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

Limei Zhang, Vito S. Hernández, David Murphy, W. Scott Young, and Lee E. Eiden

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

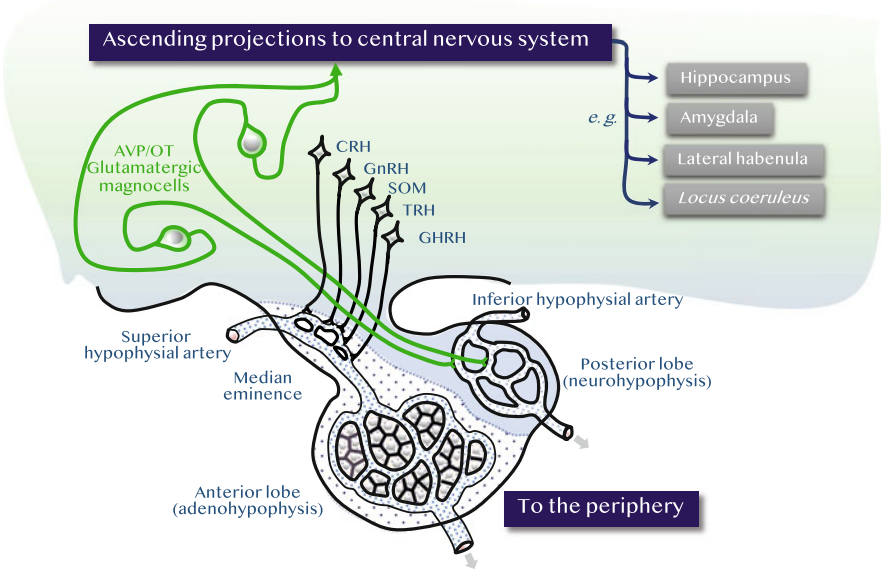

Fig. 7.1 Schematic drawing showing the gross chemo-anatomical components of the hypothalamo–hypophysis (also called pituitary gland) neuroendocrine control centres and their anatomical relationship. Two neuroendocrine sub-systems can be coarsely classified. The first is the hypothalamic–hypophysiotropic 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

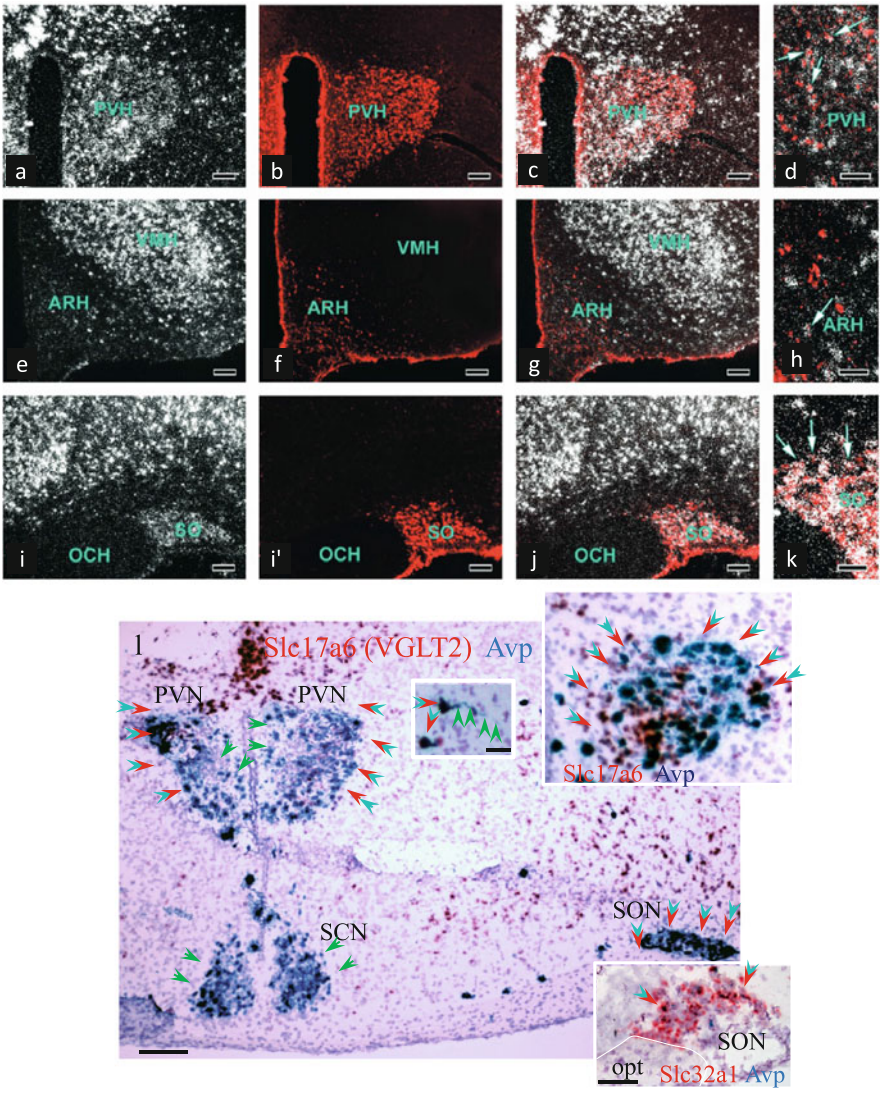

Fig. 7.2 Hypothalamic AVP-magnocells co-express VGLUT2 mRNA (see the text for details). Panels **a**, **e** and **h** show the VGLUT2 ISH in PVN, ventromedial hypothalamic nucleus (VMH) and SON. Panels **b**, **f**, **i** show FG immunohistochemistry also in the above nuclei (F: VMH as negative control), 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

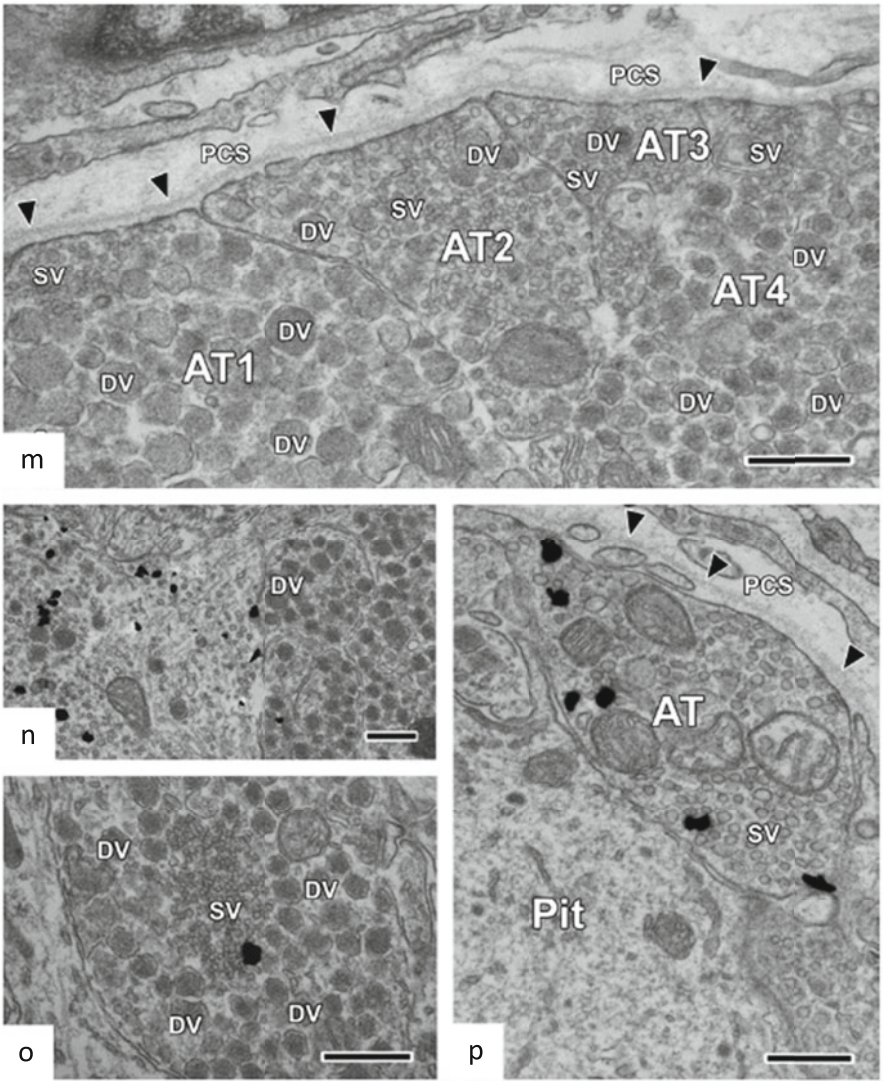

Fig. 7.2 (continued)

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

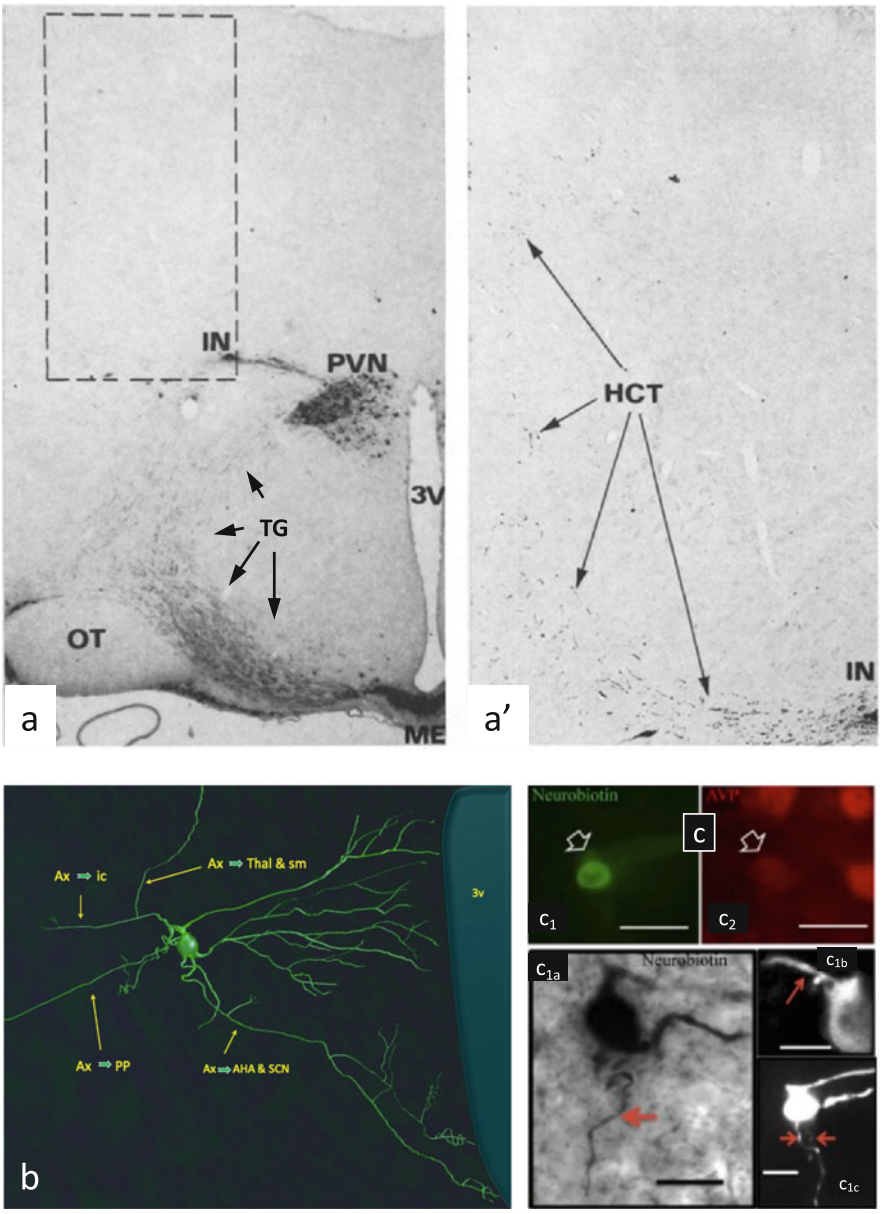

Fig. 7.3 Ascending projections to central nervous system emanate from AVP-magnocells. (**a** and **a'**) Panels from one of the earliest studies reporting that ascending neurophysin immunopositive fibres *seemed to be* emanating from hypothalamic paraventricular nucleus (PVN) and the intermediate nucleus (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

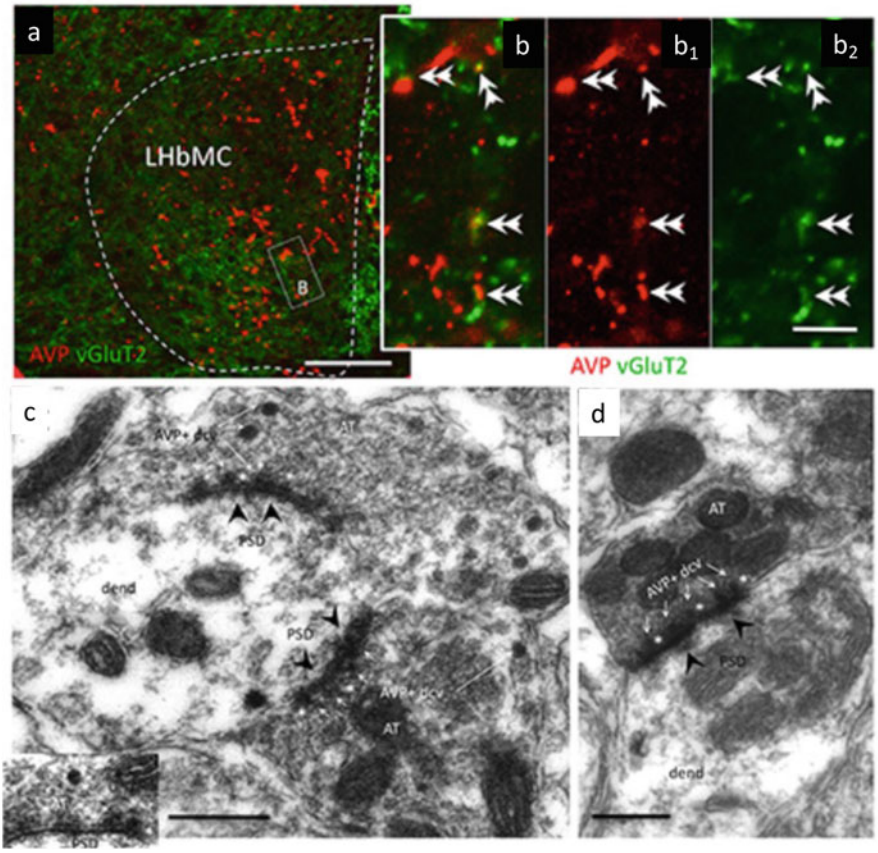

Fig. 7.4 Most AVP+ axon terminals co-expressed vesicular glutamate transporter 2 (VGLUT2) and established Gray type I synapses onto habenular neuron dendrites. **a** and **b**s: Representative confocal photomicrographs of double immunofluorescence AVP (red) and VGLUT2 (green) centred at the medio-central 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

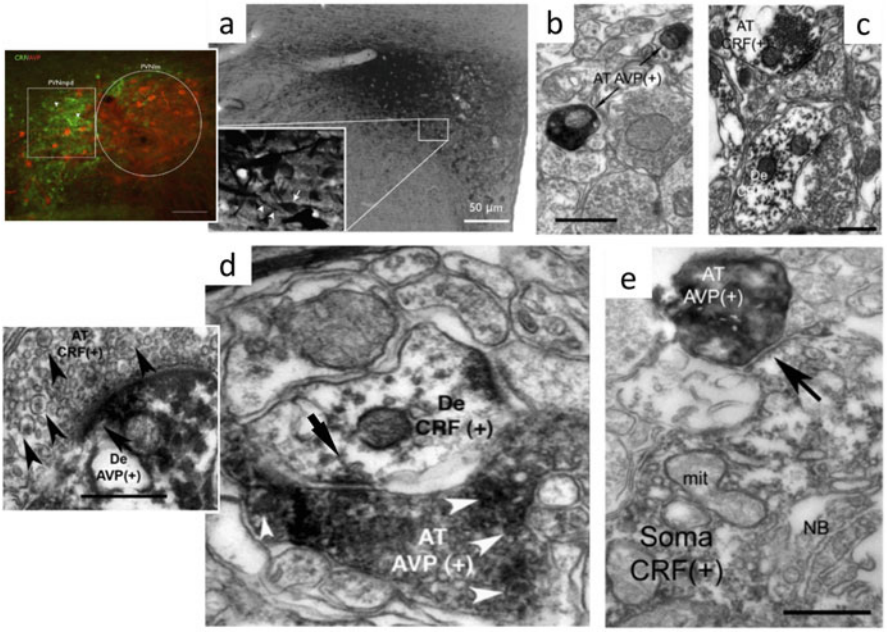

Fig. 7.5 Reciprocal synaptic connections between AVP-magnocells and corticotropin-releasing hormone (CRH) synthesizing neurons in the paraventricular nucleus, lateral magnocellular division and medial parvocellular division (PVN_{mpd} and PVN_{lmd}, respectively). (**a**) Double peroxidase-DAB reaction 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

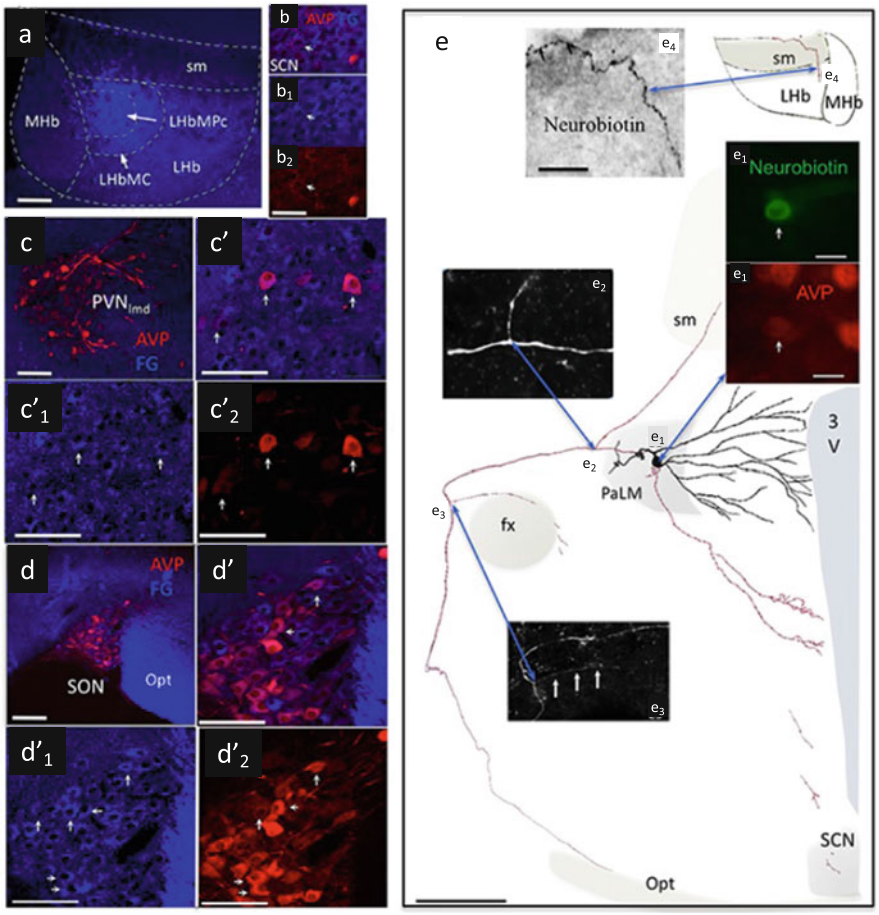

Fig. 7.6 AVP-containing magnocellular neurosecretory neurons serving as one of the sources of AVP+ axons in LHb. (**a**) Fluorogold (FG) retrograde tracer was injected into the medio-central subnucleus of the lateral habenula (LHbMC). (**b**) In the hypothalamic suprachiasmatic nucleus (SCN, panels b′a–b), 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

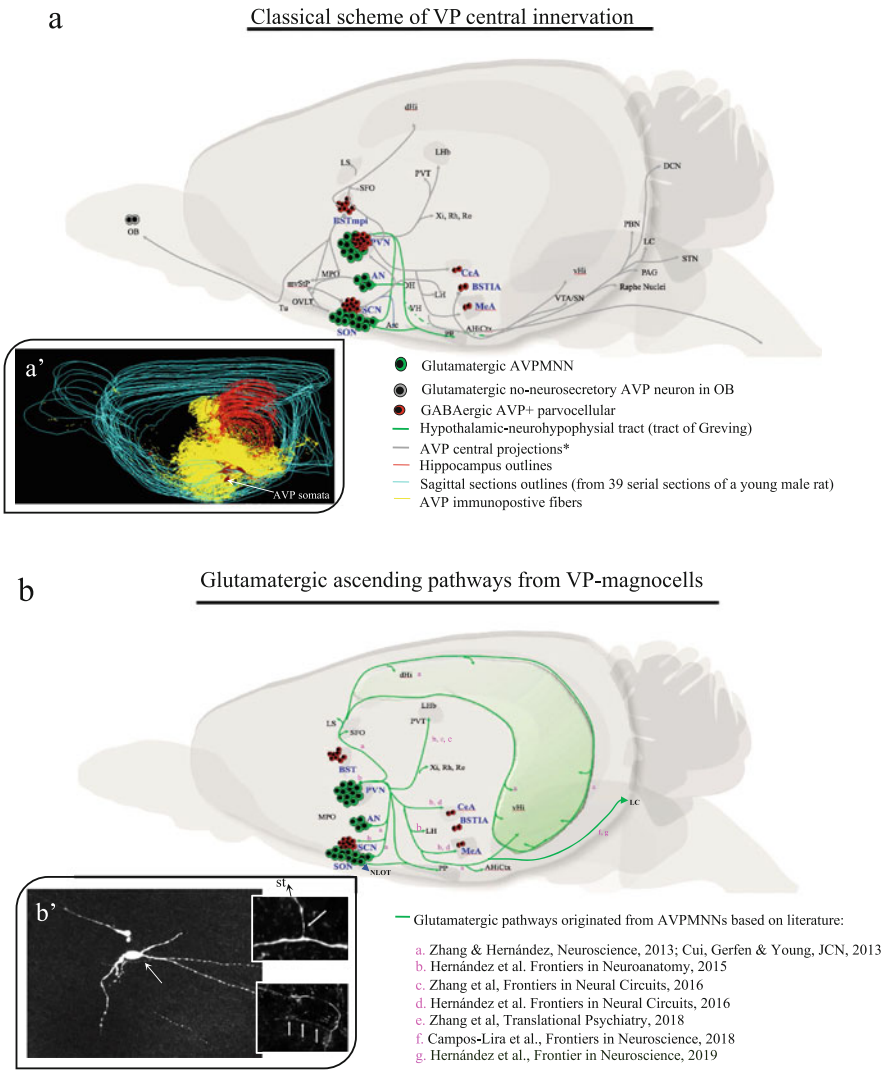

Fig. 7.7 Major central vasopressin-containing nuclei and pathways in the rodent brain. **a**: Classical scheme of AVP central innervation. **a**': Computerized 3D "one-to-one" mapping to visualize the AVP-immunopositive fibre distribution and cell bodies of a young male rat. **b**: Central projections 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

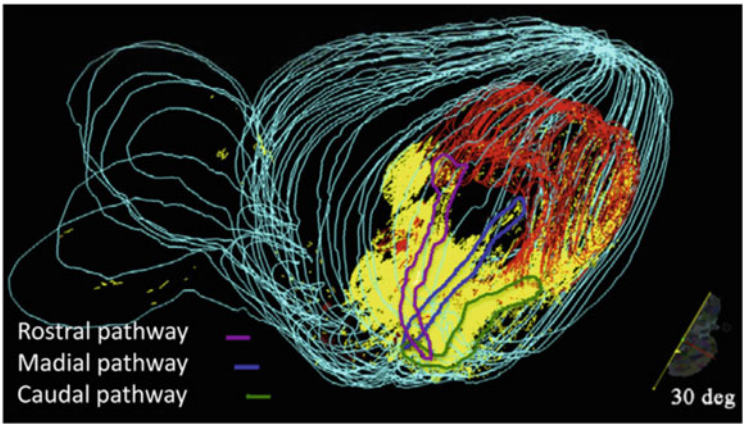

Fig. 7.8 Computerized 3D "one-to-one" mapping of AVP-ir fibres in sagittal sections (hypothalamic PVN and SON as the medial border, extending to stratum oriens of ventral CA1 as the lateral border). Three pathways are delineated as follows: the rostral pathway (purple outline, hypothalamo-septo-fimbria-dorsal 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.

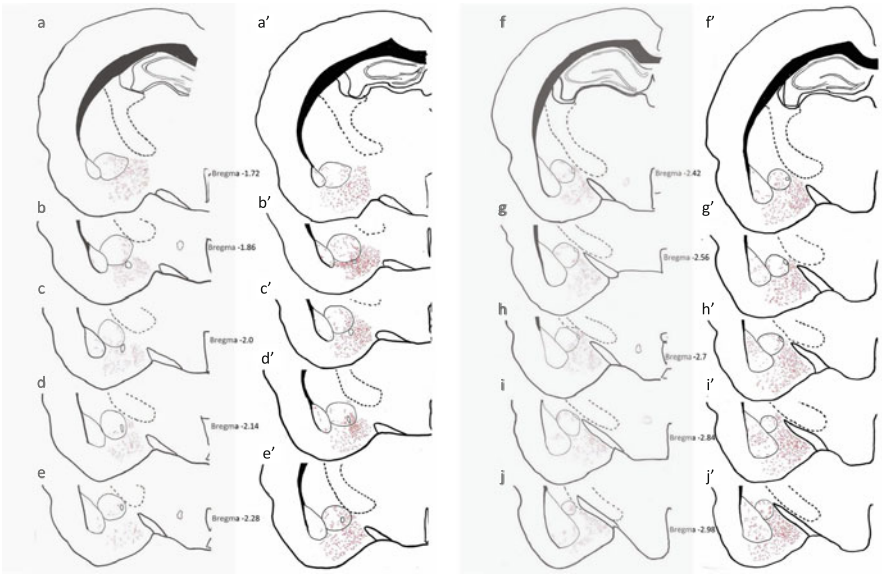

Fig. 7.9 Anatomical charting of AVPir + fibre distribution in amygdala in both control (**a**–**j**) and maternal separation (MS) male adult rat (MS, **a**'–**j**'). Chartings of coronal sections at 10 rostro-caudal levels with line drawings referenced with microscopic observation, representing AVP fibre 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

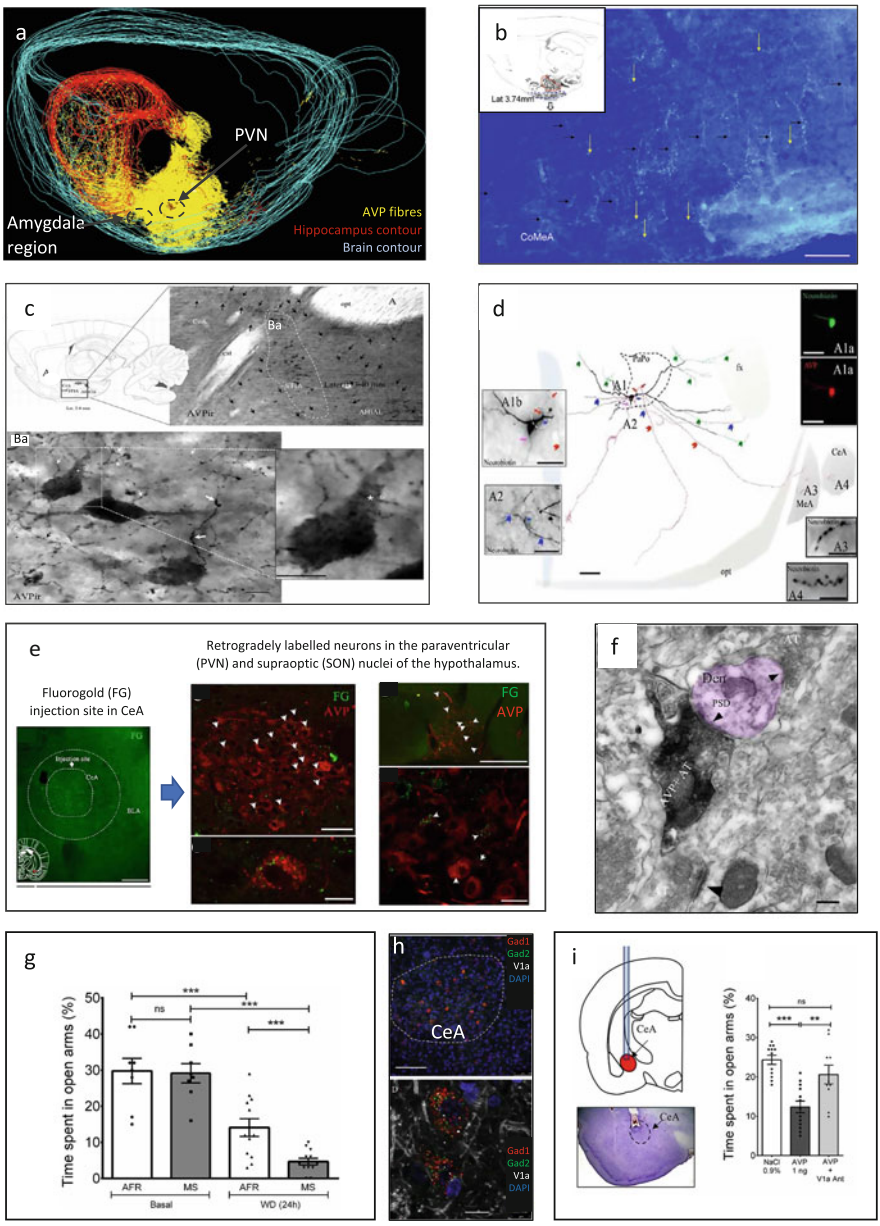

Fig. 7.10 Hypothalamic vasopressinergic magnocellular neurons innervate central amygdala. (**a**) Tracing of the AVP-immunopositive fibres (yellow traces) by *Neurolucida* showing a ventral pathway by which axons from the hypothalamic PVN and SON reach the hippocampus, during their trajectory through 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)

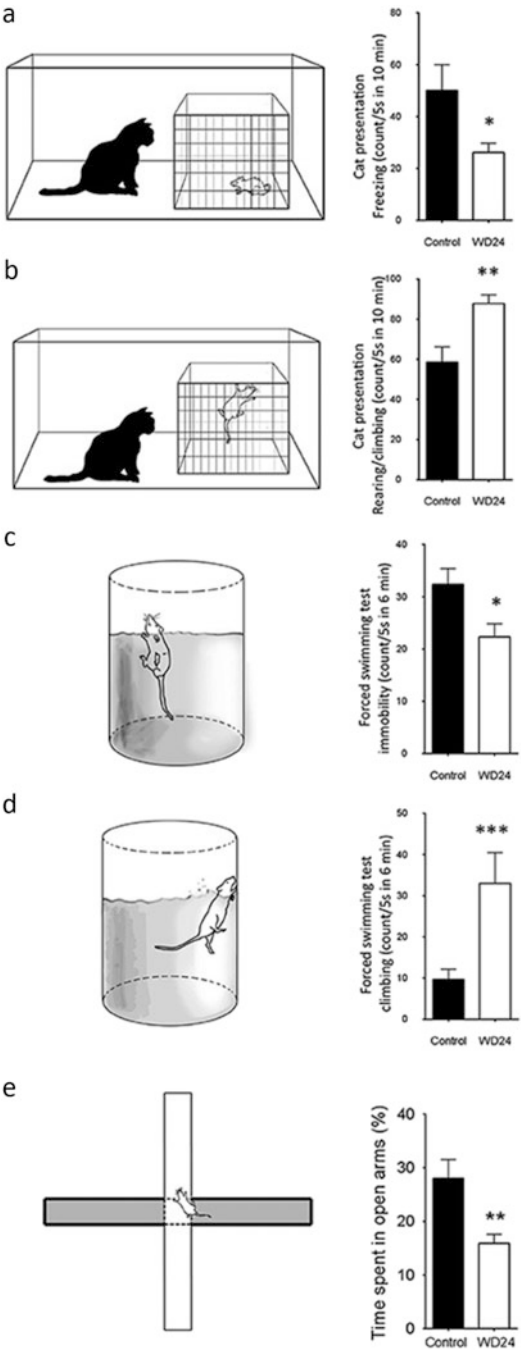

Fig. 7.11 Twenty-four hours of water deprivation (WD24) promoted active stress coping during innate fear processing (cat exposure) and behavioural despair (forced swimming test, FST). WD24 is a potent physiological stimulus to increase metabolic activities of AVP-containing magnocellular neurosecretory 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.*

Hernandez, V. S., E. Vazquez-Juarez, M. M. Marquez, F. Jauregui-Huerta, R. A. Barrio and L. Zhang (2015). "Extra-neurohypophyseal axonal projections from individual vasopressin-containing magnocellular neurons in rat hypothalamus." Front Neuroanat 9: 130. *Conclusive study using the technique of juxtacellular labelling to demonstrate that AVP-magnocells can have collateral axons, besides the one reaching the neurohypophysis, that innervate extra-neurohypophysial hypothalamic and extrahypothalamic regions.*

Hrabovszky, E., L. Deli, G. F. Turi, I. Kallo and Z. Liposits (2007). "Glutamatergic innervation of the hypothalamic median eminence and posterior pituitary of the rat." Neuroscience 144(4): 1383–1392. *Elegant and demonstrative experiment to show that AVP-magnocells or OXT-magnocells expressed VGLUT2. For this, they showed that after injecting Fluorogold in the periphery, magnocells captured the fluorogold via their axonal projections to the neurohypophysis, and these same cells expressed (by in situ hybridization) the VGLUT2 mRNA.*

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Electrophysiological study that suggested the existence of axonal collaterals from SON magnocells.

Smith, A. S., S. K. Williams Avram, A. Cymerblit-Sabba, J. Song and W. S. Young (2016). "Targeted activation of the hippocampal CA2 area strongly enhances social memory." Mol Psychiatry. *Follow-up study with an optogenetic approach, where the Young's group studied the functional/behavioral relevance of the AVP innervation.*

Zhang, B., L. Qiu, W. Xiao, H. Ni, L. Chen, F. Wang, W. Mai, J. Wu, A. Bao, H. Hu, H. Gong, S. Duan, A. Li and Z. Gao (2021). "Reconstruction of the Hypothalamo-Neurohypophysial System and Functional Dissection of Magnocellular Oxytocin Neurons in the Brain." Neuron 109(2): 331–346 e337. *Recent study by Gao's group in which, using the retrograde viral infection of hypothalamic magnocells and whole-brain imaging techniques, they reconstructed the 3D projection throughout the brain, confirming the finding of the axon collaterals projecting to multiple extrahypothalamic regions.*

Zhang, L. and V. S. Hernandez (2013). "Synaptic innervation to rat hippocampus by vasopressin-immuno-positive fibres from the hypothalamic supraoptic and paraventricular nuclei." Neuroscience 228: 139–162. *The authors made a detailed immunohistochemical study (see below) showing the synaptic innervation of diverse hippocampus regions by AVP fibres originated in AVP-magnocells. Simultaneously, Young's group used genetics and classical tracing studies to show that the hypothalamic vasopressinergic neurons innervate the CA2 region of the hippocampus (see reference above).*

Zhang, L., V. S. Hernandez, J. D. Swinny, A. K. Verma, T. Giesecke, A. C. Emery, K. Mutig, L. M. Garcia-Segura and L. E. Eiden (2018). "A GABAergic cell type in the lateral habenula links hypothalamic homeostatic and midbrain motivation circuits with sex steroid signaling." Transl Psychiatry 8(1): 50. *This study showed the existence of GABA neurons in the lateral habenula (GERNs), that express the oestrogen receptor and are sensitive to the levels of testosterone. Rats with supplemental testosterone have a higher density of GABA/ERalpha neurons, and castration reduces its density. These neurons receive input form hypothalamic magnocellular AVP neurons,*

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

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