REGULATION OF OREXIN EXPRESSION AND CHANGES IN FEEDING BEHAVIOR BY THE SEROTONIN 5-HT$_{1A}$ RECEPTOR

Tesis entregada a LA UNIVERSIDAD DE VALPARAÍSO en Cumplimiento Parcial de los requisitos para optar al grado de Doctor en Ciencias con Mención en Neurociencia

Facultad de Ciencias

Por

Cristofer Alejandro Reyes Ortiz

Agosto, 2020

Dirigida por: Dr. Pablo Moya V.
Codirigida por: Dr. Andrés Chávez N.

Acknowledgments

Dedicated

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Symbols and Abbreviations

Orx: Orexin

AC: Adenylate cyclase

LH: Lateral Hypothalamus

PFA: Perifornical area

5-HT: Serotonin

5-HT_{1A}R: Serotonin 1A receptor

5-HT_{2A}R: Serotonin 2A receptor

5-HT_{2c}R: Serotonin 2C receptor

i.c.v: Intra-cerebro ventricular

mCpp: 1-(3-chlorophenyl) piperazine

pCPA: *p*-chlorophenylalanine

ARC: Arcuate nucleus

PVN: Paraventricular nucleus

DMH: Dorsomedial hypothalamus

Foxa2: Forkhead box protein A2

VMH: Ventromedial hypothalamus

Ox_1R: Orexin receptor 1

Ox_2R: Orexin receptor 2

Prepro-Orx: Pre propeptide of orexin

Orx-A: Orexin A

Orx-B: Orexin B

PI3K: phosphatidylinositol 3-kinase

POMC: Pro-opiomelanocortin

NPY: Neuropeptide y

MSH: Melanocyte-Stimulating hormone

CRH: Corticotropin releasing hormone

TRH: Thyrotropin-releasing hormone

BNDF: Brain-derived neurotrophic factor

PoA: Preoptic area

MCH: Melanin concentrating hormone

GPCR: G-protein-coupled receptors

GIRK: G protein-coupled inwardly-rectifying potassium channel

HPRT1: Hypoxanthine phosphoribosyl transferase 1

Ct: Cycle threshold

Lep: Leptin

LepR: Leptin Receptor

STAT-3: Signal Transducer and Activator of Transcription 3

IFN-α: Interferon α

IGFBP3: Insulin-like growth factor binding protein 3

Foxa2: Forkhead Box Transcription Factor 2

AC: Adenylate cyclase

cAMP: Cyclic adenosine monophosphate

DPAT: (R)-8-hydroxy-2(di-n-propylamino) tetralin 5,7

DHT: 5,7-Dihydroxytryptamine

TPH: Tryptophan hydroxylase

aa: Amino acid

INF-αR 1/2: Interferon alpha receptor 1 and 2.

IGF-1R: Insulin-like growth factor 1 receptor

GR: Glucocorticoid receptor

SERT: Serotonin transporter

1. Abstract

Food intake is regulated by the serotonergic (5-HTergic) as well as by the Orexinergic (Orx-ergic) systems. Orexin-A and Orexin-B are hypothalamic neuropeptides synthesized by a common polypeptide precursor called prepro-Orx those that increase food intake. Serotonin (5-HT) can regulate the activity of Orexinergic neurons through the 5-HT_{1A} receptor expressed in the lateral hypothalamus (LH). 5-HT_{1A} is a $G_{i/o}$ protein coupled receptor, which canonically inhibits adenylate cyclase (AC) decreasing cyclic adenosine monophosphate (cAMP) levels, however it can activate several intracellular proteins including Akt. A relevant substrate of Akt is the transcription factor Forkhead Box A2 (Foxa2), which can control the Orx gene. In this work, we hypothesized that the 5-HT system, through 5-HT_{1A}-Akt-Foxa2 pathway, can modulate the Orx expression at transcriptional level in Orx-ergic neurons of the LH, causing a decrease in food intake in mice.

Using primary cell culture of hypothalamic neurons, we found that the application of the selective 5-HT_{1A} agonist (R)-8-hydroxy-2(di-n-propylamino)tetralin (DPAT) caused a significant decrease in the expression of prepro-Orx and the protein levels of Orx-A . We also found that the application of DPAT reduces the phosphorylation of Akt at Ser473, along with a reduced translocation to the Foxa2 into the nucleus *in vitro*; all these effects were effectively blocked by the co application of the selective 5-HT_{1A} antagonist WAY100635. Next, we evaluated the *in vivo* effects of local activation of 5-HT_{1A} intra-LH on food intake; administration of DPAT significantly decreased food intake. In addition, we found that intra-LH administration of DPAT significantly reduced Akt phosphorylation, Foxa2 nuclear translocation and Orx-A levels in the LH. To assess the direct role of Akt phosphorylation in feeding behavior, we infused an Akt antagonist into the LH of mice and found a significant decrease in food intake.

Finally, we measured the mRNA levels of prepro-Orx and 5-HT_{1A} in the serotonin transporter knockout (SERT KO) mouse, a model that has reportedly about four times higher extracellular 5-HT in the brain. We found a ~ six-fold decrease in prepro-Orx levels and a ~ four-fold decrease in 5-HT_{1A} in SERT-KO compared to control littermates.

Among the several pathways, we show that the Akt-Foxa2 pathway downstream to 5- $HT_{1A}R$ activation in LH is involved in the regulation of Orx production and controls feeding behavior.

2. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a phylogenetically ancient signaling molecule (Hay-Schmidt, 2000) and is the most widely distributed neuromodulator in the brain (Dahlström and Fuxe, 1964; Steinbusch, 1981; Ren et al., 2019). In the central nervous system (CNS), 5-HT is produced by neurons located in the raphe nuclei. The caudal raphe nuclei send predominantly descending projections to the brainstem and spinal cord, (Lam and Heisler, 2007). The rostral raphe nuclei, including the median and dorsal raphe, send predominantly ascending projections throughout the forebrain, including to the cortex, amygdala, striatum, hippocampus, and the various nuclei of the hypothalamus (Ren et al., 2020). The action of 5-HT is exerted through of activation of receptors, which are widely distributed in the brain and they are expressed both pre-synaptically and post-synaptically (Holmes, 2008). Currently about 16 types of 5-HT receptors have been described, which have been classified into sub families based on the primary signaling mechanisms they are coupled (Polter et al., 2010).

5-HT plays important roles in physiological functions such as sleep, sexual behavior, temperature regulation, pain, and cognition, all processes in which the energy balance is finely regulated (Horvitz et al., 1982; Orchard, 2006; Tecott, 2007). The maintenance of energy balance requires the regulation of many behavioral and physiological processes, including those required to forage for food, recognize nutrient sources, consume food, digest food, and utilize/store energy, where the predominant global effect of 5-HT signaling in CNS is the suppression of food intake.

2.1 Serotonin regulation of food intake

With regard to feeding, either specific lesions of raphe nuclei or acute inhibition of these neurons by raphe injection of the gamma-aminobutyric acid receptor A ($GABA_A$) agonist muscimol result in hyperphagia and obesity (Geyer et al., 1976; Klitenick and Wirtshafter, 1988). Pharmacological inhibition of 5-HT synthesis by intracerebroventricular (i.c.v.) injection of either the 5-HTergic neurotoxin 5,7-dihydroxytriptamine (5,7-DHT) or the tryptophan hydroxylase (TPH)

inhibitor p-chlorophenylalanine (PCPA) have also been found to produce hyperphagia (Breisch et al., 1976; Saller and Stricker, 1976). Conversely, central injections of 5-HT or its precursor 5-hydroxytryptophan (5-HTP) caused hypophagia (Blundell and Latham, 1979; Fletcher and Burton, 1986; Simansky, 1996; Yamada et al., 2006) as well as increased metabolic rate (Rothwell and Stock, 1987; Le Feuvre et al., 1991). Fenfluramine, a drug that increases synaptic 5-HT concentrations by inducing vesicular release and inhibiting reuptake, also produce hypophagia, reducing meal size, and increasing inter-meal intervals in rats (Blundell et al., 1975; Grinker et al., 1980), effects which are mediated by central 5-HT (Fletcher and Burton, 1986). More recently, using optogenetics to selectively activate 5-HT neurons, Nectow & cols showing that activation of 5-HT´ergic outputs significantly decreased food intake in mice. Conversely, activation of 5-HT neurons located in the local circuitry of the raphe nucleus resulted in increased food consumption (Nectow et al., 2017).

While many areas of the brain have demonstrated roles in transmitting and integrating energy balance signals and food intake, the hypothalamus is pivotal. It has been long established that most areas of the hypothalamus receive inputs from both the median and dorsal raphe (Sawchenko et al., 1983; Petrov et al., 1992; Pollak et al., 2014).

2.2 Hypothalamic network in food intake and serotoninergic modulation

In the hypothalamus, peripheral, humoral, and neural signals are processed to elaborate a response that allows maintaining energy homeostasis. The classic model of a “satiety” center, located in the ventromedial hypothalamus (VMH) and a “feeding center”, located in the lateral hypothalamus (LH), was originally proposed on the effects of local lesions (Dietrich & Horvath, 2008; Magni et al., 2009). The hypothalamic network controlling feeding and energy metabolism includes several groups of interconnected neurons located in the arcuate nucleus (ARC), ventromedial nucleus (VMN), paraventricular nucleus (PVN), dorsomedial nucleus (DMN) and LH (Berthoud et al., 2008; Dietrich & Horvath., 2008; Magni et al., 2009).

The circuit begins in the ARC, which efficiently receives peripheral signals through the 3rd ventricle and is made up mainly of two neural types: pro-opiomelanocortin (POMC) neurons and neuropeptide Y (NPY)/Agouti-related protein (AgRP) neurons (Benarroch et al., 2010). The POMC neurons are stimulated by leptin (Lep, hormone produced by adipose tissue) and insulin, and they exert an anorexigenic effect (Benarroch., 2010). Once activated, POMC neurons can produce both α and β melanocyte-stimulating hormone(α-MSH and β-MSH) that act via melanocortin 4 receptors (MC4R) distributed in the PVN, VMN, and LH to reduce food intake and increase energy expenditure (Millington et al., 2007). The NPY/AgRP neurons are in the medial portion of the ARC, and they synthesize NPY/AgRP as well as GABA (Meister et al., 2009). These neurons are mediators of the orexigenic effects of ghrelin (gastric peptide that increases food intake) and are inhibited by anorexigenic signals such as leptin, glucose, and insulin (Kageyama et al., 2006). They provide tonic GABAergic inhibition to neighboring anorexigenic POMC neurons in the lateral ARC and project to other hypothalamic areas including the PVN, VMN and DMN and LH (Dietrich et al., 2009). NPY acts via Y1, Y2, and Y5 receptors to elicit an increase in food intake and reduce energy expenditure, whereas AgRP acts as an inverse agonist of MC4R (Lechan et al., 2006), thus preventing the anorectic effect of α-MSH (Dietrich et al., 2009). The PVN contains second order parvocellular neurons that mediate the effects of both anorexigenic and orexigenic signals from the ARC and other hypothalamic areas (Berthoud et al., 2008; Dietrich & Horvath., 2008; Magni et al., 2009). PVN neurons synthesize corticotropin releasing hormone (CRH), thyrotropin-releasing hormone (TRH), and oxytocin, which reduce food intake and increase energy metabolism both via their effects on the anterior pituitary and via projections to other hypothalamic and brainstem areas controlling autonomic function (Wittmann et al., 2009). The VMN contains a large population of glucose responsive neurons that mediate the effects of Lep, inhibiting food intake and stimulating energy expenditure (King et al., 2008). VMN neurons receive inputs from POMC neurons of the ARC and synthesize brain-derived neurotrophic factor (BDNF). Experimental studies indicate that BDNF is an important mediator of Lep-induced anorexia, in part via effects on the PVN (Wang et al., 2007). The DMN is interconnected with other hypothalamic

areas controlling food intake and receives input from the suprachiasmatic nucleus the circadian pacemaker (Moriya et al., 2009). The DMN contains neurons that express circadian clock genes sensitive to daytime feeding schedules, this suggests that the DMN conveys circadian influences on thermoregulation, endocrine function, arousal, and food intake (Moriya et al., 2009). These neurons projects to the medial preoptic area (PoA), PVN, and mainly LH (Googley et al., 2006).

There are several 5-HT receptors expressed in different hypothalamic neuronal types modulating hunger/satiety balance. In the ARC nucleus, POMC neurons express and can be activated through the 5-HT_{2C} a $G_{q/11}$ protein-coupled receptor (Heisler et al., 2006) which produces hypophagia (Xu et al., 2008). In contrast, NPY/AgRP neurons are inhibited through the 5-HT_{1B}, G_i protein-coupled receptor, inhibiting food consumption (Heisler et al., 2006). Therefore, at the level of the ARC nucleus, the 5-HT tone is predominantly directed towards the inhibition of food intake, directly through the activation of 5-HT receptors. PVN neurons preferentially express the 5-HT_{2C} and its activation using meta-chlorphenylpiperazine (mCPP) produces a concomitant decrease in intake food (Heisler et al., 2007). Moreover, PVN receive inputs from the ARC nucleus (Larse et al., 1994; Hermes et al., 2006). Inputs from the ARC core include projections of NPY/AgRP neurons (Atasoy et al., 2012) y POMC (Mercer et al., 2013). Therefore, increases in 5-HT tone, indirectly through the ARC-PVN pathway, lead to activation of PVN neurons, leading to a decrease in food intake.

Another hypothalamic area implicated in energy balance that expresses a high density of 5-HT receptors is the LH or “feeding center”, which is the area most densely innervated by serotonergic inputs (Pollak et al., 2014). As such, the 5-HTergic regulation of this area turns out to be highly relevant for the control of food intake.

2.3 Lateral hypothalamic area

The LH contains two neuronal populations that are reciprocally interconnected and control feeding and energy metabolism in association with changes in arousal: one group synthesizes

melanin-concentrating hormone (MCH) and the other synthesizes orexin (Orx, also called hypocretin).

2.3.1 Melanin-concentrating hormone neurons

MCH neurons are characterized by producing two neuropeptides known as neuropeptide E-I and neuropeptide G-E (Nahon et al., 1989). Transgenic mice over expressing the MCH gene are susceptible to insulin resistance and obesity (Ludwig et al., 2001). On the other hand, MCH knock-out (KO) mice are hypophagic, lean, and show an increased energy expenditure (Himada et al., 1998). These neuropeptides can activate two MCH G-protein coupled receptors (GPCRs) (Pissios et al., 2006; Chung et al., 2011). Mice lacking the MCH receptor 1 and 2 maintain elevated metabolic rates and remain lean despite hyperphagia that is counteracted by an increased locomotion (Hen et al., 2002). These neurons are reciprocally connected to ARC neurons and are inhibited by Lep, activated by ghrelin, and promote food intake (Leinninger et al., 2009). Similarly, MCH neurons are reciprocally connected to Orx neurons, by which they are activated, stimulating the production of GABA, which in turn inhibits Orx neurons (Konadhode et al., 2015). 5-HT may cause hyperpolarization of MHC neurons; however, it is unknown which 5-HTRs are involved (Van den pol et al., 2014).

2.3.2 Orexin neurons

The other neuronal type present in the LH is the Orx neurons. They are characterized by producing two neuropeptides named Orexin A (Orx-A) and Orexin B (Orx-B) (also known as hypocretin 1 and 2, respectively) (Sakurai et al., 1998). Orx-A and Orx-B are first synthesized as a common polypeptide precursor called Orx-propeptide (prepro-Orx) of 130 amino acid residues, which has 3 proteolytic cleavage sites (Sakurai et al., 1998). Upon proteolysis, the Orx-A (33 amino acid (aa)), which has greater stability, and the Orx-B (28 aa) are produced (Sakurai et al., 1998; Sakurai., 2005; Kukkonen et al., 2002).

Since its discovery, Orx neurons were initially described as regulators of food intake; however they have also been described as crucial regulators of the sleep-wakeful cycle and have been linked to the direct control of a wide variety of behaviors including locomotive activity, fear, memory and control of the reward system, in addition to have an important regulatory role in the balance of food intake and energy metabolism (Sakurai et al., 1998; de Lecea et al., 1998; Konadhode et al., 2015; Yamanaka et al., 2003; Chemelli et al., 1999; Flores et al., 2014; Martins et al., 2016). Supporting the physiological relevance of the Orx system in the control of feeding, i.c.v. administration of Orx-A and Orx-B in rats increase food consumption in a dose-dependent manner (Sakurai et al., 2008) and conversely, the selective deletion of the Orx gene decrease food intake (Hara et al., 2011). Orx neurons receive a wide variety of inputs, the most dense in nature being 5-HT (around 66%), arising from the medial/paramedial raphe nuclei (MnR) (Sakurai et al., 2005; Sakurai et al., 2008).

2.3.4 Orexin neurons and 5-HT

The interaction between the 5-HT and the Orx systems was first suggested f by Dixon & cols, who found that the blockade of 5-$HT_{2A/C}$, but not 5-HT_{2B}, effectively inhibited the grooming behavior induced by the administration of Orx-A (Dixon et al., 2001). This was also described by Matsusaki & cols, where they demonstrated that the administration of ritanserin, a selective 5-HT_2 receptor antagonist, can reverse the increases in motor activity, grooming and shaking behavior, induced by the administration of Orx-A (Matsusaki et al., 2002). Other studies have shown that i.c.v. injection of WAY100635, a selective 5-HT_{1A} antagonist, decreases locomotor activity, an effect that was absent in the Orx/ataxin3 mouse, a model with a genetic ablation of Orx neurons (Muraki et al., 2004).

In electrophysiological studies, it has been found that 5-HT inhibits orexin neurons in a dose-dependent manner (Yamanaka et al., 2003; Muraki et al., 2004; Tsujino et al., 2005). Such hyperpolarization can also be achieved to an ~ 85% by the selective 5-HT_{1A} agonist 8-OH-DPAT

(Muraki et al., 2004). Conversely, WAY100635 fully inhibits 5-HT-induced hyperpolarization of Orx neurons (Muraki et al., 2004). Also, 5-HT indirectly inhibits Orx neurons by facilitating GABAergic inhibitory inputs without affecting excitatory inputs (Chowdhury & Yamanaka., 2016). Moreover, *in situ* hybridization and immunohistochemical studies indicate that the 5-HT_{1A} receptor is present in the soma of Orx neurons of the lateral and medial portion of the hypothalamus (Collin et al., 2002; Mannoury et al., 2006; Muraki et al., 2004). On the other hand, Saito & cols, recently generated a mouse lacking 5-HT_{1A} specifically in Orx neurons (Orx-5-HT_{1A}-KO) and found a longer NREM sleep time along with decreased wakefulness time in the later phase of the dark period. They also found that restraint stress induced a larger impact on rapid eye movement (REM) sleep architecture in Orx-5-HT_{1A}-KO mice than in controls, with a larger delayed increase in non REM sleep amount as compared with that in controls, indicating altered REM sleep homeostasis (Saito et al., 2018) and highlighting the importance of 5-HT and, in particular, of 5-HT_{1A} in the regulation of Orx neurons activity.

2.3.5 5-HT_{1A} Receptor

5-HT_{1A} is a GPCR canonically coupled to $Gi_{/o}$ protein, which can be located both pre- and post-synaptically (Pazos et al., 1985, Riad et al., 2000). At the presynaptic terminal site, 5- HT_{1A}R typically works as an autoreceptor inhibiting the 5-HT tone once the 5-HT terminal has been stimulated (Pazos et al., 1985). When located in the post-synaptic spine, 5-HT_{1A} can activate different signaling cascades depending on the neuronal type being expressed, including the canonical inhibition of AC as well as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-Akt pathways (Bayliss et al., 1995; Zhong et al., Edagawa et al., 1999). Canonically agonist binding to the 5-HT_{1A} exchanges guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the alpha subunit of $G_{i/o}$ ($G_{i\alpha}$/$G_{o\alpha}$) (Birnbaumer., 2007) which primarily functions to inhibit AC, resulting in decreased cAMP production and protein kinase A (PKA) activity (Jakbos et al., 1978). Typically, 5-HT_{1A} auto-receptors do not activate the canonical

route inhibiting AC, and rather activate alternative signaling routes (Clarke et, 1996), whereas 5-HT_{1A} heteroreceptor mainly generates inhibition by the canonical way and through the alternative signaling pathways as well. Experiments in mammalian hippocampal neurons demonstrated that 5-HT inhibited forskolin-stimulated cAMP accumulation through 5-HT_{1A} (De Vivo & Maayani., 1986), an effect replicated in cortical neuron cultures (Dumuis et al., 1989) as well as in cell lines expressing 5-HT_{1A} (Harrintong et al., 1993; Raymon et al., 1999). The activation of 5-HT_{1A} also activates G protein-coupled inward rectifying potassium channels (GIRKs) (Lüscher et al., 1997), in the hippocampus (Colino & Halliwell., 1987), dorsal raphe (Clarke et al., 1996) and Orx neurons in the LH (Muraki et al., 2004). In regard to MAPKs, ERK is particularly affected by 5-HT_{1A} in the brain; ERK is traditionally activated by growth factor tyrosine kinase receptors in pyramidal cells of the cortex (Polter et al., 2010).

The activation of 5-HT_{1A} can also activate the PI3K-Akt pathway in hippocampal neurons (Cowen et al., 2005, Polter et al., 2009). Akt is a serine/threonine protein kinase involved in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration and includes three closely related isoforms Akt1, Akt2, and Akt3 (Calera et al., 1998). Among them, Akt1 canonically exerts an anti-apoptotic action, thereby promoting cell survival (Song et al. 2005), but it can also actively participate in the neuronal synapse (Sánchez-Alegría et al., 2018). Akt1 can be activated via phosphorylation at its residue Ser473 in the carboxy-terminal regulatory region (Song et al. 2005); this activation can be carried out directly by PI3K (Tsuchiya et al., 2014) and by mammalian Target of Rapamycin (mTOR) (Bayascas & Alessi., 2005; Gao et al. 2005), both second messengers that can be activated by 5-HT_{1A} (Polter et al., 2010) Once Akt1 is activated, for example by insulin, it can phosphorylate different targets including the transcription factor Forkhead Box A2 (Foxa2) (Wolfrum et al., 2003).

Foxa2 is a member of the winged helix/Forkhead transcription factors, also called “pioneer proteins” that, by binding to tightly condensed chromatin in promoters and enhancer regions, can facilitate the access for other transcription factors (Cirillo et al., 2002). Foxa2 has been found to

play important roles in multiple stages of mammalian life, beginning in early development, continuing during organogenesis, and finally in metabolism and homeostasis in the adult (Friedman et al., 2006; Silva et al., 2009). Phosphorylation of Foxa2 by Akt1 induces its sequestering in the cytoplasm of the cell preventing its translocation to the nucleus in Orx neurons (Wolfrum et al., 2003; Silva et al., 2009). The passage of Foxa2 to the nucleus and the consequent binding to its DNA-binding sites can directly determine the transcription of different genes; among them, the Orx gene has been reported (Silva et al., 2009).

To date, it is currently unknown if the 5-HT system, and 5-HT_{1A}, can control food intake by directly regulating Orx synthesis in hypothalamic Orx neurons. As previously discussed, it has been shown that medial and lateral hypothalamic neurons co-express Orx markers and 5-HT_{1A}. As 5-HT_{1A} can activate several signal transduction pathways, the specific signal pathway involved in the modulation of Orx neurons remains unclear. Among the several proteins substrates that are known to be regulated by Akt1, the transcription factor Foxa2 arises as highly relevant for feeding behavior, since it has been demonstrated that Foxa2 directly binds to Orx gene promoter and regulates prepro-Orx production (Silva et al., 2009).

Collectively, these evidences suggest that the 5-HT_{1A}-Akt1-Foxa2 pathway is a plausible signal cascade to be involved in the regulation of hypothalamic Orx production and, therefore, feeding behavior by 5-HT activity, which remains to be demonstrated.

3. Hypothesis

5-HT via the 5-HT_{1A}-Akt-Foxa2 pathway, modulates the expression of Orexin in neurons of the lateral hypothalamus, which determines changes in food intake in mice.

4. Aims

4.1 Overall aim

To demonstrate that 5-HT through 5-HT_{1A} regulate Orx expression and feeding behavior in mice.

4.2 Specific aims

1. To measure changes in the expression of prepro-Orx mRNA, Orx-A protein levels, Akt1 phosphorylation and translocation of Foxa2 to the nucleus, in primary culture of LH neurons treated with 5-HT and selective 5-HT_{1A} ligands**.**
2. To measure changes in food intake and locomotor activity in mice infused intra-LH with selective 5-HT_{1A} ligands. In same cohort of mice, to measure Orx-A protein levels, Akt phosphorylation and Foxa2 nuclear translocation.
3. To measure changes in food intake and locomotor activity in mice infused intra-LH with selective Akt inhibitor.

5. Materials and methods

5.1 Animals

C57bl/6 mice of either sex were housed with their litter-mates with ad libitum access to food and water at a constant temperature and humidity on a 12/12 h light / dark cycle (lights on at 7:00 A.M.). Experiments were performed in compliance with the Animal Facility of the Faculty of Sciences, University of Valparaiso, act of bioethical evaluation BEA 111-2016.

Twelve litters of P1-P2 mice were used for the first objective, each one consisting of an average of ten mice. In this experiment, 6 experimental groups were generated each one with n =3-4. In the second objective, male mice, adults of 3-4 months, were used, generating 4 experimental groups each with an *n=8* and an error percentage of 15% was produced, for which 37 mice were used. Finally, in the third objective, two experimental groups were generated with n=8, a percentage of error of 15% was produced, so nineteen mice were used. Therefore, a total of one hundred and sixty-eight mice were occupied in this work.

For the first objective the mice were euthanized by cold thermal shock. After the experiments of the second and third objectives, the animals were anesthetized in the 5% isoflurane chamber. Once deeply anesthetized, the perfusion method described in the immunofluorescence section was performed.

5.2 Neuronal primary culture

From postnatal 1-2 (P1-P2) C57BL/6J mice hypothalamus was isolated. Tissue was extracted in solution of Hank's Balanced Salt Solution cold (ThermoFisher Scientific, USA) undergoing three washes. Subsequently, the samples were disrupted in presence of 0.025% trypsin and 0.01% EDTA and homogenized with sterile needles of decreasing calibers. The disintegrated tissue was then centrifuged at 3500 rpm for 5min, the supernatant was discarded and a pellet rich in cells was obtained. Then, the cells were resuspended in culture medium DMEM/F12-Dulbecco's

Modified Eagle Medium: Nutrient Mixture F-12 (cat 11320082, ThermoFisher) supplemented 10% fetal bovine serum (FBS). The cell count was performed manually in a Neubauer Chamber (Paul Marienfeld, Germany) using the four quadrants; living cells were differentiated with trypan blue staining (cat ab233465, Abcam). $2x10^5$ cells were plated p poly-(D-lysine)-coated (cat 25988-63-0, Sigma-Aldrich) 12-mm Petri dish. After 6 hours, the medium was changed and cells were maintained in serum-free medium Neurobasal™-A Medium (cat 21103049, ThermoFisher) supplemented with serum-free supplement for neural cell culture, Gibco B-27 Serum Free Supplement (cat 17504001, ThermoFisher) at 37°C (95% air, 5% CO2). Cultured cells were maintained for 7-10 days before use.

5.3 RT-qPCR

The cells were removed from the culture plates using 200µl TrypLE™ Express (cat 12604013, ThermoFisher). Cells were collected and pelleted by centrifugation at 3500 rpm for 5 min. The total supernatant was removed, and RNA was isolated using the RNA extraction kit RNeasy Mini Kit (cat 74106, Qiagen) according to the manufacturer's specifications. Reverse Transcription (RT) was done using 100ng of total RNA using the reverse transcription PrimeScript™ RT Master Mix (cat RR036A, Takara biotech) to generate cDNA. The cDNAs were stored at -80°C until use.

Quantitative polymerase chain reaction (qPCR) (AriaMx Real-time PCR System, Agilent Technologies, Inc) was performed in 0.2 ml PCR tubes containing 1 µL of cDNA, 1mM of each primer, 1x PCR master mix Brilliant III SYBR (Cat 600883, Agilent Technologies, Inc), and DEPC-Treated Water (cat 4387937, ThermoFisher) for a total volume of 20 µl. PCR conditions were as follows: 95°C for 10 min (hot start); 95°C denaturation for 10 s; 56°C annealing for 15 s, and 72°C extension for 20 s for 40 cycles. Fluorescence was measured at 72°C. Then a melting curve was performed from 72 to 95°C, collecting fluorescence every 0.5°C for 47 cycles. The PCR primers for prepro-Orx were as follows: (forward) 5'-TGGGTATTTGGACCACTGCACTGA-3´; (reverse) 5´-CAGGGAACCTTTGTAGAAGGAAAGTTC-3´ and HTR1A (5-HT_{1A} gene) (forward) 5´-

CCCCCCAAGAAGAGCCTGAA-3´; (reverse) 5´- GGCAGCCAGCAGAGGATGAA-3´. Hypoxanthine-guanine phosphoribosyl transferase (HPRT-1) was used as reference gene, primers were as follows: (forward) 5´-CAACATTTGCTTTCCCTGGT-3´; (reverse) 5´-TCTGGCCTGTATCCAACACTTC -3´. The specificity of the PCR products was determined by means of the specific melting point for each amplicon obtained (Supplementary figure 1). The efficiency (E) of the amplification reaction for each set of primers was obtained through the equation $E=10^{1/slope}$. The slope was obtained from the calibration curve constructed from serial dilutions of the PCR product for each gene and the amplification cycle or threshold cycle (Ct) (Supplementary figure 2). Data were analyzed using $2^{-\Delta\Delta Ct}$ method to compare all experimental groups (Yuan et al., 2006).

5.4 Animals and surgical procedures

C57BL/6J adult mice (25 to 30 grs) were used for intra-LH infusions and subsequent *in vivo* experiments; SERT KO mice were used to determine preproOrx and HTR1A mRNA levels. Mice were given access to standard laboratory rodent chow (cat 6973, LabDiet) and water *ad libitum*. For bilateral intracranial cannula implantation, mice were anesthetized intraperitoneally with a mixture of Xilazine 10mg/kg + Ketamine 80mg/kg, mounted in a stereotaxic frame (Kopf instruments) with a mouse adapter (RWD). An incision of < 3 cm was made and bilateral guide cannula (26 gauge, Plastics One, Roanoke, VA) were lowered into the LH (anteroposterior, −1.35 mm; mediolateral, ± 1 mm; dorsoventral, −5.4 mm to Bregma) according to (Chowdhury, et al., 2016). Cannula were anchored to the skull surface with dental cement and occluded with dummy cannula of the same length. Mice were treated post-surgically with ketoprofen (5 mg/kg, s.c.), individually housed, daily weighted and monitored by a veterinarian and allowed to recover for one week. Cannula placements were determined *post hoc* (figure 4A).

5.5 Intra LH drug infusion

Prior to measuring locomotor activity and food consumption, mice were anesthetized with 1-chloro-2,2,2-trifluoroethyldifluoromethyl ether (Isoflurane) and microinfused using 33G infusion probes that protruded 0.2 mm below the base of the guide cannula. DPAT (the active enantiomer of racemic 8-OH-DPAT, cat 78095-19-9, TOCRIS) 6.4nmol in Krebs solution, WAY100635 (cat 149007-54-5, TOCRIS) 3.7nmol in Krebs solution, Akt1 inhibitor VIII (cat 612847-09-3, Sigma-Aldrich) 6.0 nmol in Krebs-10% DMSO Krebs solution and Krebs-10% DMSO vehicle were used. Solutions were bilaterally infused into the LH (0.1 µL/minute) at a total volume 0.5 µL; infusion probes were left in place for 3 minutes following the injection and then slowly removed. Mice were returned to their home cage for 1 hour prior to the behavioral assays. Separate groups of mice received intra-LH DPAT, WAY100635, Akt1 inhibitor VIII or vehicle.

5.6 Immunofluorescence

Mice were perfused transcardially with 0.1M phosphate buffered saline (PBS) under isoflurane anesthesia followed by a mixture of 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4). The brains were removed and post-fixed in the same buffer overnight. Then, 20 µm coronal slices were sectioned on a sliding cryostat (Leyca, Biosystem). The sections were then blocked and permeabilized in PBS containing 5% normal horse serum (NHS), 5% bovine serum albumin (BSA) and 0.2% triton x-100 for 1 h. The slices were incubated for 1 day in a 4°C under continuous agitation with primary antibodies anti-Orx-A (sc-8070, Santa Cruz Biotechnology), anti-Foxa2 (ab10422, Abcam), anti-phospho Akt1 (pAkt) Serine 473 (S473) (#9271, Cell Signaling) in PBS containing 5% BSA, 5% NHS and 0.2% Triton x-100. In particular, in this work, phosphorylation in Akt S473 was selected, since it can be activated by receptors coupled to Gi protein and by PI3K (Tsuchiya et al., 2014).After washing, the slices were incubated with anti-rabbit antibody (1:1000, ab150077, Abcam) and anti-Mouse antibody Alexa 555 (1:1000, ab150106, Abcam), both diluted in blocking solution. To verify the specificity of the staining, some

sections were incubated without the primary antibody. DAPI was used as a nuclear stain. All images were obtained using a confocal microscope (Nikon, corporation).

5.7 Immunoblot

Western blot analysis was used to evaluate the expression of Orx-A, Akt1 and Foxa2 proteins in hypothalamus, prefrontal cortex and striatum. Briefly, the tissue samples were extracted and homogenized with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-base, pH 8.0). The samples were prepared at a concentration of 5 µg/µL in Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl pH 6.8). The samples were separated by electrophoresis in 8% polyacrylamide SDS gels. Then, the proteins were transferred to a nitrocellulose membrane (cat 10600002, GE Healthcare Life Sciences) and blocked in Tris-buffered saline (TBS) containing 7% dry milk for 1h in agitation and 5% bovine serum albumin (BSA). The membranes were then incubated overnight with antibodies anti-Orx-A (sc-8070, Santa Cruz Biotechnology; used at 1:1000), anti-Foxa2 (ab10422, Abcam; used at 1:1000), anti phospho Akt (#9271, Cell Signaling; used at 1:1000) in TBS containing 5% dry milk or 5% BSA and b-actin mouse monoclonal antibody (ab8227; Abcam; used at 1:5000). After incubation, the membranes were washed three times with TBST (TBS containing 0.1% Tween-20) and were then incubated again for 2 h with the appropriate anti-mouse or anti-rabbit IgG (ab6721; Abcam used at 1:5000) at room temperature. The membranes were washed again with TBST three times. Finally, the reaction was developed using a chemiluminescent reagent (cat RPN2232, GE Healthcare Life Sciences) and registered using a Licor c-digit scanner (LI-COR Biosciences, U.S.).

5.8 Calculate the Corrected Total Cell Fluorescence method

The Calculate the Corrected Total Cell Fluorescence (CFTCF) method was used to quantify the levels of Orx-A and the percentage of Foxa2 accumulation in the immunofluorescence. For

this, ImageJ software (NHI, US) was used and the results were calculated according to the formula: CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings) (Potapova et al., 2011).

5.9 Locomotor activity

The locomotor activity was at 10am measured using an open field arena, as described previously (Delgado-Acevedo et al., 2019). The open field was a square box (39 x 39 x 35 cm) with clear Plexiglas walls and floor, evenly illuminated by white overhead fluorescent lighting at ~60 lux intensity. Mice were individually placed in the center of the open field and allowed to freely explore for 10 min. Activity was measured by a computer assisted animal activity system (Ethovision version XT7, Noldus Information Technology).

5.10 Food consumption

The food intake assays were performed at 10am, in the respective home cage, where mice were given *ad libitum* access to food prior to, during, and after the assay. Measurements of food intake were made at 0, 1, 3 and 7 hours post drug administration. Bilateral LH infusions were performed as described above. Food intake was measured after drug infusion by weighing the initial content (30 grs), discounting the weight consumed at each indicated time.

5. 11 Statistical analyses

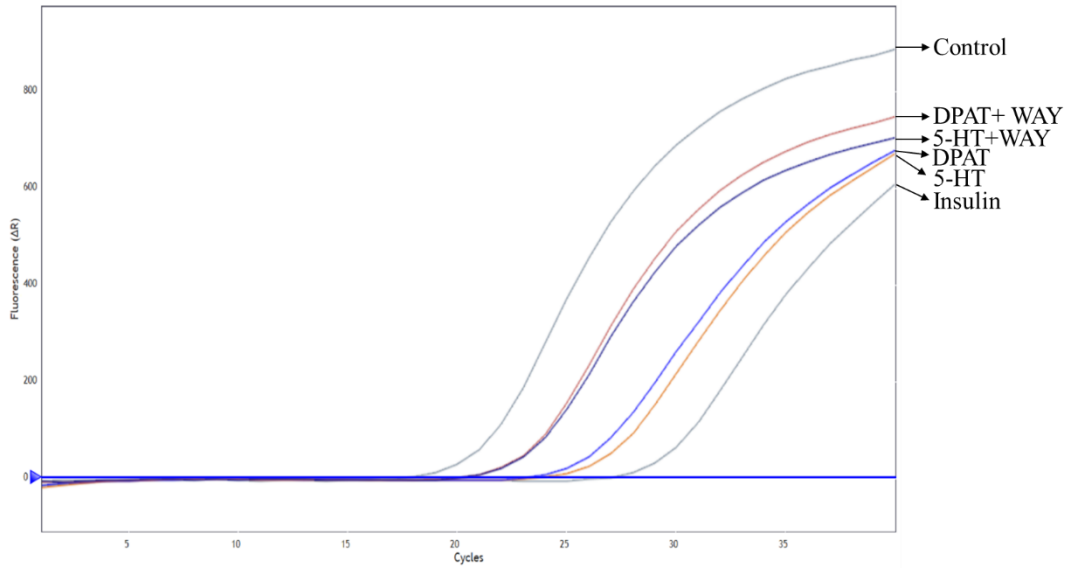
For pharmacological studies, the data obtained showed a Gaussian distribution, which was calculated according to the expression Y=Amplitude*exp(-0.5*((X-Mean)/SD)^2). We determined the mixed analysis of variance (ANOVA) of repeated one-way measures to examine the effects of 5-HT, DPAT and WAY100635 in hypothalamic neuronal primary cultures and for the analysis of protein expression in intra LH infused mice. The Sidák post hoc analysis was used to test the

differences between groups in each drug concentration when significant main effects were obtained (*P <0.05*). The Pearson correlation coefficients were calculated in all the correlations presented in this work. For feeding behavior, a Two-way repeated measures (RM) ANOVA and Sidák multiple comparison test * *P = 0.05,* ** *P = 0.01,* *** *P = 0.001* was used (supplementary figure 3). For the analysis of mRNA levels in SERT KO mice and pAkt levels with inhibitor VIII, *t student* was used for parametric variables. Statistical analyses were performed with GraphPad Prism 6 (IBM, New York NY).

6. Results

We first treated primary cultures of mouse hypothalamic neurons with 5-HT (100 µM, n=4) and with the selective 5-HT_{1A} agonist DPAT (100 µM, n=4) for 2 hours (Polter et al., 2010). Significantly decreased prepro-Orx mRNA levels were observed with the treatment with 5-HT and DPAT, reaching ~20% and ~30%, respectively of control values (Figure 1). Prepro-Orx levels were then found to be normalized in primary cultures co-treated with WAY100635 (100 µM, n=4), a selective 5-HT_{1A} antagonist (Figure 1). As a positive control, a 4h starvation challenge was used, whereas a treatment with insulin (50µM) for 2h was used as a negative control (Figure 1B).

A



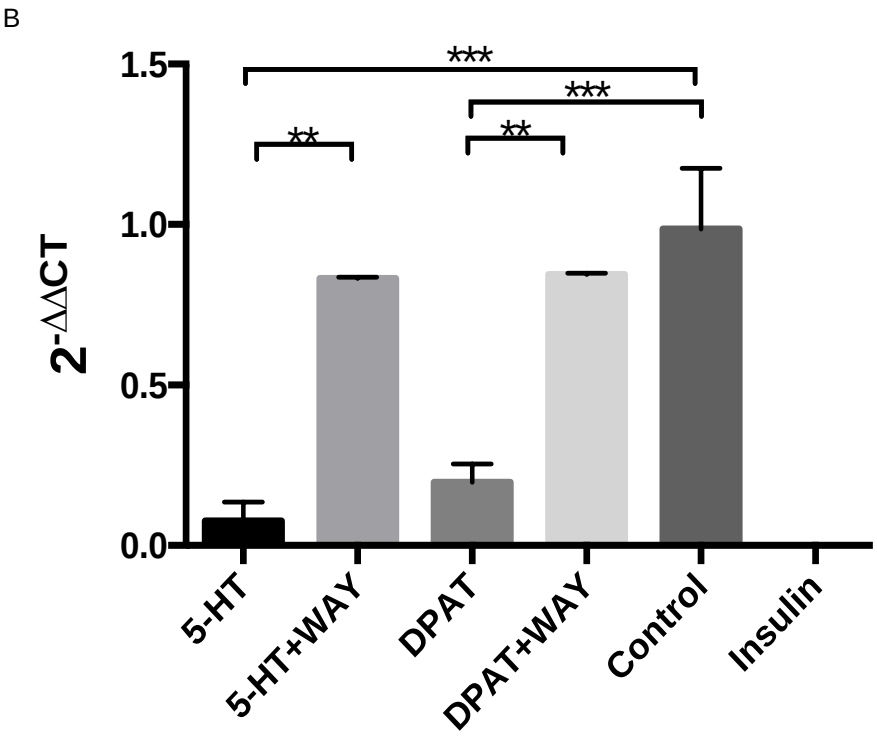
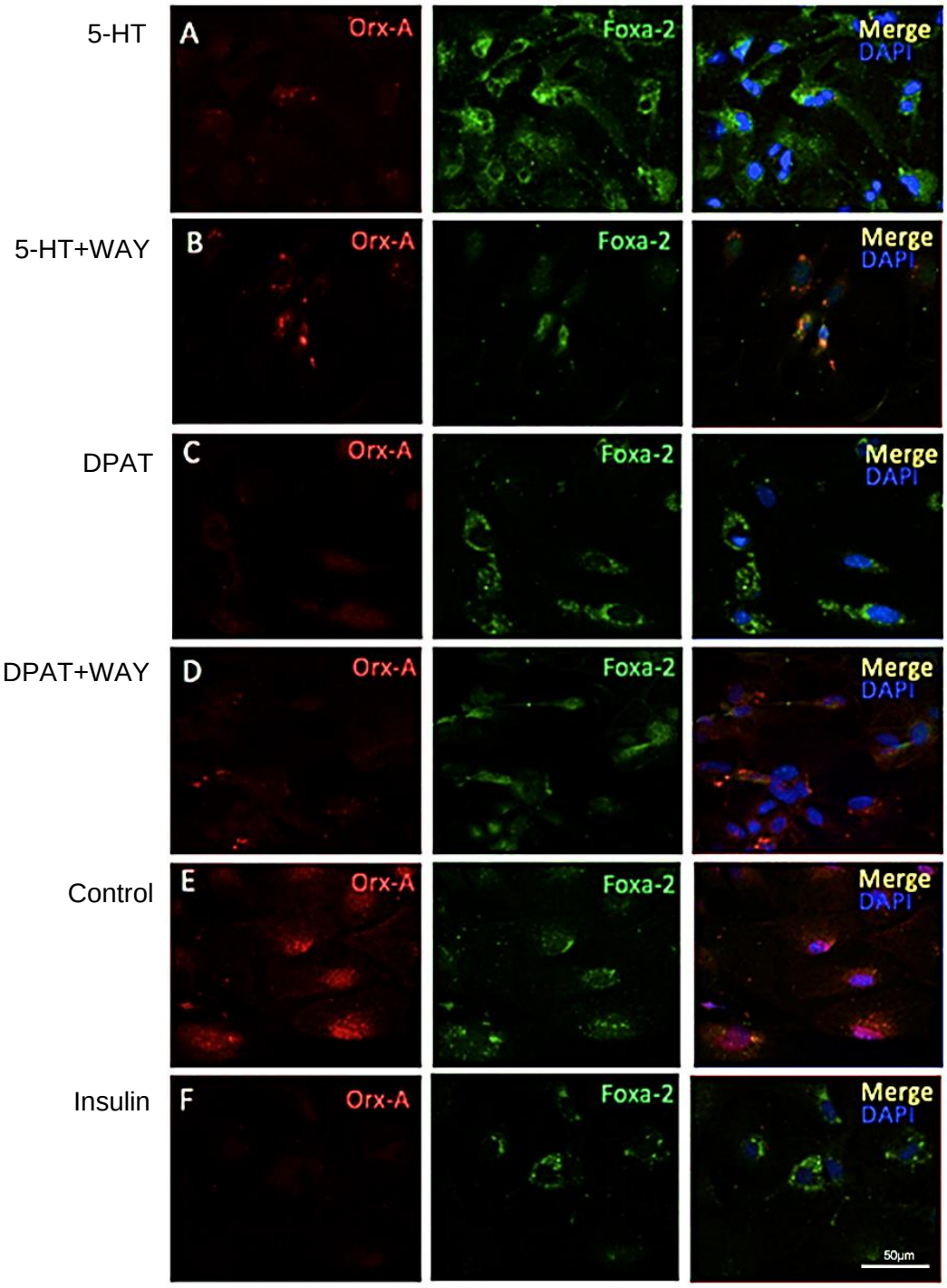


Figure 1. PreproOrx levels in primary culture of hypothalamic neurons treated with a 5-HT$_{1A}$ agonist and antagonist. (A) Amplification plot where a representative trace in cell extracts subjected to pharmacological activation of 5-HT$_{1A}$. The cultured cells were incubated with 5-HT (100µM; n=4), 5-HT (100µM); n=4 + WAY100635 (100µM); n=4, DPAT (100µM); n=4, DPAT (100µM); n=4+ WAY100635 (100µM); n=4, control starvation and Insulin (50 µM); n=4 for 2. B) It is observed that cells treated with both 5-HT and DPAT significantly decrease prepro-Orx levels (*P = 0.8826* and *P = 0.7997*), which is normalized to treatment with WAY100635. B) RT-qPCR of prepro-Orx. (One-way ANOVA, followed by Sidák's post hoc test, **P=0.05*, ***P = 0.01*, ****P = 0.001)*

To determine if the decreased prepro-Orx mRNA levels effectively results in reduced Orx levels, the Orx-A protein levels were measured, which has been reported to have greater stability than Orx-B (Kukkonen., 2012). Using immunofluorescence, we found that both treatments with 5-HT and DPAT decreased five times Orx-A immunoreactivity (n = 3) (Figure 2A, C and G), an effect that was blocked under the co-treatment with WAY100635 (Figure 2B, D and G)

Next, to determine if the molecular mechanism regulating the production of the Orx is through the transcription factor Foxa2, we determined its cellular localization after the treatments described above in primary culture of hypothalamic neurons. Treatment with 5-HT (n = 3) and DPAT (n = 3)

decreased the nuclear accumulation of Foxa2 up to 20% and 25%, respectively (Figure 2A, C and H). Then it was found that the decrease in the levels of nuclear Orx-A and Foxa2 found to be normalized in primary cultures co-treated with WAY100635 (100 μM, n=4) (Figure 2B, D and H).



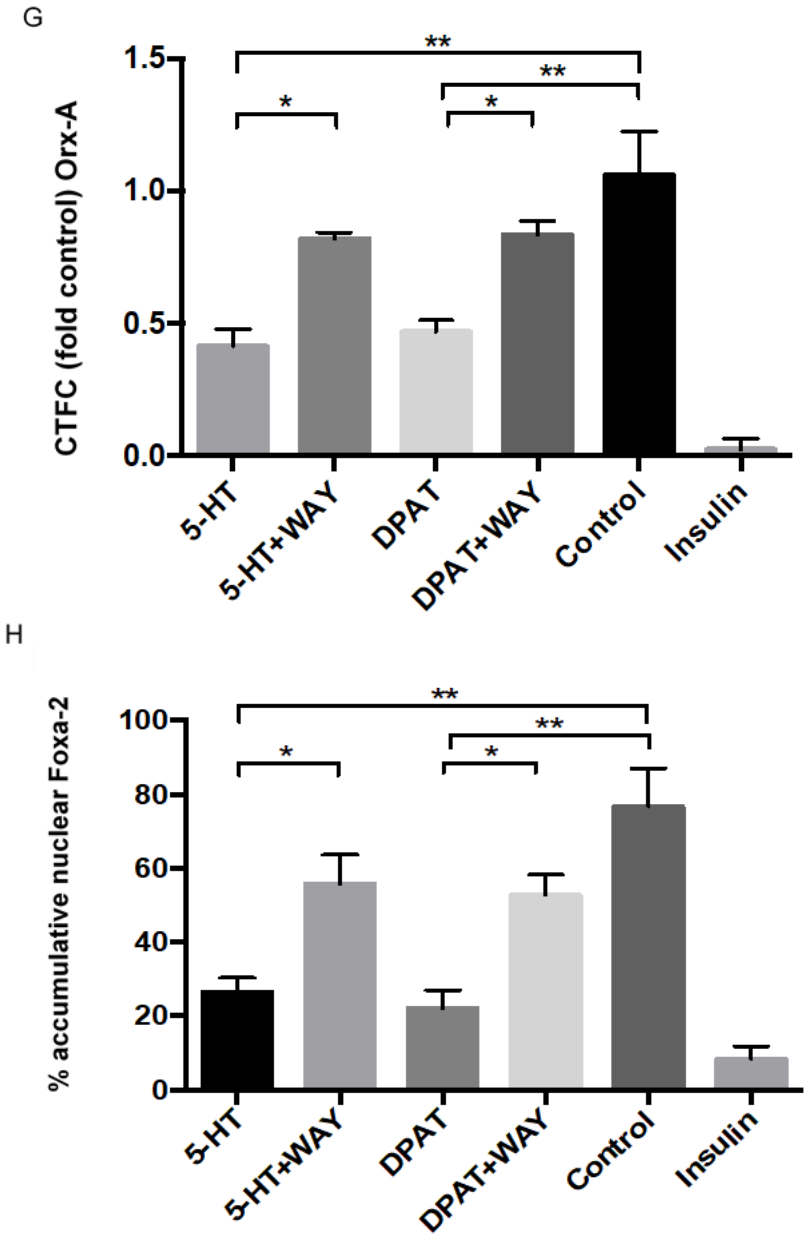
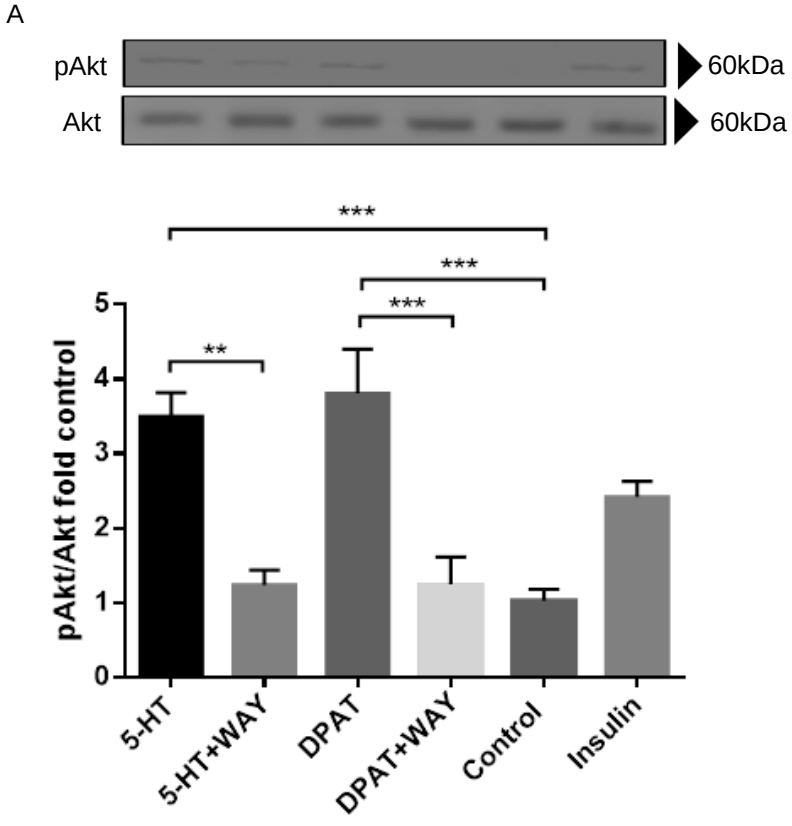


Figure 2. Orx-A levels and subcellular localization of Foxa2 in primary culture of hypothalamic neurons treated with a 5-HT$_{1A}$ agonist and antagonist. Dual immunofluorescence for Orx-A (red), Foxa2 (green) and DAPI (blue) for nuclear staining. A) Incubation with 5-HT (100µM) n=3. B) 5-HT (100µM) + WAY100635 (100µM) n=3. C) DPAT (100µM) n=3. D) DPAT (100µM) + WAY100635 (100µM) n=4. E) Control starvation. F) Insulin (50 µM). G) Quantification of the fluorescence intensity of Orx-A and H) nuclear accumulation of Foxa2 using the CTCF method. Pharmacological treatment whit 5-HT decrease Orx-A protein levels (*P= 0.0015*) and nuclear localization of Foxa2 (*P= 0.0009*) compared to control. Activation of 5-HT$_{1A}$ (*P= 0.0021*) reproduced the effect produced by 5-HT, decreasing Orx-A protein levels (*P= 0.0015*) and nuclear localization of Foxa2 (*P= 0.0021*) Scale bars: 50µm. (One-way ANOVA, followed by Sidák's post hoc test, **P=0.05*, ***P = 0.01*, ****P = 0.001).*

As previously described (Wolfrum et al., 2003), the activation of Akt1 can increase the phosphorylation of Foxa2, promoting its nuclear exclusion. Thus, to evaluate that the reduction in Foxa2 nuclear accumulation upon 5-HT_{1A} activation is a consequence of an increased Akt1 activation, we determined phosphorylated Akt1 by western blot and immunofluorescence (Figure 3). Treatment with 5-HT (n=3) and DPAT (n=3) significantly increased the phosphorylation of Akt at Ser473 compared to the control condition, measured at 5 min of drug incubation (Figure 3A and B). The increased phosphorylation of Akt1 by DPAT was also observed when colocalizing with Orx-A (Figure 3D) in comparison to the control in the starvation condition (Figure 3C).



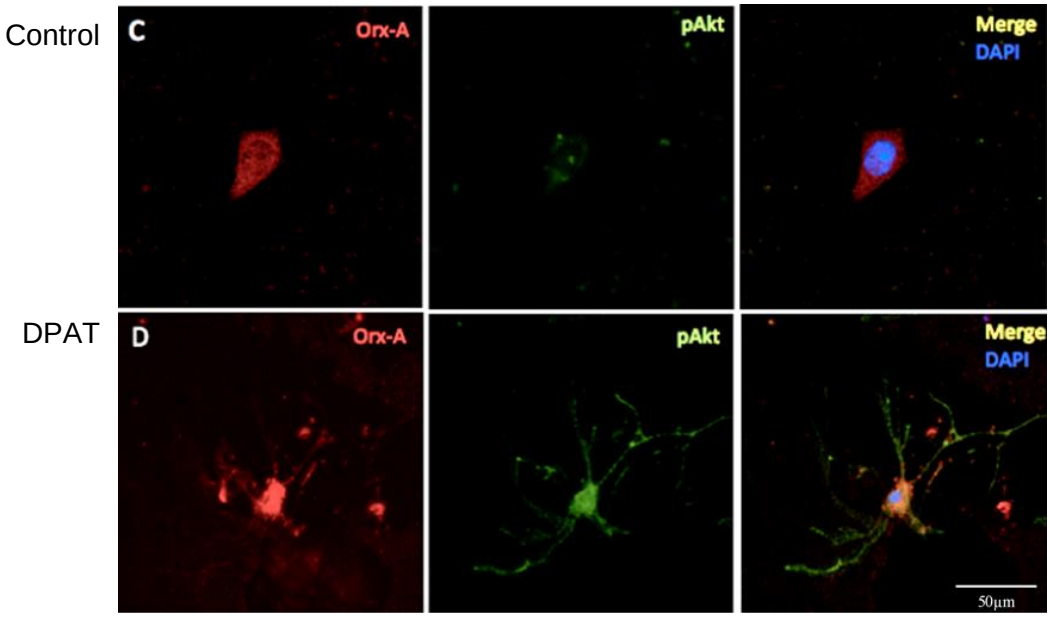
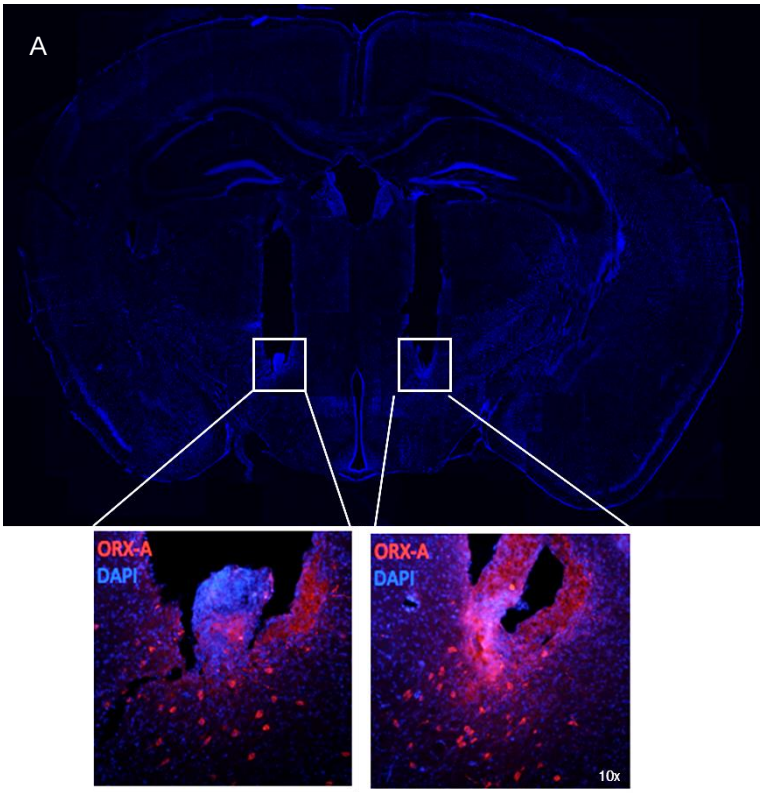


Figure 3. pAkt levels in primary culture of hypothalamic neurons treated with a 5-HT$_{1A}$ agonist and antagonist. Western blot and IF in cells subjected to pharmacological activation of 5-HT$_{1A}$. The cultured cells were incubated with 5-HT (100µM); n=4, 5-HT (100µM); n=4 + WAY100635 (100µM); n=4, DPAT (100µM); n=4, DPAT (100µM) + WAY100635 (100µM); n=4, control starvation and Insulin (50 µM); n=4 for 2 hours. A) Representative western blot of pAkt and Akt of treatment from left to right with 5-HT, 5-HT+ WAY100635, DPAT, R-OH-DPA + WAY100635, control starvation and Insulin. B) pAkt levels after previously described treatment. C) IF of Orx-A (red) and pAkt (green) in control cells and treated with DPAT. The treatment with 5-HT and DPAT increases the phosphorylation of Akt (*P=0.0012* and *P= 0.0035* respectively). (One-way ANOVA, followed by Sidák's post hoc test, **P=0.05*, ***P = 0.01*, ****P = 0.001).* Scale bars: 50µm. Note that pAkt levels were calculated as the pAkt/Akt ratio.

In vivo experiments in wild-type C57BL/6J male mice were performed to test if the activation of 5-HT$_{1A}$ can trigger changes in the feeding behavior. Confirmation of proper cannula placement was done by immunofluorescence as depicted in Figure 4A which shows the cannulation site and its vicinity with the Orx neurons in a representative picture. Administration of DPAT (n=8) significantly decreased food intake at 3 and 7 hours compared to vehicle infusion in the same animal (Figure 4C-4D) compared to vehicle infusion in the same animal. Conversely, food intake increased significantly at all tested times after administration of WAY100635 (n=8) (Figure 4E-4G). There were no differences in food intake in mice infused with DPAT + WAY100635 (n=8) comparted to control group (Figure 4H-4J).



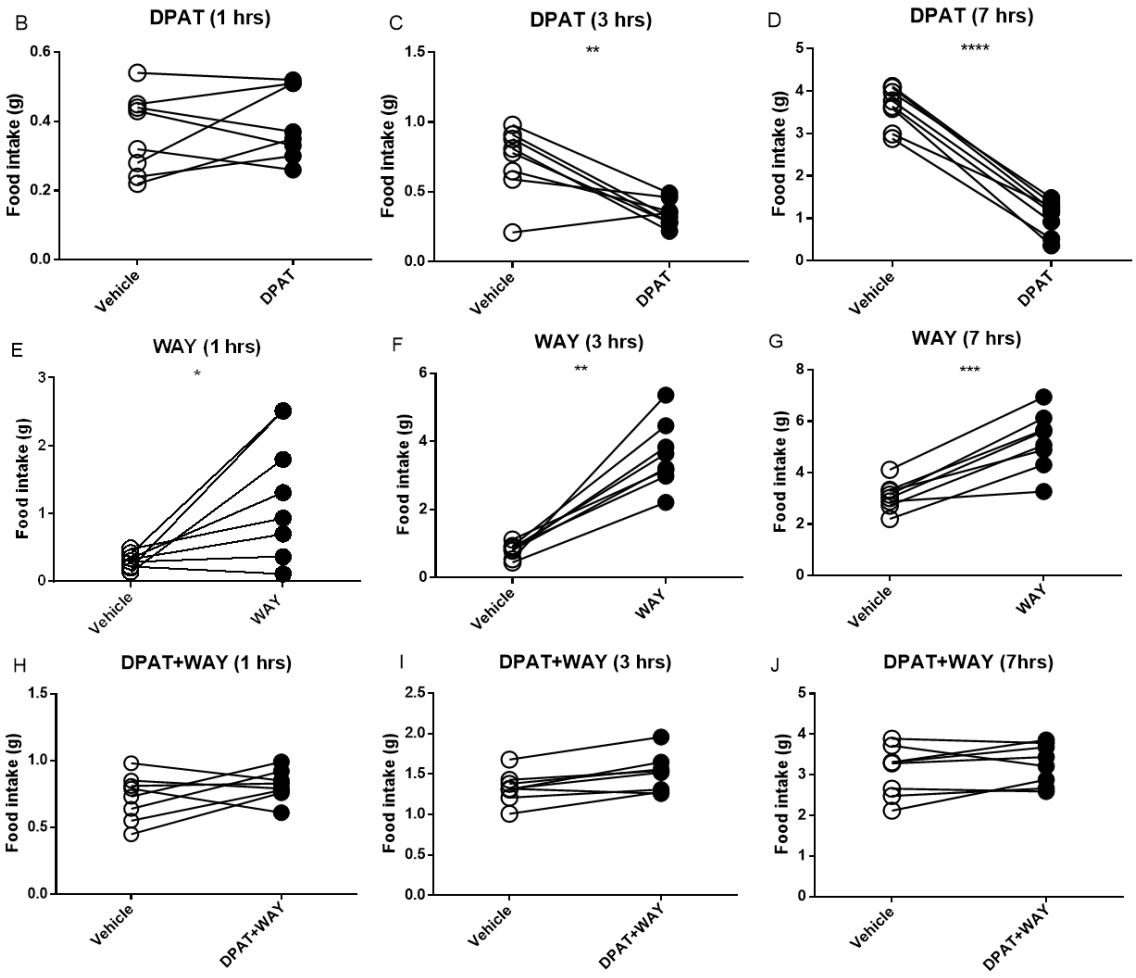


Figure 4. Effect of 5-HT$_{1A}$ agonist and antagonist infusion into LH on food intake. Adult male mice were chronically cannulated into LH and cumulative food intake was measured. A) Representative image of the stereotaxic coordinates of the LH infusion site (anteroposterior, −1.35 mm; mediolateral, ± 1 mm; dorsoventral, −5.4 mm to Bregma). Each mouse was infused with vehicle at times 1, 3 and 7hrs and food intake was measured (open circles). Then, the same mouse received one of the following drugs R-OH-DAPT; n=8 (B-D), WAY100635; n=8 (E-G) or R-OH-DAPT + WAY100635; n=8 (F-H) and food intake was measured at times 1, 3 and 7hrs (black circles). The infusion of DPAT decreases the food intake in mice compared to the vehicle at times C) 3 hours (*P= 0.0011*) and D) 7 hours (*P< 0.0001*). The infusion of WAY 100635 significantly increased the food intake E-G) from 1hrs and remain until 7h. Intake was normalized by administering H-J) DPAT followed by WAY100635. (Paired *t* test * *P = 0.05,* ** *P = 0.01,* * ** *P = 0.001*).

To determine the population effect of the activation or inactivation of 5-HT_{1A}, the food intake was measured in mice locally treated with vehicle, DPAT, WAY100635 or DPAT + WAY100635 (Figure 5). Mice treated with DPAT significantly decreased food intake at 3 hours post infusion (Figure 5A-5D). Conversely, the infusion of WAY100635 significantly increased the food intake after 3 hours compared to vehicle-infused controls (Figure 5E-5H). The co-administration of DPAT + WAY100635 did not produce changes in food intake compared to controls (Figure 5I-5L).

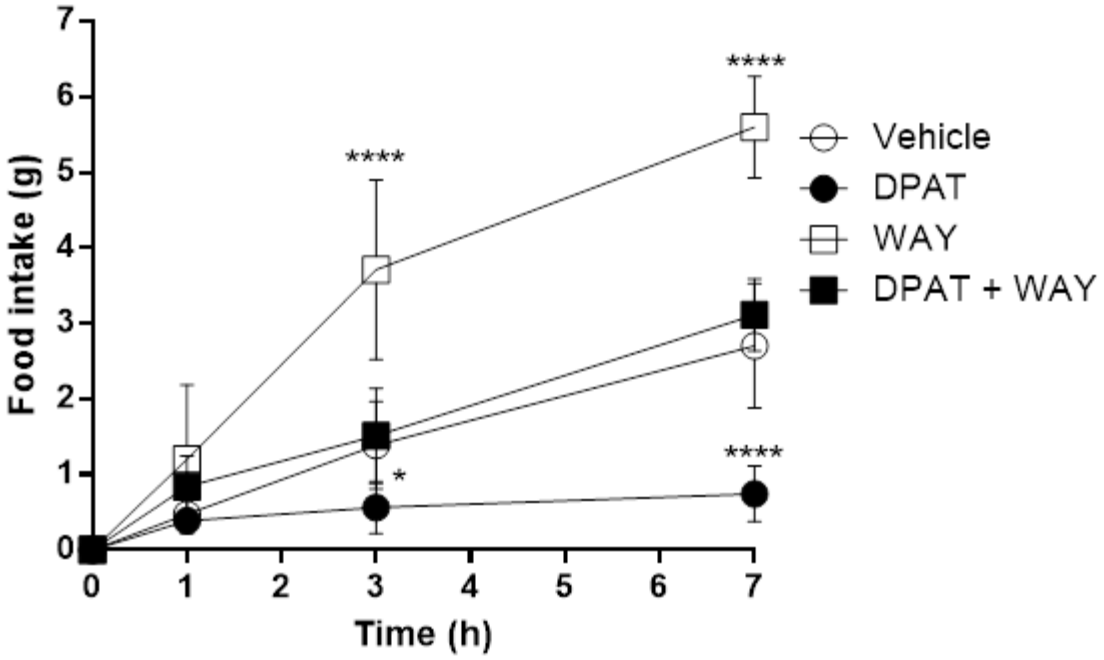


Figure 5. Population effect of 5-HT_{1A} agonist and antagonist infusion into LH on food intake. Data grouped of food intake in vehicle-treated mice, DPAT, WAY100635 and DPAT + WAY100635 at times 1, 3 and 7 hours compared to the vehicle. The infusion of R-OH-DAPT significantly decreased food intake at times 3 and 7 hours (*P= 0.0249* and *P< 0.0001* respectively). Conversely, the infusion of WAY100635 significantly increased food intake at times 3 and 7 hours (*P< 0.0001* and *P< 0.0001* respectively). Effects that were normalized when administering DPAT followed by WAY100635. (Two-way repeated measures (RM) ANOVA and Sidák multiple comparison test * *P = 0.05, ** P = 0.01, *** P = 0.001***).* Details of the analysis in Supplementary Figure 3.

It has been reported previously that the intracerebroventricular injection of orexin induces an increase in locomotor activity (in addition to an arousal response) (Matsuzaki et al., 2002). Therefore, we measured the horizontal locomotor activity in an open arena during 5 min, at 7 hours after infusion, in all the experimental groups (Figure 6A). The administration of DPAT significantly increased the total distance traveled compared to control. On the contrary, the administration of WAY100635 significantly decreased the travelled distance, which was normalized in the group receiving DPAT + WAY100635 (Figure 6).

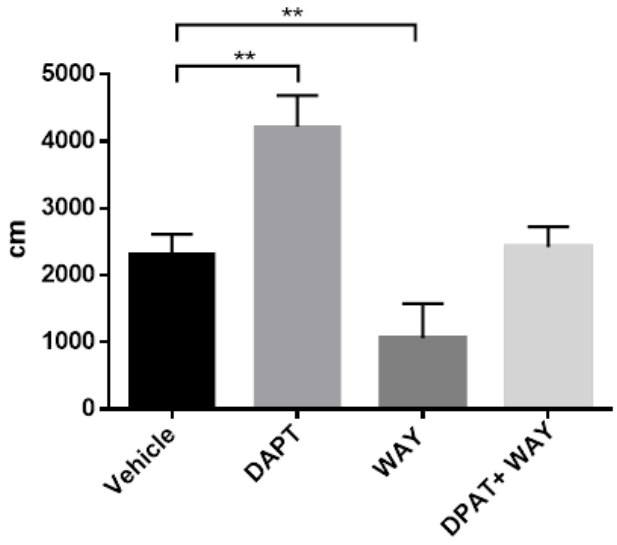
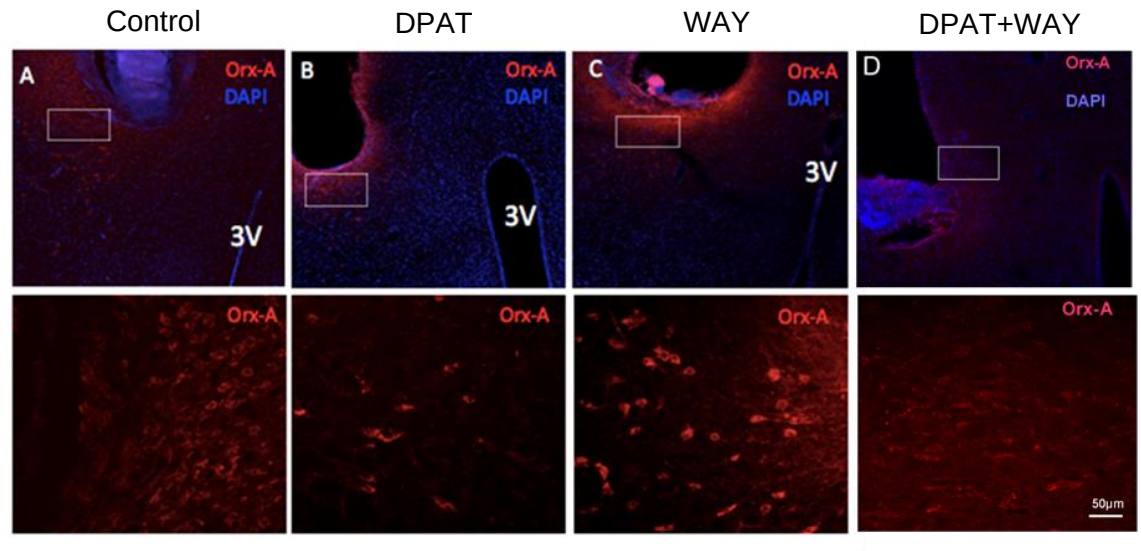
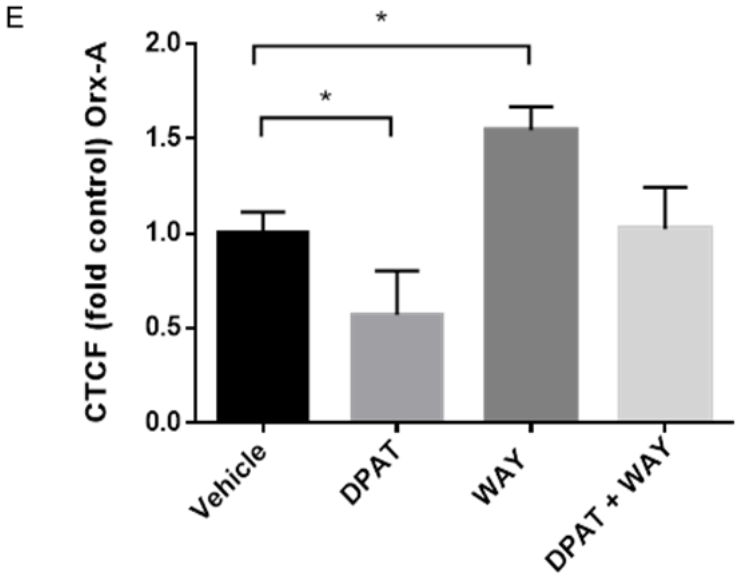


Figure 6. Effect in locomotor activity of infusion 5-HT$_{1A}$ agonist and antagonist into LH. Locomotor activity measured using open field test 3 hours after infusion of the 5-HT$_{1A}$ agonist and antagonist shows that DPAT produced a significant increase (*P= 0.0053*), whereas WAY100635 produced a significant decrease in locomotor activity compared to the vehicle (*P= 0.0055*). (One-way ANOVA, followed by Sidák's post hoc test, **P=0.05*, ***P = 0.01*, ****P = 0.001).*

Then, to evaluate if the observed change in food intake correlates with changes in orexin levels, the levels of Orx-A in the hypothalamus, prefrontal cortex and striatum of mice treated with the 5-HT_{1A} ligands were measured using immunofluorescence and western blot (Figure 7). Mice infused with DPAT significantly decreased Orx-A levels in the hypothalamus and striatum (STR) (Figure 7B-7F), two areas that receive dense inputs from Orx neurons (Sakurai et al., 2007), while there were no changes in PFC, area that is poorly innervated (Sakurai et al., 2007) (Figure 7C-7F).





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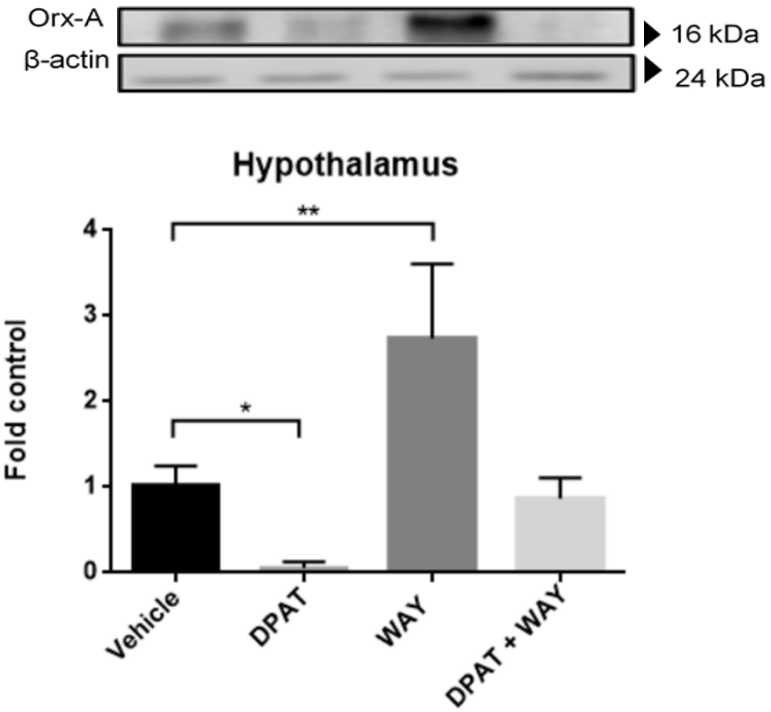
Orx-A
β-actin
16 kDa
24 kDa
Hypothalamus
Fold control
0
1
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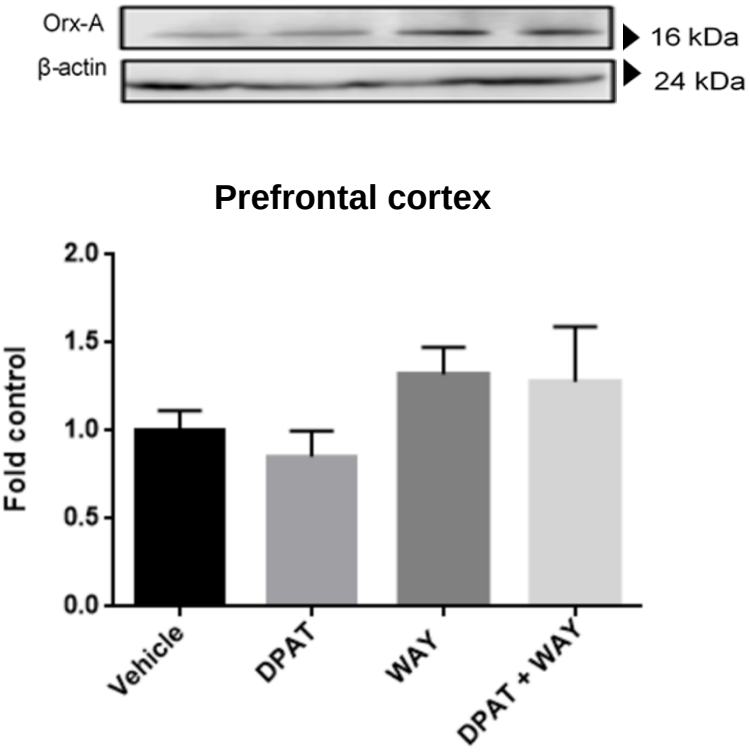
Orx-A
β-actin
16 kDa
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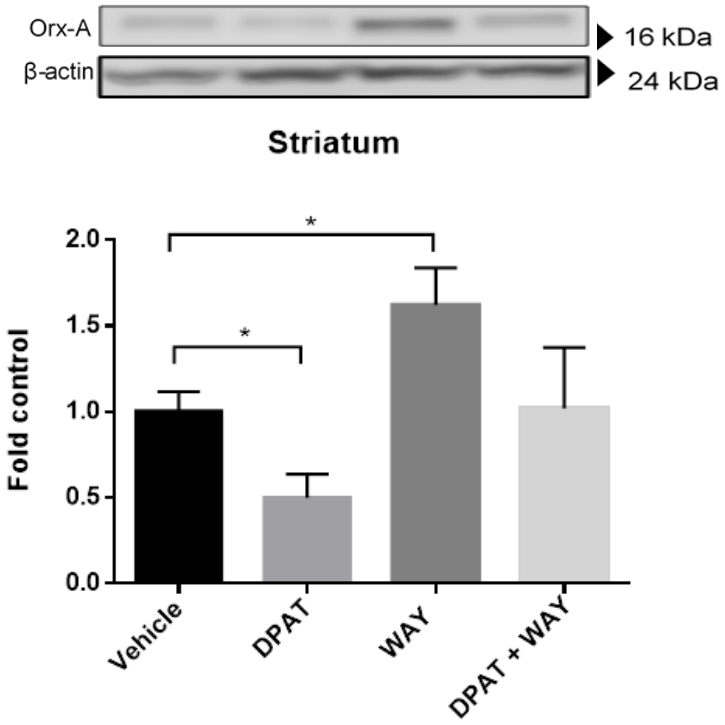
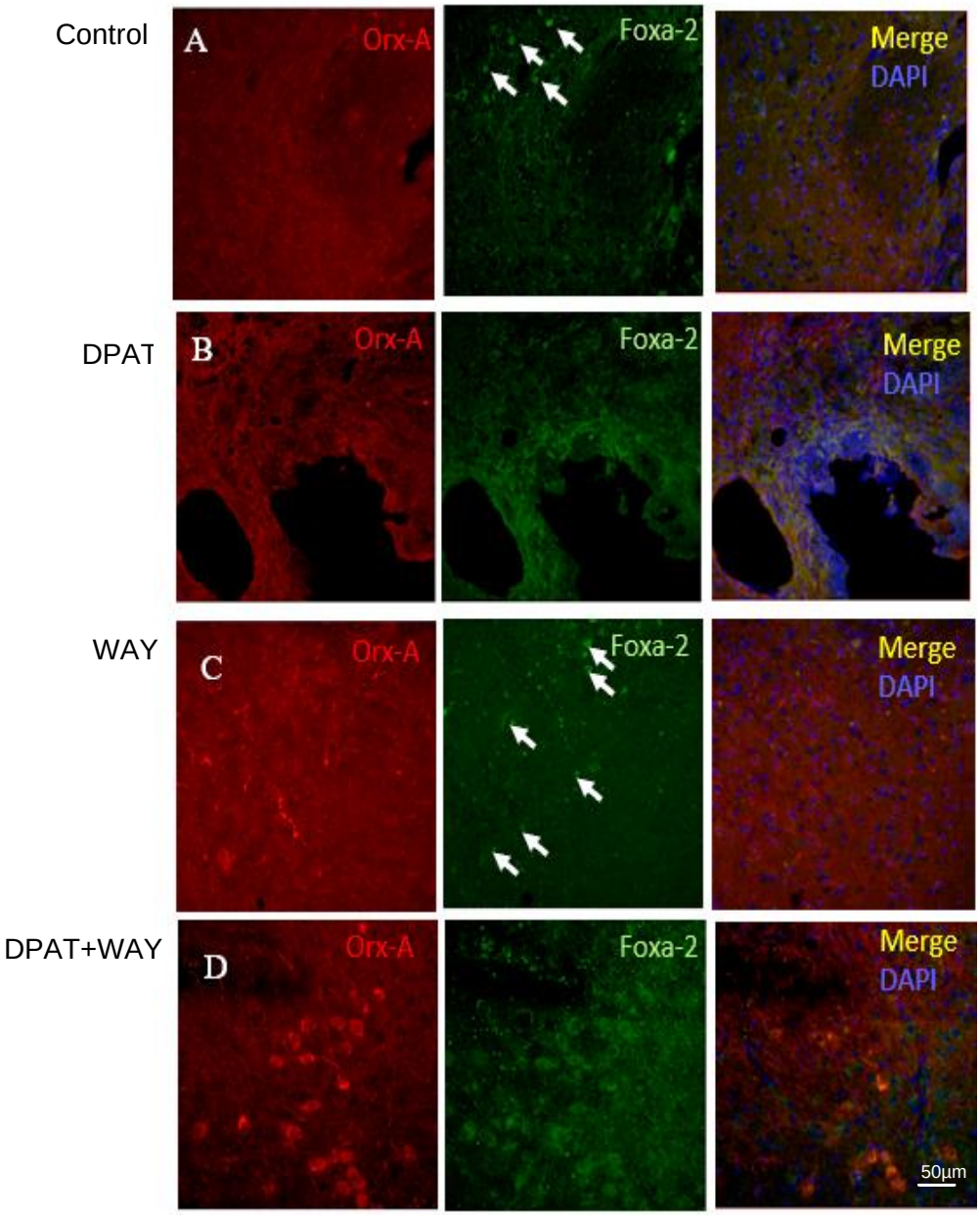


Figure 7. Orx-A levels in the hypothalamus, prefrontal cortex and striatum due to infusion of 5-HT$_{1A}$ agonist and antagonist into LH. Mice infused with vehicle (n=4), DPAT (n=4), WAY100635 (n = 4) and DPAT + WAY100635 (n = 4) in HL, samples were obtained 7 hours post infusion. Western blot and to Orx-A (red) and DAPI (blue) to nuclear staining in tissue of hypothalamus and western blot of prefrontal cortex and striatum subjected to pharmacological activation of 5-HT$_{1A}$. A) IF to Orx-A (red) in hypothalamus in mice infused with vehicle. B) IF to Orx-A in hypothalamus in mice infused with DPAT. C) IF to Orx-A in hypothalamus in mice infused with WAY100635. D) IF to Orx-A in hypothalamus in mice infused with DPAT and WAY100635. E) Quantification of the fluorescence intensity Orx-A using the CTCF method in HL. F) Western blot of hypothalamus, prefrontal cortex and striatum. It is observed that in LH the infusion of DPAT produces a decrease (*P=0.0721*) in Orx-A levels and the infusion of WAY100635 decreased Orx-A levels (*P=0.0013*). The infusion of both did not produce significant changes in the expression of Orx-A. No significant changes in Orx-A expression were observed in the cortex. In striatum, a decrease in Orx-A levels was observed after infusion of DPAT (*P=0.0477*) in LH and a significant increase after infusion of WAY100635 (*P=0.0138*). Scale bars: 50µm. (One-way ANOVA, followed by Sidák's post hoc test, **P=0.05*, ***P = 0.01*, ****P = 0.001).*

To determine whether the transcription factor Foxa2 is involved in the molecular mechanism that regulates Orx production *in vivo*, we determined its cellular localization in Orx neurons after infusion of 5-HT$_{1A}$ agonist or antagonist as described above. Treatment with R-OH -DPAT (n = 4) decreased Foxa2 nuclear accumulation by 50% compared to control (Figure 8B

and 8E). This reductions were then found to be normalized in co-treated with WAY 100635 (100 µM, n=4) (Figure 8D and 8E). infusion of WAY100635 produced an increase in Foxa2 nuclear accumulation compared to control (figure 8C and 3E).



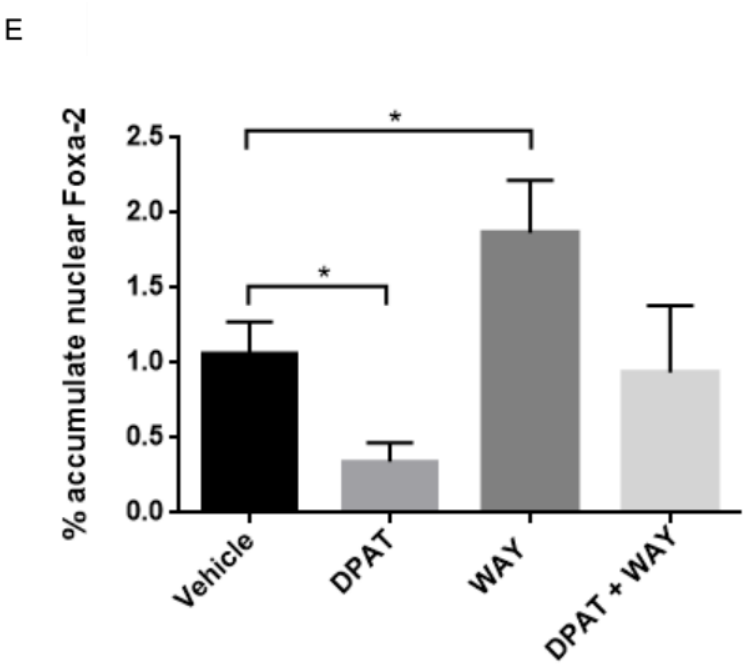


Figure 8. Subcellular localization of Foxa2 in Orx neurons due to infusion of 5-HT$_{1A}$ agonist and antagonist into LH. Mice infused with vehicle (n=4), DPAT (n=4), WAY100635 (n = 4) and R- OH-DPAT + WAY100635 (n = 4) in HL, samples were obtained 7 hours post infusion. IF to Orx-A (red), Foxa2 (green) and DAPI (blue) to nuclear staining in tissue of LH. A) Dual IF to Orx-A and Foxa2 in LH of mice infused with vehicle. B) Dual IF to Orx-A and Foxa2 in LH of mice infused with DPAT. C) Dual IF to Orx-A and Foxa2 in LH of mice infused with vehicle. WAY100635. D) Dual IF to Orx-A and Foxa2 in LH of mice infused with vehicle DPAT and WAY100635. E) % Foxa2 fluorescence intensity in the nucleus/total fluorescence. It is observed that in LH DPAT infusion significantly decreases Foxa2 levels in the nucleus of the total Foxa2 cell. It was observed that the infusion of WAY100635 significantly increased the levels of Foxa2 in the nucleus in relation to the total of Foxa2. Scale bars: 50µm. The white arrows indicate the presence of Foxa2 in the nucleus. (One-way ANOVA, followed by Sidák's post hoc test, *$P=0.05$, **$P = 0.01$, ***$P = 0.001$).

Then, to evaluate whether treatment with DPAT increases the phosphorylation of Akt at Ser473 *in vivo,* we determined Akt phosphorylation levels after infusion of 5-HT$_{1A}$ agonist or antagonist as described above. Treatment with DPAT (n = 3) increased 3-fold increase pAkt/Akt ratio compared to control (Figure 9). Then, it was observed that the treatment with WAY 100635 did not significantly increase the levels of pAkt/Akt compared to the control (Figure 9), however, the R-OH-DAPT + WAY100635 co-treatment significantly decreased the pAkt/Akt ratio compared to the group treated with DPAT (Figure 9). This finding suggests that Akt participates in the regulation of food intake by activation of 5-HT$_{1A}$.

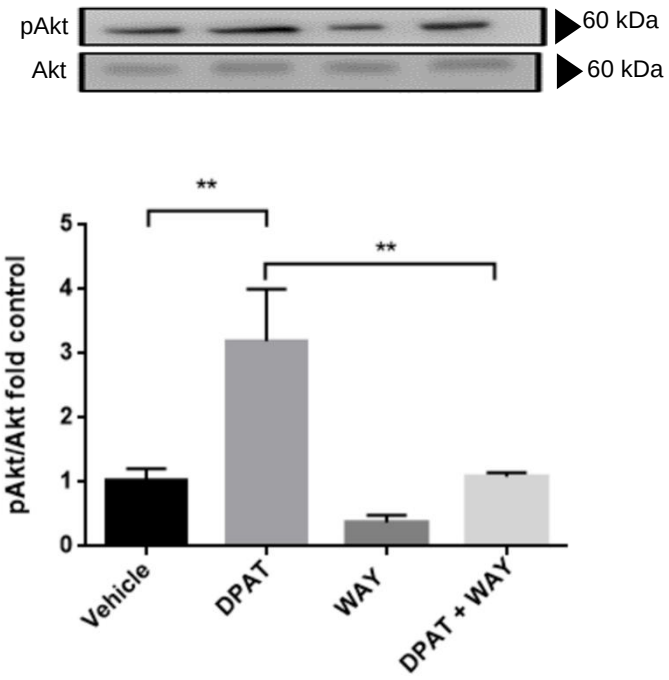


Figure 9. pAkt levels in the hypothalamus due to infusion of 5-HT$_{1A}$ agonist and antagonist into LH. Western blot in mice infused with, from left to right, DPAT (n = 3), WAY100635 (n=3) or DPAT + WAY100635 (n=3) in HL, samples were obtained 7 hours post infusion. The Infusion of DPAT significantly increase pAkt levels compared to vehicle treatment (*P= 0.0013*). The infusion of WAY100635 did not change the pAkt / Akt ratio compared to the control, but the infusion of DPAT + WAY100635 significantly decreased pAkt / Akt levels compared to the group infused with DPAT (*P = 0.0015*). (One-way ANOVA, followed by Sidák's post hoc test, **P=0.05*, ***P = 0.01*, ****P = 0.001)*. Note that pAkt levels were calculated as the pAkt/Akt ratio.

The involvement of Akt1 as the downstream signaling molecule in the pathway implicated in the 5-HT$_{1A}$-mediated control of food intake was evaluated by inhibiting Akt1 *in vivo*. For this, 60 nmol of Akt1 inhibitor VIII (in a total volume of 0.5µL) was injected into the LH of cannulated mice bilaterally; control group mice received vehicle (90% DMSO and 10% Krebs solution) (Figure 10). In mice infused with inhibitor VIII, a significant increase in food intake was observed at 3 hours compared to mice infused with vehicle (Figure 10). To demonstrate the *in vivo* inhibition of Akt phosphorylation, western blotting was performed from the hypothalamus of mice treated with the Akt inhibitor VIII, where we found a significant decrease in Akt phosphorylation (n = 4) compared to the control group treated with vehicle (n = 4) (Figure 11).

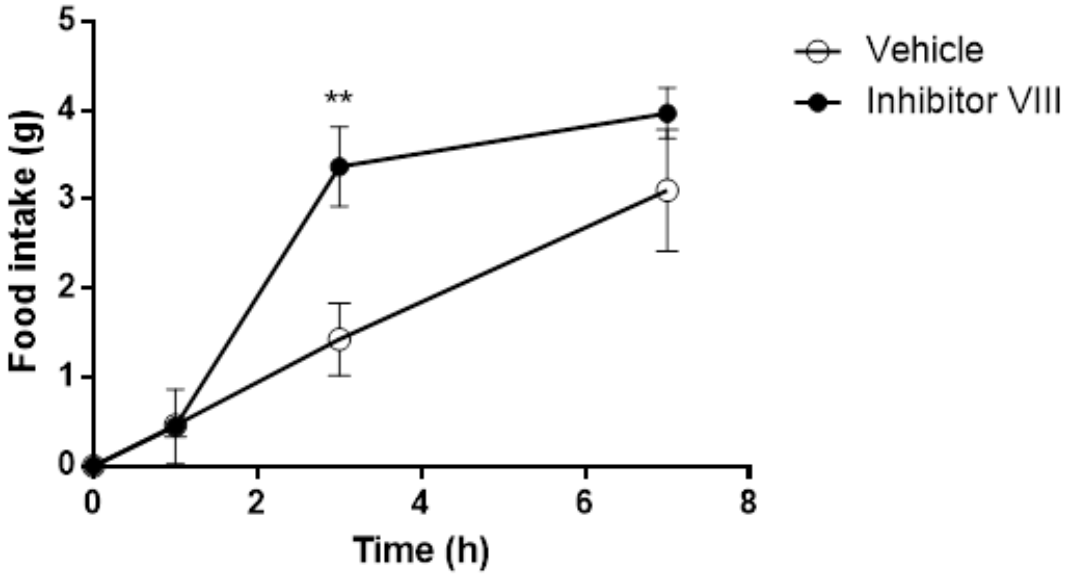


Figure 10. Effect on food intake by infusion of inhibitor VIII of Akt in LH. Adult male mice were chronically cannulated into LH and infused with vehicle and Akt inhibitor VIII (n=8) infused with inhibitor VIII (n=8). Changes in food intake were measured 1, 3 and 7hours post infusion of Akt inhibitor VIII or Vehicle. Infusion of Akt inhibitor VIII significantly decreased breath intake at 3h compared to vehicle (*P< 0.0001*) (Two-way RM-ANOVA and Sidák multiple comparison test * *P = 0.05, ** P = 0.01, *** P = 0.001**).*

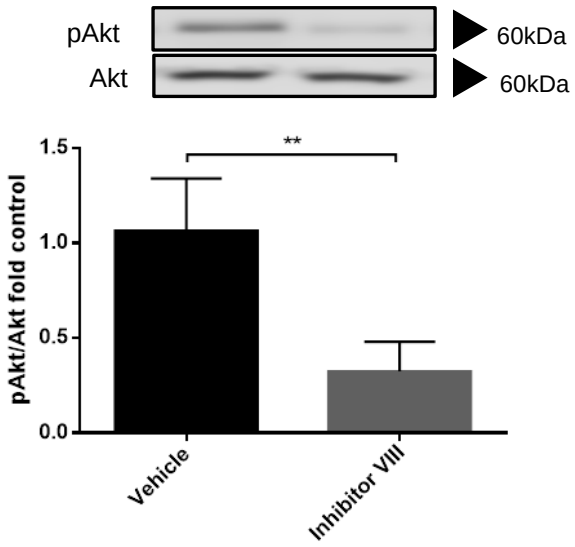


Figure 11. pAkt levels in the hypothalamus due to infusion of inhibitor VIII into LH. Western blot analysis of pAkt, Akt showing that Infusion of inhibitor VIII (60nmol) significantly reduced pAkt levels compared to vehicle treatment (*P= 0.0035*). *(*Unpaired student *t* test, * *P = 0.05, ** P = 0.01, * ** P = 0.001).* Note that pAkt levels were calculated as the pAkt/Akt ratio.

Next, to evaluate changes in locomotor activity, mice were tested in an open arena at 3 hours after a new infusion of Akt inhibitor VIII (Figure 12). In the OPT, a significant decrease in locomotor activity was observed in the mice infused with the inhibitor VIII (Figure 12A) compared to the vehicle group.

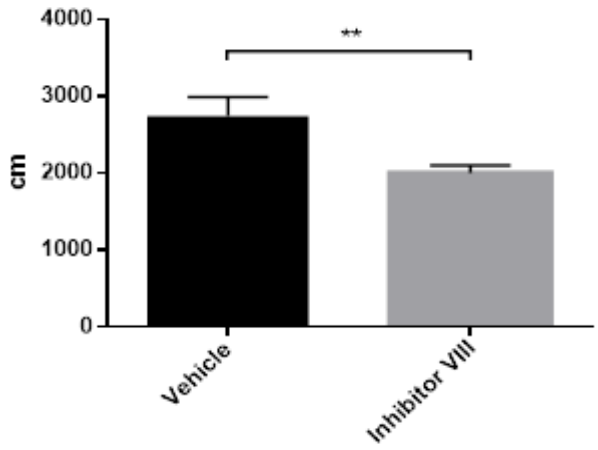


Figure 12. Effect in locomotor activity of infusion inhibitor VIII into LH. Changes in locomotor activity in mice infused with Akt inhibitor VIII (n=8) and Vehicle (n=8). It is observed that the infusion of Akt inhibitor VIII significantly decreased locomotor activity (*P=0.0013; t-student* for parametric variables, ** P = 0.05, ** P = 0.01, * ** P = 0.001*)

Finally, we sought to determine if a chronic increase of 5-HT levels could cause alterations in prepro-Orx levels. To this end, we used the serotonin transporter (SERT) KO mice available in our laboratory, which is a widely validated model that shows increases ~ 400% in extracellular 5-HT in the brain (Murphy & Lesch, 2008). Using RT-qPCR, we measured the mRNA levels of prepro-Orx and 5-HT_{1A} in the hypothalamus of SERT KO mice (Figure 13). We found a marked decrease in prepro-Orx levels about six times lower than the WT control mice (Figure 13A). Similarly, we found a significant decrease in the levels of 5-HT_{1A} in the SERT KO mouse (n = 4) respect to the WT control (n = 4) (Fig 13A-13B), in agreement with previous reports (Li Q et al., 2000)

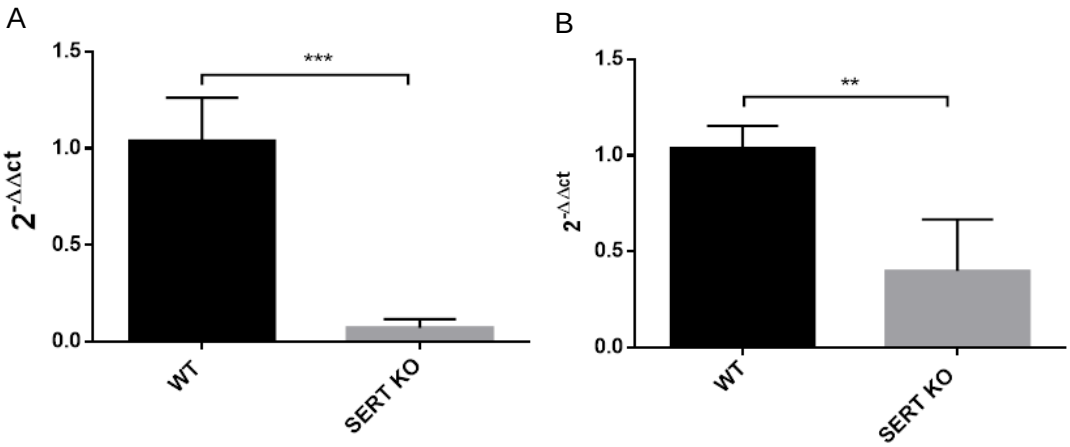


Figure 13. **Prepro-Orx and the HTR1A levels in the hypothalamus of SERT KO mice.** RT-qPCR of prepro-Orx and 5-HT_{1A} in extracts of hypothalamus from wild type (n=4) and SERT-KO mouse (n=4). A) Prepro-Orx mRNA levels in wild type and SERT-KO mice. B) HTR1A levels in wild type and SERT-KO mice. It is observed that SERT-KO mice shows significantly lower levels of the prepro-Orx and 5-HT_{1A} mRNA (*P<0.0001* and *P= 0.0047* respectively) (*t-student* for parametric variables, ** P = 0.05, ** P = 0.01, * ** P = 0.001*).

7. Discussion

Studies have established a central role of 5-HT in the regulation of food intake and satiety. The inhibition of central 5-HT synthesis with the TPH inhibitor pCPA or chemical lesion of 5-HT neurons with 5,7-DHT have orexigenic effects (Saller and Stricker, 1976). In addition, fenfluramine, a 5-HT releasing agent (i.e. increases 5-HT levels) reduces food intake; fenfluramine was used to treat obesity over 30 years before withdrawn from market for serious side-effects (Storlien et al., 1989; Zhou et al., 2007).

Several lines of evidence suggest an overlap between the activity of the 5-HT system and the modulation of the function of Orx-ergic neurons (Muraki et al., 2004; Chowdhury et al., 2016; Saito et al., 2018). 5-HT through 5-HT_{1A} mediates the inhibition of neuronal activity in Orx neurons, specifically in the LH (Muraki et al., 2004), an effect that has been so far studied in models that measure electrophysiological properties and changes in the sleep wake cycle. Therefore, we aimed to study 1) possible changes in the production of prepro-Orx mRNA and Orx-A protein levels triggered by the activation of 5-HT_{1A} in cultured hypothalamic neurons, allowing the activation of 5-HT_{1A} to be isolated only in Orx neurons; 2) changes in the feeding behavior upon local activation of 5-HT_{1A} in the LH *in vivo*.

In this thesis we found that, when 5-HT_{1A} are activated (with 5-HT and DPAT) in hypothalamic primary cells, significant reductions in prepro-Orx mRNA expression and Orx-A levels occur (Figure 1). In addition, we found a concomitant increase in the phosphorylation of Akt1 (Figure 3). Then we measured Foxa2 translocation to the nucleus and we observed a significantly greater presence in the cytoplasm in neuronal cultures treated with 5-HT and DPAT (Figure 2), which strongly suggest a role for 5-HT_{1A} via PI3K/Akt/Foxa2 pathway in the regulation of the expression of Orx´s.

To assess the role of 5-HT_{1A} activation in consequent influence on feeding behavior, in this work we infused DPAT and WAY100635 in the LH of male mice. In contrast, hyperphagia does not occur in free-feeding female rats, possibly due to the influence of ovarian hormones (Currie et

al., 2005) therefore, exclusively males were used in this study. Mice with free access to food were used, as food deprivation and the consequent lack of nutrients have been shown to alter 5-HT metabolism at the brain level (Chaouloff et al., 1988, Ebenezer et al., 1992; Li et al., 2008; Chaouloff et al., 1997). After the bilateral LH administration of 6.4nmol of DPAT, a selective 5-HT_{1A} agonist, we observed a significant decrease in food intake at 3 hours post-infusion that lasted until 7 hours (Figure 5). Conversely, local administration of WAY100635, a selective 5-HT_{1A} antagonist significantly increased food intake from 3 to 7 hours after treatment compared to control (figure 5).

Our result is consistent with a previous study performed by Steffens & cols, which shows that intra LH injection of 0.6 and 6 nmol of 8-OH-DPAT significantly decreases food intake in rats 1h post treatment, (Steffens et al., 2008). Other studies have shown that systemic treatment with 5-HT_{1A} receptor agonist 8-OH-DPAT, other 5-HT_{1A} partial agonists (buspirone, gepirone, ipsapirone) elicit hyperphagia in satiated rats (Dourish et al., 1985; Gilber et al., 1987; Fletche et al., 1990). This hyperphagic effect of 8-OH-DPAT is achieved only with the administration of a high dose (10 mg/kg) in mice, (Blanchard et al., 1997). Conversely, systemic injection of 8-OH-DPAT in low doses (100-500 µgr/Kg) enhanced the basal feeding duration (Shepherd & Rodgers., 1990; Ebenezer et al., 2000) and the total amount of food consumed (Coudereau et al., 1995) a phenomenon that also occurs after local injection of 8-OH-DAPT into the raphe (Dourish et al., 1978). This effect was antagonized by pretreatment with systemic selective 5-HT1A antagonist WAY100635 (Ebenezer et al., 2000). In this work, to demonstrate the effect of post-synaptic 5-HT_{1A} activation, it was infused a high dose of DPAT in HL, since it has been previously described that high doses of it can preferentially activate 5-HT_{1A} post-synaptic or heteroreceptor, with the intention of reducing possible activation of the 5-HT_{1A} auto receptor present in the 5-HT terminals, which has a higher sensitivity and can be desensitized early after treatment with 5-HT_{1A} agonists (Blanchard et al., 1997).

The hypophagic effects of DPAT treatment were abolished by concurrent treatment with the selective 5- HT_{1A}R antagonist WAY100635 (Figure 5). In agreement with previous reports, systemic WAY100635 had no effects on food consumption on its own in rat, mouse and pig (Fletcher et al., 1996; Swiergiel and Dunn, 2000; Ebenezer et al., 2001), but abolished the hyperphagic effects of 8-OH-DPAT in rat (Fletcher et al., 1996) and pig (Ebenezer et al., 2001). This could be because systemic treatment with WAY100635 inhibits auto 5-HT_{1A}, prevents a decrease in 5-HT tone, which has been attributed to the decrease in food intake (Currie et al., 1993; Sharp et al., 1990; Hjorth et al., 1988; Sprouse et al., 1987; Bendotti et al., 1986). However, in this work, by injecting WAY100635 directly into HL, we were able to evaluate the direct effect of post-synaptic 5-HT_{1A} inhibition, which resulted in a significantly increased food intake (figure 5).

In an immunohistochemical study, Collin and colleagues suggested that the activation of hypothalamic 5- HT_{1A}R may control food intake by affecting the expression of orexigenic and anorexigenic peptides (Collin et al., 2002). The primary aim of the study at hand was to assess whether murine postsynaptic 5-HT_{1A} are involved in feeding control and the production of Orx peptide. Then, we correlated the changes in food intake with changes with the levels of Orx-A and found that the activation of 5-HT_{1A} by infusion with DPAT significantly decreased Orx-A levels in the hypothalamus and STR (Figure 7). Several studies have suggested that the increased food intake following Orx-A administration is, at least, partially mediated by the activation of NPY neurons in the ARC nucleus (Yamanaka et al., 2000; Muroya et al., 2004). On the other hand, feeding behavior is a classic example of activating the reward system, since infusions of Orx-A into the nucleus accumbens shell (a subregion of striatum) increase feeding behavior (Thorpe et al., 2005). Mice treated with WAY100635 showed a significant increase in Orx-A levels in the striatum, which correlated with an increase in food intake- possibly due to activation of OX_1R (Martin et al., 2002), and opposite to the effect of DPAT infusion (Figure 7). The effect of 5-HT_{1A} activation on Orx-A levels was correlated with a lower translocation to the nucleus of Foxa2 in mice infused with DPAT (Figure 8). In this work, we did not find changes in Orx-A levels in the PFC. Other studies have suggested that the role of Orx in PFC is important in the regulation of

cue-induced feeding (Cole et al., 2015; Zajo et al., 2016). Therefore, we demonstrated that the activation of 5-HT_{1A} can decrease Orx levels. This suggests the participation of the 5-HT tone in the control of food intake and the energy balance that could be mediated by Orx neurons in the LH.

Other peripheral signals can also regulate Orx expression including Lep (Hakansson et al., 1999), insulin (Silva et al., 2009), glucocorticoids (Grafe et al., 2017), Interferon-α (Waleh et al., 2001) and Insulin-like growth factor binding protein 3 (IGFBP3) (Honda et al., 2009) (Figure 14).

The control of the activity of Orx neurons by Lep occurs via the direct activation of LepR (Villanueva et al., 2009), which is expressed in expressed in Orx neurons (Leinninger et al., 2011). Continuous infusion of Lep in the 3rd ventricle has been shown to decrease the levels of prepro-Orx mRNA in mice fed *ad libitum* (Yamanaka et al., 2003). Conversely, the stimulatory effect of food deprivation on the hypothalamic prepro-Orx mRNA levels can be reversed by Lep administration (Lopez et al., 2000). A crucial role for the PI3K pathway in Lep signaling was first demonstrated when i.c.v injection with PI3K inhibitors inhibited leptin's anorexigenic effects (Niswender et al., 2001). Also, the PI3K pathway is shared with other receptors, especially the insulin receptor (IR) (Wolfrum et al., 2003) (Figure 14). The IR activate a complex intracellular signaling, including PI3K and Akt (Silva et al., 2009) and the IR activation in Orx neurons produces a significant decrease in the production of prepro-Orx (Silva et al., 2009) (Figure 14). Another modulator of Orx production is IGFBP-3 which mediate effects on cells through regulating the access of insulin-like growth factors (IGFs) to the IGF receptor type 1 (IGF-1R) (De Mellow et al., 1988) IGF-1R activate multiple signaling pathways, including MAPK, phosphatidyl, PI3K and Akt (Delafontaine et a., 2003). Honda and cols shown that IGFBP-3 co-localizes in Orx producing neurons and demonstrated that IGFBP-3 is downregulated in the posterior hypothalamus of both narcoleptic *post-mortem* human brains and Orx neuron deficient mice (Honda et al., 2009). In contrast, mice over-expressing IGFBP-3 have lower levels of prepro-Orx mRNA and exhibit more sleep at the end of the dark period, compared to controls (Honda et al., 2009). Also, a

polymorphism in the IGFBP3 gene known to increase serum IGFBP3 levels has been associated with lower levels Orx- A in cerebrospinal fluid of normal patients (Honda et al., 2009) (Figure 14).

Conversely, glucocorticoids have been reported to increase prepro-Orx levels in rats, with a marked effect in the case of females. (Grafe et al., 2017). The main actions of glucocorticoid occur through the activation of glucocorticoid receptors (GRs) (Leis et al., 2004) and they activation produced inhibition of the PI3K/Akt signaling pathway in several tissues (Perez et al., 2001; Budunova et al., 2003) (Figure 14).

Therefore, a point of convergence in the mechanisms of regulation of expression of Orxs described is Akt, which, when activated, correlates with a decrease in the production of prepro-Orx or in Orx levels. (Hakansson et al., 1999; Silva et al., 2009; Grafe et al., 2017; Waleh et al., 2001; Honda et al., 2009). Therefore, Akt is likely to be a point where different signals converge that can regulate the expression of Orx, regulating food intake (Figure 14)

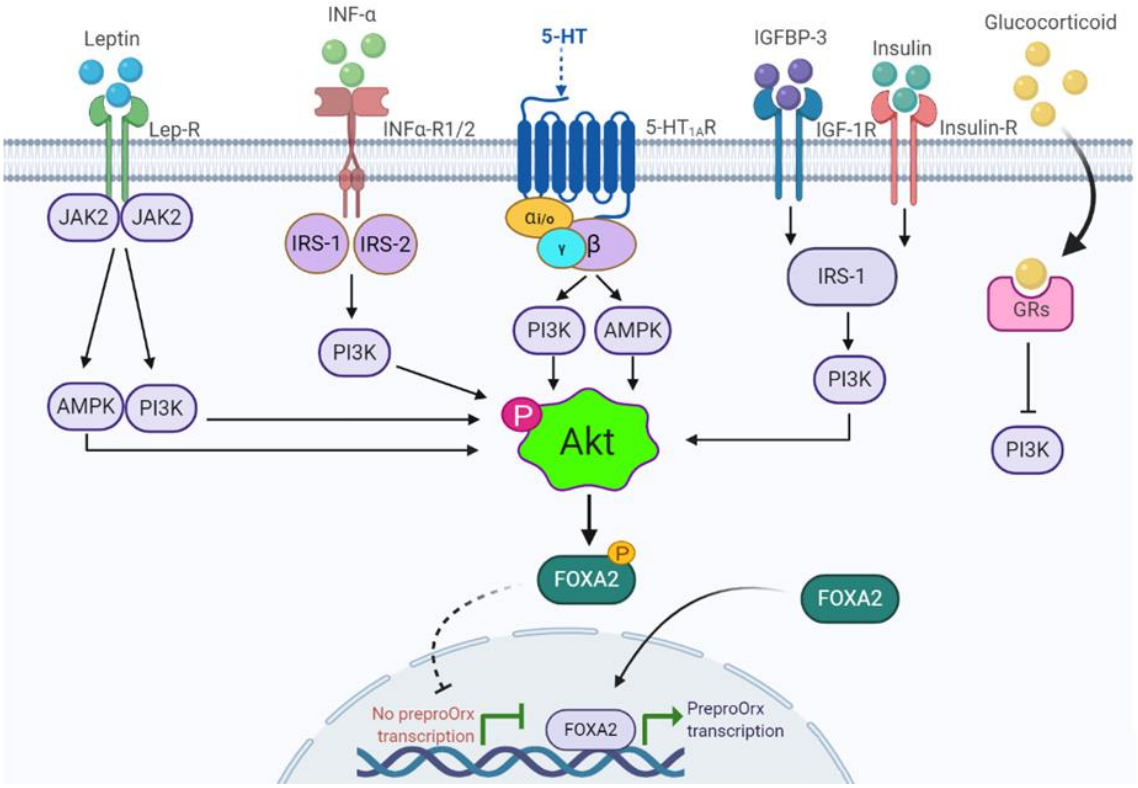


Figure 14. Signaling pathways involved in the regulation of Orexin expression. This figure summarizes the receptor activation mechanisms regulating the expression of the prepro-Orx. Its activation following LepR, INF-αR1/2, IGF-1R, insulin-R and 5-HT_{1A} activation depends on kinases' cascade such as Janus kinase 2 (Jak2), AMP-activated protein kinase (AMPK), PI3K which converge into Akt. the Phosphorylation of Foxa2 by Akt, inhibits the translocation to the nucleus of Foxa2 (transcriptional inactivation), decreasing the expression of the prepro-Orx. Conversely, glucocorticoid receptor activation drive to transcriptional activation of the prepro-Orx. See Appendix for the list of abbreviations. Dashed line, inhibition; black arrow, stimulation.

To demonstrate the involvement of the Akt pathway in the control of 5-HT_{1A} mediated food intake, we locally infused a selective inhibitor of Akt. We observed that the blockade intra-LH of Akt significantly increased food intake after 3 hours, however this effect was transient and found to be restored at 7 hours after infusion (Figure 10). Several studies have found that hypothalamic Akt-mediated signaling pathways play critical roles in the regulation of food intake and energy metabolism in mammals. The involvement of Akt in food intake has recently been reported, wherein the intra-ventricular injection of wortmannin, a selective inhibitor of Akt, significantly

increases food intake in rats (Yang et al., 2014; Yang et al., 2017). Also, i.c.v. injections of PI3K inhibitor exendin-4 and LY294002 enhanced the food intake (Seneyasu et al., 2018).

The SERT KO mouse exhibits obesity during adulthood (Holmes et al., 2002). This phenotype showing an increase in circulating levels of insulin and Lep (Murphy et al., 2008). Also, SERT KO mice show decreased food intake (Chen et al., 2013) but, to date, there are no studies addressing its relationship with Orx expression. In this work we observed that in the hypothalamus of SERT KO mice there is a significant decrease in prepro-Orx levels, likely due to an over stimulation of the 5-HT_{1A} expressed in Orx neurons (Figure 13). The selective deletion of the Orx gene produce first hypophagia initially and later a decreased metabolism that results in obesity (Hará et al., 2001). Therefore, the decrease Orx expression found in SERT KO mouse could be a contributing factor to the decreased food intake and the subsequent development of obesity observed in this mouse model. To date, no alterations have been described in any of the components of the Orx-ergic system that could be related to the decrease in both food intake and metabolism of the mouse in the SERT mouse.

8. Conclusions

In conclusion, the data presented here revealed that tonic inhibitory 5-HT inputs regulate the functioning of the LH. In addition, this work showed that the 5- HT_{1A}R located in the LH are components of the serotoninergic circuit that controls the food intake. The orexinergic circuit was affected by 5-HT_{1A} agonists and antagonists; the hypophagic effects of DPAT and hyperphagic effects of WAY100635 could increase the effectiveness of satiety influence on the LH evoked by 5-HT_{1A} through the signaling dependent on Akt. Akt could maintain a central role in the regulation of Akt production not only in that determined by the action of 5-HT, but also of Lep, insulin, INF and glucocorticoid. Therefore, the signaling mechanism that includes the activation of the 5-HT_{1A}-> Akt-> Foxa2 signaling pathway and consequent production of propepOrx and Orx-A regulates food intake in mice (Figure 15).

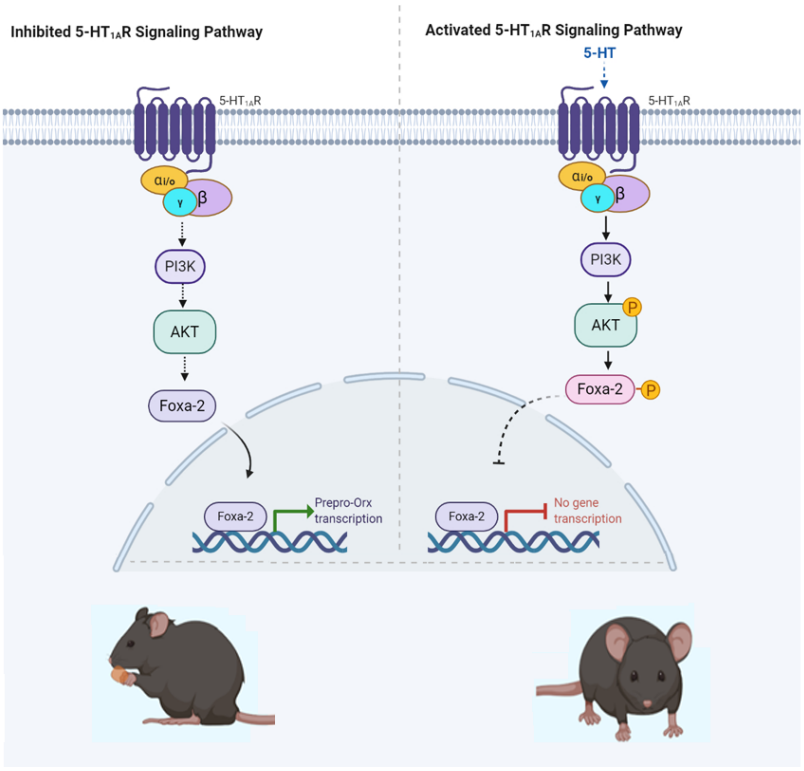


Figure 15. The 5-HT_{1A} receptor controls orexin production and food intake in mice. Inhibition of the 5-HT_{1A} receptor in Orx neurons produces a decrease in pAkt levels, an increase in Foxa2 levels in the nucleus and Orexin levels producing hyperphagia (Left). Activation of the 5-HT_{1A} receptor in Orx neurons produces an increase in pAkt levels, a decrease in Foxa2 levels in the nucleus and Orexin levels producing hypophagia (right).

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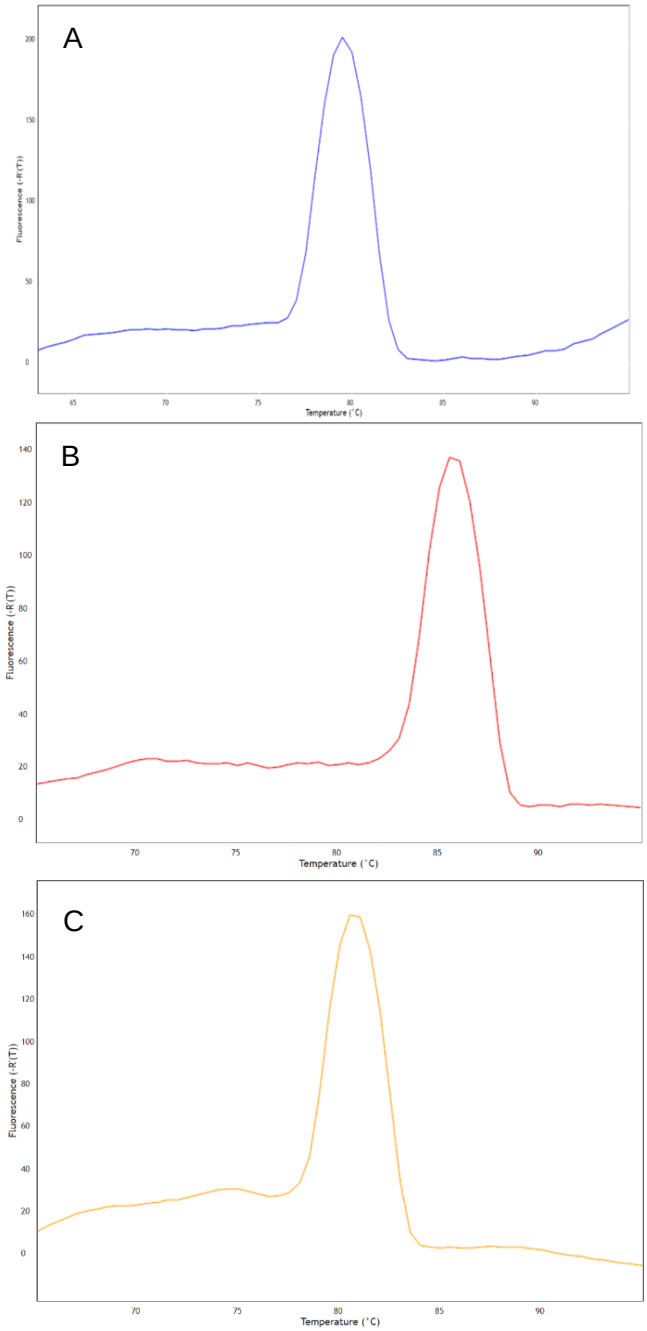
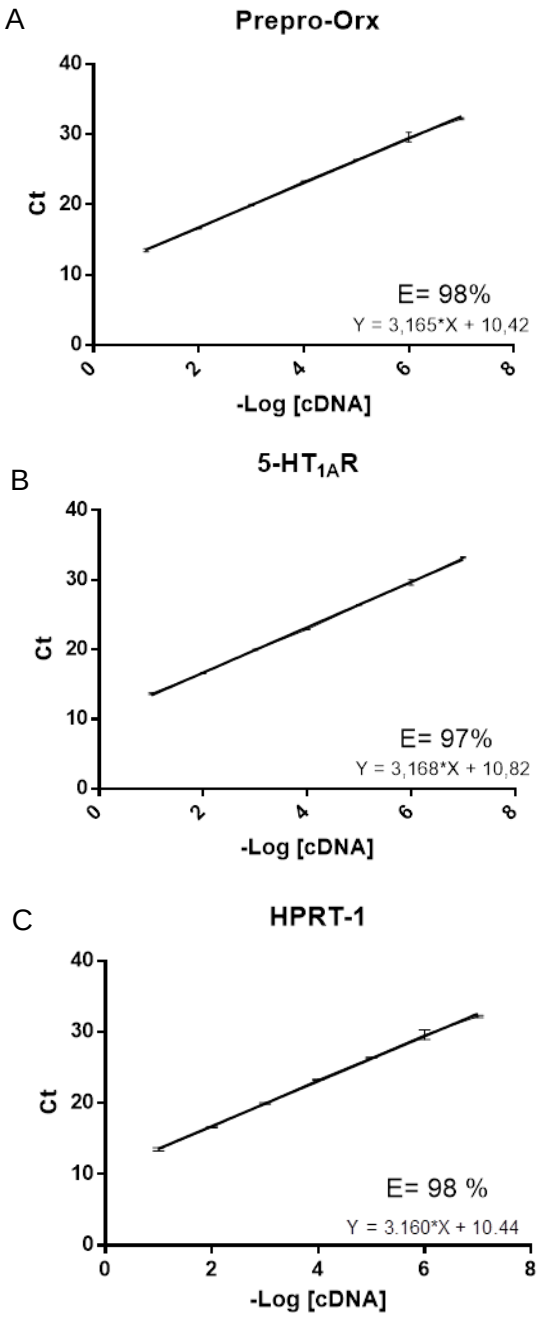
10. Supplementary figures



Supplementary figure 1. Melting curve for the PCR products to prepro-Orx, 5-HT$_{1A}$R and HPRT1. The fluorescence is plotted against temperature and then the -ΔF/ΔT (change in fluorescence/change in temperature) is plotted against temperature to obtain a melting curve. The fluorescence peak corresponds to the melting point which was specific for each of the analyzed sequences. A) Melting curve for the prepro-Orx with a melting point of 79.5 °C. B) Melting curve for 5-HT$_{1A}$R with a melting point of 85.5 °C. C) Melting curve for HPRT1 with a melting point of 80.5 °C.



Supplementary figure 2. Standard curve of qPCR data. The log of each known concentration in the dilution series (x-axis) is plotted against the Ct value for that concentration (y-axis). A) Standard curve to prepro-Orx. B) Standard curve to 5-HT_{1A}R. C) Standard curve to HPRT1. The Efficiency and slope are show in each plot.

Treatment	Time 0h				Time 1h			
	Upper limit	Lower limit	Mean	*p* value	Upper limit	Lower limit	Mean	*p* value
Vehicle vs. DPAT	0,7430568	-0,7430568	0	> 0,9999	0,833056	-0,653056	0,09	0,9997
Vehicle vs. WAY 100635	0,7430568	-0,7430568	0	> 0,9999	0,015556	-1,470557	-0,7275	0,0582
Vehicle vs. DPAT + WAY100635	0,7430568	-0,7430568	0	> 0,9999	0,376806	-1,109307	-0,36625	0,7165
DPAT vs. WAY 100635	0,7430568	-0,7430568	0	> 0,9999	-0,074443	-1,560557	-0,8175	0,0232
DPAT vs. DPAT + WAY100635	0,7430568	-0,7430568	0	> 0,9999	0,2868068	-1,199307	-0,45625	0,4786

Treatment	Time 3h				Time 7h			
	Upper limit	Lower limit	Mean	*p* value	Upper limit	Lower limit	Mean	*p* value
Vehicle vs. DPAT	1,566807	0,080693	0,823	0,0217	2,699307	1,21319	1,956	< 0,0001
Vehicle vs. WAY 100635	-1,580693	-3,066807	-2,3237	< 0,0001	-2,151943	-3,63807	-2,895	< 0,0001
Vehicle vs. DPAT + WAY100635	0,618056	-0,868056	-0,125	0,9983	0,330556	-1,15555	-0,419	0,595
DPAT vs. WAY 100635	-2,404443	-3,890557	-3,14	< 0,0001	-4,108193	-5,59430	-4,855	< 0,0001
DPAT vs. DPAT + WAY100635	-0,205693	-1,691807	-0,948	0,0052	-1,625693	-3,11180	-2,368	< 0,0001

Source of Variation	DF	Sum of Squares	Mean Square	F	*p* value
Interaction	9	61.30	6.811	F (9, 84) = 29,81	P < 0,0001
Time	3	168.1	56.04	F (3, 84) = 245,3	P < 0,0001
Column Factor	3	80.90	26.97	F (3, 28) = 49,40	P < 0,0001
Subjects (matching)	28	15.29	0.5460	F (28, 84) = 2,389	P = 0,0012
Residual (Error)	84	19.19	0.2285		
Total	127	344.8			

Supplementary figure 4. Two-way repeated measures (RM) ANOVA and Sidák multiple comparison test, to analysis of feeding behavior. Data for food intake of DPAT, WAY100635 and DPAT + WAY100635 vs. vehicle controls mice. The table (upper and middle) shows the upper, lower, and mean limits of the confidence interval, p value, at times 0, 1, 3 and 7h after treatment. The table (bottom) shows F, degrees of freedom, factor, interactions and *p value*. For the analysis, an alpha value = 0.05 was considered