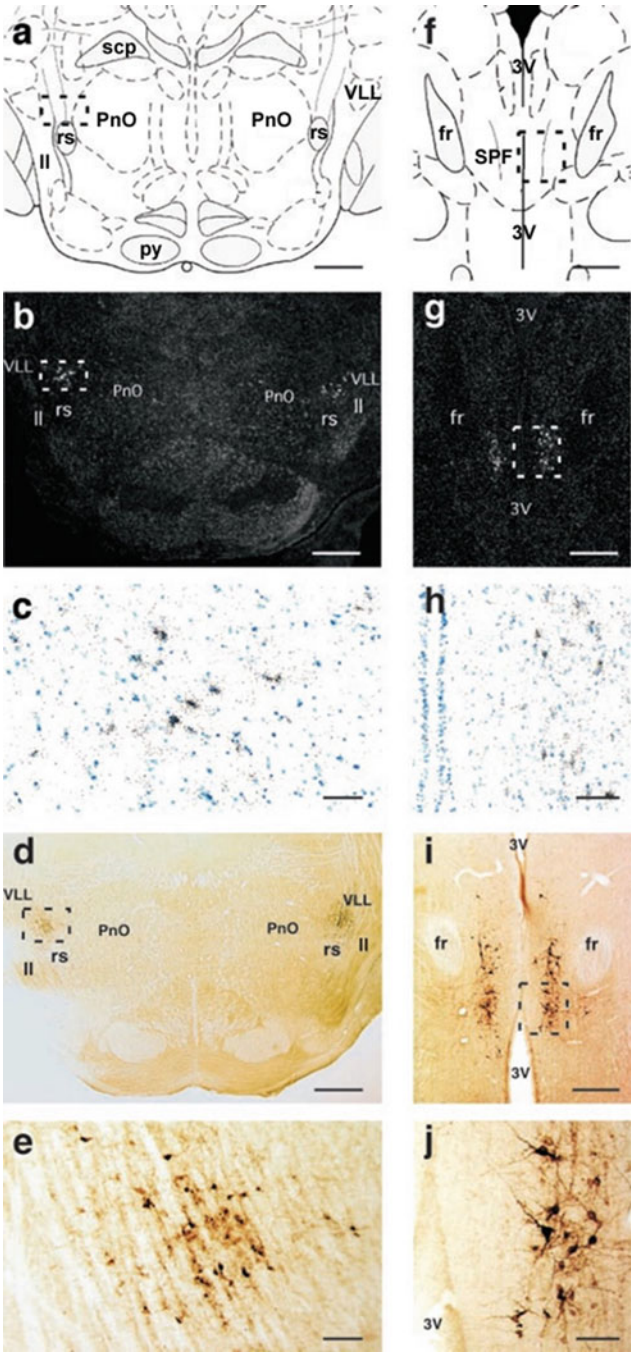

Fig. 14.2 Brain localization of TIP39-containing cells. (**a–e**) medial paralemniscal nucleus at 8.7 mm from the bregma level. (**f–j**) periventricular gray of the thalamus at 4.4 mm from the bregma. Drawings indicate the location of the micrographs (**a**, **f**). The localization of TIP39 mRNA detected by in situ hybridization histochemistry is shown at low magnification in dark-field

Cells of the MPL are distinguished from those in adjacent areas by their organization into dorsolaterally oriented cell columns separated by 20–50-μm wide cell-free zones, probably occupied by fibers of the lateral lemniscus that pass through the region (Varga et al. 2008). Thus, the cone-shaped structure of the MPL can be cytoarchitectonically distinguished from adjacent brain regions in the lateral pontomesencephalic tegmentum. The ventral border of the MPL is the rubrospinal tract. Lateral to the rubrospinal tract, the MPL extends somewhat ventrally, which gives the nucleus a triangular shape with ventral, dorsal, and medial angles. Medially, the MPL borders on the oral part of the pontine reticular formation and the pedunculopontine tegmental nucleus. The MPL narrows dorsally between the caudal part of the pedunculopontine tegmental nucleus and the dorsal nucleus of the lateral lemniscus, giving the nucleus a cone shape. The lateral border of the MPL is the intermediate nucleus of the lateral lemniscus. The caudal borders of the MPL are the region of the A7 noradrenaline cell group medially and the Kölliker-Fuse nucleus laterally. Apart from the cytoarchitectonic differences, a distinct MPL is also supported by its distinct afferent connections. The term "medial paralemniscal nucleus" was introduced by studies on TIP39 in the area (Dobolyi et al. 2003b), and has been adopted by the widely used Paxinos rat brain atlas (Paxinos and Watson 2005).

14.2.2 Identification of a Third Group of TIP39 Neurons, the Posterior Intralaminar Complex of the Thalamus (PIL)

TIP39 expression was investigated during ontogeny, which allowed the identification of a third group of cells in the posterior intralaminar complex of the thalamus (Dobolyi et al. 2006b; Brenner et al. 2008). TIP39 neurons are abundant in this brain region by embryonic day 16.5, and disappear by postnatal day 5 (Fig. 14.3a). This is in sharp contrast to the development of TIP39 neurons in the other thalamic brain region, the periventricular gray of the thalamus, where TIP39 immunoreactivity appears only in the early postnatal period (Fig. 14.3b), even though TIP39 levels also decrease in the PVG and MPL during the period of pubertal development (Dobolyi et al. 2006b). TIP39 neurons in the PIL are located in the posterior intralaminar thalamic nucleus and some adjacent brain areas including the parvicellular subparafascicular nucleus, and the lateral territory of the caudal zona incerta (Cservenak et al. 2010), which together was called as the posterior intralaminar complex of the thalamus (PIL). Since there was another neuropeptide, calcitonin gene-related neuropeptide (CGRP), with similar localization of cell bodies

Fig. 14.2 (continued) micrographs (**b**, **g**) and at greater magnification of the framed areas in bright field (**c**, **h**). The localization of TIP39 protein is demonstrated by peroxidase immunocytochemistry in colchicine-treated animals, shown at low magnification (**d**, **i**) and at greater magnification of the framed areas (**e**, **j**). Scale bars = 1 mm for **a**, **b** and **d**, 100 μm for **c**, **e**, **h**, **j**, and 500 μm for **g**, **h**, and **i**. The figure is taken from our previous publication (Dobolyi et al. 2002)

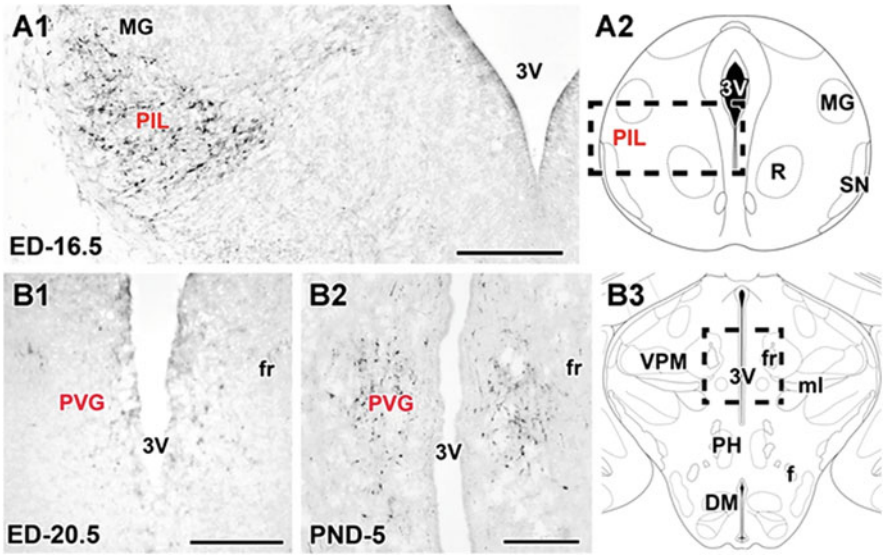

Fig. 14.3 TIP39 neurons in the posterior intralaminar complex of the thalamus at embryonic day (ED)-16.5. A1: TIP39-ir neurons are abundant by ED-16.5 in the posterior intralaminar complex of the thalamus. A2: A drawing of a coronal brain section at ED-16.5 (Paxinos et al. 1991). The framed area corresponds to panels A. B1: Only a few TIP39-ir neurons are faintly labeled in the periventricular gray of thalamus (PVG) between the third ventricle (3 V) and the fasciculus retroflexus (fr) at ED-20.5. B2: TIP39-ir neurons are distinctly labeled in the PVG at PND-5. B3: A drawing of a coronal brain section (Paxinos et al. 1991) indicates the position of the PVG at PND-1. The framed area corresponds to panels B1 and B2. Scale bars = 200 μm for A1, 250 μm for B1, and 300 μm for B2. The panels are selected from different figures of our previous publication (Brenner et al. 2008)

as TIP39, double labeling of the two peptides was performed, which showed no double-labeled cells in the PIL (Dobolyi et al. 2005). Rather, CGRP cells are located immediately lateral to the TIP39 cell group (Brenner et al. 2008).

In seeking to improve demarcation of the posterior intralaminar complex, the distribution of calcium-binding proteins was investigated in and around the PIL area in mother rats (Cservenak et al. 2017b). Parvalbumin-immunoreactive (PV-ir) neurons had low density throughout the PIL, peripeduncular area, and the triangular subdivision of the posterior thalamic nucleus. The distribution of calbindin (CB) immunoreactivity contrasted sharply with that of PV-ir. While the density of CB-ir cell bodies was low in most brain regions adjacent to the PIL, it was high in both the PIL and in the PIL's immediate dorsal neighbor, the triangular subdivision of the posterior thalamic nucleus. TIP- and CB-ir cell bodies were evenly distributed within the PIL. While almost all TIP39-ir neurons contained CB immunoreactivity, the PIL also contained neurons negative for TIP39 that were positive for CB.

Although it is an exciting question, in all three brain areas expressing TIP39, whether the TIP39 neurons contain additional major neurotransmitters, this was experimentally addressed only in the PIL. A combination of TIP39 immunolabeling

with in situ hybridization histochemistry for vesicular glutamate transporter 2 and glutamic acid decarboxylase 67 suggested that the TIP39 neurons contain glutamate but not GABA as their major neurotransmitter (Cservenak et al. 2017a). The excitatory nature of PIL TIP39 neurons was also confirmed by electron microscopy as PIL TIP39 neurons formed asymmetric synapses with their targets and contained glutamate in their terminals (Cservenak et al. 2017b).

14.3 Distribution of TIP39- and PTH2 Receptor-Containing Neuronal Fibers

TIP39 fibers are abundant in a variety of limbic, endocrine, nociceptive, and auditory brain regions including the medial prefrontal cortex, the nucleus accumbens, the lateral septum, the paraventricular thalamic nucleus, the fundus striati, a variety of hypothalamic and amygdaloid areas, the periaqueductal gray, the superior and inferior colliculi, the lateral parabrachial nucleus, the locus coeruleus and subcoeruleus areas, the paraolivary nuclei, and the nucleus of the solitary tract (Dobolyi et al. 2003b). The distribution pattern of TIP39 fibers was found to be very similar to that of the PTH2 receptor (Fig. 14.4).

The distribution of TIP39-ir and PTH2 receptor-ir fibers also shows remarkable similarities within particular brain regions (Dobolyi et al. 2006a; Faber et al. 2007) In the preoptic region, for example, a high density of TIP39- and PTH2 receptor-ir fibers is present in the medial preoptic nucleus whereas a low density of TIP39- and PTH2 receptor-ir fibers is seen in other parts of the medial preoptic area and the lateral preoptic area (Fig. 14.4). In the anterior hypothalamus, a high density of TIP39- and PTH2 receptor-ir fibers is present in the parvocellular subdivisions of the paraventricular nucleus and in the periparaventricular zone. The latter is particularly conspicuous on the side of the magnocellular subdivisions of the paraventricular nucleus, which lack TIP39- and PTH2 receptor-ir fibers. A moderate density of TIP39- and PTH2 receptor-ir fibers is present in the periventricular nucleus and the anterior hypothalamic nucleus whereas only a few immunoreactive fibers can be seen in the lateral hypothalamic area and the supraoptic nucleus, and no immunoreactive fibers are present in the suprachiasmatic nucleus. In the middle part of the hypothalamus, a high density of TIP39- and PTH2 receptor-ir fibers was found in the ventrolateral subdivision of the ventromedial nucleus, and in the dorsomedial and arcuate nuclei (Dobolyi et al. 2003b, 2006a; Faber et al. 2007).

Since PTH2 receptor-containing cell bodies are not, or only faintly, immunolabeled, X-gal histochemistry in mice expressing the beta-galactosidase enzyme driven by the PTH2 receptor promoter was used to describe the distribution of PTH2 receptor-expressing neurons (Faber et al. 2007). This distribution was essentially identical to the distribution of PTH2 receptor mRNA-expressing neurons detected by in situ hybridization histochemistry described in rodents (Dobolyi et al. 2006a; Faber et al. 2007) and also in macaque (Bagó et al. 2009). Most brain regions that contain PTH2 receptor-ir fibers also contained PTH2 receptor-expressing neurons with a very similar distribution (Dobolyi et al. 2006a; Faber et al. 2007).

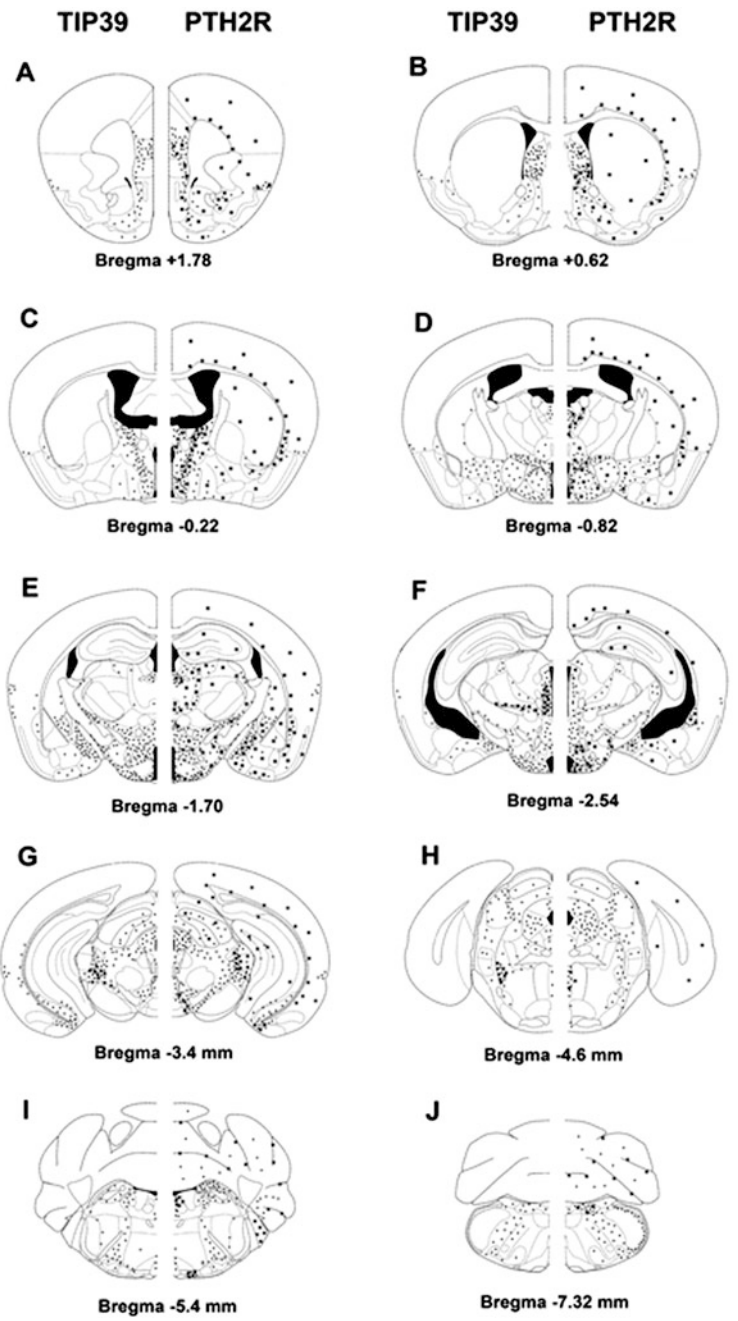

Fig. 14.4 Schematic diagrams demonstrate the distribution of TIP39 (left side) and the PTH2R (right side) in the brain of mice. The diagrams are modifications from a mouse stereotaxic atlas (Franklin and Paxinos 1997). Dots represent fibers and fiber terminals, while squares represent cell bodies. The figure is taken from our previous publication (Dobolyi et al. 2010)

For example, PTH2 receptor-expressing neurons are abundant in many regions of the hypothalamus. In the preoptic region, a high density of PTH2 receptor-expressing neurons is present in the medial preoptic nucleus whereas a low density of PTH2 receptor-expressing neurons is seen in other parts of the medial preoptic area. In the anterior hypothalamic region, a moderate density of PTH2 receptor-expressing neurons is present in the paraventricular and periventricular nuclei whereas the anterior hypothalamic nucleus contains a low density of PTH2 receptor-expressing neurons. In the middle portion of the hypothalamus, a high density of PTH2 receptor-expressing neurons is present in the arcuate nucleus whereas a moderate density was observed in the dorsomedial and perifornical hypothalamic nuclei, and some parts of the lateral hypothalamic area including the so-called far-lateral hypothalamus (Forel's field) immediately next to the internal capsule. In the posterior hypothalamus, a high density of PTH2 receptor-expressing neurons was present in the medial subdivision of the superior mammillary nucleus while its lateral subdivision, and the ventral premammillary, and the tuberomammillary nuclei contained a moderate density of PTH2 receptor-expressing neurons. In contrast, the medial and lateral nuclei of the mammillary body did not contain PTH2 receptor-expressing neurons (Dobolyi et al. 2006a; Faber et al. 2007).

14.4 Comparison of the Distribution of TIP39 to that of the PTH2 Receptor Provides Anatomical Evidence for a TIP39-PTH2 Receptor Neuromodulator System

The localization of cell bodies that express TIP39 and those that express the PTH2 receptor are profoundly different. TIP39 expression is confined to PVG, PIL, and MPL, while considerable PTH2 receptor expression is present in the infralimbic cortex, the innermost layer of other cerebral cortical areas, the basal ganglia, the lateral septum, the posteromedial part of the medial subdivision of the bed nucleus of the stria terminalis, the posterodorsal subdivision of the medial amygdaloid nuclei, the midline thalamic nuclei, the medial geniculate body, the medial preoptic, para- and periventricular, arcuate, dorsomedial, ventral premammillary, tuberomammillary, and supramammillary nuclei of the hypothalamus, and some regions of the lateral hypothalamic area, the lateral subdivisions of the interpeduncular nucleus, the sphenoid nucleus, the nucleus of the trapezoid body, and the nucleus of the solitary tract. In contrast to the profoundly different localization of TIP39- and PTH2 receptor-expressing cell bodies, the distributions of TIP39-ir and PTH2 receptor-ir fibers and cell bodies are markedly similar (Dobolyi et al. 2010). For example, the localization of TIP39 fibers is essentially the same as that of PTH2 receptor immunoreactivity at the light microscopy level in the hypothalamus (Faber et al. 2007). That means the same hypothalamic nuclei and areas contain both TIP39 and PTH2 receptor. Furthermore, their topographical distribution within the nuclei also resembles each other. Such similarities characterize most brain regions that contain TIP39 and PTH2 receptor immunoreactivity, providing anatomical evidence that TIP39 may be the endogenous ligand of the PTH2 receptor as it is

available to activate the receptor upon release from the terminals. This finding supports previous pharmacological data demonstrating that TIP39 can activate the PTH2 receptor. Still, it is worth mentioning that some brain areas such as the caudate nucleus and the cerebral and cerebellar cortices contained some PTH2 receptor immunoreactivity detectable TIP39-ir. Proposed explanations for such a mismatch, which is characteristic of several peptide-receptor systems, include long distance diffusion of the peptide, the existence of another ligand for the receptor, or the lack of sufficient sensitivity of the immunolabeling for the ligand (Herkenham 1987).

14.5 Projections of the Different TIP39 Cell Groups

Bilateral lesions of TIP39 cell groups resulted in the disappearance of TIP39 fibers from their target areas (Dobolyi et al. 2003a). Unilateral lesions also caused a reduction in the density of TIP39 fibers ipsilateral to the lesion. No obvious reduction was found contralateral to the lesion in any brain region as compared to intact animals, suggesting predominantly ipsilateral projections. The residual density was typically somewhat higher for unilateral than bilateral lesions suggesting some contribution of contralateral projection to TIP39 fibers in some brain regions (Dobolyi et al. 2003a; Cservenak et al. 2010). Still, the results of unilateral lesions are shown for demonstration because of the apparently striking difference between the two sides of the brain in the same section.

Lesion studies demonstrated that the forebrain receives most of its TIP39 fibers from the periventricular gray (PVG) and the posterior intralaminar complex of the thalamus (PIL). Lesioning of the third TIP39 cell group, the medial paralemniscal nucleus (MPL), resulted in no visible reduction of the density of TIP39 fibers in the forebrain while lesioning of the PVG and PIL were both effective albeit in different extent in different parts of the forebrain. The accumbens nucleus and the bed nucleus of the stria terminalis may receive more TIP39 input from the PVG, the medial prefrontal cortex and the lateral septum seems to receive similar input from both brain regions (Fig. 14.5). Accordingly, small but visible reductions in the density of TIP39 fibers were observed ipsilateral to the PIL lesion in the infralimbic cortex, the nucleus accumbens, the ventral subdivision of the lateral septum, the bed nucleus of the stria terminalis, and the amygdala (Dobolyi et al. 2003a). In turn, the hypothalamus is generally more abundantly innervated from the PIL than from the PVG as following lesions of the PIL, TIP39 fibers almost completely disappeared from the ipsilateral amygdala and most parts of the ipsilateral hypothalamus (Dobolyi et al. 2003a). Since TIP39 fibers could be followed from the PIL towards the supraoptic decussations (Palkovits et al. 2010) to project in a ventromedial direction, the effect of transaction of this pathway was studied in mother rats. Transections reaching of this tract resulted in the accumulation of TIP39 immunoreactivity immediately caudal to the transection within the fibers of the supraoptic decussations. In the midbrain, only the periaqueductal gray showed a moderate decrease in its density of TIP39 fibers following lesion of the PVG. Other structures of the midbrain, pons,

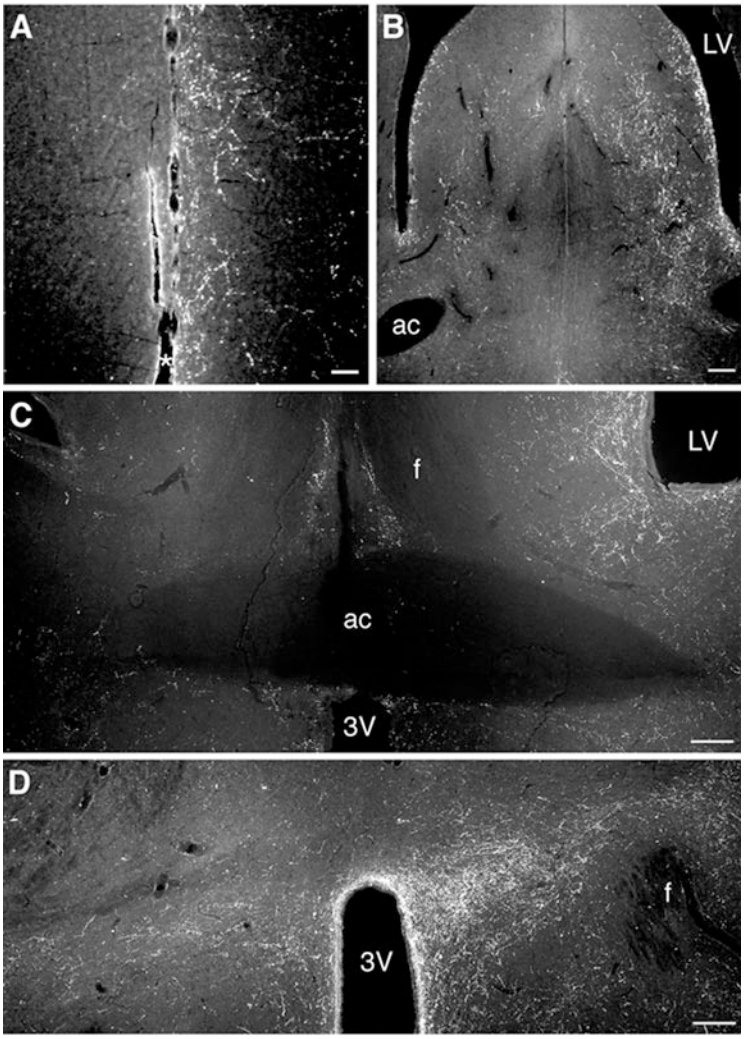

Fig. 14.5 The effect of unilateral (left side) lesions of the PVG on TIP39 fibers. Disappearance of TIP39-immunoreactive fibers ipsilateral to the lesion in the dorsal peduncular and infralimbic cortices (**a**), lateral septum (**b**), bed nucleus of the stria terminalis (**c**), and strong ipsilateral reduction in the hypothalamic paraventricular nucleus (**d**). Scale bars = 200 μm. The figure is from our previous publication (Dobolyi et al. 2003a)

and medulla did not demonstrate visible decrease in their TIP39 content following PVG or PIL lesions.

In contrast, lesions of the medial paralemniscal nucleus (MPL) were effective in reducing the density of TIP39 fibers in the lower brainstem regions. This finding suggests that the nuclei containing TIP39 in this part of the brain, such as relay nuclei of auditory, somato- and viscerosensory information, such as the external cortex of

the inferior colliculus, the spinal trigeminal nucleus, and the nucleus of the solitary tract all receive their TIP39 fibers from the MPL. It also has to be noted that the lesioning technique can detect only robust innervation. Therefore, small contribution to TIP39 fiber density from a non-detected source cannot be fully excluded based on these data.

An alternative method to detect where TIP39 fibers in a given brain regions originate from is the injection of retrograde tracer to their target area coupled with analysis of the location of retrogradely labeled cell bodies in the TIP39 cell groups. Such retrograde studies using cholera toxin beta subunit (CTB) as the retrograde tracer have been performed for the medial preoptic and arcuate nuclei (Cservenak et al. 2013). The position of the injection site was verified by double labeling with TIP39 to demonstrate that TIP39 fibers are indeed dense in the injection sites (Fig. 14.6). Following these injections, TIP39 neurons were retrogradely labeled only in the PIL but not in the PVG and MPL confirming that the hypothalamic regions receive most of their inputs from the PIL. The finding that retrogradely labeled cells were not present around the PIL regions suggests that this projection represents a specific pathway originating only from the PIL and not from other thalamic brain areas. Other retrograde studies also demonstrate the existence of projections from the PIL to the medial preoptic area (Simerly and Swanson 1986), the paraventricular hypothalamic nucleus (Campeau and Watson 2000), the arcuate nucleus (Li et al. 1999a; Szabo et al. 2010), and the amygdaloid nuclei (LeDoux et al. 1990). In these studies, double labeling with TIP39 was not performed, therefore, only the projections of neurons from the PIL can be deduced. However, it is likely that TIP39 neurons were among those (if not exclusively), which projected to the forebrain target areas.

14.6 Afferent Neuronal Connections of TIP39 Neurons

The retrograde tracer CTB was injected into the PIL to identify neurons that project there (Cservenak et al. 2017a). Most projections to the PIL were from the ipsilateral side, with the exception of the gracile and cuneate nuclei, the spinal trigeminal nucleus, and the spinal cord, where there was contralateral dominance. In the spinal cord, CTB-labeled neurons were predominantly located in Rexed laminae IV-VII. Most of the labeled thoracic cells were located in laminae IV-V and the labeled lumbar cells in laminae VI-VII. There was rarely more than one labeled cell in a coronal section. On average, every fourth 50 μm coronal section contained a labeled cell, usually characterized by oval perikarya with multiple dendrites. In the medulla oblongata, the highest density of CTB-labeled cells was in the gracile nucleus, the cuneate nucleus, and the spinal trigeminal nucleus (particularly in the deep layers of its ventral portion). Only a few upper brainstem regions contained CTB-positive neurons. The greatest number was apparent in the external cortex of the inferior colliculus. CTB-labeled neurons were far less numerous in the lateral parabrachial nucleus, periaqueductal gray, and deep layers of the superior colliculus. The infralimbic cortex contains the highest density of labeled cells within the cerebral

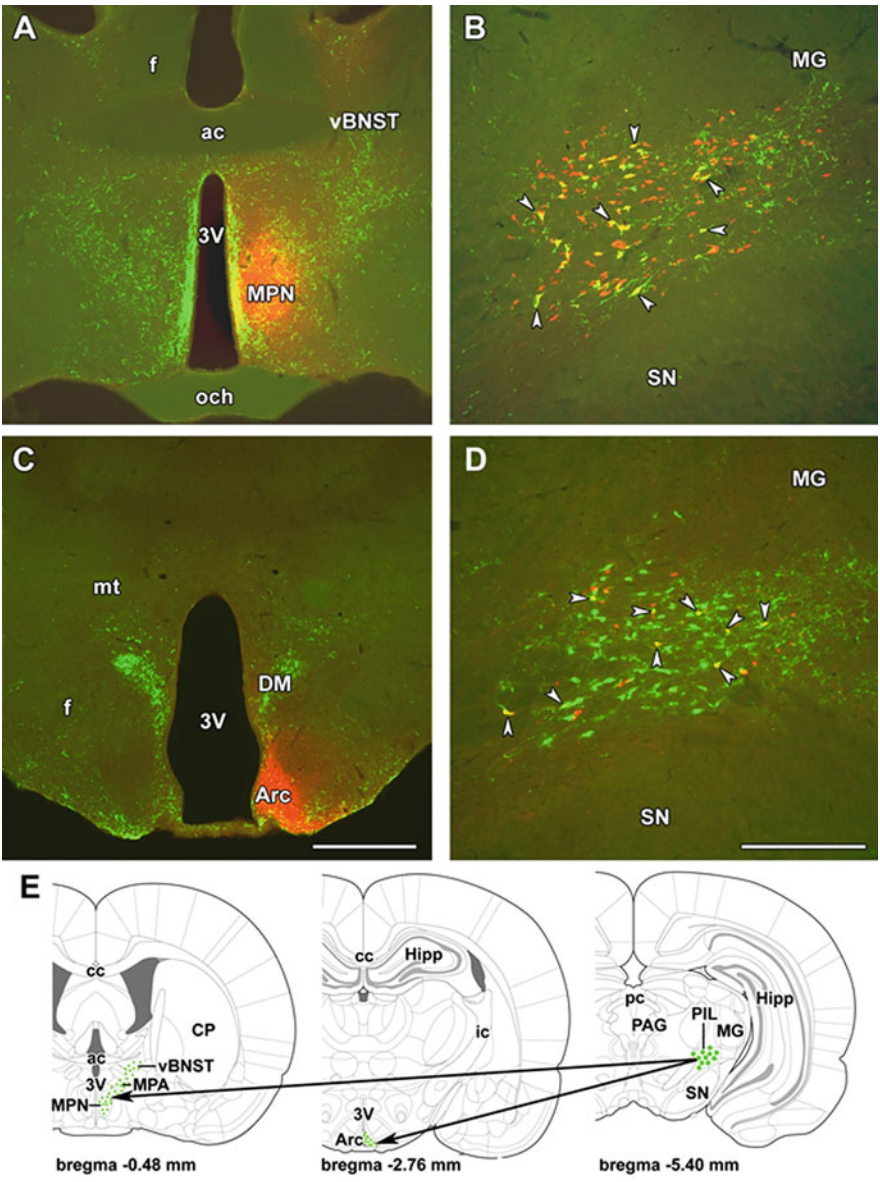

Fig. 14.6 Projections of the PIL into the medial preoptic and arcuate nuclei, demonstrated using the retrograde tracer cholera toxin beta subunit (CTB). CTB is shown in red and TIP39 in green. (**a**): A site of CTB injection into the medial preoptic nucleus (MPN) is shown in relation to TIP39 fibers. (**b**): In the PIL, the majority of TIP39 neurons are labeled with CTB following medial preoptic CTB injection (yellow; white arrowheads). In addition, a number of TIP-negative CTB-labeled neurons are also present. (**c**): A site of CTB injection into the arcuate nucleus (Arc) is shown. (**d**): A portion of the PIL TIP39 neurons are labeled with CTB (shown by white arrowheads) following its injection into the arcuate nucleus. (**e**): A drawing prepared by modifications of panels from a rat brain atlas (Paxinos and Watson 2005) shows the schematics of the PIL-hypothalamic projections. Large green dots in the PIL represent TIP39 cell bodies while small green dots in the preoptic area and the

cortex. There were also a considerable number of retrogradely labeled neurons in auditory areas. In contrast, there were few CTB-positive neurons found in the insular and medial prefrontal cortex. CTB signal was altogether absent from other cortical areas. Retrograde labeling was also largely absent from most other forebrain structures. There was a significant number of labeled neurons only in the central amygdaloid nucleus, the substantia innominata and the anterior portion of the lateral septal nucleus. Within the diencephalon, the largest number of labeled cells was in the ventromedial hypothalamic nucleus, particularly in its ventrolateral subdivision. There were also a considerable number of CTB-containing neurons in the lateral preoptic area and zona incerta.

Another study investigated the neuronal inputs of the MPL using CTB injections into the nucleus (Varga et al. 2008). The injection sites were verified using double labeling with TIP39 to demonstrate that the tracer was injected among TIP39 neurons in the MPL. As a prerequisite in precise tract tracing studies, injections into adjacent brain regions were also performed, which resulted in distinct labeling pattern in this case, too, suggesting that the retrogradely labeled brain areas project specifically into the MPL. Interestingly, a cortical brain region, the secondary auditory cortex and specifically the cortical temporal area 3 (Te3), as defined previously (Roger and Arnault 1989), projected to the MPL. However, adjacent primary (T1) and secondary auditory cortices (T2) also projected to the MPL. These projections were further verified by injecting an anterograde tracer into the primary auditory cortex, which demonstrated that TIP39 neurons in the MPL are indeed closely approached by auditory corticofugal fibers (Varga et al. 2008). In addition to the auditory cortex, another forebrain area, the ventromedial hypothalamic nucleus also projected to the MPL. Interestingly, this is a hypothalamic nucleus which received much less TIP39 innervation than the surrounding hypothalamic areas. In the cerebral cortex, CTB-containing cells were restricted to particular regions (Varga et al. 2008). In addition to these 2 sites, a thalamic auditory region, the medial subdivision of the medial geniculate body also sends descending projections to the MPL. In the midbrain, the external cortex of the inferior colliculus contains the highest density of retrogradely labeled cell bodies. Interestingly, this brain region also contains TIP39 fibers of MPL origin suggesting bidirectional connections between the 2 brain regions. While these inputs were all predominantly ipsilateral to the injection site, the MPL also receives input from the contralateral MPL. Interestingly, the contralaterally projecting neurons are typically negative for TIP39. Finally, the MPL also possesses some lower density inputs, e.g. from the lateral preoptic area, the lateral hypothalamic area, and the zona incerta, as well as periolivary regions of the medulla.

Fig. 14.6 (continued) arcuate nucleus represent TIP39 fiber terminals. The arrows show the projections from the PIL to the medial preoptic area and the arcuate nucleus, respectively. Scale bar = 1 mm for C, and 500 μm for D

14.7 Activation of TIP39 Neurons in Mothers

14.7.1 Assessment of c-Fos Activation in TIP39 Neurons of Lactating Dams

The appearance of c-Fos in response to pup exposure represents the activation of those neurons as c-Fos is the protein product of c-fos, a well-known immediate early gene that appears in activated neurons (Herdegen and Leah 1998). When pups are returned to their mothers after a 20 h separation, the dams begin care for them immediately, and suckling starts within 5 min. Following pup return, c-Fos-positive neurons appeared in a number of regions in the dams' brains including the PIL, MPL, lateral septal nucleus, anteroventral periventricular nucleus, medial preoptic nucleus, medial preoptic area, the ventral subdivision of the bed nucleus of the stria terminalis, and some parts of the periaqueductal gray, but not the periventricular gray of the thalamus. Thus, c-Fos also appears in the nuclei of TIP39 neurons of the PIL and MPL in response to pup exposure, indicating an elevated activity of TIP39 neurons in lactating rat dams in these brain areas. These findings confirmed previously reported expression of c-Fos in the PIL area of lactating rats (Lin et al. 1998) and the area corresponding to the MPL (Li et al. 1999b).

TIP39 neurons represent about half of the neurons demonstrating c-Fos activation in the PIL, as a number of TIP39-negative neurons were also activated in response to suckling (Cservenak et al. 2013). In contrast, within the MPL, c-Fos was located almost exclusively in TIP39 neurons, which is the major neuronal cell group of this nucleus (Varga et al. 2008). Based on the very low number of c-Fos-positive but TIP39-immunonegative neurons, it is likely that other cell types within the MPL are generally not activated in mother rats.

Pup exposure represents a complex stimulus for the mothers. Apart from the suckling reflex, visual, auditory, or olfactory exteroceptive stimuli or hormonal changes associated with the presence of pups could induce prolactin release and maternal behaviors (Terkel et al. 1979; Hashimoto et al. 2001). Theoretically, all these inputs derived from the pups could contribute to the activation of TIP39 neurons in the PIL and MPL by increasing their neuronal activity via specific circuitries. However, the finding that c-Fos appears in TIP39 neurons of the PIL only when physical contact is allowed suggests that TIP39 is induced in the PIL of rat dams by the suckling stimulus and not by other sensory input. These experiments have not been performed in the MPL yet, thus an adequate stimulation other than suckling is conceivable for TIP39 neurons in the MPL. In fact, auditory input could play a major role in the activation of TIP39 neurons in the MPL, because they receive massive input from the auditory cortex, the inferior colliculus, and the periolivary area (Varga et al. 2008). In addition, we have shown that highly intense noise stimulus activates paralemniscal TIP39 neurons (Palkovits et al. 2009). Furthermore, an indirect activation of paralemniscal TIP39 neurons via maternal hormones cannot be excluded either.

It is particularly striking that TIP39 neurons are activated only in the PIL and MPL while TIP39 neurons in the PVG are not activated in lactating dams, which is

consistent with the lack of TIP39 induction in that area as discussed later. Although TIP39 disappears from the PIL earlier than from the PVG and MPL during ontogeny (Brenner et al. 2008), the adult levels are markedly reduced in all three brain regions. Furthermore, brain areas that receive TIP39 axons predominantly from the PVG, including the lateral septal nucleus and the medial prefrontal cortex (Dobolyi et al. 2003b; Wang et al. 2006), also continue to possess a high PTH2 receptor level (Dobolyi et al. 2006b) suggesting that this TIP39 cell group may also be activated in response to some so far unidentified physiological stimuli.

14.7.2 Induction of TIP39 in Mother Rats

Induction of TIP39 mRNA in the PIL and the MPL of lactating mother rats was suggested on the basis of in situ hybridization histochemistry and confirmed by the independent technique of RT-PCR. The temporal pattern of activation of posterior intralaminar and paralemniscal TIP39 neurons was similar (Cservenak et al. 2010). In contrast, TIP39 expression was not changed in the third group of TIP39 neurons, the PVG in mother rats (Cservenak et al. 2010). In the PIL and the MPL, the levels of TIP39 mRNA were elevated specifically in the presence of pups while TIP39 mRNA levels were at their low, basal, non-maternal level in the absence of pups. In a more detailed study, TIP39 expression was found to be markedly upregulated on the first, ninth, and 23rd postpartum days but not on the last day of pregnancy or after weaning, which further supports the idea that elevated activity of these neurons is specific for the period of lactation (Cservenak et al. 2013). Thus, the increase in the level of TIP39 mRNA is a temporary phenomenon during lactation. The induction is likely to take place in all TIP39 neurons within the 2 cell groups as suggested by the increased autoradiography signal in the observed TIP39-expressing neurons following in situ hybridization histochemistry. In turn, the distribution of TIP39 neurons in the PIL and MPL of lactating mother rats was similar to that described previously in young rats (Dobolyi et al. 2003b, 2006b) suggesting that TIP39 reappears in the same neurons, which expressed it during earlier stages of ontogenic development and no additional, TIP39-negative cells are recruited in mothers. Furthermore, an increased TIP39 immunoreactivity was also detected in rat dams suggesting that the increase in TIP39 mRNA level translates into elevated peptide level, which in turn suggests a function of the induced TIP39 in mother rats. A function of the induced TIP39 is also conceivable because the expression level of the receptor of TIP39, parathyroid hormone 2 receptor, does not decrease during postnatal development as TIP39 does (Dobolyi et al. 2006b). Thus, parathyroid hormone 2 receptor is available for maternally induced TIP39 to exert its actions.

14.8 Neuroendocrine Functions of TIP39

14.8.1 The Effect of TIP39 on Prolactin Release

Suckling is known to elevate plasma prolactin levels within minutes of pups return to the mothers deprived of pups for 4 h, and plasma prolactin concentrations peak 30 min after the beginning of suckling (Bodnar et al. 2004; Dobolyi et al. 2020; Phillipps et al. 2020). Experiments to evaluate a potential role of TIP39 took advantage of HYWY-TIP39, a selective antagonist of the PTH2 receptor (Kuo and Usdin 2007). Injection of HYWY-TIP39 into the lateral ventricle dose-dependently blocked the elevation of plasma prolactin levels (Cservenak et al. 2010), suggesting that TIP39 acting on PTH2 receptors contributes to suckling-induced prolactin release.

To further evaluate a potential causal relationship between TIP39 signaling in the arcuate nucleus and prolactin level, cells in the mediobasal hypothalamus were infected near the arcuate nucleus with a virus encoding a secreted form of the PTH2-receptor antagonist peptide (HYWH-TIP39) and enhanced GFP (Fig. 14.7a). At least ten infected cells per injection site were seen in the most densely infected section of the animals, as illustrated in Fig. 14.7b. Mediobasal hypothalamic but not preoptic injection of the HYWH-TIP39 (Fig. 14.7c and d) markedly decreased basal serum prolactin levels and the suckling-induced prolactin release, suggesting that the mediobasal hypothalamus may be the site of action of HYWY-TIP39. PTH2 receptor-expressing neurons are abundant in the periventricular and arcuate nuclei of the hypothalamus (Wang et al. 2000; Faber et al. 2007). PTH2 receptors in these neurons are possible targets mediating the effect of HYWY-TIP39 on prolactin release. Dopaminergic neurons that control prolactin release from the pituitary are also located in the arcuate and periventricular hypothalamic nuclei. However, a direct effect of HYWY-TIP39 on dopaminergic neurons is not likely because the PTH2 receptor was not double-labeled for tyrosine hydroxylase (Usdin et al. 2003; Dobolyi et al. 2006a), and because close appositions between tyrosine hydroxylase neurons and fiber terminals projecting to the mediobasal hypothalamus from the PIL were not detected (Szabo et al. 2010). Therefore, HYWY-TIP39 might influence dopaminergic neurons in the mediobasal hypothalamus via interneurons expressing the PTH2 receptor (Cservenak et al. 2013). Dynorphin-containing neurons in the arcuate nucleus are one of the candidates because they are innervated by axon terminals derived from the PIL (Szabo et al. 2010), innervate tuberoinfundibular dopaminergic neurons (Fitzsimmons et al. 1992), and may be responsible for the effects of opioid peptides on suckling-induced prolactin release by inhibiting tuberoinfundibular dopaminergic neurons (Selmanoff and Gregerson 1986; Arbogast and Voogt 1998; Callahan et al. 2000). It is also a possibility that TIP39 evokes prolactin release by directly or indirectly stimulating prolactin-releasing substance-containing neurons (Freeman et al. 2000; Andrews 2005).

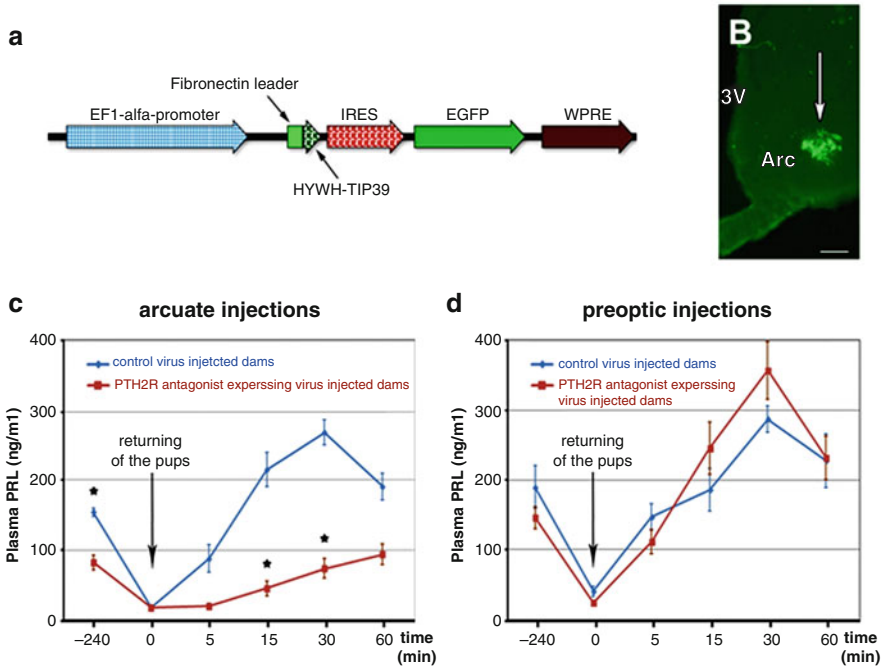

Fig. 14.7 Effect of virus encoding a peptide PTH2 receptor antagonist on prolactin release. (**a**): Structure of the viral construct expressing HYWH-TIP39, an antagonist of the PTH2 receptor. A strong mammalian promoter (EF-1-alfa) drives expression of a fusion protein between the fibronectin leader sequence with signal peptide cleavage site and the HYWH-TIP39 sequence. This is followed by an internal ribosome re-entry site (IRES) and then enhanced green fluorescent protein (EGFP) sequence and a woodchuck hepatitis post-transcriptional regulatory element (WPRE). (**b**): Hypothalamic virus injection site. The white arrow indicates cells infected by the injected virus visualized with EGFP. The injection site is located just lateral to the arcuate nucleus. (**c**): Basal plasma prolactin levels in mother rats injected with the PTH2 receptor antagonist expressing virus were significantly lower than in mothers injected with the control virus, with injections targeted to the arcuate nucleus. After returning their pups, the elevation of serum prolactin level was also blocked in the PTH2 receptor antagonist expressing virus injected mothers (*: $p < 0.01$). (**d**): Prolactin levels did not differ between PTH2 receptor antagonist expressing virus injected and control virus injected mothers with injections targeted to the medial preoptic area. Abbreviations: *Arc* arcuate nucleus, *3V* third ventricle. Scale bar = 100 μm for B (Cservenak et al. 2013)

14.8.2 The Effect of TIP39 on Oxytocin Release

Oxytocin is released in the pituitary and widespread brain areas from terminals of the magnocellular paraventricular and supraoptic neurons during parturition, in response to suckling in mothers and possibly also during adult social interactions. However, neuronal pathways that activate oxytocin neurons are not well established and TIP39-containing PIL neurons are candidates. It was shown, using double labeling in combination with electron microscopy and retrograde tracing, that oxytocin neurons are innervated by TIP39 terminals originating in the PIL (Cservenak et al.

2017a). The excitatory nature of TIP39 neurons was investigated by in situ hybridization histochemistry. Since TIP39 neurons are activated by pup exposure in mother rats as well as in adult females upon social encounter with a familiar conspecific, it was suggested that the PIL-paraventricular projection contributes to the established activation of oxytocin neurons in social contexts (Dobolyi et al. 2018; Tang et al. 2020).

14.8.3 The Effect of TIP39 on the Activity of the Corticotropin-Releasing Hormone (CRH) Neurons

Based on dense networks of PTH2 receptor- and TIP39-containing fibers in the hypothalamic paraventricular nucleus (PVN), Dimitrov and Usdin investigated a potential role of TIP39 in the control of CRH release (Dimitrov and Usdin 2010). There was a large amount of colocalization between the PTH2 receptor and the vesicular glutamate transporter VGlut2 on nerve fibers or terminals that surrounded PVN CRH neurons. TIP39-containing nerve terminals appeared to be very close to these PTH2R/VGlut2-containing processes. A hypothesis derived from these observations is that TIP39 modulates activation of CRH neurons via glutamatergic terminals in the PVN. Several observations are consistent with this idea. TIP39 infusion near the PVN causes an increase in the immediate early gene pCREB within the CRH neuron containing zone of the PVN and this stimulation of pCREB was blocked by a mixture of the glutamate receptor antagonists CNQX and AP-5. This increase in pCREB by TIP39 did not occur in PTH2 receptor knockout mice. Infusion of TIP39 near the PVN also led to an increase in plasma corticosterone that was prevented by co-infusion of glutamate receptor antagonists. Finally, consistent with a physiological role for TIP39 in control of CRH release, the normal diurnal variation in plasma corticosterone was diminished in PTH2 receptor knockout mice.

14.8.4 The Role of TIP39 in Thermoregulation

PTH receptors and TIP39 are present on or in neuronal fibers in several hypothalamic regions, including the preoptic area and one of its subregions, the median preoptic area (MnPO). The MnPO is of interest because it is an important thermoregulatory control region. A series of anatomical tract tracing experiments combined with antibody labeling and in situ hybridization histochemistry established that PTH2 receptors are present on glutamatergic terminals that are presynaptic to projection neurons in the MnPO. These projection neurons are part of thermoregulatory circuitry and provide a multisynaptic input to brown adipose tissue. Pharmacological experiments in which TIP39 was microinjected into the region of the MnPO or the lateral ventricle showed that TIP39 caused a PTH2 receptor dependent increase in body temperature that was mediated by sympathetic output. This effect was not observed when TIP39 was microinjected into the dorsomedial hypothalamic nucleus, a thermoregulatory region that mediates output from the MnPO, which

supports the suggestion that TIP39 effects on thermoregulation are specific to the MnPO. Evidence that TIP39 and the PTH2 receptor have a physiological role in control of body temperature was provided by experiments that evaluated the ability of wild-type or PTH2 receptor knockout mice to defend their body temperature in a cold environment. Wild-type mice placed in a 4° C environment for 1 hour had little change in body temperature. In contrast, the body temperature of PTH2 receptor knockout mice decreased by an average of 3.6° C during the 1-hour exposure to a 4° C environment. A qualitatively similar result was obtained by injecting a PTH2 receptor antagonist into the lateral ventricle (Dimitrov et al. 2011). Furthermore, the peripartum elevation of core body temperature was present but reduced in PTH2R KO mice, even though their locomotor activity increased as core body temperature was reduced (Gellen et al. 2017). Thus, evidence from anatomical, pharmacological, and physiological approaches supports the suggestion that TIP39 signaling plays a significant role in the homeostatic control of body temperature, likely through modulation of glutamatergic signaling in the hypothalamic MnPO. In addition, the PTH2R contributes to the maternally elevated core body temperature as well.

14.8.5 Additional Potential Neuroendocrine Roles of TIP39

There is some evidence available for a role of the TIP39-PTH2 receptor system in the regulation of arginine vasopressin (Sugimura et al. 2003) and growth hormone release (Usdin et al. 2003). Some of these actions could be part of maternal adaptations even though they are not yet proven in mothers (Dobolyi et al. 2012). Another profound neuroendocrine change in mothers is the inhibition of the gonadotropin-releasing hormone (GnRH) neurons, which leads to lactational anestrus. Although experimental data are not available yet, an action of TIP39 on GnRH neurons is plausible given the high density of TIP39 fibers in both the periventricular preoptic nucleus and the arcuate nucleus where kisspeptin neurons regulating GnRH neurons are located.

14.9 Non-neuroendocrine Functions of TIP39

The TIP39-PTH2 receptor system has been implicated in a variety of non-neuroendocrine actions. Acute injection of TIP39 into the lateral ventricle of male rats was observed to have an anxiolytic effect in an elevated plus maze test and an antidepressant-like effect in the forced swim test (LaBuda et al. 2004). Subsequently, anxiety-like behavior in TIP39 knockout (KO) animals was also demonstrated, but only if the animals were previously exposed to mild acute stress (Fegley et al. 2008) and also after fear conditioning (Coutellier and Usdin 2011). In addition, fear incubation, a time-dependent increase in fear responses to trauma-associated cues, is also affected by TIP39 (Tsuda et al. 2015).

PTH2 receptors are expressed in many CNS regions involved in the processing of nociceptive information. These include regions that are within ascending pathways

that convey nociceptive sensory information, as well as within descending pathways to regions involved in modulation of the sensitivity to peripheral stimuli or of responses to nociceptive input. The regions include the spinal cord dorsal horn, PAG, medial and intralaminar thalamic nuclei, several amygdaloid and hypothalamic nuclei, and somatosensory and anterior cingulate cortex (Dobolyi et al. 2002). The potential involvement of TIP39-PTH2 receptor signaling in pain processing was evaluated by comparing performance in several standard tests of acute nociceptive sensitivity between control or wild-type mice and mice in which PTH2 receptor signaling was inhibited either by acute administration of a PTH2 receptor antagonist, by null mutation of the PTH2 receptor or by deletion of TIP39 (Dimitrov et al. 2010). Intracerebroventricular (icv) administration of the PTH2 receptor antagonist HYWH-TIP39 increased latency in acute nociceptive withdrawal assays including the tail-flick and hotplate tests, and in both phases of the formalin test, while administration of TIP39 decreased latency in acute nociceptive sensitivity tests. Observations in the mice with constitutive genetic alterations in PTH2 receptor signaling were generally consistent with these observations. The idea that TIP39-PTH2 receptor signaling contributes to physiological modulation of nociceptive function was also evaluated in animals with more long-lasting perturbations (Dimitrov et al. 2011). Following peripheral nerve injury, both PTH2R- and TIP39-knockout mice developed less tactile and thermal hypersensitivity than controls and returned to baseline sensory thresholds faster. The effects of hind paw inflammatory injury were similarly decreased in knockout mice. Thus the TIP39-PTH2 receptor system appears to have a role in maintaining the normal sensitivity to nociceptive stimuli and modulating responses to injury.

14.10 TIP39 as a Maternal Neuropeptide

Behavioral, endocrine, and psychological changes in mothers represent one of the most profound physiological alterations in the adult central nervous system. Experimental models of maternal behaviors are well established in rodents as control females avoid or even hurt pups while mothers take care of them, retrieving them in the nest, nursing them and performing anogenital licking, etc. (Numan 2020). Additional emotional changes include maternal aggression towards intruders, decreased anxiety in general and reduced responsiveness of the hypothalamo-pituitary-adrenal axis in stress situations (Carter et al. 2001; Neumann 2003).

14.10.1 PIL TIP39 Neurons May Mediate Suckling-Induced Prolactin and Oxytocin Release

Prolactin and oxytocin are major hormones, which evoke many of the maternal adaptations in the brain in addition to their role in lactation. These hormones are released in response to suckling. Lesion and microstimulation studies suggested that the ascending reflex arch conveying sensory information from the nipples for

prolactin and oxytocin release travels through the lateral mesencephalic tegmentum and enters the zona incerta ventromedial to the medial geniculate body (Tindal and Knaggs 1977; Wakerley et al. 1978; Dubois-Dauphin et al. 1985), exactly the position where PIL TIP39 neurons reside (Dobolyi et al. 2018). Excitotoxic lesions of this area blocked the milk-ejection reflex (Hansen and Kohler 1984) and c-fos expression was detected here in lactating mothers (Lin et al. 1998) suggesting relay of the pathway in this position. PIL TIP39 neurons are candidates to be the relay neurons of these hormone-releasing reflex arcs because of a significant portion of c-Fos-positive TIP39 neurons (Cservenak et al. 2017a). Furthermore, injection of the retrograde tracer in a position of the TIP39 neurons in the PIL retrogradely labeled neurons in brainstem sensory relays nuclei as well as in the spinal cord (Cservenak et al. 2017a). In addition, PIL TIP39 neurons project to both the arcuate nucleus and the paraventricular hypothalamic nucleus, which suggests that TIP39 neurons in the PIL convey suckling information to these nuclei where dopaminergic neurons controlling prolactin release and oxytocin neurons reside, respectively. The presence of TIP39 in these neurons suggests the role of this neuropeptide in the regulation of maternal functions. While TIP39 terminals innervate oxytocin neurons, functional evidence is available for the regulation of prolactin release by TIP39. The finding that the body weight of pups is reduced in the absence of a functional TIP39 gene (Coutellier et al. 2011) and that the blockade of PTH2 receptors inhibits suckling-induced prolactin release (Cservenak et al. 2010) suggests that TIP39 plays a physiological role in the regulation of suckling-induced prolactin release. All of these data suggest that the suckling information evoking the release of both hormones in mothers is relayed by PIL TIP39 neurons (Fig. 14.8).

14.10.2 PIL TIP39 Neurons May Represent a Relay Station of Suckling-Induced Non-hormonal Brain Adaptations

Although the behavioral changes are initiated by steroid hormonal alterations in the last days of pregnancy, decreased levels of steroid hormones are detected during lactation leading to anestrous (Siegel 1986; Bridges 2020). Furthermore, maternal motivation remains high following blockade of prolactin and oxytocin actions (Lamming 1994). In addition, maternal behaviors can be induced by prolonged pup exposure even in virgin female rats. These maternally sensitized rats do not lactate and provide a model to separate metabolic regulations from regulations of maternal behaviors (Rosenblatt 1967). Somatosensory inputs derived from the pups play the most important role in maternal sensitization (Stern and Lonstein 2001). The same somatosensory inputs are thought to maintain maternal behaviors in dams after parturition (Febo et al. 2008). Since PIL TIP39 neurons project to the preoptic area, the major forebrain center controlling maternal behaviors, PIL TIP39 neurons may participate not only in the neuroendocrine responses of prolactin and oxytocin release but also in neuronal mechanisms of maternal brain adaptations (Fig. 14.8).

At the preoptic level, the anteroventral periventricular nucleus, the medial preoptic nucleus, the medial preoptic area, and the ventral subdivision of the bed

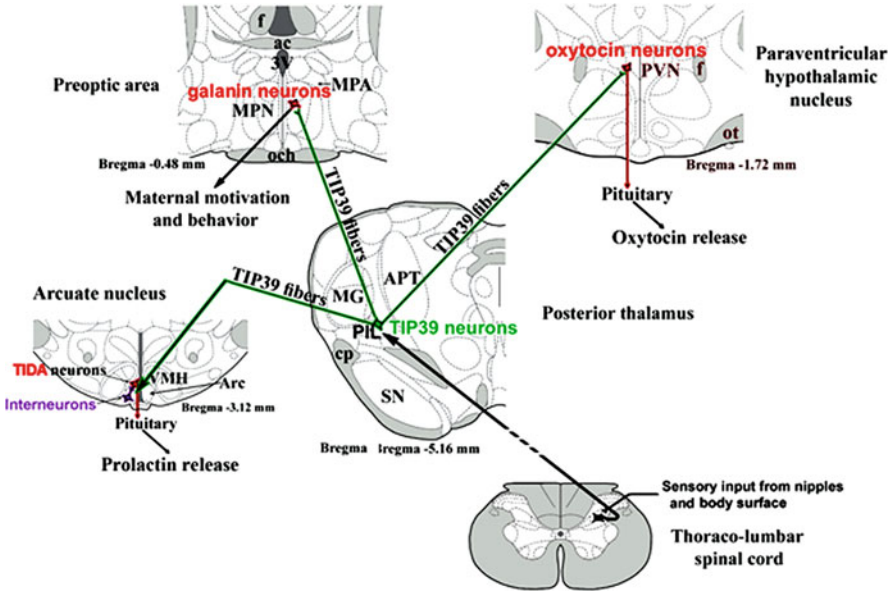

Fig. 14.8 TIP39 neurons in the PIL are proposed relay stations of maternal sensory information towards hypothalamic and limbic centers. During suckling, somatosensory information from the nipples reaches the posterior intralaminar complex of the thalamus (PIL) where TIP39 neurons reside. These neurons project to different hypothalamic sites and other limbic centers (e.g., the medial prefrontal cortex and the lateral septum, not shown in the figure). TIP39 terminals in the paraventricular hypothalamic nucleus lead to oxytocin release, e.g. for milk ejection, TIP39 terminals in the arcuate nucleus evoke prolactin release for the maintenance of lactation, while TIP39 terminals (terminating on galanin neurons) in the medial preoptic area contribute to maternal care

nucleus of the stria terminalis all contain a high density of c-Fos-expressing neurons following suckling. This is a characteristic pattern in the medial preoptic region, often referred to as the medial preoptic area in which c-Fos-expressing neurons have been implicated in pup attachment (Lonstein et al. 1998; Stack and Numan 2000). This c-Fos activation pattern differs from the distribution of prolactin-sensitive neurons in the area, as most of the neuronally activated cells are not prolactin sensitive (Olah et al. 2018). However, the distribution pattern of c-Fos-expressing neurons is very similar to the distribution patterns of TIP39 labeled fibers and terminals observed in the area. TIP39 containing fibers closely appose c-Fos-expressing neurons in all regions of the preoptic area that contain c-Fos-expressing neurons following suckling. Furthermore, galanin neurons in the preoptic area, which are known to be c-Fos activated by pup exposure as a major cell type of the preoptic neuronal network governing maternal behaviors (Wu et al. 2014), were shown to be innervated by TIP39 terminals (Cservenak et al. 2017b).

The position of the MPL immediately next to the nuclei of the lateral lemniscus and its bilateral anatomical connections with auditory brain regions (Dobolyi et al.

2003b; Varga et al. 2008) suggest some auditory functions of paralemniscal TIP39 neurons. Indeed, the paralemniscal TIP39 neurons were specifically activated by high-intensity noise (Palkovits et al. 2009). Rat pups, when isolated, are known to emit high-intensity vocalization in the ultrasonic range (Hofer 1996). Pup ultrasonic vocalizations have been reported to induce maternal behaviors in rats (Terkel et al. 1979; Hashimoto et al. 2001; Febo et al. 2008). Still, there are no data available at present on the anatomical pathway on how ultrasonic vocalization reaches limbic and hypothalamic centers responsible for maternal behavioral and neuroendocrine changes. However, results of application of retrograde tracers suggest that paralemniscal fibers may reach hypothalamic targets, such as the hypothalamic paraventricular nucleus (Palkovits et al. 2004). We hypothesize that paralemniscal TIP39 neurons could mediate pup ultrasonic vocalization towards higher brain centers of their mothers thereby contributing to central maternal adaptations.

14.10.3 Roles of TIP39 in Regulating Maternal Behavior

During the early postpartum period, pup suckling is more rewarding than cocaine (Ferris et al. 2005). A number of different approaches provide evidence that the preoptic area is critically important for maternal motivation (Numan 2020) through its projections to the nucleus accumbens and the ventral tegmental area (Numan et al. 2005). Large electrical and axon-sparing excitotoxic lesions of the MPOA eliminate all maternal behaviors without affecting other behaviors such as feeding and similar effects were found following temporal pharmacological inactivation of MPOA. In contrast to lesions, electrical stimulation of the MPOA increased maternal responsiveness (Morgan et al. 1997). In addition, brain activity is elevated in the MPOA in response to pup exposure based on c-fos (Li et al. 1999b) and fMRI techniques (Febo et al. 2005).

Virally driven constitutive release of HYWH-TIP39, an antagonist of the PTH2 receptor, in the preoptic area resulted from locally infected cells. The behavior of mother rats that received virus injections into the preoptic area was analyzed using a place preference test (Cservenak et al. 2013), which is a sensitive way to assess maternal motivation (Seip and Morrell 2009). The presence of the PTH2 receptor antagonist reduced the number of dams demonstrating preference for the pup-associated cage, and also the amount of time the dams spent in the pup-associated cage, but did not affect the time control females spent in the different cages of the test apparatus (Cservenak et al. 2013). These data provided evidence for the involvement of the TIP39-PTH2 receptor system in maternal motivation. It is also important to note that preoptic injection of the virus expressing the PTH2 receptor antagonist did not affect plasma prolactin levels. Therefore, an indirect mechanism of action on maternal motivation via prolactin can be excluded.

PTH2R KO mothers also showed anxiety-like and depression-like behaviors compared to wild-type mothers (Gellen et al. 2017). These latter data also suggest that the TIP39-PTH2R system could be involved in postpartum depression, the most

frequent psychiatric disorder after childbirth with a prevalence rate of 10% to 15% (Mallikarjun and Oyebode 2005).

14.11 TIP39 in Zebrafish Social Awareness

It was recently observed that there is a dramatic difference in the level of expression of the gene encoding TIP39 (pth2) between zebrafish maintained in social isolation and as a group (Anneser et al. 2020). While there were a number of genes with expression differences between isolated and grouped fish, a difference was only present at multiple developmental states for pth2 and several immediate early genes. The level of pth2 was directly related to the density of fish, and responded to changes in social density within 30 minutes. The signal that controlled pth2 was mediated by the lateral line, a specialized mechanosensory organ, based on the effect of its lesion. The control of pth2 expression was highly selective for agitation of water in the precise pattern created by zebrafish swimming. TIP39/pth2 is expressed in zebrafish by a small group of neurons in a lateral thalamic region and its receptor is widely expressed (estimated to be in 9% of neurons). This pattern is highly reminiscent of the pattern in mammals. It suggests that the observed response to social density may be related to the involvement of signaling by the TIP39/PTH2 receptor system in affective functions that is observed in mammals.

14.12 Perspectives

The TIP39-PTH2 receptor system is pharmacologically and histologically well characterized, which provides an excellent starting point for functional investigations. The functional studies are supported by excellent research tools available, such as new antibodies, transgenic mice lacking the peptide and its receptor, a selective and sensitive peptide antagonist and a lentivirus encoding the peptide antagonist. These research tools led to the implication of TIP39 in different hypothalamic functions, such as thermoregulation, stress response, maternal behavior, and prolactin secretion. In addition, nociceptive and auditory functions of the peptide have been reported. The recent involvement of TIP39 in the social behavior of zebra fish suggests similar functions in rodents. In fact, the established role of TIP39 in the mother–pup relationship represents a special form of social contact (Kinsley and Amory-Meyer 2011), and in addition it is possible that TIP39 may also be involved in adult social interactions in mammals. The effect of TIP39 on oxytocin (Cservenak et al. 2017a; Dobolyi et al. 2018), a well-known social neuropeptide (Neumann 2008) supports this suggestion, which is to be tested in future experiments.

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Functional Chemoanatomy of PACAP in Neuroendocrine and Neuronal Circuits

15

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Abstract

Pituitary adenylate cyclase-activated polypeptide was discovered as a peptide highly concentrated in the hypothalamus, via screening of hypothalamic extracts for their ability to affect cAMP-dependent hormone secretion from the anterior pituitary. However, PACAP is also expressed widely within specific subsets of neurons in brain and periphery in adult mammals, and before midgestation during development. Some important themes connect PACAP neuroanatomy to PACAP function: (1) PACAP is located within groups of neurons that mediate functions such as stress and threat responses, carried out through multiple circuits and even large neuronal networks, (2) PACAP may act at different receptors and via different modes of transmission depending upon location and stage of development, and (3) PACAP likely acts in concert with co-released co-transmitters both centrally and peripherally.

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Keywords

PACAP · PAC1 · GPCR · Stress · Feeding · Metabolism · Atherosclerosis · Threat responding

Abbreviations

ACA	anterior cingulate area
AHN	anterior hypothalamic nucleus
AM	anteromedial nucleus
AON	anterior olfactory nucleus
ARC	arcuate nucleus
avMLPA	anteroventral medial and lateral preoptic area
BAC	bed nucleus of anterior commissure
BNST	bed nucleus of stria terminalis
CBgcl	granular layer of cerebellum
CBpj	Purkinje layer of cerebellum
CNS	central nervous system
DB	diagonal band
DH	dorsal hippocampus proper
DISH	dual in situ hybridization histochemistry
DMH	dorsomedial nucleus of the hypothalamus
DP	dorsal peduncular area
DRG	dorsal root ganglia
FN	fastigial nucleus of the cerebellum
GCL	granule cell layer of hippocampus
GRN	gigantocellular reticular nucleus
HC	hippocampus
IC	inferior colliculus
ILA	infralimbic area
IO	inferior olivary nucleus
IPN	interpeduncular nucleus
KF	Kölliker-Fuse nucleus
LSc	lateral septum caudal
LSr	lateral septal nucleus rostral
MBO	mammillary body
MD	mediodorsal nucleus of thalamus
MEPO	median preoptic nucleus of hypothalamus
MHb	medial habenula
MOB	main olfactory bulb
MOs	secondary motor area
MLPA	mediolateral preoptic area
MPO	medial preoptic area
MS	medial septal nucleus

MV	medial vestibular nucleus
NAc	nucleus accumbens
NDB	diagonal band nucleus
NTS	nucleus tractus solitarius
ORB	orbital area
OT	olfactory tract
PACAP	pituitary adenylate cyclase-activating polypeptide
PACAP KO	PACAP knock-out mice
PAC1	PACAP receptor 1
PAG	periaqueductal gray
PBN	parabrachial nucleus
PCG	pontine central grey
PCL	Purkinje cell layer
PF	parafascicular nucleus
PH	posterior hypothalamic nucleus
PG	pontine grey
PL	prelimbic area
PMV	premammillary nucleus ventral part
PNS	peripheral nervous system
PRC	precommissural nucleus
PRNr	pontine reticular nucleus
PVN	paraventricular nucleus of hypothalamus
PVp	periventricular hypothalamic nucleus, posterior part
PVT	paraventricular nucleus of thalamus
RGC	retinal ganglion cell
RL	rostral linear nucleus raphe
RPa	raphe pallidus
RSP	retrosplenial area
SCm	superior colliculus, motor related
SCN	suprachiasmatic nucleus
SCs	superior colliculus, sensory related
SMA	somatomotor area
SO	supraoptic nucleus
SPF	subparafascicular nucleus
SN	substantia nigra
STN	subthalamic nucleus
SUM	supramammillary nucleus
TTv	taenia tecta ventral
VMN	ventromedial nucleus of hypothalamus
VTA	ventral tegmental area

15.1 The PACAP Origin Story: Actions at the Anterior Pituitary

Pituitary adenylate cyclase-activating polypeptide (PACAP) takes its name from the screening assay first used in the Arimura lab to identify, in extracts of the sheep hypothalamus, peptidic fractions that might contain additional hypophysiotropic hormones besides those already discovered: LH-RH, GH-RH, TRH, CRH, and somatostatin. With these, the regulation of secretion of the anterior pituitary hormones FSH, LH, ACTH, GH, and TSH could be largely accounted for. However, additional factors released from the hypothalamus that might accelerate prolactin production and secretion of prolactin, and modulate the secretion of other pituitary hormones, were still sought. Since the previously discovered hypophysiotropic hormones shared the property of causing cyclic AMP elevation upon engagement of their receptors on anterior pituitary cells, a generalized cAMP elevation assay was used to screen for additional hypophysiotropic hormones/factors. PACAP-38 and the smaller processed PACAP-27 were isolated from hypothalamic extracts in 1989 (Miyata et al. 1989; Arimura 1992). Synthetic PACAP was subsequently used to determine its effects on secretion and biosynthesis of the individual pituitary hormones. Rawlings and Hezarah summarized the results of a half-decade of study, showing modest and variable effects on LH, FSH, GH, prolactin, and ACTH, and no discernable effects on TSH secretion, in primary anterior pituitary cells (Rawlings and Hezareh 1996). PACAP is also expressed in the magnocellular compartment of the PVN, and can be measured in the neurohypophysis, however its role there remains unknown (Hannibal 2002). In parvocellular PVN, PACAP is virtually undetectable in parvocellular (CRH-positive) neurons, although it can be visualized after colchicine treatment in the rat (Hannibal et al. 1995). By 1998, a review of the now-burgeoning PACAP field by its discoverer described PACAP as a credible gonadotropin modulator based on both in vivo and cultured-cell studies; however, PACAP's expression throughout the nervous system was beginning to focus much more attention on its actions as a neurotransmitter (Arimura 1998).

PACAP began its neuroendocrine career, in the earliest vertebrates, as a regulatory peptide encoded on a single gene along with the structurally related GH-RH (Sherwood et al. 2000). PACAP fits the description of a classical hypophysiotropic hormone, releasing GH from the pituitary, in non-mammalian vertebrates (Lugo et al. 2008) though not in mammals (Montero et al. 2000). In the modern mammal, the PACAP gene "superfamily" consists of six genes, produced by gene duplications from the single ancestral one, which encode PACAP and PACAP-related peptide (PrP); VIP and PHM/PHI; glucagon, GLP-1 and GLP2; GH-RH; GIP; and secretin (see (Sherwood et al. 2000)). There has thus been ample opportunity within this gene family for gain and loss of function through evolutionary mutation, leading not only to specialization in signaling through the secretin family of G-protein coupled receptors (GPCRs), but also to specialization of expression within neuronal, endocrine, and neuroendocrine cell types. Evolutionary diversification of the PACAP superfamily mirrors the evolution of neuroendocrine regulatory and control mechanisms within vertebrates. This in turn matches our evolving understanding of how neuronal, endocrine, and neuroendocrine systems are constructed in

mammals, including primates. The "top-down" conceptualization of neuroendocrinology that drove early experimentation in the field distinguished between hypothalamic *neuroendocrine* cells of the SON (AVP, OT), PVN (AVP, OT, CRH, TRH), VMN (somatostatin), and medial POA (LH-RH) projecting to neurohemal sites of secretion in the median eminence and posterior pituitary and central and peripheral *neurons* projecting to synaptic or neuroeffector junction sites of secretion. These in turn are distinct from *endocrine* cells located in pituitary, pancreas, adrenal, thyroid, parathyroid, thymus, and gut epithelium, which are devoid of receptive processes with obvious morphological polarity altogether, and secrete regulatory peptides and other hormones directly into the general circulation. In addition to hypophysiotropic and neurohypophysial hormones, however, the hypothalamus contains cell groups, sometimes in the same nuclei, that project to the brain and thus are true neurons rather than neuroendocrine cells (e.g., see (Sternson 2013)). Some of these cells are directly responsive to hormones generated in gut and adipose tissue, including leptin and ghrelin, which provides "bottom-up" neuroendocrine (or endocrinoneural) regulation of appetite and feeding. PACAP represents a primordial regulatory peptide whose sparse expression in neuroendocrine cells of the hypothalamus reflects its evolutionary history of hypophysiotropic function in lower vertebrates, such as fish, while its intense expression throughout the neural axis signifies the acquisition of a new chemoanatomy and new functions, first in reptiles (Reglodi et al. 2001) and then in mammals. In this sense, a full accounting of the functional neuroanatomy PACAP in neuroendocrine systems also provides a fair description of how the concept of neuroendocrinology itself has evolved in the thirty-two years since the discovery, in the sheep hypothalamus, of this ancient, pleiotropic neuronal and neuroendocrine peptide.

15.2 Current Issues in Understanding the Functional Neuroanatomy of PACAPergic Neurons in Brain and Periphery

Clearly the definition of a neuroendocrine peptide is not restricted to synthesis in magno- or parvocellular hypothalamus and secretion into the general circulation from the neurohypophysis or the hypophysial portal circulation at the median eminence. For PACAP in particular, i.e. in this chapter, we have elected to integrate its specifically hypothalamic anatomy and function within a larger neuronal and endocrine anatomical context in Sects. 15.3 and 15.4 below, thus broadening, and hopefully enriching rather than obscuring, the definition of functional neuroendocrinology beyond the scope of the exclusively neuroendocrine cell of the hypothalamus (Korf and Usadel 1997).

What does functional neuroanatomy mean for neuroendocrine peptides in 2021? Obviously, it means first, where are the cell bodies, where are the nerve terminals, and how are they connected in circuits and subsystems; then, what are the co-transmitters; then, what do the actual collections of peptidergic neurons do? Finally, what are the post-synaptic actions of the peptides in concert with

co-released transmitters, and what signaling pathways are activated post-synaptically to affect neurotransmission in real time, and synaptic and cellular plasticity to encode experience and affect long-term behavior(s)?

A few broad observations apply not only to PACAP, but to neuropeptides in general. First, we know much more about the functions of neurons in which peptides are *located* than we do about the actual functions of the neuropeptides themselves *within* those neurons, and at the synapses formed by them. Second, while neuropeptide expression is often highly conserved across mammalian species (Elde et al. 1980), correspondence in peptide function may not be. Third, we understand that while peptides act through G-protein coupled receptors, we cannot always map a given peptide projection system to a particular receptor, and in some cases have not established which second and third messengers are activated by receptor engagement on recipient cells. Fourth, in many cases we have not established whether in a given circuit a neuropeptide acts as neurotransmitter, autocrine/paracrine factor or even hormone, and indeed whether its secretion is dendritic or axonal. Bearing in mind these gaps in knowledge is helpful in understanding why a coherent general picture of neuropeptide functional anatomy relevant not only to basic research, but also to its clinical translation, has been difficult.

Basic information about PACAP discovery, secretion and modes of signaling are not discussed here, but can be found in various reviews and references therein, as summarized below (Arimura 1992; Arimura 1998) (Mustafa and Eiden 2006; Emery and Eiden 2012; Jiang and Eiden 2016a, 2016b; Zhang and Eiden 2019).

Box 15.1 PACAP Summarized

Peptide structure	Main CNS cell populations	Biosynthesis and degradation	Receptors
PACAP38: HSDGIF TDSYSRYRKQMA VKKYLAAVLG KRYKQRVKNKamide *PACAP27: HSDGIFTDSYSR YRKQMAVKKYLAAVLamide*	Cerebral isocortex, cerebellar cortex, septum, visual and auditory thalamic divisions, hypothalamus, epithalamus (medial and lateral habenula), subthalamic nucleus, brain stem sensory centers in CNS; sensory, autonomic and enteric neurons in PNS.	PACAP38 produced from proPACAP by prohormone convertase (PC) endoproteolysis, and glycine cleavage and C-terminal amidation (PAM). PACAP27 produced from PACAP38 by second round of PC/PAM. PACAP degraded by various peptidases in several tissues and blood.	Receptors for PACAP: PAC-1 VPAC1 VPAC2

15.3 Anatomy of PACAPergic Neurons in Sensory and Autonomic Nervous Systems

Our account of PACAPergic peripheral anatomy will necessarily be cursory, as our main focus is on the role of PACAP in the central nervous system, where much more is yet to be learned about PACAP localization and function. However, the roles and functions of neuropeptides are often generally best approached by examination of the peripheral nervous system, as the function of the peripheral nervous system is more obviously parcellated anatomically among the enteric, somatic (sensory and motor) and autonomic (parasympathetic and sympathetic) nervous systems. In the enteric nervous system, PACAP is expressed in motor neurons resident in both myenteric and submucous plexus, and in interneurons (Portbury et al. 1995). As elsewhere, PACAP is neither ubiquitous to all enteric neurons, nor obviously restricted to large or small bowel or other enteric compartments. A specific functional role for the subpopulations of enteric neurons marked by PACAP expression has not yet emerged.

Box 15.2 PACAP in the Periphery

Location	Function	References
Motor neurons of the enteric system	Unknown	Portbury et al. (1995)
Somatosensory system	Nerve constriction-induced inflammation, trigeminal pain, migraine	Zhang et al. (1998), Dickinson and Fleetwood-Walker (1999), Dickinson and Fleetwood-Walker (1999), Takasaki et al. (2019b), Eftekhari et al. (2015), Moller and Baeres (2003)
Retinal ganglion cells of the retina	Light modulation of circadian rhythmicity	Hannibal et al. (1998), Beaule et al. (2009), Kawaguchi et al. (2010), Lindberg et al. (2019)
Organ of Corti	Auditory signaling	Drescher et al. (2006), Tamas et al. (2012)
Olfactory epithelium and in the ear	Neuroprotectant	Hegg et al. (2003), Hansel et al. (2001), Tamas et al. (2012)
Vagal and glossopharyngeal nerves	Processing of aversive gustatory inputs	Kano et al. (2011)
Autonomic nervous system	Innervation of the endocrine and exocrine pancreas	Onaga et al. (1996), Rudecki and Gray (2016)
Autonomic nervous system (sphenopalatine ganglion)	Trigeminal nerve-dependent pain of migraine	Elsas et al. (1996), Sundler et al. (1996)
Autonomic nervous system (superior sympathetic ganglion)	Modulation of sympathetic regulation of thermogenesis	Gray et al. (2002), Banki et al. (2014), Diane et al. (2014), Rudecki and Gray (2016)
Autonomic nervous system (cardiac postganglionic parasympathetic cells)	Co-released with acetylcholine, unknown function.	Liu et al. (2000), Tompkins et al. (2007)
Sympatho-adrenal axis	Co-released with acetylcholine. Required to sustain catecholamine secretion, and survival, during prolonged hypoglycemia	Hamelink et al. (2003), Wakade (1988), Watanabe et al. (1992), Holgert et al. (1996), Przywara et al. (1996), Hamelink et al. (2002).

Sensory nervous system expression of PACAP is marked by co-expression with glutamate and, depending on anatomical location, with CGRP and substance P (Moller et al. 1993; Goto et al. 2017). PACAP is highly induced in DRGs by chronic partial nerve constriction-induced inflammation (Zhang et al. 1998; Dickinson and Fleetwood-Walker 1999) and is implicated as well in pain of trigeminal nerve origin, such as migraine (Dickinson and Fleetwood-Walker 1999), where it represents a potential therapeutic target for neuropathic pain (Takasaki et al. 2019b). The peptide is expressed in trigeminal ganglia within sensory neurons (Eftekhari et al. 2015) and is found in nerve terminals of trigeminal sensory neuronal efferents in CNS (Moller and Baeres 2003). Whether its release also occurs from afferent terminals in the periphery, e.g. meningeal blood vessels, is not firmly established (Messlinger et al. 2020). In the eye, PACAP is present in non-visual intrinsically photosensitive retinal ganglion cells (ipRGCs) and mediates light modulation of circadian rhythmicity via synapses in SCN (Hannibal et al. 1998; Beaule et al. 2009; Kawaguchi et al. 2010; Lindberg et al. 2019). In the ear, PACAP is present in the organ of Corti and is thought to be involved in modulation of afferent auditory signaling (Drescher et al. 2006; Tamas et al. 2012). In the nose, PACAP is present during development, and in adults, in olfactory ensheathing cells of nasal epithelium and in primary sensory OSN (olfactory sensing neurons) themselves, although the latter express PAC1 receptors (Hegg et al. 2003). In both nasal epithelium and in the ear, PACAP is thought to act upon primary sensory neurons to enhance their health and lifespan, i.e., as a neuroprotectant (Hansel et al. 2001; Tamas et al. 2012). Neuroprotection may be associated with PACAP-dependent up-regulation of genes encoding proteins that protect against programmed cell death (Gonzalez et al. 1997), against calcium toxicity attendant upon chronic neuroexcitation, such as stanniocalcin and serpinb1a (Zhang et al. 2000; Holighaus et al. 2012; Seaborn et al. 2014), or against inflammation (Hori et al. 2012; Waschek 2013). In the tongue, ATP is the primary transmitter of the taste cells, along with various peptides not including PACAP, such as CCK, VIP, NPY, PYY, glucagon, and ghrelin. However, PACAP is likely involved in CNS processing of aversive gustatory inputs (vide infra) and perhaps released, along with glutamate, from the neurons of the vagal and glossopharyngeal nerves that bring taste sensation to the brain (Kano et al. 2011).

PACAP is localized to both sympathetic and parasympathetic preganglionic neurons in the autonomic nervous system, implying that PACAP is a co-transmitter with acetylcholine at autonomic synapses. PACAP appears to be absent from sympathetic postganglionic neurons, but is present in at least some postganglionic parasympathetic neurons, including those innervating the endocrine and exocrine pancreas (Onaga et al. 1996; Rudecki and Gray 2016). A second example of postganglionic parasympathetic expression of PACAP is within the sphenopalatine ganglion (Elsas et al. 1996; Sundler et al. 1996), whose neurons project to cerebral vasculature and may mediate trigeminal nerve-dependent pain of migraine (Waschek et al. 2018). The role of PACAP at sympathetic ganglionic synapses has not been well-explored, although May and colleagues have studied the effect of PACAP on cultured neonatal sympathetic neurons of the superior sympathetic ganglion and established its potential role at the sympathetic ganglion

to regulate neuropeptide expression and release (May and Braas 1995; Brandenburg et al. 1997; Beaudet et al. 1998), presumably relevant to PACAPergic modulation of sympathetic regulation of thermogenesis (Gray et al. 2002; Banki et al. 2014; Diane et al. 2014; Rudecki and Gray 2016). The effects of PACAP on postganglionic parasympathetic (cardiac ganglion cells) neurons have been thoroughly explored by electrophysiology, although its function complementarity to co-released acetylcholine at these synapses remains relatively unexplored (Liu et al. 2000; Tompkins et al. 2007).

The role of PACAP in the peripheral nervous system is perhaps best understood in the adrenal compartment of the sympathetic nervous system (Hamelink et al. 2003). The sympathetic preganglionic innervation of the chromaffin cells of the adrenal medulla by the splanchnic nerve was the first well-investigated PACAPergic synapse (Wakade 1988; Watanabe et al. 1992; Holgert et al. 1996; Przywara et al. 1996; Hamelink et al. 2002): this was also the first morphologically identified neuronal synapse outside the CNS, identified electron-microscopically by Rex Coupland 55 years ago (Coupland 1965). Cholinergic preganglionic neurons of the intermediolateral column of the spinal cord co-express PACAP, and PACAP is co-stored with acetylcholine at the nerve terminals of the splanchnic nerve that synapse upon chromaffin cells of the adrenal medulla (Hamelink et al. 2002). The interaction between acetylcholine and PACAP at this post-synapse, however, still remains something of a mystery: i.e. we know that both ACh and PACAP are critically important in basal catecholamine release (acetylcholine) and stress-induced release (PACAP). Under ex vivo conditions, both ACh and PACAP appear to be necessary for catecholamine secretion (Carbone et al. 2019), and in vivo PACAP is clearly required to sustain catecholamine secretion and animal survival, during prolonged hypoglycemia. Yet the mutual interdependence of ACh and PACAP in stress-induced secretion (i.e., under conditions in which both transmitters are released onto chromaffin cells) has not been explored sufficiently to establish the role of ACh in this situation (Eiden and Jiang 2018).

15.4 Anatomy of PACAPergic Neurons in the Brain

15.4.1 Methodologies for the Study of Distribution of PACAP-Expressing Neurons Across the Mammalian Brain

Neuropeptide chemical anatomy is the foundation for all further inquiry into physiological action and translational application. It rigorously constrains hypotheses about peptide action, and it provides the context for all meaningful experimental exploration of peptide function. This is especially true of the CNS, where anatomical location is a less straightforward indicator of function than it is in the sensory and autonomic nervous systems (see above). The PACAPergic system of the brain has been well investigated, neuroanatomically, since the peptide's discovery in 1989 (Arimura 1992) by immunohistochemistry, in situ hybridization histochemistry and radioimmunoassay after tissue dissection (Arimura et al. 1991; Ghatei et al. 1993;

Nielsen et al. 1998; Waschek et al. 1998; Hannibal 2002). Our laboratories have recently used dual in situ hybridization histochemistry (DISH), to investigate the nature of PACAPergic neurons of the adult CNS with respect to co-expression of excitatory or inhibitory co-transmitters (Zhang and Eiden 2019; Zhang et al. 2021). Table 15.1 presents a detailed accounting of PACAP-containing glutamatergic (VGLUT1 and VGLUT2 mRNA-expressing) and GABAeric (VGAT mRNA-expressing) neuronal distributions across the CNS of mouse, and Fig. 15.1 presents sections of mouse brain in coronal or sagittal section, with annotation of the relevant brain nuclei containing neurons that engage in PACAP/glutamate or PACAP/GABA co-transmission.

Single-cell transcriptomics has also taught us much about the types of neurons in cortex (Smith et al. 2019), and other brain areas (Papathanou et al. 2019) in which PACAP is expressed. This broad base of information is a foundation for conceptualizing the physiological functions of PACAP during development and in the adult mammal, and has been useful in generating Cre driver mice in which Cre expression is restricted to neurons in which the PACAP promoter (or other neuropeptide promoters, including those for CRH, galanin, neurotensin) is active. Single-cell transcriptomics (more precisely, single-nuclei transcriptomics) define approximately 120 neuronal cell types in human cortex, with about half (56) inhibitory and half (58) excitatory, based on mutually exclusive expression of mRNAs encoding either the vesicular GABA (VGAT) or glutamate (VGLUT) transporters (https://celltypes.brain-map.org/rnaseq/human_m1_10x). According to this analysis, PACAP is expressed exclusively in excitatory neurons, and in about half of the 58 excitatory subtypes defined by mRNA expression cluster (Fig. 15.2). A similar picture emerges from examination of the mouse single-cell cortical transcriptome data set (https://celltypes.brain-map.org/rnaseq/mouse_ctx-hip_10x). Conservation between human and mouse of i) restriction of cortical PACAP expression to excitatory neurons, and ii) conservation of clusters of inhibitory neurons defined by expression of *other* neuropeptides, including VIP, SST, and CRH, is striking, and consistent with the comparative neurochemistry of PACAP in human and rodent as well (Palkovits et al. 1995).

Dual in situ hybridization histochemistry (DISH) has been used to explore the co-transmission phenotypes of PACAPergic neurons which form the basis of the survey of PACAP and PAC1 distribution in excitatory and inhibitory neurons throughout the mouse brain that is summarized here. Two prominent examples shown in Fig. 15.1 are the co-localization of VGAT and PACAP in cerebellar Purkinje cells (Fig. 15.1a), and the co-localization of VGLUT2 and PACAP in most if not all of the neurons comprising the bed nucleus of the anterior commissure (BAC, see Fig. 15.1b). There are also high levels of *both* PAC1 and PACAP in BAC, with no reported PACAP afferents to BAC from other brain areas. Here, high *Acyap1* and *Acyap1r1* co-expression, combined with a lack of afferent PACAP nerve terminals, suggests that PACAP could act as an autocrine factor in neuronal autoregulation. The theme of autoregulation is one that merits active investigation in the brain PACAPergic system (vide infra, re: specific examples including

Table 15.1 Distribution, cell types, and strength of main **PACAPergic cell groups** in mouse brain with comparison of rat brain reported by Hannibal, JCN, 2002[a]

Cell group/sub-field[b]	Rat Hannibal JCN, 2002	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
Retina				
Ganglion cell layer[c]	+	–	+++	–
Cerebrum: Cortical plate				
Olfactory area				
Main olfactory bulb				
Granular cell layer	–	–	–	–
Inner plexiform layer	–	–	–	–
Mitral cell layer	+	+++	++	+
Outer plexiform layer	n.r.	+++	+++	+
Glomerular layer	–	+	+	+
Periglomerular cells	n.r.	+	+	+
Accessory olfactory bulb				
Mitral cell layer	+	++++	++	+
Glomerular layer	n.r.	+	+	+
Granular layer	n.r.	+	–	–
Other olfactory areas				
Ant olfactory n. lateral	++	++++	++	–
Ant olfactory n. medial	++	++++	++	–
Dorsal peduncular area	n.r.	+++	++	++
Taenia tecta	n.r.	+++	–	–
Piriform area: Pir2	n.r.	+	–	–
Piriform area: Pir3	n.r.	++++	+	–
N. Lat. Olfactory tract (NLOT)	++++	++++	++	+
Cortical amygdalar area (CoA)	n.r.	+	++++	–
Hippocampal formation				
Hippocampal region				
Dorsal dentate gyrus	n.r.	–	–	–
Dorsal hippocampus CA1	+	–	–	–
Dorsal hippocampus CA2	n.r.	+	–	–
Dorsal hippocampus CA3	+	–	–	–
Dorsal hilus	n.r.	++	–	–
Ventral dentate gyrus	n.r.	–	–	–
Ventral CA3vv	n.r.	+++++	–	–
Ventral hilus	n.r.	++	–	–
Retrohippocampal regions				
Entorhinal area	n.r.	+	+	–
Parasubiculum	++	+++	+++	–
Postsubiculum	++	+++	+++	–
Presubiculum	n.r.	+++	+++	–

(continued)

Table 15.1 (continued)

Cell group/sub-field[b]	Rat Hannibal JCN, 2002	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
Subiculum	n.r.	+++	+++	–
Isocortex[d]				
Layer I	+	n.a	n.a	n.a
Layer II-II	++	n.a	n.a	n.a
Layer IV	–	n.a	n.a	n.a
Layer V	++	n.a	n.a	n.a
Layer VI	+	n.a	n.a	n.a
Agranular insular cortex	n.r.	++++	++	–
Somatomotor areas				
2ry motor area, layer 2–3	n.r.	+++	–	–
2ry motor area layer 5	n.r.	++++	++	+
1ry motor area, layer 2–3	n.r.	+++	–	–
1ry motor area, layer 5	n.r.	++++	++	+
Orbital frontal cortex (OFC)				
OFC 1	n.r.	++	++	–
OFC 2/3	n.r.	+++	+	–
OFC 5	n.r.	+++	–	–
Prefrontal cortex (PFC)				
Ant cingulate cortex (ACC): **ACC 2/3:** **ACC 5:**	n.r. n.r.	++++ +++	+ +	– –
Prelimbic (PL) **PL 2/3** **PL 5**	n.r. n.r.	+++ +++	+ +	– –
Infralimbic (IL) **IL 2/3** **IL 5**	n.r. n.r.	++++ +++	+ +	– –
Cell group/sub-field[b]	**Rat Hannibal JCN, 2002**	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
Prim somatosensory a. SSp, **SSp 1** **SSp 2/3** **SSp 4 (mouth)** **SSp 5** **SSp 6a**	– – n.r. – –	+ +++ ++ ++ ++	– – – – –	– – – – –
Gustatory areas	n.r.	++++	–	–
Auditory area	n.r.	+++	–	–
Visual area	n.r.	+++	–	–
Visceral area	n.r.	+++	–	–
Temporal association area	n.r.	+++	–	–
Ectorhinal area	n.r.	+++	–	–
Perirhinal area	n.r.	++	–	–
Retrosplenial area	n.r.	++++	–	–
Post parietal association area	n.r.	++++	–	–

(continued)

Table 15.1 (continued)

Cell group/sub-field[b]	Rat Hannibal JCN, 2002	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
Cortical subplate				
Claustrum	n.r.	+	+	−
Endopiriform nucleus	n.r.	+	+	−
Lateral amygdalar nucleus	n.r.	++++	++	−
Post amygdalar nucleus (PA)	n.r.	++++	+	−
Basomedial amygdala	−	+	+	−
Basolateral amygdala	+	+	+	−
Cerebral nuclei				
Striatum				
Lateral septal nucleus	n.r.	++	++	−
Anterior amygdala area	n.r.	+	+	−
Central amygdalar nucleus	+	−	−	+
Medial amygdalar nucleus	++	+++	+++	+
Pallidum				
Bed nucleus of stria terminalis (BNST)	+	n.a	n.a	n.a
BNST oval	n.r.	−	+	+
BNST am	n.r.	−	++	++
BNST dm	n.r.	−	+	+
BNST pr	n.r.	+	++	++
Bed nucleus of anterior commissure (BAC)	n.r.	+++++	+++++	−
Brain stem, interbrain				
Thalamus				
Somatomotor related				
Subparafacicular nucleus, magnocellular part	n.r.	+	++	−
Subparafacicular area	n.r.	−	+++	−
Peripeduncular nucleus	n.r.	−	+++	−
Medial geniculate complex	n.r.	−	+++	−
Polymodal association cortex related				
Lat. Posterior n. thal	n.r.	+	++	−
Post. Limiting nucleus	n.r.	+	++	−
Supragenículate n.	n.r.	+	++	−
Anterodorsal n.	−	+++	+	−
Anteromedial n.	n.r.	++	++	−
Parataenial n.	n.r.	++	++	−
Intermedial n.	n.r.	+	+	−
Laterodorsal n.	n.r.	+	++	−
Centrolateral n	n.r.	−	++	−
Intermediodorsal n.	n.r.	+	++	−
Mediodorsal n.	n.r.	+++	+	−

(continued)

Table 15.1 (continued)

Cell group/sub-field[b]	Rat Hannibal JCN, 2002	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
Pariventricular n.	–	+	+++	–
Parateanial n.	n.r.	+	++	–
N. of reuniens	–	+	+++	–
Posterior pretectal n.	+++	–	+++	–
Precommissural n.	+++	–	+	–
Cell group/sub-field[b]	**Rat Hannibal JCN, 2002**	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
Epithalamus				
Medial habenula[e]	++++	++++	++++	–
Lateral habenula	++++	–	++++	–
Hypothalamus				
Paraventricular n	+	–	+	–
Periventricular n	+	–	++	–
Anterodorsal preoptic n.	n.r.	–	+	–
Anteroventral	n.r.	–	+++	–
Dorsomedial n.	+++	–	+++	–
Median preoptic n. (MEPO)	+++	–	++++	–
Medial preoptic area	+++	–	++	–
Vascular organ of lamina terminalis	+++	–	++++	–
Posterodorsal preoptic n.	n.r.	–	+	–
Subfornical organ	++++	–	++++	–
Lateral preoptic area	n.r.	–	++	–
Anterior hyp. Area	++	–	++	–
Premammillary n.	n.r.	–	++	+
Lateral mammillary n.	++++	–	++++	–
Medial mammillary n.	–	–	+++	–
Supramammillary n.	–	–	++	+
Median preoptic n.	++	–	++	–
Lateral hyp. Area	++	–	++	–
Preparasubthalamic n.	n.r.	–	+++	–
Parasubthalamic n.	n.r.	–	+++	–
Subthalamic nucleus	–	–	+++++	–
Retrochiasmatic area	n.r.	–	+++	–
Tuberomammillary nucleus	–	–	++	+
Zona incerta	+	–	++	–
Ventromedial hyp. n	++++	–	+++++	–
Post. Hypothalamic n.	n.r.	–	+++	–
Midbrain				
Sensorial related				
Inf. Colliculus (IC), central and external n.	n.r.	–	++	–

(continued)

Table 15.1 (continued)

Cell group/sub-field[b]	Rat Hannibal JCN, 2002	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
N. of the brachium of IC	n.r.	–	++	–
N. Saculum	n.r.	–	+	–
Parabigeminal n.	n.r.	–	+	–
Midbrain trigeminal n.	n.r.	–	++	–
Motor related				
Ventral tegmental area	n.r.	–	++	–
Midbrain reticular n.	n.r.	–	+	–
Superior colliculus, motor related	n.r.	–	+++	–
Periaqueductal gray	n.r.	–	+++	–
Cuneiform n.	n.r.	–	++	–
Edinger-Westphal n.	n.r.	–	+	–
Interfascicular n. raphe	n.r.	–	++	–
Behavior state related				
Midbrain raphe nuclei	n.r.	–	–	–
Pedunculopontine n.	n.r.	–	++	–
Dorsal n. raphe	n.r.	–	–	–
Central linear n. raphe	n.r.	–	++	–
Rostral linear n. raphe	n.r.	–	–	–
Olivary pretectal nucleus	n.r.	–	+++	–
Cell group/sub-field[b]	Rat Hannibal JCN, 2002	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
Hindbrain				
Pons				
Sensory related				
N. Lateral lemniscus	n.r.	–	+	–
Principal sensorial nucleus of trigeminal nerve	–	–	+	–
Koelliker-fuse subnucleus	n.r.	++++	–	–
Parabrachial n. lateral div.	n.r.	–	++++	–
Parabrachial n, rest subfields	n.r.	–	+++	–
Superior olivary comp (lat)	n.r.	+	–	–
Motor related				
Tegmental reticular n.	n.r.	–	+++	–
Barrington's nucleus	n.r.	–	+++	–
Dorsal tegmental n.	n.r.	–	+	–
Pontine gray	n.r.	–	+++	–
Pontine central gray	n.r.	–	+	–
Supratrigeminal nucleus	n.r.	–	+	–
Behavior state related				
Locus Coerulus (state)	+	++	+++	–
Laterodorsal tegmental n.	+	–	+++	–
Pontine reticular n.	n.r.	–	+	–
Superior central n. raphe	n.r.	–	+	+

(continued)

Table 15.1 (continued)

Cell group/sub-field[b]	Rat Hannibal JCN, 2002	Slc17a7 (VGLUT1)	Slc17a6 (VGLUT2)	Slc32a1 (VGAT)
Medulla				
N. Tractus solitarii medial	+++	++	++++	–
N. Tractus solitarii lateral	+++	–	++++	–
Hypoglossal (XII) n.	–		++	–
Dorsal motor n. of the vagus nerve (X)	+++	+++	–	–
Dorsal cochlear n.	+++	++	++	–
Ventral cochlear n.	n.r.	++	++	–
Spinal n. trigeminal	n.r.	–	++	–
N. Prepositus	n.r.	–	++	–
Inferior salivatory complex	n.r.	–	++	–
Facial motor n. (VII)	n.r.	–	++	–
N. ambiguus	+++	–	++	–
Magnocellular reticular n.	n.r.	–	++	–
Parapyramidal n.	n.r.	–	++	–
Spinal vestibular n.	+++	–	++	–
N. X	n.r.	–	+	–
N. Raphe magnus (state related)	n.r.	–	++	–
N. Raphe pallidus (state related)	n.r.	–	++	–
N. Raphe obscurus (state rel.)	n.r.	–	++	–
Cuneate n.	–	++	++	–
Inferior olivary	n.r.	–	++	–
Cerebellar cortex				
Purkinje cells[f]	++	–	–	+++++
Golgi cells	n.r.	–	–	+
Granule cells[e]	–	++	–	–
Cerebellar nuclei				
Interposed n.	++	+	–	–
Dentate n.	n.r.	–	+	–

n.a. not applicable

n.r. not reported (blue color text refers to Hannibal JCN, 2002 rat PACAPergic cell group and expression strength analysis

[a]Similar semiquantitative annotations are used here (the percentage of expressing cell/total Nissl stained nuclei: "–", not detectable; "+", weak (<20%); "++", low (40%–20%); "+++", moderate (60%–40%); "++++", intense (80%–60%); "+++++", very intense (>80%)

[b]Functional neuroanatomy order and annotations are based on Allen Institute Mouse Reference Atlas

[c]Circadian oscillating expression (Lindberg et al. 2019)

[d]Isocortex expression was regionally evaluated

[e]Dorsal half of the MHb which co-express *Calb2* (RNA encoding calretinin)

[f]Prominent in lobules paraflocculus, central and uvula. Coincide with calretinin (Calb2) expression (Table from Zhang, Hernandez et al., eLife 2021; 10:e61718. DOI: https://doi.org/10.7554/eLife.61718)

autoregulation of PACAP and PAC1 levels in BNST in Sect. 15.4.2–15.4.4), as well-documented for vasopressinergic magnocellular neurons (Brown et al. 2020).

Table 15.1, included here and abbreviated from Zhang et al. 2021, summarizes *Adcyap1* (PACAP), *Slc32a1* (VGAT), and *Slc17a6/7* (VGLUT2/1) co-expression patterns throughout the mouse brain. In the paragraphs to follow in the remainder of Sect. 15.4, the data of Table 15.1 are interpreted in the context of known facts about peptidergic and peptide receptor cell types, excitatory and inhibitory cells and synapses, and known functional brain circuits for processing of sensory input and ordering of motor output in the brain. Thus, in Sects. 15.4.2–15.4.4.2 we point out features of PACAP expression within the major divisions of the brain, and its segregation in excitatory and inhibitory neurons throughout the CNS (Fig. 15.3). In Sect. 15.5, causal and correlative relationships between PACAP-and PAC1-deficiency-related phenotypes, and PACAP's signaling roles in these circuits, will be examined.

15.4.2 PACAPergic Neurons of the Cerebrum, or Telencephalon

The cerebrum includes the cerebral cortex which comprises the isocortex, olfactory cortex, and hippocampus arising from the cortical plate, the claustrum, endopiriform cortex and amygdala complex arising from the cortical subplate, and the cerebral nuclei, comprising the dorsal and ventral striatum and the lateral septum as well as the dorsal, ventral, medial, and caudal divisions of the pallidum.

In olfactory cortex VGLUT1- and VGLUT2-positive PACAPergic neurons are found in both main and accessory olfactory bulb, in the mitral cell layer of both, more sparsely in the glomerular layer of both, and abundantly in the outer plexiform layer of the main olfactory bulb. While neurons mainly co-express VGLUT1 or VGLUT2, i.e., are excitatory, numerous sparsely distributed inhibitory PACAPergic neurons are found in the olfactory region, including anterior olfactory nuclei and dorsal peduncular area which are however also richer in excitatory PACAP-positive neurons (Table 15.1). The nucleus of the lateral olfactory tract also expresses PACAP very abundantly. The loss of avoidance responses to predator odor in NLOT-lesioned rats (Vaz et al. 2017) and the impairment in avoidance of odorant and loss of fos regulation in NLOT during odorant experiencing in PACAP-deficient mice (despite their unimpaired ability to sense odor in food foraging) suggest a potential physiological role for PACAP in mediating odor-driven aversive behavior via this pathway (Zhang et al. 2021).

Isocortical PACAPergic neurons are almost exclusively excitatory. This is in contrast to CCK and somatostatin, first identified as "markers" for subpopulations of cortical GABAergic interneurons (Somogyi, J. Neurosci. 1984), and for most all other peptidergic neurons of cortex, which also co-expresses GABA, most notably the PACAP-related neuropeptide VIP (Fig. 15.2). Potential roles for cortical PACAPergic neurons include participation in control of STN neuronal excitability for initiation of movement via layer 5 motor cortical projections ((Kita and Kita 2012) and see Fig. 15.1d and Fig. 15.3), and in modulation of stress responding via

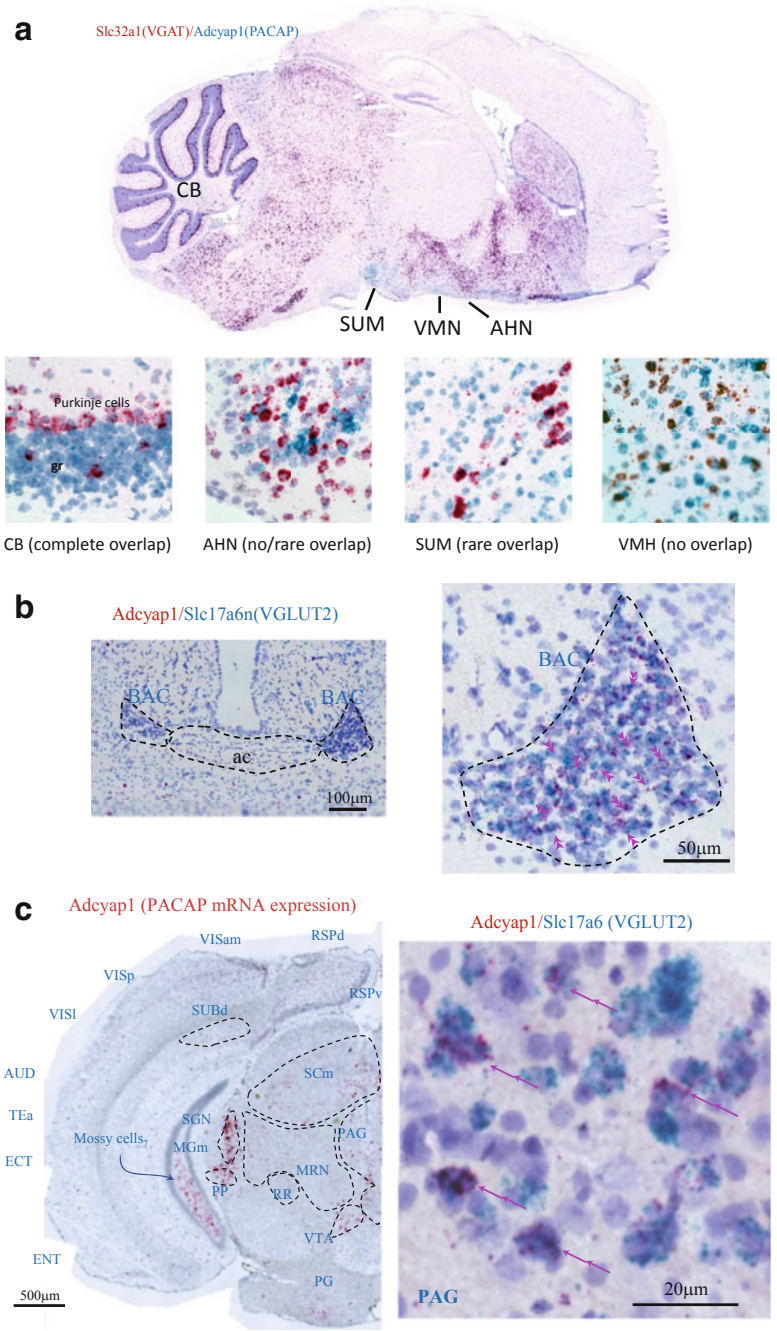

Fig. 15.1 Comprehensive DISH mapping of PACAP co-expression with VGLUT1, VGLUT2, and VGAT throughout mouse brain reveals an extensive distribution and diversity of cell types. *Adcyap1* (PACAP) mRNA mapping within glutamatergic and GABAergic subpopulations of mouse brain. *Slc17a7*, *Slc17a6* and *Slc32a1*: mRNAs encoding VGLUT1, VGLUT2, and VGAT

prefrontocortical projections to BNST targets which in turn mediate HPA activation in stress via projections to PVN, although these have not been identified as PACAPergic (Radley and Sawchenko 2011). In addition, abundant expression of Adcyap1r1 (PAC1) in isocortex suggests that PACAP-positive cortical neurons are also involved in local communication with cortical interneurons.

Two aspects of PACAP expression in the hippocampus are noteworthy, and probably merit focused investigation from the point of view of understanding the general features of PACAP neurotransmission in the brain including the unique properties of post-synaptic signaling by PACAP. First, PACAP is quite heterogeneously expressed in ventral, compared to dorsal hippocampus, with very high expression in ventral CA3vv, a subdivision characterized by high expression of the marker *Coch* (Bienkowski et al. 2018). Second, PACAP expression is also high in both dorsal and ventral hilus of the hippocampus, indicating that PACAP may function in modulation of hippocampal function via mechanisms that are yet to be elucidated and lie outside the classical trisynaptic pathway (see Fig. 15.4e and f in (Zhang et al. 2021)). The finding of a large number of excitatory PACAPergic neurons in retrohippocampal regions (para-, pre-, and post-subiculum and subiculum itself) may indicate an important role for PACAP neurotransmission in mediating hippocampal output, perhaps in regulation of the HPA axis in stress (O'Mara 2005). Although CA1 and CA3 are devoid of PACAP expression in dorsal hippocampus, there is sparse but prominent PACAP expression in CA2: given the crucial role of

Fig. 15.1 (continued) mRNAs, with respective color coding for the chromogens labeling the corresponding mRNAs. (**a**) PACAP in subpopulations of VGAT-positive neurons throughout the brain. (**b**) PACAP co-expression with VGLUT2 in BAC. (**c**) PACAP co-expression with VGLUT2 in ventral hippocampus and PAG. (**d**) PACAP co-expression with VGLUT2 in STN. (**e**) PACAP/VGLUT2-coexpressing neurons in MEPO and OVLT. (**f**) PACAP/VGLUT2-coexpressing neurons of anterior hypothalamic nuclei. (**g**) PACAP co-expression with VGLUT2 in PVN and AHN. Arrows indicate representative cells with PACAP and VGLUT2 mRNA co-expression. (**h**) PACAP co-expression with VGLUT2 in VMN and DMH. Abbreviations: *3v* third ventricle, *ACA* anterior cingulate area, *ac* anterior commissure, *AHN* anterior hypothalamic nucleus, *AI* agranular insular area, *AONpv* anterior olfactory nucleus, postero-ventral, *AUD* auditory areas, *avMLPA* anteroventral medial and lateral preoptic area, *BAC* bed nucleus of anterior commissure, *BMA* basomedial amygdala, *CA3* cornu ammonis region of hippocampus, *CB* cerebellum, *DMHa* dorsomedial nucleus of the hypothalamus, anterior part, *ECT* ectorhinal area, *ENT* entorhinal area, *GU* gustatory area, *LHb* lateral habenula, *MD* mediodorsal nucleus of the thalamus, *MEA* medial amygdala, *MEPO* median preoptic nucleus, *MGm* medial geniculate complex, medial part, *MHb* medial habenula, *MOp* primary motor area, *MOs* supplemental motor area, *MRN* midbrain reticular nucleus, *OVLT* organum vasculosum of lamina terminalis, *PAG* periaqueductal gray, *PG* pontine gray, *PH* posterior hypothalamic nucleus, *PIR* piriform area, *PP* peripeduncular nucleus, *RR* midbrain reticular nucleus, retrorubral area, *RSPd* retrosplenial area dorsal, *RSPv* retrosplenial area dorsal, *PVN* periventricular hypothalamic nucleus, *SCm* superior colliculus, motor related, *STN* subthalamic nucleus, *SUBd* subiculum dorsal part, *SGN* suprageniculate nucleus, *SUM* supramammillary nucleus, *TEa* Temporal association area, *VISam* anteromedial visual area, *VISp* primary visual area, *VISal* anterolateral visual area, *VISl* lateral visual area, *VISC* visceral area, *VMN* ventromedial hypothalamic nucleus, *VMNc* ventromedial hypothalamic nucleus, central part, *VMNdm* ventromedial hypothalamic nucleus, dorsomedial part, *VMNvl* ventromedial hypothalamic nucleus, ventrolateral part, *VTA* ventral tegmental area, *ZI* zona incerta. Figure adapted from Supplementary Fig. 15.1 of Zhang et al. 2021

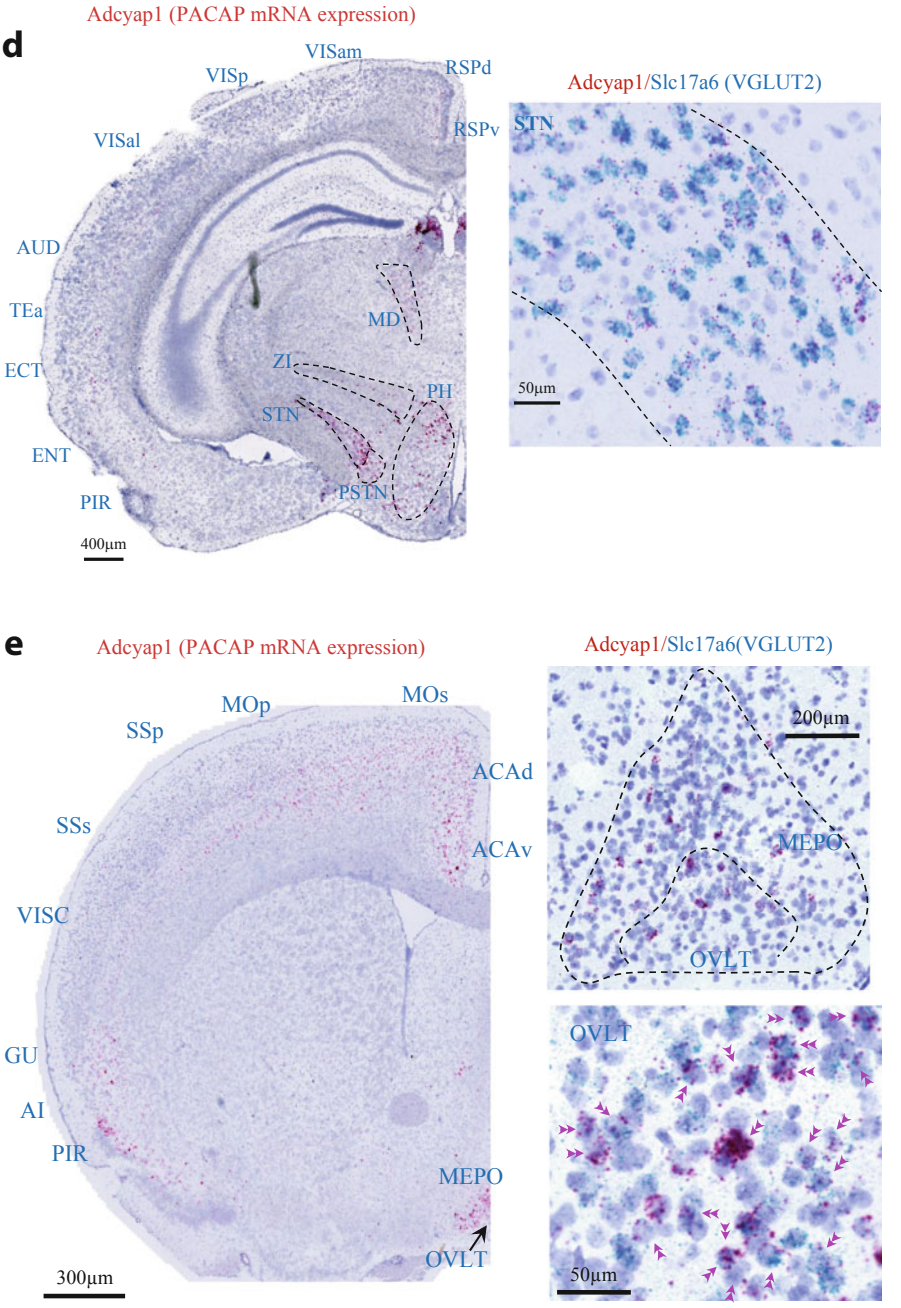

Fig. 15.1 (continued)

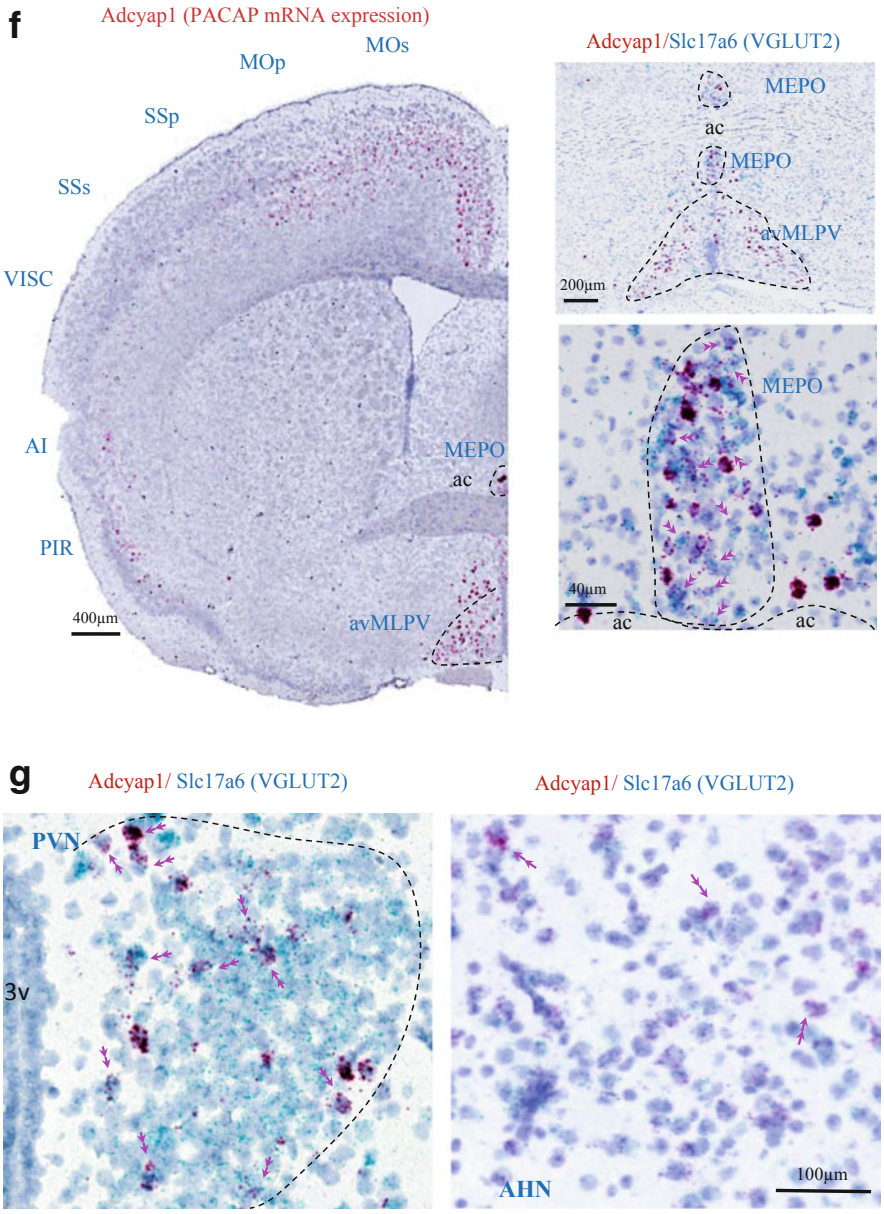

Fig. 15.1 (continued)

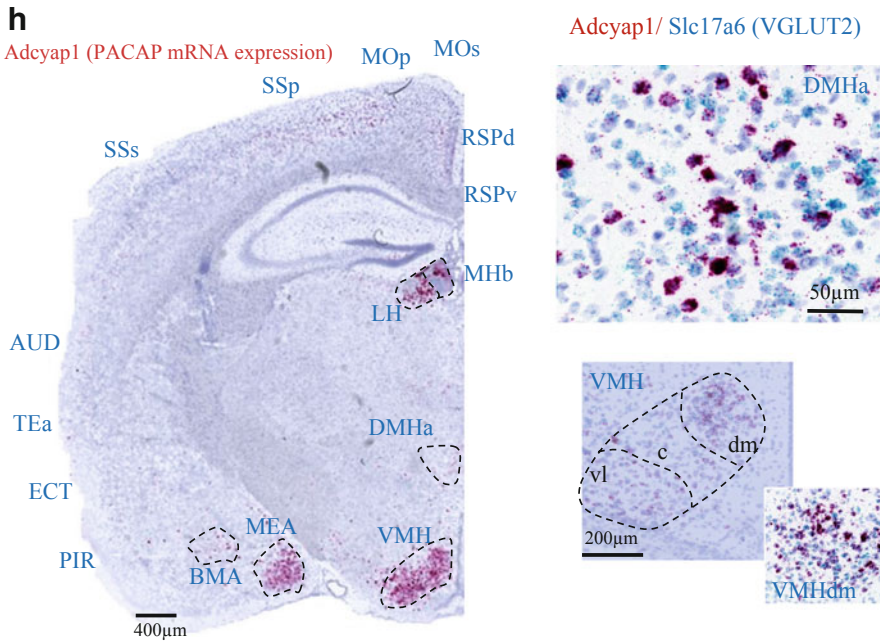

Fig. 15.1 (continued)

CA2 in mediating social recognition in mammals (Cymerblit-Sabba et al. 2020), this subpopulation may merit more thorough investigation.

In addition to PACAPergic projections to BNST from PBN (see above and Sect. 15.4) there exist PACAP cells in basolateral amygdala (BLA) ((Zhang et al. 2021) and references therein) that are reported to project either to BNST or to the intercalated cell complex (Rajbhandari et al. 2021). These ultimately modulate BNST output relevant to response to threat, pain, and other aversive stimuli. Investigation of rodent amygdalar PACAP expression has revealed some differences between rat and mouse (see, e.g., Fig. 15.3 of supplemental data (Zhang et al. 2021)). This represents an important arena in which PACAP circuitry needs to be better elucidated, and in particular as it applies to better understanding of how PACAP function in the extended amygdala might affect human fear/threat processing in the context of post-traumatic stress disorder (PTSD), other anxiety disorders (Ressler et al. 2011), and autism spectrum disorder (Goodrich et al. 2019).

The structures derived from the cerebral nuclei include the dorsal striatum, nucleus accumbens, and lateral septum, all relatively devoid of PACAP-expressing cells. Within the pallidum, PACAP is expressed only weakly in BNST of quiescent animals. However dramatic changes in expression of both PACAP and its receptor PAC1 in response to chronic stress suggest that the BNST is chemoanatomically quite plastic (Lezak et al. 2014), and that PACAPergic circuits relevant to physiological response may become functionally patent only in response to environmental

Inh_L1_LAMP5_NDNF	0.00	Inh_L1_LAMP5_GGT8P	0.00	Inh_L1_4_LAMP5_DUSP4	0.00
Inh_L6_LAMP5_C1QL2	0.00	Inh_L1_6_LAMP5_CA13	0.00	Inh_L5_6_LAMP5_SFTA3	0.00
Inh_L1_2_PAX6_SCGN	0.00	Inh_L6_LAMP5_ANKRD20A11P	0.00	Inh_L1_PAX6_CA4	0.00
Inh_L1_PAX6_GRIP2	0.00	Inh_L1_6_VIP_RCN1	0.00	Inh_L1_3_PAX6_NABP1	0.00
Inh_L1_VIP_PRSS8	0.00	Inh_L1_VIP_TNFAIP8L3	0.00	Inh_L1_ADARB2_ADAM33	0.00
Inh_L1_SST_CXCL14	0.00	Inh_L1_ADARB2_DISP2	0.00	Inh_L1_VIP_SOX11	0.00
Inh_L1_5_VIP_KCNJ2	0.00	Inh_L1_6_VIP_PENK	0.00	Inh_L1_6_VIP_RGS16	0.00
Inh_L2_6_VIP_VIP	0.00	Inh_L3_6_VIP_KCTD13	0.00	Inh_L1_VIP_PCDH20	0.00
Inh_L1_2_VIP_PPAPDC1A	0.00	Inh_L2_4_VIP_DSEL	0.00	Inh_L2_5_VIP_TOX2	0.00
Inh_L1_3_VIP_ZNF322P1	0.00	Inh_L3_VIP_CBLN1	0.00	Inh_L1_3_VIP_GGH	0.00
Inh_L1_3_VIP_ACHE	0.00	Inh_L1_2_VIP_RPL41P3	0.00	Inh_L1_3_VIP_SSTR1	0.00
Inh_L1_3_VIP_CCDC184	0.00	Inh_L2_4_VIP_LGI2	0.00	Inh_L1_4_VIP_CHRNA2	0.00
Inh_L6_SST_NPY	0.00	Inh_L5_6_SST_ISOC1	0.00	Inh_L5_6_SST_KLHL14	0.00
Inh_L4_6_SST_MTHFD2P6	0.00	Inh_L3_5_SST_MAFB	0.00	Inh_L4_5_PVALB_TRIM67	0.00
Inh_L5_6_SST_TH	0.00	Inh_L6_LHX6_GLP1R	0.00	Inh_L5_6_PVALB_FAM150B	0.00
Inh_L2_4_SST_AHR	0.00	Inh_L1_2_PVALB_TAC1	0.00	Inh_L1_3_PVALB_WFDC2	0.00
Inh_L2_4_PVALB_C8orf4	0.00	Inh_L5_PVALB_CNTNAP3P2	0.00	Inh_L5_6_PVALB_STON2	0.00
Inh_L3_4_PVALB_HOMER3	0.00	Inh_L1_6_PVALB_SCUBE3	0.00	Inh_L3_6_PVALB_MFI2	0.00

Exc_L2_4_RORB_GRIK1	**5.41**	**Exc_L3_4_RORB_RPS3P6**	**1.89**	**Exc_L4_RORB_BHLHE22**	**0.55**
Exc_L4_RORB_CCDC168	**10.19**	Exc_L4_RORB_CACNG5	0.00	**Exc_L4_5_RORB_ASCL1**	**0.34**
Exc_L4_5_RORB_AIM2	0.00	Exc_L3_LINC00507_CTXN3	0.00	Exc_L3_THEMIS_PLA2G7	0.00
Exc_L3_5_THEMIS_ELOF1	0.00	**Exc_L2_3_LINC00507_RPL9P17**	**28.71**	**Exc_L3_LINC00507_PSRC1**	**38.57**
Exc_L3_5_LINC00507_SLN	**59.51**	**Exc_L3_RORB_CARTPT**	**51.78**	**Exc_L3_4_RORB_FOLH1B**	**14.70**
Exc_L3_4_RORB_PRSS12	**40.21**	**Exc_L3_4_RORB_SEMA6D**	**21.57**	**Exc_L3_5_RORB_HSPB3**	**36.72**
Exc_L5_6_THEMIS_OR1J1	0.00	Exc_L6_THEMIS_EGR3	0.00	Exc_L5_6_THEMIS_TMEM233	0.00
Exc_L6_THEMIS_LINC00343	0.00	Exc_L3_5_RORB_CMAHP	0.00	**Exc_L3_5_RORB_CD24**	**19.20**
Exc_L4_5_RORB_LCN15	0.00	**Exc_L3_5_THEMIS_UBE2F**	**14.04**	Exc_L5_RORB_SNHG7	0.00
Exc_L4_5_RORB_RPL31P31	0.00	Exc_L4_5_RORB_HNRNPA1P46	0.00	Exc_L4_5_RORB_LINC01474	0.00
Exc_L4_6_RORB_HPCA	0.00	Exc_L5_6_RORB_LINC00320	0.00	Exc_L5_RORB_LINC01202	0.00
Exc_L6_THEMIS_C6orf48	0.00	Exc_L5_6_THEMIS_GPR21	0.00	Exc_L5_6_THEMIS_THTPA	0.00
Exc_L5_6_THEMIS_IL7R	0.00	Exc_L6_FEZF2_VWA2	0.00	Exc_L5_6_FEZF2_ANKRD20A1	0.00
Exc_L6_FEZF2_FAM95C	0.00	Exc_L6_FEZF2_CPZ	0.00	Exc_L6_FEZF2_P4HA3	0.00
Exc_L6_FEZF2_SLITRK6	0.00	Exc_L6_FEZF2_TBCC	0.00	Exc_L6_FEZF2_ETV4	0.00
Exc_L6_FEZF2_TBC1D26	**0.14**	**Exc_L6_FEZF2_KRT17**	**2.50**	Exc_L3_5_FEZF2_ONECUT1	0.00
Exc_L3_5_FEZF2_DCN	**20.69**	**Exc_L5_FEZF2_MORN2**	**5.76**	Exc_L5_FEZF2_SCN7A	0.00
Exc_L5_6_FEZF2_CABP7	0.00	Exc_L5_FEZF2_DYRK2	0.00	Exc_L5_6_FEZF2_MYBPHL	0.00
Exc_L5_6_FEZF2_CYP26B1	0.00	Exc_L5_6_FEZF2_RSAD2	0.00		

Fig. 15.2 Relative expression of PACAP (Adcyap1) mRNA in single nuclei isolated from mouse cortex. Differential expression in inhibitory (left) and excitatory (right) neuronal clusters based on transcriptome signature. Data from Allen Brain Atlas, extracted from https://molecularbrain.org/MouseCortexExcitatory/home.php and https://molecularbrain.org/MouseCortexInhibitory/home.php

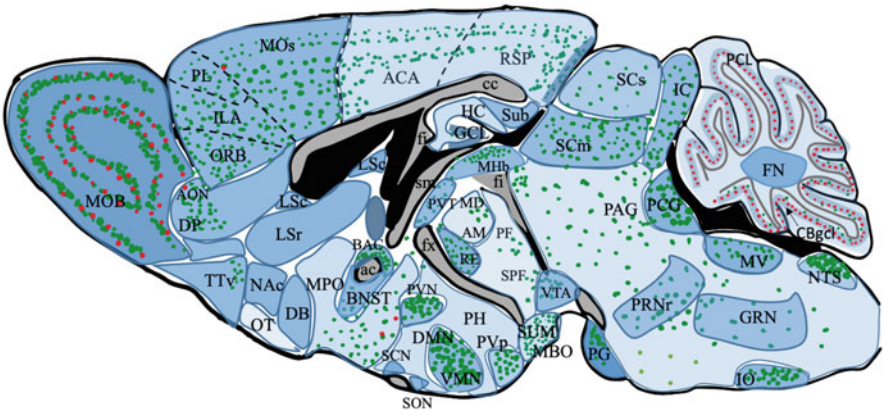

Fig. 15.3 PACAP and PAC1 co-expression with VGAT and VGluTs throughout mouse CNS. Distribution of PACAP in glutamatergic (green dots) and GABAergic (red dots) neurons, with relative proportions of positive cells given by dot density (e.g., very high in DMN and VMN hypothalamus, low in BNST, absent from lateral septum and concentrations of PAC1-positive neurons indicated by blue shading (e.g., very high in GCL and MOB, very low in cerebellum. PACAPergic cells are generally glutamatergic, except in cerebellum, where Purkinje cells are GABAergic, and olfactory bulb and preoptic area, where both VGluT- and VGAT-co-positive PACAPergic neurons are found. Scattered PACAP/GABA neurons are found elsewhere (here, and see text). Not visible at this plane is caudate-putamen, with moderate levels of PAC1 receptor expression but devoid of PACAP-expressing neurons. Abbreviations as listed elsewhere. Taken from Fig. 3, Zhang et al. 2021

inputs (King et al. 2017), underscoring the need for better understanding of the cell types in which the PACAPergic phenotype "emerges" during chronic stress.

15.4.3 PACAPergic Neurons of Interbrain, or Diencephalon: Focus on the Hypothalamus

The hypothalamus, located in interbrain (diencephalon) has the brain's highest concentration of PACAP and the densest overall investment of PACAPergic nerve terminals (Arimura et al. 1991; Hannibal 2002). This is reflected in the fact that twenty-six separate hypothalamic nuclei express Adcyap1 mRNA, and for several of these most of the cells of the nucleus (e.g., circumventricular organs, DMH, VMN, and lateral MBO) are PACAP-expressing (Zhang et al. 2021). Almost all are excitatory neurons expressing VGLUT2, although rare GABAergic/PACAPergic neurons are found in some hypothalamic nuclei, including SUM (Fig. 15.1a). The PVN, which receives PACAPergic innervation onto CRH neurons (Legradi et al. 1998) from an as-yet uncharacterized input source, also contains abundant PACAP-positive neurons. The preoptic area contains numerous PACAP-positive neurons, and their projections and functions are considered in Sect. 15.5. Expression of Adcyap1r1 is also high in hypothalamus, in both PACAP-positive and PACAP-

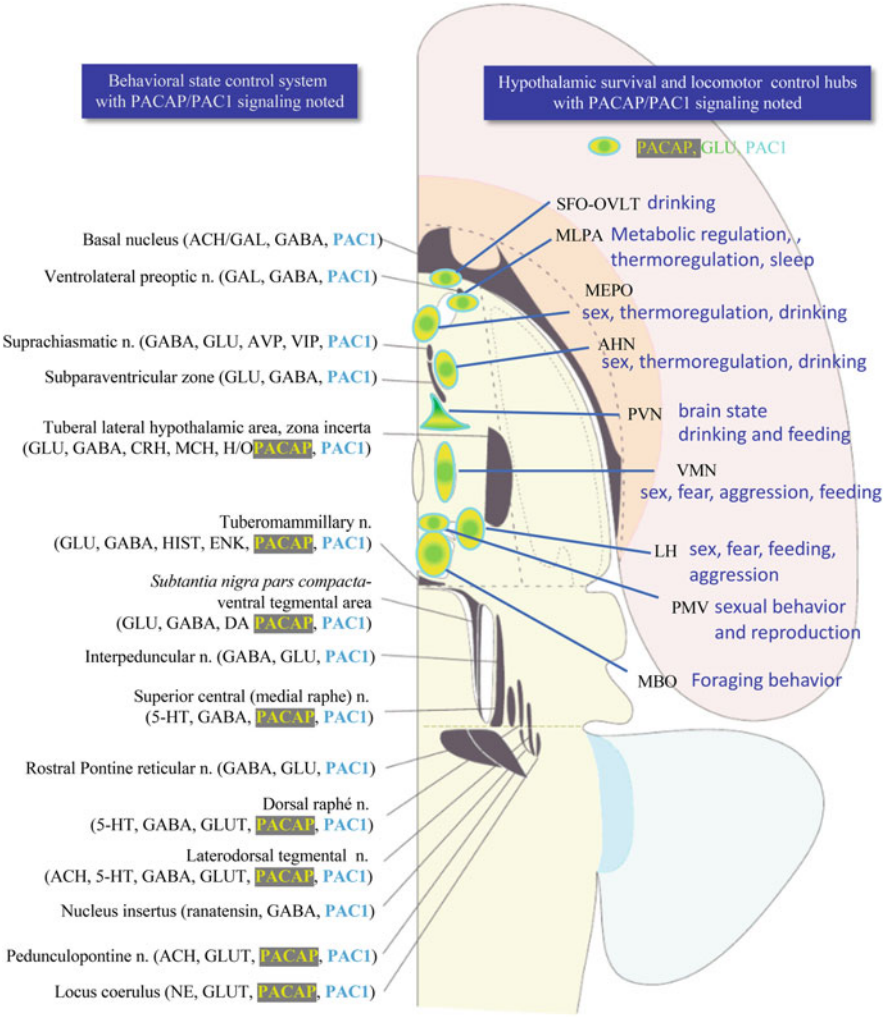

Fig. 15.4 PACAP and PACAP expression in hypothalamic and extrahypothalamic cell groups associated with behavioral state (left) and instinctual and reflex survival systems (right). Critical nodes for behavioral state, shown on a horizontal brain "flatmap," and key neurotransmitters in these nodes, are shaded in dark gray (modified from Swanson, 2012). Left column: critical nodes for behavioral state symbolized by dark gray shaded objects, modified from Swanson, 2012. In the longitudinal cell group-column of brainstem the key neurotransmitters are annotated. *ACH* acetylcholine, *CRH* corticotropin-releasing hormone, *DA* dopamine, *ENK* encephalin, *GABA* gamma-amino butyric acid, *GAL* galanin, *GLUT* glutamate, *H/O* hypocretin/orexin, *HIST* histamine, *MCH* melanin-concentrating hormone, *NE* norepinephrine, *5HT* serotonin. Right column: hypothalamic survival and locomotor control hubs that consist of discrete hypothalamic regions contain interoceptors for a variety of substances and have neuronal afferences from primary sensory systems to control the secretory and instinctive motor outputs. The rectangle in the midline represents the neuroendocrine motor zone for secretion of hypophysiotropic hormones, which include thyrotropin-releasing hormone, corticotropin-releasing hormone, growth hormone-releasing hormone, somatostatin, gonadotropin-releasing hormone, dopamine, neurotensin. *SFO* subfornical organ, *OVLT* organum vasculosum of lamina terminalis, *MEPO* preoptic nucleus, *AHN*

negative neurons. The major hypothalamic nuclei in which PACAP is expressed are depicted in the flatmap of Fig. 15.5, which functions as a guide to the description of hypothalamic circuits/projections in which PACAP participates to mediate several discrete functions, as described in Sect. 15.5. Figures 15.1e–h provide illustrations of PACAP-positive neurons of the hypothalamus, including anterior nuclei (MEPO, AHN, AVPV), the circumventricular organ OVLT, ventral and dorsomedial nuclei, and PVN).

The epithalamus (habenula) receives inputs from both dorsal striatum and limbic structures and modulates the processing of aversive information for presentation to the reward centers of the ventral striatum. PACAPergic neurons of the habenula include those in both lateral and medial habenula (Hashikawa and Stuber 2020; Wallace et al. 2020; Zhang et al. 2021) and references therein), and these two systems likely subserve very different functions, as MHb projects mainly to IPN, whereas LHb projects to VTA and rostromedial tegmental nucleus.

Thalamus receives PACAPergic innervation (principally to the PVT) and also contains PACAP-expressing cells, in PVT itself, and in suprafascicular nucleus, medial geniculate complex, nucleus reuniens, and mediodorsal thalamic nucleus (MD). PVT and MD in particular are sensory relay nuclei, participating, for example, in threat-induced hyperthermia through connections to other PACAP-expressing brain areas such as dorsal taenia tecta (Kataoka et al. 2020). PVT is also a site of apparent PACAPergic plasticity involved in addiction and reward, as chronic alcohol self-administration results in an increase in PACAP mRNA as well as PACAP-27 levels in PVT (Gupta et al. 2018). Whether the excitatory PACAPergic inputs to PVT synapse upon the excitatory PACAPergic neurons of the PVT, and what the projection fields of the latter are, has not been determined. The presence of PACAP and PAC1, in both afferents to and efferents from a given nucleus, and implicated in functionally connected circuits (PBN->PVT; NTS->PBN; PBN->BNST; cortical

Fig. 15.4 (continued) anterior hypothalamic area, *PVH* paraventricular hypothalamic nucleus, *VMM* ventromedial hypothalamic nucleus, *LH* lateral hypothalamic area, *MBO* mammillary body. In the right column, we present the main hypothalamic survival and locomotor control hubs with PACAP/PAC1 signaling noted. These hubs are SFO, OVLT, MEPO, AHN, PVN, VMH, LH, MBO, and periventricular neuroendocrine motor zone. Recent evidence suggests that these cell groups control the expression of motivated or goal-oriented behaviors, such as drinking, feeding, sex, aggression, fear, and foraging behaviors (see original Fig. 7 of Zhang et al. 2021 and references therein including Sternson et al. 2013). The rostral segment of this behavior control column has controllers for the basic classes of goal-oriented ingestive, reproductive, and defensive behaviors, common to all animals, where the caudal segment has the controllers for exploratory behavior used to obtain any goal object. Other relevant structures more caudal in this longitudinal brain stem column are the reticular part of SN (not labeled in Fig. 7), which is involved in the control of orientating movements of the eyes and head via projecting to SC, the VTA, Adcyap1-expressing, which together with ACB (intensely Acyap1r1-expressing) and STN (intensely Adcyap1-expressing), form the hypothalamic locomotor region. Other abbreviations are as used elsewhere. Figure is modified from Fig. 7 of Zhang et al. 2021

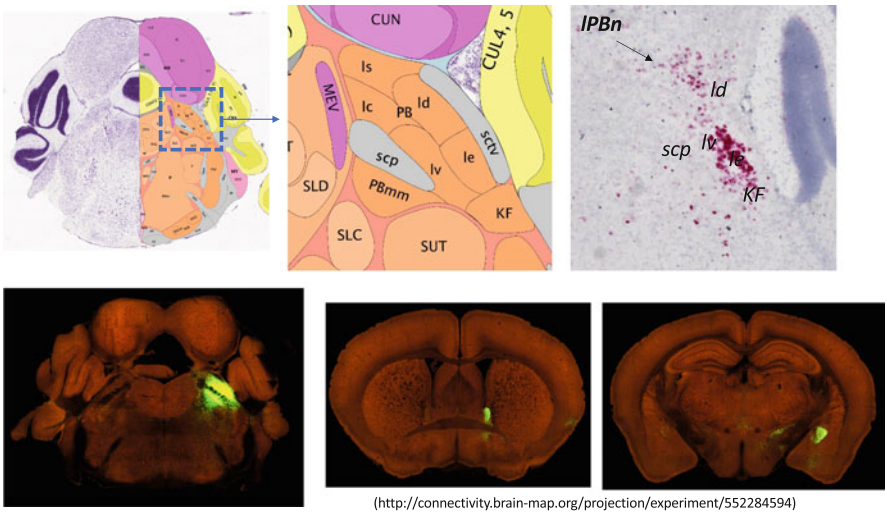

Fig. 15.5 PACAP neurons of the parabrachial nucleus. Top: Single-color Adcyap1 ISHH registered to Allen Brain Map to indicate distribution of PACAPergic neurons across the subdivisions of the parabrachial nucleus, including lateral and extended lateral (ls, lc, ld, lv, le), Koelliker-Fuse (KF), and medial (PBmm) subnuclei. Bottom: From Allen Brain Atlas, depiction of experiment 552284594 in which Cre-dependent GFP delivered into PBN (left) of Adcyap1-Cre driver mice is found in nerve terminals of BNST (middle) and central amygdala (right)

layer five->STN, see below), appears to be a general feature of the PACAP chemoanatomy of the CNS.

The subthalamic nucleus (STN) contains a large complement of PACAP-positive excitatory neurons and receives cortical layer V projection neurons that may also be PACAPergic, consistent with high expression of PAC1 mRNA in STN. In STN (see Zhang et al. 2021, and Fig. 15.1d), PACAPergic neurons are also glutamatergic and likely involved in the extrapyramidal motor system controlling locomotor activity, which coincidentally is elevated in PACAP-deficient mice (Hashimoto et al. 2001; Lehmann et al. 2013).

The circumventricular organs (CVOs) are midline structures that are contained (except for area postrema) in the diencephalon. The CVOs lack a blood-brain barrier and are classified as either secretory or sensory (Kaur and Ling 2017). The secretory CVOs include the neuroendocrine cell nerve terminals of the median eminence (ME), neurohypophysis, and pineal gland. The median eminence (ME) is specialized for secretion of the hypophysiotropic hormones TRH, LH-RH, somatostatin, CRH and GH-RH to the anterior pituitary via the hypothalamo-pituitary portal circulation. Neuroendocrine cells of the neurohypophysis (posterior pituitary) secrete oxytocin and vasopressin into the general circulation from the posterior pituitary, and to a lesser extent the ME. PACAP is contained in neuroendocrine cells projecting to both ME and neurohypophysis, in percentage and intensity of expression that differs markedly among mammalian species. The peripheral actions of PACAP released at these sites, despite PACAP's name implying hypophysiotropic function, remain

somewhat obscure (see Sect. 15.1). Neuroendocrine cells of the pineal gland are specialized for secretion of melatonin to the general circulation. The pineal represents a unique diencephalic structure in that it is innervated by neurons of peripheral origin which express PACAP (the trigeminal ganglion (Moller and Baeres 2003)) or are controlled by preganglionic sympathetic neurons of the superior cervical ganglion which express PACAP (Beaudet et al. 1998). These inputs are themselves under the control of centrally generated circadian control via the SCN, another PACAP-receptive diencephalic (hypothalamic) nucleus. The sensory CVOs include the OVLT, SFO, and area postrema (located in brainstem). Neurons of the sensory CVOs receive critical physiological information from the general circulation via receptors for a variety of hormones. The sensory CVOs also function as osmometers, sensing sodium concentration and communicating directly with neurons projecting to the secretory CVOs, to control the release of vasopressin. PACAPergic cells within the sensory CVOs are glutamatergic (Table 15.1); whether these are the same ones that project to other brain areas to regulate behaviors that enhance salt consumption and otherwise participate in regulation of thirst and salt balance (Zimmerman et al. 2017) is not yet known.

15.4.4 PACAPergic Neurons of the Brainstem

15.4.4.1 PACAP Distribution in Midbrain

Sensory-related structures including inferior colliculus, trigeminal nucleus and parabigeminal nucleus contain excitatory neurons that also express PACAP. More intense Adcyap1 mRNA expression was found, however, in motor-related structures, including superior colliculus (SCm), PAG, and VTA. The accessory oculomotor nucleus (Edinger-Westphal nucleus) expresses PACAP, which is thus a co-transmitter onto parasympathetic postganglionic cholinergic neurons innervating the iris sphincter and ciliary muscles of the eye.

15.4.4.2 PACAP Distribution in Hindbrain

In the pons, *Adcyap1* is expressed prominently in the parabrachial complex, where it is co-expressed with VGLUT1 in Kölliker-Fuse and with VGLUT2 in adjacent PBN, and in the lateral division of the superior olivary complex, lateral lemniscus nucleus and trigeminal (cranial V), abducens (cranial VI), facial (cranial VII), and vestibulocochlear (cranial VIII).

The medulla oblongata contains PACAPergic cells in the pyramid, the olive-superior olivary complex lateral part, the trigeminal nucleus (V), the nucleus ambiguous, the nucleus cuneatus, the nucleus gracilis and the hypoglossal nucleus, as well as in the major target for visceral sensory input to the brain, the nucleus tractus solitarius (NTS). As the NTS is viewed increasingly as a structure that parcellates, rather than merely relaying visceral sensory information to the brain, attention to its chemoarchitecture and its relationship to specific sensory processing functions of PACAP neurotransmission may be a fruitful experimental inquiry.

15.4.5 PACAPergic Neurons of the Cerebellum

The cerebellum comprises the flocculo-nodular lobe, the vermis, the hemi cortices, and the so-called deep cerebellar nuclei. In both neonatal animals, during which time most of the neurons of the cerebellum are generated and find their positions, and in adult mammals, PACAP is expressed in the inhibitory Purkinje cells of the cerebellar cortex. However, PACAP may have very different functions upon release from these neurons in developing and adult mammals (see Sect. 15.6).

15.4.6 PACAPergic Neurons of and Inputs to the Spinal Cord

PACAP innervation of the dorsal horn via dorsal root ganglion inputs is considered to modulate nociception, as dramatic increases in PACAP expression accompany inflammation-induced pain (Zhang et al. 1998; Dickinson and Fleetwood-Walker 1999; Jongsma et al. 2001) and a PAC1 antagonist is reported to alleviate allodynia after nerve constriction (Takasaki et al. 2019a, 2019b). PACAP is expressed in sympathetic preganglionic neurons of the intermediolateral column and is co-released from the splanchnic nerve to mediate catecholamine release associated with metabolic (hypoglycemic) (Hamelink et al. 2002) and presumably psychogenic stress as well. The chemoanatomy of the sympathetic nervous system suggests that PACAP should play an equally prominent role in stress predominantly affecting sympathetic rather than sympathoadrenal output, e.g. cold stress. However, this has not yet been investigated in vivo, although the effects of PACAP on postganglionic sympathetic neurons in culture have been studied extensively (May and Braas 1995; Brandenburg et al. 1997; Beaudet et al. 1998).

15.5 PACAP Chemoanatomy and Function within Brain Circuits

15.5.1 PACAP in Hypothalamic Circuits

We begin this section by grouping PACAPergic circuits that are primarily hypothalamic (Fig. 15.4). The hypothalamus contains a high density of PACAPergic neurons, and a high content of immunoassayable PACAP, in rat, mouse, and primate brain (Vigh et al. 1991; Ghatei et al. 1993; Palkovits et al. 1995; Hannibal 2002; Zhang et al. 2021). The nine prominent "PACAPergic" hypothalamic nuclei depicted in Fig. 15.4 (flatmap depiction after (Swanson and Bota 2010), and see (Sternson 2013)) have two prominent features in common. These are that (a) Adcyap1 mRNA is co-expressed with the glutamatergic marker VGLUT (see Table 15.1); and (b) Adcyap1r1 mRNA is expressed at high levels in each of these nuclei. What is *not* known, or only to some degree for each, is: what is the specific physiological consequence of lack of expression or antagonism of PACAP signaling in that nucleus? how does PACAP abet glutamate post-synaptically in projections from that nucleus? does PACAP act in an autocrine fashion upon dendritic release in

any of these nuclei? These questions are relevant to the ongoing inquiry into how regulatory peptides of the brain may act in concert through multiple circuits for behavioral state coding. Recently, Wu and colleagues, using CaRMA (calcium imaging and RNA multiplexed activity), examined the relationship between neuronal activation in the PVN and the subpopulations of neurons activated, across eleven behavioral states (Xu et al. 2020). Based on the behavior of cells categorized by twelve marker genes, including Crh, Pdyn, Penk, Trh, Oxt, Avp, and Sst, these authors concluded that rather than exhibiting a pattern of activation that matched cell type to physiological task or response (labeled-line coding), molecularly defined neurons of the PVN appear to respond similarly within type, but that behavioral state is likely encoded by *combinations* of cell types. Thus, critical survival-promoting physiological responses that require multiple homeostatic adjustments and allostatic adaptation of behavior also require, perhaps not surprisingly, an ensemble response within critical hypothalamic nuclei such as the PVN. This highlights the need to further expand experimental tools for neuronal "tagging," based upon neuropeptide expression, in order to fully divulge the underlying "circuit logic" encoded within the chemical neuroanatomy of neuroendocrine cell ensembles underlying neuroendocrine responses that lead to motor decisions important for survival. However, what remains to be urgently addressed, especially for translational neuroendocrinology, is the question of the post-synaptic *functions* of the regulatory peptides co-released in ensemble fashion by physiological responses to thirst, hunger, threat, stress, and sexual drives.

Sections 15.5.1.1–15.5.1.4 highlight examples of hypothalamic nuclei in which the function of PACAPergic neurons has been probed with Adcyap1-Cre driver mice in which opto- and chemogenetic manipulations have been carried out. It should be noted that an understanding of how PACAP acts as a neurotransmitter to effect function within these neurons remains to be defined. Likewise, these sections describe hypothalamic nuclei in which a function for PACAP has been identified by pharmacological receptor blockade and/or regiospecific PACAP knock-out, but for which the specific neuronal populations and PACAP's co-transmitters in them have not yet been clearly identified. Filling in the critical gaps in these studies is key to a comprehensive systems understanding of how PACAP and other regulatory peptides act to unite neural, neuroendocrine, and endocrine axes in mammalian neuroendocrinology.

15.5.1.1 Retinohypothalamic Tract

The retinohypothalamic pathway is a glutamatergic projection from melanopsin-containing retinal ganglion cells (intrinsically photosensitive, or ipRGCs) to the suprachiasmatic nucleus (Hannibal et al. 2002) whose functions are manifestly altered by genetic deletion of PACAP expression (Colwell and Waschek 2001; Colwell et al. 2004). The retinohypothalamic synapse in the SCN is one of the few central synapses at which the relative contributions of PACAP and its co-transmitter glutamate have been characterized. In vivo, light administered at early and late (subjective) night causes phase delay and phase advance (in wheel running), respectively, and in PACAP KO mice phase delay is unperturbed while phase advance is

eliminated (Kawaguchi et al. 2003; Beaule et al. 2009). In SCN slices ex vivo, application of glutamate at early and late (subjective) night causes phase delay and phase advance (in electrical activity), respectively, and in SCN slices from PACAP KO mice phase delay is unperturbed while phase advance is eliminated but can be restored by bath application of PACAP along with glutamate (Lindberg et al. 2019). As phase advance (but not delay) was similarly lost in slices from enucleated mice, the source of PACAP was confirmed as arising from the retinohypothalamic tract. Furthermore, the mechanism of interaction between glutamate and PACAP in this system must, given the ex vivo results, involve glutamate release from ipRGC projections to SCN upon late-night light exposure followed by release of PACAP from these terminals, which is somehow triggered by glutamate and leads to cooperative activation of circadian pacemaker cells of the SCN itself. This intriguing mechanism of neuropeptide-glutamate pre- and post-synaptic interaction awaits elucidation.

15.5.1.2 avMLPA: Location of PACAPergic Neurons Mediating Metabolism and Thermoregulation

The identification of specific PACAPergic neuronal groups, involved in specific physiological "tasks" in hypothalamus, has been accelerated by techniques for transcriptomic profiling of neurons activated by specific physiological events. One of these is "the phosphoTRAP" method, in which increased phosphorylation of ribosomal protein S6, associated with neuronal activation, allows ribosomally-mediated capture of mRNA transcripts engaged in translation coincident with neuronal activation (Knight et al. 2012). Using this approach, Tan et al. (2016) identified warm-activated neurons in the preoptic area, distinguished by robust co-expression of BDNF and PACAP, and distinct from other major cell groups of the preoptic area, including those expressing galanin (involved in parental behavior), LH-RH (involved in reproductive behavior), and responsive to leptin (involved in feeding behavior). Using AAV-mediated calcium sensor expression specifically in Adcyap1-expressing cells, Tan et al. were able to demonstrate the activation of these cells after warm-chamber test, and the induction of hypothermia, thermoregulatory behaviors, and inhibition of brown fat thermogenesis via a GABAergic projection to the DMH following their optogenetic activation. Whether these PACAPergic neurons are themselves the GABAergic neurons that control brown fat thermogenesis via inhibition of DMH projections to peripheral autonomic neurons, or control such GABAergic neurons, is an as-yet unresolved question. If the former, the demonstration that the PACAPergic neurons of this area by Hrvatin et al. (see Fig. 15.1f with this area labeled as "avMLPV") are in fact excitatory would imply their ability to release both GABA and glutamate depending on environmental inputs.

Torpor is a hibernation-like condition of dramatically decreased core temperature, oxygen consumption, and locomotor activity, induced by fasting. Torpor-regulating neurons lie within the hypothalamus. Hrvatin and colleagues (Hrvatin et al. 2020) have recently studied the topology of these neurons in detail, and in the process revealed that PACAPergic neurons of the preoptic area are a key component of this

microcircuitry. Genetic control (both chemical and light) over torpor-inducing neurons was achieved using FosTRAP methodology to engineer light- and designer drug-dependent protein expression into neurons in which fasting induces transient activation (fos expression). This in turn allowed identification of the anteroventral mediolateral preoptic area (avMLPA) as a site of neurons activated during torpor induction, and whose activation is in fact required for torpor induction. Single-cell transcriptomics of the avMLPA identified a cluster of neurons expressing both the vesicular glutamate transporter VGLUT2, a marker for excitatory neurons, and PACAP. Genetic engineering of these neurons in turn using an Adcyap1-Cre strategy to allow either in vivo observation, or exogenous control, of their excitability further established that this neuronal population is activated by fasting, and that silencing of this population within the avMLPA is sufficient to block torpor induction. Where do these torpor-inducing PACAPergic excitatory neurons project? Cells in avMLPA engineered to express proteins allowing exogenous control of their activity upon fos activation by fasting were also marked with mCherry to ascertain their projection fields, which were found to include PVT, PAG, MHb, DMH, ARC, and RPa, consistent with a highly divergent transmission of information within the brain to coordinate the multiple aspects of movement and metabolism that require control in order to induce torpor (Hrvatin et al. 2020).

As is true for PACAP-glutamate co-transmission (or PACAP-GABA co-transmission) in other contexts and other brain areas, and indeed at virtually all PACAPergic synapses centrally and peripherally, insight into how PACAP and glutamate (and PACAP and GABA) cooperate, or if they do, to execute physiologically relevant synaptic actions within these circuits remains to be attained.

15.5.1.3 PACAPergic PVN to ARC Projections Regulating Satiety and Feeding

Investigating hypothalamic circuits that modulate hunger motivation in mice, Krashes et al. (2014) showed first that ablation or optogenetic inhibition of AgRP arcuate neurons, normally activated by caloric deficiency, induced intense hunger and food intake in mice. These authors attempted to look upstream of these neurons to find the excitatory neurons that receive the sensory/environmental inputs normally regulating feeding motivation, and drive activation of arcuate AgRP "hunger-effector" neurons. They injected a Cre-dependent retrograde rabies virus into the arcuate nucleus of AgRP-Cre mice and found major inputs to these neurons from dorsal medial hypothalamus and paraventricular hypothalamus (PVN). With channelrhodopsin-assessed circuit mapping, it was confirmed that excitatory neurons in PVN cause activation of AgRP neurons in the arcuate nucleus. The authors used the Allen Brain Atlas to identify six neuropeptides expressed in PVN (dynorphin, oxytocin, vasopressin, CRH, TRH, and PACAP) and injected Cre-dependent ChR2 into PVN in Cre-driver mice for each of the six neuropeptides. Only the TRH-Cre and Adcyap1-Cre driver mice generated light-activated excitation of AgRP arcuate neurons after Cre-dependent ChR2 injection into PVN, and caused light-activated feeding behavior in food-sated mice. PACAP did activate AgRP neurons when infused onto synaptically isolated AgRP neurons, resulting in PAC1-dependent

cellular depolarization. This set of experiments did not address whether PACAP alone, glutamate alone, or only the combination of both cause excitation of arcuate AgRP neurons in response to hunger. Interestingly, this projection appears to be specific for AgRP neurons, and not, for example, adjacent POMC neurons, in ARC. A DREADD expressed in PVN PACAPergic neurons causes fos activation of AgRP neurons in vivo, and bilateral h3MD1-DREADD-mediated activation of PACAPergic PVN neurons also caused increased food intake during the light cycle. It is not known whether the PACAPergic neurons of PVN go *only* to AgRP neurons, or project divergently to other brain regions as well. In any event, it is of interest that a group of PACAPergic neurons in the PVN control activity in other hypothalamic areas, while at the same time PACAPergic inputs (of unknown origin) innervate the PVN itself and regulate CRH neurons there, in the context of stress modulation of the neighboring HPA axis, which acts quite independently of the PACAPergic neurons projecting to the ARC and involved in regulation of feeding.

15.5.1.4 PACAPergic VMN Projections Involved in Feeding, Thermogenesis, and Glucoregulation

PACAP-expressing neurons of the VMN project to other hypothalamic nuclei, including AHN, PVN, to thalamus, to the superior colliculus and, prominently, to the PAG, according to the distribution of fluorescent protein labeling following injection of Cre-dependent AAV vector into the VMN of Adcyap1-Cre mice (http://connectivity.brain-map.org/projection/experiment/303708513; http://connectivity.brain-map.org/projection/experiment/552759734). VMN projections to PVN, revealed as cholera toxin subunit B (CTB) in PACAP mRNA-expressing PVN neurons after CTB injection into VMN, may account for the action of PACAP in regulating food intake upon local injection into PVN (Resch et al. 2013). On the other hand, PACAP injection into VMN, which is also rich in expression of the PAC1 receptor, results in elevation of core temperature and increased UNC1 expression in brown adipose tissue, as well as suppression of feeding after fasting (Resch et al. 2011), which may in turn reflect control of VMN neuronal activity via PACAPergic afferents from medial amygdala or lateral PB (Resch et al. 2013). Intriguingly, induction of hypophagia following PACAP injection into VMN is accompanied by increased phosphorylation of NMDA receptor GluN2B subunits, and is blocked by co-administration of the NMDA antagonist APS, suggesting that the mechanism of PACAP action may be via post-synaptic augmentation of the effects of glutamate co-released from PACAP/glutamate inputs to the VMN (Hawke et al. 2009; Resch et al. 2014).

The brain contains multiple glucose-sensing neurons; those of the hypothalamus are concentrated in the VMN. The VMN also contains PACAPergic neurons which are leptin-responsive. PACAPergic neurons of the VMN are mainly if not exclusively glutamatergic (see Table 15.1; Fig. 15.1a, 15.4, and Zhang et al. 2021 and references cited therein). Using Adcyap-Cre mice for specific identification of PACAPergic cells of the VMN, Khodai et al. demonstrated that these cells, via efferents to several brain areas (see above), are intrinsically glucose-inhibited, and

that their selective chemogenetic stimulation inhibits insulin secretion (Khodai et al. 2018).

15.5.1.5 PMVv->ARC PACAPergic Projections Involved in Reproductive Development

A second PACAPergic input to ARC arises from neurons originating in the ventral premammillary nucleus (PMV) and innervating kisspeptin ARC neurons. PMV^{PACAP} neurons participate in scheduling of puberty onset in female mice by stimulation of kisspeptin arcuate neurons which in turn project to and activate the LH-RH-secreting neuroendocrine cells located in POA (Ross et al. 2018). Similarly to other investigations of this type, the question of whether PMV^{PACAP} neurons activate their targets via glutamate release alone, or via co-transmission with PACAP itself, is yet to be answered.

15.5.1.6 Progress in Unraveling the Physiological Functions of PACAPergic Cell Groups Contained in MEPO, ANH, LH and MBO of the Hypothalamus

As shown in Fig. 15.4 and mentioned in Sect. 15.4, there are four additional functionally prominent hypothalamic nuclei in which PACAP expression is prominent, yet its role is not yet defined. The median preoptic nucleus, like the nearby LPO, to which it is connected, is important in regulation of osmolality, via connections from the (also PACAP neuron-rich) anterior circumventricular organs. The MEPO (also frequently abbreviated as MnPO) contains both excitatory and inhibitory projection neurons (as well as interneurons): the fact that PACAP is found only, or almost exclusively, within excitatory (VGLUT2-expressing) neurons of the MEPO (Table 15.1) provides initial clues to their potential functions in MEPO-dependent thermoregulation, osmoregulation, and sleep regulation processing. Like the MEPO, the ANH is involved in the regulation of both body temperature and sleep, and again like the MEPO, the PACAPergic neurons of the ANH are mainly or exclusively excitatory (VGLUT2-expressing) (see Table 15.1, Fig. 15.1a). The lateral hypothalamic area (LH) is the major source of orexigenic (orexin-containing, hypocretinergic) neurons within the hypothalamus, has prominent connections to the autonomic nervous system, and has more recently been implicated in neuroinflammatory signaling, important in conveying pain signals associated with rheumatoid arthritis to the brain (Fakhoury et al. 2020). A significant fraction of the excitatory (VGLUT2-positive) neurons of the LH are PACAP-expressing (Table 15.1 and see Zhang et al. 2021). Finally, the MBO contains a very high density, compared to most other hypothalamic nuclei, of PACAP-positive neurons (Table 15.1, Hannibal 2002; Zhang et al. 2021). The MBO, through connections to the hippocampus, other limbic nuclei and thalamus, mediates important memory functions of the brain. Tracing studies using Adcyap1-Cre for Cre-dependent, fluorescent marker expression, have shown that injections into MBO result in prominent labeling of dentate gyrus, lateral septum, and the anteromedial nucleus of the thalamus (http://connectivity.brain-map.org/projection/experiment/307655867; http://connectivity.brain-map.org/projection/experiment/558673113).

In each of these nuclei, the PACAP receptor PAC1 is prominently expressed (see Zhang et al. 2021), indicating that PACAP function may include either PACAP projections to these nuclei, an autocrine function of PACAP within them, or both.

15.5.2 PACAP in Extrahypothalamic Circuits

While several hypothalamic nuclei express PACAP, and it is functionally important there, the role of PACAP extends throughout the brain axis: PACAP is by no means a "hypothalamic peptide," despite its name. In fact, as shown in Fig. 15.5, the distribution of PACAPergic neurons through the entire neuroaxis exemplifies its importance in the processing of sensory information leading to motor outputs, including neuroendocrine outputs, and behaviors that promote individual and species survival through prioritization of eating, drinking, reproduction, and avoidance of threat through aversion.

15.5.2.1 PBN->PACAPergic Projections to BNST and CeA

The parabrachial nucleus (PBN) of the hindbrain, situated near the mesencephalic-metencephalic boundary, is an important relay station for conveying viscerosensory inputs to the brain, including gustatory aversive and nociceptive inputs (Chiang et al. 2019). The PBN richly expresses a variety of neuropeptides, including PACAP, substance P, CGRP, and dynorphin. Recently, mice expressing Cre recombinase, knocked into genes encoding these peptides, have been used in intersectional and optogenetic experimental approaches designed to unravel the functional neuroanatomy of this brain region and its involvement in mediating a variety of behaviors associated with aversion, threat avoidance, and survival. Projections from PBN to extended amygdala mediate processing of sensory salience relevant to nocifensive responding, broadly defined, and including taste, sodium intake, respiration, pain, thermosensation, and appetite suppression (Carter et al. 2013). These signals are conveyed, in parallel with both input and output autonomic information relayed within the PBN, by segregated subnuclei, and even chemically coded subpopulations within specific subnuclei. For example, stimulation of CGRP-expressing neurons of PBNel (labeled as "le" in Fig. 15.5) can dramatically reduce food intake, mimicking conditions under which food intake is unfavorable, and inhibition of these neurons can enhance food intake even in mice with sharply reduced food intake due to ablation of hypothalamic AgRP neurons (Carter et al. 2013). These same neurons are required for, and can mimic, conditioned taste aversion, a learning mechanism used by mammals for future avoidance of tastes associated with gastrointestinal distress following food intake (Carter et al. 2015). CGRP-positive PBNel neurons projecting to capsular CeA also receive sensory inputs associated with pain, and their activation is required for establishment of "threat memory," i.e., association between pain and pain-attendant sensory cues that can motivate nocifensive behaviors (Han et al. 2015).

The brainstem parabrachial nucleus contains a collection of PACAP-expressing excitatory neurons (Zhang et al. 2021) that project to the extended amygdala in the

mouse (Jiang et al., unpublished observations; Allen Brain Atlas http://connectivity.brain-map.org/projection/experiment/301016900 and 552284594; (Fig. 15.5). This pathway has previously been shown, in the rat, to innervate the CeA and ovBNST and to co-express CGRP (Missig et al. 2014). Infusion of PACAP into CeA decreased latency of response to thermal stimulation, as did infusion of the PAC1-specific agonist maxadilan (Missig et al. 2014). Furthermore, chronic neuropathic pain increases PACAP expression in CeA, and infusion of the PAC1 antagonist PACAP(6–38) into the central amygdala blocks anxiety and pain sensitivity behaviors associated with chronic (sciatic nerve constrictive) pain (Missig et al. 2017). The PBN projections to BNST and CeA have received additional attention as candidates for mediating the BNST-specific effects of PACAP on behaviors associated with drugs of abuse ((Hammack and May 2015) and see Sect. 15.4.3). Recently, we have reported the effects of PACAP knock-out on defensive behavior of mice presented with predator odor. Wild-type (WT) mice respond to predator odor by freezing, avoidance, retreat after brief exploration and elevation of fos in parabrachial nucleus (PB), its targets (CeA, BNST) and other PACAPergic brain nuclei, while PACAP-deficient (KO) mice exhibit increased exploration of chamber, lack of freezing or retreat, and blunted fos activation of the same brain nuclei (Zhang et al. 2021).

PACAPergic projections to both CeA and BNST from PBn are very likely to overlap (Missig et al. 2014) with the CGRP-expressing populations involved in threat responding and described above, assuming an equivalence in rat and mouse chemoanatomy in this nucleus. Whether PACAP functions within sensory inputs to both PBN and olfactory cortex, to mediate defensive responses to threat, remains to be explored. As mentioned above, a considerable number of studies based on CGRP-Cre driver mice point to the extended lateral PBn as a source of neurons whose stimulation halts food consumption (and see (Campos et al. 2016)). These are likely to be the same as, or highly overlapping with, the PBn neurons co-expressing CGRP and PACAP and innervating CeA. However, comprehensive studies to demonstrate that these two populations are the same have not been performed. Perhaps more importantly, the actual role(s) in neurotransmission of either CGRP or PACAP in this projection have yet to be elucidated.

15.5.2.2 BNST-Intramural and Extramural "Cocaine Relapse and Stress Circuit"

The bed nucleus of the stria terminalis is a small but highly differentiated nucleus, or group of nuclei, lying above and below the anterior commissure and comprising about a dozen distinct subnuclei. It is difficult to avoid two pitfalls in approaching the BNST: one is to consider it as a single entity, and the other is to view it as a dozen disparate entities. Dumont (2009) steers an admirable middle course in considering the BNST as "a relay center within neurocircuits coordinating the activity of autonomic, neuroendocrine and somatic motor systems. . .where descending cortical information meets ascending interoceptive and exteroceptive [sensory] inputs . . .from cortical to brain stem and spinal cord [with] descending projections to motor areas of the hindbrain that. . .trigger or contributed to . . .coordinated

physiological and behavioral responses necessary for a well-balanced homeostasis". Dumont further advises conceptual division of the BNST into an anterior group specializing in energy balance and a posterior group involved in reproduction and defense, which are themselves highly interconnected. This summary, albeit more than a decade old, seems a remarkably prescient blueprint for comprehension of the role of PACAP in mediating stress and other responses (and in integration with other peptidergic systems) via synapses within the busy circuit crossroads which is the BNST.

Lebow et al. (Lebow and Chen 2016) concisely summarize (despite transposing the legends of the otherwise informative Figs. 15.1 and 15.2) most of the connections between the BNST and the rest of the brain. Thus, various subdivisions of the BNST *send* projections to amygdala, hypothalamus, lateral septum, and VTA, while *receiving* projections from olfactory bulb, frontal cortex, lateral septum, ventral striatum, amygdala, hypothalamus, locus coeruleus, dorsal raphe, VTA, and NTS. The ovBNST, in particular, receives projections from VTA and raphe nucleus, and sends projections to VTA, hypothalamus, and amygdala. These anatomical observations do not include projections from PBN to BNST, which are PACAPergic (vide infra), and also do not incorporate *intra-BNST* projections that, as pointed out by Dumont, allow further integration of the BNST as a holistic structure, with other brain areas and functions.

The ovBNST, in particular, has received attention as a "PACAPergic" brain nucleus because of the role of PACAP, acting at putative ovBNST synapses, in stress-dependent cocaine relapse in the rat (Miles et al. 2018). Previous work from the reporting laboratory had established that BNST lesions blocked behavioral manifestations of uncontrollable stress (Hammack et al. 2004); that stress modulates the PACAP mRNA content of the BNST (Hammack et al. 2010); that the PACAP content of the BNST was depleted by PBN lesions; and that PACAP infusion into the BNST mimics the cocaine-relapsing effects of stress, as does PACAP(6–38) infusion (Miles et al. 2018, 2019). On that basis, the authors confirm the role of a proposed PACAPergic circuit previously implicated in stress modulation (Hammack and May 2015) but leaving open the question(s) of the nature of PACAPergic target cells in BNST, which have been suggested to be CRH-containing neurons (Hammack et al. 2009); the role and nature of PACAPergic neurons contained within BNST; and the supplementary role of PACAPergic projections to BNST from amygdala/interstitial nucleus, relative to projections from PBN, in mediating stress-induced cocaine reinstatement.

15.5.2.3 Amygdalar->BNST

Rajbhandari and colleagues have recently posted a data set (Rajbhandari et al. 2021) that describes a function for PACAPergic neurons of the basomedial amygdala, a sparse population of neurons that co-express the vesicular glutamate transporters VGLUT1 and 2 (Zhang et al. 2021). Projections of these neurons to the medial intercalated cells (mICCs) of the amygdala are reported to modulate, through PAC1 receptor engagement, fear conditioning in mice in a sex-dependent manner, decreasing fear generalization in males, and enhancing fear acquisition in females

(Rajbhandari et al. 2021). This PACAPergic projection is conserved between rat and mouse, although sharp differences exist between these two rodent species in other amygdalar regions including, interestingly, the mICC itself, in which PACAP expression is sparse or absent in the mouse, and more abundant in rat (Zhang et al. 2021).

15.5.2.4 BAC: A PACAPergic Brain Nucleus

The BAC appears to contain a sufficiently high percentage of VGLUT2/PACAP co-expressing neurons to be considered a "PACAPergic nucleus." It is thus highly likely that its projections, determined by classical lesioning and tracing techniques, are PACAPergic, although this would need to be confirmed experimentally using neurochemically specific tracing techniques. Where does this "PACAPergic nucleus" project? Yamaguchi et al. have dissected the inputs of the adjacent triangular septum (TS) and BAC using the immunotoxin-mediated cell targeting technique. A human IL2R-GFP fusion protein was transgenically expressed under the control of the mGluR2 promoter. Immunotoxin (IT), interacting with IL2R results in the killing of mGluR2-positive cells upon injection into TS or BAC. GFP fluorescence in TS and TS projections to ventral MHb were extinguished by IT injection into TS, while GPF fluorescence in BAC and its projections to dorsal MHb were extinguished by IT injection into BAC. Specific behaviors were impaired, mutually exclusively, by IT injection into TS, including decreased open-arm exploration of the elevated plus maze and increased marble-burying, indicative of anxiety, and IT injection into TS, including decreased shock-induced avoidance learning (Yamaguchi et al. 2013). Both pathways impinge upon MHb cholinergic neurons projecting to the IPN. As yet, the specific role of PACAP, either independently of or in synergy with glutamatergic neurotransmission, is unexplored.

15.5.2.5 Projections to Hippocampus-Locus of Massive Expression of PAC1

The level of expression of *Adcyap1r1* in dentate gyrus of hippocampus is quite intense (Zhang et al. 2021), while *Adcyap1* levels are quite low. What PACAPergic projections might be operative at PAC1 receptors on granule cells of the dentate gyrus (DG)? Possibilities include PACAPergic neurons of the hilus and inputs from entorhinal cortex. PACAPergic neurons from MM also project to DG (http://connectivity.brain-map.org/projection/experiment/301016900 and 558673113). The source of PACAP input to CA1 mediating the robust electrophysiological effects in pyramidal cells after exogenous PACAP administration also remains to be identified and is of particular interest as PACAP effects appear to be mediated through the Gq- rather than Gs-GPCR signaling (Macdonald et al. 2005; MacDonald et al. 2007; Costa et al. 2009).

15.5.2.6 Cerebellum and PACAP

PACAP expressed in GABAergic Purkinje cells plays distinct roles during development and in the adult cerebellum (Coenen and Sejnowski 1995; Galas et al. 2017). During cerebellar cortical development, which occurs during early postnatal life,

PACAP is postulated to play a role in stimulating the migration of granule cells from the outer to the inner granule layer (Cameron et al. 2007), and may be involved in protection from early life neurotoxic insult (Vaudry et al. 2005). Whether PACAP release is synaptic or paracrine in this context is unknown. In adults, on the other hand, PACAP seems likely to function directly at Purkinje cell synapses, either autaptic or intercellular, since in these cells fos up-regulation attendant upon predator odor exposure is abrogated in PACAP-deficient mice (Zhang et al. 2021).

15.5.2.7 PACAP and Habenula ("Choosing a Defensive Response to Threat Is Essential for Survival")

The chemoanatomy of PACAP-positive neurons of lateral and medial habenula is described in Sect. 15.3. For both divisions of the habenula, PACAP is found in excitatory neurons (Table 15.1; Fig. 15.1h; (Zhang et al. 2021)). However as these are only a fraction of the total VGLUT-positive cells in habenula, their specific role in the general functions of the LHb and MHb via the known output pathways for each subdivision of the habenula remains uncertain.

15.5.2.8 PACAP Projections from Cerebral Cortex

As mentioned in Sect. 15.4.2, PACAP expression in isocortex is found almost exclusively in excitatory neurons, and is greatest in layers two and five, where the majority of neurons co-express *Adcyap1* and the vesicular glutamate transporter *Slc17a7* (Zhang et al. 2021). As a portion of the layer 5 Adcyap1-expressing neurons co-express PAC1, the possibility of autocrine regulation of these projection neurons exists. In addition, PACAP may also be co-released with glutamate at nerve terminals in STN and other layer 5 projection neuron targets.

15.5.3 Neuropeptide Circuits Can Be Tiny, but Meaningful, as Well as Plastic

Some brain areas have clusters of PACAP-expressing neurons whose functional significance is as yet unknown. An example is the small number of PACAPergic neurons of the mouse ventral tegmental area (VTA), which may have a special function there (Poulin et al. 2014), similar to the several clusters of galaninergic neurons in POA that control different aspects of parental behavior (Wu et al. 2014; Kohl et al. 2018). Is this subpopulation meaningful because PACAP functions as a transmitter here, or because PACAP expression "marks" the properties of this neuronal population but is not itself consequential in its function? Differences between trophic and neurotransmitter functions of PACAP may reveal themselves, in part, by differences between "acute" PACAP-regulated genes (aPRGs), transcripts induced by environmental changes such as stress in a PACAP-dependent manner, and "constitutive" PACAP-regulated genes (cPRGs) which are transcripts which are dramatically more or less abundant in PACAP-deficient compared to wild-type mice and likely represent transcripts whose expression is triggered by PACAP signaling during development. Similarly, increases in PACAP expression detectable after a

particular change in behavioral state, such as chronic stress (Hammack et al. 2009) or drug treatment, may be due to either an increase in PACAP expression within existing cells (e.g., as in PVT after ethanol administration (Gupta et al. 2018)) or represent the creation of new PACAPergic cells. The latter would mean that in some cases PACAPergic populations wax and wane physiologically, perhaps inducing new functional pathways for stress responses via circuit-level cellular plasticity.

15.6 PACAP as a "Master Regulator" of the Stress Response: Crossing CNS/PNS Boundaries

Overall, PACAP release is required for stress responses at the level of adrenocortical (corticosterone) and adrenomedullary (catecholamine) hormone output, and at the level of altered behavior(s). Peripherally, at the splanchnico-adrenomedullary synapse, PACAP is released to interact with PAC1 receptors on chromaffin cells, facilitating catecholamine release and increasing capacity for catecholamine biosynthesis required for survival from prolonged insulin-induced hypoglycemia, for example (Hamelink et al. 2002). As PACAP is required at the "final common synapse" of the so-called sympathoadrenal axis, adrenomedullary activation by either psychogenic or metabolic stress is PACAP-dependent (Fig. 15.6). Centrally, PACAP influences the activation of the hypothalamo-pituitary-(cortico)adrenal axis in response to psychogenic stressors such as restraint and social defeat, and not to systemic stressors such as inflammation, cold, or hypoglycemia (Stroth and Eiden 2010; Stroth et al. 2011; Tsukiyama et al. 2011; Lehmann et al. 2013; Stroth et al. 2013). Mammalian organismic response to psychogenic or emotional stress comprises both endocrine and behavioral limbs. PACAP affects the endocrine limb at the level of the splanchnico-adrenal synapse (vide supra) peripherally, and the activation of the HPA axis centrally. Intracerebroventricular injection of PACAP causes elevation of CRH mRNA levels in PVN (Grinevich et al. 1997). The effects of PACAP on HPA axis activation likely occur via PACAPergic synapses upon CRH neurons of the PVN (Legradi et al. 1998). These effects are probably mediated through PAC1 receptors, as the effects of PACAP deficiency on CORT elevation after chronic restraint stress are mimicked in PAC1-deficient mice (Mustafa et al. 2015), and CRH mRNA elevation after PACAP administration is blocked by the PAC1 antagonist PACAP(6–38) (Grinevich et al. 1997). It is important to emphasize, in the context of the relationship between PACAP chemoanatomy and PACAP function, that the effect of PACAP neurotransmission to CRH neurons during stress is not to mediate CRH *release* to the pituitary, for subsequent stimulation of ACTH secretion, but rather to *sustain* the ability of these neurons to release CRH by stimulation of CRH mRNA production, thus allowing a higher rate of CRH peptide biosynthesis to compensate for its depletion during high levels of secretion (Jiang and Eiden 2016a, 2016b). Thus, PACAP deficiency has no effect on CORT elevation after a short period (one to two hours) of a single restraint stress, but does attenuate CORT elevation when stress occurs continuously for three hours, or after at least two hours of restraint stress repeated over several days. In contrast, the effect of

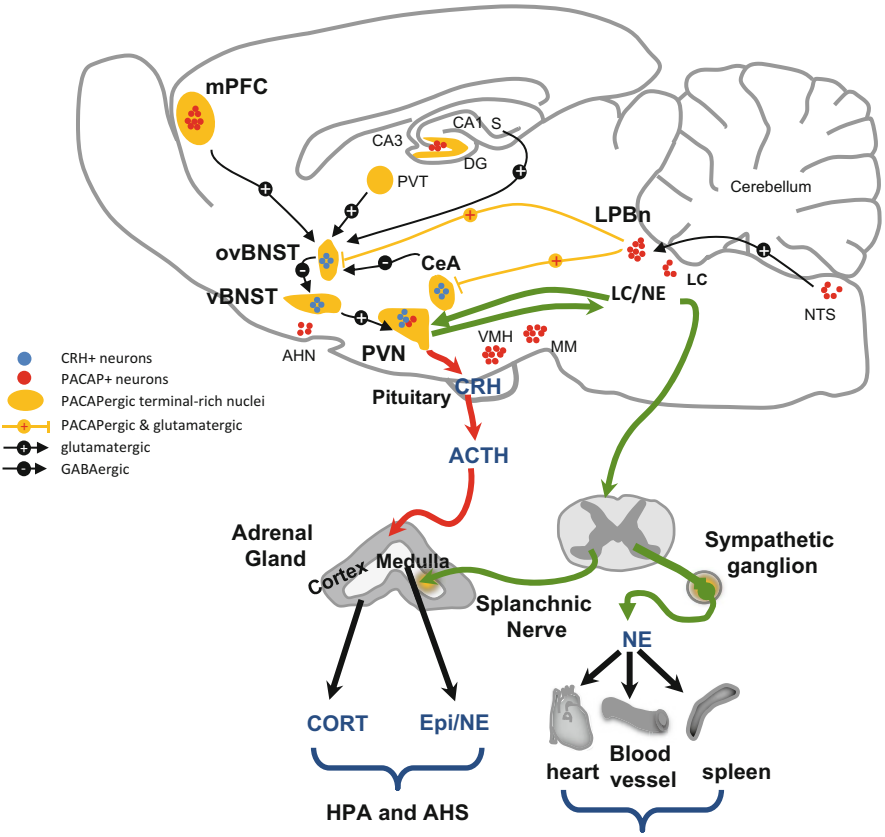

Fig. 15.6 PACAP, a master regulator of the stress response. Sites of PACAP synapses in brain and periphery are highlighted in yellow. In the periphery, these include sympathetic ganglia and adrenal medulla. In the brain, PVN-CRH and locus coeruleus-norepinephrine (LC-NE) systems are the central effectors of the stress response and stimulate each other in a positive feedback mechanism. CRH neurons in the PVN of hypothalamus are the principal starting point for activation of the hypothalamo-pituitary-adrenal (HPA) axis. CRH stimulates ACTH secretion from the pituitary, which subsequently elevates CORT release from adrenal cortex. Systemic stressors and psychogenic stressors stimulate HPA axis activation through largely distinct pathways. Systemic stressors are usually perceived as an immediate threat to survival and homeostasis. They stimulate rapid catecholamine secretion from the sympathetic nervous system (SNS) and are directly communicated to PVN to stimulate norepinephrine (NE)-directed CRH release. This process is not reinforced by higher sensory brain centers. In contrast, psychogenic stress recruits complex inputs to the PVN from multiple cortical, limbic structures as well as brain stem that mediate stress-induced adaptive/maladaptive behaviors, such as fear, anxiety, arousal, feeding response changes, and other responses. PACAPergic synapses densely innervate CRH neurons in PVN. PACAP synapses are also present in extrahypothalamic sites, such as mPFC, nucleus accumbens, BNST, amygdala, PVT, hippocampal DG, and PAG, where the PACAP-specific receptor PAC1 is highly expressed. For further details, see text Sect. 15.6

PACAP deficiency on resistance to restraint-induced hypophagia leading to acute weight loss is already evident in the PACAP-dependence of anorexia induction by as little as one hour of restraint, indicating that this effect is more likely to be triggered by acute effects of PACAP-dependent secretion at synapses elsewhere than those mediating HPA axis activation.

Figure 15.6 depicts schematically the anatomical sites at which PACAP may mediate stress responding in the CNS. In PACAP-deficient mice, aberrant fosB activation following chronic social defeat, or c-fos activation following predator odor exposure, provide important clues to the brain areas in which PACAP signaling is required for stress and threat responding (Lehmann et al. 2013; Zhang et al. 2021). The BNST acts as a gateway for cortical, limbic, and thalamus inputs (Li and Kirouac 2008; Radley et al. 2009; Radley and Sawchenko 2011) to modulate HPA axis responses through direct projections from CRH glutamatergic excitatory neurons of the ventrolateral BNST (vlBNST), onto CRH-secreting neurons of the PVN (Moga and Saper 1994; Champagne et al. 1998; Choi et al. 2007, 2008), or via vlBST GABAergic inhibitory neurons (Radley et al. 2009; Radley and Sawchenko 2011). GABA/CRH neurons from CeA and BNST project to LC facilitate stress-induced arousal and anxiety states (Valentino and Van Bockstaele 2008; McCall et al. 2015; Zitnik 2016). In hypothalamus, the classical feeding center, PACAP plays an important role in stress-induced feeding changes. Recent studies suggest that PACAP can promote both orexigenic and anorexigenic effects, depending on its neuroanatomical location within the hypothalamus. The LPBN receives direct projections from NTS neurons relaying taste and viscerosensory information in rodents. PBN^{PACAP}→CeA and PBN^{PACAP}→ovBNST (Jiang et al., unpublished), PVN^{PACAP}→ARC and VMN ^{PACAP}→ARC (Krashes et al. 2014), and NAc projections (Hurley et al. 2016, 2019) (perhaps from LH^{PACAP} neurons) have all been suggested to be related to stress-induced feeding and appetitive behavioral changes. The PAG has been characterized as an important neural substrate for defensive behaviors in response to stress (Bandler and Shipley 1994; Berton et al. 2007). Activation of neurons in the PAG in mice was sufficient to induce a series of defensive responses (including running, freezing, and avoidance) (Deng et al. 2016). PACAPergic input to PAG directly from several hypothalamic nuclei, including the preoptic area (Tan et al. 2016; Hrvatin et al. 2020) and VMN (Khodai et al. 2018), may play important roles in stress coping (Bandler and Shipley 1994; Berton et al. 2007).

What insight into the neuroanatomy of PACAP's mediation of stress responses do these observations afford us? As mentioned above, the temporal differences in the onset of PACAP action on HPA axis activation, versus stress-induced anorexia, suggest two types of synaptic action of PACAP in stress, and therefore two separate locations for those synapses. As mentioned above, the best candidate for PACAP-dependent HPA axis activation are synapses from PACAP neurons of unknown origin synapsing upon CRH-expressing neurons of the PVN. Synapses mediating PACAP-dependent stress-induced anorexia and other stress-associated behaviors have yet to be identified. Candidates for mediating PACAP-dependent stress-induced hypophagia include PACAPergic projections from PBN to extended

amygdala, PACAPergic neurons within PVN itself and projecting to ARC (Krashes et al. 2014), or even PACAPergic neurons of LH involved in reward-associated appetitive behavior(s) likely to be obtunded by stress (see Fig. 15.6). The involvement of PACAP in stress responses mediated via the extended amygdala has been considered in Sect. 15.5.2.2 and 15.5.2.3. Experiments carried out mainly in the rat have afforded additional insight into the role(s) of PACAP in the central behavioral/emotional processing aspects of the stress response and indicate that the anorexigenic effects of PACAP may occur in parallel to, but separately from, anhedonia and anxiety elicited by stress (Dore et al. 2013; Seiglie et al. 2015) with the latter potentially dependent upon processing within the central amygdala (Seiglie et al. 2019; Varodayan et al. 2020).

Delineating these multiple pathways will be quite informative about the roles of PACAP in differentially mediating, within discrete stress-responsive brain circuits, stimulus-secretion versus *stimulus-transcription coupling at post-synaptic sites, to effect the integrated stress response.*

15.7 Developmental Aspects of PACAP Function

PACAP clearly has developmental roles as well as functions within the adult nervous system. Are these functions arrayed along a continuum, or do they represent clearly distinct functions, using different modes of peptide secretion, receptor action, and metabolic and physiological consequences? PACAP expression begins as early as embryonic day 9 (E9) in rodent (Waschek et al. 1998; Skoglösa et al. 1999; Zhou et al. 1999). PACAP expression in neural tube and prominent expression of both PACAP and PAC1 in neuroepithelium focused on the possibility of a role for autocrine PACAP signaling in development of the neural axis. It is not clear how PACAP would be synthesized in, and secreted from, presumably non-secretory cells prior to neurogenesis. It has been suggested that placental as well as fetal PACAP may participate in regulating embryonic maturation (Maduna and Lelievre 2016). Waschek and colleagues note the role of PACAP in modulating the hedgehog signaling pathway (Hirose et al. 2011), a system largely devoted to development as evidenced by hedgehog-related neuroendocrine cancers associated with its aberrant regulation, including its modulation by PACAP (Lelievre et al. 2008). Altered notch/hedgehog signaling in PACAP-deficient mice has also been noted in ameloand odontoblast development in mice (Fulop et al. 2018). That the frequent co-transmitter of PACAP, glutamate, is unlikely to function until full maturation of small synaptic vesicle release competence (Verhage et al. 2000) also highlights the potential distinction between developmental, possibly hormonal, and adult synaptic PACAP function. Understanding the cellular distinctions between PACAP actions in the developing and in the mature nervous system will require a more precise neuroanatomical accounting of PACAP expression, release, and action after secretion in the developing brain. As mentioned above, the earliest appearance of PACAP and the cognate PAC1 receptor, as assessed by expression of their mRNAs by ISHH, is at day 9.5 in the mouse embryo, in neural tube, the region

fated to become hypothalamus, as well as rhombencephalon, spinal cord, and presumptive sensory and autonomic ganglia (Sheward et al. 1998; Skoglösa et al. 1999). PACAP and PAC1 are expressed in E13.5 cerebral hemispheres, and PACAP may function in regulation of cortical layer formation, as it mimics effects of cAMP on inhibition of cortical precursor mitosis (DiCicco-Bloom et al. 1998; Suh et al. 2001).

Cerebellum, as mentioned in Sect. 15.4, is also a good example of a role of PACAP in development, in this case postnatal. PAC1 receptors are highly expressed in the external granule cell layer in the first two postnatal weeks (Gonzalez et al. 1994), with PACAP expressed in Purkinje cells, while PACAP continues to be expressed in Purkinje cells in adult mice, but with PAC1 expression much greater in the Purkinje cells themselves than in granule cells, suggesting a potential intercellular role in postnatal cerebellar development (Vaudry et al. 1999; Galas et al. 2017), and a paracrine/autocrine one in adult cerebellum. Altered migration of granule cells from the external to internal granule cell layer at postnatal days 4 and 7 is observed in PACAP-deficient mice (Allais et al. 2007). However, by postnatal day 11, no differences either in layer thickness or in cell number are found in external or internal granular layers, or in the molecular layer, of the cerebellar cortex in PACAP-dependent compared to wild-type mice (Vaudry et al. 2005). In sum, these observations suggest that PACAP effects in developing cerebellum are either compensated in the absence of PACAP, or that changes in adult cerebellar structure and function in PACAP-deficient mice, while not evident as gross cytoarchitectural changes in the features of the cerebellar cortex, do nevertheless affect function. A second possibility is that effects of PACAP on granule cell migration in early cerebellar postnatal development contribute to resilience to oxidative and other insults (Vaudry et al. 2005), rather than to normal development. Similarities between developing human and rodent cerebellar PACAP and PAC1 expression suggest potential therapeutic applications for PACAP in fetal alcohol and other perinatal toxicity syndromes (Basille et al. 2006).

PACAP and PAC1 are also expressed in superior cervical ganglion cells during the time that these postganglionic neuroblasts proliferate and differentiate, and on this basis PACAP was originally proposed as an autocrine factor in autonomic development (DiCicco-Bloom et al. 2000). Likewise, early PACAP expression in dorsal root ganglia led some to propose a neurotrophic role for PACAP in sensory neuronal development, akin to that of NGF and other neurotrophic factors (Lindholm et al. 1998; Nielsen et al. 1998). A problem with postulating a developmental role for PACAP signaling through the PAC1 receptor in brain or sympathetic nervous system, whether through autocrine or paracrine mechanisms, is that aberrations in neuronal architecture have not been reported in either PACAP or PAC1 adult mice (Gray et al. 2001; Hashimoto et al. 2001; Otto et al. 2001; Hamelink et al. 2002; Colwell et al. 2004). Rather, functional deficiencies in these mice reflect impairments of adaptive responses, rather than deficiencies in basal function. An exception is spontaneous repetitive jumping behavior in PACAP knock-out mice in the absence of any provocative stimulation, and which remains unexplained at either the cellular or circuit level.

Transcriptome analysis has recently been used to evaluate differential contributions of PACAP as a developmental factor, and PACAP as a "real-time" neurotransmitter, to neuronal and neuroendocrine regulation. We have distinguished cPRGs and aPRGs—*c*onstitutive and *a*cutely *P*ACAP-*r*egulated *g*enes—as genes from which transcript production is affected by constitutive loss of PACAP in the absence of additional challenges (i.e., under "basal" conditions) (cPRGs) and those that are controlled in a PACAP-dependent manner after induction in response to specific challenges, such as stress (aPRGs) (Bakalar et al. 2022). Significantly, cPRGs appear not to be phenocopied in constitutively knocked-out PAC1-deficient mice, implying that PACAP may use a receptor other than PAC1 during development and possibly even in the adult nervous system.

15.8 Non-neuronal Aspects of PACAP Function

It would be remiss to close without recognizing that PACAP is not unique among neuropeptides in having important roles as a factor released not only from neurons and neuroendocrine cells, but also from non-neuroendocrine cells. This is important for two reasons. First, it is important to consider the genuinely non-neuronal effects of PACAP as PACAP agonists and antagonists in the context of therapeutics: immune as well as nervous system side effects of such agents, for example, will need to be monitored as these are deployed clinically. Second, some of the supposedly "neuronal" effects of PACAP (and other neuropeptides as well) may ultimately be explained by their interactions with non-neuronal cells. Two prominent examples are astrocyte-mediated effects of PACAP in mediation of inflammation-associated pain (Ohnou et al. 2016), and effects of PACAP in the modulation of microglial cells, especially in the context of neurodegenerative diseases such as multiple sclerosis (Abad et al. 2016) and amyotrophic lateral sclerosis (Ringer et al. 2013). PACAP may also be released from immunocytes during injury, inflammation, or infection, affecting both neuropathic and systemic processes (Abad et al. 2006; Armstrong et al. 2008; Waschek 2013). A recent intriguing role of PACAP is acting both to promote atherosclerosis through PAC1 receptor activation and to ameliorate atherosclerogenesis, apparently via non-PAC1 receptor signaling. In both cases, the actual source of PACAP release and action is unknown (Rasbach et al. 2019; Splitthoff et al. 2020). As the anti-atherosclerogenic effects of PACAP seem to be independent of cholesterol levels, and therefore potentially additive to anti-cholesterol treatment of cardiovascular disease, understanding the interactions between the anatomy and function of PACAP in neuronal, immune, and even endothelial cell compartments will remain clinically relevant across a broad array of human diseases and disorders.

15.9 New Discoveries, Outstanding Issues, and Perspectives

- PACAP is expressed in both excitatory and inhibitory neurons throughout the brain; in cholinergic preganglionic neurons of the autonomic nervous system; and in excitatory neurons, including neuroendocrine cells, of the hypothalamus.
- While PACAPergic neurons of the hypothalamus are implicated in regulation of torpor, feeding, and reproductive behavior, it is not yet established how PACAP itself contributes to neurotransmission in these PACAPergic neuronal clusters, situated in MLPA, MEPO, AHN, PVN, VMN, LH, PMV and MBO of the hypothalamus.
- The use of PACAP-deficient mice has established that the sympathoadrenal stress response requires PACAP release onto neuroendocrine (chromaffin) cells to maintain the adrenomedullary stress response (catecholamine release).
- PACAP is required for prolonged and chronic, but not acute, HPA axis activation in stress.
- Anxiety-associated behavioral stress responses, including hypophagia, are severely attenuated in the absence of PACAP, as is stress-precipitated relapse to cocaine self-administration in rats.
- A specific signaling role for PACAP in threat/aversive stimulus responding is emerging within a coherent framework of functional neuroanatomy within the extended amygdala and its afferent and efferent connections.
- Human genetic analysis identifies PACAP and/or PAC1 as risk factor for human diseases including PTSD (Mercer et al. 2016; Ross et al. 2020), Edward's syndrome (Pinto et al. 2014), and affective disorders (Katayama et al. 2009; Lutfy and Shankar 2019). Mapping susceptibility to these disorders to PACAP's actions within neuroendocrine circuits mediating the basic physiology of threat and stress responses, including their sex-specific features, is an important theme in PACAP research.

Key References

Referencing for all primary reports contributing to understanding PACAP functional neuroanatomy was not possible, given the broad scope of work represented. For that we apologize to colleagues whose specific contributions might not be referenced directly, but in review articles also cited. Below, a list of review and cornerstone contributions to the PACAP literature:

Miyata, A., A. Arimura, R. R. Dahl, N. Minamino, A. Uehara, L. Jiang, M. D. Culler and D. H. Coy (1989). "Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells." Biochem. Biophys. Res. Commun. 164: 567–574. Report on the discovery, identity, and characterization of PACAP-38.(Miyata, Arimura et al. 1989).

Arimura, A. (1998). "Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems." Jap. J. Physiol. 48: 301–331. A remarkably prescient overview of the state of

PACAP research and prospects for the future, eight years following the published report of its discovery. (Arimura 1998).

Hannibal, J. (2002). "Pituitary adenylate cyclase-activating peptide in the rat central nervous system: an immunohistochemical and in situ hybridization study." J. Comp. Neurol. 453(4): 389–417. The first definitive investigation of widespread expression of PACAP mRNA and nerve terminals in rodent CNS. (Hannibal 2002).

Gray, S. L., K. J. Cummings, F. R. Jirik and N. M. Sherwood (2001). "Targeted disruption of the pituitary adenylate cyclase-activating polypeptide gene results in early postnatal death associated with dysfunction of lipid and carbohydrate metabolism." Mol. Endocrinol. 15: 1739–1747. One of four independent reports on PACAP knock-out mice, each describing a unique feature for this protean peptide, in this case lipid and carbohydrate metabolism during the postnatal/weaning period. (Gray, Cummings et al. 2001).

Hashimoto, H., N. Shintani, K. Tanaka, W. Mori, M. Hirose, T. Matsuda, M. Sakaue, J. Miyazaki, H. Niwa, F. Tashiro, K. Yamamoto, K. Koga, S. Tomimoto, A. Kunugi, S. Suetake and A. Baba (2001). "Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP)." Proc Natl Acad Sci U S A 98(23): 13355–13,360. One of four independent reports on PACAP knock-out mice, each describing a unique feature for this protean peptide, in this case psychomotor beavhiors.(Hashimoto, Shintani et al. 2001).

Hamelink, C., O. Tjurmina, R. Damadzic, W. S. Young, E. Weihe, H.-W. Lee and L. E. Eiden (2002). "Pituitary adenylate cyclase activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis." Proc. Natl. Acad. Sci. USA 99: 461–466. One of four independent reports on PACAP knock-out mice, each describing a unique feature for this protean peptide, in this case PACAP function as a major sympathetic neurotransmitter mediating sympathoadrenal metabolic stress responding. (Hamelink, Tjurmina et al. 2002).

Colwell, C. S., S. Michel, J. Itri, W. Rodriguez, J. Tam, V. Lelievre, Z. Hu and J. A. Waschek (2004). "Selective deficits in the circadian light response in mice lacking PACAP." Am. J. Physiol. Regul. Integr. Comp. Physiol. 287(5): R1194-R1201. One of four independent reports on PACAP knock-out mice, each describing a unique feature for this protean peptide, in this case circadian function. (Colwell, Michel et al. 2004).

Miles, O. W., E. A. Thrailkill, A. K. Linden, V. May, M. E. Bouton and S. E. Hammack (2018). "Pituitary Adenylate Cyclase-Activating Peptide in the Bed Nucleus of the Stria Terminalis Mediates Stress-Induced Reinstatement of Cocaine Seeking in Rats." Neuropsychopharmacology 43: 978–986. Relapse to cocaine self-administration induced by foot-shock is mimicked by PACAP infusion and blocked by infusion of the PAC1 antagonist PACAP(6–38) in the BNST of the rat. (Miles, Thrailkill et al. 2018).

Krashes, M. J., B. P. Shah, J. C. Madara, D. P. Olson, D. E. Strochlic, A. S. Garfield, L. Vong, H. Pei, M. Watabe-Uchida, N. Uchida, S. D. Liberles and B. B. Lowell

(2014). "An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger." Nature 507(7491): 238–242. An Adcyap1-Cre driver mouse allows the location of a specific PACAPergic circuit from PVN to ARC optogenetic stimulation of which elicits feeding; importantly, local application of PACAP itself depolarizes post-synaptic neurons in this circuit. (Krashes, Shah et al. 2014).

Zhang, L., V. S. Hernandez, C. R. Gerfen, S. Z. Jiang, L. Zavala, R. A. Barrio and L. E. Eiden (2021). "Behavioral role of PACAP reflects its selective distribution in glutamatergic and GABAergic neuronal subpopulations." Elife 10. The use of dual in situ hybridization (DISH) provides a new perspective on PACAP co-transmission by mapping it to key glutamatergic and GABAergic neurons throughout the neural axis and demonstrating the requirement for PACAP in neuronal activation during exposure to predator odor leading to defensive response. (Zhang, Hernandez et al. 2021).

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Functional Neuroanatomy of Relaxin-3/RXFP3 Systems in the Brain: Implications for Integrated Neuroendocrine and Behavioural Control

16

Alan Kania, Anna Blasiak, and Andrew L. Gundlach

Abstract

Neuropeptides play key neuromodulatory roles in the mammalian central nervous system. Relaxin-3, a neuropeptide discovered by homology searching of the human genome 20 years ago, and its cognate G-protein-coupled receptor, relaxin-family peptide receptor 3 (RXFP3), discovered in studies of brain-enriched 'orphan' receptors, have since been shown to modulate neuronal activity in multiple brain circuits. The early anatomical association of this neuropeptide/receptor signalling system with the enigmatic *nucleus incertus* (NI) located in the pontine tegmentum of a range of mammalian brains prompted a large number of anatomical, regulatory and pharmacological studies. In this chapter, we summarize current knowledge of the neuroanatomy of the relaxin-3/RXFP3 system in the mammalian brain and detail the comprehensive studies of its functional relationship with the magnocellular and parvocellular oxytocin (OXT) and arginine-vasopressin (AVP) neurons in the paraventricular nucleus of the hypothalamus (PVN) in the rat. More generally, we review pharmacological studies using novel, chimeric and truncated peptides selective for RXFP3 compared to

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other relaxin-family receptors, which have identified several aspects of physiology and behaviour in rats and mice that are likely to be regulated by the endogenous relaxin-3/RXFP3 system; these include arousal, circadian rhythms, feeding and metabolism, social and stress-related behaviour, autonomic responses and cognition. Lastly, as a future perspective, we highlight some key issues, including the nature and regulation of neuronal relaxin-3 release and the precise location and function of RXFP3 in specific neural circuits, which require further research to improve our understanding of this complex and therapeutically relevant neuromodulatory system.

Keywords

Relaxin-3 · RXFP3 · Nucleus incertus · PVN · Arousal · Social behaviour · Anatomy · Projections

Abbreviations

Amy	amygdala
AN	anorexia nervosa
AVP	arginine vasopressin
BED	binge-eating disorder
BN	bulimia nervosa
CRH	corticotropin-releasing hormone
dHipp	dorsal hippocampus
DMH	dorsomedial hypothalamic nucleus
DpMe	deep mesencephalon
dSN	dorsal to substantia nigra
HPA	hypothalamic-pituitary-adrenal (axis)
HPG	hypothalamic-pituitary-gonadal (axis)
ICV	intracerebroventricular
INSL3–6	insulin-like peptide 3–6
IO	inferior olive
IPN	interpeduncular nucleus
LH	lateral hypothalamus
LPA	lateral preoptic area
MCH	melanin-concentrating hormone
MNCs	magnocellular neurosecretory cells
MnR	median raphe nucleus
MS	medial septum
MVe	medial vestibular nucleus
NI	nucleus incertus
NTS	nucleus of solitary tract
OX (A/B)	orexin-A/B
OXT	oxytocin

PH	posterior hypothalamic area
PnR	pontine raphé nucleus
PrH	prepositus hypoglossal nucleus
PVN	paraventricular nucleus of hypothalamus
RLN3	relaxin-3
RXFP1–4	relaxin-family peptide receptor 1–4
SON	supraoptic nucleus
SuM	supramammillary nucleus
vHipp	ventral hippocampus
vlPAG	ventrolateral periaqueductal grey

16.1 Introduction

Neuropeptides play key neuromodulatory roles in the mammalian central nervous system. The neuropeptide relaxin-3 and the $G_{i/o}$-protein-coupled receptor relaxin-family peptide receptor 3 (RXFP3) were first identified as a cognate ligand-receptor pair some 20 years ago and have since been shown to populate and modulate multiple brain circuits. In light of the extent of these studies, several aspects of relaxin-3/RXFP3 neurobiology have been reviewed in detail elsewhere, particularly its involvement in feeding and metabolism and in the modulation of learning and memory (Ryan et al. 2011; Ganella et al. 2013b; Smith et al. 2014b; Ma and Gundlach 2015; Ma et al. 2017; Olucha-Bordonau et al. 2018; Gil-Miravet et al. 2021). However, to provide a suitable overview of the topic, this chapter first introduces the various members of the relaxin-family of peptides and their cognate G-protein-coupled receptors and then reviews the neuroanatomy of the relaxin-3/RXFP3 system, including the distribution of relaxin-3-producing neurons and their widespread projections to RXFP3-enriched areas; the regulation of relaxin-3 neuron activity by neural inputs and extrinsic factors; and the pharmacological studies that inform likely physiological roles of the relaxin-3/RXFP3 system in the brain, including details of the actions of RXFP3 within the rat PVN.

Box 16.1 Relaxin-Family Peptides and Receptors

The relaxin-family peptide (RXFP) receptors are a group of four receptors that mediate the hormonal and neuropeptide actions of the relaxin-family peptides, relaxin (RXFP1), insulin-like peptide (INSL)-3 (RXFP2), relaxin-3 (RXFP3) and INSL5 (RXFP4) (Table 16.1). RXFP1 and RXFP2 are a subgroup (type C) of the family of leucine-rich repeat-containing guanine nucleotide binding (G-protein)-coupled receptors or LGRs, that include the receptors for the glycoprotein hormones FSH, LH, and TSH. Two additional orphan G-protein-coupled receptors with a short N-terminal extracellular domain, designated

(continued)

Table 16.1 Relaxin-family peptides and their G-protein-coupled receptors

Relaxin-Family Ligand	Receptor[a]	Cellular Signalling	Present in Brain
Relaxin	RXFP1	↑AC/p38MAPK/ERK1/2	Ligand/ receptor
INSL3	RXFP2	↑(↓)AC	Ligand/ receptor
Relaxin-3	RXFP3	↓AC/↑p38MAPK/JNK(1/2)/ERK1/ 2	Ligand/ receptor
INSL5	RXFP4	↓AC/↑ p38MAPK/ERK(1/2)/Akt	–
INSL4	Not known	–	–
INSL6	Not known	–	Ligand/–

[a]RXFP1-4 were formally known as LGR7, LGR8, GPCR135 and GPCR142, respectively. ↑ Increases; ↓ Decreases. Abbreviations: INSL, insulin-like peptide; AC, adenylate cyclase; p38MAPK, p38 mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; JNK1/2, c-Jun N-terminal kinases 1/2; Akt, protein kinase B.

Box 16.1 (continued)
GPCR135 (RXFP3) and GPCR142 (RXFP4), were subsequently identified. The function and cognate receptors of the more recently evolved peptides, INSL4 and INSL6 are currently unknown. RXFP1 has a widespread tissue distribution, being found in female and male reproductive tissues, the brain and numerous other non-reproductive tissues such as the kidney, heart and lung. In rodents, RXFP2 is expressed in ovary, testis and gubernaculum, and in motor and limbic circuits in the brain. RXFP3 is predominantly expressed in the brain, whereas RXFP4 is enriched in the colon and also present in kidney, testis, thymus, salivary gland and thyroid.

16.2 Relaxin-3/RXFP3 System Anatomy

The relaxin-3/RXFP3 signalling system is highly evolutionarily conserved, as reflected by the similar molecular structure of the peptide and receptor, and their consistent expression in the brain of various vertebrate species, including human (Matsumoto et al. 2000; Liu et al. 2003), macaque (Ma et al. 2009a, b), rat (Burazin et al. 2002; Liu et al. 2003), mouse (Bathgate et al. 2002; Boels et al. 2004) and fish (Donizetti et al. 2008, 2015). Relaxin-3 is the preferred native ligand for RXFP3, which activation leads to inhibition of cellular cAMP production and activation of MAP kinases. RXFP3 was first discovered by probing a human cortical cDNA library, and originally named somatostatin and angiotensin-like peptide receptor (SALPR), due to its high amino acid sequence similarity to somatostatin and angiotensin II receptors (Matsumoto et al. 2000). However, neither somatostatin

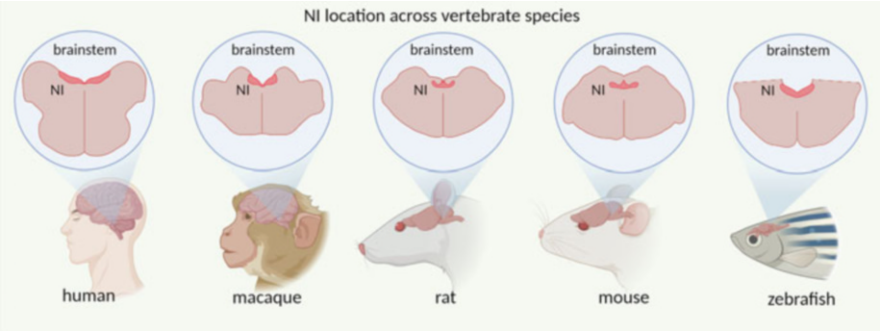

Fig. 16.1 Conserved presence of the *nucleus incertus* or a homologous area across vertebrate species. Coronal sections at the pontine level are depicted with the *nucleus incertus* (NI) region highlighted. Note the similarity of the nucleus location and its proximity to the ventricular system across all represented species. (The location of the NI region in zebrafish was based on studies in zebrafish larvae)

nor angiotensin II activated the receptor, and later studies identified the native ligand for RXFP3 (*aka* GPCR135) as the relaxin-3 peptide (Liu et al. 2003; Ma et al. 2017).

In all species tested so far, relaxin-3 synthetizing neurons are located in the brain, and only limited peripheral relaxin-3 gene expression has been reported. In humans, relaxin-3 mRNA has been detected in the brain and testis, although currently there is no detailed description of the anatomical distribution of relaxin-3-synthesizing neurons in the human brain. In the non-human primate, *Macaca fascicularis*, a small number of relaxin-3 neurons are present in the central grey of the midbrain equivalent to the periaqueductal grey of rat, while the biggest population of these peptide neurons is localised within the area of ventromedial central grey of the pons known as the *nucleus incertus* (NI) (Ma et al. 2009a). In fact, the NI was first described in human brain in 1903 by George Streeter, who named it the 'uncertain nucleus', because of its unknown function at the time (Streeter 1903).The neuroanatomical distribution of relaxin-3 neurons has been most extensively studied in the rat and mouse brain, and these histochemical studies have provided a precise description of the areas where relaxin-3 neurons are located, as well as further information about their neurochemical phenotypes. In rat and mouse, as in primates, the primary source of relaxin-3 neurons is the NI, which is located in the midline periventricular central grey, at the coronal level of the tegmentum, ventral to the fourth cerebral ventricle. Described relaxin-3 expression patterns confirm the highly conserved nature of this peptidergic system, as even in zebrafish, relaxin-3 mRNA is synthetized in a rostral portion of the pons, corresponding to the mammalian NI (Donizetti et al. 2008) (Fig. 16.1).

In fact, both early anatomical and more recent functional studies identify the NI as an important element of a behaviour control network that guides behavioural activation through integration of memory, arousal and stress-related information (Goto et al. 2001; Olucha-Bordonau et al. 2003; Ma et al. 2013; Sabetghadam et al. 2018; Szőnyi et al. 2019; Lu et al. 2020). NI can be divided into the *pars*

compacta located near the midline with densely packed neurons and the more lateral *pars dissipata*, with more loosely arranged cells (Goto et al. 2001). Relaxin-3 neurons are present in both parts of the NI, and represent a major cluster of distally-projecting GABAergic cells. In mouse brain, relaxin-3 neurons have been reported to co-express the neuropeptide, neuromedin B (Nasirova et al. 2020; Lu et al. 2020).

NI is not the sole source of relaxin-3 in the mammalian brain, and while NI relaxin-3 neurons in the rat brain number ~ 2000 cells, more diffuse and less numerous populations of relaxin-3 neurons have been detected in the rat pontine raphé nucleus (PnR, ~350 neurons), ventrolateral periaqueductal grey (vlPAG, ~750 neurons), and in the deep mesencephalon (DpMe, ~350 neurons) in the area dorsal to the substantia nigra (dSN) (Tanaka et al. 2005; Ma et al. 2007; Smith et al. 2010; Blasiak et al. 2013). A similar pattern of relaxin-3 expression has also been described in mice (Smith et al. 2010). Furthermore, a separate relaxin-3 mRNA-positive cell group has been identified in the central grey of zebrafish (Donizetti et al. 2008). Importantly, accumulating evidence indicates that relaxin-3 neurons located in different nuclei innervate different brain areas, which suggests some differences in their precise and integrative function (Blasiak et al. 2013; Nasirova et al. 2020).

16.3 Distribution of Relaxin-3 Containing Nerve Fibres and RXFP3 mRNA/Protein in Brain

Neuroanatomical studies conducted in rodents have revealed that the distribution of relaxin-3 positive nerve fibres largely but not completely overlaps with the distribution of NI-originating axons, indicating that the majority of the forebrain relaxin-3 innervation originates from the NI (Goto et al. 2001; Ma et al. 2007; Smith et al. 2010; Olucha-Bordonau et al. 2018; Nasirova et al. 2020). Similar to the distribution pattern of relaxin-3 synthetizing neurons, the distribution of relaxin-3 immunoreactive fibres in rodent and primate brain is highly comparable, which suggests the involvement of relaxin-3 signalling in the regulation of similar processes across species (Ma et al. 2009a). The existence of other sources of relaxin-3 in the brain does not contradict the concept that relaxin-3-containing nerve fibres and NI efferent projections are similarly distributed, as relaxin-3 neurons located in the vlPAG, DpMe/dSN innervate areas adjacent to their origin that generally do not overlap with the extensive ascending projections of relaxin-3 NI neurons (Nasirova et al. 2020).

A consistent feature of the relaxin-3 innervation and RXFP3 distribution across all studied species is their presence in multiple brain structures, indicating that relaxin-3/RXFP3 signalling regulates a wide variety of integrated physiological processes and behaviours. Indeed, numerous independent functional studies have demonstrated that the relaxin-3/RXFP3 system is involved in neuroendocrine control, circadian rhythmicity, arousal, anxiety, behavioural activation and locomotor activity, feeding and appetite, spatial and social memory, as well as motivation-related behaviours and drug-seeking behaviour (Ganella et al. 2013b; Smith et al. 2014b; Ma et al. 2017; Olucha-Bordonau et al. 2018) (Fig. 16.2). The diffuse nature

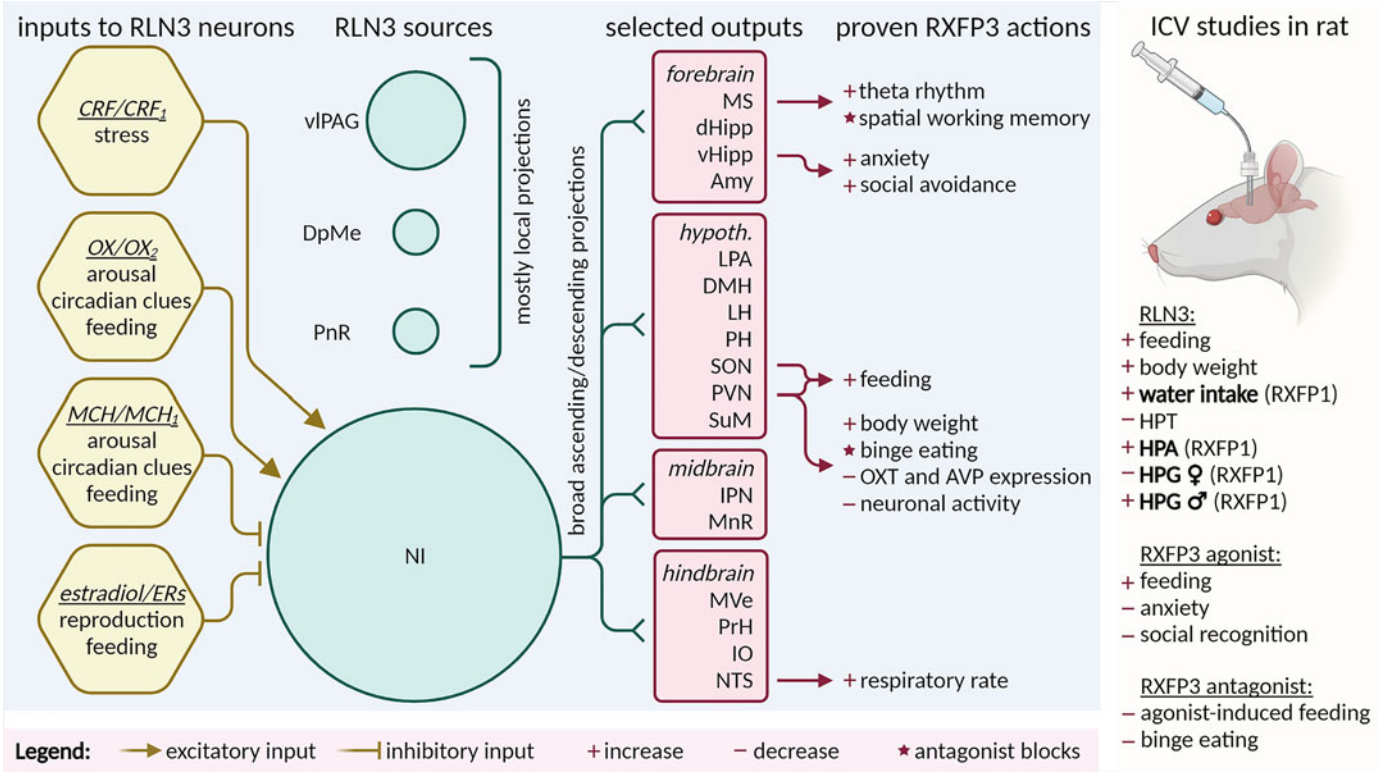

Fig. 16.2 Schematic illustration of the well-characterized aspects of relaxin-3/RXFP3 system anatomy and physiology. Yellow hexagons represent the NI input signals with putative physiological functions. Green circles depict the relaxin-3 (RLN3) cell groups in brainstem, with the size of the circle proportional to the number of RLN3 neurons in the respective area. Red squares list the brain areas that receive RLN3 projections and detail proven RLN3 or RXFP3 actions in corresponding brain regions. Yellow panel (right) summarizes effects of pharmacological modulation of RLN3/RXFP3 signalling via intracerebroventricular drug application in rat. Actions listed in bold are thought to be RXFP1-dependent

of the central relaxin-3 innervation suggests that the relaxin-3/RXFP3 network should be classified as one of the non-specific, ascending networks, which currently include the serotoninergic system originating from the neighbouring raphe nuclei, the noradrenergic, locus coeruleus system, the cholinergic dorsolateral tegmental nucleus system (Avery and Krichmar 2017) and other neuropeptide systems, such as the neuropeptide S and neuromedin B networks (Ohki-Hamazaki 2016; Botticelli et al. 2021).

16.3.1 Relaxin-3 Fibres and RXFP3 are Present in Functionally Diverse Brain Areas

A major brain area that is strongly innervated by relaxin-3 fibres and/or enriched in RXFP3 is the hypothalamus, which contains numerous key clusters of neurons (nuclei) that control multiple neuroendocrine hormone systems, feeding and metabolism, homeostatic and neurogenic stress responses and the sleep/wake cycle. Among the hypothalamic nuclei/areas containing a high density of relaxin-3 fibres are the lateral preoptic area (control of sleep/wake cycle, thirst, locomotor activity and reward-related processes), dorsomedial hypothalamic nucleus (regulation of circadian rhythms, stress responses, ingestive behaviour, reproduction and thermogenesis), lateral hypothalamus (integration of autonomic and endocrine responses, regulation of homeostatic balance and arousal, control of pain- and reward-related behaviours), posterior hypothalamic area (control of energy balance and body temperature, blood pressure, sleep/wake cycle, hippocampal/cortical function), supraoptic nucleus (SON, control of cardiovascular and body-fluid homeostasis, parturition and lactation, stress responses) and supramammillary nucleus (arousal, hippocampal theta rhythm and spatial memory modulation) (Ma et al. 2007; Smith et al. 2010) (Fig. 16.2). As suggested by the anatomical distribution of relaxin-3 fibres and RXFP3 expression within the hypothalamus, functional studies have confirmed the involvement of relaxin-3/RXFP3 signalling in the control of various autonomic and neuroendocrine components of behaviour and stress, arousal and memory-related processes, although the precise effect of physiological or pharmacological activation of RXFP3 in these specific areas, either singularly or in a coordinated, combined fashion, has only just begun to be examined experimentally.

A distinct feature of the ascending relaxin-3/NI innervation is the presence of a dense relaxin-3 fibre network in brain structures involved in memory, arousal and locomotor activity control, and in addition to the regions already mentioned above, these include the medial septum, hippocampus and the interpeduncular and median raphe nuclei (Ma et al. 2007). As implied by the distribution of relaxin-3 fibres and RXFP3 in these structures, their role in the control of the associated hippocampal theta rhythm and spatial memory has been confirmed by a series of functional experiments (Fig. 16.2).

With regard to the hippocampal formation, the ventral hippocampus is more densely innervated by relaxin-3 fibres and displays a higher density of RXFP3 than the dorsal region. The ventral hippocampus is strongly connected to subcortical

structures such as the amygdala and bed nuclei of the stria terminalis, and unlike the dorsal hippocampus, which is involved in spatial memory formation, the ventral hippocampus (anterior hippocampus in primates; also known as the temporal pole) is a principal component of the circuit controlling emotional behaviour, stress and anxiety (Bannerman et al. 2014). Relaxin-3 fibres and RXFP3 in the ventral hippocampus, the central, medial and basomedial amygdala, as well as in the specific hypothalamic nuclei, constitute a neuroanatomical basis for the relaxin-3/RXFP3 system control of affective, emotional and stress-related behaviour (Smith et al. 2010; Ma et al. 2013; Ma and Gundlach 2015; Santos et al. 2016; Kania et al. 2020) (Fig. 16.2).

In general, the distribution of relaxin-3 fibres corresponds to the distribution of RXFP3 expresssion. There are, however, some brain regions, including the paraventricular nucleus of the hypothalamus (PVN), the amygdala and the bed nucleus of the stria terminalis, in which a very high density of RXFP3 mRNA-positive neurons, but a low density of relaxin-3 fibres, are observed (Smith et al. 2010; Kania et al. 2017). These findings suggest that neuronal release of relaxin-3 is not limited to conventional synapses, and that relaxin-3 can act on RXFP3 via non-synaptic, localized volume transmission, although direct evidence of this is not yet available.

Notably, relaxin-3 fibres and RXFP3 are also present in lower abundance in the caudal brainstem in the medial vestibular, prepositus hypoglossal nucleus, inferior olive and nucleus of solitary tract (Ma et al. 2007; Smith et al. 2010; Furuya et al. 2020), and evidence is emerging of RXFP3 actions in autonomic and homeostatic functions such as respiratory control (Furuya et al. 2020) (Fig. 16.2).

16.4 Physiological Functions of the Relaxin-3/RXFP3 System

The first studies aimed at characterizing the physiological actions of relaxin-3 were conducted in rats, and in these experiments the native peptide was injected into the lateral cerebral ventricle or the PVN, a region characterized by the highest density of RXFP3 expression in rodent brain (Sutton et al. 2004; Smith et al. 2010) (Fig. 16.2).

16.4.1 Pharmacological Effects that Inform Physiological Roles of Relaxin-3/RXFP3

Intracerebroventricular (ICV) and intra-PVN injections of relaxin-3 results in a robust increase in food intake in satiated male rats (McGowan et al. 2005, 2006; Hida et al. 2006; Calvez et al. 2015; de Ávila et al. 2018). Later studies have demonstrated that selective RXFP3 agonists and antagonists mimic the action of relaxin-3 or block the agonist-induced food intake respectively, confirming the role of RXFP3 in relaxin-3-induced increase in food intake (Kuei et al. 2007; Haugaard-Kedström et al. 2011; Shabanpoor et al. 2012; Ganella et al. 2013a; de Ávila et al. 2018). Similarly, an orexigenic effect has been observed after relaxin-3 injections

into the SON, anterior preoptic area and arcuate nucleus in rats (McGowan et al. 2007) (Fig. 16.2). Notably, in mice ICV injection of relaxin-3 or RXFP3 agonist does not stimulate food intake (Smith et al. 2013); at the same time, central blockade of RXFP3 with an antagonist reduces motivated food seeking and consumption in mice (Smith et al. 2014a), pointing to possible species-specific roles of RXFP3 in the control of food intake.

As a consequence of a sustained increase in food intake, chronic ICV administration of relaxin-3 or activation of RXFP3 in the PVN leads to increased body-weight gain (Hida et al. 2006; Ganella et al. 2013a; Calvez et al. 2016a). Additionally, in diet-induced obese rats, relaxin-3 signalling seems to undergo changes, with increased expression of relaxin-3 mRNA in the NI and a refeeding-induced increased expression of RXFP3 mRNA after food deprivation, which may defend the elevated body weight, preventing weight loss (Lenglos et al. 2014). Notably, relaxin-3/RXFP3 signalling has been shown to mediate binge-eating behaviour, a symptom of the most prevalent eating disorder worldwide: binge eating disorder (Hutson et al. 2018). In bingeing female rats, relaxin-3 mRNA in the NI is elevated (Lenglos et al. 2013), and ICV/intra-PVN injections of RXFP3 antagonist block the binge-like consumption of sucrose and highly-palatable food (Calvez et al. 2016b; Kania et al. 2020) (Fig. 16.2). Importantly, female rats are more prone to binge eating than males (Klump et al. 2013; Lenglos et al. 2013) and are commonly used to model this behaviour. Similarly, in the human population, women suffer from binge eating disorder almost twice as frequently as men (Hudson et al. 2007; Kessler et al. 2013). Furthermore, ICV injections of relaxin-3 produce a stronger orexigenic effect and higher body-weight gain in female rats (Lenglos et al. 2015; Calvez et al. 2016a), which are also characterized by a denser relaxin-3 innervation of the PVN and its vicinity compared to males (Kania et al. 2020). Together, these data suggest that studies of the relaxin-3/RXFP3 system might assist our understanding of the higher susceptibility of females (including women) to develop abnormal, binge-like eating behaviour.

Box 16.2 Eating Disorders

Eating disorders are complex mental health conditions that lead to alterations in eating behaviour; insufficient or excessive food intake, resulting in an energy imbalance. Eating disorders are characterized by a high comorbidity rate with other mental disorders (e.g., anxiety, mood disorders or substance use disorders), have severe health consequences, and in serious cases, may result in death if untreated (Hudson et al. 2007; Merikangas et al. 2010; Swanson et al. 2011; Kessler et al. 2013). Common eating disorders include anorexia nervosa (AN), bulimia nervosa (BN) and binge eating disorder (BED), with the latter being the most prevalent in the human population (up to 5% of the adult population), and affecting women twice as often as men (Hudson et al. 2007; Kessler et al. 2013). Although AN is the most well-

(continued)

Box 16.2 (continued)
known eating disorder, it is the least common. It is characterized by a self-imposed food restriction, a strong desire to be thin and a fear of gaining weight. AN has the highest mortality rate among the eating disorders (Arcelus et al. 2011). Both BN and BED are characterized by recurrent episodes of binge eating, but unlike BN, in BED the episodes are not followed by compensatory behaviours (e.g. vomiting), therefore BED is often associated with overweight or obesity (Hudson et al. 2007; Merikangas et al. 2010; Swanson et al. 2011; Kessler et al. 2013).

In addition to stimulating food intake, exogenous relaxin-3 stimulates water intake. ICV injection of relaxin-3 is dipsogenic in rats and activates (i.e., enhanced c-*fos* expression) the PVN and SON, organum vasculosum of the lamina terminalis, the median preoptic nucleus, and the subfornical organ—the circumventricular organs controlling drinking behaviour (Thornton and Fitzsimons 1995; Otsubo et al. 2010; Calvez et al. 2015; de Ávila et al. 2018). Notably, water intake is not altered by ICV injection of a selective RXFP3 agonist (de Ávila et al. 2018). It is known that relaxin-3 can bind and activate the relaxin hormone receptor, RXFP1 (Sudo et al. 2003), although with lower affinity than RXFP3 (Liu et al. 2003). Nevertheless, RXFP1 is strongly expressed in the circumventricular organs (Ma et al. 2006), hence the dipsogenic effect of relaxin-3 is likely RXFP1-mediated (Fig. 16.2). While not involved in control of water intake, RXFP3 is thought to modulate sodium appetite, as ICV administration of an RXFP3 antagonist reduced the consumption of a sodium chloride (salt) solution in sodium-depleted mice (Smith et al. 2015).

Additionally, exogenous relaxin-3 displays the capacity to modulate several hypothalamo-pituitary neuroendocrine axes in rats. For instance, the hypothalamic-pituitary-thyroid axis, orchestrating key aspects of metabolism, is suppressed by exogenous relaxin-3, as plasma thyroid-stimulating hormone levels decrease after intra-PVN relaxin-3 injections (McGowan et al. 2006) (Fig. 16.2).

The relaxin-3/RXFP3 system also influences the hypothalamic-pituitary-adrenal (HPA) axis, responsible for the control of stress responses, and central injections of relaxin-3 increase plasma levels of the stress hormones adrenocorticotrophic hormone (ACTH) and corticosterone in male and female rats (McGowan et al. 2014; Lenglos et al. 2015; de Ávila et al. 2018). Moreover, central RXFP3 activation modulates anxiety-related behaviours in rats and mice (Ryan et al. 2013a; Zhang et al. 2015; Rytova et al. 2019), highlighting a putative role of relaxin-3/RXFP3 signalling in the development of stress-related psychiatric conditions, e.g., anxiety and post-traumatic stress disorder. The relationship between relaxin-3/RXFP3 system and HPA axis is bidirectional, as relaxin-3 neurons in the NI are highly sensitive to stress factors (see below), implying a key involvement of relaxin-3/RXFP3 signalling in the neural networks integrating the central reaction to stressors (Fig. 16.2).

The hypothalamic-pituitary-gonadal (HPG) axis, which governs fertility and reproduction, displays a sex-specific reaction to exogenous relaxin-3, with the relaxin-3-induced activation of HPG axis in males and its inhibition in females (McGowan et al. 2008; Calvez et al. 2016a), which again highlights the sex-specific aspects of relaxin-3/RXFP3 signalling. Notably, similar to the effects of exogenous relaxin-3 on water intake, its effects on the HPA and HPG axes seem to be mostly RXFP1-dependent, as they are not mimicked by selective RXFP3 activation (de Ávila et al. 2018) (Fig. 16.2).

Apart from demonstrating its ability to modulate hypothalamic and neuroendocrine processes, experimental studies of relaxin-3 have also shown that relaxin-3/RXFP3 signalling can modulate extra-hypothalamic functions. For instance, local injections of RXFP3 agonist into the brainstem nucleus of the solitary tract increase the respiratory rate in the perfused rat working-heart-brainstem-preparation (Furuya et al. 2020), revealing the potential of relaxin-3/RXFP3 signalling to modulate autonomic responses (Fig. 16.2).

Finally, relaxin-3/RXFP3 signalling has also been implicated in the modulation of cognitive, affective and social functions, displaying the breadth of relaxin-3/RXFP3 system actions. Acute ICV injections of RXFP3 agonist and chronic RXFP3 activation in the ventral hippocampus, respectively, impairs social recognition and promotes social avoidance in rats (Albert-Gasco et al. 2019; Rytova et al. 2019). Moreover, relaxin-3/RXFP3 signalling has been shown to modulate theta rhythm-generating nodes of the septohippocampal system involved in arousal, memory, locomotion and spatial navigation (Gil-Miravet et al. 2021) (Fig. 16.2).

16.4.2 Relaxin-3/RXFP3 Signalling in the PVN

Considering the effects of relaxin-3 and selective RXFP3 agonists in the PVN, described above, and the high density of RXFP3 expression in the PVN, this hypothalamic area appears to be a major site of relaxin-3 action. Therefore, recent research has focused on elucidating the cellular and molecular effects of relaxin-3/RXFP3 signalling in the PVN to understand the neural correlates of putative mechanisms of endogenous relaxin-3 actions on neuroendocrine control and behaviour (Fig. 16.3).

Box 16.3 Paraventricular Nucleus of Hypothalamus

The paraventricular nucleus of the hypothalamus is a hub of homeostatic control in mammals, governing a wide range of physiological and behavioural processes. Functionally and morphologically distinct PVN neurons control neuroendocrine, autonomic, cognitive and emotional processes. PVN is composed of three neuronal populations: (1) magnocellular neurosecretory cells (MNCs), secreting oxytocin (OXT) and arginine vasopressin (AVP) hormones

(continued)

Box 16.3 (continued)
into the posterior pituitary lobe and innervating many brain regions; (2) parvocellular neurosecretory neurons, secreting corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone, AVP and other neuropeptides into the portal vessels in the median eminence to control the release of anterior pituitary hormones; (3) brainstem and spinal cord projecting neurons secreting, e.g. OXT, AVP and CRH, which provide input to centres of the autonomic nervous system and nociceptive circuitry. Notably, OXT- and AVP-secreting MNCs are also present in other hypothalamic nuclei, the supraoptic nucleus and the accessory nuclei. The magnocellular and parvocellular neurons were named for their relative size difference, moreover they can be clearly distinguished by their unique electrophysiology (Luther and Tasker 2000; Armstrong 2015).

In a study in which a recombinant adeno-associated virus ($rAAV^{1/2}$) driving expression and constitutive secretion of a RXFP3 agonist (R3/I5) was injected into the PVN area, Ganella et al. (2013a) assessed the effect of chronic activation of hypothalamic RXFP3 on the expression of several peptides known to modulate food intake. The virally mediated activation of RXFP3 led to decreased levels of oxytocin (OXT) and arginine-vasopressin (AVP) mRNA, accompanied by increased food intake and weight gain (Fig. 16.2). Therefore, relaxin-3/RXFP3-mediated food intake is thought to be underlined by an inhibitory action of RXFP3 signalling on the synthesis and release of OXT and AVP, two neuropeptides known to supress eating (Meyer et al. 1989; Arletti et al. 1990; Pei et al. 2014; Yoshimura et al. 2017).

In line with the inhibitory influence of RXFP3 signalling on OXT and AVP mRNA levels (Ganella et al. 2013a), and the observation that RXFP3 couples to inhibitory $G_{i/o}$-proteins (Liu et al. 2003; Van Der Westhuizen et al. 2005, 2007), both relaxin-3 and a selective RXFP3 agonist (RXFP3-A2) strongly inhibits the electrophysiological activity of putative OXT and AVP magnocellular neurosecretory cells (MNCs) in the PVN. Additionally, the presence of RXFP3 mRNA in PVN MNCs has been demonstrated with single-cell RT-PCR, whereby the cytoplasm of single neurons was aspirated into the patch pipette and its mRNA content was subjected to PCR following reverse transcription (Kania et al. 2020) (Fig. 16.3).

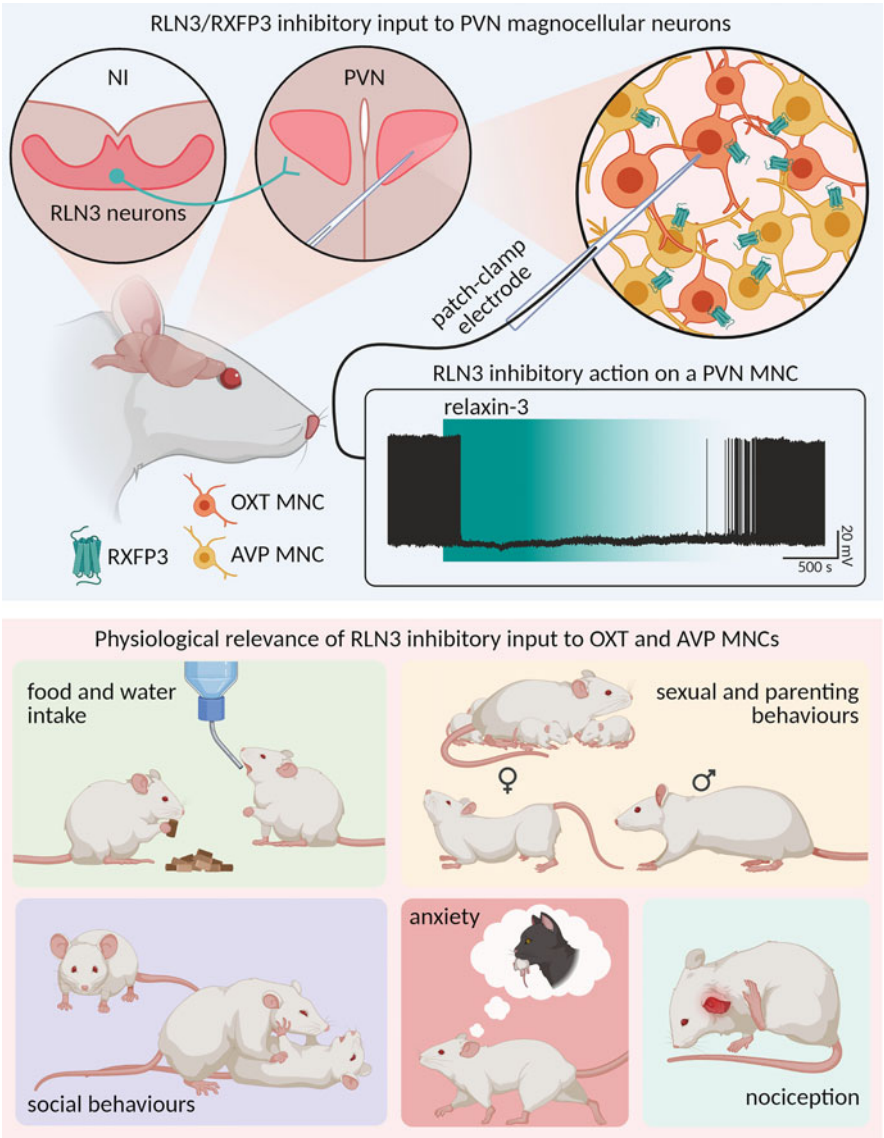

Fig. 16.3 Inhibitory input of the relaxin-3/RXFP3 system onto PVN magnocellular neurons. Top panel represents the NI RLN3 input to the PVN, where RXFP3 is expressed by almost all OXT and AVP magnocellular cells (MNCs). The inset depicts an inhibitory action of RLN3 application on the activity of PVN MNC in a patch-clamp experiment. The trace represents the membrane potential and spontaneously generated action potentials. Note that RLN3 application leads to cessation of action potential firing and membrane hyperpolarization, which was reversed after RLN3 washout. Bottom panel illustrates the range of behaviours putatively modulated by the robust RLN3-mediated inhibitory input to the PVN OXT and AVP system

Box 16.4 Patch Clamp

The patch-clamp technique is one of the most sophisticated electrophysiological tools in neuroscience. It was developed at the turn of the 70s and in the early 80s by Erwin Neher and Bert Sakmann, who were awarded the Nobel Prize in Physiology and Medicine in 1991. The patch-clamp technique allows scientists to examine electrical parameters generated by ion currents across the cellular membrane of individual neurons, dissociated cells, cell cultures, nervous tissue explants or even in vivo in behaving animals. This powerful tool enables the study of brain functions on different organizational levels, from single channel properties and neuronal excitation to synaptic connectivity and functional neuroanatomy, and is now often combined with optogenetics. Ex vivo brain sections or cell cultures recorded in a bath perfused with artificial cerebrospinal fluid are very useful for studying the electrophysiological effects and ionic and neuronal mechanisms of action of pharmacological compounds (neurotransmitters, neuropeptides, drugs) (Okada 2012; Dallas and Bell 2021).

As almost all PVN MNCs synthesize either OXT or AVP as their major neuropeptide, and ~ 90% of them are inhibited by RXFP3 activation, the OXT and AVP systems, with their plethora of physiological functions, seem to be under a robust inhibitory influence of relaxin-3/RXFP3 signalling. OXT and AVP MNCs are pivotal in food intake, water balance, reproduction, nociception and a variety of social and parenting behaviours (Koshimizu et al. 2012; Jurek and Neumann 2018), so a direct inhibitory input from the relaxin-3/RXFP3 signalling possesses broad physiological and clinical implications (Fig. 16.3).

Besides the direct influence on PVN MNCs, selective RXFP3 activation influences ~48% of putative parvocellular PVN neurons, including OXT-synthesizing cells (Kania et al. 2017). This ability of relaxin-3/RXFP3 signalling to regulate parvocellular neuron activity implies modulatory influence on of hypothalamo-pituitary axes, including stress- and autonomic responses, as well as nociception (Koshimizu et al. 2012; Jurek and Neumann 2018; Deussing and Chen 2018).

The ionic mechanisms underlying the RXFP3-induced inhibitory effect in the PVN have also been studied. A combined pharmacological and electrophysiological approach identified the activation of an M-like potassium current as responsible for RXFP3-mediated inhibition in the PVN MNCs (Kania et al. 2020). The neural M-current is mainly conducted by channels composed of members of the KCNQ transmembrane subunit family (KCNQ2–5), which are expressed in the PVN and SON (Zhang et al. 2009; Zhou et al. 2017). Indeed, KCNQ2 and KCNQ3 mRNA has been shown to co-express with RXFP3 mRNA in PVN MNCs, representing a putative molecular substrate for the modulation of the M-current by relaxin-3/RXFP3 signalling (Kania et al. 2020).

In these studies, the anatomy of the relaxin-3 innervation of the PVN has also been characterized, showing that only sparse relaxin-3-immunoreactive fibres are present within the PVN (Ma et al. 2007; Kania et al. 2017, 2020), with its immediate

surroundings more densely occupied by relaxin-3 fibres. This observation suggests that relaxin-3 might reach PVN neurons by diffusion in the extracellular matrix, following its release from nearby fibres, via the so-called volume transmission. This non-synaptic type of release has been widely reported for neuropeptides (van den Pol 2012). Additionally, retrograde neural tract-tracing studies have identified the NI as the major source of the relaxin-3 innervation in the PVN and its vicinity (Kania et al. 2017, 2020). Given the strong RXFP3 expression and sensitivity to relaxin-3 displayed by PVN neurons, a volume transmission-based relaxin-3 input provided by NI neurons should be sufficient to effectively inhibit majority of PVN MNCs (Fig. 16.3).

16.5 Regulation of Relaxin-3 Neuron Activity

Currently, little is known about the possible functional specializations and/or similarities between the four groups of relaxin-3 neurons located across the brainstem in the vlPAG, dSN, PnR and NI. Almost all regulatory studies to date have focused on the NI as the major source of relaxin-3. However, some anatomical evidence highlights possible functional specializations between different populations of relaxin-3 neurons. For example, the relaxin-3 neurons in the rat vlPAG provide the predominant relaxin-3 input to the thalamic intergeniculate leaflet, a node of the brain's biological clock circuitry, with only a sparse innervation of the region by relaxin-3 neurons from the NI and PnR (Blasiak et al. 2013). In contrast, both NI and vlPAG relaxin-3 neurons seem to robustly express the CRH type 1 receptor (CRH_1) (Tanaka et al. 2005; Blasiak et al. 2013; Ma et al. 2013), suggesting that stress sensitivity is a feature shared among different relaxin-3 neuronal populations. In this section, the physiological and pharmacological regulation of the activity of relaxin-3 neurons will be discussed. Due to the available evidence, it will focus mainly on the NI.

NI neurons express multiple neuropeptide and neurotransmitter receptors, suggesting their putative modulatory influence on the NI activity. In the rat NI, receptors for relaxin-3, neuromedin B, CRH, orexin (OX), melanin-concentrating hormone (MCH), OXT, growth hormone, serotonin, acetylcholine, glutamate and GABA have been identified (Ryan et al. 2011). Detailed description of the best known NI modulators follows.

Stress is a potent regulator of NI neurons. Several types of neurogenic stressors have been shown to induce the expression of c-Fos, a marker of neuronal activity, in the rat NI (Ryan et al. 2011). Notably, CRH/CRH_1 signalling, the main mediator of the central stress response, stimulates the electrical activity of relaxin-3 neurons under both in vivo and ex vivo conditions (Ma et al. 2013). Moreover, the expression of relaxin-3 in the NI is enhanced by physical stressors, altogether revealing the sensitivity of the relaxin-3 system to stress (Tanaka et al. 2005; Banerjee et al. 2010) (Fig. 16.2).

The aforementioned reciprocal relation between relaxin-3 signalling and stress, together with the involvement of relaxin-3 and NI neurons in the control of

cognitive, emotional and homeostatic processes (Olucha-Bordonau et al. 2018), position relaxin-3/RXFP3 signalling at the interface between stress and crucial brain processes e.g. learning and motivation. Indeed, modulation of NI CRH_1 has been shown to impair hippocampocortical plasticity, suppress cortical activation and regulate alcohol seeking in rats (Farooq et al. 2013; Rajkumar et al. 2016; Walker et al. 2017). Furthermore, the NI is involved in contextual fear memory formation, yet the direct involvement of relaxin-3/RXFP3 signalling in these phenomena remains to be verified (Szőnyi et al. 2019). Moreover, relaxin-3/RXFP3 signalling mediates stress-related alcohol consumption (Ryan et al. 2013b; Walker et al. 2015) and, as already mentioned, stress-induced binge eating (Lenglos et al. 2013; Calvez et al. 2016b; Kania et al. 2020). These findings provide a strong rationale to investigate the modulation of relaxin-3/RXFP3 signalling as a novel effective way to treat human psychiatric and comorbid conditions.

The action of two hypothalamic neuropeptides, OX and MCH, has been thoroughly examined in the rat NI. OX and MCH are synthesized by separate subpopulations of neurons in the lateral hypothalamus (LH) and both are known to stimulate food intake, yet they display opposing effects on the sleep/wake cycle and energy expenditure. OX signalling promotes wakefulness, arousal and energy expenditure, whereas MCH induces sleep and energy conservation (Adamantidis and de Lecea 2009; Diniz and Bittencourt 2017). There are two OX peptides, orexin A and B, and two types of receptors: OX_1, which binds OXA, and OX_2, which binds OXA and OXB (Sakurai et al. 1998). OXA and OXB are synthesized by the same neurons. The NI is innervated by OXA- and MCH-immunoreactive fibres, which are in close apposition to the relaxin-3 neurons (Blasiak et al. 2015; Sabetghadam et al. 2018). In addition, MCH seems to be transported from the cerebrospinal fluid into the NI area by tanycytes, specialized ependymal cells located in the walls of cerebral ventricles (Prevot et al. 2018). Moreover, NI neurons express OX (mainly OX_2) and MCH1 receptors, with OXA exciting 35–66%, and MCH inhibiting 34% of NI neurons in ex vivo patch-clamp experiments (Blasiak et al. 2015; Sabetghadam et al. 2018). Notably, both peptides affect partially distinct subpopulations of NI neurons, highlighting their functional specializations; however both directly influence relaxin-3 neurons (Blasiak et al. 2015; Sabetghadam et al. 2018). Finally, OXA injections into the NI has been shown to stimulate locomotor activity and food intake, whereas blocking the OX_2 receptor in the NI prevents stress-induced, alcohol-seeking in rats (Kastman et al. 2016). These studies have characterized the modulatory influence of OXA/OX_2 and MCH/MCH_1 signalling on the rat NI and relaxin-3 neuron system, providing further evidence for its role in the control of arousal and motivated behaviours such as food intake and alcohol seeking (Fig. 16.2).

The relaxin-3/RXFP3 signalling system is also under the modulatory influence of the female sex hormone, estradiol. The NI in female rats has been shown to express several types of oestrogen receptors, Gper1, ERα and ERβ, with Gper1, a membrane-bound oestrogen receptor, being the most abundant (de Ávila et al. 2020). In line with this observation, NI neurons, including relaxin-3-immunoreactive cells, are acutely inhibited by estradiol application during patch-clamp

recordings ex vivo. Furthermore, relaxin-3 mRNA levels in the NI are dynamically modulated across the oestrus cycle, with the lowest expression reached in proestrus, i.e., during the peak in estradiol levels in blood circulation (de Ávila et al. 2020). Intriguingly, it is well documented that in the following stage of the oestrus cycle—oestrus, food intake is significantly reduced in female rats (Drewett 1973; Eckel et al. 2000). Considering the fact that female rats receiving chronic ICV injections of relaxin-3 do not display the phasic inhibition of food intake during oestrus (Calvez et al. 2016a), one can hypothesize that estradiol partially modulates food intake via its direct inhibitory action on the relaxin-3 system. Furthermore, while the level of relaxin-3 expression fluctuates during the oestrus cycle, so does RXFP3 expression in different hypothalamic areas, including PVN, LH, medial preoptic area and the bed nucleus of stria terminalis (de Ávila et al. 2020). These dynamic changes in relaxin-3/RXFP3 signalling across hormonal states may help to ensure optimal adaptations of e.g. food intake, stress reaction and arousal, to meet physiological demands of different phases of the reproduction cycle (Fig. 16.2).

Box 16.5 Rat Oestrus Cycle

The rat oestrus cycle lasts four to five days and is characterized by cyclic patterns of hormone secretion and ovarian function. The oestrus cycle is generally divided into four stages, easily identifiable by examining the type of cells present in the vaginal smear: proestrus (12–14 h), oestrus (25–27 h), metestrus (6–8 h) and diestrus (55–57 h). Ovulation happens during oestrus, a phase of female sexual receptivity, and is preceded by the peak release of estradiol, luteinizing hormone, follicle-stimulating hormone and progesterone during proestrus. After ovulation, if the ovum does not become fertilized, in metestrus and diestrus the luteal phase occurs, during which the corpus luteum forms and regresses, and the cycle progresses again into proestrus (Levine 2015).

16.6 Summary and Perspective on Future Directions of Relaxin-3/RXFP3 Research

This chapter provides a focused summary of two decades of research by multiple laboratories, which was initially aimed at elucidating the neuroanatomy of the relaxin-3/RXFP3 system in the mammalian brain, and subsequently involved a series of pharmacological studies in rodents to identify which aspects of physiology and behaviour were modulated by acute or chronic RXFP3 activation or inhibition. The former studies were facilitated by the development of specific antisera against relaxin-3 (Tanaka et al. 2005; Ma et al. 2007) and the use of simple and advanced in situ hybridization methods and radioligand binding (Sutton et al. 2004; Ma et al. 2007; Smith et al. 2010); and the latter studies by using novel, chimeric and truncated peptides selective for RXFP3 compared to other relaxin-family receptors

(Kuei et al. 2007; Haugaard-Kedström et al. 2011; Shabanpoor et al. 2012), which enabled the distinction between RXFP3- and likely RXFP1-mediated effects. This combination of anatomical and functional studies has revealed a likely role for the endogenous relaxin-3/RXFP3 system in the modulation of arousal, circadian rhythms, feeding and metabolism, social and stress-related emotional behaviour, autonomic responses and memory. More recent experiments have sought to determine more about the neurochemical phenotype of relaxin-3 neurons (Nasirova et al. 2020) and RXFP3 target neurons in different brain regions (Albert-Gasco et al. 2019); and to understand the ionic mechanisms associated with the inhibitory effect of RXFP3 activation on neurons, in particular, the nature of relaxin-3/RXFP3 modulation of magnocellular and parvocellular OXT and AVP neurons in the rat PVN and the likely impact on various hypothalamic-pituitary-endocrine axes and intrinsic brain circuits (Kania et al. 2017; Kania et al. 2020).

These studies have established the relaxin-3/RXFP3 system as important for integrated aspects of neuroendocrine, metabolic and autonomic responses and complex behaviour, but there remain many aspects of this network that require further research and a better understanding, including the nature and regulation of neuronal relaxin-3 release and whether it involves synaptic and/or volume transmission; and the precise location (presynaptic/postsynaptic) and function of RXFP3 in specific neural circuits, which should be facilitated by viral-based and optogenetic approaches (Ganella et al. 2013a; Rytova et al. 2019; Haidar et al. 2017) and the availability of relaxin-3 and RXFP3 gene knockout mice (Smith et al. 2012; Hosken et al. 2015) and both relaxin-3- and RXFP3-Cre-recombinase mice (Nasirova et al. 2020; Voglsanger et al. 2021). There is also a need for further examinations of the plasticity of the relaxin-3/RXFP3 system under different experimental conditions (altered external/internal stimuli) and neuropathological conditions, to increase our understanding of the complex mechanisms underlying the development of health and psychiatric conditions, including eating disorders, obesity, anxiety and dementia, and any therapeutic potential of RXFP3-based treatments.

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Key Literature (5–12 Articles) (Further Recommended Reading)

Blasiak et al. (2013) The first description of the existence of specific relaxin-3 neuron projections from ventrolateral PAG (but not nucleus incertus) to the intergeniculate nucleus in the rat.

Blasiak et al. (2015) The first study to identify different electrophysiological phenotypes of nucleus incertus neurons and their responses to the arousal-related orexin neuropeptides.

Hosken et al. (2015) Description of the reduced running wheel activity of mice lacking the RXFP3 gene/protein relative to wildtype littermates, in line with a similar phenotype of mice with a relaxin-3 gene/protein deletion.

Kania et al. (2020) The first study to demonstrate an RXFP3-related blockade of binge eating in female rats via actions within the PVN on oxytocin and arginine-vasopressin neurons.
Ma S et al. (2007) Comprehensive description of the neuroanatomical distribution of relaxin-3 neurons (mRNA/peptide) and labelled fibres, and RXFP3 mRNA and binding sites in rat brain.
Ma et al. (2009) Description of the anatomy of relaxin-3 neurons and their projections in a non-human primate (Macaca fascicularis) brain.
Ma et al. (2009) Pharmacological studies demonstrating the modulation of hippocampal theta oscillations and spatial memory by RXFP3 signalling in medial septum.
Ma et al. (2013) Heterogeneous responses of nucleus incertus neurons to corticotropin-releasing hormone and coherent activity with hippocampal theta rhythm in the rat.
McGowan et al. (2005) The first description of the effect of central administration of relaxin-3 to increase feeding in rats.
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Tanaka et al. (2005) The first description of the neuroanatomical distribution of relaxin-3 neurons and immunolabelled nerve fibres in rat brain and their response to environmental stressors.

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Glossary

Actin One of the three major cytoskeletal elements found in all eukaryotic cells that creates filaments (microfilaments) and is essential for cell motility, cell division, endo- and exocytosis, and cell contractility.

Allostasis The process of maintaining balance within an organism through adaptation guided by anticipation of future requirements and changes in environmental conditions.

Anatomical Landmarks Clearly identifiable anatomic structures of the brain across individuals aiding the understanding of brain anatomy, navigation, and segmentation.

Anorexigenic A molecule, factor, or drug that decreases appetite and reduces the amount of food that an individual eats.

Antipsychotic A substance used in treating psychosis, schizophrenia, mania, depression, or paranoia, very often with severe side-effects such as hypertension or obesity.

Appetite The desire to consume food in order to satisfy homeostatic and/or hedonic needs

Ascending projections Axonal projections that emanate from the AVP-magnocellular and innervate other hypothalamic and extra-hypothalamic regions besides the neurohypophysis.

AVP-Magnocellular Magnocellular vasopressinergic neurosecretory neurons located in the hypothalamic paraventricular and supraoptic nuclei that synthesize and release vasopressin in both the neurohypophysis and within the central nervous system, classically known by their role in the control hydroelectrolyte balance.

Axons Long, thread-like parts of a nerve cell along which impulses are conducted from the cell body to other cells

Central Melanocortin System Pivotal neurocircuit in the brain controlling satiety and energy expenditure. This circuit consists mainly of two distinct, functionally opposed populations of neurons in the hypothalamic arcuate nucleus, which release their respective transmitters onto second-order neurons that express melanocortin-4 receptors and reside in the hypothalamic paraventricular nucleus. The term "melanocortin" stems from the observation that alternative cleavage

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products of POMC, a major transmitter precursor in the melanocortin circuit, convey *melanotropic* as well as *adrenocorticotropic* effects in the skin and adrenal gland, respectively.

Circadian Rhythm A daily cycle of activity, hormones, or any physiological parameter, exhibited by most organisms and based on 24-hour intervals can persist in constant dark conditions.

Circumventricular Organ A highly vascularized area bordering the cerebral ventricles, where certain substances from the blood may pass into the cerebrospinal fluid.

Cre-Driver Transgenic mouse (or rat) line expressing Cre recombinase from bacteriophage P1 under the control of a specific promoter. In combination with a "floxed" (flanked by loxP sites) allele, Cre recombinase is able to delete or invert respective DNA segments.

Dahlgren Cells Neurosecretory cells that reside along the caudal spinal cord of piscine species. These cells send axon terminals to the vascularized urophysis, forming the caudal neurosecretory system.

Developmental Programming The process by which exposure to environmental factors during prenatal or early postnatal development permanently sets the physiology, metabolism, and epigenome of an individual.

Dose-Response Relationship Quantification of dose-dependent effects of a drug, often in a non-linear fashion.

Embryonic Stem Cell Pluripotent stem cells derived from the inner cell mass of preimplantation embryos.

***En passant* Release/Volume Transmission** Another form of non-targeted release of neuropeptides, for which no true synaptic contacts or synaptic clefts are required. OT and AVP are released from non-terminal axonal compartments, while the respective axons do not terminate within the same brain region.

Expansion Microscopy A sample preparation tool that allows identification of small cellular structures, including synapses, with a wide range of microscopic techniques (including confocal microscopy) by using a polymer system that, through chemical reactions, physically expands them isotropically along with the underlying tissue.

GERNs GABA and estrogen receptor-expressing neurons. These neurons were identified first in rat lateral habenula with dual VGAT and VGLUT2 mRNA co-expression. They are located in a region densely innervated by AVP/glutamate/aromatase-expressing axons. The VGAT expression is dependent on testosterone levels in vivo.

GnRH Neurons Neurons that synthesize gonadotropin-releasing hormone and whose somata in rodents are located principally in the preoptic area (POA) of the hypothalamus, diagonal band of Broca (DBB), and medial septum (MS), and that project mainly to the median eminence (ME), where they release GnRH into the hypothalamo-hypophyseal portal circulation to control fertility.

GsPCR, GqPCR G-protein coupled receptor coupled to Gs or Gq

Hunger An internal state is actualized as the drive to eat by homeostatic feedback in response to prior caloric deprivation.

Hyperphagia An extreme unsatisfied drive to consume food, leading to excessive eating

Hypophysiotropic Neurons Hypothalamic neurons project their axons into the median eminence, where the hormone is released and transported to the anterior pituitary via the hypophyseal portal vessels, regulating the secretion of pituitary hormones.

Hypothalamic-Pituitary-Adrenal Axis The organisms' central neuroendocrine stress response system involving the sequential release of hormones (CRH, ACTH, and corticosterone/cortisol) to maintain homeostasis.

Hypothalamus A brain region, located below the thalamus and above the pituitary gland. This region regulates body homeostasis by means of various populations of neuropeptides-producing neuroendocrine cells.

Hypothalamus-Pituitary Portal Vessels Capillaries that join the venous irrigation of the base of the hypothalamus to the venous irrigation of the anterior pituitary.

Image Segmentation The process of using manual, semi-automated, and automated techniques to trace parts of an image according to the underlying anatomy or pathology.

Induced Pluripotent Stem Cell Pluripotent stem cells obtained by inducing dedifferentiation of adult somatic cells via cell reprogramming technology.

Juxtacellular Labeling Technique by which a single neuron is recorded extracellularly in vivo, labeled by neurobiotin electroporation. Perfusion/fixation is performed after allowing the neurobiotin to diffuse along the somato-dendritic and axonal arborization in vivo (usually 4–19 hours). The labeled cell is then revealed by histochemistry techniques. This technique allows the unambiguous identification of a neuron's soma and its dendritic and axonal projections to distant structures at a single-cell level, as well as determination of its chemical phenotype.

Kisspeptin Neurons Neurons that synthesize kisspeptin, which are located mainly in the arcuate nucleus (ARC) and rostral periventricular area of the third ventricle (RP3V), and that release kisspeptin onto GnRH neurons and other neurons to control fertility and other functions.

Magnocellular Neuroendocrine Cell Large hypothalamic neuron synthesizing OT or VP that is connected to the bloodstream via axonal projection to the posterior pituitary.

Magnocellular Neurons (or Magnocellular Neurosecretory Cells) Large neuroendocrine neurons located in the hypothalamic supraoptic and paraventricular nuclei and producing vasopressin and oxytocin that is secreted into the bloodstream via axonal terminals in the posterior pituitary.

Magnocellular Neurosecretory Cell Large hypothalamic neuron synthesizing OXT or AVP that is connected to the bloodstream via axonal projection to the posterior pituitary. They are localized in paraventricular, supraoptic, and accessory hypothalamic nuclei.

Medial Preoptic Area The most anterior region of the hypothalamus. It is the major regulatory center of parental behaviors in rodents. Thermosensitive neurons controlling body temperature are also located here.

Median Eminence The ventral zone of the hypothalamus where hypophysiotropic nerve terminals secrete releasing factors into portal capillaries.

Median Eminence It is located at the ventral surface of the tuberal region of the hypothalamus. It continues into the pituitary stalk and represents the contact area between nerve terminals of parvocellular neurons and capillaries of the portal vascular system of the anterior pituitary. Moreover, it is a circumventricular organ lacking a blood-brain barrier.

NCS-RapGEF2 It is a guanine nucleotide-exchange factor (*n*euritogenic *c*AMP *s*ensor-RapGEF2) and the sensor/effector for cyclic AMP that links its elevation to the activation of the MAP kinase ERK in adult mammalian neurons and endocrine cells

Neurohypophysis A neuroendocrine interface located at the posterior pituitary where neurohormones such as oxytocin and vasopressin are secreted into the blood.

Neurogenesis A cellular process by which new neurons are formed in the brain

Neuropeptide Comparatively small protein produced by neurons and stored in large dense-core vesicles. Neuropeptides act on G-protein coupled receptors and are often co-expressed with other neurotransmitters. Neuropeptides are responsible for slow-onset, long-lasting modulation of synaptic transmission.

Neuropeptides Peptides (short chains of amino acids ranging from 3 to >50 residues in length) widely expressed in neurons of the central nervous system. Neuropeptides are packaged in large dense-core vesicles, and upon synaptic or axonal release, they activate G-protein-coupled receptors to modulate rapid neuronal responses to classical neurotransmitters and produce long-term effects on intracellular signaling pathways.

Nociceptive Inputs Stimuli threaten tissue damage and typically evoke pain and fear. TIP39 affects nociceptive information transfer both in the spinal cord dorsal horn and at supraspinal levels.

Obesity A condition in which excess body fat has accumulated to the extent that it may have a negative effect on health

Optogenetics A technique that uses light to control neurons that have been genetically modified to express light-sensitive ion channels and which can be employed to perform neural circuit mapping.

Orexigenic Applied to a molecule, factor, or drug that stimulates appetite and can eventually cause hyperphagia

Osmolality A quantitative measure of the total solute concentration in a solution expressed in moles per kilogram of solution.

Osmotic Stimuli Exposing cells or tissues to a condition in which the extracellular fluid contains higher or lower concentration of membrane-impermeant solutes—hypertonic or hypotonic stimuli, respectively.

Ovulation Discharge of the ovum from the ovary.

PAC1 (encoding gene *Pac1*) is the major receptor for PACAP (VPAC1 and VPAC2 recognize both PACAP and the related peptide VIP)

Parathyroid Hormone 2 Receptor A G-protein coupled receptor, which is most abundant in the central nervous system where it is activated by tuberoinfundibular peptide of 39 residues

Paraventricular Nucleus of the Hypothalamus Hypothalamic nucleus is localized lateral to the third ventricle, which is central to the stress response. It is composed of parvocellular and magnocellular neurons. Parvocellular neurons project to the zona externa of the median eminence, liberating pituitary-releasing or -inhibiting peptides to the portal vasculature from where they reach the anterior pituitary. Magnocellular neurons project via the zona interna directly to the posterior pituitary and release their cargo to the general circulation.

Parvocellular Neuroendocrine Cells Smaller, generally spindle-shaped hypothalamic neurons synthesize a plethora of different neuropeptides, including OT and AVP, among others. These neurons project to the median eminence and other intra- and extra-hypothalamic areas but not to the posterior pituitary.

Parvocellular Neurosecretory Cell Smaller spindle-shaped hypothalamic neurons that synthesize a plethora of different neuropeptides, including OXT and AVP among others. These neurons project to the median eminence and other intra- and extra-hypothalamic areas but not to the posterior pituitary. They are localized in the hypothalamic paraventricular and arcuate nucleus, hypothalamic and preoptic stratum, medial septal nucleus and nucleus of the diagonal bed of Broca.

Perinatal Related to the time of life immediately before and after birth

Pharmacological Challenge A method to examine pharmacological effects by administering a chemical compound to a system and observing subsequent changes.

Phasic Feeding Signals feeding cessation is typically mediated by phasic "meal-control" signals from the gastrointestinal tract (gastric distension, the food-evoked release of various gut hormones, etc.); this plurality of information is sensed by afferent nerves, including the vagus nerve, and ascend to certain brainstem nuclei from where it gets relayed to the hypothalamus. Please note that an in-depth discussion of hindbrain<>hypothalamus connectivity is beyond the scope of this chapter (for further reading, see Grill and Hayes, 2012).

Pluripotent Stem Cell Specialized cells with two properties: self-renewal and pluripotency. The self-renewal is the capacity of the stem cells to divide indefinitely, producing unaltered daughter cells maintaining the properties of the progenitor cell. Under particular conditions or with specific signals, a stem cell can exit from self-renewal and engage in a program leading to differentiation into specialized cell types deriving from the three germ layers (ectoderm, endoderm, and mesoderm).

Posterior Intralaminar Complex of the Thalamus (PIL) A thalamic brain area is expressing TIP39 in and around the posterior thalamic nucleus. Its neurons project towards the hypothalamus, amygdala, and medial prefrontal cortex. The expression of TIP39 is greatly upregulated in the PIL in mother rats.

Preautonomic Neurons Neurons in the brain that connect directly with parasympathetic or sympathetic autonomic neurons located in the spinal cord or brain stem.

Presympathetic/Preautonomic Cell Neurons in different parts of the brain that send a direct projection to preganglionic sympathetic neurons in the spinal cord.

Programming The ability of exposures during the restricted period of prenatal or early postnatal life to cause permanent cellular, molecular, or health changes

Promoter-Driven Labeling Labeling of selected neuronal populations with a cell type-specific gene promoter that drives the expression of a reporter protein only in those populations and that can be used to study their anatomy and physiology.

Relaxin-3 Conserved neuropeptide is expressed mainly in the brainstem nucleus incertus across vertebrate species. Its cognate metabotropic receptor is RXFP3. Relaxin-3/RXFP3 signaling is implicated in many neuroendocrine, cognitive, and affective brain processes, with a well-established role in stimulating homeostatic and hedonic eating.

Satiation Immediate, post-prandial experience of fullness leading to meal termination.

Satiety Time period of decreased desire to eat a subsequent meal (inter-meal interval).

Somato-Dendritic Release A distinctive feature of neuroendocrine cells, whereby neuropeptides (OT and AVP) are released from somatic and dendritic compartments of the cell. This release modality is essential for autoregulation (self-feedback) of the OT and the AVP systems, but also mediates paracrine and hormone-like effects, including interpopulation crosstalk between neurosecretory and autonomic-related neurons in the PVN.

Super-Resolution Microscopy A series of imaging techniques that overcome limitation of the resolution of light microscopy by light diffraction.

Synapse A junction between two nerve cells where a nervous impulse passes from one neuron to another

Tanycytes Radial glia-like cells lining the ventral walls and floor of the third ventricle in the brain, that have long processes and large end feet that terminate close to brain capillaries.

Thyrotropin-Releasing Hormone-Degrading Ectoenzyme A TRH-specific ectopeptidase that hydrolyses TRH once released into the extracellular space.

Tissue Clearing A method of making brain tissue transparent using acrylamide-based hydrogels built from within, and linked to, the tissue, enabling highly detailed imaging of fluorescently labeled neurons.

Tonic "adiposity" Signals Signals of long-term energy availability tonically inform the adjustment of meal size and frequency according to energy reserves.

Transcription Factor A molecular factor that controls the rate of transcription of genetic information from DNA to messenger RNA by binding to a specific DNA sequence

Transcriptome The complete set of RNA transcripts in a cell.

Tuberoinfundibular Dopaminergic Neurons A dopaminergic cell group located in the arcuate nucleus of the hypothalamus. They inhibit prolactin secretion from the pituitary as the major regulators of prolactin release and consequently lactation.

Tuberoinfundiblar Peptide of 39 Residues (TIP39) A neuropeptide is also called parathyroid hormone 2. It is synthesized in only three small regions in the brain and projects widely to hypothalamic, limbic, auditory, and nociceptive regions of the brain

Tubulin/Microtubules One of the three major cytoskeleton elements found in all eukaryotic cells that assembles into tubular structures (microtubules) and is crucial for regulating cell shape, intracellular transport and cell division.

Urophysis A fish-specific neuroendocrine interface in the caudal region of the spinal cord in piscine species where urotensins are secreted into the blood.

Vasopressin Receptors Three receptors for vasopressin have been identified, V1a, V1b (once called V3) and V2. The former two use the Gq signaling pathway and have been identified in different brain regions, while the latter use the Gs signaling pathway and its expression has not been reported in brain tissue.

Vesicular GABA and Glutamate Transporters Transport proteins located in the synaptic vesicles at the axon terminal. They allow the accumulation of GABA and glutamate by synaptic vesicles. The expression of the vesicular GABA (VGAT) or glutamate (VGLUT1, VGLUT2 or VGLUT3) transporters is accepted as markers for GABAergic (inhibitory) or glutamatergic neurons (mainly excitatory; however, we have recently observed VGAT and VGLUT co-expressing neurons in limbic regions, with some showing dependence on gonadal function—see "GERNS").

VGAT The vesicular GABA transporter is a marker for inhibitory (GABAergic) neurons in the adult brain.

VGLUT1–3 The vesicular glutamate transporters type 1, 2, and 3, markers for excitatory (glutamatergic) neurons

Viral Tracing The use of a virus to trace neurons, including their projections and connections. Viruses have the advantage of self-replication over molecular tracers, but can also spread too quickly and cause degradation of neural tissue. Viruses can spread within neurons or through spatially close assemblies of neurons via *synapses*, allowing for their use in studying functionally connected neural networks.