4th

SCIENTIFIC MEETING / Training School

of the European GnRH Network

BUDAPEST
March 6-9
2016

PRESENTATION AND POSTER

ABSTRACTS

Co-sponsored by the
Hungarian Academy of Sciences (MTA) and the
European Cooperation in Science and Technology
(EU COST ACTION BM1105)

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IN SCIENCE AND TECHNOLOGY

PL 1. Olfactory receptors: from genes to behavior

Ivan Rodriguez

Laboratory of Neurogenetics, Department of Genetics and Evolution, University of Geneva (Opening lecture)

PL 2. Using population genetics studies to inform the aetiology of reproductive ageing and its links to broader health

John Perry

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As age at puberty declines and age at first pregnancy increases, the mechanisms that regulate reproductive lifespan become increasingly relevant to population health. The timing of menarche and menopause can have profound effects not only on fertility but also on the risk of diseases such as type 2 diabetes mellitus, cardiovascular disease and breast cancer. Genetic studies have identified dozens of highly penetrant rare mutations associated with reproductive disorders, and also ~175 common genetic variants associated with the timing of puberty or menopause. These findings, alongside other functional studies, have highlighted a diverse range of mechanisms involved in reproductive ageing, implicating core biological processes such as cell cycle regulation and energy homeostasis. During my talk I will review the contribution of such genetic findings to our understanding of the molecular regulation of reproductive timing, and the biological basis of the epidemiological links between reproductive ageing and disease risk.

S1/1. 3D imaging and whole-brain clearing provide novel insight onto the development of GnRH neurons in humans

Paolo Giacobini

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Traditional histological examination of brain systems and networks has long relied on tissue sectioning and analyses of thin serial slices that presents limitations for large volumetric imaging. In the past few years several groups have developed protocols to render tissues "optically transparent" thereby minimising light scatter and allowing inspection of neural networks within intact specimens usually lost in 2D optics.
Here, we used immunohistochemical approaches coupled with software-assisted 3D-reconstruction analysis of histological sections and new tissue clearing techniques to render intact human fetal brains transparent for optical imaging. These

methods allowed us to obtain exhaustive phenotypical, anatomical and quantitative descriptions of a unique population of neurons essential for vertebrate reproductive function, the gonadotropin-releasing hormone (GnRH) neurons during the first trimester of gestation.
We document for the first time an unexpected migratory process and distribution of GnRH neurons in intact human fetal brains and we show that three-dimensional imaging of solvent-cleared organs (3DISCO), coupled to laser-sheet microscopy, enables high resolution immunolabelled imaging of entire organs of human foetuses. This study provides the first high-resolution 3D-atlas of the human fetal development and opens new windows of studying neuronal network formation in physiological and pathological conditions.

S1/2. Genetics of Kallmann syndrome

Catherine Dodé[1] and Jean-Pierre Hardelin[2]

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Kallmann syndrome (KS) is a developmental disorder that associates anosmia (absence of the sense of smell), related to olfactory bulb aplasia, with congenital hypogonadotropic hypogonadism caused by gonadotropin releasing hormone (GnRH) deficiency, which is clinically characterized by absence of puberty and infertility. Hypogonadism is due to the incomplete embryonic migration of neuroendocrine GnRH-cells from the nasal epithelium to the hypothalamic region where GnRH secretion takes place. KS results from the so-called olfacto-genital pathological sequence, whereby the defective migration of GnRH-cells arises from the premature interruption of olfactory, vomeronasal and terminal nerve fibres, which normally guide these cells during their migration to the brain. This provides a developmental link between the central control of reproduction and the sense of smell, which are both affected in KS. KS can be isolated, or associated with various non-reproductive non-olfactory additional anomalies, depending on the causal genes. KS is genetically heterogeneous, with several different modes of transmission: X chromosome-linked recessive, autosomal dominant, autosomal recessive, and presumably oligogenic. The best characterized causal genes include *KAL1* (Kallmann syndrome 1), *FGFR1* (*fibroblast growth factor receptor 1*), *FGF8* (*fibroblast growth factor 8*), *PROKR2* (*prokineticin receptor 2*), *PROK2* (*prokineticin 2*), *FEZF1* (*FEZ family zinc finger 1*), *SOX10* (*sex determining region Y-box 10*, also involved in Waardenburg syndrome), and *CHD7* (*chromodomain helicase DNA-binding protein 7*, also involved in the CHARGE association). Other genes contribute to the disease phenotype, such as *HS6ST1* (*heparan sulfate 6-O-sulfotransferase 1*), *IL17RD* (*interleukin 17 receptor D*), and *SEMA3A* (*semaphorin 3A*). Notably, this list implicates at least three different signalling systems in the disease pathogenesis, *i.e.* FGF, prokineticin, and semaphorin signalling. However, mutations in any of these genes are found in less than 50% of the KS patients, indicating that either non-coding mutations in known genes or other

disease genes, remain to be discovered. The complex genetics of KS monogenic *versus* oligogenic modes of transmission, will be discussed.

S1/3. Genetic interactions in CHARGE syndrome

M. Albert Basson

King's College London

CHARGE (coloboma, heart defects, atresia of the choanae, retarded growth and development, genital and ear abnormalities) syndrome is caused by dominant loss-of-function mutations in *CHD7*, a gene that encodes the ATP-dependent chromatin remodelling enzyme, chromodomain helicase DNA binding protein 7. Mutations in *CHD7* have also been reported in Kallmann syndrome, providing a genetic explanation for the phenotypic overlap between CHARGE and Kallmann syndromes. The neurodevelopmental functions of CHD7 remain largely uncharacterised. We hypothesised that *CHD7* and other genes associated with Kallmann syndrome might interact and exhibit overlapping roles in disease aetiology. I shall describe work by my laboratory demonstrating a genetic interaction between the *Chd7* and *Fgf8* genes during cerebellar development in the mouse embryo. The potential implications of this finding to GnRH deficiency in CHARGE syndrome will be discussed. The putative involvement of other chromatin modifying factors in Kallmann syndrome and GnRH neurogenesis will be considered.

S2/1. Reproductive phenotypes, ovarian peptides and ovarian morphology in women with nIHH/Kallmann: new insights

Jacques Young

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Congenital (or isolated) hypogonadotropic hypogonadism (CHH/IHH) results from abnormal gonadotropin secretion and is characterized by a lack of pubertal development and infertility that is caused mainly by defective GnRH production or release by the hypothalamus. The prevalence of CHH, evaluated from 1/10 000 to 1/4000 in males, has been reported to be between 2 and 5 times less frequent in females. These values mainly established by specialized teams belonging to teaching hospitals are probably underestimated compared to the real frequency of CHH/IHH in the general population of women as a consequence of recruitment biases related to underestimation of milder phenotypes. CHH/IHH is revealed in the majority of female teenagers and young women by primary amenorrhea. Breast development is highly

variable and it is absent in a minority of cases but it often present and sometimes almost normal. Similarly, pubic hair may be absent, sparse or even normal. These partial forms, in majority not referred to hospital may contribute to explaining the underestimated prevalence of this condition in women. The classical hormonal signature of CHH/IHH in females is a low level of circulating estradiol together with low or "normal" levels of FSH and LH. In fact, a relationship exists between pubertal development and estradiol and pituitary gonadotropin concentrations: these hormones are often very low or undetectable in the absence of breast development, while in patients with advanced breast development stages they can reach values close to those observed in the early follicular phase of women with normal cycles.

Circulanting anti-Mullerian Hormone (AMH) and inhibin B (IB), secreted by granulosa cells of ovarian follicles, and their relationship with ovarian morphology and antral follicular count (AFC) have not been systematically studied in females with CHH/IHH. We had the opportunity to evaluate the relationship between these ovarian peptides and detailed ovarian morphology in a series of IHH/KS. More specifically, in untreated CHH/IHH women, were measured concomitantly: serum LH, FSH, estradiol (E2), androstenedione, testosterone, and serum inhibin B and AMH. At the same time, ovarian volume and antral follicular count (AFC) (small and larger follicles) were also measured by transvaginal sonography. Hormonal and ovarian morphological response to recombinant human FSH was also evaluated in a sub-group of women with complete IHH/KS.

The results of these explorations will be detailed and discussed.

S2/2. Next generation sequencing to discover novel genes in congenital hypogonadotropic hypogonadism

Nelly Pitteloud

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Over the past decade, the accelerating pace of genetic discovery has enhanced our understanding of the molecular basis of congenital hypogonadotropic hypogonadism (CHH). To date some 25 loci have been identified in relation to this condition - accounting for approximately half of cases. Previously, CHH was considered to be a Mendelian disorder. However, reports of patients who harbor pathogenic rare variants in more than one gene have challenged the long-held view that CHH is strictly monogenic. By systematically defining rare variants in large cohorts of well-phenotyped CHH patients, it has become clear that oligogenicity plays a significant role in this disease. Technologic advances such as next generation sequencing (NGS) are expanding our understanding of the molecular mechanisms underlying disorders of puberty. This presentation will provide perspectives on how NGS is beginning to further elucidate the genetic basis of CHH and shedding new light on the increasingly complex genetic architecture of this rare disorder.

S2/3. Human Genetics of complex neurodevelopmental disorders, an innovative way to characterize new mechanisms of GnRH deficiency

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The GnRH neuronal network development but also its maturation during the juvenile period represent the most accomplished process of dynamic changes of the hypothalamus. The post-natal maturation involves the changes of synaptic inputs and neuropeptide activations. It is required for the complete reactivation of the gonadotropic axis and therefore puberty. There are many clinical situations in which this program is interrupted, leading to congenital hypogonadotropic hypogonadism (CHH) and an absence of puberty. For many years, attention has mainly been focused on the genetics of isolated CHH mainly related to a defect in the development of the gonadotropic axis. More recently, the emergence of new genomics techniques has led to the description of genetic defects in very rare syndromes in which CHH is associated with complex neurological dysfunctions. Recently, *DMXL2* haploinsuffiency was found to cause a complex phenotype with both neurological and endocrine deficits. The neuronal knock-down of *Dmxl2* causes a GnRH deficiency in mice. Here, new data on the mechanism of the GnRH deficiency in *Dmxl2* knock down mice will be presented. These results highlight common mechanisms leading to GnRH deficiency and to neurodevelopmental disorders.

S3/1. Calendar cells and circannual cycle

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Living organisms show seasonality in a wide array of functions such as reproduction, fattening, hibernation and migration. At temperate latitudes, photoperiod is the main proximal cue responsible for the alignment of annual rhythms with predictable changes in the environment. Such annual rhythms are not passively driven by light. Indeed, they are usually thought as being the result of the environmental impact of light upon an endogenous component, referred to as the "circannual clock". The appropriate physiological response to changing photoperiod in mammals requires retinal detection of light and pineal secretion of melatonin. A common mechanism across all vertebrates is that these photoperiod-regulated systems alter hypothalamic thyroid hormone conversion. Here I'll review the evidence that a circadian clock within the pars tuberalis of the adenohypophysis links photoperiod decoding to local changes of thyroid hormone signalling within the medio-basal hypothalamus through a conserved thyrotropin/deiodinase axis. I will also focus on recent findings which

indicate that, beyond the photoperiodic control of thyroid hormone conversion, the pars tuberalis might host the elusive circannual timer. How such modifications may impact the GnRH neuron and seasonal breeding will be discussed.

S3/2. Maternal photoperiod programmes offspring reproductive development via the fetal pituitary

Sáenz de Miera C[1,2], Bothorel B[1], Birnie M[2], Simonneaux V[1], Hazlerigg D[2,3]

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In wild mammals, offspring reproductive development must anticipate forthcoming metabolic demands and opportunities. Within species, different developmental strategies may be employed, dependent on when in the year conception takes place. This phenotypic flexibility is initiated before birth, and is linked to the pattern of day length (photoperiod) exposure experienced by the mother during pregnancy. This depends on transplacental communication via the pineal hormone melatonin.
Here, we show that, in the Siberian hamster (*Phodopus sungorus*), the programming effect of melatonin is mediated by the pars tuberalis (PT) of the fetal pituitary gland, before the fetal circadian system and autonomous melatonin production is established. Maternal melatonin acts on the fetal PT to control expression of thyroid hormone deiodinases in ependymal cells (tanycytes) of the fetal hypothalamus, and hence neuroendocrine output. This sets the trajectory of reproductive development in pups, and has a persistent effect on their subsequent independent sensitivity to photoperiod. This programming effect depends on tanycyte sensitivity to TSH, which is dramatically and persistently increased by short photoperiod exposure *in utero*.
Our results define a novel transplacental pathway for epigenetic programming of fetal brain function, and establish programmed changes in TSH receptor signal transduction as a central feature of circannual timekeeping.

S3/3. Fasting induced neuronal plasticity in the hypothalamic paraventricular nucleus

Csaba Fekete

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The arcuato-paraventricular pathway plays critical role in the fasting induced regulation of the hypothalamic-pituitary-thyroid (HPT) axis. The fall of peripheral leptin levels during fasting inhibits the hypophysiotropic thyrotropin-releasing hormone (TRH) synthesizing neurons in the hypothalamic paraventricular nucleus

(PVN) by activating the orexigenic neuropeptide Y (NPY) and inhibiting the anorexigenic proopiomelanocortin (POMC) neurons of the arcuate nucleus. These neuronal groups directly innervate the TRH neurons and the peptides released from the arcuate-paraventricular pathway influence the TRH synthesis via the modulation of the cAMP-CREB second messenger system. This hard-wired system, however, shows high level of plasticity. Fasting increases the number of orexigenic terminals on the surface of TRH neurons and decreases the number of anorexigenic inputs of these cells. Refeeding reverses these morphological changes only 24h after the onset of food intake. The fasting induced inhibition of the TRH neurons is also reversed only after 24h refeeding despite the fact that the POMC neurons of the arcuate nucleus are already activated after 2h refeeding. These data suggest that the fasting induced morphological changes of the arcuate-paraventricular pathway causes melanocortin resistance of TRH neurons during fasting and in the early period of refeeding.
In addition to these morphological plasticity, the release of NPY also induces short term synaptic plasticity of the neuronal inputs of the parvocellular neurons of the PVN. NPY stimulates the release of endocannabinoids and nitric oxide from the postsynaptic parvocellular neurons and inhibits the neuronal inputs of these cells via the two retrograde transmitter systems. Thus, by this synaptic gating mechanism, NPY may prevent that non-feeding related signals could override the fasting induced regulation of the PVN neurons.
In summary, in addition to the classical transmitter mediated regulation, short and long term plasticity are also involved in the regulation of the TRH neurons by energy availability.

S4/1. Polycystic ovary syndrome: a common endocrine disorder with complex aetiology

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Polycystic ovary syndrome (PCOS) is the commonest endocrine disorder in women. It frequently presents during adolescence and is the commonest cause of menstrual irregularity and hirsutism. The characteristic endocrine abnormalities include hypersecretion of androgens and LH. Metabolic dysfunction is also a feature of many young women with PCOS. Hyperinsulinemia and insulin resistance, which can be regarded as an exaggeration of the normal metabolic changes that occur during puberty, are further amplified by obesity. The aetiology of PCOS is uncertain but there is evidence for a primary abnormality of ovarian androgen production that is manifest at puberty but may have its origins in childhood or even during fetal development. We have proposed that polycystic ovary syndrome has its origin in fetal life. This hypothesis is based on data from animal models and is supported by clinical studies. Animal models include Rhesus monkeys or sheep that have been exposed prenatally to high doses of androgen, and rodents given androgens before or during puberty. It is suggested that, in human females, exposure to excess androgen, at any stage from fetal

development of the ovary to the onset of puberty, leads to many of the characteristic features of PCOS, including abnormalities of LH secretion and insulin resistance. It is likely that, in humans with PCOS, the development of the PCOS phenotype results primarily from a genetic predisposition for the fetal ovary to hypersecrete androgen.

S4/2. Novel role for anti-Müllerian hormone in the regulation of GnRH neuron excitability and hormone secretion

Irene Cimino

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Fertility in mammals is dependent on a specific group of neurons secreting Gonadotropin Releasing Hormone (GnRH). Disruption of development and secretion of the GnRH neurons are shown to result in several reproductive disorders in humans. Polycystic Ovary Syndrome (PCOS) is a hyperandrogenic disease associated with chronic oligo-anovulation, polycystic ovarian morphology and other metabolic disorders, and is one of the most common causes of infertility affecting up to 10% of women. The reproductive dysfunction of PCOS is often associated with elevated luteinizing hormone (LH) levels and an altered ratio of LH to follicle stimulating hormone (FSH) suggesting an elevated GnRH pulsatility, yet it is still not clear why the GnRH-induced LH level is altered. An increased level of circulating anti-Müllerian hormone (AMH) is a hallmark of PCOS and may play a role in causing anovulation due to its inhibitory influence on follicular development. Previous studies have shown elevated plasma AMH levels in PCOS patients compared to healthy women, and this is positively correlated to the severity of PCOS phenotype. Besides the ovaries, AMH and its associated receptors (AMHRs) are expressed in various regions of the brain. However, the effect of AMH on the hypothalamic-pituitary-gonadal axis has never been investigated. Here we demonstrate that a subset of GnRH neurons expresses the AMHR in both rodents and humans, and that AMH potently activates GnRH neuron firing. Using a combination of *in-vivo* and *in-vitro* approaches, we show that AMH increases GnRH-dependent LH pulsatility and secretion, thus indicating a central role of AMH on regulating GnRH activity. Our findings raise the intriguing hypothesis that AMH-dependent regulation of GnRH release could be involved in the pathophysiology of fertility and could hold therapeutic potential for treating PCOS.

S4/3. Animal models and PCOS

Jenny A. Visser

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Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women in their reproductive age. Based on the Rotterdam criteria PCOS is diagnosed by at least two out of the following three criteria: hyperandrogenism, oligo/anovulation, and polycystic ovaries. In addition to ovarian dysfunction, women with PCOS display metabolic disturbances, such as obesity and insulin resistance, causing, women with PCOS having an increased risk for type 2 diabetes and cardiovascular disease. The subsequent increased insulin levels stimulate the ovary to further increased androgen production. Thus, the elevated androgen and insulin levels may result in a detrimental vicious circle between ovarian and metabolic functioning.
To date, the underlying cause of PCOS remains unknown, although the elevated androgen levels are suggested to play an important role in the development of PCOS. Indeed, metabolic and reproductive characteristics of PCOS can be induced in several animal models through androgenization either neonatally, prepubertally or during adult life. In rodents, testosterone, dehydroepiandrosterone (DHEA), or dihydrotestosterone (DHT) treatment are most commonly applied. We have developed a mouse PCOS model through chronic exposure of prepubertal mice to the non-aromatizable androgen DHT. DHT-treated mice were anovulatory and their antral follicles had a cyst-like structure. These mice also displayed increased adiposity and became glucose intolerant. This suggests that exposure to DHT can induce several features resembling those observed in women with PCOS. In addition, I will discuss other PCOS mouse models, induced with different androgenization schedules or through genetic manipulation, to illutrate the variable impact on reproductive and metabolic parameters.

S5/1. Stress, Metabolism and Reproduction

Julie A. Chowen

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The age at which an individual initiates puberty is determined by genetic and environmental factors, including both nutritional and social influences. Experimental models of maternal deprivation (MD) during neonatal life have been shown to induce long-term effects on numerous systems, including reproduction and metabolism. Indeed, we have shown that MD for 24 hours starting on post-natal day (PND) 9 not only reduces bodyweight throughout life, but it also affects the age at which external signs of puberty appear in rats. During the period of MD, there are changes in cell turnover, neurotrophic factors, and markers of neuronal and glial maturation in the hypothalamus, indicating effects on hypothalamic development. The fact

that this experimental manipulation abolishes the physiological neonatal leptin surge, suggests that changes in the levels of this hormone during a critical period of development could participate in the observed effects on pubertal onset. Indeed, leptin treatment of neonatal rats also modifies the timing of pubertal onset, in both control and MD rats.

Early neonatal overnutrition due to changes in litter size, and hence food availability, also modifies the timing of pubertal onset in rats. Neonatal overnutrition not only increases leptin levels during the critical period of hypothalamic development, but it also modifies the response to leptin during the peripubertal period. Early overnutrition affects hypothalamic glial cell development and function indicating that, in addition to previously reported changes in the development of neuronal circuits, modifications in glial cells may also be involved in the long-term effects observed on metabolism and reproduction. This conference will review what we have observed to date regarding the effects of MD and neonatal overnutrition on hypothalamic development and pubertal onset. The fact that these experimental paradigms differentially affect males and females will be stressed.

S5/2. Transcriptome analysis reveals the reorchestrated peptidergic signaling of the hypothalamic arcuate nucleus during pubertal development and sex steroid negative feedback

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Neuropeptides of the hypothalamic arcuate nucleus (ARC) regulate important homeostatic and endocrine functions. Pubertal maturation and negative sex steroid feedback coincide with profound transcriptional changes in this region and cause altered peptidergic signaling to the reproductive axis. To dissect the involvement of ARC neuropeptides and neuropeptide receptors in fertility regulation, we investigated the developmental and sex steroid-dependent molecular plasticity of the ARC transcriptome.

We first studied the pubertal shift in the gene expression profile of the ARC in male mice. RNA samples for quantitative RT-PCR studies were isolated from the ARC of day-14 prepubertal and day-60 adult intact male mice using laser-capture microdissection. The expression of 18 neuropeptide and 15 neuropeptide receptor mRNAs were compared between the two groups. Adult mice exhibited increased mRNA levels encoding cocaine- and amphetamine-regulated transcript (CART), galanin-like peptide (GALP), dynorphin, kisspeptin (KP), proopiomelanocortin (POMC), proenkephalin, galanin and melanocortin receptor-4 (MC4R) and reduced mRNA levels of pituitary adenyl cyclase activating peptide (PACAP), calcitonin gene-related peptide, neuropeptide Y (NPY), substance P, agouti-related protein (AGRP), neurotensin and growth hormone-releasing hormone. Many of these developmental changes are in accordance with increasing excitatory (CART, GALP, galanin, KP,

POMC, MC4R) and decreasing inhibitory (NPY, AGRP, PACAP) neuropeptidergic drive on fertility.
To test the contribution of increasing testosterone to developmental changes, the ARC transcriptome was compared between orchidectomized male mice with or without testosterone replacement. Testosterone decreased KP, GALP, CART, calcitonin gene-related peptide, somatostatin, POMC, proenkephalin, neurotensin and neurokinin 3 receptor expression and increased galanin and MC4R expression. Downregulation of KP, GALP, CART, POMC, neurotensin and neurokinin 3 receptor cause reduced excitatory drive on fertility which may contribute to negative feedback, whereas MC4R upregulation explains its developmental increase.
Developmental and testosterone-dependent plasticity of the ARC transcriptome help us understand the peptidergic mechanism(s) which shape the pulsatile pattern of GnRH/LH secretion.

S5/3. Kisspeptin and hypothalamic amenorrhea

Waljit Dhillo

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Hypothalamic amenorrhoea (HA) is a frequent cause of secondary amenorrhoea and is characterised by reduced levels of circulating LH, along with reduced LH pulse amplitude and frequency. It may be due to either a structural problem affecting the hypothalamus itself or, as more commonly occurs, due to pathology affecting hypothalamic function. Animal models have provided evidence that reproductive pathology due to hypothalamic dysfunction may be a potential therapeutic target for kisspeptin.
We have administered subcutaneous injections of kisspeptin-54 to women with hypothalamic amenorrhoea. This resulted in a significant increase serum LH levels on the first day of administration, when compared to saline ($p<0.001$). FSH and oestradiol were also shown to increase in response to kisspeptin stimulation. Women with HA appeared to be more sensitive to the stimulatory effects of kisspeptin than their healthy counterparts, with an approximate 4 fold increase in acute LH response compared to healthy women in the follicular phase of their menstrual cycle. We have also administered continuous IV infusion of kisspeptin-54 to women with HA and established a dose response with a therapeutic window within which LH pulsatility was restored, without the effect of desensitisation.
In summary, kisspeptin-54 appears able to restore LH pulsatility in women with hypothalamic amenorrhoea. However, further studies are now needed to determine therapeutic potential of chronic administration of kisspeptin to women with HA to restore fertility.

S6/1. Congenital hypopituitarism: new genes, new phenotypes

Mehul Dattani

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Congenital hypopituitarism (CH) is a rare but life-threatening condition that is associated with significant morbidity and mortality. It occurs in 1 in 4000 to 1 in 10000 live births, and may present variably. In the newborn period it is associated with conjugated hyperbilirubinaemia, micropenis with undescended testes in affected males, hypoglycaemia and possibly features of hypothyroidism including lethargy and feeding difficulties. Later on, it may present with early growth failure, or in milder cases even later. The condition includes GH, ACTH, TSH and gonadotrophin deficiencies; diabetes insipidus is usually rare unless midline abnormalities are present, as is the case with Septo-Optic Dysplasia (SOD). The diagnosis is based on a combination of auxology, biochemistry, and neuroimaging. Mutations in a number of genes have been identified in association with congenital hypopituitarism and SOD. These include a number of developmental genes such as *HESX1*, *SOX2*, *SOX3*, *OTX2*, *GLI2*, *ARNT2*, *IGSF1*, *TCF7L1*, *BRAF*, *LHX3*, *LHX4, PROP1* and *POU1F1*. Mutations have also been identified in genes that are implicated in Kallmann syndrome, such as *FGF8* and *FGFR1*. More recently, variations in *TCF7L1*, *RNPC3*, *BRAF, IFT172, GPR161, and CDON* have been associated with congenital hypopituitarism. Phenotypes, inheritance and penetrance can be variable, and much remains to be learned about the molecular basis of these conditions. In this presentation, I will summarize the current state of knowledge with respect to the molecular basis of hypopituitarism, with relevant genotype-phenotype correlations. I will discuss the use of next generation sequencing as well as the pitfalls that need to be addressed with their use.

S6/2. Pituitary Adenomas – novel aspects of genetic origin

Márta Korbonits

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The number of diseases associated with germline genetic abnormalities has grown exponentially in the last decade. Pituitary tumours are no exception, as now at least ten genes are known to predispose to pituitary tumour development: *MEN1, PRKAR1A, PRKACB, AIP, CDKN1B, SDH (A, B, C, D and AF2), GPR101* and *DICER1*. Some of these disease are associated with complex syndromes, others belong to the group of diseases named familial isolated pituitary adenomas (FIPA) where no other manifestation is expected. Although these alterations are relatively rare, recognition of the genetic alteration may have significant relevance for screening of other manifestations of the same disease, such as in MEN1 syndrome for example, or hold implications for other family members, such as for patients with *AIP* mutations, where screening may discover diseases at an early stage where therapeutic intervention maybe more advantageous.

Indeed, a seemingly far-fetched link between historical patients suffering from gigantism and current families with childhood-onset acromegaly led to the identification of an *AIP* mutation which now ties together 18 kindreds with over 100 carriers. Prospective identification of pituitary disease is emerging as a real possibility, which could potentially eradicate the development of gigantism in these families. In addition to germline abnormalities, somatic mutations in pituitary adenomas also held promise. USP8, an EGFR deubiquitinase seems to be responsible for almost 50% of cases on young female microadenomas associated with Cushing's disease. Understanding the roles of some of the novel genes in pituitary tumorigenesis may lead recognition of novel regulating pathways and possible future therapies for pituitary adenomas. Close collaboration between endocrinologists and clinical geneticists will help to recognise the patients and families who will benefit from these new exciting data.

S6/3. Functions of pituitary stem cells in turnover and disease

Cynthia L. Andoniadou

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We are interested in identifying signaling mechanisms required for correct maintenance of endocrine cells throughout life, as their disruption can underlie disease states such as organ insufficiency or the formation of tumours. Our work on the mouse anterior pituitary using genetic lineage tracing, has revealed that SOX2 positive cells can act as stem cells *in vivo* and that when mutated have the capacity to induce tumour formation. Pituitary stem cells are able to differentiate into all hormone-producing lineages and contribute to normal organ homeostasis during postnatal life. However, SOX2-expressing stem cells are not the sole source of new endocrine cells but complement contribution from more committed cell types. We find that cells providing the majority of organ turnover up-regulate the WNT signaling pathway, which promotes proliferation. Unexpectedly, we uncover that SOX2-expressing stem cells secrete WNT ligands, thus acting as critical regulators of long-term organ turnover in a paracrine manner. This represents a key step towards understanding the mechanisms controlling stimulation of new cell generation *in situ*, with an impact on future therapies for disorders of the pituitary gland.

O1; P1. Phenotype-genotype analysis in patients with GnRH deficiency in a single center

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Objective: Congenital hypogonadotropic hypogonadism (CHH) results from isolated GnRH deficiency and may present with normal sense of smell (nCHH), anosmia (Kallmann syndrome, KS) or in syndromic forms. Genetic defects are identified in approximately half of CHH cases and oligogenicity is noted in almost 10%. Further, spontaneous reversal of is seen in 15% of patients.
Methods: We analyzed the clinical characteristics of 37 Serbian CHH probands (34 sporadic, 3 familial). Genetic analyses of probands were conducted using Sanger (n=4) and exome sequencing (n=11). Rare variants (minor allele frequency <1%) were considered mutations if they were nonsense, frameshift, splice-site-altering variants or missense variants predicted to be deleterious *in silico*.
Results: In total, 11/37 (30%) had KS, 22/37 (59%) were nCHH, and 4 were syndromic (n=2 4H syndrome: HH / hypomyelination / hypodontia, n=1 CHARGE syndrome: coloboma / heart defects / atresia of choanae / retarded growth / genital anomalies / ear defects, n=1 HH + adrenal hypoplasia). Three male reversal cases were noted among the 33 KS/nCHH (10%). Genetic studies revealed mutations in 11 different loci in 12/15 (80%) unrelated probands. Two of three reversal cases were found to carry heterozygous mutations (*FGFR1* and *TACR3* respectively) and all three familial cases (2 nCHH, 1 KS) were found to harbor heterozygous mutations in *FGFR1.* Among the syndromic cases, both patients with 4H Syndrome harbor heterozygous mutations in *POLR3* while the patient with HH + adrenal hypoplasia has a hemizygous mutation in *NR0B1*. Exome sequencing revealed oligogenicity in one familial nCHH case (1/11, 10%) who harbors heterozygous mutations in *FGFR1*, *GNRH1*, and *LEP*.
Conclusions: This CHH cohort displays marked clinical heterogeneity including patients with 4H syndrome, CHARGE syndrome and congenital adrenal hypoplasia. We identified mutations in the majority (80%) of cases and those patients without mutations did not exhibit any CHH-associated phenotypes. Exome sequencing is an efficient and effective tool for exploring the complex genetic architecture of CHH.

O2; P2. A role for membrane estrogen receptor alpha in the sexual differentiation of kisspeptin neurons?

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Estrogens regulate physiology and behavior through a combination of nuclear- and membrane-initiated actions. Little is still known about the identity of the receptors involved in these responses. Here we sought to determine the role of the membrane fraction of estrogen receptor alpha (ERα) in the control of brain sexual differentiation. We investigated the expression profile of markers of brain sexual differentiation in males and females of the C451A-ERαmouse mode. In this model, ERαis unable to translocate to and signal from the membrane while retaining its transcriptional activity due to a mutation in the palmitoylation site of ERα Mice of both sexes were gonadectomized and injected for 2 weeks with estradiol benzoate to compare the

number of cells expressing tyrosine hydroxylase (TH) and kisspeptin (Kp) in the anteroventral periventricular nucleus (AVPv), two cell populations known to be more abundant in females than males. No genotype difference was found in the number of TH-immunoreactive (TH-ir) cells. However, while the number of Kp-immunoreactive (Kp-ir) cells did not differ between genotypes in females, mutant males were found to express more Kp-ir cells than wild-type males suggesting that this membrane receptor might play a role in sexual differentiation of these cell populations. By contrast, no sex difference and no genotype effect were found on the expression of kisspeptin in the arcuate nucleus as measured by the density of its immunoreactive labeling. A follow up experiment is currently investigating the effect of an early estrogenic treatment on the number of Kp-ir cells in the AVPv of both sexes to confirm the present results.

O3; P3. The involvement of the GnRH System in mediating Olfactory Bulb physiological plasticity

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The persistence of neurogenesis in the mouse Olfactory System (OS) gives a unique opportunity to investigate mechanisms/functions of neural plasticity in the adult brain. Both, the Main and the Accessory Olfactory Bulb (MOB and AOB) represent sites of adult neurogenesis and mediate social/reproductive stimuli (Schaal et al., 2003). These cues, thanks to olfactory direct connections with the amygdala and hypothalamus (Bohem et al., 2005; Yoon et al., 2005), elicit neuroendocrine responses and primary motivated behaviours (Brennan *et al.*, 2015). In turn, the internal milieu (i.e. hormones) can also impact on the modulation of the OS (Hoyk *et al.*, 2006)). In this context, we found that the pheromonal-dependent modulation of adult neurogenesis (AN) starts around puberty, a developmental critical period characterized by increased activity of the gonadotropin releasing hormone (GnRH) secretion. We argue that the process of AN in the OB region could be mediated, at least in part, by the GnRH System, through direct and/or indirect mechanisms. Accordingly, GnRH-immunoreactive fibers are present in the OB and quantitative PCR analyses on the total OB revealed the presence of mRNA of GnRH, Estrogen alpha/beta, and Androgen receptors in both sexes. Furthermore, using a transgenic mice that loses GnRH expression during postnatal development (*GnRH::Cre; DicerloxP/loxP mice*) we found that a dysfunction in the GnRH System leads to a reduced number of newly-born neurons integrating in the female MOB in basal condition, whereas no data are still available regarding this process after pheromonal exposure. Finally, in the *GnRH::Cre; DicerloxP/loxP* male mice we found defects in olfactory discrimination of both social and non-social cues, further supporting a link between GnRH system and olfactory function. In conclusion,

our data support that the GnRH system can adjust the perception of chemosensory stimuli by modulating neural plasticity in the olfactory bulb, that in turn influence the initial steps of reproductive behavior, such as the preference for opposite-sex cues and mate recognition.

O4; P4. The gonadotroph-vascular unit and its role in the pre-ovulatory LH surge in mouse models

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The hypothalamic-pituitary-gonadal (HPG) axis controls the reproductive function. Until recently, the pituitary gland was considered as a patchwork of randomly distributed cells which simply respond to hypothalamic regulation, however our investigations have shown that this organ is highly organized into structural and functional cell networks, and that dynamics of pituitary cell networks are essential for the generation of hormone pulses.
Recent preliminary data suggested the existence of a Gonadotroph-Vasculature Unit (GVU) in which crosstalk between the network of pituitary gonadotrophs and vasculature (pericytes and fenestrated endothelial cells) may be prerequisites for regulation of LH release into the bloodstream. Indeed, the gonadotroph network at proestrus displays a highly dynamic spatial reorganization which is correlated with changes in vascularisation/pericyte coverage.
Our working hypothesis is that pericytes contribute to GVU plasticity by regulating blood flow in pituitary vessels thus contributing to the generation of the pre-ovulatory LH surge.
To investigate this, we use transgenic mice and optogenetic tools to modulate the activity of pericytes in combination with gradient refractive index (GRIN) lenses (thin lens needle implanted through the cortex toward the dorsal pituitary side) to image blood flow dynamics during LH pulses (triggered upon GnRH intraperitoneal injection or endogenous pre-ovulatory surge) in conscious mice. LH levels are determined by tail vein blood sampling (3µl/sample) and ultra-sensitive hormone ELISA. We found that the onset of LH pulses is coincident with a transient increase in pituitary blood flow whilst both blood flow and LH levels are markedly altered upon optogenetic (Channelrhodopsin2) stimulation of pituitary pericytes. Hence these results suggest a role for the GVU in the LH pre-ovulatory surge.

O5. The Search for Disease-Causing Mutations in Self-Limited Delayed Puberty

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Background: Abnormal pubertal timing affects over 4% of adolescents and is associated with adverse health outcomes. Previous studies estimate 60-80% of variation in pubertal timing is genetically determined. Self-limited delayed puberty (SLDP) segregates in an autosomal dominant pattern; however, the neuroendocrine pathophysiology and genetic regulation remain unclear.

Methods: We performed whole exome sequencing (WES) in 115 members of 18 families from our large, well-phenotyped cohort with SLDP, followed by targeted exome sequencing in a further 42 families. We filtered the data returned using classic bioinformatic strategies, statistical thresholds for burden testing and by comparison with biological data on known pathways relevant to SLDP. The functional consequences of potentially pathogenic variants identified in 3 candidate genes were interrogated via tissue expression studies, in vitro assays and utilising animal models. An additional cohort of hypogonadotropic hypogonadism (HH) patients (n=334) underwent targeted exome sequencing of one gene.

Results: In ten unrelated families, we identified four rare mutations in *IGSF10*, a gene that is strongly expressed in the nasal mesenchyme during early migration of gonadotropin-releasing hormone (GnRH) neurons. *IGSF10* knockdown both *in vitro* and in our zebrafish model resulted in perturbed GnRH neuronal migration. Loss-of-function mutations in *IGSF10* were identified in five patients from our HH cohort. In our SLDP cohort, a rare, pathogenic mutation in *HS6ST1* was identified in one family and

rare variants in *FTO* were identified in 4 families. *FTO*$^{+/-}$ mice displayed delayed timing of puberty as compared to wild-type.
Conclusions: Our strategy has led to the discovery of one novel gene, *IGSF10*, as well as new variants in genes known to cause HH – *HS6ST1* – and associated with the timing of menarche – *FTO*. Whilst the genetic control of SLDP is heterogenic, we highlight the importance of developmental defects in the GnRH neuronal network in its pathogenicity and genetic overlap with HH.

O6; P6. Interactions of KNDy (kisspeptin, neurokinin B and dynorphin) neuropeptides potently stimulates LH pulsatility and gonadotrophin release in healthy male volunteers

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Background: A subpopulation of neurons in the hypothalamus co-localise three neuropeptides namely kisspeptin, neurokinin-B (NKB) and dynorphin collectively termed KNDy neurons. Data in animals shows that KNDy neuropeptides interact together to affect pulsatile GnRH release. Kisspeptin stimulates and NKB may modulate GnRH pulsatility, whilst dynorphin acting at the kappa opioid receptor (KOR) has inhibitory effects. To investigate the importance of KNDy neuropeptides in humans, we assessed for the first time the effects of co-administration of kisspeptin-54, NKB and an opioid antagonist naltrexone on LH pulsatility (surrogate marker for GnRH pulsatility) and gonadotrophin release.
Methods: We undertook an ethically approved prospective, single-blinded placebo-controlled study. Healthy male volunteers (n=5/group) attended on 8 different study visits and received a different treatment intervention at each visit: oral 50mg naltrexone (NAL), intravenous infusions (IV) of vehicle, 2.56nmol/kg/h NKB (NKB), 0.1nmol/kg/h kissspeptin-54 (KP) alone and in combinations – NAL+NKB, NAL+KP, NKB+KP, NAL+NKB+KP. After 1h of baseline blood sampling the intervention was started for 8h. Ten minutely blood sampling was performed to determine plasma gonadotrophins. LH pulsatility was determined using blinded deconvolution analysis.
Results: All kisspeptin and naltrexone containing groups caused a potent and significant rise in LH and LH pulsatility ($p<0.001$ vs vehicle). NKB alone had no effect on gonadotrophins. NKB+KP had siginicantly lower increases in gonadotrophins compared with kisspeptin alone ($p<0.001$). NAL+KP was the only group to significantly increase LH pulse amplitude ($p<0.001$ vs vehicle).
Conclusions: Our results suggest significant interactions between the KNDy neuropeptides on LH pulsatility and gonadotrophin release in humans. NKB may modulate GnRH signalling in response to an abundance of kisspeptin as shown by a lower rise in LH compared with kisspeptin alone, possibly through dynorphin

inhibition. NAL+KP significantly increased LH pulsatility, amplitude and most potently stimulated gonadotrophin release, which has important therapeutic implications in treating patients with reproductive failure and infertility.

O7; P7. Calcium-dependent exocytosis in GnRH neurons is required for sexual maturation and body weight homeostasis but not hypothalamic targeting in female mice

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Puberty is initiated by activation of the hypothalamic-pituitary-gonadal axis. The initial steps involve GnRH release by hypothalamic neurons into the pituitary portal circulation triggering of gonadotropin release by the pituitary. Intriguingly, GnRH signaling has been shown to be dispensable in the proper development and maintenance of GnRH neurons. However, whether calcium-dependent transmitter release plays a role in this process remains unclear. To address this question, we generated mice in which activity-dependent exocytosis is blocked by the Cre recombinase-dependent expression of the Clostridial botulinum neurotoxin serotype B light chain, which cleaves vesicle-associated membrane protein 2. Here we show that toxin expression in GnRH neurons promotes GnRH deficiency leading to hypogonadotropic hypogonadism in a subpopulation of female mice that also develop overweight and hyperleptinemia. This effect depends on the actual proportion of GnRH neurons expressing the transgene, which does not alter the anatomic placement and projections of GnRH neurons in the hypothalamus. These data establish the existence of a threshold effect for congenital GnRH deficiency in which small environmental changes in individuals harboring an identical pool of genes may have major consequences on their reproductive and metabolic status throughout life.

O8; P8. Impact of female hormones on functional brain networks: an in vivo MRI study in ovariectomized mice

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Introduction: Over the past years, evidence has been accumulating for the involvement of hypothalamic-pituitary-gonadal axis (HPG-axis) imbalances in the etiology of cognitive disorders, such as Alzheimer's Disease [1]. However, as many contradictory findings have been reported, it is crucial to further investigate the exact impact of the hormonal system on the brain. Resting state functional MRI (rsfMRI) is a non-invasive technique used to study functional connectivity (FC) in the brain. In the current longitudinal study, this technique was applied to examine functional brain networks in ovariectomized (OVX) and sham-operated mice.
Methods: Female C57BL/6J mice were either ovariectomized (N=18) or sham-operated (N=18) after an initial baseline scan at the age of 3 months, i.e. young adult. Afterwards, mice were followed up until 18 months in order to detect any changes in brain FC. During scanning procedures, mice were anesthetized using a mixture of medetomidine (0.3mg/kg) and isoflurane (0.3%). MR imaging was performed on a 9.4T Biospec MRI system (Bruker Biospec, Germany). RsfMRI data were analyzed by means of whole brain region-of-interest (ROI) analysis and seed-based analysis. Additionally, blood samples were collected for plasma hormone measurements.
Results: During the transition of young adult (3 months) to adult age (7 months), sham mice displayed an age-dependent maturation of FC patterns, observed as increased anterior-posterior connectivity, which was lacking in the OVX group. This effect reached significance at the age of 7 months. Analysis of plasma samples showed significantly increased LH levels in OVX mice. Histology is currently ongoing to link rsfMRI data to underlying changes in the brain.
Conclusion: These results suggest that an intact HPG-axis, i.e. physiological hormone levels, is indeed vital for regulating and maintaining the maturation of resting state networks in young adult mice.
[1] Janicki, S.C. and Schupf, N., *Curr. Neurol. Neurosci. Rep.*, 2010. 10(5): p. 359-366.

O9; P9. Modeling calcium signaling in GnRH neurons by a Piecewise Deterministic Markov Process (shotnoise) reveals the existence of an external component that synchronizes intracellular calcium

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GnRH pulsatile secretion is a compulsary property for an adequate reproductive functio. Although numerous studies on neuroendocrine cell lines have highlighted the role played by in intracellular calcium ($[Ca^{2+}]i$) in the neuroendocrine secretion, the link between $[Ca^{2+}]i$ fluctuations, Ca^{2+} entry during action potentials (APs) and GnRH secretion is far from being understood. For example, GnRH neurons derived from mouse embryos nasal placodes maintained in primary culture showed an average interval duration of 300msec for APs of, 20 sec for $[Ca^{2+}]i$ peaks and 18 min for GnRH secretory pulses. The only correlation between GnRH secretion and $[Ca^{2+}]$

i has been found at the population level, where every GnRH secretory pulse was found associated with the synchronization of $[Ca^{2+}]$i-events between more than 30% of the GnRH neurons. To better understand the dynamics of $[Ca^{2+}]$i we simulated the individual $[Ca^{2+}]$i dynamics of each neuron by a piecewise deterministic Markov process (PDMP), and compared simulated to experimental data.

Results

Recorded $[Ca^{2+}]$i –events (jumps) in control conditions followed a Poisson distribution, confirming the existence of an underlying stochastic process. Episodes of synchronization of $[Ca^{2+}]$i events (>30% of recorded neurons) were constantely observed as previously shown. Simulation of $[Ca^{2+}]$i evolution by a shotnoise process for each indivual neuron gave a good approximation of experimental data, however no high synchronization episodes were detected at the frequency observed in real experiments. In the presence of 10 nM antide, a GnRH receptor antagonist, $[Ca^{2+}]$i –events dynamic recorded in single neurons was not affected. However, at the population level, the high synchronization episodes were no more detected among the neuronal population. These results suggested that the GnRH secretion itself causes the synchronization of $[Ca^{2+}]$i-events. Graph analysis of the relationships between neurons involved in high synchronization events highlighted a „small world" organization and suggested a local paracrine GnRH regulation of $[Ca^{2+}]$i signaling.

O10; P10. Mutations in BMP4 gene network are associated with Kallmann syndrome

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Introduction: Kallmann syndrome (KS) is a genetic disorder characterized by congenital hypogonadotropic hypogonadism (CHH) and anosmia. We hypothesized that bone morphogenic proteins (BMPs) may play a role in KS as: i) *BMP4* is expressed in the olfactory placode and antagonizes fibroblast growth factor 8 (*FGF8*), a powerful morphogen for GnRH neurons; and ii) *BMP4* mutations underlie human retinal dystrophy, polydactyly, cleft lip/palate and renal dysplasia – phenotypes also observed in KS.

Methods: 214 KS were screened for *BMP4* rare sequence variants (RSV), defined as occurring <1% in ExAC and/or 1'000 Genomes. The impact of RSV was predicted using *in silico* algorithms. BMP4 mutants were mapped onto the crystal structure of

BMP4 and functionality was explored *in vitro*. BMP pathway expansion analysis using 150 CHH exomes was performed. ToppGene and Endeavour were used to prioritize the best candidate genes.

Results: Two RSVs in *BMP4* were identified in 214 unrelated KS probands (<1%) The p.R287H mutant maps to the propeptide domain of BMP4, yet exhibits similar activity to WT in BRE-Luc reporter assay. The p.T359I mutant lies in the mature peptide domain and is predicted to be deleterious in structural modeling. It shows decreased maximal activity in the reporter assay ($p<0.05$) with a right-shifted EC50 curve ($p<0.001$). Western blot analysis showed decreased expression of the mature form of T359I ($p<0.05$) consistent with defects in mature peptide cleavage and/or processing. BMP pathway expansion analysis revealed RSVs in several genes within the BMP4 signaling pathway including *BMP6*, *BMPR1* (receptor for BMP4) and *FST* (an antagonist of BMPs), all were predicted to be deleterious *in silico*.
Conclusions: We identified a loss-of-function *BMP4* mutation in KS supporting a role of BMP4 in GnRH neuron ontogeny. We also expanded the BMP network using next generation sequencing and identified several RSVs in KS probands.

O11; P11. The influence of sex hormones vs sex chromosomes on the sexual differentiation of the human brain

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Sex hormones, androgens in particular, are hypothesized to play a key role in the sexual differentiation of the human brain. However, possible direct effects of the sex chromosomes, i.e., XX or XY, have not been well studied in humans. Women with the complete androgen insensitivity syndrome (CAIS), who lack androgen action in the presence of a 46,XY karyotype, offer the unique opportunity to study isolated effects of sex hormones and sex chromosomes on human neural sexual differentiation. Therefore, we compared brain activation and structure in 46,XY women with CAIS to 46,XY men and 46,XX women. We replicated previously reported sex differences in neural activation during a mental rotation task in the control groups, with control men showing more activation in the inferior parietal lobe than control women. Women with CAIS showed a female-like neural activation pattern in the parietal lobe, indicating feminization of the brain in CAIS. At the structural level, using diffusion tensor imaging, widespread sex differences in fractional anisotropy (FA), with higher FA in control men than control women, were observed. Women with CAIS showed female-typical fractional anisotropy throughout extended white matter regions. By contrast, when looking at regional gray matter (GM) volumes, women with CAIS showed female-typical regional GM volumes in the right pre- and postcentral gyrus, arguing against a direct role for genetic sex in this region. However, based on a multivariate analysis of spatially distributed GM volume patterns, the overall pattern of regional GM volume was not female-typical in women with CAIS. These findings indicate that sex

differences in the brain do not solely reflect differences in androgen exposure, but are more likely the result of a combination of different types of sex hormone influences, and possibly also factors directly related to sex chromosome complement, with varying patterns of influence in different brain regions.

O12; P12. Zebrafish as Congenital Hypogonadotropic Hypogonadism (CHH) model system for the study of prokineticin receptor 2 (PROKR2) mutations on GNRH3 neuronal development

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Among the GnRH neurons-related genes an important and not fully understood role is played by the PROKR2, a G protein-coupled receptor. Mutations of PROKR2 in humans can cause CHH and are associated with a reproductive and olfactory phenotypic heterogeneity. The attempt to modelling PROKR2 human allelic variants using mouse model reveals phenotypic differences between these organisms, underlying some limitations of this model. The zebrafish (ZF), with its amenability to genetic manipulation and imaging, has proved to be an ideal model organism for studying the early migration of GnRH neurons and the formation of the GnRH network. However, only few data are available in the literature concerning the prokineticin-receptors in ZF. Our bioinformatics analysis using public databases (Pubmed, ZFIN and ENSEMBLE) and bioinformatics tools (UCSC BLAT alignment, Genomicus) revealed the presence of two well-conserved loci in the ZF genome, on chr1 and chr13, respectively named prokr1a and prokr1b. To better understand their expression during ZF development we performed whole mount *in situ* hybridization (WISH) and qRT-PCR experiments. WISH experiments revealed distinct patterns of expression for prok-receptors:prokr1a is mainly expressed in midbrain-hindbrain boundary at 36 and 48hpf and later in development in liver. The prokr1b presented a strong signal in olfactory placodes and hypothalamic GnRH neurons from 36hpf. The qRT-PCR results underline how prokr1b displayed higher expression level compared to prokr1a but, most interesting, the expression level start to increase at 24hpf until 72hpf, consistently with the migration pattern of GnRH3 fibers described in literature. These preliminary results suggest that prokr1b could be involved in the GnRH neurons migration process from olfactory placodes to their final hypothalamic location. Further analysis of the distinct roles of ZF prokineticin-receptors in physiological and pathological conditions could represent a good biotool to deeper understand the pathogenesis of CHH due to mutations in the PROKR2 gene.

P13. Mutations in HS6ST1 Cause Self-Limited Delayed Puberty in addition to Idiopathic Hypogonadotropic Hypogonadism

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Background: Self-limited delayed puberty (DP) often segregates in an autosomal dominant pattern, but in the majority of patients the neuroendocrine pathophysiology and its genetic regulation remain unclear. By comparison, many genes have been identified where loss-of-function mutations lead to idiopathic hypogonadotropic hypogonadism (IHH). Despite likely overlap between the pathophysiology of DP and conditions of GnRH deficiency, few studies have examined the contribution of mutations in IHH genes to the phenotype of DP.

Methods: We performed whole exome sequencing in 111 members of 18 families from our patient cohort with DP. We filtered the results, seeking potentially pathogenic mutations in genes identified in the published literature as causal in IHH. After follow-up targeted re-sequencing in a further 42 families (288 individuals), one candidate gene was identified. Developmental tissue expression studies, assessment of the enzymatic function of the mutant protein, and investigation of *Hs6st1*$^{-/-}$ mouse embryonal GnRH biology were performed.

Results: A rare variant in *HS6ST1* (Heparan sulfate 6-O sulphotransferase 1) was identified, present in 6 affected members in one family and not present in 145 controls. No other pathogenic variants in IHH genes were identified. *HS6ST1* codes for an enzyme that modifies extracellular matrix components critical for normal neural branching. It is thought to be required for the function of *FGFR1* and *KAL1 in vivo*, both of which are vital for GnRH neuronal development and normal hypothalamic-pituitary-gonadal axis function. Our variant is predicted to lie within a highly conserved coiled-coil domain and displays reduced sulphotransferase activity *in vitro*. *Hs6st1* expression was seen in human and mouse embryos in the nasal placode at e14.5, with apparently normal migration patterns of GnRH neurons in *hs6st1*$^{-/-}$ mouse embryos.

Conclusions: Mutations in *HS6ST1* contribute to the phenotype of both IHH and DP. Thus, it appears that misregulation of GnRH neuronal migration and differentiation may cause both IHH and DP. However, the overlap in the genetic basis for these two conditions appears from our study to date to be limited to a subset of IHH genes.

P14. Cannabinoid Receptor 1 knockdown induces axonal misrouting of the GnRH neurons in zebrafish embryos

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The endocannabinoid system is widely conserved in evolution and comprises several components: natural ligands, also known as endocannabinoids, enzymes and receptors. Among the receptors, Cannabinoid Receptor 1 (CB1) is the most expressed in brain and regulates various step of neuronal development. CB1 is heavily expressed in the hypothalamus, where it can negatively affect the secretion of Gonadotropin Releasing Hormone (GnRH). Previous data showed that in zebrafish embryo, CB1 knockdown causes defects in axonal fasciculation in the anterior commissure, which contains GnRH fibers. To attest whether CB1 can regulate GnRH axonal pathfinding and fasciculation in zebrafish embryos, we performed morpholino-mediated CB1 knockdown on GnRH3::GFP zebrafish embryos. We found that CB1 knockdown reduces the number of GnRH::GFP positive cells in the olfactory epithelium while not changing their position, it reduces the extension of GnRH neuropil, and causes axons misrouting in the anterior commissure. Taken together these results indicate that during early zebrafish development, CB1 acts as a regulator of axonal pathfinding on GnRH cells. Future experiments will elucidate if the CB1 miss-regulation also affects GnRH neuron migration from the olfactory placode to the hypothalamus, with consequent effects on sexual maturation and reproduction.
[Supported by PRIN 2010/2011 to PB, and CRT 2014 to PB and GRM.]

P15. Changes in basal pituitary function with time in adult patients with congenital hypogonadotropic hypogonadism

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Introduction: Functional recovery of the reproductive axis has been described in patients with congenital hypogonadotropic hypogonadism (CHH). However, the development of new pituitary deficiencies in the long-term in adult patients previously diagnosed with CHH has not been reported to date. We report the results of pituitary basal function reassessment in 20 adult CHH patients. Patients and methods: We reviewed 20 patients with CHH, examined their basal pituitary function, testing basal serum IGF-1, TSH, ACTH and cortisol levels, and performed a pituitary MRI in those showing abnormal results in at least one of them.
Results: We found 7 patients (35%) with abnormal hormonal results. All were males, with mean age 37.2 years (29-53) and followed in the adult endocrinology clinic

during an average of 21.2 years (13-37). Markedly low levels of IGF-1 were detected in 6 patients (85%), of TSH in 4 patients (57.4%) -all already treated with levothyroxine- and none of them showed abnormal ACTH or cortisol levels. Pituitary MRI revealed no changes in 2 patients, when compared to their MRIs at diagnosis, one with posterior ectopic pituitary and another with a pituitary of small size (3 mm). One had a partially empty sella (not checked at diagnosis), another 3 that previously had normal MRIs showed new-onset milimetric pituitary lesions (2, 3 and 3 mm, respectively, one of them described as a pars intermedia cyst), and another that was reported to have a 5 mm pituitary lesion in 1996 showed a normal pituitary MRI at the time of the study. Conclusions: Our results suggest that CHH might be the first step towards a combined pituitary hormonal deficiency (CPHD) and that an underlying pituitary injury/disorder (congenital or acquired) that evolves with time might be involved in the etiology of CPHD in these cases.

P16. The role WDR11 in hypogonadotrophic hypogonadism

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Proper development and coordination of the hypothalamic-pituitary-gonadal (HPG) axis is fundamental to fertility of an individual. The key factor that regulates the HPG axis is gonadotrophin releasing hormone (GnRH). Timely release of GnRH is critical for the onset of puberty and subsequent sexual maturation. WDR11 is one of the important factors mediating this process, as its mutations were found in Kallmann Syndrome (KS) and congenital normosmic hypogonadotrophic hypogonadism (CHH), but it is not clear how the mutations of WDR11 causes these diseases. We previously reported that WDR11 is expressed along the GnRH neuronal developmental niche in mouse and zebrafish and interacts with EMX1 transcription factor. To determine the role of WDR11 in HPG axis and the underlying mechanisms, we generated a genetrap knockout mouse model. Our current data indicate that homozygous null (*Wdr1-/-*) mutation is likely to be embryonic lethal with <1% survival rate, while heterozygous (*Wdr11-/+*) mice survive showing a range of phenotypes with varying penetrance. The null mice that do survive exhibit delayed growth and puberty with arrested GnRH neurones in the basal forebrain and hypothalamus at E18.5. Both null and heterozygote embryos show developmental anomalies including skeletal, eye and forebrain defects. Further characterisation of the knockout mouse phenotypes and molecular studies using cell-based assays are in progress. Our studies will confirm the effects of WDR11 deficiency upon the establishment of GnRH neurones and gonadal function *in vivo*, supporting the notion that WDR11 is an underlying genetic cause of human reproductive neuroendocrine disorders.

P17. Disruption of GnRH secretion in peripubertal female rat after early postnatal exposure to Bisphenol A and involvement of GPR151, a potential new GnRH regulator

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We studied the effects of early postnatal exposure to BPA on GnRH release (*ex vivo*) and pubertal timing. Newborn female rats were exposed from PND 1 to 15 to vehicle or BPA at 25 ng/kg/d, a dose consistent with environmental human exposure, or 5 mg/kg/d. After exposure to the low dose of BPA, the GnRH interpulse interval (IPI) was significantly increased on PND 20 (52.5 ±0.8 min vs 44.6 ±0.7 min in controls) and the vaginal opening (VO) tended to be delayed (35.3 ± 0.7 days vs 33.5 ±0.5 days in controls). In contrast, exposure to BPA 5 mg/kg.d resulted in acceleration of GnRH IPI on PND 20 (52.5 ±0.8 min vs 44.6 ±0.7 min in controls) and a trend toward early vaginal opening (32.1 ±0.6 days vs 33.5 ±0.5 days in controls). Gene expression in the retrochiasmatic hypothalamus was assessed by whole exome RNA-sequencing on PND 20. The most significantly affected gene was GPR151, with opposing changes dependent on BPA dose because mRNA levels increased after the low dose of BPA and decreased after the high dose. GPR151 is an orphan GPCR with some homology to galanin and kisspeptin receptors. We observed, by immunohistochemistry, that GPR151 was expressed in the median eminence of pubertal and adult female and male rats where some GnRH nerve terminals were found to co-express GPR151. An RTqPCR study revealed that GPR151 mRNA expression increased throughout development in the retrochiasmatic hypothalamus of female rats. In an attempt to identify specific agonist for GPR151, we performed a screening campaign using a cell-based Firefly Luciferase complementation assay between GPR151 and Arrestin. In conclusion, early postnatal exposure to BPA altered pubertal timing through disruption of GnRH release. This effect could involve changes in expression of a potential new regulator of the GnRH network, GPR151.

P18. Differential regulation of LH and FSH release by GnRH in a fish model

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Zebrafish hold a substantial promise as a model for studying the hypothalamic control of gonadotropin release since they conserve all the major functions and components of the hypothalamic–pituitary axis of higher vertebrates while possessing two unique features: LH and FSH are expressed in distinct subsets of cells and hypothalamic fibers project directly into the pituitary. These traits enable the investigation of the differential regulation of the two gonadotropins by functional imaging of the GnRH terminals and the target cells simultaneously at the same location.
The longstanding perception is that in fish the neuroendocrine control of pituitary cells is achieved via direct communication between neuroendocrine axons and their endocrine cell targets and not through the portal system as in tetrapods. We found that the permeable blood vessels entering the pituitary are surrounded by dense GnRH terminals, implicating the possible uptake of GnRH peptides. We also found distinct differences in the proximity of the two gonadotrope types to the vessels as well as to the GnRH terminals. The two gonadotrope types also differed in their level of anatomical and functional coupling. These findings have important implications regarding the differential regulation of LH and FSH and contradict the traditional view of the teleost pituitary (Golan et al, Endocrinology, 2015).
For the next stage we are using transgenic fish that express genetically-encoded calcium indicators (GCaMP6, RCaMP2) and optogenes (ChR2) and employing advanced live-imaging techniques to functionally investigate in-vivo the intricate interplay between GnRH and gonadotropes in the teleost pituitary. Our aim is to reveal how hypothalamic signals are interpreted by the gonadotropes to differentially activate LH or FSH cells. The study is expected to provide new insights into the evolution and function of gonadotropin regulation in vertebrates and yield new approaches to control and manipulate the reproductive axis of commercially important fish species.

P19. Involvement of endocannabinoids in the mediation of the effect of 17βestradiol suppressing fast neurotransmission onto GnRH neurons

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Gonadotropin-releasing hormone (GnRH) neurons play a key role in the regulation of reproduction. *In vivo*, 17αestradiol (E2) controls GnRH release in concentration and estrus cycle dependent manner. *In vitro* patch-clamp electrophysiological studies on GnRH neurons of ovariectomized, female mice showed that low physiological concentration of E2 decreased spontaneous firing rate which was eliminated by blocking fast synaptic neurotransmission. In the present study, we examined the effect of low concentration of E2 on postsynaptic currents (PSCs) in GnRH neurons of acute brain slices obtained from metestrous female mice and analyzed the putative involvement of endocannabinoid signaling in evoked E2 effect. Whole-cell

patch clamp recordings revealed that 10 pM E2 significantly diminished frequency of the PSCs, which could be abolished by the application of ERα/αblocker Faslodex. Cannabinoid receptor type-1 inverse agonist AM251 (1 μM) and the intracellularly applied endocannabinoid synthesis blocker THL (10 μM) significantly attenuated the effect of E2 on the PSCs. E2 remained effective in the presence of TTX indicating direct action of E2 in GnRH cells. The ERαspecific agonist DPN (10 pM) significantly decreased the frequency of the mPSCs. The effect of E2 was completely blocked by the selective ERαantagonist PHTPP (1 μM). In contrast, the ERαagonist PPT (10 pM) or the membrane associated G-protein coupled estrogen receptor (GPR30) agonist G1 had no significant effect on the frequency of mPSCs in these neurons. These results indicate that ERαis required for the observed rapid effect of the E2 on GnRH neurons. AM251 significantly abolished the action of E2 and the DPN on the mPSCs. Thus, involvement of the retrograde endocannabinoid mechanism has been revealed in this effect. These results indicate that an interaction exists between estradiol and endocannabinoid signaling, which represents a novel regulatory machinery in the execution of the negative estrogen feedback to GnRH neurons.

P20. T cell dependent B cell immune response induces delayed ERK1/2 phosphorylation via IL-10 in GnRH neurons in vivo

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In our study we investigated the impact of antigen-specific humoral immune challanges upon the hypothalamus-pituitary-gonad (HPG) axis. First, we compared the effect of T cell dependent and T cell independent humoral immune responses on the HPG axis by immunizig mice with FITC-KLH or FITC-dextran. We found that the estrous cycle became irregular in FITC-KLH immunized female mice while it remained unaltered in FITC-dextran immunized mice. The length of the estrous cycle became shorter and the immunized animals spent less time in the estrous phase after activation of T cell dependent immune response. Our results suggest that the shift between the estrous phases is more likely to be central than peripheral. Therefore we explored the possible central mechanism for the FITC-KLH caused estrous cycle disturbance. We showed that the FITC-KLH induced T cell dependent humoral immune response results in an antigen-specific, time-dependent, gonadotropin-releasing hormone (GnRH) neuron-specific delayed ERK1/2 phosphorylation. Using IL-10 knock-out mice we gave evidence that the anti-inflammatory cytokine IL-10 mediates the FITC-KLH-induced ERK1/2 phosphorylation. Using single cell electrophysiology study on acute brain slice on GnRH-GFP mice we demonstrated that IL-10 acts directly and indirectly on GnRH neurons. In summary, we report here that FITC-KLH induced T cell dependent humoral immune response results in time-dependent, GnRH neuron-specific, IL-10-mediated ERK1/2 phosphorylation which might lead to disordered estrous cyclicity.

P21. NO as a "volume transmitter" of neuroendocrine signals in the hypothalamic area

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Nitric oxide (NO) and its downstream signaling cascades are critical to various cellular functions in the brain, including the neuroendocrine control of reproduction. NO is produced by neuronal- NOS (nNOS) expressing neurons found in the vicinity of GnRH containing perikarya in the preoptic region of the hypothalamus (POA), and has thus been implicated in the regulation of GnRH activity (Bellefontaine et al., 2011; Clasadonte et al., 2008). Additionally, the mapping of leptin responsive cells in the hypothalamus has revealed that many LepR neurons are present in the POA, while some of them are also NO-synthesizing neurons. Recent studies from our lab have demonstrated that leptin is able to induce an acute increase in P-nNOS expression in the OVLT and MEPO hypothalamic areas, which coincide with a rise in LH levels (Bellefontaine et al., submitted). With the aim to clarify the leptin- NO crosstalk dynamics in the POA we applied our results to a mathematical model describing the active nNOS neurons of the POA as a 3-dimensional array of NO-emitting spheres. Here we propose that leptin can induce the activation of nNOS neurons, resulting to the "transformation" of NO into a "volume transmitter" able to regulate GnRH activity and hence induce the LH release. Since the biological actions of NO depend critically on its concentration, which is difficult to measure, we use a novel cGMP biosensor in combination with an ultrasensitive detector cell line in order to quantify the active concentration of NO being released in a mouse hypothalamic slice using live imaging techniques, under physiological conditions. Our results suggest that nNOS neurons within and in direct proximity to the OVLT, a site devoid of the blood brain barrier and to which GnRH neurons extend dendrites, may acutely sense changes in leptin levels and rapidly relay this information to GnRH neurons, which in turn stimulate LH release.

P22. Estradiol exerts powerful impact on the hippocampal transcriptome in middle-aged, ovariectomized female rats: implications for neurogenesis, synaptic plasticity and neuroprotection

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The hippocampus plays a pivotal role in learning and memory. It is well-known that gonadal hormones including 17αestradiol (E2) are powerful modulators of synaptic plasticity and hippocampus-dependent cognitive functions. However, the effects of E2 on the hippocampal transcriptome are mainly unexplored and the significance of genomic effects has remained elusive. In this study, we examined the regulatory role of E2 on hippocampal gene expression in a rat menopausal model. Middle-aged, ovariectomized rats were treated continuously either with E2 or vehicle for 29 days. Following dissection of the hippocampus, isolation of total RNA and quality control, we performed expression profiling studies using oligonucleotide microarray and real-time PCR. Microarray data were analyzed by Bioconductor packages and web-based software. Using a standard fold change selection criterion (FC>1.5), we identified 156 E2 responsive genes. All alterations but four were transcriptional activation. Highly responsive (FC>10) E2 target genes included transthyretin, klotho, claudin 2, prolactin receptor, Sostdc1, F5, insulin-like growth factor 2 (Igf2), Igfbp2 and Na^+/$SO4^{2-}$ symporter. Classification of the 156 E2 responsive genes revealed signaling, metabolism, extracellular matrix (ECM) and transcription as major functional groups. We confirmed differential expression of all selected genes by real-time PCR. The findings suggest that E2 increases retinoic acid and IGF2 synthesis, promotes growth factor, retinoid, neurohormone and neurotransmitter signaling, alters metabolic processes, ECM composition, transcriptional regulation, and induces protective mechanisms via genomic effects. Based on these results we propose that the hippocampal transcriptome is highly sensitive to serum E2 levels and through the putative downstream mechanisms listed above E2 promotes neurogenesis, enhances neural plasticity and improves cognitive functions in the hippocampus of aging females.

This work was supported by the Hungarian Scientific Research Fund (OTKA K100722, OTKA K115984).

P23. Testosterone-induced changes in neuropeptide gene expression of the hypothalamic arcuate nucleus in orchidectomized male mice

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Sex steroid regulation of peptidergic neurotransmission in the hypothalamic arcuate nucleus (ARC) plays a critical role in negative sex steroid feedback to the reproductive axis and in the pulsatile secretion of gonadotropin-releasing hormone (GnRH) and luteinizing hormone. Here we tested the effect of testosterone replacement to orchidectomized male mice on the neuropeptide transcriptome of the ARC.

RNA samples of the ARC were isolated with laser-capture microdissection from 60-day-old (young) and 8-10-month-old (middle-aged) adult orchidectomized and orchidectomized plus testosterone-treated mice. The expression of 18 neuropeptide, 15 neuropeptide receptor, 4 sex steroid receptor and 6 classic neurotransmitter marker mRNAs were quantified with real-time PCR and the effects of i) age and ii) testosterone-treatment assessed.

Testosterone decreased kisspeptin, galanin-like peptide, cocaine- and amphetamine regulated transcript, calcitonin gene-related peptide, somatostatin, proopiomelanocortin, proenkephalin and prokineticin expression and increased galanin expression. From the neuropeptide receptors tested, neurokinin 3 receptor, neurotensin receptor-1 and αopioid receptor mRNA was down-regulated, whereas the expression of melanocortin receptor-4 was up-regulated by testosterone. Testosterone-treated mice showed decreased androgen and estrogen receptor alpha and increased progesterone receptor expression. From the tested classic neurotransmitter markers, tyrosin-hydroxylase and glutamate decarboxylase-1 mRNA level decreased, whereas choline acetyltransferase expression increased in response to testosterone.

Age exerted relatively modest effects which were often hormone-dependent. The mRNAs encoding kisspeptin, dynorphin, agouti related protein, cocaine- and amphetamine regulated transcript, neurotensin, somatostatin, prokineticin, neurokinin 3 receptor, nociceptin/orphanin FQ receptor, estrogen receptor alpha, androgen receptor, melanocortin receptor-3 and the Y1 and Y5 neuropeptide Y receptors decreased, whereas growth hormone-releasing hormone and choline acetyl transferase mRNAs increased with aging.

Testosterone- and aging-induced changes in the neuropeptide transcriptome of the ARC will contribute to our understanding of the mechanism(s) which shape the pulsatile pattern of GnRH secretion and account for negative sex steroid feedback to the reproductive axis.

P24. Morphological evidence supporting retrograde endocannabinoid signalling between GnRH neurons and their kisspeptin afferents in mice

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Kisspeptin (KP), produced by discrete neuronal populations of the forebrain, is an essential regulator of gonadotropin-releasing hormone (GnRH) neurons. KP-producing neurons also co- synthesize GABA or glutamate (Cravo, et al.,

Neuroscience, 2011). Our laboratory has previously shown that GnRH neurons are capable of modulating their GABAergic input via endocannabinoid release (Farkas, et al., Endocrinology, 2010). The aim of this study was to determine whether KP afferents of GnRH neurons are targets of endocannabinoids and immunoreactive for CB1.
By using immunohistochemical triple labelling, we have identified KP-immunoreactive (IR) axon varicosities in apposition to GnRH neurons IR either for GAD65, or VGLUT2. To precisely determine the presumed cellular localization of CB1 immunoreactivity, the membrane contour of KP or GABAergic neurons were labelled with YFP in Kiss1-CRE-GFP or GAD2-CRE-GFP mice after its gene transfer by AAVs. Confocal microscopic analysis and 3D reconstruction of the image of immunolabelled structures allowed to visualize the whole cellular compartment of the GABAergic and KP-IR fibres and enabled intracellular localisation of CB1-IR sites. 52±1.56% of KP-IR fibres in apposition to GnRH perikarya and 47± 2.72% of KP-IR fibres in apposition to GnRH processes were found to be also IR for VGLUT2. 31±0.70% of KP-IR fibres in apposition to GnRH perikarya and 40± 2.38% of KP-IR fibres in apposition to GnRH processes were found to be also labelled for GAD65. 70±0.98% of KP-IR fibres in apposition to GnRH perikarya and 59±3.53% of KP-IR fibres in apposition to GnRH processes were found to be IR for CB1. These results indicate, that KP afferents of GnRH neurons have a mixed GABAergic and glutamatergic phenotype. They also suggest that GnRH neurons are capable of influencing their input from KP neurons via the endocannabinoid/CB1 retrograde signalling. However, the classical neurotransmitter content of the endocannabinoid-sensitive KP afferents needs to be determined.
Supported by the National Science Foundation of Hungary (K101326, K115984).

P25. Identification of differentially expressed genes encoding neurotransmitter receptors of GnRH neurons in proestrus

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Gonadotropin-releasing hormone (GnRH) neurons play an essential role in the central regulation of reproduction. In females, estradiol triggers the pre-ovulatory GnRH surge in proestrus, however, its impact on the gene expression profile of GnRH neurons has not been fully elucidated. We hypothesized that proestrus is accompanied by significant changes in the expression of neurotransmitter receptor genes in GnRH neurons. Therefore, we compared the transcriptome of GnRH neurons obtained from intact, metestrous and proestrous female GnRH-GFP transgenic mice. About 1500 individual GnRH neurons from both groups were sampled with laser capture microdissection followed by whole transcriptome amplification for microarray analysis as published recently (Vastagh et al, Neuroendocrinology, 102, 44-59, 2015).

For validation of the microarray results, quantitative RT-PCR technique was used. Differential gene expression was most apparent in receptor subunits and downstream elements of the GABA-ergic (*Gabbr1*, *Gabra3*, *Gabrb3*, *Gabrg2*), glutamatergic (*Gria1*, *Grin1*, *Slc17a6*) and cholinergic (*Chrnb2*) signaling pathways. Analysis of microarray data has revealed altered gene expression level of adrenergic (*Adra2c*) adenosine (*Adora2a*), dopaminergic (*Drd4*) and serotonergic (*Htr6*) receptors. Changes in the expression of genes involved in neurotransmitter signaling of proestrous and metestrous female GnRH neurons indicate the differential involvement of these neurotransmitter systems in the induction of the pre-ovulatory GnRH surge, the known prerequisite of the subsequent ovulation.

This work was supported by the Hungarian Scientific Research Fund (OTKA K100722, OTKA K115984).

P26. Glucagon-like peptide-1 excites firing and increases GABAergic miniature postsynaptic currents in gonadotropin-releasing hormone (GnRH) neurons of the male mice via activation of nitric oxide (NO) and suppression of endocannabinoid (EC) signaling pathways

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Glucagon-like peptide-1 (GLP-1), a metabolic signal molecule, regulates reproduction, although, the involved molecular mechanisms have not been elucidated, yet. Therefore, responsiveness of gonadotropin-releasing hormone (GnRH) neurons to the GLP-1 analog Exendin-4 and elucidation of molecular pathways acting downstream to GLP-1 receptor (GLP-1R) have been challenged. Loose patch-clamp recordings revealed that Exendin-4 (100nM–1μM) elevated firing rate in hypothalamic GnRH-GFP neurons of male mice via activation of GLP-1R. Whole-cell patch-clamp measurements demonstrated increased excitatory GABAergic mPSC frequency after Exendin-4 administration which was eliminated by GLP-1R antagonist Exendin-3(9-39) (1μM). Intracellular application of G-protein inhibitor GDP-βS (2mM) impeded action of Exendin-4 on mPSCs, suggesting direct excitatory action of GLP-1 on GnRH neurons. Blockade of nitric-oxide (NO) synthesis by L-NAME (100μM) or NPLA (1μM) or intracellular scavenging of NO by CPTIO (1mM) attenuated excitatory effect of Exendin-4 partially. Similar partial inhibition was achieved by hindering endocannabinoid pathway using CB1 inverse-agonist AM251 (1μM). Simultaneous blockade of NO and endocannabinoid signaling mechanisms eliminated action of Exendin-4 suggesting involvement of both retrograde machineries. Intracellular

application of TRPV1-antagonist AMG9810 (10μM) or FAAH-inhibitor PF3845 (5μM) impeded the GLP-1-triggered endocannabinoid pathway indicating an anandamide-TRPV1-sensitive control of 2-AG production. Furthermore, GLP-1 immunoreactive axons innervated both rodent and human GnRH neurons in the hypothalamus. RT-qPCR study confirmed the expression of GLP-1R and nNOS mRNAs in mouse GnRH neurons. These results indicate that gut- and/or brain-born GLP-1 reaches GnRH neurons, exerts facilitatory actions via GLP-1R and modulates NO and 2-AG retrograde signaling mechanisms that control the presynaptic excitatory GABAergic inputs to GnRH neurons.

This research was supported by the National Science Foundation of Hungary (OTKA K115984).

P27. Illness perceptions and quality of life in women with congenital hypogonadotropic hypogonadism (CHH)

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Background: CHH is a rare genetic disorder characterized by the lack of pubertal development and infertility. Given the striking gender discordance in CHH (3-4M:1F), women with CHH are the "rarest of the rare". This study aimed to better understand the healthcare experiences and unmet needs of women with CHH to develop more patient-centered approaches to care.

Methods: A community-based participatory research approach was used to engage patient community leaders and develop an online survey to reach CHH patients. Information on demographics, medical history, healthcare interactions and health literacy were collected and patients completed several validated questionnaires including the Illness Perception Questionnaire-Revised, Morisky Medication Adherence Scale, and the Zung Self-Rating Depression Scale. Quantitative data were analyzed using descriptive statistics and correlation analysis was performed. Qualitative responses were analyzing using thematic analysis.

Results: The 36 women with CHH had high levels of healthcare literacy and were internet power-users: 34/36 (94.4%) reported seeking CHH information on the web and 32/36 (88.9%) sought information from an online patient community. Overall, 10/36 (28%) had received fertility inducing treatment and 90% successfully conceived. However, they had poor medication adherence - only 11% had high adherence. These women report negative illness perceptions and suffer significant physical, psychological and social consequences as a result of CHH. They also have significantly increased rates of mild (11/36, 31%), moderate (6/36, 17%) and severe (6/36, 17%) depressive symptoms.

Conclusions: Women with CHH have negative illness perceptions and depressive symptoms similar to their male counterparts. Compared to males, women with CHH are

even less adherent to long-term hormonal therapy yet are more likely to have biologic children. These data underscore the importance of examining the psychological and emotional impact of living with a rare chronic condition such as CHH.

P28. Xenoestrogens Ethinyl Estradiol and Zearalenone Cause Precocious Puberty in Female Rats via Central Kisspeptin Signaling

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Xenoestrogens from synthetic or natural origin represent an increasing risk of disrupted endocrine functions including the physiological activity of the hypothalamo-pituitary-gonad axis. Ethinyl estradiol (EE2) is a synthetic estrogen 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-αand accelerate puberty. However, the neuroendocrine mechanisms that are responsible for this effect remain unknown. Immature female Wistar rats were treated with EE2 (10 µg/kg), ZEA (5 and 10 mg/kg), or vehicle for 10 days starting from postnatal day 18. On the other hand animals were decapitated at the vaginal opening. As a marker of puberty, the vaginal opening was recorded and neuropeptide and related transcription factor mRNA levels were measured by quantitative real time PCR and in situ hybridization histochemistry. The hypothalamic mRNA expressions were analyzed by NGS. Both ZEA and EE2 accelerated the vaginal opening, increased the uterine weight and the number of antral follicles in the ovary, and resulted in the increased central expression of gnrh. These changes occurred in parallel with an earlier increase of kiss1 mRNA in the anteroventral and rostral periventricular hypothalamus and an increased kisspeptin (KP) fiber density and KP-GnRH appositions in the preoptic area. These changes are compatible with a mechanism in which xenoestrogens overstimulate the developmentally unprepared reproductive system, which results in an advanced vaginal opening and an enlargement of the uterus at the periphery. Within the hypothalamus, ZEA and EE2 directly activate anteroventral and periventricular KP neurons to stimulate GnRH mRNA. However, GnRH and gonadotropin release and ovulation are disrupted due to xenoestrogen-mediated inhibitory KP signaling in the arcuate nucleus. On the other hand the results of the NGS were highlighted the new puberty related pathways.

P29. Familial Hypogonadotropic Hypogonadism: A Portuguese series

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Congenital isolated Hypogonadotropic Hypogonadism (IHH) is characterized by a partial or complete lack of pubertal development, secondary to deficient GnRH-induced gonadotropin secretion, in the absence of anatomical abnormalities in the hypothalamic and pituitary region, and normal baseline and reserve testing of the remaining pituitary hormones
We present a Portuguese series with 25 cases and 13 family members. Seventeen cases presented isolated HH while 1 had stigmata of the Charge syndrome. Seven cases had Pan-hypopituitarism. Of the cases with isolated deficiency of gonadotropins (IHH), 7 were cases of Kallmann Syndrome with anosmia or at least a clear situation of hyposmia and 10 were cases of congenital hypogonadotropic hypogonadism without afection of the olfatory structues. Six of these cases were already submitted to whole exome sequencing (NGS) and in 3 of these genetic alterations in the known loci of HH syndromes were already demonstrated. This series will next be used to cross the clinical information with the diagnosis and treatment use in Portugal. Finally, we identified a possible case of Charge syndrome that will need extended analysis both clinically and genetically.

P30. Transgenic zebrafish models to study the neurokinin B neuronal system

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Mutations in Tac3 and TacR3, encoding neurokinin B (NKB) and its receptor are the cause of normosmic idiopathic hypogonadotropic hypogonadism with complete reversal of the hypogonadotropism in adult life. These observations demonstrate the importance of the NKB pathway in the control of sexual maturation and reproduction. It has been suggested that NKB affects GnRH activity, or other factors along the hypothalamic-pituitary-gonadal axis, at early developmental stages. Nevertheless, the precise role of NKB and its receptor, and the mechanism underlying the reversible effect of their mutations are currently unknown.
NKB was recently identified as a key regulator of reproduction also in fish, which were found to possess a specific novel neurokinin termed NKF. Both NKB and BKF increase plasma levels of gonadotropins. Moreover, we have shown that pituitary

gonadotrophic cells express Tac3, and TacR3 suggesting that paracrine NKB signaling may regulate gonadotropin release in fish.
The zebrafish offers a great advantage as a genetically amenable model. The availability of genetic and molecular tools and techniques and live imaging capabilities, enable studies that are almost impossible in other vertebrate models.
We generated a transgenic zebrafish line that expresses green fluorescent protein (EGFP) specificaly under the control of the zebrafish Tac3 promoter. This transgenic line, Tg(tac3:EGFP), expresses EGFP specificaly in NKB-expressing neurons and their projection. This transgenic line, together with the other reproductive-related transgenic and mutant lines that we have previously generated [Tg(gnrh3:tdTomato), Tg(lh:mCherry;fsh:EGFP) and KO lines for GnRH and Kiss] will enable to study the interaction of the NKB system with other hypothalamic systems that regulate reproduction throughout embryonic and sexual development.

P31. NO sex without kiss: lordosis behavior depends on nitric oxide (NO) signaling downstream of kisspeptin neurons

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Sexual behavior ensures the perpetuation of a species. Lordosis in response to male mounting is the most prominent reproductive behavior in female rodents, yet its underlying neural circuits are not well understood. We have recently demonstrated that kisspeptin neurons, already well known to play a crucial role in reproduction and implicated in controlling puberty onset and ovulation by regulating gonadotropin-releasing hormone (GnRH) neurons, are activated upon mating (lordosis) in mice. We further showed that lordosis is impaired in kisspeptin knockout mice but is rescued by central or peripheral kisspeptin administration. Interestingly, acute ablation of kisspeptin neurons in the anteroventral periventricular area (AVPV) in adult females impaired lordosis, whereas optogenetic activation of these cells triggered the behavior, suggesting a prominent role of kisspeptin in the control of female sexual behavior. In order to identify candidate neurons downstream of AVPV kisspeptin neurons we expressed the transsynaptic tracer barley lectin (BL) exclusively in these cells to label synaptically connected cells (KissIC/R26-BlZ). We found BL+ neurons expressing nNOS in the paraventricular nucleus of the hypothalamus (PVN), demonstrating that kisspeptin neurons communicate with subsets of nNOS neurons in the PVN. To delineate the role of nitric oxide signaling in this neural circuit, we next analyzed nNOS phosphorylation in the PVN following lordosis. We found that nNOS was robustly

activated in the PVN following sexual behavior. We then analyzed mice deficient in nNOS. We found that nNOS-KO females showed a strong decrease in lordosis behavior compared to control littermates. Taken together, these data demonstrate that hypothalamic NO signaling is an essential mechanism downstream of kisspeptin neurons in a novel neural circuit governing lordosis behavior in female mice. Future studies will be conducted to identify the neural targets of NO signaling within the PVN and possible outputs to other brain areas important in lordosis behavior.

P32. Development of gonadotropin-releasing hormone-secreting neurons from human pluripotent stem cells

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Human pluripotent stem cells (HPSCs) have the capacity to give rise to almost all cell types of the human body, and therefore provide a tool for studying the development of cells and tissues *in vitro*. We have developed a protocol for differentiation of GnRH -expressing and -secreting cells from HPSCs. In this protocol, HPSC's were directed towards neuroectodermal lineage using dual SMAD inhibition, leading to differentiation of progenitor cells expressing anterior neuron progenitor markers *PAX6*, *EMX2*, and *FOXG1*. These cells were further treated with FGF8, and Notch inhibitor DAPT, producing TuJ1-expressing neurons that robustly expressed GnRH (15% of counted cells) and secreted GnRH decapeptide into the culture medium. These results have been repeated in three individual hPSC lines. Moreover, to facilitate the investigation of all stages of GnRH neuron differentiation, we have generated a hPSC cell line carrying a GnRH-reporter (GnRH-TdTomato) using the CRISPR-Cas9 gene editing technology. To generate the GnRH-reporter, the last exon at close proximity to the stop codon of *GNRH1* was targeted by the Cas9 endonuclease and two distinct guide oligomers. The homologous arms, which target the donor template into proper genomic location, were PCR cloned from the genomic DNA of the selected hPSCs. The donor template contains the TdTomato fluorescent protein, together with a nuclear localization signal, allowing us to identify the GnRH expressing cells with TdTomato positive nuclei, leaving the translated GnRH peptide unaffected for further processing and secretion. Several clones have been established and the TdTomato-expressing clones have been confirmed using the above mentioned differentiation protocol. Further characterization of the cell line is ongoing. This cell line will provide a useful tool for studying several aspects of GnRH neuron biology.

P33. Reversal of Congenital Hypogonadotropic Hypogonadism in a man with Kallmann Syndrome and deafness caused by a SOX10 mutation

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Key words: Waardenburg syndrome, deafness, congenital hypogonadotropic hypogonadism.

A 21-aged man was addressed for pubertal retardation. At clinical interview, it emerged a left-prevalent hear impairment and anosmia. Clinical examination revealed micropenis (3 cm stretched), low testicular volume (TV, 2 mL), a right-sided ptosis and a left iris hypopigmentation. Serum testosterone (T) was 0.2 ng/mL, with low gonadotropins (FSH 0.4 and LH 0.25 IU/L) not increasing after GnRH stimulation test. Other anterior pituitary function was normal. Hormonal treatment by testosterone enanthate was started to induce puberty (250 mg/3 weeks, i.m.). A penis enlargement (9 cm) with no TV changes were observed one year after treatment introduction. Semen fluid analysis showed azoospermia in two different samples (at the age of 24 and 25). The patient was then lost at follow-up.
At 33 he consulted again after having fathered spontaneously without previous gonadotropin treatment. At this time the mean TV was 16 mL. Seminal analysis revealed oligospermia (6.6 million sperm/mL). Circulating T and gonadotropins were found normal two months after T withdrawal. The patient finally re-consulted at the age of 40: TV was 20 mL with normal circulating hormones and normal LH pulsatility. Seminal fluid analysis showed normospermia (62 million sperm/mL). Petrosal CT Scan identified semicircular canals hypoplasia. Genetic analysis detected the p.Met108Thr inactivating *SOX10* mutation.
To our knowledge this is the first case of reversible Kallmann Syndrome associated with a *SOX10* mutation.

P34. A Novel Role for Anti-Müllerian Hormone in the Development of the GnRH System

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Gonadotropin-releasing hormone (GnRH) neurons originate in the nasal placode and must migrate during embryogenesis to reach the basal forebrain, where in postnatal life they function as the master regulators of reproductive function. Abnormalities identified in factors important in regulating this migration results in pathologies such as Kallmans Syndrome (KS) or normosmic congenital hypogonadic hypogonadism (nCHH).
Recent data from our lab have shown that the AMH receptor (AMHR2) is expressed by GnRH neurons both during development and postnatal life; whilst AMH is expressed in the olfactory epithelium and along the olfactory fibers along which GnRH neurons migrate. Further, *Amhr2*$^{-/-}$ mice display a 30% reduction in the total number of GnRH neurons located in the brain, coupled with an altered spatial distribution. Taken together these data suggest that AMH may be important in regulating prenatal GnRH cells including facilitating their correct migration. This hypothesis is further supported by the genetic screening of a large cohort of CHH patients identifying several putative mutations in AMH.
In order to functionally validate these mutations we first confirmed expression of AMHR2 and its signaling pathway at a transcript and protein level in primary and immortalized GnRH cells (Gn11). Using simple motility assays we further identified AMH as a potent pro-motility factor regulating GnRH cell motility ($p<0.01$) and using targeted gene silencing (siRNA) transfections determined that this action is specifically mediated through AMHR2 and BMPR1B (bone morphogenic protein receptor 1B). Taken together these results provide novel evidence that AMH acts as a putative regulator of this crucial developmental process that may be altered in several known human infertility pathologies.

P35. Developing zebrafish models to study disease genes and microRNA and their role in olfactory and GnRH development

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Developing zebrafish embryos with transgenic fluorescent reporters are becoming excellent tools to examine the impact of disease genes, microRNA (miR) and environmental factors on conserved processes, such as the maturation and connectivity of olfactory receptor neurons (ORN) and GnRH genesis, migration and maturation. By combining two olfactory reporters (OMP-cyan and Trcp2-yellow) with

a GnRH reporter (GnRH3-GFP) we are exploiting the system to examine early steps of olfactory/GnRH development.
In a first effort, we have used these fluorescent reporter zebrafish embryos to examine a number of coding genes that have emerged from: a) transcription profiling; b) bioinformatic analyses; c) mutation screen and/or phenotypes in mice. The knockdown of known Kallmann-causing genes (*fgfr1* and *kal1*) confirms the validity of the approach. The results obtained with the knockdown of *lrnn1*, *lingo2* and *tshz1* in the zebrafish will be presented.
In a second effort we aimed to identify miRs involved in olfactory/GnRH development. A general role of miRs in the differentiation of ORNs is known from the conditional deletion of *Dicer* in the mouse. Starting from targets of the homeogene *Dlx5,* essential for ORN differentiation and axon connectivity and for GnRH maturation, we have focused on *miR-9* and *miRs* of the -200 class, and observed delayed ORN differentiation, altered axonal trajectory/targeting, and altered genesis and position of olfactory-associated GnRH neurons. *miR-9* and *miR-200*-class negatively control *Foxg1* mRNA, a fork-head transcription factor essential for development of the olfactory epithelium and of the forebrain, known to maintain progenitors in a stem state. This work uncovers a miR-Foxg1 regulation whose alteration affects ORN differentiation, axonal targeting and GnRH neuron development, the hallmarks of the Kallmann syndrome.
To further expand this research, we will extend it to the hypothalamic neuronal network controlling the activity of GnRH neurons, using Kiss-red reporter lines. The combined Kiss-GnRH reporter system should open the way to studies on genetic and environmental factors impacting on the hypothalamic control of puberty and fertility.

P36. From trascriptomic analysis to in vivo functional experiments: zebrafish as a model for studying GnRH system

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GnRH neurons are a small group of hypothalamic neurons that controls reproductive functions and fertility in mammals. During embryonic life, GnRH neurons arise from the olfacory placode and migrate towards the hypothalamus throughout the nasal mesenchyme and the forebrain. Once they have established in their final location, they extend their axons to the median eminence where GnRH is secreted. Defects in the development of the this neuroendocrine system could lead to rare genetic disorders such as Kallmann Syndrome (KS) and Hypogonadotropic Hypogonadism (HH). To date, the genetic basis of such syndromes is largely still unknown and only few mutated genes have been identified. The study of GnRH neuron develpoment is difficult because GnRH neurons are small in numbers and scattered along the

migratory route, making complex their isolation. Here, we have taken advantage from a transgenic rat carrying GFP-labelled GnRH neurons and FACS to isolate GnRH neurons and perform a gene expression analysis to generate a trascriptome profile of GnRH neurons. Specifically, GnRH neurons at three different embryonic stages that represent the beginning, the transition from nose to forebrain and the cease of migration have been isolated. GFP-positive cells have been separated from GFP-negative cells by FACS and gene expression analysis performed using the Affymetrix GeneChip. All arrays have been normalized and data filtered by removing the probes with the smallest variances across the arrays. We have classified the differentially expressed genes into gene ontology pathways by using bioinformatic tools and selected a small number of genes associated to migration and neurite extension pathways. To study the functional relevance of these novel genes we first performed *in situ* hybridization experiments in zebrafish embryos to study their expression pattern during GnRH neuron development and started gene knock-down experiments with morfolinos.

P37. The effect of hypothyroidism on the HPG axis and seasonal neuroplasticity in European Starlings (Sturnus vulgaris)

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One of the most dramatic examples of adult neuroplasticity is the seasonal plasticity that occurs in the song control system (SCS) of songbirds. The primary factor inducing this neuroplasticity is the photoperiodic induced increase in testosterone. However, it has been shown that steroid-independent photostimulation can also induce SCS plasticity. One of the proposed alternatives is the mediating effect of thyroid hormones (TH), as THs play an important role in the regulation of seasonal reproduction and are associated with neurogenesis.

We investigated whether THs are required to induce SCS plasticity and whether TH could affect the seasonal neuroplasticity (1) directly at the level of the SCS or (2) indirectly via the HPG axis.

Photorefractory male starlings (n=30) were held on long days (LD: 16L:8D) and were divided into a hypothyroid (n=16) and a control group (n=14). Hypothyroidism was induced by adding 0.05% methimazole to the drinking water upon switching from LD to short days (SD: 8L:16D). All birds were kept on SD for 12 weeks to induce photosensitivity, after which they were photostimulated by returning to LD. All birds underwent repeated measures *in-vivo* MRI to follow up neuroplasticity at 6 time points: at the end of the photorefractory state and during the photosensitive and photostimulated phase. Each time blood samples were taken to determine plasma levels of T3, T4 and testosterone.

Methimazole treatment was successful in reducing T3 and T4, in contrast to controls that showed significant changes in TH levels between photoperiods. Preliminary analysis of the MRI data shows that both control and hypothyroid group show similar

structural changes, except for the HVC-RA tract that significantly changes in control, but not in hypothyroid starlings. Interestingly, hypothyroidism inhibited an increase in testosterone levels upon photostimulation, indicating that TH could affect the seasonal neuroplasticity in an indirect manner via the HPG axis.

P38. GnRH-positive neurons derived from human Embryonic Stem Cells (hESCs) and induced Pluripotent Stem Cells (iPSCs) of healthy individuals and patients with Kallmann Syndrome

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Little is known about the molecular ontogeny and regulation of GnRH neurons. Their anatomical localisation and small numbers (about 1000) make experimental studies extremely difficult. Immortalised GnRH-releasing cell lines have provided some functional insights but these tumour-derived cells do not represent an accurate *in vitro* model.

hESCs and iPSCs-derived neurons have been successfully used to study mechanism of neurogenesis and to model several diseases. hESC-derived GnRH neurons could provide an excellent tool for *in vitro* studies and regenerative medicine. At present however a reliable protocol for producing GnRH neurons from hESCs/iPSCs is lacking. We have created GnRH-expressing neurons from hESCs and iPSCs.

iPSCs were generated by reprogramming dermal fibroblasts from patients with Kallmann Syndrome and unaffected family members. iPSCs lines were karyotypically normal and displayed pluripotency features, including self-renewal, expression of pluripotency markers, and ability to differentiate into cells of all three germ layers.

GnRH-expressing neurons were produced by generating Neural Progenitor Cells (NPCs) through prolonged BMP inhibition over several passages in a neurogenic medium. NPCs expressed early neuronal markers such as PAX6 and Nestin and could be passaged and expanded. Terminal differentiation was achieved by incubating the cells in basal neuronal media supplemented with FGF8 with or without notch inhibition. After 21 days neurons expressed mature neuronal markers (TUJ1 and MAP2). Elevated expression of GnRH1, detected by immunocytochemistry and RT-PCR, along with markers present in mature GnRH neurons (KISS1R and TAC3R) and in the nasal placode (EYA1 and GATA2), indicated robust specification of NPCs to GnRH-like neurons.

We plan to use this system to investigate basal and stimulated GnRH release and developmental changes over time by extending the culture period.

Our model of GnRH neurons derived from hESCs/iPSCs can provide a reliable system for studying molecular mechanisms underlying developmental changes and conditions such as hypogonadotropic hypogonadism and delayed puberty.

P39. Dissecting the role of FGF signalling in human pluripotent stem-cell-derived neural crest and GnRH neurons

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The neural crest (NC) is a transient migratory cell population arising during embryonic development. NC gives rise to various adult cell types including olfactory ensheathing glia (OEG), which supports the migration of GnRH neurons. Patients with a neurocristopathy due to mutations in *CHD7* or *SOX10* also frequently have Kallmann syndrome, suggesting an intertwined fate of NC cells and GnRH neurons. In animal models, fibroblast growth factor (FGF) signalling is recognized as an important pathway for both cell types. Especially, FGF8 signalling via FGFR1 is essential for GnRH neuron specification, as demonstrated by data from animal models and humans.
To overcome the lack of suitable human disease models of Kallmann syndrome, we have developed protocols for the differentiation of NC cells and GnRH neurons from human pluripotent stem cells (hPSCs). In the current study, we will use these protocols to monitor the impact of FGF signalling on NC cells and GnRH neurons. Preliminary results show that the chemical inhibition of FGF signalling with SU5402 did not affect the proliferation or survival of NC cells but, surprisingly, accelerated the commitment of hPSCs towards NC cell fate as judged by the up-regulation of several neural crest-related genes, including *PAX3*, *SLUG*, and *TWIST1*. Noteworthy, AP-2α a marker of migratory NC cells, was only detected in SU5402-treated cells but not in the control cells, suggesting that FGF signalling controls the stage of NC cells.
Next, we will investigate the role of FGFR1 signalling more in detail during human NC cell and GnRH neuron specification. For example, we are currently constructing FGFR1 mutated hPSCs with CRISPR/Cas9 technology, and have also obtained two Kallmann syndrome patient-specific iPSC lines with mutations in *FGFR1*. We will differentiate these cells to NC cells and GnRH neurons to explore the FGFR1 signalling-mediated mechanisms in the specification of these fascinating cells.

P40. Identification of a novel kisspeptin pathway in glial cells: A new contributing circuit for kisspeptin-driven control of puberty?

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Puberty is under the control of complex neuronal circuits, which are sensitive to endogenous and environmental signals, and ultimately modulate GnRH neurosecretion. Kisspeptins, the products of Kiss1 neurons, acting via their canonical receptor, Gpr54, in GnRH neurons, have been recognized as master regulators of puberty. In addition, glial cells are known to play important roles in neuroendocrine regulation in general, and the control of GnRH neurosecretion and puberty onset in particular. However, the potential role of glial cells in mediating (at least part) of kisspeptin actions remains unexplored.
We present herein evidence for a putative kisspeptin-signaling pathway in glial cells that might play a role in the central control of puberty. Proteomic analyses revealed an acute up-regulation of glial fibrillary acidic protein (GFAP; a glial cell marker) levels in the hypothalamus of Kiss1 null mice following icv injection of an effective dose of kisspeptin-10 (Kp-10). Glial responses to Kp-10 administration in Kiss1 KO mice were confirmed by qPCR and Western blot analyses, which documented an increase in GFAP and vimentin levels in the hypothalamic preoptic area. In support of a tenable Gpr54-mediated pathway in glial cells, qPCR analyses demonstrated expression of *Gpr54* in primary cultures of rodent astrocytes, in which Kp-10 stimulation caused phosphorylation of the canonical elements of the intracellular kisspeptin-signaling pathway, ERK/MAPK and Akt. To obtain further evidence for the role of such pathway in the control of puberty, we are in the process of generation and phenotypic characterization of a novel mouse line with selective deletion of Gpr54 in glial cells, by heterozygous crossing of Gpr54-loxP and GFAP-Cre lines. Initial analyses suggest a delay in the timing of puberty in GFAP-Gpr54 null (male and female) mice; further studies are in progress in order to fully validate these initial observations and to provide conclusive evidence for a role of this novel kisspeptin-glial signaling pathway in the physiological control of GnRH neurosecretion and puberty onset.

P41. Effects of gonadectomy and testosterone replacement on number of kisspeptin-ir and NKB-ir cells in the arcute nucleus of the hypothalamus in obese and diabetic male rats

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Besides metabolic problems occurring in people with obesity and diabetes there are numerous of secondary problems, including disruptions of the reproductive system

(e.g. disruptions of menstrual cycle in women, decrease in testosterone levels and spermatogenesis in men, hypogonadism, premature child birth, miscarriages or infertility).
The reproductive system, governed by the hypothalamic-pituitary-gonadal (HPG) axis, is very sensitive to metabolic cues. Population of arcuate nucleus (ARC) neurons expressing kisspeptin, neurokinin B and dynorphin (KNDy cells) is important in GnRH pulse generation and maybe involved in control of metabolism. Moreover, sex steroids imbalance occurring along with obesity and diabetes can lead to disruptions functions of KNDy neurons.
We hypothesized that: 1) diet-induced obese (DIO), and streptozotocin-induced (STZ) diabetic rats would have altered number of kisspeptin- and neurokinin B (NKB)-immunoreactive (-ir) cells in the ARC; 2) gonadectomy (GDX) and testosterone (T) replacement would affect the number of kisspeptin-ir and NKB-ir cells in the ARC of rats.
Male rats were fed high fat diet - HFD to induce obesity (DIO) or control (C) diet for 5 weeks. Injections of STZ were performed to induce diabetes type 1, DM1 (C/STZ) or diabetes type 2, DM2 (HFD/STZ). Next animals were divided into the surgical treatment groups: 1) gonadectomy (GDX), 2) gonadectomy and T replacement (GDX+T) and 3) Sham. Immunocytochemistry for the kisspeptin and NKB was performed.
We found that: 1) In C and DIO but not in DM1 and DM2 animals, GDX caused statistically significant increase in number of kisspeptin-ir cells compared to respective Sham; 2) GDX decreased number of NKB-ir cells compared to Sham animals and this decrease was the most pronounced in DM1 and DM2 rats.
We concluded that DM1 and DM2 animals had altered response of kisspeptin-ir and NKB-ir neurons in the ARC to GDX.
Supported by NCN grant 2011/01/B/NZ4/04992.

P42. Next generation sequencing in genetic analysis of hypogonadotropic hypogonadism

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Introduction: Genetic analysis of hypogonadotropic hypogonadism (HH) has an important place in pediatrics, as it can prompt the treatment.
Subjects: 11 subjects with suspicion of congenital HH were included in our clinical series. There were 10 males and one female. Seven males had Kallmann syndrome and 3 had normosmic HH. They were aged between 16 and 67 years. The female had suspicion of Kallmann syndrome and was aged 17 years. Three subjects had other congenital anomalies associated with HH (color blindness, anomalies of

heart, learning disabilities, long limbs, sensorineural deafness, bimanual synkinesis, hypertelorism).
Methods: Targeted next generation sequencing (NGS) of 24 genes, known to be associated with HH was used. Our confirmation method was Sanger sequencing.
Results: In our subject group, we found 6 different potentially relevant mutations, using NGS and corresponding program tools. Mutations were located in genes PROK2, GNRHR, PROKR2, FGFR1, CHD7. Each mutation carrier had a single heterozygous mutation. Three of the mutations were already described and the remaining three were new. The newly discovered mutations were named as recommended by Human Genome Variation Society. All 6 mutations were confirmed with Sanger sequencing. New mutations were: c.171_172delTT (p.Ile57MetfsTer17) in 2. exon of gene PROK2, c.196T>C (p.Typ66Arg) in 4. exon of gene FGFR1 and c.5759A>G (p.Tyr1920Cys) in 29. exon of gene CHD7. In 5 subjects, no mutations were found using NGS.
Conclusion: Our subject group was genetically very diverse. Our results expand the spectrum of mutations implicated in HH. Our remaining challenge is the diagnosis of the five remaining subject in whom no mutation in selected 24 genes were found.

P43. MicroRNA in the 12q locus showing strong post-natal variations of their expression in the hypothalamus are involved in synaptic activity and axon guidance

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MicroRNAs are non-coding RNA involved in the post-transcriptional control of gene expression. Whole genome association studies have revealed the association of Lin28B polymorphisms with age at menarche. Lin28B is an inhibitor of microRNA maturation. The link between age at menarche and Lin28B was proposed to be related to the role of Lin28 in the control of let7 maturation. Central precocious puberty is associated to the maternal uniparental disomy (mUPD) of 14q32 in Humans. The maternal allele of this locus encodes a cluster of microRNAs. The double dose of these microRNAs may thus predispose to an early activation of the gonadotropic axis. In this study, we sought to analyze the changes of the post-natal expression of microRNAs of the 12q region and to determine target genes of selected microRNAs showing significant post-natal changes of gene expression in the hypothalamus after birth.
MicroRNAs with significant changes of expression were first selected by a microarray analysis of total RNA extracted from the whole hypothalamus. Ten microRNAs at the 12q locus in Mouse, which is syntenic of the 14q32 locus in Human, vary significantly between P6 and P20. The expression of few of them also varied between P20 and P60. Five microRNAs showing the highest variations of expression were then pooled together to determine their target genes by KEGG and mirGO analysis. 52 genes were found as target of these five microRNAs. These genes encode proteins involved in axon guidance and synaptic plasticity ($p < 10^{-15}$).

This study highlights the potential role of microRNAs in the maturation of hypothalamic function after birth. This maturation is potentially highly related to changes in synaptic activity of hypothalamic neurons. Additional studies are needed to confirm the link between expression variations of these microRNAs in the hypothalamus and the maturation of the gonadotropic axis leading to puberty.

P44. Expression of Kiss1 and GPR54 in the hypothalamic-pituitary-gonadal (HPG) axis and peripheral organs (fat, pancreas and liver) in obese and diabetic rats

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Kisspeptin, encoded by the KISS1 gene, could play a role in transducing metabolic information into the hypothalamic-pituitary-gonadal (HPG) axis. Negative energy balance (e.g. undernutrition) or positive energy balance (e.g. obesity and diabetes) frequently result in impairments of fertility (Pasquali et al., 2007; Wahab et al., 2015) and hypothalamic hypogonadotropic hypogonadism was reported in obese and diabetic patients (Skorupskaite et al., 2014). However, data concerning positive energy balance and the role of kisspeptin in the peripheral tissues is scant.

We hypothesized that: 1) in diet-induced obese (DIO) male rats and/or rats with diabetes type 1 (DM1) and type 2 (DM2), altered reproductive functions are related to an imbalance in Kiss1 and GPR54 expression in the HPG axis; 2) in DIO and/or DM1 and/or DM2 rats, Kiss1 and GPR 54 expression are altered in the peripheral tissues involved in metabolic functions (fat, pancreas and liver).

Animals were fed a high-fat or control diets and STZ (streptozotocin – toxin, which destroys the pancreas) was injected in high or low doses to induce diabetes type 1 (DM1) or diabetes type 2 (DM2), respectively. RT-PCR and Western blot techniques were used to assess the expression of Kiss1 and its receptor GRP54 in tissues.

At the level of mRNA, we found that diabetic but not obese rats have alterations in Kiss1 and/or GPR54 mRNA levels in the HPG axis and in peripheral tissues (fat, pancreas and liver). The most severe changes were seen in DM1 rats. However, in the case of protein levels in the peripheral tissues (fat, pancreas and liver), changes in Kiss1/GPR54 expression were noticed in DIO, DM1 and DM2 animals and were tissue-specific.

Our data support the hypothesis that alterations in Kiss1/GPR54 balance may account for both reproductive and metabolic abnormalities reported in obese and diabetic rats.

Support: OPUS grant NCN 2011/01/B/NZ4/04992 to J.H.S.

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OF THE EUROPEAN GNRH NETWORK
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