Final report

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Puberty in mammals is a complex process of sexual development which leads to complete gonadal maturation and the attainment of full reproductive capacity. Puberty takes place gonad-independently with the onset of pulsatile GnRH secretion into the hypophysial portal circulation. Human genetics provide evidence that the hypothalamic neuropeptide kisspeptin (KP) is particularly important for the pubertal awakening of the 'GnRH pulse generator'. The two populations of hypothalamic KP neurons, in the mediobasal hypothalamus and the preoptic area, respectively, also play critical roles in mediating the feedback effects of estrogens to GnRH neurons in adult mammals. The focus of this research was on understanding the contributions of GnRH neurons and KP neurons to the regulation of puberty and reproduction. Resources from this grant contributed to the following results and publications recognized in acknowledgements:

Peptidergic signaling in the arcuate nucleus (ARC) regulates important homeostatic and endocrine functions and also plays critical roles in pubertal development. To shed light on the molecular mechanisms of puberty, we studied the developmental shift in the gene expression profile of the ARC of male mice. RNA samples used for quantitative RT-PCR studies were isolated from the ARC of 14-day-old infantile and 60-day-old adult male mice with laser capture microdissection. The expression of 18 neuropeptide, 15 neuropeptide receptor, 4 sex steroid receptor and 6 classic neurotransmitter marker mRNAs was compared between the two time points. The adult animals showed increased mRNA levels encoding cocaine- and amphetamine-regulated transcripts, galanin-like peptide, dynorphin, kisspeptin, proopiomelanocortin, proenkephalin and galanin and a reduced expression of mRNAs for pituitary adenylate cyclase-activating peptide, calcitonin gene-related peptide, neuropeptide Y, substance P, agouti-related protein, neurotensin and growth hormone-releasing hormone. From the neuropeptide receptors tested, melanocortin receptor-4 showed the most striking increase (5-fold). Melanocortin receptor-3 and the Y1 and Y5 neuropeptide Y receptors increased 1.5- to 1.8-fold, whereas δ-opioid receptor and neurotensin receptor-1 transcripts were reduced by 27 and 21%, respectively. Androgen receptor, progesterone receptor and α-estrogen receptor transcripts increased by 54-72%. The mRNAs of glutamic acid decarboxylases-65 and -67, vesicular GABA transporter and choline acetyltransferase remained unchanged. Tyrosine hydroxylase mRNA increased by 44%, whereas type-2 vesicular glutamate transporter mRNA decreased by 43% by adulthood. Many of the developmental changes we revealed in this study suggest a reduced inhibitory and/or enhanced excitatory neuropeptidergic drive on fertility in adult animals and provide insight into the mechanisms of pubertal development. *(Molnár et al., Neuroendocrinology 2016).*

We have established a new Human Hypothalamic Research Unit specifically devoted to studies of the neuroendocrine network of human puberty and fertility. (http://hhru.koki.hu/).

We have studied and reported in an invited book chapter the detailed neuroanatomy of human GnRH neurons, including their development, topography, connectivity and unique anatomical features. *(Skrapits and Hrabovszky: The GnRH neuron and its control. In: Allan E. Herbison and Tony M. Plant (Editors). pp. 149-176 2018).*

We have described orexin and melanin concentrating hormone (MCH) containing afferents innervating human GnRH neurons from the lateral hypothalamus. These afferents mostly targeted the dendritic compartment and likely contribute to the metabolic regulation of fertility. *(Skrapits et al., Frontiers in Cellular Neuroscience 2015.).*

We have provided evidence that GnRH serves as a neurotransmitter in the rodent hypothalamus. Confocal and immuno-electronmicroscopic studies revealed GnRH-immunoreactive axons that formed asymmetric syapses with dopaminergic neurons of the mouse brain. The synaptic morphology suggested a glutamatergic cotransmission in this communication. *(Bardóczi et al., J. Neuroendocrinol 2018).*

The infundibular nucleus (Inf) of the human hypothalamus corresponds to the rodent ARC and serves as an important integration center for neuronal signals and hormones released by peripheral endocrine organs. KP producing neurons of the rodent and sheep ARC also synthesize neurokinin B (NKB) and dynorphins and are, therefore, commonly referred to as ’KNDy’ neurons. KNDy neurons are critically involved in puberty as well as in sex hormone signaling to GnRH neurons and also represent the long-sought GnRH/LH ’pulse generator’. We have studied the neurochemistry of the homologous KP cells in the human Inf. These studies used several new antibodies generated in our laboratory and identified NKB as an important co-transmitter in KP neurons of the human Inf. In contrast, dynorphin which occurs frequently in KP (KNDy) neurons of sheep and rodents, was rarely detectable in human KP neurons. Similarly, galanin was identified in mouse, but not the human, KP cells. To the opposite, we found that Substance P (SP) and cocaine and amphetamine-regulated transcript (CART) are often synthesized in human, but not rodent, KP neurons. In addition, human KP neurons and their fibers were devoid of immunoreactivity to proenkephalin and the dopamine marker tyrosine hydroxylase, two compounds identified previously in the rostral periventricular KP cell population of laboratory rodents. Neurochemical differences between KP cells of laboratory animals and the human indicate that species may use considerably different neurotransmitter mechanisms to regulate the GnRH neurosecretory pulses and the

negative sex steroid feedback to the reproductive axis. Species comparisons also help us identify the obligate *vs.* the interchangeable neuropeptide players in the central regulation of reproduction by these cells. *(Skrapits et al., Front. Neuroscience 2015).*

Along the same line, we have also studied the neuropeptide complement of feline and canine KP neurons. The KP neurons of these carnivores showed unique neurochemical properties and interesting species similarities as well as differences from the rodent and primate KP neurons **(***Rumpler et al, sumitted).*

Immunohistochemical studies from our laboratory characterized the basic topography, morphology, neuropeptide content and connectivity of human KP neurons. Human KP neurons differ neurochemically from their rodent counterparts and show robust aging-related plasticity. Our experiments also provided evidence for plastic changes in the hypothalamus of aging men whose NKB and KP neurons undergo hypertrophy, increase in number, exhibit increased neuropeptide mRNA expression and immunoreactivity and give rise to higher numbers of immunoreactive fibers and afferent contacts onto GnRH neurons. Furtermore, increasing percentages of KP-expressing NKB perikarya, NKB axons and NKB inputs to GnRH neurons raise the intriguing possibility that a significant subset of NKB neurons begins to co-synthesize KP as aging proceeds. Although use of post mortem tissues is technically challenging, recently-available single-cell anatomical and molecular approaches provide promising new tools to investigate the aging-related anatomical and functional plasticity of the human KP neuronal system. Technical advances in this research field, including a recently developed high-resolution anatomical and molecular techniques were discussed in a review article, also including new data about the aging-dependent anatomical and neurochemical plasticity of human KP neurons. *(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. We have prepared a 9000-word invited book chapter in which 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)

Fine structural analysis of the human KP system is complicated by the use of *post mortem* tissues. To gain better insight into the neuroanatomy of the somato-dendritic cellular compartment, we introduced the 'diolistic' labeling of immunohistochemically identified KP neurons using a gene gun loaded with the lipophilic dye, DiI. 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. 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. KP neuron synaptology was analyzed with immunoelectron microscopy on perfusion-fixed human brain specimens. KP axons established asymmetric synapses on unlabeled and on KP-immunoreactive somata, dendrites and spines. *(Takács et al., Brain Structure and Function 2018)*

Xenoestrogens from synthetic or natural origin represent an increasing risk to disrupt endocrine functions including the physiological activity of the hypothalamo-pituitary-gonad axis. Ethinyl estradiol (EE2) is a synthetic estrogen widely used in contraceptive pills, whereas zearalenone (ZEA) is a natural mycoestrogen found with increasing prevalence in various cereal crops. Both EE2 and ZEA are agonists of estrogen receptor alpha (ERα) and accelerate puberty. We studied the neuroendocrine mechanisms for these effects. Both ZEA and EE2 accelerated puberty onset and resulted in increased expression of GnRH. These changes occurred in parallel with an advanced increase of kiss1 (encoding KP) mRNA in the anteroventral and rostral periventricular (AVPV/PeV) region of the hypothalamus and increased kisspeptinergic fiber density in the preoptic area. Furthermore, the number of appositions between KP fibers and GnRH neurons were elevated following xenoestrogen treatment. Neither compound affected kiss1 expression in the KNDy neurons of the ARC nor the mRNA levels of transcriptional suppressors, makorin3, eed and cbx7. Our results implicated the central AVPV/PeV KP system in the puberty accelerating effects of the xenoestrogens EE2 and ZEA. *(Kriszt et al., Endocrinology 2015).*

The ventral tegmental area (VTA) is a main regulator of reward and integrates a wide scale of hormonal and neuronal information. Feeding-, energy expenditure-, stress, adaptation- and reproduction-related hypothalamic signals are processed in the VTA and influence the reward processes. However, the neuroanatomical origin and chemical phenotype of neurons mediating these signals to the VTA have not been fully characterized. In our study we have systematically mapped hypothalamic neurons that project to the VTA using the retrograde tracer Choleratoxin B subunit (CTB) and analyzed their putative gamma-

aminobutyric acid (GABA) and/or glutamate character with *in situ* hybridization in male rats. The analyses of vesicular glutamate transporter 2 (VGLUT2) and glutamate decarboxylase 65 (GAD65) mRNA expression revealed both aminoacid markers in different subsets of retrogradely-labeled hypothalamic neurons, typically with the predominance of the glutamatergic marker VGLUT2. Some regions including the perifornical part of the lateral hypothalamus were populated mostly by GAD65 mRNA-containing retrogradely-labeled neurons. Our results indicated that both the medial and lateral nuclear compartments of the hypothalamus provide substantial input to theVTA. *(Kalló et al., Frontiers in Neuroanatomy 2015).*

The microglial response against amyloid β, including activation of the Nlrp3 inflammasome, plays a central role in the pathogenesis of Alzheimer's disease (AD). We investigated whether intracerebroventricular treatment of middle-aged rats with Nlrp3 inflammasome inhibitor MCC950 can counteract amyloid β1-42 oligomer (AβO) induced neuroinflammation and memory impairments. AβO followed by aCSF infusion resulted in upregulation of genes encoding the microglia markers toll-like, cytokine, complement receptors, and proinflammatory mediators in the hippocampus and caused spatial memory impairments. Expression profiling demonstrated enhanced expression of genes involved in neuron-microglia interactions, especially Cx3cr1, Cd200r and Cd45, and downregulation of Scn1a encoding parvalbumin-positive basket cell-specific Na(v)1.1. These changes along with decreased serum IL-10 levels resembled characteristics of early/mild AD. Chronic MCC950 treatment attenuated microglia reactivity, completely abated memory impairment, and normalized serum IL-10 levels. These findings demonstrate that after the AβO challenge, the resolution of inflammation and restoration of memory do not take place spontaneously, but can be achieved by inhibition of the Nlrp3 inflammasome. We proposed that AβO evoked Nlrp3 inflammasome activation sustains microglia driven neuroinflammatory reactions that eventually lead to spatial memory impairments. Therefore, the Nlrp3 inflammasome is a viable molecular target for the treatment of late-onset AD. *(Fekete et al., Neuroscience 2018).*

Anatomical and molecular experiments planned on KP-GFP mice in this project have been rescheduled and will be completed in 2020. The first KP-Cre mouse strain (gift from R. Steiner) showed non-specific labeling and insufficient fluorescence for laser capture microdissection and anatomical studies. These experiments are still ongoing using new KP-Cre mice from a different laboratory (W. Colledge, Cambridge.)

The PI gave several invited symposia talks from the above results at scientific meetings and workshops *(e.g.: FAMÉ, Pécs, Hungary, 2016; 4th Scientific Meeting/Training School of the European GnRH Network, Budapest, Hungary, 2016; 9th International Congress of Neuroendocrinology, Toronto, Canada,*

2018; MEAT XXXI. Kongresszusa, Hajdúszoboszló, Hungary, 2018; 1st EUCRE Conference, Prato, Italy; 2nd EUCRE Conference, Prato, Italy, 2019.)