Final report

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In mammals, hypothalamic neurons synthesizing kisspeptin (KP) are critically involved in the central regulation of reproduction. The two populations of these cells in the preoptic area and in the mediobasal hypothalamus, respectively, play critical roles in mediating feedback effects of estrogens and establishing the pattern of gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) secretion: preoptic KP neurons trigger the preovulatory GnRH/LH surge, whereas their mediobasal counterparts (= "KNDy neurons", named after their KP, neurokinin B and dynorphin co-content) form a crucial component of the GnRH/LH "pulse generator". The focus of this research was on revealing the unique structural and neurochemical features of the pulse generator KP cells in mammalian species, including human. Resources from this grant contributed to the following results and publications recognized in acknowledgements:

We unveiled previously unexplored fine structural features of KP neurons in the mediobasal hypothalamus of humans. Confocal microscopic studies of primary dendrites in 100-µm-thick tissue sections established that 79.3% of KP cells were bipolar, 14.1% were tripolar and 6.6% were unipolar. We introduced a newly developed method in which we combined immunofluorescent staining of KP cells with random "diolistic" labeling using a gene gun loaded with the lipophilic dye, DiI. Using this method, we were able to examine the detailed dendritic morphology and the aminoacidergic and kisspeptinergic innervation of human KP neurons. Accordingly, primary dendrites branched sparsely, contained numerous appendages (9.1±1.1spines/100 µm dendrite) and received rich innervation from GABAergic, glutamatergic and KP-containing terminals. We also analyzed the human KP neuron synaptology with immunoelectron microscopy on perfusion-fixed specimens: KP axons established frequent contacts and classical synapses on unlabeled and on KP-immunoreactive somata, dendrites and spines. Synapses were asymmetric and the presynaptic structures contained round and regular synaptic vesicles, in addition to dense-core granules suggesting that KP neurons use glutamatergic, beyond peptidergic, neurotransmission. *(Takács et al., Brain Structure and Function 2018)*

Revealing anatomical and neurochemical similarities and differences in the pulse generator KP cells of mammalian species enhances our understanding of obligate and facultative players in the molecular mechanisms underlying pulsatile GnRH/LH secretion. Therefore, we characterized canine and feline KP neuron distribution, neuropeptide phenotype and connectivity to GnRH cells in ovariectomized dogs and cats. In both species, KP and neurokinin B (NKB) neurons occurred in the mediobasal hypothalamus, and the two cell populations overlapped substantially. Dynorphin was detected in large subsets of canine KP (56%) and NKB (37%) cells and feline KP (64%) and NKB (57%) cells; triple-labeled ("KNDy") somata formed ~25% of all immunolabeled neurons. Substance P (SP) was present in 20% of KP and 29% of NKB neurons in ovariectomized cats but not dogs, although 26% of KP and 24% of NKB neurons in a gonadally-intact male dog also contained SP signal. Only in cats, cocaine- and amphetamine regulated transcript was also colocalized with KP (23%) and NKB (7%). Unlike in mice, KP neurons did not express galanin in either carnivore. KP neurons innervated virtually all GnRH neurons in both species. Results of this anatomical study on ovariectomized animals reveal species-specific features of canine and feline mediobasal hypothalamic KP neurons. (*Rumpler et al., under review)*

We identified and characterized the rostral population of human KP neurons, homologous to the rodent counterpart in the rostral periventricular area (RP3V) involved in positive estrogen feedback and the generation of the the mid-cycle LH surge. The distribution of these cells in the rostral hypothalamus overlapped with different subdivision of the paraventricular nucleus. Cell numbers decreased after menopause, indicating that estrogens positively regulate KP gene expression in the rostral hypothalamus in humans, similarly to several other species. Young adult women and men had similar cell numbers, as opposed to rodents having more KP neurons in the RP3V of females. Human KP neurons differed neurochemically as well from the homologous rodent cells in that they were devoid of enkephalins, galanin and tyrosine hydroxylase. Further, they did not contain known KP neuron markers of the human infundibular nucleus (=ARC), NKB, SP and CART but received afferent input from these KP neurons. Identification and positive estrogenic regulation of KP neurons in the human rostral hypothalamus challenge the long-held view that positive estrogen feedback may be restricted to the mediobasal part of the hypothalamus in primates and point to the need of further anatomical, molecular and functional studies of rostral hypothalamic KP neurons. *(Rumpler et al., Neuroendocrinology, 2019)*

In a review article, we summarized our current knowledge of the anatomy of human KP neurons. This study focused on the basic topography, morphology, neuropeptide content, and connectivity of these cells. In situ hybridization and immunohistochemical studies on post mortem human tissues revealed that human KP neurons differ neurochemically from their rodent counterparts and show robust aging-related plasticity (hypertrophy, increased cell number, fiber density, KP-content of neurokinin B cells, neuropeptide mRNA expression and immunoreactivity, higher numbers of afferent contacts onto GnRH neurons). Recently available single-cell anatomical and molecular approaches, which provide new tools to investigate the aging-related anatomical and functional plasticity of the human KP system, have been also discussed. *(Hrabovszky et al., Neuroendocrinology 2019)*

In mammals, KP neurons are key components of the hypothalamic neuronal networks that regulate the onset of puberty, account for the pulsatile secretion of GnRH and mediate negative and positive estrogen feedback signals to GnRH neurons. Being directly connected anatomically and functionally to the hypophysiotropic GnRH system, the major KP cell groups within the preoptic area/rostral hypothalamus and the arcuate (or infundibular) nucleus, respectively, are ideally positioned to serve as key nodes which integrate various types of environmental, endocrine and metabolic signals that can influence fertility. In an invited book chapter we provide an overview of the current state of knowledge on the anatomy, functions and plasticity of brain KP systems based on the wide literature available from different laboratory and domestic species. Then, the species-specific features of human hypothalamic KP neurons were described, covering their topography, morphology, unique neuropeptide content, plasticity and connectivity to hypophysiotropic GnRH neurons. Some newly emerging roles of central kisspeptin signaling in behavior and finally, clinical perspectives were discussed. *(Hrabovszky et al. The human hypothalamic kisspeptin system. Functional neuroanatomy and clinical perspectives In: Swaab/The Human Hypothalamus: Middle and Posterior Hypothalamus. Handbook of Clinical Neurology 2020)*

ARC represents the primary anatomical site where estrogen exerts its negative feedback and regulates pulsatile GnRH secretion. In females, cyclic changes in serum estrogen levels heavily

influence the gene expression pattern of estrogen-sensitive neurons of this region. Our goal was to identify the estrogen-dependent genes and regulatory pathways of the ARC and the pulse generator KP neurons. To achieve the precise anatomical isolation of the ARC and ARC KP-zsGreen cells of ovariectomized (=low level of estrogen) and ovariectomized + estrogen-treated (=high level of estrogen) mice, respectively, we used laser capture microdissection. Subsequently, high-throughput RNA-Seq analysis was performed to compare transcript levels between animals with low and high estrogen levels. We identified >2,000 genes in both ARC and ARC KP neurons, that changed significantly (FDR<0.05) in response to the estrogen treatment. Using functional annotation, several ten pathways were found to be altered significantly. The unique database of the estrogen-responsive genes contains transcripts with currently unknown functions (e.g. lncRNAs) which will be subjects of our future studies. *[presented at the 2nd Meeting of the European Centre for Reproductive Endocrinology (Sep 2019, Prato, Italy); Manuscript in preparation will be submitted by Göcz et al. to Nature Communications in 2020]*.

The neuroendocrine control of reproduction is governed by ~2,000 hypothalamic GnRH neurons. Unexpectedly, in human, we identified 150-200,000 GnRH-synthesizing cells outside the hypothalamus, mainly in the striatum. Extrahypothalamic GnRH neurons contain authentic GnRH decapeptide derived from the proGNRH1 transcript. We determined that these cells are formed by subpopulations of cholinergic neurons. Although similar neurons could not be detected in adult rodents, the GnRH-GFP transgene is expressed transiently by caudate-putamen cholinergic interneurons in newborn transgenic mice. In slice electrophysiological studies, GnRH inhibits these interneurons via GnRHR1 autoreceptors. RNA-Seq studies revealed selective expression of GNRH1, GNRHR1, GnRH-processing enzymes and inhibitory G proteins that may bind GNRHR1 in homologous cholinergic interneurons laser-microdissected from the adult human putamen. Transcriptome profiling of cholinergic interneurons and spiny projection neurons provides novel insight into the molecular connectome of the human putamen. *[presented at the 1st European Center for Reproductive Endocrinology Conference (Mar 2018, Prato, Italy) and the Neuroscience 2018 (Nov 2018, San Diego, CA, USA); Manuscript in preparation will be submitted by Skrapits et al. to Nature Neuroscience in 2020]*.