Neuropeptides of the human magnocellular hypothalamus

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Abstract

Hypothalamic magnocellular nuclei with their large secretory neurons are unique and phylogenetically conserved brain structures involved in the continual regulation of important homeostatic and autonomous functions in vertebrate species. Both canonical and newly identified neuropeptides have a broad spectrum of physiological activity at the hypothalamic neuronal circuit level located within the supraoptic (SON) and paraventricular (PVN) nuclei. Magnocellular neurons express a variety of receptors for neuropeptides and neurotransmitters and therefore receive numerous excitatory and inhibitory inputs from important subcortical neural areas such as limbic and brainstem populations. These unique cells are also densely innervated by axons from other hypothalamic nuclei. The vast majority of neurochemical maps pertain to animal models, mainly the rodent hypothalamus, however accumulating preliminary anatomical structural studies have revealed the presence and distribution of several neuropeptides in the human magnocellular nuclei. This review presents a novel and comprehensive evidence based evaluation of neuropeptide expression in the human SON and PVN. Collectively this review aims to cast a new, medically oriented light on hypothalamic neuroanatomy and contribute to a better understanding of the mechanisms responsible for neuropeptide-related physiology and the nature of possible neuroendocrinal interactions between local regulatory pathways.

Key words: hypothalamus, magnocellular nuclei, neurochemistry, neuropeptides.

1. Introduction

The hypothalamus with its unique cellular composition still remains one of the most intriguing and engaging structures of the human brain. Despite its small volume and weight (about 4 grams), this diencephalic structure hosts important neuronal assemblies involved in the regulation of numerous fundamental life processes such as food intake, energy expenditure, osmotic balance, circadian rhythms, thermoregulation, hormonal homeostasis, stress, growth, reproductive behavior, immune response, and various emotional-affective states, in addition to orchestrating vigilance mechanisms (Hofman and Swaab, 1992; Saper and Lowell, 2014). Experimental studies on rodents and primates demonstrate that the hypothalamus performs these functions by controlling three main types of output system: behavioral, autonomous, and endocrine, above all via the activities of the hypothalamic-pituitary-adrenal axis (HPA) (Aguilera, 2011; Brown, 2016; Wilkin *et al.*, 1989).

The first merely morphological description of the hypothalamus is present in the famous treatise *De humani corporis fabrica* by Andreas Vesalius edited in 1543 (Toni, 2000). Nevertheless, only in 1877 a German neuroanatomist - Theodor H. Meynert - and a Swiss psychiatrist - Auguste H. Forel - defined more precisely the spatial coordinates of the human hypothalamus (Toni *et al.*, 2004). The name “hypothalamus”, as well as the first anatomical subdivision of this structure, was introduced into neuroscience in 1893 by the Swiss histologist Wilhelm His Jr. from the University of Leipzig (His, 1893). However, knowledge about hypothalamic functions remained purely speculative until the discovery of neurosecretion by the German physiologists Ernst and Berta Scharrer in 1928 at the University of Munich. The subsequent pioneering works on the neurosecretory centers carried out by Geoffrey Harris, Roger Guillemin, Vincent du Vigneaud and Andrew Schally, provided the fundamental findings that brought about the birth of neuroendocrinology.

Comparative anatomical investigations of this small structure of the ventral forebrain revealed common general organization of the hypothalamus among vertebrates (Ariens-Kappers, 1936; Fleming, 1939; Hofman and Swaab, 1992; Keyser, 1979; Le Gros Clark, 1938). However, variations in relative position, shape and size of the hypothalamic nuclei were observed among species (Grünthal, 1950; Hofman and Swaab, 1992). Paradoxically, although the hypothalamus is extensively explored in several vertebrates, it is still a poorly

known region of the deep human brain (Lemaire *et al.*, 2013), primarily due to the complex cellular arrangement and the multitude of afferent and efferent projections (Brockhaus, 1942; Hofman and Swaab, 1992; Saper, 1990; Saper and Lowell, 2014; Simmons, 1988; Swanson and Sawchenko, 1983). The major structures of the cell groups of the human hypothalamus are not well differentiated and defined, so that the hypothalamus is considered the most rostral part of the reticular formation (Swanson and Sawchenko, 1983). On the contrary, few cell groups are more circumscribed, and have specific structural and cytoarchitectural features, such as the magnocellular neurosecretory system of the hypothalamus (Braak and Braak, 1987). However, in more recent years immunohistochemical and cytogenetic studies have outlined various cell groups with considerably more precision.

2. Functional cytoarchitecture of the human supraoptic and paraventricular nucleus.

The hypothalamus contains highly conserved neural circuitry that controls a variety of basic life functions. Although there are some discrepancies about its regional subdivision (Brockhaus, 1942; Diepen, 1962; Gagel, 1928; Le Gros Clark, 1938), most authors agree in dividing the vertebrate hypothalamus into main tree regions, that, from rostral to caudal position, are the chiasmatic, the mammillary, and the tuberal regions (Braak and Braak, 1987). While the majorityof the hypothalamus is poorly myelinated, the mamillary region, which separates the other two regions, is rich in myelinated fibers (Braak and Braak, 1987; Diepen, 1962; Wahren, 1959).

The chiasmatic region consists of both parvocellular "preoptic" structures and partially magnocellular "supraoptic" nuclei. It is located above and anterior to the optic chiasm and includes the walls of the preoptic recess (Braak and Braak, 1992). The magnocellular neurosecretory nuclei define its posterior border (Braak and Braak, 1987; Clark, 1936). In contrast containing the most gray mass of the hypothalamus, the magnocellular nuclei of the hypothalamus are well-defined grey nuclei, including the Paraventricular (PVN) nucleus, the Supraoptic nucleus (SON), and the accessory magnocellular neurosecretory nuclei (Antunes and Zimmerman, 1978; Daniel and Prichard, 1975; Defendini and Zimmerman, 1978; Dierickx and Vandesande, 1977, 1979; Morton, 1969; Swanson and Sawchenko, 1983). Prominent components of the chiasmatic region, together with the magnocellular neurosecretory complex (supraoptic, paraventricular and accessory neurosecretory nuclei),

are the sexually dimorphic intermediate nucleus, the suprachiasmatic and retrochiasmatic nuclei (Braak and Braak, 1992).

The magnocellular neurosecretory complex is the site of synthesis of the nonapeptides arginine vasopressin (AVP) and oxytocin (OXT). Together with neurophysins, the carrier proteins of AVP and OXT (respectively neurophysin II and neurophysin I) (Breslow, 1993), are transported from the hypothalamus to the posterior pituitary via the hypothalamo-hypophyseal tract and are released into blood vessels of both the infundibulum and the neurohypophysis (Saper and Lowell, 2014). Both neurophysins are encoded as preproproteins by the OXT or AVP gene, and are cleaved during posttranslational modification (Sachs *et al.*, 1969), a process completed in the posterior pituitary lobe (Acher and Chauvet, 1988).

PVN and SON have in common a high packing density of nerve cells, a large soma size of their dominant neuronal type, and a dense capillary network (Braak and Braak, 1987). Moreover, they are in close relation with the posterior lobe of the pituitary, that contains their axonal neurosecretory endings, and in which AVP and OXT are released (Saper and Lowell, 2014). However, while the human SON is relatively well defined, homogeneous and contains the majority (50-76,000) of magnocellular neurons (Maccubbin and Vanburen, 1963; Morton, 1969, 1970), the human PVN is more heterogeneous and contains several cell types of which 30-54,000 are magnocellular neurons (Maccubbin and Vanburen, 1963; Morton, 1969, 1970).

The PVN was described as an elongated plate of nerve cells located in the wall of the third ventricle. In particular, the main mass of this nucleus appeared as an elongated structure with the lateral border crossing the medial border of the fornix, and the ventrocaudal extremity in contact with the wall of the optic recess (Braak and Braak, 1987, 1992; Dierickx and Vandesande, 1977). The boundaries of the nucleus are less well defined than those of the SON. In fact, besides this main mass of the nucleus, two additional smaller components were delineated: a more anterior filiform aggregation of neurosecretory cells linked to the main mass by scattered neurosecretory neurons, and a strand of neurosecretory neurons, lying in the roof of the optic recess and extending from the ventro-caudal extremity of the main mass towards the lamina terminalis (Dierickx and Vandesande, 1977). Additionally, ectopic paraventricular neurosecretory cells can be recognized in areas far from the parental nucleus, as within the pallidum or the bed nucleus of the stria terminalis (Braak and Braak, 1992). The medial part of the nucleus shows a lower density of nerve cell bodies than the lateral part (Braak and Braak, 1987). The SON appears divided in two to three portions: a dorsolateral part and a medial part, which can be further separated into dorsomedial and ventromedial

domains (Braak and Braak, 1987; Dierickx and Vandesande, 1977; Morton, 1969). In horizontal sections, the dorsolateral part has the aspect of a fusiform compact mass of neurosecretory neurons. The frontal sections of this mass have a triangular (anterior region) or ovoid (middle and posterior region) shape, that, throughout its length, is closely associated with the posterior portions of the optic chiasm, and is continued as a more slender part along the optic tract (Braak and Braak, 1992; Møller *et al.*, 2018). The medial part of the SON is much smaller than its dorsolateral part. It lies near the medial surface of the optic tract and consists of irregular clusters of neurons merging with each other (Dierickx and Vandesande, 1977). The fibers of the hypothalamic-pituitary tract leave the PVH in inferior-lateral directions towards the supraoptic nuclei, mostly passing ventrally to the fornix (Møller *et al.*, 2018). Close to the upper border of the SON, these fibers mingle with the supraoptico-hypophyseal tract to descend, via the antero-superior half of the infundibulum, to the neurohypophysis (Braak and Braak, 1987; Møller *et al.*, 2018).

As mentioned above, beyond the classic magnocellular nuclei of the hypothalamus, several magnocellular neurons are located outside the PVH and SON. These ectopic neurosecretory cells constitute the so-called accessory magnocellular neurosecretory nuclei of the hypothalamus, first described in the human brain by Gagel in 1928 (Braak and Braak, 1987; Gagel, 1928; Krolewski *et al.*, 2010; Saper, 2012). They are mainly located in the area between PVH and SON, where they form clusters linking the two nuclei (Braak and Braak, 1987, 1992). Near the tip of the upper boundary of the supraoptic nucleus some of these islands condense to form a saddle-like territory (Braak and Braak, 1987; Saper, 2012). Moreover, they are loosely scattered throughout the chiasmatic gray near the PVH, where they can constitute small islands (Braak and Braak, 1987; Saper, 2012). As is common, these neurosecretory cells are often arranged around the blood vessels (Braak and Braak, 1987; Saper, 2012).

Using combined Nissl staining, Braak and Braak (1987) (Braak and Braak, 1987) performed a cytoarchitectonic and pigmentoarchitectonic analysis of the human hypothalamus, differentiating four types of nerve cells found in this region. Type I cells contain coarse and intensely stained lipofuscin granules; type II cells are characterized by dense accumulations of small granules; type III neurons harbour only a fine scattering of dust-like granules; while type IV neurons are devoid of pigment. In the PVN the following three kinds of specific cells were identified. PI cells are the predominant type; they show a medium/ large size multipolar or polygonal cell body, with a small always single nucleus

compared with the soma and are accompanied by a large well-marked nucleolus. In rare occasions, small vacuoles could be seen inside the compact nucleolus (Braak and Braak, 1987), whilenear the nucleus there was a large and clear cytoplasmic area with a few scattered and faintly stained lipofuscin granules, containing large droplets of lipids. This area was limited to the soma and only occasionally extended into the proximal portions of a dendrite. Sometimes the vacuoles were large enough to cause swelling of the contour of the cell body. PII cells were smaller than PI (Braak and Braak, 1987) and their perikarya were slender generating some relatively thick dendrites. Around the eccentric nucleus there was a large area devoid of Nissl material. Here powder-like pigment granules were dispersed, which maintained a constant distance from each other. Some pigment granules often penetrated in the proximal portions of the dendrites. PIII cells had a small to medium sized cell body (Braak and Braak, 1987) while the clear nucleus was more central than the PII neurons. They housed a modest number of intensely colored, separate, knotty-looking pigment grains, surrounded by a faint halo (Braak and Braak, 1987). The cytoplasm was rich in basophilic material, mainly concentrated in the peripheral portions of the soma. Regarding the distribution of these specific neurons in the PVH, large PI neurons formed a dense portion in the center of the nucleus, small PII neurons tended to be near its edges, and PIII neurons were found in all parts of the nucleus (Braak and Braak, 1987). The SON reveal two specific neuronal types, defined as SI and SII type cells (Braak and Braak, 1987). The predominant SI cells are similar to the PI cells, showing a large rounded multipolar cell body, an eccentric nucleus and a single prominent nucleolus (Braak and Braak, 1987). The Nissl substance is peripherally arranged to form vacuoles of various sizes. The SII cells are smaller than the previous, generate only a few thick dendrites, and show numerous fine and intensely stained lipofuscin granules, evenly distributed in all parts of the soma and proximal dendrites. As to their structural features, SII-type cells closely correspond to the PII-type cells of PVH (Braak and Braak, 1987). Small cells with pigmentoarchitectonic characteristics of neurons surrounding the hypothalamic gray were occasionally encountered within the boundaries of the SON (Braak and Braak, 1987).

3. Chemoarchitecture of the human magnocellular hypothalamus

3.1 *Morphology of hypothalamic oxytocinergic and vasopressinergic neurons*

OXT and AVP are found in separate nerve cells, which differ in size and morphology, with the average size of vasopressinergic neurons being greater than that of oxytocinergic neurons. Furthermore, the reaction product against AVP and OXT or against their specific neurophysins is preferentially located in a cytoplasmic area close to the nucleus in vasopressinergic neurons, while in oxytocinergic neurons it is widespread throughout the cytoplasm (Dierickx and Vandesande, 1977, 1979). With this in mind, Brack and Brack (Braak and Braak, 1987, 1992) considered the large PI and SI as vasopressinergic neurons, and the small PII and SII as oxytocinergic.

Immunohistochemical methods with use of selective antibodies against AVP and OXT, allow the description of the magnocellular nuclei of the human hypothalamus very precisely (Saper 2012). For instance, Dierickx et al. (1977) (Dierickx and Vandesande, 1977) demonstrated shape, size and location of the magnocellular nuclei as well as localization and structural features of the AVP and OXT neurons. The PVN, unlike the SON, appeared as a heterogeneous structure consisting of magnocellular neurons and smaller neurons (Morton, 1969). The small neurons, as well as the large neurons, were immunoreactive for AVP and OXT. Among these, the AVP neurons predominated in number in the PVN, being at a ratio of vasopressinergic to oxytocinergic neurons between 7:3 / 8:2 (Dierickx and Vandesande, 1977). OXT neurons increased relatively at the upper and lower extremities of the PNV, as well as at the level of the PVN cell extensions from the main mass (Dierickx and Vandesande, 1977). From these local differences, OXT neurons showed a tendency towards a preferential location at the periphery of the PVN. Also in the SON AVP cells predominated in comparison to OXT cells: in the dorsolateral part of the SON more than 95 ~ of the neurosecretory cells were AVP positive, while in the ventromedial part of the SON the estimated ratio of vasopressinergic to oxytocinergic neurons varied between 8 : 2 and 9 : 1 (Dierickx and Vandesande, 1977). Accessory magnocellular neurosecretory cells were evident between the PVN and the SON. Furthermore, in all magnocellular hypothalamic nuclei the vast majority of neurosecretory cells were multipolar neurons, but the mean size of AVP neurons was larger than that of OXT cells (Dierickx and Vandesande, 1977). In addition, in most of the OXT perikarya the coloured reaction product was diffused over the whole cytoplasm, while in the vasopressinergic perikarya the reaction product preferentially

accumulated around the cell nucleus (Dierickx and Vandesande, 1977). Of note, the main mass of PNV contained a certain amount of medium-sized and small AVP neurons, especially in its middle and inferior areas (Dierickx and Vandesande, 1977). Unlike its other parts, the inferior region of the PVN showed a marked predominance of parvocellular neurons (Dierickx and Vandesande, 1977). This was also the case in the filiform neurosecretory cell aggregation located anteriorly to the main mass (Dierickx and Vandesande, 1977). In the strand of neurosecretory neurons, lying in the roof of the optic recess, the oxytocinergic neurons were extremely small (Dierickx and Vandesande, 1977). Immunocytochemical analysis of brain tissue of human subjects from 10 to 93 years of age, including patients with Alzheimer disease (AD), obtained results roughly comparable to previous studies (Fliers *et al.*, 1985). However, in the central part of the PVN, a lower percentage (53%) of magnocellular neurons were found to be AVP cells compared to the analysis by Dierickx et al. (70-80%) (Dierickx and Vandesande, 1977). The reason for this discrepancy could potentially be due to the PVN region where the measurements were done (Fliers *et al.*, 1985).

As mentioned above, the neurophysins are specific carrier molecules for AVP and OXT encoded as preproproteins by theirneuropeptide genes (Sachs *et al.*, 1969). Neurophysin I and neurophysin II are transport proteins for OXT and AVP respectively (their molecular weights are 10 and 19,6 kD respectively) (Acher and Chauvet, 1988). Both neurophysins undergo posttranslational modification in the neurohypohysis (Acher and Chauvet, 1988). As noted by Moller et el. (2018) (Møller *et al.*, 2018), in contrast to antibodies against the smaller primary hormones, the use of polyclonal antibodies against the neurophysin for immunohistochemistry allows you to clearly outline the axonal projections from the magnocellular nuclei, maybe due to the presence of several epitopes in the large carrier molecules which are lacking in the small hormones of the posterior pituitary, being nonapeptides.

Antibodies against neurophysins have also been used to investigate the magnocellular nuclei of the human hypothalamus (Mai *et al.*, 1997; Sofroniew and Glasmann, 1981). Unlike previous studies (Mai *et al.*, 1997; Sofroniew and Glasmann, 1981), using this technique Moller et al. (2018) (Møller *et al.*, 2018) clearly delineated not only the PVN and SON, but also the hypothalamus-pituitary tract and the accessory magnocellular neurosecretory nuclei. The results of this study showed that PVN and SON were immunoreactive for both neurophysin I and neurophysin II (Møller *et al.*, 2018). Within the PVN, neurophysin positive

neurons were less concentrated in the medial part of the nucleus with respect to the lateral part (Koutcherov *et al.*, 2000; Møller *et al.*, 2018). The number of magnocellular accessory nuclei observed in this study was high compared to many studies on the rodent brain (Rovsing *et al.*, 2013). They appeared mainly as elongated striae in the area between the PVH and the SON (Møller *et al.*, 2018; Morton, 1969; Saper, 2012). Most of the additional accessory magnocellular, located in the paraventricular neurohypophyseal tract between the immunoreactive projections from the paraventricular nucleus, were more elongated and often called intersupraoptico-paraventricular islands (Morton, 1969; Saper, 2012). Moreover, the classical circular nucleus, which has been described in the hypothalamus of many species, appeared well-defined in this study. This structure was earlier included together with the other small collection of cells in the area, as part of the so-called supraoptic accessory neurons (Bodian and Maren, 1951; Burford *et al.*, 1974) before it was finally described and named by Peterson (1966) (Peterson, 1966). The circular shape and close connection to the vascular system, as also seen in the mouse (Rovsing *et al.*, 2013), makes this nucleus easy to recognize. Finally, smaller assemblies of magnocellular neurophysin- immunopositive neurons were located in the basal part of the chiasmatic area, as well as among the fibers of the hypothalamic-pituitary tract (Møller *et al.*, 2018). The striking finding of this study was the large ovoid-shaped collections of neurophysin immunoreactive perikarya located between the nerve fibers. The neurons of magnocellular accessory nuclei showed immunoreactivity for both neurophysin I and neurophysin II, so that it is conceivable that they contain both AVP and OXT (Møller *et al.*, 2018). In addition, it was observed that the axons of most of these neurons project to the neurohypophysis, but some of the nuclei of the accessory magnocellular groups, might secrete straight into the capillary network (Møller *et al.*, 2018). Noteworthy, while in infants neurophysins immunoreactivity was present not only in the cell bodies but also in the cellular processes as well, in adults very little was detected in the magnocellular neurosecretory complex (Braak and Braak, 1992). In addition, in this study the magnocellular nuclei of the human brain seemed to be larger than that observed in rodents and other primates, and its morphology appeared to differ considerably from that of animal models (Møller *et al.*, 2018).

More recently than radioimmunoassay or immunohistochemistry techniques, the human magnocellular hypothalamus has also been studied with cytogenetic procedures (Lucassen *et al.*, 1995; Mengod *et al.*, 1990; Rivkees *et al.*, 1989; Sukhov *et al.*, 1993). In particular, gene expression of neuropeptides in the human *post-mortem* brain tissue was studied using the *in*

situ hybridization technique. With this highly sensitive method, studies determined the presence and distribution of OXT and AVP messenger ribonucleic acid (mRNA) containing cells in the human hypothalamus (Guldenaar and Swabb, 1995; Mengod *et al.*, 1990; Sukhov *et al.*, 1993). The applicability of qualitative and quantitative in situ hybridization for AVP and OXT mRNA on frozen tissue was confirmed in several studies (Lucassen *et al.*, 1995; McCabe *et al.*, 1993; Meister *et al.*, 1990; Mengod *et al.*, 1991; Murayama *et al.*, 1993; Reppert and Uhl, 1988; Sukhov *et al.*, 1993).

In accordance with previous immunohistochemical data (Dierickx and Vandesande, 1977), Mengod et al. (1990) (Mengod *et al.*, 1990) observed that OXT and AVP mRNAs are intensely present in PVN, SON and accessory magnocellular nuclei of the human hypothalamus, and that in such areas the detection of AVP transcript was greater than OXT (regardless of the probes used). In addition, the authors reported evidence that these neuropeptides are transcribed in mutually exclusive sets of neurons in mammals, and that, in agreement with initial immunohistochemical results (Agid and Javoy-Agid, 1985; Buck, 1987), these nuclei also contained transcripts for other neuropeptide genes, such as preproenkephalin A, neuropeptide Y, and somatostatin (Mengod *et al.*, 1990). Sukhov et al. (1993) (Sukhov *et al.*, 1993), revealed that the major part of medium/large sized neurons containing AVP and OXT mRNA were located in the classical magnocellular hypothalamic nuclei (SON and PVN), and only a smaller population occupied the accessory magnocellular nuclei. In these nuclei, magnocellular neurons containing AVP transcripts appeared as round or ovoid cells with large somata and tended to locate more centrally compared with those containing OTX (Sukhov *et al.*, 1993). On the other hand, according to immunohistochemical studies, OXT neurons were predominant in the peripheral parts of these nuclei, were more widely dispersed, and showed a smaller soma than AVP cells (Braak and Braak, 1987; Dierickx and Vandesande, 1977, 1979; Saper, 1990; Swaab *et al.*, 1985b). Moreover, AVP cells were more numerous than OXT cells in the SON, while their number was comparable in the PVN (Sukhov *et al.*, 1993). In the sagittal sections of PVN, AVP and OXT neurons form a continuous band running from a ventral position in the rostral hypothalamus to a more dorsal position in the caudal hypothalamus (Sukhov *et al.*, 1993). Proceeding caudally, the PVN population shifts dorsally and increases in size (Sukhov *et al.*, 1993). As mentioned above, neurons containing OXT mRNA were found throughout the PVN and are approximately equal in number to AVP neurons (Sukhov *et al.*, 1993). However, OXT neurons are more widely dispersed than AVP cells in the PVN, and typically are smaller

(Sukhov *et al.,* 1993). Analogous to the PVN of the rat and hamster, the human PVN was suggested to contain two functionally distinct populations of OXT neurons; one with a neuroendocrine role and another with central functions. However, in the human PVN these two OXT cell types could not be distinguished clearly because, unlike in other mammals, there appeared to be neither a discrete size distribution nor a regional separation of the populations (Swaab *et al.*, 1995).

In situ hybridization techniques confirmed the two portions of SON: a relatively dense cluster of cells dorsal to the optic chiasm (the dorsolateral SON), and a ventromedial cell cluster to the chiasm (ventromedial SON) (Sukhov *et al.*, 1993). When the optic tract merges with the basal forebrain, the dorsal cells assume a more lateral position and the ventral cells cluster near the medial part of the optic tract. Most neurons in both cell populations are strongly labeled for AVP mRNA (Sukhov *et al.*, 1993). The dorsolateral group contains more densely compact large and medium-sized AVP cells, with typical round or ovoid magnocellular perikarya. The ventromedial group is characterized by AVP morphologically more heterogeneous neurons (Sukhov *et al.*, 1993). Also for OXT mRNA two distinct cell populations can be distinguished on the basis of size and intensity of hybridization. One consists of typical large, round or ovoid, and lightly labeled neurons, found mainly in the dorsolateral SON, and to a lesser extent in the ventromedial SON. The other population consisted of neurons that were slightly smaller than the previous and intensely OXT mRNA positive, which are found predominantly along the medial border of the dorsolateral SON, but may also be scattered throughout the rest of the SON. Unlike the PVN, the OXT cells were fewer than those containing AVP mRNA in the SON (Sukhov *et al.*, 1993). AVP and OXT cells of the magnocellular accessory nuclei did not differ morphologically from those observed in the PVN and SON (Sukhov *et al.*, 1993). Outside the magnocellular nuclei, AVP neurons were scattered throughout the suprachiasmatic nucleus and throughout the caudolateral hypothalamus (Sukhov *et al.*, 1993), and were occasionally present within the boundaries of the other gray nuclei of the hypothalamus. In summary, both immunocytochemical (Dierickx and Vandesande, 1977, 1979; Saper, 1990; Stopa *et al.*, 1984; Swaab *et al.*, 1985b) and *in situ* hybridization studies (Lucassen *et al.*, 1995; Mengod *et al.*, 1990; Rivkees *et al.*, 1989; Sukhov *et al.*, 1993) found that AVP and OXT neurons segregate to some extent within magnocellular nuclei and differ in size, suggesting that peptides may not colocalize to a large extent in humans.

3.2 *Vasopressin/oxytocin neuronal systems and human ageing*

Different factors seem to influence AVP and OXT expression, neuronal numbers and grey volume of magnocellular nuclei, including age, sex and disease. Many studies investigated the possible age- and neurodegenerative process- related changes in human magnocellular nuclei, and some authors postulated that the hypothalamo-hypophyseal system degenerates in old age and AD, but with inconclusive results (Davies, 1987; Davies *et al.*, 1990; Goudsmit *et al.*, 1990; Goudsmit *et al.*, 1992; Hoogendijk *et al.*, 1985; Ishunina and Swaab, 2002; Legros, 1975; Li *et al.*, 1984; Mazurek *et al.*, 1986a; Mazurek *et al.*, 1986b; Raskind *et al.*, 1986; Robertson and Rowe, 1980; Sørensen *et al.*, 1985; Sørensen *et al.*, 1983; Sundquist *et al.*, 1983; Swaab, 1995; Swaab *et al.*, 1985b). Decreased levels of AVP have been reported in plasma, cerebrospinal fluid and brain tissue in AD, suggesting an involvement of both centrally and peripherally projecting vasopressin cells in this disease (Mazurek *et al.*, 1986a; Mazurek *et al.*, 1986b; Raskind *et al.*, 1986; Sørensen *et al.*, 1985; Sørensen *et al.*, 1983; Sundquist *et al.*, 1983). Goudsmit et al. (1992) (Goudsmit *et al.*, 1992) observed that the number of AVP immunoreactive cells in the PVN of AD patients was 37% lower than in age-matched controls, although values did not drop below values of young controls. The same authors also revealed that AVP cell nuclei in the PVN of AD patients were enlarged, suggesting hypertrophy of the unaffected cells, or, alternatively, a selective loss of staining in small AVP neurons (Goudsmit *et al.*, 1992). On the contrary, other works suggested that the activity of AVP cells increases in senescence (Fliers *et al.*, 1985; Frolkis *et al.*, 1982; Helderman *et al.*, 1978; Kirland *et al.*, 1984). In addition, trials with vasopressin administration to elderly and demented subjects have been rather disappointing (Jolles, 1986; Wolters *et al.*, 1990). Swaab et al. (1985) (Swaab *et al.*, 1985a) and Fliers et al. (1985) (Fliers *et al.*, 1985) found an increase in cell size in immunocytochemically identified AVP but not OXT neurons of the human SON and PVN after the age of 80 years, which was suggestive of an increased peptide production from this age onwards. In particular, Fliers et al. (1985) (Fliers *et al.*, 1985) stained post-mortem brain sections of subjects aged 10 to 93 years, including patients with Alzheimer's disease (AD), with AVP and OXT antiserum, using cell size as a parameter for peptide production. Their results revealed no significant changes in the mean OXT cell profile area with increasing age (Fliers *et al.*, 1985). In contrast, the mean profile area of AVP cells showed an initial decrease up to the sixth decade of life, after which a gradual increase was observed (Fliers *et al.*, 1985), while the size of AVP and OXT cell

nuclei did not change significantly with aging andobservations in the brains of AD patients were within the range for their age group. Therefore, their data do not support degeneration or reduced function of posterior pituitary lobe function in senescence or AD, but suggest activation of AVP cells after 80 years of age (Fliers *et al.*, 1985). The changes in the size of the cellular profiles of AVP suggested a decline in AVP production up to 60 years of age, after which activation of peptide production takes place (Fliers *et al.*, 1985). Goudsmit et al. (1990) (Goudsmit *et al.*, 1990) determined volume and total cell number in SON and PVN of 14 male and 16 aged female subjects from 10 to 93 years, and of 4 males and 6 females with AD aged from 46 to 97 years. The subjects were divided into two age groups: "young" for subjects up to 60 years, and "elderly" for subjects over the age of 60. In this study no significant differences in volume and total number of cells were found in the SON or PVN between young and elderly control subjects, and between healthy and dementia subjects, indicating that these nuclei are spared from degenerative changes in senescence and AD (Goudsmit *et al.*, 1990). Total cell number in the SON, PVN and SCN did not show any correlation with brain weight, indicating that age-related changes in the hypothalamus do not depend on changes in mass in elderly subjects (Goudsmit *et al.*, 1990). The absence of cell loss during aging and AD in the human SON and PVN was confirmed in many other studies (Goudsmit *et al.*, 1990; Hofman *et al.*, 1988; Hofman *et al.*, 1990; Van der Woude *et al.*, 1995; Vogels *et al.*, 1990; Wierda *et al.*, 1991). Hofman et al. (1990) (Hofman *et al.*, 1990) found a striking increase of SON cell number during aging: an increase in SON cell number around 30 % was found in subjects between 40 and 65 years of age, and a further increase was observed after the age of 65 years. The observation that the SON and PVN are spared from degenerative changes in AD is in line with reports on the absence of Alzheimer type neuropathological changes in these nuclei (Ishii, 1966; Saper and German, 1987). Goudsmit et al., (1992) (Goudsmit *et al.*, 1992) observed a gradual increase in the number of neurons which were immunoreactive for AVP in the human PVN with aging. This finding is in accordance with the morphological and physiological evidence of an increase in AVP synthesis in this nucleus during aging. The number of oxytocin-immunoreactive neurons in the PVN was found to remain constant during aging (Wierda *et al.*, 1991), which is coherent with the absence of morphological signs of activation in these cells in senescence (Fliers *et al.*, 1985; Hoogendijk *et al.*, 1985). The evidence for an activation of AVP cells in human senescence might be corroborated by the application of mRNA *in situ* hybridization to post-mortem human hypothalamic tissue (Mengod *et al.*, 1990). Increased levels of AVP mRNA

were demonstrated in patients with clinical evidence of ante-mortem dehydration (Rivkees *et al.*, 1989). In this small study, the highest levels of vasopressin mRNA were found in the oldest subjects, suggesting that vasopressin synthesis is indeed increased in senescence (Rivkees *et al.*, 1989). Hoogendijk et al. (1985) (Hoogendijk *et al.*, 1985) studied the nucleolar size of such cells in old age, observing an increase in the nucleolar size of AVP cells, but not OXT cells, in the PVN and SON of the same subjects of the Fliers et al. study (1985) (Fliers *et al.*, 1985). The nuclear size has proven to be a good yardstick for neurosecretory activity in these neurons (Russell, 1983; Zambrano and de Robertis, 1968). On the other hand, Mann et al. (1981) (Mann *et al.*, 1981) reported decreased nuclear and nucleolar volume of unidentified cells in the PVN and SON of demented patients as compared with age-matched controls. However, no lifespan measurements of these parameters were performed in their study. Therefore, the demented group may have been less activated, rather than 'degenerated', as compared with the age-matched controls. Theoretically, it cannot be ruled out that AVP cells are activated in senescence as a compensatory mechanism for cell loss. Although AVP cell density was found not to change significantly during aging, there may be a decrease of total AVP cell number with a concomitant decrease of volume of the PVN and SON (Goudsmit *et al.*, 1990). It was also hypothesized that activation of AVP cells observed in senescence and senile dementia can be secondary to changes in kidney function: in fact, this activation is probably related to the decreased sensitivity of the senescent kidney to vasopressin (Goudsmit *et al.*, 1990). An alternative or additional explanation for such age-related changes may involve changes in afferent innervation of the PVN and of the SON during aging (Fliers *et al.*, 1985). A number of observations indicated that neuronal loss with aging and in neurodegenerative disease might be prevented by neuronal activation (Swaab, 1991). Therefore, it could be suggested that age-related cell loss in the SON and PVN is prevented by activation of vasopressin cells in these nuclei (Goudsmit *et al.*, 1990). Conflicting results were also obtained in the peripheral dosage of posterior pituitary hormones and their carrier proteins. Legros (1975) (Legros, 1975) observed decreased blood levels of immunoreactive neurophysins between 50 and 60 years of age, a secondary increase was subsequently shown after the age of 70. Other investigators reported an age-related increase of the blood levels of neurophysins and AVP (Frolkis *et al.*, 1982; Kirland *et al.*, 1984; Rondeau *et al.*, 1982). Confirming the activation of AVP neurons during aging, Lucassen et al. (1994) (Lucassen *et al.*, 1994) showed a significant increase in area of the Golgi Apparatus (GA) with age in controls and in AD,

demonstrating an activation of the AVP neurons in the SON of the human hypothalamus in these two conditions. No changes were observed in the cellular profile areas with age, neither in the controls nor in AD, suggesting that the GA area is a much more sensitive parameter for monitoring activity changes in post-mortem material than neuronal size (Lucassen *et al.*, 1994). Different studies in humans and animals reported that neuronal profile area, nuclear volume, nucleolar profile area and GA area are sensitive parameters for detecting experimentally induced or age- or pathology- related changes in neurosecretory and cellular activity (Crespo *et al.*, 1992; Hoogendijk *et al.*, 1985; Jongkind and Swaab, 1967; Kalimo, 1975; Lafarga *et al.*, 1991; Salehi *et al.*, 1994; Silverman and Sladek, 1991; Zambrano and de Robertis, 1968). In addition, Ishunina et al. (1999) (Ishunina and Swaab, 1999) reported an age-dependent sex difference in the size of the GA, that appeared to be twice as large in the young men than women of the same age. Of note, the size of the GA increased with age exclusively in women. Again, the mean cell profile area, another measure for neuronal activity, was significantly larger in young men than in young women, as well as being larger in old women than in young women (Ishunina and Swaab, 1999).

3.3 *Sex differences in the structure of vasopressin and oxytocin neurons*

A growing body of evidence indicates the presence of sex differences in the vasopressinergic and oxytocinergic systems in mammals. In humans there are no conclusive results to date. Plasma levels of AVP were shown to be sexually dimorphic in humans (24, 25), being higher in males (Asplund and Aberg, 1991; van Londen *et al.*, 1997). However, such studies did not measure the main part of the AVP in human plasma, that is bound to platelets (van der Post *et al.*, 1993). Goudsmit et al. (1990) (Goudsmit *et al.*, 1990)found no significant differences in volume and total number of cells in SON and PVN by gender across all age groups. In addition, differences in brain weight between males and females were not reflected by differences in total cell numbers in these three hypothalamic nuclei, indicating that the sexual dimorphism which was described for both cortical and subcortical structures (Paul, 1970; Swaab and Fliers, 1985; Wessely, 1970), is not reflected in the SON, PVN and SCN (Goudsmit *et al.*, 1990). Using the same material of previous investigations (Hofman *et al.*, 1988; Hofman and Swaab, 1989), Hofman et al. (1990) (Hofman *et al.*, 1990) gave a quantitative description of the human SON morphology in normal adult subjects. In addition, they investigated the existence of sexual and age-related differences after excluding factors

that might influence the measurements, such as differences in brain size, age, postmortem delay, fixation time and hour and month of death (Hofman, 1984; Hofman *et al.*, 1990). They found no statistically significant differences for any of these parameters with the exception of the volume of the brain, which showed a marked sexual dimorphism (Hofman *et al.*, 1990). Although no significant sexual dimorphism was detected in the absolute size, shape and cellular morphology of the human SON, its volume was 11% larger in boys than girls (Hofman *et al.*, 1990). Even the average total number of cells appeared to be the same in both sexes (Hofman *et al.*, 1990). Moreover, by using the regression method this study demonstrated sexual differences in the SON internal structural organization which did not find expression when absolute measurements were used (Hofman *et al.*, 1990). Ishunina et al. (1999) (Ishunina and Swaab, 1999) investigated the possible presence of sex differences in the activity of vasopressinergic and oxytocinergic neurons of the human SON and PVN using the size of neurons as a sensitive parameter for functional differences. In particular, the minimum and maximum diameters were determined, in 15 men and 17 women ranging in age from 29–94 years, to estimate the volumes of cell somata and cell nuclei in AVP and OXT neurons stained with an antibody against human glycoprotein-(22–39), a part of the AVP precursor, and a monoclonal anti-OXT antibody (Ishunina and Swaab, 1999). The AVP neurons appeared to be larger in young men than in young women (Ishunina and Swaab, 1999), while in older women the AVP cell size considerably exceeded that in young women (Ishunina and Swaab, 1999). In older men the AVP neurons were larger than in young men and older women, although these differences they were not significant (Ishunina and Swaab, 1999). Furthermore, AVP cell size was positively correlated with age in women but not in men. No significant differences were found in the nucleus volumes of AVP cells between all four groups studied (Ishunina and Swaab, 1999). Sex differences in the size of PVN vasopressin neurons were pronounced on the left and absent on the right lateral, indicating the presence of functional lateralization in this core (Ishunina and Swaab, 1999). No difference was found in any morphometric parameters of OT neurons in the PVN among the 4 groups studied (Ishunina and Swaab, 1999). Thus, these data demonstrate sex differences in the size of AVP neurons, and therefore in their function, which were age and probably also site dependent and the absence of such changes in OT neurons in the PVN (Ishunina and Swaab, 1999).

3.4 *Hypothalamic oxytocin neurons under pathological conditions*

A decrease in OXT cell numbers had been reported in different disorders, including Parkinson's disease (22% reduction) (Purba *et al.*, 1994), aquired immune deficiency syndrome (AIDS) (40% reduction) (Purba *et al.*, 1993), and Prader Willy syndrome (PWS) (Swaab *et al.*, 1995). However, opposite or non-significant results have also been described. Guldenaar and Swaab (1995) (Guldenaar and Swabb, 1995), using *in situ* hybridization combined with densitometric image analysis for the quantitative assessment of OXT gene expression in the PVN and SON of a group of AIDS patients, found no significant difference in the amount of OXT mRNA per total PVN as compared to matched controls. In addition, no significant differences were found in the the volume of the PVN that was occupied by hybridized cells or in the mean signal density, not supporting the suggestion that the decrease in OXT-IR cell numbers of the PVN in AIDS patients would be associated with a decrease in OXT mRNA in this nucleus (Guldenaar and Swabb, 1995). Swaab et al. (1995) (Swaab *et al.*, 1995) morphometrically investigated the PVN of 5 PWS patients (2 males and 3 females), varying in age between 22-64 years, and 27 controls (14 males and 13 females) without any primary neurological or psychiatric diseases, using conventional staining with thionine and immunocytochemical staining for OXT and AVP. They found that the thionine-stained volume of the PVN was 28% smaller in PWS patients, and the total cell number was 38% lower (Swaab *et al.*, 1995). The immunoreactivity for OXT and AVP was decreased in PWS patients, although the variability within the groups was high (Swaab *et al.*, 1995). A strong and highly significant decrease was found in the number of OXT-expressing neurons of PWS patients (Swaab *et al.*, 1995), tthe volume of the PVN-containing OXT-expressing neurons decreasing by 54% in PWS patients (Swaab *et al.*, 1995), whilethe number of AVP-expressing neurons in the PVN did not change significantly (Swaab *et al.*, 1995). The OXT neurons of the PVN seem to be good candidates for playing a physiological role in ingestive behavior acting as "satiety neurons" in the human hypothalamus (Swaab *et al.*, 1995). Furthermore, it is worth noting an important difference between the results in PWS and the findings in Parkinson's disease and AIDS. In the latter two conditions, the volume of the PVN containing OXT and AVP neurons did not alter, whereas in PWS, the volume of the OXT part of the PVN declines by 54%. As the OXT and AVP cell populations only occupy some 50% of the entire PVN volume (Swaab *et al.*, 1993; Van der Woude *et al.*, 1995; Wierda *et al.*, 1991), the PVN volume of the five PWS cases was compared to that of the five

matched controls (Swaab *et al.*, 1995). Indeed, a 28% reduction was found in PWS patients, which shows that the PVN is anatomically affected in this syndrome (Swaab *et al.*, 1995).

3.5. Nesfatin-1

Nesfatin-1 is an 82-amino-acid highly anorexigenic neuropeptide which emerges as a result of the posttranslational conversion of the precursor molecule NEFA/nucleobindin-2 (NUCB2) (Schalla and Stengel, 2018). In the rat brain, neural populations with nesfain-1 expression are mainly located in the hypothalamus and the brainstem (Goebel *et al.*, 2009; Pałasz *et al.*, 2012). As a novel regulatory factor nesfatin-1 plays an important role in hypothalamic pathways regulating food intake and energy homeostasis (Wilz *et al.*, 2020). Recent findings report evidence for significant nesfatin-1 involvement in other important brain functions such as reproduction, sleep, cognition and anxiety- or stress-related responses (Pałasz *et al.*, 2018; Weibert *et al.*, 2019). Interestingly, the serum concentration of nesfatin-1 should be considered as a sensitive marker of epileptic seizures. In the rodent brain, nesfatin-1 expressing neurons form a distinct population in ARC, PVN and SON, where the peptide is colocalized with POMC/CART, NPY, oxytocin and vasopressin. Nesfatin-1 immunoreactivity was recently detected in the human hypothalamus (Psilopanagioti *et al.*, 2019) as well as in the bed nucleus of the stria terminalis (Pałasz *et al.*, 2019). Both magnocellular and parvocellular perikarya of the human PVN manifest diffuse but strong neuroplasmic nesfatin-1 expression, with the same pattern of neuropeptide immunopositivity has been found in all parts of the SON. Majority of vasopressinergic (more than 50%) and oxytocinergic (about 70%) neurons of the human SON and PVN exhibit nesfatin-1 immunoreactivity. In the PVN significant colocalization with CART additionally occurs (Psilopanagioti *et al.*, 2019). This may suggest possible synergistic effects of both regulatory neuropeptides on food intake inhibition and control of energy expenditure. In general, the pattern of nesfatin-1 expression in the human magnocellular nuclei reflects recent evidence and descriptions from animal, mainly rodent studies. Noteworthy, nesfatin-1 expression in the human hypothalamus seems to be BMI-related, subjects with obesity showed decreased neuropeptide immunoreactivity compared with normal weight individuals (Psilopanagioti *et al.*, 2019). Nesfatin-1 should be therefore considered as a new important regulator of energy homeostasis and appetitive behaviour in the human hypothalamus.

3.6. Spexin

Spexin (SPX) is a novel regulatory neuropeptide, a product of the *Ch12orf39* gene, which was firstly identified *in silico* and then detected in the rat brain (Mirabeau *et al.*, 2007; Porzionato *et al.*, 2010). The human SPX precursor molecule consists of a signal peptide, two prohormone cleavage sites, and a predicted neuropeptide (Sonmez *et al.*, 2009). SPX has a potent anorexigenic activity, decreases insulin release and also manifests some anxiolytic and antidepressive-like effects in several animal models (Mills *et al.*, 2021; Walewski *et al.*, 2014).

In the rat brain, a distinct population of SPX-expressing neurons has been found in the PVN and SON., while weaker SPX immunoreactivity occurs also in the hippocampus, amygdala, cerebellum and brainstem (Porzionato *et al.*, 2010). It was reported that SPX acts as an alternative ligand for the galanin GALR2/3 receptors (Kim *et al.*, 2014). The SPX-immunoreactive neurons have recently been identified in the human magnocellular hypothalamus (Pałasz *et al.*, 2021). The vast majority of large, oval or round SON neurons showed distinct SPX immunostaining and fluorescence. In turn, smaller multipolar, fusiform or droplet-shaped perikarya displayed medium SPX immunoreactivity. Numerous, large SPX-positive cells were observed in the posterior part of the SON sections. Of note, the medium-sized, oval-shaped perikarya of the entire SON exhibit a distinct but less intense SPX immunoreactivity. Most SPX-expressing neurons in the PVN possess large elongated, irregular-shaped, multipolar or triangular somata, however a less abundant population of small round cells was also identified. The intensity of SON cell neuroplasmic fluorescence was slightly higher when compared to the PVN, with the mean number of SPX-positive cells both in SON and PVN increased from the rostral to the caudal part of the nuclei (Pałasz *et al.*, 2021). A distribution of SPX- expressing neurons in the human hypothalamus reflects studies in animal models (Porzionato *et al.*, 2010; Wong *et al.*, 2013), with the most intense immunoreactivity in SON and reduced in PVN. To date, there is no direct evidence of SPX contribution to human hypothalamus function, although some studies may be potentially analogous to that revealed in the lower vertebrates e.g. its potent anorexigenic action (Walewski *et al.*, 2014). SPX expression in the magnocellular cells also suggests that this novel neuropeptide may be released into the blood via the hypophyseal circulation and can act as a multifunctional factor in both central and peripheral regions.

3.7. 26RFa

Neuropeptide 26RFa alternatively named QRFP (for pyroglutamilated RFamide peptide) is a 26-amino acid ligand of the human orphan G-coupled receptor GPR103. also designated SP9155 or AQ27 (Chartrel *et al.*, 2003; Ukena *et al.*, 2011). This novel orexigenic factor involved in the central regulation of food intake and glucose homeostasis was discovered via the use of bioinformatic tools (Fukusumi *et al.*, 2003; Jiang *et al.*, 2003) and subsequently identified both in the human and the rat hypothalamus (Bruzzone *et al.*, 2006; Takayasu *et al.*, 2006). In the human PVN, a population of 26RFa-expressing spindle-shaped or spiny perikarya is located in the dorsal and medial regiont of the nucleus close to lumen of the third ventricle. Of note, 26RFa- immunoreactive neurons were not detected in the SON. The PVN is involved in regulation of energy homeostasis and also in OXY-related control of male sexual responses particularly in erection triggering (Argiolas and Melis, 2005). Possibly, 26RFa-expressing neurons may play a role in analogous yet understudied regulatory mechanisms in the human brain.

3.8. Vasoactive intestinal peptide (VIP)

VIP is a 28-amino acid neuropeptide that represents a glucagon/secretin superfamily, the ligand of class II G protein–coupled receptors (Umetsu *et al.*, 2011). VIP is synthesized both in the brain and peripheral neural and glandular tissues (Delgado and Ganea, 2013). Hypothalamic suprachiasmatic nucleus consists of numerous VIP-expressing neurons that are recently considered as the master circadian pacemaker (Achilly, 2016; Ono *et al.*, 2021). The brain VIP synthesis is highly limited to the hypothalamic centres and from there the neuropepetide may affect the release of prolactin. General effects of VIP action are cardiac contractility stimulation, vasodilation, smooth muscle relaxation and stimulation of glycogenolysis (Moody *et al.*, 2011). An expression of VIP has been found in the human SON and PVN, the SON has around 2.3% of neurons showed VIP -immunoreactivity while the number of VIP-positive cells in the PVN was almost 3 times higher (5.8%). The approximate percentage of neurons with AVP coexpression was 2.1% and 5.2% respectively (Romijn *et al.*, 1999). A presence of abundant VIP-ergic neural population in the PVN may confirm a suggestion that these perikrya are an important source of neuropeptide for several brainstem and spinal cord autonomic centres of the human brain.

3.9. Galanin

Human galanin (GAL) is a 30-amino acid regulatory neuropeptide, a product of preprogalanin enzymatic cleavage. The following regulatory factors are included in the family of galaninergic neuropepetides: galanin-like peptide GALP, galanin-message associated peptide (GMAP) and alarin (Mills *et al.*, 2021). Galanin manifests a distinct affinity to its metabotropic GAL1 and GAL2 receptors (Kim *et al.*, 2014), and exposes a wide spectrum of physiological activity both in the CNS and in peripheral tissues. It is involved in the mechanisms of food and water intake regulation (Robinson *et al.*, 2006), stress responses (Tortorella *et al.*, 2007), reproduction (Lawrence and Fraley, 2011), and also play an important role in several brain functions including cognitive processing (Borbély *et al.*, 2013; Walton *et al.*, 2006). Galanin may also be involved in the pathogenesis of anxiety, depression, addiction and neurodegenerative disorders (Holmes and Picciotto, 2006). A distinctly developed population of GAL-immunoreactive perikarya was identified in the human SON and PVN (the estimated number of cells from three examined hypothalami was approximately 60 000 and 50 000 respectively) as well as in the arcuate, suprachiasmatic, and tuberomammillary nuclei. The majority of GAL-positive neurons in the SON and PVN showed AVP coexpression and both nuclei have a smaller population of perikarya which colocalize GA and OXY (Gai *et al.*, 1990). *In situ* hybridization also confirmed GAL mRNA expression in the human SON and PVN (Bonnefond *et al.*, 1990). Neuroanatomical localization of GAL-immunoreactive neurons in the human magnocellular nuclei is analogous to that observed in the rat brain. The general pattern of GAL-expressing cells in the hypothalamus strongly support the hypothesis that this mutifunctional neuropeptide may play an important role in the neuroendocrine regulatory processes in humans.

3.10 *Corticotropin releasing hormone (CRH)*

CRH is a 41-amino acid peptide derived from a 196-amino acid preprohormone. CRH is secreted by the paraventricular nucleus (PVN) of the hypothalamus in response to stress. Increased CRH production has been observed to be associated with AD and major depression (Raadsheer *et al.*, 1993). CRH expressing neurons are also present in the PVN, where CRH positive neurons were smaller in diameter than those of neurons expressing neurophysins and were located equally in the medial and lateral parts of the PVN. The vast majority of CRH-immunopositive perikarya occupy the PVN and the periventricular region delineating the 3rd ventricle, while a smaller group of CRH-IR cells can be found in the dorsomedial subdivision of the ventromedial nucleus (Dudas and Merchenthaler, 2006).

Importantly, SON did not contain any CRH-immunoreactive perikarya. Although the majority of the CRH-IR neurons are fusiform-shaped, numerous multipolar cells can be observed in the periventricular region. A dense network of CRH-positive axonal varicosities can be observed around the capillaries in the PVN and in the periventricular region (Peroski *et al.*, 2016). CRH immunoreactivity was present in neurons of the PVN and in their fibers running to the median eminence. The CRH-positive neurons were scattered throughout the PVN, but in the rostral part relatively few cells were present. There were large individual differences in the number and staining intensity of CRH neurons in the PVN and in the staining intensity of the median eminence. All discrepancies seemed not to be attributed to sex, age or *post mortem* changes. Since the distribution of CRH-immunoreactive neurons in the human PVN strongly overlap with vasopressin, colocalization of these peptides was investigated in a double label study and indeed were found in subjects ranging between 43 and 91 years of age. However, cells staining for individual peptides were also observed, while interestingly in younger subjects (23–27 years of age) no coexpression was detected. Perhaps, the age-related colocalization of CRH with vasopressin may reflect increased activation of the CRH neurons with age (Raadsheer *et al.*, 1993).

3.11 Cocaine- and amphetamine-regulated transcript (CART)

CART is a multifunctional, mainly hypothalamic neuropeptide involved in the central regulation of energy homeostasis, consummatory behaviour, thermoregulation, stress responses, reward circuits, thermogenesis (Farzi *et al.*, 2018; Lau and Herzog, 2014; Subhedar *et al.*, 2014) and possibly in cognitive processing (Bharne *et al.*, 2016). In the human magnocellular hypothalamus CART-expressing perikarya were identified both in the SON and PVN. A network of CART-positive fibers was also found throughout the hypothalamus, inside the bed nucleus of the stria terminalis (BNST), and in the amygdala. Interestingly a distinct majority of CART-immunoreactive cells (78%) in the human PVN showed coexpression with nesfatin-1 (Psilopanagioti *et al.*, 2019). The distribution of CART cell bodies and fibers in the human hypothalamus indicates that CART may also play a role in the regulation of energy homeostasis in humans (Elias *et al.*, 2001).

3.12 Dynorphin A

Dynorphins (Dyn) belong to the group of opioid peptides that are active posttranslational products of depolarization-evoked preprodynorphin (PDYN) cleavage by PC2 convertase (Schwarzer, 2009). The following dynorphin molecules are present in the brain: dynorphin A (Dyn A 1–17), dynorphin B (Dyn B 1–13), α-neoendorphin (α-NE), "big dynorphin" (Dyn A 1–32), leumorphin (Dyn B 1–29) and leucine–enkephalin–arginine (Leu–enkephalin–Arg). Dynorphins as endogenous ligands of kappa opioid receptors are widely distributed in various brain regions especially in those involved in the affective mechanisms related to reward systems and mood regulation such as the amygdala, hippocampus, hypothalamus, midbrain, striatum, and brainstem (Fallon and Leslie, 1986). To date, numerous dynorphin A (PH-8P) immunoreactive perikarya were detected in the human SON and the magnocellular part of the PVN, while most of SON dynorphin-positive neurons exhibit coexpression with AVP. PH-8P-like immunoreactive neurons in the human magnocellular perikarya send long axons to several brain regions (Abe *et al.*, 1988). Additionally, *in situ* hybridization has confirmed the presence of pre-prodynorphin (pre-PDYN) mRNA in the human hypothalamus (Sukhov *et al.*, 1995).

3.13 *Adrenomedullin-2/intermedin (AM2/IMD)*

Adrenomedullin-2/intermedin (AM2/IMD) is an endothelial 52-amino acid regulatory peptide, acting as a ligand of metabotropic calcitonin gene-receptor like receptors (CRLR) coupled with receptor activity-modifying proteins (RAMPs) (Hinson *et al.*, 2000; Kitamura *et al.*, 1993; Krzeminski, 2016). AM-2/IMD is mainly considered as a potent vasodilatatory factor, however other functions have been found such as regulation of osmotic hemostasis and natriuresis, neuromodulatory effects (Allen and Ferguson, 1996; Murphy and Samson, 1995) and antiapoptotic action. A distinct AM-2 immunoreactivity has been detected in the rat hypothalamus (Taylor *et al.*, 2005). In the human brain, neural populations occupied both magnocellular and parvocellular region of PVN showed distinct AM2/IMD expression. Conversely, SON was characterized by a small number of diffusely arranged and weakly immunoreactive cells, with strongly positive perikarya found rarely in this nucleus. The majority of AM2/IMD positive neurons in PVN revealed coexpression with AVP (Takahashi *et al.*, 2006) suggesting cautiously that this neuropeptide may be involved in the central control of electrolytic homeostasis and water intake in humans.

4. Concluding remarks

Both classical and novel neuropeptide expression in the human magnocellular hypothalamus constantly and precisely regulate a large spectrum of crucial autonomous functions, which may possibly imply overlapping roles in neuropsychiatric dysfunctions and disorders such as narcolepsy, depression and anxiety. Immunohistochemical and genetic mapping may be considered a valuable source of structural data that help to understand the neurochemical composition of the human hypothalamus. Virtually all brain neuropeptides detected are characterized by a broad spectrum of regulatory activity in the human brain and many of them may be considered as understudied but potentially important factors involved in the origin of several dysfunctions and pathologies. Theoretically, possible pharmacomodulation of central peptidergic activity may be helpful in the future treatment of certain neuroendocrinal disorders. Thus, a lot of further and wide ranging neurochemical investigations focused on various hypothalamic areas merits high attention.

CRediT authorship contribution statement

Artur Pałasz: Conceptualization, Resources, Writing - original draft**, Alessandra Della Vecchia:** Resources, Writing - original draft, **Karolina Saganiak:** Writing - original draft., **John J. Worthington**: Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing interests.

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Figure captions

Fig.1. Neuroanatomy of the human hypothalamus at the level of magnocellular nuclei, Nissl staining (A.). Histological organization of the human hypothalamus focused on neurosecretory supraoptic and paraventricular nuclei (B.). A modified structural grid is partly based on Mai et al. (2015). Morphology of the magnocellular perikarya in the SON and PVN in safranin-solochrome cyanine (C,E) and Nissl (D,F) staining. Scale bars: 400μm. Abbreviations: anterior commissure, AC; bed nucleus of the stria terminalis, BNST; medial preoptic nucleus – medial part, MPOM; lateral hypothalamus, LH; paraventricular nucleus dorsal, PaD; paraventricular magnocellular, PaMC; paraventricular nucleus parvocellular, PaPC; paraventricular nucleus, PVN; suprachiasmatic nucleus, SCh; supraoptic nucleus; supraoptic nucleus; SON.

Fig. 2. Schematic representation of noncanonical neuropeptides distribution in the human magnocellular hypothalamus. Figure shows core neuroanatomical localization and relative density of peptidergic neurons identified in supraoptic and paraventricular nuclei. Yellow dots outline spatial distribution of immunopositive perikarya but do not reflect actual cell sizes.

Fig.3. Nesfatin-1 and spexin expressing neurons in the human supraoptic (SON) and paraventricular (PVN) nuclei. Immunofluorescent reaction with goat monoclonal antibody against human/rat nesfatin-1 (1:1000, Enzo Life Sciences) and rabbit anti-rat spexin polyclonal antiserum (1:2000, Phoenix Pharmaceuticals). Brain sections were incubated with secondary antibodies labeled with TRITC or FITC (1:200, Abcam) for nesfatin-1 and spexin respectively. Scale bars: 50 μm. Original author's material based on recent unpublished data.