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Regulation of energy homeostasis by hypothalamic circuits; light- and electron microscopic studies in rodents

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Theses of the Ph.D Dissertation

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I. Introduction

The obesity epidemic is one of the major health problem of our days [1]. More than 60% of the population is overweight or obese in the USA and in Europe [2]. Obesity is not only esthetical problem, but it is also major risk factor of devastating diseases like type 2 diabetes, cardiovascular diseases *etc*… [1]. Despite the very high impact on population health and healthcare cost, efficient, non-invasive and side effect free treatment is currently not available against obesity. Large pharmaceutical companies try to develop efficient anti-obesity drugs based on the currently available drug targets without major breakthrough indicating the necessity of the discovery of novel anti-obesity drug targets. **Therefore, better understanding of the regulatory mechanisms of energy homeostasis has critical importance in the fight against obesity.**

I. 1 Role of the hypothalamic ARC in the regulation of the energy homeostasis

Circulating hormones like leptin, ghrelin, cholecystokinin, peptide YY and insulin transmit signals to the central nervous system about the actual conditions of energy stores and about the consumed food [3]. This communication is critical for the maintenance of energy homeostasis [3]. Genetic alterations in these pathways cause obesity syndrome like in leptin or leptin receptor deficient animals or humans and in mice lacking insulin receptor in the brain [4-8]. A critical brain area that can sense these energy homeostasis related humoral signals is the ARC [3]. Ablation of the ARC by neonatal monosodium glutamate treatment induces obesity and leptin resistance [9]. At least two major energy homeostasis-related neuronal groups are located in the ARC [3]. There is a ventromedially located orexigenic neuronal population that expresses two potent orexigenic peptides, the neuropeptide Y (NPY) and the agouti-related protein (AGRP) [3] and also express the classical transmitter gamma-aminobutyric acid (GABA) [10] that has been also shown to stimulate food intake [11]. NPY is one of the most potent orexigenic signals [3]. Central administration of NPY causes marked increase of food intake, weight gain and increased adiposity [12]. **The effect of NPY on the weight gain, however, cannot be exclusively accounted to its effect on the food intake.** NPY also has potent inhibitory effect on the energy expenditure [13]. NPY elicits these effect *via* the G protein coupled postsynaptic Y1 and Y5 receptors [12]. AGRP also increases the food intake and inhibits the energy expenditure [14]. The effect of AGRP on the energy homeostasis is mediated by two centrally expressed melanocortin receptors, the melanocortin 3 and 4 receptors (MC3R and MC4R). AGRP is the endogenous antagonist of these receptors [14]. Changes of energy availability regulate the expression of both peptides in the ARC neurons. While fasting stimulates the NPY and AGRP expression in these neurons, leptin administration inhibits the synthesis of the orexigenic peptides [3]. However, genetic ablation of NPY or AGRP has no major effect on the energy homeostasis; the critical importance of the orexigenic ARC neurons has been demonstrated by the life threatening anorexia of mice after ablation of the NPY/AGRP neurons [15, 16]. The proopiomelanocortin (POMC) and cocaine- and

amphetamine-regulated transcript- (CART) synthesizing neurons located in the lateral part of the ARC has opposite effect on the regulation of energy homeostasis [3]. The POMC derived α-melanocyte-stimulating hormone (α-MSH) is well known about its potent anorexigenic effect [3]. Central administration of α-MSH reduces food intake and simultaneously increases the energy expenditure [3]. The α-MSH exerts its effect as the agonist of the MC3R and MC4R [17]. CART also inhibits food intake and can completely block the NPY induced feeding response [18]. Currently, little information is available about the effect of CART on the energy expenditure. The receptor(s) of CART has not been identified yet. The POMC/CART neurons are sensitive to the effects of peripheral energy homeostasis-related hormones, like leptin and insulin [3], but these neurons are regulated differently than the NPY/AGRP neurons. Fasting inhibits the POMC and CART synthesis, while leptin administration stimulates the expression of these genes [3]. Despite the presence of leptin receptor in these cells, indirect effect of leptin that is mediated by GABAergic neurons is also critical in the regulation of the POMC/CART neurons [19]. The importance of the POMC/CART neurons in the regulation of energy homeostasis was also demonstrated by genetic studies. Genetic ablation of the POMC or MC4R genes results in morbid obesity in mice [20, 21]. Mutations of the melanocortin pathway also cause obesity in humans. Indeed, mutations of this pathway are the most frequent reason of the human monogenic obesity syndromes [22]. The absence of CART has less profound effect [23]. The CART knock out mice develop only late onset obesity [23]. In humans, a single nucleotide polymorphism (A1475G) of the CART gene is associated with human obesity syndrome [23]. The two feeding related neuronal populations of the ARC sense and integrate the energy homeostasis related signals and transmit it toward the so-called second order feeding related neuronal populations, including the hypothalamic paraventricular nucleus (PVN), the hypothalamic dorsomedial nucleus and the histaminergic neurons of the tuberomammillary nucleus (TMN). In addition to, the homeostatic regulation of the energy homeostasis, the feeding related neurons of the ARC are also involved in the regulation of food intake by adverse conditions like infection and stress [24, 25].

I. 2 Role of the PVN in the regulation of energy homeostasis

The PVN is a triangular shaped nucleus located on the two sides of the upper part of the third ventricle. It consists of magnocellular and parvocellular parts [26]. The oxytocin- and vasopressin-synthesizing neurons of the magnocellular part are involved in the regulation of the posterior pituitary function [26]. The parvocellular part can be further divided into five subdivisions: the anterior, periventricular, medial, ventral and lateral subdivisions and the dorsal cap [27]. The periventricular, and medial parvocellular subdivisions house both hypophysiotropic and non-hypophysiotropic neurons, while the other parvocellular subdivisions house only non-hypophysiotropic neurons. The hypophysiotropic neurons project to the external zone of the median eminence (ME) where they secrete their hypophysiotropic hormones into the extracellular space around

the fenestrated capillaries [26]. These hormones reach the anterior pituitary *via* the hypophyseal portal circulation and regulate the hormone production of this endocrine gland. There are three types of hypophysiotropic neurons in the parvocellular part of the PVN: the somatostatin-, the corticotropin-releasing hormone- (CRH) and the thyrotropin-releasing hormone- (TRH) synthesizing neurons. The somatostatin neurons inhibit the growth hormone synthesis of the pituitary, while the hypophysiotropic CRH and TRH neurons are the central regulators of the hypothalamic-pituitary-adrenal (HPA) and thyroid (HPT) axes, respectively. All of these neuroendocrine axes have major impact on the regulation of the energy homeostasis. A large population of the non-hypophysiotropic neurons in the parvocellular part of the PVN regulates autonomic functions. These neurons project to the intermediolateral column of the spinal cord and to brainstem nuclei involved in the regulation of energy homeostasis like the nucleus tractus solitarii, the dorsal motor nucleus of vagus, the parabrachial nucleus and the catecholaminergic neurons of the ventral medulla [28]. *Via* these nuclei, the PVN multisynaptically linked to the pancreas, white and brown adipose tissue (BAT), liver and muscle [29-31]. Thus, these so-called preautonomic neurons of the PVN can regulate the energy homeostasis by controlling the lipid metabolism and storage, thermogenesis, gluconeogenesis and insulin synthesis [30]. However, the PVN receives energy homeostasis related inputs *via* multiple neuronal pathways and also *via* hormones, one of its most important energy homeostasis related input originates from the ARC [3]. Both the orexigenic and the anorexigenic neuronal groups densely innervate the neurons of the parvocellular part of the PVN [3]. In many instances, the same parvocellular neurons are innervated by both the orexigenic and anorexigenic ARC neurons [27]. The PVN is a critical mediator of the effects of ARC neurons on the energy homeostasis [3]. Focal administration of NPY into the PVN markedly increases food intake [13], increases the carbohydrate utilization [32], decreases the energy expenditure and the uncoupling protein 1 expression in the BAT [33,34] and induces body weight gain [35].Both postsynaptic NPY receptors, the Y1 and Y5, are expressed in the PVN [36], coupled to pertussis-toxin sensitive Gi/o proteins [37], and lead to the inhibition of cyclic adenosine monophosphate (cAMP) accumulation by inhibiting adenylate cyclase [38]. Some of the effects of NPY on energy expenditure are exerted through the regulation of TRH and CRH gene expression in the PVN *via* the modulation of the cAMP pathway [39-42]. NPY also has been shown to inhibit the GABAergic inputs of the parvocellular neurons of the PVN [43]. Similarly to NPY, intraPVN administration of AGRP also increases the food intake [44].In contrast to the orexigenic peptides, α-MSH has potent anorexigenic effect when injected into the PVN [45]. Most of the effects of the α-MSH are mediated by the MC4R. The MC4R knockout (KO) mice are hyperphagic and have decreased energy expenditure [21]. Re-expression of MC4R exclusively in the PVN rescues the hyperphagic phenotype of the MC4R KO mice, but has only little effect on the energy expenditure [46] substantiating the importance of the PVN in the mediation of the effect

of melanocortins on the food intake. α-MSH also regulates the HPA and HPT axes by stimulating the CRH and TRH gene expression in the PVN [47, 48].

I. 3 Retrograde transmitter systems in the parvocellular part of the PVN

Using patch clamp electrophysiology, our laboratory showed that NPY inhibits both the GABAergic and the glutamatergic inputs of the parvocellular neurons [49]. These effects were completely prevented by the intracellular administration of the calcium chelator drug BAPTA demonstrating that NPY inhibits the inputs of the parvocellular neurons by stimulating retrograde transmitter release of the target cells [49]. The most widely utilized retrograde transmitter system in the brain is the endocannabinoid system [50]. In the central nervous system, the primary receptor of the endocannabinoid signaling system is the type 1 cannabinoid receptor (CB1) [50]. The two most abundant endogenous ligands of this receptor are the 2-arachidonoylglycerol (2-AG) and the anandamide [50]. 2-AG is synthesized by postsynaptic neurons in the perisynaptic region and acts on the CB1 located in the perisynaptic region of the presynaptic terminals [50]. Activation of the CB1 inhibits the activity of the presynaptic terminals [50]. An important regulator of the endocannabinoid synthesis is the synaptic activity. The synthesizing enzyme of 2-AG, the diacylglycerol lipase α (DAGLα), is activated in response to increased synaptic activity which effect is mediated by metabotropic receptors coupled to phospholipase C beta like metabotropic glutamate receptor 1 and 5, muscarinic acetylcholine receptor M1 and M3 [51].Our laboratory has shown that CB1 is present in both the inhibitory and excitatory terminals innervating the parvocellular neurons in the PVN [52] and the endocannabinoid system has been shown to mediate the effects of ghrelin and the glucocorticoids on the parvocellular neurons of the PVN [53, 54]. We have found that inhibition of CB1 by AM251 prevents the effect of NPY on the GABAergic input of parvocellular neurons [49]. **However, the dose of AM251 that were sufficient to prevent the effect of ghrelin on the glutamatergic inputs of the parvocellular neurons [53], did not prevent the effects of NPY on the glutamatergic inputs [49] suggesting that other retrograde signaling system(s) is also involved in the mediation of the NPY induced effects.** Nitric oxide (NO) is a gaseous transmitter [55]. NO is synthesized by a family of the NO synthesizing (NOS) enzymes: the neuronal NOS (nNOS), the inducible NOS and the endothelial NOS [55]. Among these enzymes, the nNOS is present in neurons [55]. The most sensitive receptor of NO is the soluble guanylate cyclase (sGC) [55]. In the hippocampus, both the nNOS and the sGC can be observed in both pre- and postsynaptic localization [56] suggesting that NO can serve as both anterograde and retrograde transmitter. Electrophysiological experiments also provided evidence supporting the retrograde transmitter role of NO and the interaction of the endocannabinoid and NO signaling in the presynaptic plasticity in the hippocampal formation [57].**However, nNOS is also present in the PVN, little is known about the localization of the elements of NO signaling in this nucleus and it is also unknown whether NO is utilized as a retrograde transmitter in this nucleus** [58].

I. 4 Feedback regulation of the hypophysiotropic TRH neurons

The hypophysiotropic TRH plays critical role in the regulation of the energy homeostasis [27]. These neurons control the hormone production of the thyroid gland through the regulation of the TSH secretion of the thyrotroph cells in the anterior pituitary [27]. The thyroid hormones (THs) are important regulators of the energy homeostasis [27]. In the absence of THs, the basal metabolic rate is decreased by 30% and the cold induced thermogenesis is also absent in hypothyroid animals [27]. The main regulator of the HPT axis is the negative feedback effect of THs that ensure the relatively stable circulating TH levels [27]. Thus, when the peripheral levels of THs are increased, the TRH synthesis is inhibited by THs [27]. The hypophysiotropic TRH neurons contain the thyroid hormone β2 receptor (TRβ2) that is essential for the feedback regulation of these cells [27]. In addition, implantation of T3 adjacent to the PVN inhibits the TRH expression on the side of the implantation [27]. However, restoration of the circulating T3 levels in hypothyroid animals without administration of the prohormone T4 is not sufficient to normalize the TRH expression in the PVN [27]. These data demonstrate that hypothalamic conversion of the prohormone T4 to its active form, T3, is necessary for the feedback regulation of the TRH neurons. In the hypothalamus, T4-T3 conversion is catalyzed by the type 2 deiodinase enzyme (D2) [59]. D2 activity or mRNA, however, is not present in the PVN where the hypophysiotropic TRH neurons reside [60]. D2 is synthesized in the hypothalamus by a special glial cell types, the tanycytes [60]. Tanycytes line the lateral wall and the floor of the third ventricle behind the optic chiasm. The long basal process of these cells projects to the hypothalamic dorsomedial and ventromedial nuclei, into the ARC and into the external zone of the ME [27]. Thus, the cell bodies of the TRH neurons are located relatively far from the TH activating cells of the hypothalamus. In the external zone of the ME, however, end feet processes of the tanycytes and the axon terminals of the hypophysiotropic TRH neurons are closely associated raising the possibility that T3 released from the tanycytes may be taken up by the hypophysiotropic terminals and transported to the cell bodies of TRH neurons where T3 could bind to the nuclear TRβ2 [27]. The TH transport is mediated by TH transporters [27]. The main TH transporters are the monocarboxylate transporter 8 (MCT8), organic anion-transporting polypeptide 1c1, Lat1 and Lat2 [61]. The main TH transporter of neurons is MCT8. The absence of MCT8 causes serious neurological symptom in humans and upregulation of the HPT axis in both humans and mice [62, 63] suggesting that MCT8 is involved in the feedback regulation of the HPT axis. **However, the presence of MCT8 was demonstrated in tanycytes [64], data were not available about the presence of this transporter in hypophysiotropic axons. The presence of MCT8 in the axon terminals of the hypophysiotropic TRH neurons would suggest that the axon terminals of the hypophysiotropic TRH axons are able to take up T3 in the ME. The importance of this question is underlined by the different kinetics of the T4 and T3 transport through the blood brain barrier (BBB).** T3 can far more efficiently pass through the

BBB than T4 [27]. This is however not the case in the external zone of the ME which brain region is located outside of the BBB. Thus, the site of TH uptake determines whether the hypophysiotropic TRH neurons can only sense the T3 that is activated within the BBB or these cells can sense a mixture of the T3 originating from the circulation and released by the tanycytes in the ME.

I. 5 Role of TRH neurons in the regulation of food intake

It was demonstrated that central administration of TRH decreases food intake and the time spent with feeding [65]. TRH can inhibit even the vigorous feeding when food is reintroduced after a period of fasting [65]. **Despite the very robust anorexigenic effect of TRH, very little information is available which TRH cell population and where exerts this effect.** Our laboratory has shown [66] that a seemingly continuous population of TRH neurons in the perifornical region and BNST area expresses a second anorexigenic peptide, the urocortin 3 (UCN3) and demonstrated that these neurons project to the lateral part of the ARC. **The presence of two anorexigenic peptides in the same neurons and the projection of these cells to the lateral part of the ARC where the anorexigenic POMC neurons are located raised the possibility that the TRH/UCN3 neurons of the perifornical area/BNST region could be involved in the regulation of food intake.** Another cell population that may be involved in the mediation of the anorexigenic effects of TRH is the histaminergic neurons of the TMN in the posterior hypothalamus. Similarly to TRH, central administration of histamine reduces food intake in a number of experimental models [67-69]. Furthermore, the absence of the histamine-synthesizing enzyme, the histidine decarboxylase, results in late onset obesity and hyperphagia [70] demonstrating the anorexigenic role of histamine. **Central administration of TRH not only decreases food intake in a dose-dependent manner, but also increases the concentration of histamine and t-methylhistamine (a major metabolite of neuronal histamine) in the TMN [71] suggesting that the histaminergic neurons may be involved in the mediation of TRH induced anorexia.** This hypothesis is further supported by the data that the anorexic effects of TRH could be attenuated by pretreatment with the irreversible HDC inhibitor, α-fluoro-methyl histidine [71] and that TRH can excite the histaminergic neurons [72]. **Based on these important functional data, we hypothesized that the identification of the sources of the TRH-containing inputs of the histaminergic neurons can be used to identify anorexigenic TRH cell populations. However, it was unknown, whether TRH neurons innervate the histaminergic neurons and whether TRH neurons innervate the histaminergic neurons in all five subnuclei of the TMN or the communication of these two systems is localized to only certain TMN subnuclei.** Therefore, the detailed description of the relationship of the TRH axons and the histaminergic neurons was necessary to provide anatomical data for later track tracing studies.

II. Specific aims

To better understand the hypothalamic network regulating energy homeostasis we:

1. Elucidated the ultrastructural localization of the elements of the NO transmitter system in the PVN.
2. Studied whether the NO and the endocannabinoid systems are associated to the same synapses of the parvocellular neurons of the PVN.
3. Examined the role of the endocannabinoid and the NO systems of the PVN in the mediation of the NPY induced regulation of energy homeostasis
4. Determined the presence of MCT8 thyroid hormone transmitter in the axon terminals of the hypophysiotropic TRH neurons.
5. Examined the role of TRH/UCN3 neurons of the perifornical area/BNST region in the regulation of the feeding related neuronal groups of the hypothalamic ARC.
6. Studied the TRH-containing innervation of the histaminergic neurons in TMN.

III. Materials and methods

III. 1 Animals

a. Adult, male Wistar rats (Charles River, **III.7.5-12**), CD1 mice (Charles River, **III.7.1-5**) and MCT8 KO mice [73] kindly provided by Dr. H. Heuer, Jena, Germany (**III.7.5**) were used.

III. 2 Colchicine treatment

a. 100 µg colchicine was injected into the lateral ventricle in 5 µl 0.9% saline under stereotaxic control (**III.7.8-12**)

III. 3 Fixation of animals for immunocytochemistry at light and electron microscopic levels

a. Anesthesia: ketamine 50 mg/kg, xylazine 10 mg/kg body weight, ip.
b. The animals were transcardially perfused with 10 ml phosphate buffer saline (PBS) pH 7.5 followed by fixative solution (summarized in **Table 1**).
c. After fixation, the brains were removed and postfixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB), pH 7.4 for 2 h for light microscopy or 24 h for electron microscopy.
d. Acrolein fixed tissues were treated with 1% sodium borohydride in distilled water (DW) for light microscopy or with 0.1M PB, pH 7.4 for electron microscopy for 30 min.

Table 1 *Summary of the fixation methods and the number of used animals*

Experiments	Fixative solution	No. of used animals
III.7.1-3	10 ml of 4% PFA in Na-acetate buffer, pH 6.0, followed by 50 ml of 4% PFA in Borax buffer, pH 8.5; 10 ml and 50 ml/mouse	4 mice
III.7.4	4% PFA in 0.1 M PB, pH 7.4; 50 ml/mouse	3 mice
III.7.5	4% PFA in 0.1 M PB, pH 7.4, 50 ml/mouse or 150 ml/rat	3 mice 3 rats
III.7.6	2% PFA + 4% acrolein in 0.1 M PB, pH 7.4, 150 ml/rat	3 rats
III.7.8 and 9	3% PFA + 1 % acrolein in 0.1 M PB, pH 7.4, 150 ml/rat	3 rats
III.7.10-12	2% PFA + 4% acrolein in 0.1 M PB, pH 7.4,150 ml/rat	3 rats

III. 4 Tissue preparation for light microscopic immunohistochemistry

a. The brains were cryoprotected in 30% sucrose in PBS at 4 °C overnight.
b. Serial, coronal sections (25 µm) were cut on a freezing microtome (Leica).
c. The sections were collected and stored (−20 °C) in cryoprotectant solution (30% ethylene glycol; 25% glycerol; 0.05M PB).
d. The sections were pretreated with 0.5% Triton X-100/0.5% H_2O_2 in PBS for 15 min, and with 2% normal horse serum (NHS) in PBS for 20 min.
e. The sections were placed in the appropriate serum overnight followed by the appropriate species specific biotinylated IgG (2h) (**detailed in Table 2).**
f. The sections were treated with avidin-biotin complex (ABC) (1:1000, Vector Labs; 1h)
g. The immunoreaction was developed with NiDAB developer (0.05% diaminobenzidine (DAB), 0.15% nickel-ammonium-sulfate and 0.005% H_2O_2 in 0.05M Tris buffer pH 7.6) **(III.7.5)** or DAB developer (0.025% DAB/0.0036% H_2O_2 in 0.05M Tris buffer pH 7.6) (**III.7.10**).
h. The sections were mounted on glass slides and coverslipped with DPX (Sigma).
i. The preparations were analyzed with a Zeiss AxioImager M1 microscope equipped with AxioCam MRc5 digital camera (Carl Zeiss Inc.).

Table 2 *Summary of the primary and secondary antibodies used in light microscopic studies*

Study number	Used primary antibodies and sources	Dilution	Secondary antibody	Chromogen
III.7.5	rabbit anti-MCT8 (kind gift from Dr. TJ Visser)	1:5,000 – 10,000	biotinylated donkey anti-rabbit IgG, 1:500; Jackson ImmunoResearch	Silver intensified NiDAB
III.7.10	sheep anti-TRH #08W2, 66,74	1:50,000	biotinylated donkey anti-sheep IgG, 1:500; Jackson ImmunoResearch	Silver intensified NiDAB
	sheep anti-histamine 75	1:1,000	biotinylated donkey anti-sheep IgG, 1:500; Jackson ImmunoResearch	DAB

Table 3 *Summary of the primary and secondary antibodies used in immunofluorescent studies*

Study number	Used primary antibodies and sources	Dilution	Secondary antibody
III.7.4	rabbit anti-CB1 (Abcam)	1 µg/ml	Alexa 488-conjugated donkey anti-rabbit IgG, 1:200; Life Technologies
	rabbit anti-DAGLα (Abcam)	1 µg/ml	Alexa 647-conjugated donkey anti-rabbit IgG, 1:200; Life Technologies
	mouse anti-MAP2 (Millipore)	1 µg/ml	Alexa 405-conjugated donkey anti-mouse IgG, 1:200; Life Technologies
	guinea pig anti-nNOS (Abcam)	1 µg/ml	Alexa 555-conjugated donkey anti-guinea pig IgG, 1:200; Life Technologies
	goat anti-VGLUT1 (Abcam, Cambridge UK)	1 µg/ml	Alexa 555-conjugated donkey anti-goat IgG, 1:200; Life Technologies
	goat anti-VGLUT2 (Abcam)	1 µg/ml	Alexa 555-conjugated donkey anti-goat IgG, 1:200; Life Technologies
	goat anti-VIAAT (Abcam)	1 µg/ml	Alexa 555-conjugated donkey anti-goat IgG, 1:200; Life Technologies
III.7.7	rabbit anti-MCT8 serum kind (gift from Dr. TJ Visser)	1:1,000	Alexa 555-conjugated donkey anti-goat IgG, 1:500; Life Technologies
	sheep anti-TRH serum (#08W2)	1:1,500	Fluorescein DTAF-conjugated sheep IgG 1:50; Jackson ImmunoResearch
III.7.8	rabbit anti-UCN3 serum (kind gift from Dr. WW Vale)	1:60,000	biotinylated donkey anti-rabbit IgG 1:500; Jackson ImmunoResearch; Fluorescein DTAF-conjugated Streptavidin 1:300; Jackson ImmunoResearch
	mouse anti-TRH serum	1:4,000	Alexa 555-conjugated donkey anti-mouse IgG 1:500; Jackson ImmunoResearch
	sheep anti-α-MSH serum	1:20,000	Cy5-conjugated donkey anti-sheep IgG 1:100; Jackson ImmunoResearch
	sheep anti-NPY serum (kind gift from Dr. I. Merchenthaler)	1:8,000	Cy5-conjugated donkey anti-sheep IgG 1:100; Jackson ImmunoResearch
III.7.11	mouse anti-TRH serum	1:4,000	Alexa 555-conjugated donkey anti-mouse IgG 1:500, Jackson ImmunoResearch
	sheep anti-histamine serum	1:20,000	donkey biotinylated anti-sheep IgG 1:500 fluorescein-conjugated streptavidin 1:250; Vector Lab

III. 5 Tissue preparation for immunofluorescence

a. The sections were pretreated similarly as described in **II.4 a-d** points.
b. The sections were placed in the appropriate serum overnight and the appropriate fluorochromes-conjugated species specific IgG (detailed in **Table 3**).
c. The immunostained sections were mounted on glass slides and coverslipped with Vectashield (Vector Labs).
d. The immunofluorescent preparations were analyzed with a Radiance 2000 confocal laser scanning microscope (Bio-Rad).

Table 4 *Summary of the primary and secondary antibodies used in electron microscopic studies*

Study number	Used primary antibodies and sources	Dilution	Secondary antibody	Chromogen
III.7.1	rabbit anti-nNOS serum (Zymed Lab)	1:200	donkey anti-rabbit IgG-conjugated with 0.8 nm colloidal gold, 1:100; Electron Microscopy Sciences	Colloidal gold silver intensified with the R-Gent SE-LM kit (Aurion)
III.7.2	rabbit anti-sGCα1 serum (Sigma)	1:4,000	biotinylated donkey anti-rabbit IgG, 1:500; Jackson Immunoresearch	Silver intensified NiDAB
III.7.3	rabbit anti-nNOS serum (Zymed Lab)	1:200	donkey anti-rabbit IgG-conjugated with 0.8 nm colloidal gold, 1:100; Electron Microscopy Sciences	Colloidal gold silver intensified with the R-Gent SE-LM kit (Aurion)
	sheep anti-CB1 serum (kind gift from Dr. M. Watanabe)	1:800	biotinylated donkey anti-sheep IgG, 1:500; Jackson ImmunoResearch	NiDAB
III.7.6	rabbit anti-MCT8 serum (kind gift from Dr. TJ Visser)	1:20,000	biotinylated donkey anti-rabbit IgG, 1:500; Jackson ImmunoResearch	Silver intensified NiDAB
III.7.9	sheep anti-α-MSH serum (kind gift from Dr. JB Tatro)	1:1,000	donkey anti-sheep IgG-conjugated with 0.8 nm colloidal gold (1:100; Electron Microscopy Sciences)	Silver intensified with the R-Gent SE-LM kit (Aurion)
	rabbit anti-UCN3 serum (kind gift from Dr. WW Vale)	1:1,000	biotinylated donkey anti-rabbit IgG, 1:500; Jackson ImmunoResearch	NiDAB
III.7.12	mouse anti-TRH serum	1:10,000	biotinylated donkey anti-mouse IgG, 1:500; Jackson ImmunoResearch	DAB
	sheep anti-histamine serum	1:2,000	donkey anti-sheep IgG conjugated with 0.8 nm colloidal gold, 1:100; Electron Microscopy Sciences	Colloidal gold silver intensified with the R-Gent SE-LM kit (Aurion)

III. 6 Tissue preparation for ultrastructural studies

a. Serial, coronal sections (25-50 μm) were cut on a Leica VT 1000S vibratome (Leica) and collected in PBS.
b. The sections were pretreated with 0.5% H_2O_2 in PBS for 15 min.
c. The sections were cryoprotected in 15% sucrose in PBS for 15 min and in 30% sucrose in PBS overnight at 4 °C
d. The antibody penetration was increased by freezing the sections over liquid nitrogen and then thawing them at RT. This cycle was repeated three times.
e. The nonspecific antibody binding was blocked with treatment in 2% NHS in PBS for 20 min.
f. The sections were placed in the appropriate serum for four days and then in the appropriate biotinylated species specific IgG overnight (detailed in **Table 4**).
g. The preparations were osmicated in 1% osmium tetroxide in 0.1M PB for 30 min, and then treated with 2% uranyl acetate in 70% ethanol for 30 min, dehydrated in an ascending series of ethanol and propylene oxide. Finally, the sections were flat embedded in Durcupan ACM epoxy resin (Sigma-Aldrich) on liquid release agent (Electron Microscopy Sciences)-coated slides and polymerized at 56 °C for 2 days
h. 50–70 nm thick sections were cut with Leica ultracut UCT ultramicrotome (Leica Microsystems) and collected onto Formvar-coated, single slot grids (Electron Microscopy Sciences).
i. The ultrasections were analyzed with a JEOL electron microscope.

III. 7 Experiments

The details of the experiments are summarized in Table 2-5.

III.7.1 Immunocytochemistry for ultrastructural localization of nNOS in the parvocellular part of the PVN

III.7.2 Immunocytochemistry for ultrastructural localization of sGCα1 in the parvocellular part of the PVN

III.7.3 Double-labeling immunocytochemistry for ultrastructural examination of the relationship of nNOS and CB1 in the parvocellular part of the PVN

III.7.4 Quadruple-labeling immunofluorescence of the elements of the endocannabinoid and NO signaling systems and markers of glutamatergic and GABAergic neurons in the parvocellular part of the PVN

III.7.5 Light microscopic detection of MCT8 in the ME

III.7.6 Ultrastructural detection of MCT8-immunoreactivity in the ME

III.7.7 Double labeling immunofluorescence for MCT8 and TRH in the ME

III.7.8 Triple-labeling immunofluorescence for TRH, UCN3 and α-MSH or NPY in the ARC

III.7.9 Double-labeling immunocytochemistry for ultrastructural examination of the UCN3-IR innervation of the α-MSH neurons in the ARC

III.7.10 Double labeling immunocytochemistry for TRH and histamine in the TMN

III.7.11 Double-labeling immunofluorescence for TRH and histamine in the TMN

III.7.12 Double-labeling immunocytochemistry for ultrastructural examination of the TRH-IR innervation of the histamine-IR neurons in the TMN

Table 5 *Summary of the antibodies*

Used primary antibody	***Source***	***Reference***
goat anti-VGLUT1 serum	*Abcam, Cambdridge, UK*	[76]
goat anti-VGLUT2 serum	*Abcam, Cambdridge, UK*	[76]
goat anti-VIAAT serum	*Life technologies, Waltham, MA*	[76]
guinea pig anti-nNOS serum	*Thermo Fisher, Waltham, MA*	[77]
mouse anti-MAP2 antibody	*Millipore, Billerine, MA*	[78]
mouse anti-TRH serum	*Raised in our laboratory*	[79,80]
rabbit anti- DAGLα serum	*Abcam, Cambdridge, UK*	[81]
rabbit anti-CB1 serum	*Abcam, Cambdridge, UK*	[82]
rabbit anti-MCT8 serum	*kind gift from Dr. TJ Visser, Rotterdam, The Netherlands*	[83]
rabbit anti-nNOS serum	*Zymed Laboratories, Waltham, MA*	[56]
rabbit anti-sGCα1 serum	*Sigma Aldrich, St. Louis, MA*	[56]
rabbit anti-UCN3 serum	*kind gift from Dr. WW Vale, La Jolle, CA*	[66]
sheep anti-CB1 serum	*kind gift from Dr. M. Watanabe, Sapporo, Japan*	[74]
sheep anti-histamine serum	*Raised in our laboratory*	[75,80]
sheep anti-NPY serum	*kind gift from Dr. I. Merchenthaler, Baltimore, MD*	[39,84,85]
sheep anti-TRH serum	*Raised in our laboratory, #08W2,*	[66,74,79]
sheep anti-α-MSH serum	*kind gift from Dr. JB Tatro, Boston, MA*	[86,87]

III. 8 Implantation of bilateral guide cannula in the PVN of mice

a. Bilateral 26-gauge, 0.8 mm C/C stainless steel cannula (Plastics One, Roanoke, VA, USA) was implanted over the PVN.
b. aCSF, AM251 or NPLA (0.4 µl on each side) administered to the by *via* the implanted cannula using a syringe minipump at a rate of 0.5 µl/min.
c. 10 min later, aCSF or NPY (0.4 µl on each side) was administered *via* the implanted cannula at a rate of 0.5 µl/min.
d. Body mass composition was analyzed using an Echo Medical systems' EchoMRI (Whole Body Composition Analyzers)
e. After the administration of compounds, all animals were placed inside the calorimetric cage (TSE system).
f. Energy expenditure, basal metabolism, substrate utilization (RER) and locomotor activity were calculated.

IV. Results

[C1-C5] *Thesis I.:* **The ultrastructural localization of the elements of the nitric oxide transmitter system in the parvocellular part of the PVN indicate that NO is utilized as both anterograde and retrograde neurotransmitter in this brain region.**

The nNOS and the sGC has both pre- and postsynaptic localization in the parvocellular part of the PVN indicating that the NO may serve as both anterograde and retrograde transmitter in this brain region.

[C1-C5] *Thesis II.:* **NO and the endocannabinoid systems are associated to the same synapses of the parvocellular neurons of the PVN.**

nNOS is associated to the postsynaptic side of a population of the synapses formed by CB1-IR terminals on the parvocellular neurons. These data indicate that the NO and the endocannabinoid systems may interact in the regulation of presynaptic terminals in the parvocellular part of the PVN.

[C1-C5] *Thesis III.:* **The endocannabinoid and the NO systems of the PVN are involved in the mediation of the NPY induced regulation of energy homeostasis**

Both transmitter systems have critical, but different role in the mediation of the effects of NPY on the energy homeostasis. While the NO system of the PVN mediates the effects of NPY on the food intake, the endocannabinoid system of this nucleus is involved in the mediation of the NPY induced regulation of energy expenditure. Furthermore, data of our group indicates that different neuronal circuits of the PVN are involved in the regulation of food intake, energy expenditure and locomotor activity by NPY.

[J1] *Thesis IV.:* **MCT8 thyroid hormone transporter presents in the axon terminals of the hypophysiotropic TRH neurons.**

MCT8 is present in the hypophysiotropic axon terminals in the external zone of the ME. Furthermore, specifically the axon terminals of the hypophysiotropic TRH neurons also contain this thyroid hormone transporter. Based on this finding, a novel concept of the thyroid hormone feedback regulation of the hypophysiotropic TRH axons was established.

[J2] *Thesis V.:* **The TRH/UCN3 neurons of the perifornical area/BNST region innervate the feeding related neuronal groups of the ARC.**

The axons of the TRH/UCN3 neurons juxtapose to only the minority of the NPY neurons of the ARC, but contacts more than half of the α-MSH neurons

of the nucleus. At ultrastructural level, the TRH/UCN3 axons establish asymmetric type synaptic specializations with the α-MSH neurons.

[J3] *Thesis VI.:* **TRH-containing axons innervate the histaminergic neurons in all subnuclei of the TMN.**

TRH-IR axons densely innervate the histaminergic neurons in all subnuclei of the TMN, and establish synaptic specializations on the surface of these neurons. The majority of the synaptic contacts were found to be asymmetric type, but symmetric synapses were also found between the two systems.

V. Potential application of the results

Since currently there is no efficient and side effect free medication against obesity, it is critical to uncover novel regulatory mechanisms of the energy homeostasis. Discovering novel pathways and/or novel neuronal groups that are involved in the regulation of energy homeostasis may provide new drug targets for the pharmaceutical industry in the fight against obesity.

A well-known problem is that in most cases, similar pathways regulate the food intake and the energy expenditure. The energy expenditure is stimulated by the sympathetic nervous system. However, stimulation of the sympathetic nervous system not only stimulates the energy expenditure, but also increases the blood pressure. For example, the agonists of the MC4R efficiently decrease the food intake and stimulate the energy expenditure, thus decrease the body weight [88]. However, these MC4R agonists also increase the blood pressure [89] that is serious problem as most obese patients has high blood pressure due to the metabolic syndrome [90].

Our observation that NPY regulates the food intake and the energy expenditure *via* different neuronal pathways within the PVN paws the way for new research that can identify these neuronal circuits and may provide novel drug targets that can influence the food intake without increasing the blood pressure.

Our studies showing the TRH/UNC3 innervation of the POMC neurons of the arcuate nucleus facilitated the electrophysiological studies of our groups that showed that TRH can prevent the stimulatory effect of UCN3 on the POMC neurons. Based on these data, we try to elucidate the intracellular mechanism of this antagonistic interaction that may allow to fine-tune the activity of these anorexigenic neurons.

The detailed description of the TRH-IR innervation of the histaminergic neurons will allow experiments to identify TRH-synthesizing neuronal groups that regulate the food intake. Understanding the regulation of these TRH neuronal populations may also yield novel drug targets.

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VII. Publications and conference abstracts

VII. 1 List of publications underlying the thesis

* These authors have contributed equally to the work

[J1] Kallo, I; Mohacsik, P; Vida, B; Zeold, A; Bardoczi, Z; Zavacki, AM; **Farkas, E;** Kadar, A; Hrabovszky, E; Arrojo, e Drigo R; et al. A Novel Pathway Regulates Thyroid Hormone Availability in Rat and Human Hypothalamic Neurosecretory Neurons Plos One 7: (6) Paper: e37860, 16 p. (2012)

[J2] Zoltán Péterfi*, **Erzsébet Farkas***, Kata Nagyunyomi-Sényi,Andrea Kádár, Szenci Ottó, András Horváth, Tamás Füzesi, Ronald M. Lechan, Csaba Fekete Role of TRH/UCN3 neurons of the perifornical area/ bed nucleus of stria terminalis region in the regulation of the anorexigenic POMC neurons of the arcuate nucleus in male mice and rats Brain Structure and Function Accepted (2017)

[J3] Sarvari, A*; **Farkas, E*;** Kadar, A; Zseli, G; Fuzesi, T; Lechan, RM; Fekete, C.Thyrotropin-releasing hormone-containing axons innervate histaminergic neurons in the tuberomammillary nucleus Brain Research 1488 pp. 72-80. (2012)

VII. 2 List of conferences

[C1] Zoltán Péterfi*, Imre Farkas*, Raphael Denis*, **Erzsébet Farkas***, Motokazu Uchigashima, Masahiko Watanabe, Ronald M Lechan, Zsolt Liposits6, Serge Luquet and Csaba Fekete Endocannabinoid and Nitric Oxide Retrograde Signaling Systems in the Hypothalamic Paraventricular Nucleus Have a Critical Role in Mediating the Effects of Npy on Energy Expenditure Endocrine Reviews Volume 37, Issue 2 Supplement, April 2016

[C2] **Erzsébet, Farkas,** Fekete, C; Lechan, RM. Subcellular localization of the components of the nitric oxide system in the hypothalamic paraventricular nucleus of mice Phd Proceedings Annual Issues Of The Doctoral School Faculty Of Information Technology And Bionics 10 pp. 29-32. (2015)

[C3] **Erzsébet, Farkas,** Fekete, C; Lechan, RM. Structural and functional characterization of the retograde signaling system in the hypothalamic paraventricular nucleus Phd Proceedings Annual Issues Of The Doctoral School Faculty Of Information Technology And Bionics 2014 pp. 15-18. (2014)

[C4] **Erzsébet, Farkas,** Fekete, C; Lechan, RM. Subcellular localization of the components of the nitric oxide system in the hypothalamic paraventricular nucleus of mice Pázmány Péter Catholic University Phd Proceedings pp. 21-24. (2013)

[C5] **E. Farkas**, R. M. Lechan, C. Fekete Subcellular localization of the components of the nitric oxide system in the hypothalamic paraventricular nucleus of mice. Society of Neuroscience (2012)

VII. 3 List of publications related to the subject of the thesis

Wittmann, G; **Farkas, E;** Szilvasy-Szabo, A; Gereben, B; Fekete, C; Lechan, RM. Variable proopiomelanocortin expression in tanycytes of the adult rat hypothalamus and pituitary stalk. Journal Of Comparative Neurology 525: (3) pp. 411-441. (2017)

Farkas, I; Vastagh, C; **Farkas, E**; Balint, F; Skrapits, K; Hrabovszky, E; Fekete, C; Liposits, Z. Glucagon-Like Peptide-1 Excites Firing and Increases GABAergic Miniature Postsynaptic Currents (mPSCs) in Gonadotropin-Releasing Hormone (GnRH) Neurons of the Male Mice *via* Activation of Nitric Oxide (NO) and Suppression of Endocannabinoid Signaling Pathways. Frontiers In Cellular Neuroscience 10 p. 214 (2016)

McAninch, EA; Jo, S; Preite, NZ; **Farkas, E**; Mohacsik, P; Fekete, C; Egri, P; Gereben, B; Li, Y; Deng, Y; et al. Prevalent Polymorphism in Thyroid Hormone-Activating Enzyme Leaves a Genetic Fingerprint that Underlies Associated Clinical Syndromes. Journal Of Clinical Endocrinology And Metabolism 100: (3) pp. 920-933. (2015)

Singru, PS; Wittmann, G; **Farkas, E;** Zseli, G; Fekete, C; Lechan, RM.Refeeding-Activated Glutamatergic Neurons in the Hypothalamic Paraventricular Nucleus (PVN) Mediate Effects of Melanocortin Signaling in the Nucleus Tractus Solitarius (NTS) Endocrinology 153 pp. 3804-3814. (2012)

Farkas E, Ujvarosi K, Nagy G, Posta J, Banfalvi G. Apoptogenic and necrogenic effects of mercuric acetate on the chromatin structure of K562 human erythroleukemia cells. Toxicol in Vitro. 1 pp: 267-75. (2010)

VIII. References

1 Kopelman, P. G. *Nature* 404, 635-643, (2000).
2 commission, E. <ftp://ftp.cordis.europa.eu/pub/food/docs/nutrition_obesity_examples.pdf>
3 Schwartz, M. W. *et al. Nature* 404, 661-671, (2000).
4 Zhang, Y. *et al. Nature* 372, 425-432, (1994).
5 Lee, G. H. *et al. Nature* 379, 632-635, (1996).
6 Montague, C. T. *et al. Nature* 387, 903-908, (1997).
7 Farooqi, I. S. *et al. The New England journal of medicine* 356, 237-247, (2007).
8 Bruning, J. C. *et al. Science* 289, 2122-2125, (2000).
9 Dawson, R. *et al. The American journal of physiology* 273, E202-206, (1997).
10 Horvath, T. L. *et al. Brain Res* 756, 283-286, (1997).
11 Kelly, J. *et al. Pharmacol Biochem Behav* 7, 537-541, (1977).
12 Chambers, A. P. *et al. Handbook of experimental pharmacology*, 23-45, (2012).
13 Stanley, B. G. *et al. Proc Natl Acad Sci U S A* 82, 3940-3943, (1985).
14 Wilson, B. D. *et al. Molecular medicine today* 5, 250-256, (1999).
15 Luquet, S. *et al. Science* 310, 683-685, (2005).
16 Gropp, E. *et al. Nat Neurosci* 8, 1289-1291, (2005).
17 Fan, W. *et al. Nature* 385, 165-168, (1997).
18 Kristensen, P. *et al. Nature* 393, 72-76, (1998).
19 Vong, L. *et al. Neuron* 71, 142-154, (2011).
20 Smart, J. L. *et al. Ann N Y Acad Sci* 994, 202-210, (2003).
21 Huszar, D. *et al. Cell* 88, 131-141, (1997).
22 O'Rahilly, S. *et al.* in *Endotext* (eds L. J. De Groot *et al.*) (MDText.com, Inc., 2000).
23 Larsen, P. J. *et al. Peptides* 27, 1981-1986, (2006).
24 Rorato, R. *et al. Exp Physiol* 94, 371-379, (2009).
25 Liu, J. *et al. Endocrinology* 148, 5531-5540, (2007).
26 Fink, G. *et al.* 894 (Academic Press, London, UK, 2012).
27 Fekete, C. *et al. Endocrine reviews* 35, 159-194, (2014).
28 Geerling, J. C. *et al. J Comp Neurol* 518, 1460-1499, (2010).
29 O'Hare, J. D. *et al. Am J Physiol Endocrinol Metab* 310, E183-189, (2016).
30 Hill, J. W. *Indian journal of endocrinology and metabolism* 16, S627-636, (2012).
31 Xiang, H. B. *et al. International J of clinical and experimental pathology* 7, 2987-2997, (2014).
32 Currie, P. J. *et al. Brain Res* 737, 238-242, (1996).
33 Bishop, C. *et al. Brain Res* 865, 139-147, (2000).
34 Kotz, C. M. *et al. Am J Physiol Regul Integr Comp Physiol* 278, R494-498, (2000).
35 Stanley, B. G. *et al. Physiol Behav* 46, 173-177, (1989).
36 Parker, R. M. *et al. Eur J Neurosci* 11, 1431-1448, (1999).
37 Holliday, N. D. *et al.* in *NPY and Related Peptides* (ed M.C. Michel) 45-73 (Springer, 2004).
38 Pedragosa-Badia, X. *et al. Front Endocrinol (Lausanne)* 4, 5, (2013).
39 Fekete, C. *et al. Endocrinology* 142, 2606-2613, (2001).
40 Fuzesi, T. *et al. Endocrinology* 148, 5442-5450, (2007).
41 Harris, M. *et al. J Clin Invest* 107, 111-120, (2001).
42 Spengler, D. *et al. Molecular endocrinology* 6, 1931-1941, (1992).
43 Melnick, I. *et al. Neuron* 56, 1103-1115, (2007).
44 Shrestha, Y. B. *et al. Regulatory peptides* 133, 68-73, (2006).
45 Wirth, M. M. *et al. Peptides* 22, 129-134, (2001).
46 Balthasar, N. *et al. Cell* 123, 493-505, (2005).
47 Fekete, C. *et al. J Neurosci* 20, 1550-1558, (2000).
48 Fekete, C. *et al. Neurosci Lett* 289, 152-156, (2000).
49 Péterfi, Z. *et al.* in *Endocrine Society's 98th Annual Meeting* (Boston, MA, 2016).
50 Piomelli, D. *Nature reviews. Neuroscience* 4, 873-884, (2003).
51 Araque, A. *et al. Neuropharmacology*, (2017).
52 Wittmann, G. *et al. J Comp Neurol* 503, 270-279, (2007).
53 Kola, B. *et al. PLoS One* 3, e1797, (2008).
54 Di, S. *et al. J Neurosci* 23, 4850-4857, (2003).
55 Hardingham, N. *et al. Front Cell Neurosci* 7, 190, (2013).
56 Szabadits, E. *et al. J Neurosci* 27, 8101-8111, (2007).
57 Makara, J. K. *et al. J Neurosci* 27, 10211-10222, (2007).
58 Affleck, V. S. *et al. Neuroscience* 219, 48-61, (2012).
59 Gereben, B. *et al. Endocr Rev* 29, 898-938, (2008).
60 Tu, H. M. *et al. Endocrinology* 138, 3359-3368, (1997).
61 Visser, T. J. in *Endotext* (eds L. J. De Groot *et al.*) (MDText.com, Inc., 2000).
62 Friesema, E. C. *et al. Lancet (London, England)* 364, 1435-1437, (2004).
63 Di Cosmo, C. *et al. J Clin Invest* 120, 3377-3388, (2010).
64 Heuer, H. *et al. Endocrinology* 146, 1701-1706, (2005).
65 Lechan, R. M. *et al. Prog Brain Res* 153, 209-235, (2006).
66 Wittmann, G. *et al. J Comp Neurol* 517, 825-840, (2009).
67 Itoh, Y. *et al. Neuroscience letters* 125, 235-237, (1991).
68 Ookuma, K. *et al. Brain research* 490, 268-275, (1989).
69 Yasuda, T. *et al. Endocrinology* 146, 2744-2748, (2005).

70 **Fulop, A. K.** ***et al. Endocrinology*** **144, 4306-4314, (2003).**
71 **Gotoh, K.** ***et al. J Neurochem*** **103, 1102-1110, (2007).**
72 **Parmentier, R.** ***et al. The J of Neuroscience: the official Jof the SFN*** **29, 4471-4483, (2009).**
73 **Trajkovic, M.** ***et al. J Clin Invest*** **117, 627-635, (2007).**
74 **Deli, L.** ***et al. Endocrinology*** **150, 98-103, (2009).**
75 **Fekete, C.** ***et al. Brain Research*** **969, 70-77, (2003).**
76 **Miura, E.** ***et al. J Neurochem*** **97, 1431-1446, (2006).**
77 **Narushima, M.** ***et al. J Neurosci*** **27, 496-506, (2007).**
78 **Dehmelt, L.** ***et al. Genome Biology*** **6, 204-204, (2005).**
79 **Wittmann, G.** ***et al. J Comp Neurol*** **515, 313-330, (2009).**
80 **Sarvari, A.** ***et al. Brain Res*** **1488, 72-80, (2012).**
81 **Yoshida, T.** ***et al. J Neurosci*** **26, 4740-4751, (2006).**
82 **Fukudome, Y.** ***et al. Eur J Neurosci*** **19, 2682-2692, (2004).**
83 **Kalló, I.** ***et al. PLoS ONE*** **7, e37860, (2012).**
84 **Fekete, C.** ***et al. Endocrinology*** **143, 4513-4519, (2002).**
85 **Fekete C,** ***et al. Endocrinology*** **142, 7, (2001).**
86 **Fekete, C.** ***et al. Endocrinology*** **143, 3846-3853, (2002).**
87 **Wittmann, G.** ***et al. Endocrinology*** **146, 2985-2991, (2005).**
88 **Fosgerau, K.** ***et al. J Endocrinol*** **220, 97-107, (2014).**
89 **Nordheim, U.** ***et al. Peptides*** **27, 438-443, (2006).**
90 **Han, T. S.** ***et al. JRSM cardiovascular disease*** **5, 2048004016633371, (2016).**