Imaging the Hypothalamo-Neurohypophysial System

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Abstract

The hypothalamo-neurohypophysial system (HNS) is a brain peptidergic neurosecretory apparatus which is composed of arginine vasopressin (AVP) and oxytocin (OXT) magnocellular neurones and their neuronal processes in the posterior pituitary (PP). In response to specific stimuli, AVP and OXT are secreted into the systemic circulation at the neurovascular interface of the PP, where they act as hormones, but they can also behave as neurotransmitters when released at the somatodendritic compartment or by axon collaterals to other brain regions. Because these peptides are crucial for several physiological processes, including fluid homeostasis and reproduction, it is of great importance to map the HNS connectome in its entirety in order to understand its functions. In recent years, advances in imaging technologies have provided considerable new information about the HNS. These approaches include the use of reporter proteins under the control of specific promoters, viral tracers, brain-clearing methods, genetically encoded indicators, sniffer cells, mass spectrometry imaging, and spatially resolved

transcriptomics. In this review, we illustrate how these latest approaches have enhanced our understanding of the structure and function of the HNS and how they might contribute further in the coming years.

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Introduction

The hypothalamo-neurohypophysial system (HNS) is a brain peptidergic neurosecretory apparatus responsible for the production of the peptide hormones arginine vasopressin (AVP) and oxytocin (OXT). It consists of magnocellular neurones (MCNs) of the hypothalamic supra-optic nucleus (SON), and paraventricular nucleus (PVN), the axons of which project through the internal zone of the median eminence and terminate on blood capillaries of the posterior pituitary (PP) gland. Two distinct groups of HNS MCNs synthesize the peptides AVP and OXT, which are transported down axons and stored at the neurovascular interface of the PP [1]. Upon stimulation, AVP and OXT are secreted into the systemic circulation where they act as hormones reaching remote targets and regulating essential processes for survival, including fluid ho-

Fig. 1. Schematic representation of the HNS. AVP and OXT MCNs of the hypothalamic SON and PVN project their axons to the PP gland, where AVP and OXT are released into the systemic circulation acting as hormones, and also send neuronal projections to other brain regions where AVP and OXT acts as neurotransmitters. In addition, MCNs mediate somatodendritic release of AVP and OXT. In the PVN there are also AVP- and OXT-expressing

parvocellular neurones that send their projections to other regions in the CNS (mainly to the brainstem and spinal cord) and to the external zone of the ME. Created with BioRender.com. AVP, arginine vasopressin; OXT, oxytocin; MCNs, magnocellular neurones; SON, supraoptic nucleus; PVN, paraventricular nucleus; PP, posterior pituitary; OC, optic chiasm; ME, median eminence; HNS, hypothalamo-neurohypophyseal system.

moeostasis, reproductive behaviour, and social functions. In addition to secretion from PP terminals into the systemic circulation, AVP and OXT are also released within the hypothalamic SON and PVN from dendrites and cell bodies of MCNs, hence acting as neurotransmitters. These mechanisms are usually independent of the axonal release of AVP and OXT into the bloodstream as dendritic release can be achieved by intracellular Ca^{2+} mobilization without electrical firing of these neurones [2, 3]. Somatodendritic release of AVP and OXT has important autocrine effects in MCNs and paracrine effects in nearby

cells, for example, regulating blood pressure [4] or the milk-ejection reflex [5]. In addition, MCNs from the SON and PVN also send extra-neurohypophyseal collateral projections to other brain areas to form central brain circuits that regulate pain perception [6], affective/emotional states [7, 8], social behaviour [9, 10], and fear [11, 12].

In the PVN, in addition to the MCNs that project to the PP, there is another neuronal population in the posterolateral part of the nucleus that correspond to parvocellular neurones. These neurones send their projections to other regions in the CNS, mainly to autonomic centres

in the brainstem and the spinal cord [13–15] where they are involved in the regulation of autonomic output [16, 17]. Moreover, parvocellular neurones also project to the external zone of the median eminence from where CRH and AVP are secreted into the pituitary portal circulation to mediate the secretion of adrenocorticotrophic hormone with important implications for the stress response [18, 19].

The complexity of the HNS is summarized in Figure 1. In order to understand its function and regulation, a complete and faithful description of the structure and connectivity of the HNS is essential. Brain structure-function relationships have been classically studied by histochemical and immunohistochemical techniques, but the emergence of new imaging technologies has prompted an unprecedented amount of research to detail the structure and function of the HNS. In this review, we provide an overview of novel imaging approaches that will advance our understanding of the HNS in coming years.

Genetic Labelling Methods

Specific Promoters

Most findings regarding AVP and OXT neuronal projections to other brain structures (including the amygdala, hippocampus, and spinal cord) were obtained from histological tract tracing studies decades ago [20–24]. The development of advanced fluorescent techniques and genetic labelling methods has enabled the targeting and labelling of specific neural cell populations, which is a very useful approach to map the projections and connections of individual neurones. One of these approaches is the use of fluorescent reporter proteins under the control of specific promoters of interest, the expression of which is restricted to cell types of interest. For many years, the scientific community avidly researched the cis-regulatory genomic domains that are necessary for the cell-type-specific expression of AVP or OXT MCNs by several approaches [25]. In 2012, an accurate description of the OXT and AVP cis-regulatory regions that are responsible for conferring cell-type-specific expression in either OXT or AVP MCNs was achieved using promoter-deletion studies that delivered reporter constructs in vivo using adeno-associated viruses (AAVs) [25]. Gainer and colleagues concluded that a promoter length of 2 and 563 kbp upstream the AVP and OXT transcription start site, respectively were sufficient to direct cell-type-specific expression. Further studies demonstrated that these cell

type-specific sequences could be reduced to 116 bp localized between 216 and –100 bp upstream of the OXT transcription start site [26] and to 288 bp upstream of the AVP transcription start site [27].

This knowledge triggered the use of OXT and AVP promoters to further advance our understanding of the HNS. For example, this approach was used to gain further insights regarding the long-range axonal projections of hypothalamic OXT neurones to the amygdala and other forebrain regions distant from the hypothalamus. By directing expression of the fluorescent marker Venus to OXT neurones in the PVN and SON, Knobloch et al. [28] were able to map OXT axonal projections to forebrain structures, including the taenia tecta, nucleus accumbens, lateral septum, amygdala, and hippocampus. Moreover, they were able to identify the sources of these projections demonstrating that most of them arise from PVN OXT neurones. Their findings confirmed that OXT release from local axonal endings can specifically control region-associated behaviours, such as fear in the amygdala [28]. With the same approach, a further study demonstrated that OXT neurones of the PVN project to the ipsi- and contralateral SON, whilst no intrahypothalamic connections were detected within the AVP system [6]. In addition, using an alkaline phosphatase reporter under the control of the OXT promoter, it was possible to confirm the presence of OXT fibres in several extrahypothalamic regions, including cortical and olfactory areas, the limbic system, and the hindbrain, whilst the cell bodies were exclusively detected in the hypothalamus and the bed nucleus of the stria terminalis [29].

Retrograde Tracing

The use of retrograde tracing approaches has enabled the identification of the sources of OXT and AVP projections. For example, by using retrogradely transported fluorescent dyes, it was possible to identify the neuronal populations that project to the PP, the dorsal vagal complex and spinal cord from the PVN [13, 14], and, by labelling both AVP mRNA and retrogradely transported cholera toxin subunit b, it was observed that AVP neuronal projections to the spinal cord arise mainly from parvocellular neurones in the PVN [30]. Also, by fluorogold retrograde tracing, extra-neurohypophyseal axonal projections were identified in the hippocampus from AVP MCNs [24] and in the nucleus accumbens from OXT MCNs [31].

The use of retrogradely transported viruses, such as pseudorabies or canine adenovirus 2 (CAV2), has proved to be a useful approach to trace AVP and OXT neuronal

Fig. 2. Retrograde labelling of neurones projecting to the SON. Rats were injected with a mixture of a retrograde AAV (AAV-retro-hSyn.Ruby) and an AAV-expressing eGFP (AAV1/2-CMV.eGFP). Image of the SON injection site (a) and projecting neurones in the SFO (b) and NTS are visible (c). d Higher magnifica-

tion image of the projections observed on the NTS in (c). OC, optic chiasm; SON, supraoptic nucleus; AAV, adeno-associated virus; eGFP, enhanced green fluorescent protein; SFO, subfornical organ; NTS, nucleus of the solitary tract. Scale bars, 100 μ m.

circuits. For instance, by injecting pseudotyped rabies virus into the central nucleus of amygdala, it was demonstrated that some OXT MCNs project their axons to both the PP and central nucleus of amygdala [28]. In another study, by injecting the retrogradely transported CAV2 into the SON, Eliava et al. [6], identified which neurones of the PVN project to the SON. Moreover, the systemic administration of fluorogold, in addition to CAV2 injection into the SON, demonstrated that these PVN neurones are mainly of parvocellular origin [6]. In a similar approach, Menon et al. [32], used a dual-virus approach, whereby CAV2 delivering Cre was injected into the lateral septum of mice and a Cre-dependent virus-expressing mCherry under the control of the OXT promoter was injected into the PVN and SON. They found that MCN OXT-ergic afferents to the lateral septum mainly originate in the SON. Additionally, they were able to show that these projections mediate reduced social fear in lactating mice [32].

An important recent advance has been the development of an effective retrograde AAV system based on a designer engineered capsid protein (retro-AAVs) which allows robust retrograde access to projection neurones [33]. Recently, in order to fully map the connectivity of magnocellular-OXT neurones exclusively (avoiding labelling projections from parvocellular-OXT neurones), a cre-dependent retrograde viral tracer was injected into the PP of a rat line expressing Cre under the control of OXT [10]. These authors found that OXT MCNs send projections to the nucleus accumbens, amygdala, lateral septum, piriform cortex, and for the first time, projections were also identified in the caudate putamen which suggests that OXT release facilitates social behaviours and locomotion. These findings also demonstrated that a

population of OXT MCNs collaterally project to both PP and multiple brain areas [10]. Using retrograde AAVs, we have recently obtained some preliminary data from studies where we have injected a mixture of AAV-retro-Ruby (to back-trace neuronal projections) and AAV-enhanced green fluorescent protein (eGFP) (to map the injection site) into the SON. Using this approach, we have found retrograde labelling of neurones projecting from the subfornical organ and the nucleus of the solitary tract, confirming that MCNs in the SON receive inputs from these brain structures (Fig. 2).

All the above illustrates that the use of specific promoters and viral tracers, in particular retrogradely transported viruses, is a very powerful approach to map the structure and connectivity of brain circuits. This approach has added new understanding regarding the connectivity of the HNS, in particular the OXT system, and has provided better resolution of the cell populations involved. Even though it falls outside the main scope of the present review, it is worth mentioning that the use of specific promoters and viral delivery approaches has enabled the exploration of the diversity of MCNs by means of optogenetics and chemogenetics. In these approaches, neurones are genetically manipulated to express a light-sensitive protein or a Designer Receptor Exclusively Activated by Designer Drugs that, when activated by light or a designer drug, respectively, lead to changes in the membrane potential consequently resulting in neuronal activation or inhibition. The use of AVP and OXT promoters in combination with retrogradely transported viruses, has allowed the targeted expression of opto- and chemogenetics actuators to specific neuronal populations that, for example, project to extrahypothalamic brain regions and to

decipher their physiological role [6, 9, 10, 12, 34–36]. Due to the enormous potential of these tools to give further insights into neuronal circuits and to understand the diversity of individual cells, they have already been widely used in the study of the HNS, and it is expected that they will shed more light on this system in the near future.

Brain Clearing

A detailed description of the 3-dimensional (3-D) maps of neural networks is essential to understand the structure and function of neuronal circuits. However, 3-D imaging of mammalian brains is rather challenging due to light scattering throughout this tissue, as light is dispersed by molecules, such as water and lipids [37]. Making brains transparent offers the possibility of obtaining a 3-D view of neural networks by improving light penetration. For this reason in the last few years, several techniques aiming to clear the brain (including clear, unobstructed brain imaging cocktails, and computational analysis) [38]; 3-D imaging of solvent-cleared organs [39] immunolabelling-enabled 3-D imaging of solvent-cleared organs (iDISCO) [40] and Clear, Lipid-exchanged, Acrylamide-hybridized Rigid, Imaging/immunostaining compatible, Tissue hYdrogel [CLARITY] [41]) have been developed. Amongst these, CLARITY is probably the most popular. The principle of CLARITY lies in the removal of lipid bilayers, which are responsible for making tissue poorly accessible to light and macromolecules, whilst maintaining its original structure and molecular signature. After clearing the tissue, the whole 3-D structure is imaged. This can be achieved by standard confocal microscopy or 2 photon microscopes; however, as the optical sectioning is achieved by point-by-point scanning, the processing time is rather slow and long exposure times can lead to photobleaching. Light sheet microscopes are the preferred option as they can illuminate a whole focal plane at the same time, which provides higher imaging speed [42].

In view of the abundant central projections from AVP and OXT magnocellular and parvocellular neurones, this technology is particularly attractive, as it allows axonal projections to be followed throughout the entire brain. A few studies using brain-clearing approaches have provided some insights into the neuronal networks in the hypothalamus.

The use of CLARITY in combination with immunohistochemistry allowed the depiction of the 3-D structure and distribution of OXT hypothalamic neurones at birth (at postnatal day 0, P0) and in the juvenile rat brain (P24) [43]. In another study, the distribution of OXT and AVP

neuronal populations was assessed in the perinatal mouse brain at P5 by combining whole-mount immunostaining with the iDISCO clearing method. The authors found that, at P5, the distribution of OXT and AVP neurones along the SON is not homogeneous, as both OXT and AVP cell bodies can be found at the rostral part whilst OXT neurones are more abundant in the dorsal part of this nucleus. In the PVN at P5, whilst OXT neurones were more abundant than AVP neurones, the soma volume of the former was half the size. This suggests that, in contrast to AVP-ergic cells, OXT neurones are not fully mature in the early postnatal period [44].

Spoljaric et al. [45], also labelled AVP neurons and their projections, in this case in the newborn rat brain at P0, by combining whole-tissue immunostaining and the CLARITY clearing approach. The cell bodies from AVP neurones were detected in the PVN, SON, and accessory nuclei of the hypothalamus. Moreover, AVP projections were found in several parts of the limbic system, including the amygdala, and at the hippocampal formation, including the hippocampal fissure, alveus, and fimbria. These findings indicated that AVP fibres innervate the rat hippocampus and limbic system already at birth. In combination with other findings, the authors concluded that AVP suppresses spontaneous network events in the perinatal hippocampus as a neuroprotective mechanism against the reduced oxygen supply at birth [45].

In a recent study, Madrigal and Jurado [46] used the iDISCO clearing method in combination with whole-tissue immunostaining followed by light sheet fluorescent microscopy, to generate 3-D reconstructions of the OXT and AVP systems in the mouse brain from embryonic and adult stages. They found that OXT and AVP neurones first appear in caudal nuclei, including the PVN, SON, and the retrochiasmatic area (from at least E16.5), followed by rostral areas. They also found that rostral nuclei generally show higher degree of homogeneity (with the anterodorsal preoptic nucleus and the bed nucleus of stria terminalis containing mainly OXT neurones and the suprachiasmatic nucleus containing exclusively AVP neurons), whilst caudal nuclei (including the SON, PVN, retrochiasmatic area and accessory nucleus) are more heterogeneous and contain both OXT and AVP neurones. Interestingly, they observed that in most nuclei, the number of OXT neurones increases, so the percentage from AVP to OXT neurones is higher in early developmental stages and progressively decreases throughout adulthood. In accordance with previous findings [44, 47], these results suggest that the AVP-ergic system matures earlier than the OXT-ergic [46].

Genetically Encoded Biosensors

Whilst traditional optical microscopy has enabled structural and anatomical studies, in the last years, the combination of optical microscopy and genetically encoded fluorescent indicators has allowed us to go beyond anatomy and to explore functional and physiological responses. In particular, the possibility of recording neural activity in thousands of neurons simultaneously in whole intact animals [48] has revolutionized neuroscience research, including the field of neuroendocrinology, in a way that was unimaginable 2 decades ago.

Genetically encoded calcium indicators (GECIs), in particular GCaMP, are widely used by the neuroscience community. Because neuronal activation leads to increase in intracellular levels of calcium, the use of GECIs enables the direct monitoring of neuronal activity [48]. Moreover, the improvements in instrumentation have enabled the possibility of performing calcium imaging in deep subcortical brain areas, such as the hypothalamus. Gradient-index (GRIN) lenses have unique optical properties that make them an ideal tool for imaging deep-brain regions in vivo [49]. Once the GRIN lenses are chronically implanted in the animals, the recordings of calcium signals can be performed either on head-restrained animals using a bench top microscope [50], or in freely moving animals using miniature microscopes or miniscopes [49, 51].

The availability of AVP-Cre and OXT-Cre mice has enabled the selective targeting of genetically encoded biosensors to AVP and OXT neuronal populations. In recent studies, Resendez et al. [35], injected a virus encoding Cre-inducible GCaMP6s into the PVN of OXT-Cre mice in order to selectively express GCaMP6s in OXT neurones of the PVN. By monitoring the neuronal activity of these neurones by the use of GRIN lenses in head-restrained animals, the authors were able to verify that OXT-PVN neurons respond to social stimuli and that activation of these neurones is required for social behaviour [35]. In another study, a virus encoding Cre-inducible GCaMP was injected into the SON of AVP-Cre mice leading to expression of GCaMP6s in AVP neurones in the SON exclusively. Neuronal activity recordings from freely moving animals implanted with GRIN lenses and Inscopix miniscopes placed above the lens showed that these neurones were activated by increases in blood osmolarity (in response to NaCl injection) and rapidly inhibited during drinking. They showed that this mechanism is part of a fluid homeostasis system that senses the osmolarity of ingested fluids and controls drinking behaviour accordingly [52]. One of the limitations of Cre

transgenic mouse lines is the possibility of off-target effects [53], which have been reported for AVP-Cre mice [54]. Furthermore, a recent study of AVP-IRES2-Cre mice found that AVP expression was significantly decreased in the SON, PVN, and suprachiasmatic nucleus compared with wild-type litter mates [55]. These findings suggest that a level of caution should be exercised when using specific mouse models.

Another interesting class of genetically encoded indicators are the chloride sensors. The ability to monitor changes in chloride in real time is of great interest, as chloride regulates essential processes such as cell proliferation, metabolism, synaptic transmission, and excitability in the brain. One such chloride sensor is ClopHensorN, a genetically encoded ratiometric chloride sensor that is suitable for investigating the nervous system [56]. ClopHensorN has been successfully used to explore the effects of γ -aminobutyric acid (GABA)-mediated neurotransmission in SON AVP neurons. Under resting conditions, GABA generally inhibits AVP neurones. However, in response to chronic hyperosmotic stress, GABA becomes an excitatory neurotransmitter to AVP neurones through changes in the transmembrane chloride gradient, which ultimately leads to sustained release of AVP [57]. By delivering a virus-expressing ClopHensorN under the control of the AVP promoter, Balapattabi et al. [58] demonstrated that downregulation of the K^+/Cl^- co-transporter2 and upregulation of $Na^+/K^+/Cl^-$ co-transporter1 in SON AVP neurones increases intracellular chloride following hyperosmotic stress. In other words, they pointed to the mechanisms that mediate the inhibitory-to-excitatory switch of GABA-mediated transmission in AVP neurones [58].

An interesting biosensor that has provided very valuable understanding regarding the neurovascular interface between AVP and OXT axonal terminals and the circulation expresses a vitamin D-binding protein fused with enhanced green fluorescent protein (DBP-eGFP). Transgenic zebrafish-expressing DBP-eGFP under the control of the liver-type fatty acid-binding protein promoter, secrete DBP-eGFP to the blood plasma [59]. Using this system, Anbalagan et al. [60] explored the molecular cues that drive the development of fenestrated neurohypophyseal capillaries or, in other words, how capillaries in the pituitary become permeable and allow the transfer of HNS hormones between brain and circulation across the blood-brain barrier. They found that the pituitary factors Vegf and Tgfb promote the fenestrated endothelial fate whilst Cyp26b represses the nonpermeable one. Moreover, the same group also demonstrated that the struc-

tural protein of the fenestral diaphragm, Plvap, regulates the entry of plasma proteins into the pituitary through fenestrated endothelia [61].

In addition to GECIs and chloride sensors, there are additional genetically encoded indicators that can be used to further explore the functions of the AVP and OXT systems. These include cAMP indicators to study cAMP dynamics [62], genetically encoded voltage indicators that allow to visualize action potentials, glucose indicators to study neuronal metabolism or even genetically encoded neurotransmitter indicators that monitor neurotransmitter release [48, 63]. Genetically encoded neurotransmitter indicators have been categorized by their ligand-binding scaffold into bacterial periplasmic binding proteins (PBPs) and G-protein coupled receptors (GPCRs) [64]. The first PBP-based neurotransmitter indicators were derived from *E. coli* glutamate-binding PBPs and these glutamate sensors were quickly engineered to generate several colour variants, offering the possibility to multiplex with other probes [65]. Subsequently, additional PBP-based indicators were generated to monitor GABA, acetylcholine, and serotonin as well [66]. GPCR-based indicators include dLight1 [67] and GRAB [68] sensors that initially detected dopamine but that have been engineered to expand the ligands and can now detect serotonin, norepinephrine, and acetylcholine [64]. Based on the engineering developments of PBP- and GPCR-based sensors, it is expected that before long it will also be possible to measure the spatiotemporal dynamics of neuropeptides *in vivo*, with important implications for the neuroendocrinology field.

Sniffer Cells

Most of the available techniques used to detect and measure the release of peptides or neurotransmitters from biological preparations, such as microdialysis, high-pressure liquid chromatography, or electrochemical techniques, offer great levels of sensitivity but lack the temporal and spatial resolution required to study quantal release from single cells [69, 70]. Sniffer cells can be used as biosensors to detect the release of peptides or neurotransmitters with optimal sensitivity and spatiotemporal resolution. The sniffer cell approach is based on a cell line expressing the cognate receptor of the peptide of interest and a genetically encoded fluorescent indicator. Taking into account that OXT and AVP can be released from the PP as hormones and can be also centrally released as neurotransmitters, the sniffer cells approach is one of the most promising to detect and quantify these distinct processes. Coating sniffer cells on the top of *ex*

vivo pituitary or brain explants enables direct quantification of peptide release in real time at hypothalamic or extrahypothalamic region.

Zaelzer et al. [71] generated sniffer cells by transfecting human embryonic kidney cells with the human AVP receptor, V1a VP receptor (V1aR), along with GCaMP6. As binding of AVP to its receptor leads to increases in intracellular calcium, these sniffer cells serve as AVP biosensors by monitoring changes in GCaMP6 fluorescence. By culturing sniffer cells on top of rat hypothalamic slices, the authors demonstrated that electrical stimulation of a single MCN in the SON can lead to significant AVP release from the somatodendritic compartment of the neurone, highlighting the physiological relevance of somatodendritic AVP release [71]. In a similar approach, Pitra et al. [72], genetically engineered Chinese hamster ovary cells expressing V1aR and the red fluorescent genetically encoded calcium indicator R-GECO and cultured them top of rat hypothalamic slices. With this system, they demonstrated that, unlike axonal AVP release, somatodendritic AVP release is dependent on firing activity evoked by NMDA-receptor activation. Based on these findings, they postulated that NMDA receptors might serve as a “gating mechanism” in order to regulate neuropeptide release from dendritic and axonal compartments in a compartmentalized manner [72].

The sniffer cell approach has also been used to study OXT release from neuronal projections from the PVN to parasympathetic nuclei. Pinol et al. [73], genetically engineered Chinese hamster ovary cells to express human OXT receptor and R-GECO. They activated PVN hypothalamic neuronal projections in brainstem parasympathetic nuclei and detected local release of OXT, which facilitates the excitation of cardiac vagal neurons. This has important implications as it suggests that OXT receptor-mediated facilitation of cardiac vagal neurons might be a target to mitigate deleterious cardiovascular risks [73].

Gizowski et al. [74], also used Human embryonic kidney cells co-expressing V1aR and GCaMP6 as biosensors. They showed that electrical stimulation of the suprachiasmatic nucleus leads to increases in fluorescence of sniffer cells covering the organum vasculosum lamina terminalis in rat hypothalamic slices, indicating that AVP release from axonal terminals of the suprachiasmatic nucleus activates neurons in the organum vasculosum lamina terminalis. With complementary experiments, they demonstrated this mechanism is responsible for stimulating water intake prior to sleep [74].

Mass Spectrometry Imaging

Mass spectrometry imaging (MSI) is a powerful tool used to visualize the spatial distribution of molecules by means of their molecular masses. It allows the imaging of thousands of molecules, including metabolites, peptides, proteins, lipids, and glycans in a single experiment without the need for labelling [75]. Following sample preparation, the mass spectrometer ionizes the molecules on its surface and collects a mass spectrum at each pixel. This way, a complete spectrum is collected for each pixel location over the entirety of the surface of the sample. The molecules are then identified based on the individual mass-to-charge values collected at each pixel. There are several ionization techniques compatible with MSI that offers complementary capabilities. The most common ionization approaches include secondary ion mass spectrometry, laser desorption/ionization, desorption electrospray ionization, and matrix-assisted laser desorption/ionization (MALDI) [75, 76].

Guenter et al. [77], performed MSI of neuropeptides in the mouse pituitary with cellular resolution. As expected, they found both AVP and OXT at the posterior lobe of the pituitary; however, they presented a different distribution of these peptides, with OXT localized in the outer region of the lobe, whilst AVP was mainly detected in the middle. Moreover, they also detected 2 different fragments of the peptide copeptin, which is the C-terminal peptide of the AVP pro-hormone [78], colocalizing with AVP in the centre of the posterior lobe. The distribution of all the peptides is in agreement with previous findings regarding the anatomical structure of the pituitary, demonstrating the potential of MSI in identifying the distribution of a high number of peptides in a given tissue, and the ability of the technique to detect post-translational modifications. Indeed, analysing the distribution of different peptides at the pituitary using MSI can have important implications in the clinical practice for detection and delineation of pituitary tumours and decision-making during surgery. Calligaris et al. [79] explored the possibility of applying MALDI MSI to detect and delineate the limits of pituitary adenomas, which are tumours located in the anterior pituitary gland. They found that it is possible to detect the excessive hormonal production associated to pituitary adenomas at the anterior pituitary and to identify the PP by characterizing AVP distribution. This offers the possibility of removing the adenomas whilst preserving the posterior gland cells secreting AVP. In addition, they pointed out that the possibility of detecting AVP expression may also be of clinical significance as injury to AVP-secreting cells can lead to diabetes insipidus [79].

MALDI MSI has also been successfully used to detect AVP and OXT in different brain regions. In a recent study aiming to identify the distribution of several peptides in the basal ganglia of Parkinson's disease models, it was observed that AVP and OXT were present in a discrete area of the lateral hypothalamus in the primate brain and AVP was also detected at the arcuate nucleus of the hypothalamus in the rat brain [80]. Even though MSI has not been extensively used to map the anatomy of the HNS, the possibility of providing reproducible maps of several neuropeptide distributions simultaneously and to characterize possible alterations of neuropeptides, points to MSI as an innovative approach to explore the structure and function of the HNS.

Spatially Resolved Transcriptomics

There has been an explosion of image-based single-cell transcriptomics approaches that allow the quantification of gene expression by directly imaging of individual RNA molecules within intact cells and tissues. These approaches include novel in situ hybridization technologies, in situ sequencing technologies, and in situ capturing technologies. For an overview of all the available techniques and their principles, the reader is referred to a recent review [81]. Briefly, the novel in situ hybridization technologies allow the simultaneous detection of multiple RNA molecules in their original environment using labelled probes. Whilst the ability of simultaneously detecting a large number of transcripts has historically been a limitation, nowadays, it is possible to almost reach whole transcriptomics reading capacity, although it is necessary to have previous knowledge of the targets. In situ sequencing technologies allow RNA sequencing directly in morphologically preserved cells and tissue sections; however, the number of transcripts that can be simultaneously detected is limited due to spatial limitation inherent to the size of cells. In situ capturing technologies circumvent this limitation by capturing the transcripts in situ with bar-coded mRNA capture probes, to then perform the sequencing ex situ [81].

Due to the recent development of these techniques, the requirement for specialized equipment, and in many cases the elevated costs, the use of these approaches has not been extensively used for the study of the HNS. In 2018, by the use of single-cell RNA-sequencing (scRNA-seq) followed by an image-based single-cell transcriptomics approach, it was possible to spatially resolve the cell atlas of the mouse hypothalamic preoptic region. In this work, the authors performed scRNA-seq in the preoptic region of the mouse hypothalamus which allowed for cell-type

identification and clustering of cells into classes. However, as the cell dissociation step necessary for scRNA-seq results in the loss of the spatial context, they further explored the in situ expression of 155 genes identified by scRNA-seq in the preoptic area by multiplexed error-robust fluorescence in situ hybridization [82]. Multiplexed error-robust fluorescence in situ hybridization is based on several rounds of hybridization, imaging, and signal inactivation in combination with error-robust encoding to account for error detection, resulting in the simultaneous detection of a large number of transcripts in a spatial context [83]. As a result, 70 different neuronal populations were identified in the preoptic area of the hypothalamus expanding the previously known cell types in this region, and it was possible to map these neuronal populations to specific hypothalamic nuclei [82].

Of note, scRNA-seq has also been applied to study the neuronal diversity at the hypothalamus [84], the PVN [85], and other hypothalamic nuclei, such as the supra-chiasmatic nucleus [86] and the PP [87]. However, whilst this approach is extremely useful to uncover previously unknown neuronal types, it lacks spatial resolution. It is expected that in the next coming years, we will witness an extended use of these approaches to increase the knowledge of complex multicellular biological systems, including the structure and function of the HNS.

Concluding Remarks

The advances in imaging techniques have enhanced knowledge of neuronal systems in an unprecedented way. Approaches such as the use of reporter proteins under the control of specific promoters, viral tracers, and brain-clearing methods have already provided very detailed information regarding the neuronal projections of magno-

cellular and parvocellular neurones of the HNS, unraveling the complexity of this system, especially for OXT. Other approaches, such as the use of genetically encoded indicators or sniffer cells, have evidenced how imaging techniques can be used to directly assess the function of systems and have provided valuable functional data in relation to the HNS. Moreover, the ability of detecting a large number of molecules without the need of labelling using MSI, as well as the possibility of spatially detecting a large number of transcripts by spatially resolved transcriptomics, will have an impact on the way we understand and research the HNS. The use of these technologies will shed new light on how the HNS regulates well-characterized processes, such as water balance and reproductive behaviours whilst also allowing the exploration of more novel and complex responses and behaviours mediated by this system.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

S.B-L. prepared the original draft. M.P.G. and L.S. performed the experimental part. D.M., M.P.G., and L.S. contributed to revision and editing of the manuscript. All co-authors approved the final submitted version.

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