OTKA K101326

RF-amide peptide-signalling in the hypothalamic integration of reproduction and metabolism

Final report

Content:

The role of RF-amide peptide-signalling in hypothalamic integration of reproduction and metabolism was investigated by experiments, which were focusing on the hypothalamic target cells of RFamide peptides by demonstrating cells showing c-FOS activation at different metabolic conditions, or receiving afferents from hypothalamic cells expressing KP or RFRP. The relationship of KP neurons with GnRH neurons, which form the final common pathway for the central regulation of reproduction and may also signal towards the metabolic centres via KP neurons to prepare for conception and pregnancy, has also been investigated.

I. Identification of kisspeptin responsive cells in the hypothalamus.

1. Effect of intracerebroventricular administration of KP-10 on cFOS activation of hypothalamic cells

After test treatment of animals and staining for cFOS with the (#AB-5) or (#226003) antibodies (*for details see V/1*), two experiments were carried out involving rats either receiving 1nmol KP-10 in aCSF or aCSF, intracerebroventricularly (icv) 90 minutes before sacrifice. The first included female rats, gonadectomised, estradiol replaced females (n=14), whereas the second experiment involved both gonadectomised females with and without estradiol replacement (n=10), and gonadectomised and intact males (n=10).

Briefly, adult male and female Sprague-Dawley rats were housed and accustomed to handling at standard laboratory conditions. Five male rats and all female rats were gonadectomised, and a group of the female rats (n=19) received a subcutaneous capsule of estradiol benzoate to maintain low estrogen levels. Intracerebroventricular cannula were inserted, through which the animals either received a kisspeptin solution (KP-10; 1nmol; *(Thompson, Patterson et al. 2004)*) or vehicle eighteen days after the gonadectomy. Ninety min after treatment animals were perfused with 4% PFA prepared in 0.1M PBS. The brains were removed, 30µm sections were cut and collected alternatively into sixth series. A set of sections was immunostained with either of the following antibodies; rabbit anti-cFOS antibody from Millipore (AB-5), rabbit anti-cFOS from Synaptic Systems (226003) or goat anti-cFOS antibody from Santa Cruz (sc-52G). Another set of sections containing the consecutive sections from each brain were mounted and counterstained with 1% toluidine blue.

Sections from each brain of the different animal models were incubated concurrently, mounted and matching coronal sections were photographed at 20x magnification either at bright field conditions (when the label was nickel enhanced diaminobenzidine) or in Z-series throughout their complete thickness by using a confocal microscope (NIKON A1R, when fluorescent label was used). The number of cFOS-immunoreactive (IR) nuclei were counted by using Image J in hypothalamic regions, i.e. arcuate nucleus (Arc), hypothalamic paraventricular (PVH), dorsomedial (DMN) and ventromedial nuclei (VMN) and the lateral hypothalamus (LH). For analyses, the selection frame (~ 0.365 mm^2) was positioned in the anatomical region after correlating the distribution of the counterstained cell groups with the corresponding atlas image of *Paxinos (2005*).

We have used the last aliquots of AB-5 antiserum to validate a potential replacement antibody i.e. #226003 (Synaptic Systems) for the oncoming experiments. By using AB-5, no significant difference could be detected in the number of cFOS-IR nuclei in the analyzed regions of the animals treated either with KP or aCSF (TTest, $p<0.05$). There was, however a moderate numerical increase of cFOS-IR nuclei in most analysed regions (only the Arc showed a reduction).

In contrast, by using the Synaptic Systems antibody, all analyzed regions exhibited a reduction of cFOS-IR nuclei in the animals treated with KP-10. The reduction has reached the significance level in the medial preoptic area and the lateral hypothalamus.

AB-5	ARC	VMH	DMN	Pa	MPO	LH
+aCSF	64.17±2.5	63.14±10.62	80.86±24.78	90.14±2.11	95.17±8.44	95.00±12.16
+KP	57.29±14.12	100.00±27.31	94.29±29.47	96.14±18.25	108.29±17.04	98.83±7.8
Change %	-10.72	+58.37	+16.61	+6.66	+13.79	+4.04

#226003	ARC	VMH	DMN	Pa	MPO	LH
+aCSF	113.43±23.11	113.14±22.35	146.57±21.75	260.71±20.20	196.86±19.57	84.14±7.28
+KP	65.86±6.83	93.43±13.93	100.57±14.94	204.71±87.58	140.43±12.21	63.71±5.43
TTEST (p)	0.07	0.47	0.11	0.55	**0.03**	**0.04**
Change %	-41.94	-17.42	-31.38	-21.48	**-28.66**	**-24.28**

This reduction of cFOS activation after KP-10 treatment could be observed only in the presence of gonadal steroids (One-way ANOVA, Post hoc Fisher LSD test, in females p<0,04). Similar tendency could be observed in the cFOS activation in the male gonadectomised vs. gonad-intact rats, but the difference between the KP-treated and non-treated animals remained non-significant.

Because of these non-comparable, inconclusive data obtained with AB-5 and #226003, we have suspended these studies.

2. Effect of leptin, and feeding of animals with standard chow or palatable food on cellular activation

In 2015, we have returned to the activation studies, when Este Leidmaa from the Max Planck Institute of Psychiatry, Munich joined our research team and we gained access to a reserve stock of AB-5 antibody (originally bought by the German collaborators years before from Calbiochem; PC-38). In addition, we learnt about the usability and tested the Santa Cruz antibody sc-52 antibody (raised in goat) for co-localization experiments. By using these antibodies, we have detected activated cells in various CNS regions, and quantified the number of POMC and Orexin neurons in the Arc and LH, respectively, expressing cFOS. For details see IX of the current report:

Este Leidmaa, Mary Gazea, Alexandre Patchev, Anna Pissioti, Nils C Gassen, Liposits Z, **Imre Kalló**, Osborne F.X. Almeida: Galanin gates ascending leptin and descending hedonic signals on orexin neurons in the lateral hypothalamus. ***Cell Metabolism*** (submitted) 2017

II. Projection area and target cells of kisspeptin neuronal afferents

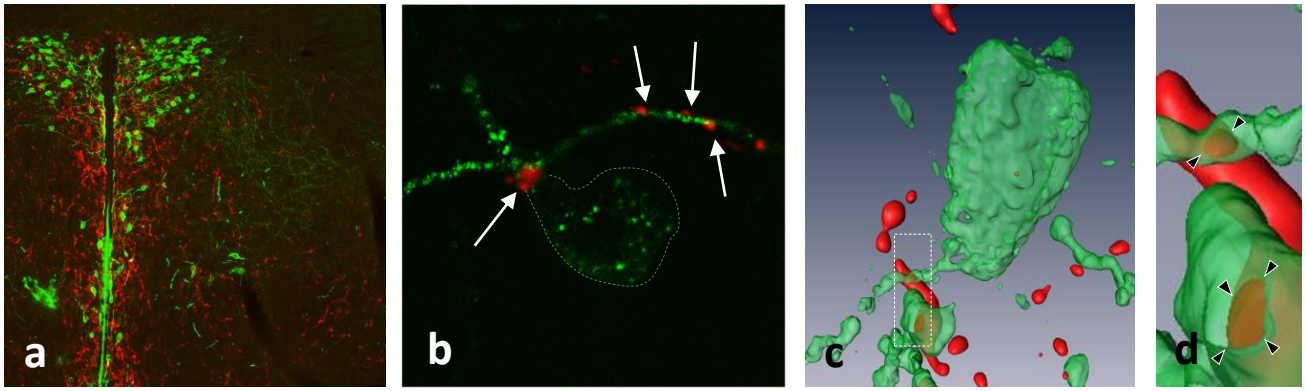
Three major groups of kisspeptin neurons were localised in the adult rodent brain i.e. (1) a group of cells in the rostral periventricular region of the third ventricle (RP3V), which play a key role in relaying the positive feedback effects of estradiol to the gonadotropin-releasing hormone (GnRH) neurons and also respond with transcriptional changes to metabolic challenges, (2) a group of cells in the arcuate nucleus, which have been associated with the mediation of the negative feedback of estrogen on the reproductive cycle as well as the metabolic status of the animals towards the GnRH neurons, and (3) a small group of animals in the medial amygdala, which receive information from the olfactory bulb and transmit these signals towards the GnRH neurons. The neuronal circuits within these kisspeptin neurons participate in regulation of reproduction and reproduction-related physiological processes i.e. food intake and metabolic processes are however only partially identified. By using immunohistochemical double labelling, combined *in situ* hybridization and immunohistochemical labelling and tract tracing approaches, we have investigated the projection area and target cells of KP afferents. KP afferents were co-labelled with the following markers identifying hypothalamic cells; oxytocin (OT), thyrotropin releasing hormone (TRH), cocaine- and amphetamine-regulated transcript (CART), tyrosine hydroxylase (TH), proopiomelanocortin (POMC) and choleratoxin B injected into the ventral tegmental area (VTA), wherefrom it retrogradely label the hypothalamic glutamatergic and GABAergic afferents.

1. Phenotypic diversity of kisspeptin neurons – kisspeptin signalling to
    - Oxytocin neurons

Central oxytocin in known to terminate food intake and a key component of mechanisms that reduce eating for pleasure and shape macronutrient preferences (for review see *Klockars et al, Front Endocrinol, 2015*)

Although the mRNA for Kiss1r (GPR54) has been demonstrated already by its discovery in the rat paraventricular nucleus in 1999 *(Lee, Nguyen et al. 199)*, it has been localised only recently in oxytocin neurons. Our observation showing a very rich network of KP-IR fibers in the PVH and KP-IR axon varicosities in apposition to OT-IR perikarya and dendrites in 3D reconstructed images of confocal Z-stacks ***(Fig.1.)*** supports the existence of a functional interaction between these two systems. These results add a new regulatory pathway to the current understanding, which suggests that kisspeptin acts peripherally, and it requires the vagus nerve, to stimulate oxytocin neurons in female rats *(Scott and Brown 2011, Scott and Brown 2013)*.

***Figure 1*.** Kisspeptin-immunoreactive (KP-IR) fibers (red) in the paraventricular nucleus of the hypothalamus in the vicinity of oxytocin (OT)-IR neurons (green). KP-IR fibers establish a rich network around the OT-IR neurons **(a)** and often establish appositions (arrows) on OT soma and dendrites **(b)**. By 3D reconstruction of the IR profiles, and visualising the OT cell in a semi-transparent way **(c)**, island of gap-free appositions (arrowheads) can be detected



between KP axons and OT soma and dendrites **(d),** indicating functional interaction at these sites. **(d)** is the magnified image of the boxed area in **(c)**.

- <u>POMC neurons</u>

POMC neurons are one of the primary, sensory cell groups of the arcuate nucleus, that are involved in the central regulation of feeding. Intracerebroventricular injection of kisspeptin has been shown to modulate POMC gene expression *(Backholer, Smith et al. 2010)*.

The hypothalamic anorexigenic POMC cells have been shown to be strongly innervated by kisspeptin neurons, and kisspeptin has been demonstrated to evoke a robust direct excitatory response by POMC neurons in mice *(Fu and van den Pol 2010)*. We have found POMC cells imbedded in a reach network of KP-IR fibres also in the rat ARC (not shown), indicating that POMC cells can be important downstream mediators of KP signalling.

In collaboration with a Spanish research team, we have demonstrated MCH1 receptors and regulatory proteins (SIRT1) in Arc POMC neurons; via this signaling pathway, MCH may exert orexinergic effects in the animals. The manuscript containing these data is under revision.

Omar Al-Massadi, Mar Quiñones, Donald A. Morgan, **Imre Kalló**, Melissa J Chee, Monica Imbernon, Daniel Beiroa, Rosalia Gallego, Miguel Lopez, Zsolt Liposits, Kamal Rahmouni, Carlos Dieguez, Ruben Nogueiras: SIRT1/FoxO1 in the arcuate nucleus underlie MCH-induced hyperphagia, adiposity and glucose intolerance. (*originally submitted to **Cell Reports**, currently transferred to **Heliyon***)

- <u>VTA neurons:</u>

The brainstem reward center, the ventral tegmental area (VTA) has been linked to influence food intake, as well reproduction-related behaviors. We have mapped the hypothalamic neurons projecting to the VTA and characterized the chemotype of these neurons for the classical neurotransmitters GABA and glutamate, as well as neuropeptides involved in the regulation of reproduction and metabolism. The GABAergic and glutamatergic hypothalamic neurons innervating the VTA has been demonstrated in a paper published in Frontiers in Neuroanatomy:

Imre Kalló; Erik Hrabovszky; **Csilla S. Molnár**; Barbara Vida; Csaba Fekete and Zsolt Liposits (2013) Projections of the hypothalamic paraventricular region to the ventral tegmental area in the rat: the integration of neuroendocrine and reward signals ***MITT***, Budapest, Hungary

Kalló I, **Molnár CS**, Szőke S, Fekete C, Hrabovszky E, Liposits Z. Area-specific analysis of the distribution of hypothalamic neurons projecting to the rat ventral tegmental area, with special reference to the GABAergic and glutamatergic efferents. ***Front Neuroanat****. 2015 Sep 4;9:112.*

By using a combined tract tracing and dual label immunohistochemical method, KP neurons were found neither in the preoptic area nor the arcuate nucleus to project to the VTA. In contrast, we identified a subpopulation of the Arc POMC neurons (30% of the VTA projecting neurons from the ARC were POMC neurons), which project the VTA. This projection to VTA dopaminergic neurons are now considered to modulate motivation for palatable food via activation of MC3R signaling. These results have been also published in collaboration with a Dutch research group:

Pandit R, Omrani A, Luijendijk MC, de Vrind VA, Van Rozen AJ, Ophuis RJ, Garner K, **Kalló I**, Ghanem A, Liposits Z, Conzelmann KK, Vanderschuren LJ, la Fleur SE, Adan RA. Melanocortin 3 Receptor Signaling in Midbrain Dopamine Neurons Increases the Motivation for Food Reward.
Neuropsychopharmacology. 2016 Feb 8.

2. Phenotypic diversity of kisspetin neurons – participation of co-synthetized peptides in the signaling:
    - Galanin:

A subset of KP neurons has been shown to express galanin (13% of KP cells in the RP3V and 88% of them in the Arc were immunoreactive for galanin, and galanin mRNAs were detected in nearly half of KP neurons, *(Kallo, Vida et al. 2012)*. The incidence of galanin-positive KP neurons was found to be higher in female than in male animals.

Kalló I, Hrabovszky E, **Molnár C S**, Caraty A, Ciofi P, Coen C W, Liposits Z, Vida B (2012) Sexual difference in the phenotypical characteristics of the mouse kisspeptin neurones ***2nd World Conference on Kisspeptin Signaling in the Brain*** – Tokyo 2012.11.06.-2012.11.09.

In collaboration with a German research team, we have initiated studies to investigate the role of galanin in food intake. During the three-month visit of Este Leidmaa, we have identified the leptin activated, pSTAT and cFOS positive cells of the hypothalamus, and phenotyped the activated cells in the brains of animals received special diet and treatment. Our results revealed, that by intact proximal leptin signaling involving Arc leptin receptor expressing neurons (including a subpopulation of KP cells), exogenous injections of leptin (to artificially increase the level of satiety) failed to curtail palatable food (PF) eating. Rather, PF eating was accompanied by activation of appetite-stimulating orexin neurons in the lateral hypothalamus (LHA). Since orexin neurons in the LH are known to be negatively regulated by galanin *(Goforth, Leinninger et al. 2014)*, we hypothesized that reduction of galaninergic inhibition of orexin neurons might, at least partially, be responsible for the strong preference for PF in defiance of leptin and other central mediators of satiety. Our hypothesis was confirmed by the finding that intra-LHA injections of a galanin receptor 2 agonist reduced PF ingestion. These results are consistent with the role of LHA orexin in determining eating behavior by integrating physiological, metabolic, environmental and hedonic information *(Yamanaka, Beuckmann et al. 2003, Berthoud and Munzberg 2011, Castro, Cole et al. 2015).*

Este Leidmaa, Mary Gazea, Alexandre Patchev, Anna Pissioti, Nils C Gassen, Liposits Z, **Imre Kalló** and Osborne F.X. Almeida: Galanin gates ascending leptin and descending hedonic signals on orexin neurons in the lateral hypothalamus. ***Cell Metabolism*** (submitted) 2017

- Dynorphin:

Besides the classical amine transmitters (GABA, glutamate), subpopulations of KP neurons also synthetize NKB and Dynorphin in the rodent Arc (KNDY neurons). Dynorphin is known to bind to the kappa opioid receptor (κOR), via it has been implicated in modulating food intake and to have inhibitory effects on GnRH pulsatility. In collaboration with the Spanish team, we have co-localized κORs and melanin concentrating hormone receptor 1 (MCH-R1) in lateral hypothalamic cells. Downregulation of κORs in these neurons may attenuate both diet-induced and MCH induced liver damage.

Imbernon M, Sanchez-Rebordelo E, Romero-Picó A, **Kalló I**, Chee MJ, Porteiro B, Al-Massadi O, Contreras C, Fernø J, Senra A, Gallego R, Folgueira C, Seoane LM, van Gestel M, Adan RA, Liposits Z, Dieguez C, López M, Nogueiras R. Hypothalamic kappa opioid receptor mediates both diet-induced and melanin concentrating hormone-induced liver damage through inflammation and endoplasmic reticulum stress. ***Hepatology.*** 2016 Jul 7. doi: 10.1002/hep.28716. [Epub ahead of print] PubMed PMID: 27387967.

III. Interaction between KP and GnRH neurons for co-ordenation of reproductive and metabolic regulation

We have postulated, that modulatory input of kisspeptin neurons on food intake and metabolism may also be influenced by direct inputs from GnRH neurons, which form the final common pathway for the central regulation of reproduction. We tested the hypothesis that GnRH neurons can influence the function of their afferent systems either at the level of axon terminals by locally released bioactive substances or at the distantly located somatodendritic region of the afferent neurons via synaptic connections, thereby providing potential feed-forward and/or feed-back information about their secretory/firing activity.

1. Alteration of KP signalling to target cells mediated by estrogen receptors

In collaboration with a Hungarian research team, the effect of the xenoestrogens ethinyl estradiol and zearalenone was investigated on the pubertal development of KP-GnRH neuronal network. We have demonstrated an increased

KP expression and fibre density, as well as increased number of KP-GnRH appositions in the preoptic area in animals received these xenoestrogens. In contrast to the preoptic area, the xenoestrogens did not cause marked alterations in Arc KP neurons.

Kriszt R, Winkler Z, Polyák Á, Kuti D, **Molnár C**, Hrabovszky E, **Kalló I**, Szőke Z, Ferenczi S, Kovács KJ. Xenoestrogens Ethinyl Estradiol and Zearalenone Cause Precocious Puberty in Female Rats via Central Kisspeptin Signaling. ***Endocrinology.*** 2015 Nov;156(11):3996-4007. doi: 10.1210/en.2015-1330. Epub 2015 Aug 6. PubMed PMID: 26248220.

We have also investigated the neurocircuitry involving the KP and GnRH neurons in the infundibular nucleus of the human, which is the corresponding area of the rodent arcuate nucleus. Our comparative study confirmed the human existence of interconnecting KP neurons, and the synaptic input of kisspeptin neurons to GnRH neurons. These results have been presented in a COST Meeting:

Zsuzsanna Bardóczi, Beáta Á. Borsay, László Herczeg, Zsolt Liposits, Erik Hrabovszky and **Imre Kalló**: Ultrastructural analysis of human kisspeptin neurons reveals features of mixed peptidergic and glutamatergic neurotransmission. ***COST Action BM1105 Meeting***: "CharitéCrossOver" at Charité University, Berlin, March 5-8, 2014

2. Endocannabinoids released by GnRH neurons target KP afferents

We have previously reported that CB1 immunoreactive afferents to GnRH neurons establish synapses with inhibitory morphological characteristics at the ultrasructural level. Furthermore, by using whole cell recordings, we have shown that GABA effects on GnRH neurons is tonically reduced by the presynaptic activation of CB1 receptors *(Farkas, Kallo et al. 2010)*. As 50 % of KP neurons in the Arc, and about 75% of KP neurons in the RP3V express the GABA synthetizing enzyme GAD67 *(Cravo, Margatho et al. 2011)* the question arises, whether KP fibers in apposition to GnRH neurons also contain CB1. We found CB1 immunoreactivity in such KP fibers. (**Fig.2.**) The significance of the co-localization, could be a possible mechanism for a differential release of the co-localizing peptides and the classical transmitters (e.g. GABA or glutamate) from KP terminals.

Imre Kalló (2015) Neuronal Feedback within the network regulating GnRH secretion. ***12th International Conference of the Polish Neuroscience Society,*** Gdansk, September 6-8, Poland

Wilheim Tamás, Watanabe Masahiko, Liposits Zsolt, **Kalló Imre** (2016) Morphological evidence supporting retrograde endocannabinoid signalling between GnRH neurons and their kisspeptin afferents in mice (P2/119) ***IBRO WORKSHOP BUDAPEST***, January 21-22, 2016

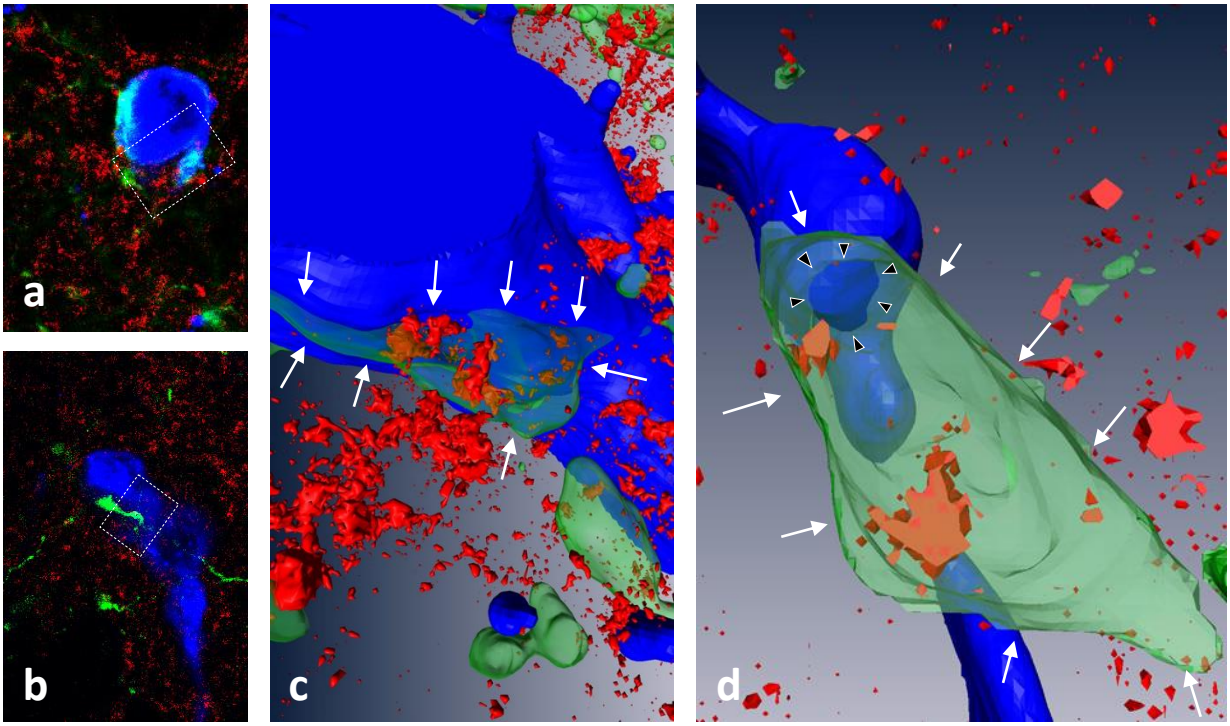


Figure 2. Kisspeptin-immunoreactive (KP-IR) axon varicosities (green) in apposition to GnRH-IR cells. **(a)** & **(b)** Dense CB1 immunoreactivity (red) can be observed in the vicinity of GnRH neurons. **(c)** & **(d)** CB1-immunoreactivity is visible

also in association with the KP varicosities (white arrows), those inside of the varicosity appear in orange-red colour due to co-localization. The kisspeptin processes were made transparent to show the CB1 immunoreactivity inside.

3. GnRH axon terminals synapse onto KP/TH, KP and TH neurons in the RP3V and Arc regions

Our aim was also to test, whether GnRH-IR neurons innervate KP neurons in the RP3V and Arc regions. We investigated therefore whether GnRH neurons in the female mouse (1) project to the RP3V and Arc and (2) establish morphological connections with the KP-and TH-IR neurons at these sites. Finally, (3) we began to study the actions of GnRH on the resting membrane potential and firing activity of KP-IR neurons in the RP3V and Arc *in vitro*.

While the dopaminergic neurons in the Arc form a separate cell population from kisspeptin neurons, there is an about 50% co-localization between these chemotypes in the RP3V. So the latter region is composed of neurons immunoreactive for either KP, or TH or both, and it is a question whether GnRH innervates each of these subgroups of cells. By using immunohistochemical triple labelling for GnRH, TH and KP, we identified GnRH-IR varicosities in apposition to all three chemotypes in the RP3V and to TH-IR and KP-IR neurons in the Arc and confirmed the presence of synaptic contacts at ultrastructural level.

Kalló I, Vida B, **Bardóczi Z**, **Szilvásy-Szabó A, Rabi F, Molnár T**, Farkas I,Caraty A, Mikkelsen J, Coen CW, Hrabovszky E, Liposits Z. Gonadotropin-releasing hormone neurones innervate kisspeptin neurones in the female mouse brain. ***Neuroendocrinology***. 2013;98(4):281-9. doi: 0.1159/000355623. Epub 2013 Sep 21.PubMed PMID: 24080803

Zsuzsanna Bardóczi and **Imre Kalló** (2015) Morphological evidence for synaptic communication between GnRH axons and dopaminergic neurons in the mouse preoptic area and arcuate nucleus, ***COST Action BM1105***, Joint Scientific Meeting & Training School, Monash University Prato Center, April 27th - 29th, 2015, Italy

To study the effect of GnRH on KP neurons *in vitro*, brain slices from mice in proestrus phase were used. Neurons for whole cell and cell-attached patch-clamp recordings were selected from the close vicinity of the ependymal layer of the third ventricle (the typical location of KP-IR neurons in the RP3V) and targeted with recording pipettes containing biocytin.

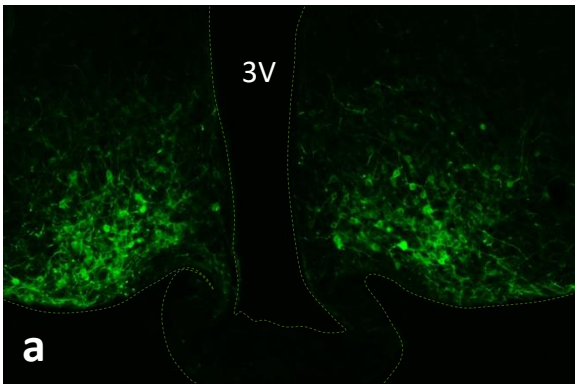
Current clamp recordings (at 0 pA) demonstrated a significant depolarization (7.45±2.157 mV; $p<0.05$) of RP3V KP-IR cells in response to the addition of 10 µM GnRH to the medium of the recording chamber. The excitatory effect of GnRH was detectable in the presence of TTX, indicating a direct effect. To ensure that these effects resulted from GnRH receptor activation, three KP-IR cells were tested with 100 nM Antide, a selective GnRH receptor antagonist. Antide blocked the effects of GnRH in these cells. To test whether the increased GnRH concentration in the extracellular milieu changed the firing activity of KP-IR neurons, action currents were recorded from RP3V neurons by using the cell-attached patch-clamp approach. An increased firing rate of KP-IR neurons was observed within 10 minutes after GnRH (10 µM) administration, compared to the pre-treatment control rate (172.7±8.37 % of the control recording, $p=0.013$). Our preliminary whole cell and cell attached recordings revealed an excitatory effect of GnRH on RP3V KP neurons.

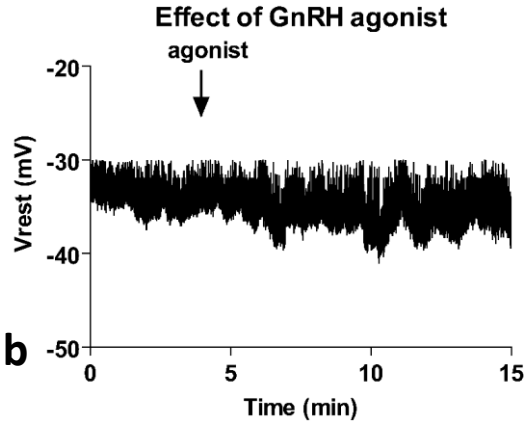
In the Arc, KP-CRE-GFP expressing neurons were selected for recording. The preliminary data show, that in contrast to RP3V KP neurons, KP neurons in the Arc do not respond to GnRH. Administration of 100 nm Antide, however, resulted in a slight membrane depolarization.

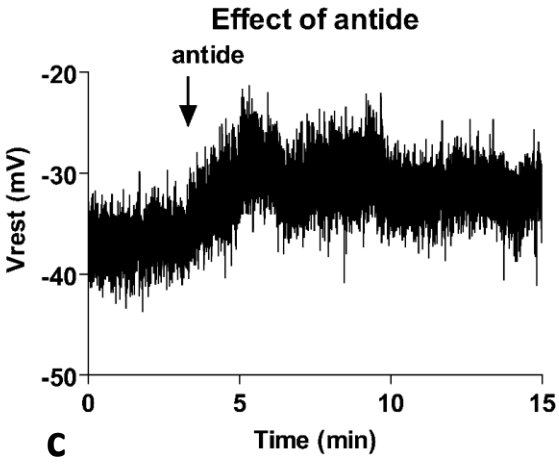
Figure 3: Recording from KP-CRE-eGFP mice infected with AAV viruses encoding channel rhodopsin-YFP. **(a)** The complete morphology of CRE expressing cells are marked by YFP in both ARC. **(b)** The membrane potential of a representative KP-CRE cells shows no change in response to GnRH agonist (100 nM), **(c)** In contrats, the GnRH antagonist Antide (100 nM) caused a slight membrane depolarization, as shown also in the graph **(d)**.

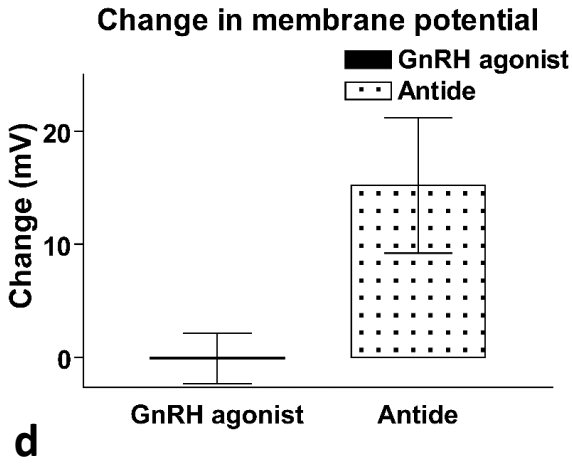
These results were presented in an international conference:

Imre Kalló (2015) Neuronal Feedback within the network regulating GnRH secretion. ***12th International Conference of the Polish Neuroscience Society,*** Gdansk, September 6-8, Poland









IV. Summary of the results and working model of neuronal circuitry

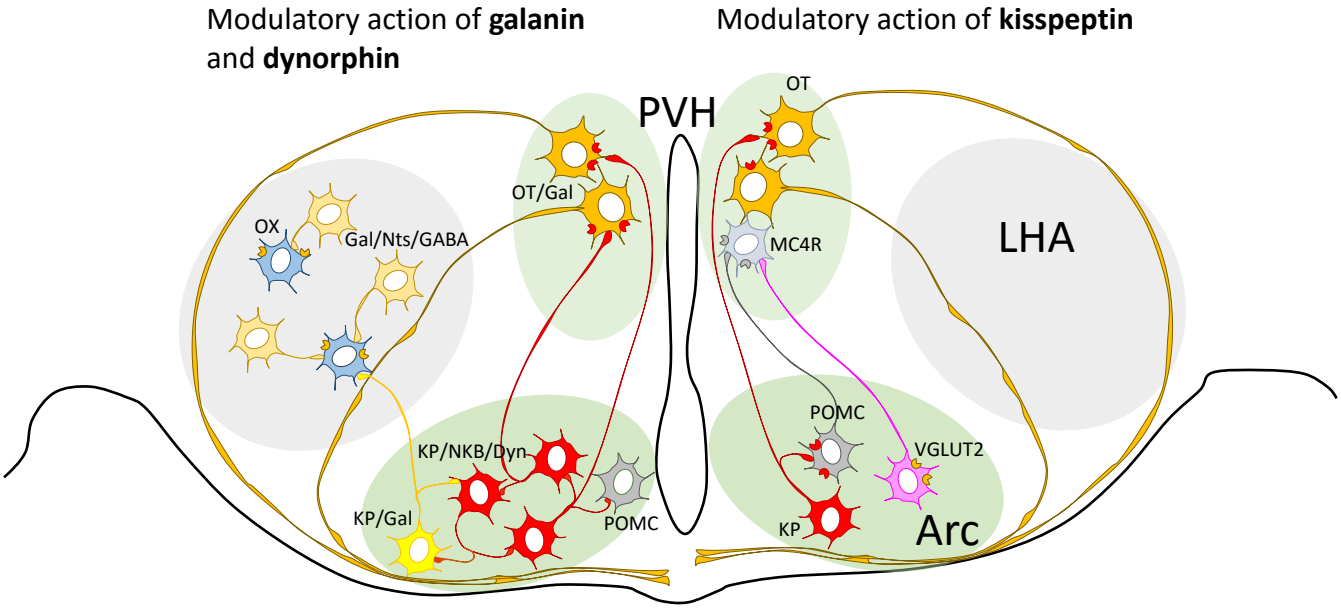


Figure 4. Working model of the circuitry upon kisspeptin (KP) neurons may modulate energy balance and food intake. Scheme of a rodent coronal hypothalamic section.

Right side of the scheme: KP directly stimulate POMC neurons in the arcuate nucleus (Arc) *(Fu and van den Pol 2010)*, which in response may increase the release of α-MSH in the paraventricular nucleus of the hypothalamus (PVH). α-MSH modulates the excitatory ARC-PVH satiety circuit via MC4R receptors *(Fenselau, Campbell et al. 2017)*. Although a subset of KP neurons are glutamatergic in the Arc, they do not participate in the excitatory stimulus to acutely suppress feeding. They may, however, stimulate oxytocin neurons (***Fig1. of this report***), and via this connection, indirectly stimulate the oxytocin receptor-expressing glutamatergic neurons in the Arc. KP targets also POMC cells, which may mediate its signal towards the VTA. (***Kalló et al, Front Neuroanat, 2015 and Pandit et al, Neuropsychopharmacol, 2016***).

Left side of the scheme: Galanin (Gal) has been recently reported to be responsible for mediating the inhibitory effects of leptin on orexin (OX) neurons *(Goforth, Leinninger et al. 2014)*. OX neurons receive dense inputs from neighbouring LepRb-positive neurons that co-express Gal, neurotensin (Nts) and γ-aminobutyric acid (GABA) *(Leinninger, Jo et al. 2009, Louis, Leinninger et al. 2010, Leinninger 2011)*. Via Gal release, a subpopulation of KP neurons may also participate in the direct regulation of OX neurons, since besides NKB and dynorphin, Gal is also synthetized in about 12% of KP neurons (***Kalló et al, 2nd World Conference on Kisspeptin Signaling in the Brain, Tokyo 2012***.) An indirect route via kiss1 receptor stimulation may be also possible by KP afferents of OT neurons, which also synthetize Gal in the PVH *(Landry, Roche et al. 1997)* and may release it in the LH from their axon varicosities (***Leidmaa et al, Cell Metabolism, 2017***). Dynorphin released from KP neurons may participate in the regulation of MCH-R1 expressing neurons in the LHA, as they express κORs (***Imbernon et al, Hepatology 64,4,2016***)

The model postulates that neuropeptides released from KP neurons can shift the balance between stimulation and inhibition on the key regulatory elements of the neural circuitry, and consequently increase or decrease the food intake and/or metabolic rate of the animals.

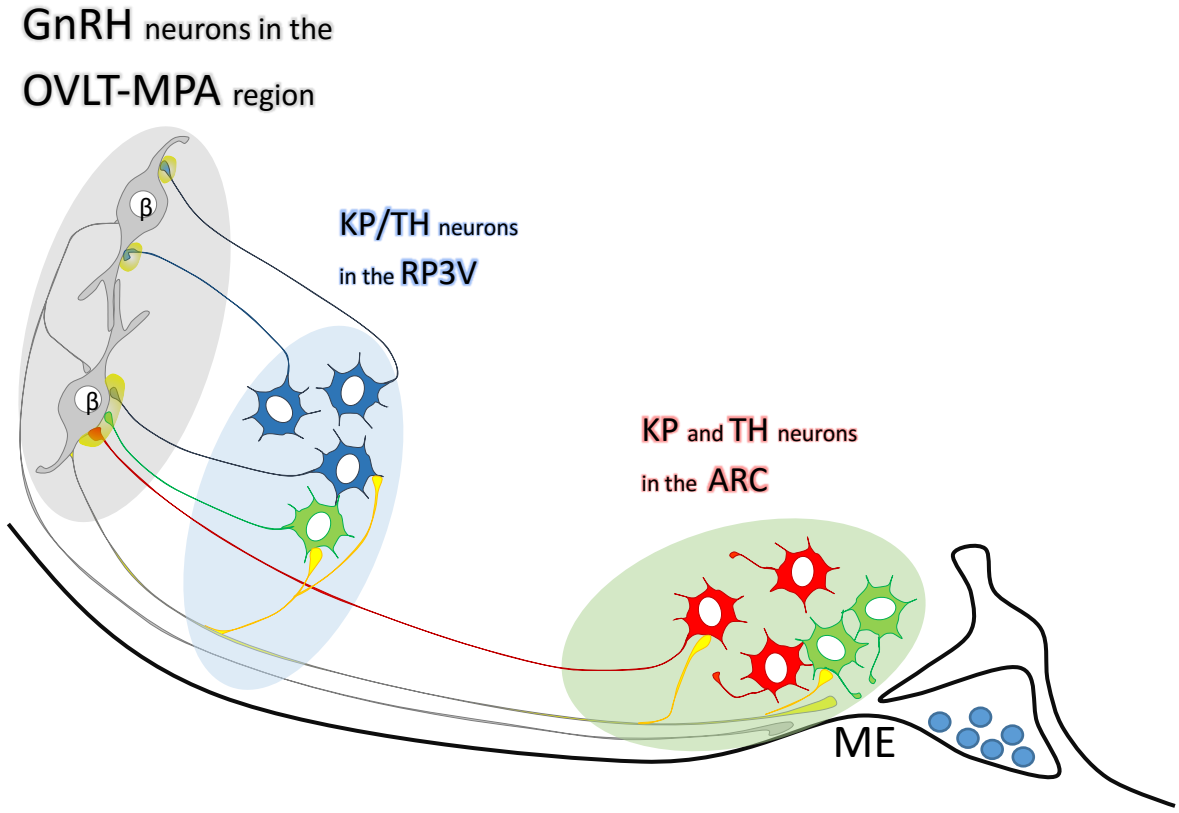


***Figure5*.**
Summary scheme depicting the circuitry upon kisspeptin (KP) and TH neurons receive potential feed-back or feed forward information from GnRH neurons to influence their downstream elements. Scheme of a rodent sagittal hypothalamic section.
The GnRH neurons release endocannabinoids (yellow area at the GnRH soma) to influence their GABAergic/KP containing afferents. (***Wilheim et al, IBRO WORKSHOP BUDAPEST, 2016***.) They also innervate RP3V and Arc KP and dopaminergic neurons (yellow axonterminals). (***Kalló et al, Neuroendocrinol, 2013; Bardóczi et al, COST meeting, 2015***) GnRH depolarise KP neurons and increase their firing frequency in the RP3V. Arc neurons may be under tonic effect of GnRH, as it was shown by preliminary records. (***Kalló et al, 12th International Conference of the Polish Neuroscience Society, 2015***)

V. Technical challenges during the supported period

1) Using cFOS antibodies from different origin

When the experiments were originally planned to detect hypothalamic cells showing cFOS immunoreactivity in response to KP-10 administration, it was done in the belief of the availability of a rabbit polyclonal antibody raised against the amino terminus of c-Fos p62 (Santa Cruz Biotechnology, USA, sc-52). This antibody was widely used in studies looking at site specific cellular activation evoked by various physiological and pharmacological challenges; we have used also successfully in our previous publication *(Halasz, Toth et al. 2006)*. This antibody was highly selective, and showed no cross-reactions with other members of the FOS protein family. When we learnt about its lost specificity (detected all cell nuclei in the tissue) by the last batches sold, we reviewed the literature and ordered another rabbit antibody (AB5 originally sold by Calbiochem) from Millipore (PC-38), which appeared in the publications as a reliable replacement for the rabbit SC-52. By using up our stock, however we faced a problem again in reordering AB5, as Millipore has discontinued selling it from 2013. To continue our immunostainings for cFOS, we had to introduce a new rabbit antibody from Synaptic Systems (226003), which was recommended by other labs and appeared in their publications. Our tests comparing AB5 and 226003 staining in consecutive sections showed similar distribution of activated cells, but perhaps with different sensitivity detecting different number of immunoreactive nuclei. To validate the specificity of staining, considering that both AB5 and 226003 was raised in rabbit, dual label immunohistochemistry could not be performed. We found by three other antibodies (SC-8047, ab102699 and ab87655) a very weak, or non-specific staining, therefore we did not use them in the experiments.

Recently in collaborating with a German group, we have received some PC-38 from their stock, and tested also a new batch of SC-52 antiserum from Santa Cruz Biotech generated in goat, and used these in our last immunohistochemical experiments (*Leidmaa et al, Cell Metabolism, 2017*).

Vendor	***Code***	***Host***	***WD using NiDAB***	***WD using IF***
Millipore (PC-38)	AB5	rabbit	1:5,000	1:5,000
Synaptic Systems	226003	rabbit	1:100,000	1:20,000
Santa Cruz Biotech (D1)	SC-8047	mouse	1:500	1:100
Calbiochem	PC-38	rabbit	1:5,000	
ABCAM	ab102699	rabbit	10µg/ml	
ABCAM	ab87655	goat	1:300	
Santa Cruz Biotech	SC-52	goat	1:10,000	

2) Characterising KP and RFRP antisera recognising different peptide fragments of the prepro-peptides

It has been reported, that some of the KP antibodies may also recognise other members of RFamide peptides *(Franceschini, Yeo et al. 2013)*. Considering additional facts, as (1) the cytoplasmic sorting and trafficking may also show differences in peptides cleaved from the same precursor molecule, and (2) kisspeptin and other members of the RFamide peptides are co-synthetized with classical neurotransmitters and other neuropeptides in different subpopulations of the neurons, we have run several experiments to compare the staining pattern of the different antibodies to be used. In concert with previous publications, we found, that the detectability of kisspeptin-immunoreactive (IR) perikarya are strongly influenced by the hormonal/physiological status of the animals. Although the perikarya detected in the two major locations can safely be regarded as KP somata, in the axonal projection area, when using certain KP antisera, one has to take a potential overlapping distribution and co-detection of KP-IR and RFRP-IR axons in account.

Antibodies tested in dual-label immunofluorescence experiments:

Antibody	***Host species***	***Antigen sequence***	***From species***
a-KP (AC566)	rabbit	YNWNSFGLRY (43-52)	Mouse
a-KP (JVL)	rabbit	(1-52)	Rat
a-KP (#053)	sheep		
a-Metastatin (#254)	mouse		Rat

Antibody	*Host species*	*Antigen sequence*	*From species*
a-Prepro RFRP (GA197)	rabbit	PSLPQRFGRTTARR (119-132)	Rat
a-RFRP1 (IF3-1)	mouse	VPHSAANLPLRF-NH2 (1-12)	Rat
a-RFRP1 (KA21-7)	guinea pig	H-VPHSAANLPLRF-NH2 (1-12)	Rat
a-RFRP3 (AC536)	sheep	VPNLPQRF-NH2 (last 8 aa)	Human

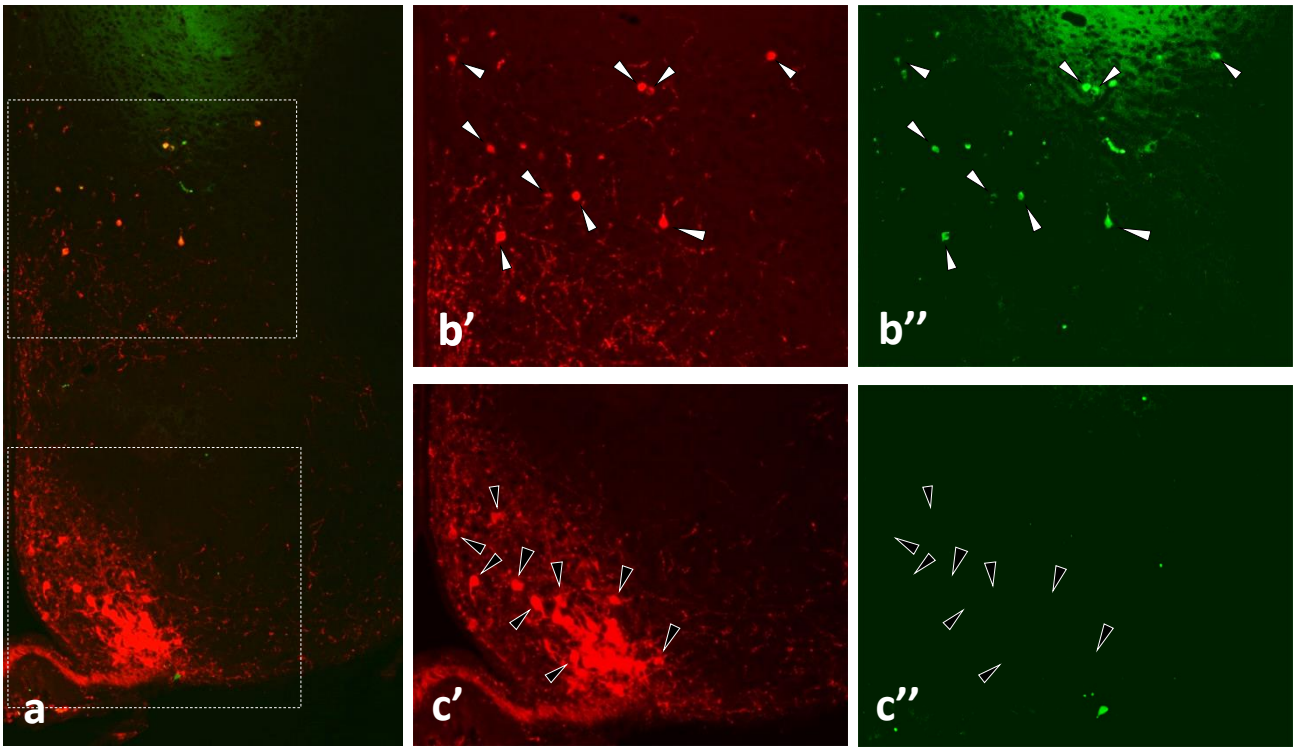


Figure 6. Immunohistochemical double labeling for kisspeptin (red) and RFRP (green) in the brain. (a) Two major locations of immunoreactive cells are enframed in the right side of the hypothalamus; the relatively compact group of neurons in the arcuate nucleus (ARC), and a few scattered cells in the area of the dorsomedial nucleus (DMN). (b', b") and (c', c") The enlarged image of the DMN and ARC regions, respectively, demonstrated in red and green channels; single labelled perikarya are marked by black arrowheads; whereas those double labelled for KP and RFRP are marked by white arrowheads. Note, that the kisspeptin neurons in the dorsomedial nucleus are also immunoreactive for RFRP.

3) Introducing genetically modified animals expressing CRE under the control of *Kiss1* promoter

To allow pre-recording identification of kisspeptin neurons in slices, and labelling of the cellular border to facilitate co-localization of molecules distributed in different cellular subdomains, we have initiated the procurement of genetically modified animals expressing CRE under the control of *Kiss1* promoter. Inspite of agreement about providing us with the Kisspeptin-IRES-Cre (KissIC) mice in 2013, the transfer arrangements were cancelled after half a year claiming a potential conflict of interest. The Kiss1-CreGFP knockin mice were sold by that time to Jackson Laboratories, so then we bought these animals and started the breeding and running immunohistochemical controls at our Institute. In these animals, heterozygotes are fertile and produce sufficient KP to maintain near normal reproductive cycle. GFP is targeted to the cell nucleus by a nuclear localization signal. RP3V and Arc distribution of cells exhibiting GFP positive nucleus correlates well with the distribution of KP-immunoreactive cells. However, cross breeding of these animals with animals bearing gene constructs encoding fluorescent reporter proteins, resulted in excessive, non-specific appearance of fluorescent cells in almost all regions of the brain. Viral injection of fluorescent transgenes, however, resulted in a more site-specific labelling of KP neurons. The tests alerted us, therefore, to target the recording as well as viral tracer holding pipettes precisely as much as possible, and label the area with the minimum amount of viral tracers.

Genetic modification	*Translated*	*Reference Lab*
Kisspeptin-IRES-Cre (KissIC) mice	Both kisspeptin and Cre recombinase	Ulrich Boehm
Kiss1-Cre transgenic mice (BACs)	Both kisspeptin and Cre recombinase	Carol F. Elias
Kiss1-CreGFP knockin mice	Cre recombinase-eGFP fusion protein	Robert A. Steiner / Michelle L. Gottsch

4) Detection of receptor mRNAs in Kiss1 hypothalamic cells

Although using dual-label in situ hybridization (ISH) or its combination with immunohistochemistry (IHC) allow the phenotypic identification of the cells within the level of a single mRNA type could be quantitatively analysed, these traditional approaches are long, composed of several technical steps each with its own risk of mistakes that can easily spoil the final results. To assess quantitatively 2-3 mRNA levels in the tissue, we have decided to introduce the RNAscope technique and use some of its big advantages to obtain data from the tissue we collected. This technique has a positive control probe set to test the mRNA integrity in the sections. In addition, by employing three target probes concurrently one can detect three different mRNAs in the tissue. The hybridization can be performed within two days. The signal appears as little dots, and quantifiable.

VI. Participants working on the project

Participants in the projects have changed unfortunately relatively frequently from the beginning. The PhD student working under my supervision, Barbara Vida left the laboratory before the start. After advertising the position, a student with MSc, Krisztina Szabadkai-Klavács was recruited on the 01.06.2012 (no PhD student submitted application for two month). She was in the lab for half a Year, then she left because of problems in her family to take care of two children. The position was taken by a *post doc* student, Balázs Barkóczi, on the 01.02.2013. Unfortunately, because of family move, his employment has ended by 31.12.2013. To fill the position, another *post doc,* Csilla Molnár was recruited on the 01.02.2014. She became pregnant and could be employed for the experiments soon only with serious limitations. She stepped down for maternity leave at the end of year 2015. For the remaining time, Barna László, and Miklós Sárvári were recruited to support the IHC and ISHH experiments.
The undergraduate students (Szilvásy-Szabó Anett, Rabi Fruzsina és Molnár Tamás) entered either a PhD program, or joined different research groups abroad. From the 01.09.2014, Tamás Wilheim joined our research team as a PhD student. In 2015 another PhD Student, Este Leidmaa joined our research team from the Max Planck Institute of Psychiatry, Munich and spent three month in our lab working on some of the experiments planned.
The frequent personal change in the supported position of the grant caused interruptions and delay in the implementation of some of the experiments. Inspite of these difficulties, projects were finished and results presented at Hungarian and International conferences, as well as in the form of submitted and accepted manuscripts.

VII. Publications and collaborations supported by OTKA K101326

Full papers

1. **Imre Kalló**; Barbara Vida; **Zsuzsanna Bardóczi**; **Anett Szilvásy-Szabó; Fruzsina Rabi; Tamás Molnár; Imre Farkas**; Alain Caraty; Jens Mikkelsen; Clive W. Coen; Erik Hrabovszky and Zsolt Liposits. Gonadotropin-releasing hormone neurones innervate kisspeptin neurones in the female mouse brain ***Neuroendocrinology***. 2013;98(4):281-9. doi: 10.1159/000355623. Epub 2013 Sep 21.

2. **Kalló I**, **Molnár CS**, Szöke S, Fekete C, Hrabovszky E, Liposits Z. Area-specific analysis of the distribution of hypothalamic neurons projecting to the rat ventral tegmental area, with special reference to the GABAergic and glutamatergic efferents. ***Front Neuroanat***. 2015 Sep 4;9:112. doi: 10.3389/fnana.2015.00112. eCollection 2015. PubMed PMID: 26388742; PubMed Central PMCID: PMC4559648.

3. Kriszt R, Winkler Z, Polyák Á, Kuti D, **Molnár C**, Hrabovszky E, **Kalló I**, Szőke Z, Ferenczi S, Kovács KJ. Xenoestrogens Ethinyl Estradiol and Zearalenone Cause Precocious Puberty in Female Rats via Central Kisspeptin Signaling. ***Endocrinology***. 2015 Nov;156(11):3996-4007. doi: 10.1210/en.2015-1330. Epub 2015

Aug 6. PubMed PMID: 26248220.

4. Rahul Pandit, Azar Omrani, Mieneke Luijendijk, Véronne Vrind, Andrea van Rozen, Ralph Oude Ophuis, Keith Garner, **Imre Kallo**, Alexander Ghanem, Zsolt Liposits, Karl-Klaus Conzelmann, Louk Vanderschuren, Susanne la Fleur, and Roger Adan. Melanocortin 3 receptor signaling in midbrain dopamine neurons increases the motivation for food reward. ***Neuropsychopharmacology*** 2016 Aug;41(9):2241-51. doi: 10.1038/npp.2016.19. PubMed PMID: 26852738; PubMed Central PMCID: PMC4946052.

5. Imbernon M, Sanchez-Rebordelo E, Romero-Picó A, **Kalló I**, Chee MJ, Porteiro B, Al-Massadi O, Contreras C, Fernø J, Senra A, Gallego R, Folgueira C, Seoane LM, van Gestel M, Adan RA, Liposits Z, Dieguez C, López M, Nogueiras R. Hypothalamic kappa opioid receptor mediates both diet-induced and melanin concentrating hormone-induced liver damage through inflammation and endoplasmic reticulum stress. ***Hepatology***. 2016 Jul 7. doi: 10.1002/hep.28716. [Epub ahead of print] PubMed PMID: 27387967.

6. Este Leidmaa, Mary Gazea, Alexandre Patchev, Anna Pissioti, Nils C Gassen, Liposits Z, **Imre Kalló** and Osborne F.X. Almeida: Galanin gates ascending leptin and descending hedonic signals on orexin neurons in the lateral hypothalamus. ***Cell Metabolism*** (submitted) 2017

*7. Omar Al-Massadi, Mar Quiñones, Donald A. Morgan, **Imre Kalló**, Melissa J Chee, Monica Imbernon, Daniel Beiroa, Rosalia Gallego, Miguel Lopez, Zsolt Liposits, Kamal Rahmouni, Carlos Dieguez, Ruben Nogueiras: SIRT1/FoxO1 in the arcuate nucleus underlie MCH-induced hyperphagia, adiposity and glucose intolerance, **Heliyon** (under revision, originally submitted to **Cell Reports**, currently transferred to **Heliyon**)*

*8. Zsuzsanna Bardóczi, Balázs Pál, Áron Kőszeghy, Tamás Wilheim, Masahiko Watanabe, László Záborszky, Zsolt Liposits and **Imre Kalló**: Glycinergic input to the mouse basal forebrain cholinergic neurons. Journal of Neuroscience (under revision)*

Conference abstracts – posters and talks

1. **Kalló I**, Hrabovszky E, Molnár C S, Caraty A, Ciofi P, Coen C W, Liposits Z, Vida B (2012) SEXUAL DIFFERENCE IN THE PHENOTYPICAL CHARACTERISTICS OF THE MOUSE KISSPEPTIN NEURONES ***2nd World Conference on Kisspeptin Signaling in the Brain*** – Tokyo 2012.11.06.-2012.11.09.

2. Z Bardóczi, B Vida, M Watanabe and **I Kalló** (2013) Morphological evidence for direct interaction between glycine transporter immunoreactive cells and GnRH neurons in the mouse brain ***95th Annual Meeting of Endocrine Society***, San Francisco, USA

3. **Imre Kalló**; Erik Hrabovszky; Csilla S. Molnár; Barbara Vida; Csaba Fekete and Zsolt Liposits (2013) Projections of the hypothalamic paraventricular region to the ventral tegmental area in the rat: the integration of neuroendocrine and reward signals ***MITT***, Budapest, Hungary

4. Zsuzsanna Bardóczi, Beáta Á. Borsay, László Herczeg, Zsolt Liposits, Erik Hrabovszky and **Imre Kalló** (2014) Ultrastructural analysis of human kisspeptin neurons reveals features of mixed peptidergic and glutamatergic neurotransmission, ***COST Action BM1105 Meeting***:"CharitéCrossOver" at Charité University, Berlin, March 5-8, 2014, Germany

5. Zsuzsanna Bardóczi and **Imre Kalló** (2015) Morphological evidence for synaptic communication between GnRH axons and dopaminergic neurons in the mouse preoptic area and arcuate nucleus, ***COST Action BM1105***, Joint Scientific Meeting & Training School, Monash University Prato Center, April 27th - 29th, 2015, Italy

6. **Imre Kalló** (2015) Neuronal Feedback within the network regulating GnRH secretion. ***12th International Conference of the Polish Neuroscience Society,*** Gdansk, September 6-8, Poland

7. Wilheim Tamás, Watanabe Masahiko, Liposits Zsolt, **Kalló Imre** (2016) Morphological evidence supporting retrograde endocannabinoid signalling between GnRH neurons and their kisspeptin afferents in mice (P2/119) ***IBRO WORKSHOP BUDAPEST***, January 21-22, 2016

MSc dissertations:

1. *Anett Szilvásy-Szabó: Sexually dimorphic expression of neuropeptides in mouse kisspeptin neurons Master's thesis, Eötvös Loránd University, Biology MSc, Neuroscience and Human Biology (2013)*

2. *Fruzsina Rabi: Investigation of the morphological relationships of the GnRH-kisspeptin system in rodent models. Master's thesis, Eötvös Loránd University, Biology MSc, Neuroscience and Human Biology (2013)*

3. *Tamás Molnár: Microscopic imaging methods for the analysis of the subcellular distribution of molecules. Joint implementation of high resolution and anatomical localization. Master's thesis, Faculty of Information Technology, Pázmány Péter Catholic University (2013)*

4. *Róbert Kemecsei: Role of kisspeptin signalization in the hypothalamic regulation of reproductions and metabolism. BSc thesis, University of Veterinary Medicine Budapest, Hungary, Institute for Biology (2016)*

VIII. References

Backholer, K., J. T. Smith, A. Rao, A. Pereira, J. Iqbal, S. Ogawa, Q. Li and I. J. Clarke (2010). "Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells." Endocrinology **151**(5): 2233-2243.

Berthoud, H. R. and H. Munzberg (2011). "The lateral hypothalamus as integrator of metabolic and environmental needs: from electrical self-stimulation to opto-genetics." Physiol Behav **104**(1): 29-39.

Castro, D. C., S. L. Cole and K. C. Berridge (2015). "Lateral hypothalamus, nucleus accumbens, and ventral pallidum roles in eating and hunger: interactions between homeostatic and reward circuitry." Front Syst Neurosci **9**: 90.

Cravo, R. M., L. O. Margatho, S. Osborne-Lawrence, J. Donato, Jr., S. Atkin, A. L. Bookout, S. Rovinsky, R. Frazao, C. E. Lee, L. Gautron, J. M. Zigman and C. F. Elias (2011). "Characterization of Kiss1 neurons using transgenic mouse models." Neuroscience **173**: 37-56.

Farkas, I., I. Kallo, L. Deli, B. Vida, E. Hrabovszky, C. Fekete, S. M. Moenter, M. Watanabe and Z. Liposits (2010). "Retrograde endocannabinoid signaling reduces GABAergic synaptic transmission to gonadotropin-releasing hormone neurons." Endocrinology **151**(12): 5818-5829.

Fenselau, H., J. N. Campbell, A. M. Verstegen, J. C. Madara, J. Xu, B. P. Shah, J. M. Resch, Z. Yang, Y. Mandelblat-Cerf, Y. Livneh and B. B. Lowell (2017). "A rapidly acting glutamatergic ARC-->PVH satiety circuit postsynaptically regulated by alpha-MSH." Nat Neurosci **20**(1): 42-51.

Franceschini, I., S. H. Yeo, M. Beltramo, E. Desroziers, H. Okamura, A. E. Herbison and A. Caraty (2013). "Immunohistochemical evidence for the presence of various kisspeptin isoforms in the mammalian brain." J Neuroendocrinol **25**(9): 839-851.

Fu, L. Y. and A. N. van den Pol (2010). "Kisspeptin directly excites anorexigenic proopiomelanocortin neurons but inhibits orexigenic neuropeptide Y cells by an indirect synaptic mechanism." J Neurosci **30**(30): 10205-10219.

Goforth, P. B., G. M. Leinninger, C. M. Patterson, L. S. Satin and M. G. Myers, Jr. (2014). "Leptin acts via lateral hypothalamic area neurotensin neurons to inhibit orexin neurons by multiple GABA-independent mechanisms." J Neurosci **34**(34): 11405-11415.

Halasz, J., M. Toth, I. Kallo, Z. Liposits and J. Haller (2006). "The activation of prefrontal cortical neurons in aggression--a double labeling study." Behav Brain Res **175**(1): 166-175.

Kallo, I., B. Vida, L. Deli, C. S. Molnar, E. Hrabovszky, A. Caraty, P. Ciofi, C. W. Coen and Z. Liposits (2012). "Co-localisation of kisspeptin with galanin or neurokinin B in afferents to mouse GnRH neurones." J Neuroendocrinol **24**(3): 464-476.

Landry, M., D. Roche, E. Angelova and A. Calas (1997). "Expression of galanin in hypothalamic magnocellular neurones of lactating rats: co-existence with vasopressin and oxytocin." J Endocrinol **155**(3): 467-481.

Lee, D. K., T. Nguyen, G. P. O'Neill, R. Cheng, Y. Liu, A. D. Howard, N. Coulombe, C. P. Tan, A. T. Tang-Nguyen, S. R. George and B. F. O'Dowd (1999). "Discovery of a receptor related to the galanin receptors." FEBS Lett **446**(1): 103-107.

Leinninger, G. M. (2011). "Lateral thinking about leptin: a review of leptin action via the lateral hypothalamus." Physiol Behav **104**(4): 572-581.

Leinninger, G. M., Y. H. Jo, R. L. Leshan, G. W. Louis, H. Yang, J. G. Barrera, H. Wilson, D. M. Opland, M. A. Faouzi, Y. Gong, J. C. Jones, C. J. Rhodes, S. Chua, Jr., S. Diano, T. L. Horvath, R. J. Seeley, J. B. Becker, H. Munzberg and M. G. Myers, Jr. (2009). "Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding." Cell Metab **10**(2): 89-98.

Louis, G. W., G. M. Leinninger, C. J. Rhodes and M. G. Myers, Jr. (2010). "Direct innervation and modulation of orexin neurons by lateral hypothalamic LepRb neurons." J Neurosci **30**(34): 11278-11287.

Scott, V. and C. H. Brown (2011). "Kisspeptin activation of supraoptic nucleus neurons in vivo." Endocrinology **152**(10): 3862-3870.

Scott, V. and C. H. Brown (2013). "Beyond the GnRH axis: kisspeptin regulation of the oxytocin system in pregnancy and lactation." Adv Exp Med Biol **784**: 201-218.

Thompson, E. L., M. Patterson, K. G. Murphy, K. L. Smith, W. S. Dhillo, J. F. Todd, M. A. Ghatei and S. R. Bloom (2004). "Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis." J Neuroendocrinol **16**(10): 850-858.

Yamanaka, A., C. T. Beuckmann, J. T. Willie, J. Hara, N. Tsujino, M. Mieda, M. Tominaga, K. Yagami, F. Sugiyama, K. Goto, M. Yanagisawa and T. Sakurai (2003). "Hypothalamic orexin neurons regulate arousal according to energy balance in mice." Neuron **38**(5): 701-713.

Galanin gates ascending leptin and descending hedonic signals on orexin neurons in the lateral hypothalamus

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Highlights

- Regardless of satiety state, mice overeat palatable food (PF)
- Appetite-driving orexin cells in the lateral hypothalamus (LH) are activated by PF
- Leptin-induced suppression of orexin neurons is blocked during hedonic eating
- Galanin2 receptors convey the inhibitory effects of leptin on orexin cells in LH

Summary

Motivation to eat is determined by the energetic and sensory properties of food; foods with high hedonic value are consumed in excess of energetic needs, presumably by initially subjugating physiological controls on hedonic eating. We observed that overeating of a highly palatable food (PF) by satiated mice is accompanied by activation of appetite-stimulating orexin neurons in the lateral hypothalamus (LHA); importantly, these effects persisted even when higher levels of satiety were simulated by exogenous leptin. Since acute PF intake did not interrupt proximal leptin signalling, we hypothesized that PF availability disinhibits LHA orexin neurons from suppression by intermediary leptin-responsive galanin neurons. Confirming that galaninergic inputs to orexin neurons are compromised during PF exposure, intra-LHA application of a galanin receptor 2 agonist restored the ability of leptin to restrain PF eating. This work thus uncovers a mechanism whereby homeostatic regulatory circuits are infringed during initial experience of hedonically-loaded foods.

Keywords: hedonic eating, palatable food, high-fat diet, leptin, orexin, galanin, galanin receptor 2, lateral hypothalamic area, mouse

INTRODUCTION

The neurocircuitry involved in the neuroendocrine and behavioural regulation of feeding serves to balance energy intake with endogenous energy depots and therefore, to maintain body mass at a level appropriate to environmental demands (Sohn et al., 2013; Morton et al., 2014). The abundance and convenience of energy-rich foods with high hedonic value poses a homeostatic challenge for modern humans and predisposes them to obesity (Swinburn et al., 2011). While recognition that overweight associates with a

spectrum of somatic and mental disorders has encouraged exploration of the maladaptive peripheral and central responses to chronic overeating (Myers et al., 2010; Morton et al., 2014), the proximal mechanisms that initiate overeating remain understudied.

Largely driven by the "hedonia hypothesis of obesity", which posits that foods imbued with pleasurable signals override (and/or reset) homeostatic set-points (Berridge and Kringelbach, 2013), the rationale behind the present work is that food preferences develop over time and depend on sensory incentive-guided motivation, learning and decision-making (Sclafani, 2004; Ferreira et al., 2012). Here, we specifically addressed the question of how the physiological mechanisms that normally curtail eating become ineffective in the presence of hedonic foods.

Our study focused on the lateral hypothalamic area (LHA) where peripheral and central signals converge to regulate eating (Berthoud and Munzberg, 2011). Briefly, the "hunger hormone" ghrelin (Ghr) triggers arousal and feeding (Diano et al., 2003; Yamanaka et al., 2003) by activating orexin (OX) neurons in the LHA (Yamanaka et al., 2003) and, under normophysiological states, feeding is terminated by leptin which signals adequate replenishment of energy depots and satiety (Diano et al., 2003; Friedman, 2014;). Orexin neurons do not bear leptin receptors (LepRb) (Louis et al., 2010) but receive indirect leptin inputs from anorexigenic (pro-opiomelanocortin, POMC) and orexigenic (neuropeptide-Y, NPY/agouti-related peptide, AGRP) neurons that originate in the arcuate nucleus (ARC) (Cone, 2005); OX neurons also receive dense inputs from neighbouring LepRb-positive neurons that co-express galanin (Gal), neurotensin (Nts) and γ-aminobutyric acid (GABA) (Lenninger et al. 2009; Louis et al., 2010; Leinninger et al., 2011; Laque et al., 2013). Despite reports that Gal may be orexigenic (Leibowitz, 2005), a recent study demonstrated that Gal is responsible for mediating the inhibitory effects of leptin on OX neurons (Goforth et al., 2014).

This work ascribes the excessive intake of calories by mice that are acutely confronted with PF to an escape of LHA OX neurons from the inhibitory actions of leptin. Having shown that PF does not disrupt proximal leptin signalling, we hypothesized that overconsumption of PF results from reduced suppression of OXergic activity by LepRb-responsive Gal neurons in the LHA. Confirmation of this hypothesis was provided by the observation that intra-LHA application of a galanin receptor 2 (Gal2R) agonist reinstates the anorexigenic potency of leptin on PF intake. These findings suggest that descending hedonic (cognitive and motivational) information impinges on Gal neurons which, in turn, gate the access of peripheral signals of satiety to OX neurons. Thus, Gal neurons may represent an arena in which hedonic and homeostatic mechanisms compete.

MATERIALS AND METHODS

Animals

Three-month old male C57Bl/6 mice (Charles River, Germany) were single-housed on arrival and acclimated (2 weeks) to local conditions before use (lights on at ZT0, lights off at ZT12; also see SI). Experiments were approved by the Government of Upper Bavaria.

Food choices

Standard chow (SC) was available throughout, unless otherwise specified. With the exception of one test when mice were presented with diluted dairy crème (1.64 kcal/g; 5% fat) and SC, animals could choose between SC, high-fat (HF, 45% fat) and/or low-fat (LF, 10% fat) foods (ResearchDiets; see SI) which varied in sweetness (LF > HF > SC). The LF option was eventually dropped since mice neglected SC and LF in favour of HF (hereinafter, "palatable food", PF) and comparisons of PF intake in the choice condition (SC/PF) were compared to SC intake when only SC (SC/SC) was provided.

Brain activation by acute hedonic food experience

Mice (n=24) were habituated to the presence of two water-containing bottles and had *ad libitum* access to SC throughout the experiment. At ZT16, 4 groups of mice (n=6) could choose between bottles containing

either water/water or water/crème (see above) for 1-24 h; one group received PF for 1 h (after 24 h access to créme). At sacrifice (1 or 24 h), brains were processed to detect *c-fos* mRNA and c-Fos protein (see SI).

Food preference as a function of natural and simulated satiety states

Using a non-randomized treatment/within-subject design, mice were repeatedly exposed to the choice paradigm (HF, LF and SC over 24 h) or control diet (SC/SC), with a 2 d "wash-out" between exposures. Animals were tested in either satiated or fasted (24 h) states (5 d recovery between fasts). Ghrelin (0.3 mg/kg) or leptin (3 mg/kg) were injected i.p. immediately (Ghr) or 30 min (leptin) before presentation of SC or SC/PF at ZT3. Ghrelin-injected mice had continuous access to SC and were naturally satiated at the time of injection; leptin was injected to mimic a state of satiety in mice that had been 24 h-fasted (Fig. S1A). Food intake was monitored after 1, 3, 6 and 24 h.

Results of the first experiment were subsequently verified by applying a between-subject factorial design to 8 groups of fasted or satiated mice (n=10; BW similar between groups) that were exposed to one of the following drug-food combinations: vehicle (saline)-SC, ghrelin (Ghr)-SC or leptin-SC, vehicle-PF, and Ghr-PF or leptin-PF (Fig. S1B). As before, mice that previously had free access to SC were injected with saline or Ghr (0.3 mg/kg) while 24 h-fasted mice were injected with saline or leptin (3 mg/kg) before presentation of test foods, the intake of which was monitored after 1 h; mice were exposed to PF (24 h, 5 d before testing) to avoid novelty confounds.

Subjugation of leptin activity by hedonia-driven eating: neuroanatomical correlates

Mice (n=56) were sacrificed 1 h after eating either SC or PF. Trunk blood was collected for determination of leptin, insulin, Ghr and brains (n=7) were used to assess expression of c-fos, orexin, melanin-concentrating hormone (MCH) and neuropeptide Y (NPY) mRNA, c-Fos and pSTAT3 (Tyr705). Brains from a subset of mice (n=6) were perfused with 4% *p*-formaldehyde and immunostained for (i) c-fos and (ii) pSTAT3, (iii) orexin-A and orexin-B, (iv) c-Fos and orexin-B, (v) c-Fos and POMC, or (vi) c-Fos, orexin-B and galanin. See SI for assay details, antibodies listed in Table S1.

Restoration of leptin responsiveness by intra-LHA galanin

Mice (n=39) were outfitted with bilateral intra-LHA injection cannulae (see SI). After 2 weeks, mice were fasted (24 h) and given leptin (3 mg/kg i.p) at ZT 3, followed by an intra-LHA microinjection (0.5 µl) of either 0.9% NaCl (vehicle) or a Gal1R or Gal2R agonist (0.1 nM each) 20 min later. Following a 10 min recovery period, mice were given the SC/PF choice (intake of each food type was measured for up to 24 h). After a 6 d rest, all procedures were repeated except that mice received intra-LHA saline or a higher dose (1 nM) of the Gal1R or Gal2R agonists.

Statistics

Statistical differences were tested using GraphPad Prism 6 software. Student's *t*-tests were used to compare data from pairs of groups. Following 2-way ANOVA, *post hoc* multiple comparisons were made using Tukey's or Sidak's (repeated measures) tests (significance level: $P < 0.05$).

RESULTS and DISCUSSION

Palatable food defies homeostatic control mechanisms in LHA

Ghrelin (Ghr) stimulated consumption of PF (but not SC) within 6 h of administration to satiated mice (Fig. 1A). Similar findings were made when another set of satiated mice were given access (1 h) to SC/SC or SC/PF (Fig. 1B). The finding that Ghr selectively increases appetite for hedonic food, matches reports that Ghr infusion into the ventral tegmental area (VTA) stimulates hedonic eating (Egecioglu et al., 2010) and that Ghr increases the salience of high-fat rewards in an OX-dependent manner (Perello and Zigman, 2012).

A converse experiment showed that systemic leptin injections suppress the intake of SC more than that of PF (Fig. 1C). Escape from the inhibitory effects of leptin during intial PF eating was demonstrated in an independent experiment in which mice were also given exogenous leptin before access to either SC/SC or SC/PF (Fig. 1D). While chronic over-eating of energy-dense foods is causally linked with leptin resistance

(Munzberg et al., 2004; Myers et al., 2010; Morton et al., 2014), these are the first demonstrations that physiological signals of satiety can be overridden during a single, brief exposure to hedonic food.

Notwithstanding the possibility that hedonic foods subdue leptin actions by acutely disrupting its ability to decrease the reward salience of foods (Hommel et al., 2006; Domingos et al., 2011), we next sought to identify the initial neural correlates of hedonic eating. In a choice paradigm (SC and milk as PF for 1 h at ZT16), satiated mice consumed more PF than SC (0.52±0.1 *vs.* 0.02±0.02 kcal/g BW) and showed activation (upregulated c-fos mRNA and protein expression) of brain regions implicated in the processing of reward/hedonic information (Fig. 1E), e.g. piriform cortex (olfaction), insular cortex (taste), ventral pallidum (hedonic, reward) orbitofrontal cortex and lateral septum (association) and amygdala (emotional learning and reward valuation).

Hedonic eating was also accompanied by strong activation of two satiety centers, the ventromedial and dorsomedial hypothalamic nuclei, as well of the posterior LHA. The LHA contains orexin (OX)-expressing cells that are activated by both, hunger (Yamanaka et al., 2003) and PF (Wortley et al., 2003; Harris et al., 2005; Valdivia et al., 2014). Consistent with those reports, consumption of PF (milk) by pre-satiated mice rapidly induced pre-pro-OX mRNA transcript levels in the LHA (Fig. 1F, *upper panel*). This response waned with time but was rekindled by introduction of a different hedonic stimulus (high-fat/high carbohydrate food, HF), suggesting that OX neurons respond to the hedonic properties of food. Ingestion of HF by fasted mice was associated with an upregulation of OX, but not of melanin-concentrating hormone, MCH (another LHA orexigenic peptide), transcription (Fig. 1F, *lower panel*). Thus, PF availability during a state of satiation triggers OX neuron activity in defiance of leptin signals (Yamanaka et al., 2003; Goforth et al., 2014).

Orexin neurons in LHA escape inhibition by leptin during hedonic eating

Prompted by the above results (Fig. 1D, 2A), examination of the regulation of orexigenic peptide expression in the LHA revealed that exogenous leptin does not influence pre-pro-OX or pro-MCH mRNA expression levels in mice exposed to either SC/PF or SC/SC (Fig. 2B and C). Predictably (Goforth et al., 2014), leptin reduced the number of c-Fos/OX-coexpressing LHA neurons in mice offered SC/SC (Fig. 2D, E). On the other hand, the number of c-Fos/OX-expressing neurons did not differ between saline- and leptin-treated mice given the SC/PF choice (Fig. 2D, E). While consistent with the behavioral data (Fig. 1D, 2A), these data are incommensurate with the known inhibitory effects of leptin on OX neuron activity (Yamanaka et al., 2003; Goforth et al., 2014). In summary, these data show that hedonic eating nullifies the anorexigenic effects of leptin and suggest that OX neurons are uniquely sensitive to the hedonic properties of food even during states of satiety (cf. Fig. 1F).

Initial contact with PF does not interrupt leptin signalling in LHA

Effective dosage of leptin was confirmed by serum leptin levels, assayed at 1.5 h post-injection (Fig. 3A). Serum insulin levels were unchanged in mice offered SC/PF but were slightly reduced in leptin-treated mice given SC/SC, consistent with the observed suppression of SC/SC intake by leptin (Fig. S2, 1D). Serum Ghr levels were affected by neither leptin administration nor PF ingestion (Fig. S2).

Measurements of pSTAT3 (Tyr705) which mediates the actions of leptin (Munzberg et al., 2004; Myers et al., 2010), revealed that leptin signaling in the brain remains intact during acute PF eating. Briefly, exogenous leptin induced pSTAT3 levels in various hypothalamic nuclei (Fig. 3B-3C), including the LHA (Fig. 3E) and arcuate nucleus (ARC) (Fig. 3D). Notably, leptin did not upregulate pSTAT3 levels in the VTA (not shown), a key component of the motivation-reward neurocircuitry.

Leptin is known to stimulate anorexigenic POMC-expressing neurons in the ARC which, in turn, inhibit orexigenic neuropeptide Y (NPY) neurons which are also located in the ARC (Myers et al., 2010; Morton et al., 2014). We found that exogenous leptin concomitantly activates POMC neurons (c-Fos expression) (Fig. 3F, 3G) and downregulates NPY mRNA expression in mice given SC/SC (Fig. 3H). Similar results were obtained in saline and leptin-treated mice offered SC/PF, indicating that PF does not disrupt the feeding circuitry in the Arc (cf. Sohn et al., 2013; Magnan et al., 2015). Strikingly, leptin injections did not add significantly to the effects of PF on POMC cell activation (Fig. 3F), indicative of a “ceiling effect”. The apparent incongruity between these findings and those showing that PF abrogates the inhibitory effects of leptin on OX neurons

(Fig. 1C, 1D, 2A) led us to consider recruitment of additional mechanisms when hedonic foods are encountered during a state of satiety.

Galanin (Gal) restores leptin efficacy during hedonia-driven overeating

We subsequently aimed to identify the mechanism(s) through which hedonic processes negate the appetite-suppressing actions of leptin during PF exposure. Given that ARC (POMC and NPY) neurons may not be the loci at which hedonic foods act to override leptin actions, our focus shifted to the LHA where Gal neurons terminate in the vicinity of OX neurons (Fig. 4A; Louis et al., 2010; Laque et al., 2013; Goforth et al., 2014). Although Gal neurons also express the inhibitory neurotransmitter GABA and the peptide neurotensin (Nts) leptin-induced hyperpolarization of OX neurons reportedly depends solely on Gal (Goforth et al., 2014).

To examine the role of Gal in the escape from leptin-induced suppression of PF consumption, galanin receptor 1 and 2 agonists (M617 for Gal1R and M1145 for Gal2R, each at 0.1 or 1 nM; Webling et al., 2012) were applied intra-LHA (Fig. 4B) immediately before introduction of PF; sub-groups of mice received i.p. injections of either saline or leptin (3 mg/kg) 30 min before PF presentation. Treatment with the low, but not high, dose of M1145 "permitted" leptin to exert its inhibitory effects on PF eating (Fig. 4C, D); in contrast, neither dose of M617 reinstated leptin potency during 24 h of PF availability (not shown). Contrasting reports that Gal stimulates eating in rats (Leibowitz, 2005) most likely reflect site-specific actions of Gal. In summary, PF overeating results from blockade of the inhibitory actions of Gal on neighbouring OX neurons.

Possible circuit mediating hedonic brain activation

The present results inform understanding of where and how hedonic foods override physiological brakes on eating. Specifically, they show that PF consumption does not disrupt leptin signaling *per se*, but rather interferes with the relay of leptin-sensitive inhibitory Gal-ergic inputs to OX neurons in the LHA. Since the LHA is innervated by the forebrain, including the nucleus accumbens (NAc) shell (Berthoud and Munzberg, 2011), our results support the view that the LHA is a node where centrally-generated hedonic signals are integrated with information on energy status to guide decisions "to eat or not to eat" (cf. Berthoud and Munzberg, 2011; Castro et al., 2015). Besides increasing appetite, LHA OX cells terminating the VTA are implicated in reward anticipation and conditioning (Castro et al., 2015) while those innervating the central amygdala contribute to flavor learning (Risco and Mediavilla, 2014).

In summary, we propose that LHA OX triggers a cycle, from the initiation of PF preference and overeating to the sensory, motivational and cognitive processes that drive excessive "wanting" of PF and ultimately, obesity. Our current working model (Fig. 4E) suggests that hedonic overeating results from the loss of Gal-mediated inhibitory control over OXergic neurons. The model does not however include other recently-proposed molecular players and switches through which PF may modulate leptin signaling in the hypothalamus (Koch et al., 2015; Fenselau et al., 2017; Campbell et al., 2017).

Author contributions

E.L., A.V.P. and O.F.X.A. conceived and planned the experiments; E.L., M.G., A.V.P., A.P., M.K. and O.F.X. performed experiments; E.L., A.V.P., Z.L. and I.K. carried out histological analyses; E.L., A.V.P., I.K. and O.F.X.A participated in data analysis and interpretation; E.L., I.K. and O.F.X. wrote the manuscript; all authors read and approved the manuscript.

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References

Berridge, K.C., and Kringelbach, M.L. (2013). Neuroscience of affect: brain mechanisms of pleasure and displeasure. Curr. Opin. Neurobiol *23*, 294-303.

Berthoud, H.-R., and Munzberg, H. (2011). The lateral hypothalamus as integrator of metabolic and environmental needs: from electrical self-stimulation to opto-genetics. Physiol. Behav. *104*, 29-39.

Castro, D.C., Cole, S.L., and Berridge, K.C. (2015). Lateral hypothalamus, nucleus accumbens, and ventral pallidum roles in eating and hunger: interactions between homeostatic and reward circuitry. Front. Sys. Neurosi. *9*, 90.

Campbell J.N., Macosko E.Z., Fenselau H., Pers T.H., Lyubetskaya A, Tenen D., Goldman, M., Verstegen, A.M.J., Resch, J.M., Rosen, E.D., et al. Nat. Neurosci. *3*, 484-496.

Cone R.D. (2005). Anatomy and regulation of the central melanocortin system. Nat. Neurosci. *8;* 571-578.

Diano, S., Horvath, B., Urbanski, H.F., Sotonyi, P., and Horvath, T.L. (2003). Fasting activates the nonhuman primate hypocretin (orexin) system and its postsynaptic targets. Endocrinology *144*, 3774-3778.

Domingos, A.I., Vaynshteyn, J., Voss, H.U., Ren, X., Gradinaru, V., Zang, F., Deisseroth, K., Araujo, I.E. de, and Friedman, J. (2011). Leptin regulates the reward value of nutrient. Nat. Neurosci. *14*, 1562-1568.

Egecioglu, E., Jerlhag, E., Salome, N., Skibicka, K.P., Haage, D., Bohlooly-Y, M., Andersson, D., Bjursell, M., Perrissoud , D., Engel, J.A. and Dickson. S.L. (2010). Ghrelin increases intake of rewarding food in rodents. Addict. Biol. *15*, 304-311.

Fenselau, H., Campbell, J. N., Verstegen, A. M. J., Madara, J. C., Xu, J., Shah, B.P., Resch, J.M., Yang, Z., Mandelblat-Cerf, Y., Livneh, Y. and Lowell, B. B. (2016). A rapidly acting glutamatergic ARC→PVH satiety circuit postsynaptically regulated by α-MSH. Nat. Neurosci. *1*, 42-51.

Ferreira, J.G., Tellez, L.A., Ren, X., Yeckel, C.W., and Araujo, I.E. de (2012). Regulation of fat intake in the absence of flavour signalling. J. Physiol. *590*, 953-972.

Franklin, K.B.J., and Paxinos, G. (2008). The Mouse Brain in Stereotaxic Coordinates (Amsterdam: Academic Press).

Friedman, J. (2014). 20 years of leptin: leptin at 20: an overview. J. Endocrinol. *223*, T1-8.

Goforth, P.B., Leinninger, G.M., Patterson, C.M., Satin, L.S., and Myers, M.G., (2014). Leptin acts via lateral hypothalamic area neurotensin neurons to inhibit orexin neurons by multiple GABA-independent mechanisms. J. Neurosci. *34*, 11405-11415.

Harris, G.C., Wimmer, M., and Aston-Jones, G. (2005). A role for lateral hypothalamic orexin neurons in reward seeking. Nature *437*, 556-559.

Hommel, J.D., Trinko, R., Sears, R.M., Georgescu, D., Liu, Z.-W., Gao, X.B., Thurmon, J.J., Marinelli, M. and DiLeone, R.J. (2006). Leptin receptor signaling in midbrain dopamine neurons regulates feeding. Neuron *51*, 801-810.

Koch, M., Varela, L., Kim, J.G., Kim, J.D., Hernández-Nuño, F., Simonds, S. E., Castorena, C.M., Vianna, C.R., Elmquist, J.K., Morozov, Y.M., et al. (2015). Hypothalamic POMC neurons promote cannabinoid-induced feeding. Nature, *519*(7541), 45–50.

Laque, A., Zhang, Y., Gettys, S., Nguyen, T.-A., Bui, K., Morrison, C.D., and Munzberg, H. (2013). Leptin receptor neurons in the mouse hypothalamus are colocalized with the neuropeptide galanin

Leibowitz, S.F. (2005). Regulation and effects of hypothalamic galanin: relation to dietary fat, alcohol ingestion, circulating lipids and energy homeostasis. Neuropeptides *39*, 327-332.

Leinninger, G.M., Jo, Y.-H., Leshan, R.L., Louis, G.W., Yang, H., Barrera, J.G., Wilson, H., Opland, D.M., Faouzi, M.A., Gong, Y., et al. (2009). Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding. Cell Metab. *10*, 89-98.

Leinninger, G.M., Opland, D.M., Jo, Y.-H., Faouzi, M., Christensen, L., Cappellucci, L..A., Rhodes, C.J., Gnegy, M.E., Becker, J.B., Pothos, E.N., et al. (2011). Leptin action via neurotensin neurons controls orexin, the mesolimbic dopamine system and energy balance. Cell Metab. *14*, 313-323.

Louis, G.W., Leinninger, G.M., Rhodes, C.J., and Myers, M.G., Jr. (2010). Direct innervation and modulation of orexin neurons by lateral hypothalamic LepRb neurons. J. Neurosci. *30*, 11278-11287.

Magnan C, Levin BE, Luquet S (2015) Brain lipid sensing and the neural control of energy balance. Mol. Cell. Endocrinol. *418*, 3–8.

Morton, G.J., Meek, T.H., and Schwartz, M.W. (2014). Neurobiology of food intake in health and disease. Nat. Rev. Neurosci. *15*, 367-378.

Munzberg, H., Flier, J.S., and Bjorbaek, C. (2004). Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology *145*, 4880-4889.

Myers, M.G., JR, Leibel, R.L., Seeley, R.J., and Schwartz, M.W. (2010). Obesity and leptin resistance: distinguishing cause from effect. Trends Endocrinol. Metab *21*, 643-651.

Perello, M., and Zigman, J.M. (2012). The role of ghrelin in reward-based eating. Biol. Psychiat. *72*, 347-353.

Risco, S., and Mediavilla, C. (2014). Orexin-1 receptor antagonist in central nucleus of the amygdala attenuates the acquisition of flavor-taste preference in rats. Pharmacol. Biochem. Behav. *126*, 7-12.

Sclafani, A. (2004). Oral and postoral determinants of food reward. Physiol. Behav. *81*, 773-779.

Sohn, J.W., Elmquist, J.K., and Williams, K.W. (2013). Neuronal circuits that regulate feeding behavior and metabolism. Trends Neurosci. *36*, 504-512.

Swinburn, B.A., Sacks, G., Hall, K.D., McPherson, K., Finegood, D.T., Moodie, M.L., and Gortmaker, S.L. (2011). The global obesity pandemic. Shaped by global drivers and local environments. Lancet *378*, 804-814.

Valdivia, S., Patrone, A., Reynaldo, M., and Perello, M. (2014). Acute high fat diet consumption activates the mesolimbic circuit and requires orexin signaling in a mouse model. PloS One *9*, e87478.

Webling, K.E.B., Runesson, J., Bartfai, T., and Langel, U. (2012). Galanin receptors and ligands. Front. Endocrinol. *3*, 146.

Wortley, K.E., Chang, G.-Q., Davydova, Z., and Leibowitz, S.F. (2003). Peptides that regulate food intake: orexin gene expression is increased during states of hypertriglyceridemia. Am. J. Physiol. *284*, R1454-1465.

Yamanaka, A., Beuckmann, C.T., Willie, J.T., Hara, J., Tsujino, N., Mieda, M., Tominaga, M., Yagami, Ki, Sugiyama, F., Goto, K., et al. (2003). Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron *38*, 701-713.

Figure Legends

Fig. 1. Role of OX cells in LHA in the overriding of leptin signals during hedonic eating. (A) Within-subject design (n=18). Ghrelin (Ghr, 0.3 mg/kg) injected in a satiated state stimulates cumulative (24 h) intake of palatable food (PF) ($F_{1,34}$=12.6, *Sidak's post-hoc *P*<0.01), but not SC consumption ($F_{1,34}$=6.6, *P*<0.05, §post-hoc *P*<0.05). **(B)** Ghr stimulates PF ingestion (n=10/group, independent set of mice cf. A; measurements 1 h-post treatment; *drug effect*: $F_{1,35}$=10.9, *P*=0.002, * Tukey's post-hoc *P*<0.05). **(C)** Within-subject design (n=18). Leptin (3 mg/kg i.p., after 24 h fast) suppresses consumption of SC ($F_{1,34}$=50.2, *P*<0.0001; *time x drug interaction*: $F_{3,34}$=33.1, *P*<0.0001, * Sidak's post hoc P<0.05) but leptin only partially reduces PF intake ($F_{1,34}$=7.1, *P* < 0.05; *time x drug interaction:* $F_{3,34}$=24.9, *P*<0.0001, §*P*<0.05). **(D)** Leptin initially suppresses SC but not PF intake (n=20-22/group; *leptin effect*: $F_{1,81}$=22.9, *P*<0.0001; *food type x leptin interaction*: $F_{1,81}$=2.96, *P*=0.09, * Tukey's post hoc *P*<0.05). **(E)** Table depicts relative changes in c-fos mRNA expression after consumption of a hedonic solution (milk) vs. water in mice already satiated on standard chow (SC); liquids were co-presented with SC. Solutions were presented at ZT16 for 1 h. Among other areas, the LHA showed increased neural activity when milk was consumed. **(F)** Expression of pre-pro-OX (but not MCH) mRNA is increased after consumption of milk or a novel solid PF (high fat, HF) for 1 h and also after 1 h of exposure to a novel solid PF (HF) (n=2-3/group) in satiated (*upper panels*) and fasted states and quantitative analysis (mean ± SEM) from overnight-fasted mice (*lower panel*) are shown (n=6-7/group; **P*<0.05).

Fig. 2. OX neurons in LHA escape inhibition by leptin. (A) Food intake by animals (z-scores in Fig. 1D) in which mRNA levels shown in **B-E** were monitored. **(B and C)** Ingestion of solid PF increases prepro-orexin (OX) ($F_{1,20}$=16.92, *P*=0.0005), but not pro-MCH, mRNA levels and were not further altered by exogenous leptin (3 mg/kg). **(D)** Immunohistochemical double labelling for cFOS (black) and orexin (brown) in the LHA. Leptin treatment significantly decreased the number of cFOS-expressing orexin neurons (*arrows*) in the LH of mice kept on SC/SC diet, but not in those provided with the SC/PF choice. The inset shows cells single labelled for cFOS (*arrowheads*), orexin (*open arrow*), and double labelled for cFOS and orexin (*arrows*) at higher magnification. **(E)** Quantitative analysis of LHA neurons co-expressing c-Fos and OX reveals leptin-

induced reductions in activation of OX cells in mice exposed to SC; leptin does not alter OX cell when PF is consumed (*drug effect*: $F_{1,21}$=27.4, *P*<0.0001; *food type effect*: $F_{1,21}$=13.9,*P*=0.0012; *drug x food type interaction*: $F_{1,21}$=12.2, *P*=0.02). Quantitative data shown as mean ± SEM; n=6-7/group; *Tukey's post-hoc *P*< 0.05. Scale bar represents 50 µm.

Fig. 3. Hedonic PF eating does not interrupt proximal leptin signalling in hypothalamus. Leptin was injected (i.p. 3 mg/kg at ZT 3) 30 min before access to SC/SC and PF/SC choice. **(A)** Serum levels of leptin 1.5 h post-injection (n=8-10/group). *P<0.05. **(B)** Leptin similarly induced phosphorylation of STAT3 (pSTAT3, Tyr705) in SC/SC- or SC/PF-exposed groups ($F_{1,20}$=59.5, *P*=0.0001). Total and pSTAT3 measured in hypothalamic lysates by capillary electrophoresis; representative results in **(C)**. Immunostaining of pSTAT3 *(green)* in the arcuate nucleus (Bregma -1.46) **(D)** and LHA (Bregma -1.82) **(E);** material was collected at end of experimental manipulations (i.p. leptin 30 min before consumption of SC/SC or SC/ PF for 1 h). **(F)** Quantitative analysis of c-Fos and POMC double-immunostained arcuate neurons shows increased activation (c-Fos) of POMC neurons after either leptin or SC/PF exposure (*main leptin and food type effects*: $F_{1,20}$=19.9, *P*=0.0002 and $F_{1,20}$=12.1, *P*=0.002, respectively; *leptin x food type interactions*: $F_{1,20}$=2.66, *P*=0.12). **(G)** Shown are POMC neurons (*red*) co-expressing c-Fos *(green)* in the arcuate nucleus of leptin-injected mice given access to SC/SC or SC/PF choices; the magnified inset shows that c-Fos expression is upregulated in a proportion of POMC-positive neurons. **(H)** Levels of NPY expression are reduced by both leptin in SC/SC mice and in mice exposed to SC/PF (*leptin X food type interaction*: $F_{1,25}$=7.5, *P*=0.01). Numerical data depicted as mean ± SEM (n=6-7/group);*Tukey's post hoc *P*<0.05. Scale bars: 50 µm in E; 20 µm in G.

Fig. 4. Galanin receptor 2 (Gal2R) agonist reinstates leptin's ability to suppress hedonic eating. (A) Galanin-immunoreactive fibers (blue) terminating in the vicinity of activated (c-Fos-immunoreactive, *green*) orexin A-immunoreactive neurons *(red)* neurons in the LHA (*arrowheads*, sites of contact). **(B)** Sites of galanin agonist microinjections were verified; correctly targeted injections of saline (*black rectangles*), Gal1R (*purple triangles*) and Gal2R (*blue circles*) are shown on coronal maps (from Franklin and Paxinos, 2008); cresyl violet-stained coronal sections with LHA outlined (*lower part,* dotted lines). **(C, D)** Intra-LHA Gal2R agonist M1145 restores suppressive effects of i.p. leptin (3 mg/kg) on PF ingestion. Compared to saline-treated (control) mice, PF intake was significantly reduced in M1145-treated mice after 1 h (**C;** $F_{1,18}$=6.9, *P*=0.017). Data are shown as mean±SEM (n=10-12/group). **P*<0.05. **(E)** Working model of the circuitry upon which leptin acts to signal energy balance and satiety (Arc nucleus is major component of the blue sphere) during SC feeding. Inhibition of OX neurons in the lateral hypothalamic area (LHA), involving leptin stimulation of Gal secretion from LepRb-bearing Gal neurons in the vicinity of OX neurons (see Fig. 4A), is considered a key mechanism in the suppression of feeding. The model postulates that besides positive emotional, cognitive and hedonic drivers, PF overeating results from a disruption of the ability of leptin to influence communication between Gal and OX cells since activation of Gal2R in the LHA reduces PF consumption (see Fig. 4C, 4D).